CHARACTERIZATION OF BROILER GUT MICROBIOMES AND PATHOGEN PREVALENCE IN CONVENTIONAL AND NO ANTIBIOTICS EVER POULTRY PRODUCTION SYSTEMS

by

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(Under the Direction of Manpreet Singh)

ABSTRACT

Poultry raised without the use of antibiotics is becoming an increasingly popular option within the poultry meat market. The transition to a no antibiotics ever (NAE) production is accompanied by substantial knowledge gaps regarding the dynamics of the gut microbiota and the occurrence of enteric pathogens. The following study assessed the effect of supplementation with antibiotics in broiler chicken diets on the gut microbiota and incidence of foodborne pathogens. A microbiome comparative analysis between conventional and NAE broilers identified distinct shifts in gut microbial populations. Chicken raised without the use of antibiotics carried an intestinal microbiota with a lower species number but a higher taxonomic diversity. Furthermore, the abundance of beneficial genera *Bifidobacterium, Ligilactobacillus*, and *Alistipes* was higher in NAE raised chicken ($P \leq 0.05$). Conventionally raised broilers harbored higher intestinal populations of *Escherichia* and *Clostridium_P*. Broilers from conventional farms also exhibited higher rates of *Salmonella* infection during early rearing stages ($P \leq 0.05$), although no major differences were reported at the end of the grow-out cycle. Greater

Campylobacter prevalence was observed on all farms, but the organism had a lower prevalence in farms with prolonged antibiotic administration ($P \leq 0.05$). The pathogen exhibited its characteristic patterns of colonization and horizontal spread, reaching a 100% positive rate and intestinal microbial loads of 7 $\log_{10}$ CFU/g during early grow-out. In addition, differential abundance analysis of microbiome data showed a higher representation of *Campylobacter* in NAE broilers ($P \leq 0.05$). The incidence of antimicrobial resistance (AMR) was higher among *Salmonella* isolates (76%) compared to that of *Campylobacter* (3.5%). However, the latter carried resistance to ciprofloxacin (1.2%), an antibiotic of clinical importance. This research identified important shifts in gut microbial community dynamics and foodborne pathogen prevalence associated with antibiotic administration within commercial poultry operations.

INDEX WORDS: Microbiome, poultry, *Campylobacter*, *Salmonella*

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DEDICATION

Á miña nai, á nai da miña nai, e á nai da nai da miña nai.

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CHAPTER 1

INTRODUCTION

Chicken meat production in the United States surpassed 20 million tons in 2020 (USDA-FAS, 2020). Furthermore, chicken is the most consumed meat among Americans, amounting to 95 pounds of broiler meat per capita (NCC, 2019). In recent years, the poultry industry has undergone a transition to limit the use of antibiotics in primary operations (FDA, 2012). In fact, recent estimates attribute over 50% of the poultry meat market to chicken raised under no antibiotics ever (NAE) practices (PHT, 2019). Antibiotics in poultry production are used as therapeutic agents to prevent or control the growth of disease-inducing bacteria, such as *Clostridium perfringens* (De Gussem, 2007; Allen and Stanton, 2014). Additionally, antibiotics may be used to increase the rate of weight gain and improve feed efficiency in broilers during grow-out (Allen and Stanton, 2014). Without efficient alternatives, the withdrawal of antibiotics from broiler meat production may pose significant challenges to the industry.

Researchers hypothesize that the growth-promoting effect associated with antibiotic administration is a consequence of the antimicrobial action on the gut microbiota (Dibner and Richards, 2005). Antibiotics frequently used in the poultry industry exhibit a wide spectrum of activity, thus leading to non-targeted inhibition of gastrointestinal microbial populations (Allen and Stanton, 2014; Zhang and Chen, 2019). This indirect impact on the gut microbiota may lead to a state of imbalance, or dysbiosis, that can impair proper host function (Teirlynck et al., 2011). Because the gut microbiota

plays an essential role in promoting host health and intestinal homeostasis, unintended deviations may lead to a higher disease susceptibility and consequential loss of productivity (Que and Hentges, 1985; Apajalahti and Vienola, 2016; Kogut, 2019). The mechanisms of this relationship remain largely uncharacterized, however, advancements in DNA sequencing technologies have revolutionized the exploration of the poultry gut microbiome, prompting the design of experiments that can illuminate our understanding of microbial community dynamics in poultry production (Danzeisen et al., 2011; Oakley et al., 2013, Ocejo et al., 2019).

From a food safety perspective, it is unclear whether feed supplementation with antibiotics can impact the populations of poultry-associated *Salmonella* and *Campylobacter* in commercial operations. Recent investigations have attempted to answer this question by performing comparative analyses of the incidence of *Salmonella* and *Campylobacter* between conventional and alternatively raised poultry (e.g., organic, pastured-raised) showing contrasting trends (Alali et al., 2010; Overbeke et al., 2006, Golden and Mishra, 2020). Alternatively raised poultry encompass an heterogeneous array of rearing practices beyond antibiotic use, which may be contributing to the differences reported. Furthermore, an analysis of foodborne pathogen prevalence on the novel no antibiotics ever (NAE) systems is lacking. The impact of antibiotic use on the emergence of antimicrobial resistant (AMR) bacteria, however, is more evident (Allen et al., 2010; Lazarus et al., 2015). Recent reports by the Centers for Disease Control and Prevention (CDC) and the Food and Drug Administration (FDA) warn of rises in the incidence of AMR *Salmonella* and *Campylobacter* from poultry processing operations

(FDA, 2018; CDC, 2019). Surveillance data from commercial broiler farms in the United States is still lacking.

This study was designed to evaluate the effects of poultry feed supplementation with antibiotics on gut microbial community dynamics, pathogen prevalence and antimicrobial resistance in large-scale broiler production systems. To our knowledge, this study is the first to carry out a comparative analysis of the gut microbiota in broiler birds raised under commercial production practices during a complete production cycle and following transportation to slaughter. By doing this study we can identify shifts in the intestinal microbiota of broiler birds associated with antibiotic administration, and characterize the prevalence and colonization patterns of foodborne pathogens in conventional and NAE farms. Data from this experiment can be used to design future investigations, in the development of competitive exclusion and probiotic strategies for effective pathogen control in the farm. This work will also provide valuable information regarding the incidence, distribution and potential drivers of antimicrobial resistance among *Salmonella* and *Campylobacter* in commercial poultry production.

CHAPTER 2

LITERATURE REVIEW

The Poultry Gut Microbiome

General Characteristics

Microbes inhabit the bodies of all animals, constituting a complex microbial ecosystem. The term microbiota is used to describe the collection of microorganisms that reside in the body, encoded by a compendium of genes known as the microbiome (Clemente et al., 2012). The gastrointestinal tract (GIT) harbors most microbial cells in the body (Savage, 1977). This microbial community is dominated by bacteria, but encompass taxa from other microbial groups (e.g., archaea, fungi and bacteriophages) (Savage, 1977; Yeoman et al., 2012). The symbiotic relationship of microbiota within itself, the host, and the environment is essential in maintaining homeostasis and promoting host health. Due to its importance, the gut microbiota is often regarded as an additional organ with complex roles in host metabolism, nutrition and immune function (O'Hara and Shanahan, 2006).

Gut microbiome research in food-producing animals has received increased attention in recent years, as it relates to issues of food safety, animal health and public health (Shang et al., 2018). This is especially important in poultry production, where the dynamics of the gut microbial populations impact nutrient digestibility, growth performance and disease resistance; ultimately affecting production efficiency (Lan et al., 2005; Apajalahti and Vienola, 2016; Kogut, 2019; Clavijo and Flórez, 2018). The

chicken gut microbiota is a highly diverse and abundant community of microorganisms, consisting primarily of bacteria, and followed by methanogenic archaea, fungi, virus and protozoa (Qu et al., 2008; Wei et al., 2013; Yeoman et al., 2012). The complexity of this microbial community can be modified by various factors, of which bird age, gastrointestinal tract segmentation and diet composition have been thoroughly characterized (Yeoman et al., 2012; Oakley et al., 2013; Pan and Yu, 2014).

Factors Affecting the Gut Microbiome

Age

Newly-hatched chicks have a sparse gastrointestinal microbial community acquired at pre-hatch, as microbes can be transmitted vertically from the mother, or horizontally from the hatchery environment (Roto et al., 2016). The composition of the gut microbial community starts to diversify quickly when newly-hatched chicks become exposed to the farm environment (Oakley et al., 2013). This community further develops as birds age, forming distinct ecosystems within each location of the chicken gastrointestinal tract (Yeoman et al., 2012). Bacteria from the *Enterobacteriaceae* family are the predominant taxa during the first 3 days of age. After, *Ruminococcaceae* groups, which belong to the Firmicutes phylum, start increasing in abundance and diversity, becoming the most prevalent taxonomic group by day 14 (Ballou et al., 2016). Firmicutes remain as the most abundant phylum in the intestinal tract for most of the growth cycle, with Lactobacillales dominating the small intestine and Clostridiales dominating the cecum (Dumonceaux et al., 2006). At later growth stages, the abundance of Firmicutes starts to decrease while members of the Bacteroidetes phylum become more prominent,

particularly in the ceca (Danzeisen et al., 2011; Kumar et al., 2018). Temporal succession of bacterial groups becomes all the more evident at the genus level (Yeoman et al., 2012).

Gastrointestinal Tract Segmentation

The chicken gastrointestinal tract is divided into six sections, crop, stomach, gizzard, small intestine, ceca and large intestine. Each section harbors a distinct microbiota, yet significant interaction occurs among these microbial populations (Gong et al., 2007). Furthermore, the spatial variation in the distribution of microbial groups is associated with its correspondent physiological function (Gong et al., 2007; Yeoman et al., 2012).

Breakdown of starch and lactic acid production are the main metabolic functions carried out in the crop, where *Lactobacillus* spp. dominate, followed by members of the *Enterobacteriaceae* family (Wei et al., 2013). The crop and gizzard have a highly acidic environment due to the metabolic activity of *Lactobacillus* (Wei et al., 2013). Both sections act as first barriers for pathogen colonization, since low pH is known to inhibit the growth of certain strains of *Clostridium* spp., *Salmonella* and *Campylobacter* in the cecum (Sekelja et al., 2012). *Lactobacillus* is also the predominant genus in the ileum and the upper gastrointestinal tract, where most of the nutrient absorption occurs (Gong et al., 2007; Kumar et al., 2018). Butyrate-producing bacteria such as *Clostridium* are also present in the ileum, yet at much lower levels (Gong et al., 2007). Microbial abundance in this section can range from 10^8 to 10^9 cells per gram of digestive material. These levels are significantly lower than those observed in the ceca, where microbial density can reach up to 10^{11} cells per gram of digesta (Yeoman et al., 2012).

The ceca, a pair of blind-ended pouches located in the lower GIT, harbor the highest diversity in microbial populations, with over 200 genera identified (Qu et al., 2008). Firmicutes is the predominant phylum in the cecum, representing between 50 and 90% of the taxa, whereas Bacteroidetes is the second most abundant group (Danzeisen et al., 2011; Qu et al., 2008). The ceca is where most microbial fermentation takes place, favored by the longer transit times of the digesta. Consequently, most microbial groups present in the ceca are obligate or facultative anaerobes as is the case of genera *Clostridium*, *Lactobacillus* and *Bacteroides* (Rehman et al., 2007). Methanogenic archaea represent a smaller percentage of the microbial population in the cecum, however they have an important role in the dissipation of hydrogen accumulated during extensive fermentation (Saengkerdsub et al., 2007). A summary of the major microbial taxa is shown in Table 2.1.

Table 2.1. Main taxonomic groups in the gastrointestinal tract of chickens[1].

Section	**Abundance (cfu/g)**	**Diversity**
Crop	10^8-10^9	Firmicutes
		Lactobacillus
		Actinobacteria
		Bifidobacterium
		Proteobacteria
		Enterobacter
Gizzard	10^7-10^8	Firmicutes
		Lactobacillus
		Enterococcus

Small Intestine	10^8-10^9	Firmicutes
		Lactobacillus
		Candidatus Arthromitus
		Clostridium
		Ruminococcus
		Proteobacteria
		Escherichia
		Enterobacter
Ceca	10^{10}-10^{11}	Firmicutes
		Clostridium
		Lactobacillus
		Megamonas
		Sporobacter
		Peptococcus
		Ruminococcus
		Faecalibacterium
		Pseudobutyrivibrio
		Subdoligranulum
		Acetanaerobacterium
		Bacteroidetes
		Bacteroides
		Proteobacteria
		Escherichia

	Bilophila
	Archaea
	Methanobacterium
	Methanothermobacter
	Methanosphaera
	Methanothermus
	Methanopyrus
	Methanococcus
	Fungi
	Candida

[1]Adapted from Yeoman et al. (2012)

Diet

Establishment of gut microbial communities is also impacted by the chemical composition of poultry feed, as nutritional requirements can vary greatly among microorganisms (Pan and Yu, 2014). Diets supplemented with whole-wheat, for example, have shown to suppress the growth of *Clostridium perfringens* and lactose-negative enterobacteria, whilst changing the dynamics among lactobacilli populations in the GIT (Engberg et al., 2004; Bjerrum et al., 2005). In contrast, diets high in non-starch polysaccharides, such as wheat, barley and rye-based diets, can be problematic to broiler health (Annett et al., 2002). These diets are known to increase viscosity in the poultry GIT, decrease the transit time of the digesta, and impair nutrient digestibility (Choct et al., 1996); conditions that can increase the susceptibility to *C. perfringens* colonization in broiler chickens and consequential development of necrotic enteritis (Annett et al., 2002).

Corn-based diets, however, do not favor the growth of *C. perfringens*, but have shown to enhance the growth of *Enterococcus* and certain *Lactobacillus* spp. (Annett et al., 2002; Hammons et al., 2010). Further, the source of dietary nutrients can impact the gut microbial community composition, as research has shown that diets high in proteins and fats from animal sources may lead to a higher abundance of *C. perfringens* in the broiler gut (Knarreborg et al., 2002; Drew et al., 2004).

Role of the Poultry Gut Microbiome

A healthy chicken gut microbiota has many essential roles, such as improving digestive function (Danzeisen et al., 2011). When compared to other farm animals, chicken have a short GIT which could potentially affect nutrient assimilation due to the shorter transit time of the digesta. However, in the ceca, the digesta can remain for up to 20 hours, potentially enhancing nutrient digestion and absorption (Kogut, 2019). The ceca harbor the highest microbial density in the chicken GIT, up to 10^{11} cells per gram of intestinal material, as well as the most diverse microbial community (Wei et al., 2013). Consequently, the cecal microbiome has been the focus of many investigations highlighting its significance in poultry nutrition (Józefiak et al., 2004; Danzeisen et al., 2011; Apajalahti and Vienola, 2016).

Metabolism

Of particular interest to the poultry industry, is the role of the chicken cecal microbiota in carbohydrate metabolism, as chickens lack many of the enzymes needed for the breakdown of dietary polysaccharides (Józefiak et al., 2004). Further, non-starch polysaccharide (NSP) fractions present in poultry feed, which are not readily digested by chickens, can be hydrolyzed and transformed into short-chain fatty acids (SCFA) by

cecal microbial groups through extensive fermentation (JøRgensen et al., 1996; Józefiak et al., 2004; Beckmann et al., 2006). Acetate, propionate and butyrate are the most abundant of the SCFAs produced by the chicken gut microbiota and can be used by the host as an additional energy source (van der Wielen et al., 2000). SCFAs also have important roles in improving intestinal health, for example, butyrate contributes to the development of intestinal epithelia by stimulating the growth of villi in the duodenum (Panda et al., 2009). Additionally, butyrate has been shown to improve feed conversion ratio and carcass yield in broiler chickens (Panda et al., 2009). Some SCFAs may exhibit bactericidal and bacteriostatic activity against enteric pathogens, such as *Salmonella* (Van Immerseel et al., 2005) and *E. coli* (Panda et al., 2009). This bactericidal action results from the accumulation and uptake of undissociated forms of the fatty acids by bacterial cells, compromising homeostasis when the acids dissociate and lower the intracellular pH, an event that leads to apoptosis (van der Wielen et al., 2000).

Nitrogen metabolism is also influenced by the gut microbiota. Metagenomic analysis of the cecal microbiome has shown a high abundance of genes involved in protein, amino acid and nitrogen metabolism (Qu et al., 2008; Danzeisen et al., 2011). Cecal microbes recycle nitrogen by breakdown of uric acid to ammonia, which is absorbed by the host and further transformed into amino acids such as glutamine (Vispo and Karasov, 1997). Other nitrogen sources that may be used for metabolism of essential amino acids include bacterial cellular proteins (Metges, 2000). Dietary protein assimilated to bacterial cells cannot be digested in the cecum and is mostly excreted with fecal material. However, the coprophagy behaviors of chickens may allow for utilization

of these bacterial proteins, since they can be digested in the upper intestine (Apajalahti and Vienola, 2016).

Immune Function

It is well-known that the intestinal microbiota supports proper immune function (Kamada et al., 2013). The chicken gut microbiota encompass bacterial groups that can be either beneficial or detrimental to the host. To maintain gut homeostasis, it is crucial that the immune system does not generate a pro-inflammatory response to commensal microbiota while maintaining the capacity to respond to pathogenic species (Clemente et al., 2012). The avian gastrointestinal immune system is comprised of organized lymphoid tissues in association with lymphocytes dispersed along various locations in the digestive tract including the epithelial lining and lamina propria (Lillehoj and Trout, 1996). The gut-associated lymphoid tissue, or GALT, is abundant in immune cells such as intra-epithelium M cells, goblet cells and T cells; as well as immunoglobulin-producing B cells in the lamina propria (Davison et al., 2008). Due to its complexity, the host immune system must elicit responses that are tightly regulated and prevent autoimmunity. Researchers have shown that birds with a depleted gut microbiota may suffer from a reduced cytokine expression and an underdeveloped gut-associated lymphoid tissue, leading to an increased susceptibility to pathogen colonization (Honjo et al., 1993). Further, microbial diversity in the gut can influence the repertoire of T cell receptors, in a way that avoids unfavorable cellular immune responses (Probert et al., 2007; Mwangi et al., 2010).

As an essential part of the avian mucosal immune system, the intestinal epithelium provides a first line of defense against entry of pathogenic microorganism into

the host (Davison et al., 2008). This mucosa is a major component of the innate immune system of chickens, and it consists of a layer of mucin glycoproteins that coat and protect the intestinal epithelium from invading microorganisms (Lievin-Le Moal and Servin, 2006). Mucin has a notable role in protecting chickens from *Campylobacter jejuni* pathogenesis (Alemka et al., 2010). Studies conducted *in vitro* have shown that intestinal mucin is able to attenuate virulence of *C. jejuni* by preventing invasion of gut epithelial cells (Byrne et al., 2007; Alemka et al., 2010). Mucin composition is also influenced by the diversity and abundance of bacterial populations in the gastrointestinal tract. For instance, in broiler chickens, the complexity of the gut microbiota, defined by a higher abundance, contributes to the early establishment of a developed mucin layer, thus providing enhanced protection against invading organisms (Forder et al., 2007).

Competitive Exclusion

Another important physiological function of the gut commensal microbiota is to exert resistance against pathogen colonization, protecting the host from infection by competitive exclusion (Lan et al., 2005). This competitive interaction between intestinal bacteria encompass several mechanisms, such as competition for nutrients, blockage of attachment sites in the intestinal epithelial wall, and production of antibacterial peptides (Lan et al., 2005). Evidence of the association between normal gut microbiota and broiler intestinal health was first provided by Nurmi and Rantala in 1973, when they demonstrated that the transfer of cecal contents from healthy adult birds to newly hatched chicks protected them from *Salmonella* colonization. Chicks hatched under commercial poultry operations can be particularly susceptible to colonization with enteric pathogens, such as *Clostridium perfringens* (Dahiya et al., 2006). Namely, the lack of contact with

adult birds and exposure to a microbially diverse environment in the hatchery may delay the establishment of a mature gut microbiota, thus providing an opportunity for enteric bacteria to invade the GIT when chicks are placed in the farm (Lan et al., 2005). As highlighted by Nurmi and Rantala (1973), the practice of administering bacterial cultures from the microflora of adult healthy birds to young chicks has been proved effective against a number of enteric pathogens including *Salmonella* Typhimurium (McReynolds et al., 2007), *Campylobacter jejuni* (Stern, 1994) and *Escherichia coli* (Snoeyenbos et al., 1982).

Competition for essential nutrients among intestinal microbiota may also limit the growth of certain pathogenic species. A study conducted by Gielda and DiRita (2012) showed that in the presence of a healthy gut microbiota, the growth of a *C. jejuni* mutant strain deficient in a high-affinity zinc transporter was restricted, suggesting that under low zinc conditions this strain was out-competed by more efficient bacteria. Another common strategy used by bacteria to suppress the growth of competing microorganisms is the production of bacteriocins, or antimicrobial peptides. The inhibitory effects of bacteriocins has long been considered a desirable trait in the search for probiotic cultures. Strains of *Lactobacillus salivarius* and *Bacillus subtilis* have been extensively studied, as they exhibit significant antimicrobial action against important poultry pathogens such as *Salmonella* Enteritidis, *C. jejuni* and *C. perfringens* (Teo and Tan, 2005; Stern et al., 2006; Messaoudi et al., 2012).

Disruptions of the normal gastrointestinal microbiota lead to a qualitatively and/or quantitatively microbial imbalance known as dysbiosis, which can negatively impact essential host function. In broilers, shifts in microbial community composition can be

caused by nutritional imbalances, host genetics and even toxic metabolites produced by pathogenic bacteria such is the case of *Clostridium perfringens* (De Gussem, 2007; Teirlynck et al., 2011; Zhao et al., 2013). Consequently, dysbiosis may result in nutrient digestibility impairment and decrease in the thickness of the intestinal epithelial wall, which leads to infiltration of immune cells in the intestinal mucosa and increased inflammatory responses (Teirlynck et al., 2011). Deviations from a balanced microbial community in broilers can impact growth performance, illness and, ultimately, the economy of the poultry industry. As the poultry industry moves towards more holistic practices it is imperative that research is carried out to achieve a deep understanding of microbiome dynamics and develop modulation strategies that will benefit poultry welfare, production efficiency and food safety.

Campylobacter

General Characteristics

Members of the *Campylobacter* genus are Gram-negative, microaerophilic bacteria exhibiting a distinct spiral morphology. *Campylobacter* species are primarily motile, by action of a polar flagella that confers a distinct corkscrew movement (Penner, 1988). Campylobacters are highly susceptible to deviations from ideal environmental conditions and often undergo a change to a coccoid morphology when microbial cells are exposed to stress (Tangwatcharin et al., 2006). Furthermore, cells often convert to a viable but non-culturable (VBNC) state to overcome unfavorable conditions, rendering it undetectable by traditional culture methods (Silva et al., 2011). Researchers often question this phenomenon due to conflicting findings related to the reversal from the VBNC state (Jones et al., 1991; Stern et al., 1994; Chaveerach et al., 2003). Nonetheless,

Campylobacter species are considered fastidious and of difficult culturability under laboratory conditions. Further, the physiology and metabolism of *Campylobacter* varies within species, which further complicates identification of the bacterium using conventional laboratory methods.

Campylobacter species *C. jejuni*, and *C. coli* have been thoroughly characterized, due to their importance as gastrointestinal pathogens with a high prevalence in food-animal production (Silva et al., 2011). The species *C. jejuni*, in particular accounts for over 80% of the reported campylobacteriois cases worldwide (WHO, 2015). In contrast to other gastrointestinal pathogens, *C. jejuni* requires an oxygen concentration of 5%, below the average concentration found in air (Smibert, 1978; Tangwatcharin et al., 2006). Furthermore, the species *C. jejuni, C. coli* and *C. lari* are considered thermophilic, growing optimally at 42°C but unable to multiply below 30°C (Penner, 1988; Tennover et al., 1990). Metabolically, *Campylobacter* spp. are non-saccharolytic and therefore rely on different energy sources than those utilized by most foodborne pathogens. *Campylobacter jejuni* for example, is incapable of metabolizing glucose or other carbohydrates (Parkhill et al., 2000; Hofreuter, 2014). Some strains can catabolize fucose through acquisition of a genomic island which encodes for a putative fucose permease (FucP) (Stahl et al., 2011). Nevertheless, *C. jejuni* preferably uses organic acids, intermediates of the citric acid cycle, and amino acids as energy substrates (Hinton, 2006; Wright et al., 2009).

Taxonomy

Campylobacter was presumably first identified in 1886 by Theodore Escherich, describing it as a curved-shaped non-culturable bacterium (Debruyne et al., 2008). It was

first isolated in 1913 by McFaydean and Stockman, as the causative agent of abortion in sheep and cattle. Finally, in 1919, Smith and Taylor named the organism *Vibrio fetus*, after isolating a spiral-shaped organism from another instance of bovine abortion (Smibert, 1978). The pathogen was attributed to a case gastrointestinal disease in cattle by Jones and collaborators (1931), who named the organism *Vibrio jejuni*. The *Campylobacter* genus was proposed in 1963, by researchers Sebald and Veron after identifying distinct genetic features in the species *V. fetus* from others in the same genus, thus proposing the reclassification of this bacterium to *Campylobacter fetus*.

For years, *Campylobacter* was classified under the *Epsilonproteobacteria*, a class of the Proteobacteria phylum, along with other genera sharing a similar morphology, such as *Helicobacter* and *Wolinella*. This taxonomic classification then changed in 2017, when the phylum Campylobacterota was proposed following a comparative genomic analysis that identified the group *Epsilonproteobacteria* as a distinct phylogenetic lineage, along with the order Desulfurellales (Waite et al., 2017). Currently, 32 species and 9 subspecies of the *Campylobacter* genus have been described. These species are further grouped under five distinct genetic clusters, all of which containing pathogenic species of clinical importance (Costa and Iraola, 2019). Much of the research focus has been placed on gastrointestinal pathogens *C. jejuni* and *C. coli* (Silva et al., 2011). However, new disease-causing species continue to emerge which are associated with a wide range of disorders (Man et al., 2010; Costa and Iraola, 2019). Further, genomic data for many of the *Campylobacter* species are lacking, thus highlighting the need for a comprehensive characterization of this genus.

Epidemiology and Disease

Many *Campylobacter* species can be pathogenic to animals and humans. The species *C. fetus*, for example, infects cattle and sheep, inducing gastrointestinal illness and at times, infection of the reproductive tract, which may result in abortion during the late stages of pregnancy (Fenwick et al., 2000). In humans, it is considered an opportunistic pathogen that can cause bacteremia in immunocompromised patients (Wagenaar et al., 2014). An emerging pathogenic species is *C. concisus*, presumably a host-restricted species, this pathogen is most often isolated from the oral cavities of humans (Zhang et al., 2010). Infection with *C. concisus* may result in gingivitis, periodontitis, and a wide range of gastrointestinal diseases including Crohn's disease (CD), ulcerative colitis (UC) and gastroesophageal reflux disease (GERD) (Liu et al., 2018).

Within the *Campylobacter* genus, the species *C. jejuni* and *C. coli* are most often associated with foodborne illness (WHO, 2015). These species are, in fact, the leading bacterial agent of enteric disease worldwide, accounting for 96 million illnesses each year (WHO, 2015). The annual burden of campylobacteriosis in the United States is characterized by more than 800,000 infections, 76 deaths and an economic impact of $1.6 billion (Scallan et al., 2011; Scharff, 2012). Campylobacteriosis has a wide range of clinical manifestations. Most often, the disease is characterized by an acute gastroenteritis with symptoms such as diarrhea, fever and abdominal cramps (Allos and Taylor, 1998). Cases are usually self-limiting, and the infection is likely to clear within a week of without antimicrobial therapy (Acheson and Allon, 2001). *Campylobacter* infections, however, have been linked to numerous chronic sequelae including Guillain-Barré

syndrome (GBS), reactive arthritis (ReA), irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (Peterson, 1994; McCarthy and Giesecke, 2001; Zhang et al., 2010; Schwille-Kiuntke et al., 2011). Guillain-Barré is the most prevalent post-campylobacteriosis complication, and recent estimates attribute more than 8% of the GBS cases in the United States to a pre-existing *Campylobacter* infection (Halpin et al., 2018).

Most cases of campylobacteriosis are linked to consumption of contaminated poultry meat, since the pathogen is highly adapted to the gastrointestinal tract of poultry (Clench and Mathias, 1995). In humans, the small intestine is the primary site of colonization (Mahendran et al., 2011). Infection with *Campylobacter* requires the ingestion of bacterial cells through contaminated food or water, at doses as low as 500 cells (Robinson, 1981). Following ingestion, *Campylobacter* is able to reach its colonization site by action of the polar flagella and a series of chemotaxis factors (Bolton, 2015). The flagella provide *Campylobacter* cells with a propulsive movement and characteristic corkscrew rotation aided by the helical shape of the cells (Ferrero and Lee, 1988). The flagella is a complex structure characterized by a hook-basal body and an extracellular filament composed of several flagellin proteins, namely FlaA (major flagellin) and FlaB (minor flagellin), (Nachamkin et al., 1993). *Campylobacter* motility is also reliant on chemotaxis, a signaling system by which the bacterium can locate its colonization site (Chang and Miller, 2006).

Campylobacter pathogenesis remains poorly understood due to the absence of well-studied virulence factors that are common in most enteric pathogens. For example, *Campylobacter* lacks a type 3 secretion system (Galán and Collmer, 1999). In turn, the

pathogen relies on the flagella for secretion of specialized proteins known as *Campylobacter* invasion antigens (Cia) which are essential for successful cell invasion (Poly and Guerry, 2008; Eucker and Konkel, 2012). Once the pathogen colonizes the gut enterocytes it can release a series of disease-inducing toxins, of which the cytolethal distending toxin (CDT) has been well-characterized (Pickett et al., 1996). The toxin is formed by three subunits encoded by genes *cdtA*, *cdtB*, and *cdtC*. Subunits cdtA and cdtC are required for transport and delivery of subunit cdtB, which is enzymatically active (Pickett et al., 1996). The cdtB subunit possesses a catalytic activity that causes cytotoxicity by inducing cell cycle arrest and DNA damage (Whitehouse et al., 1998).

Salmonella

General Characteristics

Bacteria belonging to the *Salmonella* genus are classified as Gram-negative, facultatively anaerobic bacilli which are predominantly motile by means of peritrichous flagella (Bell and Kyriakides, 2009). *Salmonella* species are mesophilic, growing optimally at 37°C, and are thus frequently isolated from the gastrointestinal tract of animals and humans (Bell and Kyriakides, 2009; Sanderson and Nair, 2013). Salmonellae have the ability to ferment glucose, mannitol and sorbotol but cannot use lactose or sucrose. The bacterium is also catalase positive and oxidase negative (D'Aoust and Maurer, 2007). Other key aspects of *Salmonella* metabolism include the production of hydrogen sulfide and utilization of citrate when growing on triple sugar iron agar (Percival and Williams, 2014). These biochemical characteristics are frequently used for presumptive identification of *Salmonella* (D'Aoust and Maurer, 2007). *Salmonella* species are highly resilient and can adapt to a number of hostile environments. For

example, *Salmonella* cells can resist desiccation and the organism is known to exhibit resistance to heat under low-moisture conditions (Finn et al., 2013). Further, *Salmonella* can multiply under a wide range of temperature (2°C to 54°C) and pH (4.5-9.5) (Percival and Williams, 2014). This inherent physiological adaptability thus makes *Salmonella* a pathogen of concern to the food industry as it can survive some common antimicrobial interventions (Wesche et al., 2005).

Taxonomy

Salmonella was first identified in 1885 by Dr. Daniel E. Salmon as the causative agent of cholera in swine, and was later recognized as a zoonotic agent of great importance to public health (Bell and Kyriakides, 2009; Scallan et al., 2011). The *Salmonella* genus belongs to the family *Enterobacteriaceae*, and consists of two species, *S. bongori* and *S. enterica* (Sanderson and Nair, 2013). The former was first identified as a distinct species by Reeves et al. (1989) using multilocus enzyme electrophoresis, and is known to primarily inhabit the bodies of cold-blooded animals (Fookes et al., 2011). The species *S. enterica*, can be further divided into six subspecies: subspecies *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI). Meanwhile, all strains of the species *S. bongori* are classified under subspecies V (Sanderson and Nair, 2013). The subspecies most often isolated from clinical specimens is *S. enterica* subsp. *enterica* (Coburn et al., 2007; Scallan et al., 2011), which encompasses over 2,600 distinct serotypes as described by the Kauffman-White protocol (Grimont and Weill, 2007; Guibourdenche et al., 2010). This classification scheme is based on the detection of O (somatic lipopolysaccharide), H (flagellar), and Vi (capsular) antigens by reaction with specific antisera (Grimont and Weill, 2007). Advancements in

DNA sequencing technology have allowed for the development of novel methodologies for subtyping of *Salmonella*. One of which, SeqSero, has emerged as a popular alternative, relying on Next-Generation Sequencing (NGS) data for the *in silico* prediction of *Salmonella* serotypes (Zhang et al., 2019).

Epidemiology and Disease

Salmonella serotypes can be further classified based on host-specificity and clinical disease manifestation as generalist, host-adapted, or host-restricted serotypes (Bell and Kyriakides, 2009). Generalist serotypes can adapt to wide variety of hosts and are most often associated with gastrointestinal illnesses (Sanderson and Nair, 2013). These serotypes are frequently identified as the causative agents of foodborne outbreaks, such is the case of *S.* Typhimurium and *S.* Enteritidis (Scallan et al., 2011). Host-adapted serotypes cause invasive systemic infections in their respective host, but can also infect others. Some examples of host-adapted serotypes include *S.* Choleraesuis in pigs and *S.* Dublin in cattle, both recognized as human pathogens (Uzzau et al., 2000). Host-restricted serotypes are exclusive to one species of host, and are frequently associated with severe systemic illness (Sanderson and Nair, 2013). Well-known host-restricted serotypes include the human pathogens *S.* Typhi and *S.* Paratyphi-A, and the poultry-specific *S.* Gallinarum (Sanderson and Nair, 2013).

Salmonella enterica subsp. *enterica* is the subspecies most often implicated in human and animal infections (Sanderson and Nair, 2013). Serotypes belonging to the subspecies enterica can be further classified as typhoidal, these are the host-restricted *S.* Typhi and *S.* Paratyphi, or non-typhodidal salmonellae (NTS), which are serotypes commonly related to foodborne disease (Gal-Mor et al., 2014). Typhoidal *Salmonella*

causes enteric fever in humans, a severe, invasive, and highly contagious disease with more than 27 million annual cases globally (Crump et al., 2015). Typhoid fever is highly prevalent in southeast and south-central Asia, having an associated incidence of over 100/100,000 cases per year (Crump et al., 2015). The disease is far less prevalent in the United States, with less than 4,000 cases reported in the last 20 years (Imanishi et al., 2015).

Non-typhoidal *Salmonella* is one of the leading foodborne zoonotic agents, causing an estimated 80 million illnesses and 59,000 deaths globally each year. (WHO, 2015). With more than 1 million illnesses annually, NTS is responsible for most salmonellosis cases in the United States, resulting in 19,000 hospitalizations and 378 deaths (Scallan et al., 2011; Scharff, 2012). Further, salmonellosis cases in the United States pose a significant cost to the national economy, estimated at $4.4 billion (Scharff, 2012). Serotypes of NTS are most often associated with poultry. This pathogen-food combination is significantly burdensome, having an associated cost of illness of $693 million (Hoffmann et al., 2012). Salmonellosis caused by NTS is manifested as a self-limiting gastrointestinal illness, characterized by diarrhea, vomiting and abdominal pain. The incubation period for salmonellosis varies from 12 to 72 hours, and clinical symptoms often last less than 7 days (D'Aoust and Maurer, 2007). Because the disease is most frequently self-limiting, the recommended treatment consists of hydration therapy and electrolyte replacement (D'Aoust and Maurer, 2007; Bell and Kyriakides, 2009). In rare occasions, *Salmonella* can enter the bloodstream and lead to a life-threatening septicemia which requires antibiotic treatment. Patients with an underdeveloped immune

system, such as children, the elderly, and individuals with autoimmune disorders, are at higher risk for development of invasive salmonellosis (Bell and Kyriakides, 2009).

Non-typhoidal *Salmonella* is an invasive pathogen possessing well-characterized virulence factors. For successful infection, *Salmonella* cells must first survive the acidic environment of the stomach (Foster and Hall, 1991). A high infectious dose, of 100,000 cells or higher, may therefore be needed to ensure that a sufficient *Salmonella* population is able to colonize the intestines (Finnlay, 1994). Once *Salmonella* reaches the small intestine, the bacteria invades enterocytes and crosses the intestinal epithelium by infection of microfold cells (M cells) of the Peyer's patches (Müller et al., 2016). In this process, *Salmonella* express flagella and adhesins which help with recognition and initial attachment to host cells (Arya et al., 2017). Invasion of non-phagocytic cells is achieved by a significant rearrangement of the cytoskeleton and formation of pseudopods, or membrane ruffles, on the host cell surface; leading to pinocytosis of *Salmonella* into the host cell. This is achieved by action of the Type III secretion system-1 (T3SS-1), a complex molecular structure that allows the injection of effector proteins into the host cell (Arya et al., 2017). In *Salmonella*, the T3SS-1 is encoded on pathogenicity island-1 (SPI-1) (Kuhle and Hensel, 2004). This sequence of events triggers a signal transduction cascade resulting in the production of IL-8 which then provokes a gut mucosal inflammatory response and consequential tissue damage (Müller et al., 2016). Following invasion, *Salmonella* cells are able to survive and replicate inside a phagosome, or *Salmonella*-containing vacuole (SCV), aided by the SPI-2-encoded T3SS-2. This virulence system secretes proteins that prevent the recruitment of lysosomal enzymes thus maintaining the integrity of the vacuole (Kuhle and Hensel, 2004). *Salmonella*

infection eventually leads to pyroptosis, a highly inflammatory response resulting in the death of the infected cell (Kuhle and Hensel, 2004). Through this process, the bacterium becomes susceptible to neutrophil-mediated phagocytosis which contributes to its elimination from the host (Sterzenbach et al., 2013).

Salmonella and *Campylobacter* in Poultry Production

Prevalence of Salmonella and Campylobacter

As the leading bacterial agents of gastrointestinal disease, *Salmonella* and *Campylobacter* cause an estimated 1.8 million illnesses each year in the United States (Scallan et al., 2011). Both microorganisms are frequent colonizers of the intestinal tract of chickens and other avian species, as a result, poultry products account for 19% and 64% of the salmonellosis and campylobacteriosis cases in the U.S., respectively (IFSAC, 2018). With a per capita consumption of poultry meat surpassing 100 pounds each year in 2019 (NCC, 2019), the association between zoonotic infections and poultry meat becomes increasingly concerning.

The prevalence of *Salmonella* and *Campylobacter* in primary production can vary greatly. Farm management practices, nutritional interventions, and vaccination strategies are all factors known to impact the rates of infection in commercial flocks (Lee and Newell, 2006; Alali and Hofacre; 2018). Further, the dissimilarity in experimental design complicates a comparative analysis among studies of prevalence. In a recent systematic review, Golden and Mishra estimated levels of *Salmonella* prevalence in environmental samples from broiler farms of 22.9% and 19.9% in conventional and alternative systems, respectively (Golden and Mishra, 2020). A study by Liljebjelke et al. (2005) surveying *Salmonella* contamination of the litter reported levels of 6 and 22% in two farms. A

prevalence of 39% in fecal samples was reported by Alali et al. (2010), and levels of 70% and 8.8% were found by Thakur et al. (2013) in fecal samples from conventional broiler farms. The prevalence of *Campylobacter* in broiler farms is, on average, higher than that of *Salmonella* (Golden and Mishra, 2020). Prevalence rates of 16% and 53% were estimated by Golden and Mishra (2020) in environmental samples from commercial farms. In a study by Thakur et al. (2013), *Campylobacter* was isolated from 40% of poultry flocks, with a contamination rate of 30% in fecal samples. Researchers frequently report levels of infection with *Campylobacter* surpassing a 70% rate (Sahin et al., 2015). While there is some variability of *Campylobacter* between flocks, within-flock prevalence is usually high (Bull et al., 2008). Consequently, flocks are often reported as either *Campylobacter*-positive or *Campylobacter*-free (Bull et al., 2008; Sahin et al., 2015).

Transmission of Salmonella and Campylobacter in Poultry Production

Both *Salmonella* and *Campylobacter* reside in the intestinal tract of poultry, with the cecum being the primary site of infection (Desmidt et al., 1997; Newell and Fearnley, 2003). Consequently, the intestinal contents of chickens are considered a major source of pathogen contamination at the pre- and post-harvest level (White et al., 1997). Many different entry routes have been characterized for *Salmonella* and *Campylobacter* within a vertically integrated system. *Salmonella* serotypes, most commonly Enteritidis, can colonize the reproductive organs of breeder hens and contaminate the eggs prior to oviposition, thus being vertically transmitted from parents to progeny (Keller et al., 1995; Miyamoto et al., 1997). In recent years, however, vertical transmission of *Salmonella* has become less likely, as a result of effective vaccination strategies (Babu et al., 2004; Dórea

et al., 2010). Eggs may also become contaminated with *Salmonella* at the hatchery, due to horizontal transmission from fecal contents of infected chickens (De Reu et al., 2006). Broiler chicks carry a sparse microbiota upon hatch, making them highly susceptible to *Salmonella* colonization (Liljebjelke et al., 2005). When infected, young birds may develop systematic infections and become long-term shedders during adulthood, further contributing to horizontal transmission within the flock (Gast and Holt, 1998; Van Immerseel et al., 2005). *Salmonella* is also able to persist in the farm environment and has been consistently isolated from the litter and feed (Davies and Breslin, 2003; Whyte et al., 2003; Buhr et al., 2007; Andino et al., 2014). Other sources of *Salmonella* contamination include insects (Wales et al., 2010) and rodents (Meerburg and Kijlstra, 2007), and has been found to be transmitted through air (Holt et a., 1998).

Vertical transmission of *Campylobacter* in broilers remains uncharacterized. Sahin et al. (2003) were unable to isolate *Campylobacter* from eggs laid by artificially-inoculated hens. In the same study, however, Sahin and collaborators showed that *Campylobacter* was able to survive in the yolks of inoculated eggs for 14 days (Sahin et al., 2003). Studies using molecular techniques have provided some evidence of vertical transmission of *Campylobacter* (Hiett et al., 2002, 2003). Based on these results, researchers have suggested that transmission of *Campylobacter* from parents to progeny is possible but has likely gone unnoticed due to the fastidious culturability of the pathogen (Cox et al., 2012). *Campylobacter* is first detected from young birds within the first 2 to 3 weeks of age (Nachamkin et al., 1993; Sahin et al., 2001). Once the first chickens become infected, the pathogen can quickly spread between birds, colonizing virtually every individual within days, thus explaining the high prevalence at the time of

slaughter (Shreeve et al., 2002; Skarp et al., 2016). Contact with infected feces is, in fact, the primary route of *Campylobacter* transmission within poultry farms (Newell and Fearnley, 2003). Because *Campylobacter* is highly sensitive to low moisture conditions, the pathogen is rarely isolated from the poultry feed (Keener et al., 2004). However, the pathogen can contaminate the water and has been isolated from the litter environment (Berndtson et al., 1996; Johnsen et al., 2006; Sparks, 2009). Beetles and flies are important vectors for *Campylobacter* transmission and have received increased attention in recent years (Skov et al., 2004; Templeton et al., 2006; Choo et al., 2011).

Salmonella and Campylobacter in Poultry Raised without Antibiotics

Alternative systems of poultry production have risen in recent years as a result of consumer awareness on antibiotic use and animal welfare (Boyer et al., 2017). Market sales of meat raised without the use of antibiotics have risen dramatically since 2009 (Perrone, 2012; PHT; 2019). Further, more than 50% of the poultry in the United States is produced under no antibiotics ever (NAE) systems (PHT, 2019). The practice of raising broilers under NAE systems involves the withdrawal of medically-important antimicrobials from all stages of the poultry production chain, including those administered *in-ovo* at the hatchery. Additionally, the administration of ionophore anticoccidials through the feed is not allowed under this system (PHT, 2019). The impact of transitioning systems to NAE on the incidence of enteric diseases of poultry remains unclear, as investigations on commercial farms have yet to be carried out. However, experts foresee the need for increased attention to biosecurity on the farm and hatchery as successful strategies for control of zoonotic agents (Cervantes, 2015).

Prevalence data on antibiotic-free broiler farms is lacking. Some studies have shown conflicting results when comparing the prevalence of foodborne pathogens in conventional and alternative systems with restricted antibiotic use. For example, Overbeke et al. (2006) reported that levels of *Salmonella* did not vary greatly between organic (13%) and conventional (9%) broilers. However, Alali et al. (2010) reported a higher prevalence of *Salmonella* in conventional flocks (39%) when compared to organic (6%). Overbeke et al. (2006), in the aforementioned study, reported that organic broilers were almost three times as likely to carry *Campylobacter* than broilers raised under conventional practices (Overbeke et al., 2006). This trend was also seen by Heuer et al. (2001). Golden and Mishra, also estimated a higher prevalence of *Campylobacter* in environmental samples from alternative farms when compared to conventional (Golden and Mishra, 2020). Researchers have yet to attribute these differences strictly to antibiotic use, the reason being that broilers raised under alternative systems frequently have access to the outdoors, thus being susceptible of pathogen colonization from the environment (Sahin et al., 2002). A comprehensive investigation of pathogen dynamics on the novel NAE poultry farms will help provide a better understanding of these systems and illuminate the path towards better on-farm interventions.

Antimicrobial Use in Poultry Production

Antibiotics were introduced in the 1940s, following the discovery of penicillin in 1928 by Alexander Fleming (Ligon, 2004). This event became one of the greatest achievements in modern medicine and since then, antimicrobials have become essential tools for the treatment of disease. In food-producing animals, antimicrobials are used therapeutically to treat and prevent disease, and as nontherapeutic agents for growth

promotion purposes (Dibner and Richards, 2005). Therapeutic treatment includes acute therapy and prophylactic therapy. The former consists of treating diseased animals for a limited time, whereas the latter is a disease prevention and management strategy in which antibiotics are administered to healthy animals at points of stress along the production chain (Dibner and Richards, 2005). The purpose of nontherapeutic use is to enhance performance and feed efficiency conversion, and the practice consists of feeding animal diets containing antimicrobials at sub-therapeutic levels (Dibner and Richards, 2005).

The practice of using Antibiotic Growth Promoters (AGPs) was introduced over 70 years ago in poultry production; when a study reported a correlation between increased weight gain and the administration of in-feed antibiotics to chickens (Moore et al. 1946). The benefits of enhancing feed-efficiency in commercial animal production led to the widespread use of AGPs. In 1951, the United States Food and Drug Administration (FDA) approved the use of antibiotics as dietary supplements without a veterinary prescription, and patent applications of AGPs started to flood the market (Jones and Ricke, 2003). A list of antimicrobials historically approved by the U.S. FDA as dietary additives for poultry can be seen in Table 2.2.

Table 2.2. Antimicrobials historically approved as dietary additives for poultry in the United States[1].

Antimicrobial	**Uses**
Clopidol, narasin, nicarbazin, robenidine, salinomycin, semduramicin	Prevent coccidiosis
Decoquinate	Prevent coccidiosis
Diclazuril, halifusinone, zoalene	Prevent coccidiosis
Amprolium	Prevent coccidiosis

Lasalocid	Prevent coccidiosis
Bacitracin methylene disalicylate, bacitracin (Zn)	Increase rate of weight gain Aid to prevent/control enteritis
Bambermycin	Increase rate of weight gain/feed efficiency
Chlortetracycline	Increase rate of weight gain/feed efficiency Control mycoplasma Control respiratory diseases Reduce mortality of infections by *Escherichia coli*
Lincomycin	Increase rate of weight gain/feed efficiency
Monesin	Prevent coccidiosis
Neomycin/oxytetracycline	Increase rate of weight gain/feed efficiency
Penicillin	Increase rate of weight gain/feed efficiency
Roxarsone	Increase rate of weight gain/feed efficiency
Sulfadimethoxine/ormetoprim	Prevent coccidiosis
Tylosin	Increase rate of weight gain/feed efficiency

	Control respiratory diseases
Virginiamycin	Increase rate of weight gain/feed efficiency Prevent necrotic enteritis (*Clostridium perfringens*)

[1]Adapted from Allen and Stanton 2014

Shortly after the implementation of AGP use, researchers expressed concerns about antibiotic resistance (AMR) in bacteria isolated from poultry that had been fed sub-therapeutic levels of antibiotics (Barnes 1958; Elliot and Barnes 1959). The increasing incidence of multidrug-resistant-bacteria in food animal production became an emerging public health concern, and governmental agencies across the world issued proposals to reduce the use of antimicrobials in livestock and poultry (Aarestrup, 2003).

In 1970, the European Council issued a directive unifying previous regulations concerning animal feed additives which was implemented among member states (Castanon, 2007). In the following years, FDA and several international organizations including the World Health Organization (WHO), the United Nations Food and Agriculture Organization (FAO) and the Institute of Medicine issued reports on antimicrobial resistance highlighting that small dosage and long-term exposure to antimicrobials seemed to have a greater effect on antimicrobial resistance than therapeutic use (WHO 1997). During this period, countries such as Sweden and Denmark issued bans on antibiotic use for growth-promotion (Castanon, 2007). Finally, in 2003, the European Union published the Regulation (EC) No 1831/2003 of the European

parliament and the council on additives for use in animal nutrition, which effectively banned the use of antibiotics as feed additives.

In the United States, feed supplementation with antibiotics remains a widely implemented practice, despite attempts to withdraw non-therapeutic antibiotic use from animal feed. In 1977, the FDA issued a proposal to prohibit the use of tetracycline and penicillin in food animal production (National Research Council, 1980). However, lack of evidence concerning the impact of resistant bacteria in human health led to its refutal (National Research Council, 1980). It is now well understood that antimicrobial use in food-animal production contributes to the emergence of antimicrobial resistance in foodborne bacteria (Allen et al. 2010). A comprehensive study published by the European Antimicrobial Resistance Surveillance Network (EARS-Net) corroborated the transmission of antimicrobial-resistant *Salmonella* and *C. jejuni* from food-animals to humans, prompting worldwide campaigns to limit antibiotic use in meat production and agriculture (FDA, 2012; ECDC, EFSA and EMA, 2017). In the US, the FDA published a guidance document on the judicious use of medically important antimicrobials in food-animals, providing industry recommendations on voluntary practices to ensure a responsible use and prevent a loss of efficacy (FDA, 2012).

Modulations of Gut Microbiomes due to Antibiotic Use

Gut Microbiota

Poultry producers rely on antibiotics to prevent the incidence of subclinical infections and enhance broiler performance and profitability (Castanon, 2007). However, the exact mechanisms by which growth promotion is achieved are not fully understood. Early studies conducted by Coates et al. showed that orally-administered antibiotics did

not induce a growth-promoting effect in chicks free from native microorganisms (Coates et al. 1955; Coates et al. 1963). This was one of the first studies to conclude that weight gain effects of dietary antibiotics were dependent on modulations of the intestinal microbiota. Since then, authors have proposed several mechanisms for growth promotion explained through the direct effects of antimicrobials on the gut microflora. These mechanisms include (a) reduction in nutrient competition between the host and the gut microflora, (b) decreased production of growth-suppressing microbial metabolites, (c) enhanced nutrient digestibility due to thinning of gut wall and intestinal villi, and (d) reduction of subclinical infections caused by opportunistic pathogens (Dibner & Richards, 2005).

Antibiotic use in food-animals may exert a beneficial effect in terms of food safety and animal health. For example, antimicrobials can prevent colonization or reduce the intestinal load of certain pathogenic bacteria, which in turn, may reduce the risk of foodborne illness in humans (Johansen et al., 2007; Ramiah et al., 2014). In broiler production, antibiotics play a crucial in controlling the intestinal populations of *Clostridium perfringens*, the causative agent of necrotic enteritis (NE) (Immerseel et al., 2004). Conversely, antimicrobials may impose non-targeted inhibitory effects due to a wide activity spectrum. Prolonged administration of these drugs may deplete the gut microbiota from certain microbial populations, leading to a potentially detrimental dysbiosis (Zhang and Chen, 2019). Que and Hentges (1985) illustrated this event in the development of a mouse infection model for *Salmonella enterica* serovar Typhimurium. The authors demonstrated that streptomycin treatment disrupted the balance of the intestinal mouse microbiota and consequently increased the susceptibility to colonization

of streptomycin-resistant *S.* Typhimurium populations in the gut (Que and Hentges, 1985).

Many studies have evaluated the impact of broad-spectrum antibiotics on the poultry gut microbiota with different outcomes (Dumonceaux et al., 2006; Neumann and Suen, 2015; Zhu et al., 2019). The use of virginiamycin, a popular feed additive in conventional broiler production, has been associated with reductions of lactic acid bacteria resulting from its inhibitory effect against Gram-positives (Hynes et al., 1997; Zhu et al., 2019). La Vorgna and collaborators (2013), while evaluating the impact of virginiamycin on broiler performance, showed an association between antibiotic use and chicken body weight gain. The authors then hypothesized that this increase in efficiency was likely attributed to the virginiamycin-induced inhibition of gut populations of Gram-positive bacteria (LaVorgna et al., 2013). This effect was also reported by Zhu et al. (2019), who observed that the relative abundance of *Lactobacillus* groups in the ceca of chickens treated with virginiamycin was three times lower (7%) than that of the control group (21%). Treatment with virginiamycin has also been associated with a decrease in the taxonomic diversity of cecal microbial communities and the enrichment of the genus *Faecalibacterium* (Neumann and Suen, 2015). Pourabedin et al. (2015), reported similar effects showing a reduction in bacterial diversity and populations of *Lactobacillus* and *Bifidobacterium* in the ceca of virginiamycin-treated chickens. In turkey production, virginiamycin feed supplementation has been reported to increase the intestinal colonization rate of *C. jejuni* (Scupham et al., 2010).

On the other hand, virginiamycin supplementation has also been linked to beneficial modifications of the poultry gut microbiota. Neumann and Suen (2015), for

example, showed an increase in the abundance of *Lactobacillus* in the small intestine of broilers receiving a virginiamycin supplemented-diet (Neumann and Suen, 2015). Similarly, Dumonceaux et al. (2006) reported a higher abundance of Lactobacillales in the ileum of chickens treated with virginiamycin but a decreased abundance of these populations in the cecum. Authors then hypothesized that virginiamycin treatment was more likely to change the abundance of specific bacterial strains rather than modify the total abundance of bacterial genotypes within the intestinal microbiota (Dumonceaux et al., 2006; Gong et al., 2008). Research conducted by Engberg et al. (2000) supports this hypothesis, as the authors showed that broiler feed supplementation with salinomycin and zinc bacitracin reduced intestinal populations of *Lactobacillus salivarius* but did not affect the total abundance of *Lactobacillus* spp. (Engberg et al., 2000). A similar observation was reported by Pourabedin et al. (2015), who concluded that antibiotics, despite having a wide activity spectrum, were more likely to modulate the gut microbiota in a species-specific manner.

Antimicrobial Resistance

There is enough evidence to attribute the increasing incidence of antibiotic-resistant infections in humans to the widespread use of sub-therapeutic antibiotics in animal production (Allen et al., 2010). A comprehensive review carried out by Lazarus et al. (2015) provided concluding evidence for the dissemination of expanded-spectrum cephalosporin-resistant *Escherichia coli* from animals to humans. The spread of this type of resistance is of great concern to public health worldwide, since extended-spectrum cephalosporins (ESCs) are used to treat severe human infections (WHO, 2019). The

cumulative incidence of drug-resistant infections paired with a slow development of novel antibiotic therapies further complicates this issue (CDC, 2019).

Antimicrobial resistance is not a modern occurrence. Microbes have evolved to naturally acquire antimicrobial resistant traits from the environment and other community members as a mechanism for adaptation and survival (D'costa et al. 2011). However, the rate at which this phenomenon develops is exacerbated by the selective-pressure imposed by antibiotic use (Andersson and Hughes, 2012). Bacteria may acquire resistance through many mechanisms, of which horizontal gene transfer (HGT) is the most common (Aminov, and Mackie 2007; Aarestrup et al. 2008). The process of HGT encompasses three basic strategies: transformation, transduction and conjugation (Tenover, 2006). Under a transformation event, bacteria are able to uptake non-bound DNA from the environment and incorporate it into their genome (Tenover, 2006). Transduction is a phage-mediated process resulting from the injection of resistant genetic determinants into the bacterial cell (Tenover, 2006). Conjugation is, likely, the highest contributor to AMR within the intestinal microbiota (Hedge et al., 2016). The process is carried out by cell-to-cell transfer of resistant genes through mobile genetic elements, such as plasmids, transposons, and integrons (Tenover, 2006). The latter are particularly concerning, being responsible for the development of multi-drug resistance (MDR) in bacteria from the *Enterobacteriaceae* family (Hedge et al., 2016). In recent years, commensal microorganisms have been recognized as important vectors of MDR within intestinal bacterial communities by transfer of resistant integrons (Marshall et al., 2009; Djordjevic et al., 2013). In poultry specifically, researchers report of an increase in the occurrence of

MDR commensal *E. coli,* acting as a significant reservoir of resistance genes in the intestinal microbiota (Marshall et al., 2009; Zhao et al., 2001; Hedge et al., 2016).

Antimicrobial Resistance in Salmonella and Campylobacter

Enteric pathogens *Campylobacter* and *Salmonella* are routinely surveyed by the Centers for Disease Control and Prevention (CDC), as part of their National Antimicrobial Resistance Monitoring System (NARMS), due to rises in resistance to drugs of clinical importance (CDC, 2019). In the case of *Salmonella*, resistance to ciprofloxacin, azithromycin, and ceftriaxone has been increasing steadily in the past decade (CDC, 2019). Consequently, more than 200,000 resistant salmonellosis cases are estimated annually in the United States (CDC, 2019). In its latest report, NARMS warns of dramatic rises in resistance to ceftriaxone within *Salmonella* isolates recovered from poultry and poultry meat sources (FDA, 2018). The reported rates of ceftriaxone-resistant *Salmonella* were 9.3% in chicken samples collected at slaughter and 12% in ceca samples from turkeys (FDA, 2019). Ceftriaxone is an extended-spectrum cephalosporin used to treat invasive salmonellosis in children (WHO, 2019). Resistance to this drug in *Salmonella* and other members of the *Enterobacteriaceae* family is achieved by production of the production of extended-spectrum β-lactamases (ESBLs) which confer resistance to a broad range of antimicrobial classes (Bradford, 2001; Chen et al., 2019). The incidence of ceftriaxone-resistant *Salmonella* has been increasing worldwide, posing a significant threat to public health (Iwamoto et al., 2017; Kuang et al., 2018; Chen et al., 2019).

Paired with rises in ceftriaxone resistance, the trend of decreased susceptibility to ciprofloxacin has also been increasing within *Salmonella* isolates (CDC, 2019). NARMS

estimate levels of ciprofloxacin resistance in *Salmonella* isolates recovered from poultry sources range from 9% to 18% (FDA, 2018). Ciprofloxacin, a fluoroquinolone, is a drug used in the treatment of acute *Salmonella* and *Campylobacter* infections (WHO, 2019). Fluoroquinolones work by inhibiting DNA gyrase, an essential enzyme of DNA replication (Drlica, 1999). Ciprofloxacin resistance is primarily achieved by double mutations in the *gyrA* gene paired with a single mutation in the *parC* gene in *Salmonella* isolates (Hooper, 2001). Whereas in *Campylobacter*, ciprofloxacin resistance results mainly from a single point mutation C257T in the *gyrA* gene, which encodes the DNA gyrase subunit A (Wieczorek and Osek, 2013). Resistance to ciprofloxacin is becoming increasingly prevalent in *Campylobacter* spp., particularly among *C. coli* isolates (Gupta et al., 2004) and is also becoming widespread (Post et al., 2017). In the United States, drug-resistant *Campylobacter* causes an estimated 448,000 annual infections resulting from a decreased susceptibility to ciprofloxacin and azithromycin (CDC, 2019). Among the two, resistance to ciprofloxacin is the most prevalence in *Campylobacter* isolates from poultry, with reported rates ranging from 15 to 28% (FDA, 2018).

Finally, the incidence of multidrug resistance (MDR) among *Salmonella* serotypes has increased dramatically in recent years (Brunelle et al., 2017; CDC, 2019). Current levels of MDR *Salmonella* isolated from chickens, range from of 18% to 25% in samples collected during processing operations in the United States (FDA, 2018). Further, numerous MDR *Salmonella* serotypes are frequently isolated from commercial broiler farms (Liljebjelke et al., 2017). Simultaneous resistance to ampicillin, streptomycin, sulfonamide, and tetracycline, or ASSuT, is an emergent and widespread MDR pattern (Barco et al., 2014; Yang et al., 2015). This is quickly becoming an issue of

clinical importance, as it is estimated that 20% of MDR *Salmonella* isolated from humans carry ASSuT resistance (CDC, 2019).

CHAPTER 3

CHARACTERIZING THE GUT MICROBIOMES OF BROILERS RAISED UNDER CONVENTIONAL OR NO ANTIBIOTICS EVER PRACTICES[1]

[1] Novoa Rama, E., Bailey, M., Kumar, S., den Bakker, H., Thippareddi, H., and Singh, M. To be submitted to *Journal of Food Protection*

Abstract

Meat from broilers raised without the use of antibiotics is increasingly becoming popular among consumers. Consequently, the interest in the microbial profiling of chickens produced under non-conventional practices is growing. Research on this topic, however, is lacking. The current study was designed to characterize the dynamics of gut microbial populations of conventional or no antibiotics ever broilers. Four commercial farms, two conventional and two no antibiotics ever (NAE) were included in this study. Within each farm, cecal (n=224) and ileal (n=224) contents were collected from birds at different stages during the grow out of a single flock and following transportation to the processing facility. The V4 hypervariable region of the 16S rRNA gene was sequenced on the Illumina MiSeq platform, and the software package DADA2 software package was used for bioinformatic analysis. The cecal microbiota was dominated by the genus *Escherichia* upon hatch, shifting with time towards a predominance of *Faecalibacterium* and *Bacteroides*. The composition of cecal microbial communities of NAE broilers was different than that of conventional chickens ($P \leq 0.05$). Conventional broilers harbored a rich, but less diverse cecal microbiota than NAE. The ileum microbiota was primarily populated with genera previously named *Lactobacillus*, which exhibited a higher abundance in NAE broilers ($P \leq 0.05$). Under both production systems, the microbiota followed a similar temporal succession that was more evident in the ceca. Transportation to the processing plant impacted the microbial composition of the ileum ($P \leq 0.05$), characterized by a sharp increase in the abundance of *Psychrobacter*. Finally, differential abundance analysis showed a positive correlation between *Campylobacter* and *Parasutterella* within the cecum microbiota, and a negative correlation with *Clostridium.*

Introduction

The gastrointestinal tract of chicken harbors a diverse and dynamic microbial ecosystem led by bacteria (Zhu et al., 2002). The role of this microbial community, or microbiota, in host metabolism, nutrition and immune function has been extensively studied (Brisbin et al., 2008; Owens et al., 2008; Yegani and Korver, 2008). Consequently, the gut microbiota plays a complex role in host health. In commercial poultry production, the host-microbiome interaction has direct implications on growth performance, disease occurrence and, ultimately, on productivity (Danzeisen et al., 2011; Singh et al., 2012; Pan and Yu, 2014). The poultry gut microbiota can also impact food safety and public health by acting as a line of defense against foodborne pathogen colonization through competitive exclusion events (DuPont, 2007; Yeoman et al., 2012). Furthermore, recent advances in DNA-based molecular techniques have allowed a deeper exploration of gastrointestinal microbiomes, highlighting the role of previously uncharacterized microbial groups (Zhu et al., 2002; Gong et al., 2007). For these reasons, microbiome research is now at the forefront of food safety and animal health.

The per-capita consumption of poultry meat in the United States surpassed 110 pounds in 2019 (NCC, 2019). Furthermore, changes in consumer behaviors have influenced the poultry meat market towards the production of "Antibiotic-Free (ABF)" or "No Antibiotics Ever (NAE)" broilers (PHT, 2019). Raising chickens under NAE production systems involves the removal of antibiotic administration, from the hatchery and primary production (PHT, 2019). This transition may pose significant challenges to poultry producers who relied on antibiotics to reduce the incidence of subclinical infections and enhance broiler performance and profitability (De Gussem, 2007). In

primary production, antibiotics such as virginiamycin are used to control the populations of *Clostridium perfringens*, thus reducing the incidence of necrotic enteritis (Allan and Stanton, 2014). These antimicrobials, however, exhibit a wide range of activity that may decrease gut populations of beneficial bacterial groups (Hynes et al., 1997; LaVorgna et al., 2013). Disruptions of the normal gastrointestinal microbiota lead to a qualitative and/or quantitative microbial imbalance, or dysbiosis. Dysbiosis can negatively affect nutrient digestibility, immune function, and disease resistance (Teirlynck et al., 2011; Zhao et al., 2013; Sommer et al., 2017).

Assessment of potential disruptors of the poultry gut microbiota and extensive characterization of a healthy microbial community composition, have not been accomplished . An impaired gut microbiota resulting from a loss of taxonomic diversity provides a niche for enhanced colonization of detrimental microorganisms (Fujimura et al., 2010; Sommer et al., 2017). For this reason, a healthy microbiota is commonly defined as a diverse microbiota. A number of factors including age, diet composition, and genetics, have been shown to impact the complexity of gut microbial groups (Gong et al., 2008; Singh et al., 2012; Danzeisen et al., 2015). Other factors such as rearing conditions and antibiotic supplementation are less-understood and data is scarce (Pourabedin et al., 2014, 2015; Crippen et al., 2019). The following study was designed to improve our understanding of gut microbial community dynamics as affected by dietary supplementation with antibiotics in a commercial production setting. For this purpose, we characterized the ceca and ileum microbiota of commercial broiler birds raised under conventional or NAE practices during a full production cycle.

Materials and Methods

Farm Characteristics

Four farms raising birds for the same integrator and classified as either conventional or no antibiotics ever (NAE) were selected. Within each farm, one flock was the subject of investigation. The Ross 708 genetic line, which is considered a high-yield variety was used in all four flocks. Broiler house specifications and flock sizes were comparable among groups, these are described in Table 3.1. All birds were raised under a similar nutritional regimen (Table 3.1), only differing in antibiotic supplementation and coccidiosis management strategies. Conventional birds received a virginiamycin-supplemented feed (20 g/ton) from day of hatch until the second withdrawal period (WD2, Table 3.2). However, antibiotic use in chickens from conventional house A was discontinued after day 1.

The diets of NAE broilers were not supplemented with antibiotics, but broilers were given a synthetic anticoccidial during the first 30 days of age. Anti-coccidiosis interventions varied among conventional broilers. Chickens raised in house C were given salinomycin between 20 and 32 days of age, whereas no form of coccidiostat was given to conventional broilers from house A. Regardless of production systems, broilers underwent two distinct feed withdrawal periods. The first withdrawal feed (WD1) was characterized by a diet of high caloric content and a reduced supplementation of lysine. The second withdrawal (WD2) was a traditional feed withdrawal during which birds are given a low-nutrient feed without additional supplementation, thus antibiotics are removed from the feed at this time.

Sample Collection

Samples were collected in two separate rounds, and two farms (one conventional and one NAE) were visited during one production cycle. Sample collection from the first two farms (houses A and B) was carried out during the summer (June to August) of 2018, and the remaining farms (houses C and D) were visited during the fall (September to November). Samples were collected from each farm at six different time points, corresponding to day of placement (hatch, 0d) and every change in feed regimen. Furthermore, broilers were sampled after transportation to the processing facility after unloading from the coops. The average transit time from farm to processing plant was approximately 1 hour for all the flocks. With each sampling day, 8 chickens were selected at random and euthanized by cervical dislocation, with the exception of 1 day-old chicks, of which 16 birds were selected and pooled in groups of two. The ceca (n=8) and ileum (n=8) were then aseptically removed using sterile tongs and scissors, placed inside sterile Whirl-Pak™ bags (Nasco Whirl-Pak™; Fisher Scientific Pittsburgh, PA) and immediately placed on ice. Upon arrival to the laboratory, intestinal contents were aseptically extracted and homogenized with buffered peptone water (BPW; Fisher Scientific, Pittsburgh, PA) using a Stomacher for 1 minute (230 rpm). For DNA extraction, a 1.5 mL portion of the sample homogenate was collected and stored in a -80 °C freezer.

DNA Extraction and 16S rRNA Sequencing

DNA extraction and 16S rRNA sequencing were carried out at the Microbiome Core Facility of The University of North Carolina at Chapel Hill (Chapel Hill, NC). Isolation of microbial DNA was achieved by bead beating in a Qiagen TissueLyser II (5

minutes at 30 Hz) using 200 mg of ≤106 μm glass beads (Sigma, St. Louis, MO). Samples were centrifuged for 5 minutes and 0.45 mL of supernatants were resuspended with 0.15 mL of Qiagen IRS solution. The suspension was incubated at 4 °C overnight. After a second centrifugation, supernatants were transferred to well plates containing 0.45 mL of Qiagen binding buffer supplemented with Qiagen ClearMag Beads. DNA was purified using the automated KingFisher™ Flex Purification System (Thermo Fisher Scientific, Wilmington, DE) and eluted in DNase-free water (Thermo Fisher Scientific, Wilmington, DE).

For 16S rRNA amplicon sequencing, sample DNA was amplified using universal primers targeting the V4 region of the aforementioned gene (Caporaso et al., 2011). Primer sequences were the following:

- Forward: 5’ CMGCCGCGGTAA 3’
- Reverse: 5’ GGACTACHVGGGTWTCTAAT 3’

The PCR reactions were carried out using 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA), 0.5 μM of the 16S rRNA gene (V4 region) forward and reverse primers, and 12.5 ng of total DNA. The cycling conditions for target amplification consisted of an initial denaturing step at 95 °C for 3 minutes, followed by 25 cycles of denaturation (95 °C, 30 seconds), annealing (55 °C, 30 seconds) and extension (72 °C, 30 seconds), a final extension at 72 °C for 5 minutes and hold at 4 °C. The AMPure XP reagent (Beckman Coulter, Indianapolis, IN) was used for purification of 16S amplicons. Following, samples were amplified by a limited cycle PCR assay to attach Illumina sequencing adapters and barcodes to the amplicon target. The thermal cycling conditions were the following: an initial 3-minute denaturing step at 95 °C, eight

cycles of denaturing (95 °C, 30 seconds), annealing (55 °C, 30 seconds) and extension (72 °C, 30 seconds), a 5-minute extension step at 72 °C and a hold stage at 4 °C. The resulting libraries were purified, quantified and normalized to create a DNA library pool. Library denaturation and dilution was carried out following specifications for the MiSeq instrument (Illumina, San Diego, CA). Cluster generation and paired–end sequencing were performed according to the instructions developed by the manufacturer.

Bioinformatic and Statistical Analysis

Bioinformatic analysis of amplicon data was performed using the software package DADA2. Developed by Callahan et al. (2016), this software was designed to overcome Illumina-sequenced amplicon errors using the Divisive Amplicon Denoising Algorithm (DADA), which avoids the use of Operational Taxonomic Units (OTUs) and allows identification of fine-scale variation in amplicon data. The Illumina paired-end sequencing data (fastq files) were preprocessed using the DADA2 software. This consisted on a filtering step to remove primers, followed by sample inference and merging of paired-end reads, and removal of chimeric sequences. Taxonomy was assigned using the assignTaxonomy function with the Genome Taxonomy Database (GTDB). The following bioinformatic analysis was carried out in RStudio (Version 1.3.1056) by adapting the DADA2 Pipeline Tutorial (1.16) to our data set, which can be accessed at https://benjjneb.github.io/dada2/tutorial.html.

Subsequent analysis was carried out in RStudio (Version 1.3.1056) using the Phyloseq v.1.19.1 package for data visualization, diversity analysis and evaluation of differential abundance (McMurdi and Holmes, 2013). Non-parametric tests Kruskal-Wallis and Pairwise Wilcoxon were carried out to test differences in alpha diversity

measures between groups. The alpha diversity indices under evaluation were Observed ASV and Chao1 for inference of species richness, and Shannon and Simpson indices for evaluation of community evenness. Beta-diversity analysis was performed by calculation of Bray-Curtis dissimilarity distances and ordination by Non-Metric Multidimensional Scaling (NMDS). Effects of age, treatment, season, and farm on microbial community composition, as evaluated by Bray-Curtis dissimilarity, were tested using PERMANOVA and betadisper from the Vegan package in R (Oksanen et al., 2017). A linear mixed model analysis was also performed within each measure of alpha and beta diversity to investigate potential confounding variables. For this analysis, the lmer function under the merTools package (Knowles and Frederick, 2016) was used, setting the variable DAY (age) as a fixed effect. Finally, differential abundance analysis was performed using the Wald test of significance with a parametric fit type and a significance level of 0.01, carried out under the DESeq2 package (Love et al., 2014). Differentially abundant taxa were identified within cecal communities of different treatment groups (conventional or NAE), as well as within *Campylobacter*-positive or -negative communities.

Results

Alpha Diversity

Alpha diversity metrics corresponding to ceca samples by age group are represented in Figure 3.1. For both conventional and NAE datasets, alpha diversity estimations followed a similar distribution with age. Species richness (Observed ASV and Chao1) increased gradually as broilers aged, reaching maximum values on the last day of WD1 (50d) and the last day of finisher (43d) for conventional and NAE communities, respectively. In ceca samples from conventional birds, species richness

increased from day of hatch (0d) to the last day of finisher (d43) ($P \leq 0.05$), and plateaued from day 50 until the end of the production cycle. Cecal communities from NAE broilers, however, exhibited a steady decrease in species richness after day 43, corresponding to the withdrawal feeds (WD1 and WD2) and transportation to the processing plant (T). These differences are highlighted in Figure 3.2, which represents the alpha diversity metrics of cecal microbial communities as grouped by treatment. When comparing conventional and NAE broilers, the former exhibited higher values of species richness ($P \leq 0.05$). Considering the impact of age on alpha diversity, the datasets were further grouped as young (d0 – d43) or old (d50 – d56) broilers (Figure 3.3). This analysis then showed that the values for species richness between conventional and NAE microbial communities started to diverge after day 43 of age.

Measurements of community heterogeneity (Shannon and Simpson indices) exhibited similar patterns in both treatment groups (Figure 3.1). The lowest values were attributed to the microbiota of young broilers, which suggests that species distribution in these communities was uneven. Similar to species richness, average Shannon and Simpson indices increased as the birds aged and reached a maximum on days 43 (NAE) and 50 (conventional). Cecal microbial communities of conventional broilers were more evenly distributed than those of NAE chickens (Figure 3.2). These differences, again, were evident in chickens older than 43 days of age (Figure 3.3).

Alpha diversity measures were compared between cecal and ileal microbial communities (Figure 3.4). Kruskal-Wallis tests indicated that the average values of species richness (Observed ASV and Chao1) and community evenness (Shannon and Simpson) were significantly higher in cecal samples ($P \leq 0.05$). Thus showing that the

ceca harbors a more rich and uniform microbiota. Figure 3.5 represents alpha diversity indices of communities in the ileum as impacted by age group. Species richness remained relatively stable throughout production for both conventional and NAE broilers ($P > 0.05$). Pairwise comparison showed a significant increase of species richness for the conventional dataset from the last day of WD1 (50d) to the maximum value of Observed ASVs achieved at post-transportation (T, 56d). Whereas in NAE broilers, diversity measures peaked on the last day of WD2 (56d) and decreased slightly after transportation. Measures of community evenness were not impacted by age group ($P > 0.05$). Further, Shannon and Simpson indices corresponding to the NAE dataset exhibited a wide range of variability between samples for days WD2 and T, as represented by the length of the boxplots. The impact of treatment on alpha diversity measures was also evaluated (Figure 3.6). Species richness estimates did not differ among treatment groups, however, microbial species in conventional communities were more evenly distributed as indicated by higher Shannon and Simpson values ($P \leq 0.05$).

Results from the linear mixed model analysis showed that there was a significant interaction between seasonality and treatment (conventional or NAE) on alpha diversity measures ($P \leq 0.05$). Figure 3.7 represents differences in alpha diversity indices of both cecal and ileal datasets as impacted by sampling season. This seasonality analysis showed that ceca samples from broilers sampled in the summer harbored a more rich microbial community as indicated by Observed and Chao1 measures, but the species present were equally abundant in both seasons (Shannon and Simpson measures). In ileum samples, seasonality impacted species evenness, but not species richness. Thus, the number of

species in microbial communities did not differ between seasons, yet in the summer, the microbiota exhibited a more equal distribution of the species present.

Beta Diversity

The differences in microbial community composition between sample groups were evaluated by the Bray-Curtis beta diversity index and visualized using non-metric multidimensional scaling (NMDS) ordination. The Jaccard index was also used for evaluation of community similarity (data not shown) and results were consistent with those obtained using Bray-Curtis. Figure 3.8 represents the level of similarity between microbial communities of different age groups for both ceca and ileum samples. Analysis with PERMANOVA showed a significant impact of age on both the ceca ($R^2 = 0.28$, $P < 0.001$) and ileum ($R^2 = 0.25$, $P < 0.001$) microbiota. Results from betadisper, however, showed that variance between individuals within age groups was significant for both ceca ($F = 2.2$, $P = 0.04$) and ileum samples ($F = 3.5$, $P = 0.003$) indicating a low homogeneity in dispersions. Age-related clustering was still observed in both datasets. In the ceca, the community structure of hatch day chicks (0d) clustered further apart from all other age groups. Whereas clusters corresponding to broilers of ages 19d to 56d, and those sampled after transportation, exhibited a considerable overlap, indicating a high level of similarity between these communities. In the ileum, communities from the last day of starter (19d) to the last day of WD1 (50d) clustered closely among them, but far from communities associated with transportation (56d) and day of hatch (0d). Samples corresponding to the last day of WD2 (56d) on the other hand, exhibited the highest within-group distance.

Microbial community composition as evaluated by Bray-Curtis dissimilarity was also compared between treatment groups, conventional and NAE, for both ceca (Figure

3.9) and ileum (Figure 3.10) samples. Results from PERMANOVA testing identified treatment as a significant factor ($R^2 = 0.072$, $P < 0.001$) for the ceca dataset (excluding hatch day samples). Yet, attention should be paid to the effect size, as illustrated by the low R-square statistic, which indicates that only 7.2% of the total variance in distances can be explained by the treatment. Further, results from betadisper analysis deemed within group variance as significant, indicating a high level of dispersion ($F = 9.5$, $P = 0.002$). Despite the lack of homogeneity, samples clustered together under each treatment group, with those from conventional broilers forming a tighter cluster. This was further corroborated by calculating the distance from the centroids of each treatment group, which has highest for the NAE dataset (data not shown).

When analyzing the diversity of individual age groups, broiler house proved a significant factor ($R^2 = 0.43$, $P < 0.001$), impacting cecal communities from broilers sampled on day WD1 (50d). The corresponding NMDS plot illustrates this effect, showing four distinct clusters associated with each farm (Figure 3.11). Previous alpha diversity analysis indicated that species richness in the ceca microbiota was highest on day WD1 (50d) (Figure 3.1). These results therefore suggest a significant differentiation of the mature cecal microbiota that is associated with the individual broiler flocks. Treatment effect on ileum samples was also deemed significant by PERMANOVA ($P < 0.001$), however, the corresponding R-square statistic was very low ($R^2 = 0.02$). This is further illustrated by the considerable overlap between communities of both treatment groups (Figure 3.10).

The impact of season on microbial community composition was also evaluated (Figure 3.12). In the ceca, the impact of season was more evident than that of treatment,

(PERMANOVA; $R^2 = 0.1$, $P < 0.001$), reinforced by a lack of variance between individuals (betadisper; $F = 0.52$, $P = 0.5$). Linear mixed model analysis was used to evaluate the potential confounding effect of variables "farm" and "season" on beta diversity computed as relative abundance data. Results from this analysis showed that both factors impacted the cecal microbial community composition ($P \leq 0.05$). Ileal microbial communities again did not differ between seasons as illustrated by the significant overlap between both clusters (Figure 3.12). Again, PERMANOVA results were deemed significant ($P < 0.001$) yet the effect size was considerably low ($R^2 = 0.015$).

Microbial Community Dynamics

The composition of cecal microbial communities of conventional and NAE broilers as represented by the most abundant taxa is shown in Figure 3.14. Firmicutes, Bacteroidota, and Proteobacteria were the most abundant phyla in the ceca, undergoing notable changes in relative abundance associated with age. Upon hatch (0d), Firmicutes and Proteobacteria represented over 50% and 36% of the microbiota in NAE broilers, and more than 17% and 70% of the cecal microbial community in conventional birds. Under both treatments, the abundance of Proteobacteria decreased sharply after the first two weeks of age, while the proportion of Firmicutes and Bacteroides increased. At the genus level, *Escherichia* was the predominant group of the cecal microbiota of young chicks (0d), accounting for 71% and 99% of all Proteobacteria in NAE and conventional birds respectively. Chicks of NAE systems hosted a more diverse cecal microbiota, represented by *Clostridium* (34%), *Escherichia* (26%), *Enterococcus* (12%), and *Psychrobacter* (8%). In comparison, the ceca of conventional chicks was characterized by a

predominance of *Escherichia* (70%). Differences in microbial community composition between production systems were evident from the day of placement on the farm.

As the birds aged, cecal microbial communities became more diverse, and changes in relative abundance followed similar patterns under both NAE and conventional production. Of the Firmicutes, Clostridiales and Lactobacillales were the most abundant groups. At the end of the starter ratio (19d), the genus *Faecalibacterium* was the most abundant of the Clostridiales, representing 17% of the total taxa in both treatment groups, followed by *Lactobacillus*, with an abundance of 9% and 6% in conventional and NAE broilers respectively. The abundance of Firmicutes decreased after the finisher ratio (43d), coinciding with an increase in the abundance of Bacteroides. Of the Bacteroides phylum, *Alistipes*, *Bacteroides*, and *Bacteroides_B* were the predominant taxa, with an average abundance of 6%, 7%, and 8% in conventional chickens and 9%, 8%, and 9% in those NAE-raised. Proteobacteria again became a prominent phylum of the cecal microbiota after birds were transported to the processing facility. Notably, the abundance of the genus *Psychrobacter* increased from 0.02% to 16%, and from 0.1% to 8% in NAE and conventional broilers, respectively.

Some of the most prevalent genera followed distinct patterns of abundance in the different treatment groups. For example, in NAE broilers, the genus *Coprobacter* was present in all age groups at an average abundance of 4%. However, in conventional broilers, *Coprobacter* represented less than 1% of the total taxa throughout production, only increasing its abundance to 6% after the second withdrawal feed (WD2, 56d). The genus *Alistipes* was also more abundant in the cecal microbiota of NAE broilers, particularly at the end of the production cycle (WD1, 50d), representing 11% of the total

taxa. Of the foodborne pathogens, *Campylobacter* was the most prevalent genus in cecal microbial communities. The highest abundance of *Campylobacter* was found in 56 day-old NAE broilers (0.4%) and 29 day-old conventional broilers (0.1%).

The dynamics of ileum microbial communities are represented in Figure 3.15 as represented by the top 10 most abundant taxa. Firmicutes, Proteobacteria, and Actinobacteriota were the dominating phyla of the ileum microbiota. There were noticeable differences in the diversity of the chick microbiota (0d) between production systems. Actinobacteriota represented over 41% of all the microbial taxa in NAE birds, having *Brevibacterium* (12%), *Corynebacterium* (10%), and *Nocardoipsis* (10%) as the most abundant genera. The level of Actinobacteriota in conventional chicks, however, did not surpass 12%. In this system, Proteobacteria was the predominant phylum, encompassing more than 51% of the total taxa. The genus *Escherichia* was the most abundant, accounting for 85% of the Proteobacteria and 44% of all the taxa in the ileum microbiota. Conversely, the presence of *Escherichia* in the microbiota of NAE chicks was below 4%. Temporal succession of ileal microbial communities was characterized by an increase in the abundance of Firmicutes to levels of 60% and 65% in conventional and NAE broilers.

The Firmicutes phylum was primarily represented by the genera *Lactobacillus*, *Ligilactobacillus*, and *Limosilactobacillus*, which underwent distinct shifts in relative abundance as the birds aged. In both treatment groups, *Lactobacillus* was the predominant genus, accounting for 32% and 31% of the ileum microbiota in conventional and NAE broilers respectively. The genus *Ligilactobacillus*, which includes the species *L. aviarius* and *L. salivarius*, was more abundant in NAE broilers, and became the

predominant taxa (53%) at the end of the first withdrawal period (WD1, 50d). On the other hand, *Limosilactobacillus* was more abundant in conventional broilers, representing 20% of the mature microbiota (WD1, 50d). Transportation to the processing facility changed the composition of the ileum microbiota, as characterized by a decrease in the presence of Firmicutes and Actinobacteriota, and a significant increase of Proteobacteria. Specifically, the genus *Psycrhobacter* became the leading taxa at levels of 37% in conventional birds and 80% in NAE-raised birds, a shift that was previously characterized in the ceca.

The complete list of genera identified as under- or overrepresented for the different treatment groups is shown in Tables 3.3 (ceca samples) and 3.4 (ileum samples). Interestingly, differential abundance analysis revealed that *Campylobacter* was, in fact, significantly more abundant in the ceca of NAE broilers (Table 3.3). The most representative groups of the cecal microbiota of NAE broilers were *Sutterella*, *Helicobacter_G*, and *Bifidobacterium*, whereas *Oxalobacter* and *Enorma* were most often associated with conventional broilers. Furthermore, microbial taxa identified as potential biomarkers of conventional chickens included the genera *Escherichia* and *Clostridium_P*. In the ileum, *Suterella*, *Helicobacter_G* and *Campylobacter* were again significantly more associated with NAE systems. Of the Lactobacillales, *Ligilactobacillus* was identified as an overrepresented genus of NAE broilers, and *Limosilactobacillus* of conventional chickens. Table 3.5 shows the list of genera differentially represented according to the presence of *Campylobacter* in the ceca. Under both production systems, the presence of *Campylobacter* was negatively correlated with that of *Clostridium*. Other groups, such as *Enterococcus*, *Yersinia* and *Salmonella* were

also less represented in the ceca of *Campylobacter*-positive broilers. In contrast, the presence of genera *Acinetobacter, Enorma, Helicobacter_G,* and *Parasutterella* was strongly correlated with *Campylobacter*.

Discussion

A comparative analysis of intestinal microbial communities between conventional and no antibiotics ever (NAE) chickens was carried out. Results from this study provided an exhaustive characterization of the microbiota in the ceca and ileum of commercial broilers for the duration of a production cycle. Further, our findings showed that transportation to the processing facility impacted the composition of intestinal communities, an effect previously not evaluated.

Broiler age was deemed a significant factor of microbial community diversity (Figures 3.1, 3.3 and 3.8). Numerous studies have elucidated the impact of age in the development of the poultry gut microbiota, undergoing well-characterized temporal changes (Danzeisen et al., 2011; Ballou et al., 2016; Kumar et al., 2018). The effect of age was prominent in the ceca as highlighted by diversity metrics and changes in microbial community dynamics. Measures of alpha diversity revealed that community richness and evenness increased sharply during the first 3 weeks of age, and reached maximum levels in 43-day-old and 50-day-old NAE and conventional broilers respectively (Figure 3.1). This rapid development of microbiota complexity has been characterized by many authors, reporting dramatic rises in community diversity as early as seven days of age (Ballou et al., 2016, Kumar et al., 2018). Beta diversity analysis further emphasized the age effect, as elucidated by the distinct clustering of the microbiota of hatch-day-broilers by NMDS of Bray-Curtis distances (Figure 3.8).

However, little temporal variation in microbial community composition occurred after 29 days of age. Our results are consistent with current literature which show a significant differentiation of the microbiota of young chicks from that of adults of ages 14 days or higher (Ocejo et al., 2019).

It is well-understood that the ceca harbors a more complex and rich microbiota, due to a longer transit time of the digesta which favors extensive fermentation activity (Qu et al., 2008). Ileal communities are less diverse, yet taxonomic representation as specified by relative abundance is more likely to diverge between individual groups (Yeoman et al., 2012). Our findings thus confirm the expected spatial variation of the microbiota within the chicken gastrointestinal tract. Unlike the cecal microbiota, ileal microbial communities did not follow a significant age-related distribution. Temporal differences were driven by the distinct microbial communities associated with hatch and transportation (Figures 3.5 and 3.8). The decrease in diversity after transportation was likely a result of the emptying of intestinal contents from the small intestine potentially depleting the microbiota from certain microbial groups. This shift in diversity was characterized by a significant increase in the abundance of Proteobacteria, a trend commonly reported in older birds (Ocejo et al., 2019).

Comparative analysis between treatment groups showed that conventional broilers harbored a more rich and even microbiota with a higher degree of similarity among members (Figure 3.2). Studies have evaluated the impact of antibiotics on gut microbial populations with different outcomes (LaVorgna et al., 2013; Pourabedin et al., 2014, 2015; Kumar et al., 2018). The current consensus is that antibiotics deplete the microbiota from certain taxonomic groups which can lead to a dysbiosis or a deviation

from a healthy microbial community (Que and Hengtes, 1985; Hynes et al., 1997; LaVorgna et al., 2013). To this date, this effect has not been fully characterized as studies have shown contrasting results despite evaluation of the same antimicrobial (Dumonceaux et al., 2006; Scupham et al., 2010). Nevertheless, antibiotic treatment is likely to exert a selective pressure on certain microbial groups, which could explain the differences in the community structure (Hynes et al., 1997).

The antibiotic virginiamycin was the subject of investigation in our study. In two separate studies, Pourabedin and collaborators (2014, 2015) reported that feed supplementation with virginiamycin did not alter the microbial diversity of cecal communities. However, these studies were designed to target the V3 and V1-V3 regions of the 16S rRNA gene, whereas our study targeted the V4 region. A comparison between findings could therefore be biased by the choice of hypervariable region. In a recent study, Zhu et al. (2019) reported that virginiamycin induced changes on the cecal microbiota of broilers. This was characterized by higher alpha diversity measures and a separation of community structure by PCoA of the virginiamycin-treated groups, similar to our observations.

Our findings identified seasonality and individual farm as confounding variables of microbial diversity analysis. The latter was particularly impactful, indicating a farm-driven differentiation in microbial community composition of adult broilers (Figure 3.11). Within each farm, only one flock from a single broiler house was subjected to microbiome analysis, thus, attributing the community structure differentiation to a farm effect can be misleading. Johnson et al. (2018) investigated this effect by characterizing the intestinal microbiota of different flocks, and flocks raised under successive cycles.

This analysis concluded that age was the main factor driving the taxonomic composition of the intestinal microbiota, reporting no significant correlation with flock or flock cycle. Conversely, Rothrock and Locatelli (2019) reported a differentiation of the fecal microbiota associated with farm environment. Geographical location and farm management practices have also been reported to impact the gut microbiota and litter community composition in poultry production (Zhou et al., 2016; Crippen et al., 2019). The four farms sampled in this study were located in different regions of the southeastern United States. While belonging to the same integrator, our observations during sampling suggested that the levels of adherence to biosecurity practices also differed between farms. These factors could be contributing to the reported distinctions of the intestinal microbiota between the four farms.

Temporal succession of microbial groups was characterized by an early dominance of Proteobacteria, followed by an increase in representation of bacteria from the Firmicutes and Bacteroides phylum, with the latter becoming predominant at the end of the production cycle. This pattern of microbial community dynamics has been consistently reported by other authors (Danzeisen et al., 2011; Ballou et al., 2016; Mancabelli et al., 2016; Xiao et al., 2017). The cecal microbiota of chicks was represented by Proteobacteria and Firmicutes with some differences in phylum distribution between treatment groups. Proteobacteria dominated the microbiota of conventional chicks, practically all belonging to the *Escherichia* genus, which has been consistently identified as a core member of the chick microbiota (Ballou et al., 2016; Ocejo et al., 2019). Further, our differential abundance analysis showed that *Escherichia* was also one of the genera most often associated with conventional production, driven by

the high levels at hatch. On the other hand, the presence of Firmicutes, namely *Clostridium*, was highest in NAE chicks, also a genus highly represented in the ceca of young birds (Ballou et al., 2016). Chicks from NAE farms also exhibited a more diverse microbiota. It is possible that the hatchery environment could be contributing to the differences observed, a hypothesis previously stated by Stanley et al. (2013), as studies have consistently shown that environmental microorganisms can colonize the chick microbiota through eggshell penetration (De Reu et al., 2006). The differences in microbial composition at this time, could also be attributed to the farm environment, particularly to the litter microbiome (Oakley et al., 2013). Intestinal samples from chicks were collected within a 6-hour timeframe from the moment of placement in the farm. Thus, exposure to the litter and early feeding could be contributing to this early variability (Oakley et al., 2013).

Development of the microbiota was characterized by a predominance of Firmicutes represented by the family *Ruminococcaceae*, specifically *Faecalibacterium* and *Ruminococcus_B*, which were also identified as components of the core microbiota of both conventional and NAE adult broilers. Members of the *Faecalibacterium* genus are classified as butyrate-producing bacteria (Polansky et al., 2016). Butyrate, a short-chain fatty acid (SCFA), is a product of cecal fermentation with an important role in poultry nutrition and regulation of immune response (Zhang et al., 2011; Zhou et al., 2014). Further, this SCFA has exhibited antimicrobial activity against certain microbes of importance to poultry production such as *Salmonella* and *Clostridium perfringens* (Van Immerseel et al., 2005; Timbermont et al., 2010). In parallel with other microbiome

studies, our study showed that the abundance of *Faecalibacterium* was not impacted by treatment, but underwent a gradual decrease as the birds aged (after 29d).

Comparison of the cecal microbiota of adult broilers (WD1, 50d) highlighted differences in the abundance of certain Bacteroides taxa between treatment groups. For example, the genera *Coprobacter* and *Alistipes* were more abundant in NAE broilers. Both groups are regarded as beneficial to the host due to production of metabolic SCFAs, specifically propionic and acetic acid (Van Immerseel et al., 2004; Du et al., 2020). Differential abundance analysis by DeSeq, however, did not recognize these genera as being overrepresented in broilers raised under NAE. The higher abundance of these SCFA-producers in NAE broilers could exert a measurable beneficial effect on the cecal microbiota. However, a functional characterization of the microbiota by a metagenomics approach is needed to test this hypothesis (Calderón-Pérez et al., 2020).

Differential abundance analysis revealead that *Bifidobacterium*, a beneficial lactic-acid producer and contributor to gut homeostasis (Binda et al., 2018), was present at a significantly higher rate in NAE broilers. Studies frequently report this as a low-abundance genera in the chicken cecum of significant importance, nonetheless (Awad et al., 2016; Mancabelli et al., 2016). Ocejo et al. (2019) showed that this group was more abundant in free-range birds compared to conventional broilers, however subject birds belonged to different breeds which further complicates effect attribution. Our observations may be explained by the effects of virginiamycin on the microbiota of conventional chickens, as this antibiotic is known to decrease populations of lactic-acid bacteria (Hynes et al., 1997; Pourabedin et al., 2014, 2015).

Campylobacter was another genus with a higher representation in NAE birds. The ecological role of *Campylobacter* in the chicken cecum needs extensive investigation. For years, this foodborne pathogen was considered a commensal organism of the poultry microbiota, numerous studies in recent years have elucidated a *Campylobacter*-induced proinflammatory response in broiler birds which suggests otherwise (Humphrey et al., 2015; Reid et al., 2016; Connerton et al., 2018). Researchers have also reported mixed findings on the impact of virginiamycin on *Campylobacter* populations (Cox et al., 2003; Baurhoo et al., 2009). The higher representation of *Campylobacter* in NAE broilers may be related to a higher abundance of SCFA-producing bacteria in the ceca of this birds, as research has shown that *Campylobacter* can utilize SCFAs as a metabolic source (Masanta et al., 2013; Awad et al., 2016). Luethy and collaborators (2017) reported that microbiota-derived SCFAs in the avian gut may facilitate a desirable niche for commensal colonization and growth of *Campylobacter*. This is an interesting correlation that requires further characterization but that may illuminate the ecology of *Campylobacter* in commercial broilers.

An analysis of differential abundance relating to the presence of *Campylobacter* in the ceca revealed a positive correlation with *Parasutterella*, a SCFA-producer (Li et al., 2020), and a negative correlation with *Clostridium* (family *Clostridiaceae*) and members of the *Enterobacteriaceae* family. Few studies have attempted to characterize this correlation with varying results. Ocejo et al. (2019) found a significant negative association between *Campylobacter* and *Sutterella* and *Parabacteroides*, but a positive relationship with *Faecalibacterium* among other members of the Firmicutes phylum. Connerton et al. (2018) reported variable responses regarding the presence of

Clostridiales groups in *Campylobacter*-colonized birds (Connerton et al., 2018). Further investigations are necessary to confirm these findings.

Analysis of microbial community dynamics in the ileum showed a clear dominance Firmicutes, specifically members of the Lactobacillales order. The dominance of *Lactobacillus* in the ileum and small intestine has been extensively characterized (Gong et al., 2007; Yeoman et al., 2012; Kumar et al., 2018). Our study also identified the different genera of this group as potential biomarkers in both NAE and conventional production. Our results, therefore, suggest a genus-specific impact of treatment on the abundance of Lactobacillales, a trend previously reported by other authors (Engber et al., 2000; Dumonceaux et al., 2006).

Tables and Figures

Table 3.1. Broiler house characteristics where broilers raised under conventional (CV) or no antibiotics ever (NAE) practices were collected for the characterization of their gut microbiome

House	Farm System	Season	House Size Width x Length (ft x ft)	Number of Birds	Flock Density (ft^2 $bird^{-1}$)
A	CV	Summer	54 x 500	26,000	1.05
B	NAE	Summer	60 x 600	33,300	1.08
C	CV	Fall	50 x 500	23,800	1.05
D	NAE	Fall	60 x 600	33,300	1.08

Table 3.2. Schedule of the nutrition regimen administered to broilers raised under conventional (CV) or no antibiotics ever (NAE) practices which were selected for the characterization of their gut microbiome

	Conventional		NAE	
Feed	Age Interval (days)	Duration (days)	Age Interval (days)	Duration (days)
Starter	1-21	20	1-19	18
Grower	22-31	10	20-29	8
Finisher	32-45	14	30-42	12
Withdrawal 1 (WD1)	46-53	8	43-48	5
Withdrawal 2 (WD2)	54-58	4	49-54	5

Table 3.3. List of taxa identified by differential abundance analysis between conventional (CV) and no antibiotics ever (NAE) treatment groups in ceca samples

Genus	Log2 Fold Change[1]
Helicobacter_G	8.75
Sutterella	6.94
Turicibacter	2.22
Bifidobacterium	1.98
Bittarella	1.91
UBA5808 (unclassified *Eggerthellaceae*)	1.89
Homeothermus	1.74
Ruminiclostridium_E	1.64
Campylobacter	1.59
Phascolarctobacterium	1.48
Yersinia	1.47
Streptococcus	1.17
Acinetobacter	1.12
Acidaminococcus	0.93
Brevibacterium	0.91
Helicobacter_D	0.91
Gordonibacter	0.90
Brachybacterium	0.86
Eubacterium	0.86
Yaniella	0.83
Eubacterium_I	0.73

Delftia	0.59
Saccharum	0.56
Salinicoccus	0.44
Corynebacterium	0.32
Clostridium_A	-0.54
Ruminiclostridium_A	-0.66
GCA-900066905 (unclassified Clostridia)	-0.68
Lactobacillus_D	-0.69
Clostridium_P	-0.71
Subdoligranulum	-0.76
Intestinimonas	-0.80
Butyricimonas	-1.21
Limosilactobacillus	-1.41
Hungatella	-1.47
Escherichia	-1.89
Eubacterium_M	-2.15
UBA1382 (unclassified *Atopobiaceae*)	-2.92
Enorma	-3.14
Oxalobacter	-3.16

[1]Log2 Fold Change indicates a differential change in abundance by comparison of treatment groups (conventional and NAE), a positive Log2 Fold Change means a higher abundance in the NAE group.

Table 3.4. List of taxa identified by differential abundance analysis between conventional (CV) and no antibiotics ever (NAE) treatment groups in ileum samples

Genus	Log2 Fold Change[1]
Turicibacter	3.59
Campylobacter	3.58
Sutterella	2.40
Bacillus	2.31
Psychrobacter	2.20
Helicobacter_G	1.99
Ligilactobacillus	1.22
Acidaminococcus	1.07
Bacillus_O	0.80
C941 (unclassified Bacteroidales)	0.78
Gallibacterium	0.78
Ruminococcus_C	0.71
Acinetobacter	0.59
Bacillus_C	0.55
Saccharum	0.51
Caulobacter	0.41
Ruminiclostridium_A	-0.37
Mycobacterium	-0.38
Clostridium_F	-0.51
Paramesorhizobium	-0.53
UBA1382 (unclassified *Atopobiaceae*)	-0.57

Tetragenococcus	-0.61
Eubacterium_R	-0.62
Agathobaculum	-0.65
Massilioclostridium	-0.76
Anaeromassilibacillus	-0.79
Desulfovibrio	-0.82
Intestinimonas	-0.82
Rhodococcus	-0.83
Enterococcus_A	-0.84
Clostridium_M	-0.85
Alistipes_A	-0.87
CAG-475 (unclassified Firmicutes)	-0.89
Rouxiella	-0.94
UBA7182 (unclassified *Lachnospiraceae*)	-0.97
Erysipelatoclostridium	-0.97
Lactobacillus_F	-0.97
Macrococcus	-0.99
Clostridium_P	-1.00
Urinacoccus	-1.04
Faecalibacterium	-1.11
Bacteroides_B	-1.16
CAG-56 (unclassified Firmicutes)	-1.19
UBA9475 (unclassified Clostridiales)	-1.25

Limosilactobacillus	-1.26
Lagierella	-1.27
Enterococcus_D	-1.44
Tidjanibacter	-1.47
Escherichia	-1.55
Yuhushiella	-1.60
Parabacteroides	-1.77

[1]Log2 Fold Change indicates a differential change in abundance by comparison of treatment groups (conventional and NAE), a positive Log2 Fold Change means a higher abundance in the NAE group.

Table 3.5. List of taxa identified by differential abundance analysis associated with *Campylobacter* presence in ceca samples

Genus	Log2 Fold Change[1]
Psychrobacter	2.91
Acinetobacter	2.83
Enorma	2.69
UBA1382 (unclassified *Atopobiaceae*)	2.33
Coprobacter	1.98
Helicobacter_G	1.77
Parasutterella	1.66
Eubacterium_M	1.66
Limosilactobacillus	1.59
Streptococcus	1.58
Subdoligranulum	1.52
Ruthenibacterium	1.40
Acidaminococcus	1.40
Faecalibacterium	1.37
Eggerthella sp. strain YY7918	1.37
Ligilactobacillus	1.35
Enterococcus_E	1.32
CAG-475 (unclassified Firmicutes)	1.29
Bittarella	1.29
Eggerthella	1.24
Clostridium_P	1.23

Eubacterium_E	1.22
Enterorhabdus	1.22
Fournierella	1.20
Intestinibacillus	1.18
UBA1191 (unclassified Firmicutes)	1.15
Anaeromassilibacillus	1.15
Ruminococcus_B	1.15
Agathobaculum	1.13
CAG-24 (unclassified Firmicutes)	1.12
Mycoplasma sp. CAG:877	1.11
Emergencia	1.11
Ruminococcus_D	1.11
Massiliomicrobiota	1.10
Gemmiger	1.10
Flavonifractor	1.09
DTU053 (unclassified Clostridiales)	1.09
CAG:41 (unclassified Firmicutes)	1.09
CAG:110 (unclassified Firmicutes)	1.08
Oxalobacter	1.07
Anaerotignum	1.07
CHKCI006 (unclassified Clostridiales)	1.06
Parabacteroides	1.06
Lactobacillus	1.06

Alistipes	1.06
Clostridium_AJ	1.05
UBA5808 (unclassified *Eggerthellaceae*)	1.05
Acutalibacter	1.04
UBA9475 (unclassified Clostridiales)	1.03
Oscillibacter	1.03
Hungatella	1.03
Eubacterium_D	1.03
Barnesiella	1.03
Bacteroides	1.00
Clostridium sp. CAG:302	1.00
CHKCI001 (unclassified Clostridiales)	0.99
Alistipes_A	0.98
UBA7182 (unclassified Clostridia)	0.97
Eubacterium_R	0.97
UBA1033 (unclassified Clostridia)	0.96
Dorea	0.96
Anaerofustis	0.95
Massilioclostridium	0.95
Sporobacter	0.94
Odoribacter	0.93
GCA-900066905 (unclassified Clostridia)	0.91
Acholeplasma sp. CAG:878	0.88

Anaerofilum	0.86
CAG-354	0.86
Soleaferrea	0.85
Butyricimonas	0.85
Blautia_A	0.85
Bacteroides_B	0.85
Intestinimonas	0.83
Eubacterium	0.83
CAG-65	0.83
Ruminiclostridium_C	0.83
Erysipelatoclostridium	0.82
Anaerorhabdus	0.81
Ruminiclostridium	0.80
GCA-900066575 (unclassified Clostridia)	0.79
Gallibacterium	0.77
UBA660 (unclassified Bacillales)	0.76
Zag1 (unclassified Cyanobacteria)	0.76
Blautia	0.76
CAG:56 (unclassified Firmicutes)	0.72
Catabacter sp. UBA4626	0.70
Butyricicoccus	0.68
Zag111(unclassified Cyanobacteria)	0.64
Syntrophomonas_B	0.61

Homeothermus	0.58
Methanomassiliicoccus	0.52
Ruminococcus	0.37
Aerococcus	0.33
Nocardiopsis	-0.34
Delftia	-0.72
Salmonella	-0.94
Enterococcus_C	-1.50
Escherichia	-1.69
Yersinia	-1.74
Enterococcus_D	-1.83
Enterococcus	-2.64
Clostridium	-4.69

[1]Log2 Fold Change indicates a differential change in abundance by comparison of *Campylobacter* presence, a positive Log2 Fold Change means a correlation with *Campylobacter* presence.

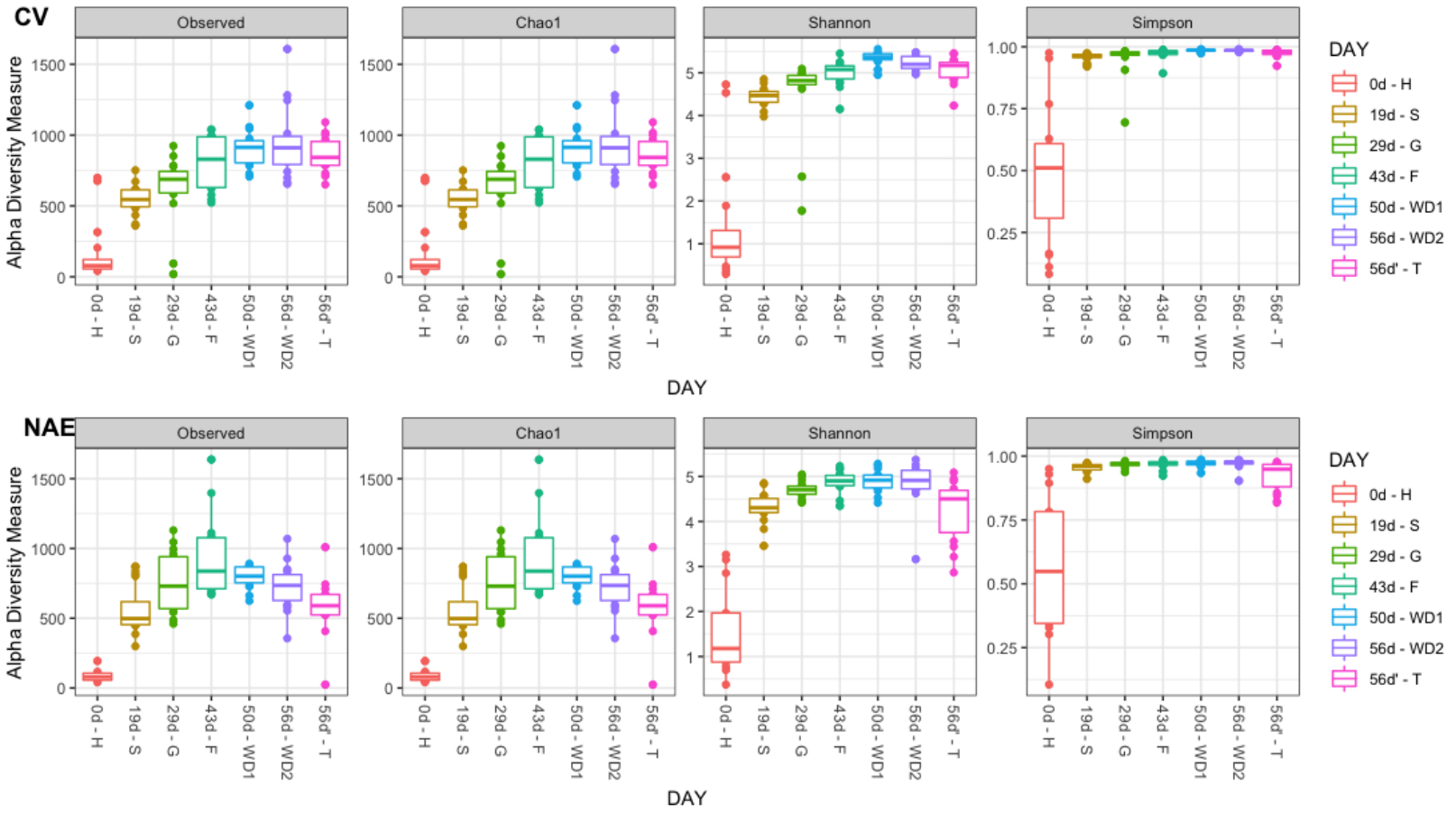

Figure 3.1. Alpha diversity metrics for ceca samples from conventional (CV) and no antibiotics ever (NAE) broilers grouped according to sampling day

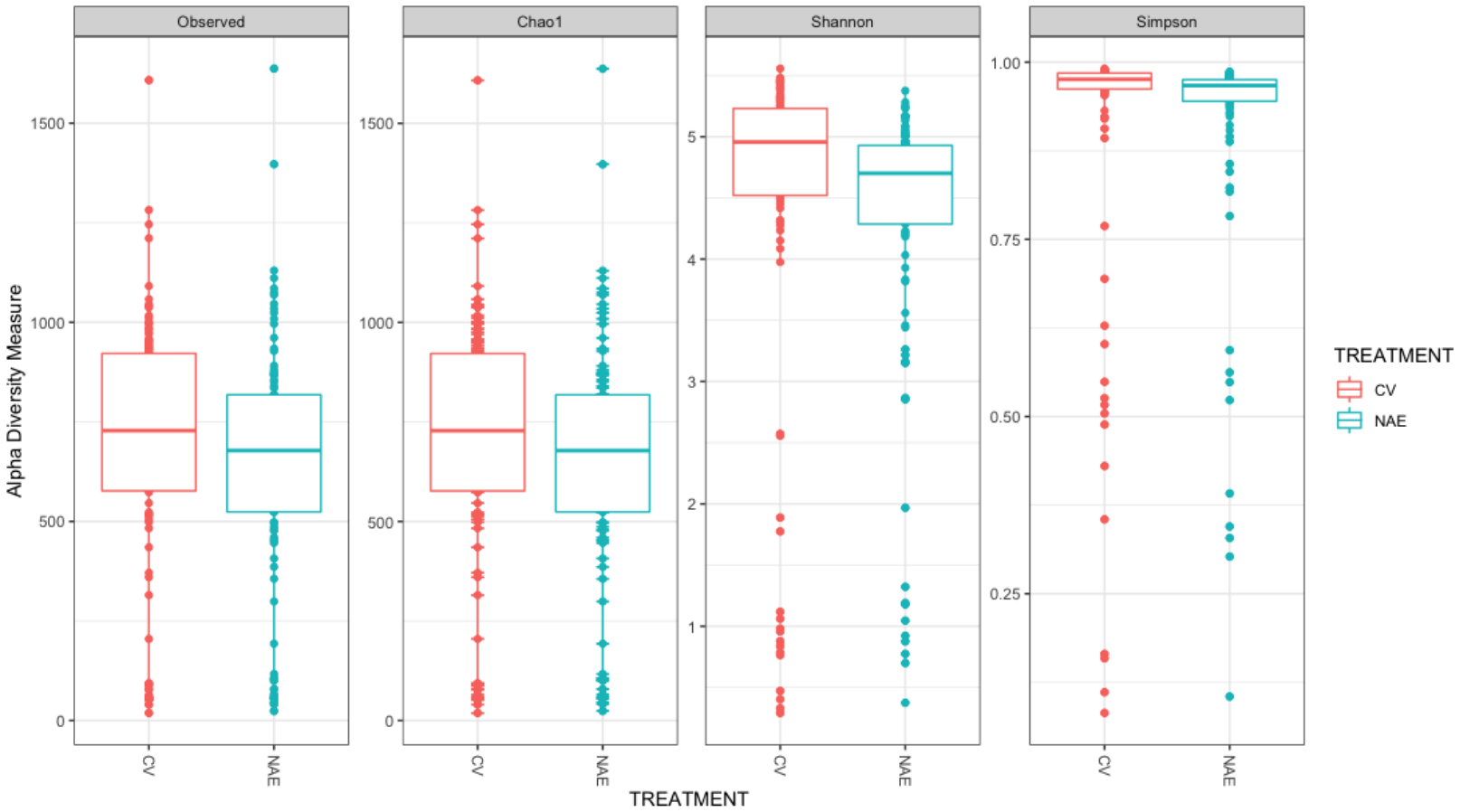

Figure 3.2. Alpha diversity metrics for ceca samples from broilers grouped according to treatments conventional (CV) or no antibiotics ever (NAE)

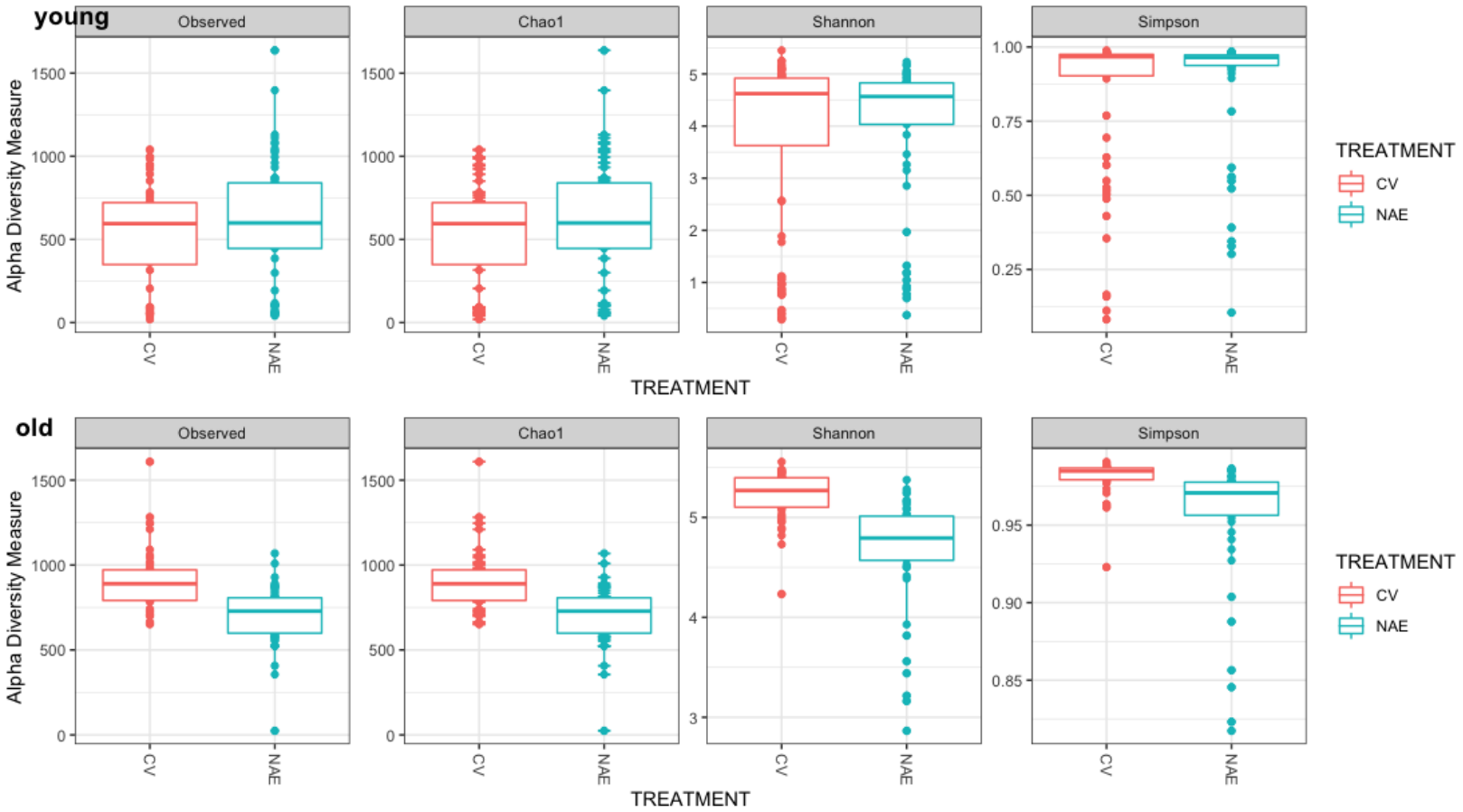

Figure 3.3. Alpha diversity metrics for ceca samples from young (d0 – d43) and old (d50 – d56) broilers grouped according to treatments conventional (CV) or no antibiotics ever (NAE)

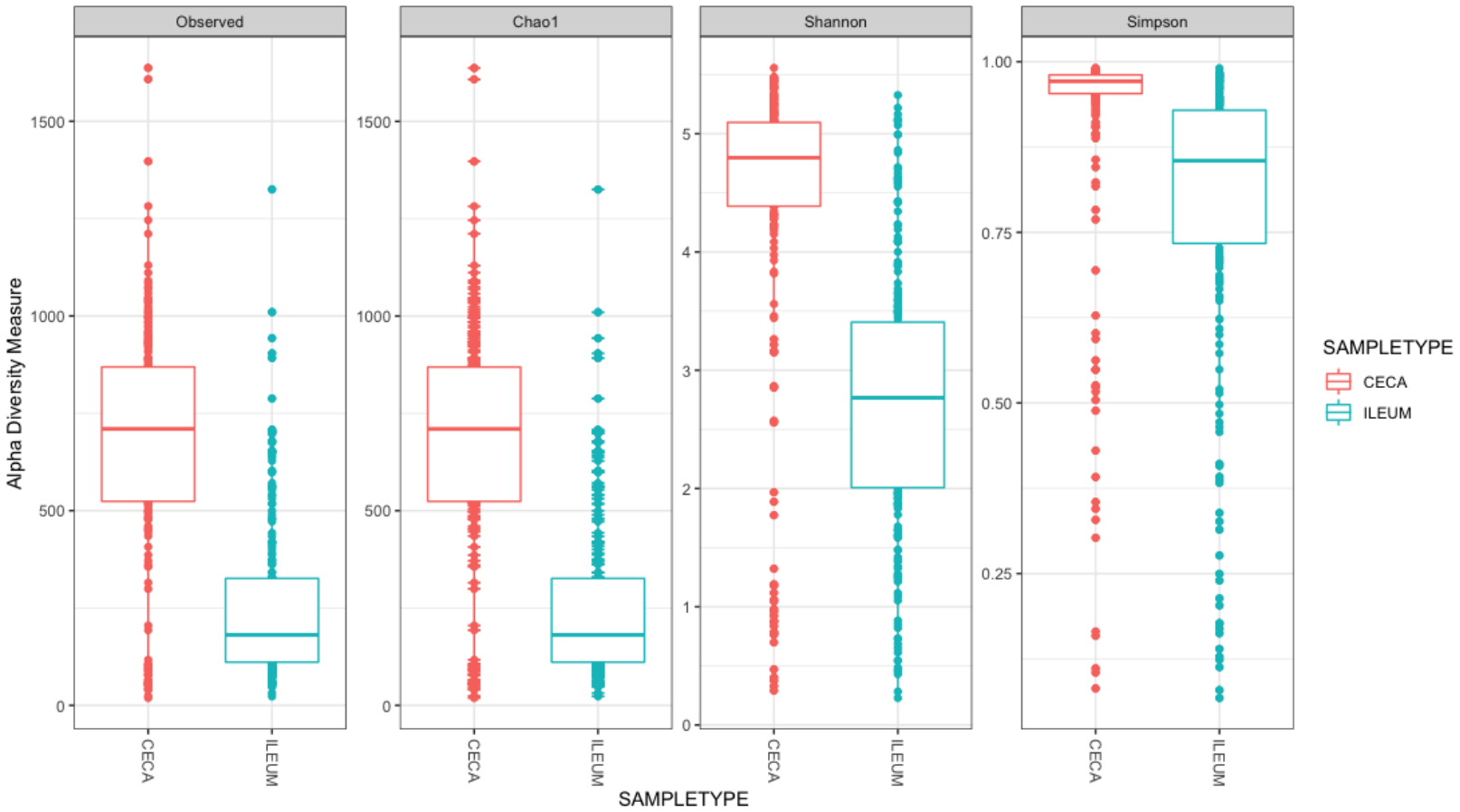

Figure 3.4. Alpha diversity metrics for intestinal samples from broilers grouped according to sample type (ceca and ileum)

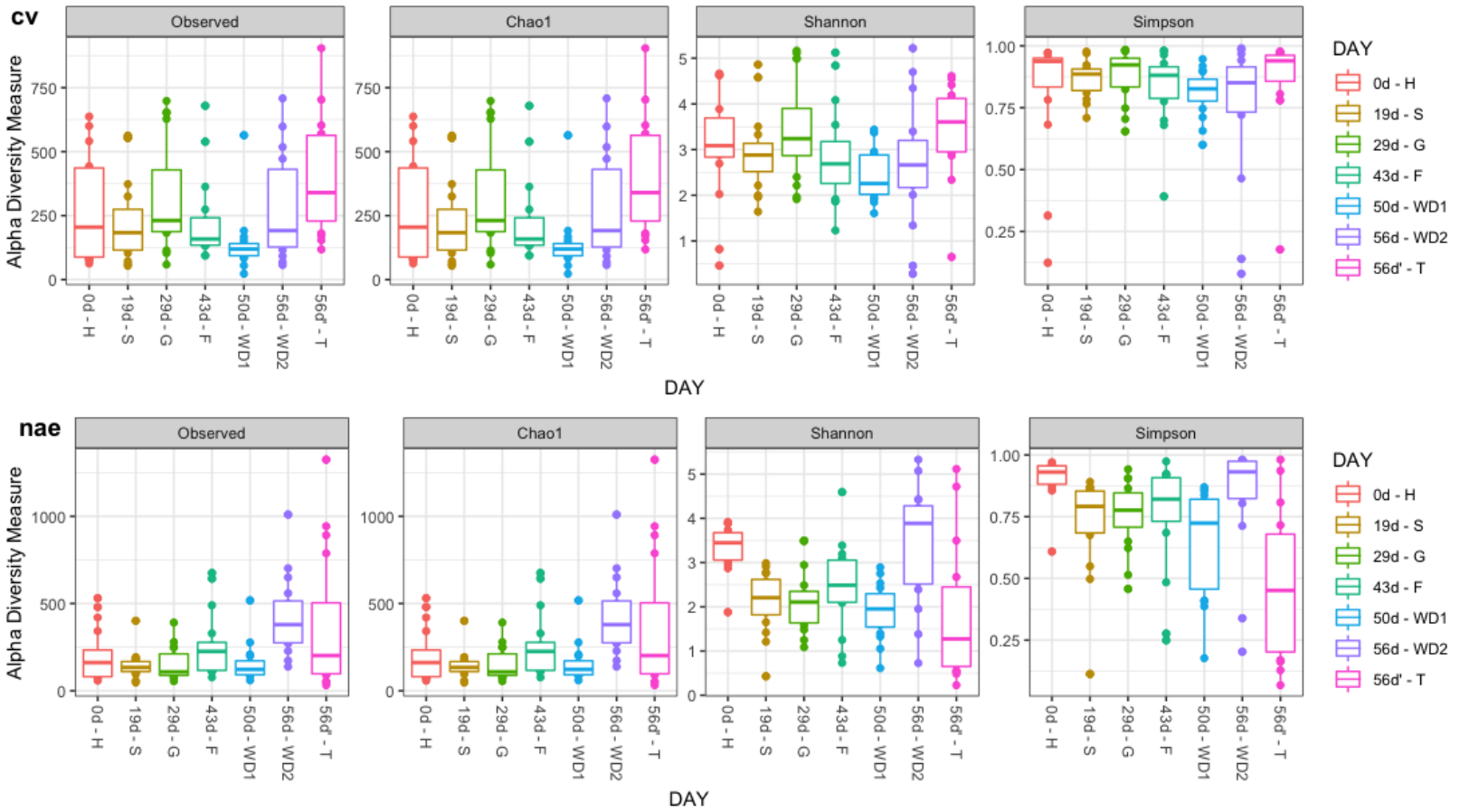

Figure 3.5. Alpha diversity metrics for ileum samples from conventional (CV) and no antibiotics ever (NAE) broilers grouped according to sampling day

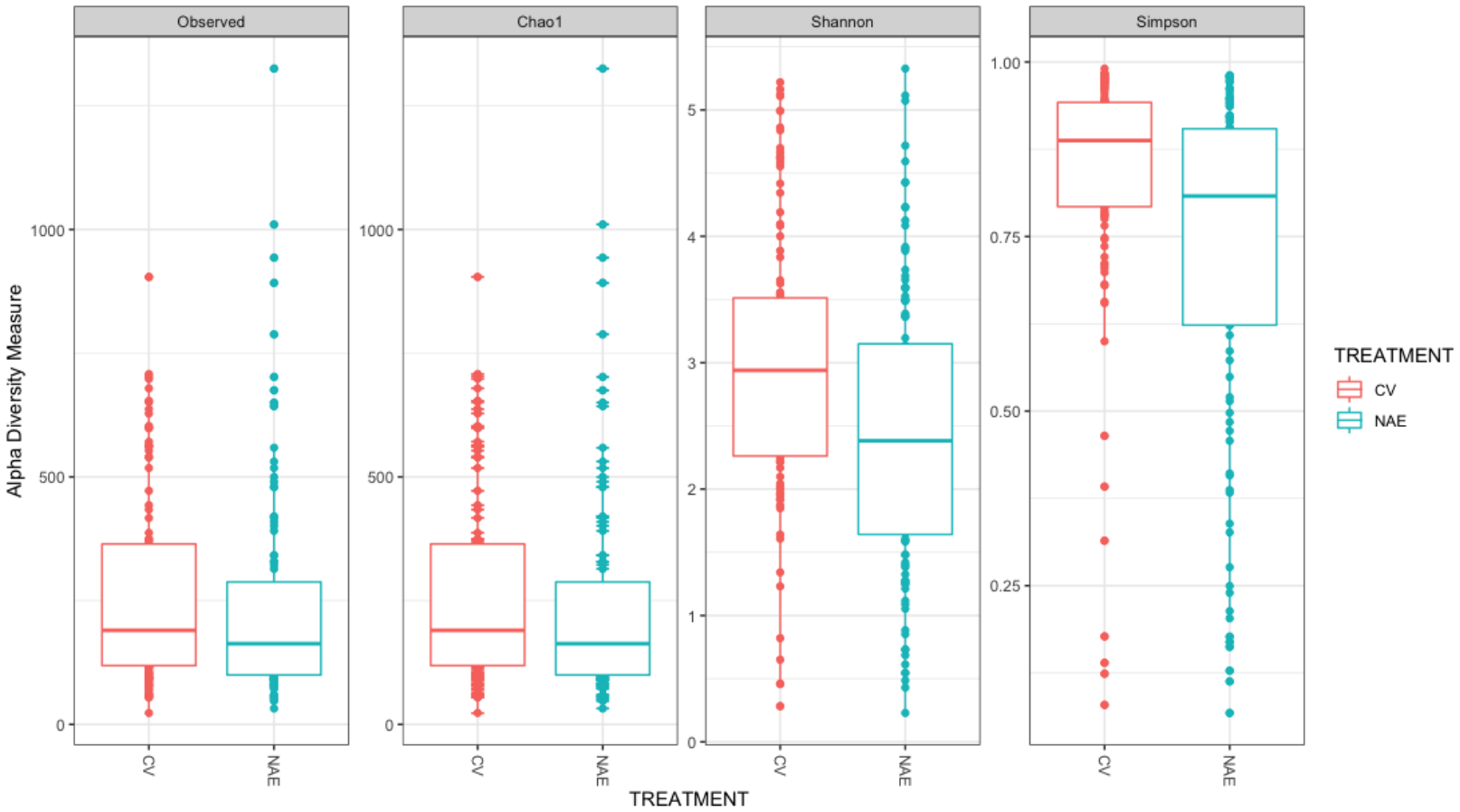

Figure 3.6. Alpha diversity metrics for ileum samples grouped according to treatments conventional (CV) or no antibiotics ever (NAE)

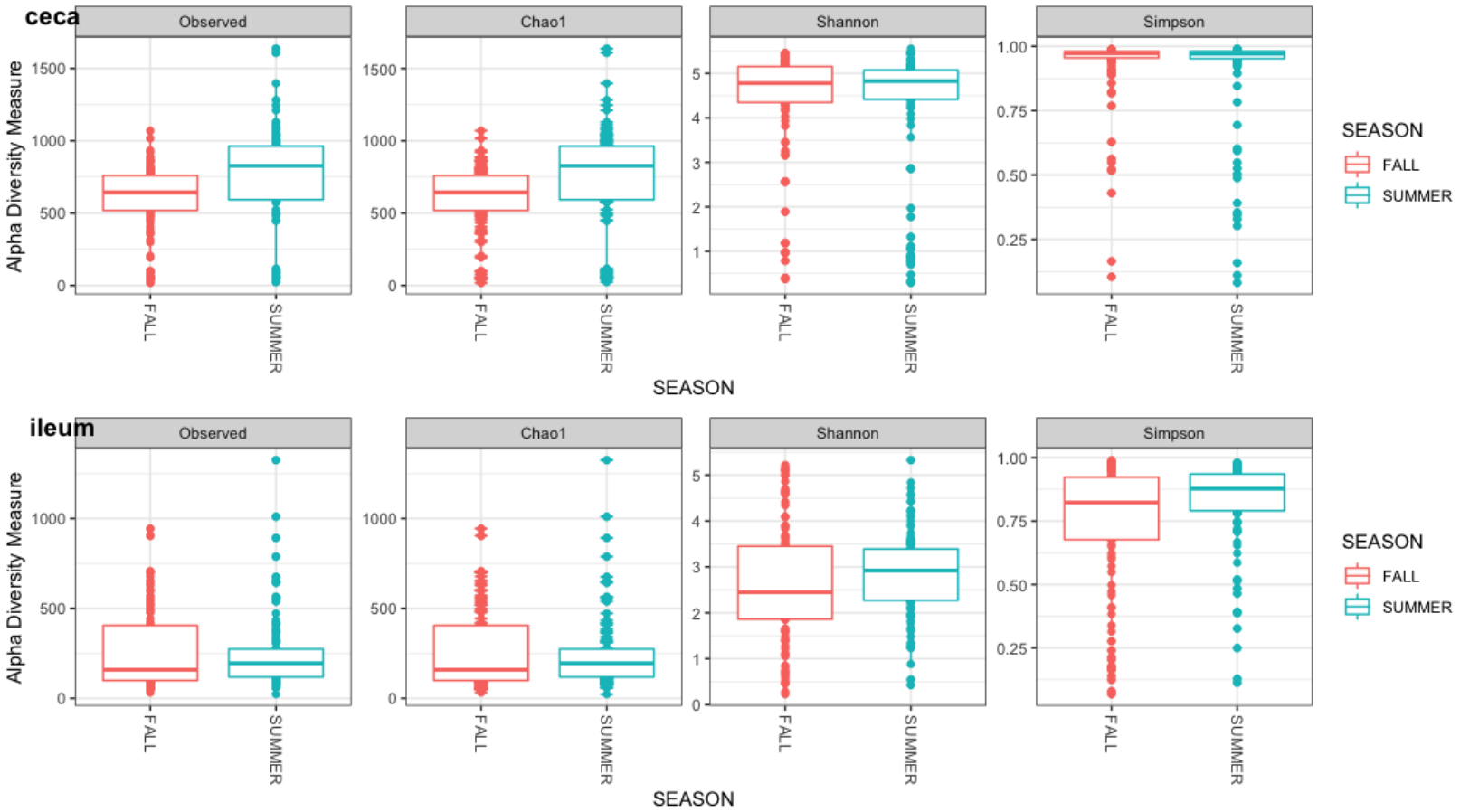

Figure 3.7. Alpha diversity metrics for ceca and ileum samples from broilers grouped according to season

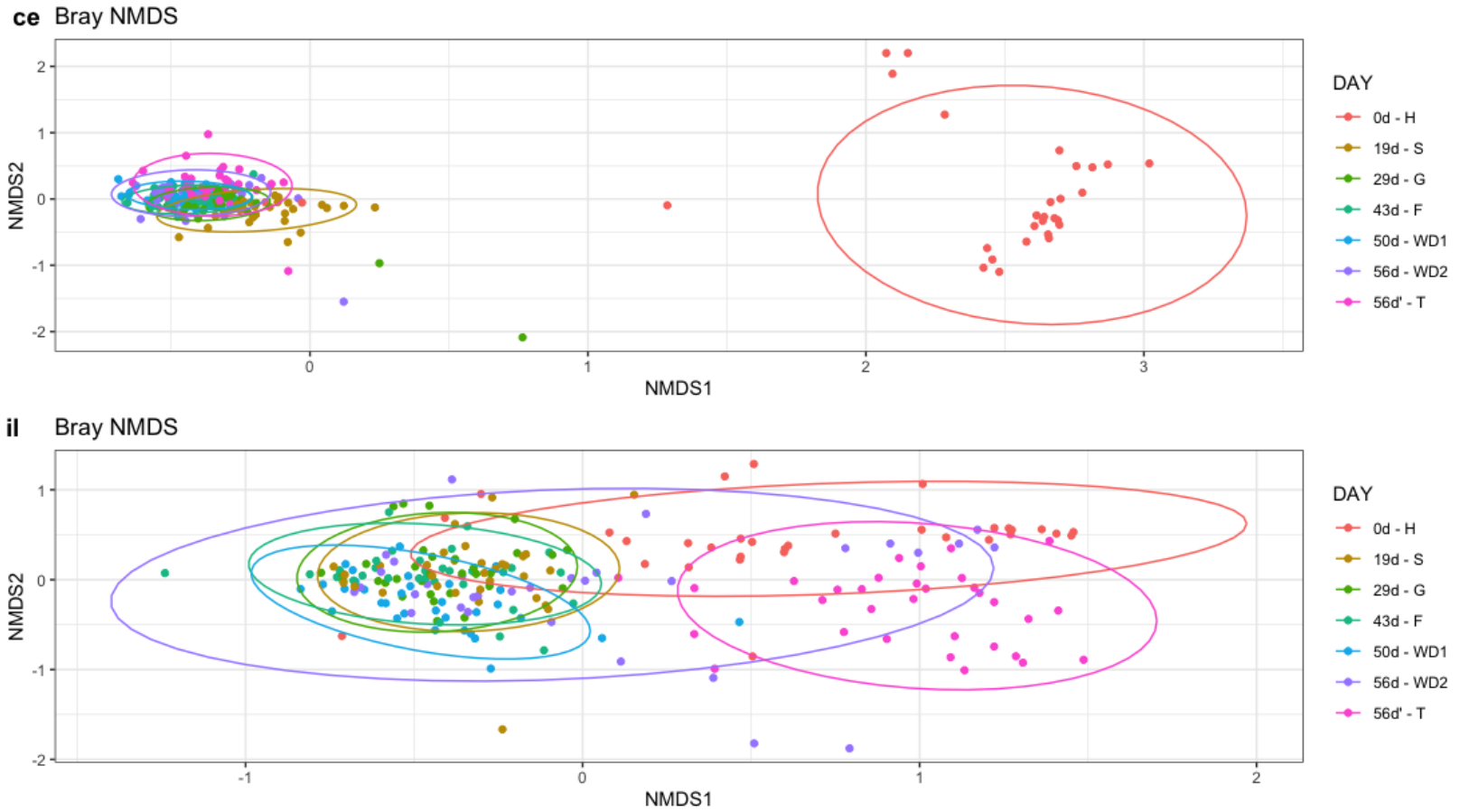

Figure 3.8. Non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarity on relative abundance data in ceca (ce) and ileum (il) samples grouped according to sampling day

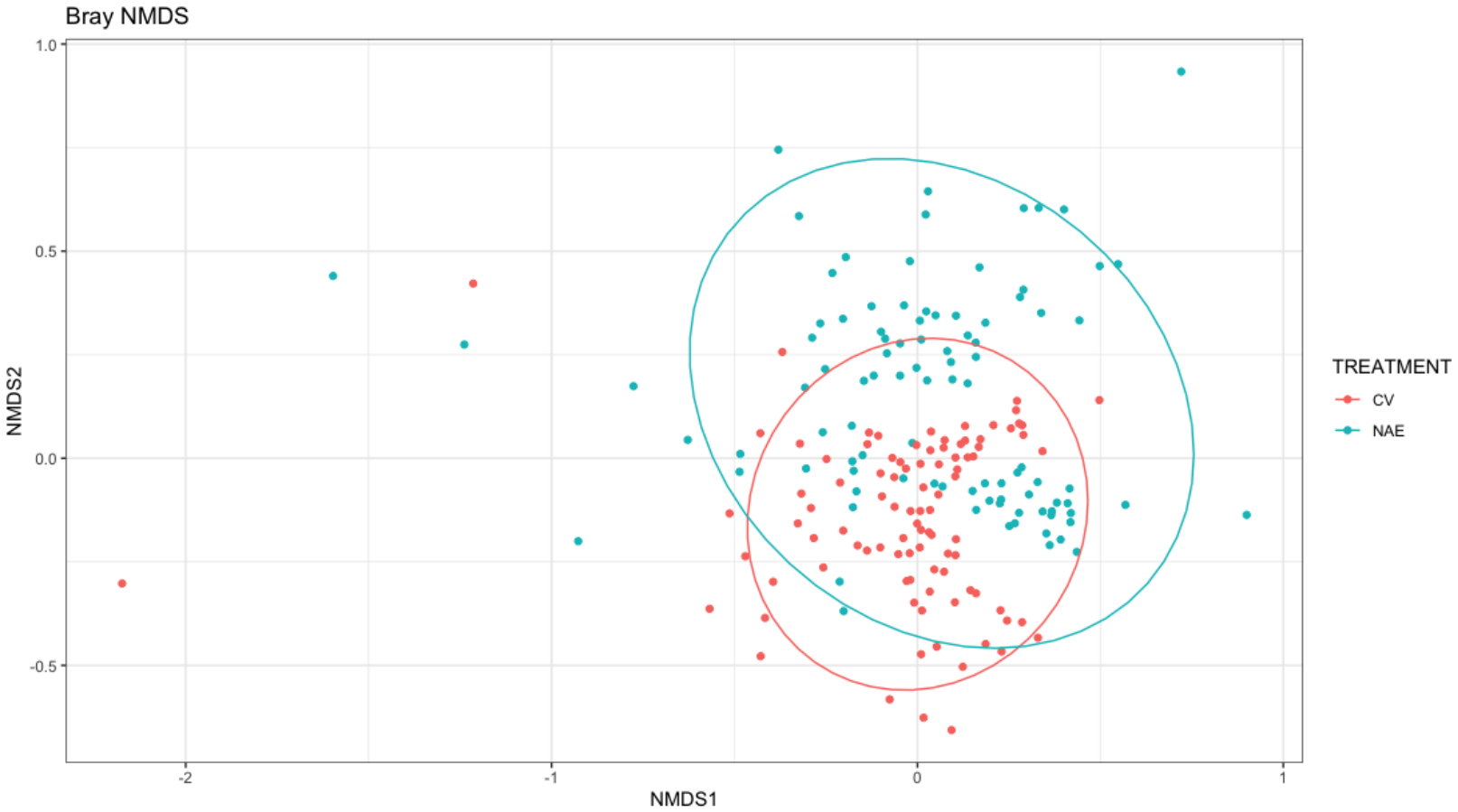

Figure 3.9. Non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarity on relative abundance data in ceca samples (excluding 0d – H) grouped according to treatments conventional (CV) or no antibiotics ever (NAE)

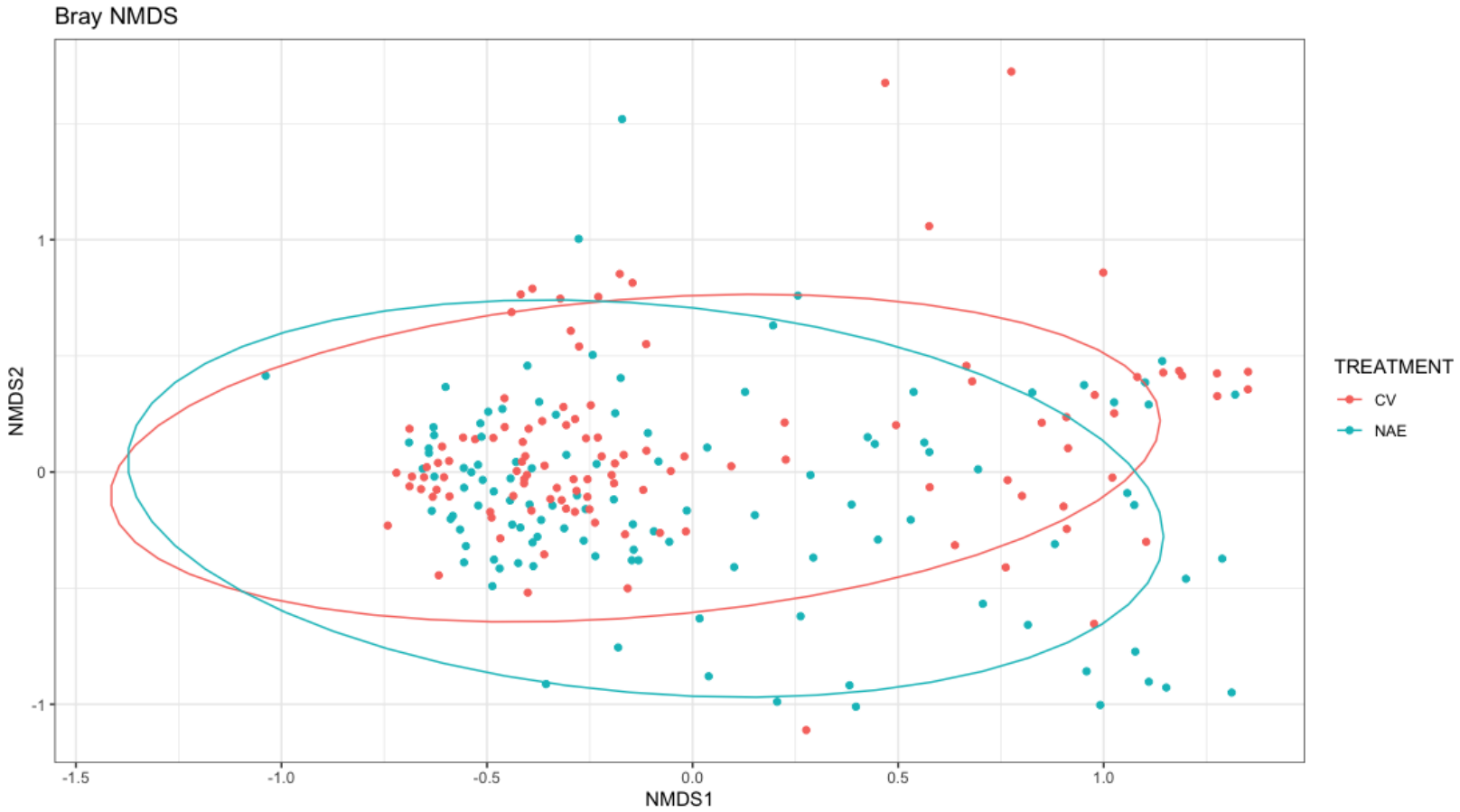

Figure 3.10. Non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarity on relative abundance data in ileum samples (excluding 0d – H) grouped according to treatments conventional (CV) or no antibiotics ever (NAE)

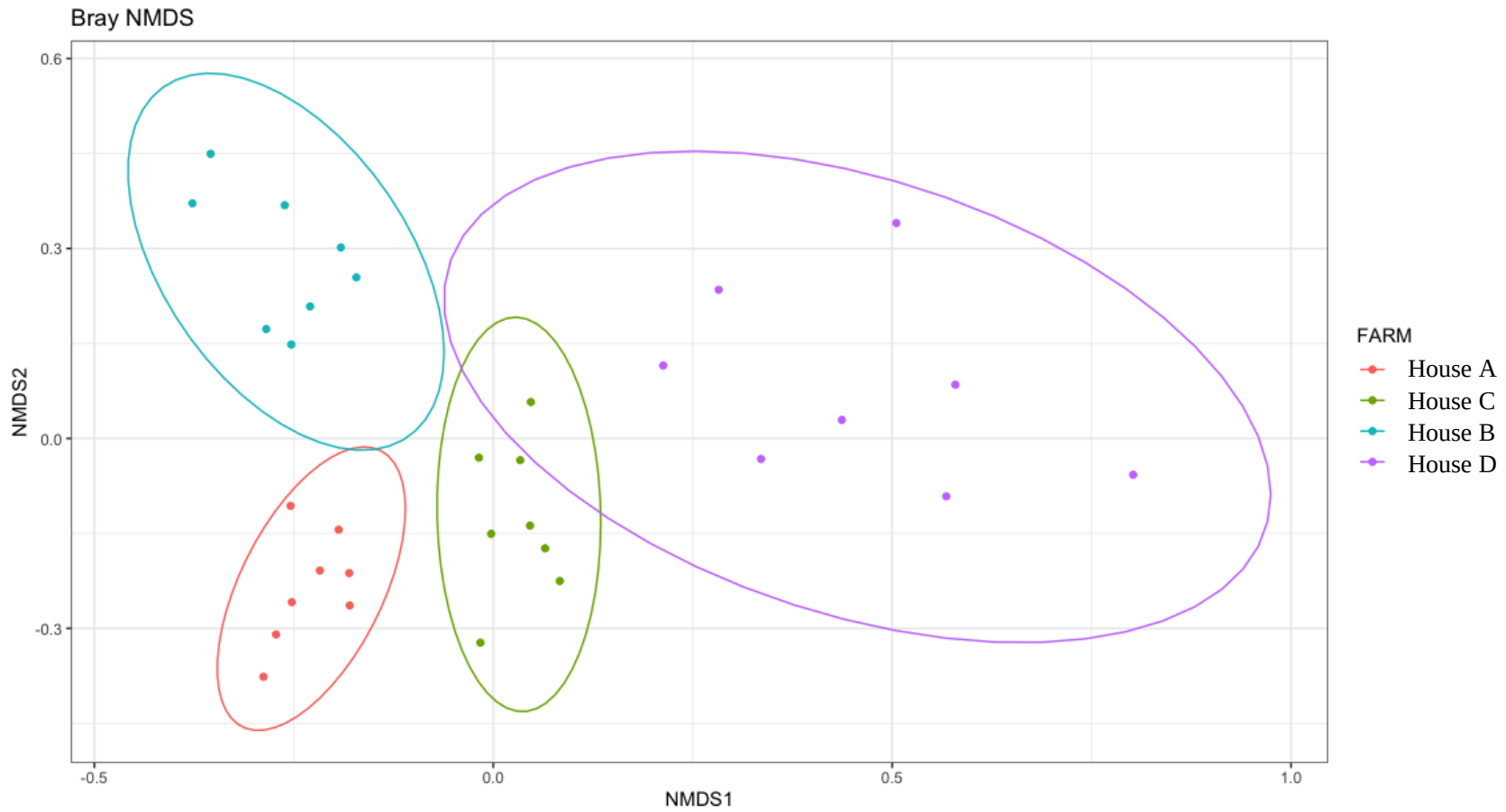

Figure 3.11. Non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarity on relative abundance data in ceca samples of day WD1 (50d) grouped according to farm

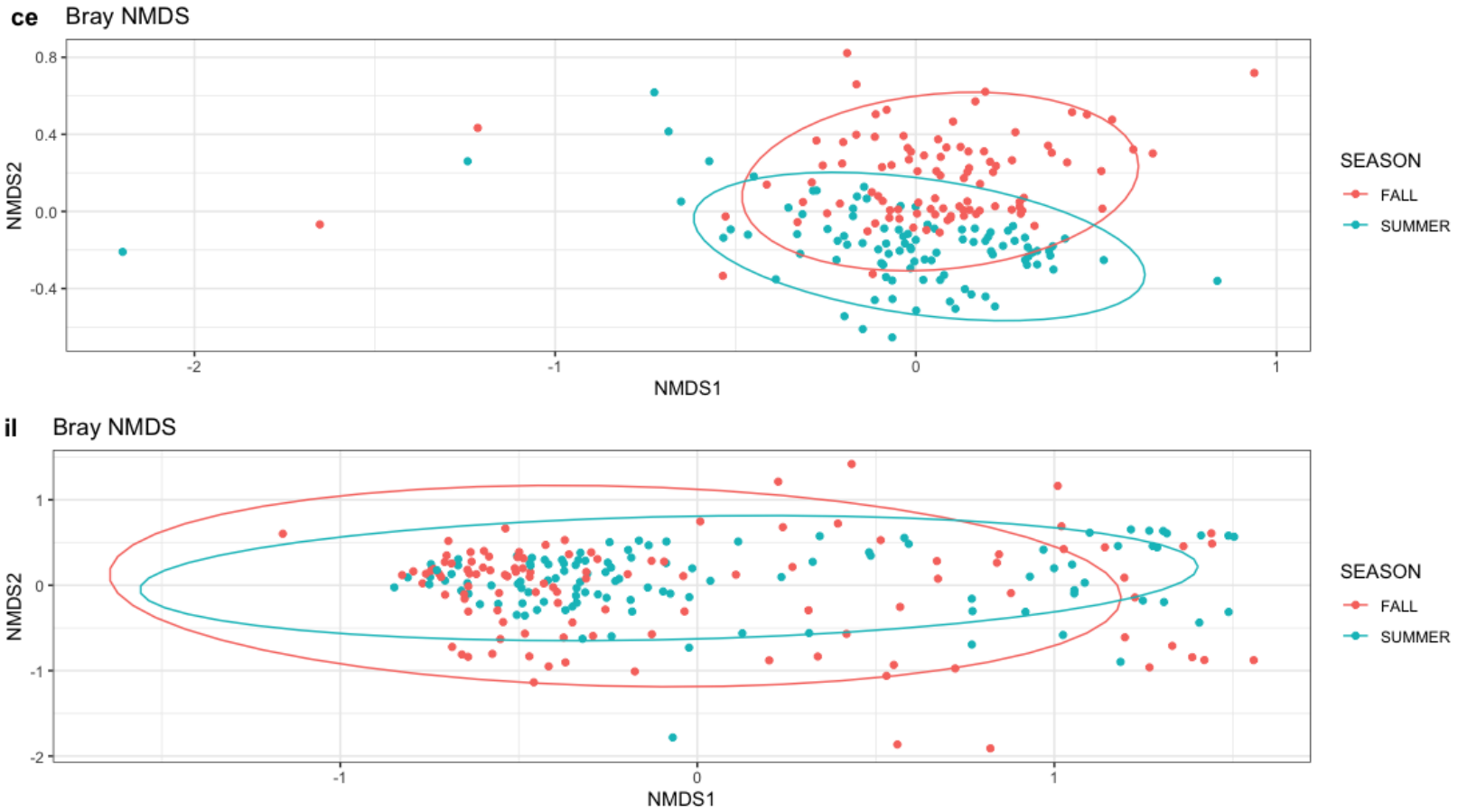

Figure 3.12. Non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarity on relative abundance data in ceca (ce) and ileum (il) samples grouped according to season

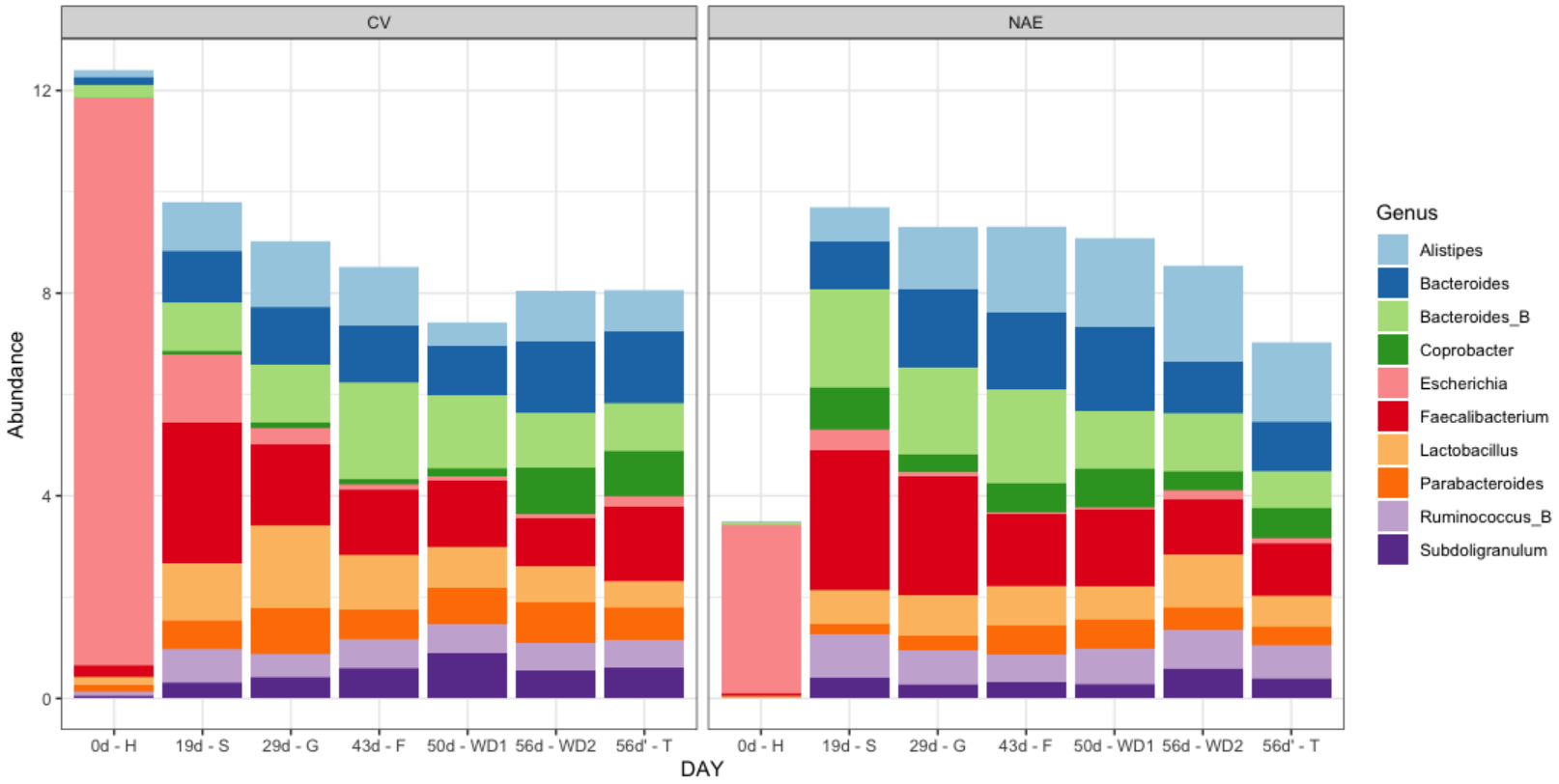

Figure 3.13. Top 10 genera by measure of relative abundance in ceca samples from conventional (CV) and no antibiotics ever (NAE) broilers grouped according to sampling day

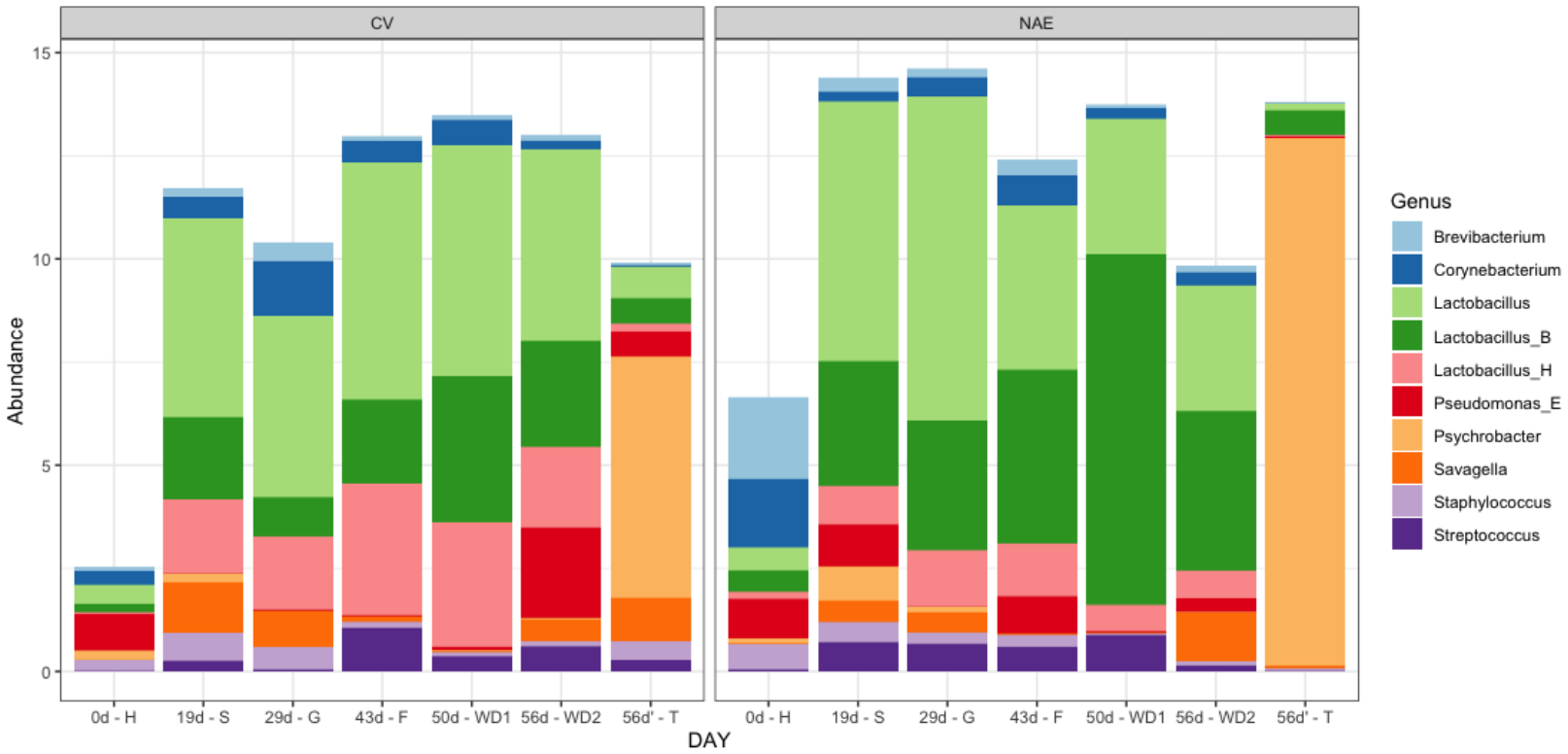

Figure 3.14. Top 10 genera by measure of relative abundance in ileum samples from conventional (CV) and no antibiotics ever (NAE) broilers grouped according to sampling day

CHAPTER 4

PREVALENCE, ENUMERATION, AND ANTIMICROBIAL RESISTANCE OF *CAMPYLOBACTER* IN CONVENTIONAL AND NO ANTIBIOTICS EVER BROILER FARMS[2]

[2] Novoa Rama, E., Bailey, M., Leone C., Thippareddi, H., and Singh, M. To be submitted to *Journal of Food Protection*

Abstract

The poultry-associated, zoonotic agent *Campylobacter* is now the leading cause of enteric disease worldwide. No antibiotics ever (NAE) poultry is becoming increasingly popular, yet little is known about the incidence and antimicrobial resistance (AMR) of *Campylobacter* in this novel production system. This study was designed to investigate the prevalence, concentration, and AMR of *Campylobacter* in conventional and NAE-raised broilers. Two conventional and two NAE commercial broiler flocks were included in this experiment. The intestinal contents from the cecum (n=420) and ileum (n=420) of chickens were collected during the broiler grow-out phase, and following transportation to the processing plant. Samples of litter (n=24), feed (n=24), and water (n=24) were also collected. Screening for *Campylobacter* positive samples was carried out using the BAX® System Real-Time PCR assay, and enumeration was performed by direct plating analysis on Campy Cefex agar. *Campylobacter* isolates were further confirmed by real-time PCR analysis and antimicrobial susceptibility was evaluated using the National Antimicrobial Resistance Monitoring System (NARMS) methods. *Campylobacter* prevalence in NAE broilers reached 100% by day 19 and day 42 in each NAE house. Conventional broilers sampled during the summer also sustained a 100% positive *Campylobacter* prevalence from day 21. The prevalence of *Campylobacter* in conventionally raised broilers sampled during the fall months was sporadic and lower compared to the summer ($P \leq 0.05$). *Campylobacter* was recovered from litter samples at the end of the production cycle. Populations were high in the cecum, carrying an average of 6.6 Log_{10} CFU/g after transportation. Antimicrobial resistant *Campylobacter* was isolated from conventional broilers sampled during the fall, coinciding with the lowest

rates of overall prevalence. Three isolates (1.2%) identified as *C. coli* carried simultaneous resistance to ciprofloxacin, tetracycline and nalidixic acid.

Introduction

Foodborne diseases pose a significant burden on public health and socio-economic development. Global estimates attribute 600 million annual illnesses to foodborne hazards, of which 96 million are caused by *Campylobacter* spp. (WHO, 2015). In the United States, more than 845,000 annual cases of campylobacteriosis are estimated (Scallan et a., 2011), with an associated economic cost of $1.6 billion (Scharff, 2012). These estimates rank *Campylobacter* as the leading bacterial agent of enteric disease worldwide (WHO, 2015). The clinical manifestations of campylobacteriosis include diarrhea, fever and abdominal cramps, and symptoms are usually self-limiting (Allos and Taylor, 1998). While rare, post disease complications may arise, such as Guillain-Barré syndrome (GBS), or irritable bowel syndrome (IBS) (Schwille-Kiuntke et al., 2011; Halpin et al., 2018). *Campylobacter* spp. may also cause systemic infections in immunocompromised patients, who often rely on antimicrobial therapy with azithromycin or ciprofloxacin (Johnson et al., 1984; Same and Tamma, 2018). The Centers for Disease Control and Prevention (CDC) with the National Antimicrobial Resistance Monitoring System (NARMS) report that 28% of clinical *C. jejuni* isolates have decreased susceptibility to ciprofloxacin, a trend that has been increasing steadily since 1997 (Gupta et al., 2004; FDA, 2018). These isolates cause an estimated 429,600 antimicrobial resistant (AMR) infections annually, in the U.S., thus, raising concerns on the therapeutic efficacy of ciprofloxacin for the treatment of severe campylobacteriosis (CDC, 2019).

Poultry are the primary reservoirs of *Campylobacter* spp. (Skarp et al., 2016). Estimates attribute over 60% of foodborne *Campylobacter* infections to poultry meat, with the consumption of raw or undercooked poultry being identified as the major risk factor in campylobacteriosis (WHO, 2013). Human pathogenic species *C. jejuni* and *C. coli* frequently colonize the gastrointestinal tract of chickens in an asymptomatic manner (Newell and Fearnley, 2003). However, recently published studies suggest that certain strains of *C. jejuni* may induce pro-inflammatory responses in young birds and impair gut barrier function (Smith et al., 2008; Connerton et al., 2018). Nonetheless, *Campylobacter* can colonize the intestinal tract of healthy broilers at levels of up to 10^9 CFU/g in the ceca (Clench and Mathias, 1995). Such high bacterial loads result in significant fecal shedding, contributing to the rapid transmission of *Campylobacter* within commercial flocks (Shreeve et al., 2002; Newell and Fearnley, 2003). Vertical transmission on the other hand, is poorly understood. Research suggests that young chicks are protected from *Campylobacter* colonization by maternal antibodies leading to the presumption that *Campylobacter* is not isolated from birds under two weeks of age (Nachamkin et al., 1993; Sahin et al., 2001). However, once the first *Campylobacter* infection occurs, a large proportion of the flock can become colonized within days and remain *Campylobacter*-positive at the time of slaughter (Shreeve et al., 2002; Skarp et al., 2016).

Campylobacter prevalence in primary broiler production can frequently surpass 70% (Sahin et al., 2015). To comply with USDA-FSIS regulations, poultry processors must reduce the prevalence of *Campylobacter* on broiler carcasses to less than 15.7% (USDA-FSIS, 2018). This can be a challenging task due to a high variability in *Campylobacter* incidence associated with seasonality and poultry management practices

(Sahin et al., 2015). Driven by consumer demand, alternative poultry production systems, such as no antibiotics ever (NAE), have become increasingly popular (PHT, 2019). Despite the rise in popularity, it is unclear how NAE production can affect the prevalence of enteric pathogens such as *Campylobacter*. Removing *in-ovo* antibiotic administration and antibiotic-supplemented feed from primary production can have negative consequences on the health and performance of broilers, as antibiotics are commonly used to prevent necrotic enteritis caused by *Clostridium perfringens* (De Gussem, 2007). However, antibiotics may also deprive the broiler gastrointestinal tract (GIT) of beneficial bacteria, inducing a dysbiosis of the intestinal microbiota (LaVorgna et al., 2013). Additionally, its continuous use has been associated with higher rates of antimicrobial resistance among the gastrointestinal bacteria of food animals (Marshall et al., 2009). It is expected that the removal of antibiotics from primary production will reduce the selection pressure for antibiotic-resistant bacteria. Thus, the objective of this study was to evaluate the impact of feed supplementation with antibiotics associated with conventional broiler production on the prevalence and antimicrobial resistance of *Campylobacter* spp.

Materials and Methods

Farm Characteristics

Four commercial broiler farms from a single integrator were included in this study. The farms were categorized as conventional or no antibiotics ever (NAE) based on the production system followed, and two farms of each system were chosen. Broiler house parameters, including size of the house and bird stocking density, were comparable among farms (Table 4.1). Chicks were placed on the houses at day 1 of age and were

raised under 5 nutrition regimens (Table 4.2) to a targeted market age of 52 or 59 days for NAE or conventional broilers, respectively. Conventional birds were routinely given virginiamycin-supplemented feed at 20 g per ton, with the exception of birds from conventional house A which only received the antibiotic supplementation at day 1 of age. Coccidiosis management strategies varied between farms. Broilers from NAE farms received a form of synthetic anticoccidial during the first 30 days of age, conventional broilers from house C were given salinomycin between 20 and 32 days of age, and finally, conventional broilers from house A were not given coccidiostats. The withdrawal 1 feed (WD1) consisted of a high calorie feed with reduced supplementation of the amino acid lysine, and the withdrawal 2 feed (WD2) was a low nutrient feed without vitamin, mineral, amino acid and antibiotic supplementation.

Sample Collection

The timeline of sampling spanned from June to August for houses A and B (summer sampling), and from September to November of that same year for houses C and D (fall sampling). Each house was visited six times during the grow-out cycle of one flock, corresponding to the day of placement, and every day prior to a change in feed regimen. Additionally, birds were followed to the processing facility and sampled upon arrival to assess the impact of transportation on pathogen prevalence. The transit time from the farm to the processing plant was approximately 1h for both conventional and NAE birds. For each sampling day, 15 birds were selected at random and euthanized by cervical dislocation, with the exception of the 1-day-old chicks, of which 30 birds were selected and pooled in groups of two. The ceca (n=15) and ileum (n=15) were then

aseptically removed, placed inside sterile Whirl-Pak™ bags (Nasco Whirl-Pak™; Fisher Scientific Pittsburgh, PA) and immediately placed on ice.

Litter, feed and water samples were also collected with each farm visit. To ensure full area coverage of the house, litter samples were collected by dividing the house into twelve equal quadrants and collecting three grab samples within each quadrant. These subsamples were then pooled to produce one composite sample (n=1, ~500 g). Two grab samples from each feed hopper inside the house were collected and pooled (n=1 composite; ~300 g). Finally, water samples were collected from the main water line as 50-ml aliquots (n=1 composite; 300 mL). Sterile Whirl-Pak™ bags (Nasco Whirl-Pak™; Fisher Scientific Pittsburgh, PA) were used for collection of litter and feed samples, whereas water samples were collected in sterile conical tubes. All samples were maintained on ice and transported to the Department of Poultry Science at the University of Georgia for microbiological analysis within 24 to 48 h of collection.

Microbiological Analysis

Cecal and ileal samples were weighed, and intestinal contents were extracted by performing a longitudinal cut with sterile scissors. The samples were then diluted in 15 mL of buffered peptone water (BPW; Fisher Scientific, Pittsburgh, PA) and homogenized using a Stomacher® (Stomacher® 400 Circulator, Seward Laboratory Systems Inc., Islandia NY) for 1 minute at 230 rpm. For feed and litter samples, 30 g of material were aseptically weighed and transferred into a sterile rinse bag containing 120 mL of phosphate buffered saline (PBS; Fisher Scientific, Pittsburgh, PA). The contents were then stomached for 14 minutes at 75 rpm. Once the samples were homogenized, a 1 mL portion of the homogenate was transferred into 10 mL of blood-free Bolton's Broth (BB;

MilliporeSigma, Burlington, MA) and enriched at 42 °C for 48 h under microaerophilic conditions. To enumerate *Campylobacter* populations, the sample was serially diluted in PBS and plated onto Campy-Cefex agar (Neogen, Lansing, MI). Enrichment and plating of water samples was carried out by adding a 1 mL portion of the original sample to 10 mL of BB and 9 mL of PBS, respectively. All samples were incubated at 42 °C for 48 h under microaerophilic conditions (5% O_2, 8% CO_2,and 87% N_2). Post-enrichment, samples were screened for *Campylobacter* spp. using the BAX® Real-Time *Campylobacter jejuni/coli/lari* assay. From the enumeration plates, a typical well-isolated colony was picked, transferred into cryotubes containing 1 mL of a 20% glycerol solution and stored at -80 °C until further analysis.

DNA Extraction

A subset of *Campylobacter*-positive samples, as determined by the BAX® Real-Time *Campylobacter jejuni/coli/lari* PCR assay, was chosen by randomly selecting 8 of the 15 birds sampled each day. The corresponding *Campylobacter* isolates, collected from the enumeration plates, were subjected to PCR speciation and analyzed for antimicrobial resistance. To perform the DNA extractions, each isolate was resuscitated from cryopreservation by streaking for isolation onto tryptic soy agar (TSA; Fisher Scientific, Pittsburgh, PA) with 5% laked horse blood (Hemostat Laboratories, Dixon, CA) (TSAB) and incubating under microaerophilic conditions for 48 h at 42 °C. Following incubation, one isolated colony was picked, and lawn-streaked onto a second TSAB plate. Bacterial lawns were harvested, resuspended in 1 mL of nuclease-free water, and centrifuged for 1 minute at 5,000 x g. DNA was isolated from the *Campylobacter* pellets using the PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific,

Wilmington, DE) and quantified using the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). A 260/280 nm absorbance ratio of 1.8-2.0 was considered as a standard for nucleic-acid purity.

PCR Speciation

Campylobacter isolates were speciated using a modified real-time PCR assay of Best et al. (2003). The assay targets genes *map*A and *ceu*E which encode a putative lipoprotein on the outer membrane of *C. jejuni* and a periplasmic-binding protein with a predicted function of iron ion transport in *C. coli*, respectively. Sequences of corresponding primers and TaqMan probes (IDT™), are specified in Table 4.3. The DNA isolated from known isolates *C. jejuni* (ATCC 33560) and *C. coli* (ATCC 33559) were used as positive controls. The PCR reactions consisted of 10 μL of 2x PrimeTime Gene Expression Master Mix (IDT), 0.5 μM of the mapA or ceuE forward and reverse primers, 0.25 μM of the corresponding mapA and ceuE probes, and 10-100 ng of the template DNA, constituting a total reaction volume of 20 μL. The PCR reaction was performed using the StepOnePlus™ Real-Time PCR System, and analysis was carried out using the presence/absence test for target genes following the default settings of the StepOne™ Software. Cycling conditions were identical for both speciation assays, consisting of a hold stage of 2 minutes at 50 °C, a polymerase activation stage of 1 cycle at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for 30 seconds, with a final extension stage of 1 cycle at 72 °C for 5 minutes.

Antimicrobial Susceptibility Testing

Within each sampling day, 8 out of 15 birds were chosen at random, and the corresponding *Campylobacter*-positive samples were subjected to antimicrobial susceptibility testing. One confirmed isolate from each *Campylobacter*-positive sample was evaluated for AMR, for a total number of 256 isolates. Antimicrobial susceptibility testing was carried out using the CAMPY2 format Sensititre™ plate (Thermo Scientific™), which contained the antimicrobials azithromycin (AZI), ciprofloxacin (CIP), erythromycin (ERY), gentamicin (GEN), tetracycline (TET), florfenicol (FFN), nalidixic acid (NAL), and clindamycin (CLI). Antimicrobial susceptibility analysis was performed following the broth micro-dilution method designed by VersaTREK™. *Campylobacter* isolates were analyzed for resistance to the antimicrobials using Minimum Inhibitory Concentration (MIC) breakpoints established by NARMS (FDA, 2011). Each isolate was grown under microaerophilic conditions on TSA supplemented with laked horse blood (5%) (TSAB), for 48 h at 42 °C. Following incubations, 3 to 5 colonies were picked using a sterile cotton swab and transferred into 5 mL tube of Mueller-Hinton Broth with TES (Thermo Scientific™). The bacterial suspension was adjusted to achieve a McFarland standard of 0.5. Then, 100 µL of the adjusted bacterial inoculum were transferred into 11 mL of Mueller-Hinton Broth supplemented with laked horse blood (Thermo Scientific™) to achieve a *Campylobacter* inoculum of $5x10^5$ CFU/mL. Finally, 100 µL of this inoculum were transferred into each well of the CAMPY2 Sensititre™ plate, on which a perforated adhesive seal was placed. The seal allowed for the diffusion of the microarophilic atmosphere into each well. The plates were incubated under microaerophilic conditions for 48 h at 42 °C. Within each testing batch, a plate inoculated with a *C. jejuni* (ATCC 33560) quality control strain was

included. After incubation, the MICs were recorded, corresponding to the lowest concentration of antimicrobial that completely hindered the growth of *Campylobacter*.

Statistical Analysis

Campylobacter prevalence was analyzed using the chi-square test of independence with production system (conventional, NAE) as the main effect following the PROC FREQ procedure in SAS 9.4. Fisher's exact test was also performed when counts were fewer than five in 25% of the cells. For each sampling day, presumptive populations of *Campylobacter* were compared between conventional and NAE intestinal samples using general linear models (SAS 9.4). Statistical significance was set at ($P \leq 0.05$).

Results

Campylobacter Prevalence

The prevalence of *Campylobacter* in ceca and ileum samples collected from conventional and NAE broilers during the summer is shown in Figure 4.1. For both conventional and NAE broilers, *Campylobacter* prevalence followed similar general trend. The prevalence of *Campylobacter* in ceca and ileum samples at the time of hatch, was 0%, and reached 100% by the end of the starter ratio, between 2 and 3 weeks of age. Broilers remained *Campylobacter*-positive during the entire production cycle and after the chickens were transported to the processing facility. For most sampling days in the summer, production system did not impact *Campylobacter* prevalence in the ceca or ileum of broilers ($P > 0.05$), except for day WD1, in which ceca samples from NAE chickens showed a lower prevalence of 40% compared to a 100% prevalence in conventional ($P \leq 0.05$). Some variability in *Campylobacter* prevalence was observed

between ceca and ileum samples from NAE chickens, as results from the BAX®Real-Time *Campylobacter jejuni/coli/lari* PCR assay suggest that ileum samples had a higher *Campylobacter* prevalence. Such differences, however, were only statistically significant for day WD1 ($P \leq 0.05$).

Figure 4.2 shows the *Campylobacter* prevalence in ceca and ileum samples corresponding to conventional and NAE broilers sampled in the fall. The prevalence of *Campylobacter* was lower in conventional broilers for all sampling days ($P \leq 0.05$) as compared to the NAE broilers in the fall. Colonization with *Campylobacter* occurred at the end of the finisher ratio (42 to 45 days of age), at levels of 33% and 100% prevalence in conventional and NAE broilers respectively ($P \leq 0.05$). Chickens raised under NAE practices remained *Campylobacter*-positive until the time of slaughter, whereas conventional chickens exhibited sporadic rates of infection. At the end of WD1 all ceca and ileum samples from conventional chickens were negative for *Campylobacter* compared to a 93-100% prevalence of *Campylobacter* in NAE birds. Prior to transportation, *Campylobacter* prevalence in conventional broilers increased to 53% and 73% in the ceca and ileum, respectively. However, all conventional broilers sampled after transport were *Campylobacter*-negative. The overall prevalence of *Campylobacter* in broilers by season is shown in Figure 4.3. The data suggest that season impacted the onset time of *Campylobacter* colonization at the farm and broilers raised in the summer became infected with *Campylobacter* after only 18 to 20 days. Whereas *Campylobacter* colonization of broilers raised in the fall occurred at 42 to 45 days. Overall, conventional broilers raised in the fall (house C) had the lowest prevalence of *Campylobacter* ($P \leq 0.05$). Table 4.4 indicates the presence of *Campylobacter* in litter samples for each house.

Water and feed samples were negative for *Campylobacter*. In litter samples, *Campylobacter* was most often isolated towards the end of the grow-out cycle of each flock.

Enumeration of Campylobacter

Presumptive *Campylobacter* populations in ceca and ileum were determined following the direct plating method using Campy-Cefex agar. In the summer, *Campylobacter* populations in the ceca ranged from 5.3 to 7.1 $\log_{10}$ CFU/g, and 5.3 to 6.6 $\log_{10}$ CFU/g in conventional and NAE broilers respectively (Figure 4.4). In conventional broilers, *Campylobacter* populations peaked after the grower ration (31 days), at 7.1 $\log_{10}$ CFU/g, and decreased after the second withdrawal period (58 days) to 5.8 $\log_{10}$ CFU/g. While, NAE broilers harbored the highest populations of *Campylobacter* after the starter ration (21 days) at 6.6 $\log_{10}$ CFU/g, decreasing slightly throughout the grow-out cycle. For most sampling days, cecal populations from conventional broilers were higher than those found in NAE broilers, whereas ileal populations, followed the reverse trend, of roughly 1-log lower in conventional broilers. These differences, however, were only significant ($P \leq 0.05$) for day of finisher and day of WD2 in ceca and ileum samples, respectively. Although statistical analyses attribute a treatment effect on *Campylobacter* populations during the summer, the observed trends are reversed for both types of intestinal samples, thus the aforementioned effect was not conclusive.

During the fall, a treatment effect was more evident (Figure 4.5). Populations of *Campylobacter* in both ceca and ileum samples of NAE birds were higher ($P \leq 0.05$) than those found in conventional broilers for all sampling days. In NAE broilers, cecal *Campylobacter* reached 7.6 $\log_{10}$ CFU/g after the first withdrawal period and remained at

the same level upon arrival to the processing facility. Ileal populations were 1 to 2-log lower than those in the ceca, reaching a maximum of 6.2 $\log_{10}$ CFU/g at the time of slaughter. *Campylobacter* populations in the ileum of conventional chickens averaged 2.4 $\log_{10}$ CFU/g after the finisher ration (45 days), and 4.5 $\log_{10}$ CFU/g after the second withdrawal period (58 days). *Campylobacter* populations in the ceca, on the other hand, were only detected after the second withdrawal period (58 days), and populations were present at 5.9 $\log_{10}$ CFU/g. Results from the fall sampling suggest that conventional broilers had a lower prevalence and lower levels of *Campylobacter*. This trend was not seen during the summer months.

Antimicrobial Resistance

A total of 256 *Campylobacter* isolates were tested for AMR, of which 96.1% (246/256) isolates were identified as *C. jejuni* and 3.9% (10/256) as *C. coli*. Figure 4.6 shows the prevalence of AMR *Campylobacter* isolates collected from conventional and NAE broilers. The majority of isolates, 96.5% (247/256), were susceptible to all nine antimicrobials tested. The prevalence of isolates exhibiting phenotypic resistance to at least one antimicrobial was 3.5% (9/256), with 1.2% (3/256) of the isolates showing MDR (Figure 4.6). Table 4.5 shows the patterns of resistance found among *Campylobacter* isolates from the different farms. All resistant isolates were collected from conventional broilers sampled in the fall (house C). The most prevalent resistance was observed for tetracycline (2.5% or 6 isolates) and the isolates carrying this resistance were identified as *C. jejuni*. The remaining 1.2% (3 isolates) were categorized as MDR exhibiting simultaneous resistance to ciprofloxacin, tetracycline and nalidixic acid, and were all identified as *C. coli* by real-time PCR. No resistant *Campylobacter* isolates were

recovered from NAE broilers or from conventional broilers sampled in the summer (houses A, B and D).

Discussion

Poultry meat is a major vehicle for human infection with *Campylobacter*. Effectively reducing the rate of *Campylobacter* contamination of chicken meat requires an understanding of the epidemiology of the pathogen on the farm, especially in the live birds. This study highlights the wide spread of *Campylobacter* in commercial broiler production. The prevalence of *Campylobacter* in the intestinal contents of broilers was 100% during the summer months, independent of the farm system in which the chickens were raised. Both conventional and NAE broilers arrived at the processing facility with populations surpassing 5 $\log_{10}$ CFU/g in the ceca, which could compromise the effectiveness of antimicrobial interventions during processing. For example, decontamination strategies such as chilling by immersion in antimicrobial solutions were shown to reduce *Campylobacter* populations by no more than 2-logs on whole carcasses or poultry parts (Smith et al., 2015; Kataria et al., 2020). An incoming load equal or higher than 5 $\log_{10}$ CFU/g of *Campylobacter*, as seen in this study, could be problematic and pose issues of cross-contamination during processing of *Campylobacter*-free flocks. However, such high levels are not uncommon, as broilers have been shown to harbor up to 9 $\log_{10}$ CFU/g of *Campylobacter* in the ceca (Clench and Mathias, 1995). It is important to consider and implement on-farm interventions that effectively reduce *Campylobacter* to ensure the safety of poultry meat.

Campylobacter prevalence levels of 100% in primary poultry production have been reported from farms around the world, particularly during warmer months (Rushton

et al., 2009). Increased populations of flies, which act as transmission vectors, could be contributing to the higher incidence of *Campylobacter* during warm seasons (Nichols, 2005; Hald et al., 2007). In this study, seasonality did not impact the overall prevalence of *Campylobacter*. However, there was a delay in *Campylobacter* colonization of chickens during the fall. Newly hatched chicks are inherently *Campylobacter*-free, as vertical transmission of this pathogen is extremely rare (Newell and Fearnley, 2003). Infection usually occurs between 2 and 3 weeks of age and is followed by a rapid horizontal transmission within the poultry house (Newell and Fearnley, 2003). Our results during the summer sampling highlight this pattern of colonization. *Campylobacter* was first detected on days 19 and 21 for NAE and conventional broilers, respectively, corresponding to the last day of the starter feed ration. Following this, the flocks remained *Campylobacter*-positive until the time of slaughter. The same was true for NAE birds sampled during the fall, which had a *Campylobacter* prevalence of 100% from the initial time of detection (days 42-45). Conventional chickens sampled during the fall, however, exhibited sporadic and overall lower rates of *Campylobacter* prevalence.

Feed supplementation with antibiotics affected the overall prevalence of *Campylobacter* in broiler chickens in the fall. During the summer, no differences in the incidence of *Campylobacter* were found between conventional and NAE broilers. Yet, during the fall, conventional broilers had a lower ($P \leq 0.05$) prevalence of *Campylobacter* compared to broilers raised without the use of antibiotics (NAE). A possible explanation might be the inconsistency of virginiamycin supplementation in conventional broilers raised in house A (summer sampling), which were only given the antibiotic on day 1 of age. In contrast, conventional chickens from house C (fall sampling) were routinely

administered the antibiotic until the time of feed withdrawal (day 58). Virginiamycin has been shown to exhibit bactericidal activity against *Campylobacter* (Baurhoo et al., 2009), thus, its continuous use during the grow-out cycle of conventional birds from house C could be effectively reducing *Campylobacter* populations. Conversely, a study by Cox et al. (2003) showed that virginiamycin supplementation of feed at 20 g per ton did not reduce naturally occurring populations of *Campylobacter* in the ceca of turkeys. This suggests that antibiotic use may not affect communities of *Campylobacter* established prior to antibiotic treatment.

Overall, data regarding *Campylobacter* prevalence on commercial broiler flocks raised under NAE practices in the US is limited, as nationwide implementation of this production system has risen only in recent years (PHT, 2019). Several authors have reported higher rates of *Campylobacter* positive flocks on organic broiler production systems, with restricted antibiotic use, compared to conventional systems (Heuer et al., 2001, Luangtongkum et al., 2006). However, a direct comparison to our own findings would be biased due to differences in management of organic and NAE flocks. For example, organic flocks have access to the outdoors potentially increasing their exposure to pests that are potential carriers of *Campylobacter*, which could in turn be contributing to the higher prevalence reported (Rodenburg et al., 2004).

Campylobacter presence in litter samples was low, and was isolated most commonly at the end of the grow-out cycle (Table 4.4). This can be surprising, considering the high loads of intestinal *Campylobacter* reported, which could be contributing to significant fecal shedding of *Campylobacter.* These results are in agreement with prior research showing that *Campylobacter* can persist in the litter

(Montrose et al., 1985; Johnsen et al., 2006), yet, its incidence is low, particularly in dry litter (Jacobs-Reitsma et al., 1995; Berndtson et al., 1996). *Campylobacter* is known to thrive in humid environments and is therefore more often isolated from wet litter (Berndtson et al., 1996) which could explain our observations.

Stringent biosecurity has also been linked to lower a prevalence of *Campylobacter* (Newell et al., 2011). In this study, NAE farms were more stringent on biosecurity practices, as observed by lower fly populations and better upkeep of the surrounding areas when compared to the conventional farms. We cannot conclude that biosecurity could be contributing to the lower levels of *Campylobacter* seen in conventional chickens during the fall. Another reason for the discrepancies in *Campylobacter* prevalence between conventional broilers from house A (sumer sampling) compared to house C (fall sampling), could be the proximity to beef cattle farming of house A. Cattle can shed *Campylobacter* through their feces for a considerable period of time (Inglis et al., 2004), which could be a consistent source of *Campylobacter* introduction to the broiler farm.

Only 3.5% of the *Campylobacter* isolates tested (9/256) exhibited phenotypic resistance to antimicrobials considered medically important by the National Antimicrobial Resistance Monitoring System (NARMS). There was an association between *Campylobacter* species and pattern of resistance i.e. *C. jejuni* isolates carried resistance to tetracycline (2.5%, 6/256 isolates) and *C. coli* isolates exhibited multi-drug resistance to ciprofloxacin, tetracycline and nalidixic acid (1.2%, 3/256 isolates). Overall, the AMR levels reported in this study were lower than the national average (FDA, 2018). In 2017, NARMS reported ciprofloxacin resistance in *Campylobacter* isolates from the

ceca of broiler birds at levels of 20% and 16% for *C. jejuni* and *C. coli*, respectively (FDA, 2018). Tetracycline resistance levels reported by NARMS were considerably higher than those observed in this study, 44% for *C. jejuni* and 38% for *C. coli* (FDA, 2018).

Evidence exists of a correlation between antibiotic use and incidence of resistance in bacteria from food-producing animals (Marshall et al., 2009). Our results, agree with literature, as emergence of AMR *Campylobacter* was associated with prolonged antibiotic use (House C). Our findings also indicated that all *Campylobacter* isolates recovered from NAE chickens and broilers raised in conventional house A (summer sampling), which only received virginiamycin supplementation on day 1, were pan-susceptible. Pan-susceptibility among *Campylobacter* isolates from poultry operations is rare, particularly pertaining to tetracycline (Luangtongkum et al., 2008). Tetracycline resistance is widespread among *Campylobacter*, and its incidence seems to be independent of antibiotic pressure (Luangtongkum et al., 2008; Kassem et al., 2017). In *Campylobacter* spp., this type of resistance is most commonly attributed to the presence of the *tet*(*O*)-containing plasmid (Gibreel et al., 2002), which encodes a ribosomal protection protein (Taylor and Courvalin, 1987). It has been suggested that carriage of *tet*(*O*)-containing plasmids poses a low fitness cost to *Campylobacter*, and that the pathogen has evolved to naturally carry this resistance (Luangtongkum et al., 2008). This could explain the high tetracycline resistance levels reported even in environments where its' use has been discontinued. The rates of tetracycline susceptibility observed in our study contradict the aforementioned trend and could be attributes to the colonization dominance of susceptible strains of *Campylobacter*. Commercial broiler flocks are often

colonized by a single *Campylobacter* strain (Ring et al., 2005; Müllner et al., 2010). However, this pathogen has an inherently high genetic variability (Parkhill et al., 2000) and colonization with multiple strains is possible (Thomas et al., 1997), particularly as the chickens age (Novoa Rama et al., 2018). Commercial broilers birds have a short lifespan (8-9 weeks), thus, single-strain colonization is a plausible argument for the results reported in our study. Alternatively, sampling methodology can potentially select for susceptible strains of *Campylobacter*. Further genetic analysis of the recovered *Campylobacter* isolates by Whole Genome Sequencing (WGS) or other molecular subtyping methods would be needed to test these hypotheses.

Similar to tetracycline, fluoroquinolone resistance was only detected in *Campylobacter* isolates from house C, corresponding to a continued selective pressure due to the use of virginiamycin and salinomycin. Resistance to fluoroquinolones (ciprofloxacin and nalidixic acid) is less prevalent than tetracycline resistance (FDA, 2018). Fluoroquinolone resistance is mediated by point mutations in the DNA gyrase A gene (*gyrA*) (Payot et al., 2006). In *Campylobacter*, ciprofloxacin resistance develops rapidly following exposure to antibiotics from the fluoroquinolone class (Han et al., 2008), including enrofloxacin (Delsol et al., 2004; Nelson et al., 2007). Ciprofloxacin resistance in *Campylobacter* is considered an issue of importance to public health (WHO, 2019). Additionally, there is abundant evidence of a direct transmission of fluoroquinolone resistance from poultry to humans (Smith et al., 1999; Gupta et al., 2004), resulting in eliminating its use in poultry production in 2005 (FDA, 2006). Research suggests that there is a low fitness cost to ciprofloxacin resistance in *Campylobacter,* as resistant mutants have been known to persist after the withdrawal of

antibiotic use (Zhang et al., 2003). Some studies have even reported higher levels of ciprofloxacin resistant *Campylobacter* in organic chicken compared to conventional (Cui et al., 2005). The levels of ciprofloxacin susceptibility seen in our study, contradict the trends reported in literature. More data from NAE production systems is needed to be able to make a reliable comparison.

Finally, there are discrepancies between the antimicrobial classes pertaining to use and resistance observed. Virginiamycin is an antibiotic of the streptogramin class, there is no scientific evidence linking virginiamycin use and emergence of resistance to drugs of the tetracycline or fluoroquinolone class. A genomic characterization of resistance determinants will provide valuable information on the development of resistance. Our study suggests that prolonged virginiamycin administration as a dietary supplement for commercial broilers results in a lower prevalence of intestinal *Campylobacter*, and a higher incidence of antimicrobial resistance. It is important to consider the deviation from expected antibiotic use in conventional house A, which could be affecting our comparative analysis.

Tables and Figures

Table 4.1. Broiler house characteristics where broilers raised under conventional (CV) or no antibiotics ever (NAE) practices were collected for evaluation of *Campylobacter* prevalence and antimicrobial resistance

House	Farm System	Season	House Size Width x Length (ft x ft)	Number of Birds	Flock Density (ft^2 $bird^{-1}$)
A	CV	Summer	54 x 500	26,000	1.05
B	NAE	Summer	60 x 600	33,300	1.08
C	CV	Fall	50 x 500	23,800	1.05
D	NAE	Fall	60 x 600	33,300	1.08

Table 4.2. Schedule of the nutrition regimen administered to broilers raised under conventional (CV) or no antibiotics ever (NAE) practices which were selected for evaluation of *Campylobacter* prevalence and antimicrobial resistance

	Conventional		NAE	
Feed	Age Interval (days)	Duration (days)	Age Interval (days)	Duration (days)
Starter	1-21	20	1-19	18
Grower	22-31	10	20-29	8
Finisher	32-45	14	30-42	12
Withdrawal 1 (WD1)	46-53	8	43-48	5
Withdrawal 2 (WD2)	54-58	4	49-54	5

Table 4.3. Genetic sequences of primers and probes used for speciation of *Campylobacter* isolates by real-time PCR

Species	Gene	Oligo	Sequence (5'/3')
C. jejuni	*mapA*	mapA-F	CTGGTGGTTTTGAAGCAAAGATT
		mapA-R	CAATACCAGTGTCTAAAGTGCGTTTAT
		mapA-P	FAM-TTGAATTCCAACATCGCTAATGTATAAAAGCCCTTT-BHQ1
C. coli	*ceuE*	ceuE-F	AAGCTCTTATTGTTCTAACCAATTCTAACA
		ceuE-R	TCATCCACAGCATTGATTCCTAA
		ceuE-P	FAM-TTGGACCTCAATCTCGCTTTGGAATCATT-BHQ1

Table 4.4. Presence of *Campylobacter* in litter samples collected from all farms

House	Sampling Day					
	Hatch	**Starter**	**Grower**	**Finisher**	**WD1**	**WD2**
A	-	-	-	-	-	-
B	-	-	+	-	+	+
C	-	-	-	-	-	+
D	-	-	-	+	+	+

Table 4.5. Antimicrobial resistance patterns of *Campylobacter* isolates collected from broilers in all farms

House	Species	Pattern[1]	Number of isolates
C	*C. jejuni*	TET	6/256
C	*C. coli*	CIP-TET-NAL	3/256

[1]Antimicrobial abbreviations stand for the following: CIP= Ciprofloxacin, NAL= Nalidixic acid, TET= Tetracycline

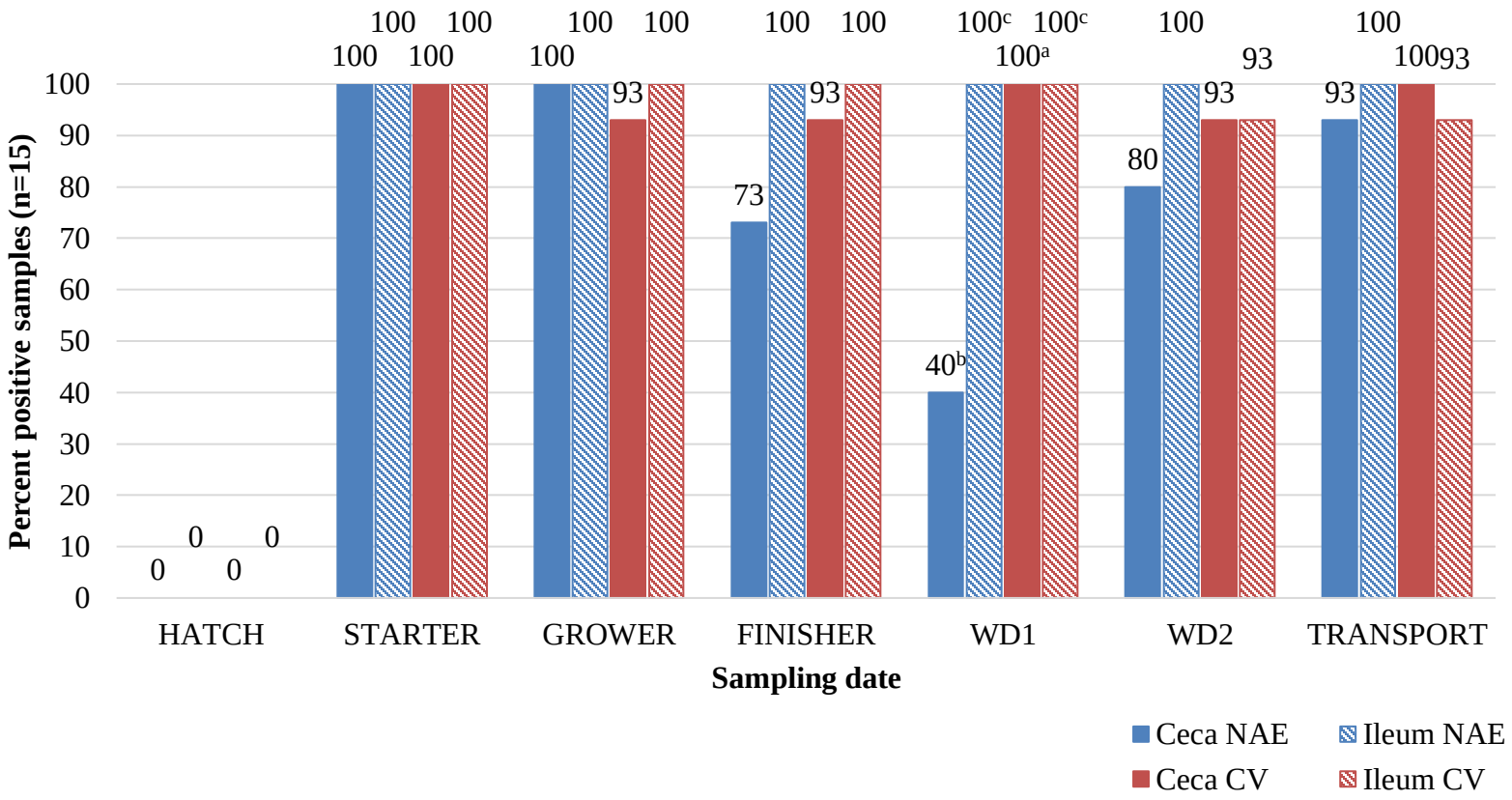

Figure 4.1. Prevalence of *Campylobacter* in ceca and ileum samples from NAE and CV birds in the summer. Superscripts "a" and "b", and "c" and "d", indicate differences in counts between production systems at a given time ($P \leq 0.05$) for ceca and ileum samples respectively, whereas absence of superscripts indicates no differences ($P > 0.05$)

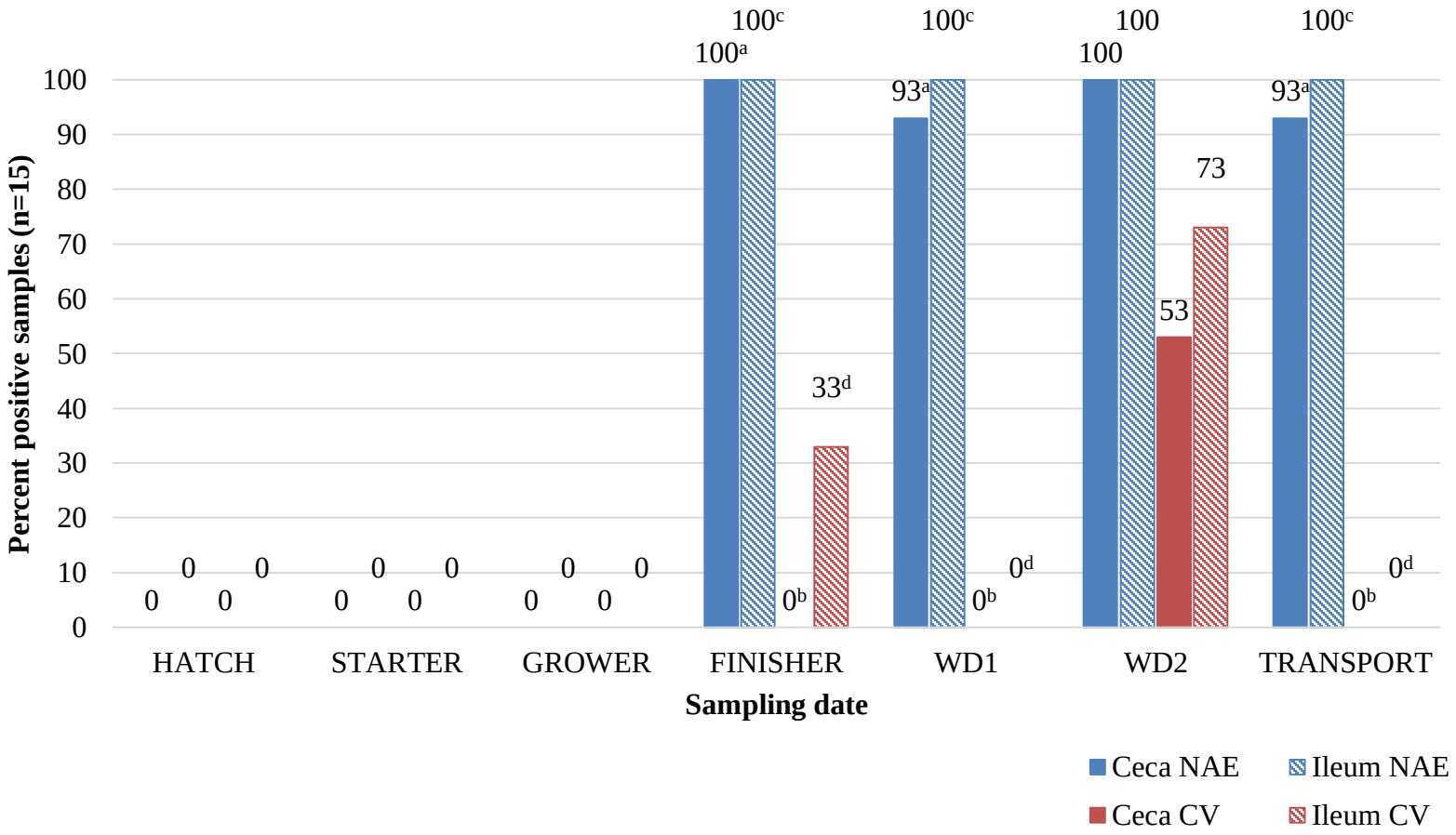

Figure 4.2. Prevalence of *Campylobacter* in ceca and ileum samples from NAE and CV birds in the fall. Superscripts "a" and "b", and "c" and "d", indicate differences in counts between production systems at a given time ($P \leq 0.05$) for ceca and ileum samples respectively, whereas absence of superscripts indicates no differences ($P > 0.05$)

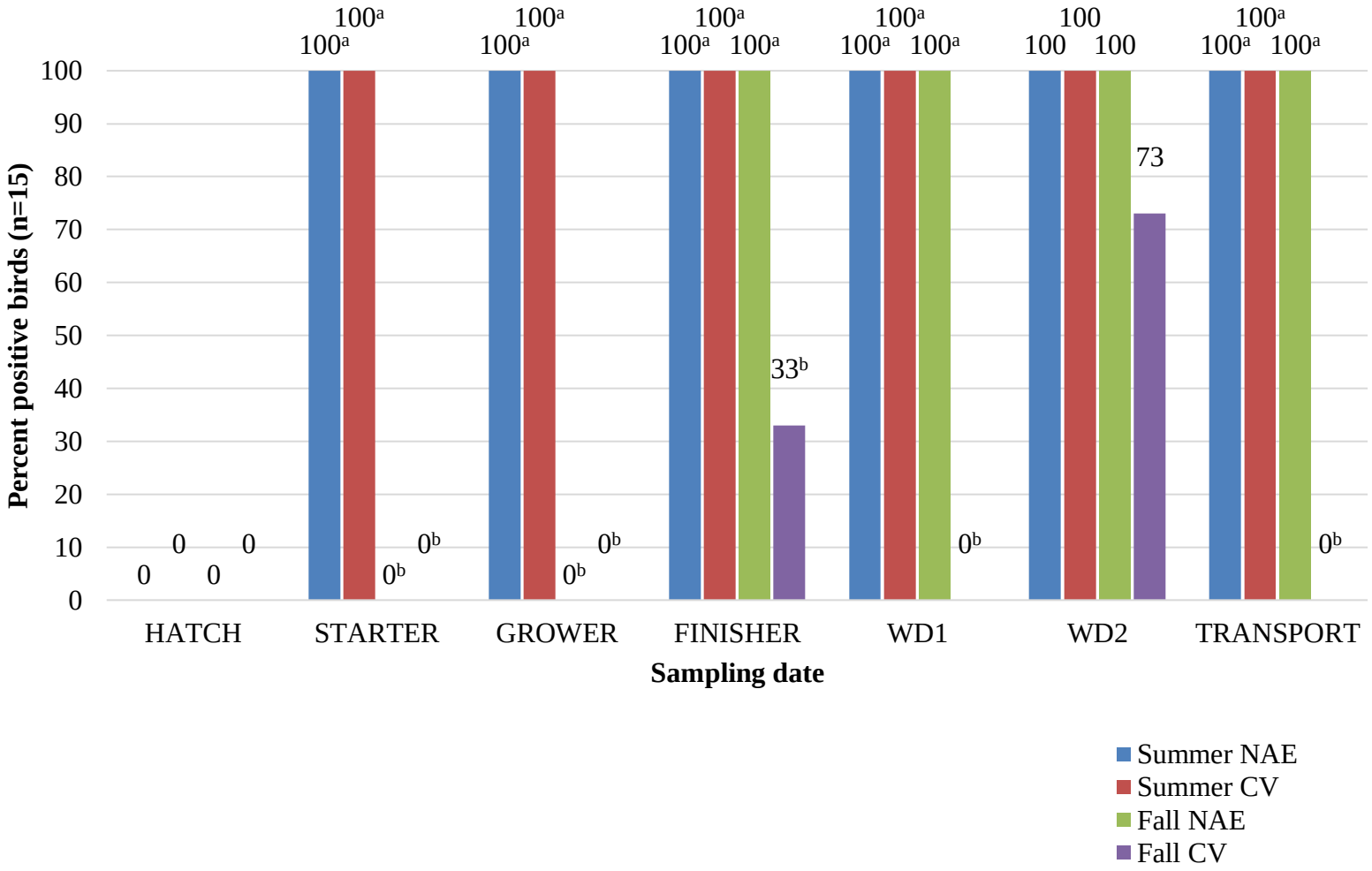

Figure 4.3. Overall prevalence of *Campylobacter* in NAE and CV broilers for seasons summer and fall. Superscripts "a" and "b" indicate differences in prevalence between production systems at a given time ($P \leq 0.05$), whereas absence of superscripts indicates no differences ($P > 0.05$)

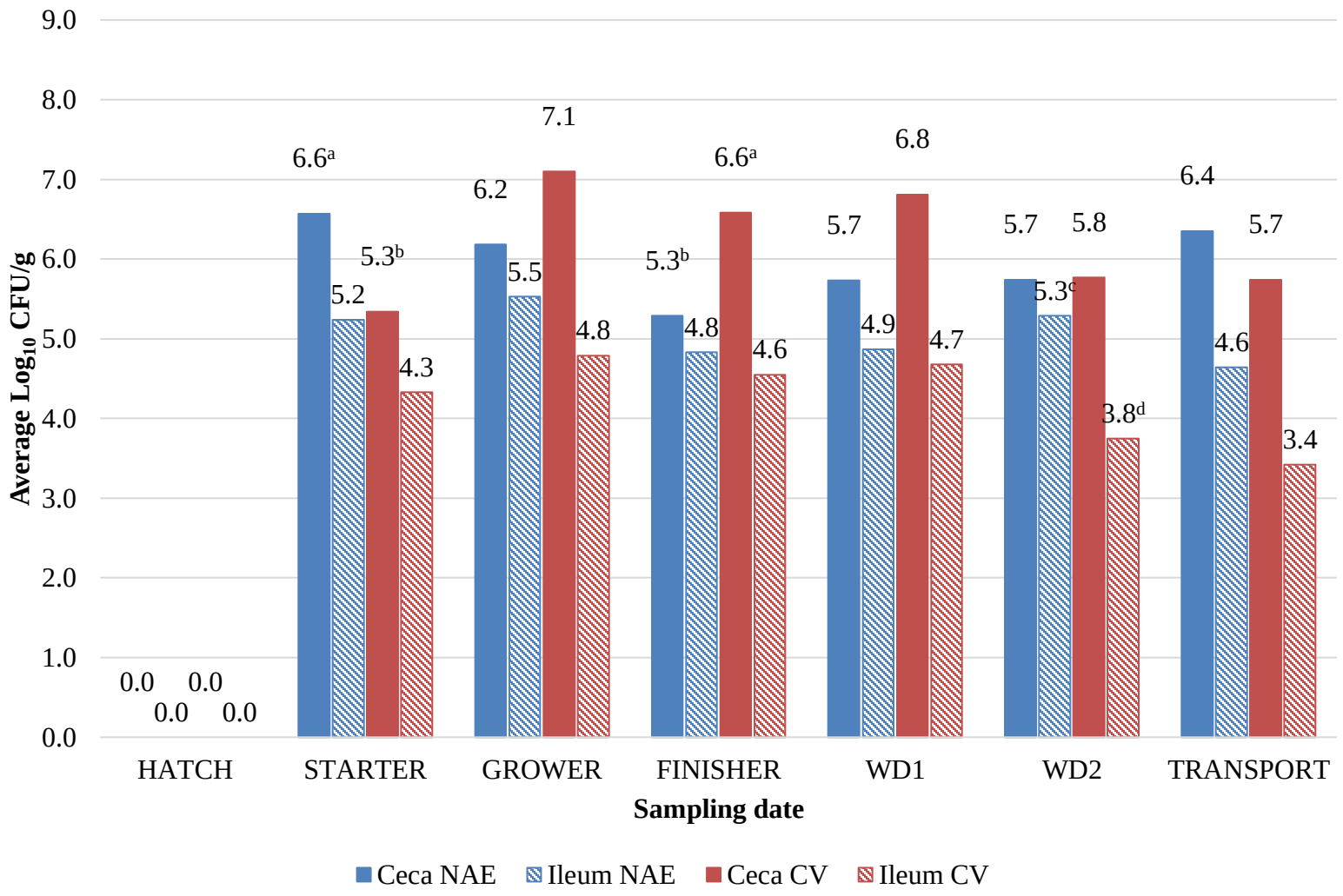

Figure 4.4. Presumptive populations of *Campylobacter* as determined by Campy-Cefex counts in ceca and ileum samples from NAE and CV birds in summer. Superscripts "a" and "b", and "c" and "d", indicate differences in counts between production systems at a given time ($P \leq 0.05$) for ceca and ileum samples respectively, whereas absence of superscripts indicates no differences ($P > 0.05$)

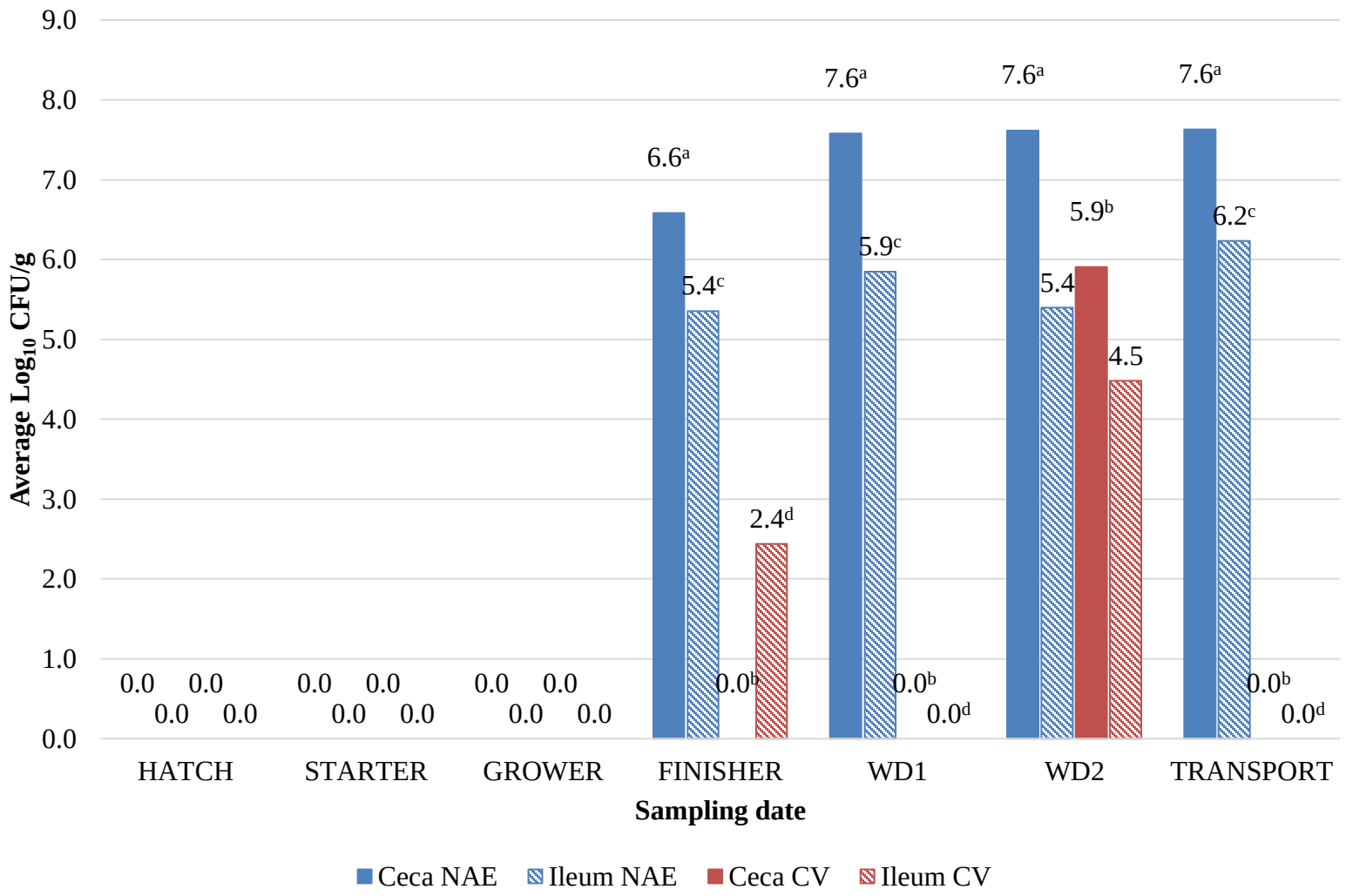

Figure 4.5. Presumptive populations of *Campylobacter* as determined by Campy-Cefex counts in ceca and ileum samples from NAE and CV birds in fall. Superscripts "a" and "b", and "c" and "d", indicate differences in counts between production systems at a given time ($P \leq 0.05$) for ceca and ileum samples respectively, whereas absence of superscripts indicates no differences ($P > 0.05$)

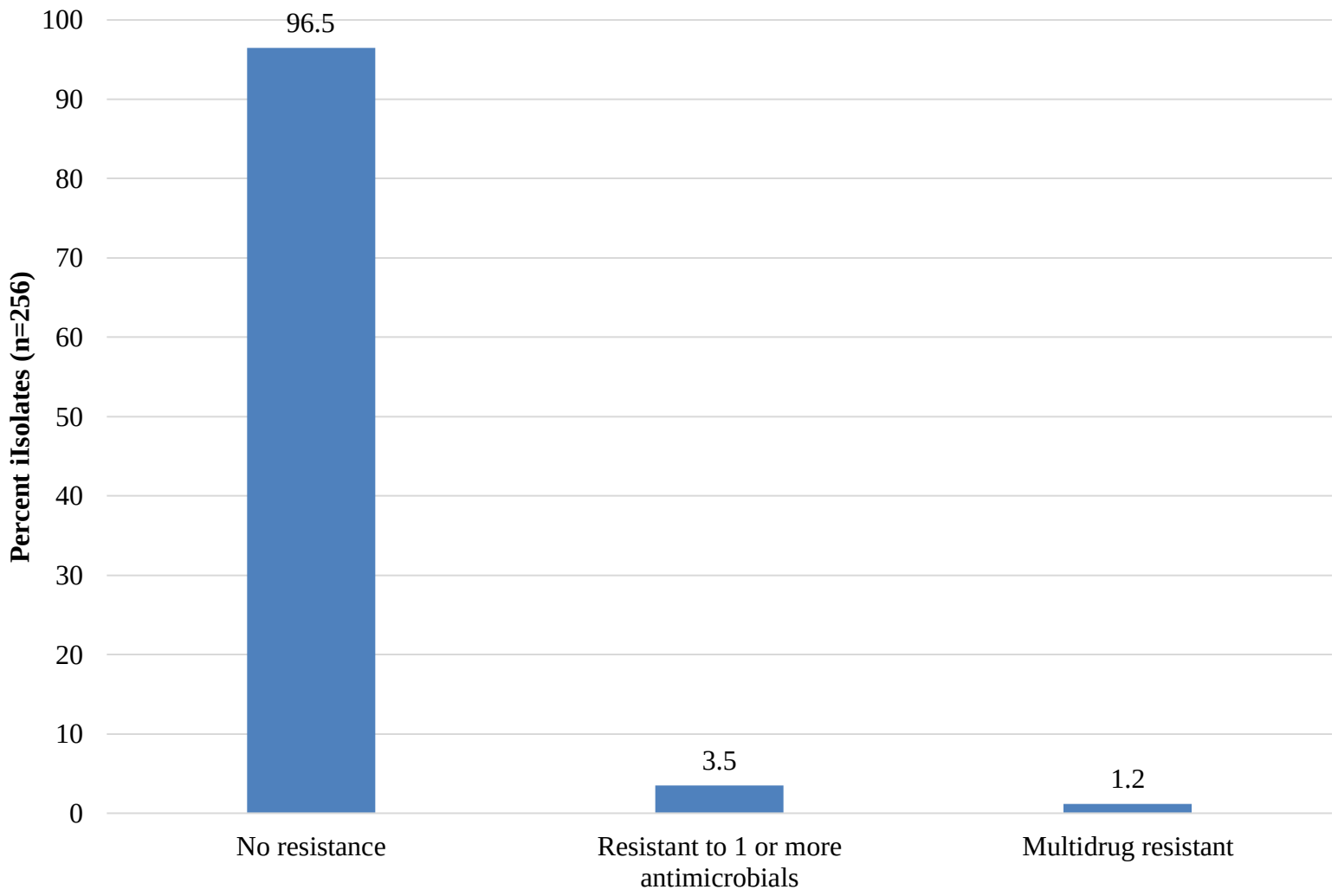

Figure 4.6. Overall prevalence of antimicrobial resistance in *Campylobacter* isolates collected from all four houses

CHAPTER 5

PREVALENCE AND ANTIMICROBIAL RESISTANCE OF *SALMONELLA* IN CONVENTIONAL AND NO ANTIBIOTICS EVER BROILER FARMS[3]

[3] Novoa Rama, E., Bailey, M., Leone C., Thippareddi, H., and Singh, M. To be submitted to *Journal of Food Protection*

Abstract

Non-typhoidal *Salmonella* is the second most common bacterial cause of foodborne illnesses in the United States. Despite regulatory and industry efforts to reduce the incidence of salmonellosis, the number of infections linked to consumption of poultry meat have not declined. More than 50% of the poultry meat sold in the United States is categorized as no antibiotics ever (NAE). Data regarding the impact of antibiotic withdrawal on the incidence of *Salmonella* in broilers is lacking. The aim of this study was to evaluate and compare the prevalence of *Salmonella* and incidence of antimicrobial resistance (AMR) in conventional and NAE broiler farms. Broiler cecal and ileal contents (n=420 per sample type), along with litter (n=24), feed (n=24) and water (n=24) samples from broiler houses were collected. Sample collection was carried out during a full production cycle and following the birds to the processing facilities. The BAX® Real-Time PCR system was used to screen *Salmonella* positive samples, and isolates were collected following USDA-FSIS guidelines. The recovered *Salmonella* isolates were confirmed using real-time PCR, and antimicrobial susceptibility was evaluated following the National Antimicrobial Resistance Monitoring System (NARMS) method. Conventional broilers had the highest *Salmonella* prevalence ($P \leq 0.05$), of 80 and 73% at day 21 of age. The rate of infections declined as the birds aged. *Salmonella* persisted in litter samples from farms with high associated prevalence. *Salmonella* was not detected in feed and water samples. Antimicrobial resistance among *Salmonella* isolates was high in all four farms (76% of all isolates), exhibiting frequent resistance to tetracycline (76%) and streptomycin (70%). *Salmonella* isolates recovered from NAE broilers carried higher

rates of AMR than isolates from conventional chickens ($P \leq 0.05$), highlighting the widespread incidence of AMR *Salmonella* in commercial broiler production.

Introduction

Non-typhoidal *Salmonella enterica* is one of the leading causative agents of diarrheal disease worldwide, causing approximately 79 million infections annually (WHO, 2015). Among the bacterial hazards, this pathogen is responsible for the highest number of bacterial foodborne deaths associated with gastrointestinal illness, an estimated 59,000 (WHO, 2015). In the United States, over 1 million cases of salmonellosis are reported each year (Scallan et al., 2011), having an economic impact of $4.4 billion (Scharff, 2012). Most cases of salmonellosis are manifested as a self-limiting diarrheal disease, yet infections can become invasive and lead to meningitis or persistent bacteremia (Vugia et al., 2004). Recent estimates report over 500,000 annual cases of invasive salmonellosis with a significant incidence in children under 5 years of age (Stanaway et al., 2019).

Poultry remains the principal vehicle of *Salmonella* transmission to humans (Jackson et al., 2013). Chickens are natural hosts of a wide variety of disease-causing serotypes of *Salmonella enterica*, such as Typhimurium and Enteritidis, hence the high incidence of salmonellosis associated with poultry products (Foley et al., 2011). At the farm, *Salmonella* can colonize broiler flocks through different routes. Pests, litter, and feed have all been identified as sources of horizontally-transmitted *Salmonella* (Heyndrickx et al., 2002). Young chicks are highly susceptible to infection, since *Salmonella* can be vertically transmitted by the parent bird (Liljebjelke et al., 2005). Further, research suggests that early infection may aid the persistence of the pathogen

(Gast and Holt, 1998). The rate of *Salmonella* infection within broiler flocks varies, and recent estimates in the United States attribute prevalence rates of 22.9% and 19.9% in conventional and alternative poultry production systems respectively (Golden and Mishra, 2020).

The consumption of poultry in the US has been rising steadily since the early 1900s (Buzby and Farah, 2006). In 2019, the per capita consumption of poultry meat among American consumers surpassed 110 pounds (NCC, 2019). With consumption levels rising every year, the potential for exposure to *Salmonella*-contaminated poultry meat increases further. The U.S. Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) has made significant efforts to reduce the incidence of *Salmonella* in poultry and poultry products. In 2011, for example, FSIS increased the stringency of performance standards for *Salmonella* and *Campylobacter* in poultry carcasses, and later introduced new performance standards for raw chicken parts and not ready-to-eat (NRTE) comminuted chicken products (USDA-FSIS, 2011, 2018). According to the latest report, the baseline *Salmonella* contamination level of broiler carcasses post-chill decreased to 3.8% from an estimated 7% reported in 2009 (USDA-FSIS, 2014). Considering an annual production of 9 billion broilers in the U.S. (USDA-FAS, 2020), a prevalence of 3.8% would translate to 342 million *Salmonella* contaminated carcasses potentially entering the market. Thus, highlighting the need for continuous improvement of *Salmonella* control programs in poultry production.

The regulatory focus in recent years has been placed on reducing the incidence of multi-drug resistant (MDR) *Salmonella* from poultry. In a recent report by the National Antimicrobial Resistance Monitoring System (NARMS), it was estimated that more than

59% of *Salmonella* isolates from the ceca of commercial broilers at the time of slaughter were resistant to medically important antibiotics. Of which, 15% were classified as MDR (FDA, 2018). Recent studies also report of the widespread incidence of extended-spectrum-lactamase (ESBL) genes in *Salmonella* isolates from poultry (Archambault et al., 2006; Silva et al., 2013). Microbial isolates carrying this gene exhibit resistance to a broad range of medically-important antibiotics, thus limiting the therapeutic efficacy of such drugs (Rupp and Fey, 2003). Consequently, regulatory agencies around the world have advocated for the withdrawal of antibiotic use in food animal production (ECDC, EFSA and EMA, 2017).

Poultry meat labeled as "no antibiotics ever" (NAE) or "raised without antibiotics" (RWA) has gained popularity among consumers, and now occupies over 50% of the market (PHT, 2019). Transitioning to NAE poultry can be challenging to producers. From a poultry health perspective, antibiotics help reduce the incidence of necrotic enteritis by controlling the populations of *Clostridium perfringens* (De Gussem, 2007). Antibiotics are also administered at the hatchery as injections *in-ovo* to prevent bacterial infections in young chicks. Thus, the complete removal of antibiotics from primary production could potentially impair broiler health and decrease performance, resulting in significant economic loses. On the other hand, antibiotic use is known to select for populations of antimicrobial resistant bacteria and induce dysbiosis in the poultry gut microbiota which can potentially make broilers more susceptible to colonization by enteric pathogens (Marshall et al., 2009; LaVorgna et al., 2013). No studies have compared the incidence of *Salmonella* in conventional and no antibiotics ever broiler farms, therefore, it is difficult to estimate whether antibiotic withdrawal from

primary production will affect the incidence of foodborne pathogens. This study was designed to evaluate the distribution and prevalence of *Salmonella* in commercial broilers as affected by feed supplementation with antibiotics.

Materials and Methods

Farm Characteristics

Two conventional and two no antibiotics ever (NAE) commercial farms raising broilers for the same integrator were selected for this study. Each farm was represented by one broiler house, with comparable size and stocking density characteristics (Table 5.1). Chicks were raised following 5 dietary rations (Table 5.2). The age of broilers at slaughter varied from 52 days to 59 days for NAE and conventional production respectively. Both conventional and NAE broilers were subjected to two feed withdrawal periods, the first, WD1, corresponded to a diet with lower lysine supplementation. The second withdrawal, WD2, was a low nutrient feed with the complete removal of antibiotics and other nutritional supplements. Chickens grown in the conventional farms received a diet supplemented with the antibiotic virginiamycin, at a dosage of 20 g per ton of feed. The antibiotic was administered from day 1 until day 53, corresponding to the start of the second feed withdrawal period (WD2). Conventional broilers raised during the summer (house A) did not receive virginiamycin supplementation after the first day of grow-out. These broilers were also not given any form of anticoccidial treatment. Conventional broilers raised during the fall (house C) were administered the ionophore coccidiostat salinomycin between 3 to 4 weeks of age. Anticoccidial treatment in NAE production consisted of the administration of a synthetic anticoccidial at about 4 weeks of age.

Sample Collection

Sample collection was carried out over two seasons during 2018, summer and fall. Broilers raised in houses A and B were sampled in the summer (June to August), whereas broilers from houses C and D were sampled during the fall (September to November). Within each house, chicks from a single flock were sampled on the day of placement, and at six subsequent times corresponding to every dietary change across one production cycle. Broilers were also sampled after transportation to the processing facility, which took place approximately 1 h after WD2. Intestinal specimens (ceca and ileum) from broilers were collected alongside feed, water and litter samples from each house. On the day of sampling, broilers were randomly selected and humanely euthanized by cervical dislocation. The ceca (n=15) and ileum (n=15) were removed aseptically from the birds, placed inside a sterile Whirl Pak bag, and maintained on ice until further processing. Due to the low intestinal contents of day-old chicks, 30 birds were selected, and samples were pooled in groups of two.

Broiler houses were visually divided into a grid of twelve equal quadrants to encompass sampling of the litter from the entire house area. Litter samples were collected by pooling three sample grabs from each quadrant in Sterile Whirl-Pak® bags (Nasco, Ft. Atkinson, WI), resulting in one composite sample of approximately 500 g. Feed samples were collected from the feed hoppers inside the broiler house (n=1 composite; ~300 g) using Sterile Whirl-Pak® bags, and water samples were collected from the main water line (n=1 composite; 300 mL) using sterile conical tubes. Samples were transported to the Department of Poultry Science at the University of Georgia in an insulated cooler with ice and were processed for analysis within 24 to 48 h of collection.

Microbiological Analysis

Intestinal samples (ceca and ileum) were weighed and the contents were extracted aseptically. The samples were homogenized by diluting the intestinal contents in 15 mL of buffered peptone water (BPW; Fisher Scientific, Pittsburgh, PA) using a Stomacher® (Stomacher® 400 Circulator, Seward Laboratory Systems Inc., Islandia NY) for 1 minute at 230 rpm. Thirty grams of feed and litter samples were stomached with 120 mL of phosphate buffered saline (PBS; Fisher Scientific, Pittsburgh, PA) for 14 minutes at 75 rpm. Sample enrichments were carried out following USDA-FSIS methods (USDA-FSIS, 2017). Briefly, 1 mL of the sample homogenate was transferred into 10 mL of BPW and enriched at 35 °C for 24 h. After enrichment, samples were tested for detection of *Salmonella* using the BAX®Real-Time *Salmonella* PCR assay. Samples screened as positive were then subjected to selective enrichment and plating for recovery of *Salmonella* isolates. Aliquots of 0.5 mL and 1 mL of the primary enrichment were transferred to 10 mL of tetrathionate broth (TT Hajna; Fisher Scientific, Pittsburgh, PA) and 10 mL of modified Rappaport Vassiliadis broth (mRV; Fisher Scientific, Pittsburgh, PA) respectively. Selective enrichments were then incubated at 42 °C for 24 h. Following selective enrichments, a loopful of each inoculum was streaked onto selective agars xylose-lysine-tergitol 4 (XLT-4; Thermo Fisher Scientific, Wilmington, DE) and brilliant green sulfa agar (BGS; Fisher Scientific, Pittsburgh, PA), and the plates were incubated at 35 °C for 24 h. The plates were examined for presumptive *Salmonella* growth. A well-isolated colony was picked from the selective agar plates for PCR confirmation and transferred into cryotubes containing 1 mL of a 20% glycerol solution. The specimens were stored at -80 °C for further analysis.

DNA Extraction

Eight of 15 broilers were randomly selected for antimicrobial resistance analysis and the corresponding *Salmonella* isolates were confirmed by PCR. Each *Salmonella* isolate was streaked onto tryptic soy agar (TSA; Fisher Scientific, Pittsburgh, PA) and incubated at 35 °C for 24 h. After the incubation period, one isolated colony was streaked onto a second TSA plate to produce a bacterial lawn. Bacterial suspensions of the presumptive salmonellae were prepared in 1 mL of nuclease-free water, and centrifuged at 5,000 x g during 1 min. The resulting pellets of *Salmonella* cells were subjected to DNA isolation using the PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, Wilmington, DE) following manufacturer's instructions. Measurements of DNA concentration and nucleic acid purity (260/280 nm absorbance ratio) were taken using the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Specimens of DNA with a 260/280 nm absorbance ratio of 1.8-2.0 were considered of acceptable quality.

PCR Confirmation

Following DNA extraction, *Salmonella* isolates were confirmed with real-time PCR using an adaptation of the assay developed by Suo et al. (2010) which targets the *invA* gene. The primers and probes used in this assay were manufactured by Integrated DNA Technologies (IDT™), and their sequences are specified in Table 5.3. The PCR reaction was designed as a 20 μL reaction containing 10 μL of 2x PrimeTime Gene Expression Master Mix (IDT), 0.5 μM of the invA forward and reverse primers, 0.25 μM of the invA TaqMan probe and 10-100 ng of template DNA. The StepOnePlus™ Real-Time PCR System was used to carry out the reactions with the following cycling

conditions: 1 cycle of denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15s, and annealing at 60 °C for 1 min. Finally, confirmation analysis using the StepOne™ Software was performed for determining the presence of the *invA* gene.

Antimicrobial Susceptibility Testing

Salmonella isolates confirmed by PCR were subjected to antimicrobial susceptibility testing by the broth micro-dilution method developed by VersaTREK™. This procedure uses the CMV3AGNF format Sensititre™ plate (Thermo Scientific™), allowing susceptibility testing against cefoxitin (FOX), azithromycin (AZI), chloramphenicol (CHL), tetracycline (TET), ceftriaxone (AXO), amoxicillin/clavulanic acid 2:1 (AUG2), ciprofloxacin (CIP), gentamicin (GEN), nalidixic acid (NAL), meropenem (MERO), sulfisoxazole (FIS), trimethoprim/sulfamethoxazole (SXT), ampicillin (AMP), and streptomycin (STR). The Minimum Inhibitory Concentration (MIC) breakpoints established by NARMS (USDA, 2011) were used for analysis of antimicrobial resistance among the *Salmonella* isolates. Each isolate was grown on tryptic soy agar (TSA; Fisher Scientific, Pittsburgh, PA) overnight, and 3-5 colonies were transferred to 4 mL of demineralized water (Thermo Scientific™) to create a bacterial suspension that matched a McFarland turbidity standard of 0.5. Following this, 10 μL of the adjusted suspension were transferred into 11 mL of cation adjusted Mueller-Hinton broth with TES buffer (CAMHBT) (Thermo Scientific™), creating a final *Salmonella* inoculum of $5x10^5$ CFU/mL. This inoculum was used to fill each well of the CMV3AGNF Sensititre™ plate with 50 μL. Finally, the inoculated antimicrobial

susceptibility plates were sealed and incubated at 35 °C for 24 h. A plate inoculated with the quality control strain *E. coli* ATCC 25922 was included with every testing batch.

Statistical Analysis

The chi-square test of independence was applied for the statistical analysis of differences in *Salmonella* prevalence between conventional and NAE production, following the PROC FREQ procedure in SAS 9.4. The same test was used to analyze differences in the prevalence of AMR *Salmonella*. Fisher's exact test was also performed when less than five counts were found in at least 25% of the cells. For both tests, statistical significance was set at $P \leq 0.05$.

Results

Salmonella Prevalence

Figure 5.1 represents the prevalence of *Salmonella* in ceca and ileum samples from conventional and NAE broilers raised during the summer. Results show a higher prevalence of *Salmonella* in conventional broilers for most sampling days in both ceca and ileum samples ($P \leq 0.05$). During the summer, samples taken from NAE chicks at the day of placement (hatch) were *Salmonella* negative. In subsequent sampling days, broilers raised under NAE practices exhibited an intermittent incidence of *Salmonella* (7%) throughout the grow-out cycle. Further, NAE broilers sampled upon arrival to the processing facility were free of *Salmonella*. Conventional broilers, on the other hand, exhibited high levels of *Salmonella* prevalence. *Salmonella* was detected in the ceca of conventional broilers on the first day of sampling (hatch), with a prevalence of 7% (1/15). Colonization rates increased after day 1, reaching a maximum of 73% in ceca samples 20 days post-hatch (starter). The prevalence of *Salmonella* then gradually decreased to 20%

and 13% in ceca and ileum samples at the end of the finisher ratio, corresponding to day 45. There was an increase in the prevalence of *Salmonella* in both intestinal samples (47%) after the feed withdrawal (WD2), followed by a decrease in prevalence to 40% and 13% in ceca and ileum samples after transportation.

The prevalence of *Salmonella* in conventional and NAE broilers sampled during the fall is shown in Figure 5.2. Broilers from conventional house C (fall sampling) exhibited a pattern of *Salmonella* infection that was comparable to that seen in conventional broilers reared in the summer. Chicks were *Salmonella*-positive upon day of hatch, with an associated prevalence of 13% (2/15). The highest prevalence of *Salmonella* was observed in 20-day-old broilers (starter), with rates of 73% and 40% in the ceca and ileum respectively. The prevalence of *Salmonella* decreased during subsequent stages of rearing, and ultimately reached a level of 0% prevalence after the second withdrawal period (WD2). After transportation to the processing facility, just hours after the WD2 sampling, the prevalence of *Salmonella* again increased to 27% in the ceca. Broilers raised under NAE practices sustained rates of *Salmonella* infection of 20% to 27% during the grow-out cycle and increased to 40% in ceca samples following transportation. When comparing the prevalence of *Salmonella* between the two systems, conventional broilers exhibited higher rates during the early stages of grow-out. This trend was reversed after the finisher ratio, when NAE broilers showed higher levels of *Salmonella* infection until arrival to the processing facility. These differences, however, were only significant for day of starter (day 20, $P \leq 0.05$).

The overall prevalence of *Salmonella* in broilers in both sampling seasons can be seen in Figure 5.3. This analysis showed no correlation between season and prevalence of

Salmonella ($P > 0.05$). Conventional broilers sampled in the summer had the highest overall rates of *Salmonella* prevalence, whereas NAE broilers raised during the same season had the lowest ($P \leq 0.05$). For both seasons, the prevalence of *Salmonella* was higher in conventional broilers during early production (days 1 to 31) ($P \leq 0.05$). Further, the levels of *Salmonella* prevalence in conventional broilers showed considerable fluctuation, contrasting with NAE broilers which sustained levels throughout grow-out of 7% and 27% during the summer and fall samplings respectively. No effect can be attributed to transportation, since there was an associated decrease in the prevalence of *Salmonella* in summer broilers and an increase during the fall.

Salmonella was detected in litter samples collected from all four farms (Table 5.4). In house B, corresponding to the NAE farm sampled during the summer, *Salmonella* was only detected once after the grower period (day 29). In the remainder houses, *Salmonella* was detected as early as the end of the starter period (days 19 or 21), and persisted in the litter until the end of the growth cycle.

Antimicrobial Resistance

The total number of *Salmonella* isolates tested for antimicrobial susceptibility was 99, of which 28 were collected from conventional house A (summer sampling), 5 from NAE house B (summer sampling), 27 from conventional house C (fall sampling) and 39 from NAE house D (fall sampling). Figure 5.4 represents the overall prevalence of AMR in *Salmonella* isolates from all production systems. The total prevalence of *Salmonella* isolates exhibiting resistance to at least one antimicrobial was 76% (76/99), yet only 1% (1/99) of isolates were classified as multi-drug resistant (MDR), showing resistance to three antimicrobials. The remaining 23% (23/99) were susceptible to all antimicrobials

tested. *Salmonella* isolates were most often resistant to tetracycline (76%), followed by streptomycin (70%), azithromycin (5%), and chloramphenicol (1%) (Figure 5.5). Simultaneous resistance to tetracycline and streptomycin was the most commonly observed resistance pattern among *Salmonella* isolates and it was present in all four farms (Table 5.5). Only one isolate was classified as MDR, with an associated resistance pattern of chloramphenicol, tetracycline and streptomycin. This MDR isolate was recovered from the NAE farm sampled in the fall.

Figure 5.6 presents data of AMR prevalence as seen among *Salmonella* isolates in the different farms. Overall, the highest rates of AMR *Salmonella* were seen in broilers raised under NAE practices ($P \leq 0.05$). In NAE farms, 97% and 90% of *Salmonella* isolates recovered during the summer and fall samplings respectively were resistant to tetracycline. Further, 80% of *Salmonella* isolates recovered from both NAE farms were resistant to streptomycin. In conventional farms, the prevalence of tetracycline resistant *Salmonella* was 64% for both farms, and a prevalence of streptomycin resistance of 59% and 48% was observed during the summer and fall samplings.

Discussion

Salmonella is one of the leading foodborne pathogens isolated from poultry meat and eggs (Foley et al., 2011). Moreover, the incidence of MDR *Salmonella* isolated from poultry meat products has increased in recent years, thus putting the health of consumers at risk for drug-resistant infections (CDC, 2019). This study illustrates the distribution and prevalence of *Salmonella* in four commercial poultry farms and the broad incidence of AMR *Salmonella* in primary production. Results from this study show that chicks from three of the four farms were infected with *Salmonella* at the moment of placement in the

broiler house. This is not uncommon, as *Salmonella* could be contaminating the eggs by vertical transmission from the parent flocks (Liljebjelke et al., 2005). Other possibilities include horizontal transmission events at the hatchery, due to contamination of the facilities, or during transportation to the farm as chicks come in contact with contaminated crates or feces from infected birds (Heyndrickx et al., 2002). Further, chicks could be colonized by *Salmonella* as a result of pecking contaminated litter once placed in the broiler house (Corrier et al., 1999). This is unlikely as litter samples collected during the day of hatch (day 0) tested negative for *Salmonella* (Table 5.4).

Differences in the prevalence of *Salmonella* between conventional and NAE broilers were more apparent during the first weeks of the production cycle. Conventional broilers had the highest prevalence of *Salmonella* at the time of starter ration (19 days), during which *Salmonella* levels reached 80% and 73% in summer and fall broilers respectively. Further, these chickens were *Salmonella*-positive upon hatch at rates of 7% during the summer and 20% during the fall. Studies show that young broilers are highly susceptible to colonization by *Salmonella*, potentially due to an underdeveloped gut microbiota which favors the establishment of *Salmonella* populations (Oakley et al., 2014). Additionally, studies using experimentally-infected chickens have shown that *Salmonella* colonization of newly hatched chickens can lead to persistent shedding of the pathogen until maturity (Gast and Holt, 1998; Van Immerseel et al., 2004). The prevalence of *Salmonella* in conventionally raised birds gradually decreased after the grower and finisher rations (days 31 to 45) (Figure 5.3). This could be explained by a competitive exclusion event between *Salmonella* and other microbial groups in the chicken gut. It is estimated that the establishment of a mature gut microbiota in the

chicken takes place after 3 weeks of age (Oakley et al., 2014). At this time, there is an increase in the relative abundance of microbial groups producing short-chain fatty acids, which could be having an antimicrobial effect against *Salmonella* (Oakley et al., 2014).

Broilers raised under NAE practices, on the other hand, had different rates of *Salmonella* infection throughout production. Birds raised in house B (summer sampling) were *Salmonella*-negative at the time of hatch, and had the lowest prevalence of all four flocks. In this farm, *Salmonella* was first detected at day 21 with a prevalence of 7% (1/15 birds). The timeline of this initial colonization, 21 days post-hatch, could be coinciding with the formation of a well-developed gut microbiota, which could then be impeding the establishment and persistent shedding of *Salmonella* populations in this farm. Thus resulting in lower prevalence rates of *Salmonella*. Conversely, NAE chickens raised in house D (fall sampling) were positive for *Salmonella* upon hatch, yet the overall flock prevalence was kept constant during production, and did not surpass a rate of 27%. These results contrast the sharp increases in *Salmonella* prevalence seen in conventional flocks early in the grow-out cycle. A possible explanation might be the increased attention to biosecurity in NAE farms, as visually noticed by a lower abundance of flies, and better upkeep of the farm surrounding areas. Such factors are known to contribute to a heightened control of *Salmonella* prevalence at the farm (White et al., 1997).

Authors also report lower levels of *Salmonella* prevalence in alternatively-raised poultry (Alali et al., 2010; Siemon et al., 2007). It is difficult to attribute these observations to the withdrawal of antibiotics from such production systems, as seasonality and biosecurity have a significant impact on *Salmonella* populations in commercial broiler production. In this study, conventional broilers received a diet

supplemented with virginiamycin, an antibiotic that targets populations of Gram-positive bacteria. Virginiamycin does not have an antimicrobial effect against *Salmonella*, yet, its use on broilers could potentially eliminate microbial populations of competitor bacteria in the gut, thus favoring the growth of the pathogen (Singh et al., 2008). The mechanisms that could explain this effect remain uncharacterized.

Feed withdrawal and transportation to the processing facility had mixed effects on the prevalence of *Salmonella* (Figure 5.8). Studies suggest that such practices can increase the prevalence of *Salmonella* within a flock, potentially due to the depletion of gut microbial populations during extended feed withdrawal periods, or cross-contamination events associated with fecal shedding of *Salmonella* during transportation (Isaacson et al., 1999). Results from this study showed no clear association of changes in *Salmonella* prevalence with such practices. For example, transportation to the processing facility seemed to decrease the prevalence of *Salmonella* in broilers raised during the summer, but had the opposite effect on fall broilers. These differences were not statistically significant ($P > 0.05$), thus no effect can be concluded.

The incidence of AMR *Salmonella* (76%) among isolates collected from all four farms in this study was higher than the national average (59%) as reported by NARMS (FDA, 2018). On the other hand, the levels of MDR reported in the is study (1%), were considerably lower than those described by NARMS (15%) (FDA, 2018). Data regarding AMR resistance in *Salmonella* isolates from primary poultry production is scarce. Siemon et al. (2007) reported a prevalence of antimicrobial resistant *Salmonella* of 51% in broilers raised in commercial broiler flocks from the Southeastern United States. Lower levels of AMR prevalence in commercial broiler farms (3% to 16%) have been

reported by Velásquez et al. (2018). This study found a higher incidence of antimicrobial resistant *Salmonella* in NAE broilers compared to that of conventional (Figure 5.6). Other studies have also shown high levels of antimicrobial resistant *Salmonella* recovered from poultry raised under organic practices in which antibiotic use is limited (Cui et al., 2005; Bailey et al., 2020). Authors, however, hypothesize that the high resistance levels seen in organic farming can be a result of exposure to outdoor environments through which resistant *Salmonella* can be transmitted (Cui et al., 2005; Bailey et al., 2020). Broilers raised under NAE practices do not have access to the outdoors thus a direct comparison with data from organic farming cannot be carried out.

Resistance to tetracycline and streptomycin were the most commonly observed AMR patterns among *Salmonella* isolates recovered from both production systems (Figure 5.5), coinciding with the national trend (FDA, 2018). As reported by NARMS, 45% and 49% of *Salmonella* isolates recovered from the ceca of broilers at the time of slaughter exhibited resistance to tetracycline and streptomycin respectively (FDA, 2018). Results from this study highlight higher than average levels of resistance to the aforementioned antimicrobials in *Salmonella* isolates (Figure 5.6). Resistance to drugs of the tetracycline and aminoglycoside class (i.e., streptomycin) is widespread among *Salmonella* isolates recovered from food-animal production (Liljebjelke et al., 2017; FDA, 2018), which could explain the high prevalence seen in both conventional and NAE broiler farms. Bacteria from the *Salmonella* genus acquire resistance to tetracycline antimicrobials through energy-dependent efflux proteins encoded most commonly by the *tet(A)* gene (Alcaine et al., 2007). This gene has been found on easily transferred mobile genetic elements such as plasmids and integrons (Briggs and Fratamico, 1999) as well as

in genomic islands (Carattoli et al., 2002). Streptomycin resistance, on the other hand, is mediated by aminoglycoside phosphotransferases encoded by genes *strA*, *strB*, *aph(3)-Ib* and *aph(6)-Id* which modify aminoglycoside hydroxyl groups (Alcaine et al., 2007). As with tetracycline resistance, *Salmonella* isolates typically acquire streptomycin resistance genes by horizontally-transmitted plasmids, integrons and genomic islands (Mulvey et al., 2006; Alcaine et al., 2007). The widespread of mobile genetic elements carrying resistance to tetracycline and streptomycin among *Salmonella* isolates and other bacteria from the *Enterobacteriaceae* family most likely explains the high incidence of resistance exhibited by the isolates tested in this study. A genomic characterization of the resistant isolates is needed to test this hypothesis.

Tables and Figures

Table 5.1. Broiler house characteristics where broilers raised under conventional (CV) or no antibiotics ever (NAE) practices were collected for evaluation of *Salmonella* prevalence and antimicrobial resistance

House	Farm System	Season	House Size Width x Length (ft x ft)	Number of Birds	Flock Density (ft^2 $bird^{-1}$)
A	CV	Summer	54 x 500	26,000	1.05
B	NAE	Summer	60 x 600	33,300	1.08
C	CV	Fall	50 x 500	23,800	1.05
D	NAE	Fall	60 x 600	33,300	1.08

Table 5.2. Schedule of the nutrition regimen administered to broilers raised under conventional (CV) or no antibiotics ever (NAE) practices which were selected for evaluation of *Salmonella* prevalence and antimicrobial resistance

	Conventional		NAE	
Feed	Age Interval (days)	Duration (days)	Age Interval (days)	Duration (days)
Starter	1-21	20	1-19	18
Grower	22-31	10	20-29	8
Finisher	32-45	14	30-42	12
Withdrawal 1 (WD1)	46-53	8	43-48	5
Withdrawal 2 (WD2)	54-58	4	49-54	5

Table 5.3. Genetic sequences of primers and probes used for confirmation of *Salmonella* isolates by real-time PCR

Gene	Oligo	Sequence (5'/3')
invA	invA-F	GTTGAGGATGTTATTCGCAAAGG
	invA-R	GGAGGCTTCCGGGTCAAG
	invA-P	HEX-CCGTCAGACCTCTGGCAGTACCTTCCTC-ZEN-IBFQ

Table 5.4. Presence of *Salmonella* in litter samples collected from all farms

House	Sampling Day					
	Hatch	**Starter**	**Grower**	**Finisher**	**WD1**	**WD2**
A	-	+	+	+	+	+
B	-	-	+	-	-	-
C	-	-	+	+	+	+
D	-	+	+	+	+	+

Table 5.5. Antimicrobial resistance patterns of *Salmonella* isolates collected from broilers in all farms

House	Farm	Season	Pattern[1]	Number of isolates
A	CV	Summer	TET-STR	18/28
B	NAE	Summer	TET-STR	4/5
C	CV	Fall	TET-STR	13/27
			AZI-TET	3/27
D	NAE	Fall	TET-STR	34/39
			AZI-TET	2/39
			TET	1/39
			CHL-TET-STR	1/39

[1]Antimicrobial abbreviations stand for the following: AZI = Azithromycin, CHL= Chloramphenicol, STR= Streptomycin, TET= Tetracycline

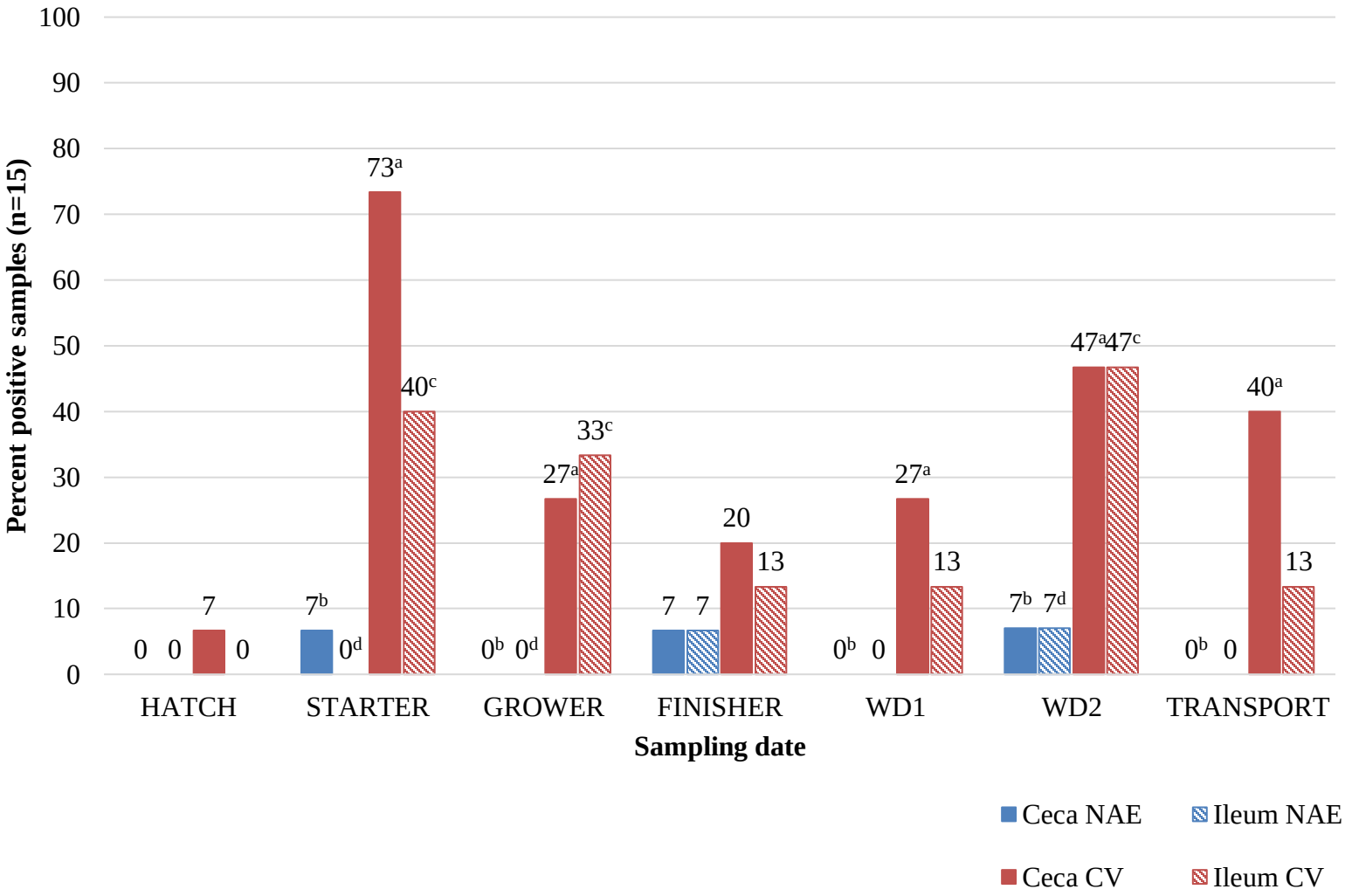

Figure 5.1. Prevalence of *Salmonella* in ceca and ileum samples from NAE and CV birds in the summer. Superscripts "a" and "b", and "c" and "d", indicate differences in counts between production systems at a given time ($P \leq 0.05$) for ceca and ileum samples respectively, whereas absence of superscripts indicates no differences ($P > 0.05$)

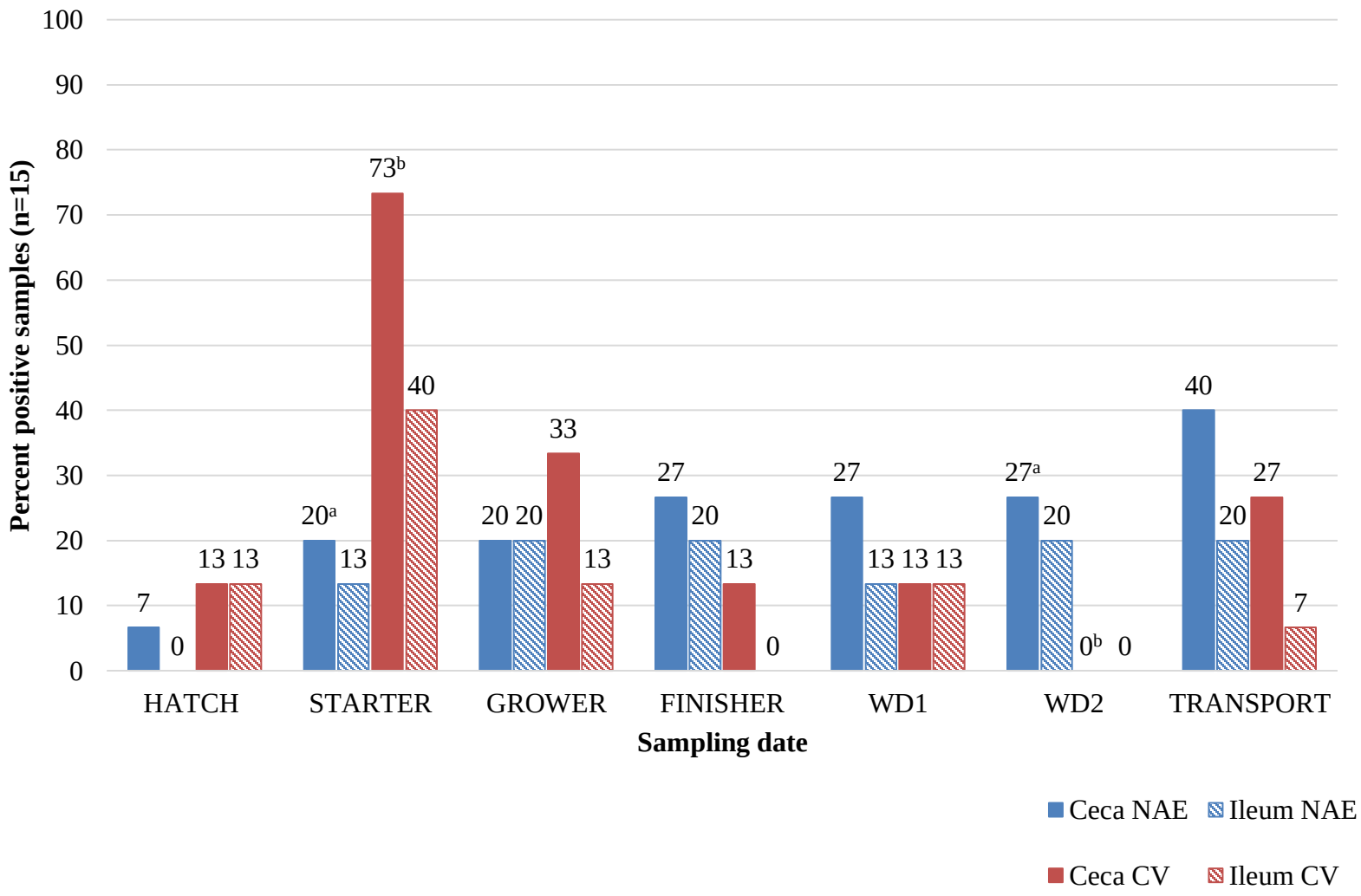

Figure 5.2. Prevalence of *Salmonella* in ceca and ileum samples from NAE and CV birds in the fall. Superscripts "a" and "b", and "c" and "d", indicate differences in counts between production systems at a given time ($P \leq 0.05$) for ceca and ileum samples respectively, whereas absence of superscripts indicates no differences ($P > 0.05$)

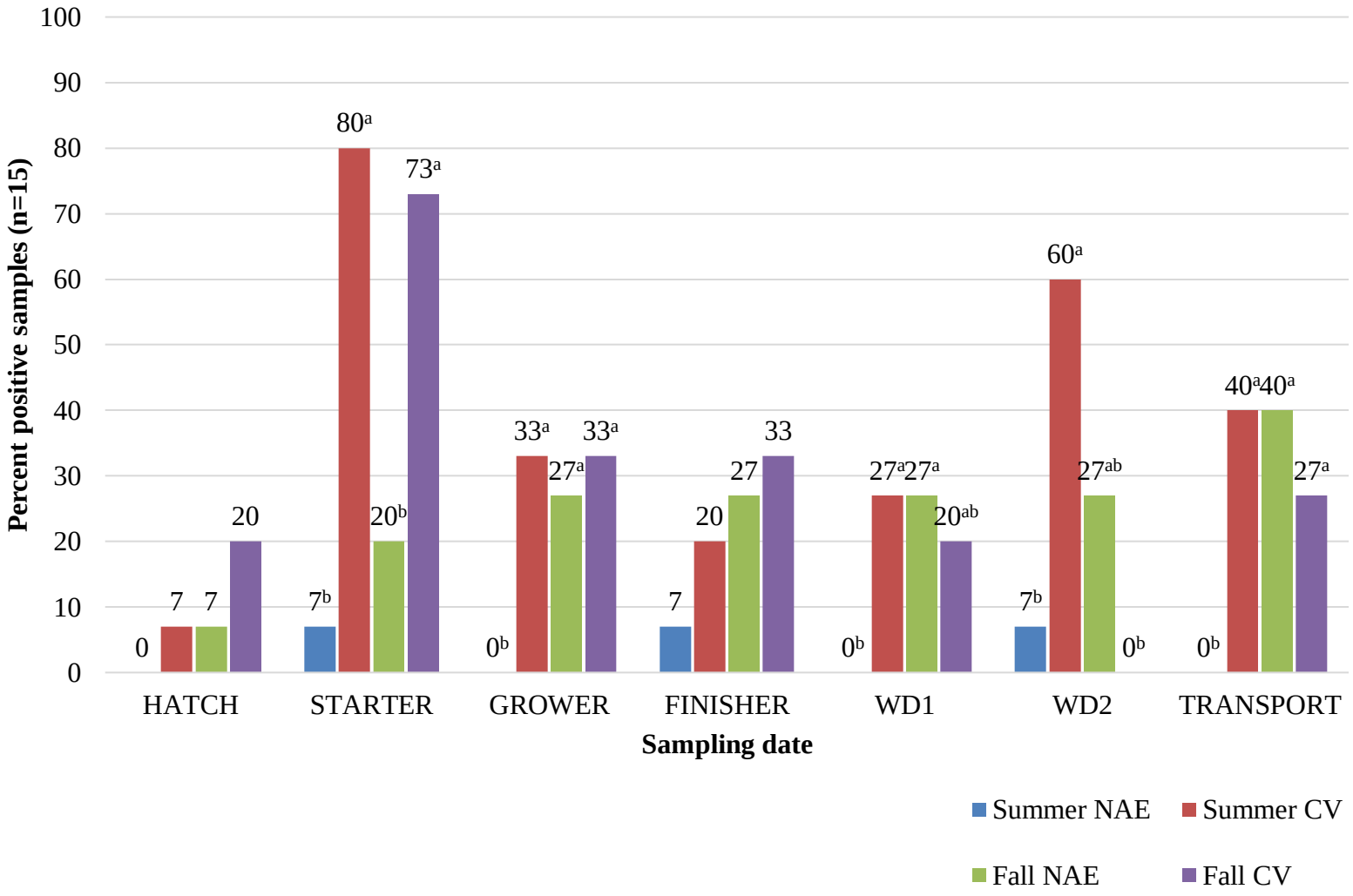

Figure 5.3. Overall prevalence of *Salmonella* in NAE and CV broilers for seasons summer and fall. Superscripts "a" and "b" indicate differences in prevalence between production systems at a given time ($P \leq 0.05$), whereas absence of superscripts indicates no differences ($P > 0.05$)

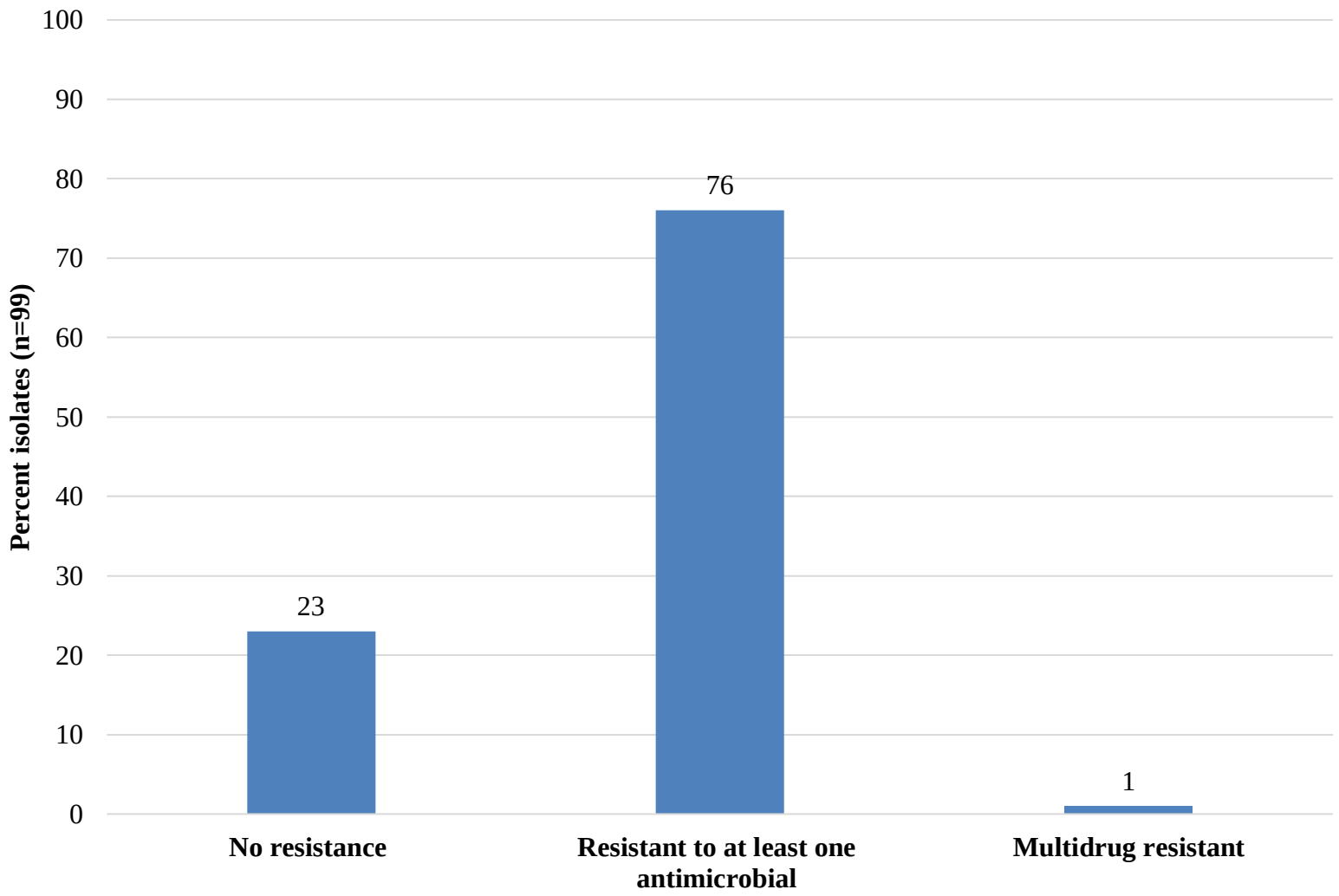

Figure 5.4. Overall prevalence of antimicrobial resistance in *Salmonella* isolates collected from all four houses

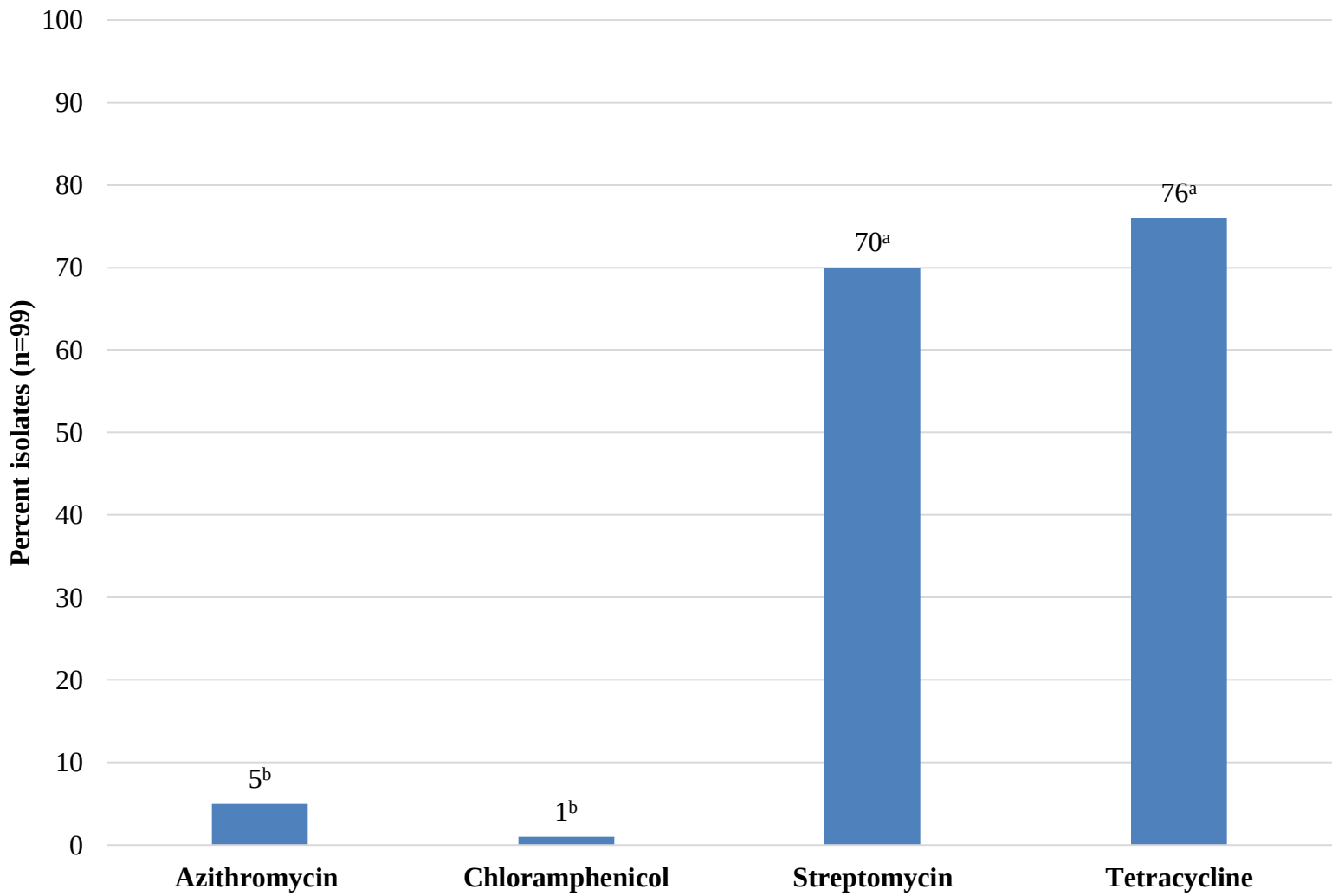

Figure 5.5. Overall prevalence of resistance to individual antimicrobials in *Salmonella* isolates collected from all four farms. Superscripts "a" and "b" indicate differences in prevalence of antimicrobial resistant isolates for a given antimicrobial ($P \leq 0.05$), whereas absence of superscripts indicates no differences ($P > 0.05$)

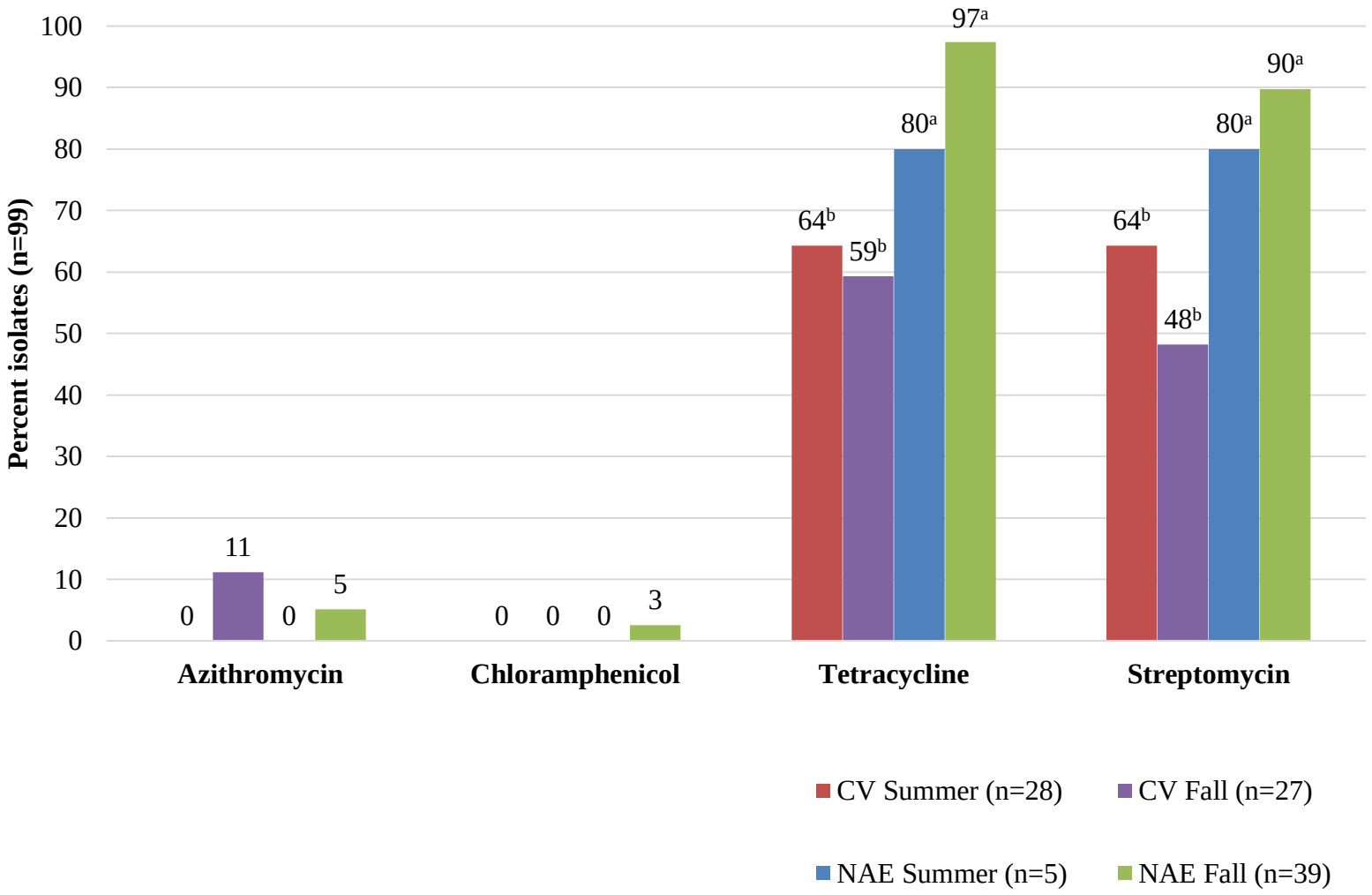

Figure 5.6. Overall prevalence of resistance to individual antimicrobials in *Salmonella* isolates recovered from each farm. Superscripts "a" and "b" indicate differences in prevalence of antimicrobial resistant isolates for a given antimicrobial ($P \leq 0.05$), whereas absence of superscripts indicates no differences ($P > 0.05$)

CHAPTER 6

SUMMARY

The poultry gut microbiome has received increased attention from the scientific community due to its importance to host health, disease incidence, and production efficiency (Shang et al., 2018). Factors affecting gut microbial communities such as age, diet, and intestinal location have been extensively characterized (Yeoman et al., 2012; Oakley et al., 2013; Pan and Yu, 2014), yet, the impact of external determinants such as antibiotic administration, and farm management practices, needs further investigation. Beyond their role as foodborne pathogens, *Salmonella* and *Campylobacter* are important members of the intestinal microbial communities of broiler birds (Newell and Fearnley, 2003; Scallan et al., 2011). Understanding the dynamics of gut microbial populations and the ecological role of foodborne pathogens can help develop better strategies for food safety control at pre-harvest. This study aimed to provide a thorough characterization of the gut microbiota of commercial broilers raised under conventional or no antibiotics ever (NAE) practices, and to further investigate the incidence and antimicrobial resistance of *Salmonella* and *Campylobacter* within these systems.

Results from this study showed a distinct temporal succession of intestinal microbial populations independent of the production system under study. However, this investigation also revealed differences between intestinal communities associated with rearing system. Overall, chickens raised without antibiotic feed supplementation harbored a more complex microbiota, with increased proportions of *Bifidobacterium*,

Ligilactobacillus, and *Alistipes*. These are microbial taxa with potential probiotic activity in broiler birds, thus suggesting that antibiotic administration may deplete the intestinal microbiota from beneficial microbial groups. Furthermore, conventional broilers harbored higher intestinal populations of *Escherichia* and *Clostridium_P*, a genus mainly represented by *Clostridium perfringens*. These are unexpected findings, which suggest that antibiotic administration may not be the panacea for enteric disease in broilers.

Microbiological analysis revealed that *Salmonella* was more prevalent in conventional broilers, whereas the prevalence of *Campylobacter* was higher in chickens raised under NAE practices. This trend was also identified through differential abundance analysis of the cecal microbiota. The presence of *Campylobacter* in the ceca was positively correlated with the presence of bacterial groups producers of short chain fatty acids (SCFAs), which could indicate a symbiotic relationship. On the contrary, a higher abundance of this beneficial microbiota could have exerted an inhibitory effect against *Salmonella* (Oakley et al., 2014). Additionally, the inhibition of Gram-positive bacteria by the antibiotic virginiamycin may have provided a niche for *Salmonella* within the gastrointestinal microbiota. However, this hypothesis requires further investigation.

The incidence of antimicrobial resistance did not follow the same trends between populations of *Salmonella* and *Campylobacter*. Antimicrobial resistant *Campylobacter* was only isolated from conventional broilers subjected to prolonged antibiotic use. However, the opposite trend was observed among *Salmonella* isolates, which exhibited higher rates of resistance in NAE broilers. Because antimicrobial resistance in certain *Salmonella* serotypes may pose a low fitness cost to the bacterium (Zhang et al., 2006), the reported findings could be attributed to an enhanced adaptability and persistence of

resistant *Salmonella* serotypes. Understanding these dynamics requires a thorough genetic characterization of microbial isolates, and the identification of evolutionary relationships among *Salmonella* isolates, which can be investigated in future studies.

Some caveats to this study include the impact of seasonality and broiler house on microbial community diversity. When reporting these findings, it is important to consider the smaller effect size attributed to production system (conventional or NAE). One of two conventional farms participating in this study discontinued antibiotic feed supplementation after day 1 of age. Furthermore, observational analysis during sample collection suggested a lower adherence to biosecurity measures in the aforementioned farm. These factors could be influencing our comparative analysis. Nevertheless, this study revealed interesting dynamics within the gut microbiota of broiler birds associated with commercial production practices, paving the way for future investigations.

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