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Dynamics of foodborne bacteria and their resistance to antimicrobials

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DEDICATION

I dedicate this work to:

My grandparents: Jaime, Clementina, Ángel y Lida

My parents and sister: Miguel, Anita y Ely

My husband: Jorge

And to my entire family for always supporting me.

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ABSTRACT

In Ecuador, there is currently no public health surveillance system dedicated to monitoring the use of antimicrobial agents in food-producing animals. The emergence of resistance in foodborne bacteria such as *Escherichia coli* and pathogenic *Campylobacter* spp. is closely linked to the use of antibiotics in these animals. Infections caused by these resistant bacteria have been well-documented in humans, and as antimicrobial resistance (AMR) continues to increase, treatment failures may become more frequent due to the reduced efficacy of commonly used antibiotics.

The epidemiology of extended-spectrum beta-lactamase (ESBL)-producing *E. coli* in primary production, along with the prevalence and transmission of *Campylobacter* spp. on farms and in retail environments in Ecuador, remains insufficiently understood.

This work is organized as follows:

- Chapter I provides a general introduction to the study, presenting the key characteristics and significance of each bacterium under investigation.
- In Chapter II, we conducted a study to analyze the prevalence of extended-spectrum β-lactamase (ESBL)-producing *E. coli* in irrigation water and agricultural products was investigated in 17 provinces of Ecuador. The genes $bla_{CTX-M-55}$, $bla_{CTX-M-65}$, and $bla_{CTX-M-15}$ were the most common. The connection between contaminated irrigation water and antibiotic resistance in agricultural products was evident
- Chapter III focuses on the transmission of *Campylobacter coli* and *C. jejuni* from farms to retail settings. In this study, 14 resistance genes and 25 plasmids were identified, along with a high prevalence of virulence factors among the isolates.
- Chapter IV presents the main conclusions of this research and offers recommendations for future studies aimed at mitigating AMR risks in Ecuador's food production systems.

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CHAPTER I: GENERAL INTRODUCTION

1. Introduction

In the 1940s, the introduction of antibiotics transformed medicine, but their misuse has also contributed to the emergence and spread of antibiotic-resistant bacteria. This resistance has resulted in treatment failures, increased hospitalization rates, higher mortality, and escalating societal costs. Antimicrobial resistance (AMR) is now a pressing global public health issue that demands urgent action. Antimicrobials encompass antibiotics, antivirals, antifungals, and antiprotozoals, all of which are designed to kill or inhibit the growth of microorganisms. AMR occurs when these microorganisms evolve to resist the effects of the drugs that once successfully targeted them (1).

Some bacteria already have resistance; these have natural or intrinsic resistance. In another case, the susceptible bacteria have become resistant over time. Bacteria are remarkably hardy and adaptable, allowing them to quickly respond to environmental pressures, such as the presence of antibiotics. Resistance can arise through mutations or through the acquisition of antibiotic resistance genes carried by mobile genetic elements via horizontal gene transfer (2).

The consequences of horizontal gene transfer are significant, as this mechanism can facilitate the simultaneous spread of resistance to multiple classes of antibiotics. Resistance genes are often located within transmissible genetic elements, making them easily shared among bacteria. This genetic process is recognized as a primary driver of the spread of antibiotic resistance genes, enabling the transfer of resistance not only within a species but also between different species, genera, families, etc (3).

Human bacteria can acquire antibiotic resistance genes through mutations in their DNA or by transferring genes from other bacteria and the food chain. The use of antibiotics in food animals for treatment, disease prevention, or growth promotion enables the accumulation and the spread of resistant bacteria and resistance genes from bacteria in these animals to humans via the food chain. Importantly, antibiotic resistance does not respect geographical or biological boundaries (4).

2. Transmission routes

The epidemiology of antibiotic resistance is exacerbated by the ability of resistance genes to transfer between different bacteria. Antibiotic-resistant bacteria can spread across various sectors, settings, and geographic boundaries. This cycle of antimicrobial resistance involves the dissemination of resistant bacteria in humans, animals, animal products, and the environment (5).

Both people and animals harbor a variety of bacteria in their guts, on their skin, and on other surfaces. As a result, even healthy individuals and animals can carry resistant bacteria, which can be transmitted within communities and across the world (6). Additionally, food products of animal origin such as meat, eggs, and milk are frequently contaminated with bacteria. This contamination is a primary route for the transmission

of resistant bacteria and resistance genes from food animals (7). Fruits and vegetables can also serve as a route for the transmission of resistant bacteria when they are contaminated by organic fertilizer or manure polluted water (8,9) (Figure 1).
Therefore, bacteria carrying resistance genes from animals can be transmitted to humans through the food chain via the consumption of raw or undercooked foods, handling of raw products, and cross-contamination with other foods. Additionally, transmission can occur indirectly through environmental exposure. Furthermore, resistant bacteria can also be transmitted directly from animals raised on farms. The spread of antibiotic-resistant genes can also occur along the food chain through direct contact with animal biological substances, such as blood, urine, milk, saliva, and semen (10).

Zoonotic foodborne bacteria, such as pathogenic *Campylobacter*, can contaminate food at various stages of the food chain. Furthermore, the use of antibiotics during the life cycle of animals intended for human consumption contributes to the development of antibiotic resistance in these bacteria. Similarly, resistant *E. coli* can also be transmitted from animals to humans through the food chain (11).

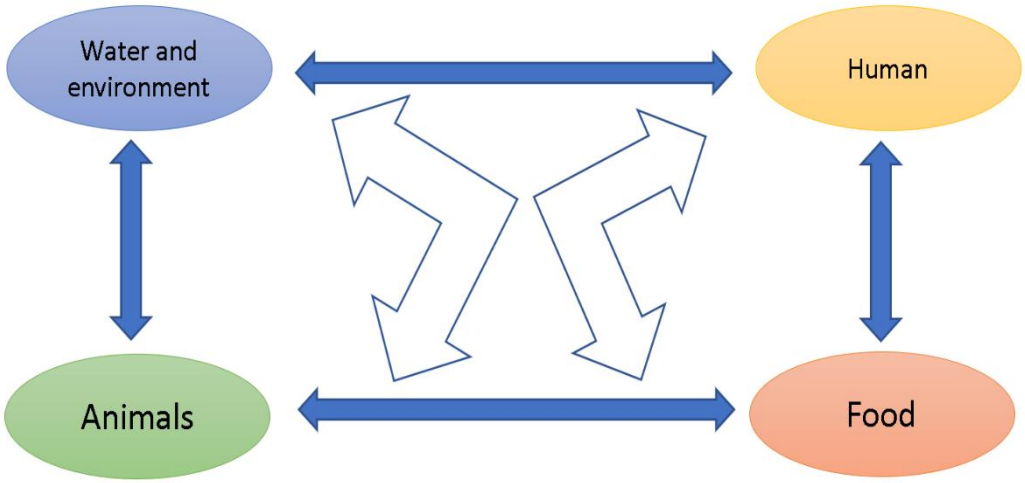

Figure 1. Potential routes of AMR in food chain

3. Antimicrobial resistance in Food

Antimicrobial resistance in food is a critical food safety issue. The use, overuse and misuse of antibiotics in livestock can lead to the development of resistant bacteria, which may be transmitted to humans through the consumption of contaminated food (11). Antibiotics are commonly used in food animals to treat or prevent respiratory and enteric infections, especially during the early stages of life. For example, broilers, pigs, and calves are often given antibiotics to manage these conditions (12).

The indiscriminate use of antibiotics in agriculture can affect the effectiveness of infection treatments in humans. Antibiotic-resistant bacteria and resistance genes can contaminate food at any point in the supply chain, from farm to retail (13). Similar to human medicine, the use of antibiotics in agriculture creates selective pressure, driving the emergence and spread of antibiotic-resistant bacteria (14).

The spread of antimicrobial resistance throughout the food chain is a cross-sectoral issue, as antibiotics are widely used in aquaculture, livestock production, and agriculture. Antibiotic-resistant bacteria and resistance genes can spread easily at every stage of the food production process (15).

Given the direct interaction between humans and the animal-ecosystem interface (Figure 1), preventing the zoonotic transmission of antibiotic-resistant bacteria and resistance genes from food to human bacteria is crucial. The rapid growth of the human population, global trade in animals and food products, international travel, and increased movement of people all contribute to the accelerated spread of resistant bacteria and their genes.

Addressing the complex issue of antimicrobial resistance requires international and intersectoral collaboration. Given its connection to food safety, these aspects must be integral to the response efforts aimed at combating this global threat (16).

Key areas to consider from a food safety perspective include:
• Regulatory framework for the use of antibiotics in animal feed
• Regulatory framework for prudent use of antibiotics in animal husbandry
• Surveillance of resistance to antibiotics in bacteria of human origin, food and animals aimed for consumption
• Education strategies (training)
•Investigation

In Ecuador, there is currently no public health information system tracking the use of antimicrobial agents in animals intended for human consumption.
Resistance in foodborne bacteria, such as *Escherichia coli* and pathogenic *Campylobacter* spp., are strongly linked to antibiotic use in food animals. Infections caused by these antibiotic-resistant bacteria are well-documented in humans. Increasing antimicrobial resistance in foodborne pathogens can result in treatment failure if the pathogen is resistant to the antibiotics commonly used for treatment (17).

In Ecuador, the epidemiology of extended-spectrum beta-lactamase-producing *E. coli* transmission in primary production, as well as *Campylobacter* in farms and retail, is not well understood.

4. E. coli

4.1. Characteristics

Escherichia coli (E. coli) is a facultatively anaerobic, Gram-negative bacillus that belongs to the Enterobacteriaceae family. It does not produce enzyme oxidase and is capable of fermenting both glucose and lactose, often with gas production. The typical *E. coli* genome is approximately 5.0 Mb in size, containing around 4,200 genes. However, the genome size can vary between strains, ranging from 4.0 to 6.0 Mb. Additionally, the *E. coli* genome has a GC content of 50.8% (18). *E. coli* is a bacterium commonly found in the digestive tract of humans and animals. Due to its high prevalence, it is frequently used as an indicator organism to detect and measure fecal contamination in food and water (19). *E. coli* is a mesophilic bacterium that thrives at the body temperature of warm-blooded animals (35–43°C) and is sensitive to temperatures above 70°C. This is why pasteurizing

foods, such as milk and juices, is crucial to ensure the elimination of the bacterium and prevent contamination (20).

Pathogenic *E. coli* differs from non-pathogenic strains in its ability to cause disease, primarily due to specific genetic factors such as toxin production, host cell attachment and invasion, and tissue destruction. The main pathogenic strains are classified based on the clinical symptoms they produce and their expressed virulence factors. These include: enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAggEC), enteroinvasive *E. coli* (EIEC), and enteropathogenic *E. coli* (EPEC). *E. coli* strains that are zoonotic: enterohemorrhagic *E. coli* (EHEC) and specifically *E. coli* O157, which are transmitted from animals to humans (18).

4.2. *Escherichia coli* in humans

E. coli is part of the normal human microbiota, however there is constant influx from humans and other animal (such as unpasteurized dairy products and juices, undercooked or processed meats, raw fruits and vegetables, and improper handling or storage of prepared foods. Factors that contribute to the persistence of *E. coli* in food systems include inadequate control of processing parameters, such as water activity, and storage at elevated temperatures for extended periods, which allows bacterial growth. *E. coli* is excreted by humans and animals in feces and can enter agroecosystems through manure, contaminated irrigation water, and tainted seeds (21).

E. coli is of fecal origin and is primarily transmitted through the oral route via fecal contamination of food and water, cross-contamination, or direct contact during food preparation and consumption. Humans and animals can excrete normally between 10^6 and 10^9 colony-forming units (CFU) per gram of feces and contribute to its spread (22). Most strains of *Escherichia coli* are harmless and do not cause illness in humans. In fact, these bacteria are naturally present in the intestines of warm-blooded animals, including humans. These animals can act as asymptomatic carriers, harboring the bacteria without showing any signs of infection. While some *E. coli* strains are benign, some can produce toxins that cause foodborne illnesses (e.g., *E. coli* O157:H7) (23).

Enterohemorrhagic *Escherichia coli* (EHEC) is primarily transmitted through the consumption of contaminated food, such as undercooked beef, raw milk, or unpasteurized juices, as well as through contact with contaminated water or surfaces. Although EHEC is typically zoonotic, originating from animals like cattle, it can also spread through human-to-human contact, especially in environments with poor hygiene.
Preventing infection by this bacterium requires control measures at every stage of the food chain, from agricultural production to processing, manufacturing, and food preparation (21).

In contrast, uropathogenic *E. coli* (UPEC) and septicemic *E. coli* are opportunistic pathogens that are mainly transmitted through direct human to human fecal-oral transmission. Both UPECs and septicemic strains colonize the intestine and gain access to bloodstream via colonization of the urinary tract, respiratory tract, wounds or babies passaging trough the birth canal colonized by this bacterium. These *E. coli* strains are

more likely to cause infections when the host's immune system is weakened or when there is an imbalance in the normal microbiota (23).

4.3. Antibiotic resistance in *E. coli*

Strains of *E. coli* from various sources that contaminate food can carry resistance genes, which may be transferred to human-adapted bacteria as they pass through the intestine. These resistance determinants can be transmitted from food animals to humans via the food supply, often through bacteria that are normally harmless, such as *E. coli*. If resistant *E. coli* colonizes a human and causes infection, or if it transfers its resistance genes to pathogenic bacteria, it could lead to treatment failure and prolonged illness (24). *E. coli* can exchange genetic material through mobile elements, such as plasmids, allowing it to adapt to new environments. It plays a crucial role in the spread of antimicrobial resistance due to its ability to accumulate resistance genes, which can be horizontally transferred to other species (24).

A growing concern is the rapid emergence of extended-spectrum beta-lactamase (ESBL)-producing strains, which exhibit transferable resistance to third- and fourth generation extended-spectrum cephalosporins. In addition, these strains often show resistance to other first-line antibiotics, such as fluoroquinolones. ESBLs can break down β-lactam antibiotics, including penicillin's and cephalosporins, which are commonly used as first-line treatments. Over the past decade, ESBL-producing *E. coli* strains have emerged worldwide, leading to infections both in hospitals and in the community, with increasing isolation of these resistant strains (25). This suggest that food and the environment could be important sources that contribute to the rise of these resistant bacteria (26).

4.3.1. B-lactam resistance

β-Lactams are commonly used to treat infections caused by *E. coli*. Extended-spectrum β-lactamases (ESBLs) are a group of enzymes that can hydrolyze and confer resistance to a wide range of β-lactam antibiotics. The most prevalent ESBLs: SHV, TEM, OXA, AmpC and CTX-M types. Among these, CTX-M β-lactamases, now numbering over 50 distinct types are classified into five groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 (25).

5. Campylobacter

5.1. Characteristics

The *Campylobacter* genus belongs to the family *Campylobacteraceae*, the order *Campylobacterales*, the class *Epsilon proteobacteria*, and the phylum Proteobacteria. Members of the genus *Campylobacter* are Gram negative spiral bacteria, microaerophilic, rod-shaped, curved bacteria with a single polar flagellum, bipolar flagella, with average G+C content of the DNA between 29-47. Also, the members of genus carry a relatively small genome with circular chromosome of 1.59-1.77 MBP in size (27).

Campylobacters populate the mucosal surfaces of the intestinal tracts of humans and animals. Human campylobacteriosis, a gastroenteritis that is transmitted through food consumption, caused by thermotolerant pathogenic *Campylobacter* species:

Campylobacter jejuni and *Campylobacter coli*. (28). Typically, *the C. fetus* and several other *Campylobacter* species (e.g. *C. coli*, *C. lari*) are implicated in extra-intestinal infections that include bacteremia, hemolytic uremic syndrome, meningitis, septicemia, reactive arthritis. Infection by *Campylobacter jejuni* is associated with the development of Guillain-Barré syndrome (GBS), a neurological disorder affecting the peripheral nervous system (29). *Campylobacter* is a naturally transformable bacterium that is able of acquiring resistance genes (30).

5.2. Epidemiology

The intestines of warm-blooded animals are the host for *Campylobacter* amplification. In poultry, microaerophilic conditions, and temperatures around 41 ^{o}C exist that allow continuous replication of *C. coli* and *jejuni*. Humans can be exposed through direct or indirect contact (consumption of raw products). Humans are the only host species to become ill after oral ingestion of *Campylobacter*; infection may result from ingesting a dose of 800 CFU (31).
Human exposure from animals is possible through a few pathways, including food (example, poultry), the environment, and direct contact with animals. *Campylobacters* survive in chicken feces after shedding and therefore chicken feces may also be a potential source of transmission to the environment or to humans when poultry feces are used as fertilizer. The lack of association of *Campylobacter* with disease or low mortality in poultry flocks means that there is no economic reason for farmers to invest in contamination prevention on their farms (32).

The surface of chicken carcasses can be contaminated during the slaughter process, due to the high concentration of *Campylobacter* in the intestines after plucking or rupture of the intestine during evisceration (33).
Campylobacter cannot multiply outside the intestines of warm-blooded animals; this feature provides an important tool to combat campylobacteriosis by removing highly contaminated products (28).

In low-income countries, *Campylobacter coli* and *C. jejuni* are endemic and infection is generally limited to children with disease rates declining with age, suggesting that exposure in the first years of life provides protective immunity and this could reflect that asymptomatic *Campylobacter* infections are common in these regions (34). In the case of campylobacteriosis, many cases the illness is self-limited and presents as 1-3 days of prodromal symptoms with fever, vomiting and headaches followed by 3-7 days of watery or bloody diarrhea with abdominal pain (35). However, a small group of people require hospitalization, the variations in clinical manifestations could be due to the wide genetic diversity of *Campylobacter*, as well as the person´s immune system. The disease mainly affects children under 5 years of age, the elderly and immunocompromised patients (35).
The usual route of transmission in the farm environment is that *Campylobacter* is introduced into a flock of chickens and spreads rapidly through the fecal matter of chickens already colonized with *Campylobacter* living on the same farm; the primary sites of colonization are the crop, cecum, and small intestine. Human and vehicular transport also appear to be an important route for transmission from the external environment (36).

5.3. Antibiotic resistance in *Campylobacter*

Campylobacteriosis is the most reported zoonosis in the Union European. This infection is acquired through ingestion of contaminated poultry meat (37). In the European Union, intestinal campylobacteriosis is acquired through ingestion of contaminated poultry meat and is the most reported zoonosis (38). The occurrence of campylobacteriosis outside industrialized countries is less known, because in many countries (such as Ecuador) it is not included in their epidemiological surveillance systems (31). Campylobacteriosis is associated with diarrhea, bloody stools, fever, and severe abdominal pain. Most cases are self-limited and do not require antibiotic therapy, when these is required, macrolides and fluoroquinolones are used. Mortality from campylobacteriosis is usually low, but it tends to be higher in people with comorbidities (36).
Campylobacters can obtain antibiotic resistance by spontaneous mutations and horizontal gene transfer via natural transformation, transduction, and conjugation (39).

5.3.1. Quinolone resistance in *Campylobacter* spp.

Fluoroquinolones exhibit concentration-dependent bactericidal activity against a Gram-negative and Gram-positive organism. Ciprofloxacin is the most extensively used antibiotic to treat diarrhea. Campylobacteriosis is clinically indistinguishable from other causes of bacterial diarrheal disease, and numerous cases are treated empirically with fluoroquinolones (40).

In *Campylobacter coli* and *C. jejuni*, there are two well-described mechanisms of resistance: inactivation of the target of fluoroquinolones and efflux of fluoroquinolones. The two intracellular enzymatic targets of fluoroquinolones are DNA gyrase (decoded by the *gyrA* and *gyrB* genes) and the structurally related topoisomerase IV (decoded by the *parC* and *parE* genes). Fluoroquinolone resistance in *C. jejuni* and *C. coli* occurs through specific point mutations in the quinolone resistance determining region (QRDR), with the Thr-86-Ile mutation being the most common in several regions. The less common Thr-86-Ala mutation confers resistance only to nalidixic acid but not to fluoroquinolones, and the Asp-90-Asn and Ala-70-Thr mutations in *gyrA* confer intermediate-level resistance to fluoroquinolones. Although mutations in the *gyrB* gene have been reported, this mutation does not confer resistance to fluoroquinolones (41). Another medium is efflux via the chromosomally decoded CmeABC multidrug efflux pump, which reduces the intracellular concentration of fluoroquinolones (41).

5.3.2. Macrolide resistance in *Campylobacter* spp

Macrolides inhibit protein synthesis by binding to the P site on the 50S subunit of bacterial ribosomes. The main mechanisms of resistance to macrolides in *Campylobacter* are target modification, efflux, and alteration of membrane permeability. Point mutations in the coding region in domain V of the 23S rRNA gene at positions 2074 and 2075 confer a high level of macrolide resistance, with the 2075 substitution being more common in some regions. *C. jejuni* and *C. coli* carry three copies of the 23s rRNA gene, all of which are usually mutated in macrolide-resistant strains (41).

Mutations in ribosomal proteins L4 and L22 lead to resistance to macrolides. The CmeABC multidrug efflux pump also contributes to macrolide resistance. A third method of resistance to macrolides involves alteration of membrane permeability mediated by the expression of outer membrane porin (MOMP), chromosomally encoded by the *porA* gene (40).

5.3.3. B-lactam resistance in *Campylobacter* spp.

Beta-lactam antibiotics include penicillin's, cephalosporins, carbapenems, and monobactams, all of which contain the beta-lactam ring necessary for antimicrobial activity (42).
Three mechanisms mediate β-lactam resistance in *Campylobacter*, enzyme inactivation by chromosomally encoded β-lactamases (OXA-61 in *C. jejuni*), reduced uptake due to alterations in outer membrane porins, and the CmeABC efflux pump (42).

5.3.4. Tetracycline resistance in *Campylobacter* spp.

In *Campylobacter*, the known mechanisms of tetracycline resistance are disruption of ribosomal targeting and tetracycline efflux. Once inside the bacterial cytoplasm, tetracyclines bind to the 30S subunit of ribosomes and inhibit protein synthesis by preventing the binding of charged aminoacyl-tRNA to the ribosomal A site. The main mechanism of tetracycline resistance in *Campylobacter* is the protection of an unoccupied A site by binding of the bacterial protein TetO to that site (43). *TetO* can be encoded on the chromosome, or generally, on the plasmids pTet (43).

5.3.5. Aminoglycoside resistance in *Campylobacter* spp.

There are two main resources by which aminoglycosides exert antimicrobial activity, interference with the translocation of the nascent peptide chain from the ribosomal A site to the P site, and interference with proofreading, leading to the incorporation of incorrect amino acids and the generation of a dysfunctional protein (40).
Aminoglycoside resistance was first detected in *C. coli* and was intermediated by a 3'-aminoglycoside phosphotransferase (decoded by *aphA-3*). This *aphA-3* gene continues the most common source of aminoglycoside resistance in *Campylobacter* (40).
Also, there is a single report of a mutation in ribosomal protein S12 (encoded by *rpsL*) in *C. coli* that confers streptomycin resistance (40).

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CHAPTER II: Extended-Spectrum Beta-Lactamase producing-*Escherichia coli* isolated from irrigation waters and produce in Ecuador

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In cities across the globe, the majority of wastewater – that includes drug resistant and pathogenic bacteria among other contaminants – is released into streams untreated. This water is often subsequently used for irrigation of pastures and produce. This use of wastewater-contaminated streams allows antibiotic-resistant bacteria to potentially cycle back to humans through agricultural products. In this study, we investigated the prevalence of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolated from produce and irrigation water across 17 provinces of Ecuador. A total of 117 vegetable samples, 119 fruit samples, and 38 irrigation water samples were analyzed. Results showed that 11% of the samples were positive for *E. coli* including 11 irrigation water samples (29%), and samples of 13 vegetables (11%), and 11 fruits (9%). Among the 165 *E. coli* isolates cultured, 96 (58%) had the ESBL phenotype, and 58% of ESBL producing *E. coli* came from irrigation samples, 11% from vegetables, and 30% from fruits. The $bla_{\text{CTX-M-55}}$, $bla_{\text{CTX-M 65}}$, and $bla_{\text{CTX-M 15}}$ genes were the most frequently found gene associated with the ESBL phenotype and coincided with the $bla_{\text{CTX-M}}$ alleles associated with human infections in Ecuador. Three isolates had the mcr-1 gene which is responsible for colistin resistance. This report provides evidence of the potential role of irrigation water in the growing antimicrobial resistance crisis in Ecuador.

Keywords: fresh produce, irrigation water, ESBL *E. coli*, CTX-M, Extended-spectrum beta-lactamase (ESBL)

INTRODUCTION

The rise of antimicrobial resistance (AMR) is one of the most serious biological threats facing modern society, and the inability to treat bacterial infections is already occurring in many nosocomial infections (Frieri et al., 2017). The World Health (WHO) has listed extended spectrum β-lactamase-producing Enterobacteriaceae (ESBL-E) as the most critical antimicrobial resistant microorganisms, among the "Highest Priority" pathogens due to the increasing prevalence in humans and livestock (Yassin et al., 2017; Shrivastava et al., 2018; Li et al., 2019; Murray et al., 2021). Globally, the majority of wastewater produced by urban settlements goes into streams without prior treatment. Only 20% of produced wastewater receives proper treatment (UNESCO, 2012), and the capacity to treat wastewater often depends on the income level of the country; treatment capacity is 70% of the generated wastewater in high-income countries, compared to ∼8% in low-income countries (Sato et al., 2013). This phenomenon is rising as urban populations grow and developing countries increasingly install pipes to channel wastewater away from communities, even before the development of wastewater treatment plants. The wastewater comes from diverse sources (e.g., homes, hospitals, and animal processing plants, etc.) and contains large quantities of antibiotic resistant bacteria (ARB), often carrying antimicrobial resistance to last-line antimicrobials, such as carbapenems (Lin et al., 2020).

These antimicrobial resistant bacteria (ARB) can cycle back to humans when wastewater-contaminated streams are used to irrigate produce or provide water to food animals (FAO and WHO, 2008; Leff and Fierer, 2013; Pigłowski, 2019); one recent example is the finding of New Delhi metallo-β-lactamases–type carbapenem-resistant *Escherichia coli* in water, domestic food animals, and humans (carbapenem, a last-line drug, is used exclusively in human medicine) (Li et al., 2019; Murray et al., 2021). Many antibiotic-resistant Enterobacterales, members of the intestinal microbiome (including *E. coli*), can survive and multiply in the environment (Vasco et al., 2015; Guerrero et al., 2020) and may colonize humans and domestic animals through the fecal-oral route of transmission. Plasmids and other mobile genetic elements (MGEs) carrying AMR genes promote the dissemination of AMR among intestinal bacteria in the intestine of vertebrates (Bonardi and Pitino, 2019), and this cycle is fundamentally captured in the One Health concept. Produce contamination can happen before pre-harvest (i.e., through contaminated irrigation water or manure fertilization) (Beuchat, 1996; Iwu and Okoh, 2019), as well as post-harvest (i.e., by washing, handling and processing food) with irrigation water (Murray et al., 2017).

Wastewater-impacted irrigation water has been identified as the main source of contamination for fresh produce with pathogenic microorganisms and ARB (Njage and Buys, 2015; Gekenidis et al., 2018a). The fecally contaminated produce can transfer ARB to the consumer especially when the produce is consumed fresh and uncooked (Pesavento et al., 2014; Araújo et al., 2017; Hölzel et al., 2018). Besides contributing to the spread of pathogens, irrigation water may potentially play a leading role in the dissemination of ARB (Moore et al., 2010; Hong et al., 2013; Gekenidis et al., 2018b; Vital et al., 2018).

The production of extended-spectrum β-lactamases (ESBL) is one of the most important mechanisms of antibiotic resistance in Enterobacteriaceae. ESBL genes can be divided into 4 groups: TEM, SHV, OXA, and CTX-M types (Bush and Jacoby, 2010); CTX-M type is the most prevalent of ESBLs described (Rossolini et al., 2008; Bevan et al., 2017). Enterobacteriaceae members are the most common bacterial agents causing foodborne outbreaks associated with the consumption of fresh produce (Cooper et al., 2007; Kilonzo-Nthenge et al., 2018; Al-Kharousi et al., 2019; McDaniel and Jadeja, 2019; Motlagh and Yang, 2019). Pathogenic E. coli is a key bacterium in foodborne illnesses, and commensal E. coli is a common indicator organism of fecal contamination in aquatic systems (Edberg et al., 2000; Rochelle-Newall et al., 2015; Motlagh and Yang, 2019). E. coli is also recognized as an important species in the spread of ARB, mainly due to a high aptitude to acquire genetic information through horizontal gene transfer (Grasselli et al., 2008; Hasegawa et al., 2018; Marlène et al., 2020). In Ecuador, an upper middle-income country, wastewater is almost entirely released untreated into streams; these streams often serve as irrigation water for produce and food-animal agriculture (Ortega-Paredes et al., 2020a,b). There are few studies about the dissemination of ESBL-E. coli from irrigation water to produce (Ben Said et al., 2015; Vital et al., 2018); most of the studies have been carried out in fresh produce from retail centers and groceries (Bhutani et al., 2015; Faour-Klingbeil et al., 2016; Ortega-Paredes et al., 2018; Al-Kharousi et al., 2019; Yang et al., 2019; Colosi et al., 2020; Richter et al., 2020; Song et

al., 2020). The aim of this study was to build upon the previous literature to understand the relationship between ARB in irrigation water and ARB on fresh produce obtaining samples from farms and their irrigation water. The study focused on the occurrence of extended spectrum β-lactamase producing *E. coli* in 17 provinces of Ecuador.

MATERIALS AND METHODS

Study Areas

This study was carried out in the following provinces of Ecuador: Manabí, Bolívar, Cañar, Loja, Guayas, Pastaza, Tungurahua, Pichincha, Azuay, Chimborazo, Cotopaxi, Imbabura, Santa Elena, Los Ríos, Morona Santiago, Orellana, and Zamora Chinchipe provinces which are mainly agrarian (**Figure 1**). The samples correspond to those that are collected as part of the national surveillance program that aims to monitor microbiological indicators and pathogens in the food supply ("Programa Nacional de Vigilancia de Microorganismos de Higiene y Control de Microorganismos Patógenos, para la Vigilancia Epidemiológica de Enfermedades Transmitidas por Alimentos de Origen Agrícola y Pecuario del país – PNVCH").

Sampling Fresh Produce

Fresh fruits and vegetables (representing 20 types) were obtained from agricultural farms in 17 provinces of Ecuador, from June to December 2019 (**Figure 1**). In total, 274 samples were analyzed (117 vegetables, 119 fruits were collected from agricultural farms. Among the vegetables consist of lettuce (*Lactuca sativa*, *n* = 43), onion (*Allium cepa*, *n* = 31), garlic (*Allium sativum*, *n* = 21), coriander (*Coriandrum sativum*, *n* = 17), cabbage (*Brassica oleracea* var. viridis, *n* = 2), spinach (*Spinacea oleracea*, *n* = 1), pepper (*Piper nigrum*, *n* = 1), tomato (*Solanum lycopersicum*, *n* = 1). The fruit samples correspond to cocoa (*Theobroma cacao*, *n* = 1), peach (*Prunus persica*, *n* = 2), strawberry (*Fregaria vesca*, *n* = 31), melon (*Cucumis melo* var. cantalupensis, *n* = 7), apple (*Malus domestica*, *n* = 1), banana (*Musa paradisiaca*, *n* = 13), blackberry (*Rubus ulmifolius*, *n* = 31), watermelon (*Citrullus lanatus*, *n* = 12), grape (*Vitis vinifera*, *n* = 1), and golden berry (*Physalis peruviana*, *n* = 20).

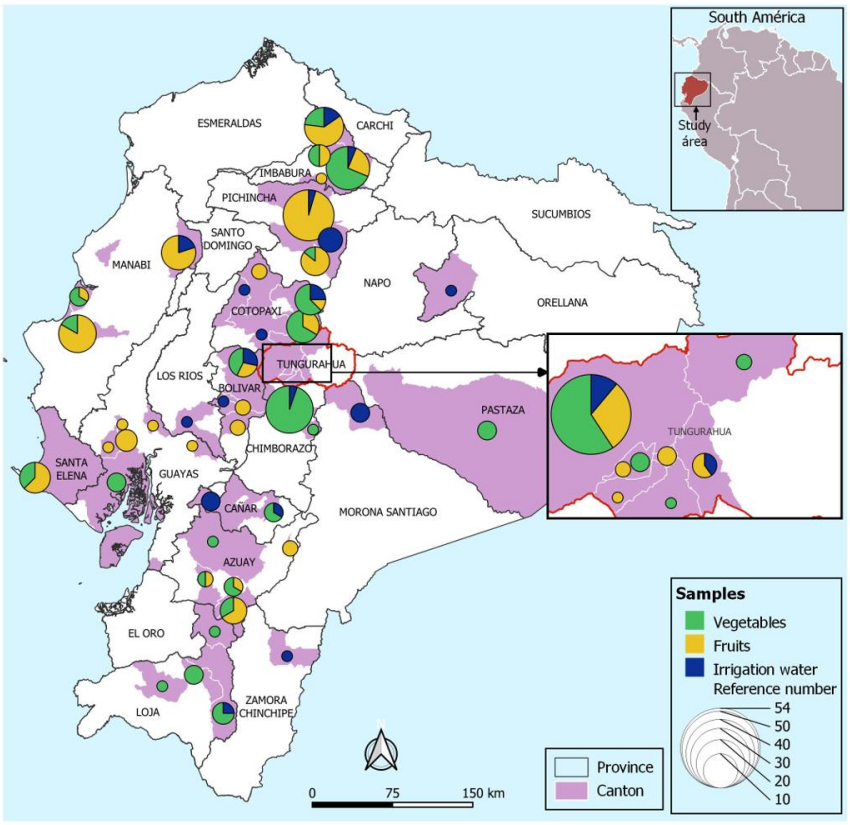

FIGURE 1 | Map of Ecuador showing the sampling locations. Map of sampling locations of irrigation water, fruits and fresh produce. The circles represent the total number of samples according to the color assigned to each sample (vegetables: green, fruits: yellow, and irrigation water: blue) collected in each canton (pink).

Isolation of *Escherichia coli* From Irrigation Water and Produce

The farmers of each crop indicated the irrigation water they used, and this water (n = 37) was collected in sterile bottles and transported to the laboratory at approximately 8°C and processed within 10 h. Five hundred milliliters of water were filtered using a 0.45 µm pore membrane filter (Millipore, United States). The filter was then incubated in Chromocult^R coliform agar (Merck, Germany) overnight at 37°C, the apparent *E. coli* colonies were taken and seeded on MacConkey agar (Difco, United States) supplemented with ceftriaxone (2 mg/L) to identify the lactose positive colonies (a maximum of five colonies were picked from each plate) (Richter et al., 2020), colonies of presumptive *E. coli* were then tested for β-glucuronidase activity using Chromocult^R medium (Merck, Germany). All *E. coli* confirmed isolates from each sample were kept frozen at −80°C in Tryptic Soy Broth medium (Difco, United States) with 15% glycerol.

The vegetable samples were collected aseptically and refrigerated until analysis (within 12 h). Ten grams of the fresh produce were weighed and placed in a sterile plastic bag and incubated with 90 ml of peptone water (Faour-Klingbeil et al., 2016) for 30 min at room temperature. In the case of fruits such as watermelon and melon, the surface was swabbed, and the swab was placed in peptone water (described above). The next day 100 µl of the liquid was taken and cultured on MacConkey agar (Difco, United States) supplemented with ceftriaxone (2 mg/L) (Botelho et al., 2015). A maximum of five

lactose positive colonies were selected from each plate sample and placed on Chromocult coliform agar after 24 h of incubation at 37◦C, colonies of presumptive E. coli, positive for β-glucuronidase, were selected for additional analyses (Lange et al., 2013). All isolates confirmed to be E. coli from each sample were kept frozen at −80◦C in Tryptic Soy Broth medium (Difco, United States) with 15% glycerol.

Antimicrobial Susceptibility Testing

Susceptibility tests were performed using the Kirby-Bauer method on Mueller-Hinton agar (Difco, United States) in accordance with Clinical and Laboratory Standards Institute (CLSI, 2019). Eleven antibiotics were used for testing and included: Cefazolin, CZ (30 μg); Ampicillin, AM (10 μg), Gentamicin, GM (10 μg), Imipenem, IPM (10 μg); Trimethropin-sulfamethoxazole, SXT (1.25/23.75 μg); Ceftazidime, CAZ (30 μg); Cefepime, FEP (30 μg); Ciprofloxacin, CIP (5 μg); Amoxicillin/Clavulanic acid, AmC (20/10μg); cefotaxime, CTX (30μg); and Tetracycline TE (30 μg). After 18 h of incubation, the *E. coli* strains were classified as susceptible, intermediate, or resistant according to the clinical interpretation criteria recommended by CLSI.

E. coli ATCC 25922 was used as quality control. To determine the ESBL phenotype, we carried out a diffusion disk method on Mueller Hinton agar as before using antibiotic susceptibility discs (Oxoid, United States) of CTX (30 μg), CAZ (30 μg). Our criterion to determine ESBL was CTX ≤ 27 mm; CAZ ≤ 22 mm (CLSI, 2019). Specifically, ESBL production was confirmed by growth in a medium with discs of ceftazidime (30 mcg) and ceftazidime + clavulanic acid (30 mcg + 10 mcg). An increase of ≥5 mm in zone of inhibition for ceftazidime + clavulanic acid compared to ceftazidime was confirmed as ESBL producers (CLSI, 2019).

PCR Amplification for Detection of β-Lactamase Genes

When samples were positive for ESBL-producing *E. coli*, one to five isolates selected per sample for further analysis. A total of 96 isolates were tested for the following resistance genes: bla_{SHV}, bla_{TEM}, bla_{CTX-M}, and bla_{OXA} (**Table 1**). Bacterial DNA was extracted by boiling (Dashti et al., 2009), and PCR amplification reactions were performed in a volume of 25 μl containing 12.5 μl of 2 × Qiagen Multiplex PCR Master Mix (Qiagen GmbH, Hilden, Germany), 0.2 μM concentrations of each primer, and 2 μl of DNA template. The cycling parameters were as follows: an initial denaturation at 95°C for 15 min; followed by 30 cycles of 94°C for 30 s, 62°C for 90 s, and 72°C for 60 s; and with a final extension at 72°C for 10 min. Amplification products were observed in agarose gel electrophoresis 1.5%, stained with Ethidium bromide at 100V for 45–60 min. The size of the amplified products was compared with the commercial (Invitrogen, United States) 100-bp ladder. The band size (bp) for each gene was: bla_{SHV}, 237; bla_{TEM}, 445; bla_{CTX-M}, 593; and bla_{OXA}: 813 (Fang et al., 2008).

DNA Sequencing and Analysis

Genomic DNA was extracted from the isolates using the Wizard^{R} Genomic DNA Purification (Promega, United States) according to the manufacturer's instructions. Sequencing was carried out at the University of Minnesota Mid-Central Research and Outreach Center (Willmar, Minnesota) using a single 2 × 250-bp dual- index run on an Illumina MiSeq with Nextera XT libraries to generate ∼30- to 50-fold coverage per genome. Genome assembly of MiSeq reads for each sample was performed using SPAdes assembler with the careful assembly option and automated k-mer detection (Bankevich et al., 2012). The identification of genus and species of the isolates was carried out using fastANI (Jain et al., 2018) with a percentage >80% of identification. Acquired AMR genes, plasmid types were identified using ABRicate tool (version 0.8.13), Resfinder was the database used for the identification of resistance genes (Zankari et al., 2012); PlasmidFinder database for plasmid replicon identification (Carattoli et al., 2014).

Phylogenetic Analysis

Pan-genomic analysis was carried out with Roary (Page et al., 2015); the core genome of the isolates analyzed was defined with at least 99%. A maximum likelihood phylogenetic tree with (1,000 bootstrap replicates) was created based on the core genomes of the isolates using RaxML-NG (Kozlov et al., 2019). The phylogenetic tree was visualized using iTOL (Letunic and Bork, 2019). Additionally, multilocus sequence typing (MLST) (Larsen et al., 2012), based on seven housekeeping genes (adk, fumC, gyrB, icd, mdh, purA, and recA) and core genome (cgMLST) (Hansen et al., 2021) were performed using the Center for Genomic Epidemiology website . The isolates also were characterized by Clermont phylogenetic typing by EzClermont web (Waters et al., 2020).

Sequence Accession Number

The sequences were uploaded to Bioproject- NCBI under the following accession numbers: SAMN20872921, SAMN20872922,

SAMN20872998,	SAMN20873936,	SAMN20873938,
SAMN20873941,	SAMN20873969,	SAMN20873994,
SAMN20874637,	SAMN20875987,	SAMN20875988,
SAMN20875992,	SAMN20875994,	SAMN20875998,
SAMN20879008,	SAMN20879962,	SAMN20879963,
SAMN20879975,	SAMN20879976,	SAMN20880112,
SAMN20880135,	SAMN20880136,	SAMN20881008,
SAMN20881023,	SAMN20881078,	SAMN20881101,
SAMN20881102,	SAMN20881103,	SAMN20881104,
SAMN20881105,	SAMN20881397,	SAMN20881398,
SAMN20881399,	SAMN20881400,	SAMN20882115,
SAMN20882121,	SAMN20882132,	SAMN20882145,
SAMN20882146,	SAMN20882147,	SAMN20882148,
SAMN20882149,	SAMN20883143,	SAMN20883144,
SAMN20883145,	SAMN20883146,	SAMN20883147,
SAMN20884528,	SAMN20884547,	SAMN20884549,

SAMN20886717, SAMN20887874, SAMN20887881,
SAMN20887882, SAMN20887901, SAMN20887904,
SAMN20887915, SAMN20887924, SAMN20887927,
SAMN20887932, SAMN20887933, SAMN20888904,
SAMN20888908, SAMN20888911, SAMN20888912,
SAMN20888913, SAMN20888914, SAMN20888915,
SAMN20888916, SAMN20888921, SAMN20888932,
SAMN20888933, SAMN20888934, SAMN20888941,
SAMN20888958, SAMN20888959, SAMN20888960,
SAMN20888962, SAMN20890819, SAMN20891007.

TABLE 1 | Primers used for detection of different β-lactamase genes in the multiplex PCR.

Genes	Primer sequence (5′ to 3′)	Size (bp)	References
bla_{SHV}	CTT TAT CGG CCC TCA CTCAA AGG TGC TCA TCA TGG GAA AG	237	Fang et al., 2008
bla_{TEM}	CGC CGC ATA CAC TAT TCT CAG AAT GA ACG CTC ACC GGC TCC AGA TTT AT	445	Monstein et al., 2007
bla_{CTX-M}	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593	Boyd et al., 2004
bla_{OXA}	ACA CAA TAC ATA TCA ACTTCGC AGT GTG TTT AGA ATG GTG ATC	813	Ouellette et al., 1987

RESULTS

Prevalence of *Escherichia coli*

In total, 274 samples were collected, including 117 vegetable samples, 119 fruit samples, and 38 irrigation water samples. Across all samples, a total of 30 (11%) were positive for *E. coli;* 11 of the irrigation water samples had *E. coli* (29%, 11/38), 13 vegetables samples had *E. coli* (11%, *n* = 13), and 11 fruits (9%, *n* = 11). In total, 165 isolates of *E. coli* were recovered from 30 samples.

Antimicrobial Susceptibility Testing

Ninety-six isolates (58% *n* = 96) showed extended-spectrum beta- lactamases (ESBL) phenotype according to the CLSI protocols; 58% of *E. coli* isolates from irrigation water were ESBL-producers, 11% from vegetables, and 30% from fruits. ESBL-*E. coli* were isolated from garlic (2 isolates), onion (9 isolates), strawberry (10 isolates), blackberry (4 isolates), banana (14 isolates), and golden berry (1 isolate).

The rate of resistance was high; more than 80% of recovered E. *coli* isolates were resistant to cefazolin, ampicillin, and cefotaxime. In the case of the *E. coli* isolates from irrigation water, 100% of the isolates were resistant to ampicillin and cefazolin. In addition, these isolates had a high prevalence of resistance to cefotaxime (96%), tetracycline (79%), and cefepime (84%) (**Table 2**).

TABLE 2 | Antibiotic susceptibility profiles of isolates ESBL- *E. coli* from irrigation water, vegetables, and fruits.

Antimicrobial categories	Antibiotics	Irrigation water *n* = 56 (frequency/percent)			Vegetables *n* = 11 (frequency/percent)			Fruits *n* = 29 (frequency/percent)		
		R	*S*	I/SDD	*R*	*S*	I/SDD	*R*	*S*	I/SDD
Cephalosporins	Cefazolin	56/100	0/0	0/0	11/100	0/0	0/0	29/100	0/0	0/0
Penicillins	Ampicillin	56/100	0/0	0/0	11/100	0/0	0/0	29/100	0/0	0/0
Aminoglycosides	Gentamicin	17/30	39/70	0/0	7/64	4/36	0/0	15/52	13/45	1/3
Carbapenems	Imipenem	2/4	49/88	5/9	0/0	10/91	1/9	0/0	20/69	9/31
Sulfonamides	Trimethropin/Sulfamethoxazole	36/64	18/32	2/4	10/91	1/9	0/0	21/72	8/28	0/0
Cephalosporins	Ceftazidime	25/45	10/18	21/38	7/64	0/0	4/36	19/66	0/0	10/34
Cephalosporins	Cefepime	47/84	2/4	7/13	10/91	0/0	1/9	22/76	0/0	7/24
Fluoroquinolones	Ciprofloxacin	36/64	10/18	10/18	7/64	2/18	2/18	15/52	9/31	5/17
Aminopenicillin + inhibitor of betalactamase	Amoxicillin/clavulanic acid	17/30	23/41	16/29	6/55	1/9	4/36	22/76	5/17	2/7
Cephalosporins	Cefotaxime	54/96	1/2	1/2	11/100	0/0	0/0	29/100	0/0	0/0
Tetracyclines	Tetracycline	44/79	12/21	0/0	11/100	0/0	0/0	29/100	0/0	0/0

R, resistant; I, intermediate; S, susceptible; SDD, susceptible-dose dependent in the case of cefepime; n, number of isolates tested.

One hundred percent of the *E. coli* isolates from vegetables and fruits were resistant to ampicillin and cefazolin, cefotaxime, and tetracycline. Ninety-one percent of *E.coli* isolates from vegetables were resistant to cefepime. Two ESBL isolates from irrigation water presented resistance to the critically important class carbapenems, however no carbapenemase gene was detected. Additionally, we observed 33 resistance profiles across all of the extended spectrum beta-lactamase-producing *E. coli* isolates. The resistance profiles with the highest number of isolates are summarized in **Table 3**. In addition, 94% (90 of 96) of the *E. coli* ESBL isolates presented multi-drug resistant (MDR) patterns, with non-susceptible to at least one antibiotic in three or more antimicrobial categories (Magiorakos et al., 2012).

TABLE 3 | The sixteen most common resistance profiles for ESBL-*E. coli* isolated from water, vegetables, and fruits in Ecuador.

Resistance profiles	Produce/Fruits	Irrigation water	Total
CZ-AM-GM-SXT-CAZ-FEP- CIP-AmC-CTX-TE	14	4	18
CZ-AM-FEP-CTX-TE	1	5	6
CZ-AM-SXT-CAZ-FEP-CIP- CTX-TE	0	4	4
CZ-AM-SXT-CAZ-FEP-CIP-AmC-CTX-TE	2	1	3
CZ-AM-GM-SXT-CAZ-FEP- CIP-CTX-TE	0	4	4
CZ-AM-GM-SXT-FEP-CIP-CTX-TE	1	4	5
CZ-AM-SXT-FEP-CIP-CTX-TE	0	4	4
CZ-AM-SXT-FEP-CIP-AmC- CTX-TE	4	2	6
CZ-AM-SXT-CAZ-FEP-CTX-TE	3	0	3
CZ-AM-CAZ-FEP-CTX-TE	1	2	3
CZ-AM-GM-CAZ-CTX-TE	2	0	2
CZ-AM-SXT-FEP-AmC-CTX-TE	2	1	3
CZ-AM-SXT-FEP-CTX-TE	4	0	4
CZ-AM-GM-CAZ-AmC-CTX-TE	4	0	4
CZ-AM-SXT-FEP-AmC-CTX	0	2	2
CZ-AM-SXT-CIP-CTX-TE	0	2	2

CZ, cefazolin; AM, ampicillin; GM, gentamicin; IPM, imipenem; SXT, trimethropin- sulfamethoxazole; CAZ, ceftazidime; FEP, cefepime; CIP, ciprofloxacin; AmC, amoxicillin/Clavulanic acid; CTX, cefotaxime; TE, tetracycline.

Genotypes of Extended-Spectrum β-Lactamase – *Escherichia coli*

We obtained high-quality genome sequences of 80 ESBL-*E. coli* isolates. MLST analysis using 7 housekeeping genes showed that 80 isolates were assigned to 37 known STs, whereas 7 isolates represented 7 novel STs. ST10 was shared by 14% (*n* = 11) of isolates from three sources, with a different province of origin: irrigation water (Pichincha), onion (Tungurahua), banana (Manabí), and strawberry (Tungurahua). ST453 (5%, *n* = 4) and ST224 (8%, *n* = 6) were shared in two sources and in different provinces of origin of the sample: ST453 (banana = Manabí, irrigation water = Pichincha), ST224 (irrigation water = Pichincha and Zamora Chinchipe, banana = Manabí) (**Table 4**).

TABLE 4 | Source and genetic characteristics of ESBL- *E. coli* isolates from different sources in Ecuador.

					Relevant antimicrobial resistance genes				
Sample (*)	Source	Location	ST	cgST	CTX-M	TEM	SHV	OXA	mcr-1
H505	Irrigation	Cañ-La Troncal	937	87149	55	141			
H719	Irrigation	Chim-Riobamba	617	93239	3				
H719	Irrigation	Chim-Riobamba	new7	143498	15	1	187	1	
H726	Irrigation	Imb-Ibarra	155	17156	55	141			
V662	Banana	Man-Portoviejo	10	15007	55	1			
V661.1	Banana	Man-Portoviejo	847	28793	55				
V662	Banana	Man-Portoviejo	6598	39050	8, 55	1			
V662	Banana	Man-Portoviejo	453	86226	8, 55	1			
V662	Banana	Man-Portoviejo	453	86226	55	1			
V662	Banana	Man-Portoviejo	453	86226	55	1	12		
V663 (3)	Banana	Man-Portoviejo	224	135673	55	1			1
V661.3	Banana	Man-Portoviejo	new3	136455	55	1	12		
HY1.3.3	Irrigation	Pich-Yaruquí	6027	2725	55	1			
HY6.5.3	Irrigation	Pich-Yaruquí	522	4492	55	1			
HP1.2	Irrigation	Pich-Yaruquí	10	5994	55,65	141			
HP6.4	Irrigation	Pich-Yaruquí	100	6271	15				
HY8.5.3	Irrigation	Pich-Yaruquí	131	9613			12		
HY3.4.3	Irrigation	Pich-Yaruquí	38	13889	9	1			
HY7.5.3	Irrigation	Pich-Yaruquí	206	17904	65	1			
HP1.4	Irrigation	Pich-Yaruquí	752	21656	65				
HY4.2.2	Irrigation	Pich-Yaruquí	224	29102	55	1			
V727 (2)	Strawberry	Pich-Yaruquí	new4	33815	65		12		
HP6.2	Irrigation	Pich-Yaruquí	1725	34210	55		5		
HY3.5	Irrigation	Pich-Yaruquí	1706	38416	15	1			
HP1.1	Irrigation	Pich-Yaruquí	155	40558	65				
HP4.3	Irrigation	Pich-Yaruquí	7290	43104	8				
HP7.2	Irrigation	Pich-Yaruquí	10	46675	55		12		
HP7.4	Irrigation	Pich-Yaruquí	10	46675	55	141	12		
HY2.4.2	Irrigation	Pich-Yaruquí	new2	79725	15				
HY4.4.2	Irrigation	Pich-Yaruquí	3944	80110	55	1			
HP4.4	Irrigation	Pich-Yaruquí	117	81681	55	141			
HP2.4	Irrigation	Pich-Yaruquí	117	82990	55	141			
HP6.3	Irrigation	Pich-Yaruquí	453	86226	55	141			
HY6 (2)	Irrigation	Pich-Yaruquí	540	96158	15	1			
HY1 (2)	Irrigation	Pich-Yaruquí	540	96158	15	1			
HP7	Irrigation	Pich-Yaruquí	124	96630	65				
HY6	Irrigation	Pich-Yaruquí	9580	96650	55	1			
HY2.3.3	Irrigation	Pich-Yaruquí	10	101136	15			1	

HY8.2.2	Irrigation	Pich-Yaruquí	9962	116134		1	12	
HP6.1	Irrigation	Pich-Yaruquí	1725	117316	55			
HY4.4 (2)	Irrigation	Pich-Yaruquí	205	117479	15	1		
HP6.5	Irrigation	Pich-Yaruquí	10340	117591	3	141		
HP2	Irrigation	Pich-Yaruquí	57	117853	55	141		
HP1.5	Irrigation	Pich-Yaruquí	57	117853	55	141		
V727.4	Strawberry	Pich-Yaruquí	new6	119048	65	176	12	
V727.5	Strawberry	Pich-Yaruquí	4541	119048	65		12	
HY6.5	Irrigation	Pich-Yaruquí	10	134002	55	1		
HY1.3.2	Irrigation	Pich-Yaruquí	2973	135505	55, 65	1		
HP1.3	Irrigation	Pich-Yaruquí	354	137556	55	1		
HY4.3.2	Irrigation	Pich-Yaruquí	224	138183	55	1		
HY1.1.4	Irrigation	Pich-Yaruquí	new1	138274		1		
HY5.2.1	Irrigation	Pich-Yaruquí	155	138689	55	1		

TABLE 4 |

					Relevant antimicrobial resistance genes				
Sample (*)	Source	Location	ST	cgST	CTX-M	TEM	SHV	OXA	mcr-1
HY3.5.2	Irrigation	Pich-Yaruquí	155	138689	55	1			
HY1.4.3	Irrigation	Pich-Yaruquí	394	142214	15				
HY6.1.2	Irrigation	Pich-Yaruquí	69	144487	55	1			1
H579.2	Irrigation	Tun-Ambato	206	4018	65				
V696 (4)	Blackberry	Tun-Ambato	5044	32678	55	1			
V698 (3)	Strawberry	Tun-Ambato	10	38518	55	1			
V1140 (2)	Onion	Tun-Ambato	4204	55533	55, 65	1			
V1140	Onion	Tun-Ambato	4204	55533	55	1			
V427.5	Onion	Tun-Ambato	58	60063	55	1			
V469.5	Onion	Tun-Ambato	10	69259	55	1			
V1147 (2)	Garlic	Tun-Ambato	973	118630	3	1			
H579.1	Irrigation	Tun-Ambato	155	138689	55	1			
V427.2	Onion	Tun-Ambato	4368	142214	15				
H430	Irrigation	Zam-Yantzaza	224	135673	55	1			1

**Number of isolates with the same cgST obtained from the same sample. Tun, Tungurahua; Pich, Pichincha; Man, Manabi; Zam, Zamora; Imb, Imbabura; Cañ, Cañar; Chim, Chimborazo.*

The application of a cgMLST scheme showed 55 cgSTs, from which only 2, cgST86226 (banana, Manabí, *n* = 5; irrigation water Pichincha, *n* = 1) and cgST135673 (banana Manabí, *n* = 3; irrigation water, Zamora Chinchipe *n* = 1) were isolates from two different sources. Several isolates belonging to the same ST (based on 7 genes) were assigned to different cgSTs based on cgMLST and some of the isolates from the same sample had the same cgST. Additionally, we constructed a maximum likelihood tree based on the core genomes to compare the phylogeny of isolates of *E. coli* from the irrigation water, vegetables, and fruits (**Figure 2**). The phylogenetic analysis showed that all isolates with the same cgMLST and obtained from different sources differed in thousands of SNPs indicating that although the isolates were genetically close, they have been evolving apart for many years (**Table 4** and **Figure 2**). The genomes of ESBL-*E. coli* isolates from irrigation and fresh produce did not cluster apart; instead, the isolates form different sources seemed to share recent common ancestry (**Figure 2**).

When ESBL-*E. coli* isolates were characterized by Clermont phylogenetic typing, 38% (*n* = 30) isolates belonged to phylogroup A: irrigation water (*n* = 21), strawberry (*n* =

3), onion (*n* = 4), banana (*n* = 2). In phylogroup B1 accounted for 35% (*n* = 28) of isolates: irrigation water (*n* = 15), banana (*n* = 7), strawberry (*n* = 1), blackberry (*n* = 4), and onion (*n* = 1). In phylogroup D it accounted for 14% of the isolates: irrigation water (*n* = 4), strawberries (*n* = 3), garlic (*n* = 2), onion (*n* = 1) and banana (*n* = 1). Phylogroups B2, E and F accounted for 3% (*n* = 2), 5% (*n* = 4) and 3% (*n* = 2) of isolates, respectively. Three (4%) isolates of irrigation water belonged to the cryptic lineage (**Figure 2**).

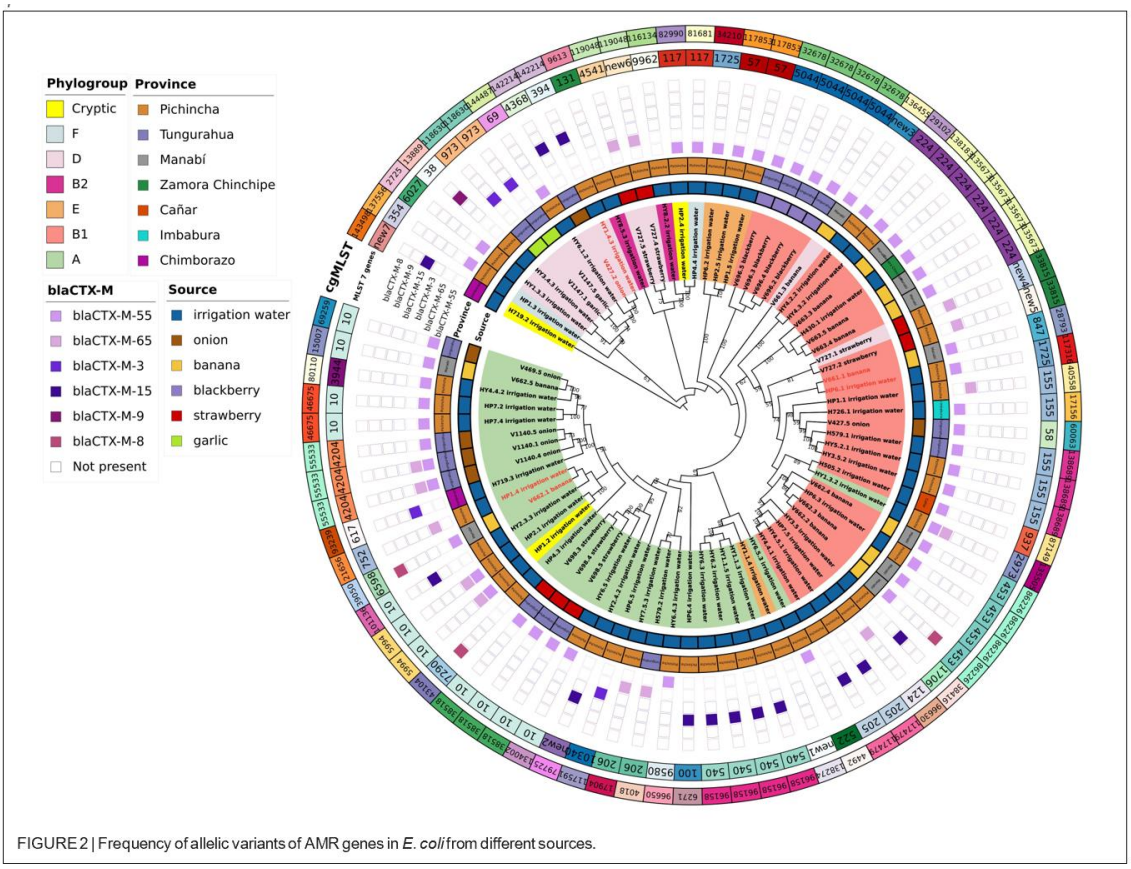

FIGURE 2 | Frequency of allelic variants of AMR genes in *E. coli* from different sources.

Detection of β-Lactamase Genes

Ninety-six *E. coli* isolates phenotypically identified as ESBL, were tested by Multiplex PCR for genes encoding SHV, TEM, CTX-M, and OXA enzymes. The CTX-M gene was detected in 98% (94 of 96) of the isolates, followed by TEM 92% (88 of 96), SHV 28% (27 of 96), and OXA 1% (1/96). Additionally, combinations of genes were present: 64% had both CTX-M and TEM; and 26% had CTX-M, TEM, and SHV.

The presence of AMR genes in the genome sequences of 80 *ESBL-E. coli* isolates was investigated by Resfinder. Several ESBL-encoding $bla_{\text{CTX-M}}$ gene variants were distributed in isolates from irrigation water and fresh produce (**Figure 3**). Among the 80 *ESBL-E. coli* isolates, we identified allelic variants of $bla_{\text{CTX-M}}$ in 77 (96%). The most common allelic variants were $bla_{\text{CTX-M-55}}$ in 49 isolates (64%) and the second most common allele was $bla_{\text{CTX-M-65}}$ in 14 isolates (18%) (**Supplementary Table 1**).

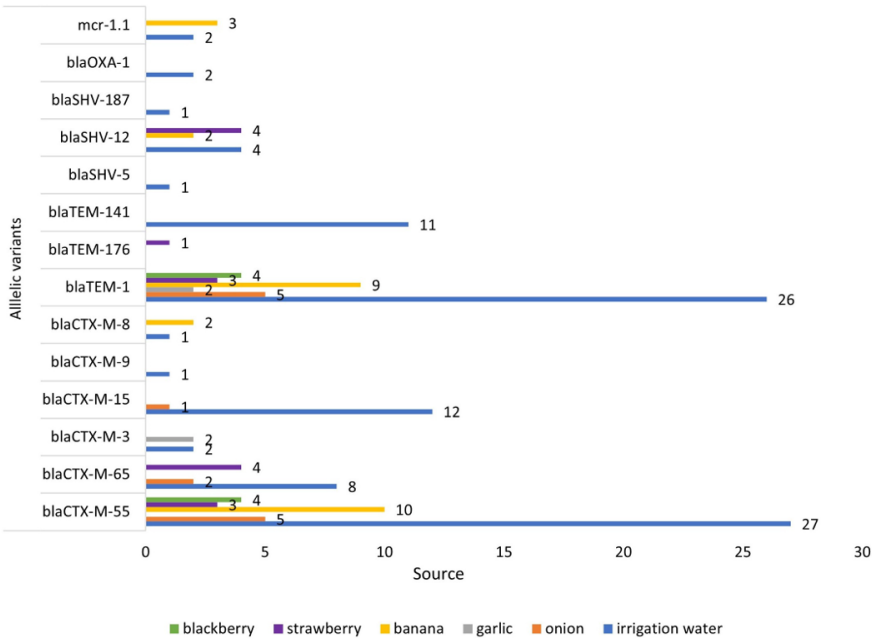

FIGURE 3 | Phylogenetic tree of ESBL-*E. coli* sequences from irrigation water, fruits, and vegetables. Maximum likelihood phylogenetic tree of the core genomes of 80 ESBL-*E. coli* isolates from irrigation water, fruits, and vegetables. The labels show the isolate ID assigned according to the sample ID, the origin of the isolate is shown by source colors (irrigation water: blue, onion: brown, banana: yellow, blackberry: purple, strawberry: red, and garlic: green). The background colors highlighted on the branches of the tree indicate the seven identified phylogroups. Numbers represent bootstrap values using 1,000 pseudo-replicates.

We found some discrepancies in some ESBL- *E. coli* isolates that were positive by PCR for some genes but negative by whole genome sequencing (WGS): 12 isolates for bla_{TEM} gene, 9 isolates for bla_{SHV} genes and bla_{CTX-M} in one gene. Additionally,

2 isolates showed bla_{SHV} and bla_{TEM} using WGS but were negative by PCR. The WGS analysis of ESBL-*E. coli* allowed us to identify 2 isolates of *E. coli* from irrigation water and 3 isolates from banana with the presence of the *mcr-1* gene that confers resistance to colistin.

DISCUSSION

In this study, we found that irrigation water, fruit, and vegetables were contaminated with ESBL-*E. coli* and the highest percentage was found in irrigation water (58%), which confirms the important and emerging role that irrigation water, contaminated with wastewater, has in the spread of ARB and ESBL *E. coli* and ESBL genes. (Gekenidis et al., 2018a; Vital et al., 2018). The major ESBL gene was the $_{CTX-M}$ (94 of 96 isolates) followed by bla_{SHV} 28% (27 of 96), and bla_{OXA} 1% (1of 96). The prevalence of bla_{CTX-M} type ESBL genes in irrigation water *E. coli* was 57%, followed by 15% in banana isolates. Additionally the most abundant allelic variants of bla_{CTX-M} found in vegetables, fruits and irrigation water ($bla_{CTX-M55}$, $bla_{CTX-M65}$, and $bla_{CTX-M15}$) (**Table 4**) are the same alleles found in children and domestic animals in Ecuador (Salinas et al., 2021), in rivers that cross cities (Ortega-Paredes et al., 2020a), and in bacteria from human infections in Ecuador (Cartelle Gestal et al., 2016; Soria Segarra et al., 2018). The presence of the same bla_{CTX-M} alleles in isolates from different sources provides strong evidence that these sources (irrigation water, domestic animals, and humans) are connected. The allelic variants of bla_{CTX-M} from isolates obtained from

same European country, but from different (unconnected) sources, animal species or time periods, have been shown to be different (Day et al., 2019; Ludden et al., 2019).

Our genomic analysis showed that most strains obtained from irrigation water and produce were genetically different with 3 exceptions (HY1.4.3 and V427.2; HP6.1 and V661.1; HP1.4 and V662.1), however the number of SNPs between these strains ranged from 9,332 to 20,310 suggesting that these strains have been evolving apart for many years (**Table 4**). As expected, some isolates from the same vegetable or fruit showed higher level of genetic closeness, for instance: V698.3 and V698.4 had 12 SNP; V663.4 and V663.5, 6 SNPs; V696.2 and V696.4, 13 SNPs; V1147.5 and V1147.1, 2 SNPs). Interstingly, 2 isolates obtaind from the same irrigation channel 1-month appart (HY3.5.2 and HY5.2.1) had 24 SNPs, suggesting that this strain was higly adapted to water. We did not find additional association of ESBL- *E. coli* clusters with provinces, which may indicate that different *E. coli* lineages have been widely distributed in the Ecuadorian territory (**Figure 2**).

These findings may indicated that *E. coli* populations in the environment are highly diverse (Day et al., 2019; Ludden et al., 2019) and *bla*$_{CTX-M}$-genes are probably disseminating in the environment mostly by mobile genetic elements and not so much by bacterial clones. The plasmids carrying *bla*$_{CTX-M}$-genes disseminate efficiently by conjugation, even between bacteria belonging to different genera (Cantón et al., 2012). Transposable elements (such as IS*Ecp1*) are also very active in *bla*$_{CTX-M}$-gene mobilization among different plasmids (Cantón et al., 2012). The activity of these MGEs conceals the source of origin of these antimicrobial resistance genes.

The majority of strains isolated from irrigation water and vegetables belonged to phylogroups A and B1 which are considered more generalists, found in most warm-blooded animals and environmental samples (Touchon et al., 2020). We found that some genetically close *E.coli* isolates, obtained from the same vegetable, had 1 or 2 additional antimicrobial resistance genes which may be a reflection of the dynamic process of antimicrobial resistance gene-turnover in the environment (Barrera et al., 2019).

The *bla*$_{CTX-M}$ type of ESBL gene is of increasing concern globally (Bevan et al., 2017), and is the predominant ESBL gene in both community and hospital-acquired infections (Manyahi et al., 2017; Fils et al., 2021). A troubling feature of *bla*$_{CTX-M}$- bearing plasmids is their ability to capture additional resistance determinants, including carbapenemase genes (Partridge et al., 2012; Potron et al., 2013). Further analysis is necessary to understand whether the plasmids carrying *bla*$_{CTX-M}$ genes, in bacteria from irrigation water and produce, are the same as those circulating in bacterial isolates from human isolates.

In our study fruits, such as bananas, we hypothesize that their contamination was due to post-harvest processes in which the food is often washed in contaminated water and reused to wash several batches of the product. Although it is true, the skin of the product protects the fruit, the transmission of resistant bacteria can occur through contact and inadequate consumer hygiene (Harris et al., 2003; Hong et al., 2013; Kawamura et al., 2017; Murray et al., 2017; Hölzel et al., 2018).

We also found a higher prevalence of ARB in vegetables in farms than in retail markets in Ecuador (Ortega-Paredes et al., 2018). However, other reports from the

Philippines, Lebanon, and Portugal have documented even higher levels (Faour-Klingbeil et al., 2016; Araújo et al., 2017; Vital et al., 2018). In most of the studies, the collection of produce samples has been carried out in groceries and wholesale markets, which makes it difficult to analyze sources of contamination (Bhutani et al., 2015; Yang et al., 2019; Colosi et al., 2020; Richter et al., 2020; Song et al., 2020). In this study, we collected produce and water from farms and their respective irrigation systems, which allowed us to study contamination at the source (i.e., not due to handling, transport, distribution, and processing). We found that MDR isolates were more prevalent in irrigation water isolates compared to fresh produce. Similar results were observed in the Philippines, where 58% of the *E. coli* isolates from irrigation water were MDR (Paraoan et al., 2017). The resistance to these antibiotics was also observed in *E. coli* isolates from irrigation water in other studies (Pignato et al., 2009; Ben Said et al., 2015; Vital et al., 2018).

Our study had some limitations; the number produce and fruit samples obtained in each location may not be representative of produce from other agricultural settings in Ecuador. Additionally, long-read sequencing of plasmids could not be carried out due to budgetary limitations.

We found evidence that fresh produce constitutes an important source of ESBL-*E. coli* and represents a route for the dissemination of resistance genes through the consumption of raw products (Rasheed et al., 2014; Hölzel et al., 2018; Al- Kharousi et al., 2019). We hypothesize that the main source of ABR contamination is irrigation water used for the cultivation of produce, which has been suggested by others as well (Pignato et al., 2009; Gekenidis et al., 2018b). In Ecuador, the lack of sewage treatment may lead to contamination of the food supply with ARB, mainly belonging to the Enterobacteriaceae family (Caicedo-Camposano et al., 2019; Ortega-Paredes et al., 2020a). Antibiotic resistant *E. coli* can transfer antibiotic resistance determinants not only to other strains of *E. coli*, but also to other species of potentially pathogenic bacteria within the gastrointestinal tract (Grasselli et al., 2008; Huddleston, 2014).

CONCLUSION

We found a high prevalence of ESBL-*E. coli* on produce and in irrigation water; bla_{CTX-M} was the main ESBL gene in these isolates. Allelic variants of the bla_{CTX-M} gene found in irrigation channels and vegetables were the same as those observed in commensal *E. coli* from domestic animals, and commensal and pathogenic *E. coli* from humans, suggesting connection between these different sources. This paradigm poses the potential risk of further spreading ARB that are resistant to last-line antibiotics such as carbapenems, which are used exclusively in serious infections in hospitals (Sheu et al., 2019). In this case, resistance goes full circle, from humans to vegetables and fruits (potentially meat and dairy), and back to human populations (Murray et al., 2021). Greater investments are needed to support the development and installation of wastewater treatment systems throughout Ecuador, as well as in other low- and middle-income countries.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

JI and LM: isolation of the *Escherichia coli* strains. LM: writing— original draft. JG, PC, and GT: review and editing. GT and LM: study design. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb. 2021.709418/full#supplementary-material

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CHAPTER III: Transmission of dominant strains of *Campylobacter jejuni* and *Campylobacter coli* between farms and retail stores in Ecuador: genetic diversity and antimicrobial resistance

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Abstract

Thermotolerant *Campylobacter* is an important zoonotic pathogen known for causing gas- troenteritis in humans, with poultry as its primary reservoir. A total of 468 samples were col- lected, of which 335 were chicken carcass samples (representing the food component), and 133 were chicken caeca samples (representing the animal component). These samples underwent culture, with colonies examined under a microscope. Species identification was achieved through multiplex PCR. Additionally, antimicrobial susceptibility profiles were determined using the Kirby-Bauer method, testing sensitivity to gentamicin, ciprofloxacin, tetracycline, and erythromycin. Additionally, 55 *C*. *jejuni* (62.5%) and 33 *C*. *coli* (37.5%) isolates were selected for whole genome sequencing (WGS). A High prevalence of *Campylo- bacter* was observed, with rates of 95.5% (n = 127, $CI_{95\%}$: 92.5% - 98.5%) in the animal component and 72.5% (n = 243, $CI_{95\%}$: 69.9% - 75.1%) in the food component. Specifically,

C. *jejuni* was detected in 33.1% (n = 42) of poultry farms and 38.3% (n = 93) of chicken carcasses, while *C*. *coli* was found in 64.6% (n = 82) of poultry farms and 60.5% (n = 147) of chicken carcasses. Antimicrobials with the highest rates of resistance (67%-100%) were ciprofloxacin and tetracycline, in both animal and food component isolates. Erythromycin resistance was notable, ranging from 22% to 33%, with only two *C*. *jejuni* isolates from retail were resistant to gentamicin. Furthermore, multidrug resistance was identified in 23% (20 isolates) of the *Campylobacter* isolates. Genetic analysis revealed the presence of fourteen resistance genes in both *C*. *jejuni* and *C*. *coli* isolates, including *tet(O)*, $bla_{OXA-460}$, $bla_{OXA-184}$, $bla_{OXA-489}$, $bla_{OXA-193}$, $bla_{OXA-784}$, $bla_{OXA-603}$, *aph(3')-IIIa*, *aad9*, *aph(2'')-If*, *aadE-Cc*, *sat4*, and *ant(6)-Ia*. Additionally, twenty-five plasmids were detected in the 88 *Campylobac- ter* isolates examined. Interestingly, most isolates also harbored genes encoding putative virulence factors associated with pathogenicity, invasion, adherence, and production of cytolethal distending toxin (cdt): *cheV*, *cheA*, *cheW*, *cheY*, *flaA*, *flgR*, *flaC*, *flaD*, *flgB*, *flgC*, *ciaB*, *ciaC*. The WGS analysis showed the presence of several cgSTs in both animal and food components, with nine of them widely disseminated between components. Moreover, *C. coli* and *C*. *jejuni* isolates from different sources presented with less than 11 single nucleotide polymorphisms (SNPs), suggesting clonality (16 isolates). Further analysis using SNP tree demonstrated widespread distribution of certain *C*. *jejuni* and *C*. *coli* clones across multiple farms and retail stores. This study presents, for the first-time, insights into the clonality, plas- mid diversity, virulence, and antimicrobial resistance (AMR) of thermotolerant *Campylobac- ter* strains originating

from the Ecuadorian poultry industry. The identification of AMR genes associated with the main antibiotics used in the treatment of campylobacteriosis in humans, highlights the importance of the prudent use of antimicrobials in the poultry industry. Additionally, this research remarks on the need for regional studies to understand the epidemiology of this pathogen.

Introduction

Thermotolerant *Campylobacter* belongs to the *Campylobacteraceae* family and is one of the most common bacterial foodborne pathogens worldwide [1]. The ingestion of as few as 500– 800 bacterial cells cause human gastroenteritis [1]. The main *Campylobacter* species associated with campylobacteriosis in humans are *C*. *jejuni* and *C*. *coli* [2]. *C*. *jejuni* is a primary causative agent of foodborne diarrheal disease worldwide [3]. On the other hand, *C*. *coli*, although less prevalent, causes an indistinguishable diarrheal illness [1]. Interestingly, in South America, *Campylobacter coli* isolated more frequently, representing about 25% of cases of diarrhea [4].

According to the Centers for Disease Control and Prevention (CDC), thermotolerant *Campylobacter* causes approximately 1.3 million cases of human illness (19.5 per 100,000 inhabitants) in the United States annually [3, 5]. Meanwhile, data from the European Food Safety Authority (EFSA), European States reveals 246,571 cases of campylobacteriosis in 2018. The highest incidence of the disease was associated with the consumption of chicken (37.5%) and turkey meat (28.2%). Consequently, controlling *Campylobacter* in poultry meat has been demonstrated as one of the most effective strategies to diminish the incidence of *Campylobacter* infection in humans [6]. Currently, chicken meat is the first source of protein consumed worldwide with special importance in developing countries where its low cost makes it an affordable option [7]. In fact, in Ecuador, the per capita consumption of chicken meat is 30.14 Kg [8] representing the most consumed type of meat in the country.

In South America, *Campylobacter coli* has been consistently identified in cases of human diarrhea [4, 9, 10], potentially indicating its presence within the food chain [1, 11]. However, few studies report clinical cases [12, 13], prevalence, genetic diversity, and antimicrobial resistance of *Campylobacter* in Ecuador [14–17].

Intestinal campylobacteriosis has an incubation period of 24 to 72 hours [1] and can cause acute bloody or watery diarrhea, fever, weight loss, and cramps [18]. The infection is self-limited most of the time, requiring antibiotic therapy only in severe instances [19]. When treatment is needed, the commonly utilized antimicrobials are macrolide and fluoroquinolone, such as erythromycin and ciprofloxacin, respectively [20]. Additionally, tetracyclines have been recommended as an alternative treatment option [19]. Antimicrobial resistance of *Campylobacter* to first line antibiotics such as ciprofloxacin, has been increasingly reported world- wide [21] prompting the need for constant monitoring of this pathogen [1].

Pulsed-field gel electrophoresis (PFGE) technique, Multilocus sequence typing (MLST), and Restriction fragment length polymorphism (RFLP-flaA) have traditionally been used to study the distribution of *Campylobacter* genotypes in various sources and reservoirs [22]. Nevertheless, these methods have limitations that have been overcome by

sequence-based protocols (such as MLST and whole genome sequencing-WGS). Although genetic typing by MLST allows the comparison of results from different laboratories, WGS provides greater genetic resolution. This technique has enabled the study of genes involved in *Campylobacter* motility, adhesion, and invasion into intestinal epithelial cells, as well as genes responsible for the expression of toxins essential for developing of infection in people [23].

In the present study we used a whole genome sequencing approach to understand the genetic diversity, distribution, virulence genes, and AMR profiles of *Campylobacter* isolates originating in poultry farms (animal component) and chicken carcasses at retail (food component).

Materials and methods

Study design and sampling

Animal component. This study was conducted in the province of Pichincha–Ecuador (0˚ 14'60.00" N -78˚34'59.99" W) from November 2017 to September 2018. In total, 133 flocks were investigated. Twenty-five poultry caeca from individual birds were randomly collected from each flock at the slaughterhouse level. These samples were aseptically transported to the laboratory at 4˚C. In the laboratory, the caeca were immersed in ethanol for 30 seconds and dried by evaporation. From each cecum, 1g of content was collected in a sterile plastic bag to obtain a pooled sample of 25g [17].

Food component. In total, 335 chicken carcasses were collected in traditional street markets, local stores, and supermarkets in Quito city. Each carcass was collected in a sterile bag and transported to the laboratory at 4˚C. In the laboratory, 25 g of breast skin from each car- cass was collected aseptically for subsequent laboratory analysis. For these samples, no ethical approvals were required under current national regulations. However, the Health Minister of Ecuador reviewed and approved the research protocol in the document MSPCURI 000234–5.

Isolation and speciation of *Campylobacter*. The isolation of *Campylobacter* was carried out using an ISO 10272–1:2017 validated culture media [24]. Briefly, 25 g of each sample was homogenized by hand for 1 min. Then, one loop of 10 μL was streaked on a RAPID Campylo- bacter Medium (BIO-RAD, California, USA) and incubated in microaerobic conditions at 42˚C for 48 h using a vacuum chamber filled with a mix of gases (N_2 92% and CO_2 8%). Pre- sumptive *Campylobacter* colonies presented a brick-red appearance and were observed by microscope after the safranin stain. Two spirally curved colonies were plated on blood agar supplemented with 5% defibrinated sheep blood (BD BBL, Maryland, USA) and incubated under microaerobic conditions at 41˚C for 48 h. A subsample of the colonies was used for DNA extraction, and the rest were cryopreserved. DNA was released by the boiling method [25] for PCR identification of *Campylobacter* species [26] (S1 Table in S1 File). Cryopreserva- tion (-80˚C) of strains was carried out in sheep blood [17, 27] for later analysis.

Antimicrobial susceptibility testing. Antimicrobial susceptibility profiles were determined by the Kirby-Bauer method, in accordance with the European Committee on Antimicrobial Susceptibility testing guidelines (EUCAST) [28]. The evaluated antimicrobials were gentamicin (10μg), ciprofloxacin (5μg), tetracycline (30μg), and

erythromycin (15 µg). Interpretation of results was based on the epidemiological cut-off values (ECOFF) recommended by EUCAST [29] (S2 Table in S1 File). The *C*. *jejuni* ATCC 33560 strain was used as quality control.

Whole genome sequencing (WGS) and genome assembly. In order to select a wide diversity of *Campylobacter* genotypes from WGS the following strategy was applied. The selec- tion of isolates from the animal component (poultry farms) was made by skipping a sampling week and considering one isolate per farm. For the food component (chicken carcasses), the first recovered isolate from each retail segment (street markets, local stores, and supermarkets) was selected skipping a sampling week. This selection delivered 88 *Campylobacter* isolates that were WGS (55 *C*. *jejuni* and 33 *C*. *coli*) as described in S3 Table in S1 File.

Extraction of genomic DNA was performed from the 88 selected *Campylobacter* isolates using the Wizard1 Genomic DNA Purification kit (Promega, USA) following the manufac- turer's instructions. DNA quantification and quality parameters were also measured using a Quantus fluorometer (Promega, MD) and NanoDrop 2000 UV-Vis (Thermo Fisher Scien- tific). Whole genome sequencing was performed using the MiSeq platform (Illumina, San Diego, CA) according to FDA GenomeTrakr/CDC Pulse Net protocols in the New York State Department of Agriculture and Markets [30]. Sequence accession numbers are available under BioProject PRJNA788759.

Bioinformatics analyses. Reads quality was assessed by FastQC V. 0.11.9 [31], the Adapter/Quality Trimming was performed using BBDuk v.38.84 [32], and assembling of reads was made using SPAdes assembler v.3.15.2 [33]. KmerFinder (www.genomicepidemiology. org) was used to identify genus and species. The tools MLST V. 2.0.9 [34] and cgMLSTFinder v.1.2 [35] from the Center for Genomic Epidemiology (www.genomicepidemiology.org) were used for Multilocus sequence typing (MLST) and Core genome MLST (cgMLST) respectively with default settings.

Pan-genome analysis was conducted for each species using Roary v.3.13.0 [36], where genes identified as core were present in at least 95% of the sequences of the isolates analyzed. After- ward, the SNPs of all core genomes were extracted by SNP-sites v.2.5.1 [37]. Finally, a maxi- mum-likelihood phylogenetic tree with 1,000 bootstrap replicates based on SNP´s was constructed using RaxML-NG v.1.1.0 [38]. The phylogenetic tree was pictured using iTOL v.6 web tool [39]. The number of SNP differences between isolates was quantified using the Snippy program with standard settings [40].

Additionally, AMRFinderPlus v.3.10.24 [41] was used to inquire about mobile genes and point mutations (SNPs) related to Antimicrobial Resistance (AMR). In addition, the ABRicate tool v.1.0 [42] with Virulence Factor Database-VFDB (dated 2022/04/27) were used to identify virulence genes using a threshold of at least 80% for identity and coverage [43].

Plasmid prediction was performed using Platon v.1.6 [44]. Complementary, the identity of the plasmid was accessed using the map to reference tool of Geneious Prime 2022.1.1. (https:// www.geneious.com) and NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis

Microsoft Excel (2022) was used to calculate the prevalence with 95% confidence intervals.

Results

Prevalence

During the study, 468 samples, 133 from feces (animal component) and 335 from carcasses (food component) were analyzed. The prevalence of *Campylobacter* was 95.5% (n = 127, $CI_{95\%}$: 92.5% - 98.5%) in the animal component and 72.5% (n = 243, $CI_{95\%}$: 69.9% - 75.1%) in the food component. We detected 33.1% (n = 42) and 38.3% (n = 93) of *C. jejuni* in poultry farms and chicken carcasses, respectively. On the other hand, *C. coli* was found in 64.6%

(n = 82) of poultry farms and 60.5% (n = 147) of chicken carcasses. The species of six isolates (three isolates from retail and three from farms) could not be identified by Multiplex PCR (S1 Table in S1 File).

Table 1. Antimicrobial resistance of *C. jejuni* and *C. coli* isolates.

Antibiotic	Number (%) of resistant isolates			
	Animal component (poultry farm) n, (%)		Food component (chicken carcasses) n, (%)	
	C. jejuni (n = 23)	*C. coli* (n = 15)	*C. jejuni* (n = 32)	*C. coli* (n = 18)
Ciprofloxacin	23 (100%)	15 (100%)	32 (100%)	17 (94%)
Tetracycline	22 (96%)	10 (67%)	30 (94%)	13 (72%)
Erythromycin	5 (28%)	5 (33%)	7 (22%)	4 (22%)
Gentamicin	0 (0%)	0 (0%)	2 (6%)	0 (0%)

Antimicrobial resistance

Antibiogram. The antimicrobials with the highest resistance rates were ciprofloxacin and tetracycline (67%– 100%). The percentage of resistance was higher in *C. jejuni* than in *C. coli* from the food and animal components. On the other hand, resistance to erythromycin ranged from 22 to 33%, while only two isolates of *C. jejuni* originating from the food component were resistant to gentamicin (Table 1).

In addition, four resistance profiles in all *C. coli* and *C. jejuni* isolates were observed. Most of isolates were resistant to ciprofloxacin and tetracycline (63%, n = 55) (S4 Table in S1 File). Multidrug resistance (resistance to more than two classes of antibiotics) was detected in nine (27%) of *C. coli* isolates and 11 (20%) of *C. jejuni* isolates (S4 Table in S1 File).

Detection of resistance genes and mutations

Fourteen resistance genes were identified in *C. jejuni* and *C. coli* isolates including *tet(O)* (resistance to tetracycline), $bla_{OXA-460}$, $bla_{OXA-184}$, $bla_{OXA-489}$, $bla_{OXA-193}$, $bla_{OXA-784}$, $bla_{OXA-603}$ (beta-lactams resistance), and *aph(3')-IIIa*, *aad9*, *aph(2'')-If*, *aadE-Cc*, *sat4*, and *ant(6)-Ia* (aminoglycoside resistance) (Table 2).

Only two *Campylobacter* (one isolate of *C. jejuni* and one isolate of *C. coli*) did not present the mutation T86I in *gyrA* gene. In addition, the mutation T86K was identified in only one *C. jejuni* isolate. The mutation in the *23S*rRNA at position A2075G linked to the erythromycin resistance was detected in 27.2% (n = 9) of *C. coli* isolates. The mutation L22: A103V in the *rplV* gene responsible for erythromycin resistance, was observed in

22% of *C*. *jejuni* isolates (n = 12). Finally, the analysis of the gene encoding the S12 ribosomal protein (*rpsL* gene) related to streptomycin resistance, showed the K88R mutation (one isolate of *C*. *coli*) and the K43R mutation (one isolate of *C*. *coli*) (Table 2).

Most of the *Campylobacter* isolates (except for two *C*. *jejuni* isolates) showed genetic resistance determinants compatible with their resistant phenotype (S5 Table in S1 File). One *C*. *jejuni* isolate that was phenotypically resistant to aminoglycosides carried more than one resis- tance gene for resistance to this group of antibiotics (*aad9*, *aph(3')-IIIa*, *aph(2'')-If*).

Genomic analysis

Core genome sequence type (cgST) designation for the 88 isolates of *Campylobacter* of *C*. *coli* and *C*. *jejuni* is presented in Fig 1A and 1B. The cgMLST analysis showed high genetic diversity among the 55 *C*. *jejuni* and 33 *C*. *coli* strains. However, cgMLST revealed that some *C*. *coli* and *C*. *jejuni* clonal or near clonal isolates (same cgST) were present in different farms and retail stores. We found cgST-22156, cgST-22408, cgST-29858, cgST-30929, cgST-31023, cgST-34079, cgST-965, cgST-6781, cgST-30698 in animal and food components (S6 Table in S1 File). We also found isolates, with less than 11 SNPs of difference in different sources (farms and retail) (S7 Table in S1 File). Furthermore, we found that two *Campylobacter coli* clones that were first isolated in farms (U1446c, U673c) were detected later in retail chicken carcasses (U1664c, U814c) (Fig 1, literal a). Additionally, one clone was found in unrelated farms (Fig 1, literal b, U969c).

MLST analysis using seven housekeeping genes assigned *C*. *jejuni* isolates to 21 known STs and one novel ST. *C*. *coli* isolates were assigned to 11 known STs. (S8 Table in S1 File). The most frequent STs were ST-607 (9 isolates), ST-829 (8 isolates), ST-7669 (7 isolates), and ST- 8317 (6 isolates). ST-607, ST-5777, ST-3515, ST-7669, ST-8316, ST-1038, ST-9336, and ST-829 were observed in both components (animal and food). On the other hand, 16STs were observed in isolates originating in either farms (8STs, n = 14) or retail stores (8STs, n = 9) (S8 Table in S1 File).

The 32 isolates of *C*. *coli* in this study belonged to the clonal complex CC-828. One isolate of *C*. *coli* belonged to a not assigned clonal complex (CC) (U658c). Regarding the isolates of *C*. *jejuni*, the most frequent CCs were CC-353 (n = 12), CC-354 (n = 10) and CC-607 (n = 10).

Furthermore, four *C*. *jejuni* isolates did not correspond to an assigned CC. Some isolates belonging to the same ST from different components had similar cgSTs assignments (S8 Table in S1 File).

SNP tree analysis also revealed that some *C*. *jejuni* and *C*. *coli* clones were distributed across multiple farms.

Table 2. Number of *Campylobacter coli* and *C. jejuni* isolates with resistance-related mutations and acquired genes.

Antibiotic class	Gene (s)	Point mutation	No. of isolates with detected genes and mutations, n, (%)	
			***C. coli* (n = 33)**	***C. jejuni* (n = 55)**
Fluoroquinolone	*gyrA*	T86I	32, (97%)	54, (98%)
		T86K	0, (0%)	1, (2%)
Macrolide	*rplV*	A103V	0, (0%)	12, (22%)
	23S	A2075G	9, (27%)	0, (0%)
Aminoglycoside	*rpsL*	K88R	1, (3%)	0, (0%)
		K43R	1, (3%)	0, (0%)
	aph(2'')-If		0, (0%)	1, (2%)
	aph(3')-IIIa		4, (12%)	2, (4%)
	aadE-Cc		1, (3%)	0, (0%)
	ant(6)-Ia		3, (9%)	0, (0%)
	sat4		3, (9%)	0, (0%)
	aad9		0, (0%)	1, (2%)
Betalactams	*blaOXA-450*		1, (3%)	0, (0%)
	blaOXA-460		0, (0%)	8, (15%)
	blaOXA-489		4, (12%)	0, (0%)
	blaOXA-193		9, (27%)	26, (47%)
	blaOXA-184		0, (0%)	5, (9%)
	blaOXA-603		0, (0%)	1, (2%)
	blaOXA-784		2, (6%)	0, (0%)
Tetracycline	*tet(O)*		23, (70%)	52, (95%)

Plasmids identification

A total of 25 plasmids were found, and 47% of isolates (n = 41) carried plasmids (Fig 1A and 1B, S9 Table in S1 File). The pTet plasmid was found in one strain (U1680c) but the tetracycline resistance gene *tetO*, was not present in this plasmid. Furthermore, four *C. coli* and one *C. jejuni* isolates carried three plasmids, while two *C. coli* isolates had four plasmids.

The isolates that carried the plasmids pR19.0802_49k-like (one isolate), pCC31-like (one isolate), pCJ14980A-like (one isolate), pCCDM224L-like (two isolates), and pD6759-1-like (one isolate), harbored the *tetO* gene. Additionally, the *aph(3')-III* gene related to resistance to aminoglycosides was found in plasmids: pCJ14980A-like (two isolates), pR19.0802_49k-like (one isolate), and pCCDM224L-like (two isolates). Plasmids pCCDM33S-like and pCC42yr- like belonging to type-2 plasmids were found in four isolates of *C. coli*.

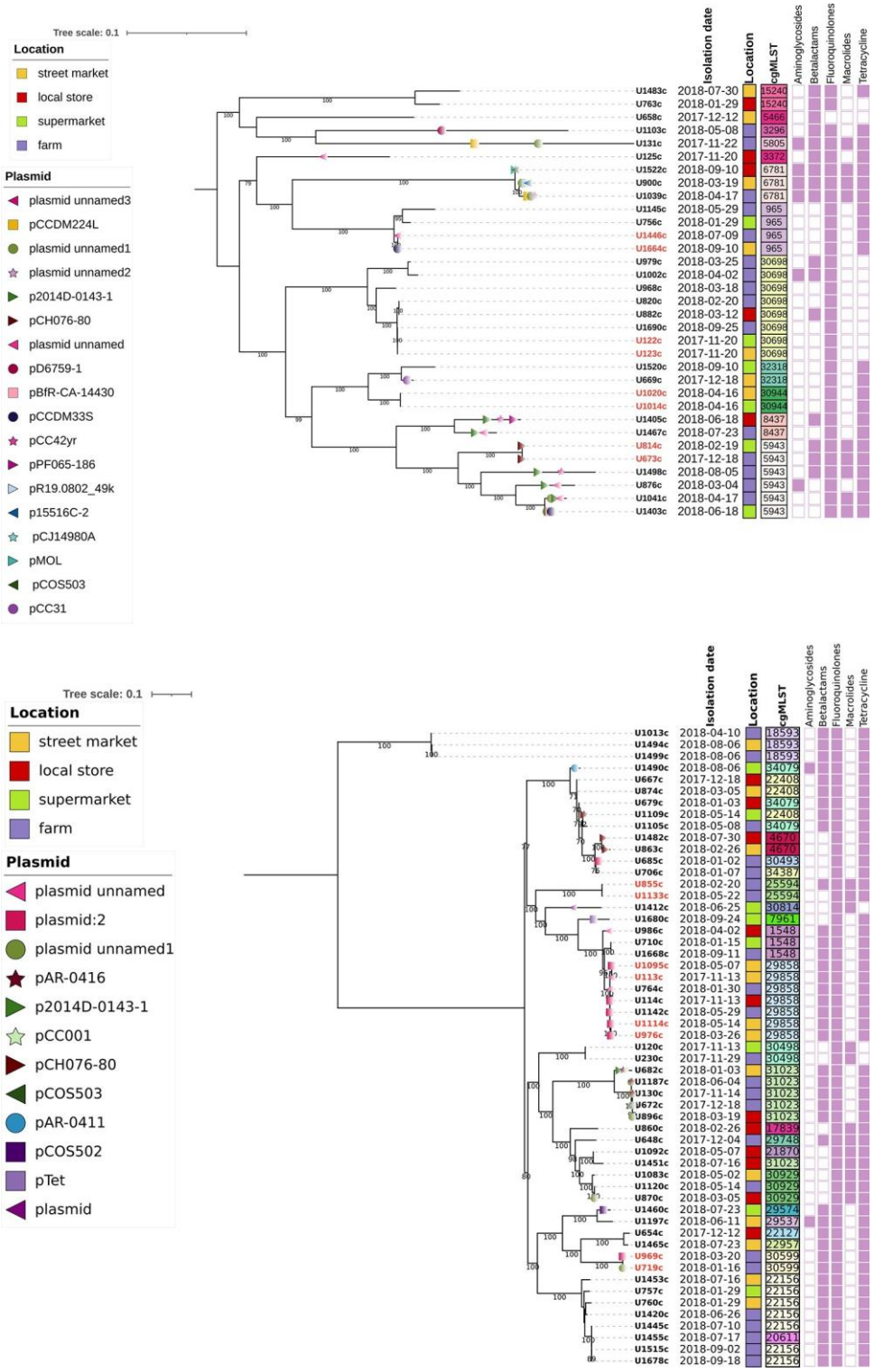

Fig 1. Phylogenetic analysis of *Campylobacter* isolates (a) 33 isolates of *C. coli* and (b) 55 isolates of *C. jejuni*. The labels show the identification of the isolate. Isolates highlighted in red are isolates with less than 11 SNPs. Isolation date (yyyy/mm/dd). Pink colored blocks represent resistance to antimicrobial classes based on identification of resistance genes. Plasmids are depicted with different figures and colors in each isolate branch.

Virulence factors

This study detected putative virulence factors associated with pathogenicity, invasion, adherence, and production of the cytolethal distending toxin (*cdt*) *Campylobacter*

isolates (S10 Table in S1 File). The putative virulence factors: *cheV*, *cheA*, *cheW*, *cheY*, *flaA*, *flgR*, *flaC*, *flaD*, *flgB*, *flgC*, *ciaB*, *ciaC* were found in most of *C. jejuni* and *C. coli* isolates (Table 3).

Discussion

The prevalence of *Campylobacter* in the animal component (poultry farms) was higher (95.5%) compared to the food component (72.5%) (chicken carcasses). However, *Campylobacter* is known to persist in large numbers of fresh foods such as chicken carcasses [45, 46]. Studies conducted in other Latin American countries have reported other rates. For example, the occurrence of *Campylobacter* in carcasses and chicken products was 32.7% in Brazil [47]. Peru also reported a prevalence of 16.7% in carcasses and 26.7% in cecum samples [48]. Besides, the prevalence of *Campylobacter* in poultry-integrated companies ranged from 7% to 10% in Colombia [49]. These variations could be explained by differences in environmental conditions in each country [50, 51], the use of different methodologies for the identification of this pathogen [24], the labile nature of *Campylobacter* in the environment [52], or the high load of *Campylobacter* in feces [53].

In this study, *C. coli* was isolated more frequently than *C. jejuni* in both components. A higher prevalence of *C. coli* (68.7%) over *C. jejuni* (18.9%) has been previously reported in Ecuadorian poultry [17]. Other countries in Latin America like Argentina [54], and Peru [55] have also reported a higher prevalence of *C. coli*. However, countries like Brazil [56] and Costa Rica [57] have reported a higher prevalence of *C. jejuni*. This variation in the ratio of *Campylobacter* species between countries has also been reported in Europe, placing climatic conditions as the probable cause of these observations [58]. Although this statement could be valid in Latin America, more studies are needed to shed light on the regional epidemiology of *C. coli* and *C. jejuni* [4].

This study revealed resistance rates in *C. jejuni* and *C. coli* for tetracycline and ciprofloxacin from 30% to 56%. This fact agrees with a previous resistance study where *Campylobacter* was isolated from Ecuadorian broilers at slaughter age [17]. This is also the case in other Latin American countries where similar antibiotic resistance rates have been reported [59, 60]. One of the main factors influencing antimicrobial resistance, especially to fluoroquinolones and tetracyclines, is the use of these antimicrobial agents in animal production. Due to their easy administration and availability without a prescription, these antimicrobials are widely used by farmers without the supervision of a veterinarian [61].

Almost all phenotypes of antimicrobial resistance (AMR) exhibited concordance between phenotypic and genotypic profiles, indicating a strong correlation between genetic determinants. However, in two cases (S5 Table in S1 File) discrepancies were observed. Further analysis suggests that those two cases could be the result of low sequence quality, demonstrating the high levels of AMR predictions reached with the WGS analysis [62].

The most common tetracycline resistance mechanism observed in *Campylobacter* is the protection of the ribosomal binding site. This mechanism is mediated by ribosomal

protection proteins encoded by the *tetO* gene, frequently encoded by the plasmid pTet— (type-1) [63].

This gene was identified in 85% of our isolates. However, in this study, only six isolates harboring type-1 plasmid carried the *tetO* gene. Although the presence of *tetO* in the chromosome of *Campylobacter* has been reported previously [64, 65], the presence of this gene in both the chromosome and the plasmids suggests that the gene was present on the chromosome and then transferred to integrated plasmids [63]. These results suggest that processes like transformation, conjugation, and transduction can occur in *Campylobacter* and most likely contribute to the horizontal gene transfer of antibiotic-resistance genes. Previous studies in Ecuador have also reported high frequencies of resistance to ciprofloxacin [14, 17, 66].

Resistance to ciprofloxacin linked to the Thr86-Ile (T86I) substitution in the *gyrA* gene was the most frequent resistance mechanism to quinolones found in this study (n = 86 isolates).

Furthermore, this mutation has been commonly observed in fluoroquinolones-resistant *Campylobacter* strains [67, 68]. Other studies conducted in Canada, Senegal, and Brazil have also reported the predominance of this mutation in their ciprofloxacin-resistant chicken isolates [69–71]. On the other hand, the mutation A2075G in 23S rRNA which confers resistance erythromycin [72], was observed in nine *C. coli* isolates despite their low phenotypic resistance to macrolides (9%-15%). This mutation has also been reported in Asian countries [73, 74].

Also, the A103V substitution was the major type of substitution in *C. jejuni* isolates in this study. This amino acid substitution in the ribosomal proteins L4 and L22 is linked with a low level of macrolide resistance in *Campylobacter* species [72].

The frequency of resistance genes to aminoglycosides in the present study was low (17%) and mainly found in *C. coli* isolates: *aph(2'')-If* (n = 1 isolate), *aph(3')-IIIa* (n = 6 isolates), *ant (6)-Ia* (n = 3 isolates), *sat4* (n = 3 isolates) and *aad9* (n = 1 isolate). Inversely, a high frequency (73%) of resistance genes of beta-lactamases was found in *C. jejuni*, with the $bla_{\text{OXA-193}}$ gene being the most frequent. The presence of beta-lactamases in a high proportion of *C. jejuni* iso- lates has been documented in some studies [75, 76]. It should be noted that *Campylobacter* exhibits intrinsic resistance to some beta-lactams [77, 78]. However, the genetic determinants of resistance to aminoglycosides and beta-lactamases found in this research should be monitored for possible frequency changes.

The low antimicrobial resistance rates to macrolides and aminoglycosides found in this study suggest that erythromycin and gentamicin can still be used when needed. The detection of AMR genes for the main antibiotics used in the treatment of campylobacteriosis raises concerns and highlights the importance of the prudent use of antimicrobials in Ecuadorian broiler production. In addition, most of the strains in this study showed multidrug resistant profiles (fluoroquinolone, tetracycline, and macrolide) which may reflect the indiscriminate use of these antibiotics [79].

Table 3. Number of isolates of *C. jejuni* and *C. coli*, by putative virulence genes.

Virulence trait	Putative virulence factor	Percentage of positive isolates n, (%)	
		***C. coli* (n = 33)**	***C. jejuni* (n = 55)**
Motility	*cheA*	28, (85%)	53, (96%)
	cheV	28, (85%)	54, (98%)
	cheW	28, (85%)	54, (98%)
	cheY	28, (85%)	53, (96%)
	flaA	21, (64%)	24, (44%)
	flaB	18, (55%)	24, (44%)
	flaC	28, (85%)	54, (98%)
	flaD	28, (85%)	53, (96%)
	flaG	30, (91%)	54, (98%)
	flgA	2, (6%)	53, (96%)
	flgB	28, (85%)	53, (96%)
	flgC	29, (88%)	55, (100%)
	flgR	32, (97%)	52, (95%)
	flgS	32, (97%)	54, (98%)
	fliA	28, (85%)	55, (100%)
	pseD/maf2	7, (21%)	22, (40%)
	pseE/maf5	23, (70%)	47, (85%)
	pseA	33, (100%)	54, (98%)
	ptmA	23, (70%)	48, (87%)
	ptmB	33, (100%)	49, (89%)
	rpoN	29, (88%)	54, (98%)
	maf4	12, (36%)	26, (47%)
Adhesion and colonization	*jlpA*	0, (0%)	53, (96%)
	porA	0, (0%)	31, (56%)
	cadF	28, (85%)	54, (98%)
	pebA	28, 985%)	53, (96%)
Cytotoxin production	*cdtA*	0, (0%)	52, (95%)
	cdtB	0, (0%)	55, (100%)
	cdtC	0, (0%)	55, (100%)
Invasiveness	*ciaB*	28, (85%)	53, (96%)
	ciaC	28, (85%)	55, (100%)
Binding and adhesion-LOS	*wlaN*	0, (0%)	3, (5%)
	neuA1	0, (0%)	3, (5%)
	neuB1	4, (12%)	3, (5%)
	neuC1	4, (12%)	3, (5%)
	cstIII	0, (0%)	2, (4%)
Immune evasion-capsule	*glf*	0, (0%)	5, (9%)
	kpsC	0, (0%)	49, (89%)
	rfbC	2, (6%)	26, (47%)
	kfiD	0, (0%)	5, (9%)
	hddA	11, (33%)	37, (67%)
	hddC	0, (0%)	37, (67%)
	gmhA2	11, (33%)	37, (67%)
	fcl	0, (0%)	7, (13%)

The results of this study underline the importance of poultry in the epidemiology of *Campylobacter* infections as some isolates with the same cgST (cgST5943, cgST965) were found in both animal and food components. It has to be noticed that the mutation rate of thermotoler- ant *Campylobacter* has been estimated to be $2.07e^{-6}$ per site per year which is close to 3.5 muta- tions in the genome per year [80]. Our study identified isolates

showing less than 11 SNPs of difference (S7 Table in S1 File), suggesting transmission events among farms of integrated poultry companies and between components (animal and food). Some clonal strains (Fig 1) seemed more successful (capable of thriving in animal intestines, spreading effectively, and surviving in the environments outside the animal host) than others because they were present in different farms and food components at different times (Fig 1A and 1B). It is critical to study whether these successful clones are also causing human disease and it´s severity. The existence of successful *Campylobacter* clones has been described previously [81].

Although having lower molecular resolution than cgMLST, MLST provides valuable information which allows us to compare our data with previous reports. The most frequent *C. jejuni* ST was ST-607. Remarkably, this ST has been previously found in chicken isolates in Ecuador [16]. In the same way, other STs (ST-353, ST-462, ST-6091, ST-6244, ST-137, ST-1233, ST-

7669, ST-464 and ST-3515) have also been described in *Campylobacter* originated in chickens and other animals in Ecuador [16, 17]. Similarly, seven STs from *C. coli* (ST-828, ST-829, ST- 902, ST-5777, ST-8316, ST-8317) have been previously found in Ecuador [17]. Some of these STs have been reported in other countries in the broiler production chain (ST-137, ST-3515) and human cases (ST-1233) of gastroenteritis [82–85]. From the 55 *C. jejuni* isolates tested, the majority belonged to the CC-353 (n = 12), CC-354 (n = 10) and CC-607 (n = 10), while the 32 out of 33 *C. coli* isolates of this study belonged to the CC-828. Predominantly distribution of *C. coli* within CC-828 has also been reported in Ecuadorian poultry [17]. Meanwhile, the results in *C. jejuni* isolates suggested a high diversity of CCs. This is in accordance with the findings of another local studies [16, 17]. Other less common CCs found in this study (CC-607, CC-574, CC-443) have also been reported in poultry from Korea [86], Thailand [87] and China [88].

Our findings emphasize the importance of studying the epidemiology of *Campylobacter* in low and middle-income countries to learn whether some genotypes might be restricted to a specific source, and whether certain genotypes are most frequently causing human disease.

The pathogenicity of thermotolerant *Campylobacter* is mediated by several virulence factors. The expression of genes that are related to the motility, adhesion, and invasion of *Cam- pylobacter* in intestinal epithelial cells, and toxin production is vital for the colonizing chicken intestines and establishing of infection in humans [23, 89]. The flagellin-coding *flaA* gene, which is the most important for bacterial motility [90] was present in 64% of *C. coli* and 44% of *C. jejuni* in this study. The low frequency of flagellin genes has already been reported in pre- vious studies [91, 92]. However, it has been reported that the *flaA* gene in *Campylobacter* ran- ged from 78%-100% [93, 94]. Remarkably, the later studies used PCR techniques that specifically target this gene, while WGS could render gaps in the consensus sequences that could sub-estimate the presence of this genetic determinants [91]. Moreover, one investigation in 40,371 *C. jejuni* genomes found that the full length flagellin locus (*flaA* and *flaB*) was present in only 35% of cases [95]. This research proposes that the low identification of these genes by WGS could be explained by recombination events that promote a high variability of these genes within the *C. jejuni* genome [95].

The *cadF* gene was detected in 98% of the *C*. *jejuni* isolates and 85% of *C*. *coli* isolates in this study. The *cadF* gene is responsible for adhesion and influencing microfilament organiza- tion in host cells [90]. Similar results were obtained in previous studies in *C*. *jejuni* of poultry origin from Japan, India, and Brazil with the *cadF* gene is present in almost of all isolates [96, 97]. Many virulence factors have been correlated with the invasion of *Campylobacter* into intestinal epithelial cells, including *ciaB* gene (*Campylobacter* invasive antigen B) [90]. This gene was present in or study in 85% of *C*. *coli* and 96% of *C*. *jejuni*. On the other hand, the *cdtA*, *cdtB*, and *cdtC* genes (cytolethal distending toxin-*cdt* operon) are required for the expres- sion of cytotoxins that damage the host's nuclear DNA and cause cell death [23]. This study found these genes in almost all (99%) *C*. *jejuni* isolates. Comparable findings were previously reported in *Campylobacter* isolates from animals, food, and humans [98]. These findings are in agreement with the notion that the *cdt* operon was more frequently present in *C*. *jejuni* than in

C. *coli* [99, 100]. It should be noted that genes associated with the occurrence of Guillan Barre´ syndrome-GBS, including *neuABC*, *wlaN*, and *cstIII*, were detected ranging from 4% to 12% of *Campylobacter* isolates in this study. These genes produce sialyltransferases (molecules resemble mammalian gangliosides), which cause the development of antibodies that could trigger an autoimmune reaction [101]. There are no statistics on Guillain Barre´ syndrome cases in Ecuador, it would be important to study the association of this syndrome with infections caused by

C. *jejuni* in the country. The presence of the GBS genes suggests that these isolates could become pathogenic in case of human infection. However, *Campylobacter´*s aptitude to cause human disease is likely multifactorial [90].

Despite the significance of these bacteria as leading causes of foodborne illness, information regarding plasmids in *C*. *jejuni* and *C*. *coli* remains poorly studied in Latin America. Previous investigations focusing on *Campylobacter* isolates from humans, poultry and pigs, revealed plasmid presence in 22% to 64% of isolates [102, 103] which agrees with our results. Moreover, several plasmids identified in this study have yet to be classified within the three recognized classes of *Campylobacter* plasmids [63].

This study showed that *Campylobacter* isolates from poultry caeca and chicken carcasses in Ecuador have high resistance to quinolones and tetracyclines, pathogenicity potential, and diverse genotypes. The coupling of antibiotic resistance and virulence poses a substantial and alarming issue to food safety and public health.

Conclusion

The present analysis sheds light on the prevalence, antimicrobial resistance patterns, genetic diversity, and virulence factors of *Campylobacter* isolates from poultry caeca and chicken carcasses in Ecuador. The higher prevalence of this pathogen in poultry farms and chicken carcasses underscores the importance of understanding the dynamics of contamination along the food production chain. Moreover, this study reports the predominance of *C*. *coli* over *C*. *jejuni*.

Our findings about the high phenotypic resistance to tetracyclines and quinolones, coupled with the presence of resistance genes and virulence factors, raise concerns regarding food safety and public health. Additionally, the detection of successful clones

across farms and food components highlights the potential for transmission of *Campylobacter* to consumers. The analysis of this data emphasizes the importance of prudent antimicrobial use in poultry pro- duction and the necessity of an active surveillance of this pathogen in Ecuador.

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CHAPTER IV: GENERAL CONCLUSION

We studied the antimicrobial resistance two intestinal bacteria that are present in food in Ecuador and found that:

I. A high prevalence of ESBL-producing *E. coli* was detected in fresh produce and irrigation water across multiple Ecuadorian provinces. The bla_{CTX-M} gene was the predominant resistance determinant.
II. Identical allelic variants of bla_{CTX-M} were found in isolates from irrigation channels, vegetables, human commensals, and animals, indicating cross-reservoir transmission.
III. *Campylobacter* spp. isolates exhibited high phenotypic resistance to key antimicrobials, including tetracyclines, quinolones, and macrolides, in both poultry farms and chicken carcasses from markets.
IV. Genomic analyses revealed clonal strains shared between farms and retail products, suggesting transmission through the poultry production chain.
V. Genes associated with Guillain-Barré Syndrome (GBS), such as *neuABC*, *wlaN*, and *cstIII*, were detected in a subset of *Campylobacter* isolates, representing a potential public health threat.
VI. These findings reflect a closed-loop transmission cycle of antimicrobial resistance involving environmental, animal, food, and human reservoirs, reinforcing the need for a One Health approach.