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Isolation and molecular characterization of *Campylobacter jejuni* and *Campylobacter coli* isolated from Ethiopian dairy supply chain and evaluation of their associated risk factors and antimicrobial resistance

Ph.D. Thesis

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Isolation and molecular characterization of *Campylobacter jejuni* and *Campylobacter coli* isolated from Ethiopian dairy supply chain and evaluation of their associated risk factors and antimicrobial resistance

By

Abera Admasie Tefera

A Ph.D. Dissertation Submitted to the Institute of Biotechnology (Health Biotechnology), Addis Ababa University, in the Partial Fulfillment of the Requirements for Degree of Ph.D. in Health Biotechnology

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PhD dissertation approval sheet

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DEDICATION

The thesis is dedicated to the all-powerful God, to whom we owe our immense wisdom and power, to Saint Merry, who has always supported me in my life, and to my family

STATEMENT OF THE AUTHOR

To begin with, I hereby state that the content of this thesis is entirely my original work and that all informational sources used in its establishment have been properly cited. This dissertation was submitted to the Institute of Biotechnology (Health Biotechnology), Addis Ababa University, in partial fulfillment of the requirements for the degree of Ph.D. in Health Biotechnology, and it will be deposited at the University/College library to be made available to borrowers following library rules. I hereby certify that this thesis is not being submitted to any other school to receive any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CDC	Centers For Disease Control
CFSAN	Center For Food Safety And Applied Nutrition
CLSI	Clinical And Laboratory Standards Institute
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats
CSA	Central Statistical Agency
DNA	Deoxyribonucleic Acid
DNase I	Deoxyribonuclease I
EDTA	Ethylenediaminetetraacetic Acid
FDA	U.S. Food And Drug Administration
GC	Guanine Cytosine
HACCP	Hazard Analysis Critical Control Point
ISO	International Organization For Standardization
Mbp	Mega Base Pairs
mg	Milligrams
ml	Milliliter
MLST	Multi-Locus Sequence Typing
NCTC	National Collection Of Type Cultures
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
PI-IBS	Post-Infection Irritable Bowel Syndrome
PubMed	Public Databases For Multi Locus Sequence Typing
RNA	Ribonucleic Acid
SNNP	Southern Nations, Nationalities, And Peoples
SPSS	Statistical Package For The Social Sciences
ST	Sequence Type
Spp	Species
TAE	Tris-Acetate-Edta
WHO	World Health Organization
μM	Micro Molar

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Isolation and molecular characterization of *Campylobacter jejuni* and *Campylobacter coli* isolated from Ethiopian dairy supply chain *and evaluation of their associated risk factors and antimicrobial resistance*

ABSTRACT

Campylobacter is among the leading bacterial foodborne pathogens, causing a high foodborne disease burden worldwide. There is a limited information on the prevalence, risk factor, and whole genome sequencing of *Campylobacter* in Ethiopian milk and milk products in major milk sheds in Ethiopia. To this end, a cross-sectional study was carried out to isolate and characterize the genomic diversity,antimicrobial resistance patterns and associated risk factors of *Campylobacter* species from milk and dairy products collected from representative regional sites (Oromia, Amhara, and SNNP). in Ethiopia. A total of 1140 dairy food samples were collected in the dry and wet seasons of which 456 samples were used for seasonal comparison. Samples were tested for *Campylobacter* by following the ISO 10272-1:2017 standard and confirmed by PCR with Illumina MiSeq instrument (v3 600-cycle cartridge) for the paired-end sequencing run. Amrfinderplus_db NCBI was used to detect *gyrA* and *50S_L22_A103V* gene mutations. NCBI Pathogen Detection database was used for the genomic similarity . A total of 141 *Campylobacter* isolates were tested for susceptibility to three antibiotics using a disk diffusion method.. The result indicated that *Campylobacter* was detected in 12% of tested food samples. The highest prevalence of *Campylobacter jejuni* and *Campylobacter coli* was found in raw milk (19%), followed by pasteurized milk (10 %) and cottage cheese (3%) (P<0.001). The prevalence did not differ significantly between the wet (20%) and dry (16%) seasons (P=0.27). However, there was a five times more chance of finding *Campylobacter* species in milk and milk products during the wet season than the dry season (COR = 4.5 (1.8-12), P = 0.002). in the Oromia region, Besides, 89% of the samples were contaminated with *C. jejuni*, and 11% with *C. coli*. Two different *C. jejuni* MLST sequence types, namely, ST 51 (clonal complex ST-443) and ST 2084 (clonal complex 353) were detected; they were clustered in different clades (B and C), respectively. Two ST 1628 and 2 ST 830 C. coli from clonal complex 828 were grouped into a single clade (C). Phenotypically, 89 %, 74%, and 57% of *Campylobacter* species were resistant to tetracycline, erythromycin, and ciprofloxacin

respectively. Moreover, 43% of the tested isolates were resistant to more than two drugs. Genomically, ten isolates of 8 *C. jejuni* ST 2084 and 2 *C. coli* ST 1628 had a T86I mutation in the gryA gene, which is associated with resistance to Quinolone (ciprofloxacin), and all 14 *C. jejuni* carry 50S_L22_A103V gene, associated with resistance against Macrolide (erythromycin). Of these, all *Campylobacter* species carried CTD genes, chemotaxis-related genes (cheA, cheB, cheR, and cheY), and invasive genes (*flaC, ciaB,* and *ciaC*). We can conclude that 12% of *Campylobacter* species were present during the dry and wet seasons. The data also showed that , 43% of the isolates acquired more than two antibiotic resistance genes and a mutation was present in the *50S_L22_A103V* and *gryA* genes in *C. jejuni*. The risk factor analysis showed that using warm water and soap for cleaning cow udders and teats on farms (AOR=0.3, P=0.023), filtering milk with a cloth, or plastic filter (AOR=0.065, P=0.005), and storing milk in an aluminum container (AOR=0.23, P=0.027) reduced the likelihood of detecting *Campylobacter* in raw milk. In contrast, *Campylobacter* detection was significantly higher in milk samples collected at collection centers with concrete floors (AOR=5.2, P=0.004). The odds of detecting *Campylobacter* in milk were 17 times greater (AOR=17, P=0.007) in milk processing facilities that did not calibrate a pasteurizer on an annual basis. Likewise,, having a separate refrigerator for milk storage reduced the occurrence of *Campylobacter* in retail (AOR=0.29, P=0.021). In conclusion, Compared to samples of pasteurized and cottage cheese, the raw milk was more contaminated. Additionally, a mutation was found in the *50S_L22_A103V* and *gryA genes* of *C. jejuni*, and 43% of the isolates that were studied possessed more than two antibiotic resistance genes. Thus, understanding the genetic composition and prevalence of Campylobacter in the dairy supply chain may help identify potential contamination sources and create effective management plans that ensure the safety and caliber of dairy products.

Keywords: Ciprofloxacin , pasteurized milk , raw milk, seasonal variation, , whole genome sequencing, contamination

CHAPTER 1

1. General Introduction

1.1. Background and justification

Campylobacter is a genus of Gram-negative, small curved spiral rods that are approximately 0.2-0.5 μm long (Wilson et al., 2021) . It is considered a microaerophilic organism and requires reduced levels of oxygen to grow (Giallourou et al., 2018). It is relatively fragile and sensitive to environmental and oxidative stresses, as well as common disinfectants (Lopez et al., 2015).

Campylobacteriosis is well recognized as the leading cause of bacterial foodborne diarrheal disease worldwide (Silva et al., 2011b). It is mainly caused by *Campylobacter jejuni* (Jackson et al., 2014b, Igwaran and Okoh, 2019). The disease is characterized by an acute gastrointestinal infection with severe abdominal pain, fever, nausea, headache, muscle pain, and diarrhea (Fitzgerald, 2015, El-Zamkan and Hameed, 2016a). *Campylobacter* infection can cause a range of complications, although most people recover without any long-term effects (Gillespie et al., 2002, Gillespie et al., 2008). Thus, it may cause Guillain-Barré syndrome (affect the nervous system and can cause muscle weakness or paralysis) (Willison et al., 2016), reactive arthritis (joint swelling and pain that lasts for 3 to 12) (Walker et al., 2022) and in rare cases sepsis (Otsuka et al., 2023b). Besides, *Campylobacter* infection can also lead to intestinal complications such as appendicitis, acute pancreatitis, or acute cholecystitis (inflammation of the gallbladder) (Kaakoush et al., 2015a).

Campylobacter can be transmitted to humans through a variety of sources. The main route of transmission is believed to be foodborne, via undercooked meat and meat products, as well as raw or contaminated food and water (Wagenaar et al., 2023). Poultry meat becomes contaminated during the slaughtering process and microaerophilic *C. jejuni* can survive ambient atmospheric oxygen tension by metabolic commensalism with *Pseudomonas* species (Giaouris, 2022). This bacterium-bacterium interaction might set the basis for the survival of *C. jejuni* in chicken meat and thus be the prerequisite step in the pathway toward human infection (Hilbert et al., 2010).

In addition to poultry meat, raw milk acts as a second main source of *Campylobacter* (Modi et al., 2015b). Milk can become contaminated with *Campylobacter* from cow feces or from colonized cow teats (Warner et al., 1986). The prevalence of *Campylobacter* species in milk and milk products poses a significant public health concern, with raw milk being identified as a key source of infection for human beings. Studies have shown that *Campylobacter* species, particularly *Campylobacter jejuni*, have been detected in raw milk samples, highlighting the potential risk associated with consuming unpasteurized milk. The presence of *Campylobacter* in raw milk indicates a hazardous situation for human health, emphasizing the importance of proper food safety measures to prevent infections caused by these pathogens (Modi et al., 2015a, El-Kholy et al., 2016).

A number of factors, including inadequate milk pasteurisation, post-pasteurization contamination, starter failure, and inadequate hygiene procedures, can increase the risk of *Campylobacter* infection in milk and dairy products (El-Kholy et al., 2016). Yet, there are additional crucial ways for diseases to spread throughout the human population, such as via drinking contaminated water (Iovine, 2013a, Dec et al., 2018). Wild birds as well as domestic and companion animals are known reservoirs for *Campylobacter* species, and shedding of the bacteria from them causes contamination of the environment (Coker et al., 2000). Direct contact with infected animals, including pets, especially puppies and kittens, is a well-documented means of disease transmission (Solomon and Hoover, 1999). Flies have been shown to carry *Campylobacter* and can infect both humans and animals (Nichols, 2005).

Campylobacter is considered to be the most common cause of bacterial gastroenteritis globally, and it is associated with 7.5 million disability-adjusted life years (Platts-Mills and Kosek, 2014). The incidence and prevalence of *Campylobacter* infection are high in both developed and developing countries (Kaakoush et al., 2015a). In developed countries, the disease occurs in all age groups, while in developing countries, it is endemic and prevalent in infants under the age of 1 year (Rushton et al., 2019). The disease occurs in 1% of the US population each year, and it is estimated to cause 1.5 million illnesses annually (Rushton et al., 2019). The treatment of campylobacteriosis poses significant economic burdens, resulting in $1.56 billion in healthcare costs in the USA (Zhang et al., 2022). Campylobacteriosis is the most commonly reported food-

borne infection in the EU, with an estimated annual number of cases of around 9 million (Lake et al., 2019). A systematic review and meta-analysis of studies conducted in West Africa and sub-Saharan Africa revealed that the pooled prevalence of *Campylobacter* infections in humans was 10% and 9.9 %, respectively (Paintsil et al., 2022a, Hlashwayo et al., 2021). The prevalence of *Campylobacter* in food of animal origin in Africa ranges from 2% in beef to 90% in chicken (Asuming-Bediako et al., 2019). Similarly, a pooled prevalence in Ethiopia found that the prevalence of *Campylobacter* species among children under 5 years of age was 10% (Diriba et al., 2021).

Clinical treatment of campylobacteriosis requires the use of fluoroquinolone (FQ) and macrolide antibiotics, mainly erythromycin and ciprofloxacin (Tang et al., 2017b). However, *Campylobacter* strains have shown increasing resistance against these drugs (Whitehouse et al., 2018, Shen et al., 2018). Resistance of erythromycin is caused by point mutations in the *23S rRNA* gene, particularly in the peptidyl transferase center, alter the ribosome's structure and function, affecting antibiotic binding and ultimately conferring resistance to antibiotics (Long et al., 2010) and *50S L22 A103V* gene, and altered membrane permeability, and efflux are the main mechanisms of resistance to macrolides in *Campylobacter jejuni* (Khan et al., 2019, Gao et al., 2023). Moreover, the primary mechanism of resistance to fluoroquinolones like ciprofloxacin is point mutations in the *GyrA* gene, which determines quinolone resistance (Shen et al., 2018).

Studying tetracycline resistance in *Campylobacter* provides insights into the evolution of resistance mechanisms and the potential for cross-resistance to other antibiotics (Premarathne et al., 2017a). The tetracycline resistance in *Campylobacter* is primarily mediated by the ribosomal protection protein TetO, which binds to an unoccupied ribosomal A site and protects it from the inhibitory effects of tetracycline (Abdi-Hachesoo et al., 2014, Rodrigues et al., 2021, Garcia-Fernandez et al., 2018). An epidemiological report revealed a high level of ciprofloxacin and erythromycin resistance in different parts of the world. For example, a CDC report showed that nearly 24% of *Campylobacter* strains tested in the USA were resistant to ciprofloxacin (Shen et al., 2018). In Europe, a study conducted in Estonia found that *Campylobacter* showed the highest resistance against ciprofloxacin (90.2%) (Tedersoo et al., 2022a). In Egypt and Ethiopia, Schiaffino et al. (2019) and Hlashwayo et al. (2020) reported 77.4% and 30% of *Campylobacter* species resisting ciprofloxacin, respectively. Similarly, during

2017-2018 in the USA, 3.3 % of *Campylobacte*r isolates were resistant to erythromycin (Ford et al., 2023). A study conducted in Poland and Italy showed that 1.1% and 0.9% of *Campylobacter* species were resistant to erythromycin, respectively (Wieczorek et al., 2020, Di Giannatale et al., 2019). A study conducted in Sub-Saharan Africa found that most *Campylobacter* isolates were resistant to erythromycin (44%) (Hlashwayo et al., 2020). Particularly in Ethiopia, 13 % of *Campylobacter* species were resistant to erythromycin (Ewnetu and Mihret, 2010). Therefore, an understanding of the antibiotic resistance mechanisms in *Campylobacter* is needed to provide proper therapy for both animal and human populations (Iovine, 2013a, Tang et al., 2017b).

The virulence of *Campylobacter* species is associated with flagellar motility, adhesion, invasion, and production of cytolethal-distending toxins (Barakat et al., 2020, Lopes et al., 2021). Flagellar motility is an important virulence factor of *Campylobacter* species, as it is required for the bacteria to establish infections in the host (Radomska et al., 2017, Reuter et al., 2020). *Campylobacter* species use several adhesive and invasive genes (*cadF, JlpA, flaC, ciaB, ciaC*) to adhere to and invade host cells (Sierra-Arguello et al., 2021, de Oliveira et al., 2019, Rubinchik et al., 2012). Moreover, CDT is considered a critical virulence factor of *Campylobacter* species and plays a significant role in the pathogenesis of the bacterium. It affects a variety of mammalian cells, including T cells, and contributes to the persistent infection and pathogenic process of *Campylobacter jejuni* (He et al., 2019, Méndez-Olvera et al., 2016, Lee et al., 2003).

Whole genome sequencing (WGS) of *Campylobacter* plays a crucial role in understanding the genetic characteristics, epidemiology, and antimicrobial resistance of this pathogen. It helps to identify genetic variations, such as single nucleotide polymorphisms (SNPs), insertions, deletions, and rearrangements, which can provide insights into the evolution and diversity of *Campylobacter* strains (Golz et al., 2020, Ghielmetti et al., 2023). It can identify clonal clusters, sources of infection, and transmission routes, aiding in outbreak investigations and surveillance efforts (Llarena et al., 2017, Joensen et al., 2020). It helps in understanding the mechanisms of resistance and tracking the spread of resistant strains, contributing to the development of effective antimicrobial stewardship strategies (Ghielmetti et al., 2023). It aids in monitoring the emergence and spread of new strains, detecting and evaluating the effectiveness of control measures (Tong et al., 2021, Ghielmetti et al., 2023).

Therefore, studying the virulence, antibiotic resistance, prevalence, and whole genome sequencing of *Campylobacter* is essential for understanding its impact on public health, developing effective treatment strategies, implementing one Health approaches, ensuring food safety, and addressing regional variations. This research can contribute to the prevention and control of *Campylobacter* infections and mitigate the global burden of this pathogen.

1.2. Statement of the problem

Campylobacter is a major global bacterial cause of gastrointestinal infections, primarily caused by commensal bacteria found in wild animals, farm animals, and companion animals. (Igwaran and Okoh, 2019). These bacteria can transmit zoonotic infections through fecal-oral routes, contaminating various animal-originated foods and dairy products. Raw milk consumption, particularly in rural areas like Ethiopia, is particularly risky due to the persistent contamination of *Campylobacter jejuni* (Kaakoush et al., 2015b). Despite the preference for raw milk, many people in the region are unaware of the benefits of pasteurization. Around 60% of respondents are at risk of contracting zoonotic infections due to their regular consumption of raw meat (Deneke et al., 2022).

Campylobacteriosis incidence and prevalence have increased in developing countries (Dec et al., 2018, Kaakoush et al., 2015b, Jackson et al., 2014b), particularly in children under five (Same and Tamma, 2018). *Campylobacter* infection is a common cause of foodborne illness, causing symptoms like diarrhea, fever, and belly pain. In Ethiopia, *Campylobacter* prevalence is 10%, 3-4 times higher than *Salmonella* or *Escherichia coli* (Facciolà et al., 2017).

Smallholder dairy farmers in Ethiopia, Zanzibar, and Kenya are reportedly practicing hand milking without washing hands before and between milking different cows and udders (Andrew et al., 2021). Only a small percentage of farmers have received training on milk quality and safety, posing a high risk of microbial contamination and transmission of pathogenic microorganisms. Informal marketing of milk poses a risk to consumers, and research on microbial evaluation of raw milk in Ethiopia is limited (Gemeda et al., 2020).

The use of molecular methods, such as whole genome sequencing, is crucial for studying *Campylobacter*, a common foodborne pathogen (Joensen et al., 2020). However, in developing countries, these methods may hinder identifying and tracking outbreaks, predicting antimicrobial resistance, and understanding the genetic diversity of *Campylobacter*. This could lead to ineffective antibiotic prescribing and the development of antibiotic resistance (Hodges et al., 2021). Additionally, sequencing could evaluate the accuracy of diagnostic assays for *Campylobacter*, thereby enhancing public health interventions and vaccine design (Jansen van Rensburg et al., 2016).

Currently, a limited information is available on milk and milk products as a source of *Campylobacter* in Ethiopia. Furthermore, a few research was done on the genomic level characterization of *Campylobacter* in milk and milk products conducted in Ethiopia to allow for pathogen source tracking and antibiotic resistance prediction. Hence, there is a need to determine the prevalence and molecular characterization of this pathogen in milk and milk products in Ethiopia to allow for dairy food safety assessment and outbreak tracking.

1.3. Research question

The research questions to be addressed in this study are the following:-

- What is the prevalence and type of *Campylobacter jejuni* and *Campylobacter coli* in the major milkshed of Ethiopia?
- What are the risk factors for the contamination of milk and milk products with *Campylobacter jejuni* and *Campylobacter coli* ?
- Is there a difference in the prevalence of *Campylobacter jejuni* and *Campylobacter coli* in the wet and dry seasons?
- What is the genotypic and phenotypic antibiotic resistance profile of *Campylobacter jejuni* and *Campylobacter coli* in the Ethiopian dairy value chain?
- What are the genomic variants of *Campylobacter jejuni* and *Campylobacter coli* in Ethiopia's dairy value chain?

1.4. Objective of the Study

1.4.1. General objective

The general objective of this study is to isolate and chatacterize the genomic diversity as well as antimicrobial resistance patterns of *Campylobacter* species found in milk and dairy products in Ethiopia.

1.4.2. Specific objectives

- To isolate and identify *Campylobacter jejuni and Campylobacter coli* from milk and dairy products
- To identify the risk factors for contamination of dairy products with *Campylobacter jejuni and Campylobacter coli* in the Ethiopian dairy value chain
- To investigate the seasonal difference in the prevalence of *Campylobacter jejuni and Campylobacter coli* in the dairy supply chain;
- To investigate the antimicrobial resistance patterns of *Campylobacter jejuni and Campylobacter coli* in the Ethiopian dairy value chain;
- To determine their pathogenicity, sequence type, virulence factor, and antibiotic resistance genes of *Campylobacter jejuni and Campylobacter coli* in Ethiopia using whole genome sequencing ;

1.5. Significance of the study

Considering the stated problem, more data are needed to assess the magnitude and relevance of campylobacteriosis infections associated with milk and dairy products in Ethiopia.Understanding the prevalence of *Campylobacter* in milk and milk products is essential for assessing the risk of foodborne infections in consumers. Investigating antibiotic resistance patterns and the presence of resistance genes in *Campylobacter* isolates from milk is vital for monitoring the emergence of antimicrobial resistance.

This information guides appropriate treatment strategies and helps in combating the spread of resistant strains. Examining virulence genes in *Campylobacter jejuni* and Campylobacter coli isolated from milk provides insights into their pathogenic potential. Moreover, understanding the virulence factors can help in assessing the severity of infections caused by these bacteria and developing targeted interventions to control their spread. Utilizing whole genome sequencing techniques allows for a comprehensive analysis of the genetic makeup of *Campylobacter jejuni* and *Campylobacter coli*. This approach provides detailed information on genetic va. riations, evolutionary relationships, and potential virulence determinants, aiding in understanding the diversity and pathogenicity of these bacteria with in three region of Ethiopia (i. e., Amhara, Oromia and SNNP).

Furthermore, the study compared different genotypes to identify the genetic diversity of *Campylobacter* recovered from milk and milk products within three regions of Ethiopia (i.e., Amhara, Oromia, and SNNP).

CHAPTER 2

2. Literature review

2.1. Taxonomy of *Campylobacter* Species

Campylobacter is a genus of gram-negative bacteria that are motile (Wilkinson et al., 2018). In 1886, Theodor Escherich was the first scientist to discover cholera infantum in a child's stool (Vandamme et al., 2010). After twenty years (1906), two British vets discovered a huge number of uncommon bacteria in sheep uterus tissue, which is now known as *Campylobacter* species (Zilbauer et al., 2008). McFaydean and Stockman isolated *Campylobacter* from aborted fetuses of aborted lambs in 1913 (Skirrow, 2006). In 1919, Smith and Taylor isolated a *Vibrio fetus* from a cow fetal tissue. Because of the bacteria's comma form, they were given the genus name *Vibrio* (El-Naenaeey et al., 2020). Smith and Orcutt in 1927 and Doyle in 1944, respectively, found a group of bacteria in the feces of cattle and pigs with diarrhea and named them *Vibrio jejuni* and *Vibrio coli* (Vandamme et al., 2010, Vandamme, 2000).

A bovine strain of the *vibrio* species was discovered in a human who had raw milk in the United States of America in 1938 (Levy, 1946). Following that, the *Vibrio* species were found in 11 gastroenteritis patients in the United States, with seven strains being *V. fetus* and four being a closely related species known as associated vibrios (King, 1957). In response to King's research, Sebald and Veron (1963) divided *Vibrio* species into two categories based on carbohydrate fermentation and DNA guanine-cytosine (GC) concentration. *Campylobacter fetus* and *C. bobulus*, a member of the current *Campylobacter* genus, were termed Vibrio fetus in 1963 after the group with the lowest GC content was allocated to a new genus *Campylobacter*, which means 'curved rods' in Greek(Sebald and Veron, 1963, Ngulukun, 2017). In 1991, a DNA-rRNA hybridization study of sixty strains representing *Campylobacter* species, *Campylobacter*-like organisms, Wolinella, Bacteroides, and Flexispira species was conducted (Romaniuk and Trust, 1987). The study discovered that *Campylobacter*, *Wolinella*, and *Flexispira* represented a separate sixth rRNA superfamily sensu De Ley within the gram-negative bacteria group (Romaniuk and Trust, 1987). The genus *Campylobacter* belongs to the family Campylobacteriaceae, the order Campylobacterales, the class

Epsilonproteobacteria, and the phylum Proteobacteria, consisting of a vast and diverse group of bacteria currently comprising 27 species, 9 subspecies, and 3 biovars (Silva et al, 2018).

2.2. Structure of *Campylobacter* species

Campylobacter species are capsulated gram-negative, microaerophilic, non-fermentative, and motile bacteria (Abdellatif et al., 2022). Most of them are rod-shaped, curved, "S"-shaped, or spiral-shaped bacteria (Borriello et al., 2005). *Campylobacter* species' virulence and persistence in host cells are influenced by their surface shape, including N-linked glycosylation, sialic acid, flagella, JlpA protein, lipooligo, lipopolysaccharides, capsules, and glycans (Kreling et al., 2020). *C. jejuni's* lipooligo and lipopolysaccharides are surface features that enhance its pathogenicity and may be important for adhesion and host cell penetration (Kreling et al., 2020).

The other surface structure is JlpA protein is a lipoprotein adhesion that promotes the interaction with host cells (Kawai et al., 2012). Sialic Acid gene sets have been identified that produce sialic acid on the cell surface, enabling *Campylobacter* to survive in the host's body by avoiding the body's immune system (Burnham and Hendrixson, 2018). *Campylobacter jejuni* is often flagellated, which allows it to move within a strongly viscous environment and plays a role in its ability to adhere to and invade host cells (Burnham and Hendrixson, 2018). N-linked Glycosylation is a process that may prevent or lessen degradation by extracellular host enzymes (Phillips et al., 2019). *Campylobacter* species, a prominent foodborne pathogen, is known to produce a polysaccharide capsule (CPS) that plays a crucial role in its virulence and ability to interact with the environment (Khemnu et al., 2023). The genome of *Campylobacter* species contains a genome size of approximately 1.6 to 1.7 million base pairs (Mbp) and a G/C content of around 30% (He et al., 2020).

2.3. Characteristics of *Campylobacter* species

Campylobacter species are fastidious consumers with severe metabolic limitations. Amino acids, citric acid cycle intermediates, and short-chain fatty acids are the main sources of energy for *Campylobacter jejuni*, the species most commonly linked to human disorders (Wagley et al., 2014). *C. jejuni* primarily depends on amino acids and

C4-dicarboxylates as a primary energy source (Yeow et al., 2020). Its energy requirements are mostly met by the citric acid cycle, which generates pyruvate, fumarate, oxaloacetate, and other intermediates in all of the pathways (Stahl et al., 2012).

They are thermotolerant, with their optimal temperature being 41.5°C, and can be grown at temperatures ranging from 37 to 42°C (Acheson and Allos, 2001). Their growth is restricted to temperatures below 30^{o}C in the absence of cold-shock proteins. *Campylobacter* species require 3 to 15% oxygen and 3 to 5% carbon dioxide for growth as they have a respiratory metabolism, resulting in the consumption of oxygen and the production of carbon dioxide (Ikeda and Karlyshev, 2012). When exposed to ambient oxygen, however, *C. jejuni* can transform into a coccal form (Ikeda and Karlyshev, 2012).

These reactive oxygen species include superoxide radicals, hydrogen peroxide, and hydroxyl radicals, which can damage bacterial DNA, proteins, and lipids (Palyada et al., 2009). To defend against oxidative stress, *Campylobacter* species have developed various mechanisms, such as the production of antioxidant enzymes and the regulation of gene expression (Kim et al., 2015, Negretti et al., 2017, Flint et al., 2014). For example, *C. jejuni* produces an iron-regulated alkyl hydroperoxide reductase (AhpC) that confers aerotolerance and oxidative stress resistance. More precisely, *C. jejuni's* continuous growth in deoxycholate induced the generation of reactive oxygen species (Palyada et al., 2009, Negretti et al., 2017). To overcome this challenge, numerous selective media including oxygen scavengers such as blood, ferrous iron, and pyruvate have been formulated for their culture (Silva et al., 2011a).

The best pH for the growth of *Campylobacter* species, particularly *C. jejuni*, is in the range of 6.5 to 7. It is important to note that *C. jejuni* is killed at pH 3.0–4.5 (Ramires et al., 2023). *Campylobacter* species, particularly *C. jejuni*, are sensitive to high salt concentrations (Oh et al., 2019).

2.4. *Campylobacter* selective culture media

There are two types of *Campylobacter* selective solid media utilized in current standard culture procedures. Skirrow agar, Blaser agar, Campy-Cefex agar, and Preston agar are among the growth media that use animal blood as a supplement. On the other hand, mCCDA (modified charcoal cefoperazone Deoxycholate agar), Karmali agar, and cefoperazone amphotericin teicoplanin agar belong to the blood-free media category (Moran et al., 2009). Blood-free media with activated charcoal has advantages over blood-containing media since sterile blood is expensive and easily contaminated (Skirrow, 1977). The use of selective agars in routine detection techniques is suggested by numerous food authorities (Karmali et al., 1986). From these, the most frequently used selective medium for the direct plating of *Campylobacters* is the charcoal cefoperazone Deoxycholate agar (CCDA) (Standardization, 2006).

This medium is usually used for the culturing of *Campylobacter* species from food and environmental samples. It contains a range of selective agents, including cefoperazone, vancomycin, and amphotericin B, which inhibit the growth of other bacteria and fungi (Soto-Beltra et al., 2023, CLSI, 2023). Bolton broth is a liquid enrichment medium that is used to increase the sensitivity of *Campylobacter* detection. It contains selective agents, including cefoperazone, vancomycin, and trimethoprim, which inhibit the growth of other bacteria and fungi (Kim et al., 2016). Preston broth is similar to Bolton broth but contains different selective agents, including polymyxin B and rifampicin. It is used for the enrichment of *Campylobacter* species from food and environmental samples with a strong background microbiota (Standardization, 2006).

Campylobacter species, particularly *C. jejuni*, can enter a viable but non-culturable (VBNC) state upon exposure to various stressors, including low temperature, oxygen, and acid (Lv et al., 2020, Pokhrel et al., 2022). The VBNC state of *Campylobacter* has been studied in various contexts, including food safety, environmental survival, and pathogenesis (Lv et al., 2020, Li et al., 2014, Reichelt et al., 2023). The challenge with the VBNC state of *Campylobacter* is that it can escape detection using traditional microbiological culturing techniques, which are still the gold standard for the detection of *Campylobacter* in food and clinical samples (Lv et al., 2020). However, molecular

methods such as PCR-based methods have been developed for the detection of VBNC *Campylobacter* species in food and clinical samples (Reichelt et al., 2023).

2.5. Molecular detection and typing of *Campylobacter* species

The first time that *Campylobacter* species were found in cow feces without enrichment was in the early 1990s using PCR (Inglis and Kalischuk, 2003). In 1997, a PCR-based method was developed for the differentiation of *Campylobacter jejuni* and *C. coli* (Reis et al., 2018). In 2002, a multiplex PCR assay was established for the identification and differentiation of *C. jejuni* and *C. coli* including *C. lari*, *C. upsaliensis*, and *C. fetus subsp. fetus* (Wang et al., 2002). Real-time PCR was then created to quickly identify and distinguish dangerous *Campylobacter jejuni* and *C. coli* from all other *Campylobacter* species (Abu-Halaweh et al., 2005). PCR-based techniques enable the detection of low concentrations of *Campylobacter* species in food and other samples because they are more quick, sensitive, and specific than conventional culture-based techniques (Singh et al., 2011). Nowadays, several studies have investigated the detection and quantification of VBNC *Campylobacter*, and qPCR assays have been developed to detect and quantify VBNC *Campylobacter* (Lv et al., 2020).

There are several molecular typing methods used to characterize *Campylobacter* species, including multi-locus sequence typing (MLST), whole genome sequencing (WGS), pulse-field gel electrophoresis (PFGE), restriction fragment length polymorphisms (RFLP), and randomly amplified polymorphic DNA (RAPD). MLST and PFGE are among the most commonly used methods for molecular typing of *Campylobacter* species (Taboada et al., 2013). MLST is a genetic typing method that identifies gene variations across bacterial strains, used for epidemiological surveillance (Noormohamed and Fakhr, 2014). PFGE separates DNA fragments based on size, providing a genomic fingerprint (Ahmed et al., 2012). RFLP detects variations in DNA sequences using restriction enzymes, allowing for analysis of genetic diversity in *Campylobacter* species. These techniques help distinguish closely related strains and investigate outbreaks (Noormohamed and Fakhr, 2014). Whole genome sequencing has been used in many public health surveillance projects recently (Cody et al., 2017). WGS has become the new reference standard for typing *Campylobacter* species due to its higher resolution, comprehensive view of the organism's genetic makeup, and ability

to extract relevant information from large quantities of complex data (Hsu et al., 2020, Ng and Kirkness, 2010).

It is also a quick and cost-effective alternative to (PFGE) and MLST, and it can overcome the constraints of these methods by assessing the full genome, including all housekeeping, virulence, and resistance genes (Hsu et al., 2020). It has also a higher discriminatory power than PFGE, RFLP, and MLST (Noormohamed and Fakhr, 2014). Overall, WGS is widely used for detecting and characterizing *Campylobacter* in various sources, and for improving surveillance and outbreak detection of the bacteria. WGS can provide valuable information for public health officials and researchers to better understand the epidemiology of *Campylobacter* and to develop strategies for reducing the risk of foodborne illness (Joensen et al., 2021, Kelley et al., 2020).

2.6. Campylobacteriosis

Campylobacter species are one of the four main causes of strong diarrheal enteritis worldwide (Kreling et al., 2020). The pathogenicity of *Campylobacter* species is multi-factorial and requires the presence of various factors, including the flagella, capsule, both O-linked and N-linked protein glycosylation, secreted proteins, and virulence genes (Young et al., 2007, Barakat et al., 2020, Ngobese et al., 2020). The pathogenesis of *Campylobacter jejuni* comprises four main stages: adhesion to intestinal cells, colonization of the digestive tract, invasion of targeted cells, and toxin production. To initiate infection, the organism must penetrate the gastrointestinal mucus, which it does by using its high motility and spiral shape (Wallis, 1994). Some of the specific virulence factors identified in *Campylobacter* species are discussed in the following paragraphs:

2.6.1. Chemotaxis and motility

The flagellum of the bacteria has a role in both bacterial adherence and host-cell invasion. Similar to the common T3SS, *Campylobacter* flagellum contains components that help transport the non-flagellar proteins (FlaC, FspA, CiaB, CiaC, and CiaI) (Guerry, 2007, Neal-McKinney and Konkel, 2012). The helical body and opposing flagella of *C. jejuni* are used to drill through the viscous mucosa of host organisms' gastrointestinal tracts (Cohen et al., 2020). Chemotaxis and motility have been identified as important virulence factors associated with *C. jejuni* colonization (Rahman

et al., 2014). *Campylobacter* species need chemotactic agents to move towards or away from specific chemical stimuli in their environment (Rahman et al., 2014, Lopes et al., 2021, Mo et al., 2022). *C. jejuni* is known to be chemotactic towards many components of the host's intestinal mucus layer such as mucin, amino acids, and organic acids. It is also chemotactic towards iron and phosphate (Chandrashekhar et al., 2018). The Genes CheP and CheQ, two novel regulators of the core chemotaxis operon cheVAW, control chemotactic motility in *C. jejuni* (Cha et al., 2019). The chemotactic control of the flagellar motor switch is directly attributed to CheO, which is necessary for colonization in several animal models. It has also been discovered to be sensitive to changes in the oxygen content of the surrounding air, playing a more significant part in energy taxis when oxygen levels are low (Mo et al., 2022).

2.6.2. Adhesins

To colonize, *Campylobacter* needs to stick to its host's intestinal wall (Hussein, 2018). These bacteria as adhesins use lipo-polysaccharides, outer membrane proteins, and flagellum (Schröder and Moser, 1997, Grant et al., 1993, McSWEEGAN and Walker, 1986). Additionally, CadF and FlpA proteins are both required for *C. jejuni* to bind to fibronectin (FN) and host cells at their maximum levels (Talukdar et al., 2020). After the bacteria attach themselves to the intestinal host cells, *C. jejuni* enters the cells mostly by endocytosis (Biswas et al., 2003). On the other hand, it has been demonstrated that *C. jejuni* traverses the epithelium through a paracellular pathway, whereby the serine protease HtrA cleaves proteins including occludin and E-cadherin to open tight and adhesion junctions (Backert et al., 2013).

2.6.3. Invasion

After adherence, the ciaC, ciaB, and ciaD are synthesized and secreted by *Campylobacter jejuni* (Rivera-Amill et al., 2001, Negretti et al., 2021). The ciaB, and ciaC proteins are used for the maximum invasion of the host cell; whereas, the *CiaD* protein is involved in promoting cell entry of *Campylobacter jejuni* and plays also a role in the intracellular survival of the bacteria (LaGier and Threadgill, 2014, Neal-McKinney and Konkel, 2012) (Negretti et al., 2021, Eucker and Konkel, 2012). After being injected into the host cell with a flagellar type III secretion system, Cia proteins manipulate MAPK signaling pathway processes in the cell and are required for the

development of the disease (Samuelson et al., 2013, Negretti et al., 2021). Overall, the Cia proteins are essential for host cell invasion and the inflammatory response of *C. jejuni* (Negretti et al., 2021).

2.6.4. The Cytolethal distending toxin (CDT)

After entering the human intestinal epithelium, *C. jejuni* synthesizes cytolethal-distending toxin (CDT) (AbuOun et al., 2005). It is produced by pathogenic bacteria like *Campylobacter* which cause foodborne disorders in the world (Pons et al., 2021). This toxin is made up of three proteins: CdtA, CdtB, and CdtC, which are all encoded by a single chromosome (Kailoo and Kumar, 2021). The two other components of the toxin (CdtA and CdtC) allow CdtB to enter cells by attaching to cholesterol-rich microdomains on the cytoplasmic membrane (Lai et al., 2016). CDT possesses a nuclease activity and is structurally similar to DNase I which is known to cause genomic DNA damage in host eukaryotic cells (Pons et al., 2019).

2.7. *Campylobacter* infection

2.7.1. Gastrointestinal infection

Campylobacter is a common source of bacterial foodborne infections (Authority, 2017). Human campylobacteriosis is dominated by two main species, *Campylobacter jejuni* and *Campylobacter coli* (Tresse et al., 2017). *Campylobacter* infection causes watery bloody diarrhea, abortion, and human acute enteritis (Boysen et al., 2014). Patients with *Campylobacter* infection experience fever, weight loss, and cramps that last, on average, 6 days (Man, 2011). The start of symptoms typically happens 24 to 72 hours after ingestion, though it can take longer for low-dose infected people to experience symptoms. Peak symptoms, may include stomach pain that resembles appendicitis and last for 24 to 48 hours (Blaser, 1997).

2.7.2. Extra gastrointestinal infection and post-infection complication

Campylobacter species have also been identified as causes of extra-intestinal infections (EI). EI are infections that occur outside the intestines, but their signs are connected to issues with the intestines themselves (Hernandez and Green, 2006). *Campylobacter* species, which are gastrointestinal pathogens, occasionally cause bacteremia, and it was

discovered that patients with this condition primarily displayed fever rather than the typical symptoms of gastroenteritis (Otsuka et al., 2023a). Bacteremia is more prevalent in kidney donation recipients who also have reactive arthritis, erythema nodosum, and multiple splenic abscesses (Coustillères et al., 2022). Additionally, in individuals with compromised immune systems, *Campylobacter* can result in myocarditis and meningitis (Mizuno et al., 2022, Jiffry et al., 2023).

Autoimmune conditions like Reactive arthritis (ReA), irritable gut syndrome, and Guillain-Barré syndrome (GB syndrome can be brought on by *Campylobacter* infection (Malik et al., 2022). Miller-Fisher syndrome, a form of GBS, is also connected to a previous *Campylobacter* infection (Havelaar et al., 2012). There are three most typical side effects of campylobacteriosis. The most prevalent and severe form of acute paralytic neuropathy is Guillain-Barré syndrome (Willison et al., 2016). The development of antibodies that cross-react with peripheral nerve gangliosides and the lipooligosaccharide from the bacterium *C. jejuni* is a key step in the immunopathogenesis of GBS (Brudvig et al., 2022). The peripheral nervous system is damaged as a result of this autoimmune response (McGrogan et al., 2009, Sejvar et al., 2011). Evidence of recent or ongoing *Campylobacter jejuni* infection has been found in approximately one out of every four cases of GBS (McCarthy and Giesecke, 2001). The organism that has most frequently been described in association with Guillain–Barré syndrome is *Campylobacter jejuni* (Rees et al., 1995). Approximately one-third of GBS patients worldwide have a history of *Campylobacte*r infection and are more severe (Poropatich et al., 2010, Yuki et al., 1993, Jacobs et al., 1996). GBS-related long-term impairment is increasingly obvious, and 20% of patients are anticipated to be moved to a critical care unit (Islam et al., 2010). *Campylobacter* species can reach the synovium and cause reactive arthritis, leading to a cascade of inflammatory responses and the initiation of painful joint symptoms. This is a painful inflammation of the joints that can last for several months. It usually occurs 3 to 40 days after the onset of diarrhea and predominantly affects the knees.

It is more common and possibly more severe in patients with HLA-B27 phenotype (Keithlin et al., 2014, Ajene et al., 2013). Additionally, *Campylobacter* enteritis can lead to post-infectious irritable bowel syndrome, which typically presents as chronic abdominal pain, bloating, and diarrhea that alternates with constipation and persists

even after the triggering pathogen has been eradicated (Thabane et al., 2007). In PI-IBS, which develops after *Campylobacter jejuni* infection (Peters et al., 2021, Takakura et al., 2022), macromolecule uptake via endocytosis was enhanced, leading to low-grade inflammation with pro-inflammatory cytokine release (Omarova et al., 2023). Recent studies suggest that low-grade chronic inflammation, autoimmunity, and bacterial translocation may be involved in the link between *Campylobacter* infection and IBS (Takakura et al., 2022). PI-IBS develops in 10% of *Campylobacter jejuni* infections, 2/3 of which are of the IBS with diarrhea subtype (IBS-D) (Spiller1, 2011).

2.8. Chemotherapeutics and antibiotic resistance genes of *Campylobacter* species

The current drugs of choice for treating *Campylobacter* infections are macrolides, such as azithromycin, and fluoroquinolones, such as ciprofloxacin and levofloxacin (Allos et al., 2013). Many *Campylobacter* isolates are becoming increasingly resistant to various antibiotics (Tang et al., 2017a). The emergence and spread of antibiotic-resistant bacteria, like *Campylobacter*, have been linked to the usage of antibiotics in cattle (Portes et al., 2023). This practice can lead to the development of antibiotic-resistant bacteria in animals, which can then be transmitted to humans through contaminated food (Portes et al., 2023). The growing isolation frequency of resistant *Campylobacter* species has motivated researchers to look into the mechanisms of resistance to various antimicrobials (Iovine, 2013b).

Tetracycline resistance in *Campylobacter* species is primarily mediated by a ribosomal protection protein (tetO), which is transferred as a plasmid-encoded gene (Abdi-Hachesoo et al., 2014, Pratt and Korolik, 2005). In addition to tetO, the cmeB gene has been identified as responsible for tetracycline resistance in *Campylobacter* species (Sierra-Arguello et al., 2015). A study conducted in China, Denmark, Peru, and Brazil found that 96-94%, 92 %, 66%, and 20%, respectively, of *Campylobacter* species isolated from different samples were confirmed to harbor the tet (O) gene.

Phenotypically, a study conducted in China, Malaysia, Peru, and Brazil inducated that 96.5%,76.9%, 73.3%, and 35.5% of *Campylobacter* species were resistant to tetracycline, respectively, (Premarathne et al., 2017b, Yao et al., 2020, Benites et al., 2022, Sierra-Arguello et al., 2015). Another study conducted in South Africa found that

all *Campylobacter* species isolated from water samples were resistant to tetracycline (Chibwe et al., 2023). In Ethiopia, a systematic review and meta-analysis of studies showed that most 41.5 % *Campylobacter* isolates were resistant to tetracycline (Zenebe et al., 2020).

Macrolides are antibiotics that inhibit protein synthesis by targeting the bacterial ribosome (Vázquez-Laslop and Mankin, 2018). Macrolide resistance has been found in high levels in *C. jejuni* and *C. coli* isolates from humans and animals (Pollett et al., 2012). Erythromycin resistance in *Campylobacter* can occur through different mechanisms. The most frequently reported mechanism of high-level erythromycin resistance in *Campylobacter* isolates is the A2075G mutation in the 23S rRNA gene (Gibreel and Taylor, 2006, Kurinčič et al., 2007). Additionally, The *50S_L22_A103V* gene mutation has been identified in various studies and is considered one of the mutations that can confer macrolide resistance in *Campylobacter jejuni* (Hull et al., 2021).

Besides, the presence of the cmeABC operon was also the most common mechanism of erythromycin resistance (Cheng et al., 2020). The rate of erythromycin resistance in *Campylobacter* varies depending on the study and location. For example, in Turkey, the rate of erythromycin resistance was found to be 4.8% among *Campylobacter* isolates (Eryıldız et al., 2022), while in China, the erythromycin resistance of *C. coli* was higher (59.23%) than *C. jejuni* (2.50%) (Gao et al., 2023). A recent study in Sub-Saharan Africa, including Ethiopia, showed that most *Campylobacter* isolates were resistant to erythromycin (44%) (Hlashwayo et al., 2020). In Ethiopia, Chala et al. (2021) identified the resistance to macrolides (erythromycin and azithromycin) as the most dominant antimicrobial class appearing in 74.1% of the 54 *Campylobacter* isolates (Chala et al., 2021).

Ciprofloxacin and other fluoroquinolones were once thought to be the most effective treatment for campylobacteriosis (McDermott et al., 2002). Fluoroquinolone resistance is mediated by changes in amino acids in the quinolone resistance-determining region (QRDR). Gyrase gene products are large quaternary enzymatic structures made up of two subunits called *gyrA* and *gyrB*. The gyrA gene, which encodes part of the gyrA subunit of DNA gyrase, provides a high level of ciprofloxacin resistance due to the point mutation Thr86Ile caused by the C257T alteration in the gyrA gene (Panzenhagen

et al., 2021). A study based in the USA found that 16% of *C. jejuni* isolated from gut and fecal samples in cattle were resistant to ciprofloxacin (Sproston et al., 2018). A recent study showed that the proportion of *Campylobacter* isolates resistant to fluoroquinolones increased from 7% in 1995 to 50% in 2018 in the UK (Veltcheva et al., 2022). A study conducted in Lithuania found that 91.4% of *C. jejuni* isolates were phenotypically resistant to ciprofloxacin (Aksomaitiene et al., 2018). In sub-Saharan Africa, the proportion of ciprofloxacin resistance in *Campylobacter* was shown to be 16% (Hlashwayo et al., 2021). A systematic review and meta-analysis conducted in Ethiopia found that over 60% of *Campylobacter* isolates were resistant to ciprofloxacin (Zenebe et al., 2020).

2.9. Prevalence of *Campylobacter* species in animal, food, and environmental sources of infection

2.9.1. Prevalence of *Campylobacter* species poultry

Poultry species are considered the major reservoirs for thermophile *Campylobacter* species, including *C. jejuni*, *C. coli*, and *C. lari* (Burnham and Hendrixson, 2018). *C. jejuni* is highly prevalent in commercial poultry farms (Sahin et al., 2002). According to research results, newly hatched chickens are free of *Campylobacter* (Kalupahana et al., 2013). It's interesting to note that chickens only start to become colonized at two to three weeks old (Hermans et al., 2014). Following the infection, broilers rapidly show a high load of *C. jejuni* in the caecal content (Shanker et al., 1990). *C. jejuni* is highly prevalent in commercial poultry farms, where horizontal transmission from the environment is considered to be the primary source of *C. jejuni* (Al Hakeem et al., 2022).

On farms, in slaughterhouses, and contaminated food, a variety of fomites and vectors have been found to support the survival and spread of the thermotolerant *Campylobacter* spp (Rossler et al., 2020). Rossler et al., (2020) reported that raw meat with the highest incidence of thermotolerant *Campylobacter* (Rossler et al., 2020) becomes the source of Campylobacteriosis in humans (Di Giannatale et al., 2019). This showed that the thermotolerant *Campylobacter* is common throughout the food chain (Rossler et al., 2020). WGS analysis to define the evidence of *Campylobacter* transfer

from the same label-tagged broiler samples during rearing to the slaughter procedure (Tang et al., 2020).

The prevalence of *Campylobacter* species in poultry in the United States and other developed countries is a significant concern. Studies indicated high prevalence rates of *Campylobacter* in poultry, including broiler chickens. For example, a systematic review and meta-analysis conducted in the United States and Canada found a pooled prevalence of 74.71% in broiler chickens (Plishka et al., 2021). Similarly, another study conducted in Ontario, Canada found that 20% of commercial broiler chicken lots sampled at federally registered slaughter were *Campylobacter* positive (Schweitzer et al., 2021). Additionally, a study conducted in Spain found a prevalence of *Campylobacter* species in poultry meat portions ranging from 60% to 100% depending on the farm (Perez-Arnedo and Gonzalez-Fandos, 2019). In Germany, the prevalence of *Campylobacter jejuni* in poultry flock samples was 42%, and the prevalence in poultry meat ranged from 14% to 34% per year (Schielke et al., 2014). In Italy, the prevalence of *Campylobacter* in poultry meat at the retail level varied from 20.7% to 81.3% in different regions, and at processing plants, the prevalence has not been widely reported (Mezher et al., 2016).

In the UK, the prevalence of *Campylobacter* in fresh chicken at retail was found to be 73.3% (Tedersoo et al., 2022b, Jorgensen et al., 2017). A study conducted in China found that 60% to 74.71% of broiler chickens were *Campylobacter* positive (Tang et al., 2020). The pooled prevalence of *Campylobacter* in poultry in Sub-Saharan Africa is reported to be 39%, with individual prevalence estimated between 4–88% in poultry (Olum et al., 2023).

Several investigations were undertaken in Ethiopia between 2008 and 2021 to examine the prevalence of *Campylobacter* in poultry feces and meat. Scholars reported that 13% to 86.6 % of the poultry feces contained *Campylobacter* species (Table 1). They showed that the prevalence of *Campylobacter* in poultry meat and reported that 21.7 % and 43.9 % of the chicken meat samples were contaminated with *Campylobacter* species, respectively. One of the most common farm operations in Ethiopia is growing scavenging chickens (Desta and Wakeyo, 2012). As a result, when children play on the ground, especially in rural and peri-urban communities, they are frequently

contaminated with chicken feces. As a result, a concerted effort should be made in Ethiopia to avoid the above-mentioned chicken management system.

Table 1. The prevalence of *Campylobacter* species in poultry in Ethiopia from 2008 to 2015.

Sample type	N	Site	n%	Reference
Chicken meat	60	Addis Ababa & Debrezeit	13 (21)	(Dadi and Asrat, 2008)
Chicken meat	66	Mekele	29 (43)	(Hagos et al., 2021)
Poultry fecal	220	Bahir Dar	160 (72)	(Ewnetu and Mihret, 2010)
Poultry fecal	97	Gambella	84 (87)	(Abamecha et al., 2015b)
Poultry fecal	69	Addis Ababa	9 (13)	(Stringer et al., 2021)
Poultry fecal	191	Jimma	130 (68)	(Nigatu et al., 2015)
Poultry fecal	90	Around Gonder	26 (29)	(Nigatu et al., 2015)

N=number of the positive sample, n=number of the sample

2.9.2. Prevalence of *Campylobacter* species Cattle

Next to poultry, Cattle are a potential source of *Campylobacter* infection in humans, and the bacteria can be transmitted to humans through contaminated meat or water. Dairy cattle may be a potential reservoir of human Campylobacteriosis, and infected dairy calves may be a direct or indirect source of *Campylobacter* infection in humans (An et al., 2018, Grout et al., 2022). The prevalence of *Campylobacter* in dairy cattle is high, and the bacterium is the most common pathogen found in dairy cattle (An et al., 2018). They can contaminate the environment by spreading harmful bacteria through feces and manure additives in agriculture because they survive for a prolonged time in the manure (Grout et al., 2022, Inglis and Kalischuk, 2003). *Campylobacter* transmission is proven to occur when people come into contact with animals directly (Hailu et al., 2021, Hansson et al., 2020). A systematic review and meta-analysis found that cross-contamination of raw milk through feces is an important vehicle for the transmission of *Campylobacter* to consumers (Knipper et al., 2022).

The prevalence of *Campylobacter* in dairy cattle in the USA is estimated to be around 29% (ranging from 23% to 36%) at the individual level and 51% (ranging from 44% to 57%) at the pooled level, according to a systematic review and meta-analysis (Knipper et al., 2022). Another study conducted in Quebec, Canada, detected *Campylobacter* species in 19.3% of dairy cattle herds (Guévremont et al., 2014). Additionally, a study conducted in France reported a prevalence of thermophilic *Campylobacter* in the global cattle population at 69.1% at the slaughterhouse level (Thépault et al., 2018). In sub-Saharan Africa, a systematic review and analysis indcated an overall mean prevalence of 17.6% for *Campylobacter* in cattle, with *C. jejuni* being more prevalent than *C. coli* (Gahamanyi et al., 2020).

In Ethiopia, different investigations have been undertaken from 2007 to 2021, *Campylobacter* species were often discovered in fecal or tissue samples of Ethiopian cattle at the farm level (Table 2). The prevalence of *Campylobacter* species in cattle feces ranges from 12.7% to 48% (Kassa et al., 2007, Nigatu et al., 2015, Abamecha et al., 2015a). However, the prevalence falls to 19 % when using a combination of culture and PCR methods (Stringer et al., 2021). Similarly, two research reports on cattle meat using the culture method and molecular method (Table 2), showed that the prevalence was lower in 2008 (6.2 %) than in 2021 (11.9%) (Hagos et al., 2021, Dadi and Asrat, 2008). However, the urban and peri-urban dairy farming community in Ethiopia's major cities is exposed to a substantial level of infectious risk as a result of their consumption habits of raw meat (Deneke et al., 2021).

Table 2: The prevalence of *Campylobacter* species in catte in Ethiopia from 2007 to 2021.

Sample Type	Sample N	Site	N%	Reference
Cattle Carcasses	177	Jimma Town	14 (7.9)	(Birhanu et al., 2017)
Cattle Carcasses	171	Jimma Town	7 (4.1)	(Debelo et al., 2022)
Cattle fecal	171	Jimma Town	22 (12.9)	(Debelo et al., 2022)
Cattle Faecal	177	Gambella	85 (48)	(Abamecha et al., 2015b)
Cattle Faecal	135	Addis Ababa	25 (18.5)	(Stringer et al., 2021)
Cattle Faecal	205	Jimma	26 (12.7)	(Kassa et al., 2007)
Cattle Faecal	270	Gonder	58 (21.5)	(Nigatu et al., 2015)
Cattle Meat	225	Addis Ababa & Debrezeit	14 (6.2)	(Dadi and Asrat, 2008)
Cattle Meat	210	Mekele	25 (11.9)	(Hagos et al., 2021)
knife swab	171	Jimma town	6 (3.5%	(Debelo et al., 2022)

2.9.3. Prevalence of *Campylobacter* species Milk and milk product

Milk contains high-quality animal protein and fats, making it a great substrate for bacteria such as *Campylobacter* (Leedom, 2006). Fecal contamination and insufficient pasteurization or post-pasteurization procedures can lead to *Campylobacter* species contamination of dairy products like milk (Taghizadeh et al., 2022). In Poland, milk taken from vending machines, cheese, and milk cream samples were all free of the *Campylobacter* bacteria. Yet, in Egypt, Pakistan, Nigeria, and India 11.8%, 10.2%, 5%, and 3% of the raw milk samples were contaminated with *Campylobacter* (Andrzejewska et al., 2019, Modi et al., 2015a, Salihu et al., 2010, Hussain et al., 2007). The prevalence of *Campylobacter* species in Egypt was observed to be 13% in raw milk, 52% in kareish cheese, 18% in Domiati cheese, and 6% in ice cream (El-Kholy et al., 2016, Modi et al., 2015a).

Inopportunely, in Ethiopia we did found a report on the frequency of *Campylobacter* in milk or milk products until 2022. Therefore, drinking raw milk is always a risk factor for enteric diseases, and contaminated unpasteurized milk has been identified as a source of *Campylobacter* outbreaks (Schildt et al., 2006, Davis et al., 2016).

Risk factors such as poor herd hygiene, the health status of milking cows, the production environment, the milking environment, and milk preservation practices at dairy farms are the main sources of milk contamination (Velazquez-Ordoñez et al., 2019). The hygienic handling of milk from milking to the time it reaches a consumer affects the safety and quality of milk and dairy products (Chatterjee, 2006, Keba et al., 2020). Proper udder and teat cleaning before milking is an important factor required for the production of safe milk according to high hygienic standards (Skrzypek et al., 2003).

Unless appropriately handled, milk can be contaminated by microorganisms at multiple points between production and consumption (Bereda et al., 2012). Moreover, containers that are used for milking, milk storage, and delivery may be possible sources of milk contamination. Under poor sanitary conditions, bacteria can contaminate milk. Additionally, the cow housing system and the environment in which milking activities are carried out have a substantial impact on the safety of milk. A dirty environment with cow dung is one of the many factors that can lead to microbial contamination of the milk during milking (Akbar et al., 2020). Milk-borne infections can be mitigated by effective pasteurization. However, mechanical errors expected to lead to inadequate pasteurization, biofilm formation, or post-pasteurization contamination can lead to persistence of contamination or re-contamination of milk and subsequent exposure of milk consumers to *Campylobacter* (Fernandes et al., 2015).

2.9.4. Prevalence of *Campylobacter* species Goat, Sheep and Pig

Similar to animal husbandry practices in other African nations, goats are typically raised close to people together with other household animals like chickens, pigs, and dogs (Salihu et al., 2009). An 18% and 8% prevalence of *Campylobacter* species in goat feces was reported in Nigeria (Salihu et al., 2009) and in Iran (Rahimi et al., 2017), respectively. In Ethiopia, In 2021, 7.1% of *Campylobacter* species were reported in goat feces in Addis Ababa, and in 2015, 47.3% of occurrence in goat feces was reported in Gambella (Stringer et al., 2021, Abamecha et al., 2015a). Besides, Hagos et al. (2021), and Dadi and Asrat (2008) reported that 7.6% and 9.3% of the goat meat, respectively, was contaminated (Hagos et al., 2021, Dadi and Asrat, 2008) (Table 3). Similarly, different studies on sheep feces were conducted in Debrebrehan, Addis Ababa, Gambella, and Jimma between 2015 and 2021, with incidences of

10.6%,13.3%,33.3%, and 38 %, respectively (Abamecha et al., 2015a, Stringer et al., 2021, Chanyalew et al., 2013b, Nigatu et al., 2015).

Pigs can also serve as a reservoir for the *Campylobacter* species (Papadopoulos et al., 2021). Kassa et al. (2007), and Dadi and Asrat (2008)) found *Campylobacter* species in 50 % and 8.5 % of pig feces and pork flesh, respectively (Kassa et al., 2007, Dadi and Asrat, 2008). Sheep and goats are kept in traditional extensive systems in Ethiopia and most undeveloped countries. Sheep and goats are mostly reared in specialized pastoral and agro-pastoral systems, as well as mixed crop-livestock systems (Gizaw, 2010) (Table 3).

Table 3: The prevalence of *Campylobacter* species in sheep, goat and pig in Ethiopia from 2008 to 2021.

Sample Type	Sample N	Sample Site	N%	Reference
Goat Carcasses	180	Debre Zeit	17 (9.4)	(Woldemariam et al., 2009)
Goat Faecal	55	Gambella	26 (47.3)	(Abamecha et al., 2015b)
Goat Faecal	14	Addis Ababa	1 (7.1)	(Stringer et al., 2021)
Goat Meat	92	Addis Ababa &Debrezeit	7 (7.6)	(Dadi and Asrat, 2008)
Goat Meat	108	Mekele	10 (9.3)	(Hagos et al., 2021)
Pig Faecal	18	Jimma Hospital	9 (50)	(Kassa et al., 2007)
Pig Meat	47	Addis Ababa & Debrezeit	4(8.5)	(Dadi and Asrat, 2008)
Sheep Carcasses	218	Debre Zeit	23 (10.6)	(Woldemariam et al., 2009)
Sheep Faecal	39	Gambella	13 (33.3)	(Abamecha et al., 2015b)
Sheep Faecal	30	Addis Ababa	4 (13.3)	(Stringer et al., 2021)
Sheep Faecal	310	Debrebrehan	33 (10.6)	(Chanyalew et al., 2013a)
Sheep Faecal	71	Jimma	27 (38)	(Nigatu et al., 2015)
Sheep Meat	114	Addis Ababa & Debrezeit	12 (10.5)	(Dadi and Asrat, 2008)

N= number of positive samples

2.9.5. Prevalence of *Campylobacter* species in water environment

Campylobacter can also be found in the water environment. The levels of ammonium and chloride ions in wastewater and surface water can be utilized to successfully forecast the future incidence of *C. jejuni* and *C. coli* (Strakova et al., 2022). Franz et al. (2020) looked into the prevalence, genotype diversity, and potential animal origins of *C. jejuni* and *C. coli* bacteria in surface water in the Netherlands (Mulder et al., 2020). Several animal sources contribute to *Campylobacter* contamination of surface water, the main contributors to *Campylobacter* contamination of surface water are wild birds and meat-producing poultry, with water type, season, and local livestock density being significant drivers of these contributions (Mulder et al., 2020). According to phylogenetic analysis, Gahamanyi et al. (2020) investigated the genetic relatedness of *Campylobacter* species from pediatric and water samples and found that *Campylobacter* infection in the studied communities may have been acquired by drinking dirty water (Gahamanyi et al., 2020).

Tap water, stored water, and surface water were all contaminated, according to Stringer et al. (2021), with the prevalence of 2(12.5), 7(8.3), 7(11.1), and 2(22.2), respectively (Stringer et al., 2021). Even though the sample size was minimal, it indicated that water in Addis Ababa, Ethiopia, was contaminated with *Campylobacter* species. A study conducted in Ethiopia found that 46.2% of dairy farmers washed the udders of lactating cows with cold water (Berhe et al., 2020). Based on the above search results, washing cow udders with cold water can be a risk factor for *Campylobacter* contamination of milk (Table 4).

Table 4. Prevalence of *Campylobacter* species in the water

Reference	Sample source	Sample size	*Campylobacter Spp.N%*
(Stringer et al., 2021)	Groundwater	16	2(12.5)
	Municipal tap water	84	7(8.3)
	Stored water	63	7(11.1)
	surface water	9	2(22.2)

2.10. Occurrence of *Campylobacter* species in Humans

The available search results provide information on the prevalence of *Campylobacter* in humans in the USA and Europe. A study conducted in the USA reported that the modeled incidence of *Campylobacter* infection adjusted for sex and age group has remained stable since 2012, with an incidence of 13.3 cases per 100,000 population in 2018 (Ford et al., 2023). In Europe, the reported incidence of *Campylobacter* infection remained relatively steady in Croatia, ranging from 38.8 per 100,000 in 2016 to 42.2 in 2019, while Germany showed a decreasing trend in the reported incidence of campylobacteriosis from 87.4 per 100,000 population in 2014 to 55.8 in 2020 (Liu et al., 2022). The prevalence of *Campylobacter* in humans in Africa varies depending upon different studies and countries. A review carried out reported the prevalence of thermophilic *Campylobacte*r in humans ranging from 9.6% to 62.7% on average in sub-Saharan Africa. Nigeria reported the highest prevalence of 62.7% in humans, followed by Malawi (21%) and South Africa (20.3%)(Olum et al., 2023). Another study conducted in South Africa found the prevalence of *Campylobacter* species to range from 23.6% to 41.8% across the country, with *C. jejuni* being the most reported species (Ramatla et al., 2022).

Since 2000, several papers have been published investigating the prevalence of *Campylobacter* in human diarrheal stool, with a focus on children under the age of five in Ethiopia. As a result, the prevalence ranged from 8.1 % to 19.9 % (Beyene and Haile-Amlak, 2004, Bukayaw and Mekonnen, 2021, Stringer et al., 2021, Ewnetu and Mihret, 2010, Lengerh et al., 2013c, Mitike et al., 2000, Tafa et al., 2014, Mulatu et al., 2015) (Table 5). The table below shows that the most recent study showed that, 8.9 percent of children under five had been infected with *Campylobacter* species (Nigusu et al., 2022).

Table 5: The prevalence of *Campylobacter* species in humans in Ethiopia from 2000 to 2021.

Sample type	Sample N	Site	N%	Reference
Human stool	430	Jimma	50(12)	(Beyene and Haile-Amlak, 2004)
	196	Jimma	39(Chatterjee)	(Bukayaw and Mekonnen, 2021)
	99	Addis Ababa	10(10)	(Stringer et al., 2021)
	210	Bahir Dar	17(8)	(Ewnetu and Mihret, 2010)
	285	Gonder	44(15)	(Lengerh et al., 2013a)
	153	Gonder	16(11)	(Mitike et al., 2000)
	227	Jimma Town	38 (17)	(Tafa et al., 2014)
	158	Hawassa	20(12.7)	(Mulatu et al., 2015)
	214	Jimma	19(8.9)	(Nigusu et al., 2022)
Hand swab	171	Jimma Town	3(1.8%	(Debelo et al., 2022)

[a]N, number of positive samples

CHAPTER 3

Prevalence of *Campylobacter* species and associated risk factors for contamination of dairy products collected in the dry season from major milk sheds in Ethiopia

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ABSTRACT

A cross-sectional study was conducted to investigate the prevalence and risk factors for contamination of Ethiopian dairy products with *Campylobacter*. A total of 912 dairy food samples were collected from establishments of 682 study participants who were interviewed. Samples were tested for *Campylobacter* by following the ISO 10272-1:2017 standard and PCR confirmation. *Campylobacter* was detected in 11% of tested food samples and all detected *Campylobacter* were *C. jejuni*. The highest prevalence of *C. jejuni* was found in raw milk (16%), followed by pasteurized milk (9%) and cottage cheese (2%) (P<0.001). Using warm water and soap for cleaning cow udders and teats on farms reduced the likelihood of detecting *Campylobacter* in milk (AOR=0.3, P=0.023). Filtering milk with a cloth, using a plastic filter (AOR=0.065, P=0.005), and storing milk in an aluminum container (AOR=0.23, P=0.027) reduced the likelihood of detecting *Campylobacter* in milk at the collection facilities. In contrast, *Campylobacter* detection was significantly more likely in milk collected at collection centers with concrete floors (AOR=5.2, P=0.004). The odds of detecting *Campylobacter* in milk were 17 times greater (AOR=17, P=0.007) in milk processing facilities that did not calibrate a pasteurizer on an annual basis. Finally, having a separate refrigerator for milk storage reduced the odds of detecting *Campylobacter* in retail (AOR=0.29, P=0.021).

Keywords: *Campylobacter jejuni*, raw milk, pasteurized milk, cottage cheese, dairy products, Ethiopia, risk factors, contamination

3.1. Introduction

Campylobacter is among the leading bacterial foodborne pathogens, causing a high foodborne disease burden worldwide (Havelaar et al., 2015, Mughini Gras et al., 2012). *C. jejuni* is responsible for the majority of campylobacteriosis cases and *C. coli* is the second most common cause of human campylobacteriosis (Lambert and Hogan, 2009, Hsieh and Sulaiman, 2018). These species are recognized as a cause of gastroenteritis that can result in severe abdominal pain, fever, nausea, headache, muscle pain, and diarrhea (Fitzgerald, 2015, El-Zamkan and Hameed, 2016a). Furthermore, infections with *Campylobacter* can cause Guillain-Barré syndrome with symptoms of muscle weakening or paralysis (Jackson et al., 2014a, El-Zamkan and Hameed, 2016b).

According to the Foodborne Disease Burden Epidemiology Reference Group (FERG) of the WHO, *Campylobacter* is one of the four (*Salmonella, E. coli* and *Shigella*) main global causes of diarrheal infections, causing an estimated 550 million foodborne disease cases annually (WHO, 2020a). In high-income countries, the incidence of campylobacteriosis is well documented through surveillance systems. For example, in the EU, 40.35 per 100,000 people in the European Union had campylobacteriosis in 2020 (Authority et al., 2021). However, due to minimal surveillance systems for *Campylobacter* in low- and middle-income countries, the incidence of campylobacteriosis in Africa remains largely unknown. A systematic review and meta-analysis reported an average campylobacteriosis incidence of 8.3% in diarrheic and non-diarrheic patients seen in hospitals, basic healthcare clinics, or community cohorts in Sub-Saharan Africa (Fletcher et al., 2011).

The ingestion of contaminated food or water and direct contact with feces from infected animals have been reported as the main modes of transmission of *Campylobacter* (Zenebe et al., 2020). *C. jejuni* is part of the normal intestinal microbiota of many wild and domesticated animals, including livestock, such as poultry, cattle, and swine (Kaakoush et al., 2015b). Among these animal reservoirs, poultry has been identified as the principal reservoir and source of human *Campylobacter* infections, followed by ruminants, including cattle and sheep (Cody et al., 2019). Raw milk was identified as the second most common source of *Campylobacter* infections, after chicken meat (Davis et al., 2016, Modi et al., 2015a). Unless appropriately handled, milk can be

contaminated by microorganisms at multiple points between production and consumption (Bereda et al., 2012). Microbial contamination of milk can originate from a variety of sources, including feed, the environment, cow's udder, milking equipment (Boor et al., 2017), and surface water (Mulder et al., 2020) utilized for cleaning milking containers (Mpatswenumugabo et al., 2019). The level of hygienic handling of milk throughout the value chain can therefore affect the safety and quality of milk and dairy products (Chatterjee, 2006, Keba et al., 2020).

Risk factors such as poor herd hygiene, the health status of the cattle, production environment, milking environment, and milk preservation practices at dairy farms have previously been associated with general bacterial contamination (Velazquez-Ordoñez et al., 2019). For example, proper udder and teat cleaning before milking plays an important role in the production of safe milk (Skrzypek et al., 2003). Similarly, an environment soiled with animal feces has been reported as one of the risk factors for microbial contamination of milk during milking (Gillespie et al., 2009). Given that milk is commonly consumed raw in Ethiopia, the introduction of pathogens at the farm level, before pasteurization, presents a considerable risk for foodborne exposure to *Campylobacter* (Davys et al., 2020a).

Several research studies on the prevalence of *Campylobacter* among humans (Asrat et al., 1999, Beyene and Haile-Amlak, 2004, Terefe et al., 2020, Budge et al., 2020, Mitike et al., 2000, Ewnetu and Mihret, 2010, Tafa et al., 2014, Getamesay et al., 2014, Mulatu et al., 2014, Gedlu and Aseffa, 1996), livestock (Kassa et al., 2007, Ewnetu and Mihret, 2010, Abamecha et al., 2015a, Nigatu et al., 2015, Hailemariam, 2014), and meat (Dadi and Asrat, 2008, Ewnetu and Mihret, 2010, Faris, 2015) have been conducted in Ethiopia. However, there is a knowledge gap in understanding the prevalence of *Campylobacter* in milk and dairy products. This study was therefore conducted to characterize the prevalence of *Campylobacter* and the potential exposure of the Ethiopian public to *Campylobacter* via the consumption of milk and cottage cheese. Importantly, this study provides insight into the regional and value chain differences in *Campylobacter* prevalence in Ethiopia. To improve the understanding of risk factors for contamination of dairy products with *Campylobacter* in Ethiopia, this study also reports findings gained through structured interviews with participating dairy farmers, milk collectors, and retailers. The results reported here can inform the development and

implementation of *Campylobacter* control measures in the dairy value chain in Ethiopia and other African countries with similar dairy value chains.

3.2. Materials and methods

3.2.1. Study areas and sample size

This study was carried out in three Ethiopian regions, including Oromia, Southern Nation Nationalities Peoples (SNNP), and Amhara during the dry season between January and March 2020 (Figure 1). The study sites in Ethiopia, specifically Oromia, Amhara, and SNNP regions, were selected based on the availability of milk value chains.

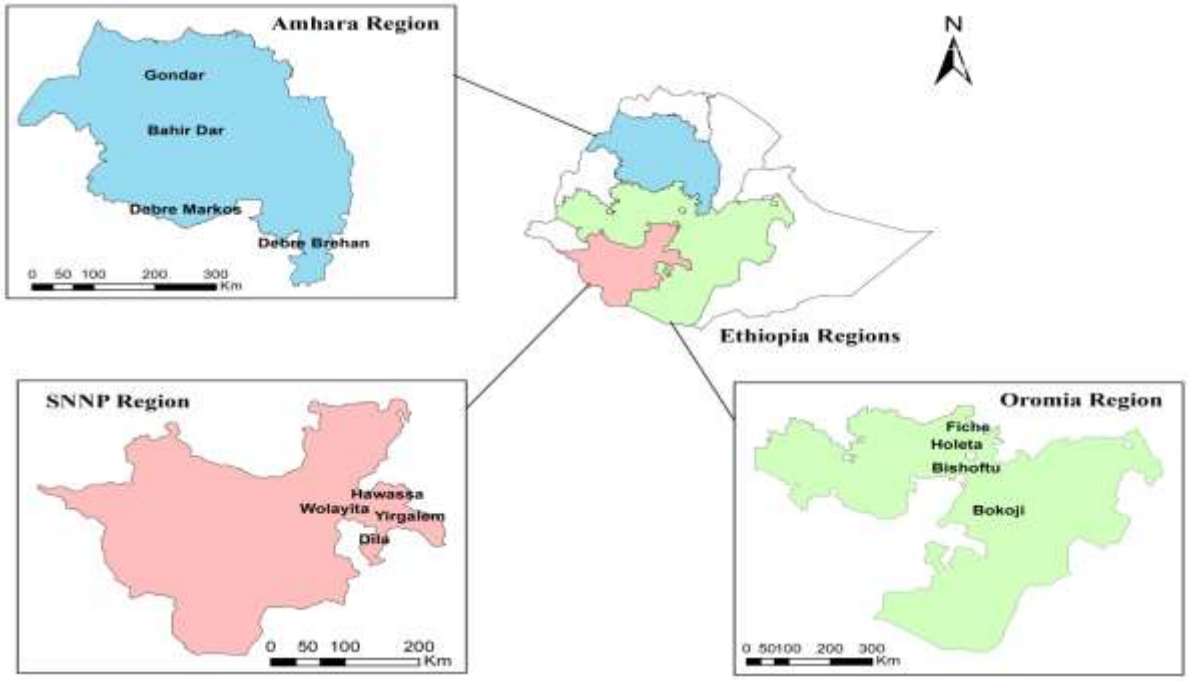

Figure 1: Map of the study areas. Milk and cottage cheese samples were collected from four sites in each of the three Ethiopian regions, including Amhara, SNNP, and Oromia. Study sites within each region are listed on maps of individual regions.

These regions were selected for inclusion in the study due to their substantial milk production potential. The sample size was calculated based on the following formula: $N = Z^2 P (1-P)/ (D^2)$, where z = 1.96 at a 95% confidence interval, D is the tolerated margin of sampling error (5% marginal error was used), p is an estimated prevalence of *Campylobacter* in the population. Since the prevalence of *Campylobacter* in dairy products in Ethiopia was not known, p was assumed to be 50% for the population. This resulted in a minimum sample size of 384.

The three study regions have different production capacities: Oromia produces an estimated ~52%, SNNP ~23%, and Amhara ~20% of milk produced in Ethiopia (CSA, 2011). The relative number of samples collected from each region was therefore proportional to the relative milk production potential. In the Oromia region, 480 samples were equaly distrubted and collected from the towns of Assela (120) , Fiche (120), Debre Zeit (120), and Walmara (120). In the SNNP region, 240 samples were collected from Wolayita (60), Dilla(60) , Hawassa(60) , and Yirgalem (60); and in the Amhara region, 192 samples were collected from Bahirdar (48), Debre Berhan (48), Gondar(48), and Debre Markos (48) (Figure 1). Thus, a total of 912 dairy food samples were collected from 682 study participants, including dairy farmers, milk collectors, processors, and retailers. Study participants were randomly selected from the list of existing potential participants that was assembled with the help of agricultural development agents. The number of participants was lower than the number of collected samples since multiple samples were collected from the same milk collectors and processing facilities.

3.2.2. Sample collection

A total of 250 ml of each raw milk sample was collected into a sterile plastic bottle at each of the 184 participating dairy farms (n = 184) and 58 participating milk collection centers (n = 184). A total of 500 ml of each pasteurized milk sample was collected from each of the 12 participating processors (n = 184) and retailers (n = 184). A total of 500 g of each cottage cheese sample was collected with a sterile plastic pouch from each participating producer (n = 88) and retailer (n = 88). All collected samples were kept at 4°C in a portable refrigerator (Dometic group) until delivery to the laboratory. After samples were delivered to the lab, the laboratory analysis was initiated within an hour. Samples were kept at 3°C in the laboratory until analyses were carried out.

3.2.3. Enrichment and isolation of *Campylobacter*

Milk and cottage cheese samples were enriched for *Campylobacter* by following the ISO 10272-1:2017 method B. This method was followed because Ethiopian milk and milk products have a relatively high concentration of background microflora (Standardization, 2017a). A total of 10 g of cottage cheese or 10 ml of milk were homogenized (Nasco, Whirl-Pak) with 90 ml of Preston broth (OXOID nutritional

broth No. 2, CM0067) supplemented with 5% laked horse blood (Hardy Diagnostics, 10052-808) and a modified Preston *Campylobacter* selective supplement (OXOID, SR0204E), by hand massaging in homogenization bags. Homogenized samples were incubated at 41.5°C for 24 ± 4 hours in a microaerobic environment (CampyGen, Oxoid AGS). A loopful of undiluted enrichment was streaked on mCCDA agar after enrichment. After 44 ± 4 hours of incubation at 41.5°C in a microaerobic environment (CampyGen, Oxoid, AGS), streaked mCCDA plates were examined for the presence of presumptive *Campylobacter* colonies.

3.2.4. DNA Extraction for PCR confirmation of *Campylobacter* species

Two presumptive *Campylobacter* colonies were collected from each mCCDA plate and streaked onto brain heart infusion (BHI) agar and incubated at 37°C for 44 hours in microaerobic conditions (CampyGen, Oxoid AGS). DNA was extracted by heat-lysing a colony of each freshly cultivated isolate in 100 µl of sterile nuclease-free water (Ambion, USA) for 10 minutes at 95°C. Cell lysis was followed by centrifugation at 13,000 g for 5 minutes to sediment cell debris (Kamei, Asakura, et al. 2014). The extracted DNA was stored at -20°C until used in a PCR reaction.

3.2.5. Confirmation of *Campylobacter* species using PCR

Multiplex PCR was used to confirm the genus and species of presumptive *Campylobacter* species isolates obtained from mCCDA agar. Table 6 lists the primer sequences as well as the size of the target PCR products (Wang, Clark, et al. 2002). PCR was performed using a thermal cycler (Bio-Rad T100TM Thermal Cycler, Singapore) in 25 µl reactions consisting of 2.5 µl of DNA template, 12 µl of GoTaq Green Master Mix (Promega), 0.125 µl of forward and reverse primers (100 µM) targeting the species specific target genes such as *C. jejuni hipO* gene, 0.25 µl of forward and reverse primers (100 µM) targeting the *C. coli glyA* gene, 0.05 µl of each forward and reverse primer (100 µM) targeting *Campylobacter genus* specific 23S rRNA sequence, and 9.65 µl nuclease-free water. The PCR thermal cycling protocol included the initial denaturation phase at 95°C for 6 minutes, followed by 30 cycles of amplification, each consisting of 0.5 minutes of denaturation at 95°C, 0.5 minutes of annealing at 59°C, and 0.5 minutes of extension at 72°C. The PCR was completed with a 7-minute final extension at 72°C. Each PCR run included a positive control (DNA

extracted from *Campylobacter jejuni* ATCC 29428) and a negative control (nuclease-free water) (Table 6).

Table 6: List of primers for confirmation of *Campylobacter* genus and species.

Primera	Size (bp)	Sequence (5′-3′)a	Accession no.	Target gene	Gene location (bp)
CJF	323	ACTTCTTTATTGCTTGCTGC	Z36940	*C. jejuni hipO*	1662-1681
CJR		GCCACAACAAGTAAAGAAGC			1984-1965
CCF	126	GTAAAACCAAAGCTTATCGTG	AF136494	C. coli glyA	337-357
CCR		TCCAGCAATGTGTGCAATG			462-444
23SF	650	TATACCGGTAAGGAGTGCTGGAG	Z29326	C. jejuni 23S rRNA	3807-3829
23SR		ATCAATTAACCTTCGAGCACCG			4456-4435

[a] Primer reference: (Wang et al., 2002)

3.2.6. Gel electrophoresis

Gel electrophoresis was performed using a 1.5% w/v agarose gel (Thermo Scientific, 17852) prepared with a trisboric acid/EDTA (TAE) buffer and 5 µl of GelRed (5 mg/ml stock concentration, Biotium) were used to stain DNA. DNA was separated at 120 volts for 40 minutes. Gel Doc EZ Gel Documentation System (Bio-Rad Laboratories) was used to view and record the gel images. Bands of 650, 323, and 126 base pairs were interpreted as a confirmation of *Campylobacter* spp., *C. jejuni*, and *C. coli*, respectively. Each electrophoresis run included a 100 bp DNA ladder, as well as positive and negative controls.

3.2.7. Questionnaire survey

A questionnaire survey was carried out face-to-face using a Kobo Toolbox by interviewing a farmer, milk collector, processor, or retailer at each sampling location. Data on pre- and post-harvest dairy product handling practices such as barn type and cleaning practices, source of water used for cleaning the udder, hygiene of a milker, sanitation of milk utensils, and housing for animal management information was collected. At each sampling location, respondents were also asked to provide information on the type of milk and milk product transportation system they use. In addition to administering a questionnaire, direct observation of general cleanliness,

hygienic practices, and pasteurized milk and cottage cheese packing material was carried out and recorded. After the questionnaires were completed, milk or cottage cheese samples were collected for laboratory analysis.

3.2.8. Data management and analysis

Descriptive statistics were performed using SPSS version 26.0 software after raw data was loaded into a Microsoft Excel spreadsheet. The chi-squared test was used to compare the prevalence of *Campylobacter* among different regions, and sample types (i.e., raw milk, pasteurized milk, cottage cheese), as well as the prevalence of *Campylobacter* at different points in the value chain. A P value of 0.05 was considered statistically significant. Unadjusted and adjusted odds ratios were calculated to investigate the associations between *Campylobacter* species contamination and contamination risk factors obtained through the survey. To calculate the unadjusted odds ratio of each variable with reference to *Campylobacter* species detection, standard logistic regression was utilized. The multivariate analysis included variables that were significant at a P value of 0.2 in the bivariate analysis. The final model that forecasts *Campylobacter* species recovery was developed using a forward selection with a P value of 0.05.

3.2.9. Ethical clearance

The Addis Ababa University Ethics Committee approved surveys used in this study (IRB/42/2019).

3. Results

3.3.1. Prevalence of *Campylobacter* species in different regions

Campylobacter species growth and morphological characteristics on selective media (i.e., glossy light gray colonies) were used to select putative *Campylobacter* colonies and confirm the genus and species using a multiplex PCR. *Campylobacter* species were confirmed in 96 samples collected in a dry season, resulting in a prevalence of 11% (Table 7). All *Campylobacter*-positive samples were contaminated with the species *C. jejuni* (*C. coli* was not detected in tested samples).

The highest prevalence of *Campylobacter* was detected in the SNNP region 15%, followed by Amhara (11%), and Oromia regional states (8%). The differences in the prevalence of *C. jejuni* among the three studied regions were statistically significant (P = 0.011).

3.3.2. Prevalence of *Campylobacter* species in different dairy food types at different points in the dairy value chain

The prevalence of *Campylobacter* species was assessed in raw milk and milk pasteurized using High-Temperature Short Time (HTST), as well as in cottage cheese (Table 7). Of the 368 raw milk samples tested, 16% were contaminated with *C. jejuni*. The prevalence of *C. jejuni* in raw milk samples collected from dairy farmers and milk collectors did not differ significantly (P = 0.88). Compared to the raw milk samples, the prevalence of *C. jejuni* was significantly lower (P = 0.004) among 368 tested pasteurized milk samples (9%) collected from milk processors and retailers. However, the prevalence (9%) of *C. jejuni* did not significantly differ in pasteurized milk samples collected from processors and retailers (P = 0.85). Lastly, the lowest prevalence of *C. jejuni* (2%) was found among 176 tested cottage cheese samples (P = 0.0001) that were collected at dairy farms and retailers. Noteworthy, the cottage cheese samples collected from retailers had a significantly lower prevalence (1%) of *C. jejuni* compared to cottage cheese samples collected from dairy farmers (3%). Overall, as outlined above and summarized in Table 7, the prevalence of *C. jejuni* differed significantly by sample type (P < 0.0001) and point in the value chain in Ethiopian dai ry value chain (P = 0.013).

Table 7: *Campylobacter* species prevalence across the Ethiopian dairy value chain.

Variable	Region, sample type, and value chain	Samples (n)	*Campylobacter* species(%)	P value[a]
Region	Amhara	192	22 (11.5)	0.011
	Oromia	480	38 (7.9)	
	SNNP	240	36 (15.0)	
	Total	912	96 (11)	
Value chain	Raw milk collectors	184	29 (15.8)	0.013
	Cottage cheese producers	88	3 (3.4)	
	Cottage cheese retailers	88	1 (1.1)	
	Milk processors	184	17 (9.2)	
	Milk retailers	184	16 (8.7)	
	Raw producers	184	30 (16.3)	
	Total	912	96 (11)	
Sample type	Cottage cheese	176	4 (2.3)	<0.0001
	Pasteurized milk	368	33 (9.0)	
	Raw milk	368	59 (16.0)	
	Total	912	96 (11)	

[a] p value indicates statistical significance.

3.3.3. Regional differences in *Campylobacter* species prevalence in different sample types

The regional differences in the prevalence of *C. jejuni* was also examined among different sample types tested in this study (Table 8). *C. jejuni* was detected in 13% of tested raw milk samples, 5% of tested pasteurized milk samples, and 3% of tested cottage cheese samples in the Oromia region. In the Amhara region, *C. jejuni* was present in 15% of the raw milk samples, 11% of the pasteurized milk samples, and 3% of the cottage cheese samples. In the Southern Nation Nationalities People region, 15% of the 240 samples tested were contaminated with *C. jejuni*. Of these 36 *Campylobacter*-positive samples, 23% were raw milk samples and 14.6% were pasteurized milk samples. Unlike in milk samples, no *Campylobacte*r was detected in any of the cottage samples collected in SNNP. Overall, in the Oromia (P = 0.003) and Amhara regions (P = 0.001), the prevalence of *C. jejuni* was significantly different among different sample types, whereas in SNNP, the prevalence of *C. jejuni* did not significantly differ among sample types (P = 0.204).

Table 8: Regional differences in *Campylobacter jejuni* prevalence among different sample types.

Region	Sample Type	Samples (n)	*Campylobacter* species(%)	P value[a]
Oromia	Cottage cheese	96	3 (3.1)	0.003
	Pasteurized milk	192	10 (5.2)	
	Raw milk	192	25 (13)	
	Total	480	38 (7.9)	
Amhara	Cottage cheese	32	1 (3.1)	0.001
	Pasteurized milk	80	9 (11.3)	
	Raw milk	80	12 (15.0)	
	Total	192	22 (11.5)	
SNNP	Cottage cheese	48	0	0.204
	Pasteurized milk	96	14 (14.6)	
	Raw milk	96	22 (22.9)	
	Total	240	36 (15.0)	
Total		912	96 (11)	

[a] p value indicates statistical significance.

3.3.4. Differences in *Campylobacter* species prevalence at different points along the dairy value chain

The prevalence of *C. jejuni* differed significantly at different points in the dairy value chain in the Oromia, Amhara, and SNNP regions ($P \leq 0.0001$) (Table 9). In Oromia, *C. jejuni* was detected in 13% of samples collected from producers (dairy farmers), 14% of samples collected from milk collectors, 5% of samples collected from milk processors, 5% of samples collected from pasteurized milk retailers, and 6 % of the cottage cheese collected from the producer. However, none of the cottage cheese collected from retailers was contaminated with *Campylobacte*r species. The prevalence of *C. jejuni* significantly differed at different points in the dairy value chain in the region ($P = 0.022$).

In Amhara, *C. jejuni* was detected in 7%, 23%, 8%, 15 %, and 6% of samples collected from milk producers (dairy farmers), milk collectors, milk processors, pasteurized milk retailers, and cottage cheese, respectively (Table 9). Unlike in Oromia, the prevalence of *C. jejuni* in the Amhara region did not significantly differ at different points in the dairy value chain ($P = 0.108$).

In the Southern Nation Nationalities Regional State, *C. jejuni* was detected in 31%, 15%, 19%, and 10 % of raw milk samples collected from producers (dairy farmers), milk collectors, milk processors, and milk retailers, respectively. Similar to Oromia, the prevalence of *C. jejuni* varied significantly at different points in the dairy value chain in the region (P = 0.001).

Table 9: The prevalence of *Campylobacter jejuni* at different points in a dairy value chain in different regions.

Region	Point in the value chain	Samples (n)	*Campylobacter jejuni* (%)	P value[a]
Oromia	Collectors	96	13 (13.5)	0.022
	Cottage cheese producers	48	3 (6.3)	
	Cottage cheese retailers	48	0	
	Pasteurized milk processors	96	5 (5.2)	
	Pasteurized milk retailers	96	5 (5.2)	
	Producers	96	12 (12.5)	
	Total	480	38 (7.9)	
Amhara	Collectors	40	9 (22.5)	0.108
	Cottage cheese producers	16	0.0	
	Cottage cheese retailers	16	1 (6.3)	
	Pasteurized milk processors	40	3 (7.5)	
	Pasteurized milk retailers	40	6 (15)	
	Producers	40	3 (7.5)	
	Total	192	22 (11.5)	
SNNP	Collectors	48	7 (14.6)	0.001
	Cottage cheese producers	24	0	
	Cottage cheese retailers	24	0	
	Pasteurized milk processors	48	9 (18.8)	
	Pasteurized milk retailers	48	5 (10.4)	
	Producers	48	15 (31.3)	
	Total	240	36 (15)	
	Total	912	96 (11)	

[a] P value indicates statistical significance.

3.3.5. Risk factors for milk contamination with *Campylobacter* at the milk production level in the Ethiopian dairy value chain

In this study, 54% of dairy farms had cattle barn floors made of concrete, while the remaining 46% had barn floors made of soil. A total of 76% of the surveyed dairy farmers had cattle barns that were in poor sanitary conditions (e.g., floor soiled with manure, contaminated feed, and accumulated dirty water). Before milking, 95% of the surveyed farmers cleaned the cow teats. Among those who cleaned cow teats, 63% used warm water, and 32% used cold water. Furthermore, 64% of the surveyed farmers used a dry cloth to dry the cleaned cow udder and teats before milking. At the time of a survey, 56% of dairy farms reported having at least one cow that was suffering from mastitis. Regarding the equipment used for milking and milk storage, 58% of farmers used tap water to clean milk storage equipment. For milk handling, 89% of the surveyed farmers used plastic containers, 7% used aluminum cans, and 4% used Mazzi cans (i.e., a wide-mouth plastic container designed to be easy to clean). Among surveyed farmers, 74% did not refrigerate milk before selling it. According to the survey results shown in (Table 10), cleaning cow udders and teats with warm water and soap reduced the risk of milk contamination with *Campylobacter* (AOR = 0.3 (0.1 - 0.8), P = 0.023).

Table 10: Risk factors associated with contamination of raw milk with *Campylobacter* species a dairy farm level in Amhara, Oromia, and the SNNP region, between January and March 2020.

Variables	Category	N (%)	*CJ.* (n)	COR[a]		AOR[a]	
				95% CI[a]	P value[a]	95% CI[a]	P value[a]
Construction material for the cattle barn floor	Concrete	100 (54)	16	0.9 (0.4 - 1.9)	0.73	-	-
	Soil	84 (46)	15				
Hygienic condition of the cattle barn	Good	45 (24)	8	0.95 (0.3 – 2.2)	0.84	-	-
	Poor	139 (76)	23				
A major source of water for washing milking equipment	Groundwater	35 (19)	8	1.4 (0.5- 3.7)	0.4	-	-
	Pump water	25 (14)	2	0.4 (0.09- 2)	0.28		
	Rainwater	2 (0)	0	0.00	0.99		
	River water	15 (8)	3	1.2 (0.3- 4.8)	0.8		
	Tap water	107 (58)	18				
Cow udder and teats are washed	No	10 1	1	1.9 (0.22 - 15)	0.55	-	-
	Yes	174 (96)	30				
Type of water used for teats and udder washing	Cold water	62 (34)	5	0.3 (0.1 – 0.8)	0.020	0.3 (0.1-0.8)	0.023
	Warm water	115 (63)	26				
Udder and teats are dried using a dry cloth	No	67 (36)	8	0.5 (0.2 - 1.3)	0.18	-	-
	Yes	117 (64)	23				
Owners' cows have been diagnosed with mastitis	No	81 (44)	10	1.8 (0.8 – 4.1)	0.15	-	-
	Yes	103 (56)	21				
Milk is being filtered	No	13 (7)	1	0.4 (0.04 – 3.1)	0.37	-	-
	Yes	171 (93)	30				
Material used for milk filtration	Piece of cloth	54 (29)	7	1.2 (0.2 - 6.3)	0.83	-	-
	Plastic filter	112 (61)	21	2.1 (0.4-9.9)	0.33		
	Wire mesh	18 (10)	2			-	-
Type of equipment used for milk handling	Aluminum cans	13 (7)	2			-	-
	Mazzi can	7 (4)	1	0.9 (0.07 - 12.3)	0.94		
	Plastic containers	164 (89)	28	1.13 (0.23 - 5.39)	0.87		
Refrigerator is available for milk cooling until sale	No	136 (74)	18	0.4 (0.18 – 0.9)	0.03		0.55
	Yes	48 (26)	13				

[a] COR, crude odds ratio; AOR, adjusted odds ratio; CI, confidence interval at 95%; P value, indicates statistical significance; CJ, *Campylobacter jejuni*, -, not calculated due to COR P value being greater than 0.2.

3.3.6. Risk factors associated with contamination of raw milk with *Campylobacter* at a milk collection point in Ethiopian dairy value chain

The majority (97%) of survey participants did not keep milk cool while transporting it to the collection center. Upon delivery to a collection center, milk was refrigerated using a refrigerator at 62% of the surveyed collection centers. The milk was filtered by 83% of the surveyed collection centers at the milk receipt. Plastic filters, bits of cloth, and wire mesh were used for milk filtering by 67%, 10%, and 5% by collectors, respectively. The majority, 91%, of the surveyed collection centers had a concrete floor, while the rest had a floor made of soil. In collection centers, 97% and 3% of collectors used tap water and groundwater for equipment washing, respectively. Plastic milk containers were used by 78% of the surveyed participants at the milk collection point. Furthermore, 26% of milk collection centers were using aluminum cans to collect milk. Mazzi can be not used in any of the surveyed collection centers. In this study, 31% of surveyed collectors cleaned their equipment with cold water and soap at the collection point. In addition to this, 47% were using warm water and soap for equipment washing. However, none of the collectors washed equipment with only water. During observation by our study team, the milk storage equipment was stored upside down on a shelf by 69% of milk collectors, 45% of the collectors stored it upright open, 66% stored it upright, and 64% stored it covered and upside down in contact with the ground.

The risk of milk contamination with *Campylobacter* at the milk collection center was lower when milk was filtered through a cloth (AOR =0.053 (0.7-0.38), P=0.003), through a plastic filter (AOR = 0.065 (0.009 - 0.04), P = 0.005), or stored in an aluminum container (AOR = 0.23 (0.064 - 0.84), P = 0.027). *Campylobacter* contamination of milk samples was also five times more likely to occur in milk collected in collection facilities with concrete floors compared to those with soil floors (AOR = 5.2 (1.7 - 16), P = 0.004) (Table 11).

Table 11: Analysis of risk factors associated with contamination of raw milk with *Campylobacter* species at the milk collection point in a dairy value chain in Ethiopia, between January and March 2020.

Variables	Category	N (%)	*CJ (n)*	COR[a]		AOR[a]	
				95% CI[a]	P value[a]	95% CI[a]	P value[a]
Temperature is kept low during transportation	No	56 (97)	21	0.16 (0.06 – 0.47)	0.001	-	0.16
	Yes	2 (3)	8				
Milk is filtered upon receipt	No	10 (17)		0.38 (0.14 – 0.98)	0.047	-	-
	Yes	48 (83)	23				
Material used for milk filtration	Piece of cloth	6 (10)	10	0.018 - 0.64	0.014	0.053 (0.7-0.38)	0.003
	Plastic filter	39 (67)	8	0.10 - 0.36	0.002	0.065 (0.009 -.04)	0.005
	Wire meshes	3 (1)	5	1	0.004	-	-
A cooling system is available for milk	No	36 (62)	11	1.3 (0.6 – 3.1)	0.461	-	-
	Yes	22 (38)	18				
Material of the collection center floor	Concrete floor	53 (91)	24	3.47 (1.2 - 9.7)	0.017	5.2 (1.7-16)	0.004
	Soil floor	5 (9)	5				
A major source of water used for equipment washing	Groundwater	2 (3)	0 (0)	0	0.99	- -	- -
	Tap water	56 (97)	29				

[a] COR, crude odds ratio; AOR, adjusted odds ratio; CI, confidence interval at 95%; CJ, *Campylobacter jejuni,* P value, indicates statistical significance; -, not calculated due to COR P value being greater than 0.2.

Table 11 continued

Variables	Category	Category	N (%)	*CJ (n)*	COR[a]		AOR[a]	
					95% CI[a]	P value[a]	95% CI[a]	P value[a]
Milk handling equipment	Plastic containers	No	13 (22)	20	20.5(0.21 – 1.15)	0.101	-	0.64
		Yes	45 (78)	9				
	Aluminum cans	No	43 (74)	6	0.39 (0.15 - 1.01)	0.054	0.23 (0.064-0.84)	0.027
		Yes	15 (26)	23				
	Mazzi cans	No	58 (100)	29	0	1	-	-
Cleaning protocol	Water only	No	58 (100)	29			-	-
	Cold water and soap	No	40 (69)	11	0.95 (0.42 - 2.1)	0.9	-	-
		Yes	18 (31)	18				
	Warm water and soap	No	31 (53)	14	0.8 (0.35 – 1.7)	0.56	-	-
		Yes	27 (47)	15				
Milk handling equipment storage	Upright and open	No	32 (55)	22	0.7 (0.26 – 1.7)	0.39	-	-
		Yes	26 (5)	7				
	Upright and covered	No	20 (34)	25	1.6 (0.5 - 5)	0.397	-	-
		Yes	38 (66)	4				
	Upside down in contact with the ground	No	21 (36)	19	3.35 (1.37 - 8.2)	0.008	-	0.3
		Yes	37 (64)	10				
	Upside down on a shelf	No	18 (31)	21	1.3 (0.57 – 3.29)	0.49	-	-
		Yes	40 (69)	8				

[a] COR, crude odds ratio; AOR, adjusted odds ratio; CI, confidence interval at 95%; P value, indicates statistical significance; -, not calculated due to COR P value being greater than 0.2.

3.3.7. Risk factors for milk contamination with *Campylobacter* species at the milk processing level in Ethiopian Dairy value chain

To investigate risk factors for *Campylobacter* contamination in milk, we used a structured questionnaire to survey an employee at 12 different milk processing plants about their milk processing practices. The survey was conducted at the time of sample collection. We found that 92% of surveyed milk processors reported that they had previously gone through food safety training. Further, we carried out an observational survey of the milk processing environment and found soiled and untidy areas (e.g., with pieces of cartons, broken plastic pouches, plastic boxes contaminated with droplets of milk, and droplets of milk on the ground) in 8% of the surveyed milk processing facilities. Among surveyed facilities, 33% used groundwater for washing equipment, while 67% used tap water. To ensure the effectiveness of pasteurization, 92% of milk processing facilities used a cleaning-in-place (CIP) system, and 92% dismantled the pasteurizer in the milk processing plant to clean it. However, we did not ask how frequently these cleaning processes were carried out. We further found that 67% of surveyed milk processors calibrated their milk pasteurizers once a year to ensure that the target temperature was reached and held for the required time during pasteurization. Among surveyed processors, 50% and 33% did microbiological and phosphate tests to assess pasteurization efficacy, respectively. Most of the surveyed milk processors (92%) reported that they prohibit milk handlers from working with milk when sick. Lastly, 50% of milk processors maintained a cold chain during distribution from the processing facility to the retailing shop, as shown in Table 12. The likelihood of detecting *Campylobacter* was 17 times higher (AOR = 17 (2.2 - 131), P = 0.007) in milk processing facilities that did not calibrate the pasteurizer annually (Table 12).

Table 12: Risk factors associated with contamination of raw milk with *Campylobacter* species during milk processing in Amhara, Oromia, and SNNP region, between January and March 2020.

Risk factor	Category	N (%)	CS (n)	COR[a]		AOR[a]	
				95 % CI[a]	P value[a]	95 % CI[a]	P value[a]
Employees attended basic food safety training	No Yes	1 (8) 11 (92)	9 8	3.69 (1.3 - 10)	0.012		0.5
The storage area is free of trash	No Yes	1 (8) 11 (92)	9 8	3.6 (1.3 - 10)	0.012		0.55
Source of water for equipment washing	Ground Water Tap water	4 (33) 8 (67)	11 6	1.72 (0.6-4.9)	0.303		
Milk handlers that are sick do not work with milk	No Yes	1 (8) 11 (92)	5 12	3.2 (1.03-10)	0.044		0.5
Cleaning in place (CIP) is applied	No Yes	1 (8) 11 (92)	9 8	3.6 (1.3-10)	0.012		0.55
Pasteurizer is dismantled and cleaned	No Yes	1 (8) 11 (92)	9 8	3.6 (1.3 - 10)	0.012		
Pasteurizer is calibrated annually	No Yes	4 (33) 8 (67)	16 1	17 (2.2-131)	0.007	17(2.2-131)	0.007
Efficacy of pasteurization is verified	No Yes	3 (25) 9 (75)	11 6	2.6 (0.9 – 7.3)	0.33	-	
Method of use for pasteurization verification	Phosphatase test	No 8 (67) Yes 4 (33)	12 5	1.8 (0.6-5.5)	0.262		
	Microbiological test	No 6 (50) Yes 6 (50)	12 5	3 (1.04 - 9.1)	0.042		0.73
A cold chain transportation system is in place	No Yes	6 (50) 6 (50)	9 8	3.1 (1.14-8.6)	0.027		0.88

[a] COR, crude odds ratio; AOR, adjusted odds ratio; CI, confidence interval at 95%; P value, indicates statistical significance; -, not calculated due to COR P value being greater than 0.2.

3.3.8. Assessment of risk factors associated with contamination of pasteurized milk with *Campylobacter* at the retail level

In terms of training, 95% of pasteurized milk retailers did not receive any milk safety training. Sixty-four percent of the pasteurized milk retailers reported transporting milk using four-wheel-drive vehicles at ambient temperature, whereas the rest of the retailers reported transporting milk by maintaining a cold chain. Furthermore, 59% of retailers did not keep milk products at a refrigerator temperature during delivery to the retailing point (a shop or a supermarket). Refrigerators and deep freezers were used for milk storage until the milk was sold by 99% and 1% of surveyed retailers, respectively. We found that 70% of retailers did not have a separate refrigerator for milk and had stored milk together with other foods. As shown in Table 13, the likelihood of *Campylobacter* contamination was lower in pasteurized milk kept in a separate refrigerator than in milk stored with other food items (AOR = 0.29 (0.1 - 0.8), P = 0.021) (Table 13).

Table 13: Assessment of risk factors associated with contamination of pasteurized milk with *Campylobacter* species at the milk retail level in Amhara, Oromia, and SNNP region, between January and March 2020.

Risk factor	Categories	N%	*CJ*. (n)	COR[a]		AOR[a]	
				95 % CI[a]	P value[a]	95% CI[a]	P value[a]
Employees attended food safety training	No Yes	174 (95) 10 (1)	14 2	0.35 (0.07 – 1.8)	0.2		0.4
Means of milk transportation	Cold trucks Four-wheel drives	67 (36) 117 (64)	9 7	0.41 (0.14 - 0.15)	0.09		-
Cold chain is maintained during transportation	No Yes	108 (59) 76 (41)	6 10	0.39 (0.13 – 1.1)	0.08		0.37
Equipment used to maintain cold chain	Deep freezer Refrigerator	1 (0.5) 183 (99)	0 16	0	0		-
A separate refrigerator is used for milk and dairy foods	No Yes	129 (70) 55 (30)	7 9	0.29 (0.1 – 0.83)	0.020	0.29 (0.1-0.8)	0.02

[a] COR, crude odds ratio; *CJ, Campylobacter jejuni*, AOR, adjusted odds ratio; CI, confidence interval at 95%; P value, indicates statistical significance; -, not calculated due to COR P value being greater than 0.2.

3.4. Discussion

This study is the first to report the prevalence of *Campylobacter* species in dairy foods collected in a dry season in Ethiopia, where *C. jejuni* was detected in 11% of tested dairy product samples. Ethiopia has a tropical climate with a dry season that typically runs from October to April (Aerts et al., 2016, Ambachew et al., 2020, Fazzini et al., 2015) and a wet season that typically runs from June to mid-September (Walker, 2016). Samples analyzed in this study have been collected exclusively in dry season months. Given that changes in temperature and precipitation have previously been shown to affect the prevalence of *Campylobacter* (Kalupahana et al., 2018), the prevalence reported here may not be representative of a wet season. Due to limited data from countries that have comparable income, level of agricultural development, livestock size, and food safety culture, we compared the prevalence of *Campylobacter* found in our study with its prevalence in other countries. The prevalence of *Campylobacter* found in this study is similar to findings reported by Zeinhom et al. (2021), who detected *Campylobacter* species in 9.5 % (n = 19/200) of the tested dairy product samples collected in Egypt (Zeinhom et al., 2021). The higher prevalence, 20.4 % (n = 51/250), of *Campylobacter* species was reported in Egypt by El-Kholy et al. (2016) (El-Kholy et al., 2016).

During the study period, of the 368 raw milk samples tested, 16% were contaminated with *Campylobacter* species, which is related to Zeinhom et al. (2021), who reported that 18% (n = 9/50) in milk samples in Egypt (Zeinhom et al., 2021). In Tanzania, Kashoma et al. (2016) reported a related finding, 13 % (n = 38/284) in raw milk samples (Kashoma et al., 2016). Furthermore, in the Eastern Cape Province of South Africa, Igwaran and Okoh (2020) observed a higher prevalence compared to our findings. They reported a 37 % (n = 59/159) prevalence of *Campylobacter* contamination in milk samples (Igwaran and Okoh, 2020). Mabote et al. (2011) reported a substantially higher prevalence of *C. jejuni* in raw milk in Koster (96 %) and Dellareyville regions (100 %) of South Africa (Mabote et al., 2011).

Pasteurization of raw milk is designed to inactivate foodborne pathogens. Gram-negative bacteria such as *Campylobacter* species are particularly susceptible to pasteurization (Mahmood et al., 2009). The fact that viable *C. jejuni* was detected in 9 % of pasteurized milk samples collected from milk processors and retailers in Ethiopia suggests that the pasteurization is not always carried out at the target temperature and/or for the recommended duration, or that cross-contamination occurs during post-pasteurization processing. Similar data was found in Nigeria, where the prevalence of

Campylobacter in pasteurized milk was even higher (16%), as reported by Ogbomon et al. (Ogbomon et al., 2018b). Several studies reported no *Campylobacter* in pasteurized milk, although there have been reports that claim that *Campylobacter* was found in patients who consumed pasteurized milk that had not been sufficiently thermally treated (Djuretic et al., 1997, Galbraith et al., 1982).

In our study, 2% of the 176 tested cottage cheese samples collected, across all three regions, were contaminated with *C. jejuni*, which is similar to the 2 % (n = 8/288) prevalence of *Campylobacter* reported by Omara et al., who analyzed Quraish cheese in Egypt (Omara et al., 2015). An even higher prevalence of *Campylobacter* (8%; n = 14/180) was recently reported in Egypt by Barakat et al. (Barakat et al., 2020). El-Kholy et al. reported that 52% of Kareish cheese, 18% of Domiati cheese, and 6% of ice cream were contaminated with *Campylobacter* species in Egypt (El-Kholy et al., 2016). The prevalence of *Campylobacter* in Egypt was high because Cheese production in Egypt involves the use of enzymes like chymosin, which is essential for the cheese-making process (Kumar et al., 2010).

We found a lower prevalence of *Campylobacter* in cottage cheese compared to milk, which is likely due to the sensitivity of *Campylobacter* to low pH. This is likely due to organic acids produced by lactic acid bacteria (e.g., lactic, acetic, and formic acids) during cottage cheese fermentation, which lower the pH of cottage cheese (Ibrahim et al., 2021). The reduction of pH due to organic acid production to 4.6 or below is likely to inactivate *Campylobacter*, which explains the low prevalence of *Campylobacter* in cottage cheese samples (Doyle and Roman, 1981).

Overall, the prevalence of *Campylobacter* detected in Ethiopian dairy products was similar or lower compared to that reported in other African countries, although other African countries may not have comparable dairy production and processing systems, or hygiene and food safety culture. Given that milk is often consumed raw in Ethiopia, the 11% prevalence of *Campylobacter* in milk represents a public health concern.

The 11% prevalence of *Campylobacter* in our study is comparable to the findings reported by Almashhadany, (2021) and Rahimi et al., (2013), who found *Campylobacter* species in 13% (n = 44/350) and 9% (n = 13/552) of tested dairy product samples collected in Iraq and Iran, respectively (Almashhadany, 2021, Rahimi et al., 2013). In Pakistan, Mahmood et al. (2015)

reported a higher prevalence of *Campylobacter*, 83% (n = 100/120) among tested milk and milk products. In India (Gujarat state), a substantially lower prevalence of *Campylobacter* compared to our finding was reported by Modi et al. who reported that 3% (n = 7/240) of tested milk and milk product samples were contaminated with *Campylobacter* species (Modi et al., 2015a).

Our study detected *Campylobacter* in 16% of the 368 tested raw milk samples from Ethiopia. This finding is similar to a recent study conducted in Iraq by Almashhadany (2021), who reported that 16% (n = 19/120) of the raw milk was contaminated with *C. jejuni*. In Pakistan, Mahmood et al. (61) reported a lower prevalence compared to us, with 12 % (n = 14/120) of the milk samples contaminated with *Campylobacter*. Another study conducted by Hussain et al. found a similar prevalence to ours in Pakistan, where 10% (n = 13/127) of raw milk samples were contaminated with *Campylobacter* species (Hussain et al., 2007, Khanzadi et al., 2010). Khanzadi et al., (2010) of Iran and Yang et al. of China reported that *Campylobacter* prevalence was 8% (n = 16/200) and 3% (n = 3/120) in milk samples, respectively, which was lower than what we found in Ethiopia (Khanzadi et al., 2010, Yang et al., 2003).

In Ethiopia, we detected *C. jejuni* in 9% of pasteurized milk samples collected from milk processors and retailers. In contrast to our finding, in Pakistan, UHT and pasteurized packaged milk samples were found to be free of *Campylobacter* (Mahmood et al., 2009). However, pasteurized unpackaged and chocolate milk samples were contaminated with *Campylobacter* at rates of 3 and 6%, respectively, in Pakistan (Mahmood et al., 2009).

In this study, *C. jejuni* was detected in 3% of the 176 cottage cheese samples across all study regions, which is comparable to the finding reported by Rahimi et al. who found that 5% of traditional cheese in Iraq was contaminated with *Campylobacter* species (70). A higher prevalence than that found in our study was reported by Hussain et al. who reported that 11% of the cheese sample in Pakistan were contaminated with *Campylobacter* species, resulting in a substantially higher prevalence as compared to our study (Hussain et al., 2007).

Lastly, in Asia, Mahmood et al. found that 6, 6, 6, 6, 4, 4, 3, and 3% of plain yogurt, ice cream, chocolate milk, mayonnaise, commercially packaged cheese, skimmed milk

powder, flavored yogurt, and pasteurized unpackaged milk were contaminated with *Campylobacter* species (Mahmood et al., 2009).

Overall, the prevalence of *Campylobacter* in Ethiopian raw milk is comparable to that reported in Asian countries. However, the prevalence of *Campylobacter* is substantially higher in Ethiopian pasteurized milk compared to pasteurized milk in Asian countries. This may be explained by economic and cultural differences among countries.

The prevalence of *Campylobacter* species in dairy foods in Ethiopia was similar to the prevalence found by Andrzejewska et al. among 454 samples of raw milk and unpasteurized milk products (12%) purchased from individual suppliers in Poland (Andrzejewska et al., 2019). We detected *C. jejuni* in 16% of the 368 raw milk samples, which is also comparable to a study reported by Bianchini et al., (2014), who reported a 12% (n = 34/282) prevalence of *Campylobacter* among tested bulk milk samples collected in Italy (Bianchini et al., 2014).

Likewise, Artursson et al. reported that 9 % of raw milk samples collected in Sweden were contaminated with *Campylobacter* species, which is again similar to what we found in Ethiopia (Artursson et al., 2018). In contrast to this study, a lower prevalence of *Campylobacter* (5%) was reported by Elmalı and Can who tested milk samples collected in Hatay, Turkey (Elmalı and Can, 2019). A lower prevalence of *Campylobacter* was also reported in Russia (5%) and Poland (5%) by Efimochkina, and Wysok et al. (Efimochkina, 2015, Wysok et al., 2011). In the USA, Jayarao et al. reported an even lower prevalence of *C. jejuni* among raw milk samples collected in Pennsylvania (2%) (Jayarao et al., 2006).

The presence of *Campylobacter* in raw milk is not surprising and it emphasizes the risks of raw milk consumption. It also points out the need for milk pasteurization. Pasteurization is one of the most effective means of controlling pathogens, such as *Campylobacter,* in milk (Robinson, 1981). Nevertheless, *C. jejuni* was found in 9% of pasteurized milk samples collected from milk processors and retailers in Ethiopia, which suggests incomplete pasteurization or potential post-pasteurization contamination. In England, Fernandes et al.(2015) tested and examined internal dairy equipment components and revealed mechanical faults that could have led to incomplete pasteurization of a portion of the milk (Fernandes et al., 2015). Fahey et al. also reported the failed milk pasteurization as a cause of an outbreak of campylobacteriosis (Fahey et al., 1995). In addition to calibration of pasteurizer, the causes for the contamination of pasteurized milk with *Campylobacter* in Ethiopia are

unclear and warrant further investigation to mitigate contamination at the milk processing level.

We discovered that farmers who wash cow udders with warm water are less likely to have milk contaminated with *Campylobacter* compared to those who wash them with cold water. Similarly, a study conducted in Ethiopia reported a reduced risk of contamination with bacteria in farms that washed milk containers using hot water with a detergent (Tigabu et al., 2015).

According to this study, at the collection center, filtering milk with pieces of cloth and plastic filter, and storing milk in an aluminum container all reduce the likelihood of finding *Campylobacter* in milk at the collection facilities. Similarly, the aluminum cans had the maximum microbial load decrease, and the type of container was significant (P = 0.001) in the reduction of microbial pollutants (Wafula et al., 2016). *Campylobacter jejuni*, a major foodborne pathogen, forms biofilms in aerobic conditions, increasing its rate to 95.5%. It forms more biofilm in mixed cultures with *Escherichia coli* or *Pseudomonas aeruginosa*. The study suggests a new approach to controlling contamination via *C. jejuni*, forming crosslinks with aerobic and facultative anaerobic bacteria (Zhong et al., 2020). The concrete floor in the milk storage area was linked with a significant increase in the odds of detecting *Campylobacter* in raw milk. Noteworthy, most collection centers had concrete flooring. According to our research, milk collected in a room with a concrete floor is 5 times more likely to be contaminated with *Campylobacter* than milk collected in a room with a soil floor. During our visit, we observed that the concrete floor in most collection centers was covered with mud, which may have contributed to this finding as a confounding factor.

At the processing level, it was found that failing to calibrate the pasteurization system on an annual basis was linked with an increased risk of detecting *Campylobacter* in pasteurized milk. Despite temperature records showing effective pasteurization, additional testing may reveal mechanical flaws likely to result in incomplete pasteurization of some of the milk (Fernandes et al., 2015). Nada et al., (2012) showed that after a dairy plant implemented a HACCP system, the presence of bacterial contaminants in pasteurized milk decreased. They had shown that the additional investments in the pasteurization unit and automated cleaning and disinfection system

resulted in a significant reduction of bacterial contaminates in pasteurized eight months after the HACCP implementation (Nada et al., 2012).

Lastly, we found that keeping pasteurized milk in a separate refrigerator at retail can reduce the risk of pasteurized milk contamination with *Campylobacte*r. This suggests that cross-contamination may be an important factor affecting the prevalence of *Campylobacter* in milk at retail in Ethiopia. Indeed, Marler (2009) showed that pasteurized milk from various sources could be cross-contaminated from other foods stored with milk (Marler, 2009).

3.5. Conclusion

The 11% prevalence of *Campylobacter* in Ethiopian dairy products presents a considerable food safety risk, particularly given that most of the milk is consumed raw in Ethiopia. Detection of *Campylobacter* in pasteurized milk suggests the need for improved manufacturing practices to ensure adequate pasteurization and prevention of post-pasteurization contamination. Our analysis of risk factors associated with increased odds of *Campylobacter* contamination suggests that simple changes in the production, collection, processing, and retailing of dairy products may lead to a reduction in contamination. These practices include cleaning cow udders and teats with warm water at the farm level, using aluminum milk can containers, and cloth and plastic filters at the collection level, annually calibrating a pasteurizer at the processor level, and storing milk and dairy products separately from other foods in retail stores. The findings reported in this study can be used to develop food safety training and prioritize investments in the dairy value chain that can result in improved dairy safety.

3.6. Recommendation

Given the prevalence of *Campylobacter* contamination in milk, awareness of the risks associated with the consumption of raw milk should be raised at a regional and national level. Producers, collectors, processors, and retailers of milk and milk products would benefit from regular training on the safe handling of milk and milk products, to contribute to the improvement of milk safety. Milk producers (dairy farmers) should be made aware of the sources of milk contamination with *Campylobacter* and provided with training on hygienic milk production. Milk processors are advised to validate and verify the pasteurizer's performance to ensure proper pasteurization. Post-pasteurization, it is advised to store milk at or below 4°C and to maintain the cold chain during transportation to retail locations. Both milk and milk-based products Customers should be informed about contaminated milk and milk products in order to protect themselves from illness. Milk vendors are advised to keep pasteurized milk in a separate refrigerator, away from other foods. Lastly, the public should be made aware of the existing health risks associated with *Campylobacter* species and encouraged to avoid the consumption of raw milk.

CHAPTER 4

Seasonal variation in the prevalence and antimicrobial resistance of *Campylobacter* species in milk and milk products in Ethiopia

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ABSTRACT

A cross-sectional study was conducted to investigate the seasonal variation in the prevalence and antimicrobial resistance of *Campylobacter* species in the Ethiopian dairy value chain. A total of 456 dairy food samples were collected in the dry (January to March 2020) and wet (May to August 2021) seasons in three regions of Ethiopia (Oromia, Amhara, and SNNP). Samples were tested for *Campylobacter species by* following ISO 10272-1:2017, and putative *Campylobacter species* isolates were confirmed using PCR. A total of 141 isolates were tested for susceptibility to three antibiotics using a disk diffusion method according to the Clinical Laboratory Standards Institute guideline. *Campylobacter* species were detected in 20% of tested samples collected in the wet season, and the overall prevalence did not differ significantly (COR=1.3 (0.9-2), P=0.27) between the wet and dry (16%) seasons. However, in the Oromia region, there was a 5 times greater chance of finding *Campylobacter* species in milk and milk products during the wet season as compared to the dry season (COR = 4.5 (1.8-12), P = 0.002). Among *Campylobacter species-positive* samples collected countrywide, 89% were contaminated with *C. jejuni*, and 11% with *C. coli*. Most tested *Campylobacter species* were resistant to tetracycline (89%), followed by erythromycin (74%), and ciprofloxacin (57%). Furthermore, 43% of the tested isolates were resistant to more than two drugs from two different classes. In conclusion, the prevalence of *Campylobacter* species did not significantly differ between the dry and wet seasons at a country level.

Keywords: *Campylobacter jejuni*, *Campylobacter coli*, Dairy products, seasonal variation, antibiotics.

4.1. Introduction

Foodborne illnesses are a major cause of diarrheal diseases, affecting 550 million people annually, including 220 million children under five. One of the main global causes of diarrheal illnesses is Campylobacter sp. (WHO, 2020b).

Campylobacter sp. is a zoonotic foodborne pathogen that is transmitted through animal-based foods, such as milk and meat (Snelling et al., 2005, Hailu et al., 2021). The most common cause of campylobacteriosis is *Campylobacter jejuni*, which has been previously detected in raw milk. *Campylobacter coli* is the second most common cause of human campylobacteriosis (European Centre for Disease Prevention and Control 2022, Davis et al., 2016). Two common risk factors associated with campylobacteriosis are the consumption of unpasteurized milk and direct contact with animals (Mughini-Gras et al., 2021, Hansson et al., 2020)

Human campylobacteriosis was linked with seasonality (Stampi et al., 1992) and outbreaks of campylobacteriosis (Gillespie et al., 2003). Additionally, outbreaks of human campylobacteriosis linked to drinking contaminated water or milk are common in autumn and spring (Snaidr et al., 1997). According to data from the European Center for Disease Prevention and Control, campylobacteriosis cases significantly increase in summer (European Centre for Disease Prevention and Control 2022). However, in temperate climates, peaks are reported in spring and autumn (Stanley et al., 1998). Ingestion of *Campylobacter* species contaminated dairy products can result in abdominal pain, watery or bloody diarrhea, headache, fever, nausea, muscle pain (Gölz et al., 2018), thrombophlebitis, endocarditis, neonatal sepsis, and pneumonia (Alnimr, 2014), and severe post-infection complications in humans, such as severe demyelinating neuropathy and Guillain-Barré syndrome (Hong et al., 2018, Scallan Walter et al., 2020, Scallan et al., 2015).

Campylobacteriosis cases were reported in 30 European countries, with 44.5 cases per 100,000 people in 2021 (European Centre for Disease Prevention and Control 2022). In African countries, the incidence of campylobacteriosis in children under the age of five

was reported to range from 2% in Sudan to 21% in South Africa (Asuming-Bediako et al., 2019). Studies on the prevalence of *Campylobacter* species in Ethiopia have identified a range of 13.8-50.0% in humans and 10.6-56.5% in animals (Chala et al., 2021). Another study found that 14.5% of children under 5 years of age with diarrheal cases had Campylobacteriosis (Behailu et al., 2022).

In southwest Ethiopia in 2022, 9% of diarrhea patients tested positive for *Campylobacter* species in their stool (Nigusu et al., 2022). Generally, a systematic review and meta-analysis of 131 studies on the prevalence of *Campylobacter* species in Ethiopia reported a pooled prevalence of 10% among children under 5 years of age. Contact with domestic animals was identified as a significant risk factor for Campylobacter infection in children (Diriba et al., 2021).

In summer, the prevalence of *Campylobacter* species in raw milk is significantly greater compared to other food products in Pakistan (Mahmood et al., 2009), suggesting that cattle may be the main source of human campylobacteriosis during summer. This could potentially be explained by enhanced *Campylobacter* species transmission with cattle feces during the wet season (Bertasi et al., 2016, Stanley and Jones, 2003, Stampi et al., 1992, Stampi et al., 1993, Biggs et al., 2011, Kwan et al., 2008)

The majority of human *Campylobacter* species infections are mild and self-limiting (Fischer and Paterek, 2019, Dai et al., 2020), and antimicrobial treatments are not generally required for the treatment of campylobacteriosis (Acheson and Allos, 2001, Dai et al., 2020). However, severe infections, particularly in children, the elderly, and those immunosuppressed require hospital care and/or antimicrobial treatment (Same and Tamma, 2018, Ammar et al., 2021, European Centre for Disease Prevention and Control 2022). These situations frequently call for the use of first-line antibiotics – macrolides (erythromycin) and fluoroquinolones (ciprofloxacin) (Dai et al., 2020). The clinical and agricultural use of these antibiotics can promote antimicrobial resistance development (Wieczorek and Osek, 2015, Regassa et al., 2009). Therefore, it is crucial to keep track of antimicrobial-resistant *Campylobacter* species to make an informed decision on which antibiotics to utilize in campylobacteriosis treatments.

This study was initiated to ascertain whether there was a seasonal variation in the prevalence of thermophilic *Campylobacter* species in raw dairy products in dry and wet seasons in Ethiopia, as well as to examine antibiotic resistance of *Campylobacter* species to antibiotics used for the treatment of campylobacteriosis.

4.2. Material and methods

4.2.1. Study areas and sample size

This study was conducted in the wet (May to August 2021) and dry seasons (January to March 2020) based on selected representative sampling sites three Ethiopian regions: Oromia, Southern Nation Nationalities Peoples (SNNP), and Amhara. The prevalence of *Campylobacte*r species. during the dry season was taken into consideration when choosing Bahidar, Hawassa, and Bishoftu study sites for sample collection in the wet season (Admasie et al., 2023).

The formula used to determine the sample size was as follows: N = Z2 P (1-P)/ (D2), where z = 1.96 at a 95% confidence interval, D is the allowed margin of error (5%), and p is the estimated prevalence of *Campylobacter* species. Due to the unknown prevalence of *Campylobacte*r species in Ethiopian dairy products, the prevalence was estimated to be 50%. This resulted in a minimum sample size of 384. Based on our previous report of the *Campylobacter* species prevalence in a dry season, three specific study sites in three regions were chosen for sample collection (Admasie et al., 2023). The three study regions have different production capacities. Oromia produces an estimated ~52%, SNNP ~23%, and Amhara ~20%, of milk produced in Ethiopia (CSA, 2011). The number of samples collected from each region was proportional to the milk production potential of each region. Thus, a total of 456 dairy products were collected from the three study sites, 240 of which were collected from the city of Debre Zeit (Bishoftu) in the Oromia region (120 in the dry season and 120 in the wet season), 120 samples from Hawassa in the SNNP region (60 in the dry and 60 in the wet seasons), and 96 samples from Bahir Dar in the Amhara region (48 in the dry season and 48 in the wet seasons).

4.2.2. Sample collection

A total of 250 ml of each raw milk sample was transferred into a sterile plastic bottle from dairy farms (n=92) and milk collection facilities (n = 92). A 500 ml plastic pouch of pasteurized milk samples was obtained from retailers (n=92) and processors (n = 92). A 500 g of cottage cheese sample was purchased from retailers (n=44) and farmers (n = 44).

All collected samples were stored at 4°C in a transportable refrigerator until delivery to the lab. Samples were stored at 3°C in the laboratory until analyses were performed.

4.2.3. Detection and confirmation of *Campylobacter* species

Milk and cottage samples were enriched for *Campylobacter* species by following the ISO 10272-1:2017 method B. Method B was followed due to the expected high level of background flora (Standardization, 2017b). The detection procedure B included enrichment in Preston broth in a microaerobic atmosphere, at 41.5°C for 24 h. The enrichment was followed by isolation on the mCCDA agar (Modified Charcoal Cefoperazone Deoxycholate Agar) (OXOID) in a microaerophilic atmosphere, at 41.5 °C for 44 h. Once isolates were obtained, a Gram staining or latex agglutination screening was carried out. Isolates that passed these screenings were confirmed using PCR that targeted the *Campylobacter* species 23S rRNA gene, as well as genes unique to individual *Campylobacter* species (Figure 1). Molecular confirmation using PCR was carried out as outlined in detail in our prior report (Admasie et al., 2023).

4.2.4. Antibiotic susceptibility testing

A total of 141 *Campylobacter* species (96 from the dry season and 45 from the wet season) were tested for antimicrobial susceptibility using a disk diffusion test. The test was performed for each isolate on Mueller-Hinton agar (OXOID, England) supplemented with 5% horse blood (Hardy Diagnostics, 10052-808). Approximately 20 ml of medium was poured into 90 mm diameter sterile Petri dishes. Isolates were grown in a brain heart infusion broth (OXOID, England) at 37°C for 24 h in microaerobic conditions (CampyGen, Oxoid, AGS). Isolates were then inoculated on Mueller-Hinton agar in the form of a lawn using sterile cotton swabs. *Campylobacter* isolates were tested for susceptibility to the following 3 antibiotics: ciprofloxacin (30 μg), tetracycline (30 μg), erythromycin (10 μg) (OXOID, England) according to guideline M45:ED3 of the Clinical Laboratory Standards Institute (CLSI-M45-Ed3, 2016). Antibiotic discs were deposited on the surface of inoculated cultures on MHA and incubated at 41.5°C for 24 hrs. The diameters of the zones of inhibition were recorded to the nearest mm and classified as resistant, intermediate, or

susceptible according to guidelines set by the Clinical Laboratory Standards Institute (CLSI M45:ED3-2016).

4.2.5. Data analysis

Microsoft Excel was used to conduct descriptive statistics of the prevalences and SPSS version 26.0 software was used to undertake the binary logistic regression. First, a chi-square test was used to assess the statistical significance of differences in *Campylobacte*r species prevalence across different regions, among sample types (raw and pasteurized milk, cottage cheese), and seasons. Then odds ratios were calculated to evaluate the relationships between the seasonal influence and the prevalence of *Campylobacter* spp in dairy products. The odds ratio for each variable affecting the detection of *Campylobacter* species was calculated using a binary logistic regression (Chala et al., 2021). Alpha level was set at 0.05.

4.3. **Results**

4.3.1. The prevalence of *Campylobacter* species in dairy products was significantly different between dry and wet seasons in Oromia, but not in Amhara and SNNP regions

Overall, *Campylobacter* species were detected in 18% of the 456 dairy product samples. In the wet season, *Campylobacter* species were detected in 20% of 228 tested samples, and in the dry season, *Campylobacter* sp. was detected in 16% of 228 tested samples (Table 14. The overall prevalence of *Campylobacter* species did not significantly differ between the wet and dry seasons in Ethiopia (COR=1.3 (0.9-2), P=0.27) (Table 15). The prevalence of *Campylobacter* species contamination was also not significantly different among regions in the wet season (from 12 – 19%, P=0.54) (Table 14), although it was significantly different among regions in the dry season (p<0.001) (Table 14).

Table 14: The prevalence of *Campylobacter jejuni* and *Campylobacter coli* in the wet and dry seasons in three Ethiopian regions.

Season	Region	Samples N	*Campylobacter* spp. n (%)	P value
Wet	Amhara	48	12 (25)	0.54
	Oromia	120	23 (19)	
	SNNP[a]	60	10 (17)	
	Total	228	45 (20)	
Dry	Amhara	48	12 (25)	0.001
	Oromia	120	6 (CLSI-M45-Ed3)	
	SNNP[a]	60	18 (30)	
	Total	228	36 (16)	

[a] Abbreviation are: SNNP, Southern Nation Nationalities, and People, N, the total number of samples, n, number of positive samples, P value indicates statistical significance.

Our findings demonstrated that the Amhara (COR=1 (0.4-2.5), P=1) and SNNP (COR= 0.5 (0.19-1.1), 0.9) regional states did not notice any seasonal variations in the incidence of *Campylobacter* species in dairy products. However, In the Oromia region, there were 5 times greater odds of detecting *Campylobacter* species in milk and milk products during the wet season as compared to the dry season (COR = 4.5 (1.8-12), P = 0.002) (Table 15).

Table 15: Prevalence of *Campylobacter jejuni* and *Campylobacter coli* in wet and dry seasons among three Ethiopian regions.

Region	Dry season		Wet season		Total		COR (95% C.I)	P-value
	Samples N	CS n (%)	Samples N	CS n (%)	Samples N	CS n (%)		
Amhara	48	12 (25)	48	12 (25)	96	24 (22)	1 (0.4-2.5)	1
Oromia	120	6 (5.0)	120	23 (19)	240	29 (12)	4.5 (1.8-12)	0.002
SNNP	60	18 (30)	60	10 (17)	120	28 (23)	0.5 (0.19-1.1)	0.9
Total	228	36 (16)	228	45 (Chatterjee)	456	81 (16)	1.3 (0.9-2)	0.27

[a] Abbreviation are: SNNP, Southern Nation Nationalities and People, CS, *Campylobacter* species, N, number of samples, n, number of positive samples, COR, crude odds ratio, CI, confidence interval at 95%, P value indicates statistical significance.

4.3.2. The prevalence of *Campylobacter* species was highest in raw milk, regardless of the sampling season.

Campylobacter sp. was detected in 33%, 13%, and 7% of raw milk, pasteurized milk, and cottage cheese samples tested in the wet season, respectively (Table 16). In contrast, the prevalence of *Campylobacter* species in these dairy products in the dry season was lower (i.e., 24%, 14%, and 2%). Regardless of the season, the prevalence of *Campylobacter* species in raw milk was 8 times higher than in other tested dairy products (COR= 8 (3-23), p<0.0001), as shown in Table 16.

Table 16: Prevalence of *Campylobacter jejuni* and *Campylobacter coli* in different sample types collected in dry and wet seasons.

Season	Sample type	Samples N[a]	*Campylobacter* species (%)	COR (95% C.I)	P value
Dry	Cottage cheese	44	1 (Spiller1)		
	Pasteurized milk	92	13 (14)	7 (0.9-56)	P=0.064
	Raw milk	92	22 (24)	13 (1.8-103)	P=0.012
	Total	228	36 (16)		
Wet	Cottage cheese	44	3 (7)		
	Pasteurized milk	92	12 (13)	3 (1.1-10)	P=0.031
	Raw milk	92	30 (33)	8 (3-23)	P<0.0001
	Total	228	45 (Chatterjee)		

Abbreviation are: N, number of samples, n, number of positive samples, COR, crude odds ratio, CI, confidence interval at 95%, and P value indicates statistical significance.

PCR analysis revealed that 89% were *C. jejuni* and 11% were *C. coli*. Our findings indicated a significant difference in the prevalence of *C. jejuni* compared to *C. coli* in the wet and dry seasons (p=0.01), which was determined by comparing the two seasons. In the dry season, all detected isolates were *C. jejuni*. In the analyzed samples from the dry season, no *C. coli* was found. In contrast, 20% of isolated *Campylobacter* species in the wet season were *C. coli* (Table 17).

Table 17: Prevalence of *C. jejuni* and *C. coli* species in the wet and dry seasons.

Campylobacter species	Isolates dry season n (%)	Isolates wet season n (%)	Isolates - total n (%)	P value
C. coli	0 (0)	9 (20)	9 (11)	0.01
C. jejuni	36 (100)	36 (80)	72 (89)	
Total	36 (100)	45 (100)	81 (100)	

[a] Abbreviation are: n, number of isolates, P value indicates statistical significance.

4.3.3. Antibiotic resistance of *Campylobacter jejuni* and *Campylobacter coli* were resistant to both erythromycin and ciprofloxacin.

A total of 141 isolates, 96 from the dry season (from chapter three)and 45 from the wet season, were examined for antibiotic susceptibility to erythromycin, ciprofloxacin, and tetracycline. Our study demonstrated a high prevalence of resistance to erythromycin (74%) and ciprofloxacin (57%) – two clinically important antibiotics for the treatment of human campylobacteriosis. The study found that tetracycline resistance was prevalent among *Campylobacter* species isolates, with 55% of the strains being resistant to tetracycline (Table 18).

Table 18: Prevalence of antimicrobial resistance in *Campylobacter* species obtained from dairy food samples collected in Oromia, Amhara, and SNNP in the dry and wet seasons.

Drug class	Antibiotic	Interpretation[a]	*C. jejuni* (%)	*C. coli* (%)	Total (%)
Quinolone	Ciprofloxacin	Intermediate	30 (23)	4 (44)	34 (24)
		Resistant	78 (59)	2 (22)	80 (57)
		Sensitive	24 (18)	3 (33)	27 (19)
Macrolide	Erythromycin	Intermediate	1 (Spiller1)	0 (Chatterjee)	1 (Spiller1)
		Resistant	97 (74)	7 (78)	104 (74)
		Sensitive	34 (26)	2 (22)	36 (25)
Tetracycline	Tetracycline	Intermediate	11 (8)	2 (22)	10 (7)
		Resistant	115 (87)	7 (78)	125 (55)
		Sensitive	6 (CLSI-M45-Ed3)	0 (Chatterjee)	6 (CLSI-M45-Ed3)
		Total	132 (100)	9 (100)	141 (100)

[a]Resistance interpretation was carried out by following the CLSI guideline M35:ED3 (CLSI-M45-Ed3, 2016). Erythromycin interpretation criteria were as follows: sensitive (≥16 mm), intermediate (13–15 mm), and resistant (≤12 mm). Ciprofloxacin interpretation criteria were as follows: sensitive (≥24 mm), intermediate (21-23 mm), and resistant (≤20 mm). Tetracycline interpretation criteria were as follows: sensitive (≥ 26 mm), intermediate (23–24 mm), and resistant (≤22 mm).

The antibiotic susceptibility testing was conducted on 141 *Campylobacter* species (132 *Campylobacter jejuni* and 9 *Campylobacter coli*) isolates stored at -80°C. Of these, 43% were found to be multidrug-resistant (i.e., resistant to three different classes of antibiotics), and 48, 52, and 65 percent, respectively, were resistant to two clinically significant classes of antibiotics for treating campylobacteriosis, such as (CIP, ERY), (CIP, TET), and (ERY, TET) (Table 19).

Table 19: Multidrug resistance patterns among isolates of Campylobacter species.

No. of antibiotics	Resistance pattern	Antibiotic resistance		
		C. jejuni	*C. coli*	Total
		N (%)	N (%)	N (%)
R2	CIP, ERY	66 (47)	1 (Spiller1)	67 (48)
	CIP, TET	73 (51)	1 (Spiller1)	74 (52)
	ERY, TET	86 (61)	5 (4)	91 (65)
R3	CIP, ERY, TET	59 (44)	1 (`0.7)	60 (43)

[a] Abbreviation are: CIP, ciprofloxacin; ERY, erythromycin; TET, tetracycline; R2, resistance to two antibiotics; R3, resistance to three antibiotics.

4.4. Discussion

4.4.1. The prevalence of *Campylobacter* species in dairy products was significantly different between dry and wet seasons in Oromia, but not in Amhara and SNNP regions

Overall, *Campylobacter* species were detected in 18% of the 456 dairy product samples. In the wet season, *Campylobacter* species were detected in 20% of 228 tested samples, and in the dry season, *Campylobacter* sp. was detected in 16% of 228 tested samples. Salihu et al. (2010) noted a comparable seasonal incidence of *Campylobacter* species in dairy products in Nigeria during the two seasons. Isolations revealed that milk samples taken during the hot, dry, and rainy seasons had the greatest incidence rates of 3 (6.1%) and 3 (6.0%), respectively (Salihu et al., 2010). The overall prevalence of *Campylobacter* species did not significantly differ between the wet and dry seasons in Ethiopia (COR=1.3 (0.9-2), P=0.27).

The prevalence of *Campylobacter* species contamination was also not significantly different among regions in the wet season (from 12 – 19%, P=0.54), although it was significantly different among regions in the dry season (p<0.001). Similar to the report of Salihu et al. (2010), our result showed that the season did not have a significant effect on the prevalence of *Campylobacter* when analyzing data for all three Ethiopian regions. On the other hand, in eastern Nigeria, the *Campylobacter* species prevalence was found to be ahigher in the summer (wet season; 94%) than in other seasons (P=0.05), whereas the lowest prevalence of *Campylobacter* species was detected in winter (dry season; 38%). In the study in eastern Nigeria, the authors found a significant difference in the prevalence of *Campylobacter* between seasons (P<0.001) (Baali et al., 2020, Dike et al., 2020).

Our findings demonstrated that the Amhara (COR=1 (0.4-2.5), P=1) and SNNP (COR= 0.5 (0.19-1.1), 0.9) regional states did not notice any seasonal variations in the incidence of *Campylobacter* species in dairy products. However, In the Oromia region, there were 5 times greater odds of detecting *Campylobacter* species in milk and milk products during the wet season as compared to the dry season (COR = 4.5 (1.8-12), P = 0.002). This finding

was similar to the report of Mahmood et al. (2009), who found that the prevalence of *Campylobacter* species in dairy products in Pakistan was highest in summer (wet season) (Mahmood et al., 2009). Consistent with our findings, the study carried out in Kenya revealed that thermophilic *Campylobacter* species prevalence peaked in both chicken and cattle during wet seasons (40%) and was significantly lower during the dry season (30%) (p=0.001) (Wanja et al., 2022). However, in Nigeria, Nwankwo et al. (2018) reported that the prevalence of this pathogen was higher in free-range chickens during the cold-dry season (Nwankwo et al., 2018).

4.4.2. The prevalence of *Campylobacter* species was highest in raw milk, regardless of the sampling season.

Campylobacter sp. was detected in 33%, 13%, and 7% of raw milk, pasteurized milk, and cottage cheese samples tested in the wet season, respectively. Regardless of the season, the prevalence of *Campylobacter* species in raw milk was 8 times higher than in other tested dairy products in the wet season (COR= 8 (3-23), p<0.0001). Our findings were in line with those of Mahmood et al. (2009) who found that the prevalence of *Campylobacter* species in raw milk was higher than in other dairy products in Pakistan during the summer season (p<0.05) (Mahmood et al., 2009). *Campylobacter* species contamination of raw milk can originate from various sources, including fecal matter contamination, wild bird droppings, poorly sanitized milking equipment, contamination during repair of milking machines, silent mastitis, contaminated water, and poor hygiene practices. It is important to follow proper hygiene practices and implement effective control measures to prevent *Campylobacter* contamination of dairy products (Davis et al., 2016, Modi et al., 2015a).

In this study, *Campylobacter* species were detected in 13% of the analyzed pasteurized milk. However, a study conducted in Pakistan by Mahmood et al. (2009) did not detect *Campylobacter* species in any of the tested pasteurized packaged milk. As previously reported, the presence of *Campylobacter* species in pasteurized milk samples could be due to insufficient heat treatment (Djuretic et al., 1997). Our previous study showed that, in Ethiopia, pasteurized milk can be contaminated due to a lack of pasteurization validation, calibration, and final product microbiological testing. The odds of detecting

Campylobacter species in milk were 17 times greater in milk processing facilities that did not calibrate their pasteurizers on an annual basis (Admasie et al., 2023). Furthermore, improper storage and lack of refrigeration can promote the contamination of *Campylobacter* species in pasteurized milk (Admasie et al., 2023). The results of our previous investigation conducted during the dry season showed that the likelihood of *Campylobacter* species contamination was lower in pasteurized milk kept in a separate refrigerator than in milk stored with other food items (AOR =0.29 (0.1–0.8), P = 0.021). This suggests that proper storage and refrigeration of pasteurized milk can help reduce the risk of *Campylobacter* species contamination. However, it is important to note that this is just one study and further research may be needed to confirm this finding. Other risk factors for *Campylobacter* species contamination in pasteurized milk in Ethiopia include bacterial contaminants, poor hygiene practices, contaminated water sources, cross-contamination, and lack of pasteurizer calibration (17 (2.2–131), P = 0.007) (Admasie et al., 2023).

PCR analysis revealed that 89% were *C. jejuni* and 11% were *C. coli*. Our findings indicated a significant difference in the prevalence of *C. jejuni* compared to *C. coli* in the wet and dry seasons (p=0.01), which was determined by comparing the two seasons. In the dry season, all detected isolates were *C. jejuni*. In the analyzed samples from the dry season, no *C. coli* was found. In contrast, 20% of isolated *Campylobacter* species in the wet season were *C. coli*. Our results were consistent with those reported by Mahmood et al. (2009) and Salihu et al. (2010) who found that *C. jejuni* prevalence was higher than that of *C. coli* in milk and milk products in Pakistan and Nigeria, respectively (Mahmood et al., 2009, Salihu et al., 2010).

4.4.3. Majority of tested *Campylobacter* species were resistant to both erythromycin and ciprofloxacin.

The rising resistance of *Campylobacter* species to clinically relevant antibiotics is a concern for public health. Since *Campylobacter* species are a zoonotic pathogen and are consequently exposed to antibiotics used in both animal production and human medicine, the development and transmission of antibiotic-resistant *Campylobacter* species are complicated (Luangtongkum et al., 2009). Therefore, a total of 141 isolates, 96 from the

dry season (Admasie et al., 2023) and 45 from the wet season, were examined for antibiotic susceptibility to erythromycin, ciprofloxacin, and tetracycline. Our study demonstrated a high prevalence of resistance to erythromycin (74%) and ciprofloxacin (57%) – two clinically important antibiotics for the treatment of human campylobacteriosis. Elmal and Can (2019) found a similarly high prevalence of ciprofloxacin resistance in 57% of the *Campylobacter* species in Turkey (Elmalı and Can, 2019). A study done in Jimma, Ethiopia, revealed that 85% (148/174) of the *Campylobacter* species from 368 cattle feces were resistant to ciprofloxacin (Abamecha et al., 2015a). In contrast, a lower prevalence of ciprofloxacin resistance 7.9% (3/38) in *Campylobacter* species from 684 live bovine, carcass swab, and separated environmental swabs samples was reported by Debelo et al. (2022)_(Debelo et al., 2022). In Spain and northern Poland, a higher prevalence of ciprofloxacin resistance in *Campylobacter* species from 78% (21/27) and 71% (70 /98) isolates, respectively was reported by Varsaki et al. (2022) and Andrzejewska et al. (2019) (Varsaki et al., 2022, Andrzejewska et al., 2019), which is higher than our study. A much lower prevalence of ciprofloxacin-resistant *Campylobacter* species was reported in Tanzania by Kashoma et al. (2016) who found that 9% (5/54) of tested *Campylobacter* species from 537 raw milk and cattle-dressed carcass swab samples were resistant, respectively(Kashoma et al., 2016). Even lower prevalence of ciprofloxacin was reported by Englen et al. (2007), who detected ciprofloxacin resistance in 3% (12/473) of tested *Campylobacter* species from 1435 dairy cows fecal (Englen et al., 2007).

In the tested *Campylobacter* species from Ethiopian dairy foods, resistance to erythromycin was found to be higher (74%) than resistance to ciprofloxacin. Our finding is comparable to that of Elmal and Can (2019), who found that 71% (10/14) of tested isolates from 192 raw milk and water samples isolated in Turkey were erythromycin-resistant (Elmalı and Can, 2019). A study conducted in Tanzania revealed that 54% (29/54) of tested *Campylobacter* species from 537 raw milk and cattle-dressed carcass swab samples were erythromycin-resistant (Kashoma et al., 2016). However, a study carried out in Spain found no erythromycin resistance among *Campylobacter* species from 520 cattle feces (Varsaki et al., 2022). In Ethiopia, Hagos et al. (2021) found that just 18% (11/64) of the examined isolates from 384 meat samples were resistant to erythromycin (Hagos et al., 2021). A low

prevalence of erythromycin resistance 2 (2/98) among *Campylobacter* species from 1058 food samples was also found in Poland (Andrzejewska et al., 2019).

The study found that tetracycline resistance was prevalent among *Campylobacter* species isolates, with 55% of the strains being resistant to tetracycline. Our results were comparable to those of Elmal and Can (2019), who reported that 64%(9/14) of the tested *Campylobacter* species from 192 milk and water samples in Turkey were tetracycline resistant (Elmalı and Can, 2019). In contrast, the prevalence determined in our study was higher than the 19% prevalence reported in Tanzania (Kashoma et al., 2016).

This study showed that, 43% were found to be multidrug-resistant (i.e., resistant to three different classes of antibiotics), and 48, 52, and 65 percent, respectively, were resistant to two clinically significant classes of antibiotics for treating campylobacteriosis, such as (CIP, ERY), (CIP, TET), and (ERY, TET). A similar study from Turkey showed that 57% of the examined isolates were multidrug-resistant (Elmalı and Can, 2019). In Ethiopia, a lower prevalence of multidrug resistance (20% and 15% respectively) was reported by Kassa et al. (2007) and Dadi et al. (2008) (Kassa et al., 2007, Dadi and Asrat, 2008). However, a higher prevalence of multidrug resistance (i.e., resistance to more than three classes of antibiotics; 69%) was reported in Ethiopia by Hagos et al. (2021) (Hagos et al., 2021). In other countries, a higher prevalence of multidrug resistance than in this study was reported in Belgium (60%), Estonia (60%), Iran (75%), and Korea (93%) (Praakle-Amin et al., 2007, Taremi et al., 2006, Hong et al., 2007).

There is limited information on the overall consumption of tested antibiotics in humans and dairy cattle in Ethiopia. However, some studies provide insights into the use of antibiotics in Ethiopia and its impact on antimicrobial resistance. A study conducted in Ethiopia found that 44% of farmers used antimicrobials in the past few years, where antibiotics (21%) and trypanocides (11%) were most commonly used (Tufa et al., 2023). Another study in Ethiopia found that tetracycline (36.4%), aminoglycosides (31.3%), and trimethoprim-sulfonamides (6.2%) were the most frequently used classes of antibiotics in extensive smallholder livestock farming systems (Gemeda et al., 2020). A study conducted in the Amhara region, northwestern Ethiopia, found that a large proportion of animal farm

owners (90.1%) had heard about antibiotics and antibiotic resistance. Generally, according to a global map of antibiotic consumption in livestock, Ethiopia consumed 174 tons of antibiotics in 2010 (Van Boeckel et al., 2015).

4.5. Conclusions

This research showed that the prevalence of *Campylobacter* species in dairy products was not significantly associated with season. The research results indicate that the relatively high prevalence of *Campylobacter* species in Ethiopian milk and dairy products is a source of human exposure to *Campylobacter* species in Ethiopia. Additionally, this study found that compared to the dry season, milk and milk products in the Oromia region were substantially more likely to be contaminated with *Campylobacter* species during the wet season. The detection of *Campylobacter* species in pasteurized milk emphasizes the need for the regulatory monitoring of the performance of pasteurizers and the implementation of their preventative and corrective maintenance. This has significant implications for public health and food safety in the country. Moreover, we found that *Campylobacter* species were resistant to three antibiotics, including the first line of clinical antibiotics used for the treatment of human campylobacteriosis. These findings warrant further monitoring and development of resistance mitigation strategies to protect public health.

In conclusion, the prevalence of *Campylobacter* species did not significantly differ between the dry and wet seasons at a country level, and over 44% of isolates of *Campylobacte*r species were resistant to three tested antibiotics. Finally, to determine the prevalence and seasonal impact on the prevalence of *Campylobacter* in milk and milk products, Ethiopia's four seasons should be taken into account. This finding warrants further investigation to identify the root causes and develop effective contamination mitigation strategies to reduce the risk of human exposure to Campylobacter through dairy food intake .

CHAPTER 5

Genomic diversity of *Campylobacter jejuni* and *Campylobacter coli* isolated from the Ethiopian dairy supply chain

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ABSTRACT

Campylobacteriosis outbreaks have previously been linked to dairy foods. The genetic diversity of *Campylobacter* is well understood in high-income countries, but it is largely unknown in low-income countries, such as Ethiopia. This study, therefore, aimed to conduct the first genomic characterization of *Campylobacter* isolates from the Ethiopian dairy supply chain to aid in future epidemiological studies. The whole genome of 14 *C. jejuni* and 4 *C. coli* isolates was sequenced using an Illumina platform (Illumina, San Diego, CA, USA). Sequences were analyzed using bioinformatics tools in the GalaxyTrakr platform to identify multi-locus sequence types, and single nucleotide polymorphisms, and infer phylogenetic relationships among the studied isolates. Assembled genomes were further screened to detect antimicrobial resistance and virulence gene sequences. Among 14 *C. jejuni*, ST 2084 and ST 51, which belong to the clonal complexes ST-353 and ST-443, respectively, were identified. Among the 4 sequenced *C. coli* isolates, two isolates belonged to ST 1628 and two to ST 830 from the clonal complex ST-828. The isolates of *C. jejuni* ST 2084 and ST 51 carried β-lactam resistance gene *blaOXA-605*, a fluoroquinolone resistance-associated mutation T86I in the *gryA* gene, and a macrolide resistance-associated mutation A103V in *50S L22*. Only ST 2084 isolates carried the tetracycline resistance gene *tetO*. Conversely, all four *C. coli* ST 830 and ST 1628 isolates carried *tetO*, but only ST 1628 isolates carried *blaOXA-605*. This finding warrants further monitoring of *Campylobacter* in dairy foods in Ethiopia to better understand and manage the risks associated with *Campylobacter* contamination and transmission.

Keywords: *Campylobacter jejuni*, *Campylobacter coli*, whole genome sequencing, multilocus sequence typing, antimicrobial resistance genes, virulence genes

5.1. Introduction

Campylobacteriosis is an infectious disease caused predominantly by *Campylobacter jejuni* and *Campylobacter coli* (Ruiz-Palacios, 2007, Amin et al., 2023). It can be particularly severe in children under the age of five (Same and Tamma, 2018). Following a *Campylobacter* infection, individuals may experience post-infection complications such as reactive arthritis, neurological disorders like Guillain-Barré syndrome, and chronic medical sequelae (Nachamkin, 2002).

In the US, *Campylobacter* causes an estimated 1.5 million illnesses, 19,300 hospitalizations, and 240 fatalities annually (Collier et al., 2021). Similarly, EU nations reported 129,960 confirmed cases of campylobacteriosis in 2021 (ECDC, 2022). Studies have also shown that *Campylobacter* species are widespread among humans in sub-Saharan Africa, with a pooled prevalence of 9.9% (Hlashwayo et al., 2021). In Ethiopia, the prevalence of campylobacteriosis reported in published studies ranged from 8% to 20% (Tafa et al., 2014, Bukayaw and Mekonnen, 2021, Chala et al., 2021, Ewnetu and Mihret, 2010, Lengerh et al., 2013b).

Campylobacter is primarily transmitted through contaminated food, particularly raw or undercooked poultry meat, as well as unpasteurized milk, contaminated dairy products and untreated water (Luangtongkum et al., 2009, Silva et al., 2011a) (Davys et al., 2020b). For example, in 2016, public health departments in England reported 69 cases of campylobacteriosis linked to the consumption of raw milk sold at dairy farms (Kenyon et al., 2020). Similarly, cases of campylobacteriosis were reported in Italy, where the consumption of raw milk resulted in an outbreak of *C. jejuni* (Amato et al., 2007). Multiple outbreaks of campylobacteriosis have also been linked to the consumption of contaminated milk and cheese in the US (Costard et al., 2017, Langer et al., 2012, Mungai et al., 2015). Due to the lack of *Campylobacter* surveillance in the Ethiopian food supply chain, there is a lack of data on foodborne outbreaks of campylobacteriosis. Furthermore, there is limited information available on the prevalence of *Campylobacter* in the dairy supply chain. In the previous cross-sectional study in major Ethiopian milk sheds, we found that 11% of all tested dairy foods collected during the dry season were contaminated with *Campylobacter*

(Admasie et al., 2023). Due to the lack of access to genotyping, it remains unknown whether *Campylobacter* isolates obtained from the Ethiopian milk supply chain resemble those that are widespread globally or if they present unique genotypes endemic to Ethiopia.

Multi-locus sequence typing (MLST) is a commonly used genotyping method that allows for the spatial and temporal comparison of the prevalence and distribution of *Campylobacter* genotypes (Maiden et al., 2013). The most prevalent MLST genotypes of *Campylobacter* reported in the global PubMLST database include sequence types (STs) 21, 45, and 50 (Wieczorek et al., 2017). While these genotypes are commonly found in high-income countries, they have not been reported in the limited published literature from sub-Saharan Africa (Quiñones et al., 2008). In order to implement *Campylobacter* control strategies, a greater understanding of the prevalence and distribution of individual *Campylobacter* genotypes in the food systems in sub-Saharan Africa is needed. Isolate genotyping, including *in silico* MLST typing through whole-genome sequencing (WGS) can enhance source tracking and identification of major sources of human *Campylobacter* in the food systems, as well as support epidemiological investigation of outbreaks (Liu et al., 2016, Tong et al., 2021, Joensen et al., 2021). Beyond tracking the transmission of *Campylobacter* among environmental, agricultural, and human sources (Kelley et al., 2020), WGS can also support the study of *Campylobacter* evolution and its antimicrobial resistance and virulence genetic determinants (Golz et al., 2020, Morita et al., 2023, Clark et al., 2018). This study, therefore, employed whole-genome sequencing to characterize *C. jejuni* and *C. coli* isolated from the Ethiopian dairy supply and generate insights into the genetic diversity and genomic potential of isolates to cause human illness and resist antimicrobial treatments.

5.2. Materials and methods

5.2.1. Isolate collection and *Campylobacter* species confirmation

Campylobacter isolates that had previously been isolated from raw milk, pasteurized milk, and cottage cheese collected from Ethiopian regions of Amhara, Oromia, and the Southern Nations, Nationalities, and Peoples' (SNNPR) during 2020 and 2021 were included in this study (Admasie et al., 2023). Isolates were preserved in brain heart infusion broth supplemented with 20% glycerol and maintained at -20°C. In 2022, 18 isolates that were successfully recovered were sent to the Pennsylvania State University for PCR confirmation (Wang et al., 2002), and whole genome sequencing was conducted at the laboratory of the Kentucky Department of Public Health.

5.2.2. DNA extraction and whole genome sequencing

DNA extraction was performed using the QIAcube Connect instrument with the DNeasy Blood and Tissue kit (Qiagen, Germantown, MD, USA). The extracted DNA was quantified using the Qubit 4 fluorometer (Thermo Fisher Scientific, Madison, WI, USA). Subsequently, library preparation was carried out using the Illumina DNA Prep kit (Illumina, San Diego, CA, USA). Nextera DNA CD indexes were used for sample indexing and indexed libraries were pooled in equimolar concentrations. Finally, the paired-end sequencing run was conducted on an Illumina MiSeq instrument, utilizing a v3 600-cycle kit (Illumina, San Diego, CA, USA).

5.2.3. Genome assembly, quality control, annotation, multi-locus sequence typing, and taxonomic identification

The quality of raw sequencing reads was assessed using FastQC (Galaxy Version 3.0.3+galaxy0) (Andrews, 2010), followed by trimming of low-quality bases and adapters with Trimmomatic by using default settings (Galaxy Version 0.39+galaxy0) (Bolger et al., 2014). The resulting trimmed sequences were assembled using Skesa (Galaxy Version 0.0.4) with default settings (Souvorov et al., 2018). The genome assembly quality was assessed with Quast (Galaxy Version 5.2.0+galaxy1) (Mikheenko et al., 2018).

SkesaMLST (Galaxy Version 0.0.4) was then applied to assemblies to determine multi-locus sequence types (MLST STs). GTDB-Tk (V2.1.0) with the reference database version R207_v2 was used for genome-based taxonomic identification (Chaumeil et al., 2019). The annotation of the 18 *Campylobacter* genomes was performed using Prokka (Galaxy Version 1.14.6+galaxy0) (Seemann, 2014), and Roary (Galaxy Version 3.13.0+galaxy2) was used to calculate the pan-genome. The output of Roary was visualized using a roary plot (Galaxy Version 1.0) (Page et al., 2015).

5.2.4. Single nucleotide polymorphism detection and phylogenetic analysis

High-quality single nucleotide polymorphisms (SNPs) were detected using the CFSAN SNP pipeline on the GalaxyTrakr platform (Gangiredla et al., 2021, Gurevich et al., 2013). For SNP analyses of *C. jejuni* isolates, we used a reference genome of *C. jejuni* SRR24187546, and for SNP analyses of *C. coli* the reference genome *C. coli* SRR24187539 was used. The reference genomes were selected based on the high quality of genome assembly, as determined using the N50 metric. The identified SNPs were used to construct a maximum likelihood phylogenetic tree using IQ-TREE (Galaxy Version 2.1.2+galaxy2) with default settings and 1,000 rapid bootstrap iterations (Nguyen et al., 2015).

5.2.5. Identification of antimicrobial resistance and virulence genes

ABRicate (Galaxy Version 1.0.1) (Seemann, 2016) was used with default settings to detect the presence of virulence factor and antimicrobial resistance gene sequences in the studied isolates by utilizing the Virulence Factor Database (VFDB) and the NCBI Bacterial Resistance Reference Gene Database, respectively. Amrfinderplus_db NCBI (Galaxy Version 3.11.11+ galaxy1) was used to detect resistance mutations T86I in *gyrA* and A103V in *50S_L22* (Feldgarden et al., 2019).

5.2.6. Comparison of genomes of the studied isolates with those available in the Pathogen Detection database

Isolates studied in this study were submitted to the NCBI Pathogen Detection database to compare their genomic similarity with the *Campylobacter* isolates available in the database. Specifically, we identified the minimum SNP distance between our isolates and *Campylobacter* isolates of the same (non-human) source and between our isolates and *Campylobacter* isolates from a human source.

5.3. Results

5.3.1. Four MLST sequence types were detected among the studied isolates

The median genome assembly size of the 14 *C. jejuni* isolates was 1.76 Mbp, the median N50 was 0.16 Mbp, and the median GC% content was 30.3%. Among the four *C. coli* genomes, the median genome assembly size, GC%, and the median N50 were 1.69 Mbp, 31%, and 0.18 Mbp, respectively. The isolate metadata and genome quality information are reported in Table 20.

Table 20: The median genome assembly size of the *C. jejuni* and *C. coli*

Campylobacter species	Accession number	Contigs N	Total length	N50	L50	GC (%)
C. jejuni	SRR24187534	15	1627086	219976	3	30.34
	SRR24187535	15	1626673	219969	3	30.33
	SRR24187536	26	1758672	161521	4	30.29
	SRR24187537	15	1627244	190374	3	30.33
	SRR24187538	23	1760003	161521	4	30.29
	SRR24187539	14	1626601	219968	2	30.33
	SRR24187542	24	1759162	161521	4	30.3
	SRR24187544	24	1758618	155689	4	30.29
	SRR24187545	24	1759042	143854	5	30.3
	SRR24187546	14	1627101	299609	2	30.34
	SRR24187547	25	1759661	155689	4	30.29
	SRR24187548	22	1760397	161521	4	30.29
	SRR24187550	21	1759529	161521	4	30.29
	Median		1758645	161521		30.30%
C. coli	SRR24187540	25	1636179	216667	3	31.41
	SRR24187541	27	1736666	141344	4	31.2
	SRR24187543	30	1741794	141344	4	31.2
	SRR24187549	25	1641406	216666	3	31.42
	Median		1689036	179005		31%

The pangenome analysis was conducted for just *C. jejuni* isolates, as there were too few *C. coli* isolates available in our dataset. Among the 14 *C. jejuni* genomes, a total of 3,412 genes were detected, of which 309 were core genes detected in all studied isolates. No softcore genes, >95% occurrence, were detected; a total of 2,822 shell genes, and 284 cloud genes, shared by a minimal subset of the genomes, were detected. Shell genes, genes shared by the majority of genomes, represented approximately 83% of the total gene count (Table 21).

Table 21: *C. jejuni* pangenome.

Genes	Identity	Genes N (%)
Core genes	(99% <= strains <= 100%)	306 (9)
Softcore genes	(95% <= strains < 99%)	0 (Chatterjee)
Shell genes	(15% <= strains < 95%)	2822 (83)
Cloud genes	(0% <= strains < 15%)	284 (8)
Total genes	(0% <= strains <= 100%)	3412 (100)

Further, a total of 2 sequence types and 2 clonal complexes were identified among *C. jejuni* isolates. Specifically, ST 2084, which belongs to the clonal complex ST-353, was the predominant sequence type. The remaining six isolates belonged to ST 51, which is part of the clonal complex ST-443. Isolates of *C. coli* clustered separately from isolates of *C. jejuni* in the constructed maximum likelihood tree (Figure 2). Specifically, isolates of *C. jejuni* clustered in two distinct clades (B and C) in the constructed maximum likelihood tree (Fig. 2) . Clade B included 6 isolates of ST 51, while clade C was comprised of 8 isolates of ST 2084. Notably, ST 51 and 2084 were detected in both raw and pasteurized milk samples. Isolates within clade B and clade C differed by 1-5 and 0 SNPs, respectively. Among *C. coli* isolates, we found two sequence types, ST 1628 and ST 830, which belonged to a single clonal complex, ST-828. Isolates within clade A differed by 0-12 SNPs. *C. coli* ST 1628 was obtained from pasteurized milk, while ST 830 was obtained from raw milk (Figure 2).

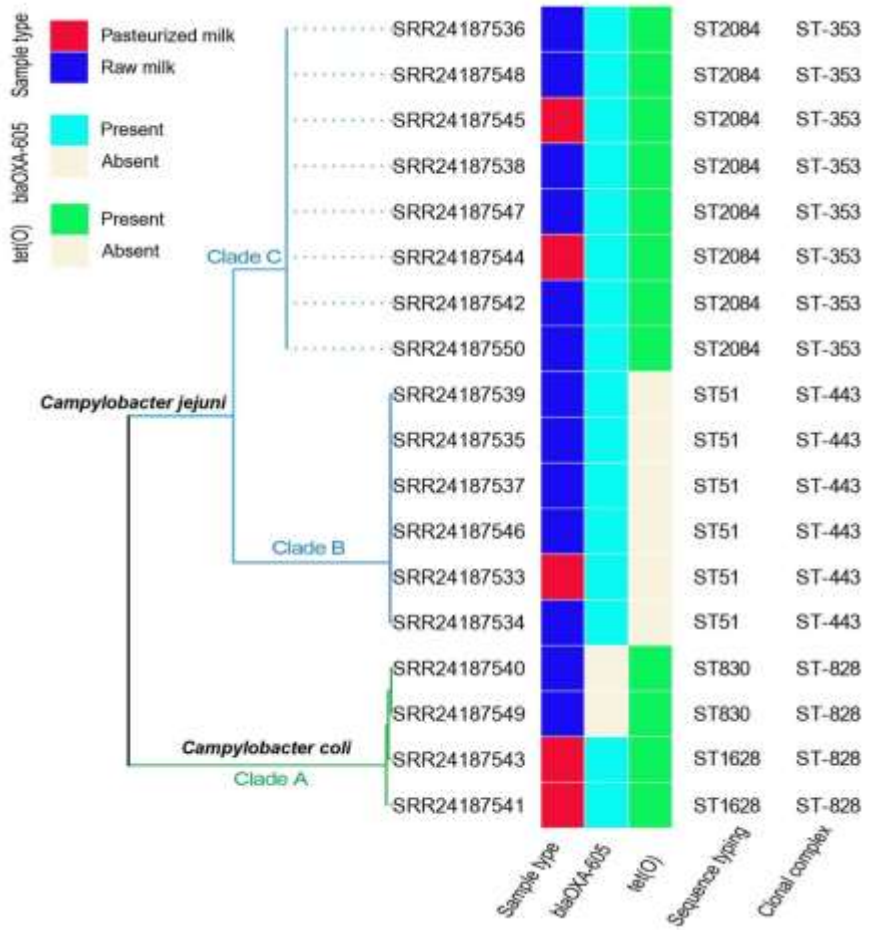

Figure 2. A phylogenetic tree constructed based on high-quality single nucleotide polymorphisms (SNPs) was identified using the FDA CFSAN pipeline for the 14 *C. jejuni* and 4 *C. coli* isolates obtained from dairy foods in Ethiopia.

5.3.2. The majority of *C. jejuni* isolates carried a fluoroquinolone resistance mutation T86I mutation in the *gryA* gene

In addition to assessing genomic similarity, we screened genomes for antimicrobial resistance gene sequences. Overall, we detected two antimicrobial resistance genes among studied *Campylobacter* isolates. All *C. jejuni* isolates carried a *blaOXA-605* gene, but only ST 2084 isolates carried the *tetO* gene. Conversely, among *C. coli*, both ST 830 and ST 1628 isolates carried *tetO*, but only ST 1628 also carried the *blaOXA-605* gene (Figure 1). Furthermore, ten isolates of *C. jejuni* ST 2084 and ST 1628 had a T86I mutation in the

gryA gene, which has been associated with resistance to quinolone antibiotics, such as ciprofloxacin and nalidixic acid. In addition to the mutation in the *gyrA* gene, all *C. jejuni* isolates carried the A103V mutation in the *50S L22* gene, which has previously been associated with the resistance of macrolide antibiotics, such as erythromycin (Table 22).

Table 22: Prevalence of antibiotic resistance gene in *Campylobacter* species

AAC. Number	MLST ST	Gene symbol	Element type	Element subtype	Class
SRR24187533	ST 51	50S_L22_A103V	AMR	Point	Macrolide
SRR24187534	ST 51	50S_L22_A103V	AMR	Point	Macrolide
SRR24187535	ST 51	50S_L22_A103V	AMR	Amr	Macrolide
SRR24187536	ST 2084	gyrA_T86I	AMR	Point	Quinolone
		50S_L22_A103V	AMR	Point	Macrolide
SRR24187537	ST 51	50S_L22_A103V	AMR	Point	Macrolide
SRR24187538	ST 2084	50S_L22_A103V	AMR	Point	Macrolide
		gyrA_T86I	AMR	Point	Quinolone
SRR24187539	ST 51	50S_L22_A103V	AMR	Amr	Macrolide
SRR24187541	ST 1628	gyrA_T86I	AMR	Point	Quinolone
SRR24187542	ST 2084	gyrA_T86I	AMR	Point	Quinolone
		50S_L22_A103V	AMR	Point	Macrolide
SRR24187543	ST 1628	gyrA_T86I	AMR	Point	Quinolone
SRR24187544	ST 2084	50S_L22_A103V	AMR	Point	Macrolide
		gyrA_T86I	AMR	Point	Quinolone
SRR24187545	ST 2084	gyrA_T86I	AMR	Point	Quinolone
		50S_L22_A103V	AMR	Point	Macrolide
SRR24187546	ST 51	50S_L22_A103V	AMR	Amr	Macrolide
SRR24187547	ST 2084	gyrA_T86I	AMR	Point	Quinolone
		50S_L22_A103V	AMR	Point	Macrolide
SRR24187548	ST 2084	50S_L22_A103V	AMR	Point	Macrolide
		gyrA_T86I	AMR	Point	Quinolone
SRR24187550	ST 2084	50S_L22_A103V	AMR	Point	Macrolide
		gyrA_T86I	AMR	Point	Quinolone

Lastly, in terms of heavy metal resistance genes, the arsenic resistance gene *arsP* was found in eight *C. jejuni* ST 2084 isolates and no other studied isolates (Table 23).

Table 23: Prevalence of arsenic resistance in Ethiopian *Campylobacter* species

Accession number	ST	Organism	Isolation source	SNP cluster	Stress genotypes
SRR24187536	ST-2084	*C. jejuni*	Raw Milk	PDS000145443.1	arsP
SRR24187538	ST-2084	*C. jejuni*	Raw Milk	PDS000145443.1	arsP
SRR24187542	ST-2084	*C. jejuni*	Raw Milk	PDS000145443.1	arsP
SRR24187544	ST-2084	*C. jejuni*	Pasteurized Milk	PDS000145443.1	arsP
SRR24187545	ST-2084	*C. jejuni*	Pasteurized Milk	PDS000145443.1	arsP
SRR24187547	ST-2084	*C. jejuni*	Raw Milk	PDS000145443.1	arsP
SRR24187548	ST-2084	*C. jejuni*	Raw Milk	PDS000145443.1	arsP
SRR24187550	ST-2084	*C. jejuni*	Raw Milk	PDS000145443.1	arsP

5.3.3. All *C. jejuni* isolates carried genes encoding cytolethal distending toxin

In this study, all *Campylobacter* isolates carried virulence genes encoding *cadF, cheAVWY, ciaBC, flaCDG, flgBCDEFHIJKMPRS, flhABFG, fliADEFGILMNPQRSWY, gmhAB, hldDE, kpsDFST, motA, pebA, pflA, pseABCEFGHI, ptmAB, rpoN, waaCFV* (Fig 2). Furthermore, all *C. jejuni* isolates carried virulence genes *flgQ, Cj1416c, cj1417c, cj1419c, cj1420c, ctdABC, cysC, eptC, flgA, fliHK, htrB, jlpA, kpsECM* and *motB*, with ST 2084 harboring two additional genes, *Cj1135* and *pseD/maf2*, compared to ST 51. All *C. jejuni* isolates carried the *cdtABC* gene cluster encoding cytolethal distending toxin (Fig 2). One isolate from ST 51 also carried the *pseD/maf2* gene, which encodes the motility accessory factor *PseD*. The genes *gmhA2* and *hddA* were detected in all studied *C. coli* isolates. Among isolates of ST 830, we detected *cj1135*, which encodes a lipo-oligosaccharide. The ST 1628 *C. coli* isolates shared the virulence genes *virB8-11, virB4,* and *virD4*, and one isolate from ST 1628 carried additional virulence genes *flaAB* and *pseD/maf2* (Figure 3).

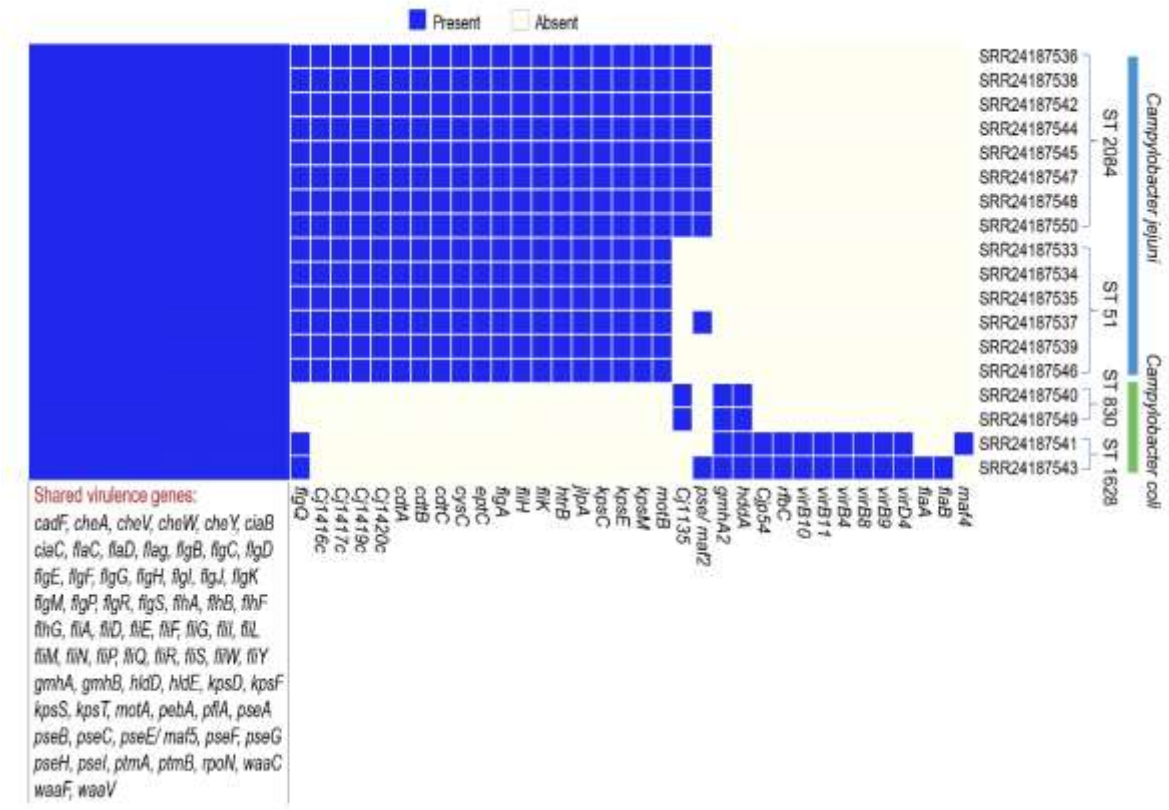

Figure 3. Virulence gene profiles detected in *Campylobacter jejuni* and *Campylobacter coli* isolates. The presence of genes is indicated in blue and their absence is denoted in light yellow.

5.3.4. *Campylobacter* isolates from Ethiopia were distinct compared to isolates from other geographical origins

Lastly, utilizing the Pathogen Detection database, we compared the 14 *C. jejuni* and 4 *C. coli* from this study with isolates from non-human (i.e., environmental, food, animal), and human sources that were available in the database. Our isolates clustered into four Pathogen Detection clusters and none of these four clusters contained isolates from other studies or countries, indicating distinct genetic profiles of isolates characterized in this study. The *Campylobacter coli* isolates from raw milk and pasteurized milk (SRR24187543 - PS02412 and SRR24187541 - PS02436) clustered in the Pathogen Detection SNP cluster PDS000145444.1. *C. coli* isolated from raw milk (SRR24187540 -

PS02419 and SRR24187549 - PS02411) clustered in the Pathogen Detection SNP cluster PDS000145445.1. Additionally, the *C. jejuni* isolates from pasteurized and raw milk clustered in the SNP cluster PDS000145443.1 (SRR24187538 - PS02422, SRR24187550 - PS02404, SRR24187536 - PS02424, SRR24187548 - PS02439, SRR24187547 - PS02440, SRR24187545 - PS02432, SRR24187544 - PS02435, SRR24187542 - PS02418) and SNP cluster PDS000145442.1 (SRR24187539 - PS02421, SRR24187537 - PS02423, SRR24187535 - PS02425, SRR24187534 - PS02426, SRR24187533 - PS02430, and SRR24187546 - PS2441). Noteworthy, isolates from pasteurized and raw milk were found in the same clusters (Figure 4).

Figure 4: Four Pathogen Detection clusters that contained isolates of *Campylobacter* isolates obtained from the Ethiopian dairy supply chain (https://www.ncbi.nlm.nih.gov/pathogens).

5.4. Discussion

5.4.1. MLST sequence types 2084 and 51 were detected in both raw and pasteurized milk

Among 14 *C. jejuni*, STs 2084 and 51 were identified in both pasteurized and raw milk, with ST-353 and ST-443 as their respective clonal complexes. There is limited information available on the global prevalence of *Campylobacter jejuni* ST-2084. Based on the PubMLST database records, it is evident that ST 2084 isolates have previously been detected in the USA, UK, and Pakistan (https://pubmlst.org/campylobacter/); however, a literature search did not yield any published papers reporting this particular sequence type (Kaakoush et al., 2015a). Nevertheless, the clonal complexes to which these two STs belong have previously been reported in studies involving both humans and chickens. For example, Kinana et al. reported that the ST-353 complex was the most common complex detected among isolates from chicken carcasses in Senegal (Kinana et al., 2006). Manning et al.(2003) identified the ST-353 complex isolate in poultry in the UK. In China, Estonia, and Poland, ST-353 *C. jejuni* was isolated from retail chicken, humans, and chicken (Ma et al., 2017, Sarhangi et al., 2021, Wieczorek and Osek, 2015). Another research has linked ST-353 isolates to human disease (Manning et al., 2003). This included a study conducted in Michigan, US, in which they found ST-353 to be the third most abundant (11.8%) clonal complex among the 214 strains of *C. jejuni* recovered from patients with gastroenteritis (Djordjevic et al., 2007).

Isolates belonging to ST-443 have previously been detected in Iran, Poland, China, as well as in the Gambia (Sarhangi et al., 2021, Sails et al., 2003, Ma et al., 2017). Among clonal complex ST-443 isolates, ST 51 was found in human feces in Poland, Germany, and Croatia, and ranked among the top 10 STs detected across Europe (Fiedoruk et al., 2019, Šoprek et al., 2022, Parker et al., 2021). ST 51 isolates were also isolated from patients undergoing treatment at the Red Cross War Memorial Children's Hospital in Cape Town, South Africa (Quiñones et al., 2008). Unlike our study, others have not previously reported isolating ST 51 from dairy food sources.

We detected *C. coli* ST 830 in raw milk and ST 1628 in pasteurized milk. Both of these sequence types belong to the clonal complex ST-828. While ST 830 has been detected in chicken meat in the Middle East, Asia, South America, and the US, the only record of its isolation in Africa (Egypt and Nigeria) from cattle and human feces exists in the PubMLST database (https://pubmlst.org/campylobacter/, Habib et al., 2023, Guk et al., 2021). Regarding ST 1628, before our study, there were no documented reports of this ST in Africa. However, the PubMLST database records suggest its detection in Europe, Asia, and North America from various sources, including cattle, chicken, beef and chicken meat, environmental water, goose, and human stool (https://pubmlst.org/campylobacter/, Asakura et al., 2019, Gripp et al., 2011).

The detection of the same STs in both raw and pasteurized milk raises contamination concerns, suggesting that *C. jejuni* is transmitted from raw milk to pasteurized milk during processing or handling and is not effectively inactivated; alternatively, the pasteurization processes may have not been effective. The presence of the same genotypes in raw and pasteurized milk suggests a common contamination source, which could be contaminated water, equipment, or processing facility environment (Calahorrano-Moreno et al., 2022, Quigley et al., 2013). Identifying and addressing the source of contamination is crucial for mitigating further contamination (Jaakkonen et al., 2020). Moreover, the presence of *C. jejuni* in pasteurized milk underscores the possibility of two critical issues. First, it raises concerns regarding the effectiveness of the pasteurization process, suggesting that it may not have achieved the required temperature and duration necessary to eliminate pathogenic bacteria like *C. jejuni* (Fernandes et al., 2015, Louwen and van Neerven, 2015, Adams et al., 2000). Second, it highlights the potential for post-processing contamination, which could occur during subsequent stages such as milk handling, filling, or packaging (Reichler et al., 2020). Previous studies conducted in Norway and Sweden have uncovered instances of spoilage bacteria recontamination during the pasteurized milk filling process (Martin et al., 2018, Eneroth et al., 1998). Further, previous research found that the detection of *Escherichia coli* in pasteurized milk may be a result of pasteurization process failures or contamination during post-pasteurization processing (Oltramari et al., 2014). This is

particularly concerning because pasteurized milk is expected to be pathogen-free due to the rigorous heat treatment it undergoes.

5.4.2. Prevalence of virulence gene in *Campylobacter* species

We have identified several virulence genes that are crucial for *Campylobacter* pathogenesis. All *Campylobacter* isolates from milk and milk products carried virulence gene involved in motility and flagellar biosynthesis (*motA, flaDG, flgABCDEFGHIJKMPQRS, flhABFG, fliADEFGHIKLMNPQRSWY, pseABC,* and *rpoN)*. The motility genes *motB, flaAB,* and *flgQ* were detected in all *C. jejuni*, *C. coli* PS02412, and all STs except ST 830 (PS02421 and PS02422), respectively. Genes *flgABCDEFGHIJKMPQRS* are involved in the assembly and structure of the flagellar rod, hook, and basal body (Cohen and Hughes, 2014), and genes *fliPQRSWY* contribute to the construction, control, and operation of the flagellar motor and filament (Bouteiller et al., 2021, Zhao et al., 2014, Schwan et al., 2022). The *motAB* encodes a stator complex protein that facilitates torque generation in the flagellar motor (Ribardo et al., 2019). Moreover, motility regulation genes that were detected included *rpoN*, the product of which promotes the transcription of rod and hook components and the minor flagellin *flaB* (König et al., 2023), and *flhFG* genes that regulate the expression of the flagellar genes and the assembly of the flagellar basal body (Sher et al., 2020, Liu and Ochman, 2007). The flagellar T3SS is located within the flagellum of *Campylobacter* (Balaban and Hendrixson, 2011). T3SS secretion system genes (*flhAB, fliPQR*) encode the export gate proteins and play important roles in the regulation and assembly of the flagellar type III secretion system in *Campylobacter* (Lopes et al., 2021, Fabiani et al., 2017, Macnab, 2004, Su et al., 2023, Hendrixson and DiRita, 2003). This system is involved in the secretion of various proteins, including the Cia proteins, Fed proteins, and flagellin C (Burnham and Hendrixson, 2018). Additionally, the type IV secretion system-related genes (*virB4*, *virB8-11*, and *virD4*) were only found in the genome of *C. coli* ST 1628. In a study conducted in Peru and Chile, T4SS genes like *virB4*, *virB9-11*, and *virD4* were present in *C. jejuni* and *C. coli* (Quino et al., 2022, Bravo et al., 2021).

Campylobacter jejuni uses flagella and chemotaxis to navigate its motility towards or away from environmental stimuli and it plays an essential role in pathogenesis during host colonization (Zautner et al., 2012). The flagellar assembly and the chemotactic motility depend on the expression of chemotaxis genes (*cheAYV,* or *cheW*) (Reuter et al., 2018). In this study, the chemotaxis-related genes (*cheAYV*) were detected in all *C. jejuni* and *C. coli* genomes.

Adhesion is a critical stage in pathogenesis before invasion and the release of toxins (Letourneau et al., 2011). The adhesins of *Campylobacter* species facilitate a specific interaction between the bacterium and host cells (Konkel et al., 2020). In our study, the genes encoding adhesion-related proteins were found in all *Campylobacter jejuni* and *Campylobacter coli*. These included outer membrane proteins (OMPs) genes (*cadF, pebA, JlpA)*), genes for synthesis of lipooligosaccharides (LOS) (*gmhA2BA, rfbC, hldDE, hddA, waaFCV,* and *htrB)*, and capsule polysaccharides (CPS) (*KpsCSDEMT, Cj1416c-Cj1420c*, and *cysC)*. This finding is consistent with other studies (Zhang et al., 2023, Neal-McKinney and Konkel, 2012, De Filippis et al.).

All of our studied *Campylobacter* isolates carried *ciaBC* genes that code for Cia proteins, which are essential for invasion and colonization (Kreling et al., 2020, Lopes et al., 2021, Gabbert et al., 2023). All of our studied *Campylobacte*r isolates carried the *flaC* gene that encodes the FlaC protein that influences cell invasion by binding to epithelial cells (Gabbert et al., 2023). The Cia proteins induce pathogen uptake and perhaps alterations in cellular responses only if delivered into the cytosol of the host target cell via bacteria-host cell contact (Rivera-Amill et al., 2001).

In addition to adhesion and invasion, the ability of *Campylobacter* species to produce the cytolethal-distending toxin (CDT) is a crucial component of their pathogenicity. All *C. jejuni* studied here carried the *cdtABC* genes encoding for CDT. This toxin causes direct DNA damage leading to the invocation of DNA damage responses in human cells and leads to cell apoptosis (Lee et al., 2003). The *cdt* gene cluster has been commonly detected in *Campylobacter* species isolated from humans (Redondo et al., 2019), poultry (Wieczorek

et al., 2018), cattle, and swine isolates and contributes to campylobacteriosis (Wysok et al., 2015, Bang et al., 2001).

Overall, the presence of multiple important virulence factor gene sequences in the genomes of the studied *Campylobacter* isolates suggests their potential to cause foodborne illness.

5.4.3. The majority of isolates carried a resistance mutation in *gryA*

Antimicrobial resistance has been emerging among *Campylobacter*, which poses a serious risk for failed antimicrobial treatment of campylobacteriosis (Luangtongkum et al., 2009, Panzenhagen et al., 2021). Concerns about public health have arisen specifically due to the rising prevalence of fluoroquinolone-resistant *Campylobacter* (Han et al., 2012, Shen et al., 2018). A study based in Oxford, UK, indicated that in 1995, 7% of the overall *Campylobacter* isolates were resistant to fluoroquinolones and by 2008 the prevalence had risen by nearly 2 percent (Veltcheva et al., 2022). We detected resistance mutations in *gryA* and *50S L22* in 55 and 78% of the isolates examined, respectively. This result is correlated to our earlier research, which found that 57% and 78% of tested *Campylobacte*r species were phenotypically resistant to ciprofloxacin and erythromycin (Admasie et al., 2024) Ciprofloxacin resistance in *C. jejuni* is mediated by mutations in the *gyrA* gene, with the point mutation T86I being the most common one (Zirnstein et al., 1999, Espinoza et al., 2020), as reported elsewhere, including in Portugal, Botswana, and Nigeria (De Vries et al., 2018, Duarte et al., 2014, Audu et al., 2022). Recently, *Campylobacter* isolated from sources of human and poultry meat in Pennsylvania, USA, showed a strong correlation between the existence of the matching known resistance genetic determinants and the phenotypic resistance of ciprofloxacin (Yan et al., 2023).

Remarkably, in all *Campylobacter jejuni* isolates tested here, an A103V mutation in the 50S L22 gene was detected. The 50S macrolide-binding site is composed of portions of the 23S rRNA subunit, ribosomal protein L4, and ribosomal protein L22 (Belanger and Shryock, 2007, Gibreel et al., 2005). The 50S L22 protein is a component of the 50S ribosomal subunit, and alterations in this protein have been associated with macrolide resistance in *Campylobacter* species (Belanger and Shryock, 2007, Gibreel et al., 2005).

Another study identified the 50S L22 point mutation as a resistance mechanism in *C. coli* isolates (Bolinger and Kathariou, 2017). These point mutations are causing the rise of macrolide-resistant *Campylobacte*r strains, and consequently, macrolides such as erythromycin and azithromycin are becoming less effective in treating *Campylobacter* infections (Bolinger and Kathariou, 2017).

Here, the genomic analysis revealed that 67% (12/18) and 89% (16/18) of studied *Campylobacter* carried *tet(O)* and *OXA-605*, which confer tetracycline resistance and beta-lactam resistance, respectively. Our previous result showed that 89% of *Campylobacter* species were phenotypically resistant to tetracycline (Admasie et al., 2024) These findings suggest a strong correlation between phenotypic tetracycline resistance and the presence of the *tet(O)* gene in *Campylobacter* species. Similarly, in a study from Korea and Peru, all tested *Campylobacter* isolates obtained from chicken carried the *tet(O)* gene (Benites et al., 2022, Kim et al., 2010). A study conducted in South Africa also found that the *tet(O)* gene was the most prevalent antimicrobial resistance gene detected in *Campylobacter* isolates from chickens and humans, with a prevalence of 68% and 64%, respectively (Reddy and Zishiri, 2017). Antibiotic resistance due to the production of D-lactamase OXA-61 was previously reported by Alfredson and Korolik (Alfredson and Korolik, 2005). Additionally, a UK investigation revealed that the isolates from both humans and poultry included OXA-61, which codes for the generation of β-lactamase and causes ampicillin resistance (Griggs et al., 2009). The function of this gene was confirmed with the insertional inactivation of blaOXA-61 which increased the susceptibility of *Campylobacter* to ampicillin, co-amoxiclav, penicillin, carbenicillin, oxacillin, and piperacillin in *C. jejuni* NCTC 11168 (Griggs et al., 2009).

In addition to antimicrobial resistance genes, we examined the presence of heavy metal resistance genes, such as arsenic resistance genes. At high levels, arsenic is toxic to most cells, including microbial organisms, and is present in the natural environment (Oremland and Stolz, 2003, Oremland and Stolz, 2005). In this study, *arsP* was found in ST2084 *Campylobacter jejuni* and this gene has previously been associated with arsenic resistance (Noormohamed and Fakhr, 2013). Exposure to arsenic can select for resistant bacteria, through horizontal gene transfer of arsenic resistance genes (Wang et al., 2009); however,

we did not collect any information about environmental levels of arsenic in areas in which samples were collected to assess whether the environmental arsenic exposure may have led to the acquisition of arsenic resistance genes by the studied *Campylobacter* isolates.

5.5. Conclusion

This study offers valuable insights into the genetic diversity, virulence, and antibiotic resistance potential of *Campylobacter* isolates collected from the dairy supply chain in Ethiopia. We identified four distinct *Campylobacter* STs, including ST-51, ST-2084, ST-830, and ST-1628. Notably, STs 51 and 2084 were found in both raw and pasteurized milk. This highlights the critical importance of assessing the risk of *Campylobacter* contamination in the dairy supply chain. Furthermore, the majority (71%) of analyzed *C. jejuni* isolates carried a fluoroquinolone-resistance-associated mutation, suggesting a concerning level of resistance to this class of clinically relevant antibiotics.

Chapter 6

6. General Discussion and Conclusion

In Ethiopia, *Campylobacte*r was detected in 12 % (141/1140) of tested Diary products. Among *Campylobacter* species-positive samples collected countrywide, 89% were contaminated with *C. jejuni*, and 11% with *C. coli*. The findings of the present study are concurrent with reports of Del Collo et al. (2017) who reported *C. jejuni* at the rate of 92% (Del Collo et al., 2017). In the dry season, all isolates were *C. jejuni*. A study conducted in India found that all the isolates identified were *C. jejuni* (Modi et al., 2015a).

The prevalence of *Campylobacter* in milk and milk products varies depending on the type of product (El-Kholy et al., 2016). For example, in Ethiopia, *Campylobacter* isolates can be found more frequently in raw milk samples than in pasteurized milk and cottage cheese ($P < 0.001$). Similarly, A study conducted in Egypt found the highest prevalence of *Campylobacter* in milk samples (18%), followed by Kareish cheese (14%) and Talaga cheese (6%) (Zeinhom et al., 2021). In our investigation, Ethiopia had the greatest incidence of *Campylobacter* species (19%) in raw milk. The prevalence of *Campylobacter* found in this study is similar to findings reported by Hussain et al. (2007), El-Zamkan and Hameed (2016), and Zeinhom et al. (2021) who detected *Campylobacter* species at 21.5%, 20 %, and 18% in raw milk samples in Pakistan and Egypt, respectively (El-Zamkan and Hameed, 2016b, Zeinhom et al., 2021, Hussain et al., 2007). However, as compared to our result a lower result, 2.91% of *Campylobacter* species in raw milk samples, was reported by Modi et al. (2015). This study revealed that using warm water and soap for cleaning cow udders and teats on farms reduced the likelihood of detecting *Campylobacter* in milk. Similar to this study, Tigabu et al. (2015) found that cleaning cow teats with warm water and soap is an important step in preventing contamination of raw milk and ensuring the safety of dairy products. In this study, storing milk in an aluminum container can reduce the likelihood of detecting *Campylobacter* in milk at the collection facilities ($P=0.027$). The result of our study was similar to Wafula et al. (2016) who found reduced odds of detection of the bacteria in raw milk from aluminum containers. In contrast to the actual reality, *Campylobacter* detection was significantly more likely in milk collected at

collection centers with concrete floors (AOR=5.2, P=0.004). It is important to have flooring that is strong, impervious, washable, and smooth to prevent bacterial growth and contamination (Rahman, 2007). However, in this study, the concrete floor of the cow house was fully covered in muck during our site visit and sample collection.

According to our analysis, 10% of pasteurized milk in the Ethiopian dairy value chain is contaminated with *Campylobacter* species. The contamination of *Campylobacter* found in pasteurized milk is similar to the finding of Ogbomon et al. (2018) who reported 16 % *Campylobacter* species in locally pasteurized milk products (Nunu) in Zaria metropolis, Kaduna State, Nigeria (Ogbomon et al., 2018a). According to Fernandes et al. (2015), the contamination of pasteurized milk with *Campylobacter* may be due to partial failure of milk pasteurization. Globally, very little report was found on the prevalence of *Campylobacter* in pasteurized milk. However, a study published by Taylor et al. (2013) identified three outbreaks of *Campylobacter* infection from dairy products that were associated with the consumption of pasteurized milk. Two of these outbreaks occurred in correctional facilities, and one was associated with a school lunch program. Besides, the odds of detecting *Campylobacter* in milk were 17 times greater (AOR=17, P=0.007) in milk processing facilities that did not calibrate a pasteurizer on an annual basis. Nada et al. (2012), Mureşan et al. (2020), and Pierson (2012) showed improvement in the microbial quality of pasteurized milk by implementing an HACCP system in a dairy plant. In addition to this, having a separate refrigerator for milk storage reduced the odds of detecting *Campylobacter* in retail (AOR=0.29, P=0.021). Marler's study in 2009 showed the importance of proper handling and storage of milk to prevent contamination. Pasteurized milk can also become contaminated if the equipment fails or if it does not reach the right temperature for long enough (Marler, 2009).

The seasonal differences in the prevalence of *Campylobacter* infections are influenced by a combination of climate, human behavior, and environmental factors. Studies have indicated that temperature, sunshine, and rainfall are important factors that determine the seasonality of *Campylobacter* infections. Of these, temperature has the greatest effect on seasonality (Djennad et al., 2019, Louis et al., 2005). These findings suggest that the seasonal differences in *Campylobacter* prevalence are driven by a complex interplay of

climatic, behavioral, and environmental factors (Baali et al., 2020). In Ethiopia, the prevalence of *Campylobacter* in milk and milk products did not differ significantly between the wet and dry seasons (20% in the wet season vs. 16% in the dry season, p=0.27). Similar results were reported by Salihu et al. (2010) who found no seasonal difference in the prevalence of *Campylobacter* species in raw cow milk samples collected from different locations in Sokoto State, Nigeria. However, in the Oromia region, the prevalence of *Campylobacter* species was significantly higher in the wet season compared to the dry season (5% vs. 19%; p=0.002). The result of this research was similar to Mahmood et al. (2009) who showed the maximum prevalence of *Campylobacter* species in milk and milk products during summer when analyzed in comparison with other seasons around the year. Similar to our study, another study conducted in the Asante Akim North Municipality of Ghana found that the frequency of *Campylobacter* was higher in the rainy season (22.2%) than in the dry season (15.1%) (Paintsil et al., 2022b). Moreover, a study conducted in the Nsukka agricultural zone, Nigeria, found that *Campylobacter* infection was more common in rainy months (58.1%) than in dry months (47.3%), autumn (31.5%), and spring (27.5%) (Njoga et al., 2019).

Furthermore, 43% of the tested isolates were resistant to more than two drugs from two different classes. A similar result was reported by Hull et al. (2021) who found that 45% of the *Campylobacter* species were resistant to more than two antibiotics (Hull et al., 2021). The result of this study is similar to Zachariah et al. (2021) who found that *Campylobacter jejuni* was resistant to more than five antibiotics used in the study, indicating a high degree of multiple drug resistance including erythromycin and ciprofloxacin in Kenya. Also, regardless of the sample size the phenotypic and genotypic resistance of *Campylobacte*r species was similar. For example, most of the tested *Campylobacter species* were resistant to tetracycline (89%), followed by erythromycin (74%), and ciprofloxacin (57%). The most common resistance mechanism of *Campylobacter* species to tetracycline is the ribosomal protection protein (tetO), which is transferred as a plasmid-encoded gene (Abdi-Hachesoo et al., 2014, Pratt and Korolik, 2005). Interestingly, Seventy-eight percent of the *C. jejuni* and *C. coli* examined carried the tetracycline gene tet (O) (89%). The relationship between the presence of Tet (o) and phenotypic resistance of tetracycline in our study is consistent

with Benites et al. (2022), and Kleinubing et al. (2021) showed that 74 % and 70% of the examined *Campylobacter* species tet(o) gene and resistance to tetracycline in Brazile and Peru, respectively (Benites et al., 2022, Kleinubing et al., 2021).

Erythromycin and ciprofloxacin are both antibiotics that have been used to treat Campylobacteriosis (Wieczorek and Osek, 2013). The 50S_L22_A103V gene is a modification of the 50S ribosomal subunit that has been associated with erythromycin resistance in *Campylobacter* (Tedersoo et al., 2022a). In our study, the presence of the 50S_L22_A103V gene in all *C. jejuni* in our study makes it fascinating and important. Additionally, the presence of the 50S_L22_A103V gene (78%) is also consistent with the phenotypic resistance to erythromycin (74%). A higher result was reported by Sithole et al. (2021) found that *Campylobacter* isolates from pigs displayed high levels of resistance to erythromycin (96.7%) in South Africa (Sithole et al., 2021). A lower phenotypic resistance of *Campylobacter jejuni* to erythromycin was reported by Papadopoulos et al. (2021) and Marotta et al. (2019) who reported 3.7% and 2.94% in Turkey and Italy, respectively.

In addition, the phenotypic analysis of this study showed that 57% of *Campylobacter* species were ciprofloxacin-resistant. This result is in line with the existence of the gyrA gene, which is responsible for *Campylobacter*'s ciprofloxacin resistance. The gyrA gene is a quinolone resistance-determining region that codes for the 'A' subunit of the enzyme DNA gyrase, which confers a decreased sensitivity to fluoroquinolones (Frasao et al., 2015, Aksomaitiene et al., 2020). The prevalence of phenotypic resistance to ciprofloxacin is consistent with Eryıldız et al. (2022) who reported 74% of *Campylobacter* isolates were resistant to ciprofloxacin in Turkey. A much higher result was reported by Yan et al. (2023), Portes et al. (2023), and Aleksić et al. (2021) who reported 93.35, 91%, and 74 of *Campylobacter* were phenotypically resistant to ciprofloxacin in South Africa, Lithuania, and China. A study on antimicrobial resistance in *Campylobacter* speciesfound that the prevalence of ciprofloxacin resistance in *Campylobacter jejuni* from humans in sub-Saharan Africa was 3.7% which was much lower than this result (Griggs et al., 2005). Finally, we showed that 90 % of *Campylobacter* species carry blaOXA- 605 gene relationship with ampicillin resistance. Similarly, of all ampicillin-resistant *Campylobacter*

isolates tested, 91% carried the blaOXA-61 gene (Zeng et al., 2014). However, *Campylobacter* is intrinsically resistant to ampicillin due to the lack of a penicillin-binding protein (Hazards et al., 2021).

6.1. Genomic diversity of *Campylobacter* species

This study showed that four sequence types were found in Ethiopia's dairy value chain, these are *C. jejuni* ST 51, *C. jejuni* ST 2084, *C. coli* ST 1628, and *C. coli* ST 830. Among these sequence types, *C. jejuni* 51 was found all over the world. Similar data was reported in Poland, Germany, and Croatia (Fiedoruk et al., 2019, Šoprek et al., 2022, Parker et al., 2021). Fiedoruk et al. (2019) reported that *C. jejuni* ST 51 is ranked among the top 10 STs detected across Europe. Additionally, *C. coli* ST 830 was found in the Ethiopian dairy value chain; this sequence type was reported by Gripp et al. (2011) and Asakura et al. (2019) who detected detections in animals, food, and human feces in Germany and Japan, respectively (Asakura et al., 2019, Gripp et al., 2011). ST 830 was also discovered in chicken meat by Habib et al. (2023) in the United Arab Emirates (Habib et al., 2023) and Guk et al. (2021) in South Korea reported finding this ST in swine feces (Guk et al., 2021). However, there is no published report on the prevalence of *C. jejuni* ST 2084 and *C. coli* ST 1628. Therefore, this ST is an uncommon *Campylobacter* Sequence type in the world.

6.2. Virulence genes present in *Campylobacter* species

Campylobacter is a foodborne pathogen that causes campylobacteriosis, which can range from mild symptoms to fatal illness. Adhesion and invasion are important virulence factors for colonizing the host's intestinal cells (Zhang et al., 2020). Various virulence factors of *Campylobacter* play a crucial role in pathogenesis, such as the chemotactically controlled cellular motility, bacterial adhesion, invasion into the host cell, and toxin formation (Kreling et al., 2020). This study showed that genes responsible for motility, adhesion, and invasion were identified in the genome of *Campylobacter* species. Of this, Cytolethal Distending Toxin (CDT) is produced by three nearby genes called cdtA, cdtB, and cdtC (Bang et al., 2003). The cdtABC was detected in 100% of the isolates tested in this study, which is consistent with previous reports by Redondo et al. (2019), Wieczorek et al. (2018),

and Wysok et al. (2015) who showed *cdt* gene cluster is common in *Campylobacter* species isolated from humans (Redondo et al., 2019), poultry (Wieczorek et al., 2018), and cattle and swine isolates (Wysok et al., 2015). Overall, the presence of virulence factors like capsular-related genes, flagellar genes, invasive genes, adhesive genes, and CDT genes in *Campylobacter* species suggests their potential to cause disease in humans and animals.

In conclusion, this research showed that 12 % of Ethiopian dairy products were contaminated with *Campylobacter* species, mainly *C. jejuni*. The contamination of raw milk, pasteurized milk, and cottage cheese was seen in three regions of Ethiopia (Oromia, Amhara, And SNNP). The detection of *Campylobacter* species in dairy products showed the need for Hygienic raw milk and cottage cheese production and improved manufacturing of pasteurized milk to ensure the safety of dairy products in Ethiopia. Additionally, we discovered that the majority of the *Campylobacter* species were resistant to ciprofloxacin and erythromycin. These results call for increased surveillance and the creation of resistance mitigation plans to safeguard the general public's health. Additionally, the diversity, virulence profiles, and antibiotic resistance resulting from mutations of *Campylobacte*r species studied in this research can help create strategies for tracing disease outbreaks and managing and treating *Campylobacte*r-related disorders. This helps to improve the safety of dairy products in Ethiopia and protect public health.

Based on the above-concluding remarks, the following recommendations are forwarded:

- Aresponsible body in the Ethiopian milk and milk product value chain should be aware of the source of contamination of milk, pasteurized milk, and cottage cheese and should provide training at regional and national levels at each value chain.
- The regulatory body should control the calibration of the milk processor at the regional and national levels to ensure the safety of pasteurized milk for the consumer.
- It is important to inform the public about the health risks linked with *Campylobacter* species and to urge them not to consume raw milk.

- Encouraging appropriate antibiotic use in both human medicine and agriculture can help in reducing the development and spread of antibiotic resistance in *Campylobacter*
- Finally, a comprehensive study of *Campylobacter* from environment and clinical isolates should involve investigating different reservoirs, comparing clinical and environmental isolates, and using DNA sequence-based methods for controlling *Campylobacter* infection.
- Due to increasing resistance rates, it is crucial to avoid using fluoroquinolones antibiotics that may not be effective against *Campylobacter* strains

You found significant level of drug resistance in these isolates? What will be your recommendation regarding treatment options?

REFERENCE

ABAMECHA, A., ASSEBE, G., TAFA, B. & WONDAFRASH, B. 2015a. Prevalence of thermophilic Campylobacter and their antimicrobial resistance profile in food animals in Lare District, Nuer Zone, Gambella, Ethiopia. *Journal of Drug Research and Development,* 1**,** 1-6.

ABAMECHA, A., ASSEBE, G., TAFA, B. & WONDAFRASH, B. 2015b. Prevalence of thermophilic Campylobacter and their antimicrobial resistance profile in food animals in Lare District, Nuer Zone, Gambella, Ethiopia. *J Drug Res Dev,* 1**,** 2470-1009.

ABDELLATIF, G. M., MOHAMED, H. A. A.-E. & ABO-ALELLA, D. 2022. Campylobacter and It's Role in Gastroenteritis. *NeuroQuantology,* 20**,** 4278.

ABDI-HACHESOO, B., KHOSHBAKHT, R., SHARIFIYAZDI, H., TABATABAEI, M., HOSSEINZADEH, S. & ASASI, K. 2014. Tetracycline resistance genes in Campylobacter jejuni and C. coli isolated from poultry carcasses. *Jundishapur journal of microbiology,* 7.

ABU-HALAWEH, M., BATES, J. & PATEL, B. K. 2005. Rapid detection and differentiation of pathogenic Campylobacter jejuni and Campylobacter coli by real-time PCR. *Research in Microbiology,* 156**,** 107-114.

ABUOUN, M., MANNING, G., CAWTHRAW, S. A., RIDLEY, A., AHMED, I. H., WASSENAAR, T. M. & NEWELL, D. G. 2005. Cytolethal distending toxin (CDT)-negative Campylobacter jejuni strains and anti-CDT neutralizing antibodies are induced during human infection but not during colonization in chickens. *Infection and immunity,* 73**,** 3053-3062.

ACHESON, D. & ALLOS, B. M. 2001. Campylobacter jejuni infections: update on emerging issues and trends. *Clinical infectious diseases,* 32**,** 1201-1206.

ADAMS, M. R., MOSS, M. O. & MOSS, M. O. 2000. *Food microbiology*, Royal society of chemistry.

ADMASIE, A., ESHETU, A., TESSEMA, T. S., VIPHAM, J., KOVAC, J. & ZEWDU, A. 2023. Prevalence of Campylobacter species and associated risk factors for contamination of dairy products collected in a dry season from major milk sheds in Ethiopia. *Food Microbiology,* 109**,** 104145.

ADMASIE, A., TESSEMA, T. S., VIPHAM, J., KOVAC, J. & ZEWDU, A. 2024. Seasonal variation in the prevalence and antimicrobial resistance of Campylobacter species in milk and milk products in Ethiopia. *International Dairy Journal,* 149**,** 105826.

AERTS, R., VAN OVERTVELD, K., NOVEMBER, E., WASSIE, A., ABIYU, A., DEMISSEW, S., DAYE, D. D., GIDAY, K., HAILE, M. & TEWOLDEBERHAN, S. 2016. Conservation of the Ethiopian church forests: threats, opportunities and implications for their management. *Science of the Total Environment,* 551**,** 404-414.

AHMED, M. U., DUNN, L. & IVANOVA, E. P. 2012. Evaluation of current molecular approaches for genotyping of Campylobacter jejuni strains. *Foodborne Pathogens and Disease,* 9**,** 375-385.

AJENE, A. N., WALKER, C. L. F. & BLACK, R. E. 2013. Enteric pathogens and reactive arthritis: a systematic review of Campylobacter, Salmonella and Shigella-associated reactive arthritis. *Journal of health, population, and nutrition,* 31**,** 299.

AKBAR, M. O., ALI, M. J., HUSSAIN, A., QAISER, G., PASHA, M., PASHA, U., MISSEN, M. S. & AKHTAR, N. 2020. IoT for Development of Smart Dairy Farming. *Journal of Food Quality,* 2020.

AKSOMAITIENE, J., NOVOSLAVSKIJ, A., KUDIRKIENE, E., GABINAITIENE, A. & MALAKAUSKAS, M. 2020. Whole genome sequence-based prediction of resistance determinants in high-level multidrug-resistant Campylobacter jejuni isolates in Lithuania. *Microorganisms,* 9**,** 66.

AKSOMAITIENE, J., RAMONAITE, S., OLSEN, J. E. & MALAKAUSKAS, M. 2018. Prevalence of genetic determinants and phenotypic resistance to ciprofloxacin in Campylobacter jejuni from Lithuania. *Frontiers in microbiology,* 9**,** 203.

AL HAKEEM, W. G., FATHIMA, S., SHANMUGASUNDARAM, R. & SELVARAJ, R. K. 2022. Campylobacter jejuni in Poultry: Pathogenesis and Control Strategies. *Microorganisms,* 10**,** 2134.

ALFREDSON, D. A. & KOROLIK, V. 2005. Isolation and expression of a novel molecular class D β-lactamase, OXA-61, from Campylobacter jejuni. *Antimicrobial agents and chemotherapy,* 49**,** 2515-2518.

ALLOS, B. M., CALDERWOOD, S. & BARON, E. 2013. Clinical manifestations, diagnosis, and treatment of Campylobacter infection. *UpToDate, Waltham, MA.*

ALMASHHADANY, D. A. 2021. Isolation, biotyping and antimicrobial susceptibility of Campylobacter isolates from raw milk in Erbil city, Iraq. *Italian Journal of Food Safety,* 10.

ALNIMR, A. M. 2014. A case of bacteremia caused by Campylobacter fetus: an unusual presentation in an infant. *Infection and Drug Resistance,* 7**,** 37.

AMATO, S., MARAGNO, M., MOSELE, P., SFORZI, M., MIONI, R., BARCO, L., DALLA POZZA, M., ANTONELLO, K. & RICCI, A. An outbreak of Campylobacter jejuni linked to the consumption of raw milk in Italy. Zoonoses and Public Health, 2007. Blackwell Publishing 9600 GARSINGTON RD, OXFORD OX4 2DQ, OXON, ENGLAND, 23-23.

AMBACHEW, S., ASSEFA, M., TEGEGNE, Y. & ZELEKE, A. J. 2020. The Prevalence of Intestinal Parasites and Their Associated Factors among Diabetes Mellitus Patients at the University of Gondar Referral Hospital, Northwest Ethiopia. *Journal of Parasitology Research,* 2020.

AMIN, S., MAHMOOD, H. & ZORAB, H. 2023. Campylobacteriosis. *One Health Triad, Unique Scientific Publishers, Faisalabad, Pakistan,* 2**,** 87-93.

AMMAR, A. M., ABD EL-HAMID, M. I., EL-MALT, R. M., AZAB, D. S., ALBOGAMI, S., AL-SANEA, M. M., SOLIMAN, W. E., GHONEIM, M. M. & BENDARY, M. M. 2021. Molecular detection of fluoroquinolone resistance among multidrug-, extensively drug-, and pan-drug-resistant Campylobacter species in Egypt. *Antibiotics,* 10**,** 1342.

AN, J.-U., HO, H., KIM, J., KIM, W.-H., KIM, J., LEE, S., MUN, S.-H., GUK, J.-H., HONG, S. & CHO, S. 2018. Dairy cattle, a potential reservoir of human campylobacteriosis: epidemiological and molecular characterization of Campylobacter jejuni from cattle farms. *Frontiers in microbiology,* 9**,** 3136.

ANDREW, R., CHUSI, T. & MWEMBEZI, G. 2021. Milking Hygiene and Handling Practices among Smallholder Dairy Farmers in Zanzibar.

ANDREWS, S. 2010. FastQC: a quality control tool for high throughput sequence data. Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom.

ANDRZEJEWSKA, M., SZCZEPAŃSKA, B., ŚPICA, D. & KLAWE, J. J. 2019. Prevalence, virulence, and antimicrobial resistance of Campylobacter spp. in raw milk, beef, and pork meat in Northern Poland. *Foods,* 8**,** 420.

ARTURSSON, K., SCHELIN, J., LAMBERTZ, S. T., HANSSON, I. & ENGVALL, E. O. 2018. Foodborne pathogens in unpasteurized milk in Sweden. *International Journal of Food Microbiology,* 284**,** 120-127.

ASAKURA, H., SAKATA, J., NAKAMURA, H., YAMAMOTO, S. & MURAKAMI, S. 2019. Phylogenetic diversity and antimicrobial resistance of Campylobacter coli from humans and animals in Japan. *Microbes and environments,* 34**,** 146-154.

ASRAT, D., HATHAWAY, A. & EKWALL, E. 1999. Studies on enteric campylobacteriosis in Tikur Anbessa and Ethio-Swedish children's hospital, Addis Ababa, Ethiopia. *Ethiopian medical journal,* 37**,** 71.

ASUMING-BEDIAKO, N., PARRY-HANSON KUNADU, A., ABRAHAM, S. & HABIB, I. 2019. Campylobacter at the human–food interface: the african perspective. *Pathogens,* 8**,** 87.

AUDU, B. J., NORVAL, S., BRUNO, L., MEENAKSHI, R., MARION, M. & FORBES, K. J. 2022. Genomic diversity and antimicrobial resistance of Campylobacter spp. from humans and livestock in Nigeria. *Journal of Biomedical Science,* 29**,** 7.

AUTHORITY, E. F. S. 2017. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSa Journal,* 15.

AUTHORITY, E. F. S., PREVENTION, E. C. F. D. & CONTROL 2021. The European Union one health 2019 zoonoses report. *EFSA Journal,* 19**,** e06406.

BAALI, M., LOUNIS, M., AL AMIR, H. L., AYACHI, A., HAKEM, A. & KASSAH-LAOUAR, A. 2020. Prevalence, seasonality, and antimicrobial resistance of thermotolerant Campylobacter isolated from broiler farms and slaughterhouses in East Algeria. *Veterinary World,* 13**,** 1221.

BACKERT, S., BOEHM, M., WESSLER, S. & TEGTMEYER, N. 2013. Transmigration route of Campylobacter jejuni across polarized intestinal epithelial cells: paracellular, transcellular or both? *Cell Communication and Signaling,* 11**,** 1-15.

BALABAN, M. & HENDRIXSON, D. R. 2011. Polar flagellar biosynthesis and a regulator of flagellar number influence spatial parameters of cell division in Campylobacter jejuni. *PLoS pathogens,* 7**,** e1002420.

BANG, D. D., NIELSEN, E. M., SCHEUTZ, F., PEDERSEN, K., HANDBERG, K. & MADSEN, M. 2003. PCR detection of seven virulence and toxin genes of Campylobacter jejuni and Campylobacter coli isolates from Danish pigs and cattle and cytolethal distending toxin production of the isolates. *Journal of applied microbiology,* 94**,** 1003-1014.

BANG, D. D., SCHEUTZ, F., AHRENS, P., PEDERSEN, K., BLOM, J. & MADSEN, M. 2001. Prevalence of cytolethal distending toxin (cdt) genes and CDT production in Campylobacter spp. isolated from Danish broilers. *Journal of medical microbiology,* 50**,** 1087-1094.

BARAKAT, A. M., ABD EL-RAZIK, K. A., ELFADALY, H. A., RABIE, N. S., SADEK, S. A. & ALMUZAINI, A. M. 2020. Prevalence, molecular detection, and virulence gene profiles of Campylobacter species in humans and foods of animal origin. *Veterinary World,* 13**,** 1430.

BEHAILU, Y., HUSSEN, S., ALEMAYEHU, T., MENGISTU, M. & FENTA, D. A. 2022. Prevalence, determinants, and antimicrobial susceptibility patterns of Campylobacter infection among under-five children with diarrhea at Governmental Hospitals in Hawassa city, Sidama, Ethiopia. A cross-sectional study. *Plos one,* 17**,** e0266976.

BELANGER, A. E. & SHRYOCK, T. R. 2007. Macrolide-resistant Campylobacter: the meat of the matter. *Journal of Antimicrobial Chemotherapy,* 60**,** 715-723.

BENITES, C., ANAMPA, D., TORRES, D., AVALOS, I., ROJAS, M., CONTE, C. & LÁZARO, C. 2022. Prevalence, Tetracycline Resistance and Tet (O) Gene Identification in Pathogenic Campylobacter Strains Isolated from Chickens in Retail Markets of Lima, Peru. *Antibiotics,* 11**,** 1580.

BEREDA, A., YILMA, Z. & NURFETA, A. 2012. Hygienic and microbial quality of raw whole cow's milk produced in Ezha district of the Gurage zone, Southern Ethiopia. *Wudpecker Journal of Agricultural Research,* 1**,** 459-465.

BERGERON, C. R., PRUSSING, C., BOERLIN, P., DAIGNAULT, D., DUTIL, L., REID-SMITH, R. J., ZHANEL, G. G. & MANGES, A. R. 2012. Chicken as reservoir for extraintestinal pathogenic Escherichia coli in humans, Canada. *Emerg Infect Dis,* 18**,** 415-21.

BERHE, G., WASIHUN, A. G., KASSAYE, E. & GEBRESELASIE, K. 2020. Milk-borne bacterial health hazards in milk produced for commercial purpose in Tigray, northern Ethiopia. *BMC Public Health,* 20**,** 1-8.

BERTASI, B., LOSIO, M. N., DAMINELLI, P., FINAZZI, G., SERRAINO, A., PIVA, S., GIACOMETTI, F., MASSELLA, E. & OSTANELLO, F. 2016. Seasonal variability of thermophilic Campylobacter spp. in raw milk sold by automatic vending machines in Lombardy Region. *Italian Journal of Food Safety,* 5.

BEYENE, G. & HAILE-AMLAK, A. 2004. Antimicrobial sensitivity pattern of Campylobacter species among children in Jimma University Specialized Hospital, southwest Ethiopia. *Ethiopian Journal of Health Development,* 18**,** 185-189.

BIANCHINI, V., BORELLA, L., BENEDETTI, V., PARISI, A., MICCOLUPO, A., SANTORO, E., RECORDATI, C. & LUINI, M. 2014. Prevalence in bulk tank milk and epidemiology of Campylobacter jejuni in dairy herds in Northern Italy. *Applied and environmental microbiology,* 80**,** 1832-1837.

BIGGS, P. J., FEARNHEAD, P., HOTTER, G., MOHAN, V., COLLINS-EMERSON, J., KWAN, E., BESSER, T. E., COOKSON, A., CARTER, P. E. & FRENCH, N. P. 2011. Whole-genome comparison of two Campylobacter jejuni isolates of the same sequence type reveals multiple loci of different ancestral lineage. *PloS one,* 6.

BIRHANU, M., LETA, S., MAMO, G. & TESFAYE, S. 2017. Prevalence of bovine subclinical mastitis and isolation of its major causes in Bishoftu Town, Ethiopia. *BMC research notes,* 10**,** 1-6.

BISWAS, D., ITOH, K. & SASAKAWA, C. 2003. Role of microfilaments and microtubules in the invasion of INT-407 cells by Campylobacter jejuni. *Microbiology and immunology,* 47**,** 469-473.

BLASER, M. J. 1997. Epidemiologic and clinical features of Campylobacter jejuni infections. *Journal of Infectious Diseases,* 176**,** S103-S105.

BOLGER, A. M., LOHSE, M. & USADEL, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics,* 30**,** 2114-2120.

BOLINGER, H. & KATHARIOU, S. 2017. The current state of macrolide resistance in Campylobacter spp.: trends and impacts of resistance mechanisms. *Applied and Environmental Microbiology,* 83**,** e00416-17.

BOOR, K. J., WIEDMANN, M., MURPHY, S. & ALCAINE, S. 2017. A 100-year review: microbiology and safety of milk handling. *Journal of Dairy Science,* 100**,** 9933-9951.

BORRIELLO, S. P., MURRAY, P. R. & FUNKE, G. 2005. *Topley & Wilson's Microbiology & Microbial Infections: Bacteriology*, Wiley Online Library.

BOUTEILLER, M., DUPONT, C., BOURIGAULT, Y., LATOUR, X., BARBEY, C., KONTO-GHIORGHI, Y. & MERIEAU, A. 2021. Pseudomonas flagella: generalities and specificities. *International Journal of Molecular Sciences,* 22**,** 3337.

BOYSEN, L., ROSENQUIST, H., LARSSON, J., NIELSEN, E., SØRENSEN, G., NORDENTOFT, S. & HALD, T. 2014. Source attribution of human campylobacteriosis in Denmark. *Epidemiology & Infection,* 142**,** 1599-1608.

BRAVO, V., KATZ, A., PORTE, L., WEITZEL, T., VARELA, C., GONZALEZ-ESCALONA, N. & BLONDEL, C. J. 2021. Genomic analysis of the diversity, antimicrobial resistance and virulence potential of clinical Campylobacter jejuni and Campylobacter coli strains from Chile. *PLoS Neglected Tropical Diseases,* 15**,** e0009207.

BRUDVIG, J. M., CLUETT, M. M., GENSTERBLUM-MILLER, E. U., CHEN, J., BELL, J. A. & MANSFIELD, L. S. 2022. Th1/Th17-mediated Immunity and Protection from Peripheral Neuropathy in Wildtype and IL10–/–BALB/c Mice Infected with a Guillain–Barré Syndrome-associated Campylobacter jejuni Strain. *Comparative Medicine,* 72**,** 63-77.

BUDGE, S., BARNETT, M., HUTCHINGS, P., PARKER, A., TYRREL, S., HASSARD, F., GARBUTT, C., MOGES, M., WOLDEMEDHIN, F. & JEMAL, M. 2020. Risk factors and transmission pathways associated with infant Campylobacter spp. prevalence and malnutrition: A formative study in rural Ethiopia. *PloS one,* 15**,** e0232541.

BUKAYAW, W., MELESE, ADDISU & MEKONNEN, D. 2021. Campylobacter Jejuni and Its Antimicrobial Susceptibility Pattern Among Under-Five Children with Gastroenteritis in Northwest Ethiopia.

BURNHAM, P. M. & HENDRIXSON, D. R. 2018. Campylobacter jejuni: collective components promoting a successful enteric lifestyle. *Nature Reviews Microbiology,* 16**,** 551-565.

CALAHORRANO-MORENO, M. B., ORDOÑEZ-BAILON, J. J., BAQUERIZO-CRESPO, R. J., DUEÑAS-RIVADENEIRA, A. A., MONTENEGRO, M. C. B. & RODRÍGUEZ-DÍAZ, J. M. 2022. Contaminants in the cow's milk we consume? Pasteurization and other technologies in the elimination of contaminants. *F1000Research,* 11.

CHA, G., CHEN, Z., MO, R., LU, G. & GAO, B. 2019. The novel regulators CheP and CheQ control the core chemotaxis operon cheVAW in Campylobacter jejuni. *Molecular microbiology,* 111**,** 145-158.

CHALA, G., EGUALE, T., ABUNNA, F., ASRAT, D. & STRINGER, A. 2021. Identification and characterization of Campylobacter species in livestock, humans, and water in livestock owning households of peri-urban addis ababa, Ethiopia: a one health approach. *Frontiers in Public Health,* 9**,** 750551.

CHANDRASHEKHAR, K., SRIVASTAVA, V., HWANG, S., JEON, B., RYU, S. & RAJASHEKARA, G. 2018. Transducer-like protein in Campylobacter jejuni with a role in mediating chemotaxis to iron and phosphate. *Frontiers in microbiology,* 9**,** 2674.

CHANYALEW, Y., ASRAT, D., AMAVISIT, P. & LOONGYAI, W. 2013a. Prevalence and antimicrobial susceptibility of thermophilic Campylobacter isolated from sheep at Debre Birhan, North-Shoa, Ethiopia. *Agriculture and Natural Resources,* 47**,** 551-560.

CHANYALEW, Y., ASRAT, D., AMAVISIT, P. & LOONGYAI, W. 2013b. Prevalence and antimicrobial susceptibility of thermophilic Campylobacter isolated from sheep at Debre Birhan, North-Shoa, Ethiopia. *Kasetsart J (Nat Sci),* 47**,** 551-560.

CHATTERJEE, S., BHATTACHARJEE, I., CHATTERJEE, S. & CHANDRA, G. 2006. 2006. Microbiological examination of milk in Tarakeswar, India with special reference to coliforms. African Journal of Biotechnology, 5.

CHAUMEIL, P. A., MUSSIG, A. J., HUGENHOLTZ, P. & PARKS, D. H. 2019. GTDB-Tk: a toolkit to classify genomes with the Genome Taxonomy Database. *Bioinformatics,* 36**,** 1925-7.

CHENG, Y., ZHANG, W., LU, Q., WEN, G., ZHAO, Z., LUO, Q., SHAO, H. & ZHANG, T. 2020. Point deletion or insertion in cmeR-box, A2075G substitution in 23s rRNA, and presence of erm (B) are key factors of erythromycin resistance in Campylobacter jejuni and Campylobacter coli isolated from central China. *Frontiers in Microbiology,* 11**,** 203.

CHIBWE, M., ODUME, O. N. & NNADOZIE, C. F. 2023. A review of antibiotic resistance among Campylobacter species in human, animal, and water sources in South Africa: a One Health Approach. *Journal of Water and Health,* 21**,** 9-26.

CLARK, C. G., CHEN, C.-Y., BERRY, C., WALKER, M., MCCORRISTER, S. J., CHONG, P. M. & WESTMACOTT, G. R. 2018. Comparison of genomes and proteomes of four whole genome-sequenced Campylobacter jejuni from different phylogenetic backgrounds. *PLoS One,* 13**,** e0190836.

CLSI-M45-ED3 2016. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. 3rd Edition.

CLSI 2023. Campylobacter Agar – CAMPY.https://anaerobesystems.com/products/plated-media/campylobacter-agar-campy/ Accessed: 25 Auguest 2023.

CODY, A. J., BRAY, J. E., JOLLEY, K. A., MCCARTHY, N. D. & MAIDEN, M. C. 2017. Core genome multilocus sequence typing scheme for stable, comparative analyses of Campylobacter jejuni and C. coli human disease isolates. *Journal of clinical microbiology,* 55**,** 2086-2097.

CODY, A. J., MAIDEN, M. C., STRACHAN, N. J. & MCCARTHY, N. D. 2019. A systematic review of source attribution of human campylobacteriosis using multilocus sequence typing. *Eurosurveillance,* 24**,** 1800696.

COHEN, E. J. & HUGHES, K. T. 2014. Rod-to-hook transition for extracellular flagellum assembly is catalyzed by the L-ring-dependent rod scaffold removal. *Journal of bacteriology,* 196**,** 2387-2395.

COHEN, E. J., NAKANE, D., KABATA, Y., HENDRIXSON, D. R., NISHIZAKA, T. & BEEBY, M. 2020. Campylobacter jejuni motility integrates specialized cell shape, flagellar filament, and motor, to coordinate action of its opposed flagella. *PLoS pathogens,* 16**,** e1008620.

COKER, A. O., ISOKPEHI, R. D., THOMAS, B. N., FAGBENRO-BEYIOKU, A. F. & OMILABU, S. A. 2000. Zoonotic infections in Nigeria: overview from a medical perspective. *Acta Trop,* 76**,** 59-63.

COLLIER, S. A., DENG, L., ADAM, E. A., BENEDICT, K. M., BESHEARSE, E. M., BLACKSTOCK, A. J., BRUCE, B. B., DERADO, G., EDENS, C. & FULLERTON, K. E. 2021. Estimate of burden and direct healthcare cost of infectious waterborne disease in the United States. *Emerging infectious diseases,* 27**,** 140.

COSTARD, S., ESPEJO, L., GROENENDAAL, H. & ZAGMUTT, F. J. 2017. Outbreak-related disease burden associated with consumption of unpasteurized cow's milk and cheese, United States, 2009–2014. *Emerging infectious diseases,* 23**,** 957.

COUSTILLÈRES, F., HANOY, M., LEMÉE, L., LE ROY, F. & BERTRAND, D. 2022. Campylobacter fetus bacteremia complicated by multiple splenic abscesses and multivisceral signs in a renal transplant recipient: a case report and review of the literature. *Brazilian Journal of Infectious Diseases,* 26.

CSA 2011. Agricultural Sample Survey, 2010/11 (2003 EC), Volume II: Report on Livestock and livestock characteristics (Private peasant holdings). Statistical Bulletin 505. Central Statistical Agency (CSA) Federal Democratic Republic of Ethiopia ….

DADI, L. & ASRAT, D. 2008. Prevalence and antimicrobial susceptibility profiles of thermotolerant Campylobacter strains in retail raw meat products in Ethiopia. *Ethiopian journal of health development,* 22**,** 195-200.

DAI, L., SAHIN, O., GROVER, M. & ZHANG, Q. 2020. New and alternative strategies for the prevention, control, and treatment of antibiotic-resistant Campylobacter. *Translational Research,* 223**,** 76-88.

DAVIS, K. R., DUNN, A. C., BURNETT, C., MCCULLOUGH, L., DIMOND, M., WAGNER, J., SMITH, L., CARTER, A., WILLARDSON, S. & NAKASHIMA, A. K. 2016. Campylobacter jejuni infections associated with raw milk consumption—Utah, 2014. *Morbidity and Mortality Weekly Report,* 65**,** 301-305.

DAVYS, G., MARSHALL, J., FAYAZ, A., WEIR, R. & BENSCHOP, J. 2020a. Campylobacteriosis associated with the consumption of unpasteurised milk: findings from a sentinel surveillance site. *Epidemiology & Infection,* 148.

DAVYS, G., MARSHALL, J., FAYAZ, A., WEIR, R. & BENSCHOP, J. 2020b. Campylobacteriosis associated with the consumption of unpasteurised milk: findings from a sentinel surveillance site. *Epidemiology & Infection,* 148**,** e16.

DE FILIPPIS, F., CASTELLANO, S. & CAPUANO, F. Isolation and characterization of Campylobacter spp. in meat products.

DE OLIVEIRA, M. G., RIZZI, C., GALLI, V., LOPES, G. V., HAUBERT, L., DELLAGOSTIN, O. A. & DA SILVA, W. P. 2019. Presence of genes associated with adhesion, invasion, and toxin production in Campylobacter jejuni isolates and effect of temperature on their expression. *Canadian journal of microbiology,* 65**,** 253-260.

DE VRIES, S. P., VURAYAI, M., HOLMES, M., GUPTA, S., BATEMAN, M., GOLDFARB, D., MASKELL, D. J., MATSHEKA, M. I. & GRANT, A. J. 2018. Phylogenetic analyses and antimicrobial resistance profiles of Campylobacter spp. from diarrhoeal patients and chickens in Botswana. *PLoS One,* 13**,** e0194481.

DEBELO, M., MOHAMMED, N., TIRUNEH, A. & TOLOSA, T. 2022. Isolation, identification and antibiotic resistance profile of thermophilic Campylobacter species from Bovine, Knives and personnel at Jimma Town Abattoir, Ethiopia. *Plos one,* 17**,** e0276625.

DEC, M., NOWACZEK, A., URBAN-CHMIEL, R., STEPIEN-PYSNIAK, D. & WERNICKI, A. 2018. Probiotic potential of Lactobacillus isolates of chicken origin with anti-Campylobacter activity. *J Vet Med Sci,* 80**,** 1195-1203.

DEL COLLO, L. P., KARNS, J. S., BISWAS, D., LOMBARD, J. E., HALEY, B. J., KRISTENSEN, R. C., KOPRAL, C. A., FOSSLER, C. P. & VAN KESSEL, J. A. S. 2017. Prevalence, antimicrobial resistance, and molecular characterization of Campylobacter spp. in bulk tank milk and milk filters from US dairies. *Journal of dairy science,* 100**,** 3470-3479.

DENEKE, T. T., BEKELE, A., MOORE, H. L., MAMO, T., ALMAW, G., MEKONNEN, G. A., MIHRET, A., TSCHOPP, R., YEHEYIS, L. & HODGE, C. 2021. Milk and Meat Consumption Patterns and the Potential Risk of Zoonotic Disease Transmission among Urban and Peri-urban Dairy Farmers in Ethiopia.

DENEKE, T. T., BEKELE, A., MOORE, H. L., MAMO, T., ALMAW, G., MEKONNEN, G. A., MIHRET, A., TSCHOPP, R., YEHEYIS, L. & HODGE, C. 2022. Milk and meat consumption patterns and the potential risk of zoonotic disease transmission among urban and peri-urban dairy farmers in Ethiopia. *BMC Public Health,* 22**,** 222.

DESTA, T. T. & WAKEYO, O. 2012. Uses and flock management practices of scavenging chickens in Wolaita Zone of southern Ethiopia. *Tropical animal health and production,* 44**,** 537-544.

DI GIANNATALE, E., CALISTRI, P., DI DONATO, G., DECASTELLI, L., GOFFREDO, E., ADRIANO, D., MANCINI, M. E., GALLEGGIANTE, A., NERI, D. & ANTOCI, S. 2019. Thermotolerant Campylobacter spp. in chicken and bovine meat in Italy: Prevalence, level of contamination and molecular characterization of isolates. *PLoS One,* 14**,** e0225957.

DIKE, V. N., LIN, Z.-H. & IBE, C. C. 2020. Intensification of summer rainfall extremes over Nigeria during recent decades. *Atmosphere,* 11**,** 1084.

DIRIBA, K., AWULACHEW, E. & ANJA, A. 2021. Prevalence and associated factor of Campylobacter species among less than 5-year-old children in Ethiopia: a systematic review and meta-analysis. *European Journal of Medical Research,* 26**,** 1-10.

DJENNAD, A., LO IACONO, G., SARRAN, C., LANE, C., ELSON, R., HÖSER, C., LAKE, I. R., COLÓN-GONZÁLEZ, F. J., KOVATS, S. & SEMENZA, J. C. 2019. Seasonality and the effects of weather on Campylobacter infections. *BMC infectious diseases,* 19**,** 1-10.

DJORDJEVIC, S. P., UNICOMB, L. E., ADAMSON, P. J., MICKAN, L., RIOS, R. & GROUP‡, A. C. S. S. 2007. Clonal complexes of Campylobacter jejuni identified by multilocus sequence typing are reliably predicted by restriction fragment length polymorphism analyses of the flaA gene. *Journal of Clinical Microbiology,* 45**,** 102-108.

DJURETIC, T., WALL, P. & NICHOLS, G. 1997. General outbreaks of infectious intestinal disease associated with milk and dairy products in England and Wales: 1992 to 1996. *Communicable disease report. CDR Review,* 7**,** R41-5.

DOYLE, M. & ROMAN, D. 1981. Growth and survival of Campylobacter fetus subsp. jejuni as a function of temperature and pH. *Journal of Food Protection,* 44**,** 596-601.

DUARTE, A., SANTOS, A., MANAGEIRO, V., MARTINS, A., FRAQUEZA, M. J., CANIÇA, M., DOMINGUES, F. C. & OLEASTRO, M. 2014. Human, food and animal Campylobacter spp. isolated in Portugal: high genetic diversity and antibiotic resistance rates. *International Journal of Antimicrobial Agents,* 44**,** 306-313.

ECDC, E. C. F. D. P. A. C. 2022. Annual epidemiological report for 2021. Accessed on 7/7/2023.

EFIMOCHKINA, N. 2015. Evaluation of the role of Campylobacter spp. in the occurrence of foodborne diseases and modern methods to detect the pathogen. *Voprosy Pitaniia,* 84**,** 5-18.

EL-KHOLY, A., MESHREF, A., EL-GEDAWY, A. & ESAM, R. 2016. Prevalence of Campylobacter species in milk and some dairy products. *Journal of Veterinary Medical Research,* 23**,** 133-142.

EL-NAENAEEY, E.-S., EL-HAMID, A. & KHALIFA, E. 2020. Foodborne Campylobacter species: Taxonomy, isolation, virulence attributes and antimicrobial resistance. *Zagazig Veterinary Journal,* 48**,** 414-432.

EL-ZAMKAN, M. A. & HAMEED, K. G. 2016a. Prevalence of Campylobacter jejuni and Campylobacter coli in raw milk and some dairy products. *Vet World,* 9**,** 1147-1151.

EL-ZAMKAN, M. A. & HAMEED, K. G. A. 2016b. Prevalence of Campylobacter jejuni and Campylobacter coli in raw milk and some dairy products. *Veterinary world,* 9**,** 1147.

ELMALı, M. & CAN, H. Y. 2019. Antimicrobial susceptibility and virulence-associated genes in Campylobacter isolates from milk and wastewater in Hatay, Turkey. *Ciência Rural,* 49.

ENEROTH, Å., CHRISTIANSSON, A., BRENDEHAUG, J. & MOLIN, G. 1998. Critical contamination sites in the production line of pasteurised milk, with reference to the psychrotrophic spoilage flora. *International Dairy Journal,* 8**,** 829-834.

ENGLEN, M., HILL, A., DARGATZ, D., LADELY, S. & FEDORKA-CRAY, P. 2007. Prevalence and antimicrobial resistance of Campylobacter in US dairy cattle. *Journal of applied microbiology,* 102**,** 1570-1577.

ERYıLDıZ, C., SAKRU, N. & KUYUCUKLU, G. 2022. Investigation of antimicrobial susceptibilities and resistance genes of campylobacter isolates from patients in Edirne, Turkey. *Iranian Journal of Public Health,* 51**,** 569.

ESPINOZA, N., ROJAS, J., POLLETT, S., MEZA, R., PATIÑO, L., LEIVA, M., CAMIÑA, M., BERNAL, M., REYNOLDS, N. D. & MAVES, R. 2020. Validation of the T86I mutation in the gyrA gene as a highly reliable real time PCR target to detect Fluoroquinolone-resistant Campylobacter jejuni. *BMC Infectious Diseases,* 20**,** 1-7.

EUCKER, T. P. & KONKEL, M. E. 2012. The cooperative action of bacterial fibronectin-binding proteins and secreted proteins promote maximal Campylobacter jejuni invasion of host cells by stimulating membrane ruffling. *Cellular microbiology,* 14**,** 226-238.

EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL , E. 2022. Campylobacteriosis. In: ECDC. Annual epidemiological

report for 2021. *Foodborne diseases.* Elsevier.

EWNETU, D. & MIHRET, A. 2010. Prevalence and antimicrobial resistance of Campylobacter isolates from humans and chickens in Bahir Dar, Ethiopia. *Foodborne pathogens and disease,* 7**,** 667-670.

FABIANI, F. D., RENAULT, T. T., PETERS, B., DIETSCHE, T., GÁLVEZ, E. J., GUSE, A., FREIER, K., CHARPENTIER, E., STROWIG, T. & FRANZ-WACHTEL, M. 2017. A flagellum-specific chaperone facilitates assembly of the core type III export apparatus of the bacterial flagellum. *PLoS biology,* 15**,** e2002267.

FACCIOLÀ, A., RISO, R., AVVENTUROSO, E., VISALLI, G., DELIA, S. & LAGANÀ, P. 2017. Campylobacter: from microbiology to prevention. *Journal of preventive medicine and hygiene,* 58**,** E79.

FAHEY, T., MORGAN, D., GUNNEBURG, C., ADAK, G., MAJID, F. & KACZMARSKI, E. 1995. An outbreak of Campylobacter jejuni enteritis associated with failed milk pasteurisation. *Journal of Infection,* 31**,** 137-143.

FARIS, G. 2015. Identification of Campylobacter species and their antibiotic resistance patterns from raw bovine meat in Addis Ababa, Ethiopia. *International Journal of Multimedia Information Retrieval,* 4**,** 1-5.

FAZZINI, M., BISCI, C. & BILLI, P. 2015. The climate of Ethiopia. *Landscapes and landforms of Ethiopia.* Springer.

FELDGARDEN, M., BROVER, V., HAFT, D. H., PRASAD, A. B., SLOTTA, D. J., TOLSTOY, I., TYSON, G. H., ZHAO, S., HSU, C.-H. & MCDERMOTT, P. F. 2019. Using the NCBI

AMRFinder tool to determine antimicrobial resistance genotype-phenotype correlations within a collection of NARMS isolates. *BioRxiv***,** 550707.

FERNANDES, A. M., BALASEGARAM, S., WILLIS, C., WIMALARATHNA, H. M., MAIDEN, M. C. & MCCARTHY, N. D. 2015. Partial failure of milk pasteurization as a risk for the transmission of Campylobacter from cattle to humans. *Clinical Infectious Diseases,* 61**,** 903-909.

FIEDORUK, K., DANILUK, T., ROZKIEWICZ, D., OLDAK, E., PRASAD, S. & SWIECICKA, I. 2019. Whole-genome comparative analysis of Campylobacter jejuni strains isolated from patients with diarrhea in northeastern Poland. *Gut Pathogens,* 11**,** 1-10.

FISCHER, G. H. & PATEREK, E. 2019. Campylobacter.

FITZGERALD, C. 2015. Campylobacter. *Clin Lab Med,* 35**,** 289-98.

FLETCHER, S. M., STARK, D. & ELLIS, J. 2011. Prevalence of gastrointestinal pathogens in Sub-Saharan Africa: systematic review and meta-analysis. *Journal of public health in Africa,* 2.

FLINT, A., SUN, Y.-Q., BUTCHER, J., STAHL, M., HUANG, H. & STINTZI, A. 2014. Phenotypic screening of a targeted mutant library reveals Campylobacter jejuni defenses against oxidative stress. *Infection and immunity,* 82**,** 2266-2275.

FORD, L., HEALY, J. M., CUI, Z., AHART, L., MEDALLA, F., RAY, L. C., REYNOLDS, J., LAUGHLIN, M. E., VUGIA, D. J. & HANNA, S. Epidemiology and Antimicrobial Resistance of Campylobacter Infections in the United States, 2005–2018. Open Forum Infectious Diseases, 2023. Oxford University Press US, ofad378.

FRASAO, B. D. S., MEDEIROS, V., BARBOSA, A. V., DE AGUIAR, W. S., DOS SANTOS, F. F., ABREU, D. L. D. C., CLEMENTINO, M. M. & DE AQUINO, M. H. C. 2015. Detection of fluoroquinolone resistance by mutation in gyrA gene of Campylobacter spp. isolates from broiler and laying (Gallus gallus domesticus) hens, from Rio de Janeiro State, Brazil. *Ciência Rural,* 45**,** 2013-2018.

GABBERT, A. D., MYDOSH, J. L., TALUKDAR, P. K., GLOSS, L. M., MCDERMOTT, J. E., COOPER, K. K., CLAIR, G. C. & KONKEL, M. E. 2023. The Missing Pieces: The Role of Secretion Systems in Campylobacter jejuni Virulence. *Biomolecules,* 13**,** 135.

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,* 2020.

GALBRAITH, N., FORBES, P. & CLIFFORD, C. 1982. Communicable disease associated with milk and dairy products in England and Wales 1951-80. *Br Med J (Clin Res Ed),* 284**,** 1761-1765.

GANGIREDLA, J., RAND, H., BENISATTO, D., PAYNE, J., STRITTMATTER, C., SANDERS, J., WOLFGANG, W. J., LIBUIT, K., HERRICK, J. B. & PRARAT, M. 2021. GalaxyTrakr: a distributed analysis tool for public health whole genome sequence data accessible to non-bioinformaticians. *BMC genomics,* 22**,** 1-11.

GAO, F., TU, L., CHEN, M., CHEN, H., ZHANG, X., ZHUANG, Y., LUO, J. & CHEN, M. 2023. Erythromycin resistance of clinical Campylobacter jejuni and Campylobacter coli in Shanghai, China. *Frontiers in Microbiology,* 14**,** 1145581.

GARCIA-FERNANDEZ, A., DIONISI, A. M., ARENA, S., IGLESIAS-TORRENS, Y., CARATTOLI, A. & LUZZI, I. 2018. Human campylobacteriosis in Italy: emergence of multi-drug resistance to ciprofloxacin, tetracycline, and erythromycin. *Frontiers in microbiology*, 9**,** 1906.

GEDLU, E. & ASEFFA, A. 1996. Campylobacter enteritis among children in north-west Ethiopia: a 1-year prospective study. *Annals of tropical paediatrics,* 16**,** 207-212.

GEMEDA, B. A., AMENU, K., MAGNUSSON, U., DOHOO, I., HALLENBERG, G. S., ALEMAYEHU, G., DESTA, H. & WIELAND, B. 2020. Antimicrobial use in extensive smallholder livestock farming systems in Ethiopia: knowledge, attitudes, and practices of livestock keepers. *Frontiers in Veterinary Science,* 7**,** 55.

GETAMESAY, M., GETENET, B. & AHMED, Z. 2014. Prevalence of Shigella, Salmonella and Cmpylobacter species and their susceptibility patters among under five children with diarrhea in Hawassa Town, South Ethiopia. *Ethiopian Journal of Health Sciences,* 24**,** 101-108.

GHIELMETTI, G., SETH-SMITH, H. M., ROLOFF, T., CERNELA, N., BIGGEL, M., STEPHAN, R. & EGLI, A. 2023. Whole-genome-based characterization of Campylobacter jejuni from human patients with gastroenteritis collected over an 18 year period reveals increasing prevalence of antimicrobial resistance. *Microbial Genomics,* 9.

GIALLOUROU, N., MEDLOCK, G. L., BOLICK, D. T., MEDEIROS, P., LEDWABA, S. E., KOLLING, G. L., TUNG, K., GUERRY, P., SWANN, J. R. & GUERRANT, R. L. 2018. A novel mouse model of Campylobacter jejuni enteropathy and diarrhea. *PLoS Pathog,* 14.

GIAOURIS, E. 2022. Relevance and Importance of Biofilms in the Resistance and Spreading of Campylobacter spp. Within the Food Chain. *Advances in Microbiology, Infectious Diseases and Public Health: Volume 17.* Springer.

GIBREEL, A., KOS, V. N., KEELAN, M., TRIEBER, C. A., LEVESQUE, S., MICHAUD, S. & TAYLOR, D. E. 2005. Macrolide resistance in Campylobacter jejuni and Campylobacter coli: molecular mechanism and stability of the resistance phenotype. *Antimicrobial agents and chemotherapy,* 49**,** 2753-2759.

GIBREEL, A. & TAYLOR, D. E. 2006. Macrolide resistance in Campylobacter jejuni and Campylobacter coli. *Journal of Antimicrobial Chemotherapy,* 58**,** 243-255.

GILLESPIE, B., HEADRICK, S., BOONYAYATRA, S. & OLIVER, S. 2009. Prevalence and persistence of coagulase-negative Staphylococcus species in three dairy research herds. *Veterinary microbiology,* 134**,** 65-72.

GILLESPIE, I., ADAK, G., O'BRIEN, S. & BOLTON, F. 2003. Milkborne general outbreaks of infectious intestinal disease, England and Wales, 1992–2000. *Epidemiology & Infection,* 130**,** 461-468.

GILLESPIE, I. A., O'BRIEN, S. J., FROST, J. A., ADAK, G. K., HORBY, P., SWAN, A. V., PAINTER, M. J. & NEAL, K. R. 2002. A case-case comparison of Campylobacter coli and Campylobacter jejuni infection: a tool for generating hypotheses. *Emerg Infect Dis,* 8**,** 937-42.

GILLESPIE, I. A., O'BRIEN, S. J., PENMAN, C., TOMPKINS, D., COWDEN, J. & HUMPHREY, T. J. 2008. Demographic determinants for Campylobacter infection in England and Wales: implications for future epidemiological studies. *Epidemiol Infect,* 136**,** 1717-25.

GIZAW, S. 2010. *Sheep and goat production and marketing systems in Ethiopia: Characteristics and strategies for improvement*, ILRI (aka ILCA and ILRAD).

GÖLZ, G., KITTLER, S., MALAKAUSKAS, M. & ALTER, T. 2018. Survival of Campylobacter in the food chain and the environment. *Current Clinical Microbiology Reports,* 5**,** 126-134.

GOLZ, J. C., EPPING, L., KNÜVER, M.-T., BOROWIAK, M., HARTKOPF, F., DENEKE, C., MALORNY, B., SEMMLER, T. & STINGL, K. 2020. Whole genome sequencing reveals extended natural transformation in Campylobacter impacting diagnostics and the pathogens adaptive potential. *Scientific reports,* 10**,** 3686.

GRANT, C., KONKEL, M., CIEPLAK JR, W. & TOMPKINS, L. 1993. Role of flagella in adherence, internalization, and translocation of Campylobacter jejuni in nonpolarized and polarized epithelial cell cultures. *Infection and immunity,* 61**,** 1764-1771.

GRIGGS, D. J., JOHNSON, M. M., FROST, J. A., HUMPHREY, T., JØRGENSEN, F. & PIDDOCK, L. J. 2005. Incidence and mechanism of ciprofloxacin resistance in Campylobacter spp. isolated from commercial poultry flocks in the United Kingdom before, during, and after fluoroquinolone treatment. *Antimicrobial agents and chemotherapy,* 49**,** 699-707.

GRIGGS, D. J., PEAKE, L., JOHNSON, M. M., GHORI, S., MOTT, A. & PIDDOCK, L. J. 2009. β-Lactamase-mediated β-lactam resistance in Campylobacter species: prevalence of Cj0299 (bla OXA-61) and evidence for a novel β-lactamase in C. jejuni. *Antimicrobial agents and chemotherapy,* 53**,** 3357-3364.

GRIPP, E., HLAHLA, D., DIDELOT, X., KOPS, F., MAURISCHAT, S., TEDIN, K., ALTER, T., ELLERBROEK, L., SCHREIBER, K. & SCHOMBURG, D. 2011. Closely related Campylobacter jejuni strains from different sources reveal a generalist rather than a specialist lifestyle. *BMC genomics,* 12**,** 1-21.

GROUT, L., MARSHALL, J., HALES, S., BAKER, M. G. & FRENCH, N. 2022. Dairy cattle density and temporal patterns of human campylobacteriosis and cryptosporidiosis in New Zealand. *EcoHealth,* 19**,** 273-289.

GUERRY, P. 2007. Campylobacter flagella: not just for motility. *Trends in microbiology,* 15**,** 456-461.

GUÉVREMONT, E., LAMOUREUX, L., LOUBIER, C. B., VILLENEUVE, S. & DUBUC, J. 2014. Detection and characterization of Campylobacter spp. from 40 dairy cattle herds in Quebec, Canada. *Foodborne pathogens and disease,* 11**,** 388-394.

GUK, J.-H., SONG, H., YI, S., AN, J.-U., LEE, S., KIM, W.-H. & CHO, S. 2021. Hyper-Aerotolerant Campylobacter coli From Swine May Pose a Potential Threat to Public Health Based on Its Quinolone Resistance, Virulence Potential, and Genetic Relatedness. *Frontiers in microbiology,* 12**,** 703993.

GUREVICH, A., SAVELIEV, V., VYAHHI, N. & TESLER, G. 2013. QUAST: quality assessment tool for genome assemblies. *Bioinformatics,* 29**,** 1072-1075.

HABIB, I., MOHAMED, M.-Y. I., GHAZAWI, A., LAKSHMI, G. B., KHAN, M., LI, D. & SAHIBZADA, S. 2023. Genomic characterization of molecular markers associated with antimicrobial resistance and virulence of the prevalent Campylobacter coli isolated from retail chicken meat in the United Arab Emirates. *Current Research in Food Science,* 6**,** 100434.

HAGOS, Y., GUGSA, G., AWOL, N., AHMED, M., TSEGAYE, Y., ABEBE, N. & BSRAT, A. 2021. Isolation, identification, and antimicrobial susceptibility pattern of Campylobacter jejuni and Campylobacter coli from cattle, goat, and chicken meats in Mekelle, Ethiopia. *PloS one,* 16**,** e0246755.

HAILEMARIAM, S. 2014. *Prevalence, associated risk factors and antimicrobial susceptibility pattern of thermophilic Campylobacter spp. of ovine carcass at Addis Ababa Abattoir Enterprise, Ethiopia.* Addis Ababa University.

HAILU, W., HELMY, Y. A., CARNEY-KNISELY, G., KAUFFMAN, M., FRAGA, D. & RAJASHEKARA, G. 2021. Prevalence and antimicrobial resistance profiles of foodborne pathogens isolated from dairy cattle and poultry manure amended farms in Northeastern Ohio, the United States. *Antibiotics,* 10**,** 1450.

HAN, J., WANG, Y., SAHIN, O., SHEN, Z., GUO, B., SHEN, J. & ZHANG, Q. 2012. A fluoroquinolone resistance associated mutation in gyrA affects DNA supercoiling in Campylobacter jejuni. *Frontiers in cellular and infection microbiology,* 2**,** 21.

HANSSON, I., OLSSON ENGVALL, E., FERRARI, S., HARBOM, B. & LAHTI, E. 2020. Detection of Campylobacter species in different types of samples from dairy farms. *Veterinary Record,* 186**,** 605-605.

HAVELAAR, A. H., HAAGSMA, J. A., MANGEN, M.-J. J., KEMMEREN, J. M., VERHOEF, L. P., VIJGEN, S. M., WILSON, M., FRIESEMA, I. H., KORTBEEK, L. M. & VAN

DUYNHOVEN, Y. T. 2012. Disease burden of foodborne pathogens in the Netherlands, 2009. *International journal of food microbiology,* 156**,** 231-238.

HAVELAAR, A. H., KIRK, M. D., TORGERSON, P. R., GIBB, H. J., HALD, T., LAKE, R. J., PRAET, N., BELLINGER, D. C., DE SILVA, N. R. & GARGOURI, N. 2015. World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. *PLoS medicine,* 12**,** e1001923.

HAZARDS, E. P. O. B., KOUTSOUMANIS, K., ALLENDE, A., ÁLVAREZ-ORDÓÑEZ, A., BOLTON, D., BOVER-CID, S., CHEMALY, M., DAVIES, R., DE CESARE, A. & HERMAN, L. 2021. Role played by the environment in the emergence and spread of antimicrobial resistance (AMR) through the food chain. *EFSA Journal,* 19**,** e06651.

HE, Y., REED, S. & STROBAUGH JR, T. P. 2020. Complete genome sequence and annotation of Campylobacter jejuni YH003, isolated from retail chicken. *Microbiology Resource Announcements,* 9**,** e01307-19.

HE, Z., GHARAIBEH, R. Z., NEWSOME, R. C., POPE, J. L., DOUGHERTY, M. W., TOMKOVICH, S., PONS, B., MIREY, G., VIGNARD, J. & HENDRIXSON, D. R. 2019. Campylobacter jejuni promotes colorectal tumorigenesis through the action of cytolethal distending toxin. *Gut,* 68**,** 289-300.

HENDRIXSON, D. R. & DIRITA, V. J. 2003. Transcription of σ54-dependent but not σ28-dependent flagellar genes in Campylobacter jejuni is associated with formation of the flagellar secretory apparatus. *Molecular microbiology,* 50**,** 687-702.

HERMANS, D., VAN STEENDAM, K., VERBRUGGHE, E., VERLINDEN, M., MARTEL, A., SELIWIORSTOW, T., HEYNDRICKX, M., HAESEBROUCK, F., DE ZUTTER, L. & DEFORCE, D. 2014. Passive immunization to reduce Campylobacter jejuni colonization and transmission in broiler chickens. *Veterinary Research,* 45**,** 1-12.

HERNANDEZ, L. & GREEN, P. H. 2006. Extraintestinal manifestations of celiac disease. *Current gastroenterology reports,* 8**,** 383-389.

HILBERT, F., SCHERWITZEL, M., PAULSEN, P. & SZOSTAK, M. P. 2010. Survival of Campylobacter jejuni under conditions of atmospheric oxygen tension with the support of Pseudomonas spp. *Appl Environ Microbiol,* 76**,** 5911-7.

HLASHWAYO, D. F., SIGAÚQUE, B. & BILA, C. G. 2020. Epidemiology and antimicrobial resistance of Campylobacter spp. in animals in Sub-Saharan Africa: A systematic review. *Heliyon,* 6.

HLASHWAYO, D. F., SIGAUQUE, B., NOORMAHOMED, E. V., AFONSO, S. M., MANDOMANDO, I. M. & BILA, C. G. 2021. A systematic review and meta-analysis reveal that Campylobacter spp. and antibiotic resistance are widespread in humans in sub-Saharan Africa. *PLoS One,* 16**,** e0245951.

HODGES, L. M., TABOADA, E. N., KOZIOL, A., MUTSCHALL, S., BLAIS, B. W., INGLIS, G. D., LECLAIR, D. & CARRILLO, C. D. 2021. Systematic evaluation of whole-genome

sequencing based prediction of antimicrobial resistance in Campylobacter jejuni and C. coli. *Frontiers in Microbiology,* 12**,** 776967.

HONG, D., YU, Y., WANG, Y., XU, Y. & ZHANG, J. 2018. Acute-onset multiple acyl-CoA dehydrogenase deficiency mimicking Guillain-Barré syndrome: two cases report. *BMC neurology,* 18**,** 1-6.

HONG, J., KIM, J. M., JUNG, W. K., KIM, S. H., BAE, W., KOO, H. C., GIL, J., KIM, M., SER, J. & PARK, Y. H. 2007. Prevalence and antibiotic resistance of Campylobacter spp. isolated from chicken meat, pork, and beef in Korea, from 2001 to 2006. *Journal of Food Protection,* 70**,** 860-866.

HSIEH, Y.-H. & SULAIMAN, I. M. 2018. Campylobacteriosis: An emerging infectious foodborne disease. *Foodborne diseases.* Elsevier.

HSU, C.-H., HARRISON, L., MUKHERJEE, S., STRAIN, E., MCDERMOTT, P., ZHANG, Q. & ZHAO, S. 2020. Core genome multilocus sequence typing for food animal source attribution of human Campylobacter jejuni infections. *Pathogens,* 9**,** 532.

HTTPS://PUBMLST.ORG/CAMPYLOBACTER/ PubMLST database (https://pubmlst.org/campylobacter/; accessed in November 2023)

HTTPS://WWW.NCBI.NLM.NIH.GOV/PATHOGENS NCBI Pathogen Detection. Acessed on 6/26/2023.

HULL, D. M., HARRELL, E., VAN VLIET, A. H., CORREA, M. & THAKUR, S. 2021. Antimicrobial resistance and interspecies gene transfer in Campylobacter coli and Campylobacter jejuni isolated from food animals, poultry processing, and retail meat in North Carolina, 2018–2019. *PLoS One,* 16**,** e0246571.

HUSSAIN, I., MAHMOOD, M. S., AKHTAR, M. & KHAN, A. 2007. Prevalence of Campylobacter species in meat, milk and other food commodities in Pakistan. *Food microbiology,* 24**,** 219-222.

HUSSEIN, M. 2018. *Further investigation of the roles of fibronectin-binding proteins CadF and FlpA during Campylobacter jejuni interactions with intestinal epithelial cells.* London School of Hygiene & Tropical Medicine.

IBRAHIM, S. A., AYIVI, R. D., ZIMMERMAN, T., SIDDIQUI, S. A., ALTEMIMI, A. B., FIDAN, H., ESATBEYOGLU, T. & BAKHSHAYESH, R. V. 2021. Lactic acid bacteria as antimicrobial agents: Food safety and microbial food spoilage prevention. *Foods,* 10**,** 3131.

IGWARAN, A. & OKOH, A. I. 2019. Human campylobacteriosis: A public health concern of global importance. *Heliyon,* 5.

IGWARAN, A. & OKOH, A. I. 2020. Occurrence, Virulence and Antimicrobial Resistance-Associated Markers in Campylobacter Species Isolated from Retail Fresh Milk and Water Samples in Two District Municipalities in the Eastern Cape Province, South Africa. *Antibiotics,* 9**,** 426.

IKEDA, N. & KARLYSHEV, A. V. 2012. Putative mechanisms and biological role of coccoid form formation in Campylobacter jejuni. *European Journal of Microbiology and Immunology,* 2**,** 41-49.

INGLIS, G. D. & KALISCHUK, L. D. 2003. Use of PCR for direct detection of Campylobacter species in bovine feces. *Applied and environmental microbiology,* 69**,** 3435-3447.

IOVINE, N. M. 2013a. Resistance mechanisms in Campylobacter jejuni. *Virulence,* 4**,** 230-40.

IOVINE, N. M. 2013b. Resistance mechanisms in Campylobacter jejuni. *Virulence,* 4**,** 230-240.

ISLAM, Z., JACOBS, B., VAN BELKUM, A., MOHAMMAD, Q., ISLAM, M. B., HERBRINK, P., DIORDITSA, S., LUBY, S., TALUKDER, K. & ENDTZ, H. 2010. Axonal variant of Guillain-Barre syndrome associated with Campylobacter infection in Bangladesh. *Neurology,* 74**,** 581-587.

JAAKKONEN, A., KIVISTÖ, R., AARNIO, M., KALEKIVI, J. & HAKKINEN, M. 2020. Persistent contamination of raw milk by Campylobacter jejuni ST-883. *PLoS One,* 15**,** e0231810.

JACKSON, B., ZEGARRA, J. A., LOPEZ-GATELL, H., SEJVAR, J., ARZATE, F., WATERMAN, S., NÚÑEZ, A. S., LOPEZ, B., WEISS, J. & CRUZ, R. Q. 2014a. Binational outbreak of Guillain–Barré syndrome associated with Campylobacter jejuni infection, Mexico and USA, 2011. *Epidemiology & Infection,* 142**,** 1089-1099.

JACKSON, B. R., ZEGARRA, J. A., LOPEZ-GATELL, H., SEJVAR, J., ARZATE, F., WATERMAN, S., NUNEZ, A. S., LOPEZ, B., WEISS, J., CRUZ, R. Q., MURRIETA, D. Y., LUNA-GIERKE, R., HEIMAN, K., VIEIRA, A. R., FITZGERALD, C., KWAN, P., ZARATE-BERMUDEZ, M., TALKINGTON, D., HILL, V. R. & MAHON, B. 2014b. Binational outbreak of Guillain-Barre syndrome associated with Campylobacter jejuni infection, Mexico and USA, 2011. *Epidemiol Infect,* 142**,** 1089-99.

JACOBS, B. C., VAN DOORN, P. A., TIO-GILLEN, A. P., VISSER, L. H., VAN DER MECHÉ, F. G., SCHMITZ, P. I., HERBRINK, P. & HOOIJKAAS, H. 1996. Campylobacter jejuni infections and anti-GM1 antibodies in Guillain-Barré syndrome. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society,* 40**,** 181-187.

JANSEN VAN RENSBURG, M. J., SWIFT, C., CODY, A. J., JENKINS, C. & MAIDEN, M. C. 2016. Exploiting bacterial whole-genome sequencing data for evaluation of diagnostic assays: Campylobacter species identification as a case study. *Journal of clinical microbiology,* 54**,** 2882-2890.

JAYARAO, B. M., DONALDSON, S. C., STRALEY, B. A., SAWANT, A. A., HEGDE, N. V. & BROWN, J. 2006. A survey of foodborne pathogens in bulk tank milk and raw milk

consumption among farm families in Pennsylvania. *Journal of dairy science,* 89**,** 2451-2458.

JIFFRY, M. Z. M., OKAM, N. A., VARGAS, J., ADEKUNLE, F. A., PAGAN, S. C., KHOWAJA, F., AHMED-KHAN, M. A. & ADEKUNLE, F. 2023. Myocarditis as a Complication of Campylobacter jejuni-Associated Enterocolitis: A Report of Two Cases. *Cureus,* 15.

JOENSEN, K. G., KIIL, K., GANTZHORN, M. R., NAUERBY, B., ENGBERG, J., HOLT, H. M., NIELSEN, H. L., PETERSEN, A. M., KUHN, K. G. & SANDØ, G. 2020. Whole-genome sequencing to detect numerous Campylobacter jejuni outbreaks and match patient isolates to sources, Denmark, 2015–2017. *Emerging infectious diseases,* 26**,** 523.

JOENSEN, K. G., SCHJØRRING, S., GANTZHORN, M. R., VESTER, C. T., NIELSEN, H. L., ENGBERG, J. H., HOLT, H. M., ETHELBERG, S., MÜLLER, L. & SANDØ, G. 2021. Whole genome sequencing data used for surveillance of Campylobacter infections: detection of a large continuous outbreak, Denmark, 2019. *Eurosurveillance,* 26**,** 2001396.

JORGENSEN, F., CHARLETT, A., ARNOLD, E., SWIFT, C., MADDEN, B. & ELVISS, N. C. 2017. Year 2 Report of a microbiological survey of Campylobacter contamination in

fresh whole UK-produced chilled chickens at retail sale.

KAAKOUSH, N. O., CASTAÑO-RODRÍGUEZ, N., MITCHELL, H. M. & MAN, S. M. 2015a. Global epidemiology of Campylobacter infection. *Clinical microbiology reviews,* 28**,** 687-720.

KAAKOUSH, N. O., CASTAÑO-RODRÍGUEZ, N., MITCHELL, H. M. & MAN, S. M. 2015b. Global Epidemiology of Campylobacter Infection. *Clin Microbiol Rev,* 28**,** 687-720.

KAILOO, S. & KUMAR, Y. 2021. Cytolethal distending toxin: from genotoxin to a potential biomarker and anti-tumor target. *World Journal of Microbiology and Biotechnology,* 37**,** 1-18.

KALUPAHANA, R., KOTTAWATTA, K., KANANKEGE, K., VAN BERGEN, M., ABEYNAYAKE, P. & WAGENAAR, J. 2013. Colonization of Campylobacter spp. in broiler chickens and laying hens reared in tropical climates with low-biosecurity housing. *Applied and environmental microbiology,* 79**,** 393-395.

KALUPAHANA, R. S., MUGHINI-GRAS, L., KOTTAWATTA, S., SOMARATHNE, S., GAMAGE, C. & WAGENAAR, J. 2018. Weather correlates of Campylobacter prevalence in broilers at slaughter under tropical conditions in Sri Lanka. *Epidemiology & Infection,* 146**,** 972-979.

KARMALI, M. A., SIMOR, A., ROSCOE, M., FLEMING, P., SMITH, S. & LANE, J. 1986. Evaluation of a blood-free, charcoal-based, selective medium for the isolation of Campylobacter organisms from feces. *Journal of clinical microbiology,* 23**,** 456-459.

KASHOMA, I. P., KASSEM, I. I., JOHN, J., KESSY, B. M., GEBREYES, W., KAZWALA, R. R. & RAJASHEKARA, G. 2016. Prevalence and antimicrobial resistance of

Campylobacter isolated from dressed beef carcasses and raw milk in Tanzania. *Microbial drug resistance,* 22**,** 40-52.

KASSA, T., GEBRE-SELASSIE, S. & ASRAT, D. 2007. Antimicrobial susceptibility patterns of thermotolerant Campylobacter strains isolated from food animals in Ethiopia. *Veterinary Microbiology,* 119**,** 82-87.

KAWAI, F., PAEK, S., CHOI, K.-J., PROUTY, M., KANIPES, M. I., GUERRY, P. & YEO, H.-J. 2012. Crystal structure of JlpA, a surface-exposed lipoprotein adhesin of Campylobacter jejuni. *Journal of Structural Biology,* 177**,** 583-588.

KEBA, A., ROLON, M. L., TAMENE, A., DESSIE, K., VIPHAM, J., KOVAC, J. & ZEWDU, A. 2020. Review of the prevalence of foodborne pathogens in milk and dairy products in Ethiopia. *International dairy journal,* 109**,** 104762.

KEITHLIN, J., SARGEANT, J., THOMAS, M. K. & FAZIL, A. 2014. Systematic review and meta-analysis of the proportion of Campylobacter cases that develop chronic sequelae. *BMC public health,* 14**,** 1-19.

KELLEY, B. R., ELLIS, J. C., LARGE, A., SCHNEIDER, L. G., JACOBSON, D. & JOHNSON, J. G. 2020. Whole-Genome Sequencing and bioinformatic analysis of environmental, agricultural, and human Campylobacter jejuni isolates from East Tennessee. *Frontiers in Microbiology,* 11**,** 571064.

KENYON, J., INNS, T., AIRD, H., SWIFT, C., ASTBURY, J., FORESTER, E. & DECRAENE, V. 2020. Campylobacter outbreak associated with raw drinking milk, North West England, 2016. *Epidemiology & Infection,* 148**,** e13.

KHAN, J. A., ABULREESH, H. H., KUMAR, R., SAMREEN & AHMAD, I. 2019. Antibiotic Resistance in Campylobacter jejuni: Mechanism, Status, and Public Health Significance. *Antibacterial Drug Discovery to Combat MDR: Natural Compounds, Nanotechnology and Novel Synthetic Sources***,** 95-114.

KHANZADI, S., JAMSHIDI, A., SOLTANINEJAD, V. & KHAJENASIRI, S. 2010. Isolation and identification of Campylobacter jejuni from bulk tank milk in Mashhad-Iran. *World Applied Sciences Journal,* 9**,** 638-643.

KHEMNU, N., SERICHANTALERGS, O., RUEKIT, S., LERTSETHTAKARN, P., POLY, F., SWIERCZEWSKI, B. E. & CRAWFORD, J. M. 2023. Description of novel capsule biosynthesis loci of Campylobacter jejuni clinical isolates from South and South-East Asia. *Plos one,* 18**,** e0280583.

KIM, J.-C., OH, E., KIM, J. & JEON, B. 2015. Regulation of oxidative stress resistance in Campylobacter jejuni, a microaerophilic foodborne pathogen. *Frontiers in microbiology,* 6**,** 751.

KIM, J., OH, E., BANTING, G. S., BRAITHWAITE, S., CHUI, L., ASHBOLT, N. J., NEUMANN, N. F. & JEON, B. 2016. An improved culture method for selective isolation of Campylobacter jejuni from wastewater. *Frontiers in microbiology,* 7**,** 1345.

KIM, J. M., HONG, J., BAE, W., KOO, H. C., KIM, S. H. & PARK, Y. H. 2010. Prevalence, antibiograms, and transferable tet (O) plasmid of Campylobacter jejuni and Campylobacter coli isolated from raw chicken, pork, and human clinical cases in Korea. *Journal of food protection,* 73**,** 1430-1437.

KINANA, A. D., CARDINALE, E., TALL, F., BAHSOUN, I., SIRE, J.-M., GARIN, B., BREUREC, S., BOYE, C. S.-B. & PERRIER-GROS-CLAUDE, J.-D. 2006. Genetic diversity and quinolone resistance in Campylobacter jejuni isolates from poultry in Senegal. *Applied and environmental microbiology,* 72**,** 3309-3313.

KING, E. O. 1957. Human infections with Vibrio fetus and a closely related vibrio. *The Journal of infectious diseases***,** 119-128.

KLEINUBING, N. R., RAMIRES, T., WÜRFEL, S. D. F. R., HAUBERT, L., SCHEIK, L. K., KREMER, F. S., LOPES, G. V. & DA SILVA, W. P. 2021. Antimicrobial resistance genes and plasmids in Campylobacter jejuni from broiler production chain in Southern Brazil. *LWT,* 144**,** 111202.

KNIPPER, A.-D., GHOREISHI, N. & CREASE, T. 2022. Prevalence and concentration of Campylobacter in faeces of dairy cows: A systematic review and meta-analysis. *Plos one,* 17**,** e0276018.

KÖNIG, F., SVENSSON, S. L. & SHARMA, C. M. 2023. Interplay of two small RNAs fine-tunes hierarchical flagellar gene expression in the foodborne pathogen Campylobacter jejuni. *bioRxiv***,** 2023.04. 21.537696.

KONKEL, M. E., TALUKDAR, P. K., NEGRETTI, N. M. & KLAPPENBACH, C. M. 2020. Taking control: Campylobacter jejuni binding to fibronectin sets the stage for cellular adherence and invasion. *Frontiers in Microbiology,* 11**,** 564.

KRELING, V., FALCONE, F. H., KEHRENBERG, C. & HENSEL, A. 2020. Campylobacter sp.: Pathogenicity factors and prevention methods—new molecular targets for innovative antivirulence drugs? *Applied Microbiology and Biotechnology,* 104**,** 10409-10436.

KUMAR, A., GROVER, S., SHARMA, J. & BATISH, V. 2010. Chymosin and other milk coagulants: sources and biotechnological interventions. *Critical reviews in biotechnology,* 30**,** 243-258.

KURINČIČ, M., BOTTELDOORN, N., HERMAN, L. & MOŽINA, S. S. 2007. Mechanisms of erythromycin resistance of Campylobacter spp. isolated from food, animals and humans. *International Journal of Food Microbiology,* 120**,** 186-190.

KWAN, P. S., BIRTLES, A., BOLTON, F. J., FRENCH, N. P., ROBINSON, S. E., NEWBOLD, L. S., UPTON, M. & FOX, A. J. 2008. Longitudinal study of the molecular epidemiology of Campylobacter jejuni in cattle on dairy farms. *Applied and Environmental Microbiology,* 74**,** 3626-3633.

LAGIER, M. J. & THREADGILL, D. S. 2014. Identification and characterization of an invasion antigen B gene from the oral pathogen Campylobacter rectus. *Indian journal of microbiology,* 54**,** 33-40.

LAI, C.-K., CHEN, Y.-A., LIN, C.-J., LIN, H.-J., KAO, M.-C., HUANG, M.-Z., LIN, Y.-H., CHIANG-NI, C., CHEN, C.-J. & LO, U. 2016. Molecular mechanisms and potential clinical applications of Campylobacter jejuni cytolethal distending toxin. *Frontiers in cellular and infection microbiology,* 6**,** 9.

LAKE, I., COLON-GONZALEZ, F. J., TAKKINEN, J., ROSSI, M., SUDRE, B., DIAS, J. G., TAVOSCHI, L., JOSHI, A., SEMENZA, J. & NICHOLS, G. 2019. Exploring campylobacter seasonality across Europe using the European surveillance system (TESSy), 2008 to 2016. *Eurosurveillance,* 24**,** 1800028.

LAMBERT, E. & HOGAN, N. 2009. The importance of job satisfaction and organizational commitment in shaping turnover intent: A test of a causal model. *Criminal Justice Review,* 34**,** 96-118.

LANGER, A. J., AYERS, T., GRASS, J., LYNCH, M., ANGULO, F. J. & MAHON, B. E. 2012. Nonpasteurized dairy products, disease outbreaks, and state laws—United States, 1993–2006. *Emerging Infectious Diseases,* 18**,** 385.

LEE, R. B., HASSANE, D. C., COTTLE, D. L. & PICKETT, C. L. 2003. Interactions of Campylobacter jejuni cytolethal distending toxin subunits CdtA and CdtC with HeLa cells. *Infection and immunity,* 71**,** 4883-4890.

LEEDOM, J. M. 2006. Milk of nonhuman origin and infectious diseases in humans. *Clinical Infectious Diseases,* 43**,** 610-615.

LENGERH, A., MOGES, F., UNAKAL, C. & ANAGAW, B. 2013a. Prevalence, associated risk factors and antimicrobial susceptibility pattern of Campylobacter species among under five diarrheic children at Gondar University Hospital, Northwest Ethiopia. *BMC pediatrics,* 13**,** 1-9.

LENGERH, A., MOGES, F., UNAKAL, C. & ANAGAW, B. 2013b. Prevalence, associated risk factors and antimicrobial susceptibility pattern of Campylobacter species among under five diarrheic children at Gondar University Hospital, Northwest Ethiopia. *BMC pediatrics,* 13**,** 1-9.

LENGERH, A., MOGES, F., UNAKAL, C. & ANAGAW, B. 2013c. Prevalence, associated risk factors and antimicrobial susceptibility pattern of Campylobacter species among under five diarrheic children at Gondar University Hospital, Northwest Ethiopia. *BMC pediatrics,* 13**,** 82.

LETOURNEAU, J., LEVESQUE, C., BERTHIAUME, F., JACQUES, M. & MOUREZ, M. 2011. In vitro assay of bacterial adhesion onto mammalian epithelial cells. *JoVE (Journal of Visualized Experiments)***,** e2783.

LEVY, A. 1946. A gastro-enteritis outbreak probably due to a bovine strain of vibrio. *The Yale journal of biology and medicine,* 18**,** 243.

LI, L., MENDIS, N., TRIGUI, H., OLIVER, J. D. & FAUCHER, S. P. 2014. The importance of the viable but non-culturable state in human bacterial pathogens. *Frontiers in microbiology,* 5**,** 258.

LIU, F., LEE, S. A., XUE, J., RIORDAN, S. M. & ZHANG, L. 2022. Global epidemiology of campylobacteriosis and the impact of COVID-19. *Frontiers in cellular and infection microbiology,* 12**,** 1666.

LIU, K. C., JINNEMAN, K. C., NEAL-MCKINNEY, J., WU, W.-H. & RICE, D. H. 2016. Genome sequencing and annotation of a Campylobacter coli strain isolated from milk with multidrug resistance. *Genomics Data,* 8**,** 123-125.

LIU, R. & OCHMAN, H. 2007. Stepwise formation of the bacterial flagellar system. *Proceedings of the National Academy of Sciences,* 104**,** 7116-7121.

LLARENA, A.-K., TABOADA, E. & ROSSI, M. 2017. Whole-genome sequencing in epidemiology of Campylobacter jejuni infections. *Journal of Clinical Microbiology,* 55**,** 1269-1275.

LONG, K. S., MUNCK, C., ANDERSEN, T. M., SCHAUB, M. A., HOBBIE, S. N., BÖTTGER, E. C. & VESTER, B. 2010. Mutations in 23S rRNA at the peptidyl transferase center and their relationship to linezolid binding and cross-resistance. *Antimicrobial agents and chemotherapy,* 54**,** 4705-4713.

LOPES, G. V., RAMIRES, T., KLEINUBING, N. R., SCHEIK, L. K., FIORENTINI, Â. M. & DA SILVA, W. P. 2021. Virulence factors of foodborne pathogen Campylobacter jejuni. *Microbial pathogenesis,* 161**,** 105265.

LOPEZ, G. U., KITAJIMA, M., SHERCHAN, S. P., SEXTON, J. D., SIFUENTES, L. Y., GERBA, C. P. & REYNOLDS, K. A. 2015. Impact of disinfectant wipes on the risk of Campylobacter jejuni infection during raw chicken preparation in domestic kitchens. *J Appl Microbiol,* 119**,** 245-52.

LOUIS, V. R., GILLESPIE, I. A., O'BRIEN, S. J., RUSSEK-COHEN, E., PEARSON, A. D. & COLWELL, R. R. 2005. Temperature-driven Campylobacter seasonality in England and Wales. *Applied and Environmental Microbiology,* 71**,** 85-92.

LOUWEN, R. & VAN NEERVEN, R. J. 2015. Milk modulates campylobacter invasion into caco-2 intestinal epithelial cells. *European Journal of Microbiology and Immunology,* 5**,** 181-187.

LUANGTONGKUM, T., JEON, B., HAN, J., PLUMMER, P., LOGUE, C. M. & ZHANG, Q. 2009. Antibiotic resistance in Campylobacter: emergence, transmission and persistence.

LV, R., WANG, K., FENG, J., HEENEY, D. D., LIU, D. & LU, X. 2020. Detection and quantification of viable but non-culturable Campylobacter jejuni. *Frontiers in microbiology,* 10**,** 2920.

MA, H., SU, Y., MA, L., MA, L., LI, P., DU, X., GÖLZ, G., WANG, S. & LU, X. 2017. Prevalence and characterization of Campylobacter jejuni isolated from retail chicken in Tianjin, China. *Journal of Food Protection,* 80**,** 1032-1040.

MABOTE, K. I., MBEWE, M. & ATEBA, C. N. 2011. Prevalence of Campylobacter contamination in fresh chicken meat and milk obtained from markets in the North-West Province, South Africa. *Journal of Human Ecology,* 36**,** 23-28.

MACNAB, R. M. 2004. Type III flagellar protein export and flagellar assembly. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research,* 1694**,** 207-217.

MAHMOOD, M. S., HUSSAIN, I., ARSHAD, M. I., ALI, S., AKTAR, M., KHAN, A. & MAHMOOD, F. 2009. Seasonal prevalence of Campylobacter species in milk and milk products in Pakistan. *Pak J Zool Suppl Ser,* 9**,** 227-31.

MAIDEN, M. C., VAN RENSBURG, M. J. J., BRAY, J. E., EARLE, S. G., FORD, S. A., JOLLEY, K. A. & MCCARTHY, N. D. 2013. MLST revisited: the gene-by-gene approach to bacterial genomics. *Nature Reviews Microbiology,* 11**,** 728-736.

MALIK, A., BRUDVIG, J. M., GADSDEN, B. J., ETHRIDGE, A. D. & MANSFIELD, L. S. 2022. Campylobacter jejuni induces autoimmune peripheral neuropathy via Sialoadhesin and Interleukin-4 axes. *Gut Microbes,* 14**,** 2064706.

MAN, S. M. 2011. The clinical importance of emerging Campylobacter species. *Nature reviews Gastroenterology & hepatology,* 8**,** 669-685.

MANNING, G., DOWSON, C. G., BAGNALL, M. C., AHMED, I. H., WEST, M. & NEWELL, D. G. 2003. Multilocus sequence typing for comparison of veterinary and human isolates of Campylobacter jejuni. *Applied and Environmental Microbiology,* 69**,** 6370-6379.

MARLER, W. 2009. Comparing the food safety record of pasteurized and raw milk products–Part 3.

MARTIN, N. H., BOOR, K. J. & WIEDMANN, M. 2018. Symposium review: Effect of post-pasteurization contamination on fluid milk quality. *Journal of Dairy Science,* 101**,** 861-870.

MCCARTHY, N. & GIESECKE, J. 2001. Incidence of Guillain-Barré syndrome following infection with Campylobacter jejuni. *American journal of epidemiology,* 153**,** 610-614.

MCDERMOTT, P. F., BODEIS, S. M., ENGLISH, L. L., WHITE, D. G., WALKER, R. D., ZHAO, S., SIMJEE, S. & WAGNER, D. D. 2002. Ciprofloxacin resistance in Campylobacter jejuni evolves rapidly in chickens treated with fluoroquinolones. *The Journal of infectious diseases,* 185**,** 837-840.

MCGROGAN, A., MADLE, G. C., SEAMAN, H. E. & DE VRIES, C. S. 2009. The epidemiology of Guillain-Barré syndrome worldwide. *Neuroepidemiology,* 32**,** 150-163.

MCSWEEGAN, E. & WALKER, R. I. 1986. Identification and characterization of two Campylobacter jejuni adhesins for cellular and mucous substrates. *Infection and immunity,* 53**,** 141-148.

MÉNDEZ-OLVERA, E. T., BUSTOS-MARTÍNEZ, J. A., LÓPEZ-VIDAL, Y., VERDUGO-RODRÍGUEZ, A. & MARTÍNEZ-GÓMEZ, D. 2016. Cytolethal distending toxin from Campylobacter jejuni requires the cytoskeleton for toxic activity. *Jundishapur journal of microbiology*, 9.

MEZHER, Z., SACCARES, S., MARCIANÒ, R., DE SANTIS, P., RODAS, E. M. F., DE ANGELIS, V. & CONDOLEO, R. 2016. Occurrence of Campylobacter spp. in poultry meat at retail and processing plants' levels in Central Italy. *Italian journal of food safety*, 5.

MIKHEENKO, A., PRJIBELSKI, A., SAVELIEV, V., ANTIPOV, D. & GUREVICH, A. 2018. Versatile genome assembly evaluation with QUAST-LG. *Bioinformatics*, 34**,** i142-i150.

MITIKE, G., KASSU, A., GENETU, A. & NIGUSSIE, D. 2000. Campylobacter enteritis among children in Dembia district, northwest Ethiopia. *East African Medical Journal*, 77.

MIZUNO, S., YOKOYAMA, K., NUKADA, T., IKEDA, Y. & HARA, S. 2022. Campylobacter jejuni bacteremia in the term infant a rare cause of neonatal hematochezia. *The Pediatric Infectious Disease Journal*, 41**,** e156.

MO, R., MA, W., ZHOU, W. & GAO, B. 2022. Polar localization of CheO under hypoxia promotes Campylobacter jejuni chemotactic behavior within host. *PLoS pathogens*, 18**,** e1010953.

MODI, S., BRAHMBHATT, M., CHATUR, Y. & NAYAK, J. 2015a. Prevalence of Campylobacter species in milk and milk products, their virulence gene profile and anti-bio gram. *Veterinary world*, 8**,** 1.

MODI, S., BRAHMBHATT, M. N., CHATUR, Y. A. & NAYAK, J. B. 2015b. Prevalence of Campylobacter species in milk and milk products, their virulence gene profile and anti-bio gram. *Vet World*, 8**,** 1-8.

MORAN, L., KELLY, C. & MADDEN, R. 2009. Factors affecting the recovery of Campylobacter spp. from retail packs of raw, fresh chicken using ISO 10272-1: 2006. *Letters in applied microbiology*, 48**,** 628-632.

MORITA, D., ARAI, H., ISOBE, J., MAENISHI, E., KUMAGAI, T., MARUYAMA, F. & KURODA, T. 2023. Whole-Genome and Plasmid Comparative Analysis of Campylobacter jejuni from Human Patients in Toyama, Japan, from 2015 to 2019. *Microbiology Spectrum*, 11**,** e02659-22.

MPATSWENUMUGABO, J. P. M., BEBORA, L. C., GITAO, G. C., MOBEGI, V. A., IRAGUHA, B. & SHUMBUSHO, B. 2019. Assessment of bacterial contamination and milk handling practices along the raw milk market chain in the north-western region of Rwanda. *African Journal of Microbiology Research*, 13**,** 640-648.

MUGHINI-GRAS, L., PIJNACKER, R., COIPAN, C., MULDER, A. C., VELUDO, A. F., DE RIJK, S., VAN HOEK, A. H., BUIJ, R., MUSKENS, G. & KOENE, M. 2021. Sources and transmission routes of campylobacteriosis: A combined analysis of genome and exposure data. *Journal of infection*, 82**,** 216-226.

MUGHINI GRAS, L., SMID, J. H., WAGENAAR, J. A., DE BOER, A. G., HAVELAAR, A. H., FRIESEMA, I. H., FRENCH, N. P., BUSANI, L. & VAN PELT, W. 2012. Risk factors for campylobacteriosis of chicken, ruminant, and environmental origin: a combined case-control and source attribution analysis. *PloS one,* 7**,** e42599.

MULATU, G., BEYENE, G. & ZEYNUDIN, A. 2014. Prevalence of Shigella, Salmonella and Cmpylobacter species and their susceptibility patters among under five children with diarrhea in Hawassa town, South Ethiopia. *Ethiopian journal of health sciences,* 24**,** 101.

MULATU, G., ZEYNUDIN, A., ZEMENE, E., DEBALKE, S. & BEYENE, G. 2015. Intestinal parasitic infections among children under five years of age presenting with diarrhoeal diseases to two public health facilities in Hawassa, South Ethiopia. *Infectious diseases of poverty,* 4**,** 1-8.

MULDER, A. C., FRANZ, E., DE RIJK, S., VERSLUIS, M. A., COIPAN, C., BUIJ, R., MÜSKENS, G., KOENE, M., PIJNACKER, R. & DUIM, B. 2020. Tracing the animal sources of surface water contamination with Campylobacter jejuni and Campylobacter coli. *Water Research,* 187**,** 116421.

MUNGAI, E. A., BEHRAVESH, C. B. & GOULD, L. H. 2015. Increased outbreaks associated with nonpasteurized milk, United States, 2007–2012. *Emerging infectious diseases,* 21**,** 119.

NACHAMKIN, I. 2002. Chronic effects of Campylobacter infection. *Microbes and infection,* 4**,** 399-403.

NADA, S., ILIJA, D., IGOR, T., JELENA, M. & RUZICA, G. 2012. Implication of food safety measures on microbiological quality of raw and pasteurized milk. *Food control,* 25**,** 728-731.

NEAL-MCKINNEY, J. M. & KONKEL, M. E. 2012. The Campylobacter jejuni CiaC virulence protein is secreted from the flagellum and delivered to the cytosol of host cells. *Frontiers in cellular and infection microbiology,* 2**,** 31.

NEGRETTI, N. M., GOURLEY, C. R., CLAIR, G., ADKINS, J. N. & KONKEL, M. E. 2017. The food-borne pathogen Campylobacter jejuni responds to the bile salt deoxycholate with countermeasures to reactive oxygen species. *Scientific reports,* 7**,** 15455.

NEGRETTI, N. M., GOURLEY, C. R., TALUKDAR, P. K., CLAIR, G., KLAPPENBACH, C. M., LAURITSEN, C. J., ADKINS, J. N. & KONKEL, M. E. 2021. The Campylobacter jejuni CiaD effector co-opts the host cell protein IQGAP1 to promote cell entry. *Nature Communications,* 12**,** 1339.

NG, P. C. & KIRKNESS, E. F. 2010. Whole genome sequencing. *Genetic variation: Methods and protocols***,** 215-226.

NGOBESE, B., ZISHIRI, O. T. & EL ZOWALATY, M. E. 2020. Molecular detection of virulence genes in Campylobacter species isolated from livestock production systems in South Africa. *Journal of Integrative Agriculture,* 19**,** 1656-1670.

NGULUKUN, S. S. 2017. Taxonomy and physiological characteristics of Campylobacter spp. *Campylobacter*. Elsevier.

NGUYEN, L.-T., SCHMIDT, H. A., VON HAESELER, A. & MINH, B. Q. 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular biology and evolution,* 32**,** 268-274.

NICHOLS, G. L. 2005. Fly transmission of Campylobacter. *Emerging infectious diseases,* 11**,** 361.

NIGATU, S., MEQUANENT, A., TESFAYE, R. & GAREDEW, L. 2015. Prevalence and Drug Sensitivity Pattern of Campylobacter jejuni Isolated from Cattle and Poultry in and Around Gondar Town, Ethiopia. *Glob Vet,* 14**,** 43-7.

NIGUSU, Y., ABDISSA, A. & TESFAW, G. 2022. Campylobacter Gastroenteritis Among Under-Five Children in Southwest Ethiopia. *Infection and Drug Resistance***,** 2969-2979.

NJOGA, E. O., NWANKWO, I. O. & UGWUNWARUA, J. C. 2019. Epidemiology of thermotolerant Campylobacter infection in poultry in Nsukka agricultural zone, Nigeria. *Int J One Health,* 5**,** 92-98.

NOORMOHAMED, A. & FAKHR, M. K. 2013. Arsenic resistance and prevalence of arsenic resistance genes in Campylobacter jejuni and Campylobacter coli isolated from retail meats. *International Journal of Environmental Research and Public Health,* 10**,** 3453-3464.

NOORMOHAMED, A. & FAKHR, M. K. 2014. Molecular typing of Campylobacter jejuni and Campylobacter coli isolated from various retail meats by MLST and PFGE. *Foods,* 3**,** 82-93.

NWANKWO, I., SALIHU, M., FALEKE, O., MAGAJI, A. & GARBA, J. 2018. Seasonal variation in prevalence and antimicrobial resistance of Campylobacter species isolates from the feces of free-range chickens and humans in Sokoto, north western Nigeria. *Animal Science Reporter,* 11**,** 11-21.

OGBOMON, E. O., AKPOMIE, O. O., ENENYA, R. P., OBANOR, O. & MORKA, E. 2018a. Prevalence and Antibiotic Susceptibility Patterns of Campylobacter Species in Locally Pasteurized Milk Product (Nunu) Sold in Zaria Metropolis, Kaduna State, Nigeria. *International Journal of Microbiology and Biotechnology,* 3**,** 89.

OGBOMON, E. O., AKPOMIE, O. O., ENENYA, R. P., OBANOR, O. & MORKA, E. 2018b. Prevalence and Antibiotic Susceptibility Patterns of Campylobacter Species in Locally Pasteurized Milk Product (Nunu) Sold in Zaria Metropolis, Kaduna State, Nigeria. *International Journal of Microbiology and Biotechnology,* 3**,** 89.

OH, E., ANDREWS, K. J., MCMULLEN, L. M. & JEON, B. 2019. Tolerance to stress conditions associated with food safety in Campylobacter jejuni strains isolated from retail raw chicken. *Scientific reports,* 9**,** 11915.

OLTRAMARI, K., CARDOSO, R. F., PATUSSI, E. V., SANTOS, A. C. B. & MIKCHA, J. M. G. 2014. Genetic heterogeneity of Escherichia coli isolated from pasteurized milk in State of Paraná, Brazil. *Brazilian Journal of Pharmaceutical Sciences,* 50**,** 337-343.

OLUM, M. O., MASILA, E., MUHOMA, V. A., TOO, E., MUNGUBE, E. O. & MAICHOMO, M. 2023. Campylobacteriosis in Sub-Saharan Africa.

OMARA, S. T., EL FADALY, H. & BARAKAT, A. 2015. Public health hazard of zoonotic Campylobacter jejuni reference to Egyptian regional and seasonal variations. *Research Journal of Microbiology,* 10**,** 343.

OMAROVA, S., AWAD, K., MOOS, V., PÜNING, C., GÖLZ, G., SCHULZKE, J.-D. & BÜCKER, R. 2023. Intestinal Barrier in Post-Campylobacter jejuni Irritable Bowel Syndrome. *Biomolecules,* 13**,** 449.

OREMLAND, R. S. & STOLZ, J. F. 2003. The ecology of arsenic. *Science,* 300**,** 939-944.

OREMLAND, R. S. & STOLZ, J. F. 2005. Arsenic, microbes and contaminated aquifers. *Trends in microbiology,* 13**,** 45-49.

OTSUKA, Y., HAGIYA, H., TAKAHASHI, M., FUKUSHIMA, S., MAEDA, R., SUNADA, N., YAMADA, H., KISHIDA, M., FUJITA, K. & OTSUKA, F. 2023a. Clinical characteristics of Campylobacter bacteremia: a multicenter retrospective study. *Scientific Reports,* 13**,** 1-6.

OTSUKA, Y., HAGIYA, H., TAKAHASHI, M., FUKUSHIMA, S., MAEDA, R., SUNADA, N., YAMADA, H., KISHIDA, M., FUJITA, K. & OTSUKA, F. 2023b. Clinical characteristics of Campylobacter bacteremia: a multicenter retrospective study. *Scientific reports,* 13**,** 647.

PAGE, A. J., CUMMINS, C. A., HUNT, M., WONG, V. K., REUTER, S., HOLDEN, M. T., FOOKES, M., FALUSH, D., KEANE, J. A. & PARKHILL, J. 2015. Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics,* 31**,** 3691-3693.

PAINTSIL, E. K., OFORI, L. A., ADOBEA, S., AKENTEN, C. W., PHILLIPS, R. O., MAIGA-ASCOFARE, O., LAMSHÖFT, M., MAY, J., OBIRI DANSO, K. & KRUMKAMP, R. 2022a. Prevalence and antibiotic resistance in campylobacter spp. isolated from humans and food-producing animals in West Africa: a systematic review and meta-analysis. *Pathogens,* 11**,** 140.

PAINTSIL, E. K., OFORI, L. A., AKENTEN, C. W., ZAUTNER, A. E., MBWANA, J., JAEGER, A., LAMSHÖFT, M., MAY, J., OBIRI-DANSO, K. & PHILIPPS, R. O. 2022b. Antibiotic-resistant Campylobacter coli and Campylobacter jejuni in commercial and smallholder farm animals in the Asante Akim North Municipality of Ghana. *Frontiers in Microbiology,* 13**,** 983047.

PALYADA, K., SUN, Y.-Q., FLINT, A., BUTCHER, J., NAIKARE, H. & STINTZI, A. 2009. Characterization of the oxidative stress stimulon and PerR regulon of Campylobacter jejuni. *BMC genomics,* 10**,** 1-19.

PANZENHAGEN, P., PORTES, A. B., DOS SANTOS, A. M., DUQUE, S. D. S. & CONTE JUNIOR, C. A. 2021. The distribution of Campylobacter jejuni virulence genes in genomes worldwide derived from the NCBI pathogen detection database. *Genes,* 12**,** 1538.

PAPADOPOULOS, D., PETRIDOU, E., PAPAGEORGIOU, K., GIANTSIS, I. A., DELIS, G., ECONOMOU, V., FRYDAS, I., PAPADOPOULOS, G., HATZISTYLIANOU, M. & KRITAS, S. K. 2021. Phenotypic and molecular patterns of resistance among Campylobacter coli and Campylobacter jejuni isolates, from pig farms. *Animals,* 11**,** 2394.

PARKER, C. T., COOPER, K. K., SCHIAFFINO, F., MILLER, W. G., HUYNH, S., GRAY, H. K., OLORTEGUI, M. P., BARDALES, P. G., TRIGOSO, D. R. & PENATARO-YORI, P. 2021. Genomic characterization of Campylobacter jejuni adapted to the guinea pig (Cavia porcellus) host. *Frontiers in Cellular and Infection Microbiology,* 11**,** 607747.

PEREZ-ARNEDO, I. & GONZALEZ-FANDOS, E. 2019. Prevalence of Campylobacter spp. in poultry in three Spanish farms, a slaughterhouse and a further processing plant. *Foods,* 8**,** 111.

PETERS, S., PASCOE, B., WU, Z., BAYLISS, S. C., ZENG, X., EDWINSON, A., VEERABADHRAN-GURUNATHAN, S., JAWAHIR, S., CALLAND, J. K. & MOURKAS, E. 2021. Campylobacter jejuni genotypes are associated with post-infection irritable bowel syndrome in humans. *Communications Biology,* 4**,** 1015.

PHILLIPS, Z. N., TRAM, G., SEIB, K. L. & ATACK, J. M. 2019. Phase-variable bacterial loci: how bacteria gamble to maximise fitness in changing environments. *Biochemical Society Transactions,* 47**,** 1131-1141.

PLATTS-MILLS, J. A. & KOSEK, M. 2014. Update on the burden of Campylobacter in developing countries. *Curr Opin Infect Dis,* 27**,** 444-50.

PLISHKA, M., SARGEANT, J. M., GREER, A. L., HOOKEY, S. & WINDER, C. 2021. The prevalence of Campylobacter in live cattle, Turkey, chicken, and swine in the United States and Canada: A systematic review and meta-analysis. *Foodborne Pathogens and Disease,* 18**,** 230-242.

POKHREL, D., THAMES, H. T., ZHANG, L., DINH, T. T., SCHILLING, W., WHITE, S. B., RAMACHANDRAN, R. & THERADIYIL SUKUMARAN, A. 2022. Roles of aerotolerance, biofilm formation, and viable but non-culturable state in the survival of Campylobacter jejuni in poultry processing environments. *Microorganisms,* 10**,** 2165.

POLLETT, S., ROCHA, C., ZERPA, R., PATIÑO, L., VALENCIA, A., CAMIÑA, M., GUEVARA, J., LOPEZ, M., CHUQUIRAY, N. & SALAZAR-LINDO, E. 2012. Campylobacterantimicrobial resistance in Peru: a ten-year observational study. *BMC infectious diseases,* 12**,** 1-7.

PONS, B. J., BEZINE, E., HANIQUE, M., GUILLET, V., MOUREY, L., CHICHER, J., FRISAN, T., VIGNARD, J. & MIREY, G. 2019. Cell transfection of purified cytolethal distending toxin B subunits allows comparing their nuclease activity while plasmid degradation assay does not. *Plos one,* 14**,** e0214313.

PONS, B. J., PETTES-DULER, A., NAYLIES, C., TAIEB, F., BOUCHENOT, C., HASHIM, S., ROUIMI, P., DESLANDE, M., LIPPI, Y. & MIREY, G. 2021. Chronic exposure to Cytolethal Distending Toxin (CDT) promotes a cGAS-dependent type I interferon response. *Cellular and Molecular Life Sciences,* 78**,** 6319-6335.

POROPATICH, K. O., WALKER, C. L. F. & BLACK, R. E. 2010. Quantifying the association between Campylobacter infection and Guillain-Barré syndrome: a systematic review. *Journal of health, population, and nutrition,* 28**,** 545.

PORTES, A. B., PANZENHAGEN, P., PEREIRA DOS SANTOS, A. M. & JUNIOR, C. A. C. 2023. Antibiotic Resistance in Campylobacter: A Systematic Review of South American Isolates. *Antibiotics,* 12**,** 548.

PRAAKLE-AMIN, K., ROASTO, M., KORKEALA, H. & HÄNNINEN, M.-L. 2007. PFGE genotyping and antimicrobial susceptibility of Campylobacter in retail poultry meat in Estonia. *International journal of food microbiology,* 114**,** 105-112.

PRATT, A. & KOROLIK, V. 2005. Tetracycline resistance of Australian Campylobacter jejuni and Campylobacter coli isolates. *Journal of Antimicrobial Chemotherapy,* 55**,** 452-460.

PREMARATHNE, J. M., ANUAR, A. S., THUNG, T. Y., SATHARASINGHE, D. A., HUAT, J. T. Y., RUKAYADI, Y., NAKAGUCHI, Y. & NISHIBUCHI, M. 2017a. Prevalence and antibiotic resistance against tetracycline in Campylobacter jejuni and C. coli in cattle and beef meat from Selangor, Malaysia. *Frontiers in Microbiology,* 8**,** 291616.

PREMARATHNE, J. M., ANUAR, A. S., THUNG, T. Y., SATHARASINGHE, D. A., JAMBARI, N. N., ABDUL-MUTALIB, N.-A., HUAT, J. T. Y., BASRI, D. F., RUKAYADI, Y. & NAKAGUCHI, Y. 2017b. Prevalence and antibiotic resistance against tetracycline in Campylobacter jejuni and C. coli in cattle and beef meat from Selangor, Malaysia. *Frontiers in Microbiology,* 8**,** 2254.

QUIGLEY, L., MCCARTHY, R., O'SULLIVAN, O., BERESFORD, T. P., FITZGERALD, G. F., ROSS, R. P., STANTON, C. & COTTER, P. D. 2013. The microbial content of raw and pasteurized cow milk as determined by molecular approaches. *Journal of dairy science,* 96**,** 4928-4937.

QUINO, W., CARO-CASTRO, J., HURTADO, V., FLORES-LEÓN, D., GONZALEZ-ESCALONA, N. & GAVILAN, R. G. 2022. Genomic analysis and antimicrobial resistance of Campylobacter jejuni and Campylobacter coli in Peru. *Frontiers in microbiology,* 12**,** 4181.

QUIÑONES, B., GUILHABERT, M. R., MILLER, W. G., MANDRELL, R. E., LASTOVICA, A. J. & PARKER, C. T. 2008. Comparative genomic analysis of clinical strains of Campylobacter jejuni from South Africa. *PLoS One,* 3**,** e2015.

RADOMSKA, K. A., WÖSTEN, M. M., ORDOÑEZ, S. R., WAGENAAR, J. A. & VAN PUTTEN, J. P. 2017. Importance of Campylobacter jejuni FliS and FliW in flagella biogenesis and flagellin secretion. *Frontiers in microbiology,* 8**,** 1060.

RAHIMI, E., ALIPOOR-AMROABADI, M. & KHAMESIPOUR, F. 2017. Investigation of prevalence of thermotolerant Campylobacter spp. in livestock feces. *Canadian Journal of Animal Science,* 97**,** 207-213.

RAHIMI, E., SEPEHRI, S. & MOMTAZ, H. 2013. Prevalence of Campylobacter species in milk and dairy products in Iran. *Revue de Médecine Vétérinaire,* 164**,** 283-288.

RAHMAN, H., KING, R. M., SHEWELL, L. K., SEMCHENKO, E. A., HARTLEY-TASSELL, L. E., WILSON, J. C., DAY, C. J. & KOROLIK, V. 2014. Characterisation of a multi-ligand binding chemoreceptor CcmL (Tlp3) of Campylobacter jejuni. *PLoS pathogens,* 10**,** e1003822.

RAHMAN, M. S. 2007. *Handbook of food preservation*, CRC press.

RAMATLA, T., TAWANA, M., MPHUTHI, M. B., ONYICHE, T. E., LEKOTA, K. E., MONYAMA, M. C., NDOU, R., BEZUIDENHOUT, C. & THEKISOE, O. 2022. Prevalence and antimicrobial resistance of Campylobacter species in South Africa: A "One Health" approach using systematic review and meta-analysis. *International Journal of Infectious Diseases*.

RAMIRES, T., WILSON, R., DA SILVA, W. P. & BOWMAN, J. P. 2023. Identification of pH-specific protein expression responses by Campylobacter jejuni strain NCTC 11168. *Research in Microbiology***,** 104061.

REDDY, S. & ZISHIRI, O. T. 2017. Detection and prevalence of antimicrobial resistance genes in Campylobacter spp. isolated from chickens and humans. *Onderstepoort Journal of Veterinary Research,* 84**,** 1-6.

REDONDO, N., CARROLL, A. & MCNAMARA, E. 2019. Molecular characterization of Campylobacter causing human clinical infection using whole-genome sequencing: Virulence, antimicrobial resistance and phylogeny in Ireland. *PLoS One,* 14**,** e0219088.

REES, J. H., SOUDAIN, S. E., GREGSON, N. A. & HUGHES, R. A. 1995. Campylobacter jejuni infection and Guillain–Barré syndrome. *New England Journal of Medicine,* 333**,** 1374-1379.

REGASSA, T. H., KOELSCH, R. K., WORTMANN, C. S., RANDLE, R. F. & ABUNYEWA, A. A. 2009. Antibiotic use in animal production: Environmental concerns.

REICHELT, B., SZOTT, V., STINGL, K., ROESLER, U. & FRIESE, A. 2023. Detection of Viable but Non-Culturable (VBNC)-Campylobacter in the Environment of Broiler Farms: Innovative Insights Delivered by Propidium Monoazide (PMA)-v-qPCR Analysis. *Microorganisms,* 11**,** 2492.

REICHLER, S., MURPHY, S., ERICKSON, A., MARTIN, N., SNYDER, A. & WIEDMANN, M. 2020. Interventions designed to control postpasteurization contamination in high-temperature, short-time-pasteurized fluid milk processing facilities: A case study on the effect of employee training, clean-in-place chemical modification, and preventive maintenance programs. *Journal of dairy science,* 103**,** 7569-7584.

REIS, L. P., MENEZES, L. D. M., LIMA, G. K., SANTOS, E. L. D. S., DORNELES, E. M. S., ASSIS, D. C. S. D., LAGE, A. P., CANÇADO, S. D. V. & FIGUEIREDO, T. C. D. 2018. Detection of Campylobacter spp. in chilled and frozen broiler carcasses comparing immunoassay, PCR and real time PCR methods. *Ciência Rural,* 48.

REUTER, M., ULTEE, E., TOSEAFA, Y., TAN, A. & VAN VLIET, A. H. 2018. Chemotactic motility and biofilm formation in Campylobacter jejuni are coordinated by the CheAYWVX system. *bioRxiv***,** 449850.

REUTER, M., ULTEE, E., TOSEAFA, Y., TAN, A. & VAN VLIET, A. H. 2020. Inactivation of the core cheVAWY chemotaxis genes disrupts chemotactic motility and organised biofilm formation in Campylobacter jejuni. *FEMS microbiology letters,* 367**,** fnaa198.

RIBARDO, D. A., KELLEY, B. R., JOHNSON, J. G. & HENDRIXSON, D. R. 2019. A chaperone for the stator units of a bacterial flagellum. *Mbio,* 10**,** 10.1128/mbio. 01732-19.

RIVERA-AMILL, V., KIM, B. J., SESHU, J. & KONKEL, M. E. 2001. Secretion of the virulence-associated Campylobacter invasion antigens from Campylobacter jejuni requires a stimulatory signal. *The Journal of infectious diseases,* 183**,** 1607-1616.

ROBINSON, D. 1981. Infective dose of Campylobacter jejuni in milk. *British medical journal (Clinical research ed.),* 282**,** 1584.

RODRIGUES, J. A., CHA, W., MOSCI, R. E., MUKHERJEE, S., NEWTON, D. W., LEPHART, P., SALIMNIA, H., KHALIFE, W., RUDRIK, J. T. & MANNING, S. D. 2021. Epidemiologic associations vary between tetracycline and fluoroquinolone resistant Campylobacter jejuni infections. *Frontiers in Public Health***,** 820.

ROMANIUK, P. J. & TRUST, T. J. 1987. Identification of Campylobacter species by Southern hybridization of genomic DNA using an oligonucleotide probe for 16S rRNA genes. *FEMS microbiology letters,* 43**,** 331-335.

ROSSLER, E., OLIVERO, C., SOTO, L. P., FRIZZO, L. S., ZIMMERMANN, J., ROSMINI, M. R., SEQUEIRA, G. J., SIGNORINI, M. L. & ZBRUN, M. V. 2020. Prevalence, genotypic diversity and detection of virulence genes in thermotolerant Campylobacter at different stages of the poultry meat supply chain. *International journal of food microbiology,* 326**,** 108641.

RUBINCHIK, S., SEDDON, A. & KARLYSHEV, A. V. 2012. Molecular mechanisms and biological role of Campylobacter jejuni attachment to host cells. *European Journal of Microbiology and Immunology,* 2**,** 32-40.

RUIZ-PALACIOS, G. M. 2007. The health burden of Campylobacter infection and the impact of antimicrobial resistance: playing chicken. The University of Chicago Press.

RUSHTON, S. P., SANDERSON, R. A., DIGGLE, P. J., SHIRLEY, M. D., BLAIN, A. P., LAKE, I., MAAS, J. A., REID, W. D., HARDSTAFF, J. & WILLIAMS, N. 2019. Climate, human behaviour or environment: individual-based modelling of Campylobacter seasonality and strategies to reduce disease burden. *Journal of translational medicine,* 17**,** 1-13.

SAHIN, O., MORISHITA, T. Y. & ZHANG, Q. 2002. Campylobacter colonization in poultry: sources of infection and modes of transmission. *Animal Health Research Reviews,* 3**,** 95-105.

SAILS, A. D., SWAMINATHAN, B. & FIELDS, P. I. 2003. Clonal complexes of Campylobacter jejuni identified by multilocus sequence typing correlate with strain associations identified by multilocus enzyme electrophoresis. *Journal of clinical microbiology,* 41**,** 4058-4067.

SALIHU, M., JUNAIDU, A., MAGAJI, A. & RABIU, Z. 2010. Study of Campylobacter in raw cow milk in Sokoto State, Nigeria. *Br. J. Dairy Sci,* 1**,** 1-5.

SALIHU, M., JUNAIDU, A., OBOEGBULEM, S., EGWU, G., TAMBUWAL, F. & YAKUBU, Y. 2009. Prevalence of Campylobacter species in apparently healthy goats in Sokoto state (Northwestern) Nigeria. *Afr. J. Microbiol. Res,* 3**,** 572-574.

SAME, R. G. & TAMMA, P. D. 2018. Campylobacter infections in children. *Pediatrics in review,* 39**,** 533-541.

SAMUELSON, D. R., EUCKER, T. P., BELL, J. A., DYBAS, L., MANSFIELD, L. S. & KONKEL, M. E. 2013. The Campylobacter jejuni CiaD effector protein activates MAP kinase signaling pathways and is required for the development of disease. *Cell Communication and Signaling,* 11**,** 1-15.

SARHANGI, M., BAKHSHI, B. & PEERAEYEH, S. N. 2021. High prevalence of Campylobacter jejuni CC21 and CC257 clonal complexes in children with gastroenteritis in Tehran, Iran. *BMC Infectious Diseases,* 21**,** 1-13.

SCALLAN, E., HOEKSTRA, R., MAHON, B., JONES, T. & GRIFFIN, P. 2015. An assessment of the human health impact of seven leading foodborne pathogens in the United States using disability adjusted life years. *Epidemiology & Infection,* 143**,** 2795-2804.

SCALLAN WALTER, E. J., CRIM, S. M., BRUCE, B. B. & GRIFFIN, P. M. 2020. Incidence of Campylobacter-associated Guillain-Barre Syndrome estimated from health insurance data. *Foodborne pathogens and disease,* 17**,** 23-28.

SCHIELKE, A., ROSNER, B. M. & STARK, K. 2014. Epidemiology of campylobacteriosis in Germany–insights from 10 years of surveillance. *BMC infectious diseases,* 14**,** 1-8.

SCHILDT, M., SAVOLAINEN, S. & HÄNNINEN, M.-L. 2006. Long-lasting Campylobacter jejuni contamination of milk associated with gastrointestinal illness in a farming family. *Epidemiology & Infection,* 134**,** 401-405.

SCHRÖDER, W. & MOSER, I. 1997. Primary structure analysis and adhesion studies on the major outer membrane protein of Campylobacter jejuni. *FEMS microbiology letters,* 150**,** 141-147.

SCHWAN, M., KHALEDI, A., WILLGER, S., PAPENFORT, K., GLATTER, T., HÄUSSLER, S. & THORMANN, K. M. 2022. Constitutive production of flagellar proteins is required for proper flagellation in Shewanella putrefaciens. *bioRxiv***,** 2022.07. 21.500047.

SCHWEITZER, P. M., SUSTA, L., VARGA, C., BRASH, M. L. & GUERIN, M. T. 2021. Demographic, Husbandry, and Biosecurity Factors Associated with the Presence of Campylobacter spp. in Small Poultry Flocks in Ontario, Canada. *Pathogens,* 10**,** 1471.

SEBALD, M. & VERON, M. Base DNA content and classification of Vibrios. Annales de l'Institut Pasteur, 1963. 897-910.

SEEMANN, T. 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics,* 30**,** 2068-2069.

SEEMANN, T. 2016. ABRicate: mass screening of contigs for antibiotic resistance genes. *San Francisco, CA: GitHub*.

SEJVAR, J. J., BAUGHMAN, A. L., WISE, M. & MORGAN, O. W. 2011. Population incidence of Guillain-Barré syndrome: a systematic review and meta-analysis. *Neuroepidemiology,* 36**,** 123-133.

SHANKER, S., LEE, A. & SORRELL, T. 1990. Horizontal transmission of Campylobacter jejuni amongst broiler chicks: experimental studies. *Epidemiology & Infection,* 104**,** 101-110.

SHEN, Z., WANG, Y., ZHANG, Q. & SHEN, J. 2018. Antimicrobial resistance in Campylobacter spp. *Microbiology spectrum,* 6**,** 6.2. 11.

SHER, A. A., JEROME, J. P., BELL, J. A., YU, J., KIM, H. Y., BARRICK, J. E. & MANSFIELD, L. S. 2020. Experimental Evolution of Campylobacter jejuni Leads to Loss of Motility, rpo N (σ54) Deletion and Genome Reduction. *Frontiers in Microbiology,* 11**,** 579989.

SIERRA-ARGUELLO, Y. M., MORGAN, R. B., PERDONCINI, G., LIMA, L. M., GOMES, M. J. P. & NASCIMENTO, V. P. D. 2015. Resistance to β-lactam and tetracycline in Campylobacter spp. isolated from broiler slaughterhouses in southern Brazil. *Pesquisa Veterinária Brasileira,* 35**,** 637-642.

SIERRA-ARGUELLO, Y. M., PERDONCINI, G., RODRIGUES, L. B., RUSCHEL DOS SANTOS, L., APELLANIS BORGES, K., QUEDI FURIAN, T., PIPPI SALLE, C. T., DE SOUZA MORAES, H. L., PEREIRA GOMES, M. J. & PINHEIRO DO NASCIMENTO, V. 2021. Identification of pathogenic genes in Campylobacter jejuni isolated from broiler carcasses and broiler slaughterhouses. *Scientific Reports,* 11**,** 4588.

SILVA, J., LEITE, D., FERNANDES, M., MENA, C., GIBBS, P. A. & TEIXEIRA, P. 2011a. Campylobacter spp. as a foodborne pathogen: a review. *Frontiers in microbiology,* 2**,** 200.

SILVA, J., LEITE, D., FERNANDES, M., MENA, C., GIBBS, P. A. & TEIXEIRA, P. 2011b. Campylobacter spp. as a Foodborne Pathogen: A Review. *Front Microbiol,* 2**,** 200.

SINGH, H., RATHORE, R., SINGH, S. & CHEEMA, P. S. 2011. Comparative analysis of cultural isolation and PCR based assay for detection of Campylobacter jejuni in food and faecal samples. *Brazilian Journal of Microbiology,* 42**,** 181-186.

SITHOLE, V., AMOAKO, D. G., ABIA, A. L. K., PERRETT, K., BESTER, L. A. & ESSACK, S. Y. 2021. Occurrence, antimicrobial resistance, and molecular characterization of Campylobacter spp. in intensive pig production in South Africa. *Pathogens,* 10**,** 439.

SKIRROW, M. 1977. Campylobacter enteritis: a" new" disease. *Br Med J,* 2**,** 9-11.

SKIRROW, M. B. 2006. John McFadyean and the centenary of the first isolation of Campylobacter species. *Clinical infectious diseases,* 43**,** 1213-1217.

SKRZYPEK, R., WÓJTOWSKI, J. & FAHR, R.-D. 2003. Hygienic quality of cow bulk tank milk depending on the method of udder preparation for milking. *Archives Animal Breeding,* 46**,** 405-411.

SNAIDR, J., AMANN, R., HUBER, I., LUDWIG, W. & SCHLEIFER, K.-H. 1997. Phylogenetic analysis and in situ identification of bacteria in activated sludge. *Applied and environmental microbiology,* 63**,** 2884-2896.

SNELLING, W., MATSUDA, M., MOORE, J. & DOOLEY, J. 2005. Campylobacter jejuni. *Letters in applied microbiology,* 41**,** 297-302.

ŠOPREK, S., DUVNJAK, S., KOMPES, G., JURINOVIĆ, L. & TAMBIĆ ANDRAŠEVIĆ, A. 2022. Resistome Analysis of Campylobacter jejuni Strains Isolated from Human Stool and Primary Sterile Samples in Croatia. *Microorganisms,* 10**,** 1410.

SOTO-BELTRA, M., LEE, B. G., AMEZQUITA-LOPEZ, B. A. & QUINONES, B. 2023. Overview of methodologies for the culturing, recovery and detection of Campylobacter. *International Journal of Environmental Health Research,* 33**,** 307-323.

SOUVOROV, A., AGARWALA, R. & LIPMAN, D. J. 2018. SKESA: strategic k-mer extension for scrupulous assemblies. *Genome biology,* 19**,** 153.

SPILLER1, C. S., E CAMPBELL1, M HASTINGS3, G DUKES4, P WHORWELL3, I HALL2, N DUROUDIER * 2011. Identifying and testing candidate genes underlying the inflammatory basis of irritable bowel syndrome.

BMJ Journals.

SPROSTON, E. L., WIMALARATHNA, H. M. & SHEPPARD, S. K. 2018. Trends in fluoroquinolone resistance in Campylobacter. *Microbial genomics,* 4.

STAHL, M., BUTCHER, J. & STINTZI, A. 2012. Nutrient acquisition and metabolism by Campylobacter jejuni. *Frontiers in cellular and infection microbiology,* 2**,** 5.

STAMPI, S., VAROLI, O., DE LUCA, G. & ZANETTI, F. 1992. Occurrence, removal and seasonal variation of" thermophilic" campylobacters in a sewage treatment plant in Italy. *Zentralblatt fur Hygiene und Umweltmedizin= International journal of hygiene and environmental medicine,* 193**,** 199-210.

STAMPI, S., VAROLI, O., ZANETTI, F. & DE LUCA, G. 1993. Arcobacter cryaerophilus and thermophilic campylobacters in a sewage treatment plant in Italy: two secondary treatments compared. *Epidemiology & Infection,* 110**,** 633-639.

STANDARDIZATION, I. O. F. 2006. *Microbiology of food and animal feeding stuffs-horizontal method for detection and enumeration of Campylobacter spp*, ISO.

STANDARDIZATION, I. O. F. 2017a. *Microbiology of the Food Chain-Horizontal Method for Detection and Enumeration of Campylobacter Spp*, ISO.

STANDARDIZATION, I. O. F. 2017b. Microbiology of the food chain—horizontal method for detection and enumeration of Campylobacter spp.—part 1: Detection method. ISO 10272-1: 2017. International Organization for Standardization Geneva.

STANLEY, K. & JONES, K. 2003. Cattle and sheep farms as reservoirs of Campylobacter. *Journal of applied microbiology,* 94**,** 104-113.

STANLEY, K., WALLACE, J., CURRIE, J., DIGGLE, P. J. & JONES, K. 1998. The seasonal variation of thermophilic campylobacters in beef cattle, dairy cattle and calves. *Journal of Applied Microbiology,* 85**,** 472-480.

STRAKOVA, N., SHAGIEVA, E., OVESNA, P., KORENA, K., MICHOVA, H., DEMNEROVA, K., KOLACKOVA, I. & KARPISKOVA, R. 2022. The effect of environmental conditions on the occurrence of Campylobacter jejuni and Campylobacter coli in wastewater and surface waters. *Journal of Applied Microbiology,* 132**,** 725-735.

STRINGER, A., CHALA, G., EGUALE, T., ABUNNA, F. & ASRAT, D. 2021. Identification and characterization of Campylobacter species in livestock, humans and water in livestock owning households of peri-urban Addis Ababa, Ethiopia: A One Health approach. *Frontiers in public health***,** 1584.

SU, Y., ALTER, T. & GÖLZ, G. 2023. Motility related gene expression of Campylobacter jejuni NCTC 11168 derived from high viscous media. *European Journal of Microbiology and Immunology*.

TABOADA, E. N., CLARK, C. G., SPROSTON, E. L. & CARRILLO, C. D. 2013. Current methods for molecular typing of Campylobacter species. *Journal of microbiological methods,* 95**,** 24-31.

TAFA, B., SEWUNET, T., TASSEW, H. & ASRAT, D. 2014. Isolation and antimicrobial susceptibility patterns of Campylobacter species among diarrheic children at Jimma, Ethiopia. *International journal of bacteriology,* 2014.

TAGHIZADEH, M., NEMATOLLAHI, A., BASHIRY, M., JAVANMARDI, F., MOUSAVIL, M. & HOSSEINI, H. 2022. The global prevalence of campylobacter spp. in milk a systematic review and meta-analysis. *International Dairy Journal***,** 105423.

TAKAKURA, W., KUDARAVALLI, P., CHATTERJEE, C., PIMENTEL, M. & RIDDLE, M. S. 2022. Campylobacter infection and the link with Irritable Bowel Syndrome: on the pathway towards a causal association. *Pathogens and Disease,* 80**,** ftac003.

TALUKDAR, P. K., NEGRETTI, N. M., TURNER, K. L. & KONKEL, M. E. 2020. Molecular dissection of the Campylobacter jejuni CadF and FlpA virulence proteins in binding to host cell fibronectin. *Microorganisms,* 8**,** 389.

TANG, Y., FANG, L., XU, C. & ZHANG, Q. 2017a. Antibiotic resistance trends and mechanisms in the foodborne pathogen, Campylobacter. *Animal health research reviews,* 18**,** 87-98.

TANG, Y., JIANG, Q., TANG, H., WANG, Z., YIN, Y., REN, F., KONG, L., JIAO, X. & HUANG, J. 2020. Characterization and prevalence of Campylobacter spp. from broiler chicken rearing period to the slaughtering process in Eastern China. *Frontiers in Veterinary Science,* 7**,** 227.

TANG, Y., SAHIN, O., PAVLOVIC, N., LEJEUNE, J., CARLSON, J., WU, Z., DAI, L. & ZHANG, Q. 2017b. Rising fluoroquinolone resistance in Campylobacter isolated from feedlot cattle in the United States. *Sci Rep,* 7**,** 494.

TAREMI, M., DALLAL, M. M. S., GACHKAR, L., MOEZARDALAN, S., ZOLFAGHARIAN, K. & ZALI, M. R. 2006. Prevalence and antimicrobial resistance of Campylobacter isolated from retail raw chicken and beef meat, Tehran, Iran. *International journal of food microbiology,* 108**,** 401-403.

TEDERSOO, T., ROASTO, M., MÄESAAR, M., HÄKKINEN, L., KISAND, V., IVANOVA, M., VALLI, M. H. & MEREMÄE, K. 2022a. Antibiotic resistance in campylobacter spp. Isolated from broiler chicken meat and human patients in Estonia. *Microorganisms,* 10**,** 1067.

TEDERSOO, T., ROASTO, M., MÄESAAR, M., KISAND, V., IVANOVA, M. & MEREMÄE, K. 2022b. The prevalence, counts, and MLST genotypes of Campylobacter in poultry meat and genomic comparison with clinical isolates. *Poultry Science,* 101**,** 101703.

TEREFE, Y., DEBLAIS, L., GHANEM, M., HELMY, Y. A., MUMMED, B., CHEN, D., SINGH, N., AHYONG, V., KALANTAR, K. & YIMER, G. 2020. Co-occurrence of Campylobacter Species in Children From Eastern Ethiopia, and Their Association With Environmental Enteric Dysfunction, Diarrhea, and Host Microbiome. *Frontiers in Public Health,* 8**,** 99.

THABANE, M., KOTTACHCHI, D. & MARSHALL, J. 2007. Systematic review and meta-analysis: the incidence and prognosis of post-infectious irritable bowel syndrome. *Alimentary pharmacology & therapeutics,* 26**,** 535-544.

THÉPAULT, A., POEZEVARA, T., QUESNE, S., ROSE, V., CHEMALY, M. & RIVOAL, K. 2018. Prevalence of thermophilic Campylobacter in cattle production at slaughterhouse level in France and link between C. jejuni bovine strains and campylobacteriosis. *Frontiers in Microbiology,* 9**,** 471.

TIGABU, E., ASRAT, D., KASSA, T., SINMEGN, T., MOLLA, B. & GEBREYES, W. 2015. Assessment of Risk Factors in Milk Contamination with S taphylococcus aureus in Urban and Peri-Urban Small-Holder Dairy Farming in Central E thiopia. *Zoonoses and Public Health,* 62**,** 637-643.

TONG, S., MA, L., RONHOLM, J., HSIAO, W. & LU, X. 2021. Whole genome sequencing of Campylobacter in agri-food surveillance. *Current Opinion in Food Science,* 39**,** 130-139.

TRESSE, O., ALVAREZ-ORDÓÑEZ, A. & CONNERTON, I. F. 2017. About the foodborne pathogen Campylobacter. Frontiers Media SA.

TUFA, T. B., REGASSA, F., AMENU, K., STEGEMAN, J. & HOGEVEEN, H. 2023. Livestock producers' knowledge, attitude, and behavior (KAB) regarding antimicrobial use in Ethiopia. *Frontiers in Veterinary Science,* 10**,** 1167847.

VAN BOECKEL, T. P., BROWER, C., GILBERT, M., GRENFELL, B. T., LEVIN, S. A., ROBINSON, T. P., TEILLANT, A. & LAXMINARAYAN, R. 2015. Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences,* 112**,** 5649-5654.

VANDAMME, P. 2000. Taxonomy of the family Campylobacteriaceae. *Campylobacter,* 2**,** 3-26.

VANDAMME, P., DEBRUYNE, L., DE BRANDT, E. & FALSEN, E. 2010. Reclassification of Bacteroides ureolyticus as Campylobacter ureolyticus comb. nov., and emended description of the genus Campylobacter. *International journal of systematic and evolutionary microbiology,* 60**,** 2016-2022.

VARSAKI, A., ORTIZ, S., SANTORUM, P., LÓPEZ, P., LÓPEZ-ALONSO, V. & MARTÍNEZ-SUÁREZ, J. V. 2022. Genetic Diversity, Antimicrobial Resistance and Survival upon Manure Storage of Campylobacter jejuni Isolated from Dairy Cattle Farms in the Cantabric Coast of Spain. *Zoonotic Diseases,* 2**,** 82-94.

VÁZQUEZ-LASLOP, N. & MANKIN, A. S. 2018. How macrolide antibiotics work. *Trends in biochemical sciences,* 43**,** 668-684.

VELAZQUEZ-ORDOÑEZ, V., VALLADARES-CARRANZA, B., TENORIO-BORROTO, E., TALAVERA-ROJAS, M., VARELA-GUERRERO, J., ACOSTA-DIBARRAT, J., PUIGVERT, F., GRILLE, L., GONZALEZ REVELLO, A. & PAREJA, L. 2019. Microbial contamination in milk quality and health risk of the consumers of raw milk and dairy products.

VELTCHEVA, D., COLLES, F. M., VARGA, M., MAIDEN, M. C. & BONSALL, M. B. 2022. Emerging patterns of fluoroquinolone resistance in Campylobacter jejuni in the UK [1998–2018]. *Microbial Genomics,* 8.

WAFULA, W. N., MATOFARI, W. J., NDUKO, M. J. & LAMUKA, P. 2016. Effectiveness of the sanitation regimes used by dairy actors to control microbial contamination of plastic jerry cans' surfaces. *International Journal of Food Contamination,* 3**,** 1-8.

WAGENAAR, J. A., NEWELL, D. G., KALUPAHANA, R. S. & MUGHINI-GRAS, L. 2023. Campylobacter: animal reservoirs, human infections, and options for control. *Zoonoses: infections affecting humans and animals*. Springer.

WAGLEY, S., NEWCOMBE, J., LAING, E., YUSUF, E., SAMBLES, C. M., STUDHOLME, D. J., LA RAGIONE, R. M., TITBALL, R. W. & CHAMPION, O. L. 2014. Differences in carbon source utilisation distinguish Campylobacter jejuni from Campylobacter coli. *BMC microbiology,* 14**,** 1-10.

WALKER, B. 2016. Seasonal Weather Assessment for Ethiopia during March–July 2016. *London: Government of the United Kingdom*.

WALKER, T. A., GRAINGER, R., QUIRKE, T., ROOS, R., SHERWOOD, J., MACKERETH, G., KIEDRZYNSKI, T., EYRE, R., PAINE, S. & WOOD, T. 2022. Reactive arthritis incidence in a community cohort following a large waterborne campylobacteriosis outbreak in Havelock North, New Zealand. *BMJ open,* 12**,** e060173.

WALLIS, M. 1994. The pathogenesis of Campylobacter jejuni. *British journal of biomedical science,* 51**,** 57-64.

WANG, G., CLARK, C. G., TAYLOR, T. M., PUCKNELL, C., BARTON, C., PRICE, L., WOODWARD, D. L. & RODGERS, F. G. 2002. Colony multiplex PCR assay for identification and differentiation of Campylobacter jejuni, C. coli, C. lari, C. upsaliensis, and C. fetus subsp. fetus. *Journal of clinical microbiology,* 40**,** 4744-4747.

WANG, L., JEON, B., SAHIN, O. & ZHANG, Q. 2009. Identification of an arsenic resistance and arsenic-sensing system in Campylobacter jejuni. *Applied and Environmental Microbiology,* 75**,** 5064-5073.

WANJA, D. W., MBUTHIA, P. G., ABOGE, G. O. & BEBORA, L. C. 2022. Seasonal Prevalence and Molecular Identification of Thermophilic Campylobacter from Chicken, Cattle, and Respective Drinking Water in Kajiado County, Kenya. *International Journal of Microbiology,* 2022.

WARNER, D. P., BRYNER, J. H. & BERAN, G. W. 1986. Epidemiologic study of campylobacteriosis in Iowa cattle and the possible role of unpasteurized milk as a vehicle of infection. *Am J Vet Res,* 47**,** 254-8.

WHITEHOUSE, C. A., ZHAO, S. & TATE, H. 2018. Antimicrobial resistance in Campylobacter species: mechanisms and genomic epidemiology. *Advances in applied microbiology*. Elsevier.

WHO 2020a. World Health Organization (WHO). Campylobacter, World Health Organisation web page, 2020. Available at: https://www.who.int/news-room/fact-sheets/detail/campylobacter. [Accessed: 9 September 2022.

WHO 2020b. World Health Organization. Campylobacteriosis.Avalable from: https://www.who.int/news-room/fact-sheets/detail/campylobacter. Acessed on 3/22/2023

WIECZOREK, K., BOCIAN, Ł. & OSEK, J. 2020. Prevalence and antimicrobial resistance of Campylobacter isolated from carcasses of chickens slaughtered in Poland–a retrospective study. *Food control,* 112**,** 107159.

WIECZOREK, K., DENIS, E., LACHTARA, B. & OSEK, J. 2017. Distribution of Campylobacter jejuni multilocus sequence types isolated from chickens in Poland. *Poultry Science,* 96**,** 703-709.

WIECZOREK, K. & OSEK, J. 2013. Antimicrobial resistance mechanisms among Campylobacter. *BioMed research international,* 2013.

WIECZOREK, K. & OSEK, J. 2015. A five-year study on prevalence and antimicrobial resistance of Campylobacter from poultry carcasses in Poland. *Food microbiology,* 49**,** 161-165.

WIECZOREK, K., WOŁKOWICZ, T. & OSEK, J. 2018. Antimicrobial resistance and virulence-associated traits of Campylobacter jejuni isolated from poultry food chain and humans with diarrhea. *Frontiers in microbiology,* 9**,** 1508.

WILKINSON, D. A., O'DONNELL, A. J., AKHTER, R. N., FAYAZ, A., MACK, H. J., ROGERS, L. E., BIGGS, P. J., FRENCH, N. P. & MIDWINTER, A. C. 2018. Updating the genomic taxonomy and epidemiology of Campylobacter hyointestinalis. *Scientific reports,* 8**,** 2393.

WILLISON, H. J., JACOBS, B. C. & VAN DOORN, P. A. 2016. Guillain-barre syndrome. *The Lancet,* 388**,** 717-727.

WILSON, M., WILSON, P. J., WILSON, M. & WILSON, P. J. 2021. Gastroenteritis Due to Campylobacter. *Close Encounters of the Microbial Kind: Everything You Need to Know About Common Infections***,** 439-450.

WOLDEMARIAM, T., ASRAT, D. & ZEWDE, G. 2009. Prevalence of thermophilic Campylobacter species in carcasses from sheep and goats in an abattoir in Debre Zeit area, Ethiopia. *Ethiopian Journal of Health Development,* 23.

WYSOK, B., URADZIŃSKI, J. & WOJTACKA, J. 2015. Determination of the cytotoxic activity of Campylobacter strains isolated from bovine and swine carcasses in north-eastern Poland. *Polish Journal of Veterinary Sciences*.

WYSOK, B., WISZNIEWSKA-ŁASZCZYCH, A., URADZIŃSKI, J. & SZTEYN, J. 2011. Prevalence and antimicrobial resistance of Campylobacter in raw milk in the selected areas of Poland. *Polish journal of veterinary sciences*.

YAN, R., M'IKANATHA, N. M., NACHAMKIN, I., HUDSON, L. K., DENES, T. G. & KOVAC, J. 2023. Prevalence of ciprofloxacin resistance and associated genetic determinants differed among Campylobacter isolated from human and poultry meat sources in Pennsylvania. *Food Microbiology,* 116**,** 104349.

YANG, C., JIANG, Y., HUANG, K., ZHU, C. & YIN, Y. 2003. Application of real-time PCR for quantitative detection of Campylobacter jejuni in poultry, milk and environmental water. *FEMS Immunology & Medical Microbiology,* 38**,** 265-271.

YAO, H., JIAO, D., ZHAO, W., LI, A., LI, R. & DU, X.-D. 2020. Emergence of a Novel tet (L) Variant in Campylobacter spp. of Chicken Origin in China. *Antimicrobial Agents and Chemotherapy,* 65**,** 10.1128/aac. 01622-20.

YEOW, M., LIU, F., MA, R., WILLIAMS, T. J., RIORDAN, S. M. & ZHANG, L. 2020. Analyses of energy metabolism and stress defence provide insights into Campylobacter concisus growth and pathogenicity. *Gut Pathogens,* 12**,** 1-13.

YOUNG, K. T., DAVIS, L. M. & DIRITA, V. J. 2007. Campylobacter jejuni: molecular biology and pathogenesis. *Nature Reviews Microbiology,* 5**,** 665-679.

YUKI, N., YAMADA, M., SATO, S., OHAMA, E., KAWASE, Y., IKUTA, F. & MIYATAKE, T. 1993. Association of IgG anti-GD1a antibody with severe Guillain–Barré syndrome. *Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine,* 16**,** 642-647.

ZAUTNER, A. E., MALIK TAREEN, A., GROß, U. & LUGERT, R. 2012. Chemotaxis in Campylobacter jejuni. *European Journal of Microbiology and Immunology,* 2**,** 24-31.

ZEINHOM, M. M., ABDEL-LATEF, G. K. & CORKE, H. 2021. Prevalence, characterization, and control of Campylobacter jejuni isolated from raw milk, cheese, and human stool samples in beni-suef governorate, Egypt. *Foodborne Pathogens and Disease,* 18**,** 322-330.

ZENEBE, T., ZEGEYE, N. & EGUALE, T. 2020. Prevalence of Campylobacter species in human, animal and food of animal origin and their antimicrobial susceptibility in Ethiopia: a systematic review and meta-analysis. *Annals of clinical microbiology and antimicrobials,* 19**,** 1-11.

ZENG, X., BROWN, S., GILLESPIE, B. & LIN, J. 2014. A single nucleotide in the promoter region modulates the expression of the β-lactamase OXA-61 in Campylobacter jejuni. *Journal of Antimicrobial Chemotherapy,* 69**,** 1215-1223.

ZHANG, D., ZHANG, X., LYU, B., TIAN, Y., HUANG, Y., LIN, C., YAN, H., JIA, L., QU, M. & WANG, Q. 2023. Genomic Analysis and Antimicrobial Resistance of Campylobacter jejuni Isolated from Diarrheal Patients—Beijing Municipality, China, 2019–2021. *China CDC Weekly,* 5**,** 424.

ZHANG, J., KONKEL, M. E., GÖLZ, G. & LU, X. 2022. Campylobacter-associated food safety. Frontiers Media SA.

ZHANG, P., ZHANG, X., LIU, Y., JIANG, J., SHEN, Z., CHEN, Q. & MA, X. 2020. Multilocus sequence types and antimicrobial resistance of Campylobacter jejuni and C. coli isolates of human patients from Beijing, China, 2017–2018. *Frontiers in Microbiology,* 11**,** 554784.

ZHAO, X., NORRIS, S. J. & LIU, J. 2014. Molecular architecture of the bacterial flagellar motor in cells. *Biochemistry,* 53**,** 4323-4333.

ZHONG, X., WU, Q. & ZHANG, J. 2020. Campylobacter jejuni biofilm formation under aerobic conditions and inhibition by ZnO nanoparticles. *Frontiers in Microbiology,* 11**,** 499084.

ZILBAUER, M., DORRELL, N., WREN, B. W. & BAJAJ-ELLIOTT, M. 2008. Campylobacter jejuni-mediated disease pathogenesis: an update. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 102**,** 123-129.

ZIRNSTEIN, G., LI, Y., SWAMINATHAN, B. & ANGULO, F. 1999. Ciprofloxacin resistance in Campylobacter jejuni isolates: detection of gyrA resistance mutations by mismatch amplification mutation assay PCR and DNA sequence analysis. *Journal of clinical microbiology,* 37**,** 3276-3280.

ANNEX

Annex 1: Questionnaire to be filled by milk producers/ farmers, milk collectors, milk processors, and cottage cheese producers and retailers (Sections A to D)

Addis Ababa University

Institute of Biotechnology

General Information and Consent

Dear respondent, good morning/Good afternoon. Thank you for your interest in talking with me today. I am Mr. Abera Admasie. I am a Ph.D. student at Addis Ababa University conducting a study to assess practices that can be risk factors for raw milk quality and safety at the collectors level. The purpose of my visit today is to collect information from you on the aforementioned issue. This study will be conducted in four regions (Oromia, Amhara, and SNNP) of Ethiopia, across all the milk value chain actors. The general conclusions of the study may be used to help formulate government policy recommendations for improving dairy production and the safety and protection of human health in the country. If you are willing to participate in the study, I will ask you a few questions for 20-30 minutes. Your name will be confidential and will never be used in connection with any of your information. You do not have to answer any question that you are not comfortable with, and you may end this task any time you want to. However, your honest answers to these questions will help us in a better understanding of the safety of milk and dairy products and will eventually help in designing and implementing appropriate interventions to alleviate related problems. Hence, we greatly appreciate it if you would be willing to participate in the

Are you willing to participate in the study?

○ Yes ○ No

Section A: Preliminary information

1) Name of interviewer_____

2) Date and time_____

3) Collection center code number_____

4) Region

○ Oromia ○ SNNP ○ Amhara

5) Town/District/Zone__

○ Selale ○ Debre Berhan ○ Hawassa

○ Wolmera ○ Debre Markos ○ Dilla

○ Debrezeit ○ Bahir Dar ○ Yirgalem

○ Assela ○ Gonder ○ Woliyta

5) Site/ Woreda/ Kebeles_____

6) Record your current location/ GPS coordinates' _____

A. Latitude (X.Y °) _____
B. Longitude (X.Y °) _____
C. Altitude (M) _____
D. Accuracy (M) _____

Section B: Transportation and collection center conditions (milk collection and transportation system infrastructure, equipment, and milk cooling and storage)

1. Do they maintain the temperature during?

○ No ○ Yes

2. Do you filter milk upon reception?

○ No ○ Yes

3. What do you use for filtration?

○ Piece of cloth ○ Plastic filter ○ Wire meshes

4. Have a cooling system to preserve your milk?

○ No ○ Yes

5. What does the collection house look like?

○ Concrete floor ○ Soil floor

6. What is your primary supply of washing water?

○ Groundwater ○ Tap water

7. What type of Milk Handling Equipment do you use?

7.1. Plastic containers

○ No ○ Yes

7.2. Aluminum cans

○ No ○ Yes

7.3. Mazzi cans

○ No ○ Yes

8. What kind of cleaning do they use?

8.1. Water only

○ No ○ Yes

8.2. Cold water and soap/detergent

○ No ○ Yes

8.3. Warm water and soap/detergent

○ No ○ Yes

9. How is the milk handling Equipment stored

9.1. Upright and open

○ No ○ Yes

9.2. Upright but covered

○ No ○ Yes

9.3. Upside down in contact with the ground

○ No ○ Yes

9.4. Upside down on the shelf

○ No ○ Yes

Section C: Milking equipment storage, cooling, and transportation in milk producers

1. What does a milking house look like?

○ Concrete floor barn ○ Soil floor barn

2. Observation: How is the hygienic condition of the cattle barn?

○ Good ○ Poor

3. A major source of water for washing

○ Groundwater ○ Rainwater ○ Tap water

○ Pump water ○ River water

4. Do you wash the udder and teats of the cows?

○ No ○ Yes

5. What do you use to wash udder and teats?

○ Cold water ○ Warm water

6. Do you dry the washed udder and teats by using a dry cloth

○ No ○ Yes

7. Did your cows ever face mastitis?

○ No ○ Yes

8. Do you filter after milking?

○ No ○ Yes

9. What do you use for filtration?

○ Piece of cloth ○ Plastic filter ○ Wire mesh

10. What type of milk handling equipment do you majorly use for milk handling?

○ Aluminum cans ○ Mazzi can ○ Plastic containers

11. Do you have a refrigerator as a cooling mechanism to store until sale?

○ No ○ Yes

Section D: Knowledge gap assessment of the processing plant and milk reception and pre-pasteurization conditions at the processing plant (food safety training, milk quality test, and hygienic condition of the processing facility).

1. Did you attend basic food safety training?

○ No ○ Yes

2. Is the storage area free of trash?

○ No ○ Yes

3. What is the source of water for washing?

○ Groundwater ○ Tap water

4. Do you restrict milk handlers who are with illnesses and infections in factories

○ No ○ Yes

5. Do you apply a cleaning-in-place (CIP) system during milk processing?

○ No ○ Yes

6. Do you dismantle and clean the processing lines during milk processing?

○ No ○ Yes

7. Do you calibrate your system to ensure the efficiency of the pasteurization?

○ No ○ Yes

8. Do you have a mechanism to check the efficiency of pasteurization?

○ No ○ Yes

9. What do you use to check the efficiency of pasteurization?

a. Phosphatase test

○ No ○ Yes

b. Microbiological test

○ No ○ Yes

10. Do you have a cold chain transportation system

○ No ○ Yes

Section E: Storage conditions level, training, and transportation at the milk retail

1. Have you attended any training related to food safety and milk handling?

○ No ○ Yes

2. What do they use as a means of transportation of milk?

○ Cold trucks ○ Four-wheel drives

3. Do they maintain the temperature during milk transportation

○ No ○ Yes

4. What do you use to maintain the temperature of the pasteurized milk?

○ Refrigerator ○ Deep freezers

5. Do you have a separate refrigerator to store the pasteurized milk?

○ No ○ Yes

Annex 2: Composition and preparation of media and standard operating procedure for detection of *Campylobacter* spp.

Equipment and materials

○ Autoclave or sterilization equipment
○ Sterile loops 10 µl
○ Sterile cylinders 225 or 500 ml
○ Pipettor 20-200 µl
○ Pipettor 100-1000 µl
○ Sterile pipette tips 1000 µl
○ Sterile pipette tips 200 µl
○ Sterile serological pipettes 25 ml
○ Serological pipette controller
○ Petri dishes
○ Refrigerator
○ Vortex
○ Stomacher
○ Scale
○ Filter stomacher bags
○ Sterile universal 9 ml tubes
○ 2 ml cryovials or cryobeads
○ Biosafety autoclave bags
○ Autoclave tape
○ Filters
○ Cotton swabs
○ Anaerobic jars
○ Autoclavable
○ Weigh boats
○ A heating block
○ Microcentrifuge tubes 1.5 ml
○ PCR tubes

1. Preston broth preparation and Primary enrichment (food samples):

Procedure:

a) The enrichment medium was taken out of the fridge at least one hour before use to adjust its temperature to room temperature (this will reduce the stress on potentially injured cells).
b) A 10 ml +/- 0.1 ml (for liquid samples) or 10 g +/- 0.1 g (for solid samples) of a food sample was aseptically measured and placed into a sterile homogenization bag with a filter. Use a sterile serological pipette or a sterile spoon to transfer samples, respectively.
c) A 10 ml or gm of food sample was added in 90 ml of Preston broth and mixed until fully dispersed by hand massaging, mixing in the bottle, or using a stomacher. Liquid milk and soft cheeses require shorter homogenization time (e.g., 10 s) compared to hard cheeses and meat (e.g., 30 s – 60 s).
d) Six enrichment bags were placed into an anaerobic jar (V = 7 l) and replaced the normal atmosphere with a microaerobic atmosphere by
e) Two microaerobic gas packs were placed inside the anaerobic jar and immediately closed the lid
f) The jar was placed in an incubator and incubated at 41.5□C for 24 hrs +/- 2 hb.

2. mCCDA media preparation and Plating onto selective agar:

a) The modified Charcoal Cefoperazone Deoxycholate agar (mCCDA) was taken out of the fridge at least one hour before use to adjust to room temperature.
b) A 10 ul loop of the enrichment was Aseptically transferred onto an mCCDA plate and streak for colony isolation.
c) Positive and negative control cultures Streaked parallelly to aid in the identification of putative *Campylobacter* colonies after incubation. A *Campylobacter jejuni*-type strain ATCC 33560 may be used as a positive control. Enterococcus faecalis WDCM 00087 may be used as a negative control.
d) The agar plates were inoculated and placed in an anaerobic jar and replaced the air with a microaerobic atmosphere as outlined in the enrichment section.
e) The inoculated mCCDA was incubated at 41.50C for 44 +/- 4hrs.

3. Selection of putative *Campylobacter* colonies:

- Pick two typical colonies and perform a PCR confirmation.

Isolation:

For presumptive positive isolation:

a) The same colony was used for Gram staining using an isolation needle and streak onto BHI, MHB
b) Incubated in microaerobic conditions at 37 ± 2.0° C for 44 ± 4 hours.

Annex 3: Molecular identification of *Campylobacter* species

DNA extraction

a) The thermal block was heated or a water bath to 95 – 100 °C.
b) A 100 µl of nuclease-free water was pipated in a microcentrifuge tube.
c) Touch a single colony with a 10 µl loop (collect just enough biomass to be able to see it with the naked eye; avoid using the whole colony).
d) Resuspend the biomass in nuclease-free water by swirling the loop in a tube 10 times.
e) Vortex the tube for 30 seconds.
f) Place the tube in a heated thermal block or a water bath (avoid cross-contamination from water by drying the tubes before opening them) and incubate for 10 min.
g) Cool down the tube for 2 min at ambient temperature.
h) Centrifuge the tube in a mini centrifuge at the highest speed for 5 min.
i) Transfer 50 µl of the supernatant to a fresh tube (carefully avoid the pellet).
j) Store the supernatant with extracted DNA at -20 °C for as long as needed. PCR confirmation may be done at a later time.
k) Use up to 5 ul of the collected supernatant per 50 µl PCR reaction or up to 2 ul per 20 µl reaction.

PCR techniques

PCR mix per one 25 µl PCR reaction:

a) 12 µl of Promega GoTaq Green Master mix

b) 0.125 µl of 100 µM working solution of *C. jejuni* F primer
c) 0.125 µl of 100 µM working solution of *C. jejuni* R primer
d) 0.25 µl of 100 µM working solution of *C. coli* F primer
e) 0.25 µl of 100 µM working solution of *C. coli* R primer
f) 0.05 µl of 100 µM working solution of 23S rRNA F primer
g) 0.05 µl of 100 µM working solution of 23S rRNA R primer
h) 0.125 µl of 100 µM working solution of *C. lari* F primer (optional)
i) 0.125 µl of 100 µM working solution of *C. lari* R primer (optional)
j) 0.25 µl of 100 µM working solution of *C. fetus* F primer (optional)
k) 0.25 µl of 100 µM working solution of *C. fetus* R primer (optional)
l) 0.5 µl of 100 µM working solution of *C. upsaliensis* F primer (optional)
m) 0.5 µl of 100 µM working solution of *C. upsaliensis* R primer (optional)
n) 2.5 9.65 µl of nuclease-free water (if using only CJ, CC, and 23S primers)+ 2.5 µl DNA template

PCR thermal cycling conditions

a) Initial denaturation step at 95°C for 6 min followed by 30 cycles of amplification (denaturation at 95°C for 0.5 min, annealing at 59°C for 0.5 min, and extension at 72°C for 0.5 min), ending with a final extension at 72°C for 7 min.
b) Include a positive control (DNA extracted from *C. jejuni* ATCC 33560) and a negative control (nuclease-free water) in each PCR run.

Annex 4: TAE buffer preparation and gel electrophoresis

a) TAE buffer (50 x, 0.04 M, pH 8.5) Preparation

a) Prepare 600 mL of dH2O in a suitable container. Add 242 g of Tris-free base to the solution. Add 18.61 g of Disodium EDTA to the solution. Add 57.1 g of Glacial Acetic Acid to the solution. Adjust the pH to 8.5 using 1 M NaOH. Add distilled water until volume is 1 L. Store the 50 x stock solution in the fridge.
b) Use 1 x TAE buffer for running gel electrophoresis and preparation of the gel. Change the buffer in gel electrophoresis once a week or as soon as you see impurities floating in the chamber.
c) Prepare 1.5 % agarose gel

d) Small gel (50 ml): Mix 0.75 g of agarose with 50 ml of 1 x TAE buffer in a glass flask.
e) Medium gel (100 ml): Mix 1.5 g of agarose with 100 ml of 1 x TAE buffer in a glass flask.
f) Close the flask (not tightly, to allow for the steam release), lightly swirl the suspension, and microwave for 30 s, carefully swirl the flask (wear the heat-protective gloves), microwave for another 30 s, and carefully swirl again (wear the heat-protective gloves) and microwave for another 15 s. There should be no visible particles of agarose floating in the suspension prior; if there are some, continue boiling the suspension.
g) Let the gel cool down for ~3 min and then pour in the cast with the well comb placed into the comb holders. Let the gel solidify for 30 min (may differ depending on size) before transferring into the electrophoresis chamber.

2. Running gel electrophoresis

a) Fill most of the tank with 1X TAE buffer.
b) Set the gel (with the holder) in the tank and finish filling the tank with 1X TAE so that the wells of the gel are covered with a thin layer of TAE and appear translucent.
c) Load the gel with your samples (5-10 µL should be sufficient for each well), controls, and a 100 bp DNA ladder, and run the gel electrophoresis for 40 min at 100 V (small gel) or 40 min at 150 V (medium gel). Always run the samples in the gel from negative to positive as the DNA in the wells is negatively charged and will migrate toward the positively charged end.
d) Check to make sure the electrophoresis is running by ensuring the electrodes are producing bubbles.
e) Prevent overheating of the buffer when running multiple runs one after another by either cooling down the buffer in the chamber or adding a cold buffer.
f) Stain the gel with gel red for 20 minutes rinse in DI water for 25 minutes (or follow a different staining protocol) and image the gel. Save the image in a file that contains the information about the experiment, the date, and the initials of the researcher.

Annex 5: Cryopreservation of isolates

- Prepare cryo-preservation medium by mixing 80 ml sterile BHI and 20 ml sterile glycerol. Aliquot 1 ml of the cryo-preservation medium in cryo tubes.
- Select an isolated colony from BHI and transfer it to a 10 ml BHI tube for cryopreservation.
- Incubate for 37 °C for 44 +/- 4 h.
- Using a cotton swab, make a lawn on a BHI (or another appropriate agar) plate.
- Incubate for 37 ± 2°C for 44 +/- 4 h. Harvest half of the plate's lawn using a sterile loop and transfer the growth into a cryo-bead tube (the other half of the lawn will be used to make a duplicate cryo-bead) or cryotube containing cryo-preservation medium.
- Twirl loop to disperse the organisms and lightly vortex if the pellet does not resuspend.
- For best long-term results, store inoculated vial at -70°C. Storage at -20°C is not acceptable due to poor survivability of *Campylobacter*.

Annex 6: Isolate storage and maintenance

a) For short-term (no longer than 2 weeks) storage, inoculate a nutrient agar slant, incubate at 35 ± 2°C overnight, and then store at 2-8°C in an anaerobic jar and microaerobic atmosphere. For long-term storage, lyophilize cultures or freeze at ≤ -70°C using cryo-beads (i.e. CryostorTM or equivalent) or cryo vials without beads.
b) Maintain "working" *Campylobacter* stock cultures on nutrient agar slants or equivalent (e.g., brain heart infusion agar (BHI) or Mueller Hinton agar . Transfer stocks biweekly onto duplicate nutrient agar slants, incubate for 44 h +/- 4 h at 37 ± 2°C and then maintain them at 2-8°C in microaerobic conditions. Use one of the slants as the working culture. Use the other slant for sub-culturing to reduce the opportunity for contamination.

Annex 7: Descriptions of Preston broth and mCCDA agar

a) Preston broth (e.g., Oxoid, Nutrient broth No. 2; 500 g/bottle; OXCM0067B)
b) Preston broth supplement (e.g., Oxoid, Modified Preston *Campylobacter* supplement; 10/pack; OXSR0204E)

c) Modified charcoal cefoperazone deoxycholate agar (mCCDA) (e.g., Oxoid; Blood-free *Campylobacte*r selective agar; 500 g/bottle; OXCM0739B)
d) mCCDA supplement (e.g., Oxoid; CCDA selective supplement; 10/pack; OXSR0155E)

Annex 8: Antimicrobial susceptibility testing *Campylobacte*r species

a) The test was performed for each isolate on Mueller-Hinton agar (OXOID, England) supplemented with 5% horse blood (Hardy Diagnostics, 10052-808).
b) Approximately 20 ml of medium was poured into 90 mm diameter sterile Petri dishes.
c) Isolates were grown in a brain heart infusion broth (OXOID, England) at 37°C for 24 h in microaerobic conditions (CampyGen, Oxoid, AGS).
d) Isolates were then inoculated on Mueller-Hinton agar in the form of a lawn using sterile cotton swabs.
e) *Campylobacter* isolates were tested for susceptibility to the following 3 antibiotics: ciprofloxacin (30 μg), tetracycline (30 μg), erythromycin (10 μg) (OXOID, England) according to guideline M45:ED3 of the Clinical Laboratory Standards Institute (CLSI-M45-Ed3, 2016).
f) Antibiotic discs were deposited on the surface of inoculated cultures on MHA and incubated at 41.5°C for 24 hrs.
g) The diameters of the zones of inhibition were recorded to the nearest mm and classified as resistant, intermediate, or susceptible according to guidelines set by the Clinical Laboratory Standards Institute

Annex 9: Methods for species-specific *Campylobacter* whole genome sequencing

For whole-genome sequencing of *Campylobacter* isolates, the following steps were performed:

a) DNA extraction: DNA extraction was performed using the QIAcube Connect instrument with the DNeasy Blood and Tissue kit, and the DNA was quantified using the Qubit 4 Fluorometer.
b) Library preparation: Library preparation was carried out using the Illumina DNA Prep kit, and Nextera DNA CD indexes were used for sample indexing.

c) Sequencing: The paired-end sequencing run was conducted on an Illumina MiSeq instrument, utilizing a v3 600-cycle cartridge.
d) Quality control: Quality of raw sequencing reads was performed using FastQC. Trimmomatic was used to remove low-quality bases and adapters with default settings.
e) Genome assembly: The resulting trimmed sequences were then assembled using Skesa, and genome assembly quality was assessed with Quast.
f) MLST: SkesaMLST was applied to determine multi-locus sequence types (MLST STs).
g) Taxonomic identification: GTDB-Tk (V2.1.0) with the reference database version R207_v2 was used for genome-based taxonomic identification.
h) Annotation: The annotation of the 18 *Campylobacter* genomes was performed using Prokka.
i) Pan-genome analysis: Roary was used to calculate the pan-genome.
j) SNP analysis: High-quality single nucleotide polymorphisms (SNPs) were detected using the CFSAN SNP pipeline on the GalaxyTrakr platform and used to construct a maximum likelihood phylogenetic tree using IQ-TREE.
k) Virulence and antimicrobial resistance gene detection: ABRicate with default settings was used to detect the presence of virulence factor genes and antimicrobial resistance genes in the studied isolates by utilizing the Virulence Factor Database (VFDB) and the NCBI Bacterial Resistance Reference Gene Database, respectively. Amrfinderplus_db NCBI was used to detect gyrA and 50S_L22_A103V mutations.
l) Plasmid detection: Staramr was used to detect the presence of plasmids.
m) Pathogen detection: The pathogen detection database was searched to find the arsenic resistance.
n) Comparison: Isolates studied here were submitted to the NCBI Pathogen Detection database to allow for the genomic similarity comparison with the *Campylobacter* isolates available in the database

Annex 10: Selected pictures while sample collection and performing laboratory analysis

Figure 5: Sample storage at a collection center at Yirgalem (SNNP)

Figure 6: Milk processing machine at milk collection center at Yirgalem

Figure 7: Interview laboratory personnel at a milk processor in Hawassa

Figure 8: Observing the sanitation of concrete floors at Hawassa

Figure 9: Site observation during transporting of pasteurized milk

Figure 10: Primary enrichment with Preston broth

Figure 11: During pouring agar plates

Figure 12: During plating on mCCDA

Figure 13: During master mix preparation for PCR

Figure 14: During running PCR

Figure 15: During gel preparation

Figure 16: During running gel electrophoresis

Figure 17: *Campylobacter jejuni* colonies

Annex 11: Ethical approval and clearance for this study from Addis Ababa University

COLLEGE OF NATURAL & COMPUTATIONAL SCIENCES
Addis Ababa University
OFFICE OF THE DEAN

Ref. No.
Date: January 23, 2020

CNSDO/296/12/2020

To Whom It May Concern

The College of Natural & Computational Sciences Institutional Review Board (**CNS-IRB**) Committee in its meeting held on 20/12/2019 Minute No. IRB/42/2019 has examined the project proposal entitled **"Assessment of Risk Factors for Microbial and Chemical Contamination of Milk and Cottage Cheese Across the Dairy Value in Ethiopia" by Dr. Ashagrie Zewdu,** from the Addis Ababa University.

The proposal is approved for implementation.

With regards,

Addisalem Anumun, PhD
Dean, College of Natural & Computational Sciences

Please Quote our reference number in you correspondence