ASSESSING THE MOLECULAR EPIDEMIOLOGY AND ANTIMICROBIAL SUSCEPTIBILITY PROFILES OF THERMOPHILIC *CAMPYLOBACTER* SPECIES FROM HUMAN AND ANIMAL FECES IN SOUTH KOREA AND TANZANIA

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EXTENDED ABSTRACT

Campylobacter species cause human gastroenteritis and have developed resistance to existing antimicrobials. The epidemiology of *Campylobacter* infections is not fully understood due to a complex genome and the existence of various reservoirs. Poultry is the primary reservoir of *Campylobacter* but other domestic and wild animals have also been reported as contributing sources. Natural products are regarded as alternative treatments in the post-antibiotic era while the whole-genome sequencing (WGS) is seen as a promising technology towards deciphering the epidemiology and evolution of *Campylobacter*. The main objective of this research was to assess the molecular epidemiology and antimicrobial susceptibility profiles of thermophilic *Campylobacter* species from human and animal feces in South Korea and Tanzania. Specifically, this study aimed at 1) determining the antimicrobial resistance (AMR) profiles, virulence genes, and genetic diversity of thermophilic *Campylobacter* species isolated from a layer poultry farm in Korea; 2) determining the susceptibility of layer chicken-derived and reference *Campylobacter* strains to selected natural products and frontline antibiotics; and 3) carrying out genomic characterization of fluoroquinolone (FQ)-resistant thermophilic *Campylobacter* strains isolated from layer chicken faeces in Gangneung, Korea by whole-genome sequencing; and 4) carrying out molecular techniques for the detection of *Campylobacter* species from human stool and cattle faecal samples in Kilosa district, Tanzania.

In the first study, 153 faecal samples were obtained from two layer chicken farms in Gangneung, South Korea. Isolation of *Campylobacter* was carried out by culture, followed by species confirmation with PCR and sequencing. Antimicrobial susceptibility tesing for six antimicrobials (sitafloxacin, ciprofloxacin, nalidixic acid, gentamicin, erythromycin, and tetracycline) was performed by broth microdilution. Three antimicrobial resistance (AMR) and nine virulence genes were screened by PCR. Genotyping was performed by flagellin A-restriction fragment length polymorphism (*fla*A-RFLP) and multilocus sequence typing (MLST). Of the 153 samples, *Campylobacter* spp. were detected in 55 (35.9%) with 49 (89.1%) and 6 (10.9%) being *C. jejuni* and *C. coli*, respectively. High-level resistance (MIC ≥ 32 μg/mL) was observed for ciprofloxacin (100%), nalidixic acid (100%), and tetracycline (*C. jejuni*: 93.9%; *C. coli*: 83.3%) but no resistance was observed for sitafloxacin. Sequencing confirmed mutations associated with quinolones (C257T in *gyr*A gene) and tetracycline [*tet*(O) gene] resistance. Multidrug resistance at a rate of 8.2% was exclusively recorded in *C. jejuni*. *cst*II, *fla*A, *dna*J, *cad*F, and *cdt*B were found in all

Campylobacter isolates while the proportions for other genes (*cia*B, *pld*A, and *csr*A) varied from 33.3 to 98 %. The *fla*A-RFLP typing resulted in 21 types for *C. jejuni* and five for *C. coli*: 5 while MLST showed 10 sequence types (STs) for *C. jejuni* and three STs for *C. coli* with CC-607 (ST 3611) and CC-460 (ST-460) being predominant. Among the 10 STs of *C. jejuni*, three were newly assigned.

The second work determined the susceptibility of layer chicken-derived and reference *Campylobacter* strains to selected natural products and frontline antibiotics. The efficacy of selected natural products was assessed by broth microdilution and the optical density recorded by a microplate reader. Antibiotic resistance genes (*tet*(O) and *gyr*A) were characterized at the molecular level. The minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) ranged from 25 to 1600 µg/mL. Cinnamon extract, cinnamon oil, (E)-Cinnamaldehyde, clove oil, eugenol, and baicalein had the lowest MIC and MBC values (25–200 µg/mL). Chicken-derived isolates were resistant to quinolones and tetracycline but sensitive to erythromycin and gentamicin. NPs were effective against both AMR and sensitive *Campylobacter* strains.

The third study characterized FQ-resistant *C. jejuni* (200605) and *C. coli* (200605) strains of layer chicken origin by whole-genome sequencing using Illumina sequencing technology. A phylogenetic relationship to existing strains was also established. WGS confirmed C257T mutation in the *gyr*A gene and the presence of *cme*ABC complex conferring resistance to FQs in both strains. Both strains also exhibited *tet*(O) genes associated with tetracycline resistance. No resistance to macrolides and aminoglycosides was found. Putative genes conferring resistance to doxycycline, minocycline; $bla_{\text{OXA-452}}$, cephalosporin, and penam were also recorded in both strains. Virulence genes associated with motility, chemotaxis, and capsule formation were found in both strains. However, the analysis of virulence genes showed that *C. jejuni* strain 200605 is more virulent than *C. coli* strain 200606.

The MLST showed that *C. jejuni* strain 200605 belongs to sequence type (ST)-5229 while *C. coli* strain 200606 belongs to ST-5935, and both STs are less common. The phylogenetic analysis clustered *C. jejuni* strain 200605 along with other strains reported in Korea (CP028933 from chicken and CP014344 from human) while *C. coli* strain 200606 formed a separate cluster with *C. coli* (CP007181) from turkey. The WGS confirmed FQ-

resistance in both strains and showed potential virulence of both strains. Further studies are recommended to understand the reasons behind the regional distribution of such rare STs.

Lastly, 70 human stool and 30 cattle faecal samples were collected in Kilosa district, Tanzania. Species confirmation was conducted by polymerase chain reaction (PCR) and 16S rRNA sequencing while the phylogenetic analysis was done with 16S rRNA sequences. *Campylobacter* species detection rates were 65.7% (46/70) and 20.0% (6/30) in humans and cattle, respectively. In humans, *C. concisus* was the predominant species 37.8% (14/37), followed by uncultured *Campylobacter* spp. 24.3% (9/37) and *C. hominis* 21.6% (8/37). The least represented species were *C. jejuni* and *C. lanienae* all occurring at 2.7% (1/37). Molecular detection methods need to be adopted in routine *Campylobacter* testing and surveillance studies because they provide results in short period of time.

The findings of this study highlight (i) the usefulness of molecular techniques in emerging *Campylobacter* detection, (ii) the molecular epidemiology and antimicrobial resistance of *Campylobacter* from layers; (iii) the importance of some natural products as alternative to conventional antimicrobials in the control of *Campylobacter* infections; and (iv) WGS data of *Campylobacter* from layer chicken for better understanding the *Campylobacter* epidemiology and serve as a baseline for future studies. This study also identified new sequence types (STs) including ST-10645, ST-10647, ST-10648 that were isolated from layer chicken in Korea. I recommend further studies on (i) the synergism of natural products and existing antimicrobials; and (ii) chemical profiling of used plant extracts and their anti- adhesion effects to both biotic and abiotic surfaces.

DECLARATION

I, Noel Gahamanyi, declare to the Senate of the Sokoine University of Agriculture that this thesis is my original work and that it has neither been submitted nor being concurrently submitted in any other institution.

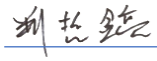
Noel Gahamanyi — 18th November 2021

(PhD Candidate) — Date

The above declaration is confirmed by

Prof. Erick V.G. Komba — 18th November 2021

(Supervisor) — Date



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DEDICATION

This thesis is dedicated to my family, sisters, brother, and parents. Without your contributions, I would not be where I am today.

LIST OF PUBLICATIONS

1. Gahamanyi, N., Mboera, L. E., Matee, M. I., Mutangana, D., & Komba, E. V. (2020). Prevalence, risk factors, and antimicrobial resistance profiles of thermophilic *Campylobacter* species in humans and animals in Sub-Saharan Africa: a systematic review. *International Journal of Microbiology* **6:** 1-12 (doi.org/10.1155/2020/2092478).

2. Gahamanyi, N., Song, D. G., Yoon, K. Y., Mboera, L. E., Matee, M. I., ... & Pan, C. H. (2021). Antimicrobial Resistance Profiles, Virulence Genes, and Genetic Diversity of Thermophilic *Campylobacter* Species Isolated from a Layer Poultry Farm in Korea. *Frontiers in Microbiology* **12:** 554 (doi: 10.3389/fmicb.2021.622275)

3. Gahamanyi, N., Song, D. G., Cha, K. H., Yoon, K. Y., Mboera, L. E., Matee, M. I., ... & Pan, C. H. (2020). Susceptibility of *Campylobacter* Strains to Selected Natural Products and Frontline Antibiotics. *Antibiotics* **9** (11): 790. (doi.org/10.3390/antibiotics9110790).

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16S rRNA	16S ribosomal ribonucleic acid
23S	Major component of the prokaryotic ribosomal subunit 50S
AFLP	Amplified fragment length polymorphism
AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
CC	Clonal Complex
*cia*B	*Campylobacter* invasion antigen B
cmeABC	*Campylobacter* multidrug efflux pump, consisting of three components cmeA, cmeB, and cmeC
DNA	Deoxyribonucleic acid
EUCAST	European Committee for Antimicrobial Susceptibility Testing
flaA	Flagellin gene subunit A
GBDP	Genome BLAST Distance Phylogeny
gDNA	Genomic DNA
*gyr*A	DNA gyrase gene encoding the gyrA subunit of the gyrase enzyme
KIST	Korea Institute of Science and Technology
LMICs	low and middle-income countries
LOS	Lipooligosaccharides
LPS	Lipopolysaccharides
MBC	Minimal bactericidal concentration
mCCDA	Modified charcoal cefoperazone deoxycholate agar
MDR	Multidrug-resistant
MEGA	Molecular evolutionary genetics analysis
MIC	Minimal inhibitory concentration
MLST	Multilocus sequence typing
NIMR	National Institute for Medical Research
NJ	Neighbor-joining
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field gel electrophoresis

RAPD	Random amplification of polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic Acid
ST	Sequence type
T3SS	Type III secretory system
TYGS	Type strain genome server
USA	United States of America
UV	Ultraviolet
WGS	Whole-genome sequencing
WHO	World Health Organization

CHAPTER ONE

General Introduction

1.1 Taxonomy and history of Campylobacter

The word "*Campylobacter*" originates from ancient Greek meaning "curved rod" referring to its shape (Tresse *et al.*, 2017). *Campylobacter* was first reported in 1886 by Theodor Escherich in the large intestine of diarrheic children who died of *Cholera infantum* (Ammar *et al.*, 2021). On 2nd February 1906, John McFadyean and co-worker (Stewart Stockman) isolated *Campylobacter* in samples from miscarrying ewes in the United Kingdom (Skirrow, 2006). In the following years, in the United States, an almost identical organism (named *Vibrio fetus*) was isolated from aborting cattle (Smith and Taylor, 1919) and it was considered as a rare, opportunistic, and invasive pathogen mainly affecting immuno-compromised people (Acheson and Allos, 2001). *Campylobacter* genus was introduced in 1973 by French workers who supported the microaerophilic nature of these vibrios constituting a distinct phylogenetic group (Véron and Chatelain, 1973).

Initially, *Campylobacter fetus* and *Campylobacter bubulus* previously called *Vibrio fetus* and *Vibrio bubulus* were the major constituents of the genus *Campylobacter* until further tests (genotyping, serological, and biochemical) were discovered which paved the way to the creation of the genus *Campylobacter* (Fonseca *et al.*, 2016; Tresse *et al.*, 2017). The first isolation of *Campylobacter* from stool used non-selective media through filtration (Dekeyser *et al.*, 1972). The development of a selective medium containing trimethoprim, polymyxin B, and vancomycin (Skirrow, 1977) paved the way to new species discovery.

Currently, the genus *Campylobacter* belongs to the phylum *Proteobacteria*, class *Epsilonproteobacteria,* order *Campylobacterales,* and the family *Campylobacteraceae* (Ammar *et al*., 2021). Of the 33 species, the predominant etiologies of human campylobacteriosis are first *C. jejuni*, and then, *C. coli* (Tresse *et al.*, 2017). However, other species like *C. fetus*, *Campylobacter hyointestinalis* subsp. *hyointestinalis*, *Campylobacter lari*, and *Campylobacter upsaliensis* have also been reported to cause human gastroenteritis and/or septicaemia (Yamazaki-Matsune *et al*., 2007). *Campylobacter concisus*, *Campylobacter ureolyticus*, *C. upsaliensis*, and *C. lari* are known as "emerging *Campylobacter* species," following advances in molecular techniques that led to their

understanding (Kaakoush *et al.*, 2015). Of veterinary importance, *C. fetus* and *C. jejuni* are known to cause abortions in ruminants (Mannering *et al*., 2004; Mshelia *et al*., 2010).

1.2 Biological features

Campylobacter spp. possess a flagellum involved in motility, and present a typical movement of corkscrew or darting Gram-negative bacteria, and they are non-spore-formers, spiral or curved bacteria (Fonseca *et al*., 2016; Zhang and Sahin, 2020). *Campylobacter* range in size from 0.5 to 5.0 μm in length by 0.2 to 0.9 μm in width while the predominant appearance is the gull-winged or S-shape (Ammar *et al*., 2021). Under stressful conditions, *Campylobacter* may form coccoidal forms that are viable but not culturable (Singh *et al*., 2011).

Campylobacter species are fastidious, slow growers which require a microaerophilic environment and a particular respiratory system for optimal growth as they are sensitive to high temperatures, low pH, oxygen, desiccation, and osmotic stress (Mshelia *et al*., 2010; Kaakoush *et al*., 2015; Zhang and Sahin, 2020). *Campylobacter concisus, C. rectus,* and *C. curvus* prefer an anaerobic environment for optimal growth (Kaakoush *et al*., 2015; Ammar *et al.*, 2021). Contrary, *C.gracilis*, *C. showae*, C. *rectus, C. mucosalis*, and *C. hyointestinalis* prefer hydrogen or formate as an electron donor for optimal growth (Kaakoush *et al*., 2015). Thermophilic *Campylobacter* species exclusively grow in the temperature range of 30-47^{o}C (Aroori *et al*., 2013; Ammar *et al*., 2021). The optimal growth temperature is 42 ^{o}C which is the chicken body temperature considered as the primary reservoir but *Campylobacter* grows also at 37 ^{o}C considered as the human body temperature (Aroori *et al.*, 2013).

Campylobacter spp. grow well between pH of 5.5 and 8.0 while pH values > 9 and <5 are lethal to them (Ammar *et al.*, 2021). *Campylobacter* is known as non-saccharolytic as it lacks the phosphofructokinase of the Embden-Meyerhof-Parnas (EMP) pathway and incomplete pentose phosphate (PPP) and Entner Doudoroff (ED) pathways (Parkhill *et al*., 2000; Fonseca *et al.*, 2016). *Campylobacter* uses amino acids and tricarboxylic acid cycle (TCA) intermediates as sources of energy instead of sugars (Ammar *et al.*, 2021).

Campylobacter spp. have small genomes (1.6–2.0 megabases). Sequencing the genome of *C. jejuni* has revealed the presence of hypervariable sequences with homopolymeric tracts,

found in genes encoding the biosynthesis or modification of surface structures like the capsule, lipooligosaccharides (LOS), or flagellum (Parkhill *et al.*, 2000; Fonseca *et al*., 2016).

Poultry is considered to be the primary source of *Campylobacter* but several other natural reservoirs have been reported (Ocejo *et al*., 2019). However, large farm animals (cattle, sheep, and pigs), and companion animals are also potential sources of *Campylobacter* infections (Epping *et al.*, 2019). The vehicles of *Campylobacter* from animals to humans include eating undercooked food, drinking unboiled milk or water (Igwaran and Okoh, 2020). Consequently, colonization of various reservoirs by *Campylobacter* is seen as a public health concern due to the shedding of the pathogen from farms in faeces and other wastes which later contaminate surface and sub-surface water sources (Oporto *et al*., 2007).

1.3 Factors required for a successful colonization

Campylobacter species possess virulence factors which contribute to their increased epidemiology compared to other members of the family *Enterobacteriaceae* (Bolton, 2015; Otigbu *et al*., 2018).

Adherence factors

Attachment is a prerequisite for infection (Castillo *et al*., 2017) where fibronectin-binding protein (CadF) facilitates the attachment of *Campylobacter* to the intestines via fibronectin (Castillo *et al.*, 2017; Otigbu *et al.*, 2018). Other factors involved in adhesion include periplasmic amino acid-binding proteins (Peb1, Peb2, Peb3, Peb4), *Campylobacter* adhesion to fibronectin (CapA), glutamine-binding protein (CjaA), fibronectin like protein A (FlpA), major outer membrane protein (MOMP), Fructose-1,6-bisphosphatase class 1 (FbpA), and surface-exposed lipoprotein (JlpA) (Konkel *et al*., 2010; Kovács *et al*., 2020). However, the process of adhesion is multifactorial as other factors like the capsules (CPS), lipooligosaccharides (LOS), and the unique O- or N-linked glycosylation systems play some roles (Kovács *et al.*, 2020).

Invasion factors

The *Campylobacter* invasion antigen (CiaB) is the main factor required for the exportation of other Cia proteins and *cia*B mutants were defective in colonizing chicken ceca (Fonseca *et al.*, 2016; Castillo *et al*., 2017). The process of invasion is also complex requiring

various flagella genes (*fla*A, *fla*B, *flg*B, *flg*E, and *fla*C) and the flagellar apparatus for the release of *cia*C and *cia*D proteins (Fonseca *et al*., 2016; Kovács *et al*., 2020).

Toxin production

Once internalized, *Campylobacter* is protected from the host's immunity until the environment is suitable for toxin production (Ammar *et al*., 2021). The Cdt complex (*cdt*A, *cdt*B, and *cdt*C) is involved in toxin production (Fonseca *et al*., 2016). The *cdt*B gene acts as the activator of the complex, and in the nucleus, it blocks the cell cycle inducing intestinal and immune cells' death (Jain *et al*., 2008). The other genes of cdt complex (A and C) encode proteins involved in attachment and invasion into the host (Fonseca *et al*., 2016). Literature showed that *cdt*B mutants exhibited a reduced host invasion beyond the gastrointestinal tract (Yamasaki *et al*., 2006).

Flagellin

Possession of flagella is an important virulence factor in *Campylobacter* spp as it is involved in chemotaxis, behavior, and survival in the intestinal tract by bacteria *(Wösten et al.*, 2004; Aroori *et al*., 2013). The three major components of the flagellum are (i) a basal body, (ii) a hook, and (iii) filament (Konkel *et al*., 2004). The FlaA considered as the main flagellin of *Campylobacter* is encoded by *fla*A genes while FlaB regarded as minor flagellin is encoded by *flaB* gene (Bolton, 2015; Fonseca *et al*., 2016).

Biofilm formation and quorum sensing

Campylobacter spp. have to withstand stressful environmental conditions through biofilm formation (Reuter *et al*., 2010). Biofilm is defined as a bacterial population enclosed in a self-produced matrix composed mainly of polysaccharides which helps them to adhere to various surfaces (Micciche *et al.*, 2019; Somrani *et al*., 2020). Once the biofilm is formed, it allows *C. jejuni* to withstand antimicrobials, host's immunity, and environmental stresses (Anja Klančnik *et al*., 2018; Shagieva *et al*., 2020), and thus becoming a threat to the food industry and human health (Srey *et al*., 2013). Generally, planktonic microorganisms are more affected by antimicrobials than are pathogens embedded in a biofilm (Somrani *et al*., 2020). The understanding of the *Campylobacter* biofilm formation process is not adequate, but the role of flagella genes (*fla*A, *fla*B, other minor components) and quorum sensing gene (*lux*S) in biofilm formation has been confirmed (García-Sánchez *et al*., 2019).

Quorum Sensing (QS), a form of communication among cells using signal molecules (autoinducers), controls biofilm formation according to cell density (Šimunović *et al*., 2020). In *C. jejuni*, *lux*S gene encodes for autoinducer (AI-2) which is crucial for motility, biofilm formation, invasion, host colonization, and virulence (Fonseca *et al.*, 2016; Šimunović *et al*., 2020).

Chemotaxis

Chemotaxis is the movement of bacteria mediated by flagella towards or away from certain stimuli (Reuter *et al*., 2020; Bolton, 2015). Methyl-accepting chemotaxis proteins (MCPs) and signal transduction pathways are the two main structures that depend on histidine kinase and consist of various proteins involved in chemotaxis like CheZ, CheY, CheW, CheR, CheB, and CheA (Bolton, 2015; Ammar *et al*., 2021). Some biomolecules like mucins and glycoproteins act as chemo-attractants of *Campylobacter* to intestines (Kreling *et al.*, 2020; Ammar *et al*., 2021). However, there is a long list of chemo-attractants belonging to protein and sugar groups (Bolton, 2015; Kreling *et al*., 2020; Ammar *et al*., 2021). Once any of the main genes regulating chemotaxis (*che*A, *che*Y, *che*V, or *che*W) is defective, the chemotactic motility and biofilm formation are impaired (Reuter *et al*., 2020).

Bile resistance

For successful colonization, *Campylobacter* needs to resist bile salts like cholates and deoxycholates (DOCs), which are bactericidal agents (Fonseca *et al*., 2016; Kreling *et al*., 2020). Bile acids kill bacteria by disrupting the cell membrane lipids and cytoplasmic proteins (Fonseca *et al*., 2016). The *cme*ABC operon encodes for the *Campylobacter* multidrug efflux pumps (CME) which allow *Campylobacter* to withstand bile salts and antimicrobials by actively pumping them out of the bacterial cells (Bolton, 2015; Fonseca *et al.*, 2016).

1.4 Isolation and identification

Although still used by some laboratories, biochemical tests seem to be outdated due to their limited reliability in identifying *Campylobacter* strains up to species level *(Fonseca et al.*, 2016). Furthermore, culture does not guarantee maximum recovery of some *Campylobacter* species due to their fastidious nature and vulnerability to temperature fluctuations (Ammar *et al.*, 2021). This is complicated by commonly used selective media

and added antimicrobials (cefoperazone, vancomycin, and cycloheximide) which limit the growth of certain *Campylobacter* species (Fonseca *et al*., 2016). Selective media commonly used were designed for the thermophilic group members that are vigorous growers when compared to emerging *Campylobacter* species (Kulkarni *et al.*, 2002; Fonseca *et al.*, 2016). However, the use of enrichment and non-selective media along with filtration (Cape Town Protocol) allows the isolation of emerging *Campylobacter* species (Fonseca *et al.*, 2016). Cape Town protocol proved to be more effective in *Campylobacter* isolation than Skirrow protocol (Jacob *et al*., 2011). Presumptive colonies (moistened, tendency to spread, grayish, and flat) are cultured onto Mueller Hinton or blood agar plates and the isolates are distinguished by Gram staining, motility, and biochemical techniques (AL-Edany *et al*., 2015; Ammar *et al*., 2021). Identification to species level by biochemical tests is difficult due to the particularity of some species like *C. jejuni* strains which cannot hydrolyze hippurate (Linton *et al*., 1997).

The integration of molecular techniques and suitable culture media in current diagnostic tests has helped in promoting the awareness of different *Campylobacter* species including the less commonly reported ones (Lastovica, 2016). The polymerase chain reaction (PCR) is suitable for prevalence studies and many species-specific PCR assays are available (Inglis and Kalischuk, 2003). However, the multiplex PCR has the advantage of detecting several species at once (Yamazaki-Matsune *et al.*, 2007). PCR and other molecular diagnostic tests based on nucleic acids are attractive due to their benefits including their higher sensitivity, ease of use, improved turnaround time, relatively low cost, and potential to be fully automated (Ghosh *et al*., 2014).

1.5 Typing methods

Typing helps in detecting the cross-transmission of nosocomial pathogens, source tracing, diagnosing virulent strains, and monitoring vaccination programs (Oline and Bean, 1999). The prevalent methods which can distinguish bacteria are phenotyping and genotyping (Eberle and Kiess, 2012). Typing is a fast and effective technique necessary for surveillance and monitoring programs (Wiedmann, 2002).

1.5.1 Phenotyping techniques

Phenotype-based typing relies on the presence or absence of a given phenotype normally expressed by the bacteria. The most known include biotyping, serotyping, and multilocus enzyme electrophoresis (Eberle and Kiess, 2012).

1.5.1.1 Biotyping

In biotyping, an isolate is identified by checking some metabolic properties like biochemical reactions, the morphology of colonies, and tolerances to environmental factors. The technique is simple and cheaper allowing rapid pathogen identification (Eberle and Kiess, 2012). Colonies on selective media are examined and suspected colonies are further analyzed (AL-Edany *et al*., 2015). Other tests include (i) direct microscopy to check motility and shape (spiral); (ii) Gram reaction (negative for *Campylobacter*), and (iii) oxidase production (positive) (Barros-Velázquez *et al*., 1999).

Biochemical tests for *Campylobacter* include catalase, oxidase, hippurate hydrolysis, indoxyl acetate hydrolysis, and hydrogen sulfide production (Fitzgerald *et al*., 2008). *C. jejuni* and *C. coli* are distinguished based on hippurate hydrolysis with *C. jejuni* being positive for the test while *C. coli* is negative (Eberle and Kiess, 2012; Fonseca *et al*., 2016). There is a long list of biochemical tests but they suffer from reduced discriminatory power and reproducibility associated with different expression levels for genes influenced by environmental factors. Therefore, biochemical tests are complemented by other phenotypic methods like serotyping (Eberle and Kiess, 2012).

1.5.1.2 Serotyping

In serotyping, bacteria are categorized based on surface antigens detected using antibodies and antisera (Wiedmann, 2002). The first serotyping method, composed of 42 antisera against *C. jejuni* and 18 antisera against *C. coli*, uses the hemagglutination of heat-stable (HS or O) antigens (Penner and Hennessy, 1980; Barros-Velázquez *et al*., 1999). Another scheme for *C. jejuni* detects heat-labile antigens that can be observed with live bacteria by slide agglutination due to the used antisera (Lior *et al*., 1982). Both methods were found to be typeable and reproducible but they are lengthy, expensive, and there exist untypeable strains (Eberle and Kiess, 2012). Both methods should be combined to obtain complementary information or be combined with the DNA-based methods (Barros-Velázquez *et al*., 1999; Eberle and Kiess, 2012).

1.5.1.3 Multilocus Enzyme Electrophoresis (MEE)

In MEE, the differentiation is based on the variation of relative mobilities under the electrophoresis of different intracellular enzymes (Wiedmann, 2002). The used enzymes differ in electrostatic charge, shape, and size, all of which affect migration rates across a gel (Eberle and Kiess, 2012). MEE may be difficult to standardize between laboratories due to low discriminatory power (Wiedmann, 2002; Eberle and Kiess, 2012).

1.5.*2* Genotyping techniques

They are more appropriate than phenotyping ones due to higher discriminatory power, reproducibility, and typeability (Wiedmann, 2002; Eberle and Kiess, 2012). There are two major categories: (i) macro-restriction mediated analyses based on separation of nucleic acids fragments after digestion with a restriction enzyme, and (ii) polymerase chain reaction (PCR)-based assays (Natsos *et al*., 2019).

1.5.2.1 Polymerase Chain Reaction (PCR)

PCR is a typing method that can distinguish *Campylobacter* to the species level and in different laboratories by amplifying target segments of DNA (Barros-Velázquez *et al*., 1999; Fonseca *et al*., 2016). The original PCR technique has been modified and currently, its variations include the multiplex PCR, reverse-transcriptase PCR, and quantitative real-time (QRT)-PCR (Eberle and Kiess, 2012). The multiplex PCR assays which detect various species of *Campylobacter* spp. have replaced the uniplex PCR that was used in the past (Yamazaki-Matsune *et al*., 2007).

1.5.2.2 FlaA-Restriction Fragment Length Polymorphism (FlaA-RFLP)

Flagellin typing is based on PCR amplification of flagellin genes, followed by restriction enzyme digestion to generate simple RFLP fingerprints (Harrington *et al*., 2003). FlaA typing is fast and known to have high discriminatory power but it is better to combine it with more suitable methods like multilocus sequence typing (MLST) for epidemiological studies (Eberle and Kiess, 2012; Natsos *et al*., 2019). PCR-RFLP analysis can be considered an effective genotyping tool in epidemiological investigations where financial resources are limited or in large-scale population surveillance (Ghorbanalizadgan *et al*., 2016).

1.5.2.3 Pulsed-field gel electrophoresis (PFGE)

PFGE refers to the electrophoretic conditions (changing the current polarity at regular intervals) used to separate large fragments of the DNA generated by digestion with a restriction enzyme which yields relatively few and larger fragments (Olive and Bean, 1999). The PFGE segregates heavy DNA fragments with high resolution, thus resulting in neat restriction profiles (Barros-Velázquez *et al*., 1999). However, a study in Chile reported that PFGE failed to distinguish *Campylobacter* isolates from broiler meat and those of human origin (González-Hein *et al*., 2013). Furthermore, PFGE is time-consuming as the time for completing the experiment may be 2 to 3 days (Olive and Bean, 1999).

1.5.2.4 Multilocus sequence typing (MLST)

MLST categorizes strains based on assessing the differences among seven housekeeping genes and it has been considered as the gold standard method for epidemiological surveillance (Dingle *et al*., 2001; Harrington *et al.*, 2003). MLST essentially mimics the MEE's multi loci principle (Natsos *et al*., 2019). In MLST, loci are given allelic numbers which are then grouped into allelic profiles known as sequence types (STs) in order of their discovery (Eberle and Kiess, 2012). The MLST is better suited for assessing the genetic relatedness among strains from various sources based on the availability of online databases like the PubMLST which allows comparative studies (Dingle *et al*., 2001; Natsos *et al*., 2019; Alaboudi *et al*., 2020). However, MLST does not include clinically important information, like the virulence or antibiotic resistance determinants, mobile genetic elements, nucleotide polymorphism, and other recombination events (Fiedoruk *et al*., 2019). The whole-genome sequencing (WGS) is expected to replace MLST as the gold standard typing method but hurdles related to the bioinformatics tools and expertise need to be first addressed (Duarte *et al*., 2016).

1.6 The use of natural products as alternative to conventional antibiotics

Campylobacter species have developed resistance to existing antimicrobials including drugs of choice like fluoroquinolones and macrolides (Hlashwayo *et al*., 2020). FQ-resistant *Campylobacter* strains have been categorized as one of the global priority pathogens requiring new drug development (Hlashwayo *et al.*, 2020). Herbal medicines and phytochemicals have been used in (i) treating various infections, (ii) food preservation, and/or (iii) drug development (Bahmani *et al.*, 2015; Wagle *et al*., 2019;

Ullah *et al.*, 2020). Various natural products have been tested for their anti-*Campylobacter* potentials (Klančnik *et al.*, 2018; Wagle *et al.*, 2019).

Plant extracts have been used in treating campylobacteriosis. The extract from *Alpinia katsumadai* was effective against both drug-sensitive and resistant strains of *Campylobacter* (Klančnik *et al*., 2012). Other plant extracts like *Mentha canadensis*, *Artemisia ludoviciana* Nutt., and *Acacia farnesiana* (L.) Willd L. used in treating human campylobacteriosis have been extensively reported (Thawkar, 2016; Ullah *et al*., 2020). In Africa, a review on plant extracts with anti-*Campylobacter* activities listed *Cryptolepis sanguinolenta*, *Terminalia macroptera*, and *Combretum woodii* as the top three species (Hlashwayo *et al.*, 2020).

Essential oils (EOs), volatile compounds protecting plants from diseases and insect attacks, have been used by humans for therapeutic and medicinal purposes (Kurekci *et al*., 2013). To date, more than 300 anti-*Campylobacter* phytochemicals can be purchased and have proved to reduce biofilm formation and *Campylobacter* load in chicken (Hassan *et al*., 2019; Micciche *et al*., 2019). Clove oil and its primary compound (eugenol) have been used to control *Campylobacter* species (Kovács *et al*., 2016; Hassan *et al.*, 2019). Other essential oils like cardamom and cumin have been tested for their anti-*Campylobacter* activities (Mutlu-Ingok and Karbancioglu-Guler, 2017). The roles of thymol, carvacrol, cinnamaldehyde in reducing *Campylobacter* concentrations have been described (Hassan *et al*., 2019; Micciche *et al.*, 2019). Further research is needed to decipher modes of action and identify appropriate infection models for *in-vivo* studies (Oh *et al*., 2017; Hlashwayo *et al*., 2020).

1.7 Problem statement and justification of the study

Campylobacter species are known as the main etiologies of human gastroenteritis (Kaakoush *et al*., 2015) and various reservoirs have been implicated (Oporto *et al.*, 2007) with poultry being the primary reservoir (Navarro *et al*., 2015). In Tanzania, the prevalence of *Campylobacter* from humans ranges between 11.4% (Komba *et al*., 2015) and 21.6% (Jacob *et al*., 2011) while the prevalence in cattle was 9.5% (Kashoma *et al*., 2016). In South Korea, human campylobacteriosis has been increasing and this was partly attributed to an escalation in chicken consumption (Wei *et al*., 2014). Thus, controlling *Campylobacter* in poultry would help reducing human campylobacteriosis incidence

(Navarro *et al*., 2015). Worldwide, research has focused on *Campylobacter* species from broilers with limited data on layers (Kassem *et al*., 2016). There is an increasing resistance of *Campylobacter* strains to existing treatment options including drugs of choice (Komba *et al*., 2015) often due to excessive use of antimicrobials in livestock production (Sproston *et al*., 2018). Following an increased reports of AMR *Campylobacter* strains, natural products are being considered as alternative sources of effective antimicrobial agents (Možina *et al*., 2018). Molecular typing helps in understanding the epidemiology of *Campylobacter* through source attribution and characterizing strains involved in human infections (Eberle and Kiess, 2012).

In South Korea and Tanzania, there is a dearth of information on the epidemiology of AMR pathogens including *Campylobacter*. Also, data on molecular detection of *Campylobacter* species are scarce in both countries. Globally, epidemiological studies have focused on broiler chicken but studies on layers are scanty. Furthermore, studies on finding alternative treatments and on whole-genome sequencing related to *Campylobacter* are rare in South Korea and Tanzania. Therefore, the main objective of this research was to assess the molecular epidemiology and antimicrobial susceptibility profiles of thermophilic *Campylobacter* species from human and animal feces in South Korea and Tanzania .

1.8 Research Questions

i. What are the antimicrobial susceptibility profiles, virulence genes, and genetic diversity of thermophilic *Campylobacter* species in layer chicken in South Korea?
ii. What are the susceptibilities of layer chicken-derived and reference *Campylobacter* strains with regards to natural products and frontline antibiotics?
iii. What special features can the whole-genome sequencing of thermophilic *Campylobacter* strains from layer chicken in South Korea reveal?
iv. What is the performance of molecular techniques in detecting *Campylobacter* species?

1.9 Study Objectives

1.9.1 Overall objective

The overall objective of this study was to assess the molecular epidemiology and antimicrobial susceptibility profiles of thermophilic *Campylobacter* species from human and animal faeces in South Korea and Tanzania.

1.9.2 Specific objectives

i. To determine the antimicrobial susceptibility profiles, virulence genes, and genetic diversity of thermophilic *Campylobacter* species isolated from layers farm in Korea.

ii. To determine the susceptibility of layer chicken derived and reference *Campylobacter* strains to selected natural products and frontline antibiotics.

iii. To genomically characterize fluoroquinolone-resistant thermophilic *Campylobacter* strains from layer chicken faeces in Gangneung, Korea by whole-genome sequencing.

iv. To estimate the detection rates of *Campylobacter* species from human stool and cattle faecal samples in Kilosa district, Tanzania using molecular techniques.

CHAPTER TWO

Paper One

Prevalence, risk factors and antimicrobial resistance profiles of thermophilic *Campylobacter* species in humans and animals in Sub-Saharan Africa: a systematic review

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Review Article

Prevalence, Risk Factors, and Antimicrobial Resistance Profiles of Thermophilic *Campylobacter* Species in Humans and Animals in Sub-Saharan Africa: A Systematic Review

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Thermophilic *Campylobacter* species are clinically important aetiologies of gastroenteritis in humans throughout the world. The colonization of different animal reservoirs by *Campylobacter* poses an important risk for humans through shedding of the pathogen in livestock waste and contamination of water sources, environment, and food. A review of published articles was conducted to obtain information on the prevalence and antimicrobial resistance (AMR) profiles of thermophilic *Campylobacter* species in humans and animals in sub-Saharan Africa (SSA). Electronic databases, namely, PubMed, Google Scholar, Research4life-HINARI Health, and Researchgate.net, were searched using the following search terms "thermophilic *Campylobacter*," "*Campylobacter jejuni*," "*Campylobacter coli*," "diarrhea/diarrhoea," "antimicrobial resistance," "antibiotic resistance," "humans," "animals," "Sub-Saharan Africa," and "a specific country name." Initially, a total of 614 articles were identified, and the lists of references were screened in which 22 more articles were identified. After screening, 33 articles on humans and 34 on animals and animal products were included in this review. In humans, Nigeria reported the highest prevalence (62.7%), followed by Malawi (21%) and South Africa (20.3%). For *Campylobacter* infections in under-five children, Kenya reported 16.4%, followed by Rwanda (15.5%) and Ethiopia (14.5%). The country-level mean prevalence in all ages and under-five children was 18.6% and 9.4%, respectively. The prevalence ranged from 1.7%–62.7% in humans and 1.2%–80% in animals. The most reported species were *C. jejuni* and *C. coli*. The AMR to commonly used antimicrobials ranged from 0–100% in both humans and animals. Poultry consumption and drinking surface water were the main risk factors for campylobacteriosis. The present review provides evidence of thermophilic *Campylobacter* occurrence in humans and animals and high levels of AMR in SSA, emphasizing the need for strengthening both national and regional multisectoral antimicrobial resistance standard surveillance protocols to curb both the campylobacteriosis burden and increase of antimicrobial resistance in the region.

1. Introduction

Diarrhoea remains the main cause of morbidity and mortality in low- and middle-income countries (LMICs) [1–3]. Worldwide, under-five children experience approximately 1.4 billion episodes of diarrhoea each year, with several medical checks, hospitalizations, and around two million deaths. Over 78% of diarrhoea cases are found in the LMICs [4]. The burden of diarrhoeal diseases is complicated by the lack of appropriate case management [5], limited ability to detect the aetiologies [6], and antimicrobial resistance [7].

The most common aetiologies of diarrhoea include bacteria such as *Escherichia coli, Vibrio cholerae*, *Campylobacter jejuni*, *Salmonella* spp., *Aeromonas* spp., and

Yersinia enterocolitica; viruses mainly rotavirus, norovirus, sapovirus, and adenovirus; and protozoa largely *Entamoeba histolytica*, *Giardia* spp., and *Cryptosporidium* spp. [8, 9]. Of the bacterial aetiologies, *Campylobacter* is a leading cause of gastroenteritis in both high-, middle-, and low- income countries, responsible for 400–500 million cases of diarrhoea each year [10]. The clinically important *Campylobacter* species are *C. jejuni* and *C. coli*, which are responsible for about 98% of all human *Campylobacter* gastroenteritis cases [11, 12].

In most cases, campylobacteriosis does not require any antimicrobial therapy except in severe cases, especially in immune-deficient or immune-suppressed individuals [13, 14]. The recommended drugs are macrolides (mostly erythromycin), fluoroquinolones (mainly ciprofloxacin), and tetracycline [10, 15, 16]. Nevertheless, there is an escalating number of *Campylobacter* isolates resistant to these drugs [17, 18] due to the immeasurable and misuse of antimicrobials [19], not only in animals but also in humans [20]. Several factors have been associated with occurrence of *Campylobacter* infections. They include consumption of different food items like undercooked poultry meat and pork, red meat at barbecue, grapes, and drinking unpasteurized milk, having a chronic illness [21–23], drinking contaminated water, type of water source, animal contact, young age, eating prepared salad, latrine usage, bottle feeding, and nutritional status [24–26]. There is a wide range of natural reservoirs for *Campylobacter* including chicken and other poultry, wild birds, pigs, dogs, cats, sheep, and cows [27, 28]. Consequently, colonization of different reservoirs by *Campylobacter* poses an important risk for humans through shedding of the pathogen in livestock waste and water sources contamination, environment, and food [29, 30].

In LMICs, studies on thermophilic *Campylobacter* species are few due to limited capacity in laboratory diagnosis [31] and lack of surveillance of enteric diseases [32]. The objective of this review was to gather information on the prevalence, risk factors, and antimicrobial resistance profiles of thermophilic *Campylobacter* species in humans and animals in SSA. The findings of this review are expected to provide evidence for policy formulation, prevention, and control of *Campylobacter* infections and increase awareness of the AMR issue.

2. Methods

The data were collected by searching articles published in English from electronic databases, namely, PubMed, Google Scholar, Research4life-HINARI Health, and Researchgate.net. The search terms were "thermophilic *Campylobacter*," "*Campylobacter jejuni*," "*Campylobacter coli*," "diarrhea/diarrhoea," "antimicrobial resistance," "antibiotic resistance," "humans," "animals," "Sub-Saharan Africa," and "a specific country name." Initially, a total of 614 articles were identified, and the lists of references were screened in which 22 more articles were identified. After screening, 33 articles on humans and 34 on animals and animal products were included in this review (Figure 1). The reviewed articles were those published from 1997 to 2018. During the review process, the data extracted included title, country, sex and age distribution, sample size, isolation and identification methods, isolation rates, and antimicrobial resistance profiles. Articles for which the sample size was not shown or which used archived *Campylobacter* cultures were excluded from this review.

3. Results

3.1. Campylobacter Infections in Humans. Of the 47 SSA countries [33], data on human campylobacteriosis were available from 15 (31.9%) countries. The prevalence of thermophilic *Campylobacter* in humans was reported in 33 articles (Table 1). Nigeria reported the highest overall prevalence of thermophilic *Campylobacter* (62.7%); followed by Malawi (21%) and South Africa (20.3%). Kenya reported the highest prevalence (16.4%) of *Campylobacter* infections in under-five children; followed by Rwanda (15.5%) and Ethiopia (14.5%). The mean prevalence in all ages and under-five children was 18.6% and 9.4%, respectively. Burkina Faso and Mozambique had the lowest prevalence of campylobacteriosis for all ages (2.3%) and under-five (1.7%), respectively. Of the 33 articles reviewed, 16 (48.5%) presented data on distribution of *Campylobacter* infections by sex but the difference was not statistically significant. Of these 16 articles, campylobacteriosis was more prevalent among males (22.7%; $n = 3966$) than females (17.7%; $n = 3705$). Culture methods on selective media, biochemical tests, molecular, and biotyping techniques were used for identification of *Campylobacter* (Table 1). Of the 33 articles, 27 studies were carried out at clinical settings (hospitals and health centres) while 6 were community-based studies. Probability sampling methods were adopted in 5 articles while the remaining used convenience sampling. Although *C. jejuni* and *C. coli* were isolated in the mentioned articles, 15 articles reported other enteric pathogens as probable aetiologies of diarrhoea. Furthermore, more than 85% of the articles considered diarrhoeic cases while the remaining included even asymptomatic participants.

Of the 33 articles, only four reported on risk factors of campylobacteriosis in humans. In Tanzania, *Campylobacter* infections were associated with sex, young age, poultry meat consumption, and eating of salads [26, 38]. In Ethiopia, human campylobacteriosis was significantly associated with nonuse of latrines, water source, drinking unboiled water, bottle feeding, nutritional status, and exposure to domestic animals including cats, dogs, poultry, and pigeons [25]. In Burkina Faso, *Campylobacter* infections were most common among under-fives and those aged 21–40 years with more pet contacts [57].

3.2. Campylobacter spp. in Animals and Contamination of Animal Products. Of the 34 articles from which data on animals were extracted, 17 collected faeces from live animals, while 16 collected samples from meat or caeca at abattoirs. In 2 articles, samples were collected from both markets and

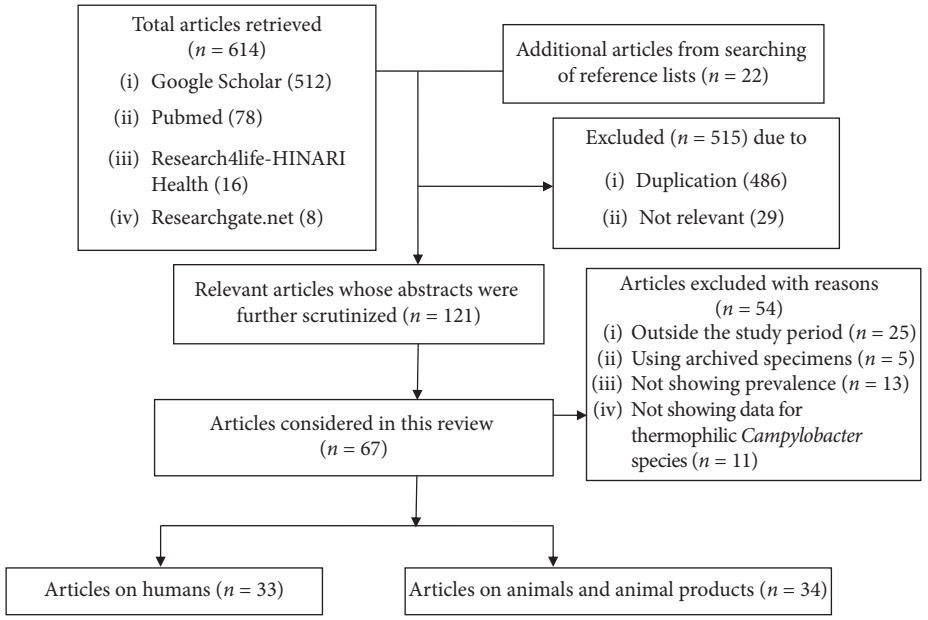

Figure 1: Flowchart showing article selection process.

Table 1: Prevalence of thermophilic *Campylobacter* spp. in humans in sub-Saharan Africa, 1997–2018.

Country	Age group (sample size)	Number of articles	Prevalence (%)	Detection method	References
Uganda	Children <5 (226)	1	9.3 (*C. jejuni*: 80.9%; *C. coli*: 4.8%)	Culture, biochemical	[34]
Tanzania	Children <5 (1,512)	5	8.8 (2.6–19) (*C. jejuni*: 89.2%; *C. coli*: 9.8%)	Culture, biochemical, Gram staining, molecular	[8, 35–38]
Kenya	Children <5 (2,550)	1	16.4	Culture, biochemical, serotyping	[39]
Rwanda	Children <5 (706)	1	15.5 (*C. jejuni*: 100%)	Molecular	[40]
Madagascar	Children <5 (5,620)	2	9.4 (9.3–9.5) (*C. jejuni*: 73.6; *C. coli*: 24.3%)	Culture, serotyping, molecular	[41, 42]
Burkina Faso	Children <5 (283)	1	2 (*C. jejuni*: 60%; *C. coli*: 40%)	Culture, molecular	[43]
Ethiopia	Children <5 (670)	2	14.5 (12.7–16.7) (*C. jejuni:* 71.1%; *C. coli*: 21.1%)	Culture, biochemical, Gram staining	[25, 44, 45]
Nigeria	Children <5 (1,311)	3	4.4 (0.5–8.2) (*C. jejuni*: 28%; *C. coli*: 72%)	Culture, biochemical, biotyping, Gram staining	[46–48]
Niger	Children <5 (260)	1	11.4 (*C. jejuni*: 100%)	Culture, biochemical, Gram staining	[49]
Mozambique	Children <5 (529)	1	1.7	Culture, biochemical, Gram staining	[50]
Cameroon	Children <5 (260)	1	9.6 (*C. jejuni*: 100%)	Culture, biochemical, Gram staining	[51]
Botswana	Under 15 years	1	14	Molecular	
Tanzania	All ages (2,487)	4	11.1 (1.9–21.6) (*C. jejuni*: 93.3%; *C. coli*: 6.1%)	Culture, biochemical, Gram staining, molecular	[26, 52–54]
Kenya	All ages (4,274)	2	9.2 (8.5–9.8) (*C. jejuni*: 76.2; *C. coli*: 12.7%)	Culture	[55, 56]
Burkina Faso	All ages (1,246)	1	2.3 (*C. jejuni*: 51.8%; *C. coli*: 13.8%)	Culture, biochemical, Gram staining	[57]
Ethiopia	All ages (640)	2	9.8 (8–11.6) (*C. jejuni*:94.1%; *C. coli*: 5.9%)	Culture, biochemical, Gram staining	[58, 59]
Nigeria	All ages (150)	1	62.7 (*C. jejuni*: 24.5%; *C. coli*: 62.3%)	Culture, biochemical, Gram staining	[60]
Ghana	All ages (202)	1	17.3 (*C. jejuni*: 42.8%; *C. coli*: 37%)	Culture, biochemical, Gram staining	[61]
Malawi	All ages (1,941)	1	21 (*C. jejuni*: 85%; *C. coli*: 14%)	Molecular	[62]
South Africa	All ages (565)	1	20.3 (*C. jejuni*: 85%; *C. coli*: 15%)	Culture, biochemical, molecular	[63]

abattoirs. Probability sampling methods were used in 6 articles while the remaining used convenience sampling.

Data on *Campylobacter* in cattle were obtained from ten articles published from studies conducted in six countries. The overall mean prevalence was 17.6% and *C. jejuni* had higher prevalence (70%) than *C. coli* (23.5%). The highest [64] and the lowest overall prevalence [52] were reported from Tanzania. Furthermore, Tanzania and Ghana showed higher prevalence for *C. jejuni* and *C. coli*, respectively (Table 2).

Data on *Campylobacter* in goats were reported in three articles from three different countries. The overall mean prevalence was 31.2%, and *C. jejuni* presented with a higher prevalence (56.2%) than *C. coli* (38.5%). The highest and lowest prevalence were reported from the Democratic Republic of Congo (DRC) [83] and Ghana [83], respectively. Ethiopia [70] and DRC [83] had the highest frequencies for *C. jejuni* and *C. coli*, respectively (Table 2). For sheep, data were reported in four articles from three countries. The overall mean prevalence was 31.8%, with *C. jejuni* being reported at a higher frequency (56.7%) than *C. coli* (35.4%). The highest and lowest prevalence were reported from Ethiopia [70] and Ghana [70], respectively. Ethiopia [70] and Tanzania [75] had the highest prevalence for C. *jejuni* and C. *coli*, respectively (Table 2).

Data on presence of thermophilic *Campylobacter* in pigs were available from six articles from five countries. The overall mean prevalence was 45.5% and contrary to other animals, *C. coli* occurred at a higher prevalence (70.1%) than *C. jejuni* (27.2%). The highest and lowest prevalence were reported from Nigeria [60] and South Africa [78], respectively. Ethiopia [69] had both higher and lower values for *C. jejuni* and *C. coli* (Table 2).

Data on thermophilic *Campylobacter* in chickens were obtained from 11 articles from five different countries. In this review, the number of articles on chickens was the highest compared to other reservoirs. The overall mean prevalence was 62.6% which was the highest in all animal reservoirs documented in this review. *Campylobacter jejuni* was reported in higher prevalence (81.0%) than *C. coli* (18.1%). The highest and lowest prevalence rates were reported in Ethiopia [70] and South Africa [18], respectively (Table 2).

As regards to animal products, data on cattle meat were reported in three articles from three countries. The overall mean prevalence was 5.5%, and *C. jejuni* had higher prevalence (95.2%) than *C. coli* (4.8%). The highest and lowest prevalence rates were reported in Ethiopia [72] and Kenya [73], respectively. For cattle carcasses, data were reported by two articles from two countries with a mean prevalence of 15.9%. Ghana [68] reported a higher prevalence of *C. jejuni* while Tanzania [74] observed a higher prevalence of *C. coli* (Table 2).

Data on sheep meat were reported by a single article from Ethiopia [72] with the prevalence of 10.5%. In sheep carcasses, the mean prevalence was 23.3% computed using two articles from two countries. Ghana [68] showed a higher prevalence of *C. jejuni* while Ethiopia [76] reported a higher prevalence of *C. coli*. In pork, the prevalence was 8.5% in one article from Ethiopia [72] with *C. coli* being more prevalent than *C. jejuni*. In pig carcasses, the prevalence was 36.3% from one article reporting a study carried out in Ghana [68]. In chicken meat, the mean prevalence was 49.4% reported by two articles from two countries. Dadi and Asrat in a study conducted in Ethiopia [72] indicated a higher prevalence for *C. jejuni* while a study in Kenya [73] found a higher prevalence for *C. coli*. For chicken carcasses, the prevalence was 50% from one article in Burkina Faso [79] and all isolates were *C. jejuni*. In goat meat, the mean prevalence was 22.5% reported by only one article from Ethiopia [72]. In goat carcasses, the mean prevalence was 16.7% reported by two articles from two countries. A study conducted in Ghana [76] reported a higher prevalence for *C. jejuni* while that in Ethiopia [76] found a higher prevalence for *C. coli* (Table 2).

The overall prevalence of thermophilic *Campylobacter* in cats [84] and dogs [84, 85] were 18.3% and 20%, respectively. Of the reviewed articles, some presented data on companion, wild, and other animals (Table 3).

3.3. Antimicrobial Resistance Profiles of C. jejuni and C. coli in Humans and Animals. In humans, the AMR profiles, determined using disk diffusion, were available in 4 articles from four different countries (Figure 2), while the remaining did not specify the species. The antimicrobials considered in this review for the ease of comparison were ampicillin (AMP), erythromycin (ERY), tetracycline (TET), cefalotin (CF), nalidixic acid (NAL), azithromycin (AZM), gentamicin (GEN), ciprofloxacin (CIP), chloramphenicol (CHL), and trimethoprim-sulfamethoxazole (TM-SFX).

The percentage of antimicrobial resistant isolates ranged from 2–100% for *C. jejuni* and 0–100% for *C. coli*. The AMR data for CIP and ERY, which are drugs of choice for treating *Campylobacter* infections, showed that Ghana [61] and Tanzania [26] reported higher values for both *C. jejuni* and *C. coli*. Resistance of *Campylobacter jejuni* to GEN was similar for both Tanzania and Ghana while for *C. coli*, it was higher in Tanzania compared eith that of Ghana [26, 61]. Higher frequencies of resistance were also reported for TET and AMP which have been in use for many years. In general, higher levels of AMR were reported in *C. jejuni* than *C. coli*.

In animal and animal products, the following antimicrobials were used in the reviewed articles: chloramphenicol (CHL), ampicillin (AMP), erythromycin (ERY), ciprofloxacin (CIP), nalidixic acid (NAL), streptomycin (STR), tetracycline (TET), gentamicin (GEN), and trimethoprim-sulfamethoxazole (TM-SFX) (Figure 3).

In animals, the percentage of resistant isolates varied from 0–100%. Resistance to CIP was in the range of 0–80.5% and 0–68.8% for *C. jejuni* and *C. coli*, respectively. Resistance to ERY varied from 0–99.5% and 0–100% for *C. jejuni* and *C. coli*, respectively. Resistance to GEN was <55.6% for both *C. jejuni* and *C. coli*. The highest resistance to most of the drugs was seen in Ghana [68] while the lowest resistance was observed in Tanzania [74, 89]. Resistance to nalidixic acid was high for both *C. jejuni* and *C. coli* in a study conducted in Tanzania [75]. Data on multidrug resistance were available from three studies in which values ranged from 23.3% to

Table 2: Prevalence of *Campylobacter* spp. in domestic animals and animal products.

Animal type	Sample type	Country	Overall prevalence	*C. jejuni* (%)	*C. coli* (%)	References
Cattle	Faeces	South Africa	19.3	72.4	27.6	[18]
		Nigeria	18.5	80	20	[65]
			12.9	65.1	23	[66]
		Tanzania	2.3	100	0	[52]
			5.6	83.3	16.7	[67]
			32.5	65.5	27.3	[64]
		Ghana	13.2	25	43.8	[68]
		Ethiopia	12.7	53.8	38.5	[69]
			48	75.3	17.6	[70]
		Mozambique	11	80	20	[71]
Average			**17.6**	**70**	**23.5**	
Cattle	Meat	Tanzania	2.8	100	0	[67]
		Ethiopia	6.2	85.7	14.3	[72]
		Kenya	2	100	0	[73]
Average			**5.5**	**95.2**	**4.8**	
Cattle	Carcasses	Tanzania	3.7	75	25	[67]
			9.5	62.5	29.2	[74]
		Ghana	34.5	84.2	13.1	[68]
Average			**15.9**	**73.9**	**22.4**	
Sheep	Faeces	Tanzania	31.6	55.6	44.4	[75]
		Ethiopia	38	59.3	40.7	[69]
			39	84.6	15.4	[70]
		Ghana	18.6	27.2	40.9	[68]
Average			**31.8**	**56.7**	**35.4**	
Sheep	Carcasses	Ethiopia	10.6	73.9	26.1	[76]
		Ghana	35.9	92.8	0	[68]
Average			**23.3**	**83.4**	**13.1**	
Sheep	Meat	Ethiopia	10.5	83.3	0	[72]
		Nigeria	92.7	14	78.7	[60]
Pig	Faeces	Ethiopia	50	0	100	[69]
		Tanzania	66.7	81.8	18.2	[77]
			32.5	2.7	91.9	[64]
		Ghana	28.7	48.2	48.2	[68]
		South Africa	2.3	16.7	83.3	[78]
Average			**45.5**	**27.2**	**70.1**	
Pig	Carcasses	Ghana	36.3	28.4	10.8	[68]
Pig	Pork	Ethiopia	8.5	25	50	[72]
		Burkina Faso	68	70	30	[79]
Chicken	Faeces	Tanzania	69.8	91.2	8.8	[53]
			42.5	87.1	12.9	[38]
			77.8	91.1	7.3	[54]
		South Africa	35.3	84.9	15.1	[18]
			49.7	100	0	[80]
			54.8	54.8	40.2	[81]
		Ethiopia	72.7	92.5	7.5	[59]
			68.1	80.8	16.2	[69]
			86.6	86.9	11.9	[70]
		Ivory Coast	63.8	51.3	48.7	[82]
Average			**62.6**	**81**	**18.1**	
Chicken	Colon	South Africa	14.2	68.8	31.2	[78]
Chicken	Carcasses	Burkina Faso	50	100	0	[79]
Chicken	Meat	Ethiopia	21.7	84	8	[72]
		Kenya	77	59	39	[73]
Average			**49.4**	**71.5**	**23.5**	
Goat	Faeces	DRC	41.7	32.7	59.4	[83]
		Ghana	18.5	36	56	[68]
		Ethiopia	33.3	100	0	[70]
Average			**31.2**	**56.2**	**38.5**	

Table 2: Continued.

Animal type	Sample type	Country	Overall prevalence	*C. jejuni* (%)	*C. coli* (%)	References
Goat	Carcasses	Ethiopia	9.4	70.6	29.4	[76]
		Ghana	23.9	81.3	0	[68]
Average			**16.7**	**76**	**14.7**	
Goat	Meat	DRC	37.3	21.3	74.7	[83]
		Ethiopia	7.6	71.4	28.6	[72]
Average			**22.5**	**46.4**	**51.7**	
Cattle	Milk	Tanzania	13.4	55.3	31.6	[74]

Table 3: Prevalence of *Campylobacter* spp. in companion, wild, and other animals.

Animal type	Specimen	Country	Overall prevalence	*C. jejuni* (%)	*C. coli* (%)	References
Companion animals						
Cat	Faeces	Nigeria	18.3	21.1		[66]
Dog	Faeces	Nigeria	27.7	23.1	0	[66]
			12.3	53.8	30.8	[85]
		Average	**20**	**38.5**	**15.4**	
Other animals						
Crow	Faeces	Tanzania	72.8	93.8	6.2	[53]
Duck	Faeces	Tanzania	80	81.5		[86]
Greater crested tern	Faeces	South Africa	16	15	1	[87]
Kelp gull	Faeces	South Africa	12.4	11.6	0.8	[87]
Quail	Caeca	Nigeria	31.1	81	19	[88]
Horse	Faeces	Tanzania	60	66.7	33.3	[75]
Guinea pig	Faeces	Tanzania	26.7	50	50	[75]
Rat	Faeces	Tanzania	1.2	66.7	33.3	[75]

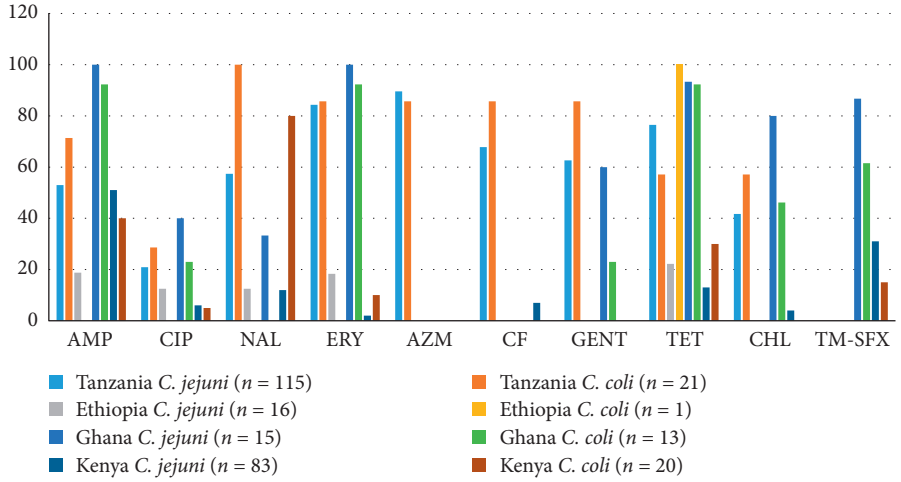

Figure 2: Antimicrobial resistance data in humans by the disk diffusion method.

63.3% for *C. jejuni* [18, 59, 74] and from 0–25% for *C. coli* [59, 70]. There were variations in resistance levels to commonly used antimicrobials in animal species depending on the species tested.

4. Discussion

The overall mean prevalence of thermophilic *Campylobacter* in humans ranged from 9.6–18.5% and is within the ranges reported elsewhere in LMICs [31] and in Poland [90]. However, the prevalence was higher than that reported from Korea [91], and was lower than that reported from the USA [92]. This variation may be attributed to the fact that campylobacteriosis is hyperendemic in LMICs probably due to poor sanitation and close proximity of humans and domestic animals [31]. The risk factors for human infections highlighted in this review partly explain this. They include consumption of poultry meat, drinking surface water, and animal contact, which is in agreement with other studies with consumption of poultry being the major risk factor [24, 93].

The prevalence of thermophilic *Campylobacter* in animals varied between 1.2% and 80%. The mean prevalence

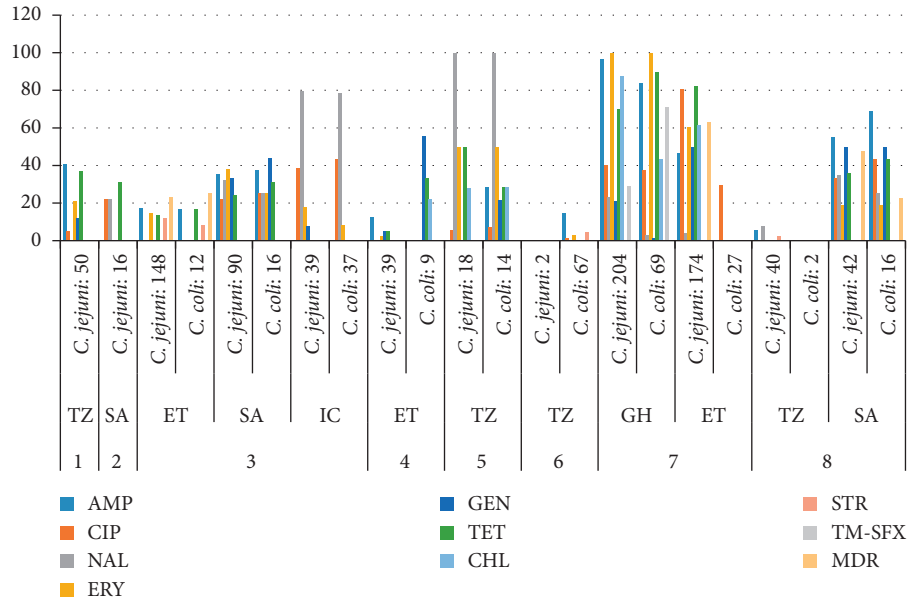

Figure 3: Antimicrobial resistance data in animals by the disk diffusion method. 1: duck, 2: sea birds, 3: chicken, 4: raw meat, 5: laboratory and farm animals, 6: pig, 7: food animals, 8: cattle; TZ: Tanzania, SA: South Africa, ET: Ethiopia, IC: Ivory Coast, GH: Ghana.

recorded in chickens (60.3%) concurs with findings from other LMICs such as Thailand [94], Sri Lanka [95], and Vietnam [96]. The mean prevalence of thermophilic *Campylobacter* in pigs was comparable to what was reported in Spain and Vietnam [30, 96] but lower than those reported in Norway and the Netherlands [97, 98]. The prevalence of *Campylobacter* in goats and sheep was slightly higher than the prevalence reported in Germany and Trinidad [99, 100] but lower than the prevalence reported in Spain [30]. The prevalence in cattle (17.6%) was lower than those reported in the USA and Iran [101, 102] but higher than the prevalence reported in another paper in the USA [103].

Although thermophilic *Campylobacter* species are frequently isolated from animal faeces, this review showed that they are also present in considerable amounts in a number of animal products. The reported prevalence of *Campylobacter* in cattle and goat carcasses in sub-Saharan Africa was higher compared to the prevalence in Poland for cattle [104] and in Canada for goat [105]. The contamination of carcasses may result from contact with gut contents during manual skin removal, cleaning, and processing in the slaughter house [106]. The prevalence rates in beef, pork, and mutton were slightly higher compared to those observed in other countries [107–109]. The variation could be influenced by the differences in husbandry practices which determine exposure of the animals to the bacteria. Partly, this could also be attributable to slaughter and animal product handling practices which enhance the contamination of the products.

Campylobacter jejuni and *C. coli* were the most frequently encountered species from both human and animals. Similar observations have been reported by other authors [30, 110]. The predominance of *C. jejuni* in various animals, other than pigs, in sub-Saharan Africa has been previously reported [31, 111]. The possible explanation is that most of the studies rely on culture and biochemical tests which may not correctly identify some species. Another reason is the use of selective media containing antibiotics to which some other *Campylobacter* species are sensitive to. Furthermore, higher incubation temperatures may limit the growth of some thermophilic *Campylobacter* species like *C. lari* and *C. upsaliensis* [112, 113].

In pigs, *C. coli* showed higher prevalence (67.4%) than *C. jejuni* (27.2%) which is in agreement with reports in Canada and the USA that *C. coli* is a normal flora of pigs' intestines [114, 115]. Furthermore, some studies show that *C. jejuni* and *C. coli* may cohabit in pigs but usually *C. jejuni* is always present in lower frequencies than *C. coli* [116, 117].

The results on AMR in both humans and animals highlight that resistance to mostly used antimicrobials is frequent. The resistance ranged from 0 to 100%, and higher resistance rates were reported in *C. jejuni* than in *C. coli*. The antimicrobials to which resistance was high included AMP, TET, ERY, and TET. The findings concur with the reports from other studies in both LMICs and high-income countries showing an increment in the number of *Campylobacter* strains resistant to most of the antimicrobials used in treating human campylobacteriosis [118–120]. The increase in resistance to most antimicrobial agents and emergence of MDR isolates could be associated with extensive use of antimicrobials not only as therapeutic agents for human infections [20] but also for prophylaxis and growth promotion in animal husbandry [68]. However, there are challenges in surveillance, differences in design and predominance of the disk diffusion method and not using globally accepted methods. These may cause differences within and between countries and certainly limit comparability with data reported in other parts of the world. The resistance to TET was comparable with the findings reported from Poland [121] and the USA [122] and the pooled estimate prevalence worldwide (94.3%) [120]. This resistance

may be due to wide use of tetracycline in both human and veterinary medicine [20]. The proportion of isolates resistant to macrolides (ERY) ranged from 0 to 100% in both humans and animals for *C. jejuni* while the range was from 0 to 92.3% for *C. coli*. The frequency of isolates resistant to fluoroquinolone was relatively lower in humans which is comparable to rates described in Western Europe [118, 121]. The resistance to both erythromycin and ciprofloxacin is of public health concern as there are currently limited options in the choice of treatment of *Campylobacter* infections. The proportion of multidrug resistance (MDR) isolates varied between 23.3 and 63.3% (Figure 3) which falls within the range of 37–90% from studies in China, Korea, and France [123–125].

There are no internationally agreed criteria of susceptibility testing and breakpoint assessment for *Campylobacter* spp. [126]. Therefore, it is difficult to interpret the available data and draw conclusion. Several laboratory standards have been applied for the susceptibility testing of *Campylobacter* species. Although disk diffusion was used in some studies, it should be used only as a screening method for resistance to erythromycin and ciprofloxacin [127].

5. Conclusion

This review indicates that *C. jejuni* and *C. coli* are frequently isolated from humans, food animals, and animal products in sub-Saharan Africa. Isolates from the different sources display varying degrees of resistance to commonly used antimicrobial agents. The findings of this review suggest that the disease burden due to thermophilic *Campylobacter* species in SSA is of public and economic importance. Therefore, routine diagnosis of *C. jejuni* and *C. coli*, appropriate use of antimicrobials, educating communities on hygienic practices, establishment of both national and regional multisectoral antimicrobial resistance standard surveillance protocols are necessary to curb both the campylobacteriosis burden, and increase of antimicrobial resistance in the region.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Paper Two

Antimicrobial resistance profiles, virulence genes, and genetic diversity of thermophilic *Campylobacter* species isolated from a layer poultry farm in Korea

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Antimicrobial Resistance Profiles, Virulence Genes, and Genetic Diversity of Thermophilic *Campylobacter* Species Isolated From a Layer Poultry Farm in Korea

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Thermophilic *Campylobacter* species are among the major etiologies of bacterial enteritis globally. This study aimed at assessing the antimicrobial resistance (AMR) profiles, virulence genes, and genetic diversity of thermophilic *Campylobacter* species isolated from a layer poultry farm in South Korea. One hundred fifty-three chicken feces were collected from two layer poultry farms in Gangneung, South Korea. The *Campylobacter* species were isolated by cultural techniques, while PCR and sequencing were used for species confirmation. Antimicrobial susceptibility testing for six antimicrobials [ciprofloxacin (CIP), nalidixic acid (NAL), sitafloxacin (SIT), erythromycin (ERY), tetracycline (TET), and gentamicin (GEN)] was carried out by broth microdilution. Three AMR and nine virulence genes were screened by PCR. Genotyping was performed by *fla*A-restriction fragment length polymorphism (RFLP) and multilocus sequence typing (MLST). Of the 153 samples, *Campylobacter* spp. were detected in 55 (35.9%), with *Campylobacter jejuni* and *Campylobacter coli* being 49 (89.1%) and six (10.9%), respectively. High-level resistance was observed for CIP (100%), NAL (100%), and TET (*C. jejuni*, 93.9%; *C. coli*: 83.3%). No resistance was observed for SIT. The missense mutation (C257T) in *gyr*A gene was confirmed by sequencing, while the *tet*(O) gene was similar to known sequences in GenBank. The rate of multidrug-resistant (MDR) strains was 8.2%, and they all belonged to *C. jejuni*. All *Campylobacter* isolates possessed five virulence genes (*cdt*B, *cst*II, *fla*A, *cad*F, and *dna*J), but none possessed *ggt*, while the rates for other genes (*csr*A, *cia*B, and *pld*A) ranged between 33.3 and 95.9%. The *fla*A-RFLP yielded 26 *fla*A types (*C. jejuni*: 21 and *C. coli*: five), while the MLST showed 10 sequence types (STs) for *C. jejuni* and three STs for *C. coli*, with

CC-607 (STs 3611) and CC-460 (ST-460) being predominant. Among the 10 STs of *C. jejuni*, three were newly assigned. The findings of this study highlight the increased resistance to quinolones and TET, the virulence potential, and the diverse genotypes among *Campylobacter* strains isolated from the layer poultry farm.

Keywords: *Campylobacter*, quinolones, antimicrobial resistance, *fla*A RFLP, multilocus sequence typing, poultry, Korea

INTRODUCTION

Globally, *Campylobacter* is the leading cause of bacterial gastroenteritis (Alaboudi et al., 2020). *Campylobacter jejuni* and *Campylobacter coli* are the species of clinical significance, being accountable for more than 95% of human campylobacteriosis (Moore et al., 2005; Fitzgerald, 2015). Globally, 96 million cases of diarrhea each year are due to *Campylobacter* (Havelaar et al., 2015). In Europe, *Campylobacter* was ranked as the second (next to *Salmonella*) etiological agent of outbreaks associated with water and food poisoning in 2018 (Klančnik et al., 2020). Contrary to European countries, reports on human campylobacteriosis in Asian countries including South Korea are limited, possibly due to low disease prevalence or the sporadic nature of infections (Kim et al., 2019; Wei et al., 2019).

Human campylobacteriosis requires antimicrobial therapy only in case of complications and in immuno-compromised people (Wieczorek and Osek, 2013; Sproston et al., 2018). Over the years, increasing rates of *Campylobacter* strains that are resistant to the drugs of choice [fluoroquinolones (FQs) and macrolides] and alternative therapies [gentamicin (GEN) and tetracycline (TET)] have been reported (Blaser and Engberg, 2008; Koolman et al., 2015), making antimicrobial resistant (AMR) *Campylobacter* strains a public health concern (Mourkas et al., 2019; Windiasti et al., 2019). The resistance to antimicrobials is partly due to their misuse in both human medicine and livestock production (Elisha et al., 2017; Sproston et al., 2018). For instance, different quinolone antibiotics have been extensively used in poultry raising, which led to an accelerated number of quinolone-resistant strains of *Campylobacter* from chicken and humans (Sproston et al., 2018).

In Korea, FQ use in livestock was banned since 2010, with a prediction to curb the increased resistance in the future (Ku et al., 2011). However, recently, FQ-resistant *Campylobacter* strains have been isolated from poultry and duck meat (Kim et al., 2019). Due to the increased resistance to quinolones throughout the world (Tang et al., 2017), erythromycin (ERY) has emerged as the recommended drug for treating human campylobacteriosis (Giannatale et al., 2019). Recently, sitafloxacin (SIT), a novel FQ drug, proved to be effective against various FQ-resistant pathogens including *Campylobacter* (Changkwanyeun et al., 2016; Chen et al., 2020), and could be a promising drug. The persistence of FQ resistance in *Campylobacter* strains could be linked to the continued use of ciprofloxacin (CIP) in human medicine, international trade, travel, and use of FQs in animal husbandry along with the circulation of resistant isolates among different reservoirs.

Poultry can carry *Campylobacter*, and chicken intestines are regarded as reservoirs for thermophilic *Campylobacter* species based on optimal conditions (high body temperature) favoring their growth (Sibanda et al., 2018). Previous reports have associated an increase in human campylobacteriosis cases with the increase in chicken consumption (Oh et al., 2017). Chickens' ceca alone are usually colonized by *C. jejuni* to levels above 10^9 colony-forming unit (CFU)/ml, posing a risk to humans (Humphrey et al., 2007, 2014). Furthermore, *Campylobacter* can stay in feces and litter for many days, and the use of these byproducts as fertilizers would aggravate the dissemination of the pathogens (Kassem et al., 2016). *Campylobacter* persistence in chicken farms and slaughterhouses is a hazard to the consumers because it is transmitted along the whole production chain up to the final product (Kim et al., 2019; Ramires et al., 2020). While the literature on broiler chicken is extensive, studies on the epidemiology of *Campylobacter* species from layer farms are limited (Kassem et al., 2016).

Campylobacter species are equipped with virulome which is used in attachment, establishment, invasion, and production of toxins, contributing to their increased occurrence and epidemiology compared to other enteric bacteria (Bolton, 2015; Otigbu et al., 2018). However, the mechanisms associated with *Campylobacter* pathogenicity are not fully understood (Nguyen et al., 2016). *C. jejuni* is known to cause Guillain–Barré syndrome, characterized by acute and progressive neuromuscular paralysis, mediated by sialyltransferases (*cst*II) (Koga et al., 2006; Humphrey et al., 2007; González-Hein et al., 2013). Sialic acid confers immune avoidance to *C. jejuni*, as a mutant lacking lipooligosaccharide sialic acid residues showed greater immunoreactivity and decreased serum resistance (Kreling et al., 2020). The CDT complex, another important factor in *Campylobacter*, codes for the cytolethal distending toxin with *cdt*B acting as the catalytic site, and in the nucleus, *cdt*B induces cell cycle arrest and leads to apoptosis of both immune and epithelial cells in the intestines (Jain et al., 2008). It has been reported that *C. jejuni cdt*B mutants had reduced extra-intestinal invasiveness (Yamasaki et al., 2006) and bowel disturbances (Pokkunuri et al., 2012). A study carried out in Poland showed that *Campylobacter* strains lacking *cdt*B and *cdt*C were non-cytotoxic, confirming their roles in toxin production (Wysok et al., 2020). The presence of *ggt* contributes to the colonization potential of *C. jejuni* in chicken and mice intestines (Barnes et al., 2007). The *fla*A gene contributes to *Campylobacter* pathogenesis as it is involved in colonization, motility, auto-agglutination, and biofilm formation (Guerry, 2007). Mutation experiments highlighted the role of *fla*A in chicken colonization (Bolton, 2015). *Campylobacter* species also possess other genes

associated with adhesion (*cad*F and *pld*A), invasion (*cia*B), thermo-tolerance (*dna*J) (Pillay et al., 2020), and stress response (*csr*A) (Fields and Thompson, 2008). Studies have shown that *Campylobacter* strain mutants for *cad*F and *cia*B exhibited a reduced attachment and invasion of INT 407 cell line along with a decline of survival potential (Kreling et al., 2020; Ramires et al., 2020).

Molecular typing methods are important not only in distinguishing bacteria at the species and subspecies levels but also in source attribution of *Campylobacter* strains (Eberle and Kiess, 2012; Lydekaitiene and Kudirkiene, 2020). Although source attribution aiming at quantifying the contribution of different reservoirs, pathways, exposures, and risk factors to the burden of human illness is difficult (Wagenaar et al., 2013), it is estimated that 80% of human campylobacteriosis are attributed to *Campylobacter* of poultry origin (Wagenaar et al., 2013; Mulder et al., 2020). Multilocus sequence typing (MLST), based on seven housekeeping genes (HKGs), is the gold-standard method used for epidemiological surveillance (Harrington et al., 2003; Lydekaitiene and Kudirkiene, 2020). Data on MLST of *Campylobacter* strains in Asia are limited (Nguyen et al., 2016), but previous studies in Korea and Japan have shown the predominance of CC-460, CC-607, CC-21, and CC-45 in poultry and human isolates (Wei et al., 2014; Ozawa et al., 2016; Oh et al., 2017). MLST data are expected to give accurate phylogenic estimation, typing, and strain relatedness (Alaboudi et al., 2020). Whole-genome sequencing (WGS) might become the preferred typing method in the future, but still, there is a need for a consensus upon bioinformatics pipelines and tools for processing WGS data (Duarte et al., 2016b).

Considering the persistence of FQ-resistant *Campylobacter* strains even in the absence of antimicrobial use and the fact that SIT has a different structure compared to other FQs, we hypothesized that *Campylobacter* species from chicken are still resistant to both ciprofloxacin and nalidixic acid (NAL) but sensitive to SIT. Furthermore, we think that the same sequence types (STs) are circulating in poultry in Korea and the region. Based on the virulence potential of *Campylobacter* and the favorable environment offered by chicken, it is most probable that *Campylobacter* species of poultry origin are hypervirulent and could be of concern. The present study aimed at assessing the antimicrobial resistance profiles, virulence genes, and genetic diversity of thermophilic *Campylobacter* species isolated from a layer poultry farm in Korea.

MATERIALS AND METHODS

Sample Collection

Fresh chicken fecal samples were purposively collected from two layer poultry farms located in Gangneung city, Republic of Korea in June 2020. The first farm uses an intensive poultry farming system with around 800 1-year-old chickens dispatched into battery cages inside a closed house. The second farm is a small one that is not for commercial purposes, where around 30 1-year-old chickens are enclosed in a cage subdivided into two blocks by a fence. A total of 133 (from the first farm) and 20 (from the second farm) pen floor fecal samples were collected using sterile swabs and transported to the laboratory under refrigeration (ice) within 1 h.

Campylobacter Isolation and Antimicrobial Susceptibility Testing

Upon arrival at the laboratory, the feces were inoculated onto modified charcoal cefoperazone deoxycholate agar (mCCDA) (Oxoid Ltd., Basingstoke, Hampshire, England) containing the *Campylobacter* mCCDA-selective supplement, SR155E (Oxoid Ltd, Basingstoke, Hampshire, England). Incubation was done as previously described (Kurekci et al., 2012) at 37°C for 48 h under microaerophilic conditions generated by CampyGen^{TM} gas sachets (Oxoid, Basingstoke, England, United Kingdom). Typical colonies of *Campylobacter*, which are grayish, flat, moistened, and with a tendency to spread (Al-Edany et al., 2015), were sub-cultured on Mueller Hinton Agar supplemented with 5% defibrinated horse blood (MHS) and incubated at 37°C for 48 h under microaerophilic conditions (Kurekci et al., 2012). *Campylobacter* isolates were preserved at −80°C in Mueller Hinton broth containing 25% glycerol (v/v).

Antimicrobial susceptibility testing was performed by broth microdilution. The isolates were tested against quinolones, namely ciprofloxacin (CIP), NAL (0.25–512 μg/ml), SIT (0.03–16 μg/ml); macrolide, ERY (0.06–64 μg/ml); and aminoglycoside, GEN (0.06–64 μg/ml), and TET (0.125–1,024 μg/ml). Apart from SIT purchased from AdooQ BioScience (Irvine, CA, United States), the other antimicrobials were supplied by Sigma-Aldrich (St. Louis, MO, United States). CIP and ERY were dissolved in 0.1 N HCl and 70% ethanol, respectively. GEN and TET were dissolved in water, while NAL and SIT were dissolved in dimethyl sulfoxide (DMSO). Except for the antibiotics dissolved in DMSO, other solutions of antibiotics were filter-sterilized before being used.

Preserved *Campylobacter* isolates were inoculated onto MHS (Oxoid Ltd, Basingstoke, Hampshire, England) and incubated at 37°C for 48 h under microaerophilic conditions (Kurekci et al., 2012). A sub-culture was performed on the same media and the same conditions to get well-grown pure colonies free from glycerol. For antimicrobial susceptibility assays, suspensions corresponding to 0.5 McFarland standard (1.5×10^8 CFU/ml) were prepared using normal saline, and the final concentration in a 96-well plate was $2–5 \times 10^6$ CFU/ml. The minimal inhibitory concentration (MIC) was determined by checking the absorbance at A_{600} nm on a microplate reader (Synergy HT; BioTek Instruments Inc., Winooski, VT, United States) and confirmed by the addition of iodonitrotetrazolium chloride to 96-well plates as previously described (Klančnik et al., 2009). The MIC was designated as the lowest concentration of the antimicrobial leading to a significant decrease (>90%) in inoculum viability after 48 h as previously described with modification on incubation time (Burt, 2004). The minimal bactericidal concentration (MBC) was determined as previously described (Dholvitayakhun and Trachoo, 2012; Duarte et al., 2016a). The concentration at which no bacterial growth was noticed after 48 h of incubation was regarded as MBC. The

MIC values were interpreted according to the standards of the European Committee for Antimicrobial Susceptibility Testing[1], except for SIT which lacks international cutoff values. The MIC values were CIP ≤ 0.5 μg/ml, NAL ≤ 16 μg/ml, ERY ≤ 4 for *C. jejuni* and ≤8 μg/ml for *C. coli*, and GEN and TET ≤ 2 μg/ml. However, all *Campylobacter* strains were sensitive to SIT (MIC ≤ 2 μg/ml) according to the literature (Huang et al., 2015; Xu et al., 2018).

DNA Extraction, PCR Confirmation of Species, and Detection of AMR and Virulence Genes

The extraction of genomic DNA from pure colonies was carried out by using the Qiagen QIAamp PowerFecal Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Then, a multiplex PCR was conducted using genus-specific primers (C412F and C1228R), *cj0414* gene primers (C1 and C3) for *C. jejuni*, and *ask* gene primers (CC18F and CC519R) for *C. coli* (Yamazaki-Matsune et al., 2007). The primers were selected based on the specificity in identifying the genus and species of *Campylobacter* (Linton et al., 1997; Pajaniappan et al., 2008). The PCR mixture (25 μl) contained 12.5 μl of 2X Master Mix (Thermo Fisher Scientific, Seoul, South Korea), 1 μl of primer (10 μM), 1.5 μl of template DNA (20 μg/ml), and 7 μl of sterile deionized water. The cycling conditions were one cycle of 95°C for 5 min, 35 cycles each of 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 7 min using MiniAmp Plus Thermal Cycler (Applied Biosystems, MA, United States). The PCR products were held at 4°C before analysis.

For the genes associated with antibiotic resistance [*tet*(O), *gyr*A, and *cme*B] and virulence (*cst*II, *cdt*B, *fla*A, *ggt*, *csr*A, *cad*F, *cia*B, *pld*A, and *dna*J), the PCR was performed using specific primers (**Supplementary Table 1**). After electrophoresis, bands of PCR products (**Figure 1**) were observed on a Dual UV Transilluminator (Core Bio System, Huntington Beach, CA, United States) under ultraviolet light. The bands of the amplification products were compared to the 100-bp DNA ladder (Dyne bio, Seongnam-si, Republic of Korea). The PCR products of antibiotic resistance genes were purified with AMPure XP beads (Beckman Coulter, Fullerton, CA, United States) and sequenced by the Sanger method at SolGent (Solutions for Genetic Technologies, Daejeon, Republic of Korea).

*fla*A-Restriction Fragment Length Polymorphism

Genetic diversity was first analyzed by *fla*A-restriction fragment length polymorphism (RFLP) using 25-μl PCR reactions (Harrington et al., 2003; Wieczorek and Osek, 2008). The *fla*A amplicon (1.7 kb) was digested for 6 h at 37°C using HpyF3I restriction enzyme (Thermo Scientific, Waltham, MA, United States), and the fragments were separated using 2.5% agarose gel (Lonza Inc., Rockland, ME, United States) in Tris–acetate–EDTA buffer at 90 V for 90 min. The bands were photographed with iBright^{TM} CL1000 Imaging System (Thermo Fisher Scientific, Seoul, Republic of Korea). The dyne 100-bp and 1-kb DNA ladders (Dyne bio, Seongnam-si, Republic of Korea) were used as standards for molecular size determination.

Multilocus Sequence Typing Analysis

Multilocus sequence typing was performed as previously described (Dingle et al., 2001; Giannatale et al., 2019) using primers available from the *Campylobacter* MLST website[2]. Briefly, the seven HKGs *asp*A (aspartase), *gln*A (glutamine synthetase), *glt*A (citrate synthase), *gly*A (serine hydroxymethyltransferase), *pgm* (phosphor glucomutase), *tkt* (transketolase), and *unc*A (ATP synthase) were PCR-amplified from genomic DNA. For *C. jejuni*, two rounds of PCR were performed (nested PCR), while for *C. coli* one set of primers was used. The PCR conditions were denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 45 s, and then a final extension at 72°C for 5 min. The purification of amplicons was performed by AMPure XP beads (Beckman Coulter, Fullerton, CA, United States) as per the manufacturer's recommendations and sequenced by the Sanger method at SolGent (Solutions for Genetic Technologies, Daejeon, Republic of Korea).

Data Analysis

GraphPad Prism 8.4.0 (GraphPad Software, La Jolla, CA, United States) was used to compute the descriptive statistics (detection rate, proportions, and frequencies of different attributes). The *fla*A restriction profiles were analyzed by pairwise comparisons and cluster analysis using the Dice correlation coefficient and the unweighted pair group method with arithmetic mean clustering algorithm in BIONUMERICS V8.0 (Applied Maths, Sint-Martens-Latem, Belgium). The optimization and position tolerance (1%) for band analysis and a cutoff of 100% were used. BioEdit software (version 7.2.6.1) was used to edit, align, and analyze the DNA chromatograms (Hall, 1999). A BLAST search was performed to compare consensus sequences [*gyr*A and *tet*(O)] with those from the GenBank database. Then, our sequences were submitted to get the corresponding accession numbers. Standard sensitive strains (L04566.1 and U63413.1) and resistant strains (KX982339.1 and MT176401.1) for *gyr*A were used for comparison. For the *gyr*A gene, the comparison was performed with Clustal Omega (Madeira et al., 2019). Amino acid sequences were deduced from the DNA sequences using the ExPASyTranslate tool (Gasteiger et al., 2003). Alleles, STs, and clonal complexes (CCs) were assigned by submitting the sequence data to the MLST database (see text footnote 2) (Maiden, 2006). A minimum spanning tree of *C. jejuni* and *C. coli* STs was created from MLST allelic profiles using BIONUMERICS 8.0 (Applied Maths NV, Saint-Martens-Latem, Belgium).

RESULTS

Out of 153 fecal samples, the detection rate of *Campylobacter* spp. was 35.9% (55), with *C. jejuni* and *C. coli* being 89.1% (49) and 10.9% (six), respectively. None of the 20 fecal samples from the second farm was positive for *Campylobacter*.

[1]http://www.eucast.org/

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

Antimicrobial Susceptibility Testing

All *Campylobacter* isolates were screened for antimicrobial susceptibility to six antimicrobials, and they showed high-level resistance to CIP, NAL, and TET. Resistance to CIP and NAL was 100%, while resistance to TET was 93.9% for *C. jejuni* and 83.3% for *C. coli* (**Table 1**). Four (8.2%) *C. jejuni* strains were multidrug-resistant (MDR), but none of the *C. coli* was MDR. Of the four MDR isolates, two were resistant to CIP, NAL, TET, and ERY, while the other two were resistant to CIP, NAL, TET, and GEN. The presence of *tet*(O) and mutation in *gyr*A were confirmed by PCR (**Figure 1**), but all strains did not show bands for the multidrug efflux pump gene (*cme*B). Sequencing revealed the presence of a missense mutation (C257T) in the quinolone resistance determining region of *gyr*A gene, causing resistance to quinolones along with other silent mutations. There was 100% sensitivity to SIT, while 4.1% of the *C. jejuni* isolates were resistant to both ERY and GEN (**Table 1**). The MBC values were as follows: CIP, 64–256 μg/ml; NAL, 128–512 μg/ml; TET, 2–1,024 μg/ml; SIT, 0.25–1 μg/ml; ERY, 1–32 μg/ml; and GEN, 1–256 μg/ml.

Upon submission of the *gyr*A and *tet*(O) sequences to the GenBank database, the following accession numbers have been assigned: MW067325–MW067370 (**Table 2**). The main mutation in *gyr*A gene is the missense mutation (C257T) associated with the codon change from ACA to ATA (*C. jejuni*) and ACT to ATT (*C. coli*) leading to *T86I* substitution. However, silent mutations were also found. It was noticed that TET-resistant strains possessed the *tet*(O) gene, which was confirmed by sequencing. The BLAST search showed similarity with known *tet*(O) gene sequences in GenBank.

Virulence Genes

The presence of selected virulence genes (*cst*II, *cdt*B, *fla*A, *ggt*, *csr*A, *cad*F, *cia*B, *pld*A, and *dna*J) was checked by PCR (**Figure 1**). We found that all isolates possessed *cdt*B, cstII, *fla*A, *cad*F, and

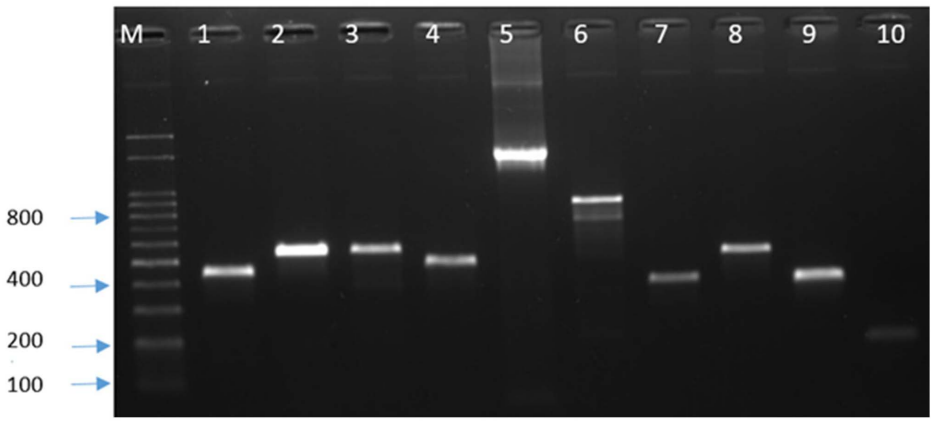

FIGURE 1 | PCR detection of antimicrobial resistance and virulence genes. M, marker; 1, *gyr*A; 2, *tet*(O); 3, *cst*II; 4, *cdt* B; 5, *fla*A; 6, *csr*A; 7, *cad*F; 8, *cia*B; 9, *pld*A; and 10, *dna*J.

TABLE 1 | Antimicrobial resistance data for both *Campylobacter jejuni* and *Campylobacter coli* species.

Anti-microbial	Class	Species	Resistance (%)	Number of isolates at the indicated minimal inhibitory concentration (μg/ml)													
				0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	512
		C. jejuni	100	0	0	0	0	0	0	0	0	1	36	10	2	0	0
CIP		*C. coli*	100	0	0	0	0	0	0	0	0	0	4	2	0	0	0
	Fluoroquinolone	*C. jejuni*	0	2	33	11	2	1	0	0	0	0	0	0	0	0	0
SIT		*C. coli*	0	0	0	5	1	0	0	0	0	0	0	0	0	0	0
		C. jejuni	100	0	0	0	0	0	0	0	0	0	1	25	17	6	0
NAL	Quinolone	*C. coli*	100	0	0	0	0	0	0	0	0	0	0	1	5	0	0
		C. jejuni	4.1	0	0	1	38	3	2	3	1	1	0	0	0	0	0
ERY	Macrolide	*C. coli*	0	0	0	0	1	0	4	1	0	0	0	0	0	0	0
	Aminoglycoside	*C. jejuni*	4.1	0	1	11	27	8	0	0	0	0	0	2	0	0	0
GEN		*C. coli*	0	0	0	2	1	3	0	0	0	0	0	0	0	0	0
	Tetracycline	*C. jejuni*	93.9	0	0	0	2	1	0	0	0	0	3	10	22	10	1
TET		*C. coli*	83.3	0	0	0	1	0	0	0	0	0	0	1	2	2	0

TABLE 2 | Accession numbers for DNA *gyr*A and *tet*(O) resistance genes.

Isolate	DNA *gyr*A accession number	*tet*(O) accession number
CJ5	MW067325	MW067326
CJ8	MW067327	MW067328
CJ17	MW067329	MW067350
CJ21	MW067330	MW067351
CJ28	MW067331	MW067352
CJ32	MW067332	MW067353
CJ37	MW067333	MW067354
CJ42	MW067334	MW067355
CJ46	MW067335	MW067356
CJ48	MW067336	MW067357
CJ50	MW067337	MW067358
CJ51	MW067338	MW067359
CJ52	MW067339	MW067360
CJ53	MW067340	MW067361
CJ54	MW067341	MW067362
CJ55	MW067342	MW067363
CJ56	MW067343	MW067364
CJ60	MW067344	MW067365
CJ71	MW067345	MW067366
CC13	MW067346	MW067367
CC45	MW067347	MW067368
CC47	MW067348	MW067369
CC2	MW067349	MW067370
CC1	MT947449	MT967270

CJ, Campylobacter jejuni; CC, Campylobacter coli.

*dna*J, but none showed the presence of *ggt*. The percentages for *csr*A, *cia*B, and *pld*A were 73.5, 95.9, and 98% for *C. jejuni* and 66.7, 33.3, and 33.3% for *C. coli*, respectively (**Figure 2**).

The *fla*A Polymorphism

Campylobacter jejuni (n = 37) and *C. coli* (n = 5) were digested with HpyF3I, yielding six to 10 fragments of DNA. There were 26 *fla*-A types (*C. jejuni*: 21 and *C. coli*: five). For *C. jejuni*, there was a predominant cluster at the top of the dendrogram. For *C. coli*, isolate numbers 45 and 47 clustered together; the other isolates had different patterns (**Figure 3**).

Multilocus Sequence Typing

Twenty-four isolates (*C. jejuni*: 19; *C. coli*: five), selected based on *fla*A RFLP profiles to maximize the diversity, were genotyped by MLST. *C. jejuni* isolates were matched with 10 STs grouped into five CCs. However, three *C. jejuni* isolates had new combinations of previously described alleles but could not be matched with any of the existing STs. Upon submission to the database (see text footnote 2) for ST assignment, the isolates CJ42 (id: 106369), CJ52 (id: 106370), and CJ71 (id: 106371) were assigned to ST-10645, ST-10647, and ST-10648, respectively. Of the 10 STs, ST-3611 was the main one with five isolates, followed by ST-460 with four isolates. The CCs with a higher number of isolates were CC-607 with nine isolates and CC-460 with five isolates. For *C. coli* (five isolates), three STs were found, with ST-5935 being the most prevalent (three isolates), and all the five isolates belonged to CC-1150 (**Table 3**).

The minimum spanning tree of *C. jejuni* and *C. coli* STs was created from MLST allelic profiles using BIONUMERICS 8.0 (Applied Maths NV, Saint-Martens-Latem, Belgium). The tree shows 78 STs grouped into 12 previously characterized CCs, including both STs identified in this study (13) and 65 STs reported in the literature as predominant in *Campylobacter* strains of human and poultry origins. ST-460 and ST-10613, belonging to CC-460, are clustered together, and they share six of the seven HKGs, with the only difference being in *gln*A allele. The STs 6238, 607, and 3611 form another cluster belonging to the same CC-607 at the center of the tree. ST-51 belonging to CC-443 is located far from the other STs identified in this study. Of the three newly assigned STs, it can be concluded that ST-10645 is closely related to ST-8994, both belonging to CC-52, while ST-10647 is closely related to ST-3611, both belonging to CC-607. We also included STs that have been previously identified in *Campylobacter* strains isolated from poultry, human, and cattle fecal samples from the database (see text footnote 2). It can be concluded that CCs of this study are closely related to other CCs (257, 353, and 354) and distanced from CC-45 and CC-21 commonly reported in *Campylobacter* strains of poultry

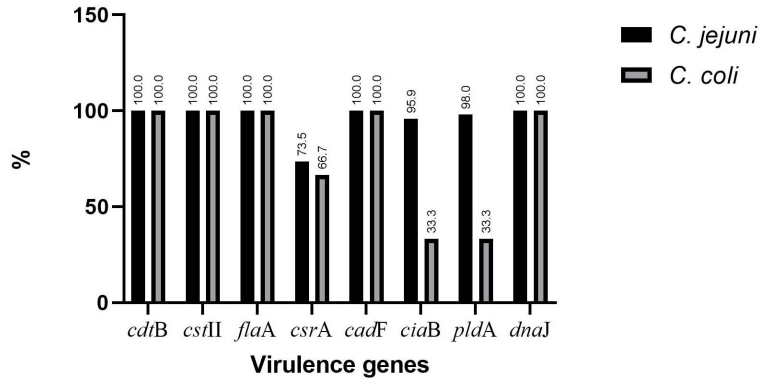

FIGURE 2 | Distribution of virulence genes in *Campylobacter jejuni* and *Campylobacter coli*.

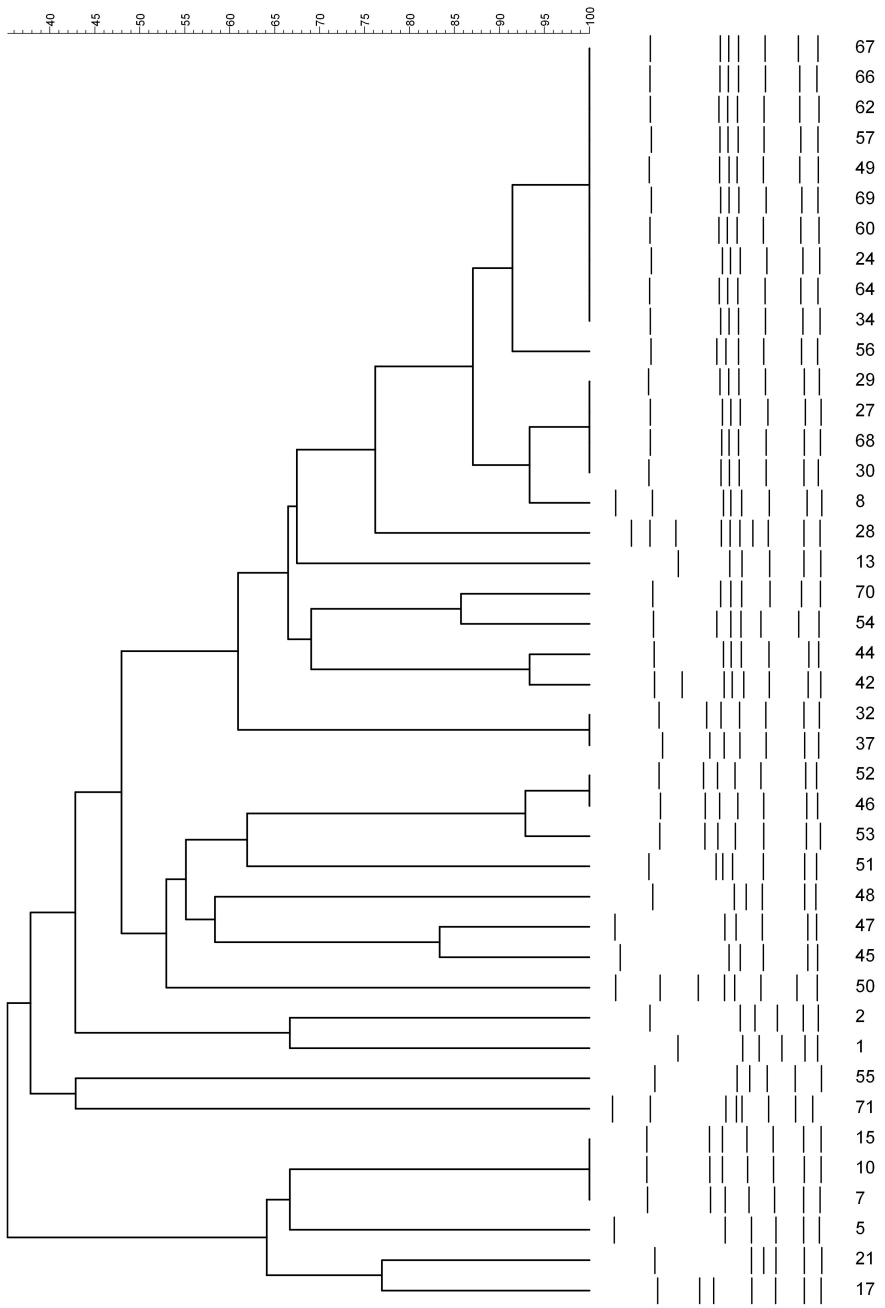

FIGURE 3 | PCR–restriction fragment length polymorphism profiles of *fla*A gene digested with HpyF31 from strains of *Campylobacter* species. Numbers represent the laboratory code for isolates.

origin in Asia and elsewhere. The STs for *C. coli* from this study all belong to CC-1150 along with other STs obtained from the database (**Figure 4**).

DISCUSSION

Chicken contamination or infection by *Campylobacter* at the farm level usually affects the whole poultry production chain from farm to fork (Giannatale et al., 2019; Tang et al., 2020), and suitable interventions have to be adopted to reduce transmission from poultry to humans (Alter et al., 2011). The detection rate of *Campylobacter* was 35.9%, with *C. jejuni* being predominant (89.1%) compared to *C. coli* (10.9%). The detection rate was slightly higher compared to the rate previously reported for layers in the United States (Rama et al., 2018) but lower than the rates reported in the Netherlands (Schets et al., 2017) and Sri Lanka (Kalupahana et al., 2013). *C. jejuni* is reported to be

TABLE 3 | Distribution of sequence types and clonal complexes among *Campylobacter* strains from chicken (n = 24).

Species	Isolate ID	Sequence type	Total number	Clonal complex
Campylobacter jejuni	CJ5; CJ50	51	2	443
	CJ8; CJ51; CJ54; CJ60	460	4	460
	CJ56	10613	1	
	CJ17; CJ32; CJ37; CJ46; CJ53	3611	5	607
	CJ21; CJ48	607	2	
	CJ55	6238	1	
	CJ52	10647	1	
	CJ28	8994	1	52
	CJ42	10645	1	
	CJ71	10648	1	446
Campylobacter coli	CC1; CC13; CC45	5935	3	1150
	CC2	8164	1	
	CC47	1121	1	

the predominant species causing human campylobacteriosis, and our results concur with the literature (Han et al., 2007; Wei et al., 2014). However, exceptions have been reported, where *C. coli* was the predominant or the only isolated species (Marinou et al., 2012; Wieczorek et al., 2020a).

Campylobacter species exhibited high-level resistance to some antimicrobials (CIP, NAL, and TET), which concurs with findings previously reported in Korea (Kim et al., 2010; Lee et al., 2017; Oh et al., 2017) and elsewhere (Elhadidy et al., 2018; Zhang et al., 2020). In Korea, the use of ciprofloxacin was banned in 2010 (Ku et al., 2011), but mass medication of poultry with FQs, especially enrofloxacin, is still allowed (Seo and Lee, 2020). We characterized the *gyr*A gene, and the point mutation (C257T) confirmed the phenotypic results. The (C257T) mutation leads to increased resistance to FQs (Frasao et al., 2015) often used in livestock (Perrin-Guyomard et al., 2020) and in humans for treating undiagnosed diarrhea cases (Sproston et al., 2018). Surprisingly, quinolone-resistant *Campylobacter* strains have been reported in Australia in the absence of their use (Abraham et al., 2020). Although the use of FQs is banned in several countries, resistant strains are maintained in bacterial populations, which may explain their continued occurrence in humans and animals (Sproston et al., 2018). The association of using FQs in animal husbandry and the increased occurrence of AMR pathogens differ depending on the poultry production system, surveillance programs, and geographic location (Hao et al., 2016). It is hypothesized that the increased rates of human campylobacteriosis in Asia, Europe, and America are partly driven by a widespread prevalence of *C. jejuni* strains resistant to quinolones *via* poultry (Oh et al., 2017).

There was high resistance to TET, which concurs with previous reports in Korea (Wei et al., 2014; Lee et al., 2017), China (Zhang et al., 2020), and India (Kabir et al., 2015). TET is often used in poultry and pig industries based on their low cost and easy administration to animals through drinking water (Jonker and Picard, 2010). The sequencing and BLAST search showed the similarity of *tet*(O) gene sequences with known plasmid-mediated *tet*(O) gene sequences in the GenBank (Elhadidy et al., 2018; Lynch et al., 2020).

All isolates were susceptible to SIT (MIC values: 0.125–1 μg/ml) which possesses specific features lacking in other quinolones like a cyclopropyl ring with fluorine at R-1 and a chloride substituent at R-8 (Changkwanyeun et al., 2016), which may explain its high effectiveness. Our results corroborate previous reports with a MIC of 0.25 μg/ml (Yabe et al., 2010; Changkwanyeun et al., 2015). SIT is a candidate for clinical trials on campylobacteriosis (Changkwanyeun et al., 2015), and it is used for the eradication of multi-drug resistant *Helicobacter pylori* (Pohl et al., 2019). This could be a breakthrough as the alternative therapies for severe campylobacteriosis are very limited (Pavlova et al., 2020).

The low resistance to ERY and GEN shown by the isolates of this study has also been reported in different countries like Korea, Vietnam, and China (Carrique-Mas et al., 2014; Wei et al., 2014; Zhang et al., 2020). Resistance to ERY has been low and stable in China (Zhang et al., 2020), the United States, and across Europe (Tang et al., 2017). The limited resistance to ERY may be partly explained by a slower process of developing resistant strains when exposed to ERY and reduced survival of resistant strains (Luangtongkum et al., 2009, 2012). Resistance to GEN has also been relatively low as it is used for treating systemic infections (Lynch et al., 2020). Prudent use of existing antimicrobials and efforts to discover new alternative treatment options would help in curbing the AMR trend.

The *Campylobacter* species virulome contributes to their pathogenicity (Han et al., 2019). In this study, *cst*II, *cdt*B, *fla*A, *cad*F, and *dna*J were detected in all isolates. The detection rates were similar to a previous report in Korea (Oh et al., 2017) but higher than those reported in South Africa and Chile (González-Hein et al., 2013; Otigbu et al., 2018; Pillay et al., 2020). *cad*F seems to be a prerequisite for the invasion of epithelial cells by any bacterial pathogen (Ramires et al., 2020). The outer membrane phospholipase A (*pld*A) was observed more in *C. jejuni* than in *C. coli* (**Figure 2**), which corroborates the study in South Africa (Pillay et al., 2020), while for *cia*B, our findings are above those of a previous report in Korea (Oh et al., 2017) and contrast with the study in South Africa (Pillay et al., 2020). The presence of *cad*F and *cia*B facilitates the adhesion and internalization of *Campylobacter* in cellular models (Ramires et al., 2020). The detection rate for *csr*A in *C. jejuni* was slightly higher than the rate in *C. coli*, but *csr*A was lacking in *Campylobacter* strains from South Africa (Otigbu et al., 2018). None of our isolates expressed *ggt*. The latter was reported to be only 5.5% in Chile (González-Hein et al., 2013), but our findings contrast with the high values (30.9–43.2%) reported in Finland (Gonzalez et al., 2009). The difference could be associated with the complexity of the colonization process involving several genes and the use of strains from a single sampling site (González-Hein et al., 2013). Furthermore, MDR and virulent *C. jejuni* strains have

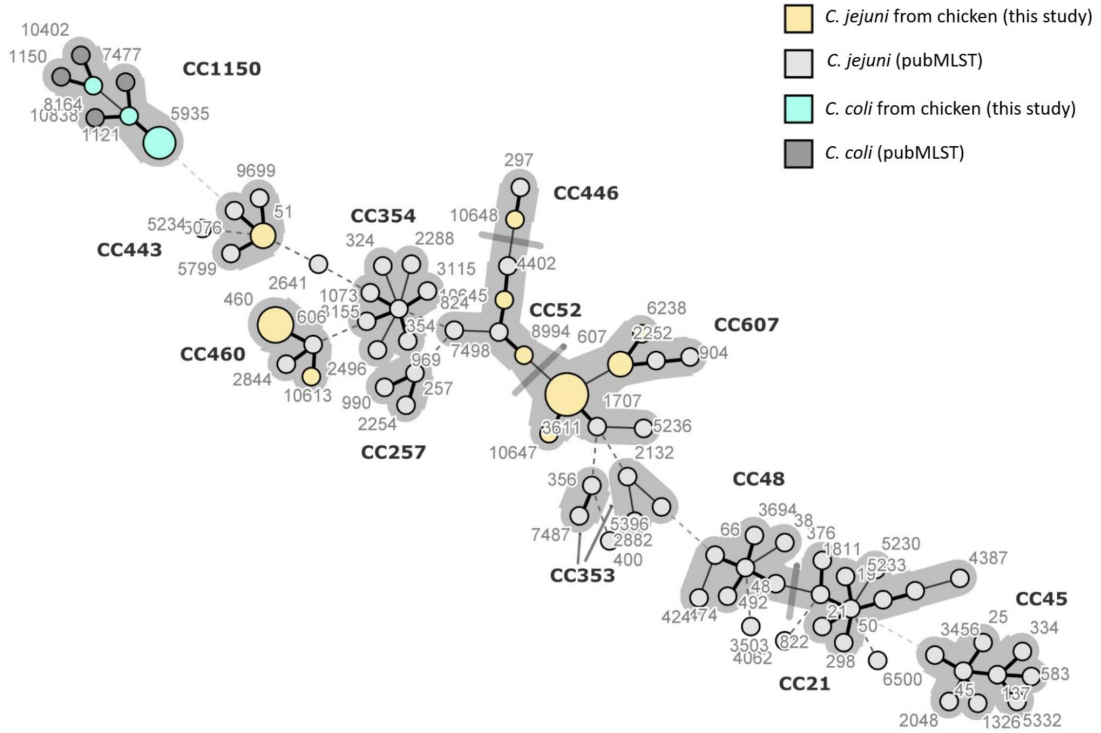

FIGURE 4 | Minimum spanning tree of *Campylobacter jejuni* and *Campylobacter coli* sequence types (STs) created from multilocus sequence typing allelic profiles. Each ST is represented as a circle, with the size of the circle proportional to the number of isolates of that ST for the isolates obtained in this study (STs colored in yellow and green). The figure also includes STs commonly reported in Korea and elsewhere from poultry and humans which were retrieved from pubMLST. The branch length and thickness represent the allelic distance: a thick short line connects single-locus variants, a thin longer line connects double-locus variants, and a dashed line connects STs separated by three or more allelic differences. Background shading highlights clonal complexes at a distance of two alleles in this dataset.

been found more in summer than in winter, suggesting the role played by climate in the expression of some genes (Kim et al., 2019). We also evoke that several virulence genes are plasmid-mediated, which may affect their presence in different strains (Oh et al., 2017). The virulence genes reported in this study have been previously reported in *Campylobacter* strains isolated from humans (González-Hein et al., 2013; Oh et al., 2017), highlighting the potential virulence of these *Campylobacter* strains in causing human infections.

The *fla*A-RFLP typing showed a considerable diversity of *Campylobacter* strains despite being from the same farm. In our study, 26 types were found by *fla*A typing of 42 *Campylobacter* isolates. In another study in Korea, 30 *fla*A types were reported for 100 *C. jejuni* from chicken (Han et al., 2007), while 19 *fla*A types were reported for 100 *C. jejuni* from Grenada, Puerto Rico, and Alabama (Behringer et al., 2011). The *fla*A typing is suitable for laboratories dealing with a small number of isolates as it is cheaper and reproducible (Behringer et al., 2011). However, the drawback of the *flaA* typing is increased recombination events in the *flaA* gene, which modifies RFLP profiles (Harrington et al., 1997). Furthermore, the *fla*A typing focuses only on a single gene from a considerable genome, and there is a lack of inter-laboratory comparison of obtained results (Nguyen et al., 2016) due to the absence of *fla*A database. Therefore, a combination of several typing methods is recommended (Wieczorek and Osek, 2008).

Molecular typing techniques showed that 80% of human campylobacteriosis is associated with *Campylobacter* of poultry origin (Newell et al., 2011). In this study, the MLST revealed 10 STs for *C. jejuni* and three STs for *C. coli*. Three STs were new but could fit in existing clonal complexes. The predominant STs (3611 and 460) have been previously isolated in *C. jejuni* from poultry, while ST-51 was found in *C. jejuni* of both chicken and human origins in various regions of Korea (Oh et al., 2017). In Korea, in addition to the CCs identified in this study, other CCs including CC-48, CC-21, and CC-45 (**Figure 4**) have been recovered from *Campylobacter* isolates of poultry and human origins (Wei et al., 2014; Oh et al., 2017). The predominance of these clonal complexes and STs could be associated with environmental factors (geography and climate) and increasing poultry consumption in Korea (Oh et al., 2017). Globally, CC-45 has been found in various hosts, including poultry, cattle, dogs, wild birds, penguins, and it was also isolated from environmental samples (Cody et al., 2012; Shin et al., 2013), while CC-607 is largely associated with chicken and humans as reported from the United Kingdom, France, Canada, Thailand, and Uruguay (Cody et al., 2012; Duarte et al., 2014; Guyard-Nicodème et al., 2015). ST-45 and ST-50, predominant in Korea, are also common

in *C. jejuni* from chickens in Europe (Wieczorek et al., 2020b). In 2019, ST-21, ST-50, and ST-137 have been isolated from wild ducks, indicating their widespread distribution (Wei et al., 2019). Three of the 10 STs (607, 443, and 51) were found in Japan and China (Ozawa et al., 2016; Ma et al., 2017), while CC-460 and CC-607 have also been reported in samples from humans and cattle, indicating their threat to public health (Ozawa et al., 2016; Kiatsomphob et al., 2019; Wei et al., 2019). CC-460 and CC-607 are thought to be virulent as they both possess many virulence genes. In Israel, type VI secretion system (T6SS), implicated in virulence, metabolism, AMR and contributing to host adaptation, has been found in both CCs (Rokney et al., 2018). In Japan, CC-21 is known as the prevalent clonal complex in human-derived *C. jejuni* isolates sharing a common genetic background and similar antimicrobial susceptibilities with *C. jejuni* strains from chicken (Ohishi et al., 2017). CC-21 (ST-50 and ST-21) could be of interest in the region as it has been found in Korea, Japan, and China from humans, poultry, and cattle (Ozawa et al., 2016; Oh et al., 2017; Kiatsomphob et al., 2019; Wei et al., 2019; Zhang et al., 2020). However, further studies are needed to confirm this hypothesis. Except for ST-257, other STs (ST-21, ST-48, and ST-353) identified in Korea have been recovered from *C. jejuni* of human origin in the United Kingdom, Brazil, and China (Gomes et al., 2019). It is known that the predominance of certain genotypes of *C. jejuni* depends on several factors such as animal reservoirs, zoonotic transmission, rates of recombination events, food source, and the first time when a given genotype is recorded in the country (Rokney et al., 2018).

Although resistance to quinolones and TET was not a particularity of certain STs, a strong correlation between CC-460, CC-607, CC-45, CC-48, CC-21, and resistance to both TET and quinolones in *C. jejuni* strains is not new (Cody et al., 2012; Shin et al., 2013; Guyard-Nicodème et al., 2015; Zhang et al., 2020). In Korea, *C. jejuni* strains of human origin with ST-607, ST-137, ST-45, ST-21, and ST-48 were found to be MDR (Shin et al., 2013). Two isolates of *C. jejuni* belonged to CC-443, which is suggested to harbor antibiotic-resistant and pathogenic *C. jejuni* strains (Kim et al., 2019). There is limited literature associating virulence genes to specific CCs and STs; thus, this study highlights the virulence potential of the presented *Campylobacter* isolates. For *C. coli*, ST-1121 has been reported from broilers in China (Tang et al., 2020). We suggest carrying out WGS to have a detailed picture of the pathogenicity by analyzing all the housekeeping, virulence, and AMR genes. The WGS would also enlighten the evolutionary pathways of the *Campylobacter* spp. used in this study to inform better practices that may lead to a reduction of campylobacteriosis cases.

The limitation of this study was the restricted access to various poultry farms across the city which would have given a broad picture of the prevalence, AMR profiles, and genotypes associated with *Campylobacter* in Gangneung. *Campylobacter* strains from the current study proved to be highly diverse considering the number of obtained STs and CCs. This is in agreement with other published studies reporting the possibility of getting several genotypes in a single poultry flock, suggesting different exposure sources *via* horizontal transmission and/or genetic drifts within the *Campylobacter* population (Alter et al., 2011; El-Adawy et al., 2013).

CONCLUSION

This study highlights the role of layers as a reservoir of *Campylobacter* spp., harboring various AMR and virulence genes. The genotyping highlighted that *C. jejuni* isolates were more diverse than *C. coli* as analyzed by MLST. The MLST revealed that CC-607 (ST-3611) and CC-460 (ST-460) were the predominant ones, while three STs were newly assigned. ERY, GEN, and SIT need to be appropriately used to prevent or delay the increasing resistance in *Campylobacter* species. The isolates of this study may present a potential hazard to public health based on their AMR profiles, virulence genes, and genotyping data.

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 in the article/**Supplementary Material**.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study.

AUTHOR CONTRIBUTIONS

NG, C-HP, and EK conceived the study. NG carried out the experiments, analyzed and interpreted the data, and wrote the manuscript. D-GS and K-YY substantially contributed to the analysis of the results. LM, MM, DM, and RA substantially revised the manuscript. All authors read and approved the final version of the 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/fmicb.2021.622275/full#supplementary-material

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Paper Three

Susceptibility of *Campylobacter* strains to selected natural products and frontline antibiotics

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Abstract: *Campylobacter* species have developed resistance to existing antibiotics. The development of alternative therapies is, therefore, a necessity. This study evaluates the susceptibility of *Campylobacter* strains to selected natural products (NPs) and frontline antibiotics. Two *C. jejuni* strains (ATCC® 33560™ and MT947450) and two *C. coli* strains (ATCC® 33559™ and MT947451) were used. The antimicrobial potential of the NPs, including plant extracts, essential oils, and pure phytochemicals, was evaluated by broth microdilution. The growth was measured by spectrophotometry and iodonitrotetrazolium chloride. Antibiotic resistance genes (*tet*(O) and *gyr*A) were characterized at the molecular level. The minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) ranged from 25 to 1600 µg/mL. Cinnamon oil, (E)-Cinnamaldehyde, clove oil, eugenol, and baicalein had the lowest MIC and MBC values (25–100 µg/mL). MT947450 and MT947451 were sensitive to erythromycin and gentamicin but resistant to quinolones and tetracycline. Mutations in *gyr*A and *tet*(O) genes from resistant strains were confirmed by sequencing. The findings show that NPs are effective against drug-sensitive and drug-resistant *Campylobacter* strains. The resistance to antibiotics was confirmed at phenotypic and genotypic levels. This merits further studies to decipher the action mechanisms and synergistic activities of NPs.

Keywords: antibiotics; *Campylobacter*; plant extracts; essential oils; phytochemicals; resistance

1. Introduction

Campylobacter species, mainly *C. jejuni* and *C. coli*, are among the major pathogens causing human gastroenteritis [1]. Campylobacteriosis is of public health concern in low-, middle-, and high-income countries [2]. Biofilm formation in *Campylobacter* contributes to its resistance to environmental stress and antibiotics [3]. Human infections with *Campylobacter* species occur via the ingestion of contaminated animal products or water [4–6].

There have been increased reports about high-level resistance to frontline and alternative antimicrobials, including macrolides, fluoroquinolones, aminoglycosides, and tetracyclines, among *Campylobacter* strains [7,8]. Increased antimicrobial resistance (AMR) among pathogens has been associated with many factors, including the unrestricted use of antimicrobials in various fields [9,10]. The main mechanisms of AMR include mutations in specific genes and acquiring efflux pumps [11]. For instance, the main resistance mechanism to ciprofloxacin is through target mutation in the DNA *gyrA* gene, along with the *CmeABC* efflux pump [4,12]; the majority (75–90%) of *Campylobacter* isolates worldwide have developed resistance to this important category of antibiotics [13]. The resistance to tetracycline is known to be either on a plasmid or bacterial chromosome [14–16]. It is estimated that by the year 2050, if no adequate actions are taken, the annual death rate due to AMR would reach 10 million people worldwide and cost USD 100 trillion [17]. Poultry has been recognized as the primary reservoir of *Campylobacter* strains that are resistant to fluoroquinolones associated with human diseases [18]. The progressive end of the traditional antimicrobial drug era as a result of the increasing number of AMR pathogens requires the development of new approaches to deal with AMR pathogens [11,19].

To improve the current trend, natural products (NPs) are good candidates in food preservation and/or drug development due to their rich composition [20,21]. Herbal medicines, generally recognized as safe, are more widely used and more affordable than synthetic ones [22,23]. NPs are also known to work in synergy with existing drugs to combat AMR pathogens [24]. Although the literature on the anti-*Campylobacter* activity of natural products is scanty, *Cinnamomum* cassia (L.) J.Presl is a known traditional Chinese medicine for treating various diseases, while *Scutellaria baicalensis* Georgi is effective against *Helicobacter pylori* [25,26], which is phylogenetically closely related to *Campylobacter* [2]. Studies have shown that cinnamon oil works well against *Campylobacter* species and several other pathogens [27–29]. *Mentha canadensis* L. proved to inhibit both *C. jejuni* and *H. pylori* [30,31] and it is also effective in the treatment of dysentery [31]. *Meehania urticifolia* (Miq.) Makino is known for its phenolic compounds but its antimicrobial activity is still poorly reported [32]. Clove oil and its major phytochemical eugenol are known for their antimicrobial and virulence-modulating activities against *Campylobacter* species [28,33]. Emodin has been found to inhibit *Pseudomonas aeruginosa* [34], while kuraridin had activity against different pathogenic bacteria [35] and reoviruses [36]. Cinnamaldehyde has been reported to possess antimicrobial properties against various pathogens [37]. Therefore, NPs could become potential sources for ensuring the safety of food items during this period when resistance to antimicrobials and tolerance to methods used in food industries are escalating [38,39].

It has been recognized that medicinal plants are equipped with bioactive compounds used for prophylaxis and therapeutic purposes [40]. It is estimated that 87% of populations from low- and middle-income countries rely on medicinal plants for their healthcare [41–43]. Several millions of NPs exist but only a small number of them have been explored for anti-*Campylobacter* activities. Considering that *Campylobacter* is a public health concern and one of the pathogens on the World Health Organization (WHO) list for which drug development is an emergency [42,43], it is imperative to explore possible alternative solutions through the use of NPs that can be candidates for drug development. With this background, the present study evaluates the susceptibility of *Campylobacter* strains to selected NPs and frontline antimicrobials.

2. Results

Each *Campylobacter* strain was confirmed to species level based on culture, PCR products (Figure 1), and sequencing. A basic local alignment search tool (BLAST) analysis showed a 99% similarity between *C. coli* from chicken (CC–CI) and *C. coli* YH502 (CP018900.1) isolated from retail chicken. The BLAST also showed a 100% similarity between *C. jejuni* from chicken (CJ–CI) and *C. jejuni* (CP047481.1) isolated from patients with gastrointestinal disease in Chile. The detection rates and species distribution related to fecal samples collected from the poultry farm are not presented in this manuscript.

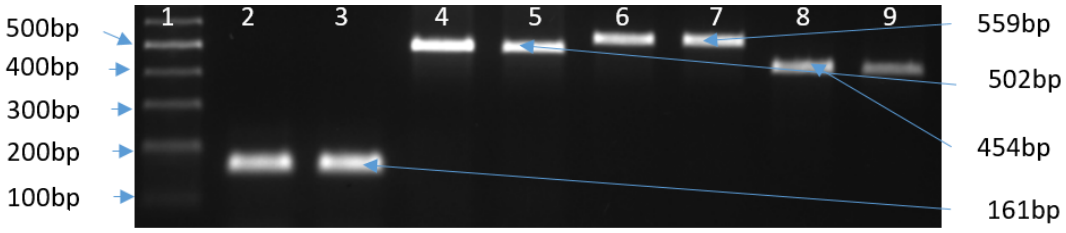

Figure 1. Agarose gel image showing bands of *C. jejuni*, *C. coli*, *tet*(O), and *gyr*A, where 1: marker; 2: CJ-RS; 3: CJ-CI; 4: CC-RS; 5: CC-CI; 6–7: *tet*(O) gene (559 bp), and 8–9: *gyr*A gene (454 bp) from antibiotic-resistant strains (CJ–CI and CC–CI).

The five plant extracts (Table 1), along with essential oils (EOs), pure phytochemicals, and antibiotics, were tested against four *Campylobacter* strains. The concentrations used ranged from 25–6400 μg/mL for plant extracts, 6.25–1600 μg/mL for EOs and phytochemicals, and 0.06–512 μg/mL for antibiotics.

Table 1. Information on used plant extracts.

Library Code	Family	Scientific Name	Common Name	Collection Site	Collection Date	Part of Plant	Extraction Solvent
BE0005B1	Lamiaceae	*Meehania urticifolia* (Miq.) Makino	Nettle-leaf mint	Gangneung, Gangwon	2016	Aerial part	Ethanol
BE0165A1	Lamiaceae	*Scutellaria baicalensis* Georgi	Skullcap	Yeosu, Jeonnam	2017	Root	Ethanol
BE0167A1	Lamiaceae	*Mentha canadensis* L.	Wild mint	Andong, Gyeongbuk	2017	Aerial part	Ethanol
BE1192A1	Lamiaceae	*Salvia plebeia* R.Br.	Common sage	Paju, Gyeonggi	2015	Whole plant	Ethanol
BEA585A1	Lauraceae	*Cinnamomum* cassia (L.) J.Presl	Cinnamon	Gyeongdong Seoul	2015	Bark	Ethyl acetate

For *C. jejuni* strains, the MIC values for extracts were from 200–800 μg/mL, with *C. cassia* being the most active against all the four strains (MIC: 200 μg/mL). The MIC value for the other extracts was 400 μg/mL, except for *Mentha canadensis* L. and *Salvia plebeia* R.Br. against CJ–CI, which had higher values (MIC: 800 μg/mL). The MIC values for EOs, pure phytochemicals, and ERY were the same for both CJ–RS and CJ–CI. In contrast, CJ–CI showed resistance to ciprofloxacin, nalidixic acid, and tetracycline while CJ–RS was sensitive to all antimicrobials (Table 2).

For the EOs, cinnamon oil, and its phytochemical (E)- Cinnamaldehyde had the lowest MIC of 25–50 μg/mL against all tested strains. For baicalein, a phytochemical from *S. baicalensis*, the MIC values were 32 and 64 μg/mL for *C. jejuni* and *C. coli*, respectively. For kuraridin, the MIC value was

48 μg/mL for all the four strains, while for emodin, the MIC values were 50 μg/mL for *C. jejuni* and 200 μg/mL for *C. coli*.

For *C. coli* strains, the MIC value for *C. cassia* was 200 μg/mL, while the MIC value for the remaining extracts was 400 μg/mL. The MIC values for EOs and pure phytochemicals were the same for both *C. coli* strains. However, for antibiotics, CC–CI was resistant to CIP, NAL, and TET, while CC–RS was sensitive to all used antimicrobials.

In general, the strains isolated from chicken showed sensitivity to gentamicin and erythromycin, but they were resistant to quinolones and tetracycline (Table 2).

The MBC values for all the strains ranged from 25–1600 μg/mL, with (E)-Cinnamaldehyde and cinnamon oil showing the lowest values (25–100 μg/mL). The MBC values for plant extracts varied between 400 and 1600 μg/mL, with *C. cassia* showing the lowest MBC of 400 μg/mL. The MBC values for ciprofloxacin and nalidixic acid varied between 64 and 256 μg/mL, while it varied between 128 and 512 μg/mL for tetracycline for the chicken isolates (Table 2). The PCR results show that chicken isolates possess *gyr*A and *tet*(O) genes, which confirm the phenotypic results from MIC determination (Figure 1). However, *cme*B was absent in both *Campylobacter* isolated from chicken by PCR. After sequencing PCR products, chicken isolates exhibited mutations for *gyr*A and *tet*(O). The Thr86Ile point mutation for *C. jejuni* and *C. coli*, associated with resistance to quinolones (CIP and NAL), was confirmed by the sequencing of PCR products (Figure 2).

Table 2. The minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) in μg/mL of different natural products (NPs) and antibiotics against *Campylobacter* strains.

NP/Antibiotic	CJ–RS		CC–RS		CJ–CI		CC–CI	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
M. urticifolia	400	800	400	800	400	800	400	800
S. baicalensis	400	800	400	800	400	800	400	800
M. canadensis	400	800	400	800	800	1600	400	800
S. plebeia	400	800	400	800	800	1600	400	800
C. cassia	200	400	200	400	200	400	200	400
Clove oil	50	100	100	400	50	100	200	400
Cinnamon oil	25	25	50	100	25	50	50	100
Eugenol	50	100	100	200	50	100	100	200
(E)-Cinnamaldehyde	25	25	50	50	25	50	50	50
Baicalein	32	64	64	64	32	64	64	64
Kuraridin	48	ND	48	ND	48	ND	48	ND
Emodin	50	ND	200	ND	50	ND	200	ND
Ciprofloxacin	0.125	1	0.5	1	32	64	64	128
Erythromycin	0.5	1	1	4	0.5	1	2	4
Gentamicin	2	8	2	8	1	2	1	8
Tetracycline	1	4	1	4	256	512	64	128
Nalidixic acid	16	32	8	32	128	256	64	128

ND = not determined

The Genbank accession numbers registered for DNA *gyr*A sequences of *C. jejuni* and *C. coli* in this study are MT947448 and MT947449, respectively. Furthermore, MT947448 and MT947449 exhibited two silent mutations each (AGT to AGC for Ser119Ser and GCC to GCT for Ala120Ala in *C. jejuni*; TTT to TTC for Phe99Phe and GCG to GCA for Ala122Ala in *C. coli*). The sequences for *gyr*A genes showed similarity to known sequences from GenBank (Figure 2).

The resistance to tetracycline was confirmed to be plasmid-mediated as the *tet*(O) gene of *C. jejuni* (MT967269) and *C. coli* (MT967270) exhibited 100% similarity with *tet* (O)-resistance genes of *C. jejuni* and *C. coli* sequences in Genbank (data not shown).

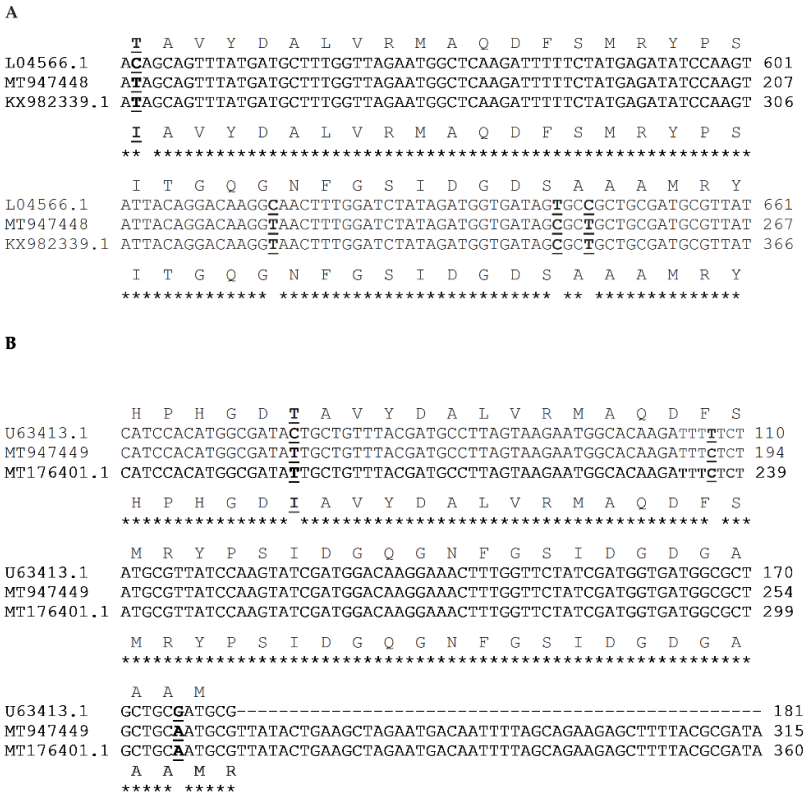

Figure 2. Mutations in *gyr*A sequences of *C. jejuni* (**A**) and *C. coli* (**B**). The mutation (Thr86Ile) is caused by the change from ACA to ATA (*C. jejuni*) and ACT to ATT (*C. coli*). Silent mutations in *gyr*A are also depicted. Mutations are bolded and underlined. L04566.1 and U63413.1 are standard strains (without mutation), while KX982339.1 and MT176401.1 are resistant strains. MT947448 and MT947449 are chicken isolates of this study.

3. Discussion

The objective of this study is to evaluate the susceptibility of *Campylobacter* strains to various NPs and frontline antibiotics. Cinnamon extract, oil, and trans (E)-Cinnamaldehyde had the lowest anti-*Campylobacter* activities, ranging from 25 to 200 μg/mL, which concurs with previous results where the range was from 46.8–600 μg/mL [28,44,45]. However, the MIC for cinnamon oil was lower than the 1000 μg/mL reported against *Campylobacter* strains in Egypt [28]. Clove oil and its major compound eugenol had MICs varying from 50–100 μg/mL, which are higher than the previously reported value of 20 μg/mL for clove oil [33] but lower than the 500 μg/mL reported for eugenol [28]. Other studies have also reported the strong anti-*Campylobacter* potential of cinnamon and clove oil [45,46]. Essential oils are given to broilers to control *Campylobacter* [46]. The difference in MIC values could be associated with the method used as some researchers used an agar-based method instead of the recommended broth microdilution [28]. Other probable reasons could be the location and extraction procedures [47] or the presence of biofilm, virulence, and antibiotic-resistance genes [3].

Except for cinnamon, other extracts had MIC values varying between 400 and 800 μg/mL. The susceptibility of screened extracts was found to be moderate to weak according to the classification of Kuete, where the activity is considered as significant (MIC < 100 μg/mL), moderate (100 < MIC < 625 μg/mL), and weak (MIC > 625μg/mL) [48]. There is a dearth of information on the biological activity of *M. urticifolia*. However, it is expected to have antimicrobial activities attributed to phenolic compounds and hyaluronidase inhibitory phenylpropanoids [32,49,50]. *Scutellaria baicalensis* Georgi is used in the treatment of *H. pylori* infections, and it is advocated to be a source of new drugs against *H. pylori, which is* closely related to *Campylobacter* [26,51]. *Scutellaria baicalensis* Georgi has also been reported to inhibit *Staphylococcus aureus* [52]. Baicalein, a major compound from *S. baicalensis* Georgi,

had a MIC of 32–64 μg/mL, which concurs with the previous report on *S. aureus* [53]. *Mentha canadensis* L., known as an antidiarrheic and antidysentery plant [31], has been reported to inhibit *H. pylori* and *C. jejuni* [30,31]. It possesses monoterpenes, mainly menthol, which increases membrane permeability, leading to the loss of intracellular contents [31]. The antimicrobial activities of *S. plebeia* on different pathogens have been extensively reported [54].

The MIC of Kuradin against all isolates was 48 μg/mL which is more or less similar to the value of 50 μg/mL reported for *S. aureus* [55], but higher than a value of 20 μg/mL previously reported for different bacteria [35]. Kuraridin, from *Sophora flavescens*, has been previously reported as a potential antimicrobial compound [35,36,56]. The MIC of emodin against *C. jejuni* was 50 μg/mL, which is slightly lower than the 70–90 μg/mL previously reported for *P. aeruginosa* and *S. aureus* [34]. However, the MIC of 200 μg/mL against *C. coli* was higher than the reported values by Basu et al. [34]. The literature on both kuraridin and emodin is scanty, and there are no previous findings against *Campylobacter* species. Further studies on *Campylobacter* strains from different sources are needed to confirm the effectiveness of both kuraridin and emodin.

The chicken isolates exhibited resistance to quinolones (CIP and NAL) and tetracycline. These results support previous reports of increased resistance to fluoroquinolones in *C. jejuni* strains from various sources, including chickens [57]. *Campylobacter* strains are becoming more resistant to drugs of choice, and this has been associated with the irrational use of various antibiotics in animal husbandry [58], mainly poultry [7,8]. Apart from the point mutation in *gyr*A, increased resistance to quinolones has been associated with the broad use of fluoroquinolones in the human population and veterinary medicine [59]. Furthermore, ciprofloxacin is used in treating diarrhea cases of unknown etiology, and once acquired, resistance to fluoroquinolones can be maintained in populations even after being banned in animal production [18]. In South Korea, the use of fluoroquinolones in veterinary medicine was banned in July 2020 [60,61]. The mutation in *gyr*A (Thr86Ile) confers resistance to ciprofloxacin and nalidixic acid [62]. However, a different mutation (Thr86Ala) in *gyr*A has been associated with resistance to nalidixic acid alone [63]. The Thr86Ile mutation was found in *Campylobacter* species isolated from chicken (this study), which is in agreement with the broth microdilution and PCR results. The same mutation has been associated with high-level resistance to quinolones [63,64].

The sequence of the *tet*(O) gene (MT967269 and MT967270) was similar to other *tet*(O) genes from the Genbank strains (81-176, NG_048260.1, CP044175.1). The high-level resistance to tetracycline is common in *Campylobacter* strains isolated from humans and broilers [6], and it has been attributed to the *tet*(O) gene found either on plasmids or bacterial chromosome [15,16]. The used NPs inhibited all the strains, including those resistant to tested quinolones and tetracycline.

The ultimate goal of screening for antimicrobial activities from plant-derived products is to avail ourselves of products with antipathogenic and anti-inflammatory potencies that can be used in either prevention or treatment of campylobacteriosis [65]. However, in vivo studies for the anti-*Campylobacter* activities of NPs are limited, possibly due to a lack of suitable infection models [66]. For instance, Hlashwayo et al. [42] recently reported that not even a single in-vivo study had been published in sub-Saharan Africa (SSA) on the antimicrobial activities of plants used to treat campylobacteriosis. The screened NPs may be candidates for in-vivo studies using different models.

The *C. jejuni* isolated from chicken (MT947450, CJ–CI) showed 100% similarity with *C. jejuni* (CP047481.1) isolated from patients with gastroenteritis in Chile. This shows the possible transmission of *Campylobacter* species from poultry to humans, and several reports have shown an association between human and poultry isolates when drinking contaminated water or eating undercooked meat [67,68]. Chicken is known as the major reservoir of human campylobacteriosis due to its high body temperature, which is suitable for *Campylobacter* growth [69], and increased poultry consumption [69,70]. Therefore, control measures and adherence to hygienic practices are required to reduce the transmission of *Campylobacter* from animals to humans. We also recommend studies on the synergistic activities of both NPs and existing antibiotics aimed at reducing the MIC values of drugs of choice and, thus, helping to slow down antimicrobial resistance and extend the effectiveness of existing antibiotics.

4. Materials and Methods

4.1. Sampling Site

Chicken fecal samples were collected from a layer poultry farm located in Gangneung city, Republic of Korea. The farm uses an intensive poultry rearing system, and chickens are dispatched into battery cages inside a closed house. The farm adheres to hygienic practices by the use of footbath disinfectant at the entrance and cleanliness inside the farm.

4.2. Sample Collection, Campylobacter Isolation, and Antimicrobial Testing

Pen floor fecal samples were collected using sterile cotton swabs, which were then placed on ice and transported to the laboratory within one hour. These samples were inoculated onto modified charcoal cefoperazone deoxycholate agar (mCCDA) (Oxoid Ltd., Basingstoke, UK) containing the *Campylobacter* mCCDA selective supplement, SR155E (Oxoid Ltd.). Plates were incubated at 37 °C for 48 h under microaerophilic conditions generated by CampyGen™ gas sachets (Oxoid Ltd.), as previously described [71]. Typical colonies of *Campylobacter*, with the features of being moistened, gray, flat, and a tendency to spread [72], were subcultured on Mueller Hinton agar supplemented with 5% defibrinated horse blood and incubated at 37 °C for 48 h under microaerophilic conditions generated by CampyGen™ gas sachets (Oxoid Ltd.). Species confirmation was performed by PCR and sequencing. *Campylobacter* isolates were preserved at −80 °C in Mueller Hinton broth (MHB) supplemented with 25% glycerol (*v*/*v*). Apart from chicken isolates, *Campylobacter jejuni* (ATCC® 33560™) and *Campylobacter coli* (ATCC® 33559™) were also used. For antibacterial activity assays, bacterial inoculum of 0.5 McFarland (1–5 × 10^8 CFU/mL) was prepared from fresh colonies taken from MHA plates supplemented with 5% defibrinated horse blood (Oxoid Ltd., Basingstoke, Hampshire, England) and dissolved in sterile normal saline (0.85%). The absorbance was recorded spectrophotometrically at 600 nm (Synergy HT; BioTek Instruments Inc., Winooski, VT, USA).

Four strains were used in this study. In the case of *C. jejuni*, the reference strain (ATCC® 33560™) and the chicken isolate (MT947450) were named CJ–RS and CJ–CI, respectively. In the case of *C. coli*, the reference strain (ATCC® 33559™) and the chicken isolate (MT947451) were named CC–RS and CC–CI, respectively.

4.3. DNA Extraction, PCR, and Sequencing

Genomic DNA was extracted from pure colonies using the Qiagen QIAamp® PowerFecal® kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, followed by multiplex-PCR using genus-specific primers (C412F; C1228R), *cj0414* gene primers (C1; C3), and *ask* gene primers (CC18F; CC519R) (Table 3), as previously described with modification [73]. *cj0414* is a conserved gene coding for a fragment of a putative oxidoreductase subunit gene (PID 6967888; Cj0414) of *C. jejuni*, while *ask* encodes aspartokinase, highly specific for *C. coli* [74–76]. The PCR mixture (25 μL) contained 12.5 μL of 2× Master Mix (Thermo Fisher Scientific, Seoul, Korea), 1 μL of each primer, 1.5 μL of DNA, and 7 μL of sterile deionized water. The cycling conditions were one cycle of 95 °C for 5 min, 35 cycles each of 94 °C for 30 sec, 55 °C for 45 sec, and 72 °C for 45 sec, and a final extension at 72 °C for 7 min using a MiniAmp™ Plus thermal cycler (Applied Biosystems, MA, USA). The PCR products were held at 4 °C before analysis.

For the antibiotic resistance genes (*tet*(O), *gyr*A, and *cme*B), m-PCR was performed using specific primers (Table 3), as previously described [77,78]. PCR products were analyzed by gel electrophoresis. The bands of the amplification products were compared to the Dyne 100 bp DNA ladder (Dyne bio, Seongnam, Korea). Bands of PCR products were observed and photographed with an iBright™ CL1000 imaging system (Thermo Fisher Scientific, Seoul, Korea). The purification of PCR products was performed with the Pure Link™ Quick PCR purification kit (Invitrogen, Vilnius, Lithuania) and sequenced by the Sanger method at SolGent (Solutions for Genetic technologies, Daejeon, Korea).

Table 3. Target genes, primer sequences, and amplification conditions.

Target Gene	Primer Name	Sequence (5′–3′)	Amplicon Size	Annealing T (°C)	Reference
16S rRNA	C412F	GGATGACACTTTTCGGAGC	816	55	[73]
	C1228R	CATTGTAGCACGTGTGTC			
cj0414	C1F	CAAATAAAGTTAGAGGTAGAATGT	161		
	C3R	CCATAAGCACTAGCTAGCTGAT			
ask	CC18F	GGTATGATTTCTACAAAGCGAG	502		
	CC519R	ATAAAAGACTATCGTCGCGTG			
tet(O)	*tet*(O)F	GCGTTTTGTTTATGTGCG	559		[77,78]
	tet(O)R	ATGGACAACCCGACAGAAG			
*cjgyr*A	QRDRF	GCCTGACGCAAGAGATGGTTTA	454		
	QRDRR	TATGAGGCGGGATGTTTGTCG			
*cme*B	*cme*BF	TCCTAGCAGCACAATATG	241		
	*cme*BR	AGCTTCGATAGCTGCATC			

4.4. Natural Products and Antibiotics

4.4.1. Plant Extracts, EOs, Pure Phytochemicals, and Conventional Antimicrobials

Plant extracts (Table 1), kuraridin and emodin, were obtained from the library of KIST Gangneung Institute of Natural Products. Except for *Cinnamomum* cassia (L.) J.Presl (BEA585A1), extracts were prepared by heat reflux extraction performed with a 10-g dried plant and 0.1 L ethanol for 2 h, twice. The ethyl acetate extract of BEA585A1 was obtained by fractionation with ethyl acetate from the water extract of dried bark of cinnamon, prepared with water reflux for 2 h. Essential oils (clove, cinnamon bark), pure phytochemicals (eugenol, trans (E)-Cinnamaldehyde, and baicalein), and antibiotics (ciprofloxacin, erythromycin, tetracycline, nalidixic acid, and gentamicin) were supplied from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of the plant extracts and EOs were dissolved in dimethylsulphoxide (DMSO). Antibiotics were dissolved as per the manufacturer's instructions. Ciprofloxacin and erythromycin were dissolved in 0.1 N HCl and 70% ethanol, respectively. Gentamicin and tetracycline were dissolved in water, while nalidixic acid was dissolved in DMSO. The solutions of antibiotics were filter-sterilized before being used.

4.4.2. Determination of MIC and MBC

The MIC and MBC for the tested NPs and antibiotics were determined by broth microdilution using 96-well plates (Greiner-bio-one, Kremsmünster, Austria) [79]. Briefly, 100 μL of the antimicrobials at working concentrations were pipetted to the first column of a plate. After two-fold serial dilutions by MHB across the plate, all wells were inoculated with 50 μL of inoculum except for the negative controls. Control wells were prepared with culture medium (sterility control), plant extract (negative control), bacterial suspension (positive control), and DMSO in amounts corresponding to the highest quantity present. The highest amount of DMSO in the test well was 2% for extracts and less than 0.5% for essential oils and pure phytochemicals. The DMSO at the highest concentration (2%) did not affect bacterial growth, as previously described [80]. Then, incubation was done at 37 °C for 48 h in microaerophilic conditions. All tests for antimicrobial susceptibility were repeated six times for reproducibility. The MICs were evaluated spectrophotometrically by measuring the bacterial concentration at an absorbance of 600 nm using a microplate reader (Synergy HT; BioTek Instruments Inc., Winooski, VT, USA). The MIC was confirmed by the addition of iodonitrotetrazolium chloride (INT), followed by agitation at 37 °C for 30 min in the dark. The MIC

was defined as the lowest concentration of the antimicrobial agent that results in a significant decrease (>90%) in inoculum viability after 48 h, as previously described, with modification on incubation time [81]. Bacterial growth was indicated by the presence of a pink color after the incubation period [82].

The MBC was determined as previously described, with modification [80]. From the wells which did not show growth, a volume of 10 μL was pipetted and streaked on the surface of MHA plates supplemented with 5% defibrinated horse blood (Oxoid Ltd., Basingstoke, Hampshire, England). The MBC was defined as the lowest concentration showing no growth after 48 h of incubation. The MIC values for antibiotics were assessed as per the epidemiological cut-off values of the European Committee for Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org).

4.4.3. Data Analysis

The MIC values were expressed as mean ± standard deviation (SD) for analysis performed in six replicates. One-way analysis of variance (ANOVA) was performed in GraphPad Prism 8.4.0 (GraphPad Software, La Jolla, CA, USA), and the differences among group means were verified by Tukey's multiple comparisons test, with p-value < 0.05 considered as significant.

After the sequencing of PCR products, BioEdit software (version 7.2.6.1) was used to edit, align, and analyze the DNA sequences [83]. The consensus sequences obtained were compared to GenBank strains by a BLAST search, and they were submitted to GenBank to get accession numbers [84]. Standard sensitive strains for *gyr*A mutation, including *C. jejuni* (GenBank accession number L04566.1) and *C. coli* (GenBank accession number U63413.1), were used for comparison with the sequences of this study. For comparison, strains with *gyr*A mutations were also included (KX982339.1 and MT176401.1). For the *tet*(O) gene analysis, different GenBank accession numbers (AM884250, 81-176, and CP044175.1) were used. For the antibiotic resistance genes, sequence alignments were performed with Clustal Omega [85]. Amino acid sequences were deduced from the DNA sequences using the ExPASyTranslate tool [86].

5. Conclusions

The isolates from chicken were sensitive to erythromycin and gentamicin, but they were resistant to quinolones and tetracycline. The mutations in *gyr*A and *tet*(O) were confirmed by DNA sequencing. The tested NPs were active against both antibiotic-sensitive and antibiotic-resistant *Campylobacter* strains. Effective NPs can be exploited by the food processing industry and poultry farms to control foodborne pathogens. There is a need to understand the mode of action of these NPs before they are used in clinical settings.

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Paper Four

Genomic characterization of fluoroquinolone-resistant thermophilic *Campylobacter* strains isolated from layer chicken faeces in Gangneung, South Korea by whole-genome sequencing

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Abstract: Thermophilic *Campylobacter* species of poultry origin have been associated with up to 80% of human campylobacteriosis cases. Layer chickens have received less attention as possible reservoirs of *Campylobacter* species. Initially, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of two archived *Campylobacter* isolates (*Campylobacter jejuni* strain 200605 and *Campylobacter coli* strain 200606) from layer chickens to five antimicrobials (ciprofloxacin, nalidixic acid, erythromycin, tetracycline, and gentamicin) were determined using broth microdilution while the presence of selected antimicrobial resistance genes was performed by polymerase chain reaction (PCR) using specific primers. Whole-genome sequencing (WGS) was performed by the Illumina HiSeq X platform. The analysis involved antimicrobial resistance genes, virulome, multilocus sequence typing (MLST), and phylogeny. Both isolates were phenotypically resistant to ciprofloxacin (MIC: 32 vs. 32 µg/mL), nalidixic acid (MIC: 128 vs. 64 µg/mL), and tetracycline (MIC: 64 vs. 64 µg/mL), but sensitive to erythromycin (MIC: 1 vs. 2 µg/mL) and gentamicin (MIC: 0.25 vs. 1 µg/mL) for *C. jejuni* strain 200605 and *C. coli* strain 200606, respectively. WGS confirmed C257T mutation in the *gyr*A gene and the presence of *cme*ABC complex conferring resistance to FQs in both strains. Both strains also exhibited *tet*(O) genes associated with tetracycline resistance. Various virulence genes associated with motility, chemotaxis, and capsule formation were found in both isolates. However, the analysis of virulence genes showed that *C. jejuni* strain 200605 is more virulent than *C. coli* strain 200606. The MLST showed that *C. jejuni* strain 200605 belongs to sequence type ST-5229 while *C. coli* strain 200606 belongs to ST-5935, and both STs are less common. The phylogenetic analysis clustered *C. jejuni* strain 200605 along with other strains reported in Korea (CP028933 from chicken and CP014344 from human) while *C. coli* strain 200606 formed a separate cluster with *C. coli* (CP007181) from turkey. The WGS confirmed FQ-resistance in both strains and showed potential virulence of both strains. Further studies are recommended to understand the reasons behind the regional distribution (Korea, China, and Vietnam) of such rare STs.

Keywords: *Campylobacter*; layer chicken; fluoroquinolone-resistant; phylogenetic analysis; whole-genome sequencing; Korea

1. Introduction

Worldwide, *C. jejuni* and *C. coli* are considered the leading etiologies of human campylobacteriosis [1,2]. Currently, most of the studies have focused on *C. jejuni*, which is associated with 85% of human infections [1]. However, *C. coli* has not received the same attention, but it is second to *C. jejuni* in causing human campylobacteriosis [2,3]. The major reservoirs include chickens and cattle, but other farm animals or food products and wild birds have been implicated in disease transmission [4–6]. Chicken ceca are colonized by high levels of *Campylobacter* which may persist in feces that are used as biofertilizers [7]. Human campylobacteriosis is of public health concern due to the increased number of *Campylobacter* strains that are resistant to both drugs of choice (macrolides and fluoroquinolones) and alternative therapies (aminoglycosides and tetracyclines) [8]. The missense mutation (C257T) in the quinolone resistance-determining region (QRDR) of *gyr*A has been associated with high-level resistance to quinolones [9]. The widespread FQ-resistant *C. jejuni* lineages via food and travel need urgent monitoring and mitigation strategies [10].

To control *Campylobacter*-related infections, it is necessary to understand virulence factors and molecular mechanisms contributing to pathogenesis [11,12]. WGS data from different pathogenic and non-pathogenic mutant strains have been used to classify virulence gene clusters linked to pathogenicity [13]. Although there are gaps in understanding the pathogenesis of *Campylobacter* [14], the roles played by several virulence factors involved in adhesion, invasion, chemotaxis, and motility are known [12,15]. However, there are various genes coding for other virulence factors, like the lipopolysaccharide (LPS), lipooligosaccharide (LOS), and capsule, which need to be well elucidated [12]. Several studies have confirmed the roles of some of the virulence genes by observing the limited capacities of mutants to attach to, colonize, and invade eukaryotic cells [15,16]. Mutant strains lacking *fla*A and *fla*B were unable to complete the colonization process in chickens [13,17]. Also, *cad*F and *cia*B mutant strains showed a reduced ability to adhere to and invade cell lines [17].

Multilocus sequence typing (MLST) has been the gold standard method used for epidemiological surveillance and source-attribution studies [18,19]. However, MLST does not include clinically important information, like the virulence or antibiotic resistance determinants, mobile genetic elements, nucleotide polymorphism, and other recombination events [20]. *Campylobacter* species can be well characterized based on their virulomes often acquired via horizontal gene transfer [21]. For instance, there are *C. coli* hybrid strains with DNA segments from *C. jejuni*, and MLST failed to genotype such strains [22].

Currently, WGS is considered the most informative and discriminative typing method of bacterial pathogens [2,23]. For instance, the WGS led to the creation of the core genome (cgMLST), a novel typing method encompassing hundreds of loci from the traditional seven loci [24]. Additionally, studies using single nucleotide polymorphism (SNP) allow the establishment of the best phylogenetic relationship among different pathogens [25]. The WGS is used for various purposes including novel antibiotic and diagnostic test development, studying the emergence of antibiotic resistance, disease surveillance, and direct infection control measures in both clinical settings and communities [26]. Next-generation sequencing (NGS) technologies are preferred in pathogen typing due to affordable cost and reduced turnaround time [27]. The NGS systems available include Illumina Genome Analyzer (HiSeq, MiSeq), Life Technologies Ion Torrent, and the PacBio RX system [28]. However, the use of WGS daily in genotyping and pathogen characterization faces hurdles related to bioinformatics, like resources, lack of validated workflows, and expertise, which are all required for data analysis [25]. This makes the efficient use of WGS data in public health investigations very hard [29]. It is important to note that some countries like the US

have incorporated the WGS in routine checking of human pathogens from clinical samples and food.

Despite the progress in understanding the complicated and multifactorial pathogenesis of *Campylobacter* as an enteric pathogen, there is a gap regarding the combination of phenotypic and genotypic characteristics [30]. Furthermore, several epidemiological studies have been carried out on *Campylobacter* species from broiler chickens [31], but there is a dearth of information on *Campylobacter* from layer chickens [7]. Layer chickens have been reported to be the source of antimicrobial-resistant *Campylobacter* strains [7,32]. The WGS allows for comprehensive phylogenetic analyses of several factors associated with virulence or antibiotic resistance [20]. Based on findings of the partial characterization of layer chicken-derived *Campylobacter* isolates, we hypothesize that the WGS-characterized isolates harbor various antimicrobial and virulence-related genes contributing to their pathogenicity. To the best of our knowledge, there are no previous reports of WGS data of *Campylobacter* from layers in South Korea. Hence, the objectives of this study were to genomically characterize two FQ-resistant *C. jejuni* and *C. coli* of layer chicken origin by WGS and to establish phylogenetic relationships of the two isolates to the existing ones.

2. Materials and Methods

2.1. Campylobacter Strains and Culture Conditions

The two *Campylobacter* strains used in this study were selected from our previously published research work [8]. For this experiment, preserved strains were revived by inoculating them onto Mueller Hinton Agar as previously described [33]. Subculturing was performed to get colonies free from glycerol.

2.2. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) against five antimicrobials, including FQs, namely ciprofloxacin (CIP) and nalidixic acid (NAL) (0.25–512 µg/mL), macrolide (erythromycin or ERY) (0.06–64 µg/mL), aminoglycoside (gentamicin or GEN) (0.06–64 µg/mL) and tetracycline (TET) (0.125–1024 µg/mL) was performed by two-fold broth microdilution [34]. The optical density was recorded spectrophotometrically at 600 nm (Synergy HT; BioTek Instruments Inc., Winooski, VT, USA). The same protocol used in our previous study was followed for minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) determination [8]. The AST procedure was done in six replicates for reproducibility. The MIC was measured spectrophotometrically with a microplate reader (Synergy HT; BioTek Instruments Inc., Winooski, VT, USA) and confirmed by the addition of iodonitrotetrazolium chloride.

2.3. DNA Extraction, Species Confirmation, and Antimicrobial Resistance (AMR) Genes Detection

The genomic DNA was extracted from pure colonies using the Qiagen QIAamp® PowerFecal® Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. For genes specific for *Campylobacter* genus and species or genes associated with antimicrobial resistance [*tet*(O), *gyr*A, and *cme*B], PCR was performed using specific primers (Table 1). After electrophoresis, bands of PCR products were observed on a Dual UV Transilluminator (Core Bio System, Huntington Beach, CA, USA) under ultraviolet (UV) light. Bands were compared to the 100 bp marker (Dyne bio, Seongnam-si, Korea). PCR products were purified with AMPure XP beads (Beckman Coulter, Fullerton, CA, USA) and sequenced by the Sanger method at SolGent (Solutions for Genetic Technologies, Daejeon, South Korea). The presence of resistance genes, as well as point mutations in the 23S rRNA and quinolone resistance-determining region (QRDR) of the *gyr*A, *rps*L, and *cme*R genes, was determined using ResFinder (Center for Genomic Epidemiology) with settings of a threshold of 85% identity and a minimum length of 60% [35].

Table 1. Primers used for species and antimicrobial resistance confirmation.

Target Gene	Direction	Sequence (5′-3′)	Amplicon Size	Annealing Temperature (°C)	Reference
16S rRNA	Forward	GGATGACACTTTTCGGAGC	816		
	Reverse	CATTGTAGCACGTGTGTC			
cj0414	Forward	CAAATAAAGTTAGAGGTAGAATGT	161	55	[8]
	Reverse	CCATAAGCACTAGCTAGCTGAT			
ask	Forward	GGTATGATTTCTACAAAGCGAG	502		
	Reverse	ATAAAAGACTATCGTCGCGTG			
tet(O)	Forward	GCGTTTTGTTTATGTGCG	559		
	Reverse	ATGGACAACCCGACAGAAG			
*cjgyr*A	Forward	GCCTGACGCAAGAGATGGTTTA	454	55	[8]
	Reverse	TATGAGGCGGGATGTTTGTCG			
*cme*B	Forward	TCCTAGCAGCACAATATG	241		
	Reverse	AGCTTCGATAGCTGCATC			

2.4. Whole-Genome Sequencing

The extraction of genomic DNA was performed as above and the sequencing library was prepared with the Illumina TruSeq Nano DNA Kit, as per the manufacturer's instructions with a library size of 350 bp. WGS was performed by Illumina HiSeq X technology at Macrogen (Seoul, South Korea) with a read length of 151 bp. The pair-ended reads passed the quality control check, followed by adapter trimming and quality filtering using Trimmomatic (v0.36) [36].

2.5. Construction of Phylogenetic Tree

The genome sequences (from our study) and those collected from public databases (Table 2 were uploaded to the Type (Strain) Genome Server (TYGS), a free bioinformatics platform available online: https://tygs.dsmz.de (accessed on 19 February 2021), for a whole genome-based taxonomic analysis [37]. TYGS employs the Genome-BLAST Distance Phylogeny method (GBDP) [38] to compare whole-genome sequences at the nucleotide level, allowing to calculate the digital DNA-DNA hybridization (dDDH) value and construct the phylogram. Submitted genomes were compared against all type strain genomes available in the TYGS database via the MASH algorithm, a fast approximation of intergenomic relatedness [39], and the 10 type strains with the smallest MASH distances were chosen per submitted genomes. An additional 10 closely related type strains selected by RNAmmer [40] were determined via the 16S rDNA gene sequences, and each sequence was subsequently BLASTed against the 16S rDNA gene sequences of type strains available in the TYGS database [41]. Intergenomic distances were used to infer a balanced minimum evolution tree with branch support via FASTME 2.1.4 including SPR post-processing [42]. Branch support was inferred from 100 pseudo-bootstrap replicates each. The trees were rooted at the midpoint [43] and visualized with PhyD3 [44]. The type-based species clustering using a 70% dDDH radius around each of the 13 type strains was done as previously described [37]. Subspecies clustering was done using a 79% dDDH threshold as previously introduced [45].

Table 2. Genomic features of strains submitted to the TYGS Database.

No	Strain number	Country/Region	Sample Type	Host	Isolation Source	Disease Association
1	*C. jejuni* (CP059968)	China/Henan	Mixed culture	Chicken	Cloacal swab	NA
2	*C. jejuni* (CP012696)	USA/Albany CA	NA	Chicken	Chicken breast from retail	NA
3	*C. jejuni* (CP048756)	China/Zhejiang	Cell culture	Duck	Meat	NA
4	*C. jejuni* (AACIWG01)	USA:TX	NA	Chicken	Feces	NA
5	*C. jejuni* (CP012213)	Finland	NA	Human	Feces	Invasive
6	*C. jejuni* (CP023866)	USA:VA	NA	Chicken	Carcass	NA
7	*C. jejuni* (CP028909)	United Kingdom: London	Mono isolate	Chicken	NA	NA
8	*C. jejuni* (CP023543)	USA:CA	NA	Chicken	Chicken breast	Missing
9	*C. jejuni* (CP017863)	USA: Tulsa	NA	Chicken	Liver	NA
10	*C. jejuni* (CP014344)	South Africa: Cape Town	NA	Human	NA	Enteritis
11	*C. jejuni* (CP053659)	Italy:Lozzo Atesino	Mono isolate	Chicken	Feces	NA
12	*C. jejuni* (CP028933)	South Korea	NA	Chicken	Meat	NA
13	*C. jejuni* (CP059966)	China/Henan	Mixed culture	Chicken	Cloacal swab	NA
14	*C. jejuni* (CP048771)	China/Zhejiang	Cell culture	Duck	Meat	NA
15	*C. jejuni* (CZHP01)	Spain/Madrid	NA	Chicken	Meat	NA
16	*C. jejuni* (CP059970)	China/Henan	Mixed culture	Chicken	Cloacal swab	NA
17	*C. jejuni* (CP059964)	China/Henan	Mixed culture	Chicken	Cloacal swab	NA
18	*C. jejuni* (CP010502)	Finland	Multi-isolate	Human	Blood	Yes
19	*C. jejuni* (CP017229)	South Korea: Seoul	NA	Human	Stool	Food poisoning
			C. coli			
1	*C. coli*(CP061537)	USA: Pennsylvania	NA	Chicken	NA	NA
2	*C. coli*(CP023545)	USA:CA	NA	Chicken	Chicken breast	NA
3	*C. coli*(CP019977)	United Kingdom: Lincolnshire		Organic chicken farm		
4	*C. coli*(CP027634)	Germany: Berlin	NA	Turkey	Meat	Colonization
5	*C. coli*(CP046317)	USA: VA	NA	Human	Gastrointestinal tract	Unknown
6	*C. coli*(CP017868)	USA: Tulsa	NA	Chicken	Chicken liver from retail	NA
7	*C. coli*(CP017873)	USA: Tulsa	NA	Chicken	Chicken liver from retail	NA
8	*C. coli*(CP027638)	Germany: Berlin	NA	Turkey	Meat	Colonization
9	*C. coli*(CP011015)	United Kingdom: Cambridge	NA	Human	Feces	NA
10	*C. coli*(CP035927)	USA	NA	Chicken	Carcass	NA
11	*C. coli*(CP018900)	USA: Wyndmoor, Pennsylvania	NA	Chicken	Carcass/Retail	NA
12	*C. coli*(CP058340)	USA	Cell culture	NA	Environmental	NA
13	*C. coli*(CP040239)	United Kingdom: Sutton Bonington	NA	Cattle	Slurry	NA
14	*C. coli*(CP006702)	United Kingdom	Monoisolate	Human	NA	Gastroenteritis
15	*C. coli*(CP038868)	China: Shanghai	NA	Chicken	Cecum	NA
16	*C. coli*(CP028187)	Denmark	NA	NA	Missing	NA
17	*C. coli*(CP017875)	USA: Tulsa	NA	Pig	Pork	NA
18	*C. coli*(CP007181)	Missing	NA	Turkey	Missing	Missing

NA: not applicable.

2.6. Data Analysis

The MIC values were interpreted using epidemiological cut-off values of the European Committee for Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org, accessed on 28 November 2020).

BioEdit software (version 7.2.6.1) (http://www.mbio.ncsu.edu/BioEdit/bioedit.html, accessed on 19 February 2021) was used to edit, align, and analyze the DNA chromatograms [46]. A BLAST search was performed to compare consensus sequences (*gyr*A and *tet*(O)) with those from the GenBank database. Standard sensitive strains (L04566.1 and U63413.1) and resistant strains (KX982339.1 and MT176401.1) for *gyr*A were used for comparison. For the *gyr*A gene, the comparison was performed with Clustal Omega [47]. Amino acid sequences were deduced from the DNA sequences using the ExPASyTranslate tool [48].

For bioinformatics analysis, the filtered reads were mapped to reference genomes (NCTC11168 and NCTC11366) using Burrows-Wheeler Aligner (BWA-MEM), followed by variants identification and annotation. Produced mass sequence data were used to search for genetic variation based on the NCBI reference genome. After removing duplicates with Sambamba (v0.6.7) [49] and identifying variants with SAMTools [50], information on each variant was gathered and classified. SnpEff [51] was used to predict the variant effect at the protein level. Data was paired and assembled using SKESA assembler [52] while Quality Assessment Tool for Genome Assemblies QUAST [53] was used for assembly statistics and the genomes were annotated using Prokka [54]. Acquired AMR genes and point

mutations conferring resistance to antimicrobials were searched using Abricate (https://github.com/tseemann/abricate, accessed on 22 January 2021) and NCBI's AMRFinderPlus database [55]. Virulence genes were screened with VFDB [56]. The genomes deposited in GenBank were further annotated with PGAP version 5.1 [57]. GenBank accession numbers JAFETJ000000000 and JAFETK000000000 for *C. jejuni* and *C. coli*, respectively, were given after submission.

3. Results

3.1. Antimicrobial Resistance Profiles

The phenotypic AMR results revealed high-level resistance of *C. jejuni* strain 200605 and *C. coli* strain 200606 to ciprofloxacin (CIP), nalidixic acid (NAL), and tetracycline (TET) with MIC values ranging between 32 µg/mL and 128 µg/mL. Also, *C. jejuni* strain 200605 and *C. coli* strain 200606 were sensitive to erythromycin (MIC: 1 vs. 2 µg/mL), and gentamicin (MIC: 0.25 vs. 1 µg/mL), respectively.

PCR confirmed the presence of DNA of *gyr*A and *tet*(O) genes, but no band was seen for *cme*B. WGS confirmed the presence of the C257T point mutation in the quinolone resistance-determining region of the *gyr*A gene of both strains. Abricate and Resfinder [35] confirmed the phenotypic data related to FQ-resistance (C257T mutation). Furthermore, *tet*(O/32/O) and *tet*(O) genes associated with resistance to doxycycline, tetracycline, and minocycline were found in both isolates by the WGS. Apart from $bla_{\text{OXA-452}}$ found in both isolates, *C. jejuni* strain 200605 also showed the $\text{bla}_{\text{OXA-521}}$ and $bla_{\text{OXA-193}}$ genes. The detection of PointFinder genes returned mutations in *gyr*A and 23S rRNA genes, but no mutations were found in *cme*R and *rps*L for *C. jejuni* strain 200605. Conversely, *cme*R was not detected, while *rps*L was found but without a mutation for *C. coli* strain 200606. The latter also showed 12 point mutations in 23S rRNA. Mass screening of contigs of both isolates using ABRicate also showed resistance to cephalosporin, penam, and the presence of *cme*B (efflux pump) conferring resistance to different antimicrobials.

3.2. Whole-Genome Sequencing Data

The annotation of the *C. jejuni* strain 200605 genome with PGAP returned 116 contigs: 1808 genes, of which 1688 were CDSs (with protein), 41 were RNAs (35 tRNAs, 3 ncRNAs, 1 rRNA), and 79 were pseudogenes (67 frame-shifted genes, 11 incompletes, 15 internal stops, and 13 multiple problems).

The annotation of the *C. coli* strain 200606 genome returned 29 contigs: 1,865 genes, of which 1743 were CDSs (with protein), 42 were RNAs (36 tRNAs, 3 ncRNAs, and 1 rRNA), and 80 were pseudogenes (62 frame-shifted genes, 16 incompletes, 12 internal stops, and 7 multiple problems). Additional details of both strains are given in Table 3 and were made publicly available on BioProject PRJNA694501.

Table 3. Genome characteristics and accession numbers of *C. jejuni* and *C. coli* strains.

Strain	SRA Accession No.	Reference Length	Mapped Site	Total Read	Mapped Read	Variant	GC (%)	Q30 (%)
C. jejuni strain 200605	SAMN17525986	1,641,464	1,596,540	9,800,132	8,152,436	22,266	30.12	97.48
C. coli strain 200606	SAMN17525987	1,938,580	1,584,482	9,922,508	8,616,294	46,102	31.19	97.27

Of the called variants (Table 3), SNPs, insertions, deletions, transitions, and transversions were 21,816; 231; 219; 18,333; and 3483, and 45,561; 284; 257; 32,766; and 12,795 for *C. jejuni* strain 200605 and *C. coli* strain 200606, respectively.

3.3. Virulence Genes

C. jejuni strain 200605 and *C. coli* strain 200606 showed 87 and 57 virulence genes, respectively (Supplementary File S1). Adhesion factors (*cad*F, *peb*A, and *jlp*A), a cytolethal distending toxin (*cdt*ABC), invasion genes (*cia*B, *cia*C), and a biofilm formation gene (*ept*C) were only found in *C. jejuni* strain 200605 and not in *C. coli* strain 200606. However, both strains harbor genes coding for lipooligosaccharides (LOS), lipopolysaccharides (LPS),

capsular (*gmh*, *waa*, and *kps* genes), chemotaxis (*che*A, *che*V, *che*W), and motility (*flh*, *fla*, *flg*, *ptm*) factors.

3.4. Phylogenetic Analysis

The Genome BLAST Distance Phylogeny (GBDP) approach used to generate a phylogenomic tree (Figure 1) shows that *C. jejuni* strain 200605 forms a cluster with CP014344, which was isolated from a human in South Africa. It is also closely related to other strains of chicken origin from several countries including South Korea (CP028933), the USA (CP023866, CP017863), and China (CP059968, CP059970). However, it is separated from another cluster of CP059964 (chicken) and CP048756 (duck), both from China (Figure 1). There were no differences among the species, subspecies, and percent G+C data of all *C. jejuni* strains used to generate the tree except for the *C. jejuni* (CP010502) strain that was isolated from human blood in Finland. The genome size was slightly higher compared to isolates from Type (Strain) Genome Server (TYGS), and it varied from 1.48–1.94 Mbp.

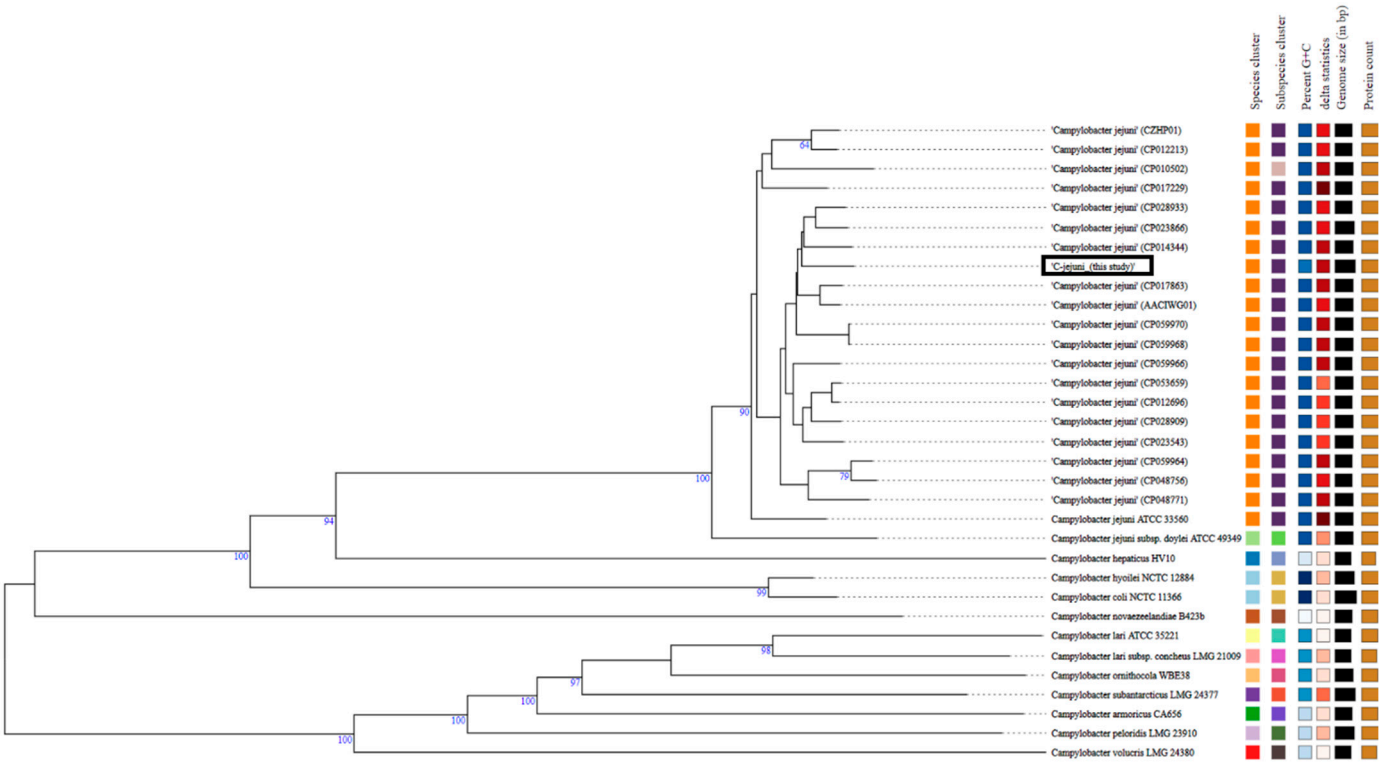

Figure 1. Type (Strain) Genome Server (TYGS) result for *C. jejuni* strain 200605 dataset. Tree inferred with FastME 2.1.4 [42] from GBDP distances calculated from genome sequences. Branch lengths are scaled in terms of GBDP distance formula d_5; numbers above branches are GBDP pseudo-bootstrap support values from 100 replications. Percent G+C (27.39–30.98); δ statistics (0.138–0.286); protein content (1379–2041).

The GBDP phylogenomic tree (Figure 2) shows that *C. coli* strain 200606 formed a separate cluster (species and subspecies) along with *C. coli* (CP007181) that was isolated from turkey and belongs to the same ST-1150 as the isolate of this study. Also, δ values were lower (0.181–0.175) than values for the cluster at the top of the tree (>0.2) (Figure 2). The overall treelikeness of the data set appeared to be high (low δ values). Briefly, δ statistics calculated using distance matrices allow for assessing the impact of individual operational taxonomic units (OTUs) on overall treelikeness (the lower the δ values, the better the treelikeness) [37].

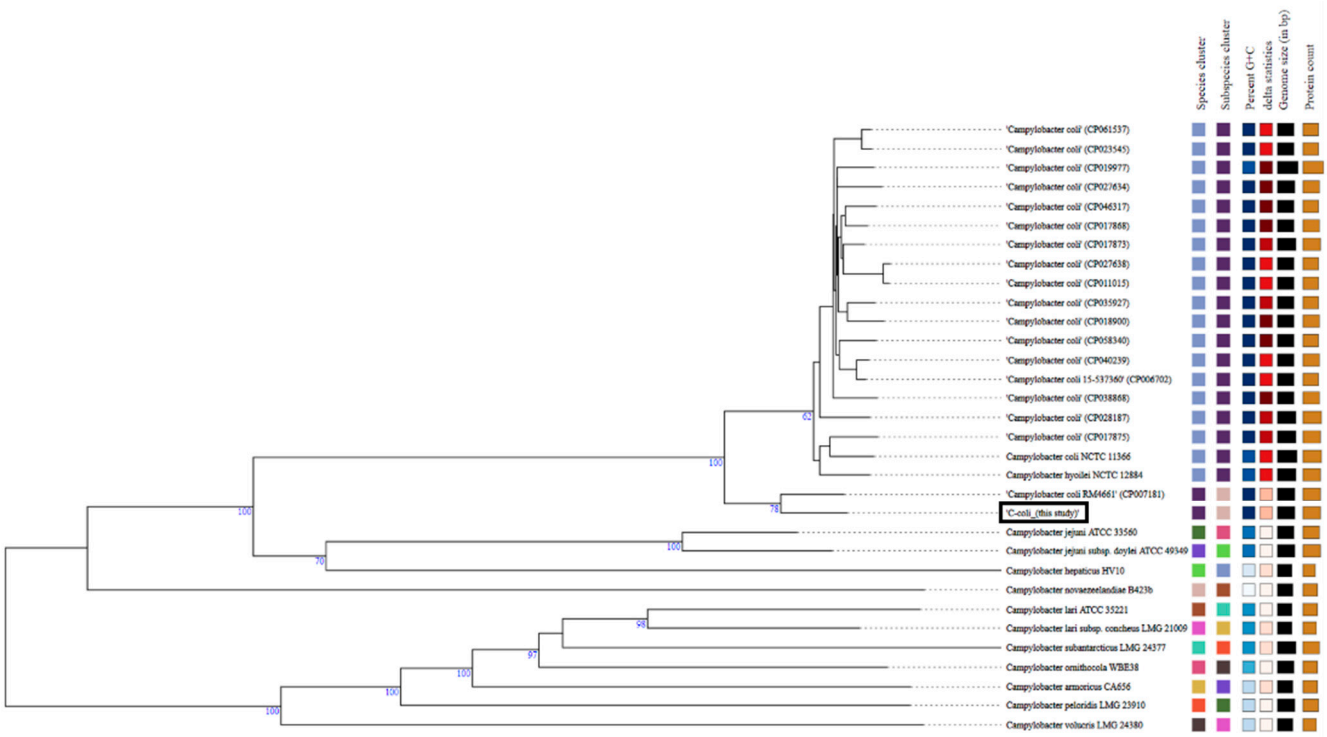

Figure 2. Type (Strain) Genome Server (TYGS) result for *C. coli* strain 200606 dataset. Tree was generated as for *C. jejuni*. Percent G+C (27.39–31.5); δ statistics (0.137–0.295); protein content (1379–2162).

3.5. Multilocus Sequence Typing (MLST)

C. jejuni strain 200605 belongs to ST-5229. So far, ST-5229 has not been assigned to a given clonal complex (CC). *C. coli* strain 200606 belongs to ST-5935, which belongs to CC-1150.

4. Discussion

Although the prevalence of *Campylobacter* spp. in table eggs is low, there is limited knowledge of their prevalence and ecology in layer chickens. Also, studies on the antimicrobial resistance profiles of layer chicken-derived *Campylobacter* isolates are limited [7,32]. This implies that the available data on whole-genome sequences of *Campylobacter* from layers important for epidemiological studies are also scanty.

This study highlights the genomic characterization and phylogenetic analysis of two FQ-resistant strains from layers in Gangneung. The isolates showed increased resistance to FQs. The resistance to ciprofloxacin has been attributed to two loci that were found in our isolates. The first one is the C257T point mutation in the *gyr*A gene, while the second factor is the *cme*ABC operon coding for an efflux pump [9,58]. Increased resistance of *Campylobacter* strains to FQs has been previously reported in Korea [59,60] and worldwide [9,61], but these strains are known to be highly persistent, even in the absence of the use of FQs [62,63]. The wide use of some FQs (enrofloxacin) in poultry farming has been associated with the spread of resistant *Campylobacter* strains and may explain the increasing resistance trend [60,64,65]. FQ-resistant *Campylobacter* strains have been classified by the World Health Organization (WHO) as high-priority antibiotic-resistant pathogens for which new drugs are required [66,67].

Ciprofloxacin and erythromycin have been used as the drugs of choice for treating *Campylobacter* infections [68]. The global distribution of ciprofloxacin-resistant strains has led to the adoption of erythromycin as the appropriate drug for campylobacteriosis therapy due to a limited number of macrolide-resistant strains [61]. Both strains of this study were sensitive to erythromycin and the WGS confirmed the results due to a lack of responsible point mutations (2074 and 2075) in the V domain of the 23S rRNA gene [69]. The reduced

resistance to macrolides in *Campylobacter* strains from poultry may be associated with the limited use of macrolides in poultry production. Tylosin is used in swine or cattle, but not in poultry [70,71]. However, Sub-Saharan Africa (SSA) has recorded a lower prevalence of *Campylobacter* strains that are ciprofloxacin-resistant compared to erythromycin-resistant ones [68,72].

Phenotypic and genomic data showed resistance to tetracycline, which concurs with previous findings all over the world [59,73,74]. Higher resistance to tetracycline has been associated with the *tet*(O) gene coding for the ribosomal protection protein TetO [19] found in various Gram-positive and Gram-negative bacteria [63]. Moreover, tetracycline is overused in chicken and swine industries due to its affordability, and simple administration via drinking water [75]. It is worth noting that the chicken body temperature (42 °C) favors the conjugation process and thus contributes to the sharing of plasmids carrying various antimicrobial-resistant genes [76].

Campylobacter spp. are known to be inherently resistant to β-lactams including ampicillin [70], and we did not test for ampicillin resistance by broth microdilution. However, the WGS showed the presence of $bla_{\text{OXA-452, 521}}$, and $_{193}$ genes which are inherent to *Campylobacter*. Ampicillin resistance is mainly due to enzymatic inactivation by $bla_{\text{OXA-61,}}$ but other factors like porins and reduced affinity to penicillin-binding protein (PBP) have also been reported [70,77]. The isolates of the current study were sensitive to gentamicin, which corroborates previous reports [78–80]. However, higher resistance was reported in China for *C. coli* strains [74]. The limited resistance to gentamicin has been associated with its limited use to only systemic infections [81,82] and it is not used in poultry production [79]. Both ABRicate and ResFinder did not yield any resistance to streptomycin, as the *rps*L was found but without mutation. Surveillance of gentamicin-resistant strains should be performed in response to the increasing number of resistant strains as reported in the USA and China [61].

This study revealed that adhesion (*cad*F, *peb*A, and *jlp*A), invasion (*cia*BC), toxin (*cdt*ABC), *flg*SR two-component system, and biofilm formation (*ept*C) factors were only found in the *C. jejuni* strain 200605 genome and not in the *C. coli* strain 200606 genome. These factors highlight the virulent nature of the *C. jejuni* strain compared to *C. coli* which concurs with the literature [83]. Both strains expressed various other virulence factors involved in pathogenesis, like chemotaxis (*che*A, V, W), LOS, LPS, and capsule formation (*gmh, waa*, and *kps* genes). The mentioned genes contribute to the pathogenicity of *Campylobacter* strains while infecting humans, as they are all required for successful colonization and survival [15,84] of the bacteria within the host. Studies demonstrated that mutant *Campylobacter* strains were negatively affected in absence of some important genes [13,66]. For instance, *Campylobacter* strains lacking *cdt*B and *cdt*C were not cytotoxic, had reduced colonization, and had extra-intestinal invasiveness [15,85]. Flagellar genes (*fla*A, *fla*B, *flg*B, *flg*E, and *fla*C) are involved in various cell functions, like motility and biofilm formation [86,87]. The presence of capsular genes (*kps* D, E, F, C, S, T) and LPS associated gene (*hld*E) in both strains underline their virulence potential. The role of the capsule in the pathogenesis of *Campylobacter* has not been well defined, but it is suspected to interact with the mucus layer during adhesion, and it helps with intracellular survival [12]. *Hld*E is involved in protein glycosylation and correct LPS configuration [88]. Surprisingly, the *C. coli* strain 200606 harbored additional genes (*cj*1420c; *cj*1419c, *cj*1417c, *cj*1416c) involved in capsule biosynthesis [89] for *C. jejuni*, suggesting an exchange of some genes between *C. jejuni* and *C. coli* species. However, the introgression of *C. coli* by *C. jejuni* is not new [90]. Taken together, WGS data highlights the virulence profiles of study strains, which may give a clue to their respective pathogenicity.

The GBDP phylogenomic tree showed that *C. jejuni* strain 200605 clustered together with another isolate previously found in chicken meat in Korea (CP028933), but it was distantly related to another strain of human origin also reported in Korea (CP017229). This suggests some host preference and adaptation in *Campylobacter*. A study in Japan highlighted a distant relationship between *C. jejuni* from wild crows and poultry, showing

the possibility of divergence due to host adaptation [91]. On the contrary, *C. jejuni* strain 200605 clustered with CP014344 collected from humans in South Africa, which could not be justified by the current study. We speculate that travel may be a predisposing factor in the occurrence of such a phenomenon. However, the phylogenetic tree (Figure 1) shows that other factors like the species, subspecies, percent G+C, and δ statistics were comparable for most of the *C. jejuni* strains used to build the tree. *C. coli* strain 200606 clustered with *C. coli* (CP007181) isolated from turkey, and this cluster was distantly related to other *C. coli* strains used to build the tree. Both chickens and turkeys are domestic poultry, and it seems common to find both strains clustering together. Also, introgression of CP007181 by *C. jejuni* would explain the clustering together with *C. coli* strain 200606 of this study in which some *C. jejuni* genes were found. Furthermore, the analysis showed that other factors like the species, subspecies, percent G+C, and δ statistics were different from the values of other *C. coli* strains used to build the tree (Figure 2). *Campylobacter* is evolving at high speed due to many recombination events that could lead to specific niche adaptation and thus justifying the obtained diversity [71]. Differential responses to environmental factors and/or management practices have also been suggested to contribute to strain distribution among various niches [92].

C. jejuni strain 200605 belongs to ST-5229 which so far has not been assigned to any clonal complex. This ST may be specific to the region, as other isolates (n = 4) of the same ST have been previously collected from chickens in Korea [60], while one isolate was isolated from swine in China, as shown by the pubMLST website. There is a shortage of information on this ST and why it has not been reported in other parts of the globe. *C. coli* strain 200606 belongs to ST-5935, which is part of the CC-1150. This ST is not common, but it has been reported in *C. coli* of chicken origin in Vietnam [93]. The CC-1150 has also been reported as the predominant clonal complex among *C. coli* from chickens in China [94]. Further studies are needed to understand the particularities of both STs and why they are not widely distributed. We also recommend studies on the roles played by indoor and cage-free laying hens along with their environment in disseminating *Campylobacter* species to the environment.

A limitation in our study was a low number of sequenced strains due to limited resources. However, the phylogenetic trees included *Campylobacter* strains from various hosts and countries to indicate the taxonomic features of isolates used in this study.

5. Conclusions

The current study describes the WGS of *C. jejuni* strain 200605 and *C. coli* strain 200606 from layer chickens in Korea. Both strains showed C257T point mutation in *gyr*A and *cme*ABC operon often associated with quinolone resistance. The two strains also carry *tet*(O) genes associated with tetracycline resistance. The presence of various virulence factors involved in motility, adhesion, invasion, toxin production, and chemotaxis shows the pathogenic potential of the studied strains. Phylogenomics revealed that the two strains resemble other strains of poultry and human origins. *C. jejuni* strain 200605 and *C. coli* strain 200606 belong to less common STs and this warrants further investigation. To the best of our knowledge, this is the first report of WGS data from *Campylobacter* species from layer chickens in Korea. Special attention should be paid to FQ-resistant strains due to a limited number of available alternative treatments.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/genes12081131/s1, Supplementary File S1: Virulence genes.

Author Contributions: N.G., C.-H.P., R.G.A. and E.V.G.K. conceived the study. N.G. and D.-G.S. collected samples. N.G. and K.-Y.Y. carried out the experiments, analyzed, interpreted the data, and wrote the manuscript. R.G.A. and D.M. substantially contributed to the analysis of the results. L.E.G.M. and M.I.M. substantially revised the manuscript. All authors read and approved the final version of the manuscript.

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Paper Five

Molecular detection of *Campylobacter* species from human and cattle faecal samples in Kilosa district, Tanzania

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2.1 Abstract

Background: Emerging *Campylobacter* species other than *C. jejuni* and *C. coli* have been reported to cause infections in both humans and animals but their contribution to human campylobacteriosis is under-reported due to difficulties in isolation procedures. The main objective of this study was to detect *Campylobacter* species from human and cattle faecal samples in Kilosa district, Tanzania using molecular techniques**.**

Methods: A total number of 100 faecal samples (70 from humans and 30 from cattle) were collected from diarrheic and non-diarrheic patients and healthy cattle in Kilosa district, Tanzania from July to October 2019. Species identification was conducted by PCR and 16S rRNA sequencing. The phylogenetic analysis was carried out by comparison of the 16S rRNA gene sequences to reference strains by the Neighbor-Joining method in MEGA X.

Results: *Campylobacter* species detection rates by PCR were 65.7% (46/70) and 20% (6/30) in humans and cattle, respectively. Of the five human diarrheic cases, four showed *Campylobacter* presence and two were from children ≤15 years of age. In humans, the 16S rRNA sequencing revealed that *C. concisus* was the most predominant species occurring at a frequency of 37.8% (14/37), followed by uncultured *Campylobacter* spp. 24.3% (9/37) and *C. hominis* 21.6% (8/37). The least represented species were *C. jejuni* and *C. lanienae* all occurring at 2.7% (1/37). In cattle, five (100%) sequenced PCR products matched with *C. lanienae*. Phylogenetic analysis revealed that *Campylobacter* 16S rRNA sequences were closely related to *C. concisus,* uncultured *Campylobacter* spp., *C. hominis,* and *C. gracilis*.

Conclusion: The non-*C. jejuni*/ *C. coli* species are present in human and cattle faecal samples and their true occurrence is probably under-reported due to shortcomings of conventional techniques used in most diagnostic microbiology laboratories. Based on our findings, we recommend that molecular techniques be adopted for the direct detection of *Campylobacter* species during routine laboratory screening and surveillance studies.

Keywords: *Campylobacter*, molecular diagnostics, polymerase chain reaction, sequencing, gastroenteritis, Tanzania

2.2 Introduction

Campylobacter species cause 96 million diarrheal cases each year in humans [1, 2]. Some *Campylobacter* species are known to cause infertility in cattle and abortions in sheep, goats, and cattle [3]. The incidence of human cases of campylobacteriosis has been increasing in many countries throughout the world [4, 5]. In Africa, the prevalence varies from 7.7–18.5%, and *Campylobacter* is persistently found in stools of both diarrheic and non-diarrheic children [6], often associated with poor hygiene and sanitation [7].

The predominant species of *Campylobacter* accounting for more than 95% of human campylobacteriosis are *C. jejuni* (85%), followed by *C. coli* (15%) [8]. However, atypical *Campylobacter* species are gaining considerable attention as important human and animal pathogens [9, 10]. Infections caused by *Campylobacter* are usually under-reported due to difficulties in isolation procedures [11]. For instance, it has been estimated that 40% of the bacteria from human faeces diagnosed through microscopy cannot be cultured in the laboratory [12]. In contrast to other gastrointestinal pathogens, the culturing of *Campylobacter* species is laborious due to their microaerophilic nature and vulnerability to temperature fluctuations [13]. Furthermore, commonly used selective media and added antimicrobials suppress the growth of certain *Campylobacter* species [14]. Moreover, *Campylobacter* species may become dormant as viable but non-culturable (VBNC) forms difficult to grow on commonly used media [15]. Consequently, the epidemiology and role of these less commonly reported species in humans are not fully understood [16].

The emerging *Campylobacter* species have been neglected but the integration of molecular techniques and suitable culture media in current diagnostic tests has helped in promoting the awareness of atypical species as relevant human and animal pathogens [11]. One of the frequently encountered emerging *Campylobacter* is *C. concisus* reported to cause diarrhea, gingivitis, periodontitis, and inflammatory bowel disease (IBD) [10, 17]. *Campylobacter hominis* was identified in a blood sample of a septicemic patient [18], while *C. gracilis* has been associated with bacteremia, head infections, periodontitis, and empyema [17, 19]. In cattle, *C. fetus*, *C. lanienae*, *C. sputorum*, *C. jejuni*, and *C. hyointestinalis* are common [20–22].

The polymerase chain reaction (PCR) and other molecular diagnostic tests based on nucleic acids are attractive due to their benefits including their higher sensitivity, ease of

use, improved turnaround time, relatively low cost, and potential to be fully automated [23]. The breakthrough in technology and easy access to commercial kits has led to shifting from traditional laboratory diagnostic techniques to newer molecular ones [24]. Amplification of the 16S rRNA gene by PCR and sequencing techniques have assisted in the phylogenetic identification of *Campylobacter* species including those unidentified by conventional techniques [25]. Nevertheless, *Campylobacter* isolation by culture is still useful as it allows the isolation of pure colonies and testing of antimicrobial susceptibilities [23].

In Tanzania and Africa in general, the reports on the role of *Campylobacter* spp. in gastroenteritis are scanty due to various reasons like lack of routine screening of *Campylobacter* by clinical laboratories and the absence of national surveillance programs [6]. Thus, the information available for both human and animal campylobacteriosis is limited [26] which undermines its importance as a public health concern. The current study aimed at molecular detection of *Campylobacter* species from human and cattle faecal samples in Kilosa district, Tanzania using PCR amplification of the 16S rRNA gene and Sanger sequencing.

2.3 Materials and Methods

Study design and sample collection

This cross-sectional study was conducted in Kilosa District of central Tanzania (6° S and 8°S, and between 36° 30’ and 38°E) from July 2019 to October 2019. Human stool samples were obtained from patients with abdominal discomfort seeking medical care at Kilosa District Hospital during the time of the study. Cattle faecal samples were randomly collected from healthy lactating cows using sterile gloves. A total of 70 human stool samples and 30 cattle rectal grab faecal samples were collected in sterile dry screw-top containers containing Dimethyl Sulfoxide (DMSO) and transported to the Sokoine University of Agriculture Microbiology laboratory on ice for DNA extraction within 8 hours of collection.

DNA extraction and *Campylobacter* species identification

Approximately, 1g faecal sample in DMSO was diluted (10% wt/vol) in buffered peptone water (BPW) (9 ml) and vortexed until the sample was thoroughly homogenized. Then, genomic DNA was extracted from 200 μL of a well-mixed faecal sample using a Zymo

Research kit (Quick-DNA™ Faecal/Soil Microbe Microprep). Eluted DNA concentration and purity were checked using a NanoDrop™ spectrophotometer (Biochrom, Cambridge, England) before storage at -20°C.

Detection of *Campylobacter* was done using specific primers (*cj0414* for *C. jejuni* and *ask* for *C. coli*) as previously described [27]. Then, PCR of the 16S rRNA gene was performed on DNA samples negative with the species-specific primers as previously described [28]. Positive and negative controls were *C. jejuni* (ATCC® 33560™) and deionized H_2O, respectively. The PCR final volume was 25 µL, including 12.5 µL of 2X Master Mix (Thermo Fisher Scientific, Seoul, South Korea), 1 µL (10 µM) of C412F primer, 1 µL (10 µM) of C1228R primer, 1 µL of genomic DNA, and 9.5 µL of sterile deionized H_2O. All primers were made by Integrated DNA Technologies, Inc. (Singapore Science Park, Singapore).

The DNA amplification was performed by PCR machine (Applied Biosystems, MA, USA). The cycling conditions used were one cycle of 95°C for 5 minutes, 35 cycles each of 94°C for 30 seconds, 55°C for 45 seconds and 72°C for 45 seconds, and a final extension at 72°C for 7 minutes. PCR products (5µl) were mixed with 2µl Loading STAR (Dyne bio, Seongnam-si, Korea) diluted with 5µl of nuclease-free water and analyzed by gel electrophoresis: 10µl of the mixture was loaded onto 1.5% SeaKem® LE Agarose gel (Lonza Inc.-Rockland, ME, USA) in 0.5X TAE buffer. After electrophoresis, PCR product bands were visualized using a Dual UV Transilluminator (Core Bio System, Huntington Beach, CA, USA) under ultraviolet (UV) light and photographed with iBright™ CL1000 Imaging System (Thermo Fisher Scientific, Seoul, South Korea). Amplicons (816bp) obtained were compared to the Dyne 100 bp DNA ladder (Dyne bio, Seongnam-si, Korea), purified using Pure Link ™ Quick PCR purification Kit (Invitrogen, Vilnius, Lithuania), and sequenced at SolGent (Solutions for Genetic technologies, Daejeon, South Korea) using the *Campylobacter* genus-specific primers by Sanger method.

Data analysis

The data were analyzed with GraphPad Prism 8.4.0 (GraphPad Software, La Jolla, CA, USA). Descriptive statistics (frequencies and percentages) were computed to determine proportions for different attributes. BioEdit sequence alignment software (version 7.2.6.1) was used for sequence trimming, alignment, and getting the consensus sequence [29]. The

NCBI BLASTN search was used to compare our sequences to those from the GenBank. Multiple sequence alignment by Muscle [30], computation of evolutionary distances by the Jukes-Cantor method [31], and the phylogenetic tree building by the NJ method [32] were done in MEGA X software (MEGA Inc, Englewood, NJ) [33]. The sequences of this study were compared to the reference strains downloaded from LPSN (https://lpsn.dsmz.de). To confirm the reliability of our analysis, bootstrap analysis was performed with 1,000 resampled datasets [34]. All the strains derived from sequencing were submitted to GenBank for obtaining accession numbers.

2.4 Results

A total of 70 (male=35; female=35) human stool samples were collected. The age of patients ranged from 2 to 89 years with 14.3% being children ≤15 years of age. Overall, the detection rate of *Campylobacter* spp. in human samples was 65.7%. The PCR products with predicted size (816bp) were obtained in some of the screened samples (Figure 2.1). Of the *Campylobacter* spp. positive samples (n=46), 24 (52.2%) were from females and 22 (47.8%) were from males. *Campylobacter* species were detected in nine of the 10 (90%) children ≤15 years old. *Campylobacter* was reported in 4/5 of the diarrheic patients. Of the diarrheic patients, two were children ≤15 years. In cattle, all 30 samples were collected from lactating cows with ages varying between 3.4-8 years. Of the 30 samples, six (20%) exhibited *Campylobacter* spp.

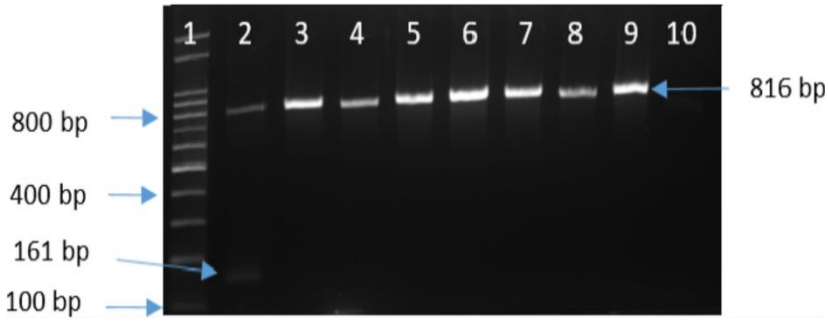

Figure 1: Amplicon showing the genus band

Label: 1: 100bp ladder; 2: *C. jejuni* sample (human), 3-5: other bands from human samples; 6-8: bands from cattle samples; 9: positive control; 10: negative control. *Campylobacter* genus band appears at 816bp.

The results of sequencing confirmed the presence of *Campylobacter* species in all submitted sequences (37 from humans and 5 from cattle). The remaining PCR products did not give enough quantity of DNA (required by the sequencing company) after the purification step. The species were confirmed based on percent identity (above 99%), the query cover, and the E-value. In humans, C. *concisus* and uncultured *Campylobacter* spp. were the most prevalent with 37.8% and 24.3%, respectively. *Campylobacter lanienae* and *C. jejuni* occurred at a frequency of 2.7% each (Figure 2.2). For cattle, all the five (100%) 16S rRNA sequences matched with *C. lanienae*.

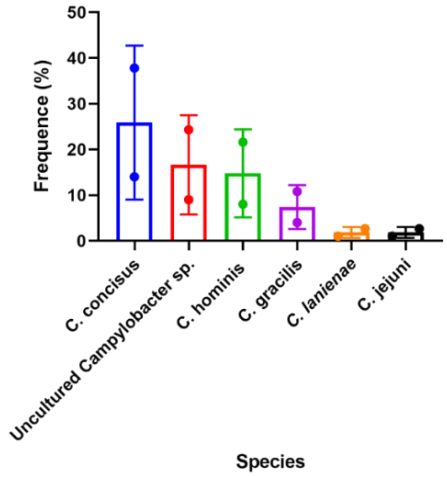

Figure 2: Distribution of *Campylobacter* species from human samples.

The 16S rRNA genes of *Campylobacter* spp. from this study were compared with 16S rRNA sequences of different strains of *Campylobacter* spp. by BLASTN search. Following submission to the GenBank, gene sequences were allocated with the following accession numbers: MT126449 to MT126453; MT130973 to MT130991; and MT131150 to MT131167.

The genetic relatedness was evaluated by comparing the sequences of this study to 16S rRNA genes of reference strains. The analysis of sequence data from *Campylobacter* species of this study revealed a high nucleotide sequence similarity to different reference strains. *Campylobacter hominis* clustered closer to *C. gracilis* than it was with *C. concisus*. It was also noted that *C. lanienae* formed a separate cluster at the bottom of the tree. Uncultured *Campylobacter* and *Campylobacter* spp. RM 12175 were also found among the sequences of this study (Figure 2.3). *H. aurati* and *A. molluscorum* were used to root the tree.

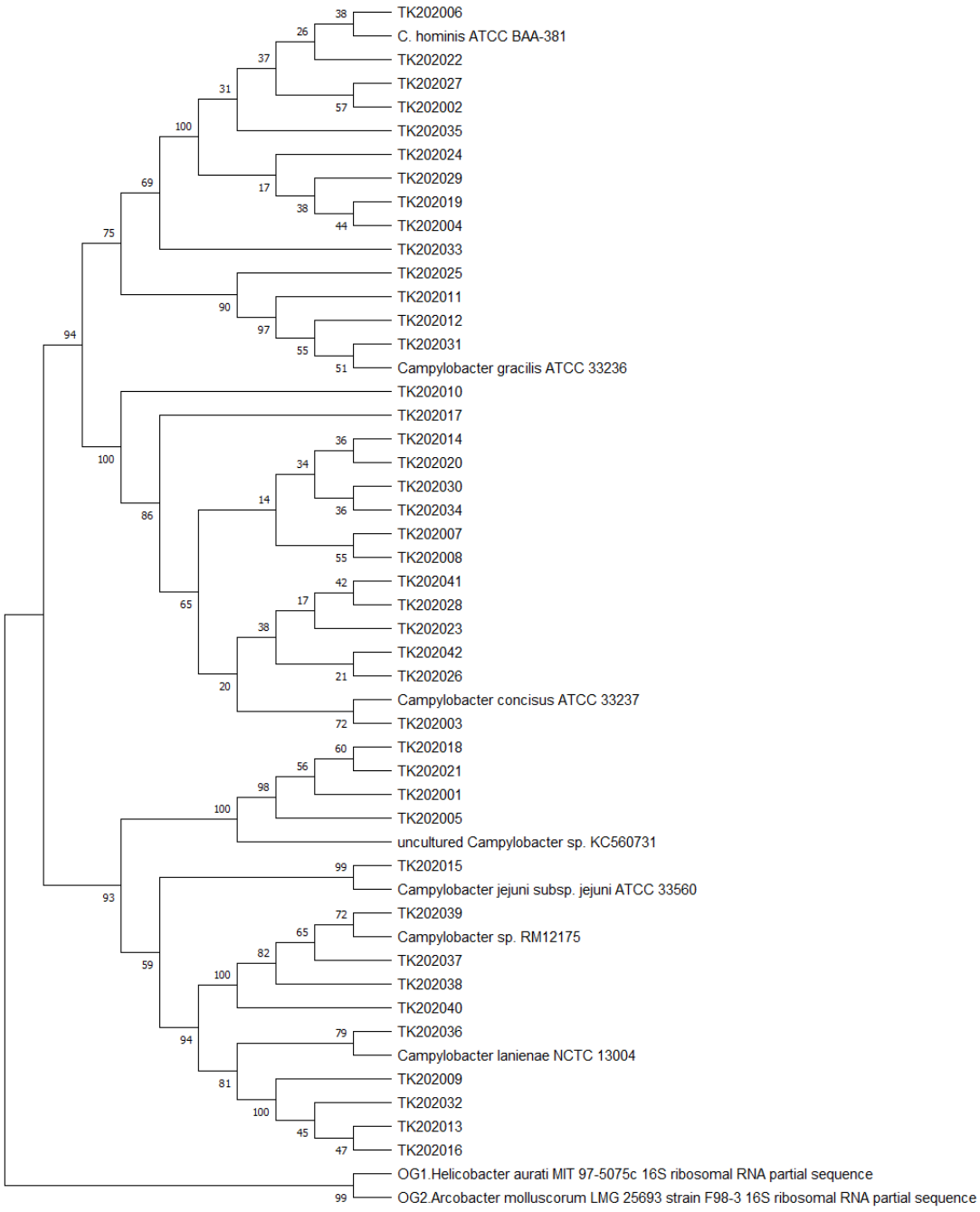

Figure 3: Evolutionary analysis of 16S rRNA sequences by the NJ method.

Evolutionary analyses were conducted in MEGA X. Reference strains were included in theanalysis. The tree was rooted using *H. aurati* and *A. molluscorum*. Bootstrap analysis was performed with 1,000 resampled datasets and it was taken to represent the evolutionary history of the taxa analyzed. The number shown for eah isolate corresponds to the laboratory code given by the researcher. TK stands for Tanzania and Kilosa while 2020 was the year sequences were analyzed and submitted. The last two digits characterize each strain in order of sampling.

2.5 Discussion

For many years, thermophilic *Campylobacter* species have been associated with human campylobacteriosis [8]. Nonetheless, discoveries in molecular diagnostic techniques proved that isolation by culture contributes to increased detection rates of thermophilic *Campylobacter* species which may bias both the outcome of the diagnosis and undermine the public health importance of emerging *Campylobacter* species [35]. Bullman et al. [35] attribute the scarcity of atypical *Campylobacter* species reported so far to the limitations and bias of culture-based methods. Therefore, this study reports the detection of *Campylobacter* spp. in both humans and cattle by molecular methods. In Tanzania, there are no previous studies on detecting the presence of *Campylobacter* species directly from faeces without a culture-based approach. Previous authors adopted cultural methods [36, 37] which could not give a true picture of *Campylobacter*-related infections as some could have failed to grow on selective media.

The reported detection rate of *Campylobacter* spp. (65.7%) in humans was similar to that reported in Nigeria [38] but higher than those reported previously in Tanzania [37, 39], Fiji [40], India [41], and Cambodia [7]. The difference could be attributed to the sampling strategy, PCR conditions, and geographically related variations. *Campylobacter concisus* and *C. hominis* were the most predominant species occurring at 37.8% and 21.6%, respectively. This concurs with the findings reported in Denmark [42] and Australia [43]. It was previously reported that *C. concisus* was the second species with a higher prevalence after *C. jejuni* [11]. However, *C. concisus* has been isolated from diarrheic patients without other pathogenic microorganisms suggesting it to be an emerging cause of human gastroenteritis [11, 43]. *C. concisus* is a normal flora of the oral cavity but may be translocated to the intestines of humans where it leads to gastroenteritis and Crohn's disease [17].

In our study, only one *C. jejuni* strain was reported. It has been reported that there is a considerable divergence in species distribution detected by culture and those detected by PCR methods [44]. Our results suggest that the reduced number of *C. jejuni* strains could be associated with the use of PCR instead of culture methods where added antimicrobials and used growth temperature favor the growth of *C. jejuni*. Our results concur with a study in Chile that failed to isolate *C. concisus* by culture but molecular methods detected it even

in higher numbers compared to *C. jejuni* in human stools [45]. Atypical *Campylobacter* species could be underreported or not detected at all.

Campylobacter was detected in 90% of the children. Although human campylobacteriosis is self-limiting, it is hyperendemic among young children and can be fatal due to their weak immunity [46]. Furthermore, children are at high risk of infection due to poor hygienic conditions predominant in rural families along with proximity with livestock and limited literacy of mothers [6, 47]. Special care is needed to protect children from *Campylobacter* infections through improving the personal hygiene, education of mothers, and availing potable water.

Campylobacter lanienae was detected in both cattle and humans. It has been previously recovered from healthy livestock [48] and it has been reported as a probable aetiology of human gastroenteritis [49]. However, it is suggested that *C. lanienae* has restricted pathogenicity or be a non-pathogenic *Campylobacter* [50]. The observed frequency of occurrence of that species in this study was higher than the one previously reported [21]. Further characterization of *C. lanienae* could shed more light on its genetic diversity and source [48].

The data on the concurrent isolation of *Campylobacter* species in both humans and cattle are limited. In this study, the detection rates were 65.7% and 20%, in humans and cattle, respectively. Our findings showed higher detection rates when compared to the rates reported earlier in Tanzania [51], and Cambodia [7]. The current study suggests other sources of human campylobacteriosis considering that most of the reported species in humans were not detected in cattle. Further comparative studies on *Campylobacter* species isolated from humans and other reservoirs are necessary to understand their epidemiology and be able to conclude on source attribution.

The evolutionary analysis using 16S rRNA sequences is of paramount importance for bacterial taxonomy [52] and it has been applied to *Brucella* [53] and *Campylobacter* identification [19]. Our findings concur with the literature highlighting *C. concisus* as the predominant species of the emerging *Campylobacter* group [54]. In cattle, our results are in agreement with previously reported occurrence where *C. lanienae* had higher proportions compared to *C. jejuni* and C. *hyointestinalis* [48]. The drawback of the 16S

rRNA gene sequence analysis is the failure to unambiguously resolve evolutionary relationships within many groups [55] and thus calling for the use of more precise techniques like the multilocus sequence typing and the whole-genome-based methods [56].

The current study had some limitations including the sample size and lack of culture-based species identification. The sample size used could not allow us to estimate the prevalence or generalize the findings at national or regional levels. Considering that we did not culture the stool samples, the comparison is made based on previous studies carried in sometimes different conditions or settings. However, this study highlights the advantages of molecular methods over culture-based ones in detecting atypical *Campylobacter* species.

2.6 Conclusion

This study highlights the higher detection rates of less frequently isolated *Campylobacter* species (*C. concisus* and *C. hominis*) in patients attending Kilosa district hospital. The *Campylobacter* detection rate in children was high compared to adults. *Campylobacter lanienae* was detected in both human and cattle faecal samples. Emerging *Campylobacter* species are often neglected due to their cultural behavior and fastidious nature but have proven to be zoonotic with a public health concern. It is therefore important that health practitioners and public health authorities recognize the possibilities of occurrence of these neglected species which are not tested on a routine basis in many countries and go unreported. Soon, molecular-based techniques may substitute the culture-based methods especially for the detection of atypical *Campylobacter* species as they provide results in a short time and up to species level.

Authors' contributions

EVGK, MIM, LEGM, and NG conceived the study. NG performed the experiments, compiled the results, and wrote the manuscript. KYY and HAM helped in some experiments. RGA, DM, CHP, and KHC helped to revise the manuscript. All authors approved the final version of the manuscript.

Declarations

Ethics approval and consent to participate

This study was approved by the Medical Research Coordinating Committee of the National Institute for Medical Research in Tanzania (Ref. No.: NIMR/HQ/R.8a/Vol. IX/3070). Stool samples from patients were collected after obtaining the informed written consent of the patient or his/her parent/guardian. Farmers consented to sample their cattle.

Availability of data and materials

Data generated during this study are available from the corresponding author on reasonable request.

Conflict of interest

The authors declare that there is no conflict of interest.

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CHAPTER THREE

General discussion, Conclusions and Recommendations

3.1 General discussion

This study investigated the molecular epidemiology and antimicrobial susceptibility profiles of thermophilic *Campylobacter* species in humans and animals in South Korea and Tanzania. I went to South Korea for a sandwhich program of two years following an agreement between my sponsor (the Partnership for Skills in Applied Sciences, Engineering and Technology or PASET), Sokoine University of Agriculture (SUA), and the Korea Institute of Science and Technology (KIST). The purpose of the agreement was to allow PASET scholars to have access to well-equipped laboratories and resources. In South Korea, I collected faecal samples from layer chicken while in Tanzania, I used human stool and cattle faecal samples. In South Korea, my research was carried out at the Korea Institute of Science and Technology (KIST). I was interested in poultry sector which is a fast growing business worldwide due to a high demand for eggs and meat products (Kyakuwaire *et al*., 2019). Poultry is also considered the main reservoir and source of campylobacteriosis in humans (Kaakoush *et al*., 2015). I collected faecal samples from layer chicken as few papers have been published on them as a possible reservoir of *Campylobacter* strains resistant to existing antimicrobials (Kassem *et al*., 2016).

Chicken droppings and litter are used as fertilizers and may contribute to the transmission of *Campylobacter* and AMR genes to the environment and humans (Kyakuwaire *et al*., 2019). However, there are few studies that address the circulating genotypes that may be associated with human infections. I faced the challenge in gaining access to poultry farms, possibly due to the ongoing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic and fear of contaminating chicken farms. Only two farmers granted access, and chicken faeces were collected using sterile swabs that were taken to the laboratory within an hour. *Campylobacter* isolates were detected only on the first farm and were highly resistant to quinolones (ciprofloxacin and nalidixic acid) and tetracycline, but sensitive to a new fluoroquinolone (sitafloxacin), erythromycin, and gentamicin (Objective 2). The drugs for which resistance was high are widely used in human and veterinary medicine to treat various infections (Sproston *et al*., 2018). The low resistance to sitafloxacin could be attributed to its special properties (fluorine and chloride) which are absent in other quinolones (Changkwanyeun *et al.*, 2016). In addition, sitafloxacin is still

new and has not been used extensively, which could justify its efficacy. The low resistance to erythromycin is attributed to the limited survival of mutant strains, while the low resistance to gentamicin is likely due to its limited use in the treatment of systemic infections (Luangtongkum *et al*., 2009; Lynch *et al*., 2020). *Campylobacter* isolates showed mutations in the *gyr*A and *tet*(O) genes conferring resistance to quinolones and tetracycline, respectively (Frasao *et al*., 2015; Elhadidy *et al*., 2018). The same isolates also showed the presence of a considerable number of virulence genes involved in adhesion, invasion, motility, and toxin production. All these genes contribute to the pathogenicity and fitness of *Campylobacter* species. As for the genotypes recorded in this study, ST-3611 and ST-460 belonging to CC-607 and CC-460, respectively, were the predominant ones. Both STs were reported to be virulent and express type VI secretion system (T6SS), which is involved in virulence, metabolism-, AMR and contributes to host adaptation (Rokney *et al*., 2018). This study suffered from the limited access to farms, but the analysis (Minimum Spanning Tree) included other STs previously reported to be prevalent in the region and elsewhere in both humans and poultry. Resistance to both erythromycin and ciprofloxacin is of a public health concern, as there are currently limited options in treating *Campylobacter* infections. Both drugs (ciprofloxacin and erythromycin) were considered the drugs of choice (Gahamanyi *et al*., 2020), but increasing resistance to ciprofloxacin has led to a decline in its use to treat campylobacteriosis in humans. Virulence genes are also of concern, as they can be exchanged between pathogens by horizontal gene transfer (Golz *et al*., 2020). In this study I obtained three new sequence types that have been deposited in the PubMLST database. This is a contribution to the literature on *Campylobacter* species especially from layer chicken.

Following the increased resistance to ciprofloxacin (previously considered as drug of choice to treat human campylobacteriosis) and the call by the WHO to find alternative drugs to FQ-resistant *Campylobacter* strains (Hlashwayo *et al.*, 2020), I worked on natural products (plant extracts, essential oils, and phytochemicals) to check which ones may work against both drug-sensitive and resistant *Campylobacter* strains (Objective 3). *Campylobacter* is one of the 12 bacteria listed by the WHO as the top threats to human health due to antibiotic resistance (Sproston *et al*., 2018; Hlashwayo *et al*., 2020). I found that *Cinnamon cassia* extract, cinnamon oil, cinnamaldehyde, clove oil, eugenol, and baicalein were effective against both chicken-derived and reference *Campylobacter* strains. However, I did not perform the phytochemistry of the used plant extracts but I included some pure compounds from these plants like cinnamaldehyde, eugenol, and baicalein that

are commercially available. The recorded MICs values for the essential oils and phytochemicals were low (25-50µg/mL) which show that the characterization of pure phytochemicals from used plants may give better results. Screening for several medicinal plants and chemical characterization to know their bioactive compounds would help in discovery of new drugs that may help to curb the current AMR trend estimated to be killing 10 million people per year by 2050 (*Burki*, 2018). Furthermore, studies on the mechanisms of action of bioactive compounds from used medicinal plants is of paramount importance towards their clinical acceptance and use. A study highlighted the synergism between natural products and conventional drugs in fighting against multidrug-resistant pathogens (Hemaiswarya *et al.*, 2008). It is suggested that the activity of some antimicrobials is enhanced by cinnamaldehyde and the combinations could be added in animal feeds or used in the food industry to deal with biofilms (Friedman, 2017). Synergistic studies between used natural products and currently used antimicrobials are recommended. This may help to delay resistance to these drugs as sub-inhibitory concentrations of NPs do not induce selection pressure for the emergence of new antimicrobial resistance (AMR) among bacteria as experienced with the use of existing antibiotics (Alibi *et al.*, 2020; Klančnik *et al.*, 2020).

Although less available in LMICs, WGS is being considered as a one-stop-shop that is embraced in foodborne pathogens surveillance, typing, outbreak investigations, and phylogenetic analyses due to the accuracy, rapidity, and high-throughput sequencing technologies (Rokney *et al*., 2018). Existing tools of genotyping (FlaA-RFLP, PFGE, and MLST) are based on a single (*fla*A) or limited number of genes (MLST) which limit the accuracy of their results considering the considerable genome and the frequency of mutations in the genus *Campylobacter*. Also, data on WGS of *Campylobacter* from layer chicken are limited in Korea and elsewhere. In a single step, WGS allows to obtain information on virulence factors, AMR, genotypes of a given pathogen but its use on *Campylobacter* is hampered by high levels of inter and intra-species recombination events resulting in a weakly clonal population (Rokney *et al*., 2018). Two strains (*C. jejuni* strain 200605 and *C. coli* strain 200606) were selected for WGS by Illumina sequencing technology (Objective 4). The analysis revealed that *C. jejuni* strain 200605 was more virulent than *C. coli* strain 200606 based on the number and types of virulence genes. Also, *C. coli* strain 200606 showed introgression of some genes (*cj*1420c; *cj*1419c, *cj*1417c, *cj*1416c) normally found in *C. jejuni*. This may be supported by the phylogenetic analysis where *C. coli* strain 200606 clustered with CP007181 from turkey which showed

introgression too. Introgression has been considered as a challenge in taxonomy and genotyping of closely related species like *C. jejuni* and *C. coli* with only 15% of nucleotide divergence (Sheppard *et al.*, 2011). *C. jejuni* strain 200605 and *C. coli* strain 200606 belong to less common STs (*C. jejuni*: ST-5229; *C. coli*: ST-5935). There is limited work on both STs and in particular, ST-5229 has not been assigned to any CC showing that it is still a new ST. Further studies may shed light on the factors influencing the regional distribution (South Korea, China, and Vietnam) of such STs. Due to limited resources, I was not able to sequence all the obtained isolates and the data provided by the sequencing of only two isolates among 55 are not enough to conclude on the epidemiology of the studied isolates. Further studies may analyze all the archived samples by WGS to get a broader picture of their virulence and antimicrobial resistance profiles.

Campylobacter is fastidious in nature and understanding its complete genome and adaptation characteristics would help to mitigate its economic and health associated consequences. Metagenomics studies are necessary to decipher the possible interactions and exchange of genes between *Campylobacter* and other microorganisms that are predominant in faeces or gut of both humans and various reservoirs. For instance, the environment provided by chicken intestine may contribute to the switching on of some virulence genes which promote *Campylobacter* survival and pathogenicity.

In Tanzania, *Campylobacter* was detected in both human and cattle faeces, but the results indicated that the predominant species in humans (*C. concisus* and *C. hominis*) might be from other reservoirs, as the only species detected in cattle was *C. lanienae*. *C. jejuni* was only detected in one sample from humans, which seems unusual. This could be due to the fact that no culture method was used, which normally favors the growth of *C. jejuni* and *C. coli* due to the antimicrobial agents added to the culture media (Bessède *et al*., 2011). In general, *C. jejuni* and *C. coli* are the predominant species associated with gastroenteritis (Wei *et al*., 2014). Emerging *Campylobacter* species, which are underrepresented in human and animal diseases, are considered as potential cause of gastroenteritis and various complications such as inflammatory bowel disease (IBD), eosophageal disease, and colorectal cancer (Kaakoush *et al*., 2015). Molecular methods are suitable for the identification of emerging *Campylobacter* species because *Campylobacter* is capable of forming coccoidal forms that are viable but cannot be cultured (VBNC) under stress conditions (Singh *et al*., 2011). The VBNC strains are not identified by culture, which may

justify their under-recording. *Campylobacter* was detected in 9/10 children under-fifteen years of age, highlighting their vulnerability, which may be due to weak immunity and increased risk of infection, often related to poor hygiene (Gahamanyi *et al*., 2020). Severe exposure of children to *Campylobacter* species has already been previously reported in various regions of Africa, Asia-, and the Middle East (Kaakoush *et al*., 2015). Detection of *Campylobacter* by molecular methods is not without drawbacks. Without isolation by culture, it is difficult to know the antimicrobial susceptibility profiles required for antimicrobial prescribing and surveillance programs. In addition, once the stock of DNA is finished or contaminated, there is no backup, whereas *Campylobacter* isolates can be preserved at – 80 ºC for years. Also, a limited availability of reagents and consumables make the use of molecular techniques difficult especially in the field. Further studies could include a comparative study where many samples from different hosts can be used for simultaneous detection of *Campylobacter* by culture and molecular methods. *Campylobacter* species surveillance program needs special encouragement in LMICs as *Campylobacter* is not on the list of routinely studied bacteria in different hospitals. This would help to know its epidemiology and develop appropriate control measures. Since *Campylobacter* is one of the major causes of diarrheal diseases, its detection can help reduce the number of diarrheal diseases in Africa.

3.2 Conclusions and recommendations

The study assessed the molecular epidemiology and antimicrobial susceptibility profiles of thermophilic *Campylobacter* species from human stool and animal fecal samples in Tanzania and South Korea.

The current research revealed that:

- Emerging *Campylobacter* species could be under or not reported as causative agents of human campylobacteriosis. I recommend the use ofolecular detection methods like PCR for such species.
- Layer chicken are reservoirs of *Campylobacter* strains that are resistant to existing antimicrobials (ciprofloxacin, nalidixic acid, and tetracycline) and potentially virulent. Despite being from the same farm, thermophilic *Campylobacter* strains were diverse as shown by genotyping methods (*fla*A-RFLP and MLST). It is high time for the prudent use of existing antimicrobials with strict restrictions of their use for growth promotion or prophylaxis in animal husbandry
- Natural products (plant extracts, essential oils, and phytochemicals) are effective against both drug-sensitive and drug-resistant *Campylobacter* strains. They may be

considered as alternative treatment options and be used in the control of *Campylobacter* in animal husbandry and food processing plants.

- Whole-genome sequencing of fluoroquinolone-resistant thermophilic *Campylobacter* strains confirmed phenotyping results and gave insights into their virulence potential. Phylogenomics showed that sequenced strains clustered together with existing strains but were of rare sequence types. I recommend the whole-genome sequencing of the remaining isolates to get a better picture of their genomic features.

Further research is needed on studying the synergism between natural products and existing antimicrobials in controlling campylobacteriosis. Also, studies in molecular mechanisms of natural products along with inhibition of biofilm formation, adhesion, and invasion of epithelial cells are paramount.

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APPENDICES

Appendix 1: Ethical clearance

THE UNITED REPUBLIC OF TANZANIA

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11101 Dar es Salaam
Tel: 255 22 2121400
Fax: 255 22 2121360
E-mail: nimrethics@gmail.com

Ministry of Health, Community Development, Gender, Elderly & Children
University of Dodoma, College of Business Studies and Law
Building No. 11
P.O. Box 743
40478 Dodoma

NIMR/HQ/R.8a/Vol. IX/3070

25th April, 2019

Gahamanyi Noel
Sokoine University of Agriculture
P.O. Box 3000
Morogoro

RE: ETHICAL CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA

This is to certify that the research entitled: Determining transmission dynamics and antimicrobial resistance of thermophilic campylobacter in humans, animals and the environment in Kilosa District, Tanzania (Noel G. et al), has been granted ethical clearance to be conducted in Tanzania.

The Principal Investigator of the study must ensure that the following conditions are fulfilled:

1. Progress report is submitted to the Ministry of Health, Community Development, Gender, Elderly & Children and the National Institute for Medical Research, Regional and District Medical Officers after every six months.
2. Permission to publish the results is obtained from National Institute for Medical Research.
3. Copies of final publications are made available to the Ministry of Health, Community Development, Gender, Elderly & Children and the National Institute for Medical Research.
4. Any researcher, who contravenes or fails to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine as per NIMR Act No. 23 of 1979, PART III Section 10(2).
5. Sites: Kilosa District in Morogoro region.

Approval is valid for one year: 25th April 2019 to 24th April 2020.

Name: Prof. Yunus Daud Mgaya

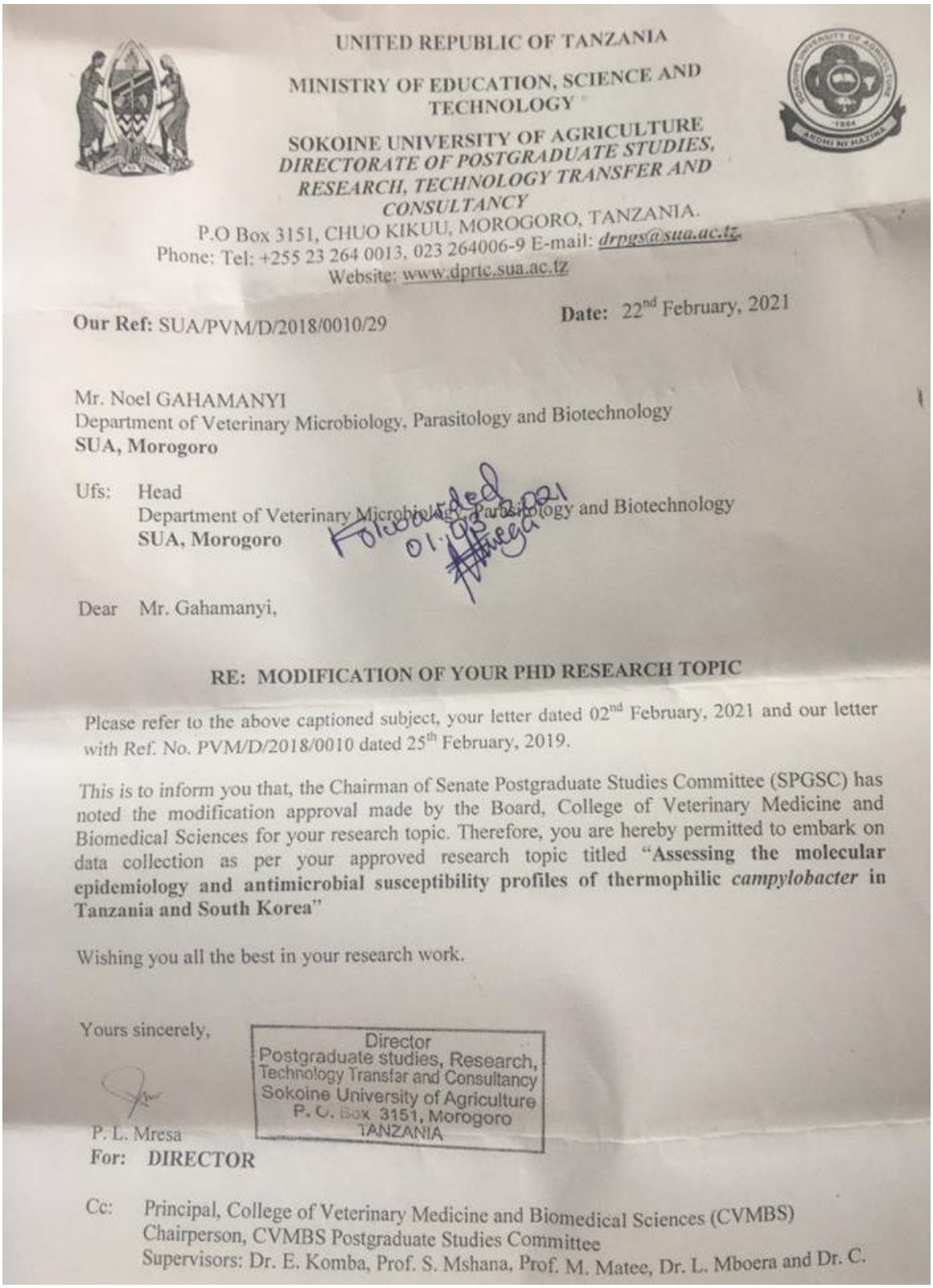
Signature
CHAIRPERSON
MEDICAL RESEARCH
COORDINATING COMMITTEE

Name: Prof. Muhammad Bakari Kambi

Signature
CHIEF MEDICAL OFFICER
MINISTRY OF HEALTH, COMMUNITY DEVELOPMENT, GENDER, ELDERLY & CHILDREN

CC: Director, Health Services -TAMISEMI, Dodoma
RMO of Morogoro region
DMO/DED of respective district

Appendix 2: Modification of research topic



UNITED REPUBLIC OF TANZANIA

MINISTRY OF EDUCATION, SCIENCE AND TECHNOLOGY

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Our Ref: SUA/PVM/D/2018/0010/29 **Date:** 22nd February, 2021

Mr. Noel GAHAMANYI
Department of Veterinary Microbiology, Parasitology and Biotechnology
SUA, Morogoro

Ufs: Head
Department of Veterinary Microbiology, Parasitology and Biotechnology
SUA, Morogoro

Forwarded 01.03.2021

Dear Mr. Gahamanyi,

RE: MODIFICATION OF YOUR PHD RESEARCH TOPIC

Please refer to the above captioned subject, your letter dated 02nd February, 2021 and our letter with Ref. No. PVM/D/2018/0010 dated 25th February, 2019.

This is to inform you that, the Chairman of Senate Postgraduate Studies Committee (SPGSC) has noted the modification approval made by the Board, College of Veterinary Medicine and Biomedical Sciences for your research topic. Therefore, you are hereby permitted to embark on data collection as per your approved research topic titled "**Assessing the molecular epidemiology and antimicrobial susceptibility profiles of thermophilic *campylobacter* in Tanzania and South Korea**"

Wishing you all the best in your research work.

Yours sincerely,

Director
Postgraduate studies, Research,
Technology Transfar and Consultancy
Sokoine University of Agriculture
P. O. Box 3151, Morogoro
TANZANIA

P. L. Mresa
For: DIRECTOR

Cc: Principal, College of Veterinary Medicine and Biomedical Sciences (CVMBS)
Chairperson, CVMBS Postgraduate Studies Committee
Supervisors: Dr. E. Komba, Prof. S. Mshana, Prof. M. Matee, Dr. L. Mboera and Dr. C.