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Evolutionary strategies to reduce antimicrobial resistance in food-animals

Proyecto de investigación y Desarrollo

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Tesis presentada en cumplimiento parcial de los requisitos para el título de Doctor (Ph.D.) en Microbiología

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Recomendamos la aceptación de esta tesis en cumplimiento parcial de los requisitos del candidato para el grado de Doctor (Ph.D.) en Microbiología

Evolutionary Strategies to Reduce Antimicrobial Resistance in Food-Animals

María Fernanda Loayza Villa

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DEDICATION

"*To become a dragon, you must suffer like an ant*"
Chinese proverb

I dedicate this work to whom have enthusiasm in the study of the dynamics of antimicrobial resistance and who are passionate about figure out a balance among animals and environment. I dedicate my efforts, personal growth, and the nights turned into days to my family Juan, María Teresa, Juan Pablo, María Alexandra, Oscar Danilo, Ricardo Xavier, Gissela Carolina, Pablito Andrés, Emily Valentina, Antonella Maite, my friends Valeria, Vanessa, Lili, Luis, Marcela, Juan, Eva Moserrath and Juan Emilio, Daniel and Salvador, Leo, Mariu, Sebas, Ismael, and Ali. To whom I had failed several times with my time limitations and who have never denied me their support, Karina Ponce, Lore Bastidas, Patty Yánez, Ana Cristina Albán, Jorge Vélez, Jorge García, Fernando and Bianca Villavicencio, Fer Guano, Marlon and Felipe Carvajal, Yanara Astudillo, Alejandra Ruiz, José and Joaquín Domínguez and María Inés Baquero.

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RESUMEN

La resistencia a los antimicrobianos está aumentando peligrosamente en todo el mundo y esta tendencia es una consecuencia del uso de antimicrobianos en la medicina humana y medicina veterinaria, así como en la industria de producción de animales de abasto. Cantidades importantes de antimicrobianos son utilizados como promotores de crecimiento y profilaxis. Se ha detectado un número creciente de resistencia a antimicrobianos y de genes de resistencia a los antimicrobianos a nivel microbiológico y molecular. La mayor parte de las investigaciones acerca del rol de los animales de abasto en la crisis de resistencia a los antimicrobianos se ha centrado en patógenos zoonóticos. Sin embargo, la gran mayoría de la resistencia a los antimicrobianos, transmitida de bacterias en animales de abasto a patógenos humanos, probablemente está mediada por bacterias comensales intestinales. En esta disertación exploré la posibilidad de aplicar principios evolutivos para reducir la resistencia a los antimicrobianos en comensales bacterianos en animales de abasto. Primero estudié la dinámica de la resistencia a los antimicrobianos, de la microbiota intestinal de cerdos, en ausencia de antimicrobianos. También evalué el efecto de los cambios en la dieta en el resistoma intestinal de pollos.

Palabras clave: *Escherichia coli*, elementos genéticos móviles, resistencia antimicrobiana, plásmidos, microbiota comensal, industria alimentaria animal, promotores del crecimiento, profilaxis veterinaria.

ABSTRACT

Antimicrobial resistance is dangerously increasing worldwide and this trend is a consequence of the use of antimicrobials in human, veterinary medicine as well as the food-animal industry. Important amounts of antimicrobials are used for growth promotion and prophylaxis. An increasing number of antimicrobial resistance genes has been detected at microbiological and molecular level. Most research regarding the role of food-animals in the antimicrobial resistance crisis has focused on the antimicrobial resistance of zoonotic pathogens. However, the vast majority of the antimicrobial resistance transferred to human pathogens, from food-animals, is probably mediated by intestinal commensals. In this dissertation I explored the possibility of applying evolutionary principles to reduce antimicrobial resistance in bacterial commensals in food-animals. I first studied the dynamics of antimicrobial resistance of intestinal pig microbiota in the absence of antimicrobials. I also assessed the effect of diet changes in the animal intestinal resistome.

Key words: *Escherichia coli*, mobile genetic elements, antimicrobial resistance, plasmids, commensal microbiota, food animal industry, growth promoters, veterinary prophylaxis.

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INTRODUCCTION

Antimicrobial resistance (AMR) is currently a big concern in public health (Nordmann, 2014) and it has been increased dangerously worldwide in clinical and non-clinical settings (Castillo N. et al 2012; Finley et al., 2013; Stedtfeld et al., 2016; Von Wintersdorff et al., 2016; Williams-Nguyen et al., 2016). This trend is thought to be associated with the growing use (and misuse) of antibiotics in human medicine and food animal industry (Andersson & Hughes, 2011; Cantas et al., 2013; Torres, 2017). Low-income countries have reported important antimicrobial consumption increase between years 2000 to 2010, nevertheless, high income countries have a higher antimicrobial per capita consumption (Gelband et al., 2015). The volume of antibiotics used for veterinary purposes could exceed 2 times the amount used in human treatment (Silva, Knobl, & Moreno, 2013), but the proportion of antimicrobials used in the agroindustry could exceed at least 8 times the use in human medicine (Aarestrup F, 2015). Antimicrobials are used in animal production settings as prophylactics or growth promotors more than therapy (Cheney et al., 2015; Cully, 2014). Bacterial transmission is possible because of the large number of animal hosts and deficient sanitary infrastructure (Finley et al., 2013; Laxminarayan, Van Boeckel, & Teillant, 2015). Also, physical stress in animals could be a factor associated with illness development in some animals (Phillips et al., 2004). Veterinarians work under high pressure to guarantee health along the productive life cycle of food animals (Coyne et al., 2014; Fleury et al., 2016). Prophylactics are used because the productive success depends on absence of infections (L. Coyne et al., 2014; Fleury et al., 2016; Gerzova et al., 2015). Moreover, industry needs to eliminate zoonotic pathogens which can be transmitted into the food chain (Stanley, Hughes, & Moore, 2014). Prophylactics may prevent infections in defined growth phases with higher morbidity rates (Butaye, Devriese, & Haesebrouck, 2003; Hao et al., 2014)

The mechanism of action for growth promotion is not completely understood but research suggests that microbiota in their host´s metabolism could use the nutrients and limits their absorption (Kim et al., 2016), which implies the growth promoter administration could raise the growth ratio of the animals by avoiding bacterial nutrient consumption in the intestine (Gonzalez Ronquillo & Angeles Hernandez, 2015). Intensive food animal husbandry

uses some antibiotics in animal feed (De Niederhäusern et al., 2013; Hölzel et al., 2012; Seiler and Berendonk, 2012), leading to the augmentation of resistant genes within commensal microbiota (Penders, Stobberingh, Savelkoul, & Wolffs, 2013). The microbiota has been characterized as a complex environment of great bacterial diversity (Eckburg et al., 2012; Hendriksen et al., 2008; Salyers, Gupta, & Wang, 2004; Schaik, 2015). The most represented phyla are Bacteroidetes, non Alphabacteria, unclassified Cyanobacteria like, Actinobacteria, Firmicutes, Fusobacterium and Proteobacterium (Eckburg et al., 2012). Some phyla are naturally resistant to antimicrobials and others have acquired antimicrobial resistance genes (ARG) (Aarestrup F, 2015; Charlett et al., 2014; Pettigrew, Johnson, & Harris, 2016; Schaik, 2015; Watkins & Bonomo, 2016). Commensal *Staphylococcus* sp. *Enterococcus* sp. and bacteria from *Enterobacteriaceae* family are frequently used as microbiological markers to monitor the spread of genetic determinants of AMR (Aarestrup, 2015; Guerra, Fischer, & Helmuth, 2014; Muzslay, Moore, Turton, & Wilson, 2013;).

Enterobacteriaceae are considered one of the most important bacterial groups in terms of AMR spread (Cardoso, Ribeiro, Aragão, Costa-Pereira, & Sarmento, 2012; Chattopadhyay, 2014; Diene & Rolain, 2013; Huttner et al., 2013; Tansarli, Karageorgopoulos, Kapaskelis, & Falagas, 2013; Ventola, 2015; R. R. Watkins & Bonomo, 2016). *Escherichia coli* (*E. coli*) o *Klebsiella pneumoniae* (*K. pneumoniae*) are family representatives, featured as common carriers of 1) the extended-spectrum β-lactamase (ESBL) enzymes (such as, SHV, TEM, and CTX-M types), 2) class A Carbapenamases: KPC type, 3) class B carbapenemases: metallo– β-lactamases type (such as, VIM, IPM or NDM), and 4) class D oxacillinases like OXA-48 and AmpC (Nordmann, 2014; Rubin & Pitout, 2014). Most of these enzymes have been described in the last 20 years (Nordmann, 2014). These resistant determinants have had impact on resistant determinants prevalence because are associated with resistance to last generation antimicrobials commonly used in human therapy (Gillings, 2013; Martínez, 2008). *E. coli* is considered an AMR indicator due to its dual nature as commensal and an important representative of animal's gut microbiota or opportunistic pathogen associated with community and hospitalized human infections. Other important features are: 1) it is easy to grow in culture plates and 2) easy genetics analysis of resistant determinants (Blount, 2015).

There are two probable mechanisms of transmission of AMR from domestic animals to humans or vice versa: cross colonization of AMR from animal microbiota to humans and

horizontal gene transference of AMR genes from domestic animal bacteria to human microbiota. So, *E. coli* is probably the most used AMR indicator to demonstrate frequency in hospitals, community, farm animals, pets, and environment (water, soil and air) and several studies aims to figure out the source or origin of AMR transmission focusing on food animal industry as a main AMR carrier niche (Hu et al., 2016; Wallinga, Rayner, & Lang, 2015). However, the complexity of *E. coli* tracking due to their plasticity and linage diversity or their linage dominance under certain niches or conditions makes it difficult to monitor its role in AMR transmission. The present study analyzed the research schemes that have been used to track AMR determinants and figure out the difficulties and biases to stablish carriers, and probable AMR sources in between humans and food animals. This analysis is described in the first chapter of this document as a review of literature examining for the factors that limits the comprehension of AMR tracking and its transmission among animals and humans.

Antimicrobial resistance is not new and has been found in natural environments (D'Costa et al., 2011). Most of antimicrobials substances were obtained from microbes (fungi or bacteria) in different habitats to compete for ecological niche (Blount, 2015; Paterson, 2006; Shier, 2011). AMR is an evolutive process, which increases at greater antimicrobials concentrations in ecological niches (Gillings, 2013). Genomic plasticity of bacteria is an advantage for adaptation to constant antimicrobial infflux into their niche (Gillings, 2013; Porse et al., 2017). Thus, resistant bacteria are selected because they can avoid the intake of antimicrobials, modifying the antimicrobials or the target molecules (Davies & Davies, 2010; Van Hoek et al., 2011). Also, mobile genetic elements (MGE), such as insertion sequences (IS), composite, complex or conjugative transposons, integrons, and bacteriophages, participate in horizontal gene transference (HGT) without specie barrier, giving carrier bacteria a large genetic diversity (Trueba, 2014; Von Wintersdorff et al., 2016). These genetic determinants (mobile elements and AMR genes) are commonly known as the "mobilome" (Gillings, 2013). Altogether, the complete set of genes, that are associated or code an AMR gene, is known as "resistome" (D'Costa et al., 2011; Wright, 2007). Genetic characterization of resistome or mobilome in animals` microbiome is possible using non invasive samples, such as stool, grabs, fecal swabs, and environmental samples associated with animal farms (Gillings, 2016; Holmes et al., 2016; Van Hoek et al., 2011)

The practice of antimicrobials is usually higher in pigs and poultry farms, which together with fish are the most important source of animal protein for humans (Coyne et al., 2016; Cully, 2014). Since 1950, different families of antibiotics are also used as growth promoters with efficient and productive overcome in animal production, but with an increased and permanent selective pressure on bacterial strains with resistant phenotypes, despite of the progressive diminishes in antimicrobial use in animal industry (Kirsty, Uwiera, Kalmokoff, Brooks, & Inglis, 2016). In pigs, the most common worldwide antimicrobial products used as growth promoters are Avilamycin, Flavomycin, Olaquindox, Spiramycin, Salinomycin, Tylosin (Tylan), Virginiamycin and Zinc bacitracin (Aarestrup et al., 1998; Cromwell, 2002; Van Cuong et al., 2016). Oral administration of antimicrobials has a strong association with the increased ratio of antimicrobial resistant bacteria isolates from fecal samples. Usually, *E. coli* is used as indicator and ratio of colony count from fecal samples and in surveillance programs (Aarestrup, 2015; E Burow, Simoneit, Tenhagen, & Käsbohrer, 2014). Just as increased AMR is associated with oral administration of antimicrobials, antimicrobial reduction should be associated with AMR remission. In fact, this principle has already been applied for periods of more than a year in many European countries (F. M. Aarestrup et al., 2001; Elke Burow et al., 2019). Antimicrobial resistance is associated with a higher fitness cost (ability to adaptation and growth in a niche) in antimicrobial free environment (Garcia-Migura, Hendriksen, Fraile, & Aarestrup, 2014). Diverse linages of the same bacterial species are present microbiota (Anderson, Whitlock, & Harwood, 2006) and, in the absence of antimicrobials, susceptible bacteria could overcome resistant ones due to fitness costs.

Nevertheless, not only antimicrobials in the niche are unique factors driving the dominance of AMR bacteria within gut microbiota as permanent residents. A compensatory evolution works to avoid the fitness cost of AMR (Aminov & Mackie, 2007; Martinez, 2009; Sundqvist et al., 2009). *E. coli* population has been estimated around 1020 linages (Tenaillon, Skurnik, Picard, & Denamur, 2010), being detected by molecular techniques and divided in phylogenetic groups or multi locus sequence typing (Gonzalez-alba, Baquero, Cantón, & Galán, 2019). Some linages could be favored under specific characteristics in the niche, such as nutrient (proteins, carbohydrates, vitamins, etc.), atmosphere, adhesion molecules, and host genetic markers (Gao, Zhao, & Huang, 2014; Matamouros et al., 2018; E. R. Watkins, Maiden, & Gupta, 2016). High throughput molecular tools have described the metagenome

evolution in bacteria groups in study subjects under different diets (Gordon, O'Brien, & Pavli, 2015). However, it is important to study how these factors could change the dominance of *E. coli* linages within the same host (Fang et al., 2018) and if this metabolic challenge affects the AMR fitness cost (Martinez, 2009).

The importance of antimicrobial resistance gene pool in *E. coli* is exacerbated by its ability to survive under extraintestinal conditions and to colonize different animal species including humans, which is a perfect example of "One Health" paradigm (Collignon et al, 2018). Under this paradox, AMR must be treated as contemporary menace and under multidisciplinary views. The high frequency of multi drug resistant (MDR) opportunistic pathogen in animals (pets and livestock) could increase AMR bacteria transmission among hosts (humans and other animals), through food chain or direct contact (Founou, Founou, & Essack, 2016; Guardabassi, Schwarz, & Lloyd, 2004; Luchao et al., 2013; Oniciuc et al., 2018). AMR transmission and dynamics of resistant determinants could be driven by MGE more than bacteria carriers, but it could be difficult to track because the high transference and the absence of barriers (Gillings, 2013; Perry & Wright, 2013).

This thesis is divided in 4 chapters in the format of scientific papers: the first chapter is a literature review, in which we analyze conflicting reports about the role of domestic animals in the current antibiotic resistance crisis; the second chapter is a manuscript describing the effects of antimicrobial restriction in pigs and its effects on intestinal resistome; the third chapter is a manuscript describing how different diets could alter the dominance of some antimicrobial resistant *E. coli* lineages in chickens; and the fourth chapter is a manuscript describing the transmission of a colistin resistance gene between domestic animals and humans in a household.

CHAPTER I

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Factors Obscuring the Role of *E. coli* from Domestic Animals in the Global Antimicrobial Resistance Crisis: An Evidence-Based Review

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Abstract: Recent studies have found limited associations between antimicrobial resistance (AMR) in domestic animals (and animal products), and AMR in hospitals. These studies have primarily used *E. coli*, which is a critically important bacterial species associated with significant human morbidity and mortality. *Escherichia coli* is found in domestic animals, the environment, and it can be easily transmitted between these compartments. Additionally, the World Health Organization has highlighted *E. coli* as a "highly relevant and representative indicator of the magnitude and the leading edge of the global antimicrobial resistance (AMR) problem". In this paper, we discuss the weaknesses of current research that aims to link *E. coli* from domestic animals to the current AMR crisis in humans. Fundamental gaps remain in our understanding the complexities of *E. coli* population genetics and the magnitude of phenomena such as horizontal gene transfer (HGT) or DNA rearrangements (transposition and recombination). The dynamic and intricate interplay between bacterial clones, plasmids, transposons, and genes, likely blur the evidence of AMR transmission from *E. coli* in domestic animals to human microbiota and vice-versa. We describe key factors that are frequently neglected when carrying out studies of AMR sources and transmission dynamics.

Keywords: commensal *E. coli*, antimicrobial resistance, food-animals, gene transfer

1. Introduction

The rapid evolution of antimicrobial resistance (AMR) in bacteria is one of the most dangerous trends in public health [1,2] causing increased morbidity, mortality [1,3–8] and

economic loss [9]. The AMR crisis is being felt more intensely in hospitals where outbreaks of pan-resistant opportunistic pathogens are emerging at an increasing pace [10–13]. Most of these drug-resistant opportunistic pathogens found in clinical settings are members of the human (or other animal) commensal microbiota [14–16]. Antimicrobial resistance in bacteria from food-animals has been reported since the 1950s when antimicrobial supplements began to be used as growth promoters in animal feed [17–21]. Currently, 75% of antimicrobials produced in the world are used in food-animals [22]; both small-scale food-animal producers and intensive food-animal operations use a variety of antimicrobials in animal feed and water, as growth promotors or prophylactics [23–27]. Performance of food-animals industry is a big pressure for veterinarians to use antimicrobial in their batch's and limited the addition to avoid their use [28,29]. Antimicrobial use in this setting causes selective pressure on the bacterial populations which accumulate AMR genes [30–35] and the large numbers and diverse AMR genes in the microbiota of domestic animals has created concern about the spread of AMR from food-animals to humans [13,36–38].

Transmission of AMR bacteria can occur through the environment [39] and food chain [40–42], especially in low- and middle-income countries (LMICs) where water, sanitation, and hygiene are inadequate [43–45]. We focus on the role of commensal *E. coli* in the AMR crisis because: *E. coli* is probably the most studied indicator [46,47] and its transmission could be tracked more easily (among animal hosts) than anaerobes which are the most abundant members of the microbiota [48,49]; *E. coli* can survive and even grow in the environment outside of the host [50]; *E. coli* may mobilize AMR genes more easily than other intestinal bacteria (such as *Bacteroides*) [51–53].

In this review, we concentrate on recent reports showing lack of relationship between antimicrobial usage in domestic animals and antibiotic resistant bacteria in humans. We postulate that the complexities due to high diversity, strain turnover, and horizontal gene transfer, hamper out ability to find greater linkage between antimicrobial resistance in domestic-animals and humans. We are including pets in this study to show how an antimicrobial resistant *E. coli* can colonize different hosts. We analyze all the potential pitfalls associated to this type of studies. To minimize the potential overestimation of human-domestic animal transmission, we focus only on reports in which whole-genome sequencing (WGS) was used, as multi locus sequence typing (MLST) can show homoplasious sequence-

types [54] or strains belonging to the same sequence-type may show many single nucleotide polymorphisms (SNPs) in other genes indicating non-recent ancestry [55].

Population genetics of E. coli and AMR

Escherichia coli is found almost exclusively in the intestines of warm-blooded animals and although it represents only around 1% of the intestinal microbiota [56], it is probably the most abundant member of the intestinal microbiota possessing the ability to survive and even grow outside the host [50]. Commensal *E. coli* is probably the most common commensal bacteria transmitted among different species of animals [48,49]. Each *E. coli* strain falls into one of the six phylogenetic groups (A, B1, B2, D, E, or F) [57]. The majority of *E. coli* clones can colonize the intestines of different animal species (generalists); however, different *E. coli* strains may display a different degree of host adaptation, and the strains belonging to some phylogroups may be better adapted to certain animal species [57–60]. *E. coli* strains with a higher degree of adaptation to a given intestinal milieu may become long-time colonizers (residents) [61] and numerically dominant [62], while strains with lower adaptation may colonize transiently and/or may become a numerical minority. Numerically dominant and resident lineages may disseminate more between different hosts. The constant competition between new arrivals with colonizing strains in the intestine is likely responsible for the rapid turnover of dominant *E. coli* strains observed in the intestines of humans [63]. Although a minority among *E. coli* lineages, pathogenic strains of *E. coli* (such as ST131 genotype) are an important category that contains virulence genes and are associated with invasive infections. Antimicrobial resistance is another layer of complexity; the transmission of AMR genes among strains of *E. coli* occurs through the movement of mobile genetic elements (MGEs; e.g., plasmids, phages, transposons, integron-cassettes, and other mosaic structures) [33,64,65]. Transposable elements and cassettes (integrons) mediate the movement of AMR genes from one MGE to another or from a bacterial chromosome to plasmids (or vice versa), whereas plasmids mediate the movement of AMR genes from one bacterium to another [33,66,67]. This phenomenon is very dynamic; it is possible to find isolates that are the same *E. coli* clone, in the same intestine with different AMR genes [68,69]. All these categories (dominant, pathogenic, and antimicrobial resistant) are very fluid as *E. coli* strains may change their status by acquiring genes (horizontal gene transfer-HGT and recombination) or by mutations.

2. Materials and Methods

2.1. Study population and outcome of interest

For this review, we considered relevant peer-reviewed literature that studied farm animals and pets carrying AMR commensal *E. coli*.

2.2. Identifying the relevant literature

The peer-reviewed literature was searched using Google, Google Scholar, MEDLINE, and PubMed using the keywords farm animals OR domestic animals in combination with antimicrobial resistance OR antibiotic resistance AND *Escherichia coli* OR *E. coli* AND human.

2.3. Eligibility assessment

Selected articles were submitted to an initial screening to determine the relevance based on title, abstract and keywords. A second full-text screening was performed to analyze methods. Those that reported whole-genome sequencing for comparison of interspecies transfer of *E. coli* or AMR determinants were selected (Table 1).

Table 1. Description of studies that applied next-generation sequencing to study interspecies transfer of *E. coli* or antimicrobial resistance (AMR) genetic determinants.

Study	Advanced Typing Methods [1]	Spatially Matched Sampling	Temporally Matched Sampling	Focused on Human Pathogens	Strong Evidence of Animal-Human Transmission	Financial Support
De Been, et al., 2014 [39]	+	–	–	+	–	Government
De Been, et al., 2014 [39]	+	+	+	–	+	Government
Hu, et al., 2016 [53]	+	–	–	–	+	Government
Salinas, et al 2019 [55]	+	+	+	–	+	Government
Ludden, et al., 2019 [70]	+	–	–	+	–	Government
Day, et al., 2016 [71]	+	–	–	+	–	Government, private, NGO
Dorado-Garcia, et al., 2018 [72]	+	–	–	+	–	Government, private
Mainda, et al., 2019 [73]	+	–	+	+	–	Government, private
Falgenhauer, 2019 [74]	+	+	+	–	+	Government
Berg, et al., 2016 [75]	+	+	+	–	+	Government
Li, et al., 2019 [76]	+	+	+	–	+	Government
Loayza, et al., 2019 [77]	+	+	+	–	+	NGO
Liu, et al., 2016 [78]	+	–	–	+	+	Government
Trung et al., 2019 [79]	+	+	+	–	–	Government
Falgenhauer, et al., 2016 [80]	+	–	–	–	+	Government
Reeves, et al., 2011 [81]	+	+	+	+	+	Government
Hedman, et al., 2019, [82]	–	+	+	–	+	Government, NGO
Trung, et al., 2017 [83]	–	+	+	–	+	Government
Valentin, et al., 2014 [84]	–	–	–	–	–	Government

[1] Advanced method include whole core-genome sequence typing and plasmid sequencing. NGO: non-governmental organization

3. Results and Discussion

3.1. Why have studies failed to show a link between antimicrobial resistance in humans and domestic animals?

Different sampling protocols often yield different results; we have identified some critical aspects that can affect the outcome of bacteriologic analysis, funding sources seems not infer on study designs, all author declare non conflict interest and financial support is general associated to academic or public health organizations (Table 1).

3.2. Inadequate sampling

Many studies failed to find a clonal relationship or AMR gene homology between AMR *E. coli* obtained from humans in hospitals (opportunistic pathogens) and domestic animals [39,70–73]. When commensal isolates were obtained from domestic animals and humans living in proximity and during the same period, however, isolates were identified that showed clear clonal relationships and the same AMR genes in *E. coli* from humans and domestic animals [74–77]. We argue that reports analyzing isolates from different locations or different time frames underestimate the *E. coli* diversity and population dynamics. Populations of *E. coli* collected from different locations and different time frames are most likely different. Despite this fact, some reports have been able to find clonal relationships between infections in hospitalized humans and fecal samples from domestic animals [74,78]. We found one exception where commensal *E. coli* from domestic animals did not show clonal similarity to human *E. coli* in the same community and during the same time period [79]. An alternative interpretation of the discrepancies between studies is that *E. coli* from domestic animals transmit to humans through the environment (people working on farms or who are in contact with animals or their waste) and not through the food-chain [39], however, it seems more likely for an enteric bacterium (like all zoonotic enteric pathogens) to enter the human gut through food than any other route.

3.3. Focus on opportunistic pathogens

The bulk of the *E. coli* transmitted from domestic animals to humans are probably numerically dominant commensals, not frank pathogens. Numerically dominant *E. coli* commensals are lineages representing the majority [62]. Pathogenic strains of *E. coli* make up a limited number of the *E. coli* lineages which may be moving from domestic animals to humans; these pathogens, however, are probably a minority in many animal intestines.

Therefore, it is no surprise that some studies failed to detect some opportunistic human pathogens (such as *E. coli* ST131) in fecal samples from domestic animals or animal products [39,70,71]. If these strains are part of the commensal *E. coli* in domestic animals, they are experiencing the same population fluctuations associated with clonal competition (described above). Assessing the prevalence of pathogenic *E. coli* in domestic animals or animal products may require massive sampling and metagenomic approaches. Nevertheless, some studies have been able to detect the same clones of opportunistic pathogens in hospitals and domestic animals or food-animal products [42,74,75].

3.4. Complex Dynamics of Mobile Genetic Elements

Transmission of AMR genes between domestic animal microbiota and human microbiota seems to occur more frequently by HGT than clonal transmission [39,53,68,77,78,80–81,85]. Nevertheless, the HGT of AMR genes complicates the identification of the source of AMR genes. It is possible that AMR *E. coli* strains (e.g., from a domestic animal) marginally colonize the human gut, but it may transfer a plasmid to a dominant bacterial strain (human-adapted) in the human intestine [68,78,86] and the same AMR gene may move via a transposon (or cassette) from the mobilized plasmid (from an animal bacteria) to a plasmid in the human bacterium [33,66] (Figure 1). Under this scenario, only a longitudinal analysis including whole plasmid sequencing of epidemiologically related (spatiotemporally linked) strains could capture this phenomenon. Identical AMR genes are carried by different *E. coli* plasmids in diverse isolates obtained from humans and domestic animals living in the same community and during the same period [55]. Recent studies (using WGS and plasmid sequencing of epidemiologically related isolates) show how transposable elements restructure plasmids with AMR genes in bacterial strains that are causing infections in one hospital over time [66], and how some plasmids can undergo rearrangements in a short period of time [67]. Plasmids carrying AMR genes also have a different ability to disseminate, such that many exhibit different levels of bacterial host specificities and cause different fitness costs in different bacterial populations [87]. Due to the complexity of the phenomena involved, the transmission of AMR genes from food-animal *E. coli* to human *E. coli* may not be possible to demonstrate molecularly but only epidemiologically (i.e., *E. coli* strains isolated from epidemiologically related sources, have the same AMR genes) [55,82,83,88,89].

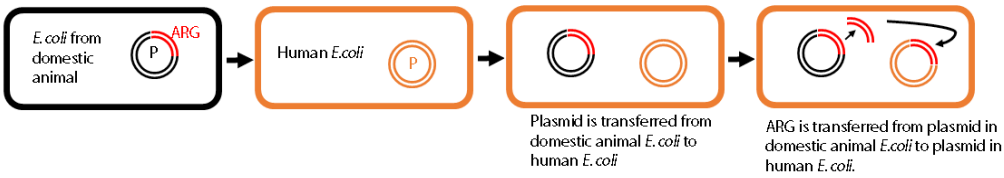

Figure 1. Example of antimicrobial resistance gene (ARG) movement that affects the complexity of studying antimicrobial resistance transmission. Plasmids (P) carried by *E. coli* from a food-animal can be transferred to human *E. coli* and the ARG can move between plasmids.

3.5. Focus on Non-Dominant Clones

Results from studies carried out on strains isolated with non-selective media (e.g., containing no antimicrobials) will differ from studies in which *E. coli* was isolated in media with antimicrobials. In the first case, we are likely assessing the numerically dominant *E. coli* [62], while in the second case we may be looking at a minority *E. coli* lineage with a specific resistance phenotype. As previously mentioned, numerical dominance may be related to some degree of adaptation of some *E. coli* lineages to an animal host; some generalist strains can thrive similarly in the intestines of different animal species, while others likely thrive in one animal species rather than in others [58,60]. We argue that a specialist *E. coli* strain colonizing the intestine of a host, for which it is not adapted, may remain a numerical minority [58] and undetectable by standard bacteriological culturing techniques (e.g., collecting 5–20 colonies from a culture plate) [62]. Conversely, when a strain is more adapted to the host, it is likely to become a numerically dominant lineage and easily detected by standard bacteriological culture. This property may then indicate that human to human *E. coli* transmission is more frequent than the transmission of *E. coli* from domestic animals to humans because of a higher exposure of human populations to human strains [72].

3.6. Different Environmental Contexts

Other factors responsible for discrepancies between studies may be associated with the environmental setting; industrialized countries have better environmental and food hygiene and sanitation than low- and middle-income countries (LMICs), and some differences in *E. coli* transmission should be expected in different contexts. Similar considerations must be made when comparing rural (farming communities) vs. urban communities [72,74,77]. For food-animal operations in low-income countries, or where there is insufficient biosecurity and

hygiene in the facilities, reducing the use of antimicrobials is perceived as a big challenge [90,91].

The review of the literature indicates that there is little doubt that cross-colonization of AMR bacteria from domestic animals to humans is occurring and many studies have shown this scenario. There is also compelling evidence that AMR genes that originated in food-animals can end up in *E. coli* strains that reside in the human gut [39,55,77,83]. However, in some instances, these phenomena may not be evident because the large diversity and constant turnover of *E. coli* strains in the intestines reduces the chances of finding a link, especially when sampling from different locations or during different timeframes as was done in many previous studies. The movement of AMR genes from one plasmid to another, or plasmids undergoing rearrangement, are also important obstacles to understanding the linkage between AMR in human and domestic animals [67].

Horizontal gene transfer acts as a mechanism that can quickly spread resistant determinants to new carriers regardless of whether they are human or animal linages of bacteria [83,92]. AMR genes and the MGEs that mobilize these genes are likely to be derived from diverse parts of the microbial biosphere [13,93]. The gut microbiome has been defined as an important source of AMR genes in both animals and humans [94], and the dynamic nature of the gut is likely complicated further by the dynamics of HGT [95,96].

There are likely major differences in the transmission of AMR in high-income, middle-income, and low-income countries. For example, poor hygienic conditions in the food-animal industry in low-income countries may accelerate the transmission of bacteria from food-animals through the food-chain; lack of wastewater treatment or lack of basic sanitation infrastructure may contaminate irrigation water or soil where crops are raised [97,98]. The latter transmission pathway is also troublesome as AMR may return to humans via food-animals and the food-chain. One potential example of this phenomenon is carbapenem resistance, which is thought to most likely originate in humans (i.e., carbapenems are not used in food-animals). A study in China found clonally related carbapenem resistant *E. coli* in backyard food-animals, humans, and the environment [76].

Finally, *E. coli* is a diverse species and shows high rates of recombination and HGT. To understand the true role of animal *E. coli* in the AMR crisis, it is necessary to take into account all the biological (population genetics and physiology) aspects of this

bacterium and to apply WGS, including whole plasmid sequencing. Fortunately, the declining costs of this technology are allowing its implementation in LMICs. The AMR crisis in human medicine is another example where the One Health paradigm is important.

4. Conclusions

We suggest that transmission of antimicrobial resistant commensal *E. coli* or AMR genes between *E. coli* from domestic animals and humans occurs frequently, however it is difficult to detect. The diversity of *E. coli* clones and the turn-over rate of *E. coli* clones in the intestines does not facilitate finding relationships between strains in domestic animals, animal products, and humans. The only way to observe this connection in is by sampling humans, animal products, and domestic animals in the same location and during the same period of time. Finding evidence of AMR gene transmission between bacteria in humans and domestic animals is made even more complex as genes frequently move from one plasmid to another. Observing transmission phenomena will likely require that studies collect spatiotemporally matched samples.

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Chapter II

Summited to BCM Veterinary research

No resistome differences between pigs treated and not treated with prophylactic antimicrobials

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ABSTRACT

Background: The use of antimicrobials in the food-animal industry has caused an increased prevalence of antimicrobial-resistant bacteria and antimicrobial resistance (AMR) genes, which can be transferred to the human microbiota through the food-chain or the environment. To reduce the development and spread of AMR, restrictions on antimicrobial use in food-animals have been implemented in different countries. We investigated the impact of an antimicrobial restriction intervention (during 2 generations of pigs) on the frequency of antimicrobial-resistant coliforms present in pigs on an Ecuadorian farm.

Results: No differences in antimicrobial-resistant coliforms or antimicrobial-resistance genes (richness and abundance) were found when we compared animals fed with or without prophylactic antimicrobials. Nevertheless, the absence of antimicrobial supplements did not negatively affect weight gain in pigs.

Conclusion: The fitness costs of antimicrobial resistance genes in intestinal bacteria may be overestimated. Avoiding antimicrobials as prophylactics in the feed may not be enough to reduce the prevalence and spread of antimicrobial resistance.

Keywords

Antimicrobial prophylaxis; pigs; resistome; fitness cost, antimicrobial resistance; mobile genetic elements; antibiotic resistance

BACKGROUND

Antimicrobials have been effective in the treatment and control of bacterial diseases and have contributed to a greater human life expectancy [1]. However, the constant use of antimicrobials has created a selective force that has increased the frequency of antimicrobial-resistant bacteria and antimicrobial resistance genes (ARGs) in animal microbiota [2, 3].

Around 80% of total antimicrobial production in the world is used in food-animals [4] and larger amounts of antimicrobials may be used in animals in less industrialized countries that lack regulatory policies for antimicrobial use [4–6]. The use of antimicrobials in food- animals causes the proliferation of commensal bacteria with ARGs, which can be horizontally transferred to many other bacterial species in the intestines [7, 8].

Antimicrobial-resistant commensals from farm animals can end up in food products, such as meat and dairy [9–11]. These bacteria can colonize human intestines and could become opportunistic human pathogens. Also, ARGs could be transferred from bacteria in food-animals to human microbiota [12–14]. For instance, *Escherichia coli* strains isolated from healthy pigs and humans carried the same mobile genetic element (MGE) with the *mcr*-1 gene, which confers resistance to colistin [15].

Reducing antimicrobial use in food-animals can contribute to the reduction of antimicrobial-resistant bacteria (ARB) [16]. In theory, if ARGs cause fitness costs in commensal bacteria in the absence of antimicrobial, the reduction in antimicrobial use in farms should cause a reduction in ARB in food-animals [17]. However, the persistence of ARB occurs due to compensatory evolution that allows bacteria to maintain ARGs without apparent fitness costs [18, 19]. Experiments, in which animals were deprived of antimicrobials (as growth promotors), retained high levels of antimicrobial resistance in numerically dominant *E. coli* [20, 21] and high abundance of ARGs [22]. More importantly, ARGs, multi-resistant, and numerically dominant bacteria have been found in animals from organic farms [23–25]. The main objective of this study was to analyze the effect of the removal of antimicrobials, administered as prophylactics (higher doses than those used for growth promotion), on the prevalence of ARB and ARGs in fecal microbiota. We withdrew antimicrobials during 2

generations of animals and kept these animals in the same building with pigs receiving antimicrobials to see if bacteria without ARGs can outcompete ARB.

RESULTS

Ratios of antimicrobial-resistance (proportion of antimicrobial resistant coliforms in MKL with and without antimicrobials) showed no significant difference between treatment groups (group A = 0.722 and group B 0.763; pa= 0.434) (table 2). Antimicrobial susceptibility tests of 537 randomly selected strains (A=266 and B= 271) showed resistance to ampicillin (n= 397; 73.9%), amoxicillin-clavulanic acid (n=188; 35%), tetracycline (n=434; 81.1%), trimethoprim-sulfamethoxazole (n=301; 56.1%), gentamycin (n=125; 23.3%), ciprofloxacin (n=71; 13.2%), chloramphenicol (n= 174; 32.4%) and ceftriaxone (n=77; 14.3%); 65.9% of the strains were multidrug resistant (resistance to 3 or more antimicrobials). There were no significant differences in these resistances between treatment groups or mothers (X^2, a≥ 0.05; table 3).
A Spearman correlation test showed no difference in ARG relative abundance profiles between samples collected during different growth phases. Pigs at day 30 showed a higher ARG relative abundance (not statistically significant), however, it declined over time (figure 3). There were no statistical differences in weight, growth, and health status among study groups (S2 supplementary materials). The ARG richness showed the same tendency in animals within a group or between groups (figure 3), however, the relative abundance of ARGs and MGEs decreased as piglets got older except for tetracycline resistance genes and MGEs, which remained stable (figure 3). Among the most abundant genes detected were aminoglycoside resistance and MGEs: Tp614, *IS*613, *tnp*A, *int*1-a-marko, *intI*2, *int*I1F165_ clinical, pBS228-IncP-1, *trb*-C, *IS*26, *IS*256, *IS*6100, *IS*91. Some of these MGEs could be responsible for the transfer of resistance genes among microbiota species (46). Genes, such as *tet* (32), were detected in all samples and colistin resistance gene prevalence was low (relative abundance 0.0000075 0.00013). A PCR amplification of the *mcr*-1 gene in fecal samples showed that 19 of the 20 sows carried bacteria with this gene (data not shown).

DISCUSSION

We expected that the absence of prophylactic doses of antimicrobials along the life period of 2 generations of pigs would cause antimicrobial susceptible bacteria to outgrow resistant ones [26, 27]. However, we did not find significant differences (a≥ 0.05) in the total number of resistant coliforms nor did we find differences in resistance genes (abundance or diversity) between groups of animals receiving antimicrobials and those not fed antimicrobials. We also failed to detect any differences in the microbiota resistome (amount and type of ARGs) between the two groups of animals (figures 2, 3 and 4). These results are in agreement with other reports showing no effect of antimicrobial restriction on the proportion of ARB of animals [28]. Similar AR phenotypes have been found in isolates from animals with and without antimicrobial restrictions (conventional vs. organic farms) [23, 29–31]. In contrast, other studies showed an important decrease in resistance in bacterial isolates or resistant gene abundance after antimicrobial removal [21, 25, 32].

Our results may indicate that ARGs are not causing a major fitness reduction in the bacterial population in pig intestines. Under laboratory conditions, it has been shown that the fitness costs associated with a plasmid carriage disappear overtime and even could transform in fitness advantage after 420 generations [33]. Furthermore, this advantage could even be transmitted to other bacterial hosts never exposed to this plasmid [33]. Reduction of antimicrobial resistance in the intestine could be a slow process as antimicrobial-susceptible bacteria may not outcompete resistant ones [19, 34].

Antimicrobial susceptibility tests showed a higher resistance frequency to tetracycline, ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol without differences among the animal groups; these resistances are common in pig farms worldwide [25]. The resistance genes with higher relative abundance were tetracycline, b lactams, and aminoglycosides (figure 3). Genes associated with tetracycline resistance have been detected in pigs feeding with or without antimicrobials which concords with the notion tetracycline resistance genes are also common in pig's intestinal resistome [21, 32, 35, 36]. Aminoglycoside and b lactam resistance genes in animals deprived of antimicrobials have been reported previously [32, 37]. These results suggest that antimicrobial restriction, in 2 generations of animals, was not enough to reduce antimicrobial resistance in this pig farm. It is possible that ARB present in the surrounding environment colonized the intestines of animals deprived of antimicrobials.

Reducing antimicrobial resistance in these environments may require antimicrobial restriction in the totality of animals and for longer periods [22]. The resilience of antimicrobial resistance in the microbiota is an important factor against the reduction of ARB transmitted to humans [38].

MGEs are important actors in antimicrobial resistance spread [39, 40] and in this study, we observed a higher relative abundance of MGEs in samples in 30 day-old pigs (figure 3), which corresponds to weaning animals. The transition from milk to solid food may cause a dysbiosis [41] and bacterial stress resulting in SOS responses which may increase dissemination of MGEs and ARGs [42, 43].

Housing the two groups of animals in the same barn may be an important factor contributing to the similarity of ARGs in bacteria from both groups. However, we hypothesized that microbiota of animals without antimicrobials should be re-colonized by antimicrobial susceptible bacteria which should be able to outcompete AR bacteria in the surrounding environment. Finally, the withdraw of antimicrobials did not have any repercussions in the growth or weight of the animals.

Our study had some limitations: first, we pooled fecal samples from 5 animals to investigate the abundance and diversity of ARGs and MGEs in samples from different time frames and the two groups limiting the possibility to carry out statistical analysis, however, phenotypic data were consistent with molecular findings; second, we did not have any way to assess the quality or bioavailability of the antimicrobials used in animals [44].

CONCLUSIONS

Our observations suggest that antimicrobial restriction did not reduce the numbers of antimicrobial-resistant bacteria in the gastrointestinal tract of pigs raised by a major producer in Ecuador. The co-evolution of bacterial chromosomes with ARGs and MGEs (such as plasmids) is a phenomenon that requires additional research.

ABBREVIATIONS

AR.- Antimicrobial resistance

ARB. - Antimicrobial-resistant bacteria

ARG. - Antimicrobial resistance gene

ARGs. - Antimicrobial resistance genes

MGE. - Mobile genetic element

MGEs. - Mobile genetic elements

MDR. - Multidrug resistance

MKL. - MacConkey Lactose

n.- Number of samples

PCR. - Polymerase chain reaction

SXT. - Trimethoprim and 76 mg /liter of sulfamethoxazole

CFU. - Colony-forming units

PO. - Phosphate-buffered saline solution

TSB. - Tryptic soy broth

mL.- Milliliter

ppm - Parts per million

METHODS

All protocols of the experimental design were approved by the ethics and biosecurity committee of the company and the Ethics Committee for Animal Protocols at Universidad San Francisco de Quito before the study.

Animals

A completely random, balanced fixed-effects double-blind study was conducted in two generations of pigs housed in a farm in Ecuador. The number of animals was calculated with a statistical power of 87% considering the probable loss of experimental units (replacement sows). The study was conducted under strict biosecurity conditions. Twenty 70-day female PCI1050 pigs were randomly assigned to each of the 2 groups; one group containing 10 female pigs (the control group) received conventional feed formulation with antimicrobial additives (group A) and, and the other 10 female pigs received feed containing no antimicrobial additives (group B) and kept in this regimen until they farrowed (S2. Supplementary materials). Piglets were placed with their corresponding mothers, weaned at 20 days, distributed into two separate pens for group A (n=40) and B (n=40), and continued with the treatments of their respective mothers. The productive performance was observed based on daily weight gain during the study period and carcasses were weighted after slaughter. Vaccines were administered to all animals and the antimicrobial treatment was administered under veterinarian supervision only to animals that had any diagnosed infection. Antimicrobial additives used are described in table 1.

During the weaning and fattening phases, each pen grouped 32 piglets. Pig density was 0.45 m^2/pig in the weaning phase and 0.90-1.0 m^2/pig at the fattening phase. Animals from each group were monitored for 150 days (figure 1). The type and antimicrobial concentrations in feed changed over time and have been used routinely in the farm for the two previous years (table 1).

Husbandry

The maternity and weaning phases (piglets until 70 days) were carried out in pens with plastic slatted flooring, the pens for the fattening phase was carried out in a different facility and had cement floor which was washed daily. In all growing phases, pens were protected from the sun with a roof, the ceiling was at 2.5 m high. The number of cages is described in figure 1.

Tap water and feed were administered *at libitum,* temperature ranges from 20 to 26°C. All experiments were performed under veterinary supervision. If any sick animal was detected, it was immediately separated in a different cage to be treated and eliminated from the study.

Both animal groups (treatment and control) were maintained at the same location with separated feeding and watering supplies. Animals of different treatment groups were fed with different instruments. Pens were kept separated by a 90cm corridor in all growing phases. There was no airflow control or physical separation of different treatment pens.

Sows that were kept out of the study were incorporated to conventional productive cycles within farm batch. On the other hand, animals were released from the experiment at day 150 and they were slaughtered (electric stunning) at day 170. After overnight rest, pigs were driven to the stunning area quietly in a single line. One by one they were moved into the stunning area and they were held with an appropriate restraining device avoiding stress. It was not permitted to beat any pig during this process.

The electrical stunning was used to cause an electro lectic shock in pig´s brain. After that, animal fell over a transport band to be immediately bleeding by neck incision. The cut of main vessels (jugular and carotid) caused a cerebral anoxia and complete bleeding procedure. Farm technicians, along all slaughtering process, were permanently trained to guarantee a performance under ethics, biosecurity, and safety standards.

Samples and phenotypic analysis

Two rectal swabs were taken from each animal and swabs were maintained on ice for transportation to the lab facilities within 2h after collection. Intestinal coliforms were used as a microbial indicator of phenotypic resistance (Figure 1). For quantification of coliform resistant bacteria in intestinal microbiota of pigs, Mac Conkey´s medium (MKL) was supplemented with 4 mg/liter of trimethoprim and 76 mg/liter of sulfamethoxazole (SXT) [45], or 32 mg/liter of tetracycline [46], or 16 mg/liter of ampicillin according to protocols described previously [47].

Swabs were eluted in 5mL of sterile tryptic soy broth (TSB) diluted in phosphate-buffered saline (0.0169M KH_2PO_4, 0.0719M K_2HPO_4, pH 7,2) as previously described [46]. A 0.1mL aliquot of this solution was serially diluted in 0.9 mL of PO until 10^{-3} and 0.1mL of each dilution was plated onto the surface of MKL plates with and without antimicrobials [47] (S3. Supplemented materials). We estimated the ratio of resistant coliforms by counting the

number of colonies in MKL plates with antimicrobials divided by the colonies in MKL plate without antimicrobials [25]. Only plates with coliform counts greater than four colony forming units (CFU) were recorded (S3, supplementary materials).

Antimicrobial susceptibility test

One lactose fermenting (coliform) colony from each plate was isolated and stored at -80°C in TSB with 30% glycerol [31]. Antimicrobial susceptibility tests were performed using AMP ampicillin (10mg), TET tetracycline (30mg), SXT trimethoprim-sulfamethoxazole (1.25/23.75mg), GEN gentamycin (10mg), AMC amoxicillin-clavulanic ac. (20/10mg), CIP ciprofloxacin (5mg), CHLOR chloramphenicol (30mg) and COX ceftriaxone (30mg) as representatives of the most used families of antibacterial drugs used in health care [48, 49]. The Kirby Bauer test was carried out following CLSI (Clinical & Laboratory Standards Institute) guidelines.

Molecular analysis

For molecular analysis, rectal swabs were frozen at -80°C until they were used, DNA was isolated using MO BIO Power Soil DNA Isolation Kit (MO BIO, Carlsbad, CA) following manufacturer instructions. Nucleic acid quality and quantity were evaluated using nanodrop (Thermo Scientific) and Qubit dsDNA HS (Thermo Fisher Scientific, Oregon, USA). For each sampling time point (6 for group A and 6 for group B), DNA extracts from fecal samples of 5 animals were combined. The concentration of each DNA sample within the pool was 10mg/mL. Each pool was analyzed in duplicate with high throughput qPCR. WaferGen SmartChip Real-time PCR system was used to detect 384 genes, 338 are informative for ARGs or MGEs. Primers for ARGs, HT- qPCR protocols and calculations of amplicon abundance and diversity were carried out as described in previous studies [35, 50–52] (Suppl. 1). Briefly, the difference between the cycle threshold (Ct) of ARGs and 16S genes (ΔCT) was used to normalize and calculate the ARGs fold change. The CT value of each amplicon was used to calculate ΔCT with this formula: $DCt = Ct_{ARG} - Ct_{16S\ rRNA\ gene}$, where ΔCT represents the proportion of ARGs vs 16S rRNA genes. To calculate the difference in ARG abundance and diversity between animal groups (DDCt) we used the formula: $DDCt = DCt_{AB\_treated} - DCt_{no\ AB}$ where: $DCt_{ARG\_Treated}$ represented the DCt value calculated for ARGs in samples from animals in treatment group A and $DCt_{no\ AB}$ represented the DCt value calculated for ARGs in samples from animals in treatment group B, without antimicrobial supplements. Finally, the fold

change of ARGs between study groups (FC) was calculated with the formula: $FC= 2^{DDCt}$. The relative abundance (RA%) of ARGs was normalized against 16S rRNA genes with a genetic copy estimate (GC); we used these formulas: $GC = 10^{((30-CT)/3.3333)}$ and $RA\% = GC_{ARG}/GC_{16SrRNA\ gene.}$ Colistin resistance was assessed in sows samples using *mcr*-1 PCR amplification [53].

Statistical analysis

All collected data were registered in MS EXCEL software and descriptive and inferential statistical analysis were performed in INFOSTAT (Statistic Software, version 2017). The impact of the antimicrobial restriction on the coliform count, antimicrobial susceptibility, and animal performance was assessed by T-test and Chi-Square respectively ($a \leq 0.05$). HT-qPCR data were analyzed according to previously established methods [32, 52].

Declarations

Ethics approval and consent to participate

All the experimental procedures were approved by the Animal Ethics Committee at Universidad San Francisco de Quito document Nr. 2016-004. A technical committee in the company provided their approval for this study (Document number Cn.Ce.Ca.16.04). The company's consent was implicit as the protocols were designed jointly between the researchers and Company's technical personnel; this agreement was confirmed in a consent letter sent from the Company to the Animal Ethics Committee at Universidad San Francisco de Quito. The number of animals under research was approved by farm managers. All facility adequation and sampling methods were performed under the farm's veterinarian supervision. Approval of all the protocols and publications were obtained from the technical managers of the company. The name of the company was kept confidential at the company's request. There is no national requirement to obtain institutional review board (IRB) approval for this type of study.

Consent to publish

Not applicable

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The research was conducted without any potential conflict of interest. The authors declare that they have no competing interests.

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Author contributions

GT Funding acquisition, Farm access agreement, and project conceptualization

FL: Experimental design, field and lab techniques, sampling procedures, data register and analysis, write and edition of the publication.

AT: experimental design, farm permissions, and animal welfare.

LZ Molecular methodology, technical support in high throughput qPCR. WaferGen SmartChip Real-time PCR system and data analysis.

LZ and GT manuscript edition

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Table 1. Antimicrobial additives used in pigs farm as prophylactics in group A.

Growth phase	Age (days)	Antimicrobial	Dose (ppm)	Administration via
0	21 - 28	Tilmicosin	200	Food
		Colistin	40	Food
1	29 - 34	Tiamulin	150	Food
		Chlortetracycline	450	Food
2	35 - 45	Tiamulin	150	Food
		Chlortetracycline	450	Food
3	45 - 70	Tiamulin	150	Food
		Chlortetracycline	450	Food
4	70 - 85	Chlortetracycline	450	Food
5	123 - 139	Chlortetracycline	450	Food
2	37 - 40	Trimetoprim-sulfamethoxazole	25mg/Kg/PV	Water
3	45 - 47	Doxicycline	10mg/Kg/PV	Water

Table 2. Total count of coliform colony forming units (CFU) in Mac Conkey lactose without antimicrobials. The average and standard deviation (SD) is shown for each treatment. Antimicrobial resistance ratios for ampicillin (AMP), cotrimoxazole (SXT) and Tetracycline (TET) were calculate using the total count of coliform colony forming units in Mac Conkey Lactosa plates with antimicrobials divided by the total count of coliform in Mac Conkey Lactosa without antimicrobials.

	Treatment A					Treatment B				
AGE (days)	TOTAL COUNT (AVERAGE)	SD	AMP	SXT	TET	TOTAL COUNT (AVERAGE)	SD	AMP	SXT	TET
5	1.73E+07	1.15E+07	0.48	1.22	2.15	2.34E+07	1.10E+13	0.39	1.55	2.38
30	6.71E+05	1.01E+06	0.78	0.56	0.66	5.15E+04	7.92E+14	0.98	0.43	0.97
50	9.17E+05	8.70E+05	0.26	0.48	0.83	8.90E+05	8.41E+14	0.48	0.48	1.01
100	2.46E+05	1.68E+05	0.38	0.94	0.99	7.40E+04	1.16E+13	0.48	1.48	0.72
140	1.08E+05	1.68E+05	0.34	0.32	1.05	2.65E+05	2.55E+14	0.25	0.14	1.2

Table 3.- Antimicrobial susceptibility test from coliform isolated. Strains are classified by sampling period (1.- 5days; 2- 30days; 3.- 50 days; 4.- 100 days; 5.- 140 days) and treatment group (A.- with antimicrobials; B.- without antimicrobials) (p = 0,77). p was calculated based on sampling period comparison. Strain with more than 2 resistances was count as multidrug resistant (MDR). AMP ampicillin (10mg), TET tetracycline (30mg), SXT trimethoprim-sulfamethoxazole (1.25/23.75mg), GEN gentamycin (10mg), AMC amoxicillin-clavulanic ac.(20/10mg), CIP ciprofloxacin (5mg), CHLOR chloramphenicol (30mg) and COX ceftriaxone (30mg) were used to perform the antimicrobial susceptibility test.

	TREATMENT A												**TREATMENT B**										
Group	1		2		3		4		5		6		1		2		3		4		5		6	
AMR	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**p**
MDR	28	60.8	28	52.8	20	95.2	29	53.7	19	51.3	49	89.1	27	58.7	39	73.5	24	92.3	31	54.4	14	45.2	46	79.3	0,989
AMP	32	69.5	29	54.7	21	100.0	25	46.3	36	97.3	52	94.5	22	47.8	40	75.5	26	100.0	30	52.6	29	93.5	55	94.8	0,957
AMC	16	34.7	11	20.7	17	80.9	3	5.5	19	51.3	29	52.7	7	15.2	16	30.1	18	69.2	5	8.8	15	48.4	32	55.2	0,497
SXT	19	41.3	32	60.3	8	38.1	31	57.4	21	56.7	40	72.7	21	45.6	35	66.0	11	42.3	34	59.6	10	32.2	39	67.2	0,654
TET	46	100.0	52	98.1	21	100.0	52	96.3	0	0.0	44	80.0	45	97.8	49	92.4	25	96.1	51	89.5	1	3.3	48	84.2	0,377
CIP	6	13.0	8	15.1	1	4.7	11	20.3	2	5.4	4	7.3	0	0.0	18	33.9	3	11.5	13	22.8	1	3.2	4	6.9	0,649
CHLOR	10	21.7	14	26.4	10	47.6	16	29.6	6	16.2	20	36.3	10	21.7	25	47.2	11	42.3	22	38.6	10	32.2	20	34.5	0,194
GEN	9	19.5	8	15.0	5	23.8	17	31.5	3	8.1	11	20.0	19	41.3	21	39.6	6	23.1	18	31.6	1	3.2	7	12.1	0,382
COX	12	26.0	4	7.5	11	52.4	5	9.2	4	10.8	5	9.1	5	10.9	7	13.2	11	42.3	7	12.2	4	12.9	2	3.4	0,365

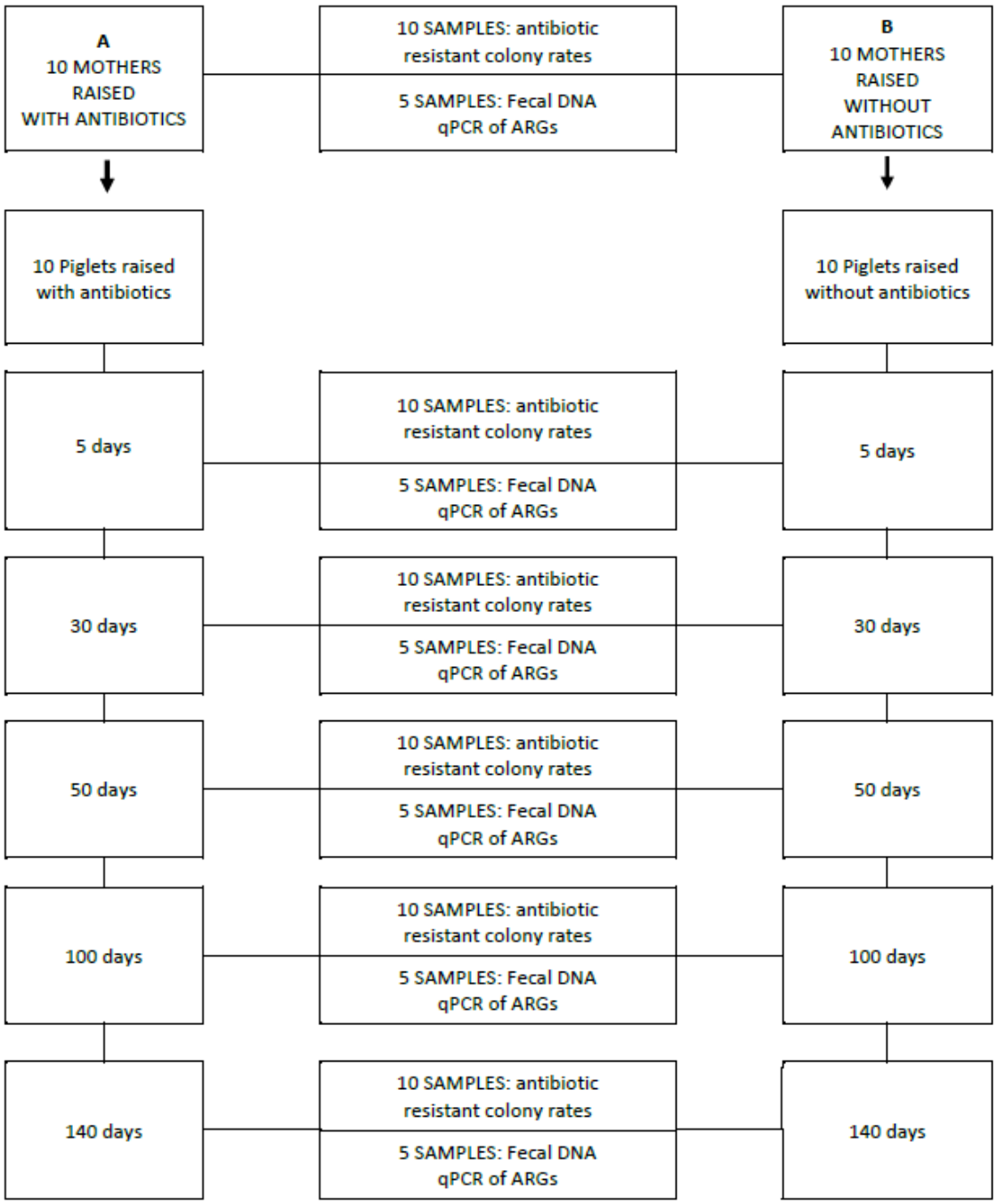

Figure 1. Workflow for each treatment group of pigs (A and B). Treatment group A was fed with antimicrobial supplements. Treatment group B was fed no antimicrobial supplements. Ten young female pigs (70d) were randomly selected for each treatment. All piglets born were maintained under the same treatment as their mothers. A homogeneous group of 32 piglets (similar age and weight) within each treatment group were selected until the end of the experiment. Rectal swab samples were collected at days: 5; 30; 50; 100; and 140. Samples from sows were taken 180 days after they were born (6).

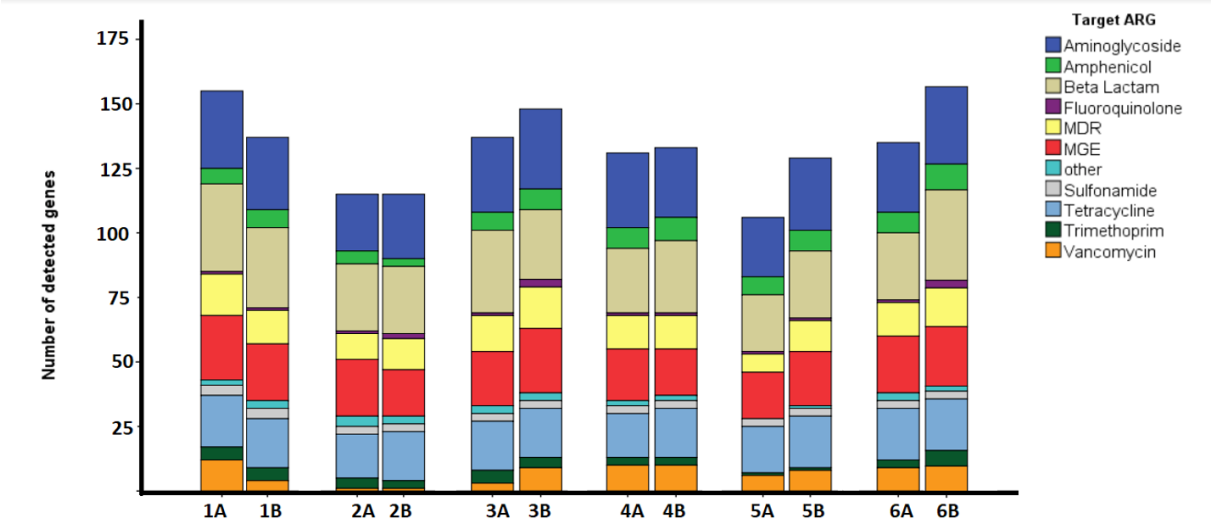

Figure 2. Antimicrobial resistance gene abundance and richness. The colors indicate gene type. Different bars represent growing phase of piglets (1: 5 days; 2: 30 days; 3: 50 days: 4: 100 days, and 5:140 days) and sows (180 days). Animals feeding antimicrobials were identified as A and animal without antimicrobial additives were identified with B.

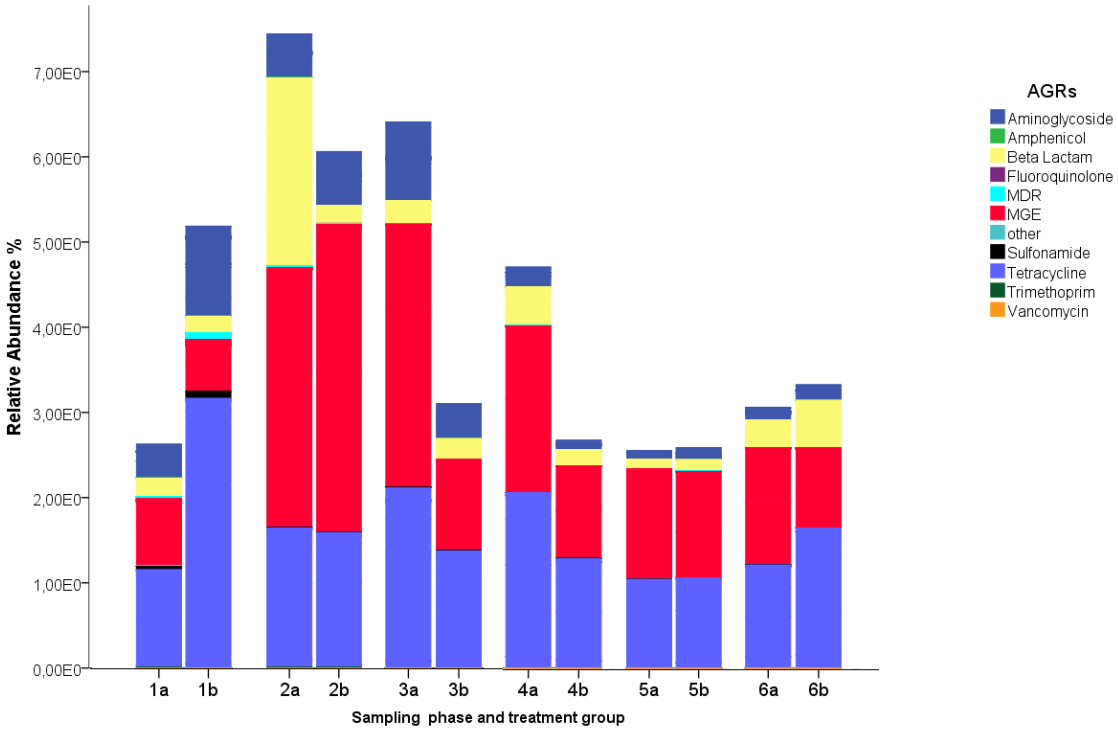

Figure 3. Relative abundance (RA%) of antimicrobial resistance genes, grouped by sampling phase and treatment group (A, feed with antimicrobials; B feed without antimicrobials; 1: 5 days; 2: 30 days; 3: 50 days; 4: 100 days, 5: 140 days and 6: sows 180 days. Different colors indicate different genes such as ARGs and MGEs. Relative abundance (RA%) measures the amount of ARGs relative to the housekeeping gene 16Sr RNA.

Chapter III

Effect of diet modification in intestinal *Escherichia coli* population and antimicrobial resistance

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Abstract

Introduction: Fluctuations in the number of some bacterial lineages in the intestine may be associated with an increased antimicrobial resistance transmission and disease. Adaptation to a given environment may select bacterial mutants that have a reduced ability to adapt to new environments. We posit that corn-rich substrate supplemented with antimicrobials may have selected certain *Escherichia coli* lineages that thrive under these conditions but have reduced adaptation to new substrates.

Methods and results: We subjected 50 chicken from an industrial operation (under corn-based diet supplemented with antimicrobials) to an alfalfa-based and added antimicrobial-free diet. Fresh feces were collected, and 5 *E. coli* colonies were obtained from each animal. Isolates were subjected to genetic typing and antimicrobial susceptibility testing. Results showed high diversity and high turnover rate of

numerically dominant *E. coli* strains from animals in either a corn-based diet or an alfalfa-based diet.

Discussion: Our results suggest that there is a high diversity of *E. coli* strains in the intestines which have the aptitude to grow efficiently in different substrates. We also found that *E. coli* strains have a high turnover rate regardless of the type of diet. Decades of the coevolution of the *E. coli* genome with some antimicrobial resistance genes have restored the bacterial aptitude to adapt to different niches.

Keywords: Commensal *E. coli,* linage diversity, antimicrobial resistance, ecology, evolution, eco-evo.

Introduction

A warm-blooded animal may harbor more than 10 different commensal *Escherichia coli* lineages in its intestine, some lineages are numerically dominant (Lautenbach et al., 2008). The relative lineage abundance of some *E. coli* lineages is critical because many lineages carry genes involved in virulence and antimicrobial resistance (Liu et al., 2018). Linage abundance in the intestine may depend on phage infection, protozoan predation, animal immunity, and lineage aptitude to use substrates in the intestine (Brito et al., 2016; Sutton & Hill, 2019; Tenaillon et al., 2010; Wildschutte et al., 2004)

It has been shown that different diets have a profound impact in the relative abundance of intestinal bacterial species (Chung et al., 2016; Frese, Parker, Calvert, & Mills, 2015; Gagnon et al., 2007; Niu et al., 2015; Rowland et al., 2018). However, little research has been carried out on the impact of diets in bacterial lineages belonging to the same bacterial species, even though different members of the same bacterial species may have lost or acquired different metabolic properties through mutation or horizontal gene transfer (Hehemann et al., 2010; Brito et al., 2016; Leiby & Marx, 2014). These properties may enable some strains to use some substrates present in the diet. Adaptation to use some substrates may reduce their ability to proliferate in other substrates (Buckling et al. 2003; Leiby & Marx, 2014). Genome analysis and culture experiments showed that different *E. coli* strains have different metabolic phenotypes with a variable aptitude to use some substrates (Baumler et al., 2011; Monk et al., 2013; Bouvet et al., 2017; Barrera et al., 2019); even a single *E. coli* strain passaged thousands

of times in culture media produce descendants with different growth rates in different substrates (Leiby & Marx, 2014).

Similarly, antimicrobial resistance may also affect the proliferation of some members of a bacterial species in the intestine. Genes involved in antimicrobial resistance cause fitness costs, and even though these costs are eventually ameliorated by compensatory mutations (MacLean et al., 2010). These compensatory mutations could also have fitness costs and may limit the bacterial ability to diversify and adapt to new environments or substrates (Buckling et al. 2003).

In this study, we aimed to observe how the *E. coli* lineages change, in chicken intestines, as diet is altered. We argue that the use of corn in animal feeds (in industrial operations) may have select some o *E. coli* variants which perform better in the presence of these substrates and should be very common in chicken fed corn-based diets. We analyzed the effects of a diet change in the relative frequency of numerically dominant *E. coli* strains. We also assessed the effect of diet change in the frequency of antimicrobial multi-resistant phenotypes in numerically dominant *E. coli*.

Methods

All protocols of experimental design were approved by the ethics and biosecurity committee of the Animals Ethics Committee of Universidad San Francisco de Quito before the study. Chicken used in this study were donated by an Ecuadorian broilers industry and were previously vaccinated against Marek Gumboro, New Castle, and Bronchitis deseases.

Animals

One hundred chicken (Cobb genetics) were donated from an industrial operation where animals are fed with a corn-based diet; all animals were kept in the same diet (without added antimicrobials) for 2 weeks before splitting chicken into different study groups. A completely randomized design was conducted with 4 groups with 25 chicken each. Animals were raised in two locations (1.- USFQ 2.- Valley farm) and at each location, one group of animals was feed with the conventional formula (Diet 1; D1), and the other group was feed with an alternative formula based in dry alfalfa pellets, none of the animals received antimicrobials (Supplementary materials Table S1). This regimen was maintained for the following 5 weeks. Water was available *at libitum*. Each chicken was an experimental subject which has identified with a mark painted in the plumage. Fecal samples were collected from ten chickens from each pen.

Samples and phenotypic analysis

Fecal samples were taken from 10 randomly selected chicken which were marked for identification. Each chick was separated in a clean cardboard box until a fecal sample was obtained in a sterile container and maintained in ice for transportation to the lab within one hour after collection. Samples were plated on MacConkey agar (Becton Dickinson) and incubated at 37°C for 18hours. Five lactose positive colonies were selected form the plate and β–glucuronidase activity was confirmed using Chromocult Agar (Merck).

Antimicrobial susceptibility test

Five confirmed *Escherichia coli* isolates from each plate were isolated and stored at -80°C in TSB with 30% glycerol (Cho et al., 2007). Antimicrobial susceptibility tests were performed using AMP ampicillin (10mg), TET tetracycline (30mg), SXT trimethoprim-

sulfamethoxazole (1.25/23.75mg), GEN gentamycin (10mg), AMC amoxicillin-clavulanic ac. (20/10mg), CIP ciprofloxacin (5mg), CHLOR chloramphenicol (30mg), IMP Imipenem (5mg), CF cefazolin (30mg), CAZ ceftazidime (30mg), FEP cefepime (30mg), and CTX cefotaxime (30mg) as representatives of the most used families of antibacterial drugs in health care (Eisenberg et al., 2012; Kozak et al., 2009). The Kirby Bauer test was carried out following CLSI (Clinical & Laboratory Standards Institute) guidelines using clinical settings for sensible or resistant phenotype interpretation.

Strain Genotyping

To determine whether the alfalfa diet could change the numerically dominant *E. coli* lineages, we analyzed the nucleotide sequences of the *fumC* gene in all isolates, as previously published (Barrera, Cardenas, Graham, & Trueba, 2019), and some strains showing identical sequence were submitted to full multilocus sequence typing (MLST) analysis. Briefly, the DNA from each isolate was released by the boiling method (Dashti, Jadaon, Abdulsamad, & Dashti, 2009), two colonies (from each isolate) were placed in a test tube with 1 mL of molecular grade water, paced in a heat block at 100°C for 10 min., transferred to an ice bath for 30 seconds, and centrifuged for five minutes at 1,680 x *g* and supernatants were stored at -20°C for further analysis. The *fumC* gene was amplified, as previously described (Wirth et al., 2006). Potential clonal isolates carrying *fumC*4 and *fumC11* were subjected to MLST to confirm clonality, as previously reported (Wirth et al., 2006). Amplicons were purified and sequenced using commercial service based on the Illumina MiSeq platform at Macrogen Inc. (Seoul, Republic of Korea). Allele and MLST were obtained using *Escherichia coli* MLST Database v1.12 (accessed on date XXX and available online: http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) (Zhou, 2020) to

define clonal relatedness, as previously done in other studies (Carattoli, 2013; do Monte et al., 2017).

Statistical analysis

Overall antimicrobial resistance was estimated at the isolate level with 95% confidence intervals. The AMR prevalence from feed treatment subgroup-collection was also estimated at the isolate level. For multidrug-resistant (MDR) estimation, isolates with resistant phenotype for three or more antimicrobial families were assigned as MDR. Prevalence estimates were carried out using the SPSS software version 24.0 (Armonk, NY: IBM Corp). The antimicrobial resistance profiles between different time points (2 weeks and 7 weeks) or feed source (corn-based vs. alfalfa-based) were compared by using the $\chi 2$ test; p values <0.05 were considered significant. For reduction analytic dimensions, principal components analysis for categoric variables were applied using CATPCA version 2.0 by Leiden SPSS Group (Leiden University, The Netherlands). Data were analyzed in a scatter plotter using XLSTAT (version 2019.4.2, Addinsoft Inc., Boston, MA, USA). Sequence types were assigned to *E. coli* isolates with a probable clonal relation and phylogenetic analysis was conducted on Mr.Bayes version 3.2 bases on MCMC algorithms (Lakner, van der Mark, Huelsenbeck, Larget, & Ronquist, 2008; Ronquist et al., 2012). Evolutionary diversity analyses were conducted using the Maximum Composite Likelihood model in MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018.; Tamura, Nei, & Kumar, 2004).

Results

We found a large diversity and high turnover rates of *E. coli* lineages, demonstrating that the 243 isolates had a limited number of *fumC* alleles (n=47), where *fumC11* (n=72) and *fumC4* (n=30) were the most common (Figure 1). We run MLST analysis in a subset of the most common alleles: *fumC11* (n=16) or *fumC4* (n=8), all of them belonged to different sequence types except for 5 ST48 (*fumC*4) isolates found in 4 different animals with corn diet at week 2 and 1 isolate, from a different animal in alfalfa diet at week 7 (Supplementary materials Table S2).

Principal components analysis did not show any association between MLST profile and diet (Figure 2). Phylogenetic analysis of concatenated MLST sequences showed that some isolates from animals feed with alfalfa of corn did not form a cluster (Figure S1). Among all analyzed sequences, the number of base substitutions per site was 0.27 from the mean of diversity calculation, the standard error estimation was 0.18 bases on the bootstrap procedure (100 replicates) (Nei & Kumar, 2000). The analysis was conducted using the maximum composite likelihood model (Tamura et al., 2004). Shannon diversity analysis of *fumC* alleles in both populations showed an H value of 2.04 for isolates from a corn-based diet and 2.43 for isolates from an alfalfa-based diet.

There was no significant difference in antimicrobial susceptibility *E. coli* from different diets (Supplementary materials Table S3) as 11. 1% of the strains from chickens with a corn-based diet and 18.7% of chickens in the alfalfa-based diet, were sensitive to all antimicrobials. After 5 weeks of intervention, 32 *E. coli* isolates from chicken with a conventional diet were MDR compared with 30 isolates from chickens with an alternative diet. The principal component analysis did not show differences between

groups when the antimicrobial resistance phenotype was analyzed (Figure 3). In location 1, chicken feed with conventional diet were more frequent carriers of *E. coli* resistant to tetracycline, cotrimoxazole, chloramphenicol, and ciprofloxacin. However, multidrug-resistant patterns were not statistically different either between sites or diet (X2; p>0,05). Isolates susceptible to all antimicrobials corresponded to 10.75% (n= 389). The most important resistance phenotype was tetracycline (TET; 11.25%) followed by tetracycline and cotrimoxazole (TET SXT; 8.25%). Antimicrobial resistance profiles *E. coli* were grouped in phenotype patterns. Some patterns were present in less than 1,00% isolates and represented the 15.75% of total phenotype patterns described (Supplementary materials Figure S2). MDR (resistance for 3 or more antimicrobials from a different family) was detected in 52.20% of *E. coli* strains. The most frequent combination was resistance to tetracycline, cotrimoxazole, chloramphenicol, and ciprofloxacin followed by tetracycline, cotrimoxazole, and ciprofloxacin combination with a 7 and 6.30% respectively.

Comparison of isolates obtained at 2 weeks and 7 weeks showed significantly less resistance, regardless of the diet (X_2; $p< 0.05$) (Supplementary materials Figures S2 and S3) (Table S1) except for cotrimoxazole, cefepime, and ceftriaxone.

Discussion

The present study showed a high diversity and a high turnover rate of dominant *E. coli* lineages associated with chicken intestinal microbiota in all groups (Figure 1). We failed to find the same clones in samples obtained from the same individuals after 5 weeks with the same diet. These findings agree with previous report showing high diversity and turnover rates of *E. coli* in intestines (Richter et al., 2018). We were not able to show any contribution of diet to strain diversity or turnover. The reason for this rapid turnover of dominant strains seems unclear but could be due to differential destruction of some *E. coli* lineages by bacteriophages, which are the most diverse and numerous members of the microbiota and known to drive bacterial diversity in many ecosystems including the gut (Sutton and Hill, 2019). To a lesser extent, protozoan predation may also influence strain turnover (Wildschutte et al., 2004). Additional contributors to diversity could be fecal material from other animals, which we cannot rule out in our experiments. Some *E. coli* lineages from other animals seem to move constantly between different species of warm-blooded animals (Salinas et al., 2019) and therefore use diverse substrates. This may be different in anaerobic bacteria, such as *Bacteroides*, which do not transmit easily among different animal hosts (Moeller, 2018) and may possess genes enabling the use of specific substrates present in the diet of a given animal species (Hehemann et al., 2010; Brito et al.,2015). We also failed to see clonal groups with a better aptitude to grow in alfalfa or corn (Supplementary materials Figure S1), which may indicate that genetically related strains may have different aptitude to grow in either of these substrates.

We did not observe any significant variation in antimicrobial resistance in any group (Supplementary materials Figure S3), which may indicate that antimicrobial susceptible strains do not have any adaptive difference when forced to grow in different substrates. Also shows that these genes are widely distributed in diverse strains of *E. coli* (numerically dominant and minority) and strains with different metabolic capabilities. The similar ability to adapt to different substrates observed in antimicrobial-resistant and sensitive *E. coli* isolates may the result of decades of antimicrobial use, which have selected strains with compensatory mutations that gradually regained metabolic diversity. Some antimicrobial resistance in *E. coli* emerged shortly after the introduction of antimicrobials, more exactly: sulfonamides were introduced in the 1930s and resistance was observed in 1950 (Tadesse et al., 2012); tetracycline was developed in 1948, resistance was observed in 1953 (Roberts, 1996); and finally, chloramphenicol was developed in 1947 (Tadesse et al., 2012) and resistance was found in 1955 (Watanabe, 1963). Our data are in concur that antimicrobial resistance genes (ARGs), such as those affecting cotrimoxazole, chloramphenicol, and tetracycline, have disseminated in most *E. coli* lineages (numerically dominant and minority) (Tadesse et al., 2012). Similarly, plasmids carrying these ARGs have evolved several mechanisms to ameliorate the fitness costs or to contain toxin-antitoxin genes (Andersson & Hughes, 2010; Bustamante & Iredell, 2017; Finn, Shewaramani, Leahy, Janssen, & Moon, 2017). Decades of co-evolution may have created a successful association of *E. coli* with plasmids and antimicrobial resistance genes, which constitutes a serious obstacle to reverse antimicrobial resistance. Contrastingly, a recent acquisition of antimicrobial resistance such vancomycin resistance in *Enterococcus faecalis* and colistin resistance in

Enterobacteriaceae (mediated by the *mcr*-1 gene) have been easily reduced by eliminating the supplementation with these type of antimicrobials (Pantosti, Grosso, Tagliabue, Macri, Caprioli, 1999; Wang et al., 2020).

Finally, we observed a statistically significant reduction of isolates displaying antimicrobial resistance after five weeks, in all groups of animals (Supplementary materials Figure S3.) Previous studies have found that the proportion of antimicrobial-resistant strains is higher in 1-day- old than in older chickens (Hedman et al., 2019; Moreno et al., 2019). It has been proposed that *E. coli* lineages carried by 1-day chickens may gradually disappear as chicken grow. This reduction may indicate that some plasmids and ARGs do cause fitness reduction.

Commensal *E. coli* plays an important role in the transmission of antimicrobial resistance from food-animals to humans (Berg et al., 2016; Hu et al., 2016). The persistence of antimicrobial resistance even in the absence of antimicrobials is a serious public health concern (MacLean et al., 2010; Andersson and Hughes, 2011).

Conclusions

We can conclude that the diversity of *E. coli* strains in chicken intestines is large and diet may not an important force driving the diversity of *E. coli* in the intestine. The diet modification that was applied in this study does not affect the presence of antimicrobial-resistant *E. coli* in the intestine**.**

Authorship

F.L. and D.G. carried out the experiments. F.L and G.T. wrote and edited the manuscript. A.T. helped supervise animal welfare, training in chicken farms, logistics, and facilities

suggestions. G.T. conceived the original idea. F.L. should be considered the first author and G.T. should be considered the senior author.

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Ethics

All the experimental procedures were approved by the Ethics Committee for Animal Research at Universidad San Francisco de Quito. Vaccines were administered to all animals. Animal welfare was supervised in each phase of the experiment, no invasive procedures were included. Fresh feces were taken in individual cages.

Conflict of interest

The authors declare that they have no conflict of interest regarding this publication.

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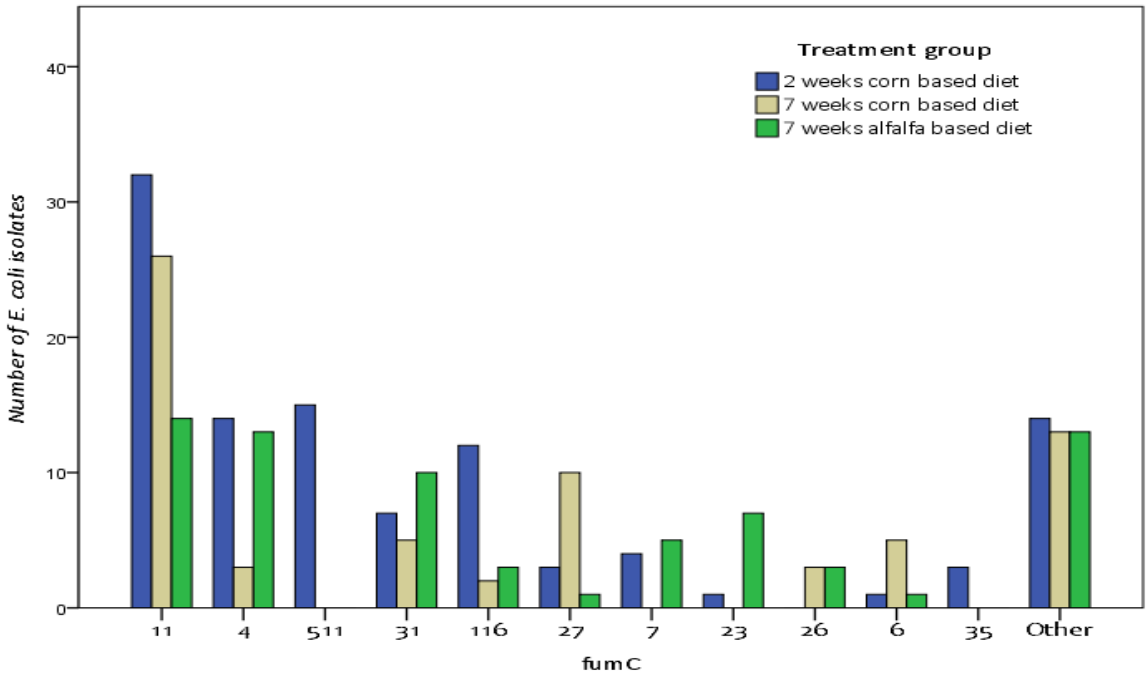

Figure 1. Number of *Escherichia coli* isolates carrying different *fumC* alleles from study groups. A total of *E. coli* isolates (n=243) were isolated from chicken feces, where 106 belonged to 2 weeks old chickens (blue bars), 67 isolates were isolated from chicken with corn-based feed (yellow bars), and finally, 70 isolates derived from chickens with alfalfa-based feed (green bars). Alleles with less than 1% of frequency where merge in "Other" category.

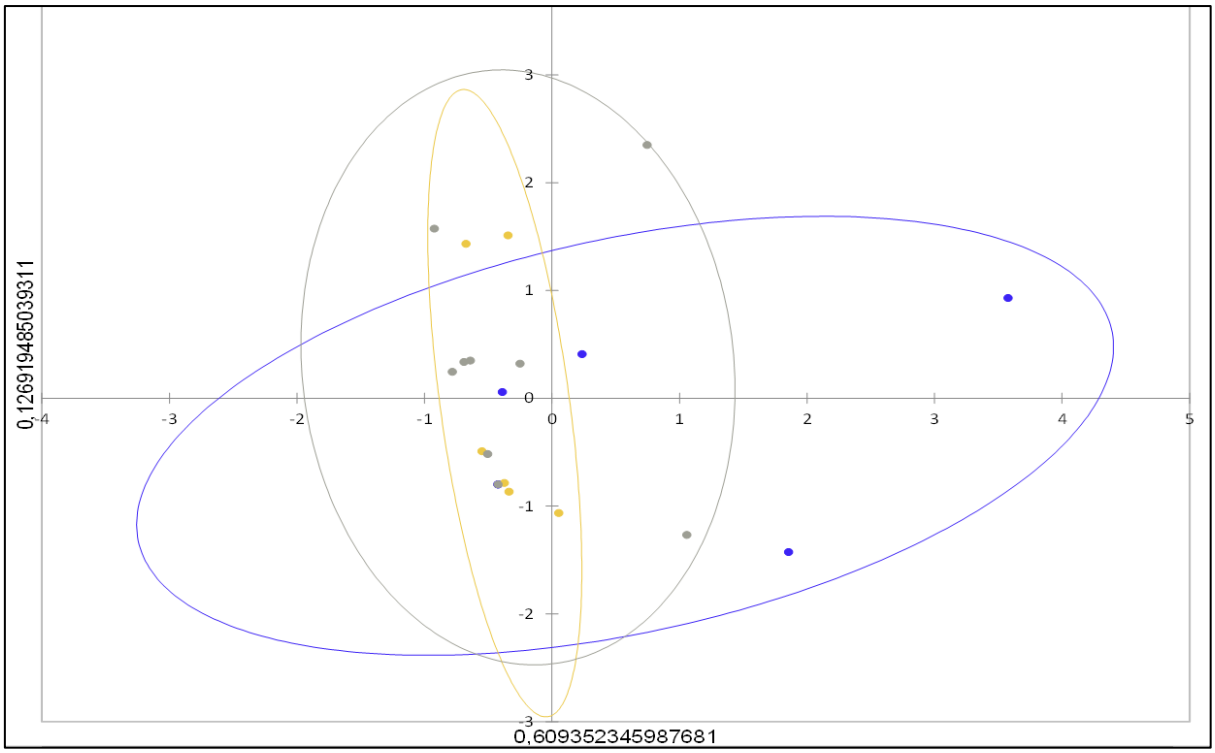

Figure 2: Principal components of 7 housekeeping genes profile of selected *Escherichia coli* isolates. 1. Blue dots represent isolates from 2 weeks chicken (basal), 2. Yellow dots represent isolated from 7-week chickens feed with a corn-based diet. 3. Grey dot represents isolates from 7-week chickens feed with an alfalfa-based diet. Confidence intervals 95% based on chi-square are graph in colored ellipses according to the origin of isolates.

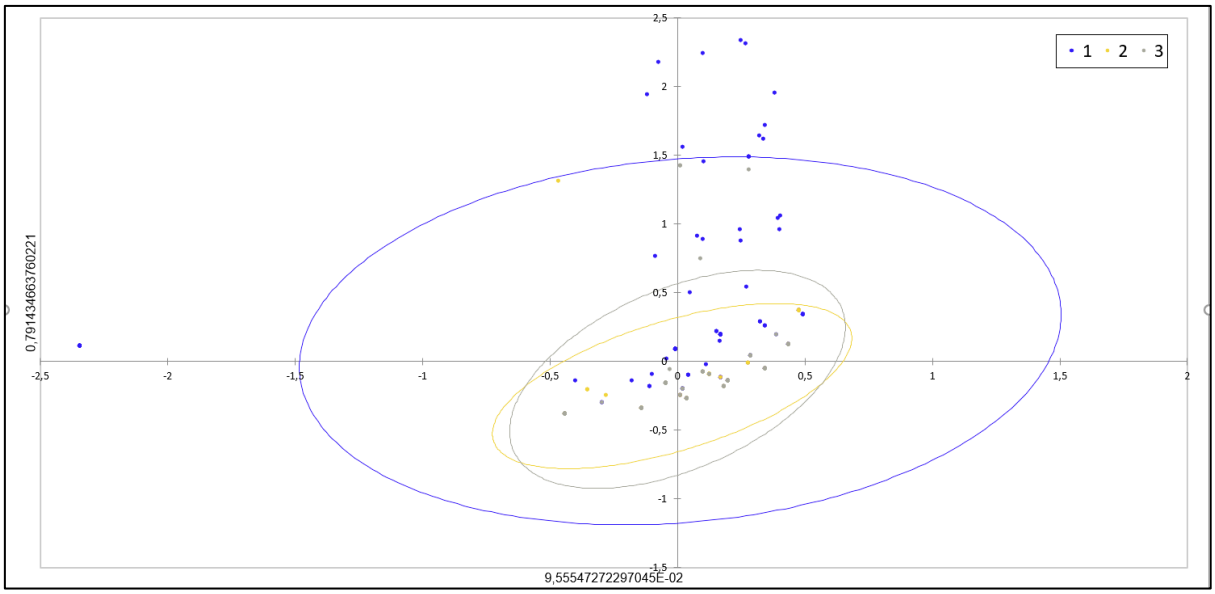

Figure 3. Principal components of phenotypic antimicrobial resistance profiles of 389 *Escherichia coli* isolates. 1. Blue dots represent isolates from 2 weeks chicken (basal), 2. Yellow dots represent isolated from 7-week chickens feed with a corn-based diet. 3. Grey dot represents isolates from 7-week chickens feed with an alfalfa-based diet. Confidence intervals 95% based on chi-square are graph in colored ellipses according to the origin of isolates.

Supplementary Materials

Table S1. Detailed analysis of diet formulation administered in each treatment groups.

	Corn-based Diet	Alfalfa-based Diet
Alfalfa pellets	0%	100%
Corn	40%	0%
Sorgo	5%	0%
Wheat	12%	0%
Rice	3%	0%
Soy	5%	0%
Soy paste	24%	0%
Fish floor	3%	0%
Bird floor	3%	0%
Salt	0.376%	0%

Table S2.- Sequence type from *Escherichia coli* strains. Alleles are described from each isolate. Treatment group: 1) 2-weeks-old chickens with a corn-based diet. 2) 7-weeks-old chicken with a corn-based diet and 3) 7-weeks-old chicken with an alfalfa-based diet. Also, phenotypic patterns of antimicrobial susceptibility from each isolate are described according to the antimicrobial discs used in Kirby Bauer test: AMP ampicillin (10mg), TET tetracycline (30mg), SXT trimethoprim-sulfamethoxazole (1.25/23.75mg), GEN gentamycin (10mg), AMC amoxicillin-clavulanic ac. (20/10mg), CIP ciprofloxacin (5mg), CHLOR chloramphenicol (30mg), IMP Imipenem (5mg), CF cefazolin (30mg), CAZ ceftazidime (30mg), FEP cefepime (30mg), and CTX cefotaxime (30mg).

			Alleles							*MLST*
Treatment group	sample ID	Antimicrobial phenotype	*adk*	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	ST
2	3.2	AMC TET SXT CHLOR AMP CIP	-196	11	55	101	113	40	38	ni
1	3.3	TET SXT CHLOR AMP CIP	6	11	3	18	70	8	6	ni
1	8.2	TET SXT CIP CHLOR	6	11	4	8	8	8	2	48
2	8.3	TET SXT CHLOR CIP	-199	11	4	8	8	1	4	ni
3	13.3	TET CHLOR AMP	6	4	4	10	7	8	14	ni
3	13.1	AMC TET CTX FEP SXT AMP	6	11	14	16	24	8	6	2497
3	15.4	TET IMP AMP	6	11	4	8	8	8	2	48
1	14.1	TET	6	4	14	16	24	8	14	155
1	16.3	AMC TET SXT AMP CIP CF	6	4	4	16	24	8	14	58
3	16.2	TET SXT	10	4	14	16	24	62	2	ni
1	18.3	TET SXT CIP AMP GN	6	11	4	8	8	8	2	48
3	18.2	TET CIP	10	4	4	8	8	8	4	ni
3	20.1	TET AMP	10	11	4	10	7	8	2	2705
1	20.3	TET SXT AMP CIP	6	4	14	1	20	62	7	345
2	21.4	TET AMP	6	11	5	8	8	8	2	6396
2	24.2	TET SXT CIP	10	4	4	10	24	8	14	5519
2	25.5	TET CHLOR CIP	24	11	4	8	8	8	73	73
2	26.4	TET SXT CIP	10	4	4	8	8	8	14	2883
2	26.5	TET SXT CIP	6	11	4	8	7	8	2	5224
1	27.4	TET SXT CIP AMP GN IMP CHLOR	6	11	4	8	8	8	2	48
1	28.5	AMC TET CTX CAZ SXT AMP CIP CF	10	11	14	8	8	8	313	4536
1	29.1	TET SXT CIP	6	11	4	8	8	8	2	48
2	30.3	TET SXT CHLOR AMP	6	11	14	10	7	8	2	ni
3	35.1	TET SXT CIP	24	11	4	8	8	8	2	43
3	39.1	TET SXT CIP	10	11	4	10	8	8	2	4704
3	40.4	TET SXT	10	11	4	8	8	1	2	1585

ni.- no identified

Table S3. Frequency of resistance phenotype analyzed by the antimicrobial disc in Kirby Bauer test. In first column antimicrobial used are listed: AMP ampicillin (10mg), TET tetracycline (30mg), SXT trimethoprim-sulfamethoxazole (1.25/23.75mg), GEN gentamycin (10mg), AMC amoxicillin-clavulanic ac. (20/10mg), CIP ciprofloxacin (5mg), CHLOR chloramphenicol (30mg), IMP Imipenem (5mg), CF cefazolin (30mg), CAZ ceftazidime (30mg), FEP cefepime (30mg), and CTX cefotaxime (30mg). The letter after the percentage of resistant isolates in each group means the category according to X2 test. The same letter in three columns means that the proportions are not significantly different under the 0.05 level.

	Treatment group										
	2 weeks Corn diet			7 weeks Corn diet			7 weeks alfalfa diet			Total		χ_2 p-Value
	n	%		n	%		n	%		n	%	
CF	17	9.0	a	1	1.0	b	3	3.0	b	21	5.4	0.08
CIP	122	64.6	a	24	24.0	b	33	33.0	b	179	46.0	0.000000000004
AMP	102	54.0	a	32	32.0	b	39	39.0	b	173	44.5	0.01
CHLOR	73	38.6	a	24	24.0	b	25	25.0	b	122	31.4	0.011
IMP	9	4.8	a	0	0.0	a	1	1.0	a	10	2.6	0.027
SXT	104	55.0	a	49	49.0	a	46	46.0	a	199	51.2	0.304
GN	33	17.5	a	0	0.0	b	0	0.0	b	33	8.5	0.000000005
CAZ	15	7.9	a	0	0.0	b	0	0.0	b	15	3.9	0.00026
FEP	4	2.1	a	0	0.0	a	3	3.0	a	7	1.8	0.252
CTX	12	6.3	a	1	1.0	a	4	4.0	a	17	4.4	0.104
TET	176	93.1	a	76	76.0	b	79	79.0	b	331	85.1	0.000073
AMC	42	22.2	a	7	7.0	b	4	4.0	b	53	13.6	0.000008

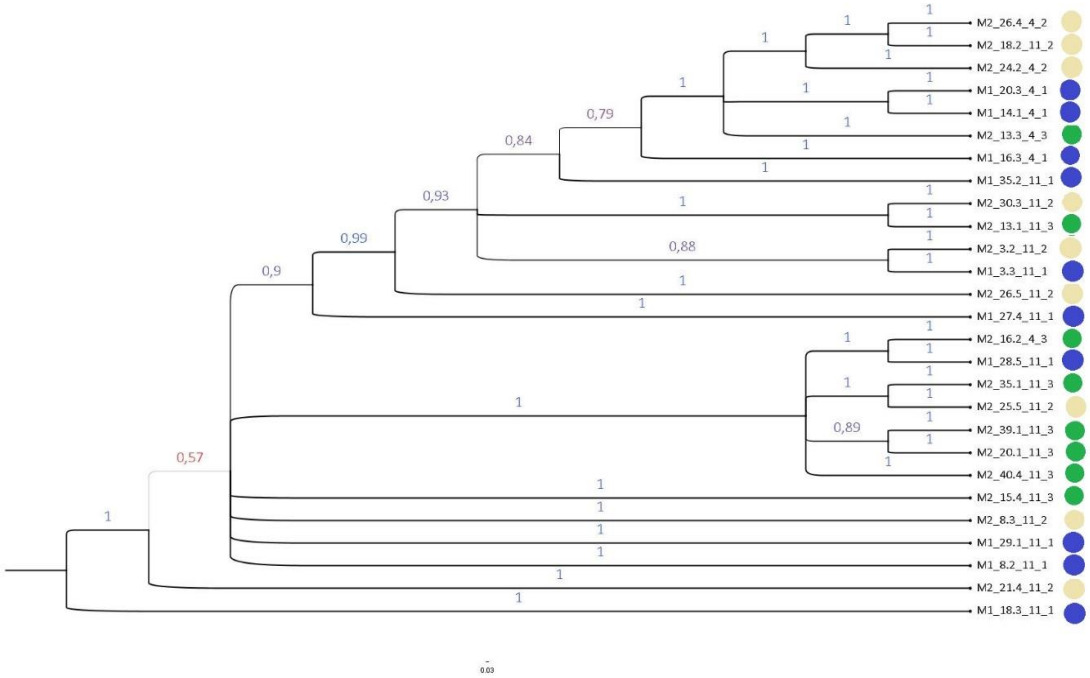

Figure S1. Bayesian phylogenetic tree was constructed from seven concatenated housekeeping genes used for MLST *E. coli* analysis. 1. Isolates from 2-week-old chicken (blue dots), 2. Isolates from 7-years-old chickens feed with a conventional corn-based formula (yellow dots) and 3. Isolates from 7-years-old chickens feed with an alfalfa-based formula (green dots). The identification label describes M1.-2 weeks old chicken, M2.- 7 weeks old chicken, followed by the ID number of each animal. After the underscore, *fum*C allele 4 or 11 are described. The last number after the second underscore confirms the treatment group. Details of the Bayesian analysis are provided in "dataset" file, which yielded 200,000 trees in the final MCMC sample. The rooted tree produced by the analysis with MrBayes software showed the posterior probabilities labelled on each branch.

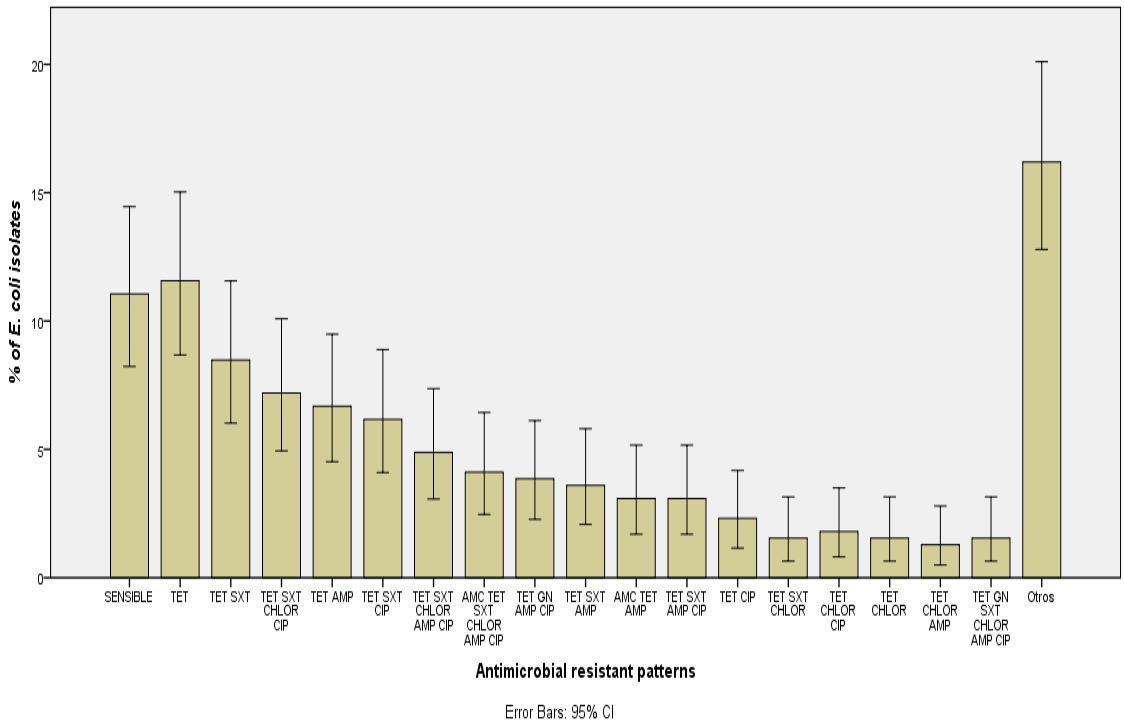

Figure S2. Overall frequency distribution of antimicrobial susceptibility patterns from 389 commensal *Escherichia coli* isolated from chicken feces in Mac Conkey Lactosa plates; 189 from 2 weeks old chickens, 100 from 7 weeks old chicken feed with a corn-based diet, and 100 from 7 week old chickens that were feed with alfalfa-based formula. Any diet has antimicrobial supplements. Kirby Bauer technique was performed for antimicrobial susceptibility testing (AST). AMP ampicillin (10mg), TET tetracycline (30mg), SXT trimethoprim-sulfamethoxazole (1.25/23.75mg), GEN gentamycin (10mg), AMC amoxicillin-clavulanic ac. (20/10mg), CIP ciprofloxacin (5mg), CHLOR chloramphenicol (30mg), IMP Imipenem (5mg), CF cefazolin (30mg), CAZ ceftazidime (30mg), FEP cefepime (30mg), and CTX cefotaxime (30mg).

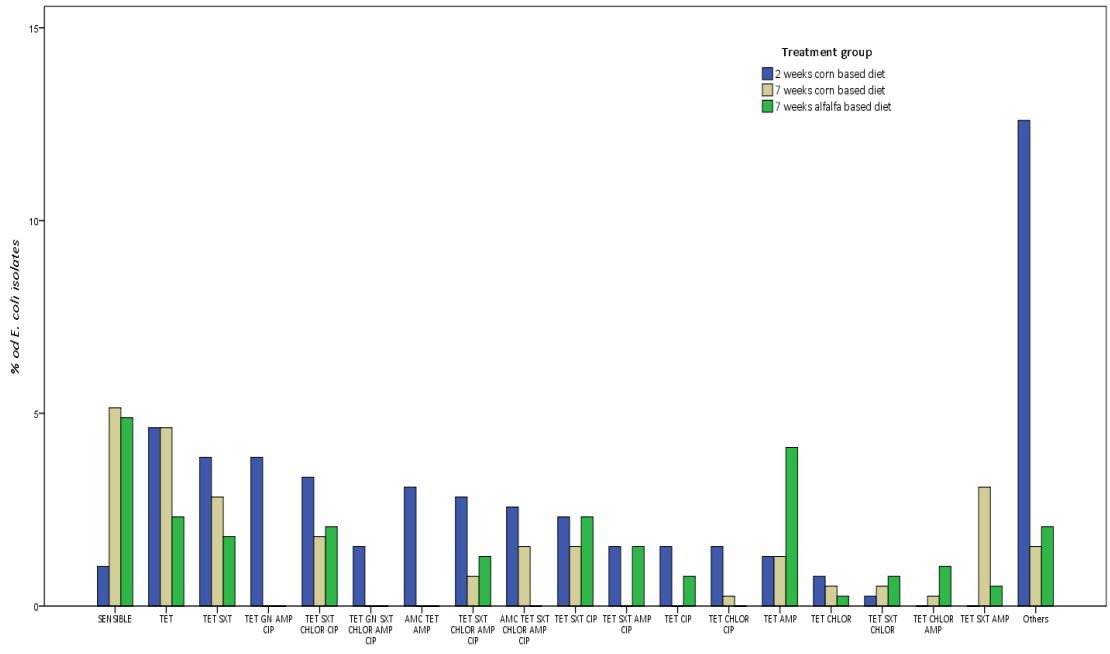

Figure S3. The number of *E. coli* isolates showing a specific phenotypic pattern of antimicrobial resistance. *E. coli* isolates (n=389) were isolated from chicken feces in Mac Conkey Lactosa plates; 189 from 2 weeks old chickens (blue bars) and 200 from 7 weeks old chickens, separated by feed administration 100 with corn-based feed (green bars), and 100 from chickens with *alfalfa-based* feed (yellow bars). Kirby Bauer technique was performed for antimicrobial susceptibility testing (AST) and antimicrobial-resistant phenotype patterns are shown. Patterns with less than 1% where merge in "Other" category. AMP ampicillin (10mg), TET tetracycline (30mg), SXT trimethoprim-sulfamethoxazole (1.25/23.75mg), GEN gentamycin (10mg), AMC amoxicillin-clavulanic ac. (20/10mg), CIP ciprofloxacin (5mg), CHLOR chloramphenicol (30mg), IMP Imipenem (5mg), CF cefazolin (30mg), CAZ ceftazidime (30mg), FEP cefepime (30mg), and CTX cefotaxime (30mg).

Chapter IV

Journal of global antimicrobial resistance

Diverse *Escherichia coli* isolates from domestic animals in the same Ecuadorian household all carry the colistin resistance gene *mcr1.1*.

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HIGHLIGHTS

- Domestic animals and a human carry colistin resistant *E. coli* in a household.
- Different *E. coli* clones carry *mrc-1.1*.
- The *mcr-1.1* gene was carried by a very similar IncI2 plasmid in different strains.

ABSTRACT

Objective: The aim of this study was to detect potential animal reservoirs of *E. coli* carrying *mcr-1* gene in and Ecuadorian household.

Methods: Colistin-resistant gene, *mcr*-1, was first detected in Ecuador in a commensal *E. coli* isolate from a boy. A cross sectional study was performed to detect the possible source of colistin-resistant *E. coli* in the boy´s household. Fecal swabs and soil fecal samples were collected from companion animals. Samples were plated on selective media to isolate colistin-resistant *E. coli* and isolates were submitted to polymerase chain reaction (PCR) detection of *mcr*-1, pulsed field gel electrophoresis (PFGE), multi-locus sequences typing (MLST). Moreover, the genomes of all the isolates were sequenced.

Results: Three different colistin resistant *E. coli* sequence types (ST3941, 1630 and 2170), corresponding to 3 PFGE patterns, were obtained from a chicken and 2 dogs; these isolates were different from the human isolate (ST609). By whole-genome sequencing, the *mcr1.1* gene was found on IncI2 plasmids with very high nucleotide identity.

Conclusions: Our results indicate a polyclonal dissemination of *mcr-1.1* in the environment surrounding the first MCR-producing *E. coli* strain reported in Ecuador. Our findings support the idea of lateral dissemination of *mcr-1.1* gene between unrelated *E. coli* isolates.

Key words: Antimicrobial resistance, *mcr-1*, Inc I2 plasmids, commensal *E. coli*, companion animals, horizontal gene transfer.

INTRODUCTION

Plasmid-mediated colistin resistance (CR), encoded by *mcr-1*gene, was reported for the first time in China in 2015 [1]. Since then, 10 *mcr* genes have been described (*mcr-1* to *mcr-10*) most of them with multiple alleles [e.g., *mcr-1.1* to *mcr-1.22*, from GenBank database, available at:

https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/gene_family:(mcr-1).

These genes code for phosphoethanolamine transferases which modify the structure of the lipid-A moiety in the outer membrane of Gram-negative bacteria and thus confers resistance to polymyxins [1,2]. Horizontal gene transfer of CR is also mediated by *mcr* genes, which contribute to the fast spread of CR among *Enterobacteriaceae* [3,4]. *Escherichia coli* plays an important role in colistin resistance because it is the main *mcr* gene carrier [4] and can be easily transferred among different animal hosts without specie barrier [5]. In Ecuador, Ortega *et al.* have described the isolation of an *E. coli* carrying *mcr-1* from a boy´s gastrointestinal tract who was admitted in a hospital in Quito, Ecuador, due to a complicated peritonitis [6]. In the present study, we screened *E. coli* isolates from domestic animals in the boy´s household, looking for the presence of *mcr1.1*.

METHODS

2.1 *E. coli* isolation and MIC determination

In June 2016, (a month after the first case were reported) a cross – sectional study was conducted to detect commensal *E. coli* carrying *mcr-1* gene. Thirty-two fecal grab samples and ten rectal or cloacal swabs were taken from animals, which were raised in the backyard of the house according to Table 1. We weren't able to obtain samples from the cat and the and the goose Soil fecal samples were placed in sterile containers and swabs were cultured in Tryptic Soy Broth (TSB, BD™) [7]. Samples were transported to the Antimicrobial Resistance Laboratory, Instituto Nacional de Investigación en Salud Pública "Dr. Leopoldo Izquieta Perez", Quito. All samples were plated in MacConkey Agar plates (MKL, BD™) supplemented with 2 µg/mL of colistin methansulfonate (RICHET®)[8].We confirmed our results using colistin sulfate salt ≥ 15,000 U/mg (Sigma). We also validated the use of MacConkey lactose supplemented with colistin methasulfonate by testing the medium with colistin-sensitive *Escherichia coli* ATCC 25923 strain and a colistin resistant *Serratia marcescens*. Strains that grew in colistin supplemented MacConkey medium were confirmed by AST 272 card, Sensititre™, and by *mcr* gene PCR. Moreover, we use different molecular analysis for confirming the colistin resistance feature associated with *mcr* gene presence.

Identification and antimicrobial susceptibility profiles of the CR isolates were assessed by using the VITEK®2 compact (bioMérieux) with AST 272 card. Sensititre™ was perform to confirm minimal inhibitory concentration (MIC) to colistin with an epidemiological cutoff value for *Enterobacteriaceae* of 2μg/mL [9].

2.2 Molecular assays

PCR was performed to detect *mcr-1* gen using primers previously described [1]. Pulsed field gel electrophoresis (PFGE; PulseNet protocol) [10] and Multi-locus sequence typing (MLST; http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) were used to define clonal relatedness [8,11].

Based in the PFGE and MLST results, we decided to sequence the whole genome of the isolates recovered from one dog, a chicken, and the child [6]. Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions and eluted in 100 µL of AE buffer. DNA concentration was determined by Qubit® 2.0 Fluorometer (Thermo-Fisher Scientific), and DNA samples were stored at −20°C until further processing. The sequencing library was prepared using the Illumina Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA) as per manufacturer's instructions. Agilent 2100 Bioanalyzer was used to determine quality of DNA library. Sequencing was performed using the Illumina MiSeq platform with 600-cycle MiSeq Reagent Kit v3. Reads were assembled using SPAdes v.3.9.0 [12]. Nanopore sequencing was performed on Oxford Nanopore Technologies (ONT) MinION device with chemistry 8 and flow cells FLO-MIN106 version R9.4. DNA extraction was made by using the MasterPure Complete DNA & RNA Purification kit (Epicenter Illumina, Wisconsin USA) with elution carried out to a final volume of 40 µL in TE buffer. Libraries for 12 isolates were prepared with the Rapid Barcoding Kit SQK-RBK004 starting with 400 ng of high molecular weight DNA from each isolate and according to Oxford Nanopore protocol (RBK_9054_V2_revE_23jan2018). Libraries were loaded and run for 48 hours. Base calling was performed while sequencing or using Guppy (available at https://community.nanoporetech.com). Nanoplot was used for quality control. Porechop (https://github.com/rrwick/Porechop) was used to split files by and to trim barcodes. Illumina-ONT hybrid assemblies were performed using Unicycler. Circular

plasmid sequences were obtained from the hybrid assemblies. Antimicrobial resistance genes were identified using the staramr pipeline, which scans genome contigs against the ResFinder, PlasmidFinder, and PointFinder databases (available at https://github.com/phac-nml/staramr).

2.3 Conjugation assay

Colistin resistant *Escherichia coli* isolated from animals were used as donors in conjugation assays. *Escherichia coli* strains 2 (Chicken), 11 (turkey) and 25 (dog). Conjugation experiments were performed in triplicate onto Luria–Bertani (LB) agar plate, using a 1:10 donor to recipient ratio [13]; sodium azide resistant *E. coli* J53 was used as recipient . Transconjugant bacteria were selected on Mueller Hinton agar plates containing colistin (1 mg/mL) and azide (100 mg/mL) [13]. Transfer frequencies were calculated based on the number of transconjugants obtained per donor.

RESULTS AND DISCUSSION

Colistin resistant *E. coli* clones from domestic animals and a human (living in the same household) were different (Table 2); however, all isolates carried the same *mcr-1.1* allelic variant and plasmids carrying *mcr-1.1* showed high nucleotide sequence identity (Figure 1). The *mcr-1.1* gene was found on IncI2 plasmids in the three *E. coli* isolates and *mcr-1.1* was the only resistance determinant in these plasmids. The conserved DNA segment containing *mcr-1.1* and *pap2* genes was not flanked by IS*ApI1* or any other transposable element. The plasmid sizes were very similar: 60,733 bp, 42.5% GC content (dog isolate); 61,412 bp, 42.4% GC content (chicken isolate); and 62,311 bp, 42.5% GC content (boy isolate). All plasmids shared an average of 90% nucleotide identity and highly conserved backbone (Figure 1). The main differences were the absence of open reading frames encoding hypothetical proteins and the *pilV* shufflon reorganization (Figure 1). It had been reported that shufflon segments are highly variable [14] and pilV C-ter is extremely important for receptor bacteria recognition during conjugation [14,15]. IncI or MOB_P according to relaxase typing, which it is a group of low copy-number, narrow-host-range, and conjugative plasmids [16]. IncI2 plasmids have been associated with different AR genes, such as $bla_{CTX\text{-}M\text{-}55}$ and $bla_{KPC\text{-}3}$, and fitness advantage

to bacteria host [17–20] , but more recently they were described carrying *mcr* genes from both human and animal sources worldwide [2,21]. Plasmids go through many DNA rearrangements in short periods [22], therefore the high nucleotide sequence identity in these plasmids may indicate that they derived from a recent common ancestor, which was moving between *E. coli* in different animal species.

The PFGE analysis of all *E. coli* isolates, including the one previously characterized by Ortega *et al*. [6], showed different genetic patterns except those from dogs´ (Figure 2); MLST agreed with the PFGE results (Table 2). The chicken isolate belonged to ST3941, which has been described in a bloodstream infection in Italy [23], and fosfomycin resistant isolate of animal origin [24]; while ST1630 was found in healthy chickens in Japan [25]. We analyzed nucleotide sequences of the genes *pmrA* and *pmrB* and we did not find any change associated with colistin resistant (Δ27–45 *pmrB*, nor L105P *pmrA*, nor G206D *pmrB*) [26]. Although we detected some changes in *pmrB* from the three isolated we analyzed (H2R substitution in boy isolate; D283G substitution in dog isolate and Y358N substitution in the chicken isolate), they haven't been associated with colistin resistance [26]. These results confirm that colistin resistance in these isolates was due to the expression of *mcr-1.1* gene. The genes *mcr-1.1* and $bla_{\text{CTX-M}}$ genes were not found in the same contig, therefore they may not be in the same replicon (Supplementary materials). The expression of *mcr-1.1* and $bla_{\text{CTX-M}}$ genes would be responsible for the levels of resistance to colistin and ceftriaxone, respectively (Table 2).

The antibiotic susceptibility profiles are shown in Table 2. All isolates had colistin minimum inhibitory concentration (MIC) >4 μg/mL and were resistant to ceftriaxone (MIC ≥64 μg/mL). Additional resistances were also described, for example: CR *E. coli* isolates from dogs were also resistant to ampicillin/sulbactam (MIC ≥32 μg/mL); and the CR *E. coli* from a poultry fecal sample to ciprofloxacin (MIC ≥4 μg/mL) (Table 2).

Whole genome sequencing was performed from each isolate, being also confirmed that mcr-*1.1* and $bla_{\text{CTX-M}}$ genes were not found in the same circular contig in separated plasmids (Supplementary materials). All the isolates were *gyrA*/*parC* double mutants, but no plasmid-mediated quinolone resistance mechanisms were found. We also found a tetracycline resistance gene in all isolates, a fosfomycin resistance gene in all isolates

from domestic animals, chloramphenicol resistance gene in the boy isolate, spectinomycin and trimethoprim-sulfamethoxazole resistance gene in the chicken and the boy isolate. The use of this antimicrobial additives on animal feed could act as selective force to maintain these resistant clones [27]. There was no evidence of colistin administration in the tested animals, however, in veterinary stores near to the household we found a water-soluble powder containing colistin for use in chickens, pigs and cows. It is unknown whether these products were administered to animals in the household we studied. There were at least 9 animal species in the backyard (12 m^2); chickens had a different water source from other animals. Food animals are considered as a source of AR bacteria, [28]. Also, foodborne *E. coli* is important due to its high potential to transfer AR genes (such as *mcr* genes) among different *E. coli* lineages and even different bacterial species [28].

Mating experiments showed a low rate of conjugation ranged among $1x10^{-3}$ to $1x\ 10^{-4}$ from dog and chicken isolate. It was not possible to conjugate the turkey isolate. Low rates of conjugation for IncI2 plasmids have been reported before [1,13]. It is possible that *mcr* gene had caused an important fitness cost in recipient bacteria because of bacteria LPS structure changes [2]. Our report supports the idea of diverse commensal *E. coli* linages, in different domestic animals are an important source of *mcr*-1.1 gene.

CONCLUSIONS

Our results suggest that polyclonal dissemination of the *mcr-1.1* gene in *E. coli* from different animal hosts occurred in the household from which the first MCR-producing *E. coli* was found in Ecuador. The fact that the three *E. coli* studied did not belong to the same clone but carried a very similar IncI2 plasmids strongly support the idea of lateral dissemination of *mcr-1.1* gene between unrelated *E. coli* isolates.

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Competing interest: The authors declare that they have no conflict of interests**.**

Ethical approval: No required

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Table 1. Description of collected samples backyard of household.

Type of sample	**Number of samples**
Grab samples	32
Rectal swabs from rabbits	2
Rectal swabs from guinea pigs	2
Rectal swabs from dogs	2
Cloacal swab from chicken	1
Cloacal swab from turkey	1
Cloacal swab from pigeon	1
Cloacal swabs from duck	1

Table 2. Susceptibility profiles (MIC, µg/mL)[d] of *mcr-1*-positive *E. coli* isolates from animal origin compared to the first case reported in Ecuador.

[a] The column indicates the origin of each isolate.

[b] Determined by MLST.

E. coli[a]	ST[b]	Resistance determinants[c]	COL	SAM	PIT	FOX	CAZ	CRO	FEP	DOR	ETP	IMP	MEM	AKN	GEN	CIP
Chicken	3941	*mcr-1.1*, $bla_{\text{CTX-M-65}}$, *fosA3*, *aadA5*, *dfrA17*, *sul1*, *tet(B)*, GyrA S83L, D87N; ParC S80I	>4 (R)	4(S)	≤4 (S)	≤4 (S)	≤1 (S)	≥64 (R)	≤1 (S)	≤0.12 (S)	≤0.5 (S)	≤0.25(S)	≤0.25 (S)	≤2 (S)	≤1 (S)	≥4 (R)
Dog1	2170	*mcr-1.1*, $bla_{\text{CTX-M-3}}$, $bla_{\text{TEM-206}}$, $bla_{\text{TEM-1B}}$, *tet(A)*, *fosA3*, GyrA S83L, ParC S80I	>4 (R)	≥32 (R)	≤4 (S)	≤4 (S)	≤1 (S)	≥64 (R)	2 (R)	≤0.12 (S)	≤0.5 (S)	≤0.25(S)	≤0.25 (S)	≤2 (S)	≤1 (S)	1 (R)
Boy[e]	609	*mcr-1.1*, $bla_{\text{CTX-M-55}}$, $bla_{\text{TEM-206}}$, *aph(3'')-Ib*, *aph(3')-Iia*, *aph(6)-Id*, *dfrA14*, *floR*, *sul2*, *tet(A)*, GyrA S83L, D87N; ParC S80I, E84G	>4 (R)	NR	NR	≤4(S)	16 (R)	≥64 (R)	2 (R)	≤0.12 (S)	≤0.5 (S)	≤0.25 (S)	≤0.25 (S)	≤2 (S)	≤1 (S)	≥4 (R)

[c] Genes were identified from WGS using the staramr pipeline, which scans genome contigs against the ResFinder, PlasmidFinder, and PointFinder databases

[d] Susceptibility interpretation (S, susceptible; R, resistant) are included. COL, colistin; SAM, ampicillin/sulbactam; PIT, piperacillin/tazobactam; FOX, cefoxitin; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; DOR, doripenem; ETP, ertapenem; IMP, imipenem; MEM, meropenem; AKN, amikacin; GEN, gentamicin; CIP, ciprofloxacin; NR, no reported

[e] Previously reported [6].

Figure 1. Circular comparison of *mcr-1.1*-carrying IncI2 plasmids. The plasmid sequence of an *E. coli* from a child [6] was used as the reference. The arrows indicate deduced open reading frames (ORFs) and their orientations.

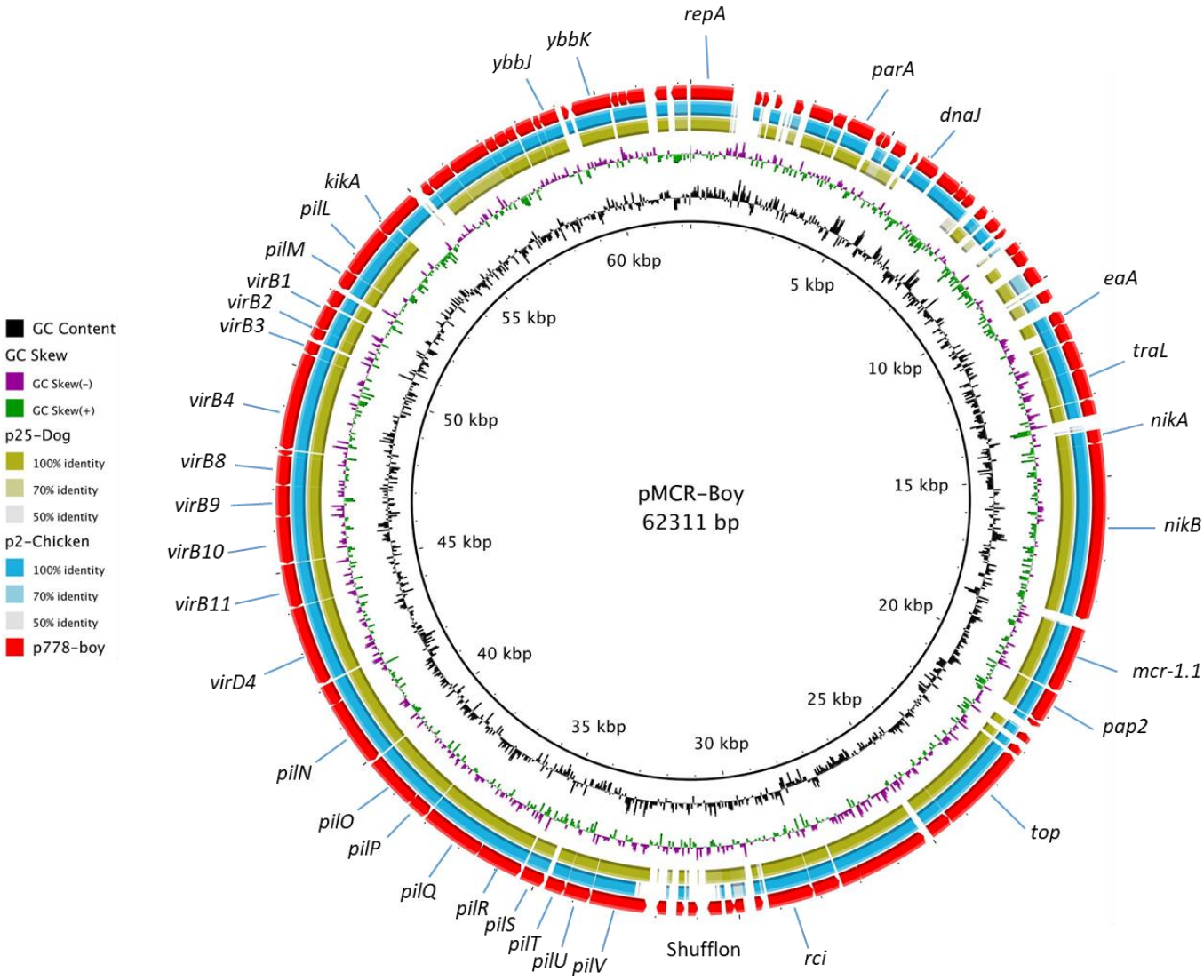
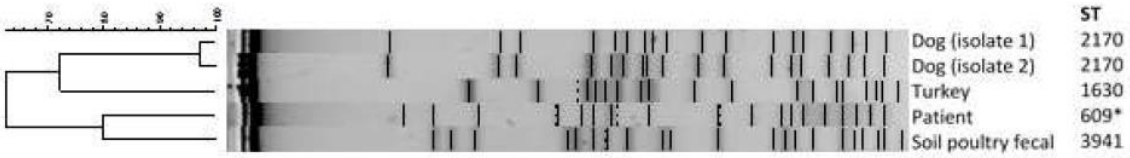


Figure 2. Pulsed field gel electrophoresis (PFGE) and sequence type (ST) description from all colistin resistant *E. coli* strains isolated from grab or fecal samples of domestic animals in the backyard of an Ecuadorian household. The household and animals were owned by the family of the boy whose case was the first description of human carrying Colistin resistant *E. coli*.



*ST published in Ortega *et al.*

Supp. Table 1. Description of circular contigs detected from Colistin resistant *Escherichia coli* (*E. coli*) isolated from domestic animals and a boy who was host for the first colistin resistant strain reported in Ecuador.

Isolate #	Species	Strain Information	Unicycler Hybrid information			Staramr information	
			# of contigs	Circular contigs (bp)	Linear contigs (bp)	Resistant Gene	Predicted Phenotype
GN2980	*E. coli*	2, chicken	8 circular	4907770			
				109881		*blaCTX-M*-65, *fosA3*	ampicillin, ceftriaxone, fosfomycin
				96285			
				74012		*aadA5, dfrA17, sul1, tet(B)*	streptomycin, trimethoprim, sulfisoxazole, tetracycline
				61412		*mcr*-1	colistin
				8910 4715 1552			
GN2982	*E. coli*	25, dog	8 circular	4861799		*tet*(A)	tetracycline
				141953		*blaCTX-M-3, blaTEM-206, fosA3*	ampicillin, ceftriaxone, fosfomycin
				106788		*blaTEM-1B*	ampicillin
				96814			
				60733		*mcr*-1	colistin
				21555 3373 2313			
GN2984	*E. coli*	778, child	9 circular	4581879 80068			
				63874		*aph(3'')-Ib, aph(3')-Iia, aph(6)-Id, blaCTX-M-55, blaTEM-206, dfrA14, floR, sul2, tet(A)*	streptomycin, kanamycin, ampicillin, ceftriaxone, trimethoprim, chloramphenicol, sulfisoxazole, tetracycline
				62311		*mcr-1*	colistin
				51257		*tet(A)*	tetracycline
				5631 4510 1927 1748			
	E. coli	2	MCR positive (chicken sample)				
	E. coli	25	MCR positive (dog sample)				
	E. coli	778	MCR positive (boy, peritoneal fluid - abscess)				

CONCLUSIONS

The transference of antimicrobial resistance from bacteria in domestic animals to human microbiota is a crucial public health problem that must be addressed to ensure the effectiveness of antimicrobials in the future. In the present research I tried to understand the interplay of population genetics of AMR *E. coli* and AMR genes in the absence of antimicrobials or in the presence of metabolic challenges. I found that neither antimicrobial resistance nor diet changes modified the resistome against antimicrobials introduced in the middle of the XX century such as tetracyclin, sulfonamide, ampicillin, amphenicols, etc. More than 50 years of co-evolution within *E. coli* may have selected mutants (in the bacterial host, mobile genetic elements, and AMR genes) that have reduced the fitness costs of AMR gene carriage. The results of these studies suggest that the effort to reduce AMR in food-animals must focused on AMR genes introduced recently in bacterial commensals such as genes coding colistin resistance and some extended spectrum β-lactamases or carbapenemases. Recent studies have demonstrated that some of these antimicrobial resistances are reduced when animals are subjected to antimicrobial restriction (Koga et al, 2015; The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme, 2005).