MICROENCAPSULATED ESSENTIAL OILS AS ANTIBIOTIC ALTERNATIVES IN BROILER CHICKENS

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ABSTRACT

Antimicrobial resistance (AMR) has become a serious problem in poultry farms that can threaten both poultry and human health. The objective of this study was to evaluate effects of encapsulated EO as antimicrobial alternatives including cinnamaldehyde (CIN) and citral (CIT) alone or in combination (CIN+CIT) on growth performance, meat quality, gut health, AMR of broiler chicken fecal *E. coli*, and zoonosis of poultry-source AMR extraintestinal pathogenic *E. coli* (ExPEC). Chapter one gave a general introduction of the study background. Chapter two provided the literature review with detailed information about poultry farms and AMR. Chapter three described the hypothesis and objectives of the study. Chapter four showed effects of bacitracin (BAC), CIN, CIT or CIN+CIT on growth performance, gut lesions, and cecal microbiota of broiler chickens receiving coccidiosis vaccines or not. The feed conversion ratio (FCR), mortality (%), and gut lesion scores were all reduced and cecal microbiota was modulated in birds fed BAC, CIN, CIT, and CIN+CIT compared to birds fed basal diets. Chapter five investigated effects of CIN, CIT, CIN+CIT on AMR phenotypes and genotypes of *E. coli* in broiler chicken feces. The AMR levels (%) of chicken fecal *E. coli* to most tested antimicrobials were lower in birds fed CIN or CIN+CIT which also showed reduced prevalence (%) of some antimicrobial resistance genes (ARGs) and plasmids. Chapter six determined effects of CIN on breast meat quality and gut health parameters of broiler chickens. The CIN improved intestinal nutrient digestibility, morphology, gene expressions for nutrient transporters, and changed cecal and ileal microbiota. In Chapter seven, zoonosis of poultry-source AMR ExPEC was evaluated by measuring the survival (%) of *Caenorhabditis elegans* when exposed to different ExPEC. ExPEC from poultry meat and feces had significant effects on reducing survival (%) of *C. elegans* but relationships between antimicrobial susceptibility or number of virulence genes with pathogenicity of *E. coli* were not conclusive. In conclusion, encapsulated CIN has the potential to improve growth performance, gut health, meat quality, and reduce AMR in chicken fecal *E. coli*. Additionally, ExPEC isolated from poultry meat or feces may possess zoonotic potential to cause human infections.

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This thesis was prepared following a manuscript format and consists of four manuscripts. Part of this thesis has been presented as poster presentations or oral presentations at the ASAS-CSAS Annual Meeting & Trade Show in Vancouver, Canada, on July 8-12, 2018; the PHRN Research Day in Guelph, Canada, on June 2, 2018; the PSA Annual Meeting in Montreal, Canada, on July 15-18, 2019; the Animal Nutrition Conference of Canada in Winnipeg, Canada, on May 26 - June 11, 2020; the Sixth International Animal Intestinal Ecology and Health in China Summit Forum in Zhengzhou, China, on November 6 - 8, 2020. All the manuscripts published or prepared to be published have been listed as follows:

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3. **Yang, C**., M. S. Diarra., J. Choi., A. Rodas-Gonzalez., D. Lepp., S. Liu., P. Lu., M. Mogire., Q. Wang., J. Gong., C. Yang. 2021. Effects of encapsulated cinnamaldehyde on growth performance, intestinal digestive and absorptive functions, meat quality, and gut microbiota in broiler chicken. Transl. Anim. Sci. 5 (3): txab099. doi: 10.1093/tas/txab099.
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CONTRIBUTIONS OF AUTHORS

The thesis was overall prepared by Chongwu Yang and reviewed, revised, and approved by internal and external examiners including Dr. Chengbo Yang (advisor), Dr. Joshua Gong (co-advisor), Dr. Moussa S. Diarra (committee), Dr. Karmin O (committee), Dr. Claudia Narvaez (committee), and Dr. Rajesh Jha (External Examiner).

For manuscripts (chapter four-seven), all the listed authors have read and approved the final manuscripts and their specific contributions were well-described below.

Chapter Four: Manuscript I. The study was designed by Dr. Moussa S. Diarra, Dr. Joshua Gong, Dr. Chengbo Yang, and Chongwu Yang. The animal trial was conducted by Yan Martel Kennes and the essential oils were prepared and encapsulated by Dr. Qi Wang and Dr. Joshua Gong. The samples were analyzed by Chongwu Yang, Dr. Dion Lepp, and Dr. Xianhua Yin. The statistical analysis was conducted by Chongwu Yang and Dr. Hai Yu.

Chapter Five: Manuscript II. The study was designed by Dr. Moussa S. Diarra, Dr. Joshua Gong, Dr. Chengbo Yang, and Chongwu Yang. The essential oils were prepared and encapsulated by Dr. Qi Wang and Dr. Joshua Gong. *E. coli* was isolated, cultured, and identified by Chongwu Yang and their DNA was extracted by Dr. Xianhua Yin and Chongwu Yang. The antimicrobial resistance (AMR) phenotypes were analyzed by Dr. Muhammad Attiq Rehman and Chongwu Yang. The AMR genotypes were analyzed by Dr. Muhammad Attiq Rehman, Dr. Catherine D. Carrilloc, and Chongwu Yang. The data was analyzed by Chongwu Yang and Dr. Moussa S. Diarra.

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LIST OF ABBREVIATIONS

ADG	Average daily gain
AMR	Antimicrobial resistance
APEC	Avian pathogenic *E. coli*
ARGs	Antimicrobial resistance genes
ASTs	Antimicrobial susceptibility
AVI	Avilamycin
BAC	Bacitracin
BCAA	Branched chain amino acids
BW	Body weight
CF	Crude fat
CFC	Chicken farmers of Canada
CFU	Colony forming unit
CIN	Cinnamaldehyde
CIN+CIT	Cinnamaldehyde and citral combination
CIT	Citral
CP	Crude protein
DM	Dry matter
EO	Essential oils
ExPEC	Extraintestinal pathogenic *E. coli*
FCR	Feed conversion ratio
GIT	Gastrointestinal tract
MDR	Multi-drug resistance
MGE	Mobile genetic elements
MIC	Minimum inhibitory concentration
MLST	Multilocus sequencing typing
NE	Necrotic enteritis
NGM	Nematode growth media
NSP	Non starch polysaccharide
OTUs	Operational taxonomic units
PCR	Polymerase chain reaction
QIMME	Quantitative Insights Into Microbial Ecology

qPCR	Quantitative PCR
rRNA	Ribosomal RNA
SCFA	Short chain fatty acids
SEM	Standard error of the means
SPPMP	Soy protein-polysaccharide Malliard Reaction
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infection
VGs	Virulence factor
WGS	Whole genome sequencing

CHAPTER ONE: GENERAL INTRODUCTION

Broiler chicken meat, with its high nutrient value, including high-quality proteins and micronutrients, generated $2.8 billion in Canada in 2019, which contributed to up to 4.3% of the farming operations income. Due to the increasing demand of broiler chicken products, the intensive poultry farming systems have been popular in commercial farms with its advantages in maximizing meat production and lowering feeding costs (Abudabos et al., 2013). Nevertheless, when birds grow quickly at high stock density in these systems, they are sensitive to stressors and diseases that could compromise growth performance, gut health, and meat quality (Bessei, 2006). These stressors such as heat, ammonia, and oxidative stress could cause poor growth performance and oxidative damage in muscle proteins and lipids (Estévez, 2015). As well, high rates of infectious diseases including coccidiosis, necrotic enteritis (NE), and colibacillosis caused by *Eimeria* species, *Clostridium perfringens*, and *Escherichia coli*, respectively, could result in elevated mortality (%) of broiler chicken and jeopardize their welfare (Shojadoost et al., 2012; Chapman et al., 2013). Among these microorganisms, *E. coli* is distributed across chicken of all ages and is a natural inhabitant of the bird's gastrointestinal tract. Even though many *E. coli* strains are non-pathogenic, poultry-source extraintestinal pathogenic *E. coli* (ExPEC) such as avian pathogenic *E. coli* (APEC) and Uropathogenic *E. coli* (UPEC) are capable of inducing colibacillosis with prevalences of around 1% in broiler chicken farms (Matin et al., 2017). Additionally, some poultry-source ExPEC exhibited similar virulence genes with human-source ExPEC, which may suggest zoonotic potentials of poultry-source ExPEC (Mitchell et al., 2015). Since the zoonotic potentials to humans have not been fully elucidated, the objective of the fourth study presented in Chapter Seven aimed to predict zoonosis of ExPEC from poultry retail meat and feces based on survival (%) of *C. elegans* as an animal model.

Feed intervention is one of the most efficient strategies that can be used to reduce infectious incidences and stresses in poultry farms and industries. Antimicrobials such as bacitracin (BAC) and avilamycin (AVI) with high efficacy in killing or stopping growth of microorganisms have been commonly supplemented at subtherapeutic concentrations in chicken feeds to prevent infectious diseases and promote growth for many years. However, the use of antimicrobials could proliferate the growth of resistant strains with antimicrobial resistance genes (ARGs) and resistance plasmids which would induce antimicrobial resistance (AMR). The increasing pressures of limitation on addition of antimicrobials as poultry feed supplementations for growth promotion or disease prevention calls for investigations into antimicrobial alternatives such as essential oils

(EOs), vaccines, organic acids, probiotics, and prebiotics. Among all antimicrobial alternatives, EOs are natural plant extracts with volatile aroma compounds that have no or low toxicity and exert antimicrobial properties against pathogenic microorganisms (Zhai et al., 2018). In addition, biological chemical compounds in EOs such as phenolics, epicatechin, and carvacrol have been proven to have anti-oxidative (Alizadeh et al., 2013), anti-inflammatory (Andrade and De Sousa, 2013), and anti-quorum sensing (Camele et al., 2019) properties in previous *in vitro* studies, respectively. Interestingly, EOs such as thymol oil as feed additives have been proven to promote growth performance (Wade et al., 2018) and reduce NE in broiler chickens challenged by *Clostridium perfringens* (Yin et al., 2017). Among over 90 types of EOs, cinnamaldehyde (CIN) and citral (CIT), which are extracted from bark of cinnamon trees and lemon grass, respectively, have been proven to possess anti-microbial (Si et al., 2009; Hong et al., 2013), anti-oxidative (Sang-Oh et al., 2013; Bouzenna et al., 2017), and anti-inflammatory functions (Muhammad et al., 2015). Despite that the potential of many types of EOs have been demonstrated in previous studies, applications of EOs in chicken farms are limited due to their instabilities during prolonged storage, feed processing, and gastric transition (Yang et al., 2015b). Therefore, studies regarding efficient ways to protect EOs have drawn the attention of scientists. Interestingly, a recent study using soy protein-polysaccharide Maillard reaction product (SPPMP) emulsions to encapsulate CIT have been proven to be stable in stimulated gastric fluids and upon heat treatment, which may indicate SPPMP could retard release of EOs from droplets (Yang et al., 2015b). However, no studies were conducted to assess effects of SPPMP-encapsulated EOs on broiler chicken parameters *in vivo*. Therefore, the objectives of the first, second, third, and fourth studies in Chapter Four, Five, Six, and seven, respectively, were to investigate the effects of encapsulated CIN and CIT alone or in combination (CIN+CIT) on broiler chicken growth performance, gut health, meat quality, AMR phenotypes, genotypes, and virulence of chicken fecal *E. coli*, and zoonosis of AMR ExPEC from poultry meats and feces.

CHAPTER TWO: LITERATURE REVIEW

2.1 Current challenge in poultry industry

Broiler meat is important to human diets as it provides high-quality protein and micronutrients such as vitamin A, riboflavin, and iron. Due to genetic selection and improved management, the body weight of a broiler at 56-day-age has increased by nearly 5 times from 900 g in the 1950s to 4,500 g in the 2010's (Zuidhof et al., 2014). The fast growth rate and high feed efficiency decreased the feeding costs which increased profits for farmers and decreased prices of broiler meat in markets. The low price and high nutrient values allow broiler meat to become one of the most popular and indispensable animal-derived foods. Additionally, due to the growth of the global population, the demand for broiler meat is growing at 5% annually compared to 3.1% and 1.5% for pork and beef, respectively (Alexandratos and Bruinsma, 2012). It has been estimated that the global production of poultry meat is approximately 100 million tons per year (Food and Agriculture Organization of the United Nations, 2020). To satisfy the high demands of broiler meat, the intensive poultry farming system has been applied in recent years. Compared to the traditional free-range system, the intensive system is more feasible since it feeds birds at a high density which allows the production of a high quantity of meat at a low cost. Another reason for the popularity of the intensive system is that this system provides a convenient way for monitoring bird performance and health (Elson, 2015). However, broilers in this system could be susceptible to stressors such as heat, ammonia, moisture, and transportation (Moura et al., 2006). These stressors make birds more vulnerable to undergoing inflammatory and oxidative stresses, resulting in compromised performance and high incidences of diseases (Abudabos et al., 2013). For instance, the prevalence of infections including coccidiosis and necrotic enteritis could be higher in the intensive system, which results in a high mortality rate, particularly in farms with poor hygiene (Hermans and Morgan, 2007; Wondimu et al., 2019). Additionally, compared with the free-range system, fast-growing broilers in intensive farms are more susceptible to myopathies, which could reduce meat quality. For example, Sandercock et al. (2009) indicated that fast-growing broilers in commercial intensive farms may exhibit pre-slaughter stress with increasing incidences of pale-soft-exudative meat (PSE).

It is meaningful to find strategies to improve the growth performance and meat quality of broilers in the intensive poultry farming system. It has been well-known that any disease caused by pathogenic infection is more likely to happen in a warm environment with high nutrient contents and water activity (a_w) (Sandle, 2016). In the intensive poultry farming system, moderate

temperature (≈ 20-32°C), high humidity (≈ 40-70%), and spilled feeds on litter provide an optimal condition for the growth of pathogens. As well, litter contaminated by feces from infected birds are reservoirs of pathogenic microorganisms including *Eimeria* species (Arabkhazaeli et al., 2011), pathogenic *Escherichia coli* (Stromberg et al., 2017), and *Clostridium perfringens* (Craven et al., 2001b), resulting in the spread of infections. A high stock density is also accompanied by high ammonia and moisture, which may proliferate the growth of pathogens and compromise growth performance (Tsiouris et al., 2015). Additionally, a study found that the meat quality of broilers feeding at higher density was inferior to those grown at lower density (Feddes et al., 2002).

Accordingly, modifications in management by reducing stock density, increasing ventilation, and improving litter sanitation could be one of the strategies to control avian diseases and improve performance (Adhikari et al., 2020). For instance, superphosphate or metabisulfide litter amendments could inhibit accumulations of *Eimeria* species, *E. coli*, and *Salmonella Typhimurium* by lowering litter moisture, pH and ammonia release (Soliman et al., 2018). A study also found that a better ventilation system could improve feed efficiency and industrial production index (IPI) (Samadpour et al., 2018). Additionally, the application of vaccines such as commercial live attenuated coccidiosis vaccine could be an efficient method to control the growth of *Eimeria* species (Lillehoj and Lillehoj, 2000). Another strategy to prevent avian infections and promote growth is feed intervention, which is being widely applied in intensive farms to prevent avian diseases, promote performance and meat quality (Adhikari et al., 2020). Feed additives including enzymes, amino acids, and antimicrobials have been proven to efficiently control avian diseases and promote growth performance. It has been demonstrated that non-starch polysaccharide (NSP) degrading enzymes such as xylanases as in-feed additives could increase feed efficiency (Selle et al., 2009), enhance nutrient digestibility (Cozannet et al., 2017), and reduce incidences of avian diseases including coccidiosis and necrotic enteritis (Broom, 2017). Besides, dietary supplementations of amino acids including arginine, glutamine, and branched-chain amino acids (BCAA) could lower productions of pro-inflammatory cytokines (Vermeulen et al., 2011), facilitate intestinal amino acid absorptions (Ebadiasl, 2011), and enhance muscle growth rate (Wu, 2009). Among all these additives, antimicrobials have been widely used in poultry farms due to low prices and high efficacy. For example, supplementations of antimicrobials at subtherapeutic concentration such as bacitracin have the potential to improve growth performance and increase villus length of small intestines of broilers (Miles et al., 2006). Due to the prevalence of antimicrobial resistance (AMR), the application of antimicrobials has been prohibited by many

countries. However, elimination of antimicrobials as growth promoters may increase incidences of diseases and compromise the performance of poultry in the intensive systems, suggesting the importance of exploring antimicrobial alternatives. Therefore, studies on evaluating the effects of in-feed antimicrobial alternatives including vaccines, probiotics, prebiotics, organic acids, and essential oils on broilers are necessary for increasing bird performance and reducing the prevalence of AMR (Griggs and Jacob, 2005). For instance, the mixtures of essential oils and organic acids have been found to improve feed efficiency, intestinal morphology, and meat quality of broilers, as efficient as antimicrobials (Isabel and Santos, 2009; Liu et al., 2017). However, there are still some challenges for the application of antimicrobial alternatives such as high cost, no fully known mechanisms, and instability (Yang et al., 2015a).

2.2 Chicken gut health and microbiota

Gut health, as a complicated concept involving nutrient digestion and absorption, intestinal morphology, immunology, and microbiota, is crucial for chickens, as it affects their performance and health in poultry farms. Impaired gut health could compromise feed efficiency, induce high incidences of gut infections, and cause economic losses. It has been demonstrated that nutrient metabolism in the gut could account for 20-36% of whole-body energy expenditure (Cant et al., 1996). Interestingly, the structure and functionality of the gut barrier and microbiota are vital factors that influence chicken performance. Interestingly, gut microbiota could affect nutrient digestibility, gut integrity, and pathogen exclusion (Shang et al., 2018). In modern intensive poultry farming systems, chicken gut microbiota could be altered by host and environmental factors such as species, types of feeds, and temperature (Kers et al., 2018).

The gut microbiota is a community with high diversity and richness in chicken gastrointestinal tracts which is dominated by bacteria (Wei et al., 2013). It has been demonstrated that chicken ceca harbor the largest quantities of bacteria with 10^{10}-10^{11} colony-forming unit per gram (CFU/g) of digesta following by small intestines, crop, and gizzard, with 10^{8}-10^{9}, 10^{8}-10^{9}, 10^{7}-10^{8} CFU/g, respectively (Shang et al., 2018). A balanced gut microbiota community could benefit the host by preventing the colonization of pathogenic microbes, producing antimicrobials such as bacteriocin, and modulating the development of innate and adaptive immunity, producing essential nutrients such as short-chain fatty acids (SCFA), controlling gut epithelial cells turnover, and regulating gut-brain axis (Diaz Carrasco et al., 2019). Furthermore, gut microbiota could be affected by bird age and species, GIT segments, feed intervention, and environmental factors such as temperature, humidity, and management (Diaz Carrasco et al., 2019). However, an imbalanced

gut microbiota could cause deleterious effects on the host including increased permeability of gut barrier, reduced nutrient digestibility and absorption, simulated inflammatory responses, and bacterial translocation (Kogut, 2019). The imbalanced gut microbiota community is referred as 'dysbiosis' which could be induced by poor sanitation, high crude protein diets, and pathogenic gut infections translocation (Kogut, 2019). In addition to the importance of balanced microbiota, monitoring the initial colonization of gut microbiota in young chicks plays key roles in bird's health and performance. This is because young chicks could be easily affected by pathogens from the environment since their gastrointestinal tracts and immune systems have not been fully developed yet (Ballou et al., 2016). To observe the characteristics of gut microbiota, a single species could be isolated or cultured in nutrient media. Although culture-dependent techniques could facilitate studying the physiological characteristics of a specific bacterium, the majority of microbial species remains unculturable (Rappé and Giovannoni, 2003). With the development of molecular techniques, the composition of gut microbiota could be analyzed by culture-independent methods such as quantitative PCR (qPCR) or the 16S ribosomal RNA (rRNA) sequencing, which help scientists to understand the diversity and richness of gut microbiota (Gong and Yang, 2012). Interestingly, with developments of 'omics' technologies including metagenomics, transcriptomics, proteomics, and metabolomics, the functions of gut microbiota could be revealed according to their genetic information (Deusch et al., 2015). The microbiota, combined with its genetic information (genes, proteins, metabolites), is referred to as a new term, 'microbiome' (Berg et al., 2020). The gut microbiota/microbiome could be modulated by management and feed intervention. Interestingly, a growing number of studies have found that the supplementation of in-feed antimicrobials may cause dysbiosis due to the influences on microbiota or microbiome (Zhang and Chen, 2019). Hence, investigation on the effects of antimicrobial alternatives on modulating chicken gut microbiota or microbiome has become important in the area of poultry science.

2.3 Pathogens and poultry infections

Pathogens are special groups of microorganisms that have the ability to induce poultry infections. The dysbiosis of microbiota in gastrointestinal, respiratory, and urinary tracts of poultry could cause the overgrowth of pathogens, compromising growth performance and health (Mor-Mur and Yuste, 2010). Pathogens including pathogenic *Escherichia coli*, *Clostridium perfringens*, and *Eimeria* species, are responsible for inducing colibacillosis, necrotic enteritis, and coccidiosis, respectively. Additionally, pathogens in poultry could be released from feces which may infect

healthy individuals and contaminate the environment, inducing the spread of diseases (Guran and Oksuztepe, 2013; Stromberg et al., 2017). As well, zoonotic potentials of avian pathogens such as avian pathogenic *Escherichia coli* could threaten human health, accounting for the economic loss of up to billions of dollars (Food and Agriculture Organization of the United Nations, 2020). Understanding the characteristics of major pathogens in poultry could be helpful for disease prevention and diagnoses.

2.3.1 *Clostridium perfringens*

Clostridium perfringens is a Gram-positive anaerobic bacterium that can be classified into five types (A, B, C, D, E) based on toxins (α, β, ε, ι) produced (McDonel, 1980). It has been found that *Clostridium perfringens* type A and C strains are related to poultry diseases by producing α- and ß- toxins, sloughing gut epithelial cells by hydrolyzing lecithin in the cell membrane and inducing hemorrhagic necrosis of intestinal mucosa (Fernandez Miyakawa and Redondo, 2016). Necrotic enteritis is one of the most common enteric diseases caused by *C. perfringens* in the intensive poultry farming system with clinical symptoms of severe depression, poor growth performance, diarrhea, and ruffled feathers, lasting for 5-10 days and resulting in a high mortality rate (2-50%) (Timbermont et al., 2011). It was first discovered by Parish in 1961, and it has become a widespread disease in poultry farms with an estimated over $6 billion global economic loss annually (Parish, 1961; Wade and Keyburn, 2015). Previous studies have demonstrated that a β-pore-forming toxin (PFTs), known as necrotic enteritis B-like toxin (NetB), which was discovered in avian *C. perfringens* type A strains are the main toxin for causing necrotic enteritis (Keyburn et al., 2008; Keyburn et al., 2010). However, recent studies have demonstrated that the type G of *C. perfringens* is responsible for inducing necrotic enteritis and its adhesive pilus is related to pathogenesis (Zhou et al., 2021). Interestingly, Zhou et al. (2021) also indicated that a two-component system (PilR/PilS) can regulate the production and binding of pilus in *C. perfringens* which may provide an extra target for developing antimicrobial alternatives. There are a lot of predisposing factors that have been described to induce necrotic enteritis in poultry farms, reflecting the complexity of its controlling and prevention (Moore, 2016). For example, feeding birds with high dietary crude protein was considered as one of the main factors for increasing incidences of necrotic enteritis (Shojadoost et al., 2012). Additionally, it has been also demonstrated that diets with high levels of indigestible NSP could increase the number of *C. perfringens* via elevating digesta viscosity (Kocher, 2003). In addition to dietary factors, healthy birds are prone to infection by necrotic enteritis through a contaminated environment in poultry

farms. It has been indicated that *C. perfringens* could be found everywhere in intensive poultry farms such as eggshell, broiler carcass, litter, fly strips, walls, fans, work boots, and contaminated feeds and water (Craven et al., 2001b). Feeding birds with antimicrobials such as bacitracin at subtherapeutic concentrations is one effective way of preventing and controlling necrotic enteritis. However, supplementation of antimicrobials in animals has been regarded as the major factor resulting in the prevalence of antimicrobial resistance and the increasing proportion of 'superbugs' with the ability to render antimicrobials ineffective. For example, *bcrABDR* as bacitracin resistance genes have been detected in *C. perfringens* from poultry farms (Charlebois et al., 2012). Therefore, it is necessary to find antimicrobial alternatives with similar efficacy as antimicrobials to prevent and control necrotic enteritis.

2.3.2 *Eimeria* species

Eimeria is a genus of intracellular parasites that is associated with the prevalence of coccidiosis in poultry farms which impairs growth performance and leads to high mortality. Although nine *Eimeria* species have been identified in chicken, only five of them including *E. necartix*, *E. maxima*, *E. acervulina*, *E. tenella*, and *E. brunetti* were reported to induce avian coccidiosis. *Eimeria* species are site-specific in the intestinal tract of broilers: *E. acervuline* and *E. tenella* are located in the duodenum and cecum, respectively; *E. brunetti* is found in the terminal ileum, cecum, and rectum; while *E. necatrix* and *E. maxima* are detected only in the middle part of the intestines (Joyner et al., 1974). Coccidiosis may occur when birds swallowed un-sporulated oocyste of *Eimeria* from a contaminated environment such as feeds, drinking water, and litter. Once entering the intestinal tract, gut environmental conditions including heat, moisture, bile salts, and digestive enzymes, facilitate the sporulation of oocytes which could further release sporozoites that invade epithelial cells to induce coccidiosis (López-Osorio et al., 2020). Coccidiosis, with symptoms such as diarrhea, fever, and emaciation, was estimated to cause a global economic loss of up to $3 billion annually (Dalloul et al., 2006). Additionally, it has been illustrated that intestinal damage by *Eimeria* species could result in the accumulation of plasma proteins in the intestinal lumen, providing a growth substrate for the proliferation of *Clostridium perfringens*, which may induce necrotic enteritis (Al-Sheikhly and Al-Saieg, 1980). Therefore, it is of great significance to eliminate oocysts of *Eimeria* in poultry farms to prevent coccidiosis and necrotic enteritis. Although antimicrobials such as ionophores could effectively control coccidiosis, finding alternatives is very important due to the prevalence of antimicrobial resistance.

2.3.3 Pathogenic *Escherichia coli*

Escherichia coli is a commensal bacterium that is ubiquitously distributed in the gastrointestinal tract of poultry among all ages. Since *E. coli* belongs to coliform with the ability to ferment lactose and produce ß-D-galactosidase, it is easy to isolate *E. coli* by selective media such as MacConkey agar (pink colonies) and Chromogenic Coliform agar (dark blue colonies). With the development of technologies, the isolated *E. coli* could be further identified by many tools such as Analytical Profile Index 20 E Test (API 20 e), quantitative polymerase chain reaction (qPCR), or 'Omics' technologies (Lupindu, 2017; Gong et al., 2018). According to pathogenic profiles (virulence factors (VG), clinical symptoms, and phylogenetic trees), pathogenic *E. coli* can be classified into two groups: intestinal pathogenic *E. coli* (IPEC) and extraintestinal pathogenic *E. coli* (ExPEC). Many serotypes of IPEC strains such as O9:H4 are beneficial to their hosts by facilitating nutrient digestion, producing micronutrients such as vitamin B12 (Lawrence and Roth, 1996), and suppressing the growth of pathogenic gut bacteria (Blount, 2015), but serotypes such as O157:H7 are pathogenic which could cause severe enteric infections in poultry, causing huge economic losses for poultry farms (Clements et al., 2012). Additionally, multilocus sequencing typing (MLST) such as ST1, 11, 25, 270, 280, 582, 731 and 1283 are mainly represented in IPEC. However, unlike IPEC, serotypes of ExPEC are not always related to the pathogenicity and only a few MLST including ST567, 1219, 1386, and 2012 only belong to ExPEC (Köhler and Dobrindt, 2011). Interestingly, ExPEC mainly belongs to phylogroup B2 while IPEC mainly exists in phylogroup A and B1 (Boyd and Hartl, 1998). Therefore, increased knowledge of phylogenetic structures, virulence genotypes, and developments of molecular typing are required to better define ExPEC.

IPEC includes 6 major pathotypes: enteroaggregative *E. coli* (EAEC), enterohamerrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), diffusely adherent *E. coli* (DAEC), and shiga toxin-producing *E. coli* (STEC). Mechanisms for causing diseases are different among pathotypes (Samanta and Bandyopadhyay, 2019): ETEC possess abilities to secrete heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST), causing diarrhea via activating cyclic AMP (cAMP), and cyclic GMP (cGMP); EIEC and EPEC could trigger actin polymerization in gut epithelial cells mediated by attaching and effacing (A/E) protein, which causes retraction of microvilli and diarrhea; EAEC and STEC produce shiga toxin (STx) that induce gut lesions by impairing protein synthesis of host cells via preventing binding of aminoacyl-tRNA with 28S ribosomal RNA; DAEC has abilities to diffuse and adhere

to epithelial cells, resulting in watery diarrhea. In contrast, ExPEC is a group of pathogenic *E. coli* that causes extraintestinal diseases. It includes pathotypes such as Uropathogenic *E. coli* (UPEC), Sepsis-associated *E. coli* (SEPEC), and Meningitis-associated *E. coli* (MNEC), which could induce urinary tract infections (UTIs), sepsis, and meningitis (Smith et al., 2007). It has been well-known that the ExPEC could cause diseases through virulence factors that are responsible for adhering, capturing iron from the host environment, and promoting inflammation.

The infections caused by pathogenic *E. coli* could be controlled by improving sanitation in poultry barns and feed interventions. The application of sanitizer, good ventilation, and collecting eggs frequently are efficient methods to reduce *E. coli* contamination (Nolan et al., 2020). The supplementations of antimicrobials such as aminoglycosides (gentamicin, linomycin, streptomycin), fluoroquinolones (enrofloxacin), and ß-lactam drugs (amoxicillin, ceftiofur) are efficient methods to control intestinal infections and colibacillosis due to pathogenic *E. coli* (Samanta and Bandyopadhyay, 2019). Due to the emergence of antimicrobial resistance, drugs such as enfloxacin and linomycin have been banned in many countries and some recent studies found that the supplementations of antimicrobial alternatives such as probiotics and essential oils can effectively control the growth of pathogenic *E. coli*. It has been reported that *Lactobacillus johnsonii* can significantly reduced the adherences and invasions of *E. coli* in small intestines (Zou et al., 2020) and a mixture of *L. planetarium* and *Pediococcus pentosaceu*s can eliminate the *E.* coli within 24 hours (Nolan et al., 2020). Additionally, oregano oil has been reported to prevent the growth of extended-spectrum ß-lactamase (ESBL) - producing *E. coli* (Si et al., 2008).

2.3.4 Avian extraintestinal pathogenic *Escherichia coli*

Avian extraintestinal pathogenic *Escherichia coli* (APEC) is a pathogenic *E. coli* in poultry that belongs to extraintestinal pathogenic *E. coli* (ExPEC) (Antao et al., 2008). In recent years, APEC with abilities to infect birds has been considered as one of the major threats in poultry farms. It has been demonstrated that APEC could be detected in fecal samples and post-slaughtering meats of poultry, possessing capacities to induce colibacillosis with symptoms including respiratory distress, reduced appetite, poor growth performance, elevated mortality, and post-mortem lesions (Nolan, 2013; Nolan et al., 2020). Interestingly, the ExPEC could be classified as APEC based on the presence of functional virulence gene (VG) categories such as *iss*, *tsh*, *fim*, *iroN*, and *kpsMII*, which are molecules that enable APEC to survive, colonize, and invade the host cells (Bonnet et al., 2009). Virulence factors (VF) are encoded by VG and can be classified into four major types including toxins (e.g., hemolysin), adherence factors (e.g., fimbriae), iron intake

proteins (e.g., ferric enterobactin), and secretion systems (e.g., T3SS), which are responsible for causing infections, facilitating adhesion and invading host cells (Di Martino, 2018), intaking iron for survival and growth (Frawley and Fang, 2014), and secreting proteins for promoting bacterial virulence (Green and Mecsas, 2016). With the development of molecular technologies, an increasing number of VG were detected in APEC such as *pap, afa-8, ibeA, sitABC,* and *iss*, encoding fimbriae, adhesins, invasin, iron acquisition systems, and outer membrane proteins (LeStrange et al., 2017). For example, VG with different frequencies including *iutA* (80%), *fimH* (33.3%), *hlyF* (24.4%), vat (17.8%), *sitD* (13.3%), *sitA* (11.1%), *frz* (8.9%), *uvrY* (4.4%), *kpsM* (2.2%), *ompT* (2.2%), *pstB* (2.2%), and *sopB* (2.0%) were detected in APEC by PCR multiplex polymerase chain reaction (PCR) assay (Mbanga and Nyararai, 2015). Interestingly, since APEC is close to human ExPEC in phylogenetical trees, the zoonotic potentials of APEC have been recognized as a great threat to food safety and human health (Mitchell et al., 2015). However, only a few *in vivo* studies were conducted to predict zoonotic potentials of APEC. Therefore, it is of great significance to use tools to predict the zoonotic potentials of APEC.

2.4 Zoonosis of poultry-source extraintestinal pathogenic *Escherichia coli*

Poutry-source extraintestinal pathogenic *Escherichia coli* (ExPEC) has been well-recognized as the primary pathogen that impairs poultry health (Antao et al., 2008). Apart from high mortality and economic losses in poultry farms, poultry-source ExPEC may threaten human health but its zoonotic potentials are still controversial (Manges and Johnson, 2012). For example, poultry meat contaminated by poultry-source ExPEC may infect humans since many poultry-source ExPEC shared similar virulent genes (VG) with human ExPEC (Jakobsen et al., 2011). It has also been demonstrated that human ExPEC are closely related to some poultry-source ExPEC in phylogenetic trees (Johnson et al., 2007; Starčič Erjavec et al., 2017; Logue et al., 2017). For example, a study found that over half of tested poultry-source ExPEC and human ExPEC strains belonged to the same subcluster (B2-1) and all expressed the K1 antigen without significant differences among the frequency of other virulence factors (Moulin-Schouleur et al., 2007). Furthermore, zoonotic risks of poultry-source ExPEC may be also related to some VG located in mobile genetic elements (MGE) which could be transmitted to human ExPEC (Rodriguez-Siek et al., 2005). In contrast, one study discovered that although poultry-source ExPEC had a large number of overlapping virulent genes and MGE with human uropathogenic *E. coli* (UPEC), other traits that are associated with pathogenicity such as serotypes and percentage of some VG were significantly different (Rodriguez-Siek et al., 2005). The zoonotic risks of poultry-source ExPEC

are not fully elucidated since only a few *in vivo* studies were available to better predict their abilities to infect humans.

It is worthwhile to conduct *in vivo* experiments to predict the zoonosis of APEC strains. There is no question that humans are the best experimental subject for *in vivo* studies. However, due to economic and ethical limitations on conducting infectious studies in humans, it is necessary to select other animal models for the experiments (Apfeld and Alper, 2018). The animal models are selected by genetic, anatomical, and physiological similarities with humans (Conn, 2017). It has been well-known that mice are the most frequently used animal models for predicting human diseases since over 85% of their genomes are similar to humans and they share similar dominant intestinal microbial phyla (Nguyen et al., 2015). However, conducting experiments only on mice poses some challenges: firstly, due to limited finances and time, only immature mice are used for predicting human diseases, which may cause inconsistencies in results; secondly, infectious experiments on mice sometimes are inhumane, which may break requirements of animal welfare (Perlman, 2016). To overcome the drawbacks of using mice to studying human diseases, other animal models such as *Canorhabditis elegans* (*C. elegans*) were selected. *C. elegans* is a tiny (< 1 mm for adults) nematode with a short reproductive cycle (3-5 days) (Hope et al., 1999). It can be easily cultured on nematode growth media (NGM) agar seeded with *Escherichia coli* OP50 in an aerobic incubator at 16^{o}C and observed easily by a dissecting stereomicroscope. Despite it being evolutionarily distant from humans, genomes of *C. elegans* have been completely identified in 1998 and are approximately 60-80% similar to human genomes (Shaye and Greenwald, 2011). Interestingly, over 40% of genomes of *C. elegans* were related to human diseases (Culetto and Sattelle, 2000). *C. elegans* also swallow bacteria as their feed and their death rates are associated with the pathogenicity of bacteria. Based on the advantages listed above, *C. elegans* life-span assay has been applied for identifying zoonotic potentials of pathogens isolated from farm animals such as *Enterococcus faecalis* (Garsin et al., 2001) and *Salmonella typhimurium* (Labrousse et al., 2000). The *C. elegans* life-span assay is one of the most frequently used approaches to predict zoonosis of pathogens by observing the death rate of worms after being incubated with pathogenic bacteria for 10-14 days. During life-span assay, death rates of *C. elegans* are positively related to severities of bacterial pathogenicity. In this assay, *E. coli* OP50 as the feed of *C. elegans*, has been frequently used as negative control (Brenner, 1974). Besides, porcine ETEC K88^{+} JG280 (O149: K88) has been selected as positive control, containing several VGs including *elt, estA, estB,* and *astA* and multiple ARGs that confer resistance to ampicillin, apramycin, gentamicin, neomycin,

spectinomycin, ceftiofur, tetracycline and trimethoprim/sulfonamide (Noamani et al., 2003). Studies have reported that ETEC K88^{+} JG280 could induce rapid deaths of *C. elegans* after three-day incubation and could cause 80% of worm death rate after eight days (Wang et al., 2011; Zhou et al., 2014; Zhou et al., 2018). Despite the above advantages, there are still some drawbacks for *C. elegans* as an animal model: firstly, missing organs including internal organs, blood vessels, and immune systems, make it hard to do anatomical studies; secondly, with their small body size, it is difficult to do biochemical analysis (Tissenbaum, 2015).

2.5 In-feed antimicrobials and challenges

The high stock density and environmental conditions in intensive poultry farming systems facilitates the growth and spread of pathogens among chickens, resulting in the spread of infections such as coccidiosis, necrotic enteritis, and colibacillosis (Hermans and Morgan, 2007; Wondimu et al., 2019). Additionally, the zoonotic potentials of some pathogenic bacteria from farm animals triggered the urgency for controlling the growth of pathogens in poultry farms (Manges and Johnson, 2012). The supplementations of in-feed antimicrobials have been proven to be an efficient strategy to prevent poultry infections and promote growth performance (McEwen and Fedorka-Cray, 2002). Antimicrobials are natural, semisynthetic, or synthetic substances that inhibit the growth of microorganisms with little or no damage to the host. After the first antimicrobial 'penicillin' discovered in 1928, antimicrobial consumption for animal husbandry has been increasing annually and will reach over 200,000 tons by 2030 (Van Boeckel et al., 2017). In Canada, antimicrobials used in farm animals consist of tetracycline (44%), pencillins/ß-lactams (19%), macrolide (11%), trimethoprism (7%), and lincosamides (5%) (Canadian Antimicrobial Resistance Surveillance System Report, 2016). Generally, the purposes of antimicrobial applications for poultry farms include: 1) treating poultry diseases; 2) reducing the spread of infections; 3) preventing the occurrence of diseases; 4) promoting growth performance (Samanta and Bandyopadhyay, 2019). The first three purposes are accomplished by antimicrobial properties including: 1) inhibiting nucleic acids synthesis (e.g., fluoroquinolones, rifampicin); 2) interfering with protein synthesis (e.g., aminoglycosides, tetracycline, macrolide, phenicol); 3) disrupting cell membrane (e.g., colistin, polymyxin B); 4) inhibition of cell wall synthesis (e.g., cephalosporin, carbapenem, penicillin, bacitracin, vancomycin). For example, bacitracin is an antimicrobial with a mixture of polypeptides that functions to inhibit phosphorylation of lipid carriers which transfers peptidoglycan subunits to the outer membrane for cell wall formation. Interestingly, previous studies indicated that in-feed antimicrobials such as bacitracin at subtherapeutic concentrations

could increase feed efficiency and enhance meat quality (Abdulrahim et al., 1999; Knarreborg et al., 2002; Singh et al., 2008). It is thought that enhancement of growth and meat quality is accomplished by improving gut health such as elevating villus/crypt ratio, increasing intestinal digestibility, enhancing tight junction gene expressions, and modulating gut microbiota (Hughes and Heritage, 2004). However, there is a lack of scientific explanation on revealing how in-feed antimicrobials actually work.

Despite antimicrobials having high efficacy to prevent disease and promote growth, prolonged applications of antimicrobials at subinhibitory concentrations for poultry have been regarded as the most important driver for the prevalence of antimicrobial resistance (AMR). The AMR is defined as the situation where microbes are able to survive when exposed to antimicrobials that can previously kill them effectively. This is because subinhibitory concentration of antimicrobials can increase mutations in genomes, accelerate genome horizontal transfers, and regulate quorum sensing and biofilm formations, resulting in altering antimicrobial susceptibilities (Andersson and Hughes, 2014). The AMR is achieved by mutation in the genome or acquisition of genetic determinants, which increase the capacity to survive when exposed to antimicrobials. It was first discovered in the 1940's in *Staphylococcus* strains, showing abilities to produce penicillinase to degrade penicillin (Kirby, 1944). Because of the rise of AMR over many years later, governments and organizations from different regions banned or are restricting antimicrobials at subinhibitory usage for growth promoters. In Europe, the application of antimicrobials for growth promotion was eliminated in 2006. In Canada, Chicken Farmers of Canada (CFC) have decided to stop the use of Category I, II, and III antibiotics as growth promoters by the end of 2014, 2018 and 2020, respectively (Chicken Farmers of Canada, 2017). The prevalence of AMR would cause treatment failure and economic losses of up to billions of dollars annually (Food and Agriculture Organization of the United Nations, 2020). Microorganisms develop strategies to reduce or eliminate the efficacy of drugs by 1) modifying or enzymatic degradation of antimicrobials; 2) reducing entry of antimicrobials by altering transport channels; 3) pumping out antimicrobials by efflux systems; 4) altering binding targets to reduce the affinity of antimicrobials (Reygaert, 2018). Interestingly, antimicrobial resistance is not a modern phenomenon since antimicrobial resistant genes (ARGs) were discovered in some ancient microbial DNA samples from over hundred-thousands years ago (D'Costa et al., 2011). However, the over- and mis- use of antimicrobials throughout these past years, positively selects resistant microorganisms to survive and speeds up the transmission of ARGs to other microbial species

(D'Costa et al., 2011; Llor and Bjerrum, 2014). It has been indicated that ARGs could be transmitted generation-to-generation (vertical transmission) and species-to-species (horizontal transmission). Nowadays, resistance to antimicrobials such as ß-lactams drugs, fluoroquinolone, tetracycline, macrolide, and colistin have been detected in poultry farms (Agyare et al., 2018). Additionally, antimicrobial residues in poultry products have become a serious problem, which may positively select the growth of resistant microorganisms in humans (Ramatla et al., 2017). Due to the prevalence and spread of antimicrobial resistance, it seems like humans are slipping back to the pre-antimicrobial era. Therefore, it is important to prevent AMR and explore antimicrobial alternatives.

2.6 Phenotype and genotype of antimicrobial resistance

An understanding of the relationship between antimicrobial resistance (AMR) phenotype and genotype of microorganisms would be necessary for better prevention and control of antimicrobial resistance. The AMR phenotype is observable growth characteristic of bacteria in the presence of an antimicrobial agent which could be measured by the determination of the minimum inhibitory concentration (MIC), describing the lowest concentration of an antimicrobial in which no visible growth is observed (Corona and Martinez, 2013). Several antimicrobial susceptibility test methods have been widely applied with low cost and high accuracy (Qi et al., 2006). Disk diffusion and microdilution are two commonly used methods for identifying susceptibilities of a microorganism to antimicrobials by detecting the sizes of inhibition zones and checking for bacterial growth under varying concentrations of antimicrobials, respectively. For example, a study using a disk diffusion method found that all 73 avian pathogenic *Escherichia coli* (APEC) displayed high resistances against penicillin (100%), followed by cefepime (96%), sulfamethoxazole (90%), neomycin (89%), deoxycycline (70%), nalidixic acid (49%) and chloramphenicol (22%) (Younis et al., 2017). With advances in technology, an automated system called 'Sensititre™ Complete Automated AST System', which offers more rapid and flexible detection of broad-spectrum antimicrobials in 96-well microtitre plates, has become popular in phenotypic testing (Puttaswamy et al., 2018). For example, a recent study using this automated system demonstrated that among 234 *E. coli* isolates in a poultry farm water system, approximately 70% were susceptible to all tested antimicrobials, 24% were resistant to one or two antimicrobials, and 5% were resistant to three or more antimicrobials (Taggar et al., 2018). Despite phenotypic tests that could directly describe phenomena of antimicrobial resistance, analysis of genotypic characteristics are still necessary to reveal mechanisms of the prevalence and spread of AMR.

The AMR genotype refers to genetic information that could explain the prevalence and spread of AMR, such as profiles of antimicrobial resistance genes (ARGs) and mobile genetic elements (MGEs). ARGs encode proteins that cause AMR, while MGEs in the cytoplasm contain ARGs which are responsible for ARGs transmission (Stokes and Gillings, 2011). With the development of molecular technologies such as whole genome sequencing (WGS), scientists revealed that there are three types of AMR in microorganisms: intrinsic resistance, acquired resistance, and adaptive resistance (Schroeder et al., 2017). The intrinsic resistance is commonly due to natural impermeability and efflux pump. In contrast, adaptive and acquired resistance indicate microorganisms obtained ARGs through mutation and acquisition, respectively. The MGEs including plasmids, integrons, transposons (Tns), genomic islands (GIs), and integrative and conjugative elements (ICEs), are responsible for acquired resistance by transmitting ARGs horizontally from resistant microorganisms to sensitive microorganisms (Manyi-Loh et al., 2018). Interestingly, it has been demonstrated that the presence of environmental ARGs is insufficient for entering and spreading among microorganisms because of high fitness cost owing to the expenditure of energy to maintain the burden of new genes (Bengtsson-Palme et al., 2018). Nevertheless, the fitness cost could be reduced when the gene is secured in MGEs or under selective pressure. The prolonged exposure to subinhibitory concentration of antimicrobials has been recognized as one of the major selective pressures which facilitate entry of ARGs and their securing in MGEs (Bengtsson-Palme et al., 2018). In addition to selective pressures, horizontal transfer of ARGs could be successful in microorganisms with phylogenetic similarities (Smillie et al., 2011). Hence, due to phylogenetic similarities among bacterial strains in poultry and human, ARGs in antimicrobial resistant bacteria from poultry can be transferred to humans (Bengtsson-Palme and Larsson, 2015). Additionally, MGEs in microorganisms may contain more than one type of resistance genes which could lead to co-resistance. For example, Tn21 contains six ARGs including *Intl1*, *Attl1*, *aadA*, *oxa*, *sul1*, and *qacΔE*, which are responsible for resistance to sulfonamide, aminoglycosides, ß-lactam drugs, sulphonamide, and quaternary ammonium (Nikaido, 2009). As well, cross-resistance could be detected in some microorganisms, resulting in resistance to different antimicrobials belonging to the same class due to similar resistant mechanisms. For example, some *Escherichia coli* from the urinary tract detected with resistance to tetracycline are also insensitive to fluoroquinolone due to the shared efflux pumps (Hwang and Hooper, 2014). In addition to acquired resistance, prolonged usage of antimicrobials at subinhibitory concentrations can prompt adaptive resistance by elevating mutation rates in

microorganisms (Andersson and Hughes, 2012). Therefore, trying to explore antimicrobial alternatives to reduce or eliminate the application of subtherapeutic usage of antimicrobials is important in farms to decrease incidences of acquired and adaptive resistances.

2.7 Antimicrobial alternatives

Antimicrobial resistance could bring about huge economic loss annually and threaten human health and many countries have banned or are banning the usage of antimicrobials. However, the elimination of antimicrobials as feed additives could compromise poultry performance, resulting in unintended impacts on poultry health and production. To overcome adverse effects caused by antimicrobial eliminations, antimicrobial alternatives have been proposed in poultry farms including vaccines (e.g., coccidiosis vaccine), essential oils (e.g., cinnamaldehyde), organic acids (e.g., propionic acid), probiotics (e.g., *Bifidobacterium logum*), prebiotics (e.g., fructooligosaccharide), and synbiotics (e.g., *Lactobacillus salivarius* with galactooligosaccarides) (Cheng et al., 2014). For example, coccidiosis vaccines and essential oils have the potential in poultry to prevent and control coccidiosis and necrotic enteritis and to improve growth performance and meat quality (Yang et al., 2015a).

2.7.1 Coccidiosis vaccine

Due to the prevalence of antimicrobial resistance (AMR), the applications of anticoccidial drugs such as sulphonamides and ionophores in poultry feeds have been limited. Alternation of anticoccidial drugs with vaccines has been proven to be a possible way to control coccidiosis and avoid AMR (Chapman and Jeffers, 2014). Vaccines are live attenuated or killed vaccines with abilities to stimulate immune responses to produce antibodies in the host. Coccidiosis vaccine belongs to live attenuated vaccines for preventing and controlling coccidiosis, which has been used for over half a century in poultry farms. The live *Eimeria* species in the coccidiosis vaccine are responsible for inducing a robust protective immune response to produce antibodies against *Eimeria* species (Rose, 1963). However, it has been demonstrated that birds must receive vaccines containing different *Eimeria* species to get full protection due to minor protection against heterologous species (Joyner, 1969). Therefore, an efficient coccidiosis vaccine contains a blend of at least four live *Eimeria* species including *E. acervulina*, *E. mivati*, *E. maxima*, and *E. tenella*. Coccidiosis vaccine could be applied in poultry farms via spraying on feed, drinking water, litter, or surface of day-old hatched chicks (Jenkins et al., 2013). However, there are still some challenges for applying coccidiosis vaccine in poultry farms including: 1) factors such as environmental conditions, the timing of challenge doses, and chicken breed could alter the efficacy of the vaccine

(Soutter et al., 2020); 2) antibodies produced by immune responses are only targeted on *Eimeria* species in the vaccine; 3) infections induced by live *Eimeria* species could cause gut lesions that may compromise growth performance of poultry (Willians, 2003). In accordance with the above drawbacks, the coccidiosis vaccine has been proposed in combination with other in-feed antimicrobial alternatives such as essential oils to promote bird growth performance and gut health (Yang et al., 2015a).

2.7.2 Essential oils

Essential oils (EO) are mixtures of aromatic and volatile compounds which are extracted mostly from plants (Preedy, 2015). Interestingly, only three or fewer of these compounds in the mixture could account for up to over 70% of the total compounds which contribute to major properties of EO. These compounds such as aldehyde, phenols, and terpenes provide EO with antimicrobial, antioxidant, or anti-inflammatory properties. For instance, citral (>81%), an unsaturated aldehyde that is a major component of lemongrass oil, has anti-inflammatory potential by reducing pro-inflammatory cytokines via inhibiting nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) (Andrade and De Sousa, 2013). Compounds in thyme oil including terpenoid (>12%), *p*-cymene (>23%) and thymol (>40%) have antioxidant capacities through reducing free oxygen radicals (Grassmann et al., 2005; Baschieri et al., 2017). As well, it has been demonstrated that oregano oil possesses antimicrobial abilities since its major constituent called carvacrol (>85%) could enter the cytoplasm which can kill bacteria by collapsing bacterial membrane potential and depleting the intracellular ATP pool (Ultee et al., 1999). Additionally, some research also revealed other beneficial properties of EO such as disruption in quorum sensing (QS) and improvement in palatability (Kroismayr et al., 2006). For example, a study has found that geranium, lavender, and rosemary oils could disrupt QS by inhibiting acyl-homoserine lactones (AHL) produced by bacteria such as pathogenic *Escherichia coli* (Szabó et al., 2010). Inhibition of QS could reduce the production of virulence factor since QS is a microbe-microbe interaction system that regulates the expressions of virulent genes based on molecules produced by microorganisms such as AHL in response to cell density (Nazzaro et al., 2019).

Another interesting phenomenon is that EO may have synergistic effects when combined with other chemical compounds. This synergistic effect refers to the fact that two or more substances in combination could produce an effect greater than the sum of their individual effects. The synergistic effects of EO and antimicrobials may be because EO can disrupt the cell wall which can facilitate the penetration of drugs. For example, Talei et al. (2017) indicated that *Carum*

copticum EO combined with vancomycin could reduce minimum inhibitory concentration (MIC) of *Staphylococcus aureus* from 0.5 to 0.12 μg/mL in response to vancomycin. As well, previous studies have found that blends of EO may possess higher efficiency in controlling the proliferation of bacteria such as *Clostridium perfringens* compared with single EO applications (Mitsch et al., 2004). Besides, the synergistic effects of thymol and eugenol have been revealed which may be explained that thymol can disrupt the cell membrane which can help eugenol to enter the cytoplasm to bind to protein (Bassolé and Juliani, 2012). However, limited *in vivo* research was conducted on revealing the synergistic effects of two or more EOs as feed supplementations on poultry. Based on the properties of EOs, it is assumed that two or more EOs in combination may possess more beneficial effects on poultry compared with their individual effects. For example, cinnamaldehyde may have synergistic effects with citral when fed to animals because: 1) both cinnamaldehyde and citral have antimicrobial, anti-inflammatory, and antioxidant capacities via different mechanisms which may elevate the efficacy (Amalaradjou et al., 2014; Shi et al., 2017); 2) cinnamaldehyde combined with citral has been shown to have a stronger antimicrobial ability against pathogens such as *Penicillium expansum* compared to individual effects or when combined with other EOs such as perillaldehyde (Loeffler et al., 2014; Wang et al., 2018). However, it is still necessary to conduct *in vivo* experiments in poultry to investigate the synergistic effects of cinnamaldehyde and citral as feed supplementations.

2.7.3 Other antimicrobial alternatives

In addition to EOs and vaccines, there are still many other popular alternatives such as probiotics, prebiotics, and organic acids. Probiotics are living beneficial bacteria while prebiotics are types of undigested carbohydrates that may favor the growth of good bacteria. For example, a combination of *Lactobacillus* strains could effectively reduce expressions of cytokines that help alleviate Salmonellosis in chicken (Hu et al., 2015). Prebiotics such as xylo-oligosaccharide and fructo-oligosaccharide could alleviate colonic inflammation and favor the growth of intestinal beneficial genera including *Lactobacillus* and *Bifidobacterium*, respectively (Xu et al., 2003; Fei et al., 2020). Organic acids are carboxylic acids including formate, acetate, propionate, butyrate, and lactate, which may disrupt the cell membrane and deplete the ATP of bacteria. Interestingly, a synergistic effect of organic acids and EO on poultry has been demonstrated by some previous studies. For example, an *in vivo* study demonstrated that the combination of a blend of EO (clove and cinnamon) and a mixture of organic acids (propionate and formate) had better efficacy in

improving feed efficiency and breast weights of broilers compared to individual supplementation (Isabel and Santos, 2009).

2.7.4 Challenges of in-feed essential oils

An essential oil (EO) is expected to meet the following requirements before its application: 1) similar efficiency as antimicrobial in preventing disease and promoting growth; 2) not toxic to humans and animals; 3) low price; 4) environmental-friendly. Despite that most of the above requirements have been tested and verified by scientists, there are still some challenges regarding in-feed EO. Firstly, the mechanisms surrounding how EO promote growth are not fully understood. For example, some EO have been proven to have antimicrobial, antioxidant, and anti-inflammatory properties *in vitro*, but there is a lack of evidence showing mechanisms about how they improve growth performance, gut health, and meat quality *in vivo*. Secondly, most of EO are not stable during prolonged storage and their compounds could be damaged by acidic gastric fluids before entering the small intestines, thus requiring efficient protection before adding them in feeds. Thirdly, the microorganisms that EO target are not fully elucidated. Furthermore, the results of antimicrobial alternatives in animal trials in previous studies are inconsistent, which may be because the results are affected by various conditions such as animal breed, age, feed formula, management, and duration of storage.

2.8 Cinnamaldehyde and citral

Cinnamaldehyde and citral are essential oils (EOs) that have antimicrobial, anti-inflammatory, and antioxidant properties (Figure 2). Cinnamaldehyde is an aldehyde with pale yellow color occurring naturally in aromatic trees and shrubs that belong to the genus *Cinnamomum* (Tisserand and Balacs, 1995). Although pure cinnamaldehyde (>95%) was used mainly as a flavoring additive in a variety of human foods including chewing gums, breakfast cereals, and snacks, growing attention has arisen from scientists based on its antimicrobial ability (Nabavi et al., 2015). Studies *in vitro* have reported that cinnamaldehyde inhibits the growth of Gram-positive pathogenic bacteria such as *Listeria monocytogenes* and Gram-negative pathogenic bacteria such as *Salmonella Typhimurium* (Pina-Pérez et al., 2012; Hong et al., 2013). The antimicrobial activities of cinnamaldehyde could be accomplished by: 1) damaging the integrity of bacterial membrane after penetrating to membrane bilayer (Zhang et al., 2015a); 2) decreasing mitochondrial ATP synthesis and depleting intracellular ATP pools (Nowotarska et al., 2017); 3) interfering with the cellular biological function of nitrogen-containing biomacromolecules such as nucleic acids and proteins (Fadli et al., 2012). Cinnamaldehyde also inhibits host inflammation by

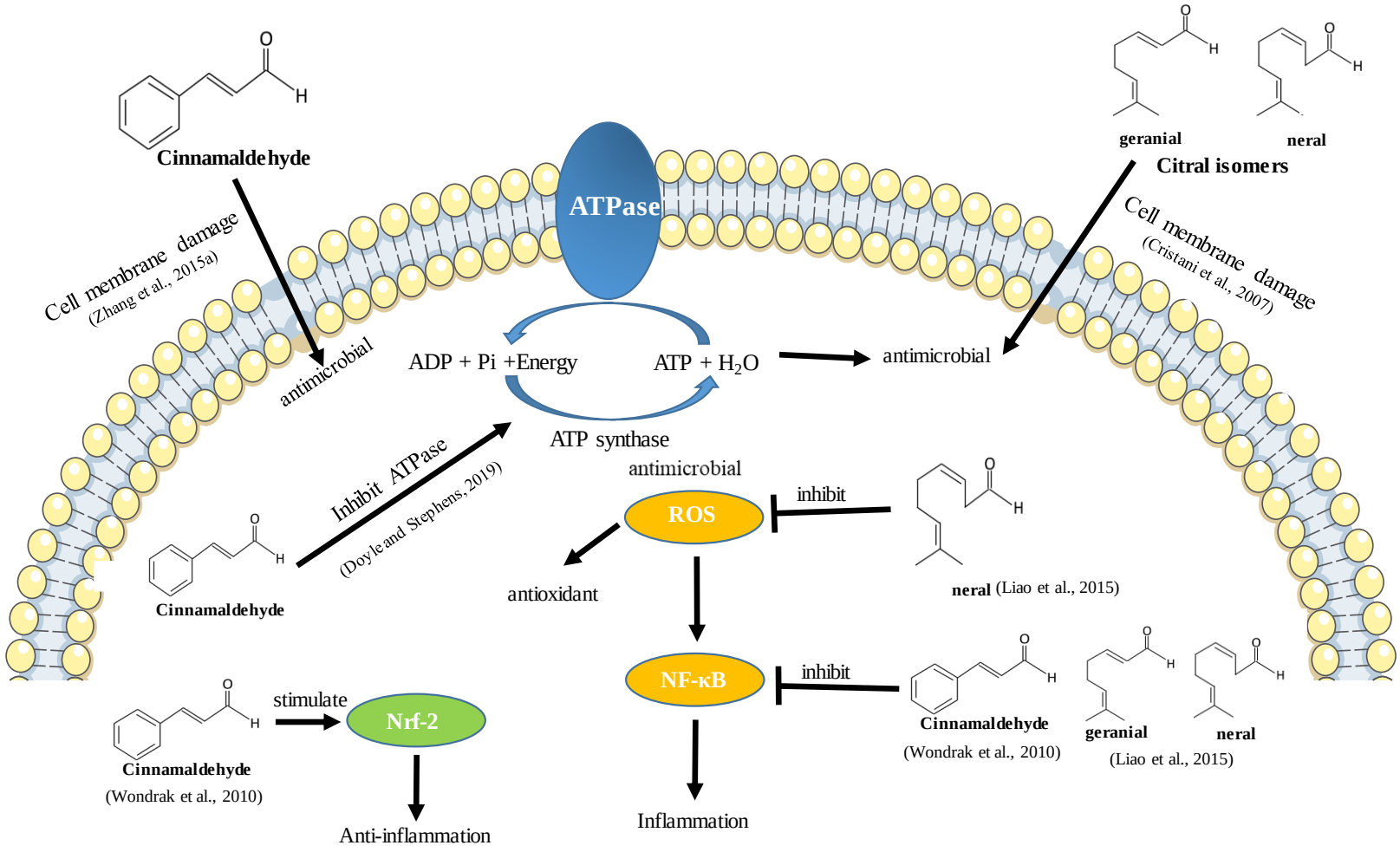
inhibiting nuclear factor-κB (NF-κB) and stimulating nuclear factor erythroid 2–related factor 2 (Nrf-2) (Wondrak et al., 2010). Additionally, cinnamaldehyde could reduce oxidative stress in chicken meat by reducing thiobarbituric acid reactive substances (TBARS) as a byproduct of lipid peroxidation (Sang-Oh et al, 2013).

Citral (3,7-dimethyl-2,6-octadienal) is a mixture of *cis* and *trans* isomers of terpenoids extracted from plants such as lemongrass. The strong antimicrobial potentials of plant extracts that contain high proportions of citral such as citral oils (>95%), lemongrass oils (75-85%), and lemon myrtle oils (>90%) have been discovered in previous studies (Si et al., 2009; Adukwu et al., 2016). As a derivative of terpenes, citral could inhibit the growth of microorganisms by: 1) disrupting lipid section in cytoplasmic membrane; 2) interfering with mitochondrial enzymes for ATP synthesis (Cristani et al., 2007; Nikbakht et al., 2014). Furthermore, an *in vitro* study demonstrated that citral at alkalic pH had higher efficacy in controlling the growth of *Candida albicans*, *Staphylococcus aureus, E. coli* compared to those at acidic pH (Onawunmi, 1989). Since animal intestines is a weakly acidic environment with a pH around 6-7 which may stabilize citral, it was hypothesized that citral as feed additives may possess a strong antimicrobial ability to regulate gut microbiota. In addition to antimicrobial activity, citral may prevent oxidative stress by reducing the formation of oxygen and nitrogen reactive species and alleviate inflammation via inhibiting nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways (Andrade and De Sousa, 2013; Bouzenna et al., 2017).

Despite lots of beneficial potentials, cinnamaldehyde and citral have not been widely applied in poultry farms to prevent disease and promote growth. This is because cinnamaldehyde has high volatility and poor water solubility, which makes its application difficult (Ashakirin et al., 2017). Additionally, cinnamaldehyde is unstable during heat processing, prolonged storage, and gastric transition, even after being stabilized by oil-in-water emulsions, resulting in flocculation and oil sedimentation (Tian et al., 2016; Chen et al., 2017; Yuliani et al., 2018). The limitations of citral are that it easily undergoes cyclization and oxidation to produce *p*-cresol and *p*-methylacetophenone that could generate off-odor during prolonged storage and feed processing (Baines et al., 1970; Ikenberry and Saleeb, 1993; Iwanami et al., 1997; Schieberle and Grosch, 1988). Studies have reported that the strong unpleasant off-odor could reduce palatability that may decrease feed intake of farm animals including swine and broilers (Amad et al., 2011; Omonijo et al., 2018). Similar to cinnamaldehyde, citral is easily degraded under conditions during the gastric transition, resulting in insufficient amounts of active compounds reaching the small intestines

(Djordjevic et al., 2007). Interestingly, it has been indicated that the rate of citral degradation in an acidic environment could be reduced by it being incorporated into a material such as medium-chain triacylglycerols and triacetin (Choi et al., 2009). The process in maintaining the stabilities of EO is called ‘encapsulation’. Hence, finding an efficient way to stabilize EO before their in-feed applications is of great significance.

Figure 2. 1 The mechanisms of antimicrobial, anti-inflammatory, and antioxidant properties of cinnamaldehyde and citral.



2.9 Encapsulation of essential oils

Due to the instabilities of essential oils (EO), the protection of EO by encapsulation is important to extend its shelf life and activities. Based on this purpose, many encapsulation techniques such as emulsification, coacervation, spray drying, complexation, ionic gelation, nanoprecipitation, and film hydration have been investigated in recent years (Maes et al., 2019). Based on these techniques, types of micro (1 - 1000 μm) or nano (1 - 1000 nm) encapsulation including particles (a matrix with dispersed EO), capsules (a core with surrounding membrane), complexes (EO interacted with chemicals), and droplets (fine bubbles in the solvent) have been designed to protect EO (Bouguéon et al., 2019; Maes et al., 2019). However, different EO encapsulation types exhibit different potentials in protecting EO under various conditions. For example, due to the presences of digestive enzymes, irons and mucin in chicken gastric fluid, a large proportion of citral (CIT) encapsulated in droplets by emulsification technique could be degraded before reaching the small intestines (Kim et al., 2019). Hence, it is necessary to conduct studies to analyze the stabilities of EO with different encapsulation types or technologies. For example, quantities of encapsulated EO released in different conditions could be detected by gas chromatography (GC) coupled with mass spectrometry (MS). Interestingly, a study by Yang et al. (2015b) showed that CIT encapsulated by soy protein-polysaccharide Maillard reaction product (SPPMP) could be more stable during a prolonged storage and heating processing compared to soy protein-polysaccharide mixture (SPP) or soy protein alone (SP). Additionally, encapsulated CIT by SPPMP had a slow-releasing rate during the gastric transition, indicating the potentials in protecting CIT from gastric fluids.

2.10 Summary

The intensive poultry farming system characterized by high stock density is popular to satisfy the increasing demands of chicken meat. However, the growth performance, health, and post-slaughtered meat quality of broiler chickens could be compromised when fed at high density. The imbalanced gut microbiota caused by the outgrowth of pathogenic microorganisms such as *Eimeria* species, *C. perfringens*, and pathogenic *E. coli* is one of the main factors compromising chicken performance and health. Additionally, the zoonotic potentials of avian pathogenic *E. coli* may threaten human health through contaminated chicken products and the environment. To prevent bird diseases and promote growth performance, in-feed antimicrobials such as bacitracin and avilamycin at subtherapeutic concentration have been widely applied in poultry farms. However, the prevalence and spread of antimicrobial resistance have become a serious problem

that reduces the efficacies of drugs. Analyses on antimicrobial resistance phenotypes and genotypes of commensal microorganisms such as *Escherichia coli* are important to understand antimicrobial resistance mechanisms and transmissions. Despite the elimination of antimicrobials that could alleviate antimicrobial resistance, it could also compromise growth performance and cause economic loss. Hence, exploring in-feed antimicrobial alternatives such as coccidiosis vaccines and essential oils is necessary. Cinnamaldehyde and citral, which are two essential oils with antimicrobial, antioxidant, and anti-inflammatory properties, are not widely applied in poultry farms due to limited *in vivo* studies and their instabilities during the gastric transition and prolonged storage. Therefore, investigating the effects of cinnamaldehyde and citral with encapsulation on growth performance, gut health, and meat quality is very necessary.

CHAPTER THREE: HYPOTHESES AND OBJECTIVES

Research gap:

Based on the background knowledge described previously (Chapter One and Two), citral (CIT) encapsulated with soy protein-polysaccharide Maillard products (SPPMP) can be used to control necrotic enteritis in broiler chicken. Additionally, another study also encouraged the potential use of encapsulated cinnamaldehyde (CIN) for control of animal enteric pathogens by oral in-feed administration. However, further studies are still needed to evaluate the efficacy of the encapsulated CIN and CIT as antimicrobial alternatives and elucidate the underlying molecular mechanisms in broiler chickens.

The studies tested the following hypotheses:

Encapsulated CIN and CIT can replace in-feed antimicrobials by controlling necrotic enteritis, improving intestinal digestive and absorptive function, modulating gut microbiota, and improving meat quality in broiler chicken. In addition, encapsulated CIN and CIT can also reduce the prevalence of antimicrobial resistance (AMR) and virulence of chicken fecal *E. coli*, which may reduce the risks of extraintestinal pathogenic *E. coli* (ExPEC) infections in humans.

The specific objectives were:

1. To investigate the changes of growth performance, gut lesions due to coccidiosis and necrotic enteritis, and cecal microbiota in vaccinated or non-vaccinated broiler chickens fed bacitracin (BAC), SPPMP-encapsulated CIN, CIT, or CIN+CIT;

2. To estimate alternation of phenotypes and genotypes of fecal *E. coli* isolated from broiler chickens fed BAC, SPPMP-encapsulated CIN, CIT, or CIN+CIT;

3. To evaluate effects of CIN at low (EOL) or high (EOH) concentrations on meat quality, ileal microbiota, intestinal morphology, gene expressions, and nutrient digestibility; and

4. To predict the zoonotic potentials of ExPEC from poultry retail meat or feces and to find out the relationship between AMR profiles and/or numbers of virulence genes (VGs) with pathogenicity to *C. elegans* by a life span assay.

CHAPTER FOUR: MANUSCRIPT I

Effects of Encapsulated Cinnamaldehyde and Citral on the Performance and Cecal Microbiota of Broilers Vaccinated or not Vaccinated Against Coccidiosis[1]

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4.1 Abstract

Due to the emergency of antimicrobial resistance, it is urgent to evaluate the potentials of other products such as essential oils as antimicrobial alternatives. Based on the objectives described previously (Chapter Three), this study firstly examined the effects of encapsulated cinnamaldehyde (CIN) and citral (CIT) alone or in combination (CIN+CIT) on the growth performance, gut lesions, and cecal microbiota of nonvaccinated broilers and broilers vaccinated against coccidiosis. Vaccinated (1,600) and nonvaccinated (1,600) 0-day-old male Cobb500 broilers were randomly allocated to five treatments: basal diet (control) and basal diet supplemented with bacitracin (BAC, 55 ppm), CIN (100 ppm), CIT (100 ppm), and CIN (100 ppm) + CIT (100 ppm). In general, body weight (BW) and feed conversion ratio were significantly improved in birds treated with BAC, CIN, CIT, and CIN+CIT ($P < 0.05$) but were all decreased in vaccinated birds compared with nonvaccinated birds ($P < 0.05$). Significant interactions ($P < 0.05$) between vaccination and treatments for average daily gain during the periods of starter (day 0-9) and BW on day 10 were noted. Broilers receiving vaccines ($P < 0.01$) or feed supplemented with BAC, CIN, CIT, or CIN+CIT ($P < 0.01$) showed reductions in mortality rate from day 0 to 28. The incidences of minor coccidiosis were higher ($P < 0.05$) in vaccinated birds than in nonvaccinated birds. Diet supplementation with BAC or tested encapsulated essential oils showed comparable effects on the coccidiosis incidences. Similar to BAC, CIN and its combination with CIT reduced both incidence and severity of necrotic enteritis ($P < 0.05$). No treatment effects were observed on the cecal microbiota at the phyla level. At the genus level, significant differences between vaccination and treatment groups were observed for five (*Lactobacillus*, *Ruminococcus*, *Faecalibacterium*, *Enterococcus*, and *Clostridium*) of 40 detected genera ($P < 0.05$). The genus *Lactobacillus* was more abundant in broilers fed with CIT, while *Clostridium* and *Enterococcus* were less abundant in broilers fed with CIN, CIT, or CIN+CIT in both the vaccinated and nonvaccinated groups. Results from this study suggested that CIN alone or in combination with CIT in feed could improve chicken growth performance to the level comparable with BAC and could alter cecal microbiota composition.

Key words: broiler chickens, cecal microbiota, coccidiosis, essential oil, growth performance

4.2 Introduction

Intestinal diseases such as coccidiosis and necrotic enteritis (NE) are responsible for immense economic losses (several billion USD annually) to the poultry industry worldwide

(Dalloul and Lillehoj, 2006). The common *Eimeria* species involved in coccidiosis include *Eimeria tenella*, *Eimeria maxima*, and *Eimeria acervulina*. These species can multiply and damage the intestine epithelial layer of the chicken duodenum, mid-intestine, and ceca, reducing feed intake and nutrient digestibility (Long and Jeffers, 1986; Martin et al., 1997; Dahiya et al., 2006). The NE caused by *Clostridium perfringens* is an enteric disease characterized by severe necrosis of intestinal mucosa, which impairs broiler productivity (McDevitt et al., 2006). Necrotic enteritis B-like toxin produced by *C. perfringens* is considered the major toxin responsible for NE (Keyburn et al., 2008), although many other toxins such as Beta 2, Tpel, and virulent factors also play a role (McDonel, 1980; Keyburn et al., 2008; Lepp et al., 2013; Prescott et al., 2016). Traditionally, coccidiosis and NE have been effectively controlled by the application of antimicrobials in feed (Reid, 1990). However, due to the misuse and overuse of antimicrobials, resistance to antibiotics has become a public health issue, leading to new challenges to urgently develop effective alternatives to antibiotics to control enteric diseases in broilers (Casewell et al., 2003; Agyare et al., 2018). Recently, the Chicken Farmers of Canada revised its antimicrobial use to eliminate the preventive use of category II antibiotics in 2018 and that of category III antibiotics by the end of 2020 (Chicken Farmers of Canada, 2019).

To overcome the potential increase in mortality and morbidity of broilers due to the ban of in-feed antimicrobial use, probiotics, prebiotics, organic acid, essential oils (EOs), and vaccines are becoming studied as alternatives to antibiotics (Griggs and Jacob, 2005; Diarra et al., 2007; Osman and Elhariri, 2013). In the 1970's, coccidiosis vaccines containing mixtures of living *Eimeria* species were introduced to control coccidiosis by stimulating intestinal immune T-cells of broilers (Lillehoj and Lillehoj, 2000; Wallach, 2010). The EOs are aromatic compounds derived from plants, and many are potent inhibitors of bacterial growth (Si et al., 2006; Bakkali et al., 2008). Natural EOs are extracted from parts of trees such as the leaves, roots, and bark. Synthetic EOs may contain toxins although the purity could reach more than 95%. Although no studies have been conducted to compare the effects of natural and synthetic EOs on the performance of poultry, cinnamaldehyde (CIN) used in the present study was synthetic while citral (CIT) was a natural EO.

CIT and CIN are two EOs that have been used in medication over the last century (Burt, 2004). CIN compounds displaying a pale-yellow color are extracted from the bark of cinnamon trees or from other species of the genus Cinnamomum (Tisserand and Balacs, 1995). CIN has antimicrobial effects but has additionally been used for food flavoring in sweets and chewing gum (Nabavi et al., 2015). It has been demonstrated that cinnamon powder in feed could improve meat

quality and growth quality of broiler chickens (Sang-Oh et al., 2013) and alleviate intestinal injury (Wang et al., 2015). CIT (3,7-dimethyl-2-6-octadienal) is extracted from different plants including lemongrass (around 76% by gas chromatography) (Silva et al., 2008), lemon myrtle (90%) (Tisserand and Balacs, 1995), and Lindera citriodora (about 65%) (Ohtsuru et al., 1967). The antimicrobial activities of CIT have been documented as effective against several bacterial pathogens including *C. perfringens* (Onawunmi, 1989; Si et al., 2006, 2009; Yang et al., 2016). Previous studies have demonstrated that protection is required for effective delivery of EOs to the animal gut (Zhang et al., 2014, 2015b; Ma et al., 2016; Yang et al., 2015a; Omonijo et al., 2017). The use of CIT and CIN in feed as antibiotic alternatives could be limited because of physical and chemical instabilities during storage and in the gastrointestinal tract of poultry (Kimura et al., 1981; Tian et al., 2016). A recent study indicated that the incorporation of a soy protein-polysaccharide Maillard reaction product stabilized CIT and offered protection to CIT during the storage, upon low pH in the stimulated gastrointestinal tract fluid and heat treatment (Yang et al., 2015b). The protection could be due to the incorporation of soy protein-polysaccharide Maillard reaction product (SPPMP) that may have shield peptide bonds against proteolysis and thus retard the release of CIT from the droplets (Yang et al., 2016).

In this study, according to the objectives described previously (Chapter Three), the effectiveness of encapsulated CIT and CIN alone or in combination in feed on growth performance, gut health, and cecal microbiota was firstly evaluated in broilers that were either vaccinated or not against coccidiosis.

4.3 Materials and methods

4.3.1 Essential oils

CIT (a mixture of cis and trans isomers, 95% purity) and CIN (≥ 95% purity) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). The CIN and CIT were encapsulated separately using the materials and methods described previously with minor modifications (Yang et al., 2015b; Ma et al., 2016)

4.3.2 Experimental design

A total of 3,200 0-day-old male Cobb 500 broiler chickens were housed in 40 floor pens (80 birds per pen). The pens were assigned to two groups; half of the birds (1,600) received a commercial coccidiosis vaccine via spraying at 0-day of age and the other half (1,600) did not receive the vaccine. Pens in each of these two groups were randomly allocated to 5 dietary treatments (4 pens per treatment) in a completely randomized design. The dietary treatments were:

1) basal diet (enriched with animal by-products) serving as the control; 2) basal diet with 55 mg/kg BAC (positive control); 3) basal diet with 100 mg/kg encapsulated CIN; 4) basal diet with 100 mg/kg encapsulated CIT; and 5) basal diet with a combination of 100 mg/kg encapsulated CIN and 100 mg/kg CIT (CIN+CIT). Broilers were fed a starter diet from the age of 0 to 9 D, grower diet from age 10 to 19 D, and a finisher diet from age 20 to 28 D. The EOs were fed from day 10 to 28 (grower, finisher), and BAC was fed from day 0 to 28 (starter, grower, and finisher). The starter, grower, and finisher diets (Table 4.1) were formulated and pelleted with wheat and corn as the principal cereals and soybean meal, fish meal, and meat meal as protein sources according to the nutritional recommendation by Cobb 500 (Cobb-Vantress Inc., 2012). No coccidiostats were provided in the diets to prevent coccidiosis.

Table 4. 1 Feed ingredients (a) and nutrient composition (b) for starter (days 0-9), grower (days 10-19), and finisher (days 20-28) of chicken (%, as feed basis otherwise indicated).

Ingredients	Inclusion in basal diet		
	Starter	**Grower**	**Finisher**
Wheat	30.00	35.00	35.00
Soybean Meal	27.79	21.89	16.76
Vegetable Oil	3.74	3.54	4.41
Corn	23.39	24.81	29.15
Corn Gluten Meal	5.00	5.00	5.00
Limestone	0.96	0.96	0.92
Biofos[1]	0.31	0.11	0.00
Mineral and Vitamin Mix[2]	0.25	0.25	0.25
L-Lysine HCl	0.12	0.13	0.25
Sodium Chloride	0.10	0.10	0.10
DL-Methionine	0.23	0.21	0.19
Avizyme 1502[3]	0.05	0.05	0.05
Sodium Bicarbonate	0.44	0.34	0.31
Phytase[4]	0.01	0.01	0.01
Choline	0.12	0.10	0.10
Fish meal	4.50	4.50	4.50
Meal meal	3.00	3.00	3.00
Calculated Nutrients			
Crude Protein	25.30	23.30	21.30
Methionine	0.62	0.58	0.53
Methionine & Cysteine	1.08	1.01	0.94
Lysine	1.37	1.23	1.19
Metabolisable Energy, kcal/kg	3022	3063	3152
Crude Fat	6.19	6.06	7.00
Crude Fiber	2.31	2.24	2.11
Calcium	0.90	0.85	0.80
Total Phosphorus	0.66	0.60	0.55
Available Phosphorus	0.40	0.35	0.32
Sodium	0.20	0.17	0.16

[1]Feed-grade monocalcium phosphate.

[2]Supplied per kilogram of diet: vitamin A, 9000 IU; cholecalciferol, 5000 IU; vitamin E, 30 IU; vitamin K, 0.5 mg; cobalamin, 0.007 mg; thiamine, 0.4 mg; riboflavin,6 mg; folicacid,1 mg;

biotin,0.15 mg; niacin,135 mg; pyridoxine, 4 mg; Fe, 125 mg; Mn, 60 mg; Cu, 5 mg; Se, 0.10 mg; I, 0.35 mg; Zn, 50 mg.

[3]Multi-Enzyme System for Wheat-Based Poultry Feed (Halchemix Canada Inc., Toronto, ON, Canada) containing 600 U/g of xylanase, 8000 U/g of protease and 800 U/g of amylase.

[4]Ronozyme P5000 (DSM Nutritional Products Canada Inc., Ayr, ON, Canada)

4.3.3 Animals and management

All experimental procedures performed in this study were approved by the Animal Care Committee of the Centre de recherche en sciences animales de Deschambault (CRSAD; Deschambault, QC, Canada) according to guidelines from the Canadian Council on Animal Care (Canadian Council on Animal Care, Ottawa, ON Canada, 1993). The clean and disinfected wood floor was covered with approximately 3 in. (7.6 cm) of clean softwood shavings, and the bird density was approximately 0.087 m^2 per bird. Ventilation was provided by negative pressure with fans. The heat was provided by gas-fired brooders in each pen; water and feed were offered ad libitum through nipple drinkers and tube feeders, respectively, throughout the entirety of the experiment. The temperature was set at 33℃ on day 0 and then was reduced by 2.5℃ each week. Chicks were exposed to light for 24 h for the first day, 20 h for the second and until day 9, then 18 h thereafter. Birds were fed ad libitum with free access to water throughout the whole experiment. Birds were weighed at the start of the trial (day 0); body weight (BW) and feed intake were measured on day 10, 20, and 28 from each pen, and average daily feed intake (ADFI) and feed conversion ratio (FCR) were calculated. Birds were inspected at least twice per day, and mortalities or culls were removed and necropsied by the "Services Vétérinaires Ambulatoires Triple-V Inc." (Acton Vale, QC, Canada). The mortality rate was calculated based on the average mortality in each pen on day 0 to 28.

4.3.4 General and gut health

Fecal samples were collected on days 6, 9, 13, 16, 20, 23, and 27 from each pen (3/pen; 60/vaccination group/sampling time for a total of 420 samples) for oocyst counts (log_{10}/g). At day 21–22, 4 birds/pen (80 per vaccinated or nonvaccinated group for a total of 160 birds) were sacrificed for necropsy by the "Services Vétérinaires Ambulatoires Triple-V Inc." The intestines of all sacrificed birds (4 birds per pen: 16/treatment) were examined for evidence coccidiosis and NE. The intestinal health was scored for NE and coccidiosis lesions according to the study by Collier et al., (2003). Intestines were longitudinally opened to score mucosa on a scale of 0 to 3 for NE lesions for each of the upper gut and lower gut (including ceca). Coccidiosis lesions were scored on a scale of 0 to 4 for each of E. maxima which induces bleeding in the middle of the small intestines, mucosa, *E. tenella* causing severe inflammation of ceca and *E. acervulina* causing white plaques in the duodenum. The body weights of killed birds were determined.

4.3.5 Genomic DNA isolation and 16S ribosomal RNA gene sequencing

Cecal contents of the aforementioned 160 sacrificed birds (4 birds/treatment for a total of 40 pooled contents) were used for genomic DNA extraction. Genomic DNA was extracted from frozen cecal content using the QIAamp DNA Stool Mini Kit (QIAGEN, Toronto, Canada) according to the manufacturer's instructions. The purity and concentrations of the extracted DNA were determined using an Invitrogen Qubit 2.0 Fluorometer (Life Technologies Inc., Carlsbad, CA). Sequencing libraries of the 16S ribosomal RNA gene (rRNA) were prepared according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide Rev. B and sequenced on a MiSeq instrument (Illumina). Briefly, a 444-bp fragment spanning the V3-V4 hypervariable region (*Escherichia coli 1*6S rRNA position 340 - 784) was amplified with primers Bakt_341F (50 -CCTACGGGNGGCWGCAG-30) and Bakt_805R (50 -GACTACHVGGGTATCTAATCC-30) (Klindworth et al., 2013) containing 50 Illumina overhang adapter sequences (50 -TCGTCGGCAG CGTCAGATGTGTATAAGAGACAG-30 and 50 -GTCTCGTGGGCTCGGA GATGTGTATA AGAGACAG-30 , respectively) using 2x KAPA HiFi HotStart ReadyMix (VWR, CA89125- 042) and purified with AMPure XP beads (Beckman by PCR using the Nextera XT Index Kit (Illumina Inc., FC-131-1002), and PCR products were cleaned up with AMPure XP beads. Samples were pooled together at equimolar concentrations and sequenced using a 600-cycle v3 reagent kit (Illumina, MS-102-3003). The sequencing data were analyzed by Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.1; Caporaso et al., 2010). Paired-end reads were joined with fastq-join (Aronesty, 2011), and quality filtered and demultiplexed in QIIME using default settings. The reads were clustered at 97% sequence identity (similar as the species level) using uclust (Edgar, 2010), and operational taxonomic units (OTUs) were picked against the Greengenes database (gg_otus_13_8) using an open-reference approach (DeSantis et al., 2006). Taxonomic assignment of the sequences was performed using the uclust consensus taxonomy assigner. Taxa that could not be assigned were presented as 'unclassified' using the highest taxonomic level that could be assigned to them. The sequences were aligned against the Greengenes core set with PyNast (Caporaso et al., 2010), and a phylogenetic tree was constructed with FastTree (Price et al., 2009). Alpha-diversity (within groups) metrics were then calculated by QIIME, and a beta diversity (between groups) distance matrix based on unweighted UniFrac metric (Lozupone and Knight, 2005) was calculated, which was used for principal co-ordinate analysis (PCoA).

4.3.6 Data analysis

The experiment was arranged as a 2 × 5 factorial design with 2 groups (vaccinated or nonvaccinated) and 5 feeding treatments (Control, BAC, CIN, CIT, CIN+CIT). Effects of vaccination and feeding treatments on growth performance, oocysts count (log_{10}/g), gut lesions, and incidences and the relative microbial abundances and diversity were analyzed as a randomized complete block design (RCBD) by the ANOVAs using the MIXED procedure followed by Tukey's multiple comparison test of SAS 9.4 (SAS Institute Inc., Cary NC). Pens (as replicates) were included as the block and incidence of gut lesions were analyzed using Proc Freq of SAS. Only taxa with > 0.05% mean relative abundance in at least one treatment group were included in the analysis. Block, vaccination, treatments, and the interaction between vaccination and treatments were considered as fixed effects. The results were expressed as the least square means and standard error of the means (SEM). A P value < 0.05 was used to declare significance.

4.4 Results

4.4.1 Growth performances

In general, vaccination significantly decreased ($P < 0.05$) BW on days 20 and 28, while BAC, CIN, CIT, and CIT+CIN significantly increased ($P < 0.05$) BW compared with the control on day 28. At day 10, only BAC increased ($P < 0.05$) the BW compared with the control. The interactions ($P < 0.05$) between vaccination and treatments (control, BAC, CIN, CIT, and CIN+CIT) on affecting BW were noted on day 10. For ADFI, no differences were observed between vaccinated and nonvaccinated broilers in the starter, grower, finisher, and the whole phase (day 0- 28). Similarly, the broilers fed CIN, CIT, and CIN+CIT showed ADFI that is similar to the control and BAC. In this study, birds vaccinated against coccidiosis showed a decreased ($P < 0.05$) ADG in the whole phase. During the grower, a significant effect on ADG was noted only with BAC and CIN+CIT ($P < 0.05$). Vaccination was found to reduce ($P < 0.05$) FCR only in the starter, grower, and whole phase. The CIN, CIT, and CIN+CIT showed the effects similar to the BAC treatment group, whereby there was a significant reduction ($P < 0.05$) of FCR in the grower, finisher, and whole phase. Like BAC, tested encapsulated oils (either alone or in combination) significantly reduced mortality rates compared to the control ($P < 0.01$, Table 4.2).

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Table 4. 2 Effect of bacitracin, encapsulated cinnamaldehyde and citral on growth performance of broilers vaccinated (PV) or non-vaccinated (PNV).

Phases	Items[1]	Vaccination[2]		Treatments[3]					SEM[4]	Effects[5]		
		PNV	PV	Control	BAC	CIN	CIT	CIN+ CIT		Vac	Trt	Vac × Trt
Starter, 0-9 d	BW (0 d, g)	45	45	45	45	45	45	45	-	-	-	-
	ADFI, g/d	30	30	29	30	29	30	30	0.452	ns	ns	ns
	ADG, g/d	24	24	23	25	23	24	24	0.639	ns	ns	*
	FCR, g/g	1.23^{b}	1.26^{a}	1.26^{a}	1.21^{b}	1.26^{ab}	1.26^{a}	1.25^{ab}	0.019	*	*	ns
Grower, 10-19 d	BW (10 d, g)	282	286	280^{b}	293^{a}	281^{b}	283^{b}	283^{b}	4.071	ns	*	*
	ADFI, g/d	99	100	102	104	94	95	101	3.369	ns	ns	ns
	ADG, g/d	67	64	64^{b}	67^{a}	66^{ab}	63^{b}	67^{a}	1.591	ns	*	ns
	FCR, g/g	1.58^{a}	1.48^{b}	1.60^{a}	1.55^{ab}	1.43^{c}	1.53^{b}	1.52^{b}	0.054	*	*	ns
Finisher, 20-28 d	BW (20 d, g)	960^{a}	928^{b}	931^{bc}	971^{a}	947^{b}	914^{c}	958^{b}	18.954	*	*	ns
	ADFI, g/d	158	157	150	162	159	160	157	3.499	ns	ns	ns
	ADG, g/d	94	90	80^{b}	102^{a}	95^{ab}	94^{ab}	90^{ab}	6.197	ns	*	ns
	FCR, g/g	1.754	1.723	1.94^{a}	1.59^{c}	1.69^{b}	1.71^{b}	1.76^{b}	0.097	ns	*	ns
Whole phase, 0-28 d	BW (28 d, g)	1727^{a}	1662^{b}	1593^{c}	1787^{a}	1732^{ab}	1680^{b}	1683^{b}	56.97	*	*	ns
	ADFI, g/d	99	97	102	98	97	96	96	1.959	ns	ns	ns
	ADG, g/d	63^{a}	60^{b}	60^{b}	64^{a}	63^{ab}	61^{b}	60^{b}	1.591	*	*	ns
	FCR, g/g	1.61^{a}	1.57^{b}	1.69^{a}	1.54^{c}	1.54^{bc}	1.58^{b}	1.60^{b}	0.049	*	*	ns
	Mortality, %	9.31^{a}	6.88^{b}	15.16^{a}	3.61^{d}	10.31^{b}	5.31^{c}	6.09^{c}	18.702	*	**	ns

[a, b, c, d] Means in rows with different letters differ ($P < 0.05$).

[1]BW, body weight; ADFI, average daily feed intake; ADG, average daily gain; FCR, feed conversion ratio.

[2]PV, birds vaccinated with a live coccidiosis vaccine; PNV, birds not vaccinated with vaccine.

[3]Control, basal diet; BAC, 55 ppm bacitracin; CIN, 100 ppm encapsulated cinnamaldehyde; CIT, 100 ppm encapsulated citral; CIN+CIT, a combination of 100 ppm encapsulated cinnamaldehyde and citral.

[4]SEM, standard error of the means.

[5]Vac, main effect of vaccination; Trt, main effects of treatments; Vac × Trt, interaction between vaccination and treatments. Asterisks indicate significant statistically differences (one asterisk means a significance level of 0.05 and two asterisks 0.01).

4.4.2 General and intestinal health

No treatment effects were noted on the oocyst counts in fecal materials collected from 6 to 27 D (Figure 4.1). As expected, birds that received vaccines showed a significantly higher oocyst count compared with birds that did not receive this vaccine ($P < 0.01$). In nonvaccinated birds, the highest oocyst counts were observed on day 27, while increased oocyst counts were observed in vaccinated birds from days 6 to 27. On day 27, the nonvaccinated group showed the highest oocyst counts in the control and CIN+CIT fed birds ($P < 0.01$).

Coccidiosis: In general, subclinical (minor low lesion scores) coccidiosis, which was more prevalent in vaccinated than nonvaccinated birds, were observed (Figure 4.2 A and B). In the nonvaccinated group, coccidiosis lesions due to *E. acervulina* were observed in the control, BAC-, CIN-, CIT-, and CIN+CIT-treated birds with an incidence being 37.5%, 6.3%, 6.3%, 18.8%, and 56.3%, respectively. Birds with a lesion core of 3 (numerous coalescent lesions in the duodenum) were found only in the control. Data suggested that encapsulated CIN at 100 ppm in feed could provide the results similar to BAC at 55 ppm in controlling coccidiosis due to *E. acervulina* in nonvaccinated broiler chicken. In vaccinated birds, *E. acervulina* was the most prevalent *Eimeria* species examined (regardless of treatments) with more than 80% of birds in each treatment group showing intestinal lesions due to this parasite. However, the average lesion scores due to *E. acervulina* were 1.3, 1.2, 1.0, 1.6, and 1.3 for the control, BAC-, CIN-, CIT- and CIN+CIT-treated groups, respectively. Low incidence and minor coccidiosis lesion scores by E. maxima (6.3 to 18.8% of birds with average lesion scores of 0.2 to 0.1) and *E. tenella* (6.3 to 12.5% of birds with average lesion scores of 0.1) were observed. No lesions due to *E. maxima* were found in CIN+CIT-treated birds and no lesions due to *E. tenella* were observed in each of CIN and CIT-treated birds.

Necrotic enteritis: Significant effects of the tested EOs were observed on reducing NE incidence and severity, which were comparable to the effects of bacitracin (Figure 4.2 C).

The nonvaccinated control birds showed a 19% incidence of NE with an average lesion score being 0.2 of severity (the maximum severity was 3 found mainly in the control and CIT-fed birds). In general, the CIT treated birds showed a similar level of NE incidence and severity as the control birds, while birds treated with CIN showed 0% NE incidence and severity. An incidence of 6.3% and an average severity lesion score of 0.1 of NE were observed in the BAC-treated group. The CIN+CIT- treated birds resulted in a similar level NE incidence and severity as the BAC-treated group.

Among the birds vaccinated against coccidiosis, the control birds (no supplemented feed) showed an NE prevalence of 63% with an average lesion severity score of 1 (occasional lesion consisting of small areas of erosion, necrosis, or hemorrhage). Nineteen percent of birds (average lesion score = 0.2) of the BAC-treated birds presented NE while 56% (average lesion scores = 0.9) and 31% (average lesion scores = 0.3) of CIT- and CIN-treated birds showed NE lesion scores. The CIN+CIT-treated birds resulted in 6% incidence and 0.1 average severity of NE, which suggested a better control of NE disease than BAC (19% and lesion score of 0.9). It appeared that encapsulated CIN at 100 ppm was more active than CIT at the same dose in controlling NE, although an additive effect appeared to exist when the 2 oils were combined. Overall, the data indicated that the encapsulated essential CIN at the test dose and its combination with CIT could be used as an alternative to dietary bacitracin to combat NE disease in broiler production.

Figure 4. 1 Effects of vaccination and encapsulated cinnamaldehyde and citral in diets of broilers on oocysts counts in fecal samples at days 6, 9, 13, 16, 20, 23 and 27 of age.

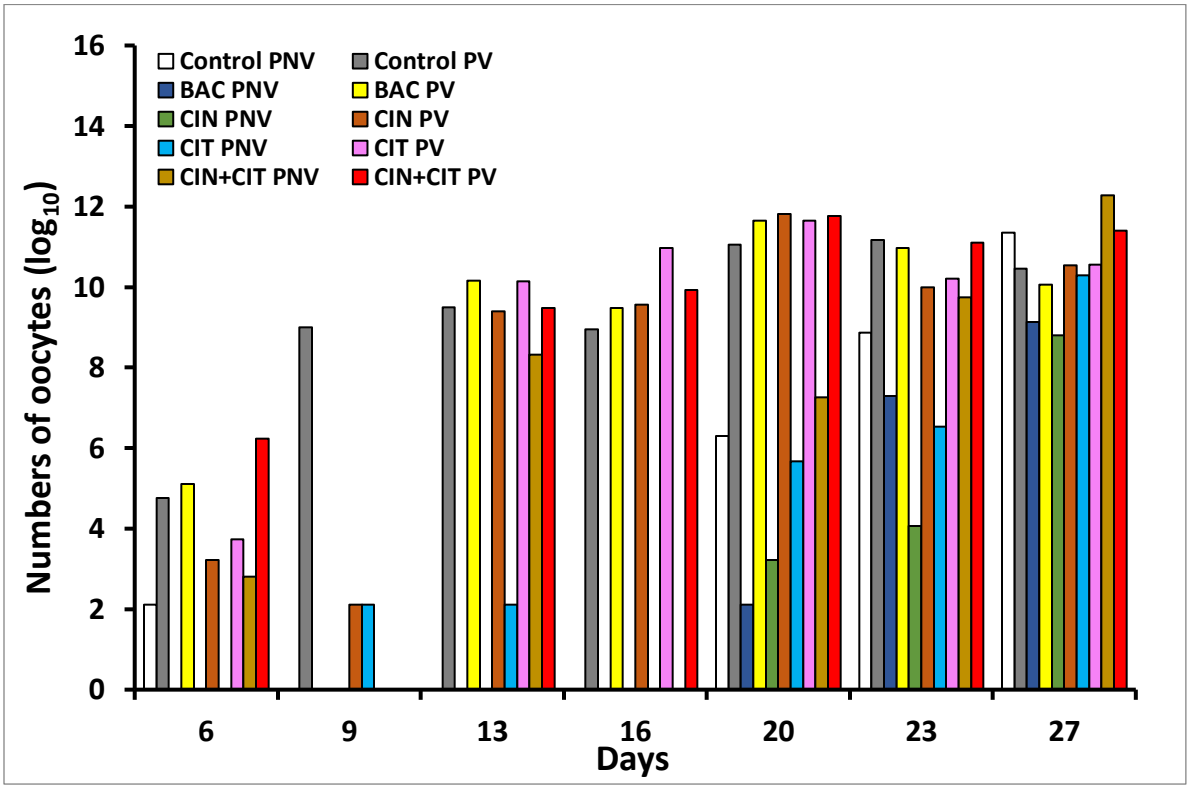
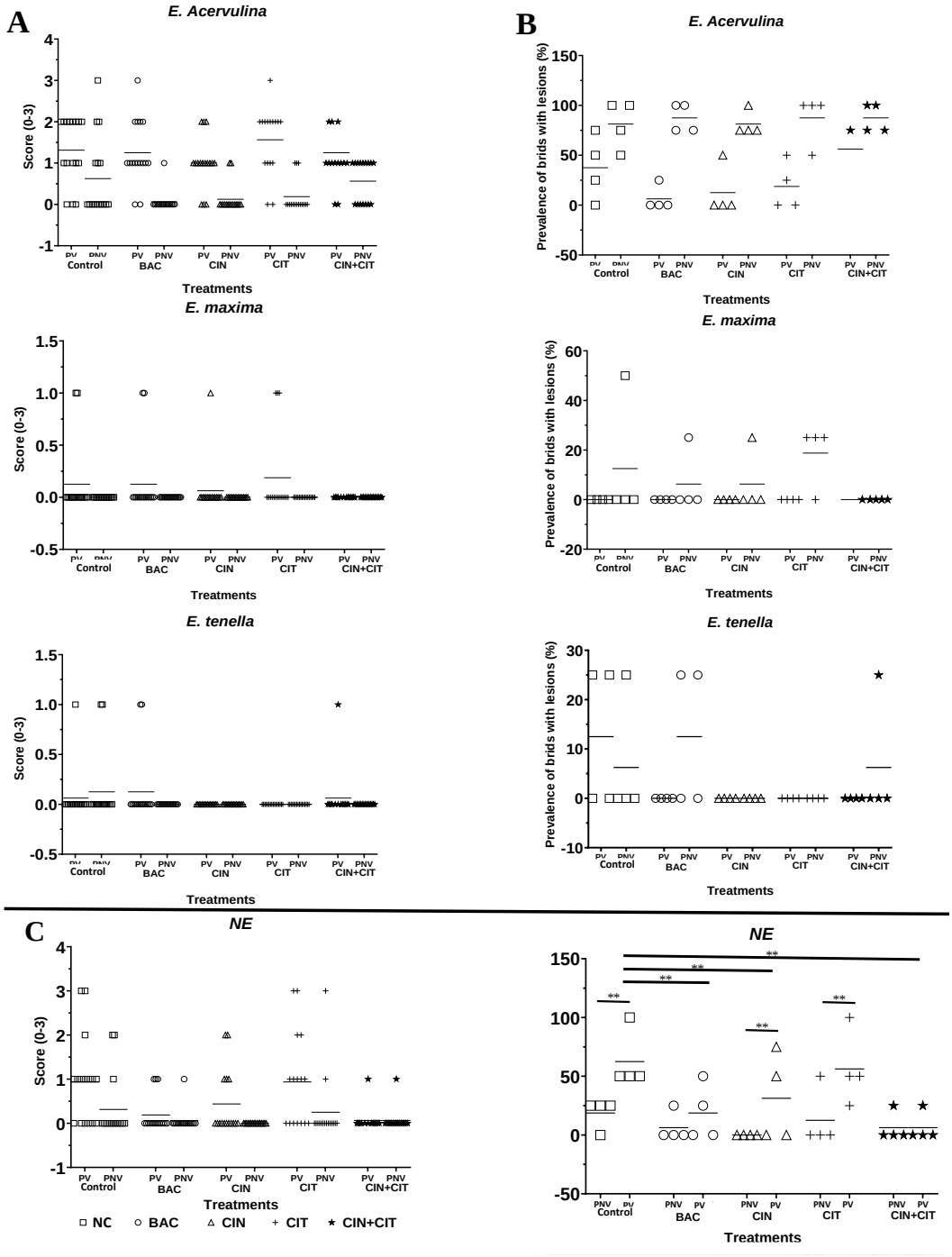


Figure 4. 2 Effects of bacitracin and encapsulated cinnamaldehyde and citral in diets of broilers on severity (panel A) and prevalence of (panel B) coccidiosis lesions due to *E. acervuline*, *E. maxima*, *E. tenella* and necrotic enteritis lesions and prevalence (panel C) due to *C. perfringens* (panel B) in both vaccinated (PV) or non-vaccinated (PNV) chickens at 21-22 d of age.



4.4.3 Cecal microbiota

Four bacterial phyla (*Firmicutes, Proteobacteria*, *Tenericutes*, and *Actinobacteria*) were detected by 16S rRNA gene sequencing analysis, with *Firmicutes* (90%) being the predominant phylum. Vaccination and treatments alone or their interaction did not affect the relative abundance of phyla (Table 4.3). At the genus level, a significant reduction in the relative abundance of *Lactobacillus* ($P < 0.05$) in the vaccinated group was observed. Ceca from broilers received feed supplemented with CIT, showed an increased relative abundance of *Lactobacillu*s ($P < 0.01$) but a decreased *Ruminococcus* ($P < 0.05$) compared with the control and BAC-fed birds. In addition, feed supplemented with CIN, CIT, and CIN+CIT reduced relative abundances of *Enterococcus* ($P < 0.05$) and *Clostridium* ($P < 0.01$) in ceca of birds compared with control feed which is similar to BAC. In addition, *Clostridium* was reduced ($P < 0.05$) in the vaccinated group with interactions between vaccination and treatments being found. However, interactions between vaccination and treatments were observed for the relative abundances of *Ruminococcus* ($P < 0.05$), and *Faecalibacterium* ($P < 0.01$), but not for, *Oscillospira* and *Dorea* (Table 4.3). The microbiota richness was estimated using observed OTUs, and the diversity was evaluated by Chao1, Shannon and Simpson indices. Vaccination did not affect microbiota richness and diversity. Feed supplemented with BAC, CIN, CIT alone showed lower ($P < 0.05$). Simpson compared to the control and BAC supplemented feed (Table 4.4). The PCoA of the microbiota based on unweighted UniFrac phylogenetic distances followed with PERMANOVA showed that the majority of the samples from the nonvaccinated group (red circle) and vaccinated groups (green circle) clustered separately (Figure 4.3 A); however, no significant differences between dietary treatments in the microbiota composition were observed (Figure 4.3 B).

Table 4. 3 Relative abundance of phyla and major genera (each representing > 1.0% of total sequences on average) in ceca.

Phyla/Genera	Vaccination[1]	Treatments[2]					SEM[3]	Effects[4]		
		Control	BAC	CIN	CIT	CIT+CIN		Vac	Trt	Vac × Trt
p_*Actinobacteria*	PV	0.02	0.01	0.01	0.03	0.04	0.019	ns	ns	ns
	PNV	0.02	0.03	0.03	0.03	0.02				
p_*Firmicutes*	PV	97.44	94.16	96.71	96.76	94.98	0.485	ns	ns	ns
	PNV	96.88	96.26	92.62	93.71	95.13				
p_*Proteobacteria*	PV	1.44	2.89	2.23	2.28	4.53	4.460	ns	ns	ns
	PNV	2.14	2.84	6.51	5.94	3.82				
p_*Tenericutes*	PV	0.83	2.86	0.81	0.80	0.39	1.415	ns	ns	ns
	PNV	0.91	0.73	0.79	0.46	0.91				
p_*unassigned*	PV	0.26	0.08	0.24	0.13	0.12	0.120	ns	ns	ns
	PNV	0.06	0.14	0.05	0.06	1.63				
g_*Lactobacillus*	PV	10.70	8.90	3.76	17.79	3.09	0.637	*	**	ns
	PNV	5.82	5.80	3.57	11.11	1.74				
g_*Ruminococcus*	PV	13.05	11.60	11.10	7.42	12.33	2.230	ns	*	*
	PNV	11.39	12.25	11.33	10.33	7.53				
g_*Oscillospira*	PV	8.87	11.53	11.02	9.62	12.80	3.114	ns	ns	ns
	PNV	10.18	10.71	9.08	9.96	8.06				
g_*Faecalibacterium*	PV	2.78	7.55	4.61	6.09	6.46	2.940	ns	ns	*
	PNV	8.62	5.36	8.11	5.25	3.18				
g_*Dorea*	PV	1.26	1.27	1.14	0.69	1.40	0.498	ns	ns	ns
	PNV	1.35	1.42	1.29	1.56	0.87				
g_*Enterococcus*	PV	1.21	0.04	0.05	0.13	0.08	0.107	ns	*	ns
	PNV	1.04	0.08	0.07	0.05	0.02				
g_*Clostridium*	PV	1.00	0.17	0.20	0.15	0.19	0.214	*	**	ns
	PNV	1.29	0.37	0.23	0.34	0.16				

[1]PV, birds vaccinated with a live coccidiosis vaccine; PNV, birds not vaccinated with vaccine.

[2]BAC, 55 ppm bacitracin; CIN, 100 ppm encapsulated cinnamaldehyde; CIT, 100 ppm encapsulated citral; CIN+CIT, a combination of 100 ppm encapsulated cinnamaldehyde and citral.

[3]SEM, standard error of the means.

[4]Vac, main effect of vaccination; Trt, main effects of treatments; Vac × Trt, interaction between vaccination and treatments. Asterisks indicate significant statistically differences (one asterisk means a significance level of 0.05 and two asterisks 0.01).

Table 4. 4 Summary of alpha-diversity measurements of microbiota in ceca of vaccinated (A) and non-vaccinated (B) broilers treated with bacitracin, cinnamaldehyde, citral alone or in combination.

	Vaccination[1]	Treatments[2]					SEM[3]	Effects[4]		
		Control	BAC	CIN	CIT	CIN+CIT		Vac	Trt	Vac × Trt
Observed OTUs	PV	393.38	396.95	409.50	389.60	383.88	18.88	ns	ns	ns
	PNV	398.67	384.78	399.75	391.20	400.10				
Chao1	PV	431.91	436.71	444.70	427.54	419.26	15.72	ns	ns	ns
	PNV	437.41	421.36	436.50	428.08	438.48				
Shannon	PV	6.47	6.34	6.67	5.85	6.60	0.36	ns	ns	ns
	PNV	6.54	6.42	6.42	6.22	6.61				
Simpson	PV	0.97	0.97	0.98	0.94	0.98	0.016	ns	*	ns
	PNV	0.98	0.97	0.97	0.96	0.98				

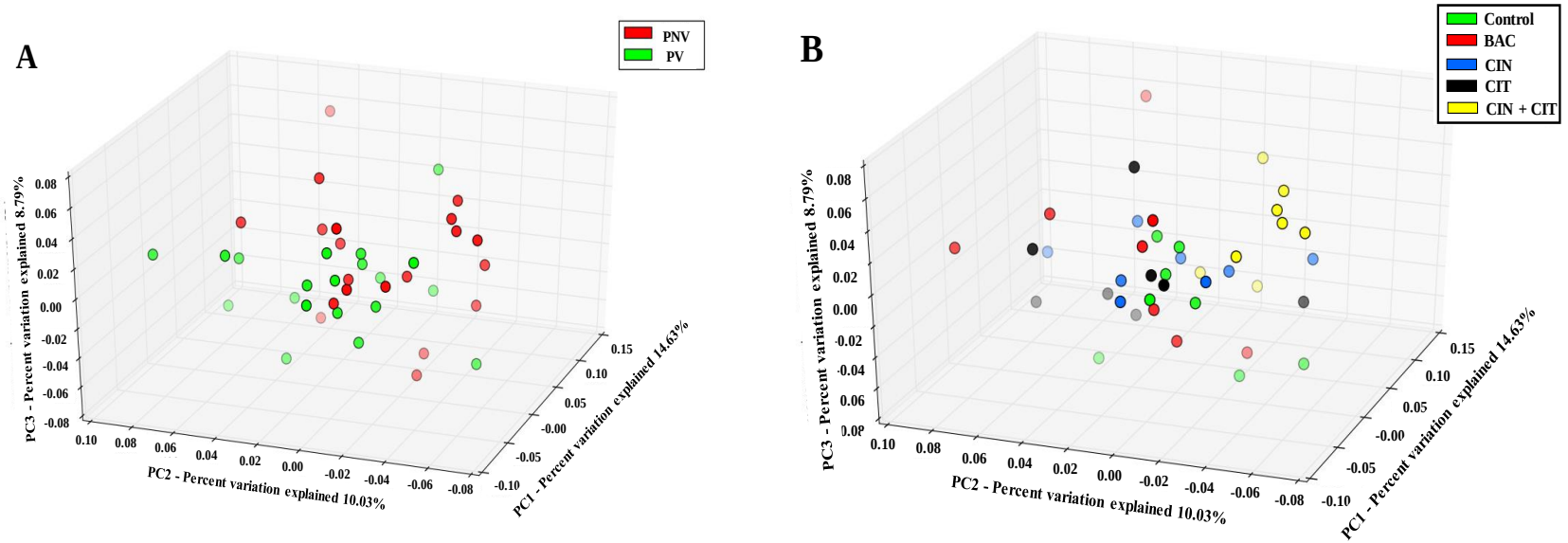
[1]PV, birds vaccinated with a live coccidiosis vaccine; PNV, birds not vaccinated with vaccine.

[2]BAC, 55 ppm bacitracin; CIN, 100 ppm encapsulated cinnamaldehyde; CIT, 100 ppm encapsulated citral; CIN+CIT, a combination of 100 ppm encapsulated cinnamaldehyde and citral.

[3]SEM, standard error of the means.

[4]Vac, main effect of vaccination; Trt, main effects of treatments; Vac × Trt, interaction between vaccination and treatments. Asterisks indicate significant statistically differences (one asterisk means a significance level of 0.05 and two asterisks 0.01).

Figure 4. 3 The 3D principal coordinate analysis (PCoA) graph shows the variation among distance matrixes (unweighted UniFrac) of cecal microbiota in vaccinated or non-vaccinated status (A) and treatments (B) with bacitracin, encapsulated cinnamaldhyde and citral, alone or in the combination.



4.5 Discussion and conclusion

Since the restrictions on antibiotic use in feed-additives, the search for affordable alternatives has been a growing discipline (Manges et al., 2007). In this study, vaccine and encapsulated CIN and CIT were used alone and in combination as alternatives for preventing natural incidences of coccidiosis and NE in broilers. Compared with previous studies (Aguilar et al., 2013; Khattak et al., 2014), the EOs in this research were fed at the beginning of the grower (day 10-28) instead of the starter. This is based on our previously reported experimental infection studies, in which EOs were applied from day 10 to day 28 and NE was reduced to the level similar to the treatment with antibiotics in the feed (Liu et al., 2016; Yang et al., 2016). In addition, we speculated that feeding EOs to broilers from the grower instead of starter could receive higher efficacy to promote the growth of broilers because the gut microbiota is not established and stable in the starter (Lu et al., 2003; Gong et al., 2008). As the cecum harbors more diverse and stable microbial communities than the ileum in broilers (Gong et al., 2002; 2007), we selected cecal digesta for analyzing microbiota in the present study.

The results suggested that vaccinations and EOs have interactions on increasing ADG in starter broilers and BW in grower broilers, respectively. This could be explained by the relationship between coccidiosis and NE, which reports that coccidiosis due to *Eimeria* could enhance mucus production and release plasma proteins by damaging gut epithelial cells, thus increasing nutrient availability for *C. perfringens* to grow (Williams et al., 2003). The results indicated that the combination of vaccines and EOs may improve the performance of broilers by controlling pathogens including *Eimeria* and *C. perfringens*. In the whole phase, the broilers supplemented with CIN, CIT, and CIN+CIT had similar effects as the BAC treatment, whereby BW was increased and FCR was decreased compared with the control. A previous study has shown that the higher nutrient digestibility associated with growth performance was due to increased secretions of endogenous digestive enzymes stimulated by EOs in the whole phase (Lee et al., 2003). Whether this is applied to the observation reported in the present study remains to be determined. Studies regarding the synergistic effects of EOs containing terpenes on growth performance have been conducted but no studies have examined synergism in an aldehyde and terpene blend, which were the compounds of CIN and CIT, respectively (Siani et al., 2013). In the present study, however, there were no significant synergistic effects of CIN and CIT on growth performance compared with CIN or CIT alone on growth performance. In addition, there were no observed differences among treatment groups regarding ADFI, suggesting that chickens may not

sense the flavor of EOs because of their encapsulation. This finding is consistent with previously published studies reporting that feed intake was not significantly reduced by dietary inclusion of EOs (Brenes and Roura, 2010; Bozkurt et al., 2014). This study also indicated that CIN, CIT, and CIN+CIT could reduce mortality, similar to the BAC treatment. In contrast, the mortality in the control was 15%. It could be associated with the environment, management, and diet during the animal trial. For example, high dietary protein content in this study (25.30, 23.30, and 21.30% CP in the starter, grower, and finisher, respectively) may have increased morality, as reported previously in both broilers (Gibson et al., 1989) and laying hens (Pearson and Herron, 1982).

Coccidiosis is a major parasitic infection in broilers, which increases mortality (Wealleans et al., 2017). In this study, the mortality rate was reduced after administering the oocyst vaccine, indicating its effectiveness in controlling coccidiosis. The lower BW and ADG in vaccinated broilers may have been due to a cell-mediated immune response to the oocyst vaccine that contains Eimeria species (Johnson, 1997). It has been reported that the immune response could affect the growth performance of chickens, resulting in reduced ADG (Ahmad et al., 2016). In addition, previous research also found that the coccidial vaccination may depress the growth of broilers as birds may not be able to protect themselves against Eimeria infections (Williams, 2002). The lower FCR in the grower and whole phase indicated that vaccination could increase feed efficiency. The results were in accordance with a previous study indicating that the Eimeria challenge induced by vaccination could improve the growth performance of broilers by lowering the FCR (Lee et al., 2011). However, higher FCR in starter suggested that vaccination may reduce FCR in young birds (Yang et al., 2011).

The minor coccidiosis incidences and lesions due to *E. maxima* and *E. tenella* may be due to the high hygienic and biosecurity practice in this study (Diarra et al., 2007). However, coccidiosis due to *E. acervulina* and NE incidences increased after vaccination. The appearance of coccidiosis lesions could be explained by the fact that the immune response to the live coccidiosis vaccine could repeat reinfection through the ingestion of sporulated oocysts sprayed over the surface of feed (Reid, 1990). In addition, it has been reported that factors including *Eimeria* infection and dietary fish meal could be responsible for inducing NE lesions (Stanley et al., 2014; Wu et al., 2014). In this study, the increased NE incidences in vaccinated birds could be at least partially due to the appearance of *Eimeria* infection (coccidiosis lesions) and fish meal applied in the diet.

Many EOs including CIN and CIT have been studied in vitro for their potential to inhibit pathogens that cause diseases in chickens (Friedman et al., 2002, 2004; Si et al. 2009; Giteru et al., 2015). The reduction in coccidiosis incidences and lesions caused by *E. acervulina* after feeding CIN at 150 ppm has been demonstrated by a previous study (Orengo et al., 2012). However, in the present study, the coccidiosis incidence and lesions due to *E. acervulina* decreased in birds fed 100 ppm CIN. The results indicated that the protection of the EOs through encapsulation could result in lowering the concentration of CIN to decrease coccidiosis incidence and severity. Accordingly, the results in this study also indicated that both CIT and CIN supplementation possess the ability to reduce coccidiosis. As coccidiosis has been reported to promote NE (Williams, 2003), the decreased NE incidence in the present study could be due to the reduction of coccidiosis by CIN and CIN+CIT. Besides, CIN (aldehyde), and CIT (terpene) have been reported to act synergistically (Caldas et al., 2015). To fully understand the molecular mechanisms underlying the effects of CIN and CIT individually or in combination in promoting chicken gut health, more studies are required.

Oocyst counts in feces from broilers are the reflection of coccidial infection in the birds (Hodgson, 1970). The higher fecal oocyst counts in vaccinated birds was consistent with the results of coccidiosis lesions.

The cecal microbiota of broilers can reflect feed digestion and nutrient absorption (Rinttilä and Apajalahti, 2013), which is related to urine recycling and gut health (Karasawa, 1999). In the present study, 4 phyla of microbiota were detected, with *Firmicutes* (90%) being the predominant phylum, followed by *Proteobacteria*, *Tenericutes*, and *Actinobaceria*. The results were in agreement with previous studies on broilers (Sakaridis et al., 2018; Biasato et al., 2019), although a higher relative abundance of phylum *Firmicutes* was observed. No *Bacteroidetes* were detected in the present study, which was also reported by others previously (Han et al., 2016; Pedroso et al., 2016; Lucke et al., 2018).

At the genus level, *Lactobacillus*, *Ruminococcus*, *Faecalibacterium*, *Enterococcus*, and *Clostridium* showed significant differences in relative abundance between the control and treatment groups in the present study. Previously *Lactobacillus* (*L. aviarius* in particular) has been reported to negatively correlate with the abundance of *C. perfringens* in broilers with NE disease (Feng et al., 2010). In addition, the reduction of *Lactobacillus johnsonii* by NE (Stanley et al., 2012) and independently by fish meal (Wu et al., 2014) has also been described. Stanley et al. (2012) observed a significant reduction of *Wissella confusa* in C. *perfringens* challenged birds of

Weissella confusa, a heterofermentative lactic acid-producing bacterium that was previously classified as a member of *Lactobacillus* but later relocated to *Leuconostoc* (Collins et al., 1993; Bjorkroth et al., 2002). Antonissen et al. (2016) reported a shift in species composition of *Lactobacillus* and reduction in butyrate-producing strains that belong to the *Ruminococccceae* family in broilers treated with *Eimeria* and fish meal. A decrease of *Faecalibacterium* and *Oscillospira* in ceca were also detected in broilers with NE disease (Stanley et al., 2012; Lacey et al., 2018). In the present study, the CIT or vaccine treatment alone increased *Lactobacillus* abundance compared with control and bacitracin treatments, which could suggest a potential benefit to chicken gut health according to the reports above. In contrast, the CIT and vaccine treatments decreased *Ruminococcus* abundance compared with the control. *Ruminococcus* is responsible for the degradation of cellulolytic fibre in ruminants, but its role in broilers remains to be further clarified (Koike and Kobayashi, 2009; Mondot et al., 2016). *Facecalibacterium* is a group of bacteria able to produce butyrate, which is a source of energy in broilers (Mondot et al., 2016; Bortoluzzi et al., 2017) and benefits animal gut health in general (Bedford and Gong, 2017). In the present study, neither vaccination nor EOs alone changed the abundance of *Faecalibacterium*, but their combination did. The low relative abundance of *Enterococcus* may imply less potential for chicken infection, as the density of *Enterococcus* in feces and digesta has been considered to be an indicator of fecal contamination that is associated with infections such as septicemia in poultry (Gilmore, 2002; Boehm and Sassoubre, 2014). *Clostridium* contains some pathogenic, but largely nonpathogenic species (Num and Useh, 2014). Although it was proposed to be a factor for predicting the potential of infections (Udaondo et al., 2017), whether it can be well-established needs to be determined. The changes in microbiota composition beneficial to chicken gut health in response to EO treatment have also been suggested previously (Cooper et al., 2013; Rehman et al., 2018). To determine the cause-effect relationship, further studies are required.

In conclusion, encapsulated CIN alone or in combination with encapsulated CIT in feed altered cecal microbiota composition and improved the intestinal health and performance of broiler chickens similar to BAC. Further studies are required to determine if the cecal microbiota changes contribute to the improvement of intestinal health and performance.

CHAPTER FIVE: MANUSCRIPT II

Antimicrobial Resistance Phenotype and Genotype of Generic *Escherichia coli* from Encapsulated Cinnamaldehyde and Citral Fed-Broiler Chicken[2]

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5.1 Abstract

The results in the previous study (Chapter Four) have demonstrated that encapsulated cinnamaldehyde (CIN) and citral (CIT) alone or in combination (CIN+CIT) as feed supplementations can improve growth performance, reduce gut lesions, and alter cecal microbiota. However, it is still unknown whether in-feed encapsulated CIN, CIT, or CIN+CIT can affect antimicrobial susceptibilities of commensal bacteria in broiler chickens. Therefore, this study investigated the effects of in-feed encapsulated CIN, CIT, or CIN+CIT on antimicrobial resistance (AMR) phenotypes and genotypes of *E. coli* isolated from feces of 6-, 16-, 23- and 27-days old broiler chickens. The five dietary treatments included the basal diet (control; NC), the basal diet supplemented with 55 ppm bacitracin (BAC), and 100 ppm encapsulated CIN, CIT, or CIN+CIT. Antimicrobial susceptibility using a Sensititre method of 240 *E. coli* isolates showed that the most frequent resistances were against β-lactam, aminoglycoside, sulfonamide, and tetracycline; however, the prevalence of AMR decreased ($P < 0.05$) when birds aged in general. The prevalence of resistance to amoxicillin-clavulanic acid, ceftiofur, ceftriaxone, cefoxitin, gentamicin, and sulfonamide was lower ($P < 0.05$) in isolates from CIN or CIN+CIT compared to those from NC or BAC. The whole-genome sequencing analysis of 227 of the 240 isolates detected 26 AMR genes (ARGs) and 19 plasmids but the prevalence of some ARGs and plasmid numbers were lower ($P < 0.05$) in *E. coli* isolated from CIN or CIN+CIT than NC or BAC. The most prevalent resistance genes included *tet(A)* (n=108), *aac3_Vla* (n=91), *aadA1* (n=86), *bla*$_{\text{CMY-2}}$ (n=78), *sul1* (n=77), *aph3_lb* (n=58), *aph6_ld* (n=58), and *sul2* (n=24). Interestingly, the number of most virulence genes (VGs) increased ($P < 0.05$) over time from 6 to 27 days of age. The prevalence of isolates of serotype O21:H16 was lower ($P < 0.05$) in CIN and CIN+CIT while colibacillosis-associated multi-locus sequence typing (ST117) was the most prevalent in isolates from day 23. A whole genome-based phylogenetic tree revealed a close relationship of 25 of 227 isolates to human or broiler extraintestinal pathogenic *E. coli*. This study indicates that AMR and virulence genotypes of *E. coli* could be modulated by encapsulated CIN or CIN+CIT feed supplementations and prompt further investigations on the involved mechanisms.

Key words: antimicrobial susceptibility, broiler chickens, *E. coli*, encapsulated essential oils, phenotypes and genotypes, whole genome sequencing

5.2 Introduction

The global poultry production increased from 250,000 to 1,250,000 tons annually in the last 50 years due to increased meat demands for humans (Food and Agriculture Organization of the United Nations, 2020). To promote growth and prevent or treat infectious diseases, antimicrobials have been widely used in poultry farms (McEwen and Fedorka-Cray, 2002; Butaye et al., 2003). This practice decreases incidences of bacterial infections, promotes growth performance, and reduces the mortality of poultry (Škrbić et al., 2009). For instance, bacitracin (BAC) at a sub-inhibitory concentration has been commonly supplemented in feeds as a growth promoter that increased body weight, feed efficiency, and altered intestinal microbiota of chickens in previous studies (Abdulrahim et al., 1999; Knarreborg et al., 2002; Singh et al., 2008). However, overuse or misuse of antimicrobials increases selective pressure to enrich resistant bacteria with antimicrobial resistant genes (ARGs), resulting in the prevalence of antimicrobial resistance (AMR) (Nhung et al., 2017). Additionally, ARGs could be transmitted horizontally from one bacterial species to another through mobile genetic elements (MGEs) such as plasmids (Muloi et al., 2018). Due to the prevalence and spread of AMR, Chicken Farmers of Canada (CFC) has decided to stop the preventive uses of category I to III antibiotics from 2014 to the end of 2020 (Chicken Farmers of Canada, 2017). Since the elimination of antimicrobials as feed additives may compromise the growth performance and increase incidences of bacterial infections in poultry production, it is necessary to develop antimicrobial alternatives and investigate their effects on the AMR prevalence and spread (Tsiouris et al., 2015).

Essential oils (EO) are aromatic and volatile liquids and their potentials as antimicrobial alternatives have been investigated (Preedy, 2015). Cinnamaldehyde (CIN) and citral (CIT) are two EO extracted mainly from trees, which possess antimicrobial, antioxidant and/or anti-inflammation properties (Yang et al., 2015b). Due to their instabilities during prolonged storage, feed processing, and gastric transition, their application in poultry production as feed supplements has been limited (Tian et al., 2016; Chen et al., 2017; Yuliani et al., 2018). Our previous study reported that broiler chickens fed CIN, CIT alone or in combination (CIN+CIT) encapsulated by a soy protein-polysaccharide Maillard reaction product (SPPMP) induced comparable effects as BAC in improving growth performance, reducing gut lesions, and modulating cecal microbiota (Yang et al., 2020). However, it is unknown whether in-feed CIN, CIT, or CIN+CIT could affect AMR genotypes and phenotypes of fecal bacteria in broiler chickens. Additionally, the synergistic effects of CIN (aldehyde) and carvacrol (terpene) on inhibiting the growth of food-born bacteria

including *E. coli* and *Staphylococcus aureus* have been reported previously (Ye et al., 2013). Although a recent study reported no significant synergistic effects of CIN and CIT on broiler chicken growth performance (Yang et al., 2020), no studies to date have evaluated the synergism of CIN and CIT (terpene) on AMR profiles of bacteria from chicken feeding trials.

Escherichia coli is a Gram-negative bacterium that is distributed ubiquitously in the chicken intestinal tract. Although most commensal *E. coli* strains are harmless to the host, pathogenic *E. coli* such as Extraintestinal Pathogenic *E. coli* (ExPEC) could cause extraintestinal infections, resulting in compromised chicken performance and an increased mortality rate, inducing important economic losses (Clements et al., 2012). The pathogenicity of such *E. coli* is mediated by several virulence genes (VGs) (Samanta and Bahdyopadhyay, 2019). Additionally, ARGs such as those conferring resistance to tetracycline [*tet(A)*], ampicillin *(bla*$_{\text{TEM}}$*)*, and sulfonamide *(sul1)* have been observed in *E. coli* isolates from chicken ceca, cloacae, or feces (Bonnet et al., 2009; Wang et al., 2013). Besides, mobile genetic elements like some plasmids carrying ARGs have been detected in chicken *E. coli* isolates, highlighting their transmission ability (Stokes and Gilling, 2011). Interestingly, it has been reported that the prolonged usage of antimicrobials as feed additives could promote the prevalence of ARGs and VGs in broiler chickens (Rehman et al., 2018) while increasing transmissions of ARGs via plasmids (Bengtsson-Palme et al., 2018).

Based on the results in the previous study (Chapter Four), feed supplementations of cinnamaldehyde (CIN) and citral (CIT) alone or in combination (CIN+CIT) can improve growth performance, reduce gut lesions, and alter cecal microbiota of broiler chickens (Yang et al., 2020). However, as stated above, relatively limited studies have been conducted on the AMR profile of commensal bacteria such as *E. coli* from broilers fed EO. The objective of this study was to examine the longitudinal effects of feed supplemented with encapsulated CIN, CIT, alone or in combination (CIN+CIT), compared to those of BAC on the AMR genotypes and phenotypes of fecal *E. coli* isolates from broiler chickens under a controlled trial.

5.3 Materials and methods

5.3.1 Bacitracin and essential oils

The CIN (catalogue no. W228613; ≥ 95% purity) and CIT (a mixture of cis and trans isomers; Catalogue no. C83007; 95% purity) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). They (CIN and CIT) were encapsulated separately in a soy-derived

polysaccharide called soy protein-polysaccharide Maillard reaction product (SPPMP) by emulsification and spray drying technology as previously described (Yang et al., 2015b).

5.3.2 Animal trial

The detailed information of animal trial design and management was described in Yang et al. (2020). Briefly, a total of 1,600 1-day-old male Cobb 500 broiler chickens were allocated in 20 floor pens (80 birds per pen) and vaccinated against coccidiosis using a commercial vaccine. Birds in each group were distributed to five dietary treatments (4 pens/treatment) including 1) basal diet as a negative control (NC); 2) basal diet with bacitracin methylene disalicylate at 55 ppm (BAC); 3) basal diet with encapsulated CIN at 100 ppm (CIN); 4) basal diet with encapsulated CIT at 100 ppm (CIT); 5) basal diet with a combination of 100 ppm encapsulated CIN and CIT (CIN+CIT). Birds were fed from day 0 to 9 (starter), day 10 to 19 (grower) and day 20 to 28 (finisher). The diet, with principal cereals and soybean meals, was formulated and pelleted according to Cobb 500 guidelines as previously described (Yang et al., 2020). All experimental procedures in this study were approved by the Animal Care Committee of the Centre de recherche en sciences animales de Deschambault (CRSAD, Deschambault, QC, Canada) based on the Canadian Council on Animal Care guidelines (Canadian Council on Animal Care, Ottawa, Ontario, Canada).

5.3.3 Sample collection, *E. coli* isolation and identification

Fresh fecal samples were collected on day 6, 16, 23 and 27 from all 20 pens (3 birds per pen for a total of 60 samples) in sterilized "whirl-pack" plastic bags. All fresh feces were immediately kept in an insulated foam container with dry ice and transported to the lab and then stored at -20^{o}C immediately for further analysis. Fecal *E. coli* were isolated using Chromocult® coliform agar (CCA). Briefly, 1 g of fecal samples were weighted in 15 mL sterilized plastic centrifuge tubes with 9 mL of 0.85% saline and vortexed for 5 mins to get a homogenous solution. Approximately 100 µL of ten-fold (10 ×) serial dilutes of homogenate were spread on CCA and incubated aerobically at 37^{o}C for 16-24 h. Three presumptive colonies were purified from each analyzed sample and stored at -80^{o}C in 25% glycerol in TSB. API 20E strips (BioMerieux, St. Laurent, QC, Canada) were used to confirm *E. coli* identity according to the manufacturer's specifications.

5.3.4 Antimicrobial susceptibility test (AST)

Minimal inhibitory concentrations (MICs) of antimicrobials were determined by broth-dilution method using the Sensititre automated system (Trek Diagnostic Systems, Cleveland, OH, USA) as previously described by Taggar et al. (2018). The following 14 antimicrobial agents were

included in the test panel: amoxicillin-clavulanic acid, ampicillin, ceftiofur, ceftriaxone, cefoxitin, chloramphenicol, ciprofloxacin, gentamicin, azithromycin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole. The MIC values were interpreted according to the breakpoints of the Clinical Laboratory Standards Institute's guidelines (Clinical and Laboratory Standards Institute, 2015) and the Canadian Integrated Program for Antimicrobial Resistance Surveillance guidelines (Canadian Integrated Program for Antimicrobial Resistance Surveillance, 2018).

5.3.5 Whole genome sequencing (WGS) and phylogenetic analysis

To get a better insight into the AMR genotype, all *E. coli* isolates were submitted for WGS as described (Rehman et al., 2017; Taggar et al., 2018). Briefly, DNA was extracted from each *E. coli* isolates using the DNeasy Blood & Tissue Kits (QIAGEN, Toronto, ON, Canada) and quantified using Qubit Fluorometer (Invitrogen). The sequencing libraries were established using Illumina Nextetra XT DNA sample preparation kit (Illumina, Inc., CA, USA) and a 600 cycle MiSeq reagent kit (v3) was used to perform paired-end sequencing on a Miseq Platform (Illumina, Inc.). For genome sequence analysis, the reads were checked using FastQC and combined using Fast Length Adjustment of SHort reads v1.2.9 (Magoc and Salzberg, 2011). The high-quality reads were assembled using SPAdes genome assembler version 3.9.0 software (Bankevich et al., 2012). The assembled genomes were annotated by Prokka version 1.11 (Seemann, 2014). The genome sequences were assembled in Spades v. 3.0 using The Integrated Rapid Infectious Disease Analysis (IRIDA) platform. The Resistance Gene Identifier version 4.0.2 (Comprehensive Antibiotic Resistance Database, 2018) platform, PlasmidFinder 1.3 (Center for Genomic Epidemiology, 2018a), and VirulenceFinder (Center for Genomic Epidemiology, 2018b) databases were used to identify AMR, plasmid replicon, and VGs in sequenced genomes.

The phylogenetic analysis was conducted using core genome single nucleotide variant phylogenomics (SNVphyl) pipeline with SNVs calling at a minimum mapping quality of 30, minimum base quality of 30, minimum alternate fraction of variant bases in agreement 80%, and minimum depth of coverage of 15x (Petkau et al., 2017). The reference used in this analysis was obtained from GenBank under the accession # NZ_CP008957.1. A maximum likelihood phylogeny with bootstrap support was built in FigTree.1.4.4.

5.3.6 Data analysis

Data on the number of antibiotics against which isolates were resistant (resistance spectrum), MGEs found in isolate, Multilocus sequence typing (MLST), and VGs categories were

analyzed a randomized complete block design using the General Linear Mixed Model (GLMM) procedure of the Statistical Analysis System version 9.4 (SAS Institute Inc., 2016, Cary North Carolina, United States). Treatments and day (bird's age) were used as sources of variation and the individual pens as experimental units. Least significant difference (LSD) was used to separate treatment means whenever the F value was significant. The association Cochran-Mantel-Haenszel test was used to determine the relationship between treatment or days and the prevalence of antibiotic resistance phenotype and genotype as well as specific MGEs (plasmids) using the FREQ procedures. The difference between treatment was considered significant at $P < 0.05$.

5.4 Results

5.4.1 Bacterial isolation

No significant differences ($P > 0.05$) were noticed between dietary treatments regarding *E. coli* concentration (CFU/g of the sample). From 6 to 27 days of age, a total of 240 *E. coli* isolates were obtained (48/treatment, 12/sampling day) and the subject of characterizations.

5.4.2 Antimicrobial susceptibility (ASTs)

All 240 *E. coli* isolates were susceptible to ciprofloxacin, azithromycin, and nalidixic acid. Birds fed CIN showed a lower ($P < 0.05$) prevalence of AMR to seven of the 14 antimicrobials (including amoxicillin, ampicillin, ceftiofur, ceftriaxone, streptomycin, sulfonamide, and tetracycline) than birds fed NC and BAC (Figure 5.1 A). Additionally, birds fed CIN+CIT showed a lower ($P < 0.05$) prevalence of AMR to five of the 14 antimicrobials (including amoxicillin, ceftiofur, ceftriaxone, cefoxitin, and sulfonamide) compared to BAC birds, while a higher prevalence of resistance to streptomycin and trimethoprim/sulfamethoxazole was noted in bird fed CIT-supplemented diet ($P < 0.05$) than the NC and BAC groups. Of the 240 isolates, 125 (52.08%) were resistant to gentamicin regardless of the treatment. Bird's age effect was also noted on the prevalence of resistance to this antibiotic ($P < 0.05$). Prevalence of AMR to six of 14 antimicrobials (including amoxicillin, ceftiofur, ceftriaxone, cefoxitin, gentamicin, and sulfonamide) decreased ($P < 0.05$) when bird aged (Figure 5.1 B).

Multi-drug resistance was observed among all 240 E. coli isolates with more than 70% of them being multiresistant to ≥ 3 antibiotics. The mean numbers of antibiotics to which isolates were resistant, varied from five to three with isolates from CIN-fed birds tending to present the lowest resistance spectrum ($P = 0.06$). Over 41% of *E. coli* from CIN treatment were pan-susceptible or resistant to only one drug compared to 27% from CIN+CIT, 21% from CIT, 21% from NC, or 19% from BAC treatment (Figure 5.2 A). The highest number of multi-drug resistant

isolates were found in 6-days old birds with the BAC and CIN treatment showing the highest and the lowest number ($P < 0.05$), respectively. At this sampling time (day 6), 40% of studied *E. coli* were resistant to nine antimicrobials followed by 20%, 7%, and 7% on days 16, 23, and 27, respectively (Figure 5.2 B). Except in BAC treated birds, lower numbers of multi-drug resistance isolates were found in 27-days-old birds ($P < 0.05$).

Generally, birds fed EOs (CIN or CIN+CIT) and increased age of birds are two factors that could reduce the prevalence of resistance to antimicrobials. Of the 240 *E. coli* isolates submitted for sequencing, 13 (NC=4, BAC=1, CIN=3, CIT=2, CIN+CIT=3) showed a low quality sequence and were not included in the final analysis (MLST, ARGs, VGs and plasmid) as below on the remaining 227 isolates.

Figure 5. 1 Effects of dietary treatments (A) and ages (B) on the prevalence of antimicrobial resistance (%) in 240 *E. coli* isolated from broiler chickens. Asterisks indicate the antimicrobials against which the resistance percentages between treatments were statistically different (One asterisk is $P < 0.05$ and two asterisks are $P < 0.01$). AMOCLA, amoxicillin; CEFTIF, ceftiofur; CEFTRI, ceftriaxone; AMPICI, ampicillin; CEFOXI, cefoxitin; GENTAM, gentamicin; STREPT, streptomycin; TRISUL, trimethoprim/sulfamethoxazole; CHLORA, chloramphenicol; SULFIZ, sulphonamide; TETRA, tetracycline.

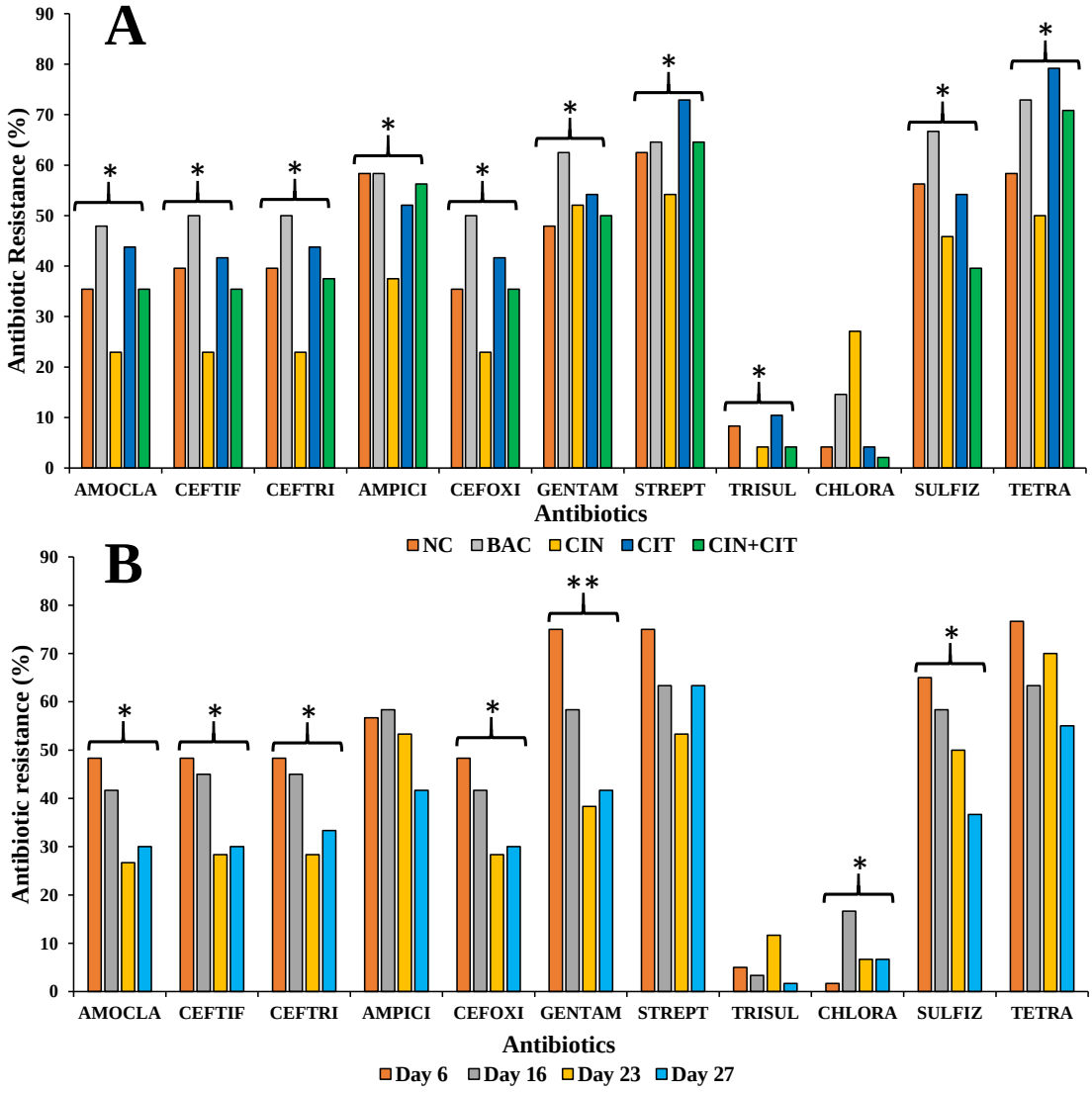
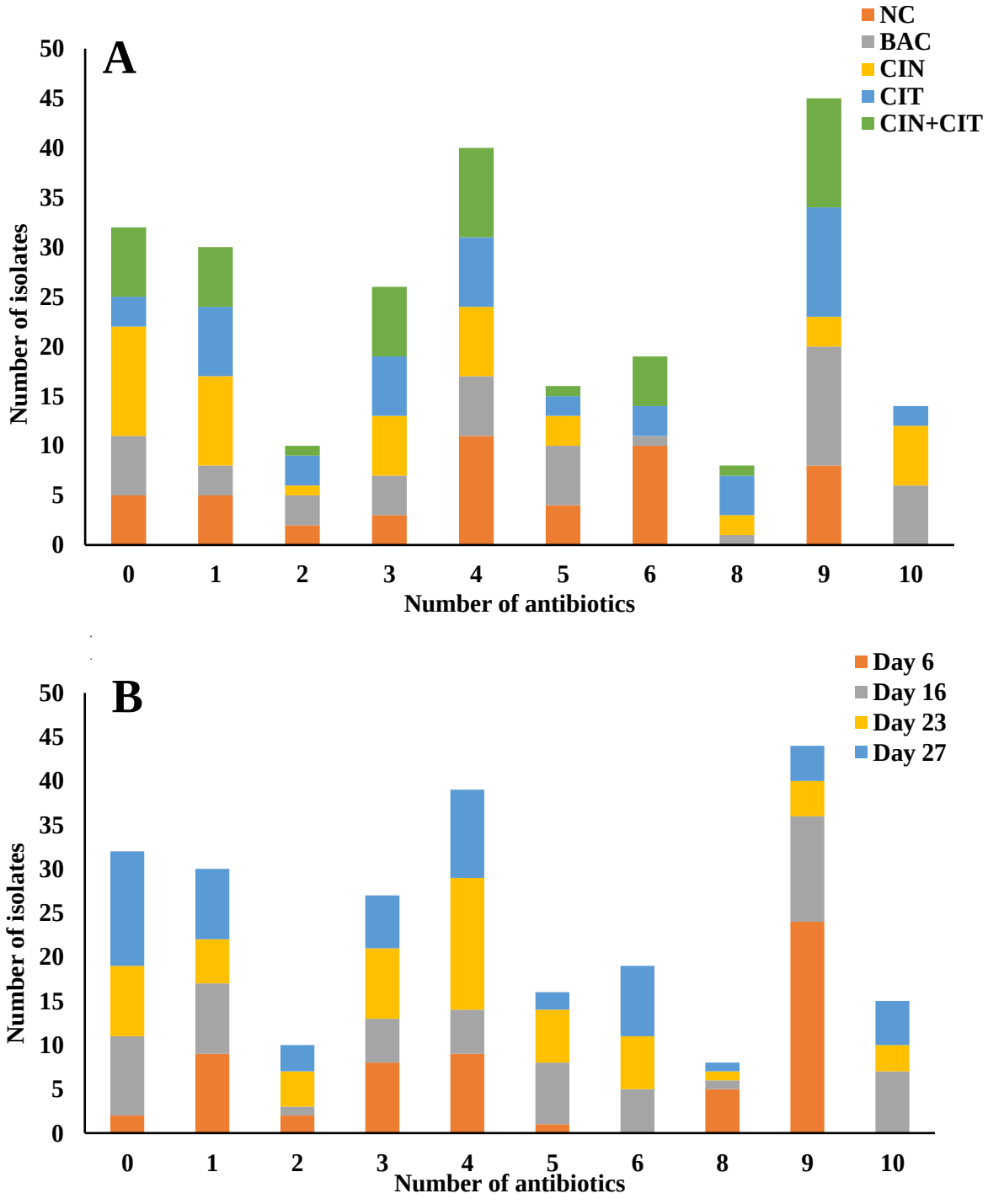


Figure 5. 2 Effects of dietary treatments (A) and ages (B) on the resistance spectrum of 240 *E. coli* isolated from broiler chickens.



5.4.3 Serotypes and multilocus sequencing typing (MLST)

The 227 *E. coli* isolates belonged to 66 serotypes with serotype O21:H16 (n = 22), O99:H15 (n = 16), O8:H19 (n = 14), O77:H18 (n = 10), O91:H7 (n = 9), O?:H21 (n = 8), O39:H7 (n = 7), and O15:H6 (n = 7) being the most detected serotypes (Table 5.1). The distribution of 28 serotypes was affected ($P < 0.05$) by dietary treatments and/or days (Table 5.1). Interestingly, serotype O21: H16 was less prevalent in isolates from birds fed CIN or CIN+CIT compared to those from NC, BAC, or CIT but more prevalent in younger birds (6 and 16 days of age) than in older birds (23 and 27 days of age). Besides, two isolates from birds fed BAC on day 23 belonged to serotype O78. A total of 40 MLST were observed and the distribution of 20 MLST was affected ($P < 0.05$) by dietary treatments, days, and/or their interactions (Table 5.2). Each MLST contained one or more serotypes and interestingly, some colibacillosis-associated MLST such as ST38 and ST117 showed higher ($P < 0.05$) numbers at day 23 than at day 6, 16, and 27. A significant lower number of ST38 was observed in CIN-fed birds ($P < 0.05$) than in those in the control birds as well as in CIT CIN+CIT treatments.

The above data indicated that CIN or CIN+CIT in feed could decrease the prevalence of avian colibacillosis associated with MLST and serotypes compared to birds fed NC or BAC. Interestingly, bird age could also be a factor influencing the prevalence of MLST and serotypes.

Table 5. 1 Serotypes (n=66) of *E. coli* showing significant dietary treatments and/or days effects.

Serotypes	Treatments[1]					Days[2]				SEM[3]	*P*-value[4]		
	NC	BAC	CIN	CIT	CIN+CIT	D6	D16	D23	D27		Trt	Day	Trt × Day
O?:H21(n=8)	4	2	2	0	0	0	1	7	0	0.12	ns	**	ns
O?:H31 (n=3)	0	0	2	1	0	3	0	0	0	0.07	ns	*	ns
O?:H7 (n=3)	0	0	1	0	2	1	2	0	0	0.07	ns	ns	*
O103:H21 (n=3)	0	0	2	1	0	0	0	0	3	0.07	ns	*	ns
O108:H21 (n=3)	0	0	0	0	3	0	0	0	3	0.07	**	*	**
O149:H23 (n=3)	0	1	1	1	0	3	0	0	0	0.07	ns	*	ns
O15:H6 (n=7)	2	1	1	3	0	0	5	2	0	0.11	ns	*	*
O153:H4 (n=4)	0	2	2	0	0	0	4	0	0	0.08	ns	**	ns
O16:H48 (n=3)	0	3	0	0	0	0	0	0	3	0.07	**	*	**
O160:H4 (n=4)	0	0	3	1	0	0	0	3	0	0.08	*	**	**
O180:H14 (n=2)	0	0	0	0	2	0	2	0	0	0.06	ns	ns	*
O184:H4 (n=2)	0	0	0	0	2	0	2	0	0	0.06	ns	ns	*
O21:H12 (n=5)	0	0	1	3	1	0	0	2	3	0.09	ns	ns	**
O21:H16 (n=22)	6	6	1	6	3	18	4	0	0	0.19	ns	**	ns
O21:H21 (n=2)	0	0	0	0	2	0	0	0	2	0.06	ns	ns	*
O22:H16 (n=4)	0	0	0	1	3	0	3	1	0	0.08	*	ns	**
O25:H18 (n=4)	0	1	2	1	0	0	0	4	0	0.08	ns	**	ns
O39:H7 (n=7)	2	0	4	0	1	3	3	1	0	0.11	ns	ns	*
O4:H16 (n=3)	0	0	0	0	3	0	0	3	0	0.07	**	*	**
O4:H45 (n=2)	0	0	0	0	2	0	0	0	2	0.06	ns	ns	*
O45:H32 (n=2)	0	0	2	0	0	0	0	0	2	0.06	ns	ns	*
O77:H18 (n=10)	0	4	4	1	1	0	6	1	3	0.13	ns	ns	**
O78:H4 (n=2)	0	2	0	0	0	0	0	2	0	0.06	ns	ns	*
O8:H19 (n=14)	6	0	1	5	2	2	2	6	4	0.15	*	ns	**
O9:H21 (n=2)	0	2	0	0	0	0	2	0	0	0.06	ns	ns	*
O91:H7 (n=9)	2	3	2	2	0	3	2	3	1	0.12	ns	ns	**

O96:H5 (n=2)	0	2	0	0	0	0	0	0	2	0.06	ns	ns	*
O99:H15 (n=16)	8	0	0	3	5	0	3	5	8	0.16	**	*	**

[1]NC, negative control; BAC, 55 ppm bacitracin; CIN, 100 ppm encapsulated cinnamaldehyde; CIT, 100 ppm encapsulated cinnamaldehyde; CIN+CIT, a combination of100 ppm encapsulated cinnamaldehyde and citral.

[2]D6, day 6; D16, day 16; D23, day 23; D27, day 27.

[3]SEM, standard error of the means.

[4]Trt, main effect of dietary treatments; Day, main effects of days; Trt × Day, the interaction effect between treatments and days.

Table 5. 2 Multilocus sequence typing (MLST) of *E. coli* showing significant dietary treatments and/or days effects on numbers.

MLST[1]	Number of Serotypes[2]	Treatments[3]					Days[4]				SEM[5]	*P*-value[6]		
		NC	BAC	CIN	CIT	CIN+CIT	D6	D16	D23	D27		Trt	Day	Trt × Day
NS (n=25)	8	6	5	5	4	5	12	5	4	4	0.20	ns	*	ns
new (n=34)	6	8	11	5	6	4	26	6	1	1	0.23	ns	**	**
10 (n=11)	5	0	3	5	0	3	0	2	3	6	0.14	*	*	**
38 (n=16)	1	8	0	0	3	5	3	5	8	0	0.16	**	*	**
40 (n=3)	1	0	0	0	0	3	0	0	0	3	0.07	**	*	**
69 (n=20)	2	2	7	5	5	1	0	12	4	4	0.18	ns	**	**
101 (n=5)	2	0	0	2	1	2	0	0	0	5	0.09	ns	**	ns
117 (n=15)	6	1	3	4	4	3	0	1	11	3	0.16	ns	**	ns
155 (n=6)	2	1	0	1	3	1	1	0	2	3	0.10	ns	ns	**
162 (n=11)	2	2	0	0	6	3	4	2	5	0	0.14	*	ns	*
191 (n=1)	1	0	0	0	0	1	0	0	1	0	0.04	ns	ns	ns
201 (n=5)	1	4	0	1	0	0	0	0	1	4	0.09	**	*	**
206 (n=3)	2	0	2	0	0	1	0	1	0	2	0.07	ns	ns	*
453 (n=4)	2	0	0	0	1	3	0	3	1	0	0.08	*	ns	**
1163 (n=4)	2	0	1	1	1	1	4	0	0	0	0.09	ns	**	ns
1286 (n=2)	2	0	0	2	0	0	0	0	0	2	0.06	ns	ns	*
1304 (n=10)	2	2	3	3	2	0	3	3	3	1	0.13	ns	ns	**
1564 (n=7)	1	4	2	1	0	0	0	1	6	0	0.11	ns	**	**
1594 (n=4)	1	0	2	2	0	0	0	4	0	0	0.08	ns	**	ns
2161 (n=2)	1	0	0	0	0	2	0	2	0	0	0.06	ns	ns	*
2485 (n=2)	1	0	0	0	0	2	0	0	0	2	0.06	ns	ns	*
5897 (n=3)	1	0	0	2	1	0	3	0	0	0	0.07	ns	*	ns

[1]MLST, multi-locus sequencing typing.

[2]The number of serotypes in each of multi-locus sequencing typing.

[3]NC, negative control; BAC, 55 ppm bacitracin; CIN, 100 ppm encapsulated cinnamaldehyde; CIT, 100 ppm encapsulated cinnamaldehyde; CIN+CIT, a combination of100 ppm encapsulated cinnamaldehyde and citral.

[4]D6, day 6; D16, day 16; D23, day 23; D27, day 27.

[5]SEM, standard error of the means.

[6]Trt, main effect of dietary treatments; Day, main effects of days; Trt × Day, the interaction effect between treatments and days.

5.4.4 Antimicrobial resistance genes (ARGs) and plasmids

Among all 227 sequenced genomes, 26 ARGs and 19 plasmids were detected. In agreement with AMR phenotypes, dietary CIN or CIN+CIT showed a low prevalence of ARGs including those conferring resistance to aminoglycoside (*aac3_Vla*, *aadA1*, *aadA2b*), ß-lactam (bla_{CARB}, bla_{CMY-2}, $bla_{CTX-M-1}$), sulfonamide (*sul2*, *sul3*), and tetracycline [*tet(A*)]. The prevalence of 18 of the 26 (69.2%) ARGs was significantly influenced ($P < 0.05$) by the dietary treatments, days, or their interactions (Table 5.3). These 18 genes included seven aminoglycosides (*aac3_Vla*, *aadA1*, *aadA2b*, *aadA23*, *aph3_la*, *aph3_lb*, and *aph6_ld*), three ß-lactam (bla_{CARB}, bla_{CMY-2}, and bla_{CTXM-1}), three sulfonamides (*sul1*, *sul2*, and *sul3*), two trimethoprim (*dfrA15* and *dfrA16*), one of each erythromycin (*ereA*), fosfomycin (*fosA7*), and tetracycline (*tet(A)*) resistance genes. The lowest ($P < 0.05$) prevalence of *aac3_Vla*, *aadA1*, *aadA2b*, bla_{CARB}, *dfrA15*, *dfrA16*, *ereA*, *tet(A)*, and *sul3* was found in CIN and/or CIN+CIT treated birds mainly. The prevalence of some ARGs including *aac3_Vla*, *aadA1*, bla_{CMY-2} , *sul1*, and *tet(A)* decreased ($P < 0.05$) from day 6 to 27, while the prevalence of *aadA2b*, *aph3_lb*, *aph6_ld*, *blaCARB*, *dfrA16*, and *ereA* increased ($P < 0.05$) from day 6 to 27. Additionally, significant interactions ($P < 0.05$) between dietary treatments and days for *aadA1*, *aadA2b*, *aadA23*, *aph3_la*, *dfrA16*, *ereA*, and *tet(A)* were observed.

The distribution of ARGs was influenced by serotypes. None of the *aac3_Vla* gene was detected in the 16 and 14 isolates of serotypes O99:H15 and O8:H19, respectively, while all O77:H18 (n=10) and 21 (95.45%) O21:H:16 isolates carried this gene. Of the 86 *aadA1* positive isolates, 20 (90.91%) of O21:H16, none of O77:H18 and O99:H15 were positive, while the 17 *aadA2* positive isolates were all 16 O99:H15 and the single isolate of serotype O99:H38. All O99:H15, O77:H18 and 21 O21:H:16 and none of O8:H19 carried bla_{CMY-2} gene.

A total of 15 plasmids were affected but their distribution was influenced ($P < 0.05$) by dietary treatments (Figure 5.3 A) and/or days (Figure 5.3 B). Compared to NC or BAC, a lower ($P < 0.05$) prevalence of *Inc*FIB, *Inc*I1, *Inc*Y, and *Inc*AC2 were observed in *E. coli* isolated from CIN or CIN+CIT birds. The prevalence of eight plasmids including *Inc*FII, *Inc*FIIA, *Inc*FIB, *Inc*H, *Inc*I1, *Inc*X1, *Col*RNAl _1778, and *Col*RNAl_1857 decreased ($P < 0.01$) from day 6 to 27, while the prevalence of *Col*RNAl_1993, *Col*RNAl_1885, and *Col*RNAl_1291 increased ($P < 0.05$) from day 6 to 23 but decreased ($P < 0.01$) thereafter from day 23 to 27. Additionally, interactions ($P < 0.05$) between dietary treatments and days on the prevalence of nine plasmids including *Inc*FII, *Inc*FIIA, *Inc*FIB, *Inc*H, *Inc*X1, *Inc*Y, *Inc*AC2, *Col*RNAl_1857, and *Col*RNAl_1885 were found. As for ARGs, the prevalence of plasmids was also serotype dependent. A clear relationship

between some plasmids and resistance genes was found. For example, 74 of the 91 (81.31%) and 72 of 86 (83.72%) isolates harboring *aac3_Via* and *aadA1* respectively also carried the plasmid *Inc*FII. Furthermore, 63 of 78 (80.77%) and all 5 of $bla_{\text{CMY-2}}$, $bla_{\text{CTX-M-1}}$ and 34 of the 40 (85.0%) bla_{TEM} positive isolates carried *Inc*FII.

Table 5. 3 Prevalence of detected antimicrobial resistance genes in *E. coli* showing significant dietary treatments and/or days effects.

ARGs[1]	Treatments[2]					Days[3]				SEM [4]	*P*-value[5]		
	NC	BAC	CIN	CIT	CIN+CIT	D6	D16	D23	D27		Trt	day	Trt × day
aac3_Vla (n=91)	38.64	51.06	40	47.83	22.22	75	38.98	23.33	30	0.33	*	**	ns
aadA1 (n=86)	50	36.17	35.56	47.83	20	66.67	30.51	35	25	0.32	*	**	*
aadA2b (n=17)	18.18	2.13	0	6.52	11.11	0	5.08	8.33	15	0.18	*	*	**
aadA23 (n=13)	2.27	10.64	8.89	4.35	2.22	2.08	10.17	3.33	6.67	0.15	ns	ns	**
aph3_la (n=18)	0	19.15	11.11	6.52	2.22	4.17	13.56	6.67	6.67	0.18	*	ns	**
aph3_lb (n=58)	22.73	21.28	26.67	21.74	35.56	10.42	25.42	30	33.33	0.29	ns	*	ns
aph6_ld (n=58)	22.73	21.28	26.67	21.74	35.56	10.42	25.42	30	33.33	0.29	ns	*	ns
bla$_{CARB}$ (n=16)	18.18	2.13	0	6.52	8.89	0	5.08	8.33	13.33	0.17	*	*	**
bla$_{CMY-2}$ (n=78)	36.36	46.81	22.22	41.3	24.44	56.25	30.51	26.67	28.33	0.32	*	**	ns
bla$_{CTX-M-1}$ (n=5)	4.55	2.13	0	0	4.44	0	6.78	1.67	0	0.10	ns	*	ns
dfrA15(n=7)	4.55	0	0	10.87	0	0	3.39	8.33	0	0.12	*	*	ns
dfrA16 (n=17)	18.18	2.13	0	6.52	11.11	0	5.08	8.33	15	0.18	*	*	**
ereA (n=17)	18.18	2.13	0	6.52	11.11	0	5.08	8.33	15	0.18	*	*	**
fosA7 (n=4)	0	2.13	2.22	2.17	2.22	8.33	0	0	0	0.09	ns	**	ns
sul1(n=77)	36.36	40.43	33.33	36.96	22.22	70.83	27.12	20	25	0.31	ns	**	ns
sul2 (n=24)	18.18	17.02	6.67	6.52	4.44	2.08	8.47	21.67	8.33	0.20	ns	*	ns
sul3 (n=6)	4.55	0	0	8.7	0	0	3.39	6.67	0	0.11	*	ns	ns
tetA (n=108)	50	55.32	35.56	52.17	44.44	60.42	49.15	55	28.33	0.33	ns	**	**

[1]ARGs, antimicrobial resistance genes.

[2]NC, negative control; BAC, 55 ppm bacitracin; CIN, 100 ppm encapsulated cinnamaldehyde; CIT, 100 ppm encapsulated cinnamaldehyde; CIN+CIT, a combination of100 ppm encapsulated cinnamaldehyde and citral; ARGs, antimicrobial resistance genes.

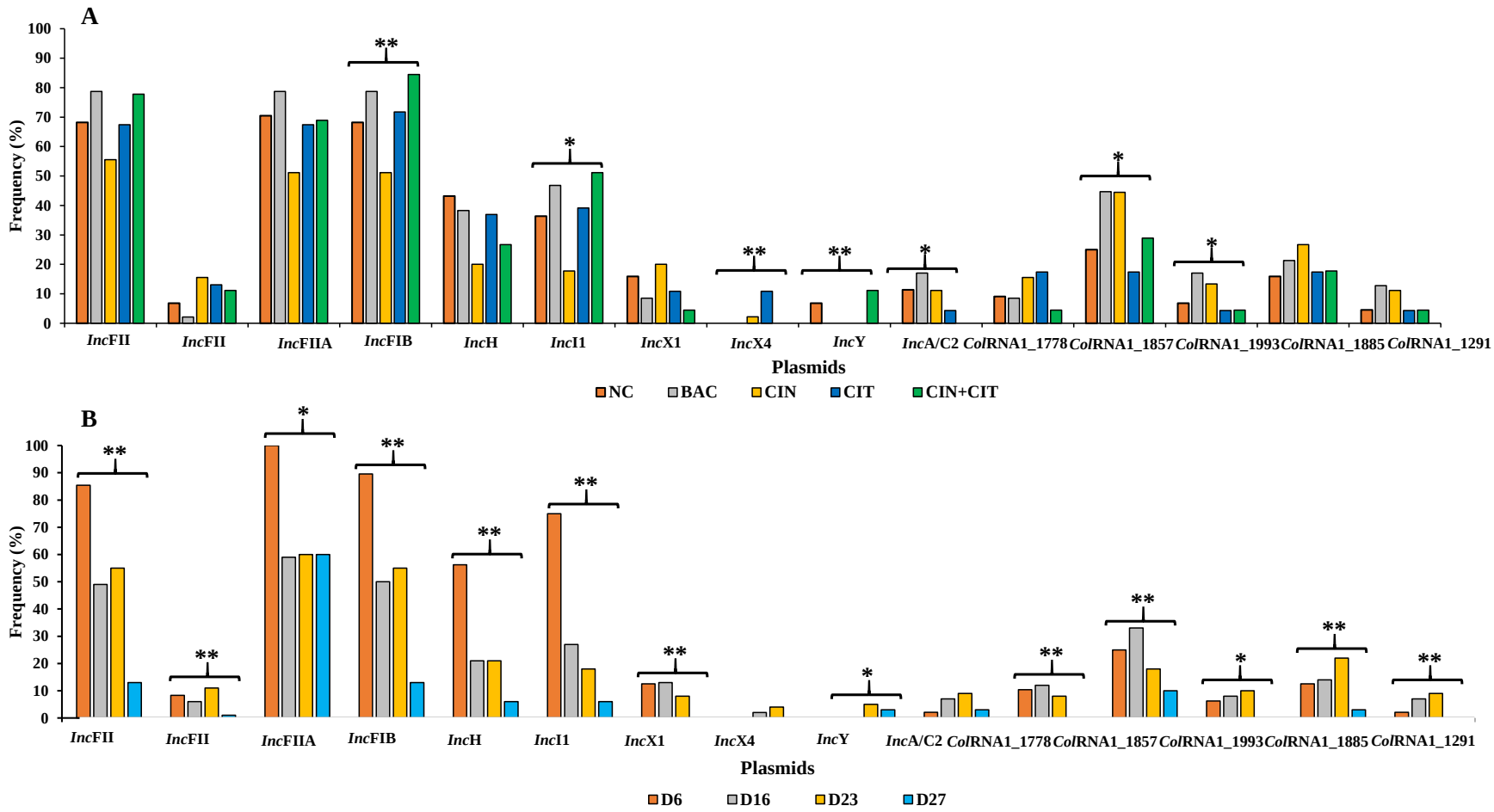
[3]D6, day 6; D16, day 16; D23, day 23; D27, day 27.

[4]SEM, standard error of the means.

[5]Trt, main effect of dietary treatments; Day, main effects of days; Trt × Day, the interaction effect between treatments and days.

One asterisk (*) indicates *P*-value smaller than 0.05 ($P < 0.05$); two asterisks (**) indicates *P*-value smaller than 0.01 ($P < 0.01$); ns indicates no statistically significant difference ($P > 0.05$).

Figure 5. 3 Effects of dietary treatments (A) and ages (B) on frequency of plasmids in 227 *E. coli i*solated from broiler chickens (One asterisk is $P < 0.05$ and two asterisks are $P < 0.01$).



5.4.5 Virulence genes (VGs)

A total of 430 VGs, belonging to 11 virulence factor categories including flagella, chemotaxis, fimbriae, pilus, curli, iron intake proteins, toxins, secretion systems, enzymes, regulators, and miscellaneous were detected. (Table 5.4). No significant differences ($P > 0.05$) between dietary treatments on the average number of virulence factor categories were observed. However, the average number of VGs in each virulence category was lower ($P < 0.01$) in isolates collected from day 6 compared with those isolated from day 16, 23, and 27. Interestingly, the interactions ($P < 0.05$) between dietary treatment and days on five virulence categories (pilus, toxins, secretion systems, enzymes, and regulators) were noted.

Table 5. 4 Virulent genes categories showing significant dietary treatments and/or days effects.

Virulence Categories	Treatments[1]					Days[2]				SEM[3]	*P*-value[4]		
	NC	BAC	CIN	CIT	CIN+CIT	D6	D16	D23	D27		Trt	Day	Trt × Day
Flagella (n=227)	35.3	37	36.8	38.1	37.2	31.7	38.5	38.3	38.7	0.6	ns	**	ns
Chemotaxis (n=227)	6.4	6.3	6.6	6.7	6.6	5.6	6.8	7	6.8	0.11	ns	**	ns
Fimbria (n=227)	16	15.5	16.1	18.7	17.8	13.7	17.6	17.6	18	0.39	ns	**	ns
Pilus (n=227)	10.2	12.2	10.9	11.3	10.7	8.7	11.8	11.7	11.8	0.28	ns	**	*
Curli (n=227)	6.4	6.8	6.5	6.7	6.6	5.6	6.9	7	7	0.1	ns	**	ns
Iron Intake Proteins (n=227)	21.9	23	21.1	23	22	18.3	23.1	25	22.3	0.47	ns	**	ns
Toxins (n=227)	4.8	4.5	4.2	4.6	4.3	3.8	4.9	4.3	5	0.11	ns	**	*
Secretion Systems (n=227)	23.7	22.5	21.2	24.3	23.7	18.3	25.4	24.6	24	0.67	ns	**	**
Enzymes (n=227)	6.4	6	6	6.6	5.6	4.1	6.7	7.2	6.8	0.22	ns	**	**
Regulators (n=227)	5.4	4.7	4.7	5.2	6	4	5.3	5.4	6.2	0.17	ns	**	**
Miscellaneous (n=227)	30	30.7	34.1	35.5	34.7	23.2	34.2	38	36.7	0.89	ns	**	ns

[1]negative control; BAC, 55 ppm bacitracin; CIN, 100 ppm encapsulated cinnamaldehyde; CIT, 100 ppm encapsulated cinnamaldehyde; CIN+CIT, a combination of 100 ppm encapsulated cinnamaldehyde and citral.

[2]D6, day 6; D16, day 16; D23, day 23; D27, day 27.

[3]SEM, standard error of the means.

[4]Trt, main effect of dietary treatments; Day, main effects of days; Trt × Day, the interaction effect between treatments and days.

One asterisk (*) indicates *P*-value smaller than 0.05 ($P < 0.05$); two asterisks (**) indicates *P*-value smaller than 0.01 ($P < 0.01$); ns indicates no statistically significant difference ($P > 0.05$)

5.4.6 Phylogenetic analysis

The phylogenetic tree was built using all 227 sequenced *E. coli* genomes based on single nucleotide polymorphism (SNP) (Figure 5.4 A). Three avian pathogenic *E. coli* (APEC) strains and 11 human ExPEC strains were used for comparison and the reference gene used in this analysis was obtained from GenBank (accession # NZ_CP008957.1). As the result, 20 *E. coli* isolates in five dietary treatments including NC (n = 1), BAC (n = 5), CIN (n=7), CIT (n = 5), and CIN+CIT (n = 2) at three different ages including day 16 (n = 7), 23 (n = 5), and 27 (n = 8) showed high genetic relatedness (distance of SNVs < 30) to human ExPEC. Five *E. coli* isolates in two dietary treatments including BAC (n = 3) and CIN+CIT (n = 2) at two different ages including day 23 (n = 3) and 27 (n = 2) had high relationship (distance of SNP < 30) with both human and broiler chicken ExPEC. To clearly show the AMR and virulence genotype of the 25 isolates with high relatedness to human ExPEC and broiler APEC, a reduced phylogenetic tree was established (Figure 5.4 B) and detailed information of their AMR phenotypes, genotypes, and virulence were shown in Table 2.5. Nine serotypes including O15: H6 (n = 6), O16: H48 (n = 3), O21: H21 (n = 2), O25: H18 (n = 4), O78: H4 (n = 2), O81: H39 (n = 2), O103: H21 (n=3), O184: H4 (n = 2) were determined in 24 *E. coli* isolates showing phylogenetic association with human or chicken ExPEC. Additionally, four MLST including ST10 (n = 5), ST69 (n = 6), ST101 (n = 5), and ST117 (n = 4) were found in 20 *E. coli* isolates that were related to human or broiler chicken ExPEC. Interestingly, all 25 *E. coli* isolates that were phylogenetically related to human or chicken ExPEC were observed in five dietary treatments including NC (n = 1), BAC (n = 9), CIN (n = 6), CIT (n = 5), and CIN+CIT (n = 4) or three ages including day 16 (n = 7), 23 (n = 8), and 27 (n = 10).

Table 5. 5 The serotypes, multi-locus sequencing typing, antimicrobial resistance spectrum, resistance genes, plasmids, and virulence genes of 25 *E. coli* isolates from chicken fecal clustering with human or chicken avian pathogenic *E. coli* (reference genomes) in phylogenetic tree.

ID[1]	OR[2]	Serotype	MLST[3]	ASTs[4]	ARGs[5]	Plasmids	VGs[6]
Reference isolates							
2792	CF	O24:H4	117	STR, SXT, CHL, FIS, TET	*sul1, aadA1, blaEC, dfrA15, tet(A)*	IncI1_Alpha, IncFIC(FII), IncFIB, ColRNAI, Col156	183
2793	CF	O24:H4	117	Susceptible	*blaEC-18*	IncFIC(FII), IncFIB, IncFII, Col156	192
5301	HP	O11:H18	69	AMP, STR, SXT, CHL,FIS, TET	*aadA5, aph(3'')-Ib, aph(6)-Id, blaEC-18, blaTEM-1, catA1, dfrA17, mph(A), mdf(A), sul1, sul2, tet(B)*	Col156, Col8282, Col, IncFIB, IncFII, IncY, IncQ1	241
5302	HB	O17:H18	69	FIS, TET	*aph(3'')-Ib , aph(6)-Id , blaEC-8, sul2, tet(A)*	Col, IncFIB, IncFII, IncN	237
5303	HB	O?:H2	69	SXT, CHL, FIS, TET	*aadA5, blaEC-18, catA1, dfrA17, mph(A), mdf(A), sul1, tet(B)*	Col156, Col8282, ColpVC, IncFIB, IncFII	242
5306	HU	O2:H18	963	Susceptible	*blaEC-8*	Col8282, IncFIB, IncFIC	218
5307	HU	O159:H45	101	AMP, NAL, STR, SXT, FIS, TET	*aadA2, blaEC-18, blaTEM-1, dfrA12, mph(A), mdf(A), sul1, tet(A)*	Col, IncFIB, IncFII, IncI1	177
5309	HU	O114:H4	117	Susceptible	*blaEC-18*	Col156, ColRNAI, IncFIB, IncFIC	200
5298	HU	O11: H4	10	SXT, FIS	*aph(3'')-Ib, aph(6)-Id, blaEC, dfrA14, sul2*	IncFIB, IncFIC, Col156, ColpVC, Col, IncX1, ColRNAI	205
Chicken fecal isolates in the present study							
6907	CF	O15: H6	69	AMP, GEN, STR, SUL, TET	*aac(3)-Vla, aadA1, blaTEM, mdf(A), sul1, tet(A)*	IncFII, IncFIIA, IncX1, IncFIA, IncFIB, Col1778	233
6919	CF	O81: H39	10	GEN, STR, TET	*aac(3), aph(3'')-Ib, aph(4)-Ia, aph(6)-Id, mdf(A), tet(B)*	IncH; Col1778, Col1088, Col1857, Col1987	154
6920	CF	O81: H39	10	GEN, STR, TET	*aac(3), aph(3')-la, aph(3'')-Ib, aph(4)-Ia, aph(6)-Id, mdf(A), tet(B)*	IncH; Col1778, Col1088, ColRNAIr_1857, Col1987	154
6926	CF	O15: H6	69	GEN, STR, SUL	*aac(3)-Vla, aadA1, blaTEM, mdf(A), sul1, tet(A)*	IncFII, IncFIIA, IncFIA, IIncFIB, IncX1, Col1778	232
6927	CF	O15: H6	69	AMP, GEN, STR, SUL, TET	*aac(3)-Vla, aadA1, blaTEM*	IncFII, IncFIIA, IncFIA, IIncFIB, IncX1, Col1778	232

6928	CF	O15: H6	69	AMP, TET	*blaTEM, mdf(A), tet(A)*	IncFIA, IncFIB, IncFII, IncX1, Col1778	222
6929	CF	O15: H6	69	AMP, GEN, STR, SUL, TET	*aac(3)-Vla, aadA1, blaTEM, mdf(A), sul1, tet(A)*	IncFII, IncFIIA, IncFIA, IIncFIB, IncX1, Col1778	232
6960	CF	O15: H6	69	AMP, GEN, STR, SUL, TET	*aac(3)-Vla, aadA1, blaTEM, mdf(A), sul1, tet(A)*	IncFII, IncFIIA, IncFIA, IncFIB, IncX1, Col1778	234
6966	CF	O25: H18	ND	Susceptible	*mdf(A)*	IncFIA, IncFII, IncFIIA, Col1291, Col1885, Col1993, ColRNAI1778	211
6969	CF	O78: H4	117	STR, SUL, TET	*aph(3')-la, aph(3'')-lb, aph(6)-ld, mdf(A), sul2, tet(B)*	IncFIIA, IncFII, IncFIB, Col1857, Col1291	199
6971	CF	O78: H4	117	STR, SUL, TET	*aph(3')-la, aph(3'')-lb, aph(6)-ld, mdf(A), sul2, tet(B)*	IncFIIA, IncFII, IncFIB, Col1857, Col1291	194
6979	CF	O25: H18	new	Susceptible	*mdf(A)*	IncFIA, IncFII, IncFIIA, IncFIB, Col1885, Col1993,Col1778	208
6980	CF	O25: H18	ND	Susceptible	*mdf(A)*	IncFIA, IncFII, IncFIIA, IncFIB, Col1885, Col1993,Col1778	213
6991	CF	O25: H18	ND	Susceptible	*mdf(A)*	IncFIA, IncFII, IncFIIA, IncFIB, Col1885, Col1993,Col1778	210
7004	CF	O184: H4	117	AMP, STR, TET	*aph(3'')-lb, aph(6)-ld, blaTEM, mdf(A), tet(B)*	IncFII, IncFIIA, IncFIB	189
7009	CF	O184: H4	117	AMP, STR, TET	*aph(3'')-lb, aph(6)-ld, blaTEM, mdf(A), tet(B)*	IncFII, IncFIIA, IncFIB	193
7030	CF	ND: ND	ND	STR, SUL, TET	*ND*	ND	205
7032	CF	O16: H48	10	GEN, STR, SUL, TET	*aac(3)-Vla, aadA1, mdf(A), sul1, tet(A)*	IncFIIA, IncFII, IncI1, IncI2, IncH, Col1857	128
7033	CF	O16: H48	10	GEN, STR, SUL, TET	*aac(3)-Vla, aadA1, mdf(A), sul1, tet(A)*	IncFIIA, IncFII, IncI1, IncI2, IncH, Col1857	129
7034	CF	O16: H48	10	GEN, STR, SUL, TET	*aac(3)-Vla, aadA1, mdf(A), sul1, tet(A)*	ND	132
7038	CF	O103: H21	101	GEN, STR, SUL, TET	*aac(3)-Vla, aadA1, mdf(A), sul1, tet(A)*	ND	188
7039	CF	O103: H21	101	AMP, GEN, STR, SUL, TET	*aac(3)-Vla, aadA1, mdf(A), sul1, tet(A)*	ND	181
7053	CF	O103: H21	101	Susceptible	*mdf(A)*	ND	181
7062	CF	O21:H21	101	Susceptible	*mdf(A)*	IncFIIA, IncFII, IncFIB, Col1857	179
7064	CF	O21:H21	101	Susceptible	*mdf(A)*	IncFIIA, IncFII, IncFIB, Col1857	179

[1]ID, strain ID.

[2]OR, origin of isolate; CF, chicken feces; HP, human pyelonephritis; HB, human blood; HU, human urinary tract infections.

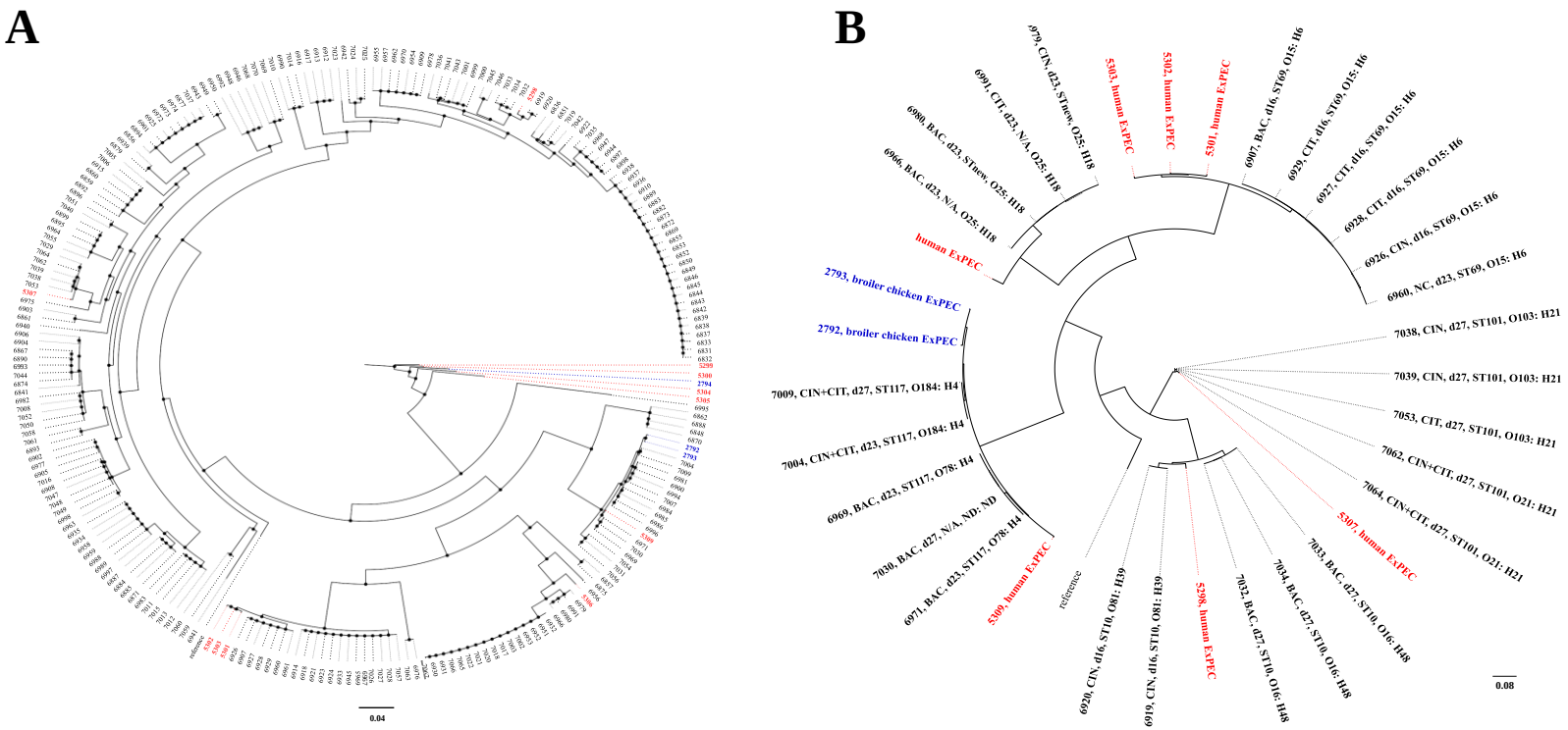
[3]MLST, multi-locus sequencing typing.

[4]AST profile, antimicrobial susceptibility profiles; Pan-susceptible, susceptible to all tested antimicrobials; AMP, ampicillin; CHL, chloramphenicol; GEN, gentamicin; FIS, sulfisoxazole; SUL, sulfonamide; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline.

[5]ARGs, antimicrobial resistance genes.

[6]VGs, number of virulence genes in each of the isolate.

Figure 5. 4 Phylogenetic trees of *E. coli* isolates based on core genome single nucleotide variant phylogenomics using SNVphyl for analysis. The entire tree (A) showed the phylogeny of all 227 *E. coli* isolates with strain ID only. The abbreviated tree (B) showed 25 isolates which were highly related to human or broiler ExPEC isolates (reference genomes) with detailed information including strain ID, dietary treatments, days, serotypes, and MLST. Red and blue colors were the reference genomes of human and broiler ExPEC isolates, respectively.



5.5 Discussion and conclusion

Avian pathogenic *E. coli* is one of the most significant pathogenic bacteria in broiler chickens, causing colibacillosis characterized by acute septicemia or subacute airsacculitis, high mortality, compromised bird's performance, and economic loss (Kabir, 2010). Additionally, AMR *E. coli* from chicken gastrointestinal tracts and feces have been proved to have the potential to cause human infections through contaminated chicken meat and carcasses, which present a food safety risk (Rasheed et al., 2014). The present study reported longitudinal (6, 16, 23, and 27 days of ages) effects of in-feed encapsulated Eos (CIN, CIT, CIN+CIT) compared to those of BAC on AMR phenotypes and genotypes of fecal *E. coli* from broiler chickens. Knowing that various factors including age and diets can affect gut microbiota AMR levels (Apajalahti et al., 2004; Diarra et al., 2007), isolates at these four days (d6, 16, 23, 27) were investigated based on the nutritional phases (starter: d 0-10; grower: d 11-20; finisher: d 21-28). Day 6 and 16 were in the middle of starter and grower, day 23 and 27 represent the beginning and end of the finisher close to harvest.

Over 85% of *E. coli* isolates in this study were resistant to at least one of the tested antimicrobials. A higher prevalence of resistance to ampicillin, gentamicin, and sulphonamide was observed in *E. coli* isolated from birds fed BAC compared to other dietary treatments. BAC has been commonly used in poultry farms to prevent or treat necrotic enteritis caused by *Clostridium perfringens* (Diarra and Malouin, 2014), an increase AMR *E. coli* a Gram-negative bacterium in BAC fed-birds need further investigations since this antibiotic target generally Gram positive. However, the effects of BAC supplementation on antimicrobial resistance in *E. coli* via possible plasmid transfer or altering resistance gene expressions have been reported (Mathers et al., 2004; Thibodeau et al., 2008). This suggests the urgency of reducing commonly used antimicrobials in poultry production and exploring their alternatives. In the present study, dietary CIN or CIN+CIT as alternatives showed a lower prevalence of resistance to most of the tested antimicrobials such as ceftriaxone, cefoxitin, and sulphonamide indicating the potential of CIN to breakdown the spread of AMR. It has been reported that successive *in vitro* exposures of Gram-negative bacteria such as *Serratia marcescens* and *Proteus mirabilis* isolated from clinical human samples to CIN and oregano oil increased MICs values of antimicrobials such as ampicillin, nalidixic acid, and tetracycline against them (Becerril et al., 2012). The present reports for the first-time effects of encapsulated CIN, CIT, and CIN+CIT in chicken feed on antimicrobial susceptibility of *E. coli* from their faeces. A higher prevalence of resistance to streptomycin was detected in *E. coli* isolated

from CIT fed birds. This phenomenon may be related to terpenes as the active compounds in CIT since increased MIC values of streptomycin against *E. coli* isolated from fecal samples of broiler chickens fed oregano oil, which contains high terpene compounds similar to CIT, have been reported (Horošová et al., 2006). The mode of action of CIN or CIT against bacteria has been reviewed and it has been suggested that these compounds interact with *E. coli* cell membrane inducing a rapid inhibition of energy metabolism resulting in leakage of small ions without the leakage of larger components adenosine triphosphate (Cristani et al., 2007; Friedman, 2017). However, the mechanisms of CIN, CIT, and CIN+CIT on modulating the prevalence of AMR are still unknown.

In agreement with our present study, a high prevalence of AMR in young birds has also been previously reported in broiler chickens or laying hens (Diarra et al., 2007; Braykov et al., 2016; Moreno et al., 2019). The possible explanations for the decline of AMR with increasing bird age may be due to the decrease of their fitness in the gut environment when birds increase their body mass (Khachatryan et al., 2004; Braykov et al., 2016). The decrease of AMR levels with increasing bird age could be explained by the fact that young birds are colonized with some resistant strains and could be replaced by susceptible bacteria with increasing bird age (Khachatryan et al., 2004). Accordingly, a similar study also found that AMR levels could be affected by bird ages (Diarra et al., 2007). Besides, dietary changes among stages (starter, grower, and finisher), altered room temperature for birds, and alterations in richness and diversity of gut microbiota at different ages could be all factors that affect AMR in bacteria (Mackowiak et al., 1982; Oakley et al., 2014; Klugman and Black, 2018; Sánchez et al., 2019). However, all these possibilities remain to be further determined.

A clear correlation between AMR phenotype and genotype has been observed (Schelz et al., 2006; Rosengren et al., 2009). The low AMR found in CIN-fed birds could be due to the damage of bacterial cell membranes resulting from its interaction with membrane-proteins or interferences with cellular energy, which could consequently result in the disruption of efflux pumps and ARGs expressions (Utchariyakiat et al., 2016; Yuan and Yuk, 2019). Furthermore, four of the studied *E. coli* isolated from day 6 contained previously described fosfomycin resistance gene at a similarity of 94% highlighting the importance of monitoring the emergency of fosfomycin resistance (Cassir et al., 2014).

It is well known that MGEs such as plasmids play an important role in the spread of AMR via horizontal gene transfers (HGT). Therefore, new strategies able to decrease HGT need to be

developed in the present study, a low prevalence of plasmids Inc*FIB*, Inc*1*, and Inc*A/C*$_2$ carrying ARGs observed especially in CIN fed-birds suggesting the potential anti-plasmid activities of this essential oil in *E. coli*. Little is known about the anti-plasmid activity of CIN; however, this activity has been reported in compounds such as promethazine and menthol (Schelz et al. 2006). Since plasmids can be horizontally transferred from one bacterial species to another, our study indicates that in-feed CIN may help to slow down the transmission and spread of AMR. Data from the present study also indicates that the efficacy of EO on chicken could be altered by bird age but the mechanism is still unknown (Cabuk et al., 2006).

Virulence factors allow attachment and colonization to host cells, iron uptake (Andrews et al., 2003), host cell and tissue damage (Do Vale et al., 2016, Green et al., 2016). In the present study, the average numbers of VGs in studied *E. coli* were not impacted by dietary treatments. It has been reported that the expressions of VGs could be affected by several factors such as pH, environmental conditions, and oxygen tensions (Zhu et al., 2011; Thomas and Wigneshweraraj, 2014). Data from the present study showed that the number of VGs encoding flagella, chemotaxis, fimbria, pilus, curli, iron intake proteins, toxins, secretion systems, enzymes, regulators, and miscellaneous were lower in young birds compared to old birds. The results were inconsistent with a previous study using pulsed-field gel electrophoresis (PFGE) showing that the frequency of VGs was higher in young birds compared to old birds (Kemmett et al., 2013). But it was at least proved that age is an essential factor that could modulate the number of VGs, suggesting the importance of further studies on this factor.

Escherichia coli can be classified into several serotypes based on somatic (O) and flagella (H). Certain serotypes such as O1, O2, O4, O8, O15, O35, O78, O88, O109, and O115 have been associated with colibacillosis in poultry (Huja et al., 2015). In the present study, a total of 66 *E. coli* serotypes were detected among 227 sequenced *E. coli*. Isolates of serotype O21:H16 frequently associated with zoonotic infections (Ren et al., 2008), were the most detected (22 isolates: NC, n=6; BAC, n=6; CIT, n=6; CIN+CIT, n=3; CIN, n=1). The virulence and pathogenicity of isolates O21:H16 were not evaluated in this study. However, about 159-167 different virulence genes were detected in these isolates, which were classified as APEC acceding to Bonnet et al. (2009) criteria. The low number (an isolate) of this serotype in CIN-fed birds suggests the potential of CIN in decreasing the number of the potential pathogenic *E. coli*. Interestingly, two isolates of serotype O78: H4, which is highly related to avian colibacillosis in poultry (Ewers et al., 2004; Ronco et al., 2017), were detected in BAC-fed birds on day 23. These

two isolates (APEC) carried 6 ARGs and more than 199 virulence genes. Additionally, bacteremia and urinary tract infection associated serotypes including O4 and O15 (Poolman and Wacker, 2015) were more prevalent in 16 or 23 days-old birds.

Some MLST such as ST38 and 117 have been associated with avian colibacillosis (Chattaway et al., 2014; Ronco et al., 2017). As well, MLST including ST69, 73, 95, and 131 are predominant in ExPEC isolated from human infections (Tartof et al., 2005). In the present study, a high number of ST38 and 117 were detected in *E. coli* isolated from day 23 which indicates a risk of colibacillosis. Interestingly, it appeared that CIN reduced the number of ST38 which may demonstrate its potential in preventing colibacillosis. The phylogenetic tree also suggests that *E. coli* of serotypes O15:H6, O16:H48, O25:H18, O78:H4, O81:H39, O103: H21, and O184:H4 clustered closely with clinical *E. coli* isolates used as control in this study, indicating their potential pathogenicity in human or broiler infections. Multidrug resistant *E. coli* of ST10 or ST101 have been reported to carry ß-lactamase ($bla_{\text{CTX-M}}$, $bla_{\text{NDM-1}}$) or colistin resistance (*mcr-1)* genes (Oteo et al., 2009; EI Garch, 2017; Ashcroft et al., 2019). The present study revealed that AMR *E. coli* ST10 or ST101 from chicken faeces may be potential pathogens.

In conclusion, the present study reported that the dietary CIN affected AMR phenotypes and genotypes of *E. coli* in chicken feces. The underlying mechanisms need to be elucidated; however, the anti-plasmids carriage could not be excluded. This study also confirmed that bird age can affect AMR phenotypes and genotypes of *E. coli* in broilers. Although no significant dietary treatment effect was observed on the number of detected virulence genes, bird age appeared to influence the prevalence of VGs, serotypes, and MLST. Dietary CIN affected the presence of various plasmids harboring ARGs in studied *E. coli* isolates, indicating the ability of this essential oil to decrease a potential lateral antibiotic resistance gene transfer between this bacterium and other chicken gut bacteria. Future studies are necessary to determine the *in vitro* and *in vivo* activities of CIN against AMR for its deployment as an alternative to decrease food safety risks in broiler production.

CHAPTER SIX: MANUSCRIPT III

Effects of Encapsulated Cinnamaldehyde on Growth Performance, Intestinal Digestive and Absorptive Functions, Meat Quality and Gut Microbiota in Broiler Chickens[3]

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6.1 Abstract

It has been demonstrated in Chapter Five that encapsulated cinnamaldehyde (CIN) alone had higher efficacy in reducing antimicrobial resistance of chicken fecal *E. coli* compared to birds fed citral (CIT) alone or in combination (CIN+CIT). However, to further evaluate potentials of encapsulated CIN as antimicrobial alternatives, it is necessary to measure its effects on gut health and breast meat quality at different concentrations. Thus, this study investigated the effects of encapsulated CIN at 50 mg/kg or 100 mg/kg on the growth performance, organ weights, meat quality, intestinal morphology, jejunal gene expression, nutrient digestibility, and ileal and cecal microbiota. A total of 320 male day-old broiler Cobb-500 chicks were randomly allocated to 4 treatments with 8 pens per treatment (10 birds per pen): 1) basal diet (negative control, NC); 2) basal diet supplemented with 30 mg/kg avilamycin premix (positive control, PC); 3) basal diet with 50 mg/kg encapsulated CIN (EOL); 4) basal diet with 100 mg/kg encapsulated CIN (EOH). Despite that birds fed EOH tended to increase ($P = 0.05$) meat pH at 24 h, all pH values were normal. Similar to the PC group, meat from birds fed EOL and EOH showed a reduced ($P < 0.05$) Warner-Bratzler force shear (WBFS) compared to the NC group. The highest villus to crypt ratio (VH/CD; $P < 0.05$) was observed in broiler fed either EOL or EOH, with an average being 14.67% and 15.13% in the duodenum and 15.13% and 13.58% in the jejunum, respectively. For jejunal gene expressions, only six out of the 11 studied genes showed statistical differences among the dietary treatments. Gene expressions of cationic amino acid transporter 1 (CAT-1) and neutral amino acid transporter 1 (B^0AT-1) were upregulated in EOH-fed birds compared to PC and NC-fed birds ($P < 0.05$), respectively; while expression of proliferating cell nuclear antigen (PCNA) was downregulated in EOL-fed birds when compared to NC birds ($P < 0.05$). Nonetheless, the expressions of cadherin 1 (CDH-1), zonula occludens 1 (ZO-1), and maltase-glucoamylase (MG) were all upregulated ($P < 0.05$) in EOH-fed birds compared to PC-fed birds. The apparent ileal digestibility (AID) of dry matter, crude protein, crude fat and of all 18 tested amino acids increased in EOL-fed birds ($P < 0.01$). Additionally, relative abundances (%) of ileal Proteobacteria decreased, while ileal and cecal *Lactobacillus* increased in EOH-fed birds ($P < 0.05$). In conclusion, dietary encapsulated CIN improved meat quality and gut health by reducing meat WBFS, increasing VH/CD in intestines, jejunal gene expressions, AID of nutrients and beneficially ileal and cecal microbiota composition.

Key words: antimicrobial alternatives, broiler chickens, encapsulated essential oils, gut health, meat quality, performance

6.2 Introduction

Poultry products are essential components of a well-balanced human diet due to their nutrient richness in highly digestible proteins, B-group vitamins, and minerals (Marangoni et al., 2015). In Canada, it has been reported that broiler meats account for a large portion of total poultry products with over 130,000 metric tons in 2019 (Bedford, 2019). However, broiler could be a reservoir of pathogens such as *Eimeria* spp., *Clostridium perfringens*, nontyphoidal *Salmonella enterica* serovars, and extraintestinal pathogenic *Escherichia coli* (ExPEC) (Craven et al., 2001a; Bergeron et al., 2012; Györke et al., 2013). These pathogens can be transmitted horizontally from feces or feathers to healthy birds through contaminated feeds, drinking water or beddings, and vertically from infected maternal breeders to their offspring (Liljebjelke et al., 2005). Additionally, due to the increased consumption of broiler meat, broiler farming has become extremely intensive which may induce poor bird performance, high incidences of infections, and mortality, causing economic losses and poor welfare (Dawkins et al., 2004; Škrbić et al., 2009). Traditionally, antimicrobials were supplemented in feed as sub-therapeutic antimicrobial growth promoters (AGP) to prevent infections and improve performance (Wellenreiter et al., 2000). However, overuse and misuse of antimicrobials have been linked to the development of antimicrobial resistance (Ventola, 2015). Thus, Chicken Farmers of Canada (CFC) eliminated category I antibiotics in 2014 and the preventive use of Category II antibiotics in 2018 and proposed to stop the use of category III antibiotics by end of 2020 (Chicken Farmers of Canada, 2017). However, withdrawal of AGP in feed may have adverse effects on broiler chicken such as reduction in performance (Kumar et al., 2018). Therefore, it is necessary to explore antimicrobial alternatives such as probiotics, prebiotics, organic acids, and plant extracts for improving chicken production and health (Casewell et al., 2003; Osman and Elhariri, 2013).

Essential oils (EOs) are aromatic and volatile liquids extracted mainly from plants by steam distillation (Preedy, 2015). It has been reported that EOs from star anise oil, ginko biloba, and oregano had beneficial impacts on enhancing nutrient utilization, immunity, and liver antioxidant status in broilers (Galal et al., 2016; Ding et al., 2017; Ren et al., 2018). Encapsulated cinnamaldehyde and citral alone or in combination were shown to reduce necrotic enteritis (NE), improve chicken growth performance as bacitracin, and beneficially alter cecal microbiota composition (Yang et al., 2020). However, limited studies were conducted on the effects of cinnamon oil on performance, meat quality, and intestinal histology and microbiota of broilers.

Cinnamaldehyde (CIN; from *Cinnamonmum*) has been used for food flavorings and medications for many years without receiving much attention of their potential effects as antimicrobial alternatives on broilers (Burt, 2004; Nabavi et al., 2015). CIN powder has shown antimicrobial activities against some other pathogenic bacteria including *Listeria monocytogenes* and *Bacillus cereus* in laboratory media and rice cakes (Hong et al., 2013). It also demonstrated high antimicrobial efficacy against *C. perfringens*, *S. typhimurium* DT104, *E. coli* O157: H7, and enterotoxigenic *Escherichia coli* (ETEC) with little inhibition towards *Lactobacillus* and *Bifidobacterium in vitro* (Si et al., 2006; Si et al., 2009). However, CIN should not be applied directly to broilers as feed additives due to their instability during feed processing and gastric transition (Tian et al., 2016). In this study, the CIN was encapsulated by the soy protein polysaccharide reaction products to maintain its stability during prolonged storage, feed processing, gastric transitions (Yang et al., 2015a).

The results from the previous study (Chapter Five) have demonstrated that encapsulated CIN alone showed the higher efficacies in reducing AMR levels (%) and prevalences (%) of antimicrobial resistance genes (ARGs) and plasmids of broiler chicken fecal *E. coli* when compared to birds fed CIN+CIT or CIN (Yang et al., 2021a). To further test whether CIN alone at different concentrations can affect gut health and breast meat quality, the objective was to evaluate the effects of encapsulated CIN at 50 mg/kg or 100 mg/kg on growth performance, organ weight, meat quality and gut health of broilers.

6.3 Materials and methods

6.3.1 Preparation of encapsulated materials

CIN (catalogue no. W228613; ≥95% purity; 828$/5 kg) was purchased from Sigma Aldrich Chemical Co. (St. Louis, MO) and encapsulated separately in a soy-derived product called soy protein-soy polysaccharide Maillard reaction product (SPPMP) by emulsification and spray drying technologies (20% CIN in the capsules) (Yang et al., 2020). Surmax 100 Premix (avilamycin premix, 100 g/kg) was purchased from Elanco Canada Co. Ltd (Guelph, Ontario, Canada).

6.3.2 Experimental design

A total of 320 1-day-old male Cobb 500 broiler chickens obtained from a local hatchery in Manitoba (Carleton Hatchery, Grunthal, Manitoba) were housed in 32 pens with 10 birds per pen (University of Manitoba, Winnipeg, MB, Canada). The pens were randomly allocated to four dietary treatments (8 pens/treatment): 1) basal diet as negative control (NC); 2) basal diet with 30 mg/kg avilamycin premix as a positive control (PC); 3) basal diet with encapsulated CIN at 50

mg/kg (EOL); 4) basal diet with encapsulated CIN at 100 mg/kg (EOH). The birds were fed a starter diet from day 1 to 14, a grower diet from day 15 to 28 and a finisher diet from day 29 to 41. The diets were provided in mash form and formulated (Table 6.1) according to the nutritional recommendation by Cobb 500 guidelines (Cobb-Vantress Inc., 2012) and prepared in Glenlea Research Station (Manitoba, Canada), which were described by a previous study (Mogire et al., 2021). For diets in PC, EOL and EOH groups, antibiotics and EOs were added by replacing equal amounts of corn.

6.3.3 Animals and management

All procedures involving birds in this experiment were approved by the Animal Care and Welfare Committee of the University of Manitoba according to animal use protocol (#. F18-024). The birds were weighed on day 1 and distributed into floor pens of identical size (81.5 inches × 59 inches = 4,808.5 square inches) in a deep litter system with a wood shaving floor. The size of the floor pen was 2 m^2, and 4 cm of low floor straw was provided for chickens. Chickens were allowed *ad libitum* access to feed and drinking water (water cups were 6.5 inches in diameter and 2.25 inches deep) during the experiment. The temperature was maintained at 31°C from day 1 to 3, 30°C from day 3 to 7, 28°C from day 7 to 14, 25°C from day 21 to 28, 24°C from day 28 to 35, and 22°C from day 35 to 41. The lighting program during the study was as follows: 24L:0D from day 0 to 3, 22L:2D from day 4 to 7 and 18L:6D in the period from day 8 to 39 and 23L:1D in the period of day 40 to 41. The birds were handled according to the guidelines of the Canadian Council on Animal Care (Canadian Council on Animal Care, 2009).

Table 6. 1 Feed ingredients and nutrition composition for starter (d 1-14), grower (d 15-28) and finisher (d 29-41) of chicken (g/kg, as feed basis otherwise indicated).

Ingredients	Inclusion in basal diet		
	Starter	Grower	Finisher
Corn	522.29	529.38	563.00
Soybean meal	305.00	261.00	225.00
Corn gluten meal	35.00	35.00	35.00
Wheat	25.00	30.00	30.00
Canola meal	25.00	30.00	30.00
Soy oil	22.60	43.80	45.80
Corn DDGS	20.00	30.00	30.00
Limestone	15.00	13.00	13.00
Vitamin premix[1]	10.00	10.00	10.00
21% Monocalcium phosphate	9.00	7.00	5.00
Mineral premix[2]	5.00	5.00	5.00
99% DL-methionine	2.65	2.37	2.05
Lysine-HCl	2.25	2.46	2.31
Threonine	0.71	0.49	0.34
Xylanase 8000G[3]	0.20	0.20	0.20
Phytase 5000G[4]	0.30	0.30	0.30
Calculated composition			
ME (Kcal/kg)	3000.00	3150.00	3200.00
CP (%)	22.30	20.80	19.40
Ca (%)	0.86	0.74	0.70
Total P (%)	0.60	0.54	0.52
SID Lys (%)	1.18	1.10	1.00
SID Met (%)	0.68	0.55	0.50
SID Met + Cys (%)	0.88	0.84	0.77
SID Thr (%)	0.78	0.74	0.67

[1]Provided per kilogram of diet: vitamin A, 8,255 IU; vitamin D3, 3,000 IU; vitamin E, 30 IU; vitamin B12, 0.013 mg; vitamin K3, 2.0 mg; niacin, 41.2 mg; choline, 1300.5 mg; folic acid, 1.0 mg; biotin, 0.25 mg; pyridoxine, 4.0 mg; thiamine, 4.0 mg; calcium pantothenic acid, 11.0 mg; riboflavin, 6.0 mg.

[2]Provided per kilogram of diet: manganese, 70.0 mg; zinc, 80.0 mg; iron, 80.0 mg; iodine, 0.5 mg; copper, 10 mg; selenium, 0.3 mg.

6.3.4 Growth performance and organ weights

All individual birds were weighed on day 1 and allotted with similar initial body weight (BW; 48.3 ± 3.3g) in a randomized complete-block (RCB) design. Feed intake and BW were recorded weekly throughout day 1 - 41 to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR). Any injured, malformed, or suffering bird was euthanized by carbon dioxide (CO_2) and confirmed by cervical dislocation. Euthanized birds were recorded to calculate mortality (%). On day 41, a total of 32 birds (1 bird per pen) were sacrificed to collect heart, liver, spleen, and bursa. The relative organ weights (%) were calculated as follows:

$$\text{Relative organ weights (\%)} = \frac{\text{Organ weight (g)}}{\text{chicken BW (g)}} \times 100$$

6.3.5 Intestinal morphology

Intestinal sections were collected from the duodenum (0.5 cm, the middle of the descending duodenum), jejunum (1.5 cm, midway between entry of bile ducts and Meckel's diverticulum), and ileum (0.5 cm, mid-ileum at the Meckel's diverticulum) on day 41 from 32 randomly selected euthanized birds (1 bird per pen) for morphology. The collected sections were flushed gently using ice-cold physiological saline solution, dehydrated with alcohol, fixed in 10% neutral buffered formalin, and embedded in paraffin. Approximately 6 µm of each section was cut, mounted on slides, deparaffinized in xylene, rehydrated and stained with hematoxylin and eosin (H&E) for histology analysis (Fischer et al., 2008). Villus height (VH) and crypt depth (CD) were measured by Carl Zeiss MicroImaging equipped with a computer-assisted morphometric system (Carl Zeiss Ltd., Göttingen, Germany) and calculated villus/crypt ratio (VH/CD).

6.3.6 Apparent ileal nutrient digestibility

An indigestible analytical marker, 0.3% chromium oxide (Cr2O3), was added to the feed at the last 4 days of the trial for analysis of apparent ileal digestibility (AID) of dry matter (DM), crude protein (CP), crude fat (CF), and amino acids (AA). Approximately 1 kg of diets in each treatment were collected and kept in a cold room at 4°C. Ileal digesta were collected from 4 birds per pen (pool digesta) on day 41 and then freeze-dried. The dried digesta samples were kept in airtight bags and stored at 22°C for further analysis. Before analyzing, the dried digesta and feedstuff were finely grounded by a grinder (CBG5 Smart Grind; Applica Consumer Products, Inc., Shelton, CT, USA) in duplicates.

The DM was measured by Official Methods of Analysis (AOAC, 2000; procedure # 934.01), CP was analyzed by a Leco NS 2000 Nitrogen Analyzer (Leco Corporation, St. Joseph, MI, USA) and calculated based on nitrogen content (CP = Nitrogen × 6.25), and CF was

determined using ANKOM Extraction System. Samples for AA analysis were prepared by acid hydrolysis according to the method of AOAC (2006; procedure # 994.12). Samples for methionine and cysteine analysis were oxidized with performic acid (AOAC, 2006; procedure # 985.18) before acid hydrolysis. Samples for tryptophan analysis were determined by the method of Commission Directive (2000) after hydrolyzing with barium hydroxide octahydrate for 20 h at 110 °C. The AA were analyzed using an Amino Acid analyzer (SYKAM, Germany). The Cr content was measured by inductively coupled plasma spectrometer (VarianInc., Palo Alto, CA, USA):

$$\text{AID (\%)} = (1- \frac{\text{ND} \times \text{CF}}{\text{NF} \times \text{CD}}) \times 100$$

Where ND is the nutrient concentration in digesta; CF is the chromium concentration in feed; NF is the nutrient concentration in diet; CD is the chromium concentration in digesta.

6.3.7 Gene expression

The jejunum from 32 random-selected euthanized birds (1 bird per pen) were flushed with phosphate buffered saline (PBS) and immediately stored in liquid nitrogen in 15 mL tubes.

Total RNA was extracted from jejuna using Trizol reagents according to the manufacturer's protocol. The extracted RNA concentrations were measured by a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Ottawa, ON, Canada). The RNA quality was visually checked by 1% agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized from RNA using iScriptTM cDNA Synthesis kit (Bio-Rad, Mississauga, ON, Canada). Quantitative Real-time PCR (RT-PCR) of genes, including cationic amino acid transporter (CAT-1), neutral amino acid transporter (B^0AT-1), glutamate transporters excitatory amino acid carrier 1 (EAAC-1), cysteine/glutamate antiporter (xCT), sodium-dependent glucose transporter 1 (SGLT-1), peptide transporter 1 (PepT-1), maltase-glucoamylase (MG), zonula occludens 1 (ZO-1), cadherin 1 (CDH-1), claudin 1 (CLDN-1) , and proliferating cell nuclear antigen (PCNA) were determined by iQ™ SYBR® Green Supermix (Bio-Rad) in a CFX ConnectTM Real-Time PCR Detection System (Bio-Rad). The detailed primer information was shown in Table 6.2. The cycling conditions were 95°C for 3 min, 40 cycles at 95°C for 20 s, 60°C for 30 s and 72°C for 30 s. Relative gene expressions were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

6.3.8 Genomic DNA extraction and 16S ribosomal RNA gene sequencing

Ileal and cecal digesta were collected from four birds per pen (pooled digesta) at day 41 and stored at -80ºC for genomic DNA extraction using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Toronto, Canada). The quantity and quality of DNA were determined by Nanodrop 2.0

and 1.0% agarose gel electrophoresis, respectively. The sequencing of the 16S ribosomal RNA gene (16S rRNA) was prepared according to Illumina 16S Metagenomics Sequencing Library Preparation Guide Rev. B and sequenced using a MiSeq instrument (Illumina). Briefly, the amplicon library of V3-V4 hypervariable region (444 bp) was amplified, and sequencing libraries were prepared as previously described (Yang et al., 2020). A 600-cycle v3 reagent kit (Illumina, MS-102-3003) was used to sequence after pooling equimolar quantities of each sample together. The sequencing data was analyzed by Quantitative Insights Into Microbial Ecology 2 (QIIME 2) (Bolyen et al., 2018). Briefly, 300 bp paired-end reads were processed with DADA2 (Callahan et al., 2016) to denoise reads, remove chimeric sequences and singletons, join paired-ends and de-replicate sequences to produce unique amplicon sequence variants (ASVs). Taxonomic classification of the resulting feature table was performed with VSEARCH (Rognes et al., 2016) and the Greengenes 99% OTU sequences (McDonald et al., 2012) as reference. ASVs were discarded if they had fewer than 10 instances across all samples, were present in fewer than two samples, or were not assigned taxonomy at the phylum level. Multiple sequence alignment of ASV representative sequences was performed with MAFFT (Katoh et al., 2009) and a rooted phylogenetic tree constructed with FastTree (Price et al., 2009). Core diversity analysis was performed using a sampling depth of 10,000 sequences to plot taxonomic relative abundances, calculate alpha-diversity metrics, and generate dissimilarity matrices based on Bray-Curtis, Jaccard, and UniFrac distances, which were used for principal coordinate (PCoA) analyses.

6.3.9 Meat quality

The birds were fed finisher diets until day 49 for collecting breast meat (*Pectoralis major* muscle) for meat quality analysis as described previously (Lu et al., 2020). The breasts from euthanized birds were carefully split, deboned, and trimmed of extra-muscular fat and connective tissues without damaging the exposed surface. The split breasts were placed on white Styrofoam trays (foam meat tray, 8.25 × 5.75 × 1, Pack. All Manufacturing Inc, Rockland, ON, Canada) containing soaking pads and covered with oxygen permeable polyvinyl chloride films (PVC; 037242 PUR Value Polyvinylchloride Standard Meat Films, AGL, Richmond Hill, Ontario, Canada). The trays with breast samples were placed on a retail display cabinet (Model MI, Husmann) at 2°C under LED lighting (light emitting diodes; Acuity Brands Dimmable Rigid 30-LED Light Strip Board HTG S7 - 94v-0 – 4000k) with an intensity of about 1240 lx. The cabinet was rotated every 24 h to minimize the temperature and lighting variations of the machine.

White striping (WS) and woody breast (WB) were evaluated visually and scored by a well-trained technician at a processing plant. The WS was scored as normal (0; no distinct white lines), moderate (1; visible white lines with <1 mm thick), and severe (2; large white lines 1-2 mm thick) as previously described (Kuttappan et al., 2012). The WB was scored as normal (0; fillets are flexible), mild (1; hard in the cranial region but flexible), moderate (2; hard throughout but flexible in mid to caudal region), and severe (3; extremely hard and rigid throughout from cranial region to caudal tip) based on tactile evaluation (Tijare et al., 2016).

Breast pH values at 24 h and 96 h post-slaughter were recorded by a waterproof meter (HI 99163, HANNA Instruments, Carrollton, TX). Meat color was measured at three locations by a colorimeter (Chroma Meter CR-410, Minolta Canada Inc., Mississauga, ON) using CIELAB systems for determining lightness (L*), redness (a*), and yellowness (b*). Dripping loss (%) was measured as previously described (Wang et al., 2016). Briefly, a small piece (approximately 5 g) of each breast sample on the caudal portion of the breast was cut and weighted. The pieces were hung perpendicularly to the ground in a flat-bottomed volumetric flask without touching the surface of the flask. The samples were suspended at 2°C and final weights were measured after 48 h to calculate dripping loss (%):

$$\text{Dripping loss (\%)} = \frac{\text{weight before suspension (g)} - \text{weight after suspension (g)}}{\text{weight before suspesion (g)}} \times 100$$

Myofibrillar fragmentation index (MFI) was measured as previously described (Culler et al., 1978). Firstly, a 4 g of breast sample was minced at 2°C, suspended in 40 mL of cold MFI buffer (100 mM KCL, 20 mM KPO4, 2 mM MgCl2, 2 mM EGTA, 1 mM NaN3, pH 7.0), and homogenized for 30 secs. The homogenate was transferred in a 50 mL sterilized tube to centrifuge at 2°C with a speed of 1000 × g for 15 min (Thermo Scientific™ Sorvall™ RC6 plus Centrifuge). After centrifugation, the pellet was re-suspended by a 10 mL cold MFI buffer and mixed by a vortex mixer. The mixture was poured through a strainer to remove connective tissues. Then, the protein concentration of each suspension was determined using bovine serum albumin (BSA) as the standard. Briefly, 0.25 mL suspension was added in tubes with 0.75 mL MFI buffer and 4 mL of biuret reagent. Then the suspension was placed in the dark for 30 min at room temperature. Simultaneously, serial dilutions of BSA were made to generate a standard curve by measuring the optical density (OD) at 540 nm (OD_{540nm}) using a spectrophotometer (GENESYS 30 visible spectrophotometer). The protein concentration of each suspension was determined by OD_{540nm} measurements using the BSA standard curve. Finally, based on the protein concentration, the

suspension was diluted to 8 mL of 0.5 mg protein per mL solution and mixed well. The MFI was calculated after the OD_{540nm} determination (Culler et al., 1978):

MFI (mg/mL) = 200 × Absorbance

After the measurements of WS, WB, pH, color, dripping loss (%), and MFI, the rest of the breast samples were cut into 5 pieces, vacuum packed (6" × 10" FlairPak Vacuum Pouch, Flair Flexible Packaging Corporation, Canada/USA), and stored in a -40°C freezer for analyzing cooking loss (%) and Warner-Bratzler Shear Force (WBSF). Frozen breast samples were thawed overnight in a cold room at 2°C, sealed in plastic bags, and cooked in a water bath at 85°C. A thermometer was inserted immediately in breast samples to monitor the internal temperatures until reaching 75 - 78°C and the cooking times were recorded. Then breast samples were moved from the water bath to the cold room (2°C) for 2 h cooling. The cooled samples were weighted to calculate cooking loss (%):

$$\text{Cooking loss (\%)} = \frac{\text{weight before cooking (g)} - \text{weight after cooking (g)}}{\text{weight before cooking (g)}} \times 100$$

The cooked samples were placed in the cold room at 2°C overnight and were moved to room temperature for 30 mins to measure WBSF by an analyzer (TA-XT Plus, Texture Technologies). Before analysis, the analyzer was calibrated with a 2 kg weight using a 10 kg loading cell. Then, 5 rectangular strips (2-4 cm long, 1 cm wide, 1 cm height) were cut along the fiber by a ruler and a knife. The strips were placed in the analyzer with the fiber perpendicular to the blade in order to record WBSF values (kg).

Table 6. 2 Primer sequences for Real-time quantitative PCR analysis.

Gene[1]	Genbank accession number		Primer sequences (5′-3′)
β-actin	NM_205518.1	FP	AATGGCTCCGGTATGTGCAA
		RF	GGCCCATACCAACCATCACA
B^0AT-1	XM_419056	FP	GCTCTACAGTGTTTGGAACCC
		RF	AAACTAGGCACACCAGCGAT
CAT-1	NM_001145490.1	FP	AACTGGGTTTCTGCCAGAGG
		RP	AACCCATGATGCAGGTGGAG
CDH-1	NM_001039258.2	FP	GGCAAGCCGTTTACCACATC
		RP	ATAATCCAGGCCCTTGGCTGT
CLDN-1	NM_001013611.2	FP	GGTATGGCAACAGAGTGGCT
		RP	CAGCCAATGAAGAGGGCTGA
EAAC-1	XM_424930.5	FP	GATTGTTCTGAGCGCTGTCG
		RP	ACCAAAGGCATCTCCCAAG
MG	XM015273018.1	FP	AAGAACCTCTGCAACCTCCG
		RP	TCTCCGTCCACCCTATAGC
PCNA	NM_204170.2	FP	GCCATGGGCGTCAACCTAAA
		RP	AGCCAACGTATCCGCATTGT
PepT-1	NM_204365	FP	CTTTGGCTACCCCTTGAGCA
		RP	AAAGTTGTCATCCCACCGCA
SGLT-1	NM_001293240.1	FP	ATGCTGCGGACATCTCTGTT
		RP	TCCGTCCAGCCAGAAAGAAT
xCT	XM_426289.5	FP	TGAGCTGGGAACGTGCATTA
		RP	AGGGCGAATAACCAGCAGTT
ZO-1	XM_015278981.1	FP	TATGCACAAGGAGGTCAGCC
		RP	TTGGCCGAAGCATTCCATCT

[1]B^0AT-1, neutral amino acid transporter; CAT-1, cationic amino acid transporter; CDH-1, cadherin 1; CLDN-1, claudin 1; EAAC-1, glutamate transporters excitatory amino acid carrier 1; MG, maltase-glucoamylase; PCNA, proliferating cell nuclear antigen; PepT-1, peptide transporter 1; SGLT-1, sodium-dependent glucose transporter 1; xCT, cysteine/glutamate antiporter; ZO-1, zonula occludens 1.

6.3.10 Data analysis

The experiment was analyzed as a complete random design (CRD) and pens were considered as experimental units. The growth performance, organ weight, intestinal morphology, ileal digestibility, and gene expressions obtained in each treatment were evaluated using PROC ANOVA followed by Tukey's multiple comparison test (SAS 9.4) with the model: $Y_{ij} = \mu + T_i + e_{ij}$, where μ is the total means, T_i is the fixed treatment effects, e_{ij} is residual of the model. The relative abundance of microbial taxa and diversity, and meat quality including meat color (a*, b*, L*), pH, purge loss, cooking loss, MFI, and shear force were analyzed using PROC MIXED followed by the Tukey's multiple comparison test (SAS 9.4). Chi-square analysis was analyzed using PROC FREQ to check differences in the distribution of severity scores in WS and WB. A *P*-value of 0.05 was used to declare significance.

6.4 Results

6.4.1 Growth performance and organ weights

Results demonstrated that encapsulated CIN at 50 ppm or 100 ppm had no significant effects on BW, ADFI, and FCR compared to the controls for either each growing stage and for the overall feeding trial (Table 6.3). However, both EOL- and EOH-fed birds showed a lower mortality rate (%) than birds in NC and PC. Similarly, no significant differences were noted between treatments for the weights of heart, liver, spleen, and bursa (Table 6.4).

Table 6. 3 Effects of encapsulated cinnamaldehyde at either 50 ppm or 100 ppm in feed on growth performance of broiler chickens.

Items[1]	Treatments[2]				SEM[3]	*P*-value
	NC	PC	EOL	EOH		
Starter (d 1-14)						
BW (14 d, g)	514.83	501.14	505.03	497.36	4.802	0.63
ADG, g	33.32	32.34	32.62	32.07	0.337	0.61
ADFI, g	44.99	46.07	46.63	45.42	0.401	0.51
FCR, g/g	1.35	1.43	1.43	1.42	0.015	0.19
Grower (d 15-28)						
BW (28 d, g)	1749.33	1784.13	1728.14	1696.89	15.793	0.26
ADG, g	88.18	91.64	87.37	85.68	1.067	0.25
ADFI, g	118.64	119.03	118.70	116.37	1.152	0.85
FCR, g/g	1.35	1.30	1.36	1.36	0.011	0.12
Finisher (d 29-41)						
BW (41 d, g)	3274.39	3319.32	3305.02	3185.67	34.625	0.54
ADG, g	116.54	118.09	121.3	114.52	2.146	0.74
ADFI, g	185.56	182.63	186.18	179.37	2.348	0.75
FCR, g/g	1.60	1.55	1.54	1.57	0.015	0.54
Whole phase (d 1-41)						
ADG, g	76.57	77.88	77.54	74.70	0.825	0.54
ADFI, g	110.06	108.87	110.66	108.12	0.982	0.81
FCR, g/g	1.44	1.40	1.43	1.45	0.010	0.32
Morality, %	16.25	10	12.5	10	-	-

[1]BW, body weight; ADFI, average daily feed intake; ADG, average daily gain; FCR, feed conversion ratio.

[2]NC, negative control, birds fed with basal diet; PC, positive control, birds fed with 30 ppm avilamycin premix; EOL, birds fed 50 ppm encapsulated cinnamaldehyde; EOH, birds fed 100 ppm encapsulated cinnamaldehyde. [3]SEM, standard error of the means.

[3]SEM, standard error of the means.

Table 6. 4 Effects of encapsulated cinnamaldehyde at either 50 ppm or 100 ppm in feed on organ weights of broiler chickens.

Items	Treatments[1]				SEM[2]	*P*-value
	NC	PC	EOL	EOH		
Heart	0.42	0.43	0.45	0.45	0.007	0.28
Liver	2.25	2.16	2.25	1.97	0.053	0.22
Bursa	0.15	0.14	0.15	0.15	0.005	0.55
Spleen	0.08	0.08	0.09	0.08	0.003	0.89

[1]NC, negative control, birds fed with basal diet; PC, positive control, birds fed with 30 ppm avilamycin premix; EOL, birds fed 50 ppm encapsulated cinnamaldehyde; EOH, birds fed 100 ppm encapsulated cinnamaldehyde.

[2]SEM, standard error of the means.

6.4.2 Meat quality

No treatment effects were found on cooking time (min), purge loss, dripping loss, pH values (96 h), color (L^*, a^*, b^*), white striping (WS), and woody meat (WB). Despite meat pH at 24 h post slaughter broilers fed EOH tended to be higher ($P = 0.05$) than other treatments, all pH values were at the normal range. However, birds fed in PC and EOH treatments yielded ($P < 0.05$) the WBSF (kg) in breast meat compared to NC birds (Table 6.5).

Table 6. 5 Effects of encapsulated cinnamaldehyde at either 50 ppm or 100 ppm in feed on breast meat quality of broilers.

Variables[1]	NC[2]	PC	EOL	EOH	SEM[3]	*P*-value
WBSF (kg)	1.95[a]	1.46[b]	1.59[ab]	1.33[b]	0.16	0.04
Cooking loss (%)	23.11	23.63	23.24	23.04	0.85	0.96
Cooking time (min)	37.52	37.96	35.85	35.8	1.47	0.31
pH $_{24\ h}$	6.12[a]	6.18[ab]	6.20[ab]	6.21[b]	0.078	0.05
pH $_{96\ h}$	5.41	5.61	5.56	5.52	0.316	0.54
Dripping loss (%)	1.34	1.26	1.29	0.78	0.37	0.69
L*	58.68	59.86	58.49	59.62	0.57	0.23
a*	11.52	10.91	11.56	11.09	0.24	0.14
b*	18.69	19.06	17.56	18.02	0.45	0.09
MFI (mg/mL)	39.54	37.99	40.28	37.54	0.91	0.69
WS % (n)						
WS scores	0.19	0.32	0.33	0.27	0.06	0.52
Normal	95.24 (20)	90.91 (20)	87.10 (27)	89.29 (25)	-	0.88
Moderate	4.76 (1)	9.09 (2)	12.9 (4)	10.71 (3)	-	-
Severe	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	-	-
WB % (n)						
WB scores	0.08	0.27	0.35	0.34	0.08	0.28
Normal	100 (21)	86.36 (19)	80.65 (25)	85.71 (24)	-	0.38
Mild	0.00 (0)	13.64 (3)	16.13 (5)	14.29 (4)	-	-
Moderate	0.00 (0)	0.00 (0)	3.23 (1)	0.00 (0)	-	-
Severe	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	-	-

[1]WBSF, Warner-Bratzler shear force (kg); WS, white striping; WB, woody breast; L*, lightness; a*, redness; b*, yellowness; MFI, Myofibril Fragmentation Index.

[2]NC, negative control, birds fed with basal diet; PC, positive control, birds fed with 30 ppm avilamycin premix; EOL, birds fed 50 ppm encapsulated cinnamaldehyde; EOH, birds fed 100 ppm encapsulated cinnamaldehyde.

[3]SEM, standard error of the means.

6.4.3 Intestinal morphology, jejunal gene expression and ileal digestibility

Feeding EOL or EOH to birds had no significant effect on VH and CD of duodenum jejunum and ileum of day-41 old broilers. However, EOL and EOH feeding increased ($P < 0.05$) VH/CD compared to NC and PC birds, with average increases by EOL and EOH respectively, being 14.67% and 15.13% in the duodena and 15.13% and 13.58% in the jejuna (Figure 6.1).

For jejunal gene expression, 6 of the 11 studied genes, including CDH-1, PCNA, ZO-1, B^0AT-1, CAT-1, and MG showed significant ($P < 0.05$) effects of dietary treatments. Birds fed EOL or EOH increased ($P < 0.05$) expressions of CDH-1 and B^0AT-1 compared to PC and NC, respectively. The PCNA expression was significantly downregulated ($P < 0.05$) with the EOL supplemented diet compared to the NC treatment. The ZO-1 expressions were significantly upregulated in EOH treatments compared to PC ($P < 0.05$) but not different from NC treatment. Nonetheless, EOH fed birds showed a higher expression ($P < 0.05$) of CAT-1 and MG compared to PC (Figure 6.2).

The AID of DM, CP, CF, and 18 amino acids (alanine, arginine, aspartate, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylamine, proline, serine, threonine, tryptophan, tyrosine, and valine) were improved in birds fed EOL compared to NC birds ($P < 0.05$). However, when birds were fed EOH, no increases in AID of DM and CP were observed, and only eight amino acids (alanine, arginine, glutamine, isoleucine, leucine, phenylamine, tyrosine, and valine) showed a higher ($P < 0.05$) AID than NC birds. Additionally, as shown in Table 6.6, birds on PC showed an increased ($P < 0.01$) AID of CF and digestibility of 13 amino acids (alanine, arginine, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, phenylamine, threonine, tyrosine, and valine) compared to NC birds.

Table 6. 6 Effects of cinnamaldehyde at either 50 ppm or 100 ppm in feed on dry matter, crude fat, crude protein, and amino acid digestibility.

Item[1]	Treatments[2]				SEM[3]	*P*-value
	NC	PC	EOL	EOH		
DM	71.26^{b}	71.96^{b}	76.21^{a}	69.39^{b}	0.514	< 0.01
CF	81.51^{b}	87.18^{a}	89.70^{a}	86.36^{a}	0.765	< 0.01
CP	76.88^{b}	79.83^{b}	84.03^{a}	79.14^{b}	0.632	< 0.01
Ala	78.12^{b}	84.78^{a}	87.25^{a}	82.94^{a}	0.905	< 0.01
Arg	79.51^{c}	87.27^{ab}	89.76^{a}	85.49^{b}	0.915	< 0.01
Asp	74.10^{bc}	79.15^{ab}	81.35^{a}	77.11^{ab}	0.933	0.03
Cys	70.25^{b}	79.00^{a}	80.15^{a}	73.32^{ab}	1.188	< 0.01
Glu	82.42^{c}	87.23^{ab}	88.94^{a}	86.13^{ab}	0.680	< 0.01
Gly	71.16^{c}	77.75^{ab}	80.20^{a}	75.10^{abc}	1.035	0.01
His	54.48^{bc}	62.91^{a}	67.68^{a}	59.54^{ab}	1.301	< 0.01
Ile	64.74^{c}	80.48^{ab}	82.47^{a}	78.87^{ab}	1.547	< 0.01
Leu	78.24^{c}	85.54^{ab}	89.12^{a}	84.67^{b}	0.867	< 0.01
Lys	82.60^{c}	87.88^{ab}	89.15^{a}	83.12^{bc}	0.791	< 0.01
Met	89.65^{b}	92.10^{ab}	94.38^{a}	90.38^{b}	0.546	0.01
Phe	77.12^{c}	84.62^{ab}	88.13^{a}	83.64^{b}	0.874	< 0.01
Pro	79.57^{b}	83.89^{ab}	86.36^{a}	81.92^{ab}	0.812	0.02
Ser	75.55^{b}	80.95^{ab}	83.11^{a}	79.10^{ab}	0.862	0.01
Thr	65.94^{b}	76.35^{a}	78.78^{a}	71.39^{ab}	1.374	< 0.01
Trp	78.26^{b}	80.70^{ab}	84.81^{a}	79.82^{ab}	0.869	0.04
Tyr	73.11^{c}	84.79^{ab}	88.45^{a}	83.16^{b}	1.212	< 0.01
Val	62.35^{c}	79.13^{ab}	84.27^{a}	77.32^{b}	1.701	< 0.01

[1]DM, dry matter; CF, crude fat; CP, crude protein.

[2]NC, negative control, birds fed with basal diet; PC, positive control, birds fed with 30 ppm avilamycin premix; EOL, birds fed 50 ppm encapsulated cinnamaldehyde; EOH, birds fed 100 ppm encapsulated cinnamaldehyde.

[3]SEM, standard error of the means.

Figure 6. 1 Effects of encapsulated cinnamaldehyde at either 50 ppm or 100 ppm on villus height (A), crypt depth (B) and ratio of villus height and crypt depth (C) on duodenum, ileum, and jejunum in broilers. Significant differences are indicated by letters (a, b).

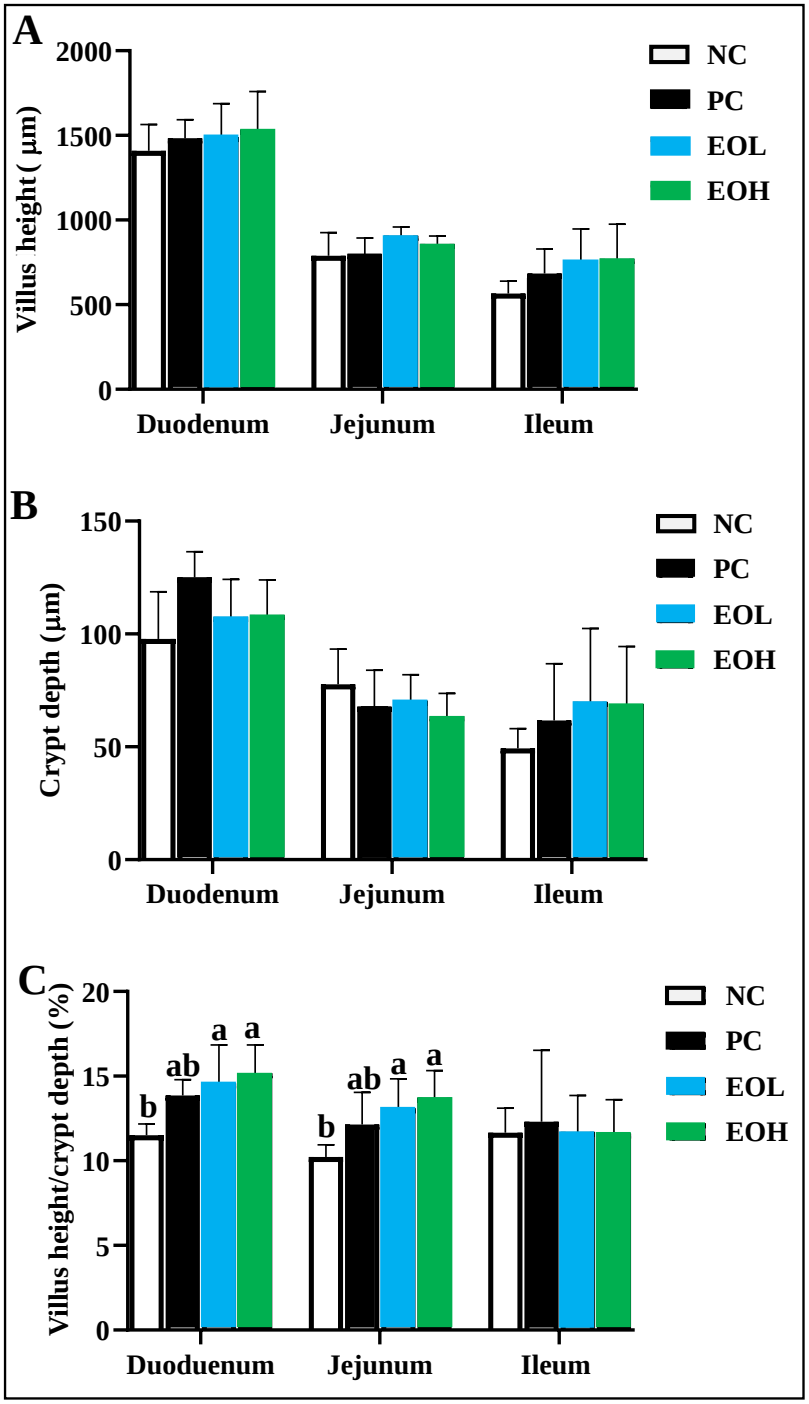
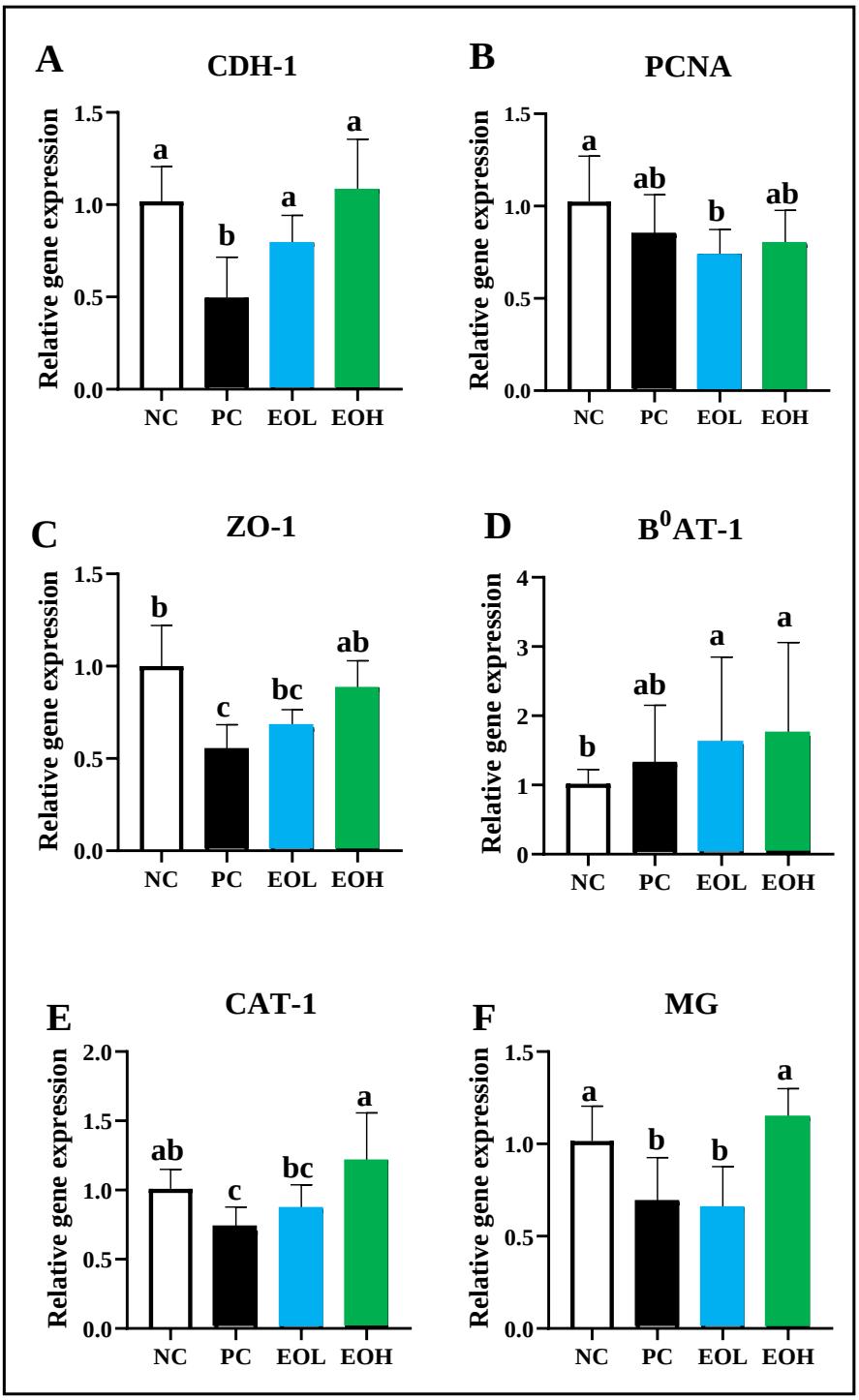


Figure 6. 2 Effects of cinnamaldehyde at either 50 ppm or 100 ppm on jejunal gene expressions of CDH-1 (A), PCNA (B), ZO-1 (C), B^0AT-1 (D), CAT-1 (E) and MG (F). Significant differences are indicated by letters (a, b, c).



6.4.3 Ileal and cecal microbiota

Four bacterial phyla (Firmicutes, Bacteroidetes, Proteobacteria, and Cyanobacteria) with greater than 1% relative abundance were observed in both ileal and cecal digesta (Figure 6.3). A lower relative abundance of Proteobacteria was observed in ileal digesta of EOH-fed birds than in NC birds ($P < 0.01$) while a higher proportion of Bacteroidetes were observed in cecal digesta of EOL- and EOH-fed birds than in those of NC and PC birds ($P < 0.01$). At the genus level, ileal and cecal digesta from EOH-fed birds showed the highest relative abundance of *Lactobacillus* population ($P < 0.01$) compared to other treatments, but a higher relative abundance of *Turicibacter* was observed in ileal digesta in EOL-fed birds ($P < 0.01$), than those in NC, PC and EOL groups. No significant differences were observed between treatments for alpha diversity (richness and diversity) including Chao1, Shannon, and Simpson indices (Table 6.7). Principal Coordinates Analysis (PCoA) of the microbiota according to weighted UniFrac phylogenetic distances showed that the majority of samples from ileal (blue) and cecal (red) digesta clustered separately ($P < 0.01$). In ileal samples, EOL was separated from NC ($P < 0.05$), PC ($P < 0.05$), and EOH ($P < 0.01$). In cecal samples, EOL was separated from NC ($P < 0.05$) and PC ($P < 0.05$). However, there were no significant differences between NC, PC and EOH in both ileal and cecal samples (Figure 6.4).

Table 6. 7 Summary of alpha-diversity measurements of microbiota in ileum and cecum of broilers treated with avilamycin premix or cinnamaldehyde.

α-diversity	Gut segments	Treatments[1]				SEM[2]	*P*-value
		NC	PC	EOL	EOH		
Observed OTUs	ileum	32.14	32.86	32.13	34.14	1.490	0.97
	cecum	100.50	89.17	92.75	110.29	4.952	0.82
Chao1	ileum	33.29	37.75	38.14	34.60	1.854	0.79
	cecum	100.14	93.63	94.57	110.13	6.200	0.78
Shannon	ileum	4.38	4.50	4.39	4.73	0.079	0.43
	cecum	6.27	6.28	6.03	6.12	0.071	0.82
Simpson	ileum	0.94	0.94	0.94	0.95	0.003	0.39
	cecum	0.98	0.98	0.98	0.98	0.001	0.91

[1]NC, negative control, birds fed with basal diet; PC, positive control, birds fed with 30 ppm avilamycin premix; EOL, birds fed 50 ppm encapsulated cinnamaldehyde; EOH, birds fed 100 ppm encapsulated cinnamaldehyde.

[2]SEM, standard error of the means.

Figure 6. 3 The 3D principal coordinate analysis (PCoA) graph shows the variation among distance matrixes (unweighted UniFrac) of ileal (A) and cecal (B) microbiota alone or together (C) in birds treated with avilamycin premix or encapsulated cinnamaldhyde. Percentages shown are percentages of variation explained by the PC1 (28.42%), PC2 (6.15%), and PC3 (3.94%).

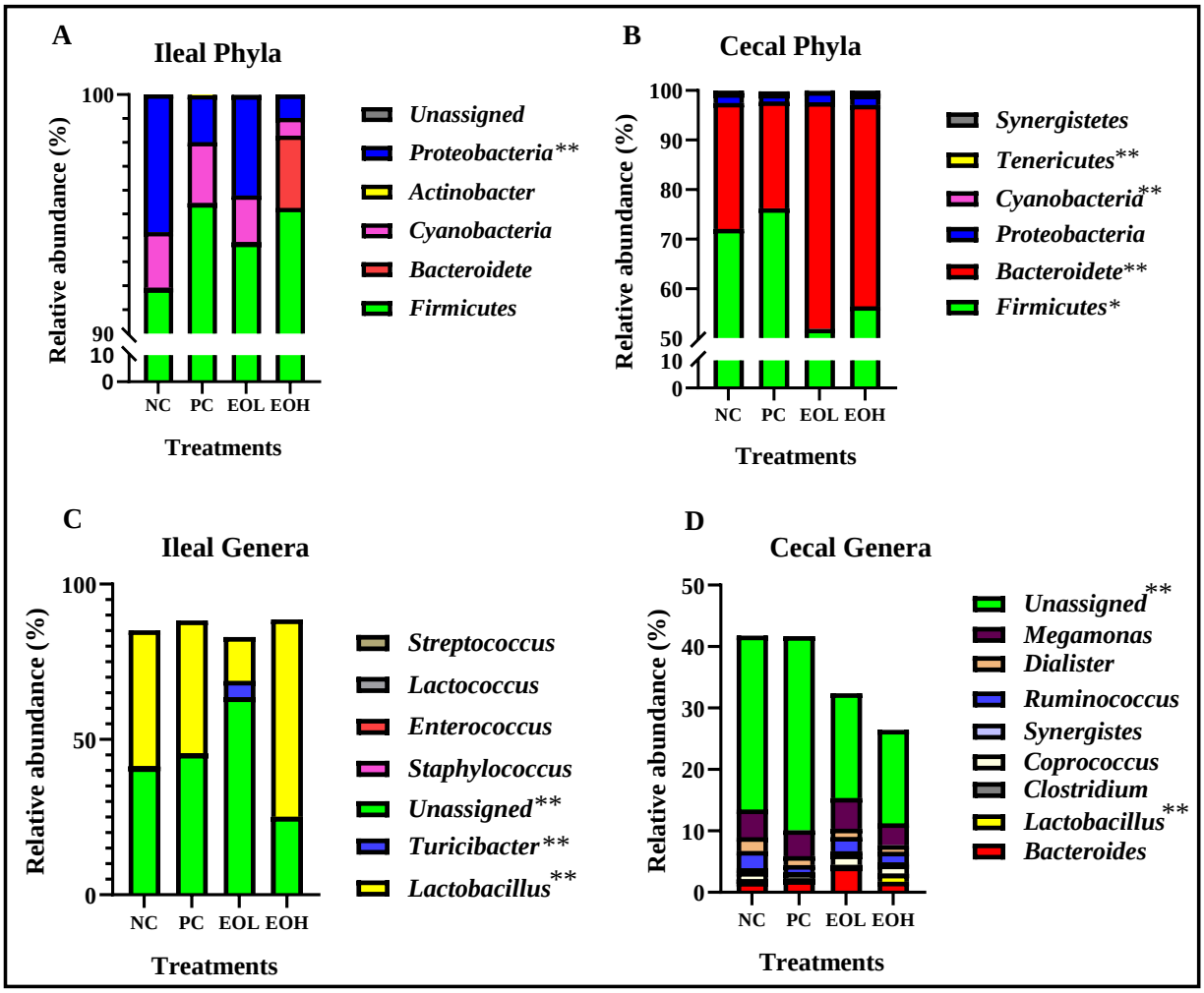
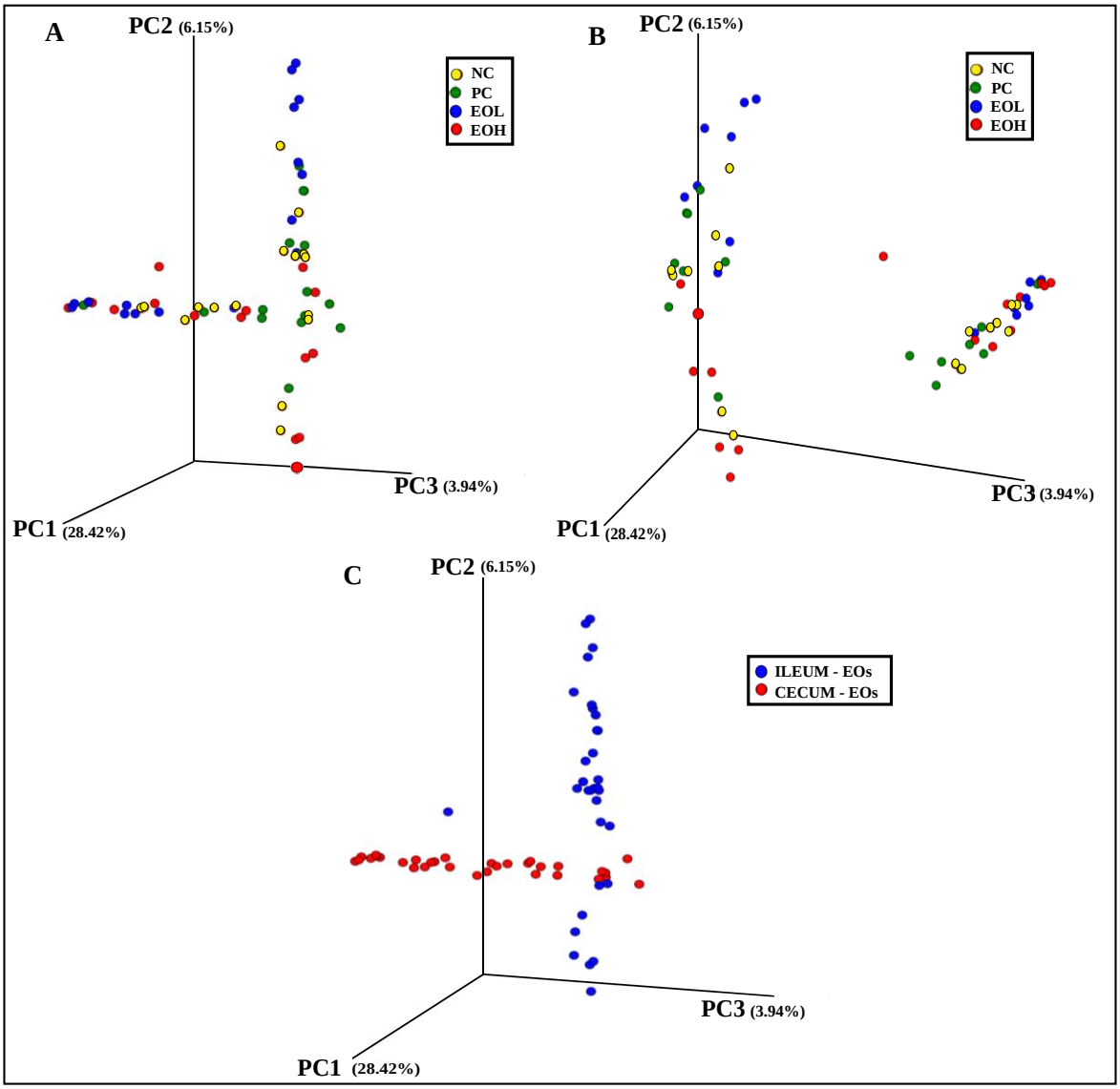


Figure 6. 4 The 3D principal coordinate analysis (PCoA) graph shows the variation among distance matrixes (unweighted UniFrac) of ileal (A) and cecal (B) microbiota alone or together (C) in birds treated with avilamycin premix or encapsulated cinnamaldhyde and citral in combination. Percentages shown are percentages of variation explained by the PC1 (28.42%), PC2 (6.15%), and PC3 (3.94%).



6.5 Discussion and conclusion

A recent study found that dietary supplementations of encapsulated CIN (100 mg/kg) improved growth performance, reduced gut lesions caused by *Eimeria* spp. and *C. perfringens*, and modulated cecal microbiota in broiler chickens that received coccidiosis vaccine (Yang et al., 2020). In the present study, no vaccination was applied and a lower dosage of CIN (50 mg/kg) was selected in addition to the 100 mg/kg to evaluate their effects on performance and gut health parameters including some organ's weight, meat quality, intestinal morphology, jejunal gene expression, and ileal nutrient digestibility. Avilamycin (30 mg/kg), which is another common antibiotic besides bacitracin (55 mg/kg) used in broiler chicken production to prevent NE lesions caused by *C. perfringens*, was supplemented in diets as the PC (Canadian Food Inspection Agency, 2020).

Data showed no significant improvement of BW, ADG, and FCR by the inclusion of either CIN (50 or 100 mg/kg) or 30 mg/kg avilamycin in feeds. The result was inconsistent with a previous study (Yang et al., 2020), which may be due to the feeding conditions since it has been reported that the growth performance of broilers could be altered by different broiler-housing conditions (Mesa et al., 2017). The high mortality rate in the present study could be ascribed to a high rate of culling birds due to injuries according to the guidelines (Canadian Council on Animal Care, 2009). High mortality could be explained by high BW (> 3,000 g in average at day 41) in this study, which could have increased the incidence of injuries (Martins et al., 2016). Notably, birds fed EOL and EOH had a relatively lower mortality than those in NC. In poultry barns, enteric diseases of broilers including coccidiosis and NE could be the main cause of high mortality (Christaki et al., 2004; Cooper et al., 2013). In this study, the lower mortality in birds fed EOL and EOH may suggest that enteric infections could have been controlled by the supplementation of CIN at 50 or 100 mg/kg when compared to the previous study (Yang et al., 2020).

Any abnormal changes to organ weights including heart, liver, spleen, and bursa are indicators of chicken health disorders (Bowes and Julian, 1988). No changes in bursa and spleen weights may suggest that no disorders were generated after feeding CIN since bursa and spleen could get larger due to inflammation (Cazaban et al., 2015). No differences in liver and heart weights could suggest that CIN may not be toxic to birds since toxicity could be one of the major causes of abnormal liver and heart weight gains (Zaefarian et al., 2019), which was also consistent with results from a previous study in which birds were fed CIN powder (Najafi and Torki, 2010). However, toxic assessment is required in further studies.

Intestinal morphology, gene expression, nutrient digestibility, and gut microbiota, are four major parameters to reflect gut health. In this study, the higher VH/CD in duodena and jejuna detected in broilers fed EOL and EOH indicated the potential of CIN in improving nutrient digestion, absorption, and gut barrier function. Higher VH/CD could explain increased AID of DM, CP, CF, and AAs when birds were fed EOL or EOH. The improvement of AID in broiler chickens was also reported in a previous study when birds were fed with mixtures of oregano, CIN, and pepper oils (Hernandez et al., 2004). Interestingly, lower AID of DM, CP, and AAs such as lysine and tryptophan were shown in birds fed EOH compared to EOL. This may be due to high concentrations of chemical compounds (aldehyde) in EOH that can block lysine and tryptophan residuals from digestive enzymes (Rawel et al., 2002). Another interesting finding in the current study was that mRNA expression of some proteins for nutrient absorption, gut barrier integrity, and DNA repair was altered by EOL or EOH. In this study, higher mRNA expressions for B^0AT-1 (compared to NC) and CAT-1 (compared to PC) in birds fed EOH were observed, suggesting improvements in neutral amino acids and cationic amino acids absorption (Gilbert et al., 2007). Additionally, higher MG expressions in birds fed EOH compared to PC and EOL indicated higher maltase-glucoamylase expressions for carbohydrate digestion (Diaz-Sotomayor et al., 2013). Since lower AID of DM was observed in birds fed EOH compared to EOL in this study, upregulated mRNA expression of MG in birds fed EOH could be compensatory feedback to maximize carbohydrate digestion (Ebrahimi et al., 2015). Tight junctions are multi-protein complexes that regulate ion and water transportations and prevent the entry of harmful substances such as pathogens and endotoxins (Pitman and Blumberg, 2000). A recent study on IPEC-J2 cells in our lab reported that thymol oil could enhance intestinal barrier function by increasing gene expression of ZO-1 (Omonijo et al., 2019). The present study suggested that birds fed EOH could improve gut barrier function when compared to birds fed PC. Additionally, lower mRNA expressions of PCNA may suggest alleviated pathogenic inflammations in birds fed EOL. This is because mRNA expression of PCNA was higher in animals with inflammation compared to those without inflammation (Manohar and Acharya, 2015). For ileal microbiota, lower phylum Proteobacteria and higher genus *Lactobacillus* indicated that broilers fed EOH may possess the ability to control the growth of pathogenic bacteria. This is because phylum Proteobacteria includes pathogenic genera such as *Salmonella* and *Campylobacter*, which are associated with inflammatory disorders in hosts (Moon et al., 2018), while many species in genus *Lactobacillus* such as *L. acidophilus* are beneficial bacteria (Azad et al., 2018). For cecal microbiota, higher phylum Bacteroidetes

suggested increased carbohydrate degradation and propionate synthesis via succinate pathway in birds supplemented with CIN, and higher genus *Lactobacillus* demonstrated improvements of beneficial bacterial populations (Glendinning et al., 2019). The current results differ from the previous study in which that genus *Lactobacillus* was found to be more abundant in the cecum of broilers on citral, but not on CIN compared to birds fed a basal diet (Yang et al., 2020). Additionally, no significant differences of alpha diversity suggest that broilers fed EOL or EOH had minor effects on the richness and diversity of microbiota. The PCoA results showed that the diversity of ileal microbiota was significantly different from cecal microbiota, which is consistent with a recent study (Rios-Covian et al., 2017).

Increased AID, improved intestinal morphology, enhanced expressions of nutrient transporters, and altered ileal and cecal microbiota in birds fed EOL or EOH did not seem to promote growth performance in this study. This may be because other factors such as genetics, management, and environment can affect the growth performance of broilers (Craig et al., 2016). Additionally, in comparison to the recommendation in Cobb 500 guidelines (Cobb-Vantress Inc., 2012), the FCR values in each treatment were lower than provided in the recommendation, indicating the growth performance in the present study was reaching the optimum genetic potential and there is little room for improvement.

In addition to the importance of bird growth performance and gut health, post-slaughtering meat quality is essential in ensuring consumers' satisfaction and it could be judged by parameters including color, pH, water holding capacity (WHC), and scores of myopathies (Baracho et al., 2006). Since muscle myopathies such as WS and WB are frequently observed in heavy birds (> 3,500 g live weight), four birds per pen were fed finisher diet until d 49 to investigate the effects of CIN on breast meat quality (Kuttappan et al., 2013; Mogire et al., 2021). Because the fast-growing birds may be exposed to heat stress and pre-slaughtering stress in the current intensive poultry farming system that often reduces meat quality (Sandercock et al., 2009), we tested the effects of CIN on breast meat quality of broiler chickens in the present study. Meat color is a critical parameter affecting consumer selection of deboned and skinless raw meats in markets (Qiao et al., 2001). In this study, all meat color parameters were normal and no color changes were found in breast meat from broilers fed EOL and EOH compared to NC and PC. This may be due to antioxidant compounds (aldehyde) in CIN, which could maintain meat color by preventing further oxidation of lipids and myoglobins caused by air exposure (Kanani et al., 2017). Additionally, no changes in dripping loss (%) and cooking loss (%) may suggest that feeding birds

with EOL or EOH did not have adverse effects on WHC of meat during storage, thawing and cooking, thereby reflecting no alterations to meat juiciness (Bowker, 2017). Despite the observation of a higher pH value in EOH compared to NC at 24 h post-slaughter, no pH differences among treatments were detected at 96 h. This result was in line with lack of alternations in meat color, cooking loss (%) and dripping loss (%) among treatments since any abnormal meat pH could cause alterations of these parameters (Mir et al., 2017). Interestingly, a similar study did not detect changes in pH, cooking loss (%) and dripping loss (%) in broiler chickens that were fed cinnamic bark powder at 200 mg/kg (Logaranjani, 2014). Myopathies on breast meat, such as WB and WS, reflect histological stress including lipolysis, fibrosis, necrosis, and myo-degeneration (Kuttappan et al., 2012; Kuttappan et al., 2013). In this study, we did not observe any differences in WB and WS scores among treatments. This may be due to better management and relatively lower stock density at the research barn used in the current study, compared to commercial farms (Kuttappan et al., 2016). Thus, a future study with higher bird numbers and a density comparable to commercial farms is required to test the effects of CIN on WB and WS in broiler chickens. Additionally, the WBSF and MFI are another two meat quality parameters in predicting tenderness (Lyon and Lyon, 1990). The lower WBSF in birds treated with EOH compared to NC suggested that CIN may have the potential to enhance meat tenderness, which is consistent with the results obtained in a previous study when birds were fed CIN (Gomathi et al., 2018).

In conclusion, this study indicated that encapsulated CIN in broiler feed could promote meat tenderness by reducing WBSF and gut health by improving AID, intestinal morphology and microbiota, and expressions of nutrient transporters. The impacts of CIN on growth performance were not significant probably due to the birds having already obtained their optimum performance. Future studies are necessary to conduct a complete assessment to investigate the toxicity, safety, and economic impacts of encapsulated CIN in broiler chickens.

CHAPTER SEVEN: MANUSCRIPT IV

Virulence Potential of Antimicrobial Resistant Extraintestinal Pathogenic *Escherichia coli* from Poultry in a *Caenorhabditis elegans* Model[4]

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7.1 Abstract

Encapsulated cinnamaldehyde has high potential as antimicrobial alternatives to improve chicken health and antimicrobial susceptibilities of commensal bacteria according to previous studies (Chapter Four, Five, Six). However, even healthy poultry can also be a reservoir for extraintestinal pathogenic *Escherichia coli* (ExPEC) and these ExPEC strains could contaminate the environment and/or food chain posing food safety and human health risk. Moreover, zoonotic potentials of poultry-source AMR ExPEC are still controversial. This study investigated virulence potentials of poultry-source antimicrobial resistant (AMR) extraintestinal pathogenic *Escherichia coli* (ExPEC). A total of 46 *E. coli* isolates from chicken or turkey retail meats and feces (chicken), or humans were sequenced and identified as ExPEC. Based on their characteristics, eight of these ExPEC isolates with various antimicrobial resistance genes (ARGs) and virulence genes (VGs) were evaluated for their pathogenic potentials using a *Caenorhabditis elegans* infection model. Except eight, all remaining 38 ExPEC were resistant to at least one antibiotic and carried corresponding ARGs. About 27 ExPEC were multidrug resistant ($\geqslant$3 antibiotic classes). Eight ExPEC were of serotype O25: H4 and Sequence Type (ST) 131. Interestingly, all ST 131 isolates from chicken or turkey meats clustered with an isolate from a human urinary tract infection (UTI) case having the same serotype and ST. The human UTI isolate induced a similar effect as the K88+ ETEC strain JG280 on reducing ($P < 0.05$) survival of *C. elegans*. Chicken and turkey meat and fecal ExPEC, caused similar negative impacts on the survival of worms as the human UTI isolate, compared to OP50 (negative control). However, the survival of *C. elegans* was not reduced with an increasing number of VGs and did not seem to be affected by antimicrobial susceptibility profiles. The data from this study indicated the virulence potential of AMR ExPEC isolates from retail poultry meat or feces. Relationships between specific AMR profiles and/or VGs with pathogenicity in these *E. coli* isolates deserve further instigations.

Key words: antimicrobial resistance, *Caenorhabditis elegans*, extraintestinal pathogenic *E. coli*, poultry, virulence gene

7.2 Introduction

Poultry meat products provide essential nutrients for humans, which are worth over 2.8 billion Canadian dollars in 2019 in Canada (Canada's Chicken Industry, 2019). Contamination of poultry meat by a variety of pathogens pose a human health risk (Aslam et al., 2014). *E. coli* is a Gram-negative bacterium that is a common inhabitant of gastrointestinal tracts (GIT) in poultry.

Despite that most of *E. coli* are harmless commensals in the GIT, pathogenic *E. coli* can cause enteric or extraintestinal infections (Clements et al., 2012). In poultry, colibacillosis is caused by avian pathogenic *E. coli* (APEC) belonging to Extraintestinal pathogenic *E. coli* (ExPEC) pathotype which also includes uropathogenic *E. coli* (UPEC) which cause urinary tract infections (UTI) (Bergeron et al., 2012). Interestingly, it has been suggested that some poultry-source ExPEC share similar virulence genes (VGs) as human ExPEC which indicate their potential to cause human infections such as UTI (Colobătiu et al., 2014). It was reported that some ExPEC from contaminated poultry meats could form biofilm and survive through human GIT (Mitchell et al., 2015). Additionally, ExPEC in poultry is increasingly recognized as a reservoir of antimicrobial resistance genes (ARGs) for other bacteria (Brook et al., 2013; Coleman et al., 2013).

There are serious ethical limits to performing experimental infections in humans to analyze virulence potential microorganisms (Apfeld and Alper, 2018). Due to limitations of testing poultry-source ExPEC on humans, we explored the use of C. elegans life-span model for evaluating the infection of ExPEC. Since *C. elegans* is easy to culture, its genome has counterparts in humans and shares many similar biological pathways with humans, there are a number of studies that use the nematode as an animal model to predict human diseases (Corsi et al., 2015, Schifano et al., 2019). In addition, the *C. elegans* model has already been applied for studying the relationship between human infections and pathogens from poultry or other livestock animals including *Staphylococcus aureus* (Sifri et al., 2003), *Enterococcus faecalis* (Garsin et al., 2001), *Salmonella enterica* serovar *Typhimurium* (Liu et al., 2020; Zhou et al., 2021), and *E. coli* (Zhou et al., 2014).

The results in previous studies (Chapter Four, Five, and Six) have indicated that encapsulated cinnamaldehyde (CIN) can improve health status of broiler chickens by improving growth performance, gut health, meat quality, and reducing AMR levels (%) of fecal *E. coli* (Yang et al., 2020; Yang et al., 2021a, b). Nonetheless, healthy poultry can also be the reservoir of AMR ExPEC that may contaminate human food chains. Besides, no strong evidence has shown zoonotic risks of poultry-source AMR ExPEC. In addition, whether antimicrobial susceptibility tests (ASTs) and number of VGs profiles of isolates affect zoonotic potentials are unclear (Mellata, 2013). Hence, it is important to determine the zoonosis of poultry-source AMR ExPEC containing various VGs and ASTs. The objectives of this study were: 1) to compare and analyze AMR phenotypes, genotypes and virulence of 46 ExPEC from human, chicken or turkey meat, and chicken feces; 2) to establish *C. elegans* life-span assays that allows the measurement of nematode responses to

AMR ExPEC isolate from human UTI, chicken or turkey retail meats, and chicken fecal samples in comparison with the human ExPEC isolate; 3) to determine whether ASTs and number of VGs in poultry-source AMR ExPEC affect *C. elegans* survival rate.

7.3 Materials and methods

7.3.1 Bacterial strains

A total of 46 *E. coli* isolates were used in this study. Isolates from chicken (n = 30) and turkey (n = 11) meats were partially described as ExPEC by Aslam et al. (2014). An ExPEC from human UTI isolate was collected in Montréal (Quebec, Canada) during 2005-2007 described by Bergeron et al. (2012). Four fecal isolates were recovered on ChromoCult coliform agar (CCA) after incubation at 37ºC for 16 h and dark blue colonies were further identified by API-20E Biochemical Test Strip (Yang et al., 2021a). Additionally, K88+ ETEC strain JG280 and *E. coli* OP50 were cultured in Luria-Bertani (LB) medium at 37ºC for 16 h prior to use as positive and negative control, respectively (Zhou et al., 2014; Zhou et al., 2018). All isolates were stored in sterilized screw-capped tubes with 20% glycerol in a -80°C freezer before use.

7.3.2 Antimicrobial susceptibilities, whole genome sequencing, and phylogenetic analysis

All isolates were grown on Muller Hinton agar (MHA) for 16 h at 37°C and were prepared for analyzing ASTs by Sensititre^{TM} Complete Automated Microbiology System in Guelph Research and Development Centre, Agriculture Agri-Food Canada. The Clinical and Laboratory Standards Institutes (CLSI) breakpoints (CLSI, 2015) and the Canadian Integrated Program for Antimicrobial Resistance Surveillance guidelines (CIPAS, 2008) were used to interpret minimal inhibitory concentration (MIC). All isolates were tested against a panel of antimicrobials in three categories (I, II, and III). The category I antimicrobials (amoxicillin, ceftiofur, ceftriaxone, and ciprofloxacin) represented very high importance in human medicine; while the category II (ampicillin, azithromycin, cefoxitin, gentamicin, nalidixic, streptomycin, and trimethoprim) and category III (chloramphenicol, sulfonamide, and tetracycline) represented high and medium importance, respectively.

The presence of ARGs, plasmids, and VGs of all 46 isolates were determined by whole genome sequencing (WGS) as described (Yang et al., 2021a). Briefly, genomic DNA was extracted from 16 h-grown cultures using DNeasy Blood & Tissue Kits (QIAGEN). The DNA quality was checked by 1.0% gel electrophoresis and stored at -20°C before performing WGS. The sequencing libraries were prepared using the Illumina Nextetra XT DNA sample preparation kit (Illumina, Inc., CA, USA) and paired-end sequencing was performed using MiSeq reagent jit (v3)

on an Illumina Miseq Platform (Illumina Inc., CA, USA). The DNA sequences were analyzed and qualities were checked using FastQC. The reads were combined by Fast Length Adjustment of Short reads (FLASH) c1.2.9 (Magoc and Salzberg, 2011). High-quality reads were assembled in Spades v. 3.0 integrated in The Integrated Rapid Infectious Disease Analysis (IRIDA) platform (Bankevich et al., 2012). The assembled genomes were annotated by Prokka version 1.11 (Seemann, 2014). The Resistance Gene Identifier version 4.0.2 platform (Comprehensive Antibiotic Resistance Database, 2018), PlasmidFinder 1.3 (Center for Genomic Epidemiology, 2018a), and VirulenceFinder (Center for Genomic Epidemiology, 2018b) databases were used to identify ARGs, plasmid replicon, and VGs in the assembled sequenced genomes.

The phylogenetic analysis was conducted using core genome single nucleotide variant phylogenomics (SNVphyl) pipeline with SNVs calling at a minimum mapping quality of 30, minimum base quality of 30, minimum alternate fraction of variant bases in agreement 80%, and minimum depth of coverage of 15x (Petkau et al., 2017). The human ExPEC strain was used as a reference in this study. A maximum-likelihood phylogeny with bootstrap supports was built in FigTree. 1.4.4.

7.3.3 *C. elegans* and bacterial preparation

A temperature-sensitive mutant *C. elegans* SS104 was obtained from Caenorhabditis Genetics Center, University of Minnesota (Minneapolis, MN, USA). The frozen *C. elegans* solution from -80°C was revived and maintained on a nematode growth media (NGM) agar seeded with *E. coli* OP50 in an aerobic incubation at 16°C (Stiernagle, 2006).

C. elegans life-span assays were performed using the method described previously (Ikeda et al., 2007). Briefly, to synchronize *C. elegans* to the same stage, adult worms were treated with sodium hydroxide (final concentration is 0.5 M) and fresh sodium hypochlorite (bleach, final concentration is 0.5%), followed by vortexing at 30 secs intervals to release eggs (Stiernagle, 2006). The released eggs were washed by sterilized water three times and were hatched on NGM agar at 16°C for 60 h until they reached larval stage 4 (L4). The L4 worms were washed three times in S medium via centrifugation (1300 × g for 2 minutes) and suspension. The formula of S medium was described in a previous protocol (Cold Spring Harbor Protocols, 2019). Approximately 15-20 worms were transferred to each well of a 24-well plate containing 2 mL of S medium and incubated at 25°C. The worms were then mixed with the selected bacterial isolates that have been washed twice in S medium. To determine the survival rate of *C. elegans*, the live worms were counted and recorded daily for 12 days. The percentage of surviving worms was calculated by the following

formula: survival (%) = (live worms/total worms used) × 100. A worm was considered dead when it failed to respond to touch by a sterilized steel loop.

Life-span assay I. To investigate the survival (%) of *C. elegans* in response to human ExPEC infection, an ExPEC isolate (ID # 5305) from human UTI at three different concentrations (1×10^8, 5×10^8, and 1×10^9 CFU/mL) were incubated with worms in three replicates in 24-well plates in an aerobic incubator at 25°C. In parallel, both *E. coli* OP50 (negative control) and $K88^+$ ETEC strain JG280 (positive control) were treated in the same manner with the same concentrations.

Life-span assay II. To estimate the responses of *C. elegans* to the infection of AMR ExPEC isolates from poultry retail meat, two AMR ExPEC isolates (ID # 4557 and 4873) from turkey meat and one ExPEC isolate (ID #4604) from chicken meat at two concentrations (5×10^8, 1×10^9 CFU/mL) were selected for life-span assays. The *E. coli* OP50 (negative control), JG280 (positive control), and the human ExPEC isolate (ID # 5305) were parallel treated with the same concentrations.

Life-span assay III. To evaluate the possible effects of chicken fecal AMR ExPEC isolates with different ASTs and number of VGs on the survival (%) of *C. elegans*, four AMR ExPEC isolates (ID # 2448, 6841, 6941, and 6859) at 5×10^8 CFU/mL were selected for the life-span assay along with *E. coli* OP50 (negative control), JG280 (positive control), and the human ExPEC isolate (ID # 5305).

7.3.4 Data analysis

All data analyses were performed using SAS 9.4 (SAS Institute Inc., Gary NC). Survival (%) curves for *C. elegans* life-span assay were compared by Kaplan–Meier survival analysis followed by a log-rank test. A P-value < 0.05 was used to declare significance.

7.4 Results

7.4.1 Antimicrobial minimal inhibitory concentrations (MICs)

The distribution of MICs and the levels of resistance against 14 antimicrobials for all isolates as well as the resistance genotype are presented in Table 7.1 and Figure 7.1. In general, none of the isolates were resistant to ciprofloxacin, azithromycin, and nalidixic acid. Among the 14 antimicrobials, resistances to tetracycline (70%), ceftiofur (48%), ampicillin (48%), amoxicillin (43%), ceftriaxone (41%), cefoxitin (41%), streptomycin (35%), sulfonamide (22%), gentamicin (15%), chloramphenicol (9%), and trimethoprim-sulfamethoxazole (4%) were the most frequent.

Additionally, 38 isolates (83%) were resistant to at least one antimicrobial and 27 (71%) of these resistant isolates showed multidrug resistant phenotypes (≥ three antibiotic classes).

Chicken meat isolates. Among all 30 isolates from chicken meats, 22 (73%), 16 (53%), 15 (50%), nine (30%), five (17%), and one (3%) isolates showed resistance to tetracycline, ampicillin, amoxicillin, streptomycin, sulfonamide, and chloramphenicol, receptively; 14 (47%) were resistant to ceftiofur, ceftriaxone, or cefoxitin, and two (7%) were resistant to gentamicin or trimethoprim-sulfamethoxazole. Overall, a total of 24 (80%) isolates from chicken meat were resistant to at least one antimicrobial, 19 of these 24 isolates (79%) showed multiple resistance (≥ three antibiotic classes). Whereas five of chicken meat isolates (17%) (4747, 4822, 4906, 4960, 4961) were sensitive to all tested antimicrobials (Table 7.1). As shown in Figure 7.1, no antimicrobial-specific resistance genes were detected in 4822, showing sensitivity to all 14 antimicrobials. The highest number (*n* = 11) of antimicrobial-specific resistance genes were observed in 4926 with resistance to five antimicrobials (gentamicin, streptomycin, chloramphenicol, sulfonamide, and tetracycline). The ß-lactamase genes (*ampC*, $bla_{\text{CMY-2}}$, $bla_{\text{TEM-1}}$), aminoglycoside resistance genes [*aac(3)-VIa*, *aadA1*, *aph(3'')-Ib*, *aph(3')-Ia*, *aph(6)-Id*)], sulfonamide resistance genes (*sul1*, *sul2*), and tetracycline resistance genes (*tetA*, *tetB*) were observed in 14 (47%), 8 (27%), 22 (73%), and five (17%) isolates, showing resistance to ß-lactam drugs (48%), aminoglycoside (28%), tetracycline (76%), and sulphonamide (17%), respectively (Figure 7.1). Interestingly, aminoglycoside resistance genes [*aph(3'')-Ib*, *aph(6)-Id*] were observed in an ExPEC (5006) that was not resistant to aminoglycosides and ß-lactamase genes (*ampC*, $bla_{\text{CMY-2}}$) were found in five isolates (4747, 4822, 4906, 4960, 4961) with sensitivity to ß-lactam drugs. Additionally, a chloramphenicol resistance gene (*dfrA1*) was detected in two isolates (4954, 4956) without showing resistance to chloramphenicol. Interestingly, Tn21-associated ARGs including *sul1* and *qacEdelta1* were detected in two isolates (4926, 4941).

Turkey meat isolates. Among all 11 isolates from turkey meats, seven (64%), five (45%), and four (36%) were resistant to tetracycline, streptomycin or ampicillin, amoxicillin, ceftiofur, ceftriaxone, or cefoxitin, respectively while, three (27%) and two (18%) were resistant to sulfonamide or gentamicin, respectively. Overall, about nine (82%) isolates were resistant to at least to one antimicrobial, five of these nine (56%) isolates showed multiple resistance (≥ three antibiotic classes), and three (25%) isolates were sensitive to all tested antimicrobials (Table 7.1). The highest number (*n* = 10) of antimicrobial-specific resistance genes was detected in 4971 and 4973 with resistance to nine (amoxicillin, ampicillin, ceftiofur, ceftriaxone, cefoxitin, gentamicin,

streptomycin, sulphonamide, tetracycline) and eight antimicrobials (amoxicillin, ampicillin, ceftiofur, ceftriaxone, cefoxitin, streptomycin, sulphonamide, tetracycline), respectively (Fig 1). The ß-lactamase (*ampC*, $bla_{\text{CMY-2}}$, $bla_{\text{TEM-1}}$), aminoglycoside [*aac(3)-VIa, aph(3'')-Ib, aph(6)-Id*], sulphonamide (*sul1*), and tetracycline [*tet(A), tet(B)*] resistance genes were observed in four (36%), six (55%), two (18%), and eight (73%) isolates showing corresponding resistance phenotype, respectively. Similar to chicken meat isolates, there was agreement between AMR phenotypes and genotypes. Additionally, Tn21-related ARGs including *sul1* and *qacEdelta1* were detected in two isolates (4971, 4973).

Chicken fecal and human isolate. The ExPEC isolate (5305) from the human case was only resistant to cefoxitin but no antimicrobial-specific resistance genes were detected in these isolates (Table 7.1 and Figure 7.1). In the four isolates from chicken fecal samples also classified also as ExPEC, and resistance to gentamicin (75%) chloramphenicol (75%), tetracycline (75%), streptomycin (50%), sulfonamide (50%), amoxicillin (25%), ceftiofur (25%), ceftriaxone (25%), and ampicillin (25%) were observed. Multiple resistances to six (amoxicillin, ampicillin, ceftiofur, ceftriaxone, gentamicin, tetracycline), five (gentamicin, streptomycin, chloramphenicol, sulphonamide, tetracycline), one (chloramphenicol), and five (gentamicin, streptomycin, chloramphenicol, sulphonamide, tetracycline) antimicrobials were observed in fecal isolate. Twelve, eight, one, and six antimicrobial-specific resistance genes were detected in isolates 2448, 6841, 6859, and 6941, respectively. Interestingly, no chloramphenicol resistance gene was observed in the isolates (6841, 6859) which showed resistance to this antibiotic. In addition, Tn21-related ARGs including *sul1* and *qacEdelta1* were detected in two isolates (2448, 6941).

Table 7. 1 Minimal inhibitory concentrations (MICs) of the 46 studied *E. coli* isolates isolated from chicken (*n* = 30) or turkey (*n* = 11) retail meats, chicken fecal samples (*n* = 4) and, and human UTI case (*n* = 1).

Class	AM[1]	OR[2]	Percentiles[3]		AMR (%)[4]	Number of strains with MICs (µg/mL)												
			MIC50	MIC90		0.015	0.03	0.12	0.25	0.5	1	2	4	8	16	32	64	256	>256
I	AMOX	HU (*n* = 1)	4	8	0 (0)									1					
		CF (*n* = 4)	4	8	1 (25)							2		1		1			
		TM (*n* = 11)	4	8	4 (36)								6	1		4			
		CM (*n* = 30)	4	8	15 (50)							2	11	2		15			
	CEFO	HU (*n* = 1)	0.25	0.5	0 (0)					1									
		CF (*n* = 4)	0.25	0.5	1 (25)				2	1				1					
		TM (*n* = 11)	0.25	0.5	4 (36)			1	4	2			1	3					
		CM (*n* = 30)	0.25	0.5	14 (47)				8	8				14					
	CEFT	HU (*n* = 1)	<=0.25	<=0.25	0 (0)					1									
		CF (*n* = 4)	<=0.25	<=0.25	1 (25)				4					1					
		TM (*n* = 11)	<=0.25	<=0.25	4 (36)				7					3		1			
		CM (*n* = 30)	<=0.25	<=0.25	14 (47)				16					12	2				
	CIPR	HU (*n* = 1)	<=0.015	<=0.015	0 (0)		1												
		CF (*n* = 4)	<=0.015	<=0.015	0 (0)	5													
		TM (*n* = 11)	<=0.015	<=0.015	0 (0)	11													
		CM (*n* = 30)	<=0.015	<=0.015	0 (0)	30													
II	AMPI	HU (*n* = 1)	2	4	0 (0)									1					
		CF (*n* = 4)	2	4	1 (25)						2		1			1			
		TM (*n* = 11)	2	4	5 (45)								2	4		5			
		CM (*n* = 30)	2	4	16 (53)						1	1	12			16			
	AZIT	HU (*n* = 1)	>8	128	0 (0)								1						
		CF (*n* = 4)	>8	128	0 (0)								3	1					
		TM (*n* = 11)	>8	128	0 (0)								9	2					
		CM (*n* = 30)	>8	128	0 (0)								16	14					
	CEFX	HU (*n* = 1)	4	8	1 (100)										1				
		CF (*n* = 4)	4	8	0 (0)						3			1					
		TM (*n* = 11)	4	8	4 (36)							1	6			4			

		CM (*n* = 30)	4	8	14 (47)					1	11	4		14		
	GENT	HU (*n* = 1)	0.5	1	0 (0)			1								
		CF (*n* = 4)	0.5	1	3 (75)				1				3			
		TM (*n* = 11)	0.5	1	2 (18)			1	8				2			
		CM (*n* = 30)	0.5	1	2 (13)			1	27				2			
	NALI	HU (*n* = 1)	2	4	0 (0)					1						
		CF (*n* = 4)	2	4	0 (0)				3		1					
		TM (*n* = 11)	2	4	0 (0)				7	4						
		CM (*n* = 30)	2	4	0 (0)			1	19	10						
	STRE	HU (*n* = 1)	<=0.32	64	0 (0)							1				
		CF (*n* = 4)	<=0.32	64	2 (50)							2			2	
		TM (*n* = 11)	<=0.32	64	5 (45)						4	1	1		5	
		CM (*n* = 30)	<=0.32	64	9 (30)						5	12	4	1	8	
	TRIM	HU (*n* = 1)	<=0.12	0.25	0 (0)	1										
		CF (*n* = 4)	<=0.12	0.25	0 (0)	4										
		TM (*n* = 11)	<=0.12	0.25	0 (0)	10	1									
		CM (*n* = 30)	<=0.12	0.25	2 (7)	27			1		2					
	CHLO	HU (*n* = 1)	4	8	0 (0)							1				
		CF (*n* = 4)	4	8	3 (75)					1			1	2		
		TM (*n* = 11)	4	8	0 (0)					4	3	4				
		CM (*n* = 30)	4	8	1 (3)					5	11	13		1		
III	SULF	HU (*n* = 1)	<=16	32	0 (0)										1	
		CF (*n* = 4)	<=16	32	2 (50)								2			2
		TM (*n* = 11)	<=16	32	3 (27)								8			3
		CM (*n* = 30)	<=16	32	5 (17)								21	4		5
	TETR	HU (*n* = 1)	<=4	>32	0 (0)						1					
		CF (*n* = 4)	<=4	>32	3 (75)						1			3		
		TM (*n* = 11)	<=4	>32	7 (64)						4			7		
		CM (*n* = 30)	<=4	>32	22 (73)						7	1		22		

[1]AM, antimicrobials; AMOX, amoxicillin; CEFO, ceftiofur; CEFT, ceftriaxone; CIPR, ciprofloxacin; AMPI, ampicillin; AZIT, azithromycin; CEFX, cefoxitin; GENT, gentamicin; NALI, nalidixic acid; STRE, streptomycin; TRIM, trimethoprim-sulfamethoxazole; CHLO, chloramphenicol; SULF, sulfonamide; TETR, tetracycline.

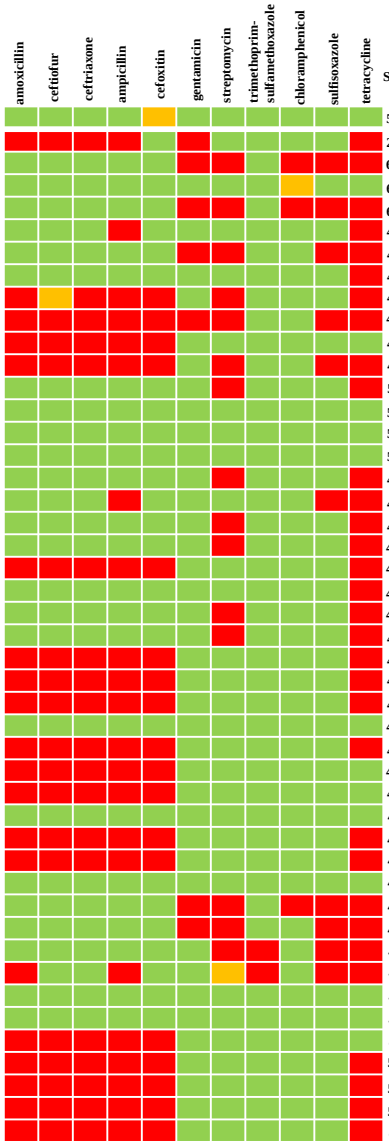
[2]OR, origin of isolates; HU, human urinary tract infection; CM, chicken retail meat; TM, turkey retail meat; CF; chicken fecal samples.

[3]The MIC50 represents the MIC value at which 50% of the strains within a test population are inhibited; The MIC90 represents the MIC value at which 90% of the strains within a test population are inhibited.

[4]AMR (%), antimicrobial resistance levels (%).

Dotted and solid bars indicate the breakpoints for intermediary and complete resistance, respectively. No breakpoints for azithromycin for *E. coli*.

Figure 7. 1 Antibiotic resistance phenotypes and genotypes of the 46 studied *E. coli* isolates: human urinary tract infection (*n* = 1), chicken feces (*n* = 4), turkey meat (*n* = 11), and chicken meats (*n* = 30). Asterisks are isolates that were selected for C. elegans life-span assays. Green: susceptible; yellow, intermediate susceptibility; red, resistant.



Strain ID	OR[1]	Serotypes	MLSTs[2]	Antimicrobial resistance genes (ARGs)
5305*	HU	O25: H4	ST131	ND
2448*	CF	O81: H51	ST155	*aac(3)-Vla – aadA24 - ampC – aph(3'')-Ia – aph(3'')-Ib – bla$_{CMY-2}$ – qacEdelta1 – sul1 – sul2 - bla$_{TEM-1}$ – tet(A) – tet(B)*
6841*	CF	O8: H20	ST155	*aac(3)-Vla - aadA22 – ampC - aph(3'')-Ib - aph(6)-Id – floR – sul2 – tet(A)*
6859*	CF	O39: H7	STnew	*ampC*
6941*	CF	O81: H25	ST57	*aac(3)-Vla – aadA – ampC - bla$_{CMY-2}$ – qacEdelta1 – sul1*
4557*	TM	O25: H4	ST131	*bla$_{TEM-1}$ – tet(A)*
4694	TM	O11: H16	ST93	*aac(3)-Vla – aadA – ampC – tet (A)*
4724	TM	O50: H1	ST429	*ampC – tet(B) – tet(R)*
4839	TM	O39: H7	ST6096	*ampC - aph(3'')-Ib - aph(6)-Id – tet(A)*
4971	TM	O25: H4	ST131	*aac(3)-Vla – aadA24 – aph(3'')-Ib - aph(6)-Id – bla$_{CMY-2}$ – qacEdelta1 – sul1 – sul2 - bla$_{TEM-1}$ – tet(A)*
4972	TM	O50/O2: H6	ST141	*ampC – bla$_{CMY-2}$*
4973*	TM	O25: H4	ST131	*aac(3)-Vla – aadA24 – aph(3'')-Ib – aph(6)-Id – bla$_{CMY-2}$ – qacEdelta1 – sul1 – sul2 - bla$_{TEM-1}$ – tet(A)*
5022	TM	O182: H7	ST1148	*ampC – aph(3'')-Ib – tet(A)*
5142	TM	O182: H7	ST1148	*ampC*
5143	TM	O182: H7	ST1148	*ampC*
5144	TM	O25: H4	ST131	*ampC*
4470	CM	O19: H34	ND	*ampC - aph(3'')-Ib – aph(6)-Id – tet(B)*
4487	CM	O21: H16	ST93	*sul2 - bla$_{TEM-1}$ – tet(A)*
4566	CM	O39: H18	ST38	*aph(3'')-Ib – aph(6)-Id – tet(B)*
4604*	CM	O25: H4	ST131	*aph(3'')-Ib – aph(6)-Id – tet(B)*
4618	CM	O71: H10	ST93	*ampC – bla$_{CMY-2}$ – tet(A)*
4619	CM	O71: H10	ST93	*ampC – tet(A)*
4627	CM	O15: H6	ST69	*ampC – aph(3'')-Ib – aph(6)-Id – tet(B)*
4628	CM	O7: H18	ST38	*ampC – aph(3'')-Ib – aph(6)-Id – tet(B)*
4701	CM	O11: H52	ST373	*ampC – bla$_{CMY-2}$ – tet(A)*
4702	CM	O11: H52	ST373	*ampC – bla$_{CMY-2}$ – tet(A)*
4703	CM	O11: H52	ST373	*ampC – bla$_{CMY-2}$ – tet(A)*
4747	CM	O21: HM	ND	*ampC – bla$_{CMY-2}$*
4767	CM	O71: H10	ST93	*ampC – bla$_{CMY-2}$ – tet(A)*
4819	CM	O25: H4	ST131	*bla$_{CMY-2}$*
4821	CM	O139: NM	ST10	*ampC – bla$_{CMY-2}$*
4822	CM	O25: H4	ST131	ND
4859	CM	O71: H10	ST93	*ampC – bla$_{CMY-2}$ – tet(A)*
4860	CM	O71: H10	ST93	*ampC – bla$_{CMY-2}$ – tet(A)*
4906	CM	O7: H18	ST38	*ampC*
4926	CM	O15: H5	ST2309	*aac(3)-Vla – aadA – ampC – aph(3'')-Ib – aph(6)-Id – bla$_{CMY-55}$ – floR – qacEdelta1 – sul1 – sul2 – tet(A)*
4941	CM	O21: H16	ST93	*aac(3)-Vla – aadA – ampC – aph(3'')-Ia – aph(3'')-Ib – aph(6)-Id – qacEdelta1 – sul1 – tet(A) – tet(B)*
4954	CM	O7: H18	ST38	*aadA – ampC – aph(3'')-Ib – dfrA1 – sul2 – tet(A)*
4956	CM	O22: H2	ST1914	*aadA – ampC – dfrA1 – sul2 – tet(A)*
4960	CM	O22: H2	ST1914	*ampC*
4961	CM	O16: H34	ST1158	*ampC*
5006	CM	O21: H4	ST93	*ampC – aph(3'')-Ib – aph(6)-Id – bla$_{CMY2}$ – floR – sul2 – tet(A)*
5082	CM	O71: H10	ST93	*ampC – bla$_{CMY2}$ – tet(A)*
5083	CM	O71: H10	ST93	*ampC – bla$_{CMY2}$ – tet(A)*
5084	CM	O71: H10	ST93	*ampC – tet(A)*
5101	CM	O2: H1	ST1354	*ampC – bla$_{CMY2}$ – tet(A)*

7.4.2 Plasmids

The plasmid profiles of all 46 studied isolates are shown in Table 7.2. Additionally, the characteristic of the multidrug resistance plasmid *Inc*A/C2 found in chicken (4926, 5006) or turkey meat ExPEC (4971, 4973) is described in Figure 7.2. Overall, the highest (n = 11) and the lowest (n = 2) plasmid numbers were detected in the turkey meat isolate 4694 and the chicken fecal isolate 6859, respectively. Interestingly, plasmid *Inc*A/C2 carried multiple ARGs including *aph(6)-Id*, *aph(3'')-Id*, *bla*$_{\text{CMY-2}}$, *sul2*, *tet(A)*, *tet(R)*, *floR*, and *ParB* (Figure 7.2).

Chicken meat isolates. Briefly, plasmids *Inc*FIB, *Inc*FII, or *Inc*FIIA were detected in 25 (83%) of the 30 studied isolates. Additionally, plasmids *Col*RNAI_488, 1088, 1291,1527, 1704, 1778, 1857, 1885, 1993, 1994 were also detected in 24 (80%) of them, and plasmids *Inc*A/C2, *Inc*H, *Inc*I1, or *Inc*I2 were found in 25 (83%) of isolates.

Turkey meat isolates. All 11 (100%) isolates carried *Inc*FIB, *Inc*FII, or *Inc*FIIA. In addition, plasmids *Col*RNAI_1088, 1291, 1527, 1704, 1778, 1857, and 1885 were observed in nine (73%) isolates. Interestingly, plasmids *Inc*A/C2, *Inc*I1, or *Inc*I2 were found in isolate 4694,4971, 4972, 4973, and 5022.

Chicken fecal and human isolate. Plasmids *Inc*FIB, *Inc*FII, or *Inc*FIIA were detected in three fecal isolates (2448, 6841, 6941) and the human ExPEC (5305). Besides, plasmids ColRNAI_1527, 1778, 1857, or 1885 were detected in isolate 6841, 6859, and 6941. In addition, plasmids *Inc*I1, *Inc*H, or *Inc*X1 were detected in fecal isolates 6841, 6859, and 6941.

Table 7. 2 Plasmid profile of the 46 studied *E. coli* isolates from chicken or turkey retail meats, chicken fecal samples, and a human UTI case.

ID[1]	OR[2]	Representative plasmids	Total number of plasmids
5305*	HM	*Inc*FIB; *Inc*FII; *Inc*FIIA	3
2448*	CF	*Inc*FIIA; *Inc*FII; *Inc*FIB	3
6841*	CF	*Inc*FII; *Inc*FIIA*; Inc*FIB; *Inc*I1; *Inc*H; *Col*RNA_(1527, 1778, 1857, 1885)	9
6859*	CF	*Inc*X1; *Col*RNAI_1857	2
6941*	CF	*Inc*FII; *Inc*FIIA; *Inc*FIB; *Inc*I1; *Inc*H; *Col*RNAI_(1527, 1778, 1857, 1885)	9
4557*	TM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Col*RNAI_(1857, 1885)	5
4694	TM	*Inc*13;*Inc*FIB; *Inc*FII; *Inc*FIIA;;*Inc*H; *Inc*I1;*Col*RNAI_(1291, 1704, 1778, 1857, 1885)	11
4724	TM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Col*RNAI_1088	4
4839	TM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Col*RNAI_1885	4
4971	TM	*Inc*A/C2; *Inc*FIB; *Inc*FII; *Inc*FIIA; *Col*RNAI_(1857, 1885)	6
4972	TM	*Inc*FIB; *Inc*FII; *Inc*I2; *Col*RNAI_(1857, 1885)	5
4973*	TM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*A/C2; *Col*RNAI_(1857; 1885)	6
5022	TM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*I1; *Col*RNAI_1885	5
5142	TM	*Inc*FIB; *Inc*FII; *Inc*FIIA	3
5143	TM	*Inc*FIB; *Inc*FII; *Inc*FIIA	3
5144	TM	*Inc*FIB; *Inc*FII; *Inc*FIIA	3
4470	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*H; *Col*RNAI_1885	5
4487	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Col*RNAI_(488, 1704)	5
4566	CM	*Inc*FIB; *Inc*FIIA; *Inc*FII; *Col*RNAI_1704	4
4604*	CM	*Inc*FII; *Inc*FIIA; *Inc*H; *Col*RNAI_(1088, 1885, 1993)	6
4618	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*I1; *Inc*l2; *Col*RNAI_(1291, 1885, 1993)	8
4619	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*I1; *Col*RNAI_(1291, 1993)	6
4627	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*H; *Col*RNAI_1885	5
4628	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*H; *Col*RNAI_1885	5
4701	CM	*Inc*H; *Col*RNAI_(1778, 1857, 1885, 1993, 1994)	6
4702	CM	*Inc*H; *Col*RNAI_(1857, 1778, 1857, 1885, 1993, 1994)	7

4703	CM	*Inc*H; *Col*RNAI_(1857, 1778, 1857, 1885, 1993, 1994)	7
4747	CM	*Inc*H; *Col*RNAI_(1857, 1778, 1857, 1885, 1993, 1994)	7
4767	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*I1; *Col*RNAI_(1291, 1993)	6
4819	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*I1; *Co*lRNAI_1857	5
4821	CM	*Inc*FII; *Inc*FIIA; *Col*RNAI_1857	3
4822	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*I1; *Inc*X1; *Col*RNAI_1885	6
4859	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*I1; *Inc*X1	5
4860	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*I1; *Inc*X1	5
4906	CM	IncFIB; IncFIIA; IncH; *Col*RNAI_(1291, 1778, 1857, 1885)	7
4926	CM	*Inc*A/C2; *Inc*FII; *Inc*FIIA; *Inc*I1; *Inc*I2; *Col*RNAI (488, 1778, 1857, 1885)	9
4941	CM	IncI1; IncH; IncFII; IncFIIA; *Col*RNAI_(1291, 1778, 1857, 1885)	8
4954	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*H; *Inc*I2; *Col*RNAI (1291, 1527, 1778, 1857, 1885)	10
4956	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*H; *Inc*I2; *Col*RNAI (1291, 1527, 1778, 1857, 1885)	10
4960	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Col*RNAI_(1857, 1885)	5
4961	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Col*RNAI_(1857, 1885)	5
5006	CM	*Inc*A/C2; *Col*RNAI_(1857, 1885)	3
5082	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*I1; *Inc*I2	5
5083	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*I1; *Inc*I2	5
5084	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*I1; *Inc*I2	5
5101	CM	*Inc*FIB; *Inc*FII; *Inc*FIIA; *Inc*I1	4

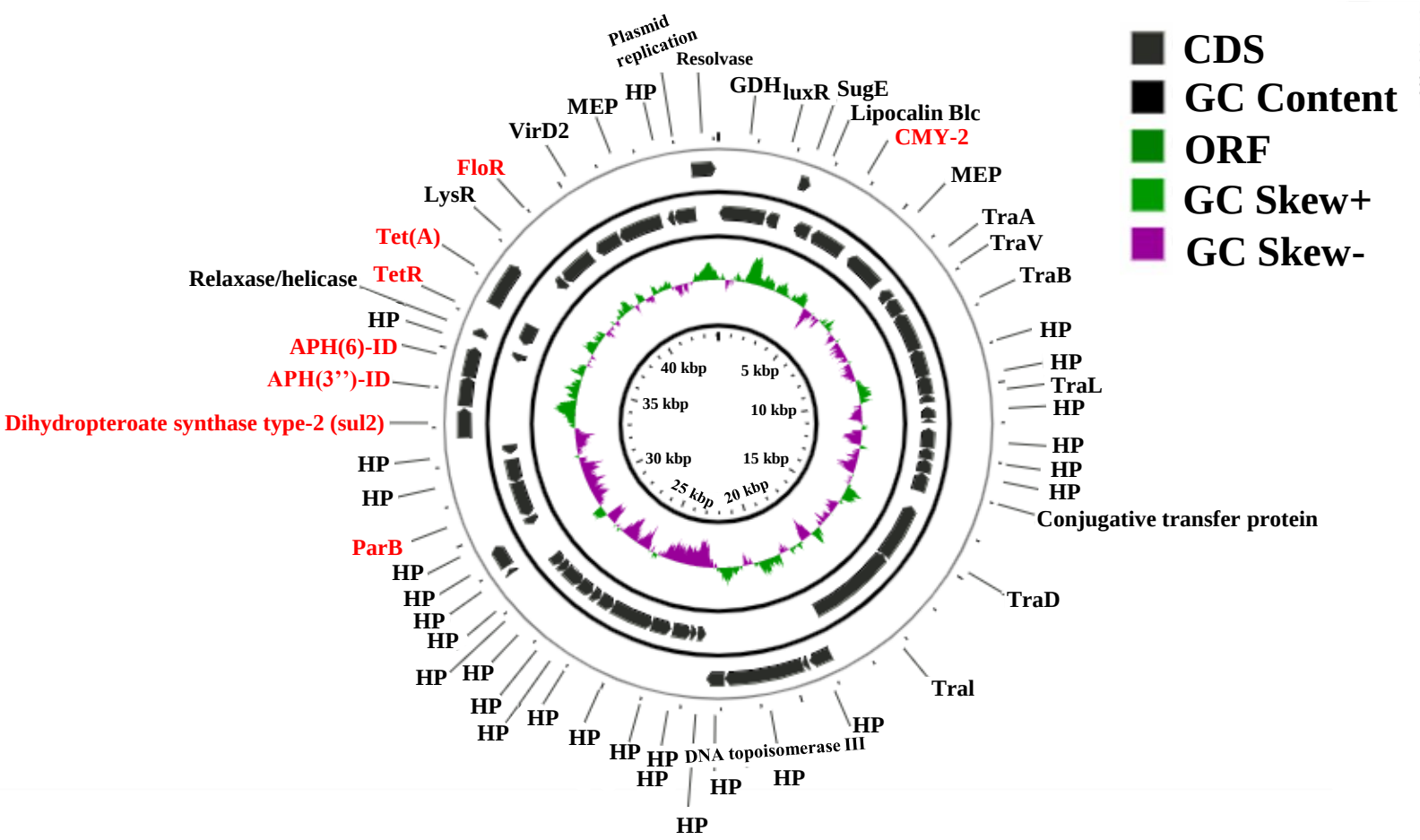
Bold and asterisks are isolates selected for *C. elegans* life-span assays.

[1]ID, strain ID.

[2]OR, origin of isolates; HU, human urinary tract; CM, chicken retail meat; TM, turkey retail meat; CF; chicken fecal samples.

Bold and asterisks indicate the isolates selected for the *C. elegans* life-span assay.

Figure 7. 2 Characteristic of the multi-drug resistance (MDR) *Inc*A/C2 plasmid found in several of studied *E. coli*. Red are antimicrobial resistance genes (ARGs); HP, hypothetical proteins; MEP; mobile element Protein; CDS, coding sequences; ORF, open reading frame. Red are antimicrobial resistance genes (ARGs); HP, hypothetical proteins; MEP; mobile element Protein; CDS, coding sequences; ORF, open reading frame.



7.4.3 Serotype, multilocus sequencing typing, virulence gene

The serotypes, MLSTs, and VGs numbers of all 46 isolates were shown in Table 7.3. Overall, the highest number (*n* = 106) and the lowest (*n* = 52) numbers of VGs was observed in the isolate 4487 from chicken meat and the isolate 6859 from the chicken fecal samples. A total of 24 serotypes and 18 MLSTs were detected among all 46 isolates with O25: H4 (17%) and ST93 (26%) being the most frequent.

Chicken meat isolates. A total of 14 serotypes with O71: H10 (27%), O7: H18 (10%), O11: H52 (10%), O25: H4 (10%), O21: H16 (7%), and O22: H2 (7%) being the most detected serotypes. The 30 isolates belonged to 11 sequence types including ST93 (37%), ST38 (13%), ST131 (10%), and ST373 (10%) being the most detected MLSTs. A total of 129 VGs, belonging to six categories including adherence, flagella, iron utilization, secretion system, toxin, and miscellaneous, were detected in 30 chicken meat isolates. The highest (*n* = 106) and lowest (n = 53) numbers of VGs were detected in isolates 4487 and 4821, respectively. Interestingly, among all 30 chicken meat isolates, most of VGs belonged to iron utilization (34%), followed by adherence (28%), secretion system (21%), flagella (11%), miscellaneous (4%), and toxin (2%).

Turkey meat isolates. The 11 isolates belonged to six serotypes including O25: H4 (36%), O182: H7 (27%), O11: H16 (9%), O39: H7 (9%), O50: H1 (9%), and O50/O2:H6 (9%). The most frequent sequence types were ST131 (36%), ST1148 (27%), ST93 (9%), ST141 (9%), ST429 (9%), and ST6096 (9%). The highest (*n* = 100) number of VGs was detected in 4971, 4972, and 4973. Among all turkey meat isolates, most of VGs belonged to iron utilization (38%), followed by adherence (28%), secretion system (16%), flagella (11%), miscellaneous (4%), and toxin (1%).

Chicken fecal isolates. Serotypes including O81: H51, O8: H20, O39: H7, and O81: 25 and MLSTs including ST155, ST155, STnew, and ST57 were detected in fecal isolates 2448, 6841, 6859, and 6941, respectively. Additionally, fecal isolates 2448, 6841, 6859, and 6941 contained 59, 56, 52, and 81 VGs in chicken fecal isolates belonging to categories including adherence (28%), flagella (11%), iron utilization (38%), secretion system (16%), toxin (1%), and miscellaneous (4%).

7.4.4 Phylogenetic tree

The phylogenetic relationships between all 46 isolates are shown in Figure 7.3. All isolates clustered into three major clades (A, B, C): containing 25 isolates in clade A (subclades A1-A9), 11 in clade B (subclades B1-B3), and 10 in clade C (subclades C1-C5). Interestingly, seven isolates (4557, 4566, 4604, 4822, 4819, 4973, 4971) from chicken or turkey retail meats were clustered

together (clade B; subclade B1) with the human ExPEC (5305) and shared the same serotype (O25: H4) and ST131.

Table 7. 3 Serotypes, multilocus sequence types (MLSTs), and number of virulence genes in the 46 studied *E. coli* isolates from chicken (*n* = 30) or turkey (*n* = 11) retail meats, chicken fecal samples (*n* = 4), and a human case (*n* = 1).

Category	OR[1]	Serotype	MLSTs[2]	Adherence	Flagella	Iron Utilization	Secretion Systems	Toxin	Miscellaneous[3]	Total number of virulence genes
5305*	HU	O25:H4	131	24	11	36	11	2	3	87
2448*	CF	O81: H51	155	21	8	12	16	1	1	59
6841*	CF	O8:H20	155	12	8	17	16	2	1	56
6859*	CF	O39: H7	STnew	14	8	12	16	2	0	52
6941*	CF	O81: H25	57	21	8	30	19	1	2	81
4557*	TM	O25:H4	131	23	11	41	11	1	3	90
4694	TM	O11:H16	93	21	9	17	16	2	1	66
4724	TM	O50:H1	429	25	9	41	11	1	3	90
4839	TM	O39:H7	6096	21	8	17	16	1	1	64
4971	TM	O25:H4	131	31	11	41	11	1	5	100
4972	TM	O50/O2:H6	141	24	9	41	11	1	14	100
4973*	TM	O25:H4	131	31	11	41	11	1	5	100
5022	TM	O182:H7	1148	18	9	33	16	2	1	79
5142	TM	O182:H7	1148	21	8	22	16	1	1	69
5143	TM	O182:H7	1148	21	8	22	16	1	1	69
5144	TM	O25:H4	131	21	8	22	16	1	1	69
4470	CM	O19:H34	ND	23	8	30	22	1	2	86
4487	CM	O21:H16	93	34	7	41	21	1	2	106
4566	TM	O39:H18	38	23	11	41	11	1	3	90
4604*	CM	O25:H4	131	24	11	41	11	1	3	91
4618	CM	O71:H10	93	14	9	22	12	2	4	63
4619	CM	O71:H10	93	14	9	22	12	2	4	63
4627	CM	O15:H6	69	24	8	30	22	1	2	87
4628	CM	O7:H18	38	24	8	30	22	1	2	87
4701	CM	O11:H52	373	23	9	28	17	2	2	81

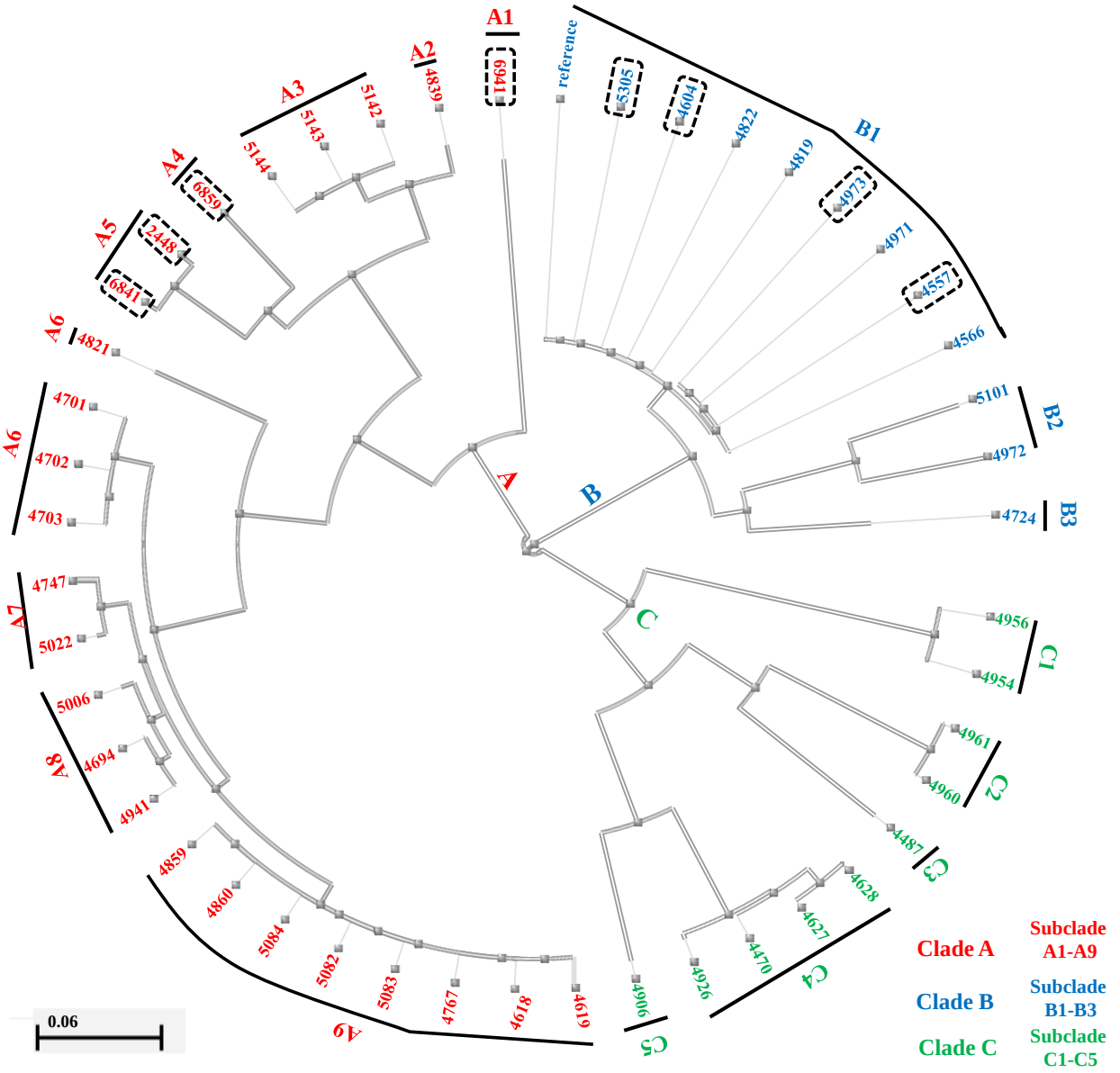
4702	CM	O11:H52	373	23	9	28	17	2	2	81
4703	CM	O11:H52	373	23	9	28	17	2	2	81
4747	CM	O21:NM	ND	23	9	17	16	2	1	68
4767	CM	O71:H10	93	14	9	22	12	2	4	63
4819	CM	O25:H4	131	24	11	41	11	1	3	91
4821	CM	O139:NM	10	21	7	12	9	1	3	53
4822	CM	O25:H4	131	24	11	41	11	1	3	91
4859	CM	O71:H10	93	14	9	22	12	2	4	63
4860	CM	O71:H10	93	14	9	22	12	2	4	63
4906	CM	O7:H18	38	23	8	36	22	2	2	93
4926	CM	O15:H5	2309	24	8	25	22	2	2	83
4941	CM	O21:H16	93	21	9	17	15	2	1	65
4954	CM	O7:H18	38	25	8	23	22	2	2	82
4956	CM	O22:H2	1914	25	8	23	22	2	2	82
4960	CM	O22:H2	1914	31	7	25	22	2	4	91
4961	CM	O16:H34	1158	31	7	25	22	2	4	91
5006	CM	O21:H4	93	24	9	17	16	2	2	70
5082	CM	O71:H10	93	14	9	22	12	2	4	63
5083	CM	O71:H10	93	14	9	22	12	2	4	63
5084	CM	O71:H10	93	14	9	22	12	2	4	63
5101	CM	O2:H1	135	24	12	40	11	2	3	92

[1]OR, origin of isolates; HU, human urinary tract; CM, chicken retail meat; TM, turkey retail meat; CF; chicken fecal samples.

[2]MLSTs, multilocus sequencing typing.

Bold and asterisks indicate the isolates selected for the *C. elegans* life-span assay.

Figure 7. 3 The phylogenetic tree of 46 ExPEC isolates based on core genome single nucleotide variant phylogenomics using SNVphyl for analysis. The selected eight ExPEC isolates (5305, 4604, 4557, 4973, 2448, 6841, 6859, 6941) that belongs to three clades (A, B, C) for conducting *C. elegans* life-span assay were labeled by black dashed retangular.

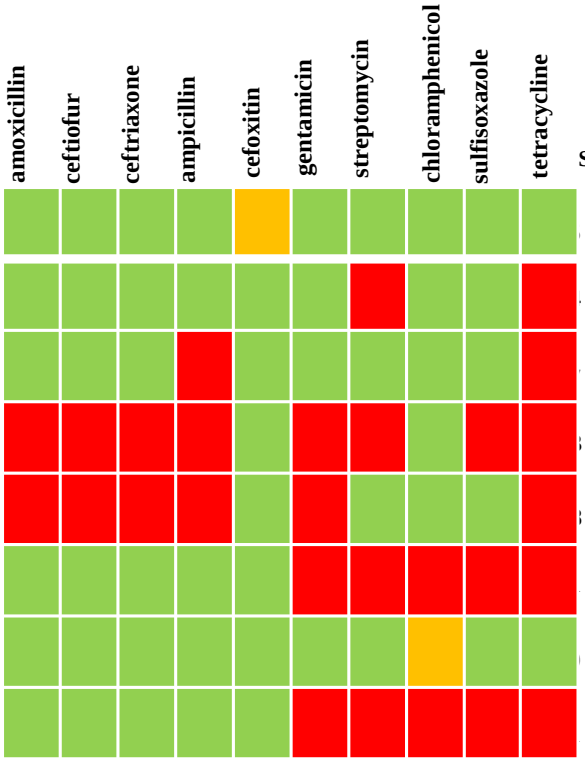


7.4.5 *E. coli* selection for *C. elegans* life-span assay

Eight of 46 *E. coli* isolates were selected for *C. elegans* life-span assay. The heatmap describing serotypes, MLSTs, AMR profiles and VGs and genome comparisons of the eight *E. coli* are shown in Figure 7.4 and 7.5, respectively. The ExPEC isolate (5305) from human UTI that belongs to clade B was firstly selected to determine pathogenicity. Once the assay model was established, one ExPEC isolate (4604) from chicken meat and two ExPEC isolates (4557, 4973) from turkey meat with close relatedness in the phylogenetic tree (clade B; subclade B1) to the human ExPEC (5305) of serotypes (O25: H4) and MLSTs (ST131) were selected as representatives to evaluate their effects on *C. elegans* survival (%) to predict zoonotic potentials.

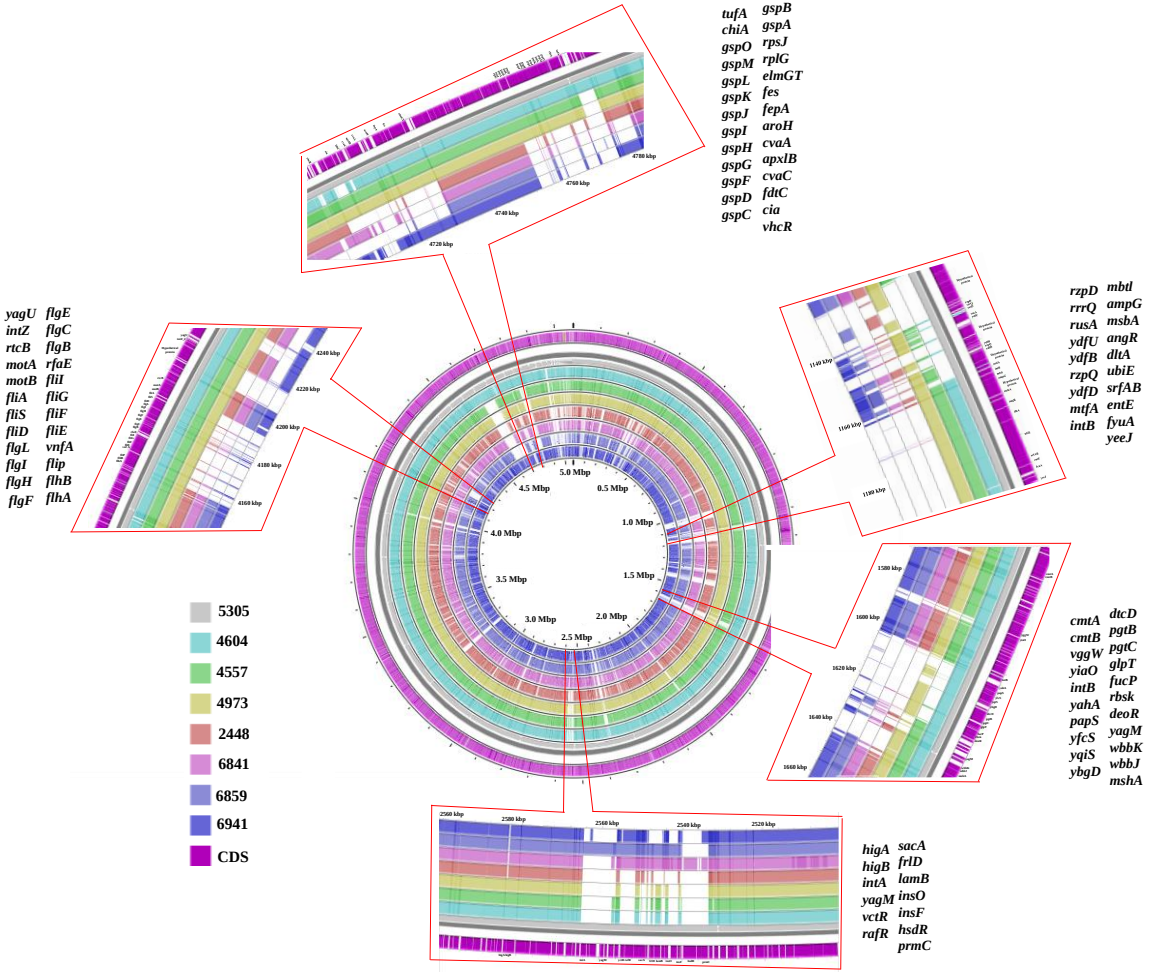
To determine potential virulence of isolates from chicken fecal samples with different ASTs and number of VGs, all four fecal isolates belong to clade A in the phylogenetic tree including 2448 (subclade A5), 6859 (subclade A4), 6841 (subclade A5), and 6941 (subclade A1) were included in the life-span assay. As described above, isolates 2448, 6841, 6859 and 6941 were all multi-resistant and carried varying numbers of VGs.

Figure 7. 4. Antibiotic resistance and virulence genotype of eight *E. coli* for the *C. elegans* life-span assay: human urinary tract infection (5305), chicken meat (4604), turkey meat (4557, 4973), and chicken fecal samples (2448, 6841, 6859, 6941). Green: susceptible; yellow, intermediate susceptibility; red: resistant. [1]OR, origin of isolates; HU, human urinary tract; CM, chicken retail meat; TM, turkey retail meat; CF; chicken fecal samples. [2]MLSTs, multilocus sequencing typing.



Strain ID	OR[1]	Serotypes	MLSTs[2]	Antimicrobial resistance genes (ARGs)	Virulence genes (VGs)
5305	HU	O25: H4	ST131	*None*	*aslA – cheBW – chuASTUVWXY – flhC – fyuA – ibeA – irp1,2 – kpsDMT – ybtAEPOS – ybtTUX*
4604	CM	O25: H4	ST131	*aph(3'')-Ib – aph(6)-Id – tet(B) – tet(OE)*	*aslA – cheBW – chuASTUVWXY – flhC – fyuA – ibeA – irp1,2 –iucABCD – iutA – kpsDMT - ybtAEPOSTUX*
4557	TM	O25: H4	ST131	*bla_{TEM-1} – tet(A)*	*aslA – cheBW – chuASTUVWXY – flhC – fyuA – ibeA – irp1,2 –iucABCD – iutA – kpsDM – ybtAEPOSTUX*
4973	TM	O25: H4	ST131	*bla_{CMY-2} – bla_{TEM-2} – aac(3)-Vla – aadA24 – aph(3'')-Ib – aph(6)-Id – sul1 – sul2 – tet(A) – qacEdelta1*	*aslA – cheBW – chuASTUVWXY – flhC – fyuA – ibeA – irp1,2 –iucABCD – iutA – kpsDM – papBCDEFHJK – sfaCX - ybtAEPOSTUX*
2448	CF	O81: H51	ST155	*aac(3)-Vla, aadA24, ampC, – aph(3'')-Ia – aph(3'')-Ib, bla_{CMY-2} – bla_{TEM-2} – qacEdelta1 – sul1 – sul12 tet(A) – tet(B)*	*cheY – espL1R1X1X4X5 – fdeC – fimABCDEFGHI – yagV/ecpE – yagW/ecpD – yagX/ecpC – yagY/ecpB – yagZ/ecpA – ykgK/ecpR*
6841	CF	O8: H20	ST155	*aac(3)-Vla - aadA22 - ampC – aph(3'')-Ib – aph(6)-Id – floR - sul12 – tet(A) – tet(A)*	*astA – cheY – espL1R1X1X4X5 – fdeC – iucABCD – iutA - yagV/ecpE – yagW/ecpD – yagX/ecpC – yagY/ecpB – yagZ/ecpA – ykgK/ecpR*
6859	CF	O39: H7	STnew	*ampC*	*astA – cheY – espL1R1X1X4X5 – fimABCEFGHI*
6941	CF	O81: H25	ST57	*aac(3)-Vla, aadA, bla_{CMY-2} – qacEdelta1 – sul1*	*aslA – cheY – chuUVW – espL1R1X1X4X5Y1Y3 – fdeC – fimABCDEFGHI – iroBCDEN – iucABCD – iutA – shuASTXY - yagV/ecpE – yagW/ecpD – yagX/ecpC – yagY/ecpB – yagZ/ecpA – ykgK/ecpR*

Figure 7. 5 Genome comparison of the eight selected *E. coli* isolates from human (5305), chicken feces (2448, 6841, 6859, 6941), turkey meat (4557, 4973), and chicken meats (4604) for *C. elegans* life-span assay. The gaps in the alignment represent the regions missing in the genomes in comparison to the human. The listed genes are the missing genes in the gap from five selected regions.



7.4.6 Life-span assay I

The initial effort was made to establish the life-span assay of *C. elegans* to investigate the nematode response to *E. coli* OP50 (negative control), $K88^+$ ETEC strain JG280 (positive control), and an ExPEC isolate from human infection (5305) at three different concentrations (1×10^8, 5×10^8 and 1×10^9 CFU/mL) of each isolate. As shown in Figure 7.6, the treatment with OP50 at each concentration did not result in any significant reductions in the survival (%) of *C. elegans* after 12-day incubation. Additionally, at 1×10^8 CFU/mL, nearly no death of worms was observed in the groups treated with JG280, and more than 10% of worm death was observed in the group treated with isolate 5305 on day 12. In contrast, the treatments with JG280 or isolate 5305 at either 5×10^8 or 1×10^9 CFU/mL caused significant ($P < 0.05$) death of worms within 3 to 5 days compared to OP50 at the same concentration, in which over 60% of the worms were dead after 12-day incubation. Interestingly, the survival (%) of *C. elegans* was lower ($P < 0.05$) when fed isolate 5305 compared to JG280 at the same concentration from day 5 to 12. Additionally, when the nematode was fed isolate 5305 at 1×10^9 CFU/mL, it caused the death of all worms on day 12.

7.4.7 Life-span assay II

Figure 7.7 shows the response of *C. elegans* to AMR ExPEC isolates from chicken (4604) and turkey (4557, 4973) retail meats. Like the results from Experiment I, when *C. elegans* were fed OP50, minimal death of worms was observed after 12-day incubation. Additionally, when nematode was fed JG280, 5305, 4604, 4557, or 4973 at either 5×10^8 CFU/mL or 1×10^9 CFU/mL, a significant lower ($P < 0.05$) survival (%) of *C. elegans* was observed compared to OP50. Interestingly, a similar survival (%) of *C. elegans* was detected when worms were fed isolate 5305, 4557, 4604, or 4973 at 5×10^8 CFU/mL (Fig 7A), while lower ($P < 0.05$) survival (%) of *C. elegans* fed isolate 4604 and 4557 at 1×10^9 CFU/mL was detected compared to other isolates (Fig 7B).

7.4.8 Life-span assay III

The responses of *C. elegans* to the infection of chicken fecal ExPEC isolates (2448, 6841, 6941, and 6859) with different ASTs and number of VGs were shown in Figure 7.8 with the OP50, JG280, and 5305 as the control. At 5×10^8 CFU/mL, all chicken fecal ExPEC isolates caused a significant reduction ($P < 0.05$) of *C. elegans* survival (%) compared to OP50, which is similar to JG280 or 5305. Interestingly, 2448 with 59 VGs showed lower ($P < 0.05$) survival (%) of worms compared to JG280 and 6941 (81 VGs), which was similar to 5305 (87 VGs), 6841 (56 VGs), and 6859 (52 VGs). In addition, *C. elegans* treated with isolate 6859 that is resistant to one

antimicrobial had the lower ($P < 0.05$) survival (%) (< 10% after 12-day incubation) compared to JG280 and 6941 (resistance to five antimicrobials), which was similar to 5305 (resistance to one antimicrobial), 2448 (resistance to six antimicrobials), and 6841(resistance to five antimicrobials).

Figure 7. 6 Establishment of *C. elegans* life-span assay. The life-span assay was expressed as the survival (%) of *C. elegans* after incubation with three *E. coli* isolates including OP50, JG280, and 5305 at 1×10^8, 5×10^8 or 10^9 CFU/mL for 12 days.

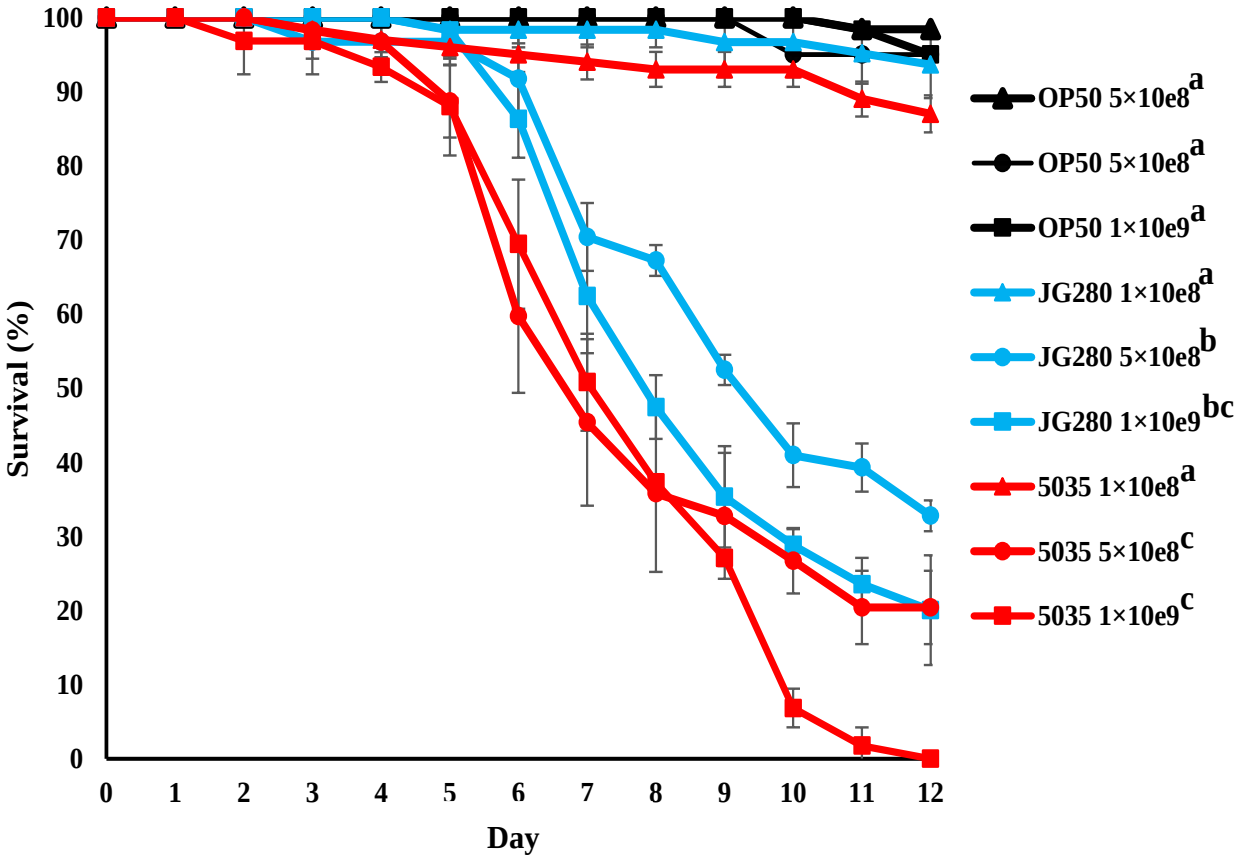


Figure. 7 Effects of feeding isolates OP50, 5305 and three extraintestinal pathogenic *E. coli* from poultry retail meats including 4604, 4557, and 4973 at 5 × 10^8 (Fig. A) or 10^9 CFU/mL (Fig. B) on survival (%) of *C. elegans* for 12 days.

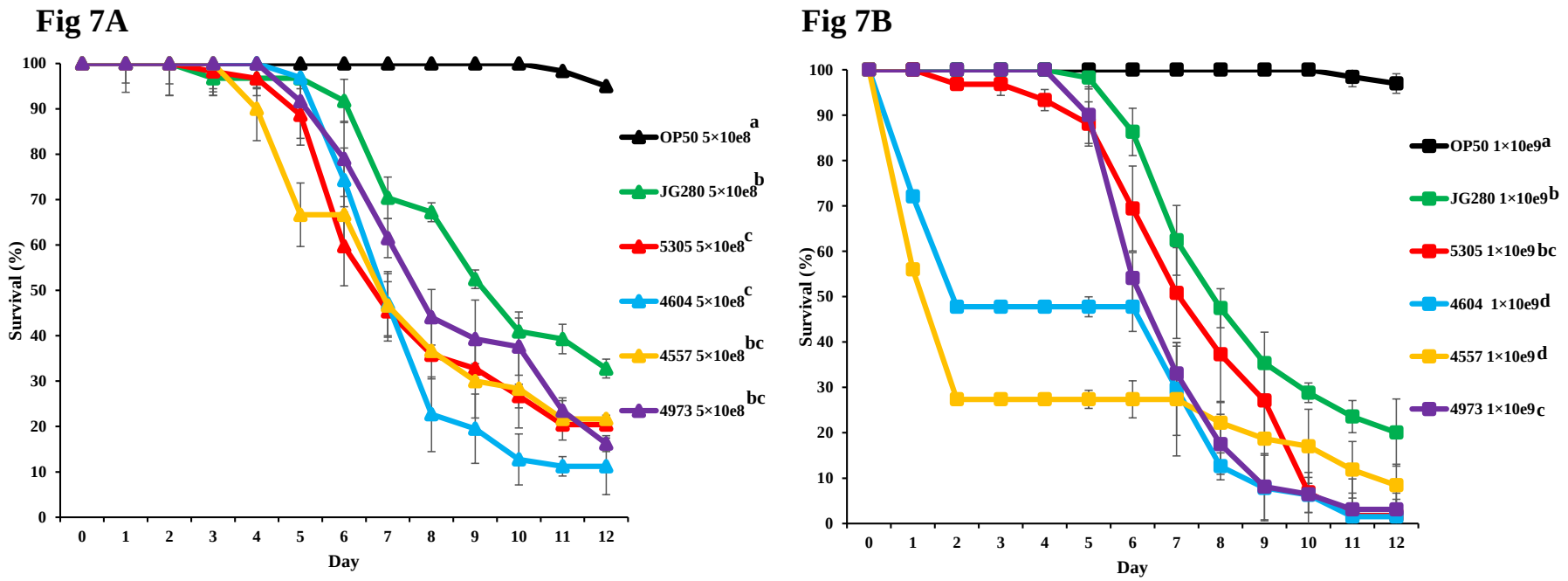
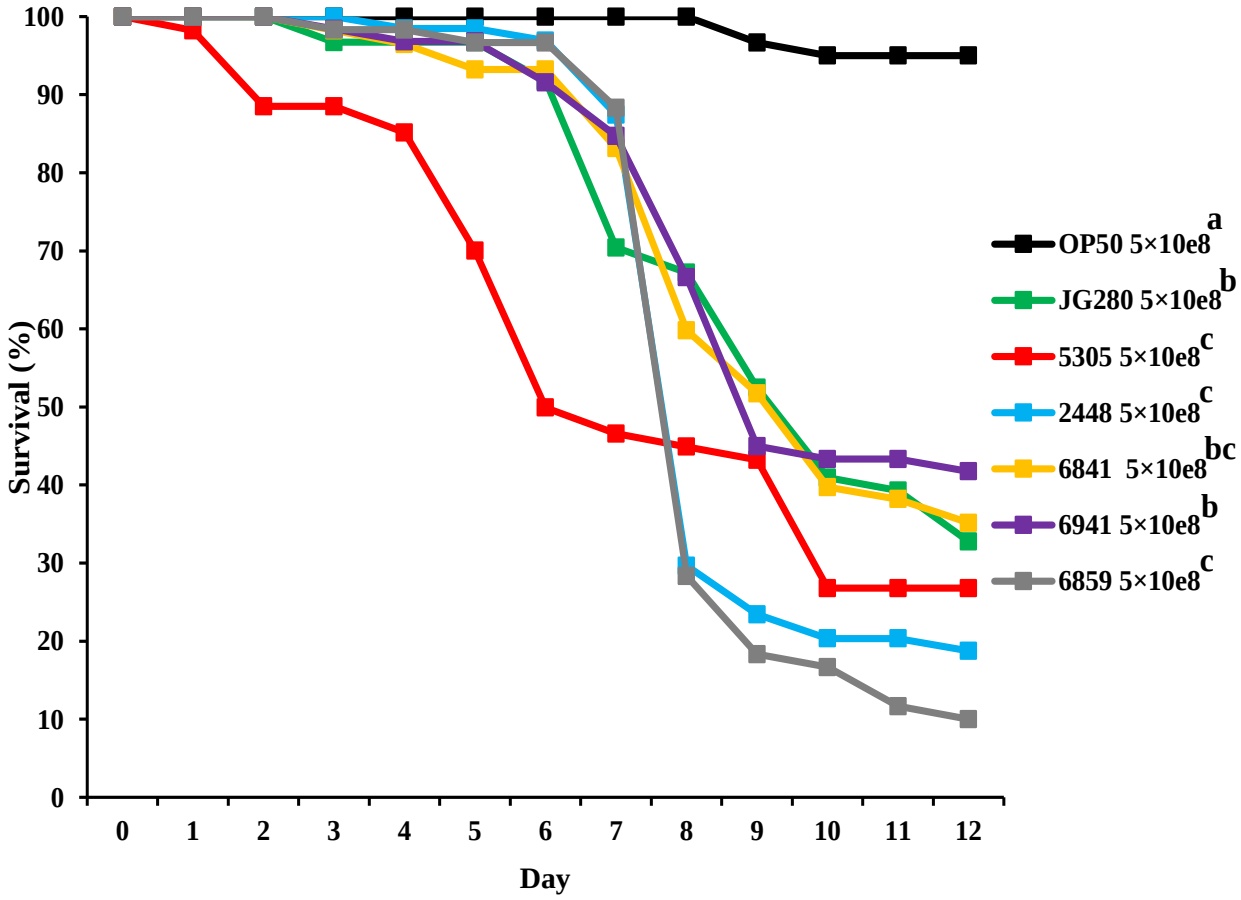


Figure 7. 8 Effects of feeding isolates OP50, 5305 and four extraintestinal pathogenic *E. coli* from chicken feces including 2448, 6841, 6941, and 6859 at 5 × 10^8 CFU/mL on survival (%) of *C. elegans* for 12 days.



7.5 Discussion and conclusion

Avian pathogenic *E. coli* that can cause extraintestinal infections contains VGs which enable their survival and invasion (Rosengren et al., 2009) and have been increasingly reported to be a reservoir of ARGs with potentials of food contamination during processing (Osman et al., 2018; Mpundu et al., 2019; Yang et al., 2021a). The present study evaluated the virulence potential of APEC and whether the pathogenicity is associated to or influenced by their AMR profiles.

High frequency of multidrug resistances among *E. coli* were found in major chicken and turkey meat production categories including conventionally-raised, organic, and "raised without antibiotics" (Davis et al., 2018). The most resistance frequency was observed against tetracycline, one of the common antimicrobials in animal farms, which belongs to Category III drugs. Our data strongly highlighted the health risks associated with the tetracycline use in poultry farms as also reported by Granados-Chinchilla and Rodríguez (2017). In *E. coli*, tetracycline resistance can be mediated by genes including *tet (A, B, C, D, E)* (Grossman, 2016). These genes can co-locate (link) with other antimicrobial determinants leading to multiple resistance, which is becoming a major health concern worldwide in *E. coli* (Licker et al., 2015). Nonetheless, the discrepancy of AMR genotype and phenotype in *E. coli* has been also reported in a previous study and it may be due to environmental factors that can affect the emergence, mobilization, and maintenance of ARGs in bacteria (Bengtsson-Palme et al., 2018).

In this study, two ß-lactam antibiotic resistance genes (*ampC, bla*$_{\text{CMY-2}}$), two aminoglycoside resistance genes [*aph(3'')-Ib, aph(6)-Id*], and a dihydrofolate reductase gene (*drfA1*) coding for an insensitive dihydrofolate reductase which confers 190- or 1000- fold resistance to trimethoprim, were detected in studied poultry isolates. Additionally, no specific ARGs were detected in the human ExPEC (5305) even showing a cefoxitin resistance phenotype. This may be due to the presence of non-specific mechanisms such as changes in the outer membrane and/or multidrug resistant efflux pumps code by genes such as *emr*, *mdt*, and *TolC* (Lomovskaya and Lewis, 1992; Perreten et al., 2001; Zgurskaya et al., 2011).

Plasmids in bacteria are well-known to play an important role in horizontal gene transfers (Muloi et al., 2018). Interestingly, a recent report using Oxford Nanopore sequencing technology to investigate plasmidome revealed that ARGs were preferentially located on bacterial plasmids, contributing to their transmissibility (Kirstahler et al., 2021). *Inc*FII, *Inc*FIIA, *Inc*FIB in the studied isolates indicated their capabilities to transfer and spread AMR. Additionally, the presence of multiple ARGs carrying plasmid *Inc*A/C2 demonstrated the potentials in inducing the spread of

multidrug resistance to aminoglycosides, ß-lactam, sulfonamide, tetracycline, and chloramphenicol since plasmid *Inc*A/C2 has been reported to disseminate ARGs such as $bla_{\text{CMY-2}}$ globally among bacteria (Guo et al., 2014; Humbert et al., 2019). Interestingly, the multidrug resistant turkey meat isolate 4973 harboring *Inc*A/C2 plasmid, showed the reduction in *C. elegans* survival (%) like the human ExPEC, indicating its virulence potential (zoonosis). Furthermore, the presences of *sul1* and/or *qacEdelta 1* (non-motile) in six isolates (4926 and 4941 from chicken, 4971 and 4973 from turkey meat, 2448 and 6941 from feces) associated with the transposon *Tn21* (class 1 integron) indicated the potential of these isolates disseminate antimicrobial resistance (Nikaido, 2009). Interestingly, isolates 4973, 2448, and 6941 reduced the *C. elegans* survival (%) similar to the human ExPEC, suggesting their zoonotic potentials. Whether the cause of worm death was associated with ARGs in *Inc*A/C2 or *Tn21* remained to be further determined.

The defense system in *C. elegans* is highly homologous to the innate immune system in humans, indicating that nematode is the reliable animal model to study human infections (Kong et al., 2016; Poupet et al., 2020). Since virulence of many pathogenic bacteria of animal origin have been studied using nematodes, *C. elegans* as an animal model was used in this study (Aballay et al., 2000; Ikeda et al., 2007; Wang et al., 2011; Zhou et al., 2014; Zhou et al., 2018). To assess the virulence potentials of poultry-source AMR ExPEC, seven multi-resistance isolates from poultry retail meat (n = 3) and feces (n = 4) were compared to a human UTI isolates on *C. elegans* life-span (Noamani et al., 2003; Zhou et al., 2014; Zhou et al., 2018). These multidrug resistant isolates from poultry retail meats were selected based on their close relationship, serotype (O25: H4), and sequence type (ST131) with the human isolate. It has been previously reported that poultry-source *E. coli* may possess zoonotic potentials (Johnson et al., 2007; Starčič Erjavec et al., 2007; Logue et al., 2017). A recent study demonstrated the global expansion of *E. coli* ST131 causing increasing incidences of human infections (Matsumura et al., 2016; Manges et al., 2019; Peirano et al., 2020). Additionally, since chicken fecal *E. coli* have been reported to possess potentials in contaminating poultry products and food chain during processing, four chicken fecal AMR ExPEC with different ASTs and number of VGs were included in the *C. elegans* model (Mellata, 2013).

The worm death by human ExPEC in the first *C. elegans* life-span assay suggested that this animal model is successful to predict zoonosis of human infections, which was in agreement with a recent study reporting that ExPEC from human UTI could kill the nematode (Schifano et al., 2019; Mageiros et al., 2021). This virulence could be attributed to colonization and invasion factors encoded by genes such as *kspM* and *kspT* (Schifano et al., 2019). Furthermore, detected

iron acquisition systems related factors in studied isolates have been shown to be involved in virulence (Okeke et al., 2004; Anderson and Leibold, 2014). Interestingly, the reduced survival of *C. elegans* by ExPEC from poultry meat and feces in the second and third *C. elegan*s life-span assays indicated their zoonotic potentials due to the presence of several virulence factors which need to be further specified (Rodriguez-Siek et al., 2005; Jakobsen et al., 2011).

The increasing AMR has become a major challenge and an interesting phenomenon is that the prevalence of AMR and increased pathogenicity of bacteria often arise simultaneously (Guillard et al., 2016). Despite the genetic relationship between some ARGs and VGs on chromosomes and plasmids, the specific role of AMR in pathogenicity remains elusive. An increased pathogenicity is often concurrent with increased AMR and both intrinsic and acquired antibiotic resistance could contribute to a positive fitness advantage to bacteria during infection (Roux et al., 2015; Schroeder et al., 2017). Thus, the present work showed the ability of multi-resistant ExPEC from poultry to induce death of *C. elegans*. Although the relationship between specific antimicrobial resistance phenotype and genotype in the pathogenicity in studied isolates remains to be established, whether the death of worms is associated with any types of ARGs and plasmids remains to be further determined.

This study provided the genome sequence analysis of multidrug resistant ExPEC isolated from a chicken and turkey. Several ARGs and VGs were found along with various plasmids indicating their possible transfer to disseminate their carrying resistant and virulence determinants. The emergency to pandemic lineages such as *E. coli* sequence type 131 (ST131), along with its highly drug-resistance is of concern. The reduction of *C. elegans* survival by the multidrug resistant ST131 isolates from poultry described in the present study was comparable to a human clinical isolate, indicating their zoonotic potential. Further studies on the role of specific ARGs and relation with factors allowing their persistence, predominance, and competitiveness involved in their virulence are warranted. Data from the present study highlighted food safety concerns about AMR *E. coli* from poultry.

CHAPTER EIGHT: GENERAL DISCUSSION

The prevalence and spread of antimicrobial resistance (AMR) have become a major threat to animal and human health, and usage of antimicrobials as feed supplementations for farm animals is one of the greatest contributors (Ma et al., 2020). Despite the elimination of using antimicrobials as an effective strategy to control AMR, it could compromise animal performance and elevate incidences of diseases (Abudabos et al., 2013). In recent years, the supplementations of antimicrobial alternatives in poultry feed such as essential oils (EOs) have been extensively studied (Yang et al., 2015a). Since one of limitations on the application of EOs in poultry farms is their instabilities, a recent study used soy protein-polysaccharide Maillard Reaction product emulsions to protect citral (CIT), enhanced stabilities during prolonged storage, feed processing, and gastric transition (Yang et al., 2015b). In this project, we tested cinnamaldehyde (CIN) and CIT in our studies since their high antimicrobial, anti-inflammatory, and antioxidant potentials have been demonstrated in previous studies, but limited research was conducted to investigate the effects of encapsulated CIN and CIT on growth performance, gut health, meat quality, and AMR phenotypes and genotypes of fecal *E. coli* in broiler chickens.

Our study demonstrated that encapsulated CIN (100 ppm), CIT (100 ppm), or CIN+CIT (100 ppm CIN + 100 ppm CIT) could improve growth performance, gut lesions, and modulate cecal microbiota (Chapter Four). The selected concentrations of EOs used in this animal trial were lower than MICs of CIN (100 - 600 ppm) (Adukwu et al., 2016) and CIT (600 - 2500 ppm) (Adukwu et al., 2016), suggesting the antimicrobial properties should not be the only criteria of selection for antimicrobial alternatives in poultry production. Pathogens and endotoxins can stimulate a low-grade inflammation in broiler chickens and one of the negative consequences of inflammation at the intestinal level is increased intestinal permeability, or “leaky gut”, associated with impaired nutrient absorption and increases in diarrhea incidence. In addition to antimicrobial properties, EOs can reduce inflammation associated with lipopolysaccharide and prevent “leaky gut” (Omonijo et al., 2019). Since the industry’s acceptance of EOs to optimize chicken performance and health also depends on inclusion cost, using lower doses of CIN and CIT to prevent inflammation will be encouraging for the poultry industry.

The reason for evaluating the effects of vaccination alone or in combination with EOs was because live or attenuated coccidiosis vaccines have been marketed and used commercially since the 1950s, showing potential as antimicrobial alternatives due to their high efficacy in controlling drug-resistant *Eimeria* species (Chapman et al., 2014; Soutter et al., 2020). Furthermore, it was

unknown if vaccine efficacy could be affected by the presence of EOs. The inhibition of drug-resistant *Eimeria* species by coccidiosis vaccine has been largely associated with the replacement of resident resistant oocysts by vaccine-derived drug-sensitive species due to a low rate of genetic recombination in avian coccidia and stimulating the chicken immune system to produce antigens (Dalloul and Lillehoj, 2006; Chapman et al., 2013). Nevertheless, the coccidiosis vaccine may compromise the performance of young chicks and increase susceptibility to other infections such as necrotic enteritis (NE) (Li et al., 2005). Therefore, spraying coccidiosis vaccines is an alternative strategy to challenge birds to cause minor gut inflammations (coccidiosis and NE), while providing a way to observe the effects of tested EOs on gut lesions. Additionally, results of the coccidiosis vaccine alone or in combination with EOs on affecting bird performance and gut health are inconsistent in the previous research (Oviedo-Rondón et al., 2006). As per the results shown in Chapter Four, the interactions between coccidiosis and EOs on increasing average daily gain (ADG) and body weight (BW) were observed in the starter and grower phases, respectively, indicating the ability of EOs to improve the growth performance of birds after vaccination. We selected bacitracin (BAC) as a positive control since BAC was commonly used to prevent NE when the animal trial was conducted. However, BAC, which is categorized as Category III in Canada, was restricted in usage as feed supplementations and was planned to be eliminated by the end of 2020 (Chicken Farmers of Canada, 2020). This could also explain the selection of avilamycin (AVI) as a positive control in the second chicken trial (Chapter Six) conducted in 2018 instead of BAC, an antimicrobial that is still allowed in poultry farms (Canadian Food Inspection Agency, 2020). The results from the first animal trial also suggested that tested EOs, especially CIN alone, could reduce NE lesions and relative abundance (%) of cecal *Enterococcus* and *Clostridium* as infectious predictors (Boehm and Sassoubre, 2014; Num and Useh, 2014), and increased cecal *Lactobacillus* as beneficial genera in broiler chickens (Aziz et al., 2019). The reduction of NE lesions may be due to their inhibition on spore germination and outgrowth of *C. perfringens* by CIN as phenolic compounds (Juneja and Friedman, 2007). However, the mechanisms for modulating cecal microbiota and its impacts on chicken gut health are still not clear. Furthermore, no significant synergism has been found to affect growth performance, gut lesions, and cecal microbiota, which remains to be further determined.

Despite the beneficial effects of encapsulated CIN, CIT, and CIN+CIT on growth performance, reducing gut lesions, and altering gut microbiota as discussed in Chapter Four, the data was still not enough to develop a viable alternative to in-feed antimicrobials. One of the most

important things to know was whether tested EOs have impacts on AMR phenotypes, genotypes, and virulence of chicken commensal bacteria such as *E. coli*. It has been well-known that AMR was caused by positive selections on bacteria with antimicrobial resistance genes (ARGs) and resistance plasmids due to prolonged, overuses, or misuses of antimicrobials (Von Wintersdorff et al., 2016). Nonetheless, no analyses on the effects of EOs supplemented in broiler chicken feeds on AMR phenotypes and genotypes of *E. coli* isolated from chicken fecal samples have been conducted. Thus, the study in Chapter Five was the first of its kind, reporting a full investigation on the effects of BAC, encapsulated CIN, CIT, and CIN+CIT on AMR levels, and genetic information in chicken fecal *E. coli* including ARGs, resistance plasmids, virulence genes (VGs), serotypes, and multilocus sequence typing (MLST). Interestingly, the results showed that encapsulated CIN reduced the AMR levels and prevalence (%) of ARGs and resistance plasmids compared to both NC and BAC in chicken fecal *E. coli*, while encapsulated CIN+CIT could reduce AMR levels and ARGs only compared to BAC. This new finding suggested that CIN could be the effective compound that contributes to the increase in antimicrobial susceptibilities (ASTs) of chicken commensal *E. coli*. However, the mechanisms of CIN or CIN+CIT on modulating AMR prevalence are still unknown. Regarding the effects of tested EOs on virulence potentials, despite that the number of VGs was not altered, colibacillosis-associated serotypes such as O21: H16 and MLST, including ST38, showed lower numbers in fecal *E. coli* isolated from CIN or CIN+CIT-treated birds. This finding suggested that not only CIN or CIN+CIT could reduce AMR, but they may also decrease the prevalence of colibacillosis. Interestingly, higher AMR levels and number of ARGs were observed in fecal *E. coli* isolated from birds treated with BAC compared to other treatments. This result was interesting since BAC has been frequently used to control the growth of Gram-positive bacteria such as *C. perfringens* instead of *E. coli* by inhibiting cell wall formation before 2020 (Diarra and Malouin, 2014). The mechanism is not quite clear but it at least suggests the urgency of reducing commonly used antimicrobials as poultry feed supplementations. Interestingly, despite there being no significant synergistic effects of CIN and CIT on broiler chicken growth performance in Chapter Four, the interaction between CIN and CIT was detected in reducing resistance levels and the number of ARGs. This may suggest the potential of synergism between CIN and CIT on the AMR profiles of chicken fecal *E. coli*. However, CIN possesses the highest efficacy in reducing AMR levels and ARGs numbers. In addition, the study found that bird age is also a factor that can modulate the AMR phenotypes and genotypes of chicken fecal *E. coli*. Although the most pronounced effects of antimicrobials for improving growth performance were

observed in young animals (Butaye et al., 2003; Yang et al., 2020), this result indicated the importance of eliminating supplementations of antimicrobials in young birds. Additionally, we observed that some chicken fecal *E. coli* had a close evolutionary distance to human extraintestinal pathogenic *E. coli* (ExPEC) in the phylogenetic tree, indicating a close relationship between some poultry-isolated *E. coli* and human ExPEC (Park, 2019). This generated our interest in evaluating whether poultry-isolated *E. coli* could have zoonotic potentials.

Due to the AMR and virulence of poultry-isolated ExPEC shown in Chapter Five, it is necessary to control the growth of pathogens by improving chicken gut health. This is because a healthy chicken gut plays an important role in preventing the colonization of pathogens (Diaz Carrasco et al., 2019). Additionally, chicken breast meat quality is another important factor that could reflect gut health since pathogens present in meat products originated partly from the gastrointestinal tract which can affect meat quality traits (Chaillou et al., 2015; Rouger et al., 2017). Since we found that tested EOs, especially encapsulated CIN alone as feed supplementations, could reduce gut NE lesions, alter cecal microbiota, and reduce prevalence and spread of AMR (Chapter Four and Five), in Chapter Six, it was hypothesized that encapsulated CIN could promote chicken gut health and improve meat quality. While conducting the animal trial, we selected four parameters to fully predict gut health including ileal apparent nutrient digestibility (AID), intestinal morphology, jejunal gene expressions of specific molecules, ileal and cecal microbiota (Jeurissen et al., 2002), and five parameters reflecting chicken breast meat quality including pH, meat color, myopathies (white stripping, WS; woody breast, WB), tenderness (myofibrillar fragmentation index, MFI; Warner-Bratzler shear force, WBSF), and water holding capacity (dropping loss and cooking loss) (Baracho et al., 2006). In comparison to Chapter four, we selected encapsulated CIN alone at lower concentrations including 50 ppm and 100 ppm since no coccidiosis vaccine was applied in this second animal trial and it was necessary to test the efficacy of the tested EOs at lower concentrations. Additionally, as mentioned above, AVI was selected as a positive control instead of BAC due to the new regulations in Canada regarding restrictions on BAC as feed supplementations. Briefly, the results in Chapter Six demonstrated that gut health could be improved by increasing the AID of nutrients, enhancing duodenal and jejunal villus/crypt ratio (VCR), elevating jejunal gene expressions for amino acid transporters (B^0AT-1, CAT-1), and increasing either ileal or cecal relative abundance of genus *Lactobacillus*. Despite results that were similar to previous studies with EOs, the mechanisms of encapsulated CIN on modulating chicken gut health are unclear. It may be due to antimicrobial, anti-inflammatory, and antioxidant

potentials of CIN found *in vitro* studies as discussed in Chapter Six (Onawunmi, 1989; Wondrak et al., 2010; Pina-Pérez et al., 2012; Andrade and De Sousa, 2013; Sang-Oh et al, 2013; Yang et al., 2015a; Yang et al., 2015b; Bouzenna et al., 2017). In contrast, most of the meat quality parameters were not affected by encapsulated CIN but reduced WBSF was observed by CIN at 100 ppm, indicating the elevated meat tenderness. No differences in meat color, myopathies, cooking loss (%) and dripping loss (%) may be due to good management and low stock density at the University's poultry barn, which reduced pre-slaughter stress of all birds. Nevertheless, the meat quality results at least indicated that CIN has the potential to improve meat tenderness by reducing WBSF. However, it is unknown whether improved meat quality and gut health have a relationship with the controlled growth of pathogens by CIN. Further studies are still necessary to find the relationship by comparing the number of pathogens in chicken digesta and retail meat among treatments. By combining the results from Chapter Four to Six, it is noted that encapsulated CIN as chicken feed supplementations could be an efficient way to promote chicken growth performance, improve gut health and meat quality, and modulate the AMR and virulence genotypes of chicken fecal *E. coli*. However, toxic and economic assessments are still necessary for future studies.

As per the results observed in Chapter Five, some chicken fecal ExPEC had a close relationship with human ExPEC in the phylogenetic tree. This generated our interest to investigate whether poultry-isolated ExPEC has zoonosis by using animal models since the phylogenetic tree only showed the revolutionary distance between bacterial isolates. Although this study did not aim to further evaluate our tested EO, it is still meaningful to know whether poultry-isolated ExPEC could infect humans. The *C. elegans* was selected because its life-span assay has been proven to be a useful tool in predicting zoonosis of other pathogens isolated from farm animals including *E. coli* as discussed in Chapter Seven (Zhou et al., 2014). In addition to chicken fecal ExPEC, muti-drug resistance ExPEC isolated from turkey and chicken retail meats were also selected for performing life-span assay due to increasing concerns about food safety of poultry products caused by AMR ExPEC (Mellata, 2013). Since the present study was the first report on predicting zoonosis of poultry-isolated AMR ExPEC by *C. elegans*, the first life-span assay using *E. coli* OP50, K88+ enterotoxigenic *E. coli* (ETEC), and an ExPEC isolated from human urinal tract infection (UTI) was established to determine whether the model is successful in predicting the pathogenicity of human ExPEC. Additionally, we tested the effects of AMR ExPEC with a different number of VGs or ASTs on *C. elegans* survival rate (%). Despite there being no

relationship between pathogenicity and ASTs or number of VGs, the results at least revealed that our established *C. elegans* life-span assay is a successful model to predict human pathogenicity and all tested AMR ExPEC isolated from poultry retail meat or fecal samples may have zoonotic potentials. Since the tested poultry-isolated AMR ExPEC were selected with high evolutionary relatedness to human ExPEC in the phylogenetic tree, our results also indicated that zoonotic potentials could be also predicted by comparing evolutionary distance in the phylogenetic tree (Jakobsen et al., 2011; Logue et al., 2017). No conclusive relationship between ASTs or number of VGs with *C. elegans* survival rate (%) shown in this study may be due to small sample sizes, expressions of VGs oxygen exchange interference of *C. elegans* by attached *E. coli* (Chisholm and Hsiao, 2012), and differences in immune system between *C. elegans* and human in response to ExPEC (Poupet et al., 2020), as discussed in Chapter Seven. Therefore, it is still difficult to conclude that *E. coli* with different ASTs and number of VGs had no impact on pathogenicity. Nevertheless, this study was the first step for evaluating and predicting the zoonotic potentials of poultry-isolated AMR ExPEC. This suggested that poultry-isolated ExPEC could have associations in inducing diseases and it is important to control the prevalence of poultry-isolated ExPEC. Additionally, in Chapter Five, we also observed that prevalence (%) of chicken fecal ExPEC was 86.55, 81.25, 73.61, 87.50, and 83.33 in birds when fed NC, BAC, CIN, CIT, and CIN+CIT, respectively (data not shown). This may indicate CIN could be a potential antimicrobial alternative that may reduce the risk of human infections caused by poultry-isolated ExPEC, but further investigations are required.

CHAPTER NINE: SUMMARY AND FUTURE STUDIES

9.1 Summary

The major findings that can be drawn from the present research (Chapter Four – Eight) are:

1. Encapsulated cinnamaldehyde (CIN) alone or in combination with citral (CIN+CIT) appeared to improve the general performance of broilers similar to bacitracin (BAC).
2. Encapsulated CIN and CIN+CIT had similar effects on controlling necrotic enteritis (NE) due to *C. perfringens* and coccidiosis caused by *Eimeria* species.
3. Combination of vaccine and encapsulated essential oils (EOs) could modulate the composition of cecal microbiota by increasing *Lactobacillus* and decreasing *Enterococcus* and *Clostridium*.
4. Dietary CIN affected antimicrobial resistance (AMR) phenotypes and genotypes of *E. coli* in chicken feces.
5. Bird age could also be a factor that affects AMR phenotypes and genotypes of chicken fecal *E. coli* and appeared to influence the number of virulence genes (VGs), serotypes, and multilocus sequencing typing (MLST)
6. Dietary CIN affected the presence of various plasmids harboring antimicrobial resistance genes (ARGs) in studies of *E. coli* indicating the ability of CIN to decrease a potential lateral AMR gene transfer between this bacterium and other chicken gut bacteria.
7. Dietary encapsulated CIN improved meat quality by reducing meat Warner-Bratzler force shear.
8. Dietary encapsulated CIN increased villus to crypt ratio in intestines, and apparent ileal digestibility of nutrients, and altered ileal and cecal microbiota composition.
9. The extraintestinal pathogenic *E. coli* (ExPEC) isolated from poultry meat and chicken fecal samples tested in this study may have the potential for human infections.
10. Additionally, the relationship between AMR and VGs profiles with pathogenicity remains to be further determined.

9.2 Future studies

The present research showed the potentials of encapsulated CIN as antimicrobial alternatives in broiler chickens. However, more investigations are still needed before encapsulated CIN can be adopted by the poultry industry. The future research directions are to investigate:

1) the toxicity and safety of encapsulated CIN in broiler chickens by a complete assessment and,

2) the effectiveness of encapsulated CIN in controlling necrotic enteritis in on-farm trials (e.g. raised without antibiotics) and its economic impact under the current commercial production of broiler chickens (real world) and,

3) the effects of encapsulated CIN as antimicrobial alternatives on other poultry and livestock species such as turkey, layer hens, swine, ruminants.

CHAPTER TEN: LITERATURE CITED

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