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Salmonella and *Campylobacter* in broilers at slaughter age: a possible source for carcasses contamination in Ecuador

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List of abbreviations

ARB	Antimicrobial Resistant Bacteria
BPW	Buffered Peptone Water
BRICS	Brazil, Russia, India, China, and South Africa
CC	Clonal Complex
CI	Confidence interval
CLSI	Clinical & Laboratory Standards Institute
DALYs	Disability-Adjusted Life-Years
DNA	Deoxyribonucleic Acid
CDC	Center of Disease Control of The United States
CFU	Colony-Forming Unit
DD	Disk Diffusion Test
ECDC	European Centre for Disease Prevention and Control
ECOFFs	Epidemiological Cut-Off Values
EFSA	European Food Safety Authority
EMA	Ethidium Monoazide
ERIC	Enterobacterial Repetitive Intergenic Consensus
ESBL	Extended-Spectrum Β-Lactamases
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
ESCs	Extended-Spectrum Cephalosporins
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization of the United Nations
ISO	International Organization for Standardization
mCCDA	Modified Charcoal-Cefoperazone-Deoxycholate Agar
MSRV	Modified Semi-Solid Rappaport-Vassiliadis medium
MIC	Minimal Inhibitory Concentration
MLST	Multi Locus Sequence Typing
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis

PICs	Poultry Integrated Companies
PMA	Propidium Monoazide
qPCR	Real-Time Polymerase Chain Reaction
REP	Repetitive Extragenic Palindromic
RFLP	Restriction Fragment Length Polymorphism
ST	Sequence Type
XLD	Xylose lysine deoxycholate agar
WHO	World Health Organization

GENERAL INTRODUCTION

1. *Salmonella enterica*

1.1. Taxonomy and characteristics of *Salmonella* spp.

Salmonella was first discovered from abdominal lymph nodes and the spleen of typhoid patients by Karl Joseph Eberth and Rudolf Virchow in 1879. This discovery was confirmed by Robert Koch but it was only in 1884 when Salmon and Smith isolated the bacillus from hogs that the genus *Salmonella* was named (Meštrović, 2015). *Salmonella* belongs to the family *Enterobacteriaceae.* There are 2 species of *Salmonella*: *Salmonella enterica* and *Salmonella bongori*. The former species is subdivided in 6 subspecies, namely *S. enterica* subspecies *enterica, S. enterica* subspecies *salamae*, *S. enterica* subspecies *arizonae*, *S. enterica* subspecies *diarizonae*, *S. enterica* subspecies *houtenae* and *S. enterica* subspecies *indica* (Win *et al*., 2008).

Salmonella are Gram-negative rods of 0.7 to 1.5 µm wide and 2.0 to 5.0 µm long. These bacteria are motile due to the presence of peritrichous flagella. Only *S*. Gallinarum and *S*. Pullorum (responsible of fowl typhoid and pullorum disease respectively) are not motile serotypes. Biochemical identification of the *Salmonella* spp. can be performed (Table 1). Most clinical important *Salmonella* are positive for methyl red, citrate, fermentation of glucose, arginine dihydrolase, and decarboxylation of lysine and ornithine. Additionally, they are negative for indol, Voges Proskauer and urease test (Stanchi *et al*., 2007).

Table 1. Biochemical tests for identification of Salmonella spp.

Biochemical tests	***Salmonella enterica***						***Salmonella bongori***
	enterica	*salamae*	*arizonae*	*diarizonae*	*houtenae*	*indica*	
Lactose	-	-	- (75%)	+ (75%)	-	V	-
H_2S	+	+	+	+	+	+	+
Gelatine	-	+	+	+	+	+	-
KCN	-	-	-	-	+	-	+
ONPG	-	-	+	+	-	V	+
Dulcitol	+	+	-	-	-	V	+
Malonate	-	+	+	+	-	-	-
Sorbitol	+	+	+	+	+	-	+
L(+) tartrate	+	-	-	-	-	-	-
Mucate	+	+	+	- (70%)	-	+	+
Salicin	-	-	-	-	+	-	-

V: variable reaction
Source: Stanchi *et al*. (2007)

The Kauffman-White scheme, first published in 1929, classifies *Salmonella* in more than 2600 serotypes based on somatic lipopolysaccharide (O), flagellar (H) and capsular (Vi) antigens (Grimont and Weill, 2008).

Most clinically relevant *Salmonella* serotypes are included in *S. enterica* subspecies *enterica* (from now on *S. enterica* subspecies *enterica* will be referred as *Salmonella*). Within this group *Salmonella* serotypes can be divided in two groups: typhoidal salmonellae and non-typhoidal salmonellae. Typhoidal salmonellae are human restricted or human adapted serotypes and include *S.* Typhi, *S.* Paratyphi A, *S.* Paratyphi B and *S.* Paratyphi C. The nontyphoidal salmonellae group includes host generalist *Salmonella* (human and animal hosts) as *S.* Typhimurium and *S.* Enteritidis, host-adapted *Salmonella* (which have a small number of hosts) as *S.* Choleraesuis (swine host) and host-restricted *Salmonella* (with a specific host) as *S.* Gallinarum or. *S.* Pullorum (Barrow and Methner, 2013).

1.2. Epidemiology in humans

1.2.1. Importance and disease burden

Foodborne infections in humans caused by *Salmonella* are of primary importance around the world (Majowicz *et al*. 2010). The WHO (2015b) estimated that worldwide non-typhoidal *Salmonella* is the cause of 93.8 million cases of gastroenteritis and 155,000 deaths yearly. Moreover, it was estimated that foodborne salmonellosis caused by nontyphoidal *Salmonella* resulted in 6.43 million disability-adjusted life-years (DALYs) in 2010 (WHO, 2015a).

EFSA and ECDC reported that within the European Union non-typhoidal salmonellosis occupies the second place of human cases, after campylobacteriosis, regarding to reported hospitalizations (EFSA and ECDC, 2015a). On the other hand, the Center of Disease Control of United States (CDC) estimates that non-typhoidal *Salmonella* is the leading cause of hospitalization and deaths attributed to foodborne bacteria (Scallan *et al*., 2011).

In Latin America, some *Salmonella* outbreaks in humans linked to chicken consumption have been published. For example, food preparations containing chicken meat have been incriminated in *Salmonella* outbreaks in Colombia and Peru (Pazzaglia *et al*., 1992; Mercado *et al*., 2012; Díaz *et al*., 2013). In Mexico and Brazil, *S*. Enteritidis outbreaks associated with the consumption of foodstuffs prepared with eggs have been reported (Carneiro *et al*., 2015; Shane *et al*., 2002). Another study carried out in Chile, found a relation between *Salmonella* strains isolated from human salmonellosis cases and poultry samples (Fernandez *et al*., 2003). In

Ecuador, there are no reports of poultry-associated salmonellosis, but 3,373 human cases of salmonellosis (21.6 cases/100,000 inhabitants) were reported in 2014 (Ministerio de Salud Pública del Ecuador, 2014).

Studies on *Salmonella* in industrially reared poultry and its epidemiology in Latin America are limited (Donado-Godoy *et al*., 2012; Donado-Godoy *et al*., 2012; Pulido-Landínez *et al*., 2013). Additionally, most of the countries in the region lack of a system to report comprehensive data about *Salmonella* in animals, foodstuffs and foodborne salmonellosis.

1.2.2. Clinical manifestations and complications

Salmonellosis is characterized by acute onset of fever, abdominal pain, diarrhea and nausea. Other symptoms include headache, myalgia, malaise and chill (WHO, 2015b). Bacteremia is not a common consequence of salmonellosis caused by non-typhoidal *Salmonella*. However, when it occurs, it has been associated with the occurrence of meningitis (in infants younger than 5 months of age), bronchopneumonia, soft tissue infection, aortic mycotic aneurysms, endocarditis, septic arthritis, splenic or hepatic abscesses and osteomyelitis (Bope and Kellerman, 2015).

Normally, gastroenteritis caused by *Salmonella* does not need antimicrobial medication and can be resolved with symptomatic treatment. However, salmonellosis can be life-threatening in children, elderly and immunocompromised patients (CDC, 2015a). Additionally, antimicrobial resistant *Salmonella* strains pose a global concern since their occurrence has increased over the last years (MacFadden *et al*., 2016).

From all *Salmonella* serotypes, *S.* Enteritidis and *S.* Typhimurium are the two most important serotypes causing human infections in most parts of the world (WHO, 2015b).

1.2.3. Sources of *Salmonella* for human infection

Salmonella are ubiquitous organisms that are widely distributed in animals and the environment. *Salmonella* can contaminate soil, vegetables and fruits at pre-harvest stage and may be the source for human infection. Risk factors for *Salmonella* contamination of fruits and vegetables related to the use of contaminated manure and/or water have been described (Park *et al*., 2012).

Nonetheless, domestic animals are considered as the most important source of human infection. Importantly, *Salmonella* serotypes have been found in pigs, cows and companion

animals (de Jong *et al*., 2014; Helke *et al*., 2016; Kuang *et al*., 2015; Lambertini *et al*., 2016), but poultry is an important vehicle of these pathogens in the food chain (FAO, 2002; Herman *et al*., 2015; Antunes *et al*., 2016), especially in countries where the control of *Salmonella* in the poultry sector has not be addressed.

Salmonella has been largely studied in poultry. *S*. Pullorum and *S*. Gallinarum are only poultry related *Salmonella* serotypes and are not a cause of zoonotic diseases.

On the other hand, the presence of other non-typhoidal *Salmonella* in poultry is an important threat to public health. In EU and USA, *S*. Typhimurium and *S*. Enteritidis are the most reported serotypes in human salmonellosis (CDC, 2014a; EFSA and ECDC, 2015a). Several studies have investigated the impact of poultry and poultry products on human salmonellosis (FAO, 2002; Barrow *et al*., 2012). For example, in USA 29% of foodborne salmonellosis cases were associated with poultry. From these cases, 71% were chicken related (Scallan *et al*., 2011a).

Factors as handling live backyard poultry and tenancy of chickens as pets have been linked to human salmonellosis, especially in children younger than 5 years of age (CDC, 2014b; Bula-Rudas *et al*., 2015). The latter has resulted in recommendations for proper manipulation of these animals in USA (CDC, 2016a). Chicken meat consumption is also identified as an important risk factor for *Salmonella* infections. Cross-contamination of foodstuffs as well as transfer of *Salmonella* from chicken meat to cooking materials may play an important role in the occurrence of salmonellosis in both household and commercial kitchens (Ravishankar *et al*., 2010; Soares *et al*., 2012). It must be taken into account that, despite *Salmonella* counts could be low in chicken carcasses, small number of *Salmonella* (10 cells for susceptible individuals) are enough to cause disease in humans (Cosby *et al*., 2015).

In Latin-America studies aiming to establish the dynamics of *Salmonella* contamination in the poultry industry have been conducted. At farm level, it was demonstrated that the introduction of *Salmonella* in broiler flocks can be linked to poor biosecurity and rearing practices (Donado-Godoy *et al*., 2012). At slaughterhouse level, the following risky steps for *Salmonella* cross-contamination and/or recontamination of broilers carcasses have been identified: bleeding, evisceration and spray washing (which are mostly manual processes in developing countries) (Rivera-Pérez *et a*l., 2014). At retail level it was found that factors as store type (i.e., wet markets and supermarkets), type of poultry company (integrated or non-integrated companies) or storage temperature (i.e., frozen, chilled, or ambient) have an impact on the prevalence and counts of *Salmonella* in chicken carcasses (Donado-Godoy *et al*., 2014).

The globalization of food markets has made poultry meat to be under extensive movements along several counties and continents which poses a necessity to control the safety of poultry products (Allerberger, 2016). Ultimately, measures to prevent foodborne *Salmonella* contamination must include a farm-to-table approach, which not only include the whole poultry production sector but also food handlers and education of consumers (Kimura *et al*., 2004).

2. *Campylobacter* spp.

2.1. Taxonomy and characteristics of *Campylobacter* spp.

In 1886 Theodore Escherich described for the first time non-culturable spiral shaped bacteria. After this report, *Campylobacter* was identified in 1906 from uterine mucus of a pregnant sheep by two veterinarians. They described the bacteria as "peculiar organisms" (Skirrow, 1977). These bacteria were also found in other animals and classified as *Vibrio jejuni* and *Vibrio coli* for several decades (Moore *et al*., 2005). However, it was only in 1963 when, due to the DNA characteristics, metabolism and microaerophilic growth conditions, the genus *Campylobacter* (meaning "curved rod") was proposed (On, 2001).

Campylobacter spp. are a member of the family *Campylobacteraceae* which belongs to the *Proteobacteria* class. At the moment, the *Campylobacter* genus contains 34 species and 14 subspecies. (http://www.bacterio.net/campylobacter.html, last accessed September 12, 2016).

Campylobacter spp. are Gram-negative bacteria with a spiral or "S" shape (0.2-0.9 µm wide and 0.2-5.0 µm long). These bacteria are mobile due to one flagella in monotric or amphitric disposition (Gentilini *et al*., 2007). *Campylobacter* spp. have oxidase activity and are negative for fermentation and oxidation of carbohydrates. *Campylobacter* spp. does not form spores, but in stress conditions they can form coccoid bodies that can persist in the environment (Silva *et al*., 2011).

Some biochemical characteristics within the *Campylobacter* genus can be useful for phenotypical identification of the most important species (Fernandez *et al*., 2016). However, the complexity of the genus, the presence of related genera (*Arcobacter* and *Helicobacter*) and the characteristic growth conditions of *Campylobacter* implies disadvantages in phenotypical identification. Therefore, molecular techniques as multiplex PCR (Vandamme *et al*., 1997) have become widespread tests to effectively identify a number of *Campylobacter* species.

Thermotolerant *Campylobacter* are able to grow at a temperature range from 30°C up to 42-43°C. *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter lari* and *Campylobacter upsaliensis* belong to this group. The optimal temperature for these *Campylobacter* species ranges between 37 – 42 °C. This is important from the public heath perspective since, unlike other pathogens, *Campylobacter* is not able to grow outside of the intestinal tract of warm-blood animals. Therefore, *Campylobacter* can only survive in foodstuffs.

Thermotolerant *Campylobacter* are labile organisms and need especial requirements to survive. Therefore, stress caused by processes related to the food production can affect the survival of *Campylobacter*. Normal air conditions with oxygen concentrations of about 20% are toxic for *Campylobacter* (Gharst *et al*., 2013), leading to a reduction of the *Campylobacter* counts over time (Habib *et al*., 2010). The optimal water activity (a_w) for growth of *Campylobacter* is 0.997 while below a_w 0.987 *Campylobacter* cannot growth (Burgess *et al*., 2016). Desiccation has been demonstrated to be fatal for *Campylobacter*. The lack of a suitable nutrients is also a deleterious factor which can explain the absence of *Campylobacter* on clean surfaces (Mihaljevic *et al*., 2007). It has also been shown that freezing can inactivate *Campylobacter* cells by oxidative damage, which plays an important role on the prevalence of *Campylobacter* in processed foods (Archer, 2004).

In response to unfavorable conditions, *Campylobacter* may form viable but non-culturable (VBNC) cells (Rollins and Colwell, 1986). These forms can survive and eventually transform into cultivable cells after passing the intestinal tract of chickens (Silva *et al*., 2011).

2.2. Epidemiology in humans

2.2.1. Importance and disease burden

Thermotolerant *Campylobacter* spp. are a major cause of zoonotic gastrointestinal infections in both developed and developing countries. The WHO (2015a) estimated that diseases caused by thermotolerant *Campylobacter* affect 95.6 million people and result in 37,600 deaths per year worldwide.

Globally, more than 1 million DALYs can be attributed to *Campylobacter*. In developed countries it ranges from 1,568 in New Zealand to 22,500 in the USA (Skarp *et al*., 2015). On the other hand, in the European Union (EU) it is estimated that *Campylobacter* causes 9 million cases yearly (Havelaar *et al*., 2009). Within the EU member states campylobacteriosis is the most reported gastrointestinal disease and the occurrence of *Campylobacter* infections showed an increasing

trend since 2005. However, mortality due to campylobacteriosis in EU is rarely reported (0.01%) (EFSA and ECDC, 2015a). In USA the Centers for Disease Control and Prevention estimates that campylobacteriosis affects 1.3 million persons every year. Here, *Campylobacter* is the second cause of hospitalization attributed to foodborne bacteria (Scallan *et a*l., 2011b; CDC, 2014c)

Children malnutrition, deficient food safety controls and lack of public awareness about *Campylobacter* are some factors that influence the occurrence of campylobacteriosis in developing countries (Platts-Mills and Kosek, 2014).

Studies in Latin America report *Campylobacter* in both human and animal cases, however these investigations are sporadically conducted and many countries lack structured monitoring programs for these bacteria (Fernández, 2011). These facts could lead to a lack of data with a consequent sub-estimation of campylobacteriosis in Latin America (WHO, 2015a). Still, diarrheal diseases caused by *Campylobacter* are especially important in developing countries where infection in children under the age of two years is frequent and may lead to death (WHO, 2011).

In Ecuador data about *Campylobacter* infections in humans are very limited. Guderian *et al*. (1987) found that the most prevalent bacteria in diarrheal diseases in 2-years old children from low-income households in the city of Quito was *Campylobacter*. On the other hand, a more recent study in Ecuadorian low income communities could not establish an association between the presence of *Campylobacter* and the occurrence of diarrheal diseases in humans (Vasco *et al*., 2014). The conclusions of the later study are not new. Since 1980 asymptomatic *Campylobacter* carriers have been reported in Latin America (Oberhelman *et al*., 2006; Fernández, 2011). In the same way, a study carried out in the Netherlands estimated that once in a year an asymptomatic *Campylobacter* infection occurs in an adult person, suggesting that only an small fraction of infections leads to a symptomatic disease and that the carrier state could be frequent in both developed and developing countries (Teunis *et al*., 2012; Wagenaar *et al*., 2013).

2.2.2. Clinical manifestations and complications

Thermotolerant *Campylobacter* spp. are a major cause of foodborne gastrointestinal infections worldwide. Specially, *C. jejuni* and *C. coli* have been linked to human infections, *C. jejuni* being the most frequently isolated species (Altekruse *et al*., 1999). The incubation period of human campylobacteriosis ranges between 1-5 days. Symptoms are diarrhea, fever, abdominal cramps and vomiting that last for 5-7 days (Skarp *et al*., 2015). *Campylobacter* infections do not require

antibiotic treatment. However the use of antibiotics is recommended in invasive cases or to eliminate the carrier state (WHO, 2016).

Some complications of human campylobacteriosis include Guillain-Barré syndrome, reactive arthritis and irritable bowel syndrome (EFSA, 2011; WHO, 2015a). From these complications, Guillain-Barré syndrome is strongly linked to campylobacteriosis and it is estimated to present in 3/10 000 of *Campylobacter* infections (Skarp *et al*., 2015). Although the nature of this complication is not completely clear, several studies attribute it to the similitude of *Campylobacter* antigens to human gangliosides. The immune response to *Campylobacter* can lead to a reaction against human gangliosides, determining a disruption in the muscular motor response (Loshaj-Shala *et al*., 2015).

2.2.3. Sources of *Campylobacter* for human infection

Campylobacter infection routes have been extensively studied. These bacteria can be found in a wide range of foods of animal origin as milk and, meat from sheep, pigs and cattle (Moore *et al*., 2005). *Campylobacter* can also be found in companion animals which are mostly asymptomatic carriers and might represent a source of infection (Fernández *et al*., 1994; Toledo *et al*., 2015; Rijks *et al*., 2016). However, poultry is estimated to be the main source of *Campylobacter* for humans. Handling, preparation and consumption of broiler meat are recognized to account for 20% to 30% of human campylobacteriosis. Moreover, it has been estimated that 50%-80% of campylobacteriosis cases may be attributed to the chicken reservoir as a whole (EFSA, 2011; Skarp *et al*., 2015). On the other hand the human health risk is directly linked to the consumption of highly contaminated food products (Wagenaar *et al*., 2013).

In order to reduce the risk of *Campylobacter* infections, intervention measures from the farm to the kitchen are proposed. Increased biosecurity and abandoning thinning of flocks during the rearing period are proposed to prevent *Campylobacter* contamination of flocks (Skarp *et al*., 2015). Several intervention measures are recommended to decrease the impact of *Campylobacter* at slaughterhouse level. Logistic slaughtering, that means scheduling contaminated flocks to be slaughtered after non-contaminated ones is one option (Havelaar *et al*., 2007). Other studies also show that procedures on the slaughter chain could determine the contamination of the final product and that evisceration could be critical on the control of *Campylobacter* in poultry carcasses (Havelaar *et al*., 2007; Seliwiorstow *et al*., 2015a, 2016). However, more studies are necessary to generalize these conclusions in developing countries, were different broiler meat production conditions are applied.

3. Antimicrobial resistance

3.1. Antibiotics for treatment of human salmonellosis and campylobacteriosis

In most cases, infections caused by non-typhoidal *Salmonella* and *Campylobacter* do not require the prescription of antibiotics. In the management of human salmonellosis and campylobacteriosis, symptomatic treatment and fluid therapy are the most important therapeutic actions. However, when these infectious diseases threaten the life of certain population groups as young, old, pregnant women and immunocompromised persons (YOPI), it might be necessary to treat patients with antibiotics. In such cases the WHO (2016) recommends the use of antibiotics for the treatment of campylobacteriosis. In cases of salmonellosis fluoroquinolones, amoxicillin or sulfamethoxazole-trimethoprim are usually used to start the treatment, but should also be accompanied with a susceptibility test (i.e., MIC, Kirby-Bauer test)(Bope and Kellerman, 2015).

Nonetheless, the WHO-AGISAR (2011) has listed the above mentioned antibiotics as critically important antimicrobials for human medicine. Importantly, fluoroquinolones and macrolides are classified in the highest priority group. The criteria for this classification is based on the importance of these antibiotics for the treatment of serious human diseases and microorganisms originated from other sources which could harbour important resistance genes.

3.2. World concern about antimicrobial resistance

In 1928, Alexander Fleming discovered a substance produced by a fungus that had antimicrobial activity, and called it Penicillin. Penicillin was the first antibiotic known by the mankind. Since its discovery, new antimicrobials, natural and synthetic, were rapidly discovered giving rise to the hope of a world without infectious diseases. However, the use of the antibiotics led to the development of antimicrobial resistant bacteria (ARB) worldwide (Brandt *et al*., 2014).

The WHO has highlighted the critical importance of antimicrobial resistance in the world. Indeed, it is proposed that in the 21th century humankind might face a post-antibiotic era, in which now common infections and minor injuries can kill thousands of people (WHO, 2014). This problem will not only result in increased illness, disabilities and deaths but it also puts at risk the achievement of Sustainable Development Goals for the next 30 years (United Nations, 2016; WHO, 2016). It is estimated that only in the USA ARB will lead to $34 billion dollars extra costs, and more than 8 million additional days in hospitals (WHO, 2014).

In its last report about the global situation of antimicrobial resistance, the WHO (WHO, 2015c) showed that this phenomenon is triggered by the misuse of antimicrobial medicines, poor-quality medicines, weak laboratory capacity and surveillance, insufficient regulation on the use of antibiotics and inadequate programs to address infection prevention and control of pathogens. These limitations are especially patent in low- and middle-income countries where financial resources destined to surveillance programs, as well as the public awareness about antimicrobial resistance are lower.

The spread of ARB within the food chain, particularly food producing animals, is an essential part of these problems. Although some bacteria as *E. coli* are mainly commensals in the human gut, they can carry resistance genes that could be transfer to pathogenic bacteria (Wellington *et al*., 2013; FAO, 2016). Moreover, foodborne ARB like *Salmonella* or *Campylobacter* represent a direct danger to human health that can be worsened by the difficulty of treating patients with common used antibiotics (Andersson and Hughes, 2014).

The global antimicrobial consumption report that in 2010 63,151 tons were used in livestock (FAO, 2016). It has also been calculated that by 2030 the global use of antibiotics in food animals will rise by 67%. Moreover, in Latin America, countries as Peru and Brazil will have a significant increase on antibiotics usage (160%) due to a shift to intensive food animal production systems (Van Boeckel *et al*., 2015). The poultry production sector will be one of the most impacted ones for this increase. BRICS countries (Brazil, Russia, India, China, and South Africa) and countries with emerging market economies are expected to greatly contribute to these projections (Van Boeckel *et al*., 2015).

It must be taken into account that for the misuse of antibiotics different aspects have to be addressed. For example, the use of antibiotics as prophylactic drugs is a common practice in the developing world. Moreover, antibiotics are still used as growth promotors in many countries (Van de Venter, 2000; Butaye *et al*., 2003; FAO, 2016b; Castanon, 2007).

Additionally, several studies showed that direct contact with birds and consumption of contaminated food can lead to the transmission of ARBs to humans (Graham *et al*., 2009; Ferro *et al*., 2015; Mattiello *et al*., 2015; Sierra–Arguello *et al*., 2016).

These issues have raised the concern to the development of new antibiotic resistances. A recent case is the new type of resistance found towards colistin in *E. coli* strains isolated from an intensive pig farm in China. This resistance was mediated by a plasmid gene named *mcr-1* (Liu *et al*., 2016). Far from being an isolated case, since its publication *mcr-1* started to be found in

collections of *E. coli* and *Salmonella* isolated from humans and food animals (including poultry) in different continents (Hasman *et al*., 2015; Malhotra-Kumar *et al*., 2016; Doumith *et al*. , 2016; Fernandes *et al.,* 2016; Lentz *et al.,* 2016; Quesada *et al*., 2016; Rapoport *et al*., 2016; Webb *et al*., 2016). These data indicate that the genetic determinant of this resistance was not only previously present in bacteria from different parts of the world but also that *mcr-1* was able to move between different genera of Gram negative bacteria. Additionally, a second plasmid-mediated colistin-resistance gene called *mcr-2* was reported in Belgium from *E. coli* isolated in porcine and bovine samples (Xavier *et al*., 2016).

It has to be mentioned that colistin is one of the last therapeutic resources to treat complicated infections caused by multi-resistant Gram negative bacteria (WHO and AGISAR, 2011). Nonetheless, colistin, among other antibiotics, is still largely used as a grow promotor in many countries (Hao *et al*., 2014). This example shows the global impact that husbandry activities can have on public health if no worldwide measures are taken in the middle term to rationalize the use of antibiotics.

4. Methods to study *Salmonella* and *Campylobacter*

4.1. Methods for detection of *Salmonella* and quantification of *Campylobacter*.

4.1.1. Detection of *Salmonella*

Salmonella is able to grow in foodstuffs at temperatures as low as 10°C and at a water activity of at least 0.95, meaning that *Salmonella* may be able to grow in a wide range of foodstuffs during storage and some environments (Oscar, 1999). These facts, and the low infective dose of *Salmonella*, have made the presence/absence approach the most accepted criteria to study this pathogen. For this purpose, highly specific and sensitive isolation protocols have been developed. Commonly, *Salmonella* isolation starts with a preenrichment step carried out in non-selective media as buffered peptone water. Thereafter, selective enrichment and selective media are used. The ISO 6579 (Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.) recommend the simultaneous use of Rappaport-Vassiliadis medium with soya (RVS broth) and Muller-Kauffmann Tetrathionate Novobiocin broth (MKKTn) as enrichment broths for the isolation of *Salmonella* from food and feeding stuffs, while the annex D of this ISO method recommends the use Modified Semi-Solid Rappaport-Vassiliadis medium (MSRV) as enrichment medium for samples collected in the primary production stage. The latter medium allows the migration of motile *Salmonella* in the medium.

RVS, MKKTn and MSRV, showing migration zone after incubation, has to be plated out on the selective medium Xylose Lysine Deoxycholate agar (XLD). Suspect colonies have to be confirmed by biochemical tests (ISO, 2002).

Several alternatives for the detection of *Salmonella* in foodstuffs have been proposed. For instance, PCR-based techniques have been proven to be efficient for the detection of *Salmonella* cells in food matrixes (Tsen *et al*., 2013; Singh and Mustapha, 2013). More recently, a molecular technique based on the detection of *Salmonella* DNA by real-time PCR has shown to have high sensitivity and specificity levels. Moreover, this protocol showed high concordance and accordance results in several European laboratories (Delibato *et al*., 2014). Lately, electrochemical immunosensors have been used for "real-time" diagnostics of *Salmonella* in food, showing that these devices can be a reliable alternative for controlling pathogens in the food industry. The robustness and the possibility to collect isolates for further studies by the ISO 6579 standards makes them suitable for both research and clinical applications (Schatten and Eisenstark, 2015; Cloke *et al.*, 2016).

4.1.2. Quantification of *Campylobacter*

The public health risk of *Campylobacter* has been linked to the number of cells present in foods. EFSA has stated that if *Campylobacter* levels in broiler neck and breast skin are limited to 1000 or 500 CFU/gram, a health risk reduction of > 50% or > 90% respectively could be achieved (EFSA, 2011). Additionally, it has been demonstrated that 500 *Campylobacter* cells are enough to cause infection in humans (Gentilini *et al*., 2007). Therefore, techniques for the quantification of *Campylobacter*, rather than presence-absence tests, give reliable data to assess the public health risk by consuming contaminated poultry meat (Callicott *et al*., 2008; Nauta and Havelaar, 2008).

In order to evaluate the level of the *Campylobacter* contamination in foodstuffs, selective agars have been developed. Seliwiorstow *et al*. (2014) conducted a comparison of chromogenic media for quantification of *Campylobacter.* In this research RAPID® Campylobacter agar (Bio-Rad, Marnes-la- Coquette, France), mCCDA® (Oxoid, Basingstoke, England), and CFA® (bioMérieux, Marcy l'Etoile, France) agars where evaluated with artificial and naturally contaminated poultry meat samples. Results showed a high level of agreement between tested media. RAPID® *Campylobacter* agar was the best option since it could efficiently inhibit background microflora, making plate readings reliable.

Alternatives for *Campylobacter* quantification are based on the presence of bacterial DNA (Melero *et al*., 2011). The limitation of these techniques is the presence of DNA from dead and not viable cells which can lead to an overestimation of *Campylobacter* counts. To overcome this constraint DNA-binding molecules such as propidium monoazide (PMA) or ethidium monoazide (EMA) have been used (Elizaquível *et al*., 2014). These dyes enable quantitative PCR (qPCR) protocols to detect DNA from living cells and correlate the intensity of the qPCR signal with the number of live *Campylobacter* in a given food matrix (Vondrakova *et al*., 2014; Seliwiorstow *et al*., 2015b). However, Pacholewicz *et al*. (2013) demonstrated that PMA do not fully eliminate the signal of dead cells when quantifying *C. jejuni*, *C. coli* and *C. lari* on broiler chicken carcasses with a qPCR method.

4.2. Characterization of isolates

Traditionally *Salmonella* isolates are further characterized by serotyping used the Kaufmann-White scheme. DNA-based typing methodologies offer higher resolution outputs allowing further characterization of isolations below the species and subspecies level. Such methods are very useful when carrying out epidemiological studies. For example, it is possible to attribute human infections to the consumption of contaminated foodstuffs, the spreading of pathogens in the primary production and tracing the source of contamination of food products in the food chain.

Another application of subtyping methodologies is the study of antimicrobial resistance. Antibiotic resistant phenotypes can be explained by the presence of determined genes or mutations in DNA. Therefore, the analysis of DNA sequences has become an indispensable tool in molecular epidemiology (Zankari *et al*., 2012).

Several genetic methods have been proposed for different bacteria. The methodologies used in this research for the characterization of collected *Salmonella* and *Campylobacter* isolates are summarized below.

4.2.1. Macrorestriction analysis by pulsed-field gel electrophoresis (PFGE)

PFGE is a method used to analyze DNA fingerprints of different bacteria. Identical DNA fingerprints (patterns) are supposed to be representative for a clonal bacterial population. Therefore, the PFGE type assigned to a given strain could be used to relate it with isolates from other sources (e.g., food, water, clinical or human infections, etc.) (Parizad, 2016).

This methodology is based on the digestion of the complete DNA of a bacterial culture by a restriction enzyme (Figure 1). For *Salmonella*, the most commonly used restriction enzyme is *Xba*I*,* but when two or more isolates are indistinguishable the use of secondary (*Bln*I/*Avr*II) or tertiary (*Spe*I) enzymes can be useful. Afterwards, fragments of the cut DNA are separated according to their size by a slow electrophoresis (18-19 hours). Electric fields used in PFGE are emitted as pulses in three directions. This particularity allows the separation of fragments larger than 20.5 Kb (kilo base pairs). Finally, a picture of the processed gel showing patterns of bands is analyzed with suitable software (CDC, 2016b).

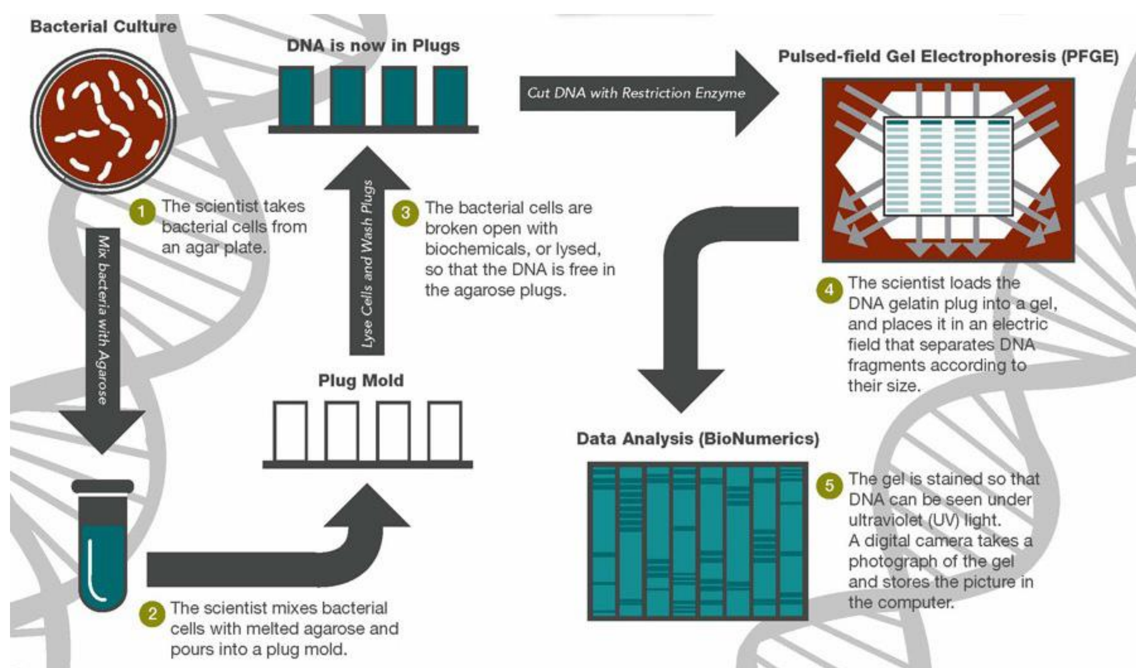

Figure 1. Schematic overview of the Pulse Field Gel Electrophoresis Process.
Source: CDC (2016b)

The high levels of reproducibility (within and between laboratories) of this technique has been largely reviewed and is considered to be the gold standard for fingerprint analysis of *Salmonella* and other microorganisms (Barrett *et al.*, 2006).

In order to explain the diversity of PFGE patterns, several criteria have been proposed. The so called *Tenover* criteria state that a single genetic event may result in up to three-band differences. Therefore, profiles differing in the position of up to three bands should be considered as related while profiles differing up to six bands should be considered as possibly related (Tenover *et al*., 1995). This can be explained by the fact that deletions, insertions and point mutations in restriction sites result in the cleavage or junction of DNA fragments. However,

this concept does not take into account that differences in a single band are common in foodborne bacteria. The latter can be explained by the presence of acquired plasmids with a large diversity in size. Another possibility is that the fragments resulting from deletions, insertions or point mutations could be so small that they are not detectable in the gel (Barrett *et al*., 2006). In consequence, single-band variations should be considered when analyzing the output data of PFGE in epidemiological research. Nonetheless, some *Salmonella* serotypes like *S.* Enteritidis and *S*. Infantis are very clonal, resulting in similar PFGE profiles (De Cesare *et al.*, 2015; Franco *et al*., 2015). In such cases, the interpretation of PFGE results could be more difficult and data from other approaches, such as whole genome sequencing, could be required to have deeper epidemiological insights.

4.2.2. Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR)

Repetitive sequencing-based PCR (REP-PCR) are molecular techniques based on the natural occurrence of repetitive DNA in bacterial genome (Versalovic *et al.*, 1994). As the number and location of those repetitions can be different for bacteria belonging to the same species, they can be used for genotyping purposes. There are 3 families of repetitive sequences: 1) the 35–40-bp repetitive extragenic palindromic (REP) sequence, 2) the 124–127-bp enterobacterial repetitive intergenic consensus (ERIC) sequence and 3) the 154-bp BOX element sequences (Li *et al*., 2009).

ERIC-PCR is based on the presence of short, repeated and scattered extragenic DNA sequences in *Enterobacteriaceae*. Primers are designed to anneal to these sequences. The resulting PCR amplification gives bands in an electrophoresis gel that represent regions separating those sequences. Thus, the pattern generated will depend of the number and extension of the sequences between primers (Lupski and Weinstock, 1992).

This technique has been used to study the molecular epidemiology of *Campylobacter* in order to support the existence of a genetic relationship between human and poultry isolates (Ahmed *et al*., 2015). However, it is reported that the reproducibility of the results obtained with combined techniques is still low (Wassenaar and Newell, 2000). ERIC-PCR has also been used for typing *Salmonella* in combination with other techniques (Campioni *et al*., 2014; Turki *et al*., 2014). However, Rasschaert *et al*. (2005a) showed that discrimination of ERIC-PCR alone is not adequate to differentiate strains within serotypes. Those authors proposed to use this genetic method to group *Salmonella* isolates allowing to reduce the number of isolates to be serotyped.

4.2.3. Restriction fragment length polymorphism PCR (RFLP-PCR).

RFLP-PCR has been used for the detection of intraspecies and interspecies variation. This technique is based on the cleavage of DNA by restriction enzymes. DNA fragments generated are representative of the polymorphisms present in a given organism. These fragments can be visualized after a gel electrophoresis (Magdeldin, 2012).

An important advantage of RFLP-PCR is the lack of requirement for advanced instruments. However, some RFLP-PCR assays may have a low reproducibility between laboratories and need to be precisely standardized in all its steps. Nonetheless, RFLP-PCR has been used to type a wide variety of prokaryotic and eukaryotic organisms (Berg, 2012). For example, a RFLP-PCR using *Dde*I enzyme to digest the *fla*A gen of *Campylobacter* has been designed (Nachamkin *et al*., 1993). This method has shown to be robust and has good reproducibility (Harrington *et al*., 2003). Additionally, RFLP-PCR of the *fla*A gene (RFLP-*fla*A) has shown to be a cost-effective technique and has acceptable discrimination to make epidemiological inferences (Djordjevic *et al*., 2007). RFLP-*fla*A has been used in studies on both human and animal isolates, giving meaningful insights in the epidemiology of *Campylobacter* strains in the short term (Kashoma *et* al., 2014; Ghorbanalizadgan *et al*., 2016). Nevertheless, RFLP-*fla*A has been questioned due to the fact that intra- and intergenomic recombination within the flagellin gene can make comparisons of isolates difficult on the long term (Lukinmaa et al., 2004; Eberle and Kiess, 2012). Moreover it has been shown that a combination of *fla*A and *fla*B in a RFLP-PCR analysis can have a higher discriminatory power that the RFLP-PCR on *fla*A alone (Petersen and Newell, 2001).

4.2.4. Multi locus sequence typing (MLST)

This genetic technique uses the internal fragment sequences of house-keeping genes (genes required for the maintenance of basic cellular functions). Differences of the house-keeping genes sequences are used to designate identification numbers of those alleles. These differences occur when single or multiple nucleotides have changed in the DNA by means of single mutations or recombinational replacement (that will often change multiple sites). Gene regions of approximately 450–500 bp are sequenced to obtain this information. For every bacterial isolate the allele at each of the seven loci define the allelic profile, also called sequence type (ST) (Pérez-Losada *et al*., 2013). MLST is especially useful for bacteria with weak clonal population structure. This is the case of *Campylobacter,* where STs that share four or more alleles belong to the same lineage, called clonal complex (CC) (Taboada *et al*., 2013).

The high reproducibility of the MLST approach has allowed to generate a worldwide bacteria-typing database. Results obtained globally can be submitted to an international database that conglomerate results from many bacteria (e.g., *Campylobacter*, *Salmonella*, *E. coli*, etc.). This information is freely available from the website of this initiative, and can be retrieved using filters of epidemiological relevance (MLST.net, 2016).

The fact that MLST information is widely spread, comparable and reliable has made this technique useful in fields like molecular epidemiology and public health, phylogenetics and taxonomy, and population structure and dynamics. (Pérez-Losada *et al*., 2013).

4.3. Antimicrobial resistance tests.

4.3.1. Disk diffusion test

Disk diffusion test (DD), also known as the Kirby-Bauer method is an *in vitro* technique for testing the antimicrobial sensitivity of bacteria. Although factors like immunological competence and *in vivo* inactivation of antibiotics are not taken into account, bacterial response to tested antibiotics correlate well with the clinical outcome of infected patients treated with those agents (Biemer, 1973).

DD test involves the preparation of a homogenous bacterial inoculum on nonselective media (Mueller-Hinton agar). Later, paper discs impregnated with antimicrobials are placed onto the agar and plates are incubated. The antibiotic diffuses throughout the agar where it may inhibit the bacterial growth in a zone surrounding the disk. The inhibition zones are measured and interpreted according reference values (Bauer *et al*., 1966).

Despite the simplicity of the method, factors like the amount of bacterial inoculum, rate of bacterial growth and pH and depth of media can influence the size of the inhibition zone. Resulting differences can make intra- and inter-laboratory comparison of results impracticable. For these reasons, standardized protocols must be strictly followed in order to obtain comparable and realistic results (CLSI, 2015; EUCAST, 2016a). However, the low-cost and easy-to-do characteristics of this method have made it extensively used in clinical and research laboratories.

4.3.2. Broth dilution test

This method is based on the growth of a standardized bacterial suspension in a series of tubes containing nutritive broth with two-fold dilutions of antibiotics. Visual inspection of turbidity as

proof of bacterial growth indicates the antibiotic concentrations at which bacteria are inhibited. The lowest concentration of antibiotic that prevent bacterial growth represent the minimal inhibitory concentration (MIC). The precision of this technique is considered to be +/- one-fold concentration (Jorgensen and Ferraro, 2009). The advantage of this technique is the generation of quantitative data which can be used to perform further statistical analysis and have insights in the level of resistance present in bacterial isolates (Jorgensen and Ferraro, 2009).

Broth dilution tests can be performed using in-laboratory prepared materials and dilutions, which makes the technique laborious and prompt to mistakes. However, nowadays there are microdilution-based kits that use small quantities of reagents in ready-to-use plastic plates for rapid and reliable testing of several antibiotics (Cavalieri *et al*., 2005).

4.3.3. Comparison of breakpoint values from CLSI/EUCAST and ECOFFs

The Clinical & Laboratory Standards Institute (CLSI) is an organization with base in USA that aims to develop standardized clinical and laboratory practices (CLSI, 2016a). For more than 30 years this institution has provided guidelines for antibiotic resistance testing that have been used extensively worldwide in both human and animal medicine. Documents generated by CLSI are accessible under a commercial price from the website www.clsi.org. Specifically, standards regarding antibiotic resistance tests and their interpretation are sold for 140-180 USD (last reviewed: 10/10/2016).

On the other hand, The European Committee on Antimicrobial Susceptibility Testing (EUCAST) is a standing committee organized by the European Centre for Disease Prevention and Control (ECDC), European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and European national breakpoint committees. EUCAST continuously works to update and set breakpoints and technical aspects of phenotypic *in vitro* antimicrobial susceptibility testing (EUCAST, 2016a). Tables of breakpoint, standards, procedures and all material published by EUCAST are freely available on its website.

In their documents, both CLSI and EUCAST publish clinical breakpoints (cut-off values) that aim to give reliable criteria to interpret the results of MIC or DD tests. Clinical breakpoints serve as clinical guidelines to select antibiotics for treatment of bacterial infections (CLSI, 2015). However, these data do not always relate to the presence of antimicrobial resistance in a given bacteria. For example, if a pathogen that has acquired a resistance mechanism can result in a phenotype with a MIC value below the clinical breakpoint, it will not be identified as a resistant strain.

To overcome this limitation, EUCAST has developed epidemiological cut-off values (ECOFFs) for the early detection of antimicrobial resistances. ECOFFs are parameters that separate microorganisms without (wild type) and with acquired resistance mechanisms (non-wild type) to an antibiotic according to the MIC distribution of reported isolates (EUCAST, 2016a).

The existence of different standards evidence the necessity of generating universal criteria for antimicrobial resistance tests interpretation. In Table 2 an example of the concordance/discrepancy of different breakpoints for *Enterobacteriaceae* is shown.

Table 2. Example of differences in MIC clinical breakpoints (CLSI and EUCAST) and ECOFF values (EUCAST) for Enterobacteriaceae.

Antimicrobial Family	Antimicrobial agent	CLSI Clinical breakpoints (μg/ml)			EUCAST Clinical breakpoints (μg/ml)			EUCAST ECOFF[a] μg/mL (≥)
		S (≤)	I	R (≥)	S (≤)	I	R (≥)	
Penicillins	Ampicillin*	8	16	32	8	-	16	16
Aminoglycosides	Gentamicin*	4	8	16	2	4	8	4
	Tobramycin*	4	8	16	2	4	8	4
	Amikacin*	16	32	64	8	16	32	16
Carbapenems	Doripenem	1	2	4	1	2	4	0.25
	Ertapenem	0.5	1	2	0.5	1	2	0.128
	Imipenem*	1	2	4	2	4-8	16	1
	Meropenem*	1	2	4	2	4-8	16	0.25
Cephalosporins	Cefazolin*	2	4	8	-	-	-	-
	Cefuroxime*	8	16	32	8	-	16	16
	Cefepime*	2	4-8	16	1	2-4	8	0.25
	Cefotetan*	16	32	64	-	-	-	-
	Cefoxitin*	8	16	32	-	-	-	16
	Cefotaxime	1	2	4	1	2	4	0.5
	Ceftazidime*	4	8	16	1	2-4	8	1
	Ceftaroline*	0.5	1	2	0.5	-	1	1
Fluoroquinolones	Ciprofloxacin*	1	2	4	0.5	1	2	0.128
	Levofloxacin*	2	4	8	1	2	4	0.5
Folate pathway inhibitors	Trimethoprim-sulfamethoxazole*	2/38	-	4/76	2/38	4/76	8/152	2/38
Monobactams	Aztreonam*	4	8	16	1	2-4	8	0.5
Phenicols	Chloramphenicol*	8	16	32	8	-	16	32
Tetracyclines	Tetracycline*	4	8	16	-	-	-	-
β-lactam/β-lactamase inhibitor combinations	Amoxicillin-clavulanate*	8/4	16/8	32/16	8/4	-	16/4	-
	Ampicillin-sulbactam*	8/4	16/8	32/16	8/2	-	16/2	16/2

[a] ECOFF values correspond to *E. coli.*

* Different values reported from CLSI and EUCAST

Source: CLSI (2016b), EUCAST (2016a)

5. Poultry meat production in Ecuador

Poultry meat is an important source of animal proteins for humans worldwide. In many developing countries poultry meat is largely consumed and it expected that by 2025 (together with the pork sector) it will be the most important sector of meat production in both developed and developing countries (OECD and FAO, 2016). It is estimated that by 2016 44 million tons of poultry meat will be produced in the Americas (Evans, 2015) .

Additionally, due to its efficiency in feed conversion and growth rate, poultry will remain to be the cheapest source of animal protein (OECD and FAO, 2016). These factors will promote the production of broiler meat in the developing world (Skarp *et al*., 2015).

5.1. Generalities of Ecuador

Ecuador is a South American country that is situated at the Pacific Ocean and has borders with Colombia at the north and Peru at the south. Some important indicators that characterize the economy and social standards of Ecuador are given in Table 3.

Table 3. Economic and social characteristics of Ecuador.

Indicator	Data	Reference
Area	256,370 Km^2	IGM 2013
Population (2016)	16,610,280	INEC 2016
Density (2016)	64.79 h/Km^2	Direct research
Gross domestic product (GDP - 2015)	$100.872 billions	WB 2016b
GDP per capita (2015)	USD 6248,111	WB 2016b
Family basket	USD 691.38	INEC 2016
Agriculture (% of GDP) (2015)	9.55 %	WB 2016a
Human Development Index (HDI)	0.73	UNDP 2015
HDI world ranking	88	UNDP 2015
Life expectancy at birth (years)	75.9	UNDP 2015
Expected years of schooling	14.2	UNDP 2015
Mean years of schooling	7.6	UNDP 2015
Gross national income (GNI) per capita	USD 10,605	UNDP 2015
GNI per capita Rank minus HDI rank	7	UNDP 2015
Dow Jones Index (19-oct-2016)	18202.62	BCE 2016
Country risk (19-oct-2016)	757.00	BCE 2016

Geographically, Ecuador is divided in 4 regions: Amazon Region, Andes Region, Coast Region and Galapagos Islands (Figure 2). The presence of the Andes, with some of the highest volcanos in America, allow the existence of a variety of microclimates along the foothills of the Andes region (IGM, 2013).

Figure 2. Geographical regions of Ecuador. Galapagos Islands are not shown in scale.

From the administrative point, Ecuador is divided in 24 provinces (Figure 3). In terms of population and economy, the provinces of Guayas and Pichincha are the two biggest ones. Quito, the capital city of Ecuador is located in the province of Pichincha while the city of Guayaquil (harboring the main port of Ecuador) is located in the province of Guayas. These provinces contain 42.96% of the Ecuadorian population (INEC, 2016).

Figure 3. Provinces of Ecuador. Green and red colors denote the provinces of Guayas and Pichincha respectively.
Galapagos province is not shown in scale.

5.2. Broiler production industry in Ecuador

Ecuador produces approximately 220.000.000 broilers yearly. This production is only destined for local consumption. The per capita consumption of broiler meat in Ecuador was estimated to be 32 Kg/year in 2014 (CONAVE, 2014). However, taking into account the current broiler production and projected population, it can be assumed that by 2016 the per capita consumption of broiler meat is 33.1 Kg/year. Although poultry meat is the main source of meat in Ecuador, its consumption is still lower than in other countries like Peru (41 Kg/head/year) or Brazil (45 Kg/head/year) (AVEC, 2015; SENASA, 2015)

5.2.1. Integrated poultry companies as a model of poultry production

It is estimated that 90% of broilers in Ecuador are produced in integrated companies (PICs). PICs in Ecuador manage several stages in the production of broilers which can include: breeders, hatcheries, feed plants, broiler farms, slaughterhouses and shops. A scheme of how PICs are organized and the main channels of commercialization of poultry meat is shown in Figure 4 (Egas, personal communication). It is important to mention that PICs depend on the provision of raw materials for feed preparation. Some raw materials like maize and soy are mainly produced in Ecuador with high quality standards, while other ingredients like vitamins and amino acids are imported from industrialized countries. Some PICs also produce hatching eggs and/or one-day-old chicks in order to occupy their capacity at broiler farms. Finally, when broilers have reached the slaughter age, they can be processed in the slaughterhouse within the PICs. Another possible route for the commercialization of broilers is the sale of live birds at the farms to traders who sell these birds at open markets. Once broilers are slaughtered either in a commercial slaughterhouse or in informal slaughterhouses, carcasses are sold to supermarkets, open markets, food factories or restaurants for final consumption.

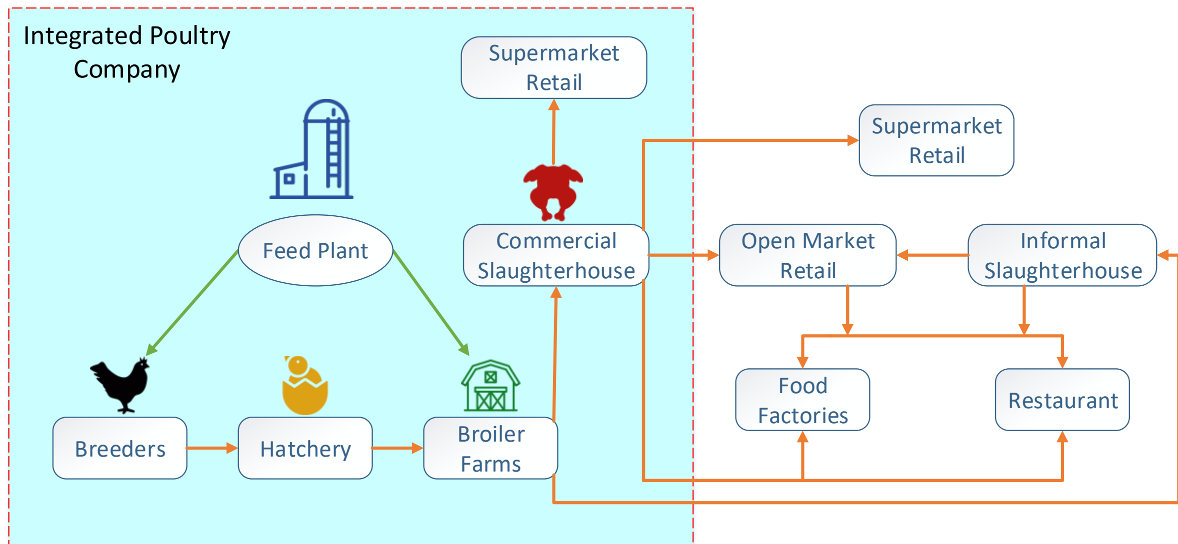

Figure 4. Integrated poultry company and commercialization channels.
Dashed line encloses productive stages within the PICs.

5.2.2. Breeders and hatcheries

In Ecuador, broiler breeders are mainly imported from Brazil. According to the Husbandry and Agriculture Ministry of Ecuador (CGSIN and MAGAP, 2015a) there are 2.426.888 broiler breeders distributed over 54 farms (Figure 5). Breeders farms are mainly located in the provinces of Guayas (23.7%) and Pichincha (12.8%), near to the main centers of broiler production (CGSIN and MAGAP, 2015a). The distribution of broiler breeders per province and region is shown in Table 4.

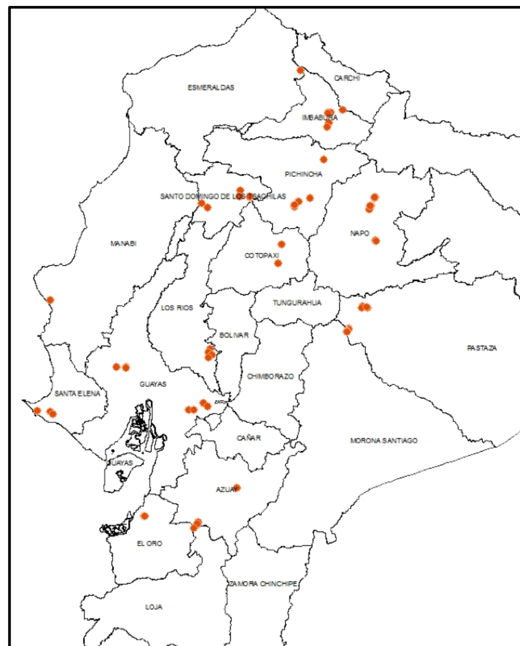

Figure 5. Distribution of the 52 breeder farms in Ecuador. Some farms are overlapping.
Source: (CGSIN and MAGAP, 2015a).

Table 4. Distribution of broiler breeders in Ecuadorian provinces and regions.

Sector	Farms	Broiler breeders	%
COASTAL REGION			
Guayas	7	576,000	23.7
Santa Elena	4	165,000	6.8
Manabí	1	91,227	3.8
Los Rios	1	34,000	1.4
Esmeraldas	1	18,868	0.8
El Oro	2	14,900	0.6
Total	16	899,995	37.1
ANDES REGION			
Pichincha	8	311,388	12.8
SD Tsachilas	4	227,500	9.4
Azuay	3	202,000	8.3
Cotopaxi	2	172,000	7.1
Imbabura	5	162,894	6.7
Loja	1	128,000	5.3
Carchi	1	32,000	1.3
Total	24	1,235,782	50.9
AMAZON REGION			
Napo	6	120,000	4.9
Pastaza	8	171,111	7.1
Total	14	291,111	12.0
Total National	**54**	**2,426,888**	**100**

Source: (CGSIN and MAGAP, 2015a).

Nonetheless, local breeders do not supply all hatching eggs needed by the poultry industry. It is estimated that 2 to 5% of those eggs are imported from neighbor countries, especially from Peru. Most of PICs own hatcheries but there are also hatcheries out of the PICs. In Ecuador there are 30 hatcheries that are mainly located in subtropical areas where climatic conditions are suitable for this stage (Egas. Personal communication). Also the presence of broiler farms has influenced the location of hatcheries. Therefore, most of the hatcheries are located around the provinces of Pichincha and Guayas.

5.2.3. Broiler farms

Climatic conditions and the availability of cheap workforce in Ecuador allow the use of basic infrastructure for rearing broilers. Houses with an "open" configuration and in many cases built with local materials are common in poultry production (Figure 6). While it is true that this has enabled a reduction of costs in the poultry industry, it is also a factor that hinders the control of pathogens associated with the rearing of broilers.

Figure 6. Open poultry house in coastal region.

The presence of microclimates and the weather stability allows the poultry industry to rear broilers throughout the whole year with a minimal invest on heating. Although broilers are reared all over Ecuador, the main centers of broiler production are located near of populated cities (Figure 7). Thus, nearly 60% of broilers farms are located in the provinces of Imbabura, Pichincha and Sto. Domingo in the Andes region; and Manabí, Guayas and El Oro in the coastal region (Table 5). Farms located in these provinces account for 77.7% of broiler production in Ecuador (CGSIN and MAGAP, 2015a).

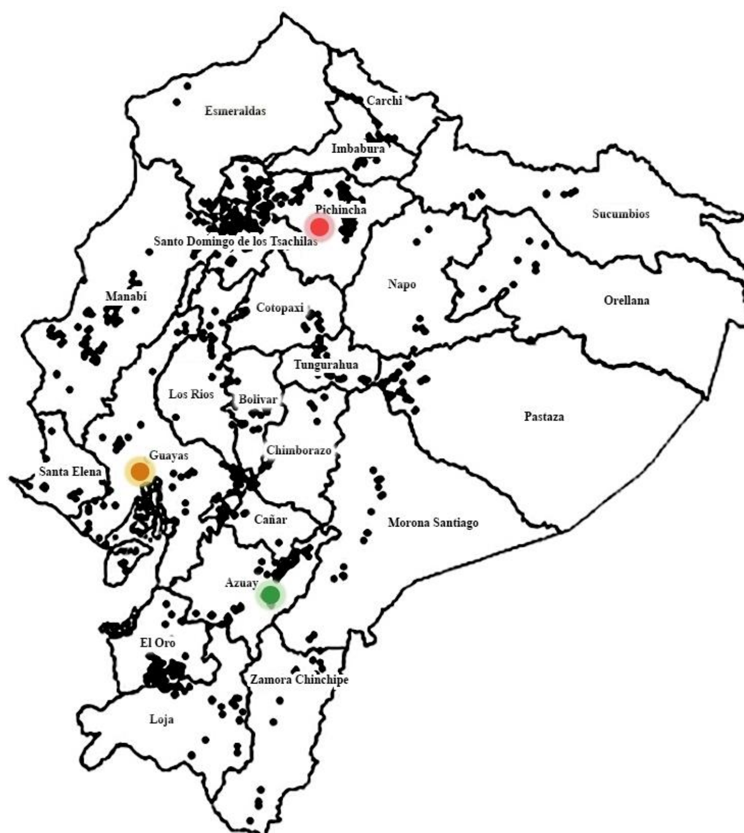

Figure 7. Location of broiler farms in Ecuador. Colored dots indicate the three most populated cities of Ecuador.
Red: Quito. Yellow: Guayaquil. Green: Cuenca. Some farms are overlapping.
Source: MAGAP (2015a)

Table 5. Number of farms and housing capacity of broilers in provinces and regions in Ecuador.

Sector	Number of farms	Capacity	
		Broilers/farm	**%**
COASTAL REGION			
Guayas	59	9,347,100	22.3
El Oro	253	4,245,000	10.1
Manabí	98	3,604,100	8.6
Los Ríos	27	686,000	1.6
Santa Elena	15	617,990	1.5
Esmeraldas	6	458,600	1.1
Total	458	18,958,790	45.2
ANDES REGION			
Pichincha	220	6,756,008	16.1
SD Tsáchilas	155	6.113.100	14.6
Imbabura	48	2.488.200	5.9
Chimborazo	39	1.855.500	4.4
Azuay	210	1.042.240	2.5
Carchi	11	903.000	2.2
Tungurahua	44	847.850	2.0
Loja	47	845.500	2.0
Cañar	32	505.900	1.2
Cotopaxi	20	338.300	0.8
Bolívar	26	278.800	0.7
Total	852	21.974.398	52.4

AMAZON REGION			
Pastaza	45	727.100	1.7
Morona Santiago	19	76.900	0.2
Orellana	17	47.594	0.1
Napo	8	40.000	0.1
Sucumbíos	11	38.400	0.1
Zamora Chinchipe	24	34.950	0.1
Total	124	964.944	2.3
Total national	**1.434**	**41.898.132**	**100**

In Ecuador the use of antibiotics in the poultry industry is common. Antibiotics are used as growth promotors as well as prophylactic (within the first week of broiler production) and curative treatments.

In Ecuador broilers are commonly slaughtered at 38-49 days of age depending on the requirements of the market (e.g. restaurant chains buy smaller broiler carcasses that supermarkets). Thinning is not a common practice in broiler farms but can be done under some commercial circumstances. When broiler houses are depopulated the removal of the litter is generally performed by other companies. Cleaning and disinfection of the houses is done by the personnel of the PICs and a down period of 8-15 days is implemented.

5.2.4. Slaughterhouses

Slaughtering of broilers in Ecuador is carried out in both industrialized and informal slaughterhouses. In Ecuador, industrialized slaughterhouses are controlled by sanitary authorities (INEN, 2012; AGROCALIDAD and MAGAP, 2013). However, only about 35% of broilers in Ecuador are slaughtered in industrialized facilities. Due to commercial reasons, slaughterhouses are located near to the main centers of consumption and production of broiler meat and broiler meat products.

In Ecuadorian industrialized slaughterhouses a number of processes are performed manually (Figure 8). In most of the cases chilling of carcasses is carried out in water tanks in which chlorine may be added to reduce bacterial counts on the final product. However, most of slaughterhouses lack of a system to monitor the concentration of chlorine in chilling water. Other products based in organic acids and hydrogen peroxide are allowed to be used in chilling water, but their high price prevents their use in most slaughterhouses.

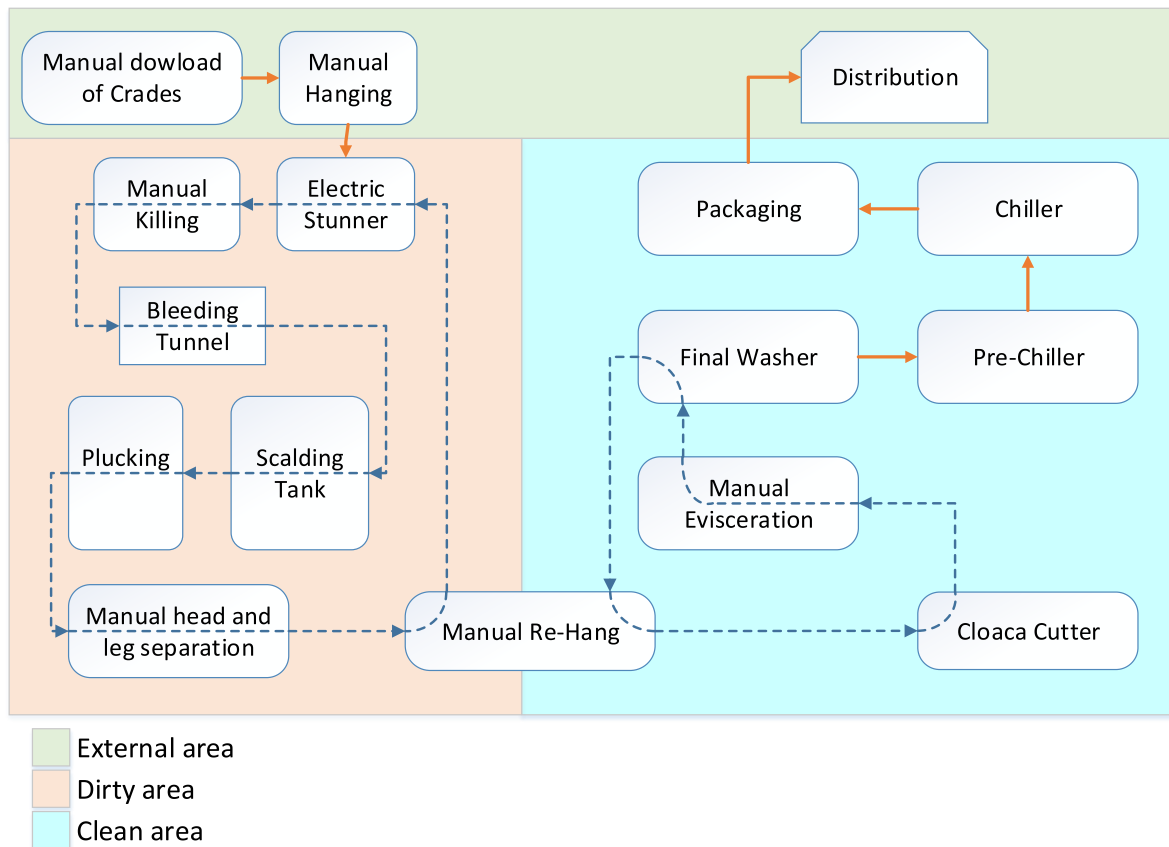

Figure 8. Industrial slaughter process of broilers in Ecuador.

5.3 Other poultry types

In the last years, turkey consumption in Ecuador has become more popular. Yet, turkey production is marginal compared to broiler production. Official reports indicate that there are 17 turkey farms in Ecuador with a housing capacity of 500.000 birds (CGSIN and MAGAP, 2015a). Production of other poultry species is even smaller. Some farmers rear ducks and quails under the requirements of specific markets and there are not official reports on the quantity of farms and birds of these species.

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AIMS

Campylobacter and *Salmonella* are major foodborne pathogens worldwide. Not only the gastro enteric diseases that they cause but also the complications linked to their occurrence are of primary importance in both developed and developing world. Although some food products are recognized to transmit these bacteria, poultry as a whole is the main source of contamination of *Campylobacter* and *Salmonella* for humans. Moreover, the antibiotic resistance in these bacteria are of general concern since failure of treatments may occur in the presence of multi drug resistant bacteria.

In Latin America, poultry meat is a principal source of animal protein for human consumption. However, little is known about the epidemiology of these bacteria in Latin America and especially in the Ecuadorian poultry industry.
In order to contribute to close this gap, the general aim of this thesis was to obtain data about *Campylobacter* and *Salmonella* in the poultry industry in Ecuador.
To fulfill this aim the following specific objectives were formulated:

1. For *Salmonella*
    a. Estimation of the prevalence in broilers slaughtered at the province of Pichincha (Chapter 1).
    b. Further characterization of isolates using phenotypic and genotypic methods (Chapter 1).
2. For *Campylobacter*
    a. Estimation of the prevalence in broilers slaughtered at the province of Pichincha (Chapter 2).
    b. Further characterization of isolates using phenotypic and genotypic methods (Chapter 2).
    c. To assess the contamination dynamics during the slaughter of *Campylobacter* positive batches in commercial slaughterhouses (Chapter 3).

CHAPTER 1: Prevalence and Diversity of *Salmonella* serotypes in Ecuadorian Broilers at Slaughter Age.

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1. Abstract

Salmonella is frequently found in poultry and represent an important source for human gastrointestinal infections worldwide. The aim of this study was to investigate the prevalence, genotypes and antimicrobial resistance of *Salmonella* serotypes in broilers from Ecuador. Caeca content from 388 at random selected broiler batches were collected in 6 slaughterhouses during one year and analyzed by the ISO 6579/Amd1 protocol for the isolation for *Salmonella*. Isolates were serotyped and genotypic variation was acceded by pulsed field gel electrophoresis. MIC values for sulfamethoxazole, gentamicin, ciprofloxacin, ampicillin, cefotaxime, ceftazidime, tetracycline, streptomycin, trimethropim, chloramphenicol, colistin, florfenicol, kanamycin and nalidixic acid were obtained. Presence of $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV} and bla_{CMY}; and *mcr-1* plasmid genes was investigated in resistant strains to cefotaxime and colistin respectively. Prevalence at batch level was 16.0%. The most common serotype was *S*. Infantis (83.9%) followed by *S*. Enteritidis (14.5%) and *S*. Corvallis (1.6%). The pulsed field gel electrophoresis analysis showed that *S*. Corvallis, *S*. Enteritidis and *S*. Infantis isolates belonged to 1, 2 and 12 genotypes respectively. *S*. Infantis isolates showed high resistance rates to 12 antibiotics ranging from 57.7% (kanamycin) up to 98.1% (nalidixic acid and sulfamethoxazole). All *S*. Enteritidis isolates showed resistance to colistin. High multiresistant patterns were found for all the serotypes. The $bla_{\text{CTX-M}}$ gene was present in 33 *S*. Infantis isolates while *mcr-1* was negative in 10 colistin resistant isolates. This study provides the first set of scientific data on prevalence and multidrug-resistant *Salmonella* coming from commercial poultry in Ecuador.

2. Introduction

Foodborne infections in humans caused by *Salmonella* are of primary importance around the world. Majowicz *et al*. (Majowicz *et al*., 2010) estimated that non-typhoidal *Salmonella* was the cause of 93.8 million cases of gastroenteritis, with 155.000 deaths yearly worldwide. For 2010 it was estimated that foodborne salmonellosis caused by non-typhoidal *Salmonella* resulted in 6.43 million Disability-Adjusted Live Years (WHO, 2015a). Salmonellosis is characterized by acute onset of fever, abdominal pain, diarrhea and nausea (WHO, 2015b). Salmonellosis is especially important in susceptible groups such as young, elderly and immunocompromised patients (WHO, 2015a). In Ecuador 3373 human cases or 21.6 cases/100,000 inhabitants of foodborne salmonellosis were reported in 2014 (Ministerio de Salud Pública del Ecuador, 2014).

Although *Salmonella* contaminated vegetables and fruits may be the source for human infection, several domestic animal species are considered as the most important source of human infection, since such animals are often colonized by this pathogen. Poultry is by far the main vehicle of these pathogens in the food chain (Antunes *et al*., 2015; FAO, 2002). In Latin America some *Salmonella* outbreaks in humans linked to chicken consumption are published (Fernandez *et al*., 2003; Mercado *et al*., 2012; Pazzaglia *et al*., 1992). However data on the prevalence of *Salmonella* in industrial reared poultry in Latin America is limited (Donado-Godoy *et al*., 2012a; Donado-Godoy *et al*., 2012b; Pulido-Landínez *et al*., 2013).

Worldwide the use of antibiotics in husbandry practices is a major concern since this may promote the development of multidrug-resistant bacteria. Antibiotics in poultry production systems are widely used to prevent, control and treat bacterial infections as well as growth promoters (Seiffert *et al*., 2013). These facts are of special relevance in developing countries where misuse of antibiotics and the lack of control over their usage is a problem to be addressed (Reardon, 2014). Resistant bacteria can cause human diseases or transmit its resistance genes to pathogenic bacteria (Andersson and Hughes, 2014).

In Ecuador chicken meat is frequently consumed and its demand increased over the years (CONAVE, 2014). Although Ecuadorian poultry industry only provides chicken meat for local consumption, it is expected that in the future it can have access to international markets once sanitary conditions are better understood and controlled. Moreover, despite the importance of non-typhoidal *Salmonella* as a foodborne pathogen, little is known about its epidemiology on poultry farms, in slaughterhouses and retail stores in the main centers of production and

consumption of poultry products. This Information may help to establish surveillance programs and interventions measures regarding the presence and antimicrobial resistance of *Salmonella.*

The aim of this study was to investigate the prevalence, genetic profiles and antimicrobial resistance of *Salmonella* in broilers slaughtered in industrial facilities located in the province of Pichincha in Ecuador.

3. Materials and Methods

3.1. Study design and sampling

Pichincha, the province where Quito the capital city of Ecuador is located, was selected as the area to collect samples since it is an important region within Ecuador for the production of broiler meat. Big slaughterhouses were contacted and asked for their willingness to cooperate in the study. Based on these results sampling was performed in 6 slaughterhouses. From June 2013 to July 2014, a total of 388 batches (birds coming from one broiler house and slaughtered on the same day) were sampled. Each batch originated from a different epidemiological unit. All sampled batches were commercially reared and slaughtered at the age of 6 to 7 weeks.

From each batch one caecum from 25 randomly selected chickens were collected, and transported in an ice box within 1 hour to the laboratory for bacteriological analysis.

3.2. Isolation and identification of *Salmonella*.

From each of the 25 caeca content was aseptically pooled. Therefore, all caeca were immersed in ethanol, and after evaporation of the ethanol approximately 1 g content/cecum was collected in a sterile plastic bag. All samples were homogenized by hand during 1 min. after the addition of 225 ml Buffered Peptone Water (BPW; Difco, BD, Sparks, MD). After the incubation of the preenrichment media at 37 ^{o}C for 20 hours 3 drops of each culture medium were spotted onto a Modified Rappaport-Vassiliadis agar plate (MSRV; Oxoid, Basingstoke, UK) and incubated at 42°C for 24 hours. Plates were examined for migration and if present a loopful from the edge of the migration zone was streaked onto a Xylose Lysine Deoxycholate agar plate (XLD, Difco) and incubated at 37 ^{o}C for 24 hours. Two presumptive *Salmonella* colonies were tested using Triple Sugar Iron agar (Difco, BD), Lysine Iron agar (BBL, BD), Urea agar (BBL, BD) and Sulfur Indole Motility medium (BBL, BD) for confirmation.

3.3. Characterization of *Salmonella* isolates

One *Salmonella* isolate per positive sample was further characterized. To limit the number of *Salmonella* strains to be serotyped, isolates were grouped by an enterobacterial repetitive intergenic consensus (ERIC) PCR as described by Rasschaert *et al*. (2005). ERIC PCR was performed on 59 isolates within the same run. Based on ERIC PCR profiles 16 isolates were selected for serotyping. All these selected isolates and the 3 isolates not included in the ERIC PCR run were serotyped according to the Kauffmann-White scheme.

To characterize the *Salmonella* strains within each serotype, all isolates were genotyped by pulse field gel electrophoresis (PFGE) after digestion with *XbaI* enzyme (CDC, 2015b). The relatedness among the PFGE profiles was analyzed with GelCompar II software v. 6.6 (Applied Maths, Sint-Martems-Latem, Belgium). Bands representing fragments between 35 kb and 1140 kb in size were included in the analysis. A similarity dendrogram was constructed by the unweighted pair group method using arithmetic averages algorithm (UPGMA). DICE similarity coefficient with a position tolerance of 1.4 was calculated. A PFGE genotype was assigned on the basis of the difference in the presence of at least one band in the *XbaI* fingerprint (Barrett *et al*., 2006). Genotypes were identified by numerical suffixes after a capital indicating the serotype (e.g. I-1 refers to serotype Infantis).

3.4. Antimicrobial resistance

Antimicrobial resistance was evaluated by determining the minimum inhibitory concentration (MIC) using the EUMVS2 plates (Thermo Scientific, West Palm Beach, USA). The tests were performed according to the manufacturer instructions. The following antibiotics were evaluated: sulfamethoxazole, gentamicin, ciprofloxacin, nalidixic acid, ampicillin, cefotaxime, ceftazidime, tetracycline, streptomycin, trimethoprim, chloramphenicol, colistin, florfenicol and kanamycin. *Escherichia coli* ATCC 25922 was used as the quality control strain. Clinial breakpoints values from the Clinical and Laboratory Standards Institute (CLSI, 2014) were considered to determine bacterial antibiotic resistance for kanamycin and sulfamethoxazole. For all other antibiotics epidemiological breakpoint values from the European Committee on Antimicrobial Susceptibility Testing were considered (EUCAST, 2015). *Salmonella* isolates resistant to cefotaxime where further examined for the presence of ESBL or AmpC phenotypes by disk diffusion tests (Song *et al*., 2007; CLSI, 2015). According to the disk diffusion results PCR tests were performed to identify $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV} genes in ESBL isolates and bla_{CMY} in AmpC isolates. PCR conditions and primers were the ones described by Hasman *et al*. (2005) for

bla$_{CTX-M}$, Olesen *et al*. (2004) for *bla*$_{TEM}$, Arlet *et al*. (1997) for *bla*$_{SHV}$ and; Hasman *et al*. (2005) and Kruger *et al*. (2004) for *bla*$_{CMY}$.

Isolates with phenotypic resistance to colistin were tested for the presence of the new described *mcr-1* plasmid gene by primers described by Liu *et al*. (Liu *et al*., 2016). For the PCR reaction mixture the Maxima Hot Start Green PCR Master Mix (Promega, Madison, WI, USA) was used. The total volume of 25 µl contained 1 X hot start PCR buffer, 400µM of each nucleotide (dNTP), 4mM $MgCl_2$, 0.2 µM of each primer and 1 µl of the template DNA obtained after boiling 1 colony of the bacteria in 100 µl of DNA free water during 10 minutes. The following PCR program was used: a denaturation step at 95°C for 5 minutes, 35 cycles of 1 minute at 95°C, 0.5 minutes at 60°C and 1 minute at 72°C; and finally 10 minutes at 72°C. After the PCR, the amplification products were confirmed by gel electrophoresis using a 2% agarose gel. A PCR amplicon of 308 bp was expected. As positive control the *Salmonella* autoagglutinable strain S15FP06306 was used (strain isolated from poultry and confirmed to have the *mcr-1* gene by sequencing of the PCR product and by performing whole genome sequencing).

3.5. Statistical analysis

Prevalence of *Salmonella* positive batches was estimated using a random-effects logistic regression model with farms and the sampling occasions per farm as random factors. The 95% confidence interval ($CI_{95\%}$) for the prevalence was calculated once the regression model fit the intercept. Variance components and their standard deviations and the intraclass correlation coefficient (ICC) are reported. Function *glmer* from *lme4* package (Bates *et al*., 2016) in R environment version 3.3.1 (R Core Team,) was used to estimate the fixed and the random factors. *Salmonella* prevalence in farms and its $CI_{95\%}$ were estimated under independence assumption for farms and considering a farm positive when at least one of the sampled batches was positive.

Results

In total 388 batches originated from 119 farms (1 to 9 flocks per farm) were sampled. From all tested batches 62 (16.0%; $CI_{95\%}$: 12.6-24.5) were *Salmonella* positive. The variance component for farms was 0.0237 (SD: 0.154) and 0.0345 (SD: 0.185) for sampling occasions per farm. Thus, the ICC estimated was 0.5928 as a measure of reproducibility in the sample results. Positive batches originated from 50 (42.0%; $CI_{95\%}$: 33.1-51.4) farms (Table 1). For 87 farms, more than one batch was sampled. One, two and three batches were found *Salmonella* positive on 41, 6

and 3 of those farms respectively.

Table 1. Salmonella positive batches in relation to the number of tested batches per farm.

Number of batches/farm sampled	Number of farms	Number of farms with 0, 1, 2 or 3 positive batches			
		0	1	2	3
1	34	27	7		
2	18	12	6		
3	12	7	5		
4	19	10	8	1	
5	17	5	10		2
6	15	6	4	4	1
7	2	1		1	
8	1		1		
9	1	1			
Total	**119**	**70**	**41**	**6**	**3**

ERIC-PCR of the 59 *Salmonella* isolates delivered 2 patterns. Serotyping demonstrated that pattern 1 corresponded to *S*. Enteritidis and pattern 2 to *S*. Infantis (Figure A in S1 File). Direct serotyping of the other 3 *Salmonella* strains resulted in 2 strains belonging to *S*. Infantis and 1 strain to *S*. Corvallis. In total 52 isolates (83.9%) were *S*. Infantis, 9 (14.5%) *S*. Enteritidis and 1 (1.6%) *S*. Corvallis.

The PFGE analysis (Figure B in S1 File) showed that *S*. Corvallis, *S*. Enteritidis and *S*. Infantis isolates belonged to 1, 2 and 12 genotypes respectively (Table 2).

Table 2. Salmonella genotypes present in each serotype.

Serotype	Genotype	No. of strains
S. Corvallis	C-1	1
S. Enteritidis	E-1	5
	E-2	4
S. Infantis	I-1	21
	I-2	6
	I-3	2
	I-4	6
	I-5	1
	I-6	1
	I-7	1
	I-8	10
	I-9	1
	I-10	1
	I-11	1
	I-12	1
Total		62

Within the *S.* Infantis strains the genetic similarity was high (at least 87% similarity) and the different genotypes were due to the presence or absence of one band in the obtained profiles.

The genotype I-1 was the dominant genotype (40.4%) within this serotype. *Salmonella* isolates from 9 farms with more than 1 *Salmonella* positive batch, belonged to different serotypes (2 farms), genotypes (5 farms) or serotypes and genotypes (1 farm) (Table 3).

Table 3. Salmonella serotypes and genotypes found in farms with multiple positive batches.

Farm	**Serotypes-genotypes**									**Total**
	C-1	E-1	E-2	I-1	I-2	I-8	I-9	I-10	I-11	
A							1		1	2
B				1		1				2
C				2						2
D		1	1							2
E		1						1		2
F	1				1					2
G				1	2					3
H				1	2					3
I			1	1			1			3

Antimicrobial resistance rates within each *Salmonella* serotype against the 14 tested antibiotics are shown in Table 4 and the MIC distributions for the different antibiotics are shown in Table B in S1 File. *S*. Infantis isolates showed a resistance rate of 5.8% and 1.9% for ceftazidime and colistin respectively, whereas for the other 12 tested antibiotics the resistance rates varied from 57.7% (kanamycin) up to 98.1% (nalidixic acid and sulfamethoxazole). In contrast, all *S*. Enteritidis isolates showed resistance to colistin. The resistance rate for the other antibiotics ranged from 11.1% up to 33.3%.

Table 4. Number of Salmonella strains resistant to each tested antibiotic.

	Number (%) of resistant isolates		
Antibiotic	*S*. Infantis	*S*. Enteritidis	*S*. Corvallis
Sulfamethoxazole	51 (98.1)	3 (33.3)	1 (100)
Nalidixic acid	51 (98.1)	2 (22.2)	
Ciprofloxacin	49 (94.2)	2 (22.2)	1 (100)
Tetracycline	49 (94.2)	1 (11.1)	
Trimethoprim	47 (90.4)	2 (22.2)	1 (100)
Streptomycin	47 (90.4)	2 (22.2)	
Cefotaxime	42 (80.8)	2 (22.2)	
Ampicillin	41 (78.8)	1 (11.1)	
Florfenicol	40 (76.9)	2 (22.2)	
Gentamicin	39 (75)	2 (22.2)	
Chloramphenicol	39 (75)	1 (11.1)	
Kanamycin	30 (57.7)	2 (22.2)	

	Number (%) of resistant isolates	
Colistin	1 (1.9)	9 (100)
Ceftazidime	3 (5.8)	1 (11.1)

S. Infantis isolates showed 19 resistance patterns in which resistance from 2 up to 13 antibiotics were involved (Table 5). The resistance pattern 2 (38.5%) was the most frequent one within *S*. Infantis isolates. *S*. Enteritidis isolates presented 4 antibiotic resistance patterns containing 1 (pattern 24, 6 strains), 2 (pattern 21, 1 strain) and 12 (patterns 4 and 5, both one strain) antibiotics. Two *S*. Enteritidis isolates were resistant to 12 antibiotics. The *S*. Corvallis isolate was resistant to 3 antibiotics.

Table 5. Antibiotic resistance patterns of Salmonella strains and phenotypes of cefotaxime resistant strains.

Pattern	Resistance pattern	No. Antibiotics	*S.* Infantis	*S.* Enteritidis	*S.* Corvallis	Rate (%)	ESBL + strains*	*bla*$_{CTX-M}$	AmpC + strains*
1	SGCAFZTRMHNKL	13	2			3.2%	2	2	
2	SGCAFTRMHNKL	12	20			32.3%	15	15	5
3	SGCAFTRMHONL	12	1			1.6%			1
4	SGCAFTRMONKL	12		1		1.6%			1
5	SGCFZRMHONKL	12		1		1.6%			1
6	SGCAFTRMHNL	11	6			9.7%	6	5	
7	SGCAFTMHNKL	11	2			3.2%	2	2	
8	SGAFTRMHNL	10	1			1.6%	1	1	
9	SGCAFTRMKL	10	1			1.6%	1	1	
10	SGCAFTRHNL	10	1			1.6%	1	1	
11	SGCAFMHNKL	10	1			1.6%	1	1	
12	SGCTRMHNKL	10	3			4.8%	NA	NA	NA
13	SCAFTRMNL	9	1			1.6%			1
14	GCAFMHNKL	9	1			1.6%	1	1	
15	SCTRMHNL	8	1			1.6%	NA	NA	NA
16	SCAFZTRL	8	1			1.6%	1	1	
17	SCAFTRL	7	3			4.8%	3	3	
18	SCFTRML	7	1			1.6%			1
19	SCTRML	6	4			6.5%	NA	NA	NA
20	STRML	5	1			1.6%	NA		NA
21	SCM	3			1	1.6%	NA		NA
22	SO	2		1		1.6%	NA		NA
23	SM	2	1			1.6%	NA		NA
24	O	1		6		9.7%	NA		NA
Total			52	9	1		34	33	10

Sulfamethoxazole (S), ciprofloxacin (C), nalidixic acid (L), tetracycline (T), trimethoprim (M), cefotaxime (F), ampicillin (A), florfenicol (N), gentamicin (G), chloramphenicol (H), kanamycin (K), streptomycin (R), colistin (O) and ceftazidime (Z).
NA: Not Applicable.
*Number of strains with ESBL or AmpC phenotype according to disk diffusion test.

From the 44 *Salmonella* isolates that showed resistance to cefotaxime 34 presented an ESBL phenotype and were *S*. Infantis, while 10 presented an AmpC phenotype with 2 *S*. Enteritidis and 8 *S*. Infantis. None of the ESBL isolates were positive by PCR for the bla_{TEM} or bla_{SHV} genes, while 33 of these isolates were positive for the $bla_{CTX\text{-}M}$ gene. None of the AmpC isolates were positive for the bla_{CMY} gene. None of the 10 colistin resistant strains were positive for the *mcr-1* plasmid gene by PCR.

Discussion

To our knowledge, this is the first study about *Salmonella* in commercial reared broiler batches at slaughter in Ecuador. Results indicate that 15.9% of the batches slaughtered in the province of Pichincha are *Salmonella* positive. This result is similar to the prevalence reported in Venezuela (23%; n=332) (Boscán-Duque *et al*., 2007). In contrast prevalence in Brazil was only of 5% (n=40) (Giombelli and Gloria, 2014) and in Colombia 65% (n=315) (Donado-Godoy *et al*., 2012c). On the other hand, for the European Union member states and 3 European non-member states an overall *Salmonella* prevalence of 3.37% at farm level was reported with rates varying from 0.08% in Norway to 13.48% in Hungary in 2014 (EFSA and ECDC, 2015b).

Only *S*. Infantis (83.9%), *S*. Enteritidis (14.5%) and *S*. Corvallis (1.6%) were found in positive batches. These findings contrast with data from Colombia, where a wider diversity of *Salmonella* serotypes were reported in broilers at slaughter age (Donado-Godoy *et al*., 2014). These authors found 31 serotypes among 378 examined *Salmonella* strains with the most common serotypes being *S*. Paratyphi B dT+, *S*. Heidelberg, *S*. Enteritidis and *S*. Typhimurium. Similarly, data from Venezuela indicated that the most prevalent *Salmonella* serotypes at slaughterhouse level were *S*. Paratyphi B and *S.* Heidelberg (Boscán-Duque *et al*., 2007). On the other hand, in Brazil the most prevalent serotypes in chicken carcasses were *S*. Enteritidis, *S*. Infantis, *S*. Typhimurium and *S.* Heidelberg (Medeiros *et al*., 2011). In the European Union the most reported serotypes at farm level were *S*. Infantis (43.4%) followed by *S*. Mbandaka (13.5%), *S*. Livingstone (7.3%) and *S*. Enteritidis (7.3%) in 2014 (EFSA and ECDC, 2015b). Accordantly, the emergence of *S*. Infantis in human salmonellosis has been reported (Hendriksen *et al*., 2011). The role of poultry in human salmonellosis caused by *S*. Infantis in Ecuador needs further research.

Moreover, PFGE analysis demonstrated that the *S*. Infantis strains were genetically very similar. Although there were 12 identified genotypes within *S*. Infantis, most of them varied in 1 to 2 bands with similarities above 88%, which suggest that these strains are highly related (Barrett *et al*., 2006). This is in accordance with other studies that showing a high similarity of *S*. Infantis

within poultry, other animal and human isolates (Hauser *et al*., 2012; Rahmani *et al*., 2013; Velhner *et al*., 2014; Franco *et al*., 2015).

The reason why only 3 *Salmonella* serotypes were found and the *S*. Infantis strains showed a high genetic similarity in the present study is not clear and need further research for clarification. In a first step collection of samples from all over Ecuador may give a broader view of *Salmonella* serotypes present in broilers at national level. Moreover, such a study may also confirm the prevalence of *Salmonella* in broilers observed in the present study.

High antibiotic resistance rates were shown against most of the tested antibiotics within *S*. Infantis strains. *S*. Infantis strains showed also higher multiresistant patterns than *S*. Enteritidis. Of the *S*. Infantis strains 44.2% showed resistance to at least 12 antibiotics, whereas 22.2% of *S*. Enteritidis strains presented resistant patterns to 12 antibiotics. In concordance, for Brazil 71.3% (n=87) of *Salmonella* strains isolated from poultry houses were reported to be resistant to chloramphenicol, ampicillin, ceftazidime, ciprofloxacin, nalidixic acid, tetracycline, sulfamethoxazole, and trimethoprim/sulfamethoxazole (Mattiello *et al*., 2015). Although *S*. Enteritidis has been found to be susceptible to most antibiotics (Hur et al., 2012; EFSA, 2015), antibiotic resistance has also been reported to β-lactam antibiotics, sulfonamides, quinoxalines, fluoroquinolones and tetracyclines (Diarra *et al*., 2014; Turki *et al*., 2014; Kuang *et al*., 2015). Moreover, 2 *S.* Enteritidis isolates presented resistance towards 12 antibiotics which is in accordance with previous findings (Hur *et al*., 2011). This is of special interest since it suggests that in high antibiotic pressure environments, non-classical multidrug resistant (MDR) *Salmonella* serotypes can emerge.

In the present study 85.5% and 83.9% of *Salmonella* strains were resistant to nalidixic acid and ciprofloxacin respectively. High resistance rates to fluoroquinolones have been reported in *Salmonella*. For example, EFSA and ECDC reported for 2013 high to extremely high levels of resistance to these 2 antibiotics in *Salmonella* from broilers (EFSA, 2015). A study in Serbia showed that 100% of *S*. Infantis strains were resistant to ciprofloxacin and nalidixic acid (Velhner *et al*., 2014) while Rahmani *et al*. (2013) demonstrated high fluoroquinolone resistance in both, *S*. Infantis and *S*. Enteritidis. High fluoroquinolone resistance rates reported in our study may be explained by the selective pressure of resistant strains under the common use of fluoroquinolones as therapeutics in Ecuadorian broiler farms.

Low rates of colistin resistance in *Salmonella* has been described before (Lu *et al*., 2011; Rahmani *et al*., 2013; Olaitan *et al*., 2015). However, it has been suggested that *S*. Enteritidis may have

increased colistin MIC values (Agersø *et al*., 2012). This is in accordance with our results where 77.8% of *S*. Enteritidis and 1.9% of *S.* Infantis strains presented a colistin resistant phenotype. On the other hand, other studies have reported that resistance to colistin in *Salmonella enterica* isolated from food animals was mainly presented in *S*. Typhimurium but not in *S*. Enteritidis or S. Infantis (Morales *et al*., 2012; de Jong *et al*., 2014). Since the resistance in the phenotype positive *Salmonella* strains was not attributable to the *mcr-1* plasmid gene, it may be assumed that mutations in the chromosomal genes were the source for the observed resistance (Liu *et al*., 2016). Even though the *mcr-1* plasmid gene has been mainly described in *E. coli* from Latin America, Europe and Asia (Hasman *et al*., 2015; Liu *et al*., 2016; Malhotra-Kumar *et al*., 2016; Rapoport *et al*., 2016) this gene has also been observed in *Salmonella enterica* from European countries like UK, Spain and France (Doumith *et al*., 2016; Quesada *et al*., 2016; Webb *et al*., 2016). These data suggest that *mcr-1* gene might be present in *Salmonella enterica* in Latin America, but further research is needed to confirm this assumption.

In accordance with findings from other studies carried out in Latin America, β-lactam-resistant *Salmonella* isolates were identified (Winokur *et al*., 2001; Donado-Godoy *et al*., 2012c; Gelinski *et al*., 2014). Although bla_{TEM} and bla_{SHV} are reported as common genes in resistant *Salmonella* (Miriagou *et al*., 2004; Mattiello *et al*., 2015), these resistance genes were not found in our strains. However, studies in Brazil and USA have identified the $bla_{CTX\text{-}M}$ genes as the most prevalent ESBL genes in *Salmonella* recovered from poultry (Wittum *et al*., 2012; Fitch *et al*., 2015) which is in accordance with our results. It should be taken into account that, even though the main families of beta-lactamases were included in this study, resistance to beta-lactams present in the negative strains could be mediated by other ESBL or AmpC genes (Bush and Jacoby, 2010; Seiffert *et al*., 2013). The presence of these strains in Ecuadorian broilers is of public health concern since resistance to β-lactam antibiotics, listed as WHO Essential Medicines (WHO, 2015d), may limit the options to treat human *Salmonella* infections .

Moreover, *Salmonella* isolates showed high rates of antimicrobial resistance for all antibiotics (with exception of colistin and ceftazidime), indicating the necessity of a better use of antibiotics and biosecurity implementation in the primary sector to reduce the multidrug-resistant bacteria loads in broilers reared in Ecuador. It is worth to mention that there is a global trend towards an increase of antimicrobials consumption in the animal production sector (Van Boeckel *et al*., 2015). This place a concern since the misuse of antibiotics in livestock production can lead to the occurrence of MDR bacteria, especially in low- and middle-income countries frequently

lacking a clear legislative framework about the use of antibiotics in the animal production sector (WHO, 2015c).

In conclusion, this study provides the first set of scientific data on prevalence and multidrug-resistant *Salmonella* originating from commercial poultry in Ecuador. This evidence may be useful for implementation of official policies aiming to decrease the prevalence of *Salmonella* in poultry farms.

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Supplementary Information (S1 File)

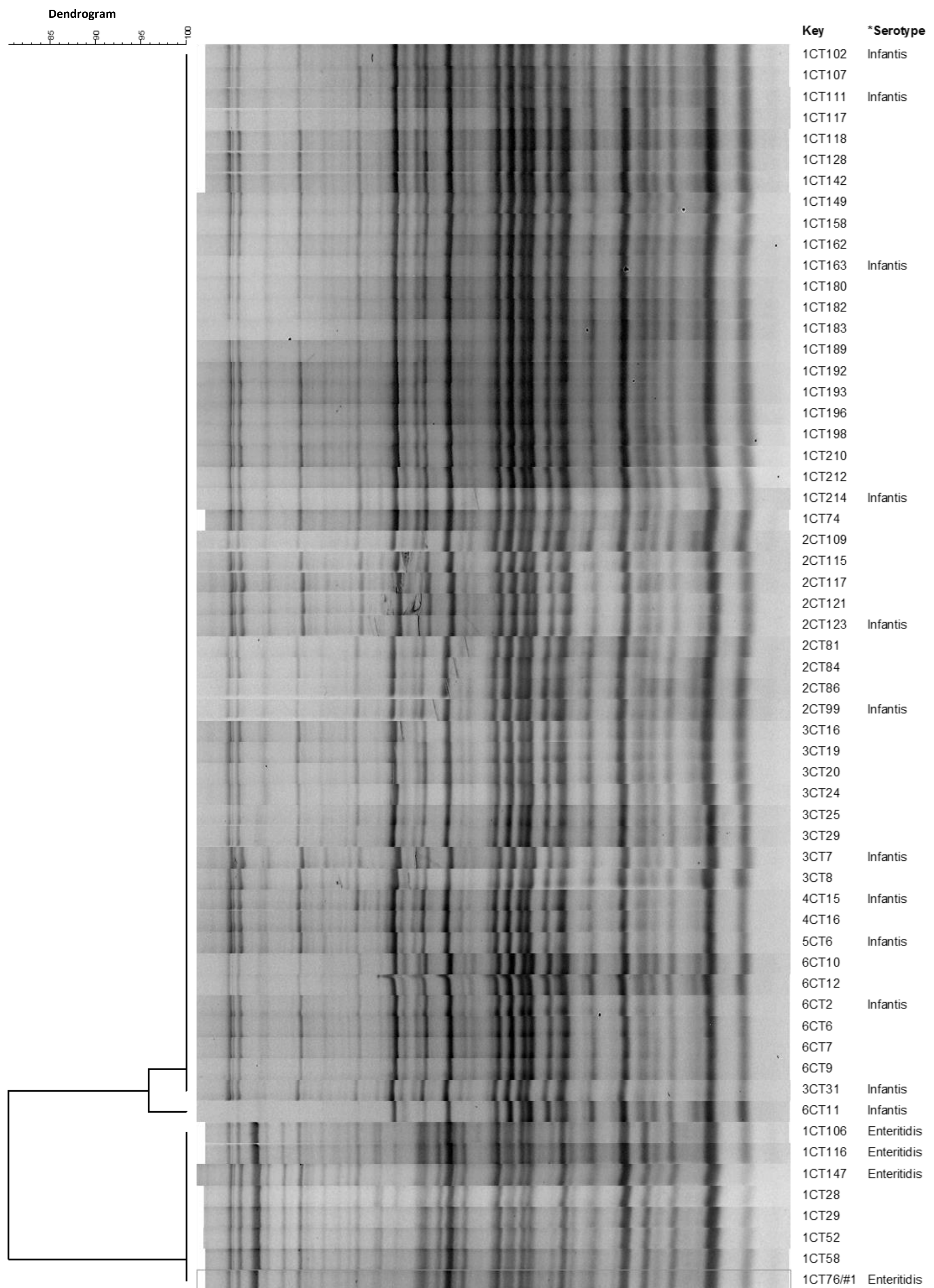

Figure A. ERIC-PCR profiles of the 59 tested Salmonella isolates.
** Selected isolates for serotyping according to the Kaufmann-White scheme*

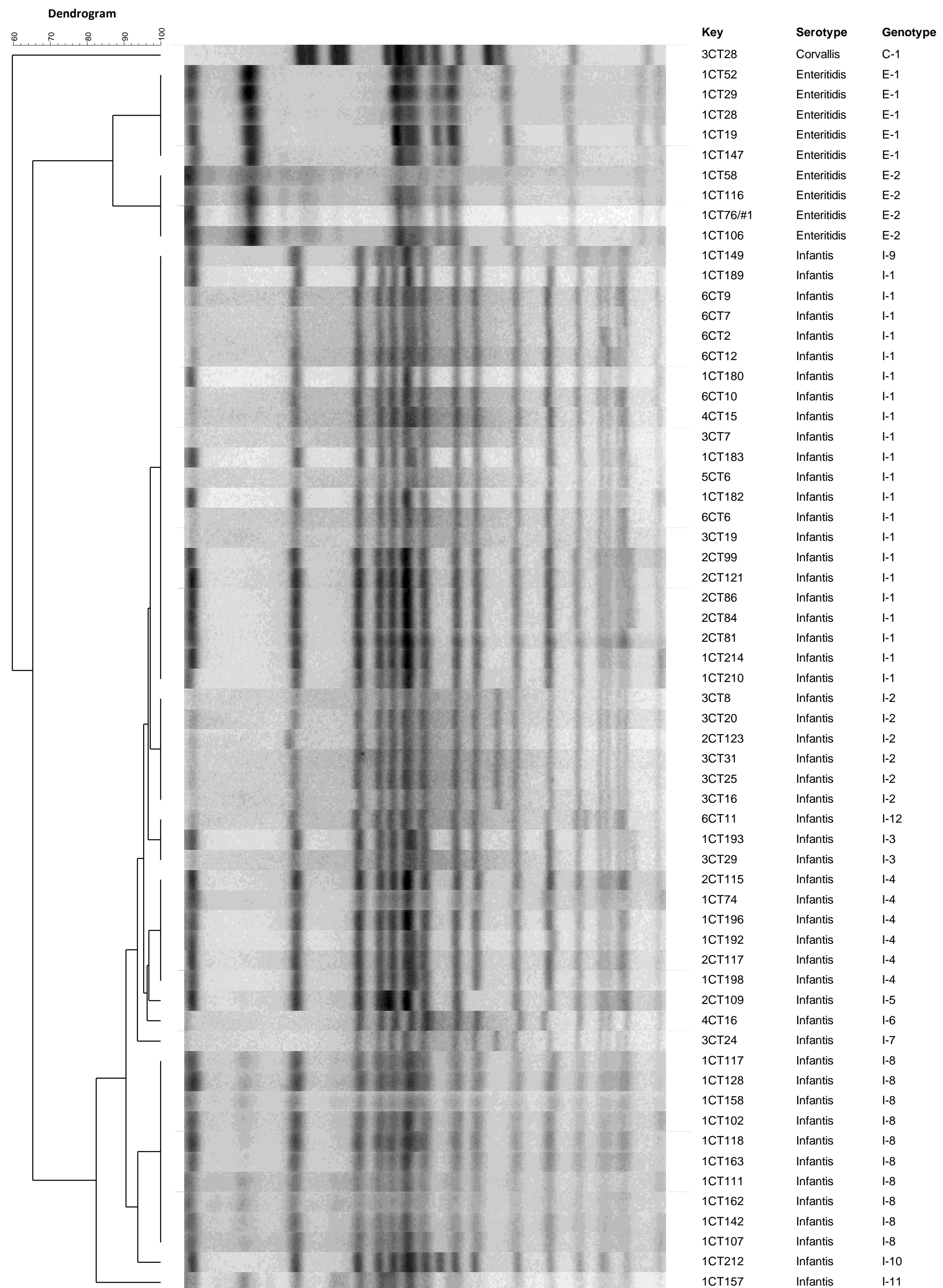

Figure B. PFGE profiles of the 62 Salmonella isolates collected from the positive broiler batches.

Table A. Distribution of the minimal inhibitory concentration values for the 62 Salmonella isolates collected from the positive broiler batches.

Antibiotic	Number of *Salmonella* isolates with minimal inhibitory concentrations (µg/ml)																
	0,02	0,03	0,06	0,12	0,25	0,5	1	2	4	8	16	32	64	128	256	512	1024
Sulfamethoxazole													2	2	3	1	54
Gentamicin					12	8		1	15	23	3						
Ciprofloxacin	5	2	3	20	26	6											
Ampicillin						4	9	6	1			42					
Cefotaxime			8	8	1	1	1		42			1					
Ceftazidime					11	5	6	36	4								
Tetracycline							5	4	2	1	1	48	1				
Streptomycin								3	5	2	3	31	15	3			
Trimethropim						11	1			2	1	47					
Chloramphenicol								13	6	2	1	1	39				
Colistin								52	10								
Florfenicol								14	4	2		1	41				
Kanamycin									30				2	30			
Nalidixic acid									7	1	1		53				

Full vertical lines indicate epidemiological break points for resistance described by European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2016a).
Clinical break points for resistance described by the Clinical and Laboratory Standards Institute (CLSI, 2014) were used for Kanamycin and Sulfamethoxazole.

CHAPTER 2: Prevalence, antimicrobial resistance and genetic diversity of *Campylobacter coli* and *Campylobacter jejuni* in Ecuadorian broilers at slaughter age

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1. Abstract

Thermotolerant *Campylobacter* spp. are a major cause of foodborne gastrointestinal infections worldwide. The linkage of human campylobacteriosis and poultry has been widely described. In this study we aimed to investigate the prevalence, antimicrobial resistance and genetic diversity of *C. coli* and *C. jejuni* in broilers from Ecuador. Caecal content from 379 randomly selected broiler batches originating from 115 farms were collected from 6 slaughterhouses located in the province of Pichincha during 1 year. Microbiological isolation was performed by direct plating on mCCDA agar. Identification of *Campylobacter* species was done by PCR. Minimum inhibitory concentration (MIC) values for gentamicin, ciprofloxacin, nalidixic acid, tetracycline, streptomycin and erythromycin were obtained. Genetic variation was assessed by RFLP-*flaA* typing and Multilocus Sequence Typing (MLST) of selected isolates. Prevalence at batch level was 64.1%. Of the positive batches 68.7% were positive for *C. coli*, 18.9% for *C. jejuni* and 12.4% for *C. coli* and *C. jejuni*. Resistance rates above 67% were shown for tetracycline, ciprofloxacin and nalidixic acid. The resistance pattern tetracycline, ciprofloxin and nalidixic acid was the dominant one in both *Campylobacter* species. RFLP-*flaA* typing analysis showed that *C. coli* and *C. jejuni* strains belonged to 38 and 26 profiles respectively. On the other hand, MLST typing revealed that *C. coli* except one strain belonged to CC-828, while *C. jejuni* except 2 strains belonged to 12 assigned clonal complexes (**CCs**). Furthermore 4 new sequence types (**STs**) for both species were described, whereby 2 new STs for *C. coli* were based on new allele sequences. Further research is necessary to estimate the impact of the slaughter of *Campylobacter* positive broiler batches on the contamination level of carcasses in slaughterhouses and at retail in Ecuador.

2. Introduction

Thermotolerant *Campylobacter* spp. are a major cause of foodborne gastrointestinal infections worldwide. Human campylobacteriosis in its acute phase is characterized by diarrhea, fever, abdominal cramps and vomiting and has been linked to the development of Guillain-Barré syndrome, reactive arthritis and irritable bowel syndrome as complications after the acute phase of the disease (Loshaj-Shala *et al*., 2015). The WHO (2015) estimated that *Campylobacter* caused 37.600 deaths per year worldwide. For 2014, 237,642 campylobacteriosis cases were registered in the European Union (EFSA and ECDC, 2015a). However it has been estimated that the real number of cases occurring yearly may be 9 million cases (Havelaar *et al*., 2009). Diarrheal illness caused by these pathogens are especially important in developing countries where the infection in children under the age of two years is frequent and may lead to death (WHO, 2011). *Campylobacter* has been associated to 11.3-21% of diarrhea episodes in children from low-income countries (Platts-Mills and Kosek, 2014). However, the lack of studies on the epidemiology of *Campylobacter* in developing countries could lead to the underestimation of the burden of *Campylobacter* infections in these regions (Platts-Mills and Kosek, 2014). In Ecuador data about *Campylobacter* infections in humans is very limited. *Campylobacter* has been reported in Ecuadorian low income communities as a possible cause of diarrhea in humans (Vasco *et al*., 2014). Furthermore, it has been estimated that 50%-80% of campylobacteriosis cases may be attributed to the chicken reservoir as a whole being poultry the main source of *Campylobacter* transmission within the European Union (Skarp *et al*., 2015).

In general, *Campylobacter* infections do not require antibiotic treatment, however the use of erythromycin, tetracycline and quinolones is recommended in severe cases (WHO, 2011). Worldwide the use of antibiotics in husbandry practices is a major concern since this may promote the development of resistant and even multidrug-resistant bacteria. Antibiotics in poultry production systems are widely used to prevent, control and treat bacterial infections as well as growth promoters in a large number of countries (Seiffert *et al*., 2013). These facts are of special relevance in developing countries where misuse of antibiotics and the lack of control over their usage is a problem to be addressed (Reardon, 2014). In Latin-American countries, increased rates of antimicrobial resistant *Campylobacter* have been reported (Pollett *et al*., 2012; Sierra–Arguello *et al*., 2016).

In Ecuador chicken meat is frequently consumed and its demand increased over the years (CONAVE, 2014). Although Ecuadorian poultry industry only provides chicken meat for local consumption up to now, it is expected that in the future it can have access to international

markets once sanitary conditions are better understood and controlled. Despite of the importance of *Campylobacter* as a foodborne pathogen, little is known about its epidemiology in poultry farms, slaughterhouses and retail stores in the main centers of production and consumption of poultry products in Ecuador. This information may help to establish surveillance programs and intervention measures regarding to the presence and antimicrobial resistance of *Campylobacter* in Ecuadorian poultry.

The aim of this study was to investigate the prevalence, antimicrobial resistance and genetic profiles of *Campylobacter* in broilers slaughtered in industrial facilities in the province of Pichincha in Ecuador.

3. Materials and methods

3.1. Study design and sampling

Pichincha, the province where Quito the capital city of Ecuador is located, was selected as the area for the collection of samples since in this province and the surrounding ones 36% of the total Ecuadorian broiler production is located (CGSIN and MAGAP, 2015b).

In Pichincha 8 big slaughterhouses are located (CGSIN and MAGAP, 2015a). All of them were contacted and asked for their willingness to cooperate in the study. Based on these results sampling was performed in 6 slaughterhouses. From June 2013 to July 2014, a total of 379 batches (birds coming from one house and slaughtered on the same day) were sampled. All sampled batches from a same farm originated from different houses or birds reared on different periods in the same house. In Ecuador commercial broiler management at the farm includes total depopulation of houses, removal of the litter after every reared batch, cleaning and disinfection of the house followed by a down period of 8-15 days. All sampled batches were commercially reared and slaughtered at the age of 6 to 7 weeks. From each batch, one caecum from 25 randomly selected chickens was collected, and transported in an ice box within 1 hour to the laboratory for bacteriological analysis.

3.2. Isolation and identification of *Campylobacter* spp.

The content from the 25 collected caeca was aseptically pooled. Therefore, all caeca were immersed in ethanol, and after evaporation of the ethanol approximately 1 g content/cecum was collected in a sterile plastic bag. The pooled sample was homogenized by hand during 1 min. after the addition of 225 ml Buffered Peptone Water (218103, Difco, BD, Sparks, MD, USA) and

a loopful (10 µl) from each homogenate was directly streaked onto a modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) (*Campylobacter* blood free selective medium CM0739 plus selective supplement SR0155H [Oxoid, Cheshire, UK]). Plates were incubated under microaerobic conditions at 41.5 °C for 48 h. Three presumptive *Campylobacter* colonies per plate were confirmed by Gram staining and microscopic observation. Colonies containing bacteria with a typical shape were subcultured on mCCDA. After incubation under microaerobic conditions at 41.5°C for 48 h the DNA of one colony per plate was extracted by boiling for 10 minutes in 100 µl of DNA free water. The rest of the culture was transferred into sheep blood and stored at -80°C. Multiplex PCR described by Vandamme *et al*. (1997) was performed for identification of *Campylobacter* species. PCR results indicating the presence of both *C. jejuni* and *C. coli* were retested after sub-culturing of one colony on mCCDA until only one species was detected.

From each positive batch one isolate was randomly selected for further characterization.

3.3. Antimicrobial resistance

Antimicrobial resistance was evaluated in one isolate per sample. The minimum inhibitory concentration (MIC) was determined using the EUCAMP2 plates (Thermo Scientific, West Palm Beach, Florida). The tests were performed according to the manufacturer instructions. The following antibiotics were evaluated: gentamicin, ciprofloxacin, nalidixic acid, tetracycline, streptomycin and erythromycin. *Campylobacter jejuni* ATCC 33560 was used as the quality control strain. Epidemiological breakpoint values from the European Committee on Antimicrobial Susceptibility Testing were considered to determine bacterial antibiotic resistance (EUCAST, 2015).

3.4. Restriction fragment length polymorphism of the *fla*A gene (RFLP-*fla*A)

One *Campylobacter* isolate per positive batch was tested. For the PCR the consensus pair of primers for the *flaA* gene described by Wassenaar and Newell (Wassenaar and Newell, 2000) and the reagents and conditions described by Nachamkin *et al*. (Nachamkin *et al*., 1993) were applied. For restriction fragment length polymorphism (RFLP) analysis *flaA* PCR amplicons were treated with restriction enzyme *DdeI* (Thermo Scientific, West Palm Beach, Florida). PCR amplicons (7 µl) were digested according to the manufacturer's instructions and then separated by electrophoresis for 1:40 hours at 120 V. The gels were stained and photographed. The relatedness among the RFLP-*flaA* profiles was analyzed with GelCompar II software v. 6.6

(Applied Maths, Sint-Martems-Latem, Belgium). Bands representing fragments between 200 bp and 1100 bp in size were included in the analysis. A similarity dendrogram was constructed by the unweighted pair group method using arithmetic averages algorithm (UPGMA). DICE similarity coefficient with a tolerance position of 1% was calculated. A RFLP-*flaA* genotype was assigned on the basis of the difference in the presence of at least one band in the *DdeI* fingerprint.

3.5. Multilocus sequence typing

Multilocus Sequence Typing (MLST) was carried out on all *C. jejuni* isolates that still could be subcultured (40 isolates). For *C. coli*, 40 randomly selected isolates representing 40 farms were typed by MLST.

MLST was performed by the protocol previously described (PubMLST.org, 2016). Sequence types (STs) and clonal complexes (CCs) were assigned by submitting DNA sequences to the *Campylobacter* MLST database website (http://pubmlst.org/campylobacter). Novel alleles and STs were submitted to the MLST database for the assignation of new numbers.

3.6. Statistical analysis

Statistical analysis was carried out with STATA/IC 11.0 (StataCorp LP, College Station, USA). The survey design corrected prevalence estimates of *Campylobacter* at batch level were obtained using the linearized Taylor series method. Farms was identified as first-stage cluster. To determine the prevalence of *Campylobacter* at farm level, a farm was considered positive when at least one of the sampled batches was positive. Farms were assumed to be independent.

Differences of antibiotic resistances between *C. coli* and *C. jejuni* were calculated by the Chi square test. Proportions were considered statistical different when the p value was below 0.05.

4. Results

4.1. Prevalence of *Campylobacter* spp.

The 379 sampled batches originated from 115 farms (1 to 9 batches per farm). From all tested batches 243 (64.1%; $CI_{95\%}$: 58.7%-69.6%) were *Campylobacter* positive and originated from 97 farms (84.4%; $CI_{95\%}$: 77.6%-91.1%). From 84 farms, more than one batch was sampled. The number of times that those farms had *Campylobacter* positive batches ranged from 1 to 6 (Table

1). Initial PCR speciation demonstrated that 167 batches (68.7%; CI$_{95\%}$: 62.9%-74.6%) were positive for *C. coli*, 46 (18.9%; CI$_{95\%}$: 14.0%-23.9%) for *C. jejuni* and 30 (12.4%; CI$_{95\%}$: 8.2%-16.5%) for *C. coli/C. jejuni*. Subculturing of the mixed cultures yielded 22 *C. coli* and 8 *C. jejuni* isolates.

Table 1. Campylobacter positive batches in relation to the number of tested batches per farm.

Number of sampled batches/ farm	**Number of farms**	**Number of farms with 0 to 6 positive batches**						
		0	1	2	3	4	5	6
1	31	15	16					
2	19	2	7	10				
3	12	1	2	8	1			
4	18		1	3	9	5		
5	15		2	3	1	5	4	
6	16			4	4	4	3	1
7	2		1			1		
8	1							1
9	1							1
Total	115	18	29	28	15	15	7	3

4.2. Antimicrobial resistance

Twenty-five isolates (19 *C. coli* and 6 *C. jejuni*) could not be sub-cultured from -80°C for MIC test; hence 218 isolates were tested (170 *C. coli* and 48 *C. jejuni*). The MIC distributions for the different antibiotics of *C. coli* and *C. jejuni* are shown in Tables 2 and 3 respectively. *C. coli* and *C. jejuni* showed very low resistance rates for gentamicin and the resistance rate was not statistically different between both species (P=0.752). For streptomycin the resistance rates were 11.2% and 8.3% for *C. coli* and *C. jejuni* respectively (P=0.199). Resistance rate for erythromycin was statistically higher for *C. coli* (25.9%) compared to *C. jejuni* (4.2%) (P=0.024). In contrast the resistance rates for tetracycline was statistically higher for *C. jejuni* (83.3%) than for *C. coli* (67.6%) (P=0.016). Resistance rates of *C. coli* for ciprofloxacin and nalidixic acid were 100% and 99.4% respectively (P=0.086). Similarly, *C. jejuni* presented resistance rates of 97.9% and 100% for ciprofloxacin and nalidixic acid respectively (P=0.558).

Table 2. Distribution of the minimal inhibitory concentration values for 170 C. coli isolates collected from broiler batches.

Antibiotic	Number of *C. coli* isolates with minimal inhibitory concentrations (µg/µl)[1]										
	0,12	0,25	0,5	1	2	4	8	16	32	64	128
Gentamicin	106	49	8	5	1		1				
Streptomycin			1	120	30			19			
Erythromycin			38	33	33	22	6		31		7
Tetracycline		34	15	6		1	1	89		24	
Ciprofloxacin			1		2	134	5	28			
Nalidixic acid						1		1	11	157	

[1]Full vertical lines indicate epidemiological break points for resistance described by European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015).

Table 3. Distribution of the minimal inhibitory concentration values for 48 C. jejuni isolates collected from broiler batches.

Antibiotic	Number of *C. jejuni* isolates with minimal inhibitory concentrations (µg/µl)[1]										
	0,12	0,25	0,5	1	2	4	8	16	32	64	128
Gentamicin	42	3	2			1					
Streptomycin		2	5	35	2	2		2			
Erythromycin			23	21	2				1		1
Tetracycline		6	2		1		1	28		10	
Ciprofloxacin	1				2	33	3	9			
Nalidixic acid								1	1	46	

[1]Full vertical lines indicate epidemiological break points for resistance described by European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015).

C. coli and *C. jejuni* isolates showed 8 and 6 different resistance patterns respectively. *C. coli* presented resistance against 1 up to 6 antibiotics, whereas for *C. jejuni* resistance against 2 up to 6 antibiotics were involved. The resistance pattern 5 (*C. coli*: 42.9%; *C. jejuni*: 72.9%) was the most frequent one for both species (Table 4). Pattern 3 and, pattern 1, 4 and 6 were presented exclusively for *C. jejuni* and *C. coli* respectively.

Table 4. Antibiotic resistance patterns of C. coli and C. jejuni isolates

Pattern	Resistance pattern[1]	*C. coli* (%)	*C. jejuni* (%)
1	C	1 (0.6)	0
2	CN	49 (28.8)	8 (16.7)
3	TN	0	1 (2.1)
4	CEN	5 (2.9)	0
5	CTN	73 (42.9)	35 (72.9)

6	CTEN	23 (13.5)	0
7	SCTN	3 (1.8)	2 (4.2)
8	SCTEN	14 (8.2)	1 (2.1)
9	GSCTEN	2 (1.2)	1 (2.1)
Total		170 (100)	48 (100)

[1]Gentamicin (G), ciprofloxacin (C), nalidixic acid (N), tetracycline (T), streptomycin (S) and erythromycin (E)

4.3. RFLP-*flaA* typing

For RFLP-*fla*A typing 38 isolates (26 *C. coli* and 12 *C. jejuni*) could not be sub-cultured from -80°C; hence 163 *C. coli* and 42 *C. jejuni* isolates were tested. From all tested isolates *1 C. coli and* 7 *C. jejuni* did not present bands in RFLP-*fla*A typing. For *C. coli* 38 profiles were obtained, from which 19 profiles contained more than one strain. Each of the later profiles contained 2 up to 25 strains. For *C. jejuni* 26 profiles were obtained, from which 7 profiles contained 2 to 7 strains. Most of the strains within a RFLP-*flaA* profiles originated from different farms. However, for profile 5, 9, 18, 19, 21, 22 (C. *coli*) and 20 (*C. jejuni*) two strains were found in a single farm, and for profile 16 (*C. coli*) two farms yielded 2 and 3 strains respectively (Table 5).

Table 5. Campylobacter spp. RFLP-flaA profiles with more than one isolate.

Species	**ID of RFLP-*flaA* profiles**	**Number of isolates within each profile**	**Number of origin farms**
C. coli	22	2	1
	7, 17, 25, 31	2	2
	1, 11, 32	3	3
	21	5	4
	5	6	5
	3, 30	7	7
	6	9	9
	8	10	10
	18	10	9
	9	12	11
	29	12	12
	16	22	19
	19	25	24
C. jejuni	7, 8, 9, 16, 19	2	2
	14	4	4
	20	7	6

4.4. MLST typing

From the 40 *C. coli* isolates selected for MLST 39 belonged to CC-828 and 1 did not have an assigned CC (ST-1581). The most frequent STs were ST-5777 (9 isolates), followed by ST-829 (8 isolates) and ST-828 (6 isolates) (Figure 1).

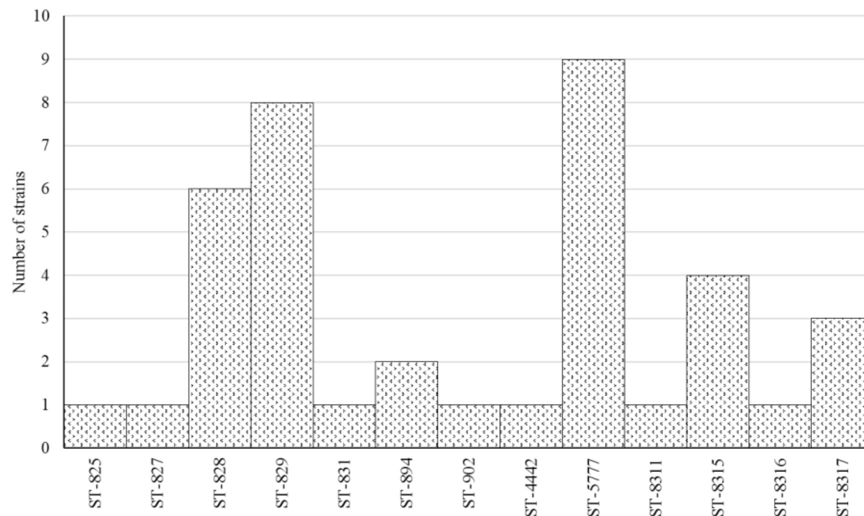

Figure 1. Distribution of STs among the 39 C. coli strains belonging to clonal complex 828.

From the 40 *C. jejuni* isolates selected for MLST the most common CCs were CC-574 (9 isolates), CC-257 (7 isolates), CC-353 (5 isolates) and CC-354 (5 isolates) (Figure 2). Two *C. jejuni* isolates did not correspond to an assigned CC. The most ST-diverse CC was CC-353 (4 STs) followed by CC-257 (3 STs), CC-52 (2 STs), CC-354 (2 STs), CC-464 (2 STs) and CC-21 (2 STs).

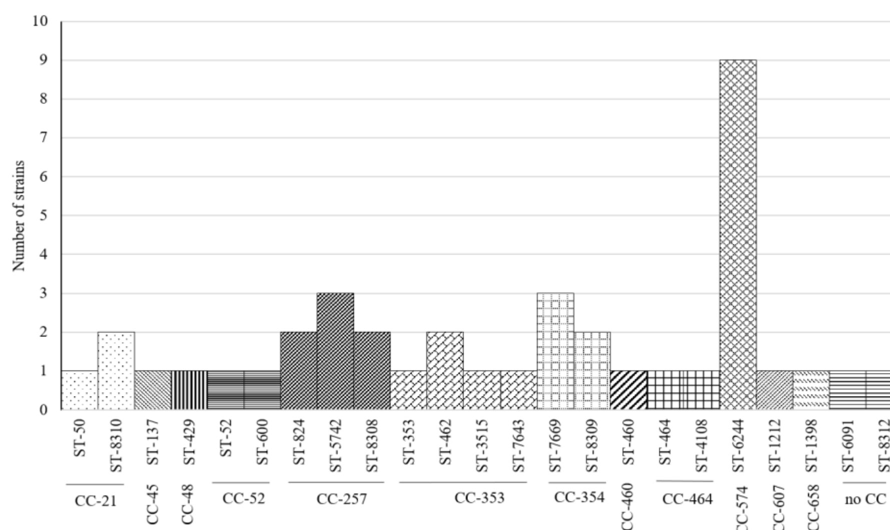

Figure 2. Distribution of STs and clonal complexes among the 40 C. jejuni strains.

In total, 9 *C. coli* and 7 *C. jejuni* strains belonged to STs which were not reported previously. Sequence data from those strains were submitted to the *Campylobacter* MLST database (PubMLST.org, 2016) leading to the assignation of 8 novel ST numbers (4 STs for each species) (Table 6). Two novel STs within *C. coli* (ID PubMLST 48107 and 48108) resulted from novel allele sequences: 5 strains had a novel allele sequence for *aspA*, of which one strain had also a novel allele sequence for *tkt*.

Table 6. MLST profiles of novel STs identified in Campylobacter strains.

Species	No. of isolates	Clonal complex	Sequence type	MLST allelic profile[a]							ID on the PubMLST
				aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*	
C. jejuni	2	257	8308	9	2	4	62	606	5	6	48096
	2	354	8309	8	10	95	2	10	12	6	48097
	2	21	8310	2	1	5	672	11	1	5	48113
	1	NA[b]	8312	2	84	5	10	11	3	6	48099
C. coli	1	828	8311	33	39	30	82	373	56	17	48106
	4	828	8315	**441**	39	30	82	373	47	17	48107
	1	828	8316	**441**	39	30	82	113	**641**	17	48108
	3	828	8317	33	39	30	82	373	47	17	48110

[a] *New allele sequences are given in bold.*
[b] *NA, not assigned.*

4.5. Comparison of RFLP-*flaA* profiles and MLST data.

When comparing MLST data with RFLP-*flaA* profiles, *C. coli* STs 8315, 8317, 828, 5777 and 829 included 2, 2, 3, 4 and 5 RFLP-*flaA* profiles respectively, while RFLP-*flaA* profiles 18, 21, 16 and 19 included 2, 2, 4 and 4 different ST types. For *C. jejuni* 4 STs (6244, 8308, 8309 and 8310) had two RFLP-*flaA* profiles and only the RFLP-*flaA* profile 14 included 2 ST types. No association of RFLP-*flaA* profiles within STs was found regarding the origin of the isolates.

5. Discussion

Our findings demonstrated that the prevalence of *Campylobacter* in broiler batches at slaughter age was 64.1%. Studies from other Latin American countries showed different prevalences. From Brazil and Costa Rica, 100.0% respectively 80.0% of the flocks were reported to be positive for *Campylobacter* when ceca samples were studied (Zumbaco-Gutiérrez *et al*., 2014a; Giombelli and Gloria, 2014). On the other hand, in Argentina and Chile *Campylobacter* was found in 33.3% of samples (Rivera *et al*., 2011; Zbrun *et al*., 2013a) while in Peru Tresierra-Ayala *et al*. (1995) reported a prevalence of 35%. Other tropical countries such as Vietnam and South Africa have reported a prevalence of 31.9 and 14.2% respectively (Jonker and Picard, 2010; Carrique-Mas *et*

al., 2014). Although different prevalences are shown in developing countries, it should be kept in mind that differences in methodologies can make direct comparison of results difficult. Moreover, obtained data indicated that at least 84.3% of farms delivered *Campylobacter* positive batches. For farms delivering only *Campylobacter* negative batches only a maximum of 3 batches were tested. For those farms it can be hypothesized that when more batches would be sampled also these farms would deliver *Campylobacter* positive batches for slaughter. On the other hand, the number of positive batches per farm variated considerably which is in concordance with the observations described by McDowell *et al*. (2008). This variation may be attributed to different risk factors for the introduction of *Campylobacter* in broilers (Adkin *et al*., 2006; Torralbo *et al*., 2014; Sandberg *et al*., 2015).

Considering *Campylobacter* species, *C. coli* was the dominant species in positive batches. This contrasts with other studies from Latin America where *C. jejuni* has been demonstrated to be the most prevalent species in broilers (Tresierra-Ayala *et al*., 1995; Rivera *et al*., 2011; Zbrun *et al*., 2013b; Zumbaco-Gutiérrez *et al*., 2014b; Giombelli and Gloria, 2014). *C. jejuni* has also been demonstrated as the most common *Campylobacter* species from broilers at slaughter age in China and South Africa (Jonker and Picard, 2010; Ma *et al*., 2014). Meanwhile, the European baseline study on *Campylobacter* in broilers indicated that the proportion of *C. coli*/*C. jejuni* varied considerable between countries and this proportion was generally higher in southern countries than in northern countries (EFSA, 2010).

In this study, *C. coli* and *C. jejuni* presented high resistance rates to ciprofloxacin, nalidixic acid and tetracycline while erythromycin, gentamicin and streptomycin showed lower resistance rates. This is in accordance with a study in Brazil where high resistance rates to ciprofloxacin, nalidixic acid and tetracycline, and low resistance rates to erythromycin and gentamicin were reported (Ferro *et al*., 2015). Besides, a similar low resistance rate for erythromycin, a low resistance rate for ciprofloxacin (11,8%) was reported from Chile (Rivera *et al*., 2011).

In contrast with the data reported in EU, this study showed that *C. jejuni* presented higher resistance rates for tetracycline than *C. coli* (EFSA, 2015). On the other hand, a higher resistance rate to erythromycin was shown for *C. coli*, which is consistent with data from China and South Africa that showed higher erythromycin resistance rates for *C. coli* (92.0% and 72.7% respectively) than for *C. jejuni* (18.8% and 20% respectively) (Jonker and Picard, 2010; Ma *et al*., 2014).

High resistance rates for (fluoro)quinolones and tetracycline found in the present study may be explained by the common use of these antibiotics as therapeutics in Ecuadorian poultry farms. However, it is not clear why the resistance rate to tetracycline was higher for *C. jejuni* than for *C. coli* in Ecuador. The low antimicrobial resistance rates to aminoglycosides and macrolides for *C. jejuni* found in this study indicates that gentamicin and erythromycin can still be used for the treatment of human campylobacteriosis when necessary (WHO, 2011). However, changes in resistance rates presented in this research have to be monitored by the implementation of antimicrobial resistance surveillance on *Campylobacter* in Ecuador.

Campylobacter typing by RFLP-*fla*A has been used based on the highly diverse character of this gene. It has also been shown to be a cost-effective alternative to more costly methodologies (Djordjevic *et al*., 2007). The use of RFLP-*flaA* as the only typing method is questioned due to intra- and intergenomic recombination within the flagellin genes (Eberle and Kiess, 2012) which can make the comparison of isolates over time difficult. In contrast MLST typing is a more reliable method since it is based on changes in allele sequences of determined housekeeping genes and a library of MLST types is available to compare results from all over the world (PubMLST.org, 2016). Our results showed that the combination of RFLP-*flaA* and MLST typing led to a further differentiation of a number of isolates. This is in concordance with the results of Duarte *et al*. (2016) who demonstrated that the combination of both RFLP-*flaA* and MLST had a higher discriminatory power than both methods separately.

Based on one isolate per batch, our results indicated that a large variation of genetic types were present in Ecuadorian broiler batches. Some genetic types seemed to be more widespread than other ones. Additionally, RFLP-*flaA* data suggested that over time the persistence of specific genetic types on farms is limited. Analyses of the variable region in the *flaA* locus (*flaA*-SVR) have demonstrated that more than one *Campylobacter* genotype may be present in the same farm (Jorgensen *et al*., 2011; O'Mahony *et al*., 2011; Prachantasena *et al*., 2016). Moreover, some batches were simultaneously colonized with *C. coli* and *C. jejuni* in the present study.

To the best of our knowledge, this study is the first report that showed *Campylobacter* MLST types from commercial broiler batches in Andes region of Latin America. In this study 39 out of 40 *C. coli* isolates belonged to CC-828. Predominant distribution of *C. coli* within CC-828 has also been reported in Europe (Levesque *et al*., 2013; Piccirillo *et al*., 2014). It is suggested that the low diversity of CCs in *C. coli* can be attributed to the presence of a 3-clade *C. coli* population structure. In this genetic structure, horizontal gene transfer within each clade would be more

common than among members of different clades (Sheppard and Maiden, 2015), resulting in a limited number of CCs.

Interestingly, the new reported ST-8315 was present in 4 (10%) *C. coli* isolates. The implication of this ST in the epidemiology of *Campylobacter* needs further research. From the 40 *C. jejuni* isolates tested, the majority belonged to CC-574 (9 isolates), CC-257 (7 isolates), CC-353 (4 isolates), CC-354 (5 isolates) and CC-21 (3 isolates). In Great Britain, an important number of *C. jejuni* strains were grouped in CC-257, CC-353 and CC-574 (Jorgensen *et al*., 2011). Meanwhile, CC-354 has been found in commercial poultry in Thailand (Prachantasena *et al*., 2016). This is in accordance with our results where these CCs were found in 72.5% of the tested samples. Additionally, a Canadian study reported CC-353 in *C. jejuni* isolates from chickens originated in Peru, Bolivia and Argentina (Lévesque *et al*., 2008). Other less common CCs found in this study (CC-45, CC-48, CC-52, CC-460, CC-658, CC-464 and CC-607) have also been reported in poultry from Europe, Africa, Asia and North America (Lévesque *et al*., 2008; Griekspoor *et al*., 2010; O'Mahony *et al*., 2011; Kittl *et al*., 2013; Ngulukun *et al*., 2016; Zeng *et al*., 2016).

Moreover, a study in Ecuador demonstrated that CC-353, CC-354 and CC-607 were present in *C. jejuni* isolates from backyard poultry and other domestic animals kept in households (Graham *et al*., 2016). A query in the *Campylobacter jejuni/coli* PubMLST database (PubMLST.org, 2016) (Last accessed: 21/07/2016) showed that in Latin America, Brazil and Uruguay reported *Campylobacter* MLST profiles from chicken samples. These isolates belonged to CC-257, CC-52 (*C. jejuni*) and CC-828 (*C. coli*) in Uruguay, while in Brazil a no-determined CC (ST-7370) was reported.

Although there are new STs in some of our strains, the most of CCs found in this study have been reported in chicken samples (PubMLST.org, 2016).

This study gives insights on the epidemiology of *Campylobacter* in commercial reared poultry in Ecuador. Since high levels of *Campylobacter* on carcasses has been linked to an increasing risk of *Campylobacter* infections in humans (EFSA, 2011), it would be interesting to collect data about the contamination of broiler meat and related risk factors for contamination at the following stages of the broiler meat chain. *Campylobacter* types and its antimicrobial resistance have not been studied from humans in Ecuador. Therefore, it is not possible to link human campylobacteriosis to the genotypes found in this study. Therefore, further research on *Campylobacter* isolates from the broiler meat chain and humans may give more insights on the epidemiology of *Campylobacter* in Ecuador.

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CHAPTER 3: Quantification of *Campylobacter* on broiler carcasses throughout the slaughter process in Ecuadorian slaughterhouses

Vinueza-Burgos, C., Cevallos, M., Martiny, D., Cisneros, M., Van Damme, I., & De Zutter, L. (2016). Quantification of *Campylobacter* on broiler carcasses throughout the slaughter process in Ecuadorian slaughterhouses. Manuscript in preparation.

Abstract.

Campylobacter contamination of broiler carcasses has not been studied in Ecuadorian slaughterhouses, where several steps are carried out manually or with limited technology. In this study, we performed quantification of the *Campylobacter* contamination on carcasses at four steps in the slaughter process in three Ecuadorian slaughterhouses. Therefore, 15 *Campylobacter* positive batches were sampled in three commercial slaughterhouses. For every batch, caecal content and five samples of breast skin were taken and examined for *Campylobacter* counts at the following steps: after plucking, after evisceration, after final washing and after water chilling. Slaughterhouse C was the only slaughterhouse in which *Campylobacter* counts increased significantly after evisceration. No significant differences were found between counts after evisceration and after final washing ($P>0.05$). In all slaughterhouses, a significant reduction of *Campylobacter* counts (0.11 to 2.55 log_{10} CFU/g) was found after the chilling step. The presence of chlorine in the chilling water was associated with the highest reduction in *Campylobacter* counts on the carcasses. A high variability of *Campylobacter* counts was found within and between batches slaughtered in the same slaughterhouse. *Campylobacter* counts in caecal content samples were not correlated with counts on carcasses after plucking nor after evisceration.

1. Introduction

The global food demand for a population of more than 7 billion people has led to the need of providing safe food in all nations. However, foodborne infections are of worldwide concern, especially in developing countries where the lack of epidemiological data and resources to control foodborne diseases needs to be addressed (Newell *et al*., 2010; WHO, 2015a). Thermotolerant *Campylobacter* spp. are a major cause of foodborne gastrointestinal infections worldwide (WHO, 2015a). Human campylobacteriosis is characterized by diarrhea, fever, abdominal cramps and vomiting and has been linked to the occurrence of Guillain-Barré syndrome, reactive arthritis and irritable bowel syndrome (Loshaj-Shala *et al*., 2015). The WHO (2015) estimated that *Campylobacter* caused 37.600 deaths per year globally. Furthermore, disability-adjusted life-years (DALYs) attributed to campylobacteriosis in developed countries is calculated to range from 1.568 DALYs in New Zealand to 22.500 in the USA (Skarp *et al*., 2015). It is estimated that in the European Union 50%-80% of campylobacteriosis cases may be attributed to the chicken reservoir as a whole while 20-30% is linked to poultry meat consumption (EFSA, 2011; Skarp *et al*., 2015). Additionally, if the infective dose of *Campylobacter* (≥ 500 bacteria) is taken into account, the consumption of poultry meat contaminated with these bacteria may pose a public health concern (Nachamkin *et al*., 2008). *Campylobacter* loads on poultry meat are related to the level of contamination in processing plants (Pacholewicz *et al*., 2015a; Seliwiorstow *et al*., 2015a). Several studies reported that a reduction of *Campylobacter* counts on chicken carcasses leads to a risk reduction of campylobacteriosis cases associated with handling and consumption of chicken meat (Uyttendaele *et al*., 2006; Havelaar *et al*., 2007; Nauta *et al*., 2009). More specifically, production of batches of broiler carcasses with *Campylobacter* counts on neck and breast skin of maximal 500 to 1000 CFU/g may reduce the health risk by more than 50% (EFSA, 2011). To date, the Ecuadorian poultry industry only provides chicken meat for local consumption. The annual broiler meat consumption per capita is estimated to be 32 Kg (CONAVE, 2014). Despite the importance of the poultry industry in Ecuador, there are no studies that show the dynamics of *Campylobacter* contamination in industrialized slaughterhouses.

In this study, we aimed to provide insights in *Campylobacter* counts throughout different steps of the slaughter process of *Campylobacter* positive batches in three slaughterhouses in Ecuador.

2. Materials and methods

2.1 Slaughterhouse profiles

Three poultry slaughterhouses, each belonging to a different integrated company, were included in this study. The characteristics of each slaughterhouse are listed in Table 1.

Table 1. Characteristics of selected slaughterhouses.

Slaughterhouse	**A**	**B**	**C**
Line speed (carcasses/hour)	3000	3000	1000
Stunning	Electrical	Electrical	Electrical
Evisceration	Manual	Manual	Manual
Water temperature during scalding (°C)	56,9	52,9	64
Scalding time (seconds)	180	180	45
Plucking time (seconds)	180	180	40
Final inside-outside washer	Present	Present	Present
Water chilling tanks	Present	Present	Present
Temperature (°C) of the chilling water	Tank 1: 22 Tank 2: 17 Tank 3: 8	Tank 1: 25 Tank 2: 3	Tank 1: 7 Tank 2: 3
Free chlorine concentration in chilling water (ppm)	Tank 1: 0.5 Tank 2: 0.5 Tank 3: 0	Tank 1: 17 Tank 2: 20	Tank 1: 0* Tank 2: 0*
Chilling time (minutes)	Tank 1: 10 Tank 2: 20 Tank 3: 45	Tank 1: 11 Tank 2: 60	Tank 1: 8 Tank 2: 50
Addition of water in chilling tanks (l/carcass)	Tank 1: NA** Tank 2: NA** Tank 3: NA**	Tank 1: 1.5 Tank 2: 1.5	Tank 1: 0 Tank 2: 0

* Only potable water was used in slaughterhouse C.

**NA: not available

2.2. Identification of *Campylobacter* positive broiler flocks.

Identification of *Campylobacter* positive flocks (birds reared in the same house) was performed one week before the chickens were slaughtered. Therefore, caecal droppings were collected in the broiler house at the farm and transported to the laboratory within 6 hours. Direct plating of caecal droppings was performed on modified Cefaperazone Charcoal Desoxycholate Agar (mCCDA; *Campylobacter* blood free selective medium CM0739 plus selective supplement SR0155H [Oxoid, England]). Plates were incubated under microaerobic conditions at 41.5°C for 24 h. Presumptive *Campylobacter* colonies were confirmed by Gram staining and microscopic observation. Only *Campylobacter* positive flocks were sampled during the slaughter process.

2.3. Caeca and carcasses sampling during slaughter.

In each of the three slaughterhouses, five batches originating from five *Campylobacter* positive flocks, were sampled, resulting in 15 visits in the period from July 2014 to April 2015. During each visit, five broiler carcasses were aseptically collected after each of the following slaughter steps: plucking, evisceration, final washing and water chilling. Additionally, one caecum from each of 25 chickens were collected. The first samples were collected 30 min after starting the slaughter process of the batch. Sample collection was performed in a consecutive way over 1.5 h of slaughter. All samples were placed in sterile plastic bags and transferred to a clean area in the slaughterhouse. There, approximately 10 g of breast skin was aseptically sampled for *Campylobacter* enumeration (Baré *et al*., 2013), placed in sterile plastic bags with filter (BagPage®, Interscience, Paris, France) and transported to the laboratory under cool conditions within two hours.

2.4. Sample preparation and enumeration of *Campylobacter* spp.

From each of the 25 caeca, the content was aseptically pooled. Therefore, all caeca were immersed in ethanol, and after evaporation of the ethanol approximately 1 g of content was collected in a sterile plastic bag. The pooled caecal content and the breast skin samples were homogenized in bacteriological peptone (Lab M, Lancashire, UK) at a ratio of 1:10, plated on Rapid Campylobacter Agar (Bio-Rad, California, USA) and incubated under microaerobic conditions at 41.5°C for 48 h. After incubation, colonies with typical *Campylobacter* morphology were counted and at least two colonies per sample were confirmed by microscopic observation.

2.5 Data analysis

The detection limit of enumeration was 10 CFU/g for breast skin samples and 100 CFU/g for caecal samples. Quantification of breast skin samples that were below the enumeration limit was set to one-half of the enumeration threshold (Rosenquist *et al*., 2006). *Campylobacter* counts were log_{10}-transformed prior to analysis.

Differences in *Campylobacter* counts were tested using random-effects generalized least squares regressions, including the batch as group variable. Differences in *Campylobacter* counts on carcasses between the different steps (after plucking, after evisceration, after final washing and after chilling) were determined for each of the three slaughterhouses. Bonferroni corrections were applied for multiple testing. Differences between slaughterhouses were

determined for each of the different steps separately. The relation between caecal content and the contamination level of carcasses was assessed using the mean caecal content counts of the batch as explanatory variable and carcass counts after plucking or counts after evisceration as response variable. A significance level of 5% was used. Statistical analyses were performed using STATA/IC 14.1 (StataCorp LP, TX, USA).

3. Results

During this study, 315 samples (15 caecal and 300 breast skin samples) were collected from 15 *Campylobacter* positive batches slaughtered in three Ecuadorian slaughterhouses. *Campylobacter* counts in pooled caecal content samples varied considerably between batches (from 6.2 up to 11.1 log_{10} CFU/g; Table 2).

Table 2. Campylobacter counts (log_{10} CFU/g) in the caeca content of sampled batches.

Batch number	**Slaughterhouse**		
	A	B	C
1	9.91	7.51	11.12
2	10.25	10.48	10.11
3	6.94	6.20	10.09
4	9.44	7.64	10.21
5	10.03	9.59	10.20

In order to get insight of the impact of the slaughter process on the *Campylobacter* contamination, quantification of *Campylobacter* was carried out after four processing steps.

The mean *Campylobacter* counts per sampling step in the three slaughterhouses is presented in Figure 1. After plucking, mean counts in slaughterhouse C were significantly higher than in slaughterhouse A ($P<0.05$). After evisceration, slaughterhouses B and C had significantly higher mean counts than slaughterhouse A ($p<0.001$), while the difference between slaughterhouse B and slaughterhouse C was not significant ($P>0.05$). After final washing, counts in slaughterhouse C were significantly higher than in slaughterhouse A ($P<0.05$). Finally, after chilling slaughterhouse C had higher counts than slaughterhouse A ($P<0.05$) and slaughterhouse B ($P<0.001$).

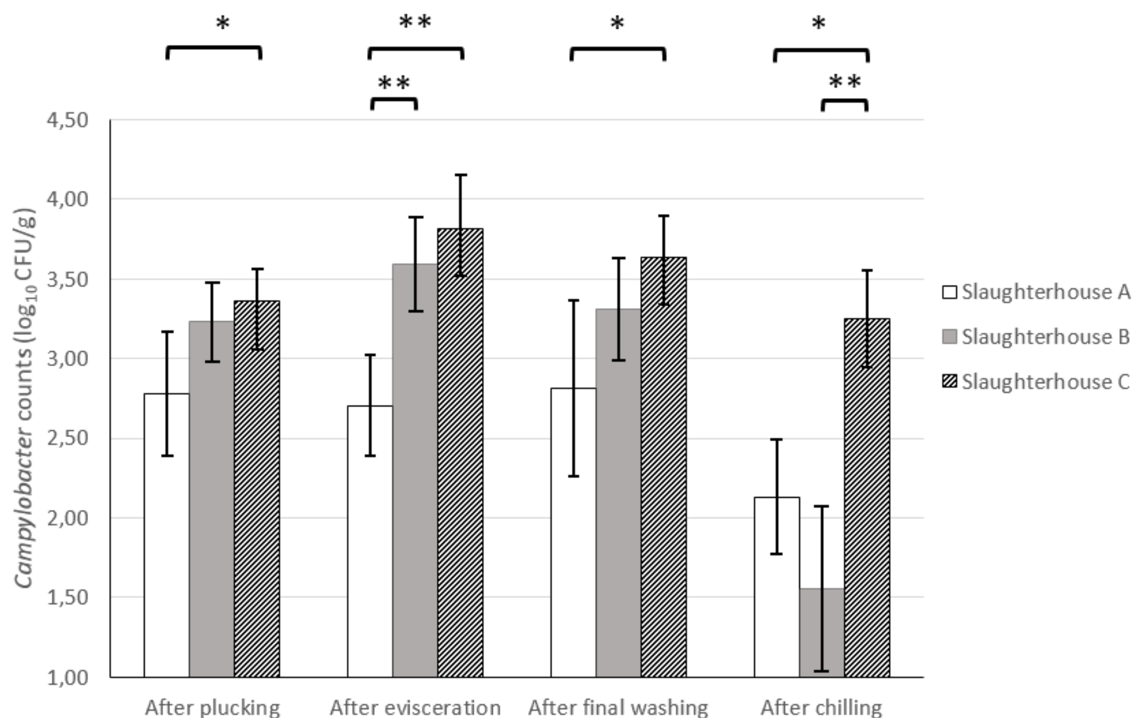


Figure 1. Comparison of the mean Campylobacter counts (log10 CFU/g ±SD) in 3 slaughterhouses for each of 4 tested steps during the slaughter process.
Significant differences of the mean count between slaughterhouses are indicated by an asterisk (equals* P<0.05*, ** equals* P<0.001*).*

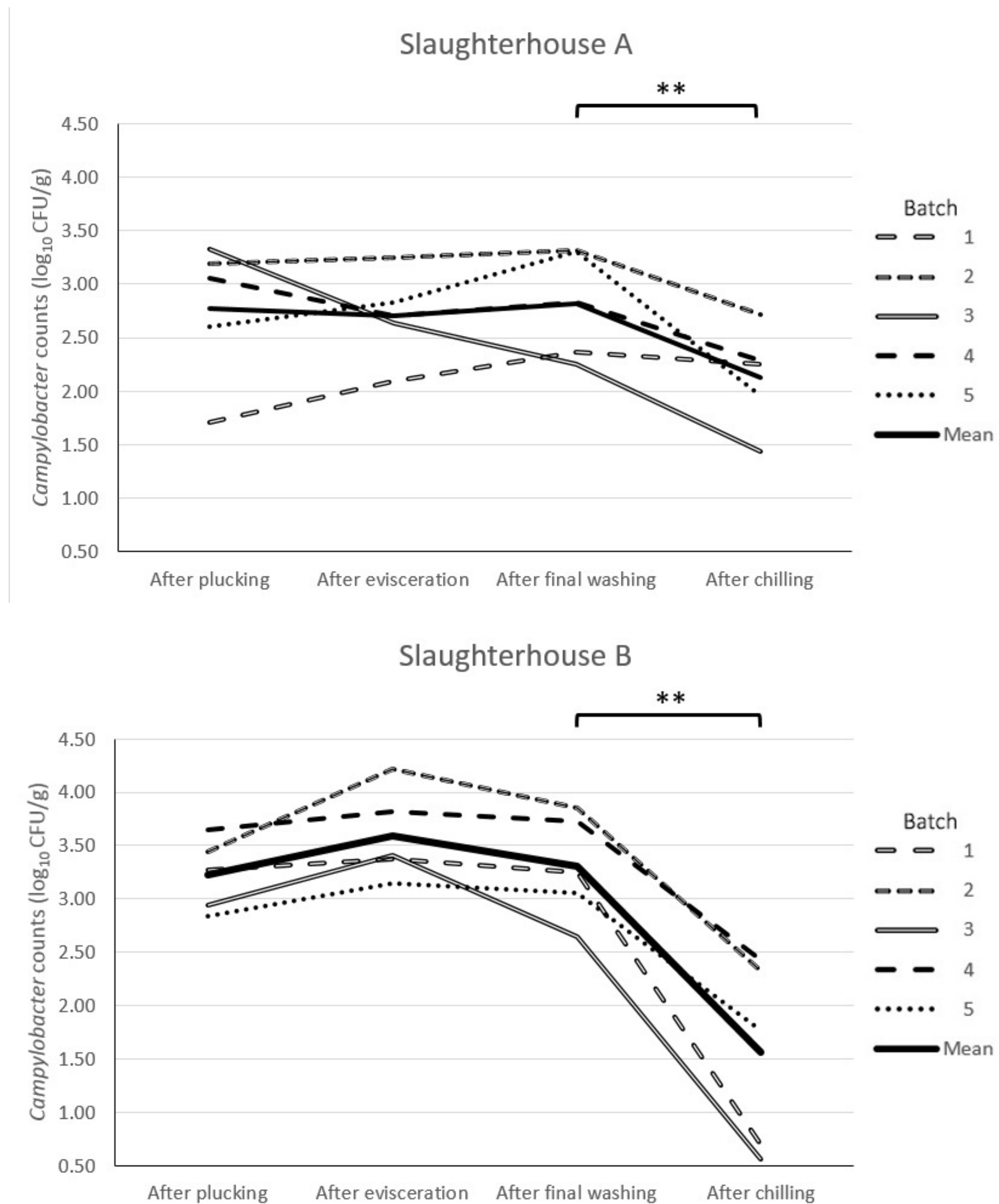
The mean counts per process step within each batch are shown for each slaughterhouse in figure 2. The mean *Campylobacter* counts and corresponding standard deviations within each step of the slaughter process per batch is given in the supplementary Table.

Campylobacter counts on carcasses within a batch generally variated considerably at the 4 tested processing steps (standard deviations ranged between 0.11 and 1.12 log_{10} CFU/g). Also the mean counts at the different slaughter steps for the 5 tested batches in each slaughterhouse showed a variation (Figure 1).

After plucking, the mean *Campylobacter* count was 2.81 log_{10} CFU/g, 3.23 log_{10} CFU/g and 3.36 log_{10} CFU/g breast skin, while after water chilling the mean counts were 2.16 log_{10} CFU/g, 1.77 log_{10} CFU/g and 3.25 log_{10} CFU/g in slaughterhouse A, B and C respectively. In slaughterhouse C, a significant increase of the mean *Campylobacter* count was observed after evisceration compared to after plucking step (0.46 log_{10} CFU/g; $CI_{95\%}$ [0.17; 0.74]; P<0.05), though this was not observed in the other slaughterhouses (P>0.05). Final washing had no significant effect on the contamination level of the carcasses in any of the 3 slaughterhouses (P>0.05). Water chilling led to a significant decrease of the mean count in all slaughterhouses. This step caused a mean

decrease of 0.71 log_{10} CFU/g ($CI_{95\%}$ [-1.09 ; -0.34]; P<0.05) and 0.39 log_{10} CFU/g ($CI_{95\%}$ [-0.67 ; -0.10]; P<0.05) in slaughterhouse A and C, respectively. In slaughterhouse B, chilling reduced the mean *Campylobacter* count by 1.59 log_{10} CFU/g ($CI_{95\%}$ [-1.86 ; -1.30]; P<0.001).

There was no significant relation between caecal counts and *Campylobacter* counts on carcasses after plucking (p = 0.110) nor after evisceration (p = 0.696).



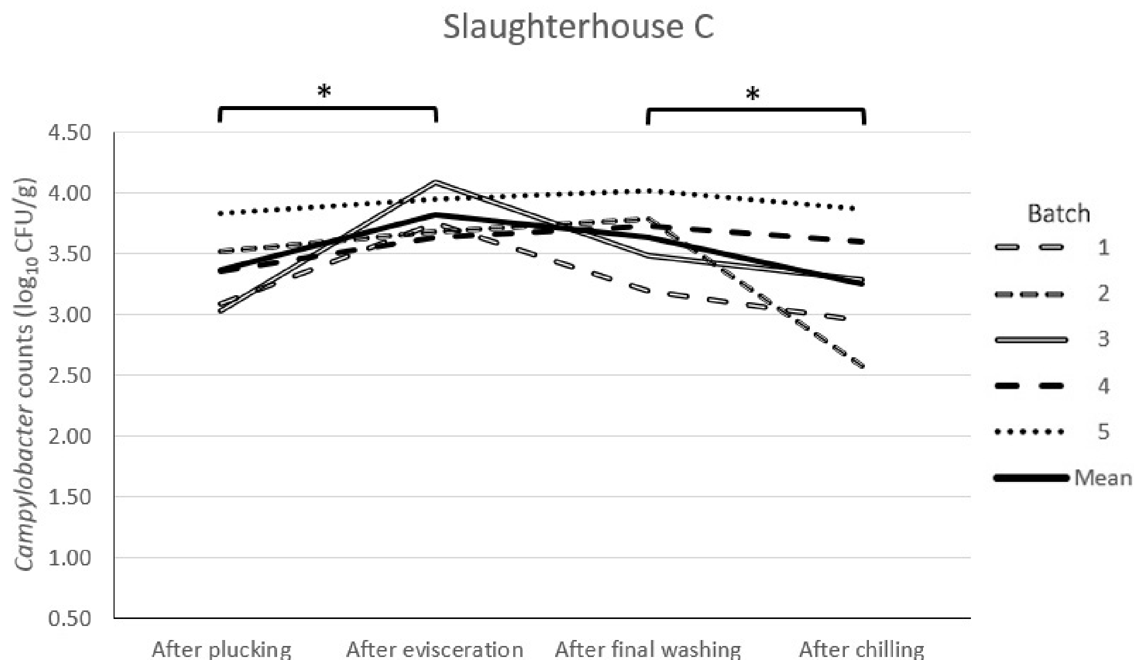


Figure 2. Campylobacter mean counts (log10 CFU/g) on carcass breast skin collected at 4 steps of the slaughter process in slaughterhouses A, B and C.
Significant differences of the mean count between 2 consecutive steps are indicated by an asterisk (equals* P<0.05*, ** equals* P<0.001*).*

4. Discussion

In this study we present results of *Campylobacter* quantification in 3 Ecuadorian slaughterhouses where critical steps as hanging, rehanging and evisceration are done manually. *Campylobacter* counts higher than 6 log_{10} CFU/g were found in pooled caecal content samples, which is consistent with values reported in other studies (Stern *et al*., 2005; Allen *et al*., 2007; Seliwiorstow *et al*., 2016).

After plucking the mean *Campylobacter* counts in the different slaughterhouses ranged from 2.81 up to 3.36 log_{10} CFU/g breast skin. Those levels are similar to those obtained in other studies (Seliwiorstow *et al*., 2015a)

Although evisceration has been described as a critical step for *Campylobacter* contamination of carcasses (Keener *et al*., 2004; Figueroa *et al*., 2009; EFSA, 2011), it has also been described that an increase is not always observed in this step (Rosenquist *et al*., 2006; Pacholewicz *et al*., 2015b). In our study, only slaughterhouse C showed a small but significant increase of *Campylobacter* counts after evisceration. This observation could indicate that intestinal leakage caused by manual evisceration in these slaughterhouses has a minimal contribution to *Campylobacter* contamination.

Final washing of the carcasses showed no significant decrease in *Campylobacter* loads. A possible explanation could be related to the use of water without disinfectants and an insufficient amount of water used in this slaughter step (Bashor *et al*., 2004). Nevertheless, a lack of effect of final washing before chilling has been observed even when chlorine is used in this washing (Berrang *et al*., 2007).

The reduction of *Campylobacter* counts after chilling in slaughterhouse C was less than in slaughterhouses A and B. In the former slaughterhouse no potable water was added during the chilling process, probably leading to a lower washing effect on the carcasses. In slaughterhouse A, a larger reduction of the mean *Campylobacter* count was observed. The reason for this reduction is unclear: the amount of drinking water added was not available and it is unknown what may be the effect of the low concentration of free chlorine (0.5 ppm) on the reduction of *Campylobacter* on broiler skin. The large reduction of the mean *Campylobacter* count in slaughterhouse B may be explained by the addition of a high concentration of chlorine (17-20 ppm) in the chilling water. Chlorine compounds can reduce the number of *Campylobacter* on carcasses up to 2.9 log_{10} CFU/g (Berrang *et al*., 2007; Berghaus *et al*., 2013; Duffy *et al*., 2014). Besides, the washing effect of the addition of drinking water in chilling tanks (three liter/carcass) may have decreased the amount of *Campylobacter* as shown elsewhere (Figueroa *et al*., 2009).

In the present study, intra-batch variation of *Campylobacter* counts was observed. This is in concordance with other studies that have shown similar results (Pacholewicz *et al*., 2015b; Seliwiorstow *et al*., 2015a). Concordantly, variability of *Campylobacter* counts among slaughterhouses within sampling steps was observed. This variation shows that some slaughterhouses are more able to control *Campylobacter* contamination levels during the slaughter of broilers than others, which has been described before (EFSA, 2010; Seliwiorstow *et al*., 2015a).

A positive correlation between *Campylobacter* counts of positive batches and counts on broiler carcasses at slaughterhouse has been reported (Berghaus *et al*., 2013). However, in this study such correlation was not observed. Factors as the variable number of visceral rupture and leakage during evisceration have been mentioned to explain the lack of association of *Campylobacter* counts in feces and carcasses (Allen *et al*., 2007). Further research including other steps of the slaughtering process should be done to understand this result.

Although the slaughterhouses studied are small in a global context, they are important for the Ecuadorian food industry since a considerable portion of chicken meat sold at retail comes from

these facilities. The same situation can be considered for other developing countries where high technology processes are not applied yet. In order to tackle the *Campylobacter* contamination of broiler carcasses at slaughterhouse level, global measures should be taken. Increased biosecurity at farm level, adoption of rational interventions with *Campylobacter* positive batches and hygienic slaughtering must be considered (Wagenaar *et al*., 2013).

Data provided in this research may contribute to the understanding of the impact of the slaughter process on the *Campylobacter* contamination level on broiler carcasses in developing countries. Moreover, this study will help national authorities and private companies to implement corrective measures at poultry slaughterhouses in order to obtain safer poultry meat for the Ecuadorian market.

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Supplementary table

***Campylobacter* counts and standard deviation in caeca and different steps during the slaughter process**

Batch	Caeca	After plucking	After evisceration	After final washing	After chilling
			Slaughterhouse A		
1	9.91	1,71 ± 1.07	2.09 ± 0.37	2.36 ± 1.41	2.25 ± 0.44
2	10.25	3,2 ± 0,12	3.25 ± 0.58	3.31 ± 0.15	2.72 ± 0.28
3	6.94	3,32 ± 0,40	2.64 ± 0.44	2.26 ± 0.35	1.43 ± 0.81
4	9.44	3,06 ± 0,38	2.71 ± 0.43	2.83 ± 1.89	2.29 ± 0.67
5	10.03	2,61 ± 0,27	2.83 ± 0.80	3.31 ± 0.55	1.96 ± 0.78
Mean	9.32 ± 1.36	2.78 ± 0,78	2.7 ± 0.63	2.81 ± 1.1	2.13 ± 0.72
			Slaughterhouse B		
1	7.51	3.27 ± 0.61	3.38 ± 0.17	3.25 ± 0.19	0.70 ± 0.32
2	10.48	3.45 ± 0.18	4.22 ± 0.90	3.86 ± 0.57	2.32 ± 0.89
3	6.20	2.94 ± 0.21	3.41 ± 0.29	2.65 ± 0.55	0.56 ± 0.81
4	7.64	3.65 ± 0.63	3.82 ± 0.32	3.72 ± 0.57	2.43 ± 0.20
5	9.59	2.84 ± 0.28	3.15 ± 0.43	3.06 ± 0.51	1.77 ± 0.33
Mean	8.28 ± 1.72	3.23 ± 0.5	3.59 ± 0.59	3.31 ± 0.64	1.56 ± 1.04
			Slaughterhouse C		
1	11.12	3.08 ± 0,28	3.74 ± 0.12	3.19 ± 0.39	2.94 ± 0.28
2	10.11	3.51 ± 0,13	3.67 ± 0.43	3.79 ± 0.11	2.57 ± 0.35
3	10.09	3.02 ± 0,34	4.09 ± 0.95	3.47 ± 0.65	3.28 ± 0.67
4	10.21	3.35 ± 0,26	3.63 ± 0.37	3.72 ± 0.61	3.59 ± 0.25
5	10.20	3.83 ± 0,40	3.95 ± 1.12	4.02 ± 0.41	3.87 ± 0.37
Mean	10.35 ± 0,44	3.36 ± 0,41	3.82 ± 0.67	3.64 ± 0.52	3.25 ± 0.60

GENERAL DISCUSSION

1. General situation in Ecuador

Campylobacter and *Salmonella* are important causes of human gastroenteritis worldwide. Nonetheless, the diseases caused by these bacteria have been poorly studied and reported in Ecuador. The Ecuadorian Ministry of Health releases a weekly bulletin with information about the most important diseases such as data on foodborne *Salmonella* cases in Ecuador (Ministerio de Salud Pública del Ecuador, 2016). However, these data are not based on an active surveillance program but on reports passively generated by hospitals. Additionally, *Salmonella* serotypes and results of other typing methods are not reported in official documents and are scarcely carried out. Besides, infections caused by *Campylobacter* are not reported in these documents since screening of *Campylobacter* is not mandatory in hospitals. Antimicrobial resistance tests are carried out on *Salmonella* isolates from humans and in some cases from animals. Further research is needed to put that data in an epidemiological context (Reyes, personal communication).

A similar situation is presented in the husbandry sector, where public policies on the surveillance of foodborne bacteria and their antimicrobial resistance are not clear or cannot be implemented due to shortage of funds.

In Ecuador, as in other American countries, antibiotics are largely used in the commercial rearing of broilers. For example, quinolones are commonly used in the first week of life of broilers as prophylactics (Vinueza-Burgos. unpublished data). Like in many countries, antibiotics are used as growth promotors in the production of broilers (Hao *et al*., 2014; Kempf *et al*., 2016). In Ecuador, the prescription of antibiotics for animal use is not necessarily mediated by a veterinarian. Indeed, antibiotics can be bought freely from veterinary stores. This has led to an indiscriminate use of antibiotics in food animals, which has been recognized as a factor that greatly drives the emergence of ARB (WHO, 2016). Besides, surveillance of ARB in the animal and food sector in Ecuador is weakly executed.

Some studies aiming to describe *Salmonella* and *Campylobacter* in backyard poultry and other food animals in Ecuador have been done (Vasco *et al*., 2014; Escudero and Casierra, 2015; Graham *et al*., 2016). However, this thesis is the first attempt to understand the epidemiology of these bacteria in industrial poultry from Ecuador.

2. *Salmonella* and *Campylobacter* in broilers

The prevalence of *Salmonella* and *Campylobacter* in broilers at slaughter age has been presented in Chapter 1 and Chapter 2. Despite the importance of these pathogens, data about their presence in poultry farms is only available from a limited number of Latin American countries. Data indicate that the *Salmonella* and *Campylobacter* prevalence in the primary sector of these countries ranges widely (Table 1).

Table 1. Prevalence of *Salmonella* and *Campylobacter* in poultry farms in Latin American countries.

Country	*Salmonella*	Reference	*Campylobacter*	Reference
Peru	48.7%	SENASA, 2015	35.0%	Tresierra-Ayala *et al*., 1995
Argentina	45.0%	Bueno and Soria, 2016	33.0%	Zbrun *et al*., 2013
Brazil	5.4%	Voss-Rech *et al*, 2015	100%	Giombelli and Abreu, 2014
Ecuador	16.0%	Vinueza-Burgos *et al*., 2016	64.1%	Vinueza-Burgos *et al*., 2017
Venezuela	23.0%	Boscan-Duque *et al*., 2007		
Colombia	65.0%	Donado-Godoy *et al*, 2012		
Chile			34.0%	Rivera *et al*., 2011
Costa Rica			80.0%	Zumbaco-Gutiérrez *et al*., 2014

The epidemiology of non-typhoidal *Salmonella* in poultry at the farm level has been extensively studied in the developed world (Cosby *et al*., 2015; Chousalkar and Gole, 2016). From the more than 2500 serotypes of non-typhoidal *Salmonella* reported worldwide, *S.* Enteritidis and *S.* Typhimurium are the most important ones resulting in legislation and control for both serotypes in many countries (WHO, 2015b). In Latin American countries a variety of *Salmonella* serotypes have been found. For example, in broiler farms of Colombia and Venezuela *S.* Paratyphi B variant Java and *S.* Heidelberg have been mostly identified (Boscán-Duque *et al*., 2007; Donado-Godoy *et al*., 2012c) while in Brazil *S*. Enteritidis, *S.* Infantis, *S.* Typhimurium and *S.* Heidelberg were reported as the most prevalent at farm level (Medeiros et al., 2011). These studies report other *Salmonella* serotypes in lower proportions.

In **Chapter 1** we not only reported a narrower variety of *Salmonella* serotypes (only *S*. Infantis, *S*. Enteritidis and *S*. Corvallis) but also that *S.* Infantis was by far the most prevalent one (83.9%). This is a novel issue in the region since *S.* Infantis has been reported with lower prevalence rates in commercial poultry from Brazil (ranging from 0.6-7.6%) but not in other Latin American

countries (Medeiros *et al*., 2011; Pulido-Landínez *et al*., 2013). However, in Peru 90% of *Salmonella* isolates at farm level have been identified as *S.* Infantis (SENASA, 2015). Considering that trade of poultry (broilers and hatching eggs) in the border with Peru is more important than the one with Colombia, these findings could be related to our results. Additionally, we identified one *S*. Infantis isolate (n=21) in one-day old chickens from an integrated company whose PFGE genotype was found during the rearing and slaughter of those broilers (Vinueza-Burgos, unpublished data). Therefore, it would be probable that the presence of *Salmonella* in broilers at slaughter age may have an origin in earlier stages of the production chain (breeders or hatcheries), but more research is needed to confirm this hypothesis.

In Ecuador, epidemiological data about the role of *S.* Infantis in human cases of salmonellosis are not available. However, there are sporadic notifications of *S.* Infantis implicated on foodborne salmonellosis (Zurita, Personal communication) which indicates that further research is needed to understand the epidemiology of this serotype in Ecuador.

A similar situation for *Campylobacter* was described in **Chapter 2.** Here, *C. coli* was the most prevalent species (81.4%) which is different of what has been reported in other Latin American countries where *C. jejuni* prevails at farm level (Tresierra-Ayala *et al*., 1995; Rivera *et al*., 2011; Zbrun *et al*., 2013b; Zumbaco-Gutiérrez *et al*., 2014a; Giombelli and Abreu, 2014).

The explanation of the differences in both prevalence and serotypes/species in the studied pathogens is not straightforward. Therefore, a sampling representing other Ecuadorian regions would give a broader picture of these bacteria. However, it must to be taken into account that the current sampling included farms from 5 climatic zones representing 36% of Ecuadorian poultry production. Consequently, the contribution of a bigger sampling to new insights on the epidemiology of these bacteria could be limited.

Another implicated factor could be the different methodologies applied to detect and to identify these pathogens. For instance, *Campylobacter* detection has been carried out with techniques that include enrichment (Zbrun *et al*., 2013b), direct plating (Silva *et al*., 2016) and DNA screening (Giombelli and Abreu, 2014), while the identification has been done with phenotypic (Simaluiza *et al*., 2015) and DNA-based methods (Graham *et al*., 2016). In the same way, *Salmonella* detection and characterization has been carried out with different phenotypic and genotypic approaches (Góis *et al*., 2015; Jarquin *et al*., 2015; Donado-Godoy et al., 2015). Additionally, it has to be considered that countries in South America have a large variety of climatic conditions. The presence of four seasons (Argentina, Chile), one season (Brazil and

Ecuador) along with micro-climate conditions in all the Andes region could also determine differences in the epidemiology of *Salmonella* and *Campylobacter.*

All these variations within the research methodologies and conditions makes the direct comparison of results difficult and could have influenced *Salmonella* and *Campylobacter* prevalence data in the region. However, outcomes presented in this thesis proved the importance of these pathogens in the Ecuadorian poultry sector.

Beyond the prevalence of *Salmonella* and *Campylobacter*, a number of genotypes of both pathogens seemed to be widespread in poultry farms of different integrated companies (**Chapter 1** and **Chapter 2**), which is in accordance with previous studies (Allen *et al*., 2007; Campioni *et al*., 2014). Several risk factors for the contamination of broiler with these pathogens at farms are described and are mainly linked to the implementation of biosecurity measures (Newell *et al*., 2011; EFSA, 2011; Berghaus *et al*., 2012). As it has been mentioned in this thesis, due to climatic conditions, Ecuadorian poultry houses are setup with an open configuration. In these kind of buildings, implementation of rigorous biosecurity is difficult. Additionally, some services as delivery of fuels and removal of litter are offered by companies that operate in different integrations which might lead to a carriage of bacterial strains from one farm/PIC to another.

The information shown in these chapters highlights the necessity of the implementation of a stricter biosecurity at farm level. Actions like better cleaning and disinfection of poultry houses, pest control and hygienic management of farms should be implemented in order to decrease the contamination of *Salmonella* and *Campylobacter* in the long term (Rose *et al*., 2000; Newell *et al*., 2011; Bahrndorff *et al*., 2013). For example, the implementation of flies screens and control of rats have demonstrated to be useful since flies and rodents have been shown to be carriers of these pathogens (Bahrndorff *et al*., 2013; Sandberg *et al*., 2015). These animals could be a source of contamination even when a broiler house has been correctly disinfected. Additionally, control of *Salmonella* in breeders and hatcheries should be addressed in order to avoid the possibility of recontamination of farms through one-day old chickens.

3. Antimicrobial resistance of *Salmonella* and *Campylobacter*.

The appearance of multidrug resistant bacteria (MRB) in the food chain is of global concern (WHO, 2015e, 2016). The diseases and complications caused by *Salmonella* and *Campylobacter* would be worse if, in the case that antibiotics are needed, they would not have the expected

effect. In our study we demonstrated that both *Campylobacter* and *Salmonella* presented high antimicrobial resistant phenotypes (**Chapter 1** and **Chapter 2**).

In the case of *Salmonella* most of isolates were resistant to more than 3 classes of antibiotics. Colistin and ceftazidime were the antibiotics with lowest resistance rates. In general, *S*. Enteritidis showed less multiresistant phenotypes than *S*. Infantis which has also been seen in the EU (EFSA and ECDC, 2016). Although *S.* Enteritidis was 100% resistant to colistin, we did not find the *mcr-1* gene in these strains. It has been proposed that *S.* Enteritidis is constitutively resistant to colistin without harboring a related plasmidic gene (Agersø *et al*., 2012) which is consistent with our findings.

Regarding to *Campylobacter* strains, they presented high resistance rates for (fluoro)quinolones and tetracycline while for other antibiotics as streptomycin and erythromycin resistance rates ranging from 4.2% to 25.9% were found. Multiresistant phenotypes were also evident among *Campylobacter* strains since 24.7% of *C. coli* and 8.4% of *C. jejuni* presented resistant phenotypes to more than 2 groups of antibiotics. The presence of *Campylobacter* phenotypes highly resistant to nalidixic acid, ciprofloxacin and in a lower extend to tetracycline, is not a new phenomenon and has been reported worldwide (Gyles, 2008; Dallal *et al*., 2010; Fraqueza *et al*., 2014). Moreover, our results are in accordance with the ones presented in Brazil and Chile (Rivera *et al*., 2011; Ferro *et al*., 2015).

It has been pointed out that the absence of clear legislative frameworks on the usage of antimicrobials in livestock may result in its irrational consumption (Van Boeckel *et al*., 2015). This is the case of Ecuador, where the extensive use of antimicrobials in the poultry production may have led to the resistance presented in this thesis. As already mentioned in the introduction, there were no data available regarding the use of antibiotics in the poultry sector at the beginning of this research. Therefore, from each of the sampled batches, data were collected about the moment and the types of antibiotics used. Obtained results showed that antibiotics are used as growth promotors, prophylactic treatments and curative treatments in 43.8%, 73.2% and 55.9% of sampled batches, respectively. The most commonly used drugs were bacitracin and halquinol as growth promotors; enrofloxacin, tiamulin and tylosin in prophylactic treatments and; tilmicosin, fosfomycin and florfenicol in curative treatments (Table 2). In some batches, more than one antibiotic were used as growth promotor whereas a number of batches received different curative treatments.

Table 2 Antimicrobials used in sampled batches (n = 388).

	Growth promoter (%)	Prophylactic (%)	Curative (%)
Number of treated batches (%)	170 (43.8%)	284 (73.2%)	217 (55.9%)
Antibiotics used			
Enrofloxacin		193 (67.9%)	26 (11.9%)
Tiamulin		29 (10.2%)	
Tylosin		23 (8.1%)	
Tilmicosin		19 (6.7%)	122 (56.2%)
Fosfomycin		12 (4.2%)	89 (41.0%)
Ciprofloxacin		3 (1.1%)	13 (5.9%)
Doxycycline		2 (0.7%)	36 (16.6%)
Florfenicol		2 (0.7%)	78 (35.9%)
Norfloxacin		2 (0.7%)	34 (15.7%)
Sulfamethoxazole + trimethoprim			19 (8.8%)
Neomycin			1 (0.5%)
Bacitracin	107 (62.9%)		
Halquinol	85 (50.0%)		
Virginiamycin	14 (8.2%)		
Avilamycin	6 (3.5%)		
Salinomycin	6 (3.5%)		
Olaquindox	4 (2.3%)		
Gentamicin	2 (1.2%)		
Flavomycin	1 (0.6%)		
Vancomycin	1 (0.6%)		

The common use of fluoroquinolones and fosfomycin in batches could be related to the high resistance rates to these drugs found in the present study. On the other hand, other antibiotics, such as tetracycline and sulfamethoxazole+trimethoprim are now rarely used in broiler production since these antimicrobials do not have the expected effect in the field anymore (López, personal communication), an assumption that is supported by our findings. Factors driving to the antimicrobial resistance towards other antibiotics such as cephalosporins and aminoglycosides should be further studied. However, a link to other aspects like the overuse of antibiotics in other food animals and humans, and the presence of antibiotic residues in the environment should not be discarded (Andersson and Hughes, 2014).

It has been seen that actions aiming to rationalize the use of antibiotics in food animals lead to a decrease of the prevalence of ARB in developed countries (Johnsen *et al*., 2009; Wegener, 2012). Thus, the implementation of measures to reduce the selective pressure of ARB should be placed in the Ecuadorian poultry sector.

Data about antibiotic resistance of *Salmonella* and *Campylobacter* reported in this thesis may convince public authorities and the private sector to generate strategies to tackle ARB in Ecuadorian broiler farms.

It is worth to mention that in order to determine resistant phenotypes we used epidemiological cutoffs (ECOFFs) from EUCAST (EUCAST, 2016a). These values make sense in an epidemiological context since ECOFFs are not intended to assess the concentration of an antibiotic at which an isolate is inhibited in a host. Instead, ECOFFs evidence when a non-resistant bacteria (wild type) has acquired resistance determinants (EUCAST, 2016d). Yet, many studies on antimicrobial resistance in *Salmonella* and *Campylobacter* use clinical breakpoints (commonly from CLSI) to interpreted results. This is understandable when there are not enough reference values from EUCAST (e.g. some breakpoints for disk diffusion tests). However, the dissimilitude of reference values can result in biased overviews when analyzing epidemiological or clinical data (Hombach *et al*., 2013; Wolfensberger *et al*., 2013). A global effort to standardize these aspects should lead to protocols for the research of ARB without ambiguities.

4. *Salmonella* and *Campylobacter* contamination in slaughterhouse.

While it is true that *Salmonella* and *Campylobacter* contamination at farm level can influence the exposure risk for consumers to these pathogens, the role of slaughtering in the microbiological quality of chicken carcasses is crucial (Heyndrickx *et al*., 2002; Berrang *et al*., 2007). Therefore, in **Chapter 3** we tried to evaluate the dynamics of *Campylobacter* contamination during the slaughter process in representative slaughterhouses of Ecuador. In the three selected slaughterhouses, some processes were carried out manually which differs from large slaughterhouses in developed countries. Another characteristic of the studied slaughterhouses was the addition of different levels of chlorine to the chilling water. In the Ecuadorian context, this is a cheap and feasible way to improve the microbiological quality of chicken carcasses. However, we could evidence a lack of standardization when applying chlorine and in the measurement of chlorine concentrations in chilling water.

In Ecuador, there are norms on the slaughter processes in Ecuadorian slaughterhouses (JMG, 1964). However, there are some gaps concerning to the microbiological control in the processing of broilers. By assessing *Campylobacter* counts in four steps of the slaughter line we evaluated three processes: 1) manual evisceration, 2) final showers and 3) water chilling.

Excluding manual evisceration, that did not significantly increase *Campylobacter* counts (with exception of slaughterhouse C), we could propose some actions in the other two steps. Final

showers could be adjusted to work at a higher pressure and/or large amount of water in order to reduce *Campylobacter* contamination on carcasses, as has been described before (Bashor et al., 2004). Additionally, it has been shown that the implementation of sprays with chlorinated water can effectively reduce bacterial loads on chicken carcasses (Pissol *et al*., 2013). Regarding water chilling, our results demonstrated that in the slaughterhouse that used a higher level of chlorine a mean *Campylobacter* reduction of 1.75 log_{10} CFU/g was achieved, which is consistent with the findings of Demirok *et al*. (2013). In contrast, a study carried out in Belgian slaughterhouses demonstrated that air chilling of broiler carcasses did not lead to a significant reduction of *Campylobacter* counts (Seliwiorstow *et al*., 2015). However, when comparing the *Campylobacter* counts after evisceration and after chilling, the counts were significantly lower after the latter step for certain batches, which was supposed to be partially attributed to the effective implementation of a final washing step (Pacholewicz *et al*., 2015; Seliwiorstow *et al*., 2015). These factors should be considered when comparing results from Ecuador and the ones from industrialized countries, where chlorine is not allowed in the chilling step.

Furthermore, other compounds as paracetic acid and cetylpyridinium chloride allow an effective reduction of *Campylobacter* counts on chicken carcasses (Bauermeister *et al*., 2008; Wideman *et al*., 2016) and could be used in Ecuador. However, their price could limit the use of these disinfectants especially in small slaughterhouses.

It is worth to mention that correlation between *Campylobacter* counts and loads of other bacteria, like *E. coli* has been reported (Pacholewicz *et al*., 2015b). This finding supposes that it may be possible to follow up the *Campylobacter* contamination during the slaughter of *Campylobacter* positive flocks by the quantification of *E. coli*. However, up to now it is unknown if water chilling has the same effect on *Campylobacter* and *E. coli* counts.

We have seen that carcasses originating from positive and negative *Salmonella* broiler batches were *Salmonella* positive after chilling in the 3 slaughterhouses (Vinueza-Burgos, unpublished data). These findings indicate that cross contamination may occur during the slaughter process. Moreover, also carcasses chilled in water containing a high concentration of chlorine (slaughterhouse B) were positive. This suggests that, in contrast to the large reduction observed for *Campylobacter*, the reduction rate for *Salmonella* may be lower so that a total elimination is not obtained. Further research is needed to evaluate the dynamics of *Salmonella* contamination in broiler slaughterhouses.

Finally, it must to be considered that most of the broilers consumed in Ecuador are slaughtered in “informal” slaughterhouses, which lack hygienic procedures and are not sufficiently controlled by local authorities. Poultry meat released to the market from these sites could have higher bacterial loads as demonstrated previously by Donado-Godoy *et al*. (2012). Efforts directed to study and improve the microbiological quality of poultry meat in Ecuador should also consider this section of the market to address the production of safe poultry meat.

Conclusions

In this thesis we have reported for first time the prevalence and antimicrobial resistance data for *Campylobacter* and *Salmonella* in Ecuadorian broilers at slaughter age and *Campylobacter* loads on carcasses in Ecuadorian slaughterhouses. The observed prevalences are comparable with the ones in other developed and developing countries but some peculiarities can be highlighted.

The predominant presence of *S*. Infantis is outstanding since this serotype is not the most prevalent one reported in Latin America. In the same way, *C. coli* was the most prevalent *Campylobacter* species which differs from reports of other countries in the region. The reason of why *S*. Infantis and *C. coli* are dominant in Ecuadorian poultry farms needs further research.

Salmonella and *Campylobacter* genotypes also demonstrated to be widely distributed in poultry farms and even in different PICs which could be explained by defects in the implementation of biosecurity strategies in the poultry sector.

We found high rates of antimicrobial resistance and multiresistant phenotypes in both *Campylobacter* and *Salmonella* isolates. Unfortunately, the antibiotics affected by these resistances are among those of critical use in humans. These findings could represent a human health concern if these bacteria are able to survive in the chicken carcasses sold at retail.

Finally, we showed that some processes at the slaughtering of broilers are critical for contamination/decontamination of broiler carcasses. Corrections and improvements in final showers and chlorinated chilling water could lead to lower bacterial loads in chicken carcasses.

6. Further perspectives

In Ecuador epidemiological information on foodborne bacteria and antimicrobial resistance in the food production chain is very scare. So further research is needed to better understand the impact of broiler meat on human health and to support the food sector and the government in the development of efficient intervention measures and controls in order to produce safe meat.

Therefore, I would like to focus future research on the following subjects:

- The study of foodborne bacteria at retail level. Since the commercialization of chicken meat occurs by supermarkets and open markets, both sectors should be covered.
- Characterization of *Salmonella* on different stages in the production of broilers to elucidate a possible explanation of why *S*. Infantis is predominant in Ecuadorian poultry.
- ESBL bacteria in poultry and poultry meat
- Since the Galapagos Islands are a different ecological niche, it may be interesting to investigate *Campylobacter*, *Salmonella* and *E. coli* ESBL in poultry reared on these islands.
- The microbiological loads and antimicrobial resistances of foodborne bacteria in other food animals (e.g. cattle, pigs) at the primary sector, transformation and retail level.

In order to focus on the mentioned subjects within the Faculty of Veterinary of the Central University of Ecuador, the 'Unity for Research of Foodborne Diseases and Antimicrobial Resistance' (UNIETAR for its acronym in Spanish) was recently created.

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SUMMARY

Salmonella and *Campylobacter* are major foodborne pathogens worldwide and are especially prevalent in the poultry meat chain. Their epidemiology has been extensively studied in developed countries but there is a gap of knowledge about these pathogens in many countries of Latin America. Although poultry meat is a principal component of the diet in Ecuador, little is known about the presence of *Campylobacter* and *Salmonella* in this food chain. Moreover, the antimicrobial resistance of these bacteria has been scarcely studied in the poultry sector of this country. Climatic conditions in Ecuador lead to poultry production systems which make the implementation of strict biosecurity protocols difficult (e.g. open broiler houses). Additionally, the use of antibiotics as growth promotors and prophylactics is a common practice.

In the introduction of this thesis, generalities of *Salmonella* and *Campylobacter* and the techniques and concepts used for isolation and characterization of these pathogens are described.

The aim of this thesis was to obtain data about *Campylobacter* and *Salmonella* in the broiler meat chain in Ecuador. To fulfill this objective, we assessed the prevalence and characterized these pathogens in broiler batches at slaughter age and studied the contamination dynamics during the slaughter of *Campylobacter* positive batches in commercial slaughterhouses. Therefore, six slaughterhouses located in the province of Pichincha were involved. In a first stage, caeca were collected from broiler batches that originated in 119 farms at slaughterhouse level during one year.

Chapter 1 focusses on *Salmonella*, for which samples from 388 randomly selected broiler batches were collected. These samples were analyzed by the ISO 6579/Amd1 protocol for the isolation of *Salmonella*. Isolates were further characterized by pulsed field gel electrophoresis (PFGE). Antimicrobial resistance was assessed by MIC determination using broth dilution for sulfamethoxazole, gentamicin, ciprofloxacin, ampicillin, cefotaxime, ceftazidime, tetracycline, streptomycin, trimethoprim, chloramphenicol, colistin, florfenicol, kanamycin and nalidixic acid. Finally, the presence of β-lactam genes ($\mathit{bla}_{\text{CTX-M}}$, $\mathit{bla}_{\text{TEM}}$, $\mathit{bla}_{\text{SHV}}$ and $\mathit{bla}_{\text{CMY}}$) and the colistin-resistance plasmidic gene *mcr-1* were investigated by PCR. *Salmonella* prevalence at batch level was 16.0%. The most common serotype was *S*. Infantis (83.9%) followed by *S*. Enteritidis (14.5%) and *S*. Corvallis (1.6%). *S*. Infantis isolates showed high resistance rates to 12 antibiotics ranging from 57.7% (kanamycin) up to 98.1% (nalidixic acid and sulfamethoxazole). Resistance to colistin was present in all *S*. Enteritidis isolates and one *S*. Infantis isolate, though all isolates were *mcr-1* negative. The PFGE analysis showed that *S*. Infantis had a highly clonal structure among strains and that some *Salmonella* genotypes were widely distributed in farms.

In order to determine the prevalence, antimicrobial resistance and characterization of *Campylobacter* described in **Chapter 2,** caecal content samples from 379 randomly selected broiler batches were analyzed. Microbiological isolation was performed by direct plating on mCCDA agar and confirmed by PCR. MIC values for gentamicin, ciprofloxacin, nalidixic acid, tetracycline, streptomycin and erythromycin were obtained. In order to assess the genetic variation of the isolates, two approaches were applied: 1) RFLP-flaA typing and 2) Multilocus Sequence Typing (MLST) of selected isolates. The prevalence of *Campylobacter* at batch level was 64.1%. Positive batches delivered *C. coli* (68.7%), *C. jejuni* (18.9%) and *C. coli* and *C. jejuni* mixed isolates (12.4%). MIC values showed resistance rates above 67% for tetracycline, ciprofloxacin and nalidixic acid while the resistance pattern tetracycline, ciprofloxacin and nalidixic acid was the dominant one in both *Campylobacter* species. MLST typing revealed that, with exception of one strain, *C. coli* belonged to clonal complex (CC) CC-828, while *C. jejuni*, with the exception of 2 strains, belonged to 12 CCs. Furthermore, 2 new sequence types (STs) within each species were described (2 new STs for *C. coli* were based on new alleles). When combining these results with RFLP-flaA types, additional profiles were identified. However, no association of RFLP-*flaA* profiles within STs was found regarding the origin of the isolates. These findings demonstrate the wide distribution of *Campylobacter* genotypes among sampled farms.

In a second stage of this thesis, described in **Chapter 3,** the dynamics of *Campylobacter* contamination were studied in three representative slaughterhouses. In these slaughterhouses, several processes (e.g. hanging, evisceration) were carried out manually and water was used in the chilling step. The impact of evisceration, final washing and water chilling was evaluated by quantifying *Campylobacter* contamination on carcasses at different steps of the slaughtering process. Five *Campylobacter* positive batches were followed in every slaughterhouse. For every batch, caecal content and 5 samples of breast skin were taken and exanimated for *Campylobacter* counts after plucking, after evisceration, after final washing and after water chilling. A small but significant increase of *Campylobacter* counts was detected after evisceration in one slaughterhouse only. No significant differences were found between *Campylobacter* counts after evisceration and after final washing (*P>0.05*), denoting a lack of efficiency of this step. In all slaughterhouses, a significant reduction of *Campylobacter* counts (0.13 to 2.13 log_{10} CFU/g) was found after the chilling step, presumptively due to the presence of chlorine in the chilling water or to the washing effect in this step. Additionally, high variability of *Campylobacter* counts was found within and between batches of the same slaughterhouse.

In the general discussion, the outcomes of the different studies are reviewed, with especial attention to the particular situation of Ecuador and countries in the region. Regarding *Salmonella*, we reported not only a narrower variety of *Salmonella* serotypes but also that *S.* Infantis was by far the most prevalent one. This differs to what has been reported in other Latin American countries where more serotypes have been found and other serotypes are dominant. The same observation was stated for *Campylobacter* since, unlike other Latin American countries, *C. coli* was the most prevalent species. Differences of prevalence data could be influenced by the application of different methodologies when isolating *Campylobacter*. However, outcomes presented in this thesis proved the importance of both pathogens in Ecuadorian poultry.

Moreover, high resistance rates to antibiotics used in human medicine were found in these pathogens. This could be provoked by the extensive use of antimicrobials in poultry production and might pose a human health concern. The attribution of *S*. Infantis to human salmonellosis has been reported unofficially, though there are no data of human campylobacteriosis in Ecuador. Further research is needed to elucidate the high prevalence of *S*. Infantis and *C. coli* found in this study and their role in human infections.

Characterization of *Salmonella* and *Campylobacter* by genetic methods showed that genotypes of both pathogens are widely distributed in broiler farms. This distribution could be attributed to ineffective biosecurity on farms. In this case improvement of biosecurity measures is recommended in order to decrease the contamination of farms in a long term.

Evaluation of *Campylobacter* counts during different processing steps at slaughterhouses showed that final washing and addition of chlorine in chilling water may be used to decrease *Campylobacter* numbers on carcasses, though should be improved in certain slaughterhouses. Actions aiming to monitor and standardize these processes are essential for decreasing bacterial loads on chicken carcasses. This is of special relevance if we take into account that cross contamination is likely to occur in the studied slaughterhouses. More research is needed to establish the dynamics of other pathogens like *Salmonella* in these slaughterhouses.

This thesis represents the first study on the epidemiology of *Campylobacter* and *Salmonella* in the poultry meat chain in Ecuador. Further projects are suggested to understand the extent of foodborne bacteria and their antimicrobial resistance in the food chain, and their role in public health in Ecuador.

SAMENVATTING

Salmonella en *Campylobacter* zijn wereldwijd belangrijke voedselpathogenen en zijn frequent aanwezig in de pluimveevleesketen. Hun epidemiologie werd uitgebreid bestudeerd in ontwikkelde landen, maar in veel Zuid-Amerikaanse landen is er een gebrek aan kennis omtrent deze pathogenen. Niettegenstaande pluimveevlees een belangrijk onderdeel van het dieet is in Ecuador, is er weinig kennis beschikbaar omtrent het voorkomen van *Campylobacter* en *Salmonella* in deze voedselketen. Bovendien is de antimicrobiële resistentie van deze bacteriën nauwelijks bestudeerd in pluimveevlees geproduceerd in dit land. Klimatologische omstandigheden in Ecuador hebben geleid tot productiesystemen waarbij toepassing van strikte bioveiligheidsmaatregelen bemoeilijkt worden (o.a. open pluimveestallen). Bovendien worden antibiotica als groeipromotor en profylactie frequent gebruikt.

In de inleiding van deze thesis wordt een overzicht gegeven omtrent de problematiek van *Salmonella* en *Campylobacter* en de methoden voor de isolatie en karakterisatie van de pathogenen.

De doelstelling van deze thesis was gegevens te verzamelen omtrent *Campylobacter* en *Salmonella* in de productieketen van braadkipvlees. Daartoe werd de prevalentie in braadkiptomen op het moment van slachting bepaald, de bekomen isolaten werden gekarakteriseerd en de *Campylobacter* contaminatie tijdens het slachten van positieve tomen in commerciële slachthuizen werd onderzocht.

Voor dit onderzoek werden monsters verzameld in 6 slachthuizen gelegen in de provincie Pichincha. In de eerste fase werd gedurende één jaar ceaca van braadkiptomen, afkomstig van 119 vleeskippenbedrijven, tijdens het slachten verzameld.

Hoofdstuk 1 handelt over het onderzoek omtrent *Salmonella*. In totaal werden van 388 at random geselecteerde braadkiptomen monsters verzameld. De monsters werden onderzocht gebruik makend van de ISO 6579/Amd1 protocol voor de isolatie van *Salmonella*. Bekomen isolaten werden genetisch onderzocht met behulp van *pulsed field gel electrophoresis* (PFGE). De antimicrobiële resistentie, gebaseerd op MIC bepalingen d.m.v. microdilutie, werd nagegaan voor sulfamethoxazol, gentamycine, ciprofloxacine, ampicilline, cefotaxime, ceftazidime, tetracycline, streptomycine, trimethoprim, chloramphenicol, colistin, florfenicol, kanamycine and nalidixinezuur. Bovendien werd de aanwezigheid van β-lactamase genen (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CMY}$) en het plasmidegebonden colistine resistentiegen *mcr-1* opgespoord gebruikmakend van PCR. De PFGE analyse toonde aan dat de onderzochte *S*. Infantis isolaten

een hoge klonale structuur vertoonden en dat een aantal *Salmonella* genotypes voorkomen op meerdere landbouwbedrijven.

Voor de studie omtrent de prevalentiebepaling, antibiotica resistentie en karakterisatie van *Campylobacter* (**hoofdstuk 2**), werd de cecuminhoud afkomstig van 379 at random geselecteerde braadkiptomen geanalyseerd. *Campylobacter* isolatie werd uitgevoerd door directe uitplating op mCCA en kolonies werden bevestigd met PCR. MIC waarden werden bepaald voor gentamicine, ciprofloxacine, nalidixinezuur, tetracycline, streptomycine and erythromycine. Voor de bepaling van de genetische variatie binnen de bekomen isolaten werd gebruik gemaakt van RFLP-flaA en Multilocus Sequence Typing (MLST). Op toomniveau bedroeg de *Campylobacter* prevalentie 64.1%. Van positieve tomen werden respectievelijk *C. coli* (68.7%), *C. jejuni* (18.9%) en *C. coli* en *C. jejuni* gemengde isolaten (12.4%) bekomen. Met de MIC bepaling werd een resistentiepercentage van minstens 67% bekomen voor tetracycline, ciprofloxacine en nalidixinezuur. Het resistentieprofiel tetracycline, ciprofloxacine en nalidixinezuur was het meest voorkomend profiel in beide *Campylobacter* species. MLST typering toonde aan dat, met uitzondering van één isolaat, alle onderzochte *C. coli* isolaten tot het klonaal complex CC-828 behoorden, terwijl *C. jejuni*, met uitzondering van 2 isolaten, tot 12 CC's behoorden. Bovendien werden 2 nieuwe sequentietypes binnen ieder species beschreven (2 nieuwe sequentietypes binnen *C. coli* waren gebaseerd op nieuwe allelen). Door combinatie van deze resultaten met de bekomen RFLP-flaA profielen werden nieuwe genetische profielen bekomen. RFLP-flaA profielen binnen de verschillende ST types konden niet geassocieerd worden met de herkomst van de isolaten. Deze bevindingen wijzen op de wijde verspreiding van *Campylobacter* genotypes in bemonsterde braadkipbedrijven.

In de tweede fase van het onderzoek, beschreven in **hoofdstuk III**, werd de *Campylobacter* contaminatie van braadkipkarkassen tijdens het slachten opgevolgd in 3 slachthuizen. In deze slachthuizen werden sommige slachthandeling (o.a. ophangen en evisceratie) manueel uitgevoerd en werden de karkassen uitgekoeld met behulp van koud water. De impact van de evisceratie, de finale wasstap en de waterkoeling werd nagegaan aan de hand van *Campylobacter* tellingen op de karkassen. Karkassen van 5 *Campylobacter* positieve tomen werden opgevolgd in ieder slachthuis. Van iedere toom werd een cecuminhoudmonster en na het plukken, de evisceratie, de finale wasstap en het koelproces 5 borsthuidmonsters genomen voor de kwantificatie van *Campylobacter*.

Er werd slechts in één slachthuis een kleine maar significante stijging in het aantal *Campylobacter* waargenomen na de evisceratie. De finale wasstap had geen significant effect

op het aantal *Campylobacter* (P>0,05), wat wees op een gebrek aan efficiëntie van deze stap. In alle slachthuizen leidde de waterkoeling tot een significante reductie van het aantal *Campylobacter* (0,13 tot 2.13 log_{10}kve/g) op de karkassen, mogelijks veroorzaakt door de toevoeging van chloor aan het koelwater of het waseffect in deze stap. Bovendien werd een hoge variabiliteit in het aantal *Campylobacter* binnen en tussen tomen in eenzelfde slachthuis waargenomen.

In de **discussie** worden de onderzoeksresultaten besproken waarbij in het bijzonder aandacht wordt besteed aan de situatie in Eucador en andere landen in deze regio. Met betrekking tot *Salmonella*, werden niet alleen een beperkt aantal serotypes gevonden, maar tevens werd duidelijk dat *S*. infantis het meest voorkomend serotype was. Dit verschilt van wat gerapporteerd wordt voor andere Latijns Amerikaanse landen, waar meer serotypes worden gevonden en andere serotypes domineren. Een zelfde bevinding werd waargenomen voor *Campylobacter*, waar in tegenstelling met andere Latijns Amerikaanse landen, *C. coli* het meest voorkomend species is. Verschillen in prevalenties kunnen onder meer verklaard worden door het gebruik van verschillende methoden voor de isolatie van *Campylobacter*. Bevindingen vermeld in deze thesis duiden op het belang van beide pathogenen in pluimvee gekweekt in Ecuador.

Bovendien werd bij deze pathogenen veel resistentie gevonden tegen antibiotica gebruikt in de humane geneeskunde. Dit verschijnsel kan verklaard worden door het frequent gebruik van antimicrobiële stoffen in de pluimveesector en is een bezorgdheid voor de volksgezondheid. Het belang van *S*. Infantis in humane infecties is gesteund op niet officiële rapporten, terwijl er geen gegevens beschikbaar zijn omtrent *Campylobacter* infecties in Ecuador. Verder onderzoek is nodig om de oorzaken voor de hoge prevalenties gevonden in deze studie te achterhalen en hun rol in humane infecties te bepalen.

Genetische karakterisatie van *Salmonella* en *Campylobacter* stammen toonde aan dat genotypes van beide pathogenen wijd verspreid voorkomen op pluimveebedrijven. Dit kan verklaard worden door gebrekkige bioveiligheidmaatregelen op landbouwbedrijven. Toepassen van efficiënte maatregelen zou de contaminatie op landbouwbedrijven op lange termijn kunnen reduceren.

Evaluatie van het effect van het slachtproces op de *Campylobacter* contaminatie van karkassen in slachthuizen toonde aan dat de finale wasstap en de toevoeging van chloor in het koelwater kan verbeterd worden. Acties met het doel om deze stap op te volgen en te standaardiseren zijn

essentieel om de bacteriële contaminatie van karkassen te verlagen. Deze acties zijn ook relevant om kruiscontaminatie tijdens het slachtproces te voorkomen. Verder onderzoek is noodzakelijk om de karkascontaminatie van braadkippen met andere pathogenen, zoals *Salmonella*, tijdens het slachtproces in kaart te brengen.

Deze thesis omvat de resultaten van een eerste studie naar de epidemiologie van *Campylobacter* en *Salmonella* in de pluimveevleesketen in Ecuador. Andere studies worden voorgesteld om de omvang van voedselgebonden pathogenen en hun antimicrobiële resistentie in de voedselketen en hun rol voor de volksgezondheid in Ecuador in kaart te brengen.

CURRICULUM VITAE

Christian Vinueza was born on January 8th 1976 in Quito (Ecuador). In 1995 he started his studies at the Faculty of Veterinary Medicine and Animal Husbandry at the Central University of Ecuador in Quito. In 2007 he moved to Antwerp to start the master program in Tropical Animal Health at the Institute of Tropical Medicine. One year later he started the master program in Veterinary Research at the Universidad Complutense de Madrid (Spain).

After his master studies he worked in Ecuador and Peru for the private and public sector. In November 2012 he was selected as scholarship holder by the Ecuadorian Secretariat of Higher Education, Science, Technology and Innovation (SENESCYT). In February 2013 he started his PhD study at the Department of Veterinary Public Health and Food Safety at the Faculty of Veterinary of Ghent University. His research was focused on *Salmonella* and *Campylobacter* in the broiler production chain.

Christian Vinueza has presented his research at national and international conferences and published his results in international peer-reviewed journals. Nowadays he is lecturer at the Faculty of Veterinary Medicine and Animal Husbandry at the Central University of Ecuador in Quito, and leads the 'Unity for Research of Foodborne Diseases and Antimicrobial Resistance'.

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