

**IMPACTS OF ANTIMICROBIAL GROWTH PROMOTERS
USED IN BROILER CHICKEN PRODUCTION ON THE
EMERGENCE OF ANTIBIOTIC RESISTANCE IN
COMMENSAL *E. COLI* AND *SALMONELLA***

by

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Abstract

Despite their beneficial effects, concerns have been raised about the role of antimicrobial growth promoters (AGP) in the emergence of antibiotic resistant bacteria. This study evaluated the effects of approved AGP on the emergence of antibiotic resistance in commensal *E. coli* and foodborne pathogen *Salmonella*. A survey of antibiotic resistance levels in commercial broiler chicken farms in the Fraser Valley (B.C.) and an experimental feeding trial were conducted from May 2004 to February 2005 and May to November 2005, respectively. The latter examined the effects of ten AGP formulations (bambermycin, penicillin, salinomycin, bacitracin, combination of salinomycin and bacitracin, chlortetracycline, virginiamycin 11ppm, virginiamycin 22ppm, monensin and narasin) on bird performance as well.

Multiple antibiotic resistant commensal *E. coli* and *Salmonella* carrying virulence genes were found at commercial broiler chicken farms and therefore may serve as reservoirs for these genes. There was no significant difference between feed formulations on the phenotypic or genotypic characteristics of the isolates, except for tetracycline resistance gene *tet(B)*.

In the experimental feeding trial, broiler chickens were fed a diet including or excluding AGP. Birds were sampled prior to and weekly during feeding of the control and the AGPP containing diets. Although not detected on day 0, *E. coli* increased after day 7 to more than $9.9 \log_{10}$ CFU/g in ceca. Multi-drug resistant *E. coli* were isolated from birds fed the ten AGP containing diets as well as the control diet. Except for penicillin, none of the AGP containing diets significantly improved bird performance compared to the control diet ($P > 0.05$). Good management practices can significantly improve broiler chickens performance and decrease the mortality rate.

Keywords: Broiler chicken, commensal *E. coli*, *Salmonella*, antibiotic resistance, virulence genes, class 1 integron, growth promoters, chicken performance, food safety, foodborne diseases.

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List of abbreviations

AAFC: Agriculture and Agri-Food Canada
ABC: ATP-binding cassette
AGP: Antimicrobial used as Growth Promoters
AMR: Antimicrobial resistance
BAC: Bacitracin
BBM: Bambermycin
C: Control
CDC: Center for Disease Control
CE: Competitive Exclusion
CFC: Chicken Farmers of Canada
CFIA: Canadian Food Inspection Agency
CHLOT: Chlorotetracyclin
CIPARS: Canadian Integrated Program for Antimicrobial Resistance Surveillance
CRSAD: Centre de Recherche en Sciences Animales de Deschambault
FDA: U.S. Food and Drug Administration
FSIS: Food Safety Infection Service
HACCP: Hazard Analysis Critical Control Point
MATE: Multidrug and Toxic Compounds Extrusion family
MDR: Multi-drug resistance
MIB: Medicating Ingredient Brochures
MIC: Minimum Inhibitory Concentration
MFS: Major Facilitator Superfamily
mg: Milligrams
MON: Monensin
NAR: Narasin
PARC: Pacific Agri-Food Research Centre
PBP: Penicillin Binding Proteins
PEN: Penicillin
PHAC: Public Health Agency of Canada
PMF: Proton motive force-dependant family
PTC: peptidyl transferase catalytic
RND: Resistance/Nodulation/Cell Division family
SAL: Salinomycin
SAL+BAC: Salinomycin + Bacitracin combination
SMR: Small Multi-drug Resistant family
VIR11: Virginiamycin 11 ppm
VIR22: Virginiamycin 22 ppm
WHO: World Health Organization

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Dedication

This thesis is dedicated to:

My darling Dad (Soumana) who was gone so soon

This thesis is for you Dad. You are not physically here, but I know you will be always by my side, watching over me as you have always done. I love you
papa.

My dear mom (Assitan) and bothers (N’Faly and Ibrahim)

You have been supportive all these years. You gave me the strength to keep going, even during my saddest moments. Thank you so much. I love you.

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My son, the sun of my life Soumana Apho Ly

I enjoyed every second of being with you. You are a blessing, a gift and I already anticipate all the happiness and joy of watching you grow.

Chapter I. Review of the literature

I.1 Introduction

Chicken production constitutes the most important part of the worldwide poultry industry (AAFC 2008). The threat of the avian flu pandemic seems not to have dulled the ebullience for chicken meat, which remains the favorite meat for Canadians. The Canadian poultry industry is proliferating; in 2005, Canada was thirteenth in chicken production in the world (AAFC 2008). British Columbia is third, after Ontario and Quebec in chicken production (AAFC 2008). This fast growing industry is led by the Chicken Farmers of Canada (CFC) which elaborated a well-organized and efficient production system and supply management to meet the ever increasing requirements of the consumers. In fact, current dietary trends involve the consumption of lean, protein-rich and low-carbohydrate meats. Chicken meat is the first choice because, in addition to its lower price, 100 g of roasted breast contains only 2.1 g of fat (CFC 2005).

The chicken industry operates under the Farm Products Agencies Act administrated by the National Farm Products Council which reports to Parliament through the Minister of Agriculture and Agri-Food Canada. CFC, in accordance with the federal government, provincial agriculture ministers and chicken producers in member provinces, strictly supervises the industry in a supply management system (AAFC 2008). In this orderly system, the chicken industry stakeholders, i.e. farmers, processors and restaurant traders from all ten provinces, decide the market requirements, determine the allocations

for producers and then agree on the price per kilogram, according to the cost of production (AAFC 2008).

The chicken industry is regulated by supply management to meet or exceed consumer's needs and expectations for safety, quality and to make chicken products affordable for consumers. Food safety and quality are the top priorities of the Government for Canada. The Canadian Food Inspection Agency (CFIA) works with provincial and territorial governments and the chicken industry to maintain the production of one of the safest chicken production systems in the world. This is achieved by the implementation of the On-Farm Food Safety program for all commodity boards (AAFC 2008). CFC's safety program is called *Safe, Safer, and Safest* and was accredited by CFIA in 2002 (CFC 2005).

The chicken industry also aims at insuring proficiency and economic returns to producers. This requires low inputs and greater economic and animal performance outcomes. To reach this objective, producers significantly intensified chicken production by improving breeding expertise and changes in rearing practices. The changes include the use of pharmaceutically active substances to treat and prevent diseases and to enhance growth performance (NRC 1999). Clinical and sub-clinical infections are controlled and prevented in chickens by administration of antimicrobials in feed or water. Mass treatment is preferred because of the large size of flocks in barns and mainly because of the fact that diseased chickens could passively contaminate neighbors which will not necessarily present the symptoms at the same time. During the production cycle, chickens are fed

three different grain-based diets including the starter, grower and finisher, each one supplemented with a combination of antimicrobials (NRC 1999). The starter diet is higher in proteins and fats to allow fast growth, then the concentration of these components are slightly decreased to balance the weight for age and to lower the fat content in the finisher diet.

About 90% of the antimicrobials used in agriculture are administered for growth promotion and prophylaxis (Khachatourians 1998). Many theories have been suggested to explain the positive effects of antimicrobials used as growth promoters (AGP). The major reasons include the reduction of sub-therapeutical infections that alter the immune system of chickens, making them more vulnerable to various bacterial, viral and parasitic infections. A further explanation could be the decreased competition between the host and the microbial flora for nutrients, which are now available in sufficient quantity for better feed intake, feed conversion and growth (Khachatourians 1998; NRC 1999). Healthy chickens are better able to resist pathogens and therefore face less stress and perform better. Consumers are also more confident to consume safe and high quality chicken products.

The standard formulation for each diet comprises an antibiotic, a coccidiostat and an arsenical for reduced morbidity and mortality and for weight gain and efficient feed conversion (Tables I.1; I.2) (NRC 1999). CFIA and the provincial governments firmly monitor antibiotic medication and withdrawal periods (CFIA 2008b). It is well recognized that antibiotic use in food-animal production is responsible for the emergence of bacteria

resistant to these antibiotics and has also led to development of cross resistance with their analogues used for human therapy (Wegener 1999). Foodborne diseases are caused by five groups of pathogens including multicellular animal parasites, protozoa, fungi, bacteria and viruses (Jay 2000). Fecal-oral transmission is the most common way of transmission to humans. Pathogens or contaminated food must first be ingested to allow the microbe to endure the different hurdles (e.g. gastric acidity, intestinal mucus barrier, host immune defence systems, competitive natural microflora and low oxygen tension in intestines), proliferate and then start their virulence activities (e.g. release of toxins). Some pathogens need to attach to intestine walls and colonize (e.g. entero-hemorrhagic *E. coli*, *Listeria monocytogenes*, *Salmonella*) while others can multiply without being attached on any intestinal tissue (e.g. *Clostridium perfringens*). Adhesion factors such as intimin (encoded by *eae* gene), virulence factors such as *Salmonella* plasmid virulence (encoded by *spv* gene), toxins such as Stx (encoded by *Stx1* and *2*) and acid tolerance factors such as RpoS (encoded by *rpoS*) are usually transported on mobile genetic elements such as plasmids, which are horizontally transmitted between bacteria species. This allows non-harmful bacteria to develop pathogenic characteristics and thus become a serious health concern (Jay 2000). Each year, about 76 million illnesses, 325,000 hospitalizations and 5,000 deaths are caused by foodborne diseases in the U.S. and the costs in terms of pain, suffering, reduced productivity and medical costs was estimated to range from U.S.\$10 to 83 billion (Mead 1999).

Although the spread of foodborne pathogens is an important health and food safety issue, it is less worrisome than the emergence and dissemination of resistant pathogens

into the environment. In Canada, the current overall medical costs of antibiotic resistance to the Canadian health care system, predominantly in the institutions, is approximately \$200 million per year, while in U.S., it is an estimated US\$100 million to 10 billion (OTA 1995; Mead 1999; Conly 2002). Chicken producers and the chicken industry also face significant economic losses due to widespread pathogenic and resistant bacteria in chicken and rearing environments (Nurmi and Rantala 1973; Mead 2002). These microorganisms could further contaminate chicken carcasses at the slaughter house, reach the retail market and cause millions of deaths globally each year (Yogarathnam 1995; Zhao et al. 2006).

Antimicrobial resistance (AMR) is a natural or acquired defence mechanism of bacteria when threatened by substances that can kill or inactivate them. Natural resistance is transferred vertically through chromosomes among bacteria of the same species, whereas the acquired resistance could be horizontally transferred among various bacteria groups. The latter is mainly achieved by mobile genetic elements such as plasmids, transposons and integrons (Levesque et al. 1995). More recently, a resistance mechanism called efflux was discovered (Sapunaric et al. 2005). This mechanism decreases the concentration of an antimicrobial inside the target cell by pumping out the antibiotic. It has been described for tetracycline and last resort antibiotics such as fluoroquinolones (Sapunaric et al. 2005; Vila 2005).

Interestingly, the World Health Organisation (WHO) recommends that AMR surveillance and monitoring for foodborne pathogens include a tripartite approach taking into account isolates from human clinical cases, food animals and retail meats (WHO

2001). Therefore, more emphasis was put on limiting contaminated carcasses from entering the food supply chain whereas monitoring systems aiming at eliminating the contamination source at the farm level are only slowly being implemented. The Canadian Integrated Program of Antimicrobial Resistance Surveillance (CIPARS) was developed in 2002 to provide AMR trends among some enteric organisms from humans, animals and animal-derived food sources across Canada (PHAC 2008). Adhering to WHO recommendations, the CIPARS focus is mainly on clinical samples, putting very little emphasis on husbandry of healthy broiler chickens, which could asymptotically carry resistant pathogens, especially since certain antimicrobials are recognized to competitively inhibit Gram positive microflora in favour of Gram negative pathogens (Callaway et al. 2003; PHAC 2008).

Education of the public, producers, processors and food service facility employees, in addition to efficient quality assurance and control systems in conjunction with accurate detection methods, are extremely important in controlling and reducing the emergence of antibiotic resistant pathogens. To reach this objective, there is an increasing need for more reliable data for evaluating the current situation regarding the impact of the use of antimicrobials in food-animal production, more specifically in chicken production, on the emergence of antibiotic resistant bacteria.

The present study aims at identifying the circumstances leading to the emergence of pathogenic and antimicrobial resistant commensal bacteria isolated from healthy broiler chickens raised on commercial farms and in an experimentally controlled barn. The

findings of this thesis may allow the identification and modification of current management practices that can potentially cause bacteria-related economic losses and favour the emergence of antibiotic resistance in broiler chicken production.

I.2 Bio-security procedures on broiler chicken farm

I.2.1 Cleaning and sanitation of broiler chicken barn

A clean environment is necessary to raise clean, quality chickens and to break the cycle of contamination (CFC 2005). CFC's *Safe, Safer, Safest* program issued guidelines about proper cleaning and disinfecting procedures that are to be used according to provincial environmental regulations and adapted to individual barns. In Canada, after each flock, the barn is often thoroughly cleaned as soon as possible to have it ready for the new flock. Three steps are involved in this process: cleaning, disinfecting and resting period.

Cleaning requires that all organic material be removed (i.e. blown or brushed) from the floors, walls, ceiling, fans and equipment as thoroughly as possible (CFC 2005). The cleaning starts by manure removal immediately after shipping of the flock and storage of the manure far enough away so that no possible contamination of the feed or barns can occur. Complete washing then follows, which includes thorough washing with water under high pressure (CFC 2005). Disinfection must follow the complete washing to

ensure effective cleaning procedures. Furthermore, disinfectants work better in a clean environment since organic matter can neutralize disinfectants rendering them inefficient in sanitizing surfaces. Disinfectant wash or fumigation can be used for this step (CFC 2005). Lastly, a resting period is necessary to optimize the sanitation protocol. This period after disinfection and before the next flock needs to be prolonged, if possible, because microorganisms which may have survived the disinfection process could be destroyed by natural dehydration (CFC 2005). Recontamination of the barn can be minimized by reducing all access to the barn.

CFC may request proof that farmers are using proper and efficient cleaning procedures. A method to ensure effectiveness of cleaning and also to assess the validity of corrective actions taken is to routinely check the flock microbial status by testing for pathogens during the growth period and after cleaning and disinfecting (CFC 2005). Another measure to control the spread of disease between flocks is to use an ‘all-in all-out’ system. Flow-through barns using different aged birds are more challenging due to difficulty in effectively controlling disease outbreaks.

In the case of Pacific Agri-Food Research Centre (PARC), the Standard Operating Procedure for cleaning out and disinfecting the floor pens was issued and adapted from CFC’s *Safe, Safer, Safest* program. The manual describes the procedure used to remove the litter and disinfect pens before the barn is set up for the next flock. Manure is composted in a designated area. Composted manure is environmental friendly and more easily stored and constitutes a valuable by-product. The disinfection is achieved using an

appropriate disinfectant at the proper dilution, spraying down the entire barn and all equipment inside, covering all surfaces. A resting period lasting at least 48 hours allows the barn to dry out. Water lines are disinfected by allowing the disinfectant to remain in the lines for at least 1 hour before flushing to remove the product.

Foot baths are located near the doorways with easy access for all visitors and staff. This measure contributes to maintaining bio-security of the barn. Potassium monopersulfate solution at a concentration of 1 to 2% is used for foot baths and is replaced daily with fresh disinfectant solution. Other parameters contributing to the spread of diseases such as staff clothing (coveralls, hats and footwear), the direction of movement of station personnel that are not poultry staff (electricians, plumbers, general maintenance workers), disposal of dead or euthanized birds, rodents, insects and pest control are all submitted to strict scrutiny and procedures are described in the PARC's SOP brochure. Keeping a clean environment in the barn is the key to reducing the potential for infestation. However, if rodents, insects and pests are out of control, an exterminator should be called.

Despite these bio-security procedures, life threatening bacteria such as *Salmonella*, *E. coli* O157:H7, *Campylobacter* and other pathogens are routinely detected on chicken carcasses at slaughter houses and in fecal droppings collected at the farm level (Barrios et al. 2006). Numerous studies suggested that measures should be implemented at farm level to prevent bird contamination (Guerin et al. 2007; Huneau-Salaün et al. 2007).

Implementing effective bio-security measures implies identification of risk factors for flock colonization at the broiler chicken farm level.

I.2.2 Risk factors for flock bacterial contamination

Tremendous amounts of work have been dedicated to delineating risk factors for broiler chicken contamination with pathogens on the farm. Although results are sometimes conflicting, certainly due to differences in production systems, flock numbers and sampling methods, most studies agree that risk factors are strongly associated with the lack of proper bio-security measures, flock age and size, unofficial or untreated water source, presence of other farm animals on the same farm or in close proximity (regardless of the animal type), ventilation systems, the presence of rodents, insects, wild birds and poor manure management practices (Bouwknegt et al., 2004; Cardinale et al. 2004; Stern et al. 2005; Barrios et al. 2006; Arsenault et al. 2007; Guerin et al. 2007).

Among the different available studies on the risk factors of on-farm flock contamination, the Icelandic study led the way in explaining risk factors that specifically influence the proportion of positive flocks on a farm (Barrios et al. 2006; Guerin et al. 2007). The authors sampled fecal materials from all Icelandic broiler farms and sampled over 1,090 flocks (Barrios et al. 2006). In addition, Iceland does not import poultry meat nor meat products thus offering an ideal setting for an all-inclusive epidemiological study investigating the factors that affect the prevalence of *Campylobacter* spp. at all production

levels, from the breeder flocks to the consumer (Barrios et al. 2006; Guerin et al. 2007). Although this study was based on identifying risks factors associated with *Campylobacter* contamination, it considered pertinent aspects that could be applied to other pathogens and thus could constitute a basis for farm-level interventions.

The odds of a flock being positive for *Campylobacter spp.* increased with chicken age (Barrios et al. 2006). Younger chickens (less than 2 weeks old) may owe their apparent protection to maternal immunity (Kaino et al. 1988; Bouwknecht et al. 2004). In fact, high titers of maternal antibodies were found in sera from 1-day- and 7-day-old chickens, which subsequently decreased significantly (Sahin et al. 2001). Additionally flocks were more frequently colonized in the warmer months of summer and autumn (Refrégier-Petton et al. 2001). Ventilation systems seem to play an important role in the latter factor. Vertical ventilation systems, entrance for infected insects and droppings from migratory birds, barns with poor air circulation and/or static air distribution were associated with elevated contamination risks (Barrios et al. 2006). Increases in temperature related to problems of air circulation in the barn can generate favourable growth conditions for *Campylobacter* (low oxygen content and high temperature) (Refrégier-Petton et al. 2001).

The risk for flock infection with bacteria increased with flock size and the number of broiler barns on the farm regardless of bird density (Refrégier-Petton et al. 2001; Barrios et al. 2006). This is believed to be attributable to difficulties in maintaining strict hygiene or biosecurity practices and to the fact that bigger flocks represent increased

chances for introduction of bacteria because of increased personnel movements, or larger volume of water and air used (both potential carriers of the pathogen) (Barrios et al. 2006). Other authors did not find any association between the size of the flock and the increased risk of contamination (Bouwknegt et al. 2004).

The list of risk factors for on-farm bacterial contamination is not exhaustive. However adhering to strict bio-security practices such as improved maintenance of poultry-house surroundings in addition to the observation of simple hygienic practices by farm employees (coveralls, footbaths, hand cleaning and sanitation for each premise) would significantly reduce the long list of risk factors.

I.3 Chicken gastrointestinal microbiology

Scientists recognized the importance of the gastrointestinal (GI) microflora on chicken health and its immune system since the late 1890s (Shapiro and Sarles 1949). This marked the beginning of numerous studies aiming at elucidating the intestinal flora composition of the chicken and its relationship with the host (Knarreborg et al. 2002; Zhu et al. 2002; Jiangrang et al. 2003).

Several studies proved that the bacterial composition of the GI tract varies according to the diet, location and age of chickens (Shapiro and Sarles 1949; Knarreborg et al. 2002; Zhu et al. 2002; Jiangrang et al. 2003; Lu et al. 2008). It has been reported that newly hatched chicks harbor bacteria only in their cecal pouches before receiving any

feed. After consumption of feed and water, the total bacterial number significantly increased, most importantly in the ceca and progressively decreased in the colon, ileum and duodenum (Shapiro and Sarles 1949).

In general, the most important constituent of the chicken GI tract are anaerobic lactic acid bacteria, followed by coliforms and enterococci. Almost all obligate anaerobes are composed of *Clostridium perfringens* and the facultative bacteria include *Escherichia coli* (Shapiro and Sarles 1949). Apajalathi et al., found 10^8 CFU bacteria per gram of ileal content and 10^9 CFU bacteria per gram of cecal content from one day old chicks. These numbers increased steadily to 10^9 and 10^{11} bacteria per gram of ileal and cecal digesta, respectively (Apajalahti et al. 2004). Although, these results reflect the bacterial community of indoor chickens they are similar to the proportion and composition of microorganisms enumerated in free range chickens. However, free range chickens could harbor, in addition to those bacteria detected in indoors ones, other bacterial species, such as *Aerobacter aerogenes* that might be present in soil and plants and contaminate the feed (Shapiro and Sarles 1949).

Chicken GI microbial community increases in the first days after hatching to reach a peak, then slightly decreases during the next four to five weeks and then stabilizes as the bird matures (Apajalahti et al. 2004). The changes not only affect the bacterial density, but the bacterial composition also changes as the chicken ages. The transient bacterial community is progressively replaced by a more stable and varied composition between each region of the GI tract (Jiangrang et al. 2003). These differences could be explained

by the variability in bacteria substrate preference and growth requirements (Knarreborg et al. 2002). Hence the microbial community of chicken GI tract is greatly affected by the chemical composition and constitution of the gut contents and is distributed accordingly. Therefore, modifications in chicken diet and environment could influence the bacterial density and diversity in the gut, and consequently, affect the chicken health status and later human health.

It is well established that understanding the biological significance of the chicken normal intestinal flora is of supreme importance. Indeed, elucidating the composition of the gut microflora is necessary to understand the physiology and life cycle of the chicken, in addition to predicting the pharmacokinetic activity of antibiotics administered for treatment, prophylactic or growth promotion purposes. Knowing the exact composition of chicken GI tract is an essential component of antimicrobial resistant bacteria monitoring systems, because it provides information on resistance reservoirs and the shedding of resistant bacteria into the environment via manure. Life threatening diseases caused by foodborne pathogens such as *Campylobacter*, *Clostridium* and *Salmonella*, commonly found in the chicken GI track, are widespread in chicken flocks and rearing environments and are responsible for significant economic losses, for the producer and for the chicken industry (Mead 2002). Furthermore, the pathogenic bacteria can contaminate chicken carcasses at the slaughter house, reach the retail market and cause millions of deaths globally each year (Yogarathnam 1995; Mead 2002).

Undoubtedly, the ideal situation remains to eliminate contamination sources and prevent contaminated carcasses from entering the food supply chain. Many solutions to this issue are being developed to reach this objective. Modifying the gut microflora is one such strategy. In fact, elucidation of part of the bacterial composition of the GI tract allowed the initiation of strategies such as the concept of Competitive Exclusion (CE) (Nurmi and Rantala 1973; Mead 2002). CE is based on the principal of initiating an early immunity to bacteria of health concern in newly hatched chicks or chicks still in shells (Mead 2002). In other words, chicks are protected from deadly diseases by exposure to a bacterial preparation that contains a mixture of defined or unknown species. It is more likely that pathogens are excluded from the GI track by physical (physical barrier of the glycocalyx) and chemical (volatile fatty acids such as propionic and acetic acids) responses within the host or a combination of both. Further competition between the bacterial preparation and the native microflora for the short supply of nutrients and/or strategic targets may occur (Mead 2002). Pathogens like *Salmonella*, native intestinal flora such as *Lactobacillus* and *Bifidobacterium* and other combinations of bacteria were extensively studied to understand the mechanism underlining the CE phenomenon (Mead 2002). However, the mechanism of CE remains uncertain. In the mean time, pressure is put on enhancing biosecurity through improving hygiene by implementation of monitoring systems such Hazard Analysis Critical Control Point (HACCP) in rearing environments and throughout processing plants (CFIA 2008a).

I.4 Antibiotic use in animal production

I.4.1 Microbial challenge

It is possible to influence and control the gut microflora by better bio-security measures, bio-control systems and diet choice. An improved knowledge of the chicken gut microorganisms, their genetic variability and evolution allows prediction of their interaction with each other and with the host. Moreover, cloning allows structure-function analysis of gene products. A cloned gene or set of genes can be introduced into a new host to create a new metabolic pathway or to modify an existing pathway by large-scale production of proteins (Ping et al. 2007). Despite this powerful and constantly improving technology, numerous microorganism groups in the GI tract still remain undiscovered and their physiological characteristics unknown (Head et al. 1998; Lu et al. 2003; Apajalahti et al. 2004).

Most importantly, to control well defined microorganism species, animal food producers and processors depend on the use of substances to inhibit or kill the majority of microbial groups that could be harmful for both animals and humans. These substances, commonly called antimicrobials, have been used since the late 1940s in animal production and have evolved along with the development of molecular biology methods and surprisingly, with the improvement of those same antimicrobials. It seems the more efficient a detection and identification tool is, the more adapted and stronger microorganisms become each time they are challenged. However, this battle against the clock should be carried on because, in the absence of any winner, both parties should stay

at least in equal positions or maybe with a slim light of hope for the hosts to significantly obtain an advantage.

I.4.2 Purpose of antibiotic use in food-animals

Food-animals or food-producing animals are essentially raised for meat, milk or egg production (NRC 1999). They constitute an important source of protein, vitamins and minerals for human nutrition. On the other hand, food-producing animals could bear diseases or disease-causing microorganisms that could threaten human life. Therefore, maintaining health and well-being of food animals is fundamental for human health. One way of assuring a good health status for these food animals is the use of pharmacologically active substances also called antibiotics.

For over fifty years, there has been an intensification of food-animal production along with an increasing use of antibiotics. A conjugation of several factors contributed to this situation. The pressure of consumers' demand for high quality and quantity of a safe product at a reasonable price required producers to find economical approaches to meet the demands. Furthermore, producers' intent for increased productivity, efficiency and more economic return encouraged mass production in less time and increased the need for antibiotics to enhance growth. Confined housing of animals allows more mingling between animals and fosters the emergence and rapid spread of diseases among flocks;

and also contribute to the important demand for antibiotics to contain and eliminate any source of contamination (NRC 1999).

Most importantly, scientific discoveries regarding breeding, nutrition and animal health brought new perspectives to rearing practices. For instance, farmers select breeds that allow astonishing growth and also create a variety of genetically modified animals with remarkable performances. In 1928, average broiler chickens required 112 days and 22 kg of feed to reach 1.7 kg of weight. In 1990 the average weight was 2.0 kg after 42 days and less than 4 kg of feed. The average number of eggs per hen was 93 per year in 1930 and this number almost tripled to 252 in 1993 (NRC 1999). Although chickens can very rapidly double their weight this sometimes can affect their immune system and general health status. The use of antimicrobial agents for maintaining equilibrium between the increased growth for age seems to be necessary.

1.4.3 Antibiotics used as growth promoters

It is well established that the use of antibiotics in food-producing animals is fundamental to animal health, welfare and to the economic of the industry (Samanidou and Evaggelopoulou 2008). In this optic, five main classes of drugs are used: (i) topical antiseptics, bactericides and fungicides to treat skin infections; (ii) ionophores which alter GI tract microorganisms to provide more feed efficiency and conversion and also protect against certain parasites; (iii) hormones and hormonelike production enhancers for increased milk and meat production; (iv) antiparasite; and (v) antibiotics used to control

diseases and to promote growth (NRC 1999; Samanidou and Evaggelopoulou 2008). The last category will be the focus of this work.

Antimicrobial agents refer to all types of natural and synthetic antibiotics which may kill or slow down the growth of microorganisms. These include anti-fungals, household disinfectants and antibiotics. Antibiotics are natural substances produced by microorganisms. Presently, most antibiotics used are semi-synthetic, produced in large-scale microbial cultures, and then transformed by chemical processes (Samanidou and Evaggelopoulou 2008). Hundreds of antimicrobials have been produced since their discovery. However, for more than sixty years, bacteria have been challenging human ingenuity in the field of antimicrobial agents. They seem invulnerable and always appear to have developed the most effective protective armour.

The era of antibiotic growth promotion began in 1946 with the recognition of a substantial growth response to the inclusion of streptomycin in chicken feed (Jukes and Williams 1953). In 1949 it was shown that pigs and chickens consuming a diet supplemented with the dried mycelial mass, recovered from *Streptomyces aureofaciens* fermentation cultures, had significantly improved daily gain in bodyweight (Jukes and Williams 1953). At a time when livestock management was changing rapidly from low-performance, high-morbidity and free-range farming to more controlled and intensive production, and when post-war demands on increased food production were high, the discovery of an unexpected way to accelerate growth was received with enormous interest and enthusiasm by scientists and the public. Antimicrobial growth promoters (AGP) are

antibiotics added to the feed of food animals at subtherapeutic levels in order to enhance their growth rate and production performance (Samanidou and Evaggelopoulou 2008). According to the U.S. Food and Drug Administration (FDA), subtherapeutic concentrations are those inferior to 200 g/t of feed; although various antibiotic concentrations below this threshold could be used for different animal species (NRC 1999).

Many studies have demonstrated the benefits of these antimicrobials in livestock (Samanidou and Evaggelopoulou 2008). These benefits consist mostly of increased growth rate, increased feed conversion, improved egg production in laying hens, increased litter size in sows and increased milk yield in dairy cows. The favourable ways in which AGP favourably affect the intestinal flora and growth are not exactly known. However, some theories have been proposed: (i) nutrients may be protected against bacterial destruction; (ii) absorption of nutrients may improve because of a thinning of the small intestinal barrier; (iii) antibiotics may decrease the production of toxins by intestinal bacteria; and (iv) there may be reductions in the incidence of sub-clinical intestinal infections (Butaye et al. 2003). Clearly, animals are helped in fighting competitive microbial populations and thus, save a larger portion of consumed nutrients that could be efficiently used for maintenance, growth and productive functions. Tremendous amounts of antimicrobials are used for food producing animals and have exceeded by far (100 to 1000 times) human antimicrobial consumption (Khachatourians 1998; Levy 1998; Conly 2002). Consequently, in respect of the classical concept of survival of the fittest,

microorganisms exposed to this increasing pressure of antimicrobials developed and mastered strategies for survival.

I.4.4 Emergence of resistance

Antimicrobials are indispensable to both agriculture and human health. Some antibiotics are exclusively used in animal production (e.g. avilamycin, bambarmycin, tylosin), others only for human medicine (e.g. vancomycin, quinupristin, dalfopristin) and some antibiotics routinely used in human are also administered in large quantities to animals (e.g. erythromycin, gentamicin, tetracycline, penicillin, ciprofloxacin) (Khachatourians 1998). Prophylactic and growth promotion are the essential use of antimicrobials in agriculture, representing about 90% of overall use and highly contributing to the promotion of bacterial resistance (Khachatourians 1998). In fact, from the 1950s to now, the recommended levels of antibiotics in feeds increased 10 to 20 fold. This behaviour may promote the emergence of antibiotic resistant bacteria. Another important factor in agricultural practice that could encourage the emergence of resistance among microbial populations is the use of antimicrobials to control surface bacteria on crops, fruit trees and plants (Khachatourians 1998; Conly 2002).

Since the late 1960s, concerns have been raised regarding the possible emergence of bacteria, of both human and animal origin, resistant to therapeutic antibiotics. This concern was over food-borne infections with multi-antibiotic-resistant *Salmonella typhimurium* (strain DT 29) (Khachatourians 1998). The “Swann Report” recommended that antibiotics for promoting livestock growth should be restricted to those with limited

or no human therapeutic uses (Swann 1969; Samanidou and Evaggelopoulos 2008). Unfortunately, this report was not based on full scientific certainty and its recommendations were not totally taken into account (Khachatourians 1998). The foresight of the “Swann Report” is currently being confirmed worldwide by numerous peer-reviewed papers. Bacterial resistance to antimicrobials, especially those used for growth promotion, has now become a universal concern (Conly 2002; Bywater 2005)

Bacteria exposed to antimicrobials develop strategies for survival and resistance is one such strategy. To exert its activity, an antimicrobial should be able to enter into a specific bacterium, find its target and bind to it (Khachatourians 1998; Conly 2002). If any one of these steps is impaired, an antibiotic sensitive bacterium could become resistant to that antibiotic. Besides naturally occurring resistance, mutations might modify the target and/or the uptake of the antibiotic. These mutations could also lead to an efflux of the antibiotic as well as an inactivation or a modification of the antibiotic (Conly 2002). In reality, the relationships between an antibiotic and bacteria resistant to that antibiotic are not as simple as it seems. Antibiotics have different mechanisms of action and the bacterial reactions are varied. Antibiotics can target six different sites within the bacterium: (i) cell wall (e.g. beta-lactams, polypeptides), (ii) cell membranes (e.g. ionophores), (iii) protein synthesis sites (e.g. tetracycline, streptogramins), (iv) RNA (e.g. rifamycins), (v) DNA (e.g. quinolones), and (vi) folate synthesis (e.g. sulfonamides) (Khachatourians 1998; Conly 2002).

Antibiotic resistance mainly results from four events (Conly 2002). Expression of intrinsic resistance genes are responsible for naturally occurring resistance such as penicillin resistance in *Staphylococcus aureus* or naturally occurring resistance plasmid R (R-factors) conferring resistance to tetracycline (Sapunaric et al. 2005). Besides, genetic mutations could lead to the structural transformation of the antibiotic target in such a manner that the antibiotic could not bind (ribosomal mutations creating resistance to tetracyclines) to it or simply could not recognize it (alteration in DNA gyrase and topoisomerase IV inducing resistance to quinolones) (Conly 2002; Sapunaric et al. 2005; Vila 2005). In addition, existing resistance mechanisms could be modified by mutations making them more active or providing them with a broader spectrum of activity. This is the case in plasmid-mediated β -lactamases that results in extended-spectrum β -lactamases (Gold and Moellering 1996). Furthermore, antibiotics could be pumped out of the cytoplasm of target bacteria by active transportation through proton pumps. This mechanism, called active efflux, decreases the concentration of the antibiotic in the cell rendering it ineffective because the effective dose will not be reached (e.g. tetracycline and quinolone resistance) (Gold and Moellering 1996; Conly 2002; Sapunaric et al. 2005; Vila 2005). Most importantly, a greater concern resides in the potential for bacteria to acquire multiple antibiotic resistance genes carried on mobile genetic elements such as transposons, plasmids and integrons (Levesque et al. 1995; Gold and Moellering 1996; Conly 2002). This last mechanism which allows exchange of resistance genes between various bacteria species strongly contributes to the dissemination of antibiotic resistant genetic determinants among different environments, animal species and humans.

Although assaulted by as many as 150 available antimicrobial agents, bacteria succeed in finding ways to subsist and become resistant to existing antibiotics, consequently making it more difficult to treat diseases. Therefore, the lack of appropriate treatment, in addition to the inefficiency of former effective antibiotics, engenders treatment failure that causes millions of deaths each year in the world. Mead et al., estimated that 76 million people become sick and of those, 325,000 are hospitalized and 5,000 die in the United States each year from foodborne illness (Mead 1999). Significant economic costs are also inevitably related to these illnesses (OTA 1995; Conly 2002).

I.4.5 Antibiotic classes in chicken production

I.4.5.1 Trends in drug use in chicken production

As presented in Tables I.1 and I.2, standard broiler chicken feed rations contain three different classes of antimicrobials for growth promotion, prophylaxis and treatment: a coccidiostat, an arsenical and an antibiotic (NRC 1999; Samanidou and Evaggelopoulou 2008). During chicken production individual treatment is almost impossible due the large number of flocks and the large numbers of chickens in each flock. In addition, an individual bird may asymptotically carry diseases. Therefore, mass medication, called metaphylaxis, is the most practical and efficient way to administer any antimicrobial (Health Canada 2002).

Broiler chickens are fed three different diets: pre-starter and starter during the first two weeks after hatching, then the grower diet for the two next weeks and the finisher is

given until slaughter. Each diet is supplemented with a combination of antimicrobials in accordance with the withdrawal periods, especially the finisher diet. This safety measure allows the removal of any antimicrobial residue in tissues before shipping the chickens for processing. Water soluble antimicrobials are used in water systems for systemic or intestinal medication, in case of flock illness (NRC 1999). Day old chicks are injected with antibiotics such as ceftiofur or gentamicin to prevent further infections after vaccination against Marek's disease virus that causes a common lymphomatous (tumor raised in lymphatic tissues) and neuropathic disease in domestic chickens (NRC 1999).

The Canadian Food Inspection Agency (CFIA) has listed, in the Compendium of Medicating Ingredient Brochures (MIB), approved antimicrobials for use in animal feed (Tables I.1 and I.2). The major claims are their use against Gram-positive *Enterococcus* that cause colitis necrotitis as well as an aid in the prevention of coccidiosis in broiler chickens caused by *Eimeria necatrix*, *E. tenella*, *E. acervulina*, *E. brunette*, *E. mitis*, and *E. maxima* (CFIA 2008b). However, not every antibiotic used in food-animal production is approved for use in poultry production. The following antibiotics are used in broiler chicken feed, but can also be used for egg laying hens and breeding stock in the United States of America and in Canada (Samanidou and Evaggelopoulos 2008; CFIA 2008b).

I.4.5.2 Antimicrobials of interest

I.4.5.2.1 Ionophores

I.4.5.2.1.1 Mode of action

Ionophores are hydrophobic molecules that selectively bind to a given metal ion (e.g. sodium, potassium) and increase its cell permeability. Ionophores shield the charge of the ion to be transported, enabling it to penetrate the hydrophobic interior of the lipid bi-layer. Once they cross the cell membranes of Gram-positive bacteria, they dissipate ion gradients and uncouple energy expenditures from growth, killing the bacteria (Callaway et al. 2003). Ionophores are antibiotics not related to drugs used in human medicine. Their use results in an alteration of the microbial ecology in chicken GI tract.

Ionophores are also used as an aid in the prevention of coccidiosis in broiler chickens (CFIA 2008b). Coccidiosis is caused by a single-celled parasite called coccidian, which belongs to the genus *Eimeria*. The coccidian is a parasite that produces spores and causes disease in the small intestine. Coccidiosis is faecally-orally transmitted and is an important concern in animal husbandry, particularly in poultry production (NRC 1999). Ionophores are also used to improve feed efficiency in cattle and pigs. Some examples of inclusion in complete feed are salinomycin used at 60mg/kg, monensin at 99mg/kg and narasin at 70mg/kg feed. Most ionophores are compatible with virginiamycin and bacitracin methylene disalicylate in feed for broiler chickens (CFIA 2008b).

I.4.5.2.1.2 Mechanism of Resistance

Cases of resistance to ionophores have been reported in coccidial species (NRC 1999). Although the resistance mechanism is not really understood, some theories suggest that the parasites might have developed certain mechanisms such as improved membrane ion control systems or decreased affinity for binding ionophore molecules (Zhu and McDougald 1992). The cell wall of Gram-negative bacteria (*E. coli* and *Salmonella*) does not allow penetration of hydrophobic molecules with molecular weights over 600 and is thus resistant to ionophores. Resistance to ionophores has been reported in Gram positive bacteria, including *Staphylococcus* spp and *Enterococcus* spp isolates from animals but the mechanism of this resistance remains to be established (Butaye et al., 2003).

I.4.5.2.2 Virginiamycin

I.4.5.2.2.1 Mode of action

Virginiamycin, which belongs to the group of streptogramins, is produced as a fermentation product of *Streptomyces virginiae* which was isolated in 1955 (Rogers et al. 1995; Schwarz et al. 2006). The bacterial inhibitory activity of this antibiotic family relies upon the synergic effect of two groups: macrocyclic lactone peptides, called peptolides A and B (Schwarz et al. 2006). The A-group streptogramins contains polyunsaturated cyclic peptolides while the B-group consists of cyclic hexadepsipeptides. Both groups competitively inhibit protein synthesis by causing a conformational modification of the

peptidyl transferase catalytic (PTC) centre of the 50S ribosome subunit (Cocito 1979) which results in the inhibition of cell growth.

The PTC is the centre that catalyses the peptide bond synthesis by first positioning a peptidyl-tRNA molecule (donor of the carboxyl group) at the P-site and aminoacyl-tRNA molecule (donor of the NH₂ group) at the A-site (Cocito et al. 1997). The peptidyl-tRNA is then dislocated to the A-site, after a link is formed between the carboxyl terminus of the peptide chain and the NH₂ group of the amino terminus of AA-tRNA at the A-site. A peptide bond is formed resulting in the elongation of the peptide by one unit. The last step of the elongation is the translocation of the peptide from the A-site back to the P-site. Type A-streptogramins inhibit substrates from attaching to both P and A-sites by competitively modifying the PTC centre (Chinali et al. 1984). This results in inhibition of the elongation step of the peptide. The conformational modification of the PTC centre enhances the affinity of the type-B streptogramins (Cocito et al. 1997). Both types individually are bacteriostatic, but once combined, they are bactericidal (Cocito 1979; Cocito et al. 1997).

Virginiamycin is composed of two components M and S. Virginiamycin M belongs to the A-group whereas virginiamycin S is part of the B-group. It has been used for therapeutic and growth promotion purposes in chickens for more than 60 years (Smith and Tucker 1975). Virginiamycin has also been used for similar purposes in swine, cattle and turkeys. CFIA approved the use of virginiamycin in the meal or pellet feed for broiler chickens and for swine (CFIA 2008b). Virginiamycin is used as an aid in prevention of

necrotic enteritis in broiler chickens caused by *C. perfringens* which are inhibited by virginiamycin. The antibiotic level is 22mg/kg (0.0022%) of complete feed. This amount is within the range that Rogers et al. found to be effective for increasing average daily gain (19.3 to 27.3 mg/kg), but exceeds the dose for improving feed conversion (13.2 to 19.3 mg/kg) and reduces liver abscess incidence (16.5 to 19.3mg/kg) in feedlot cattle (Rogers et al. 1995). Virginiamycin is compatible with monensin sodium, narasin and salinomycin sodium used in broiler feed (CFIA 2008b). Virginiamycin has been banned in Europe since 1999 because they belong to classes of antimicrobials also used in human therapy (Dibner and Richards 2005).

I.4.5.2.2.2 Mechanism of resistance

Like all streptogramins and related antibiotics, lincosamins (e.g. lincomycin) and macrolides (e.g. tylosin, erythromycin), virginiamycin is mostly effective in inhibiting Gram-positive bacteria. Gram-negative bacteria are naturally resistant to this antibiotic due to the constitution of their membrane which does not allow it to penetrate the cell (Cocito et al. 1997). Whereas the resistance mechanism to type-A streptogramins has not been totally elucidated, the resistance mechanism to the type-B has been widely studied (Cocito et al. 1997; Leclercq 2002; Rice and Bonomo 2005). Streptogramins type-B and related antibiotics, marolides and lincosamides (MLS_B) have overlapping binding sites in the 23S rRNA located in the 50S ribosome subunit. This confers a cross resistance to the three classes of antibiotic (Leclercq 2002). So far three resistance mechanisms have been identified: (i) target-site modification, (ii) efflux of the antibiotic and (iii) antibiotic

inactivation (Cocito et al. 1997; Leclercq 2002; Rice and Bonomo 2005). These resistance phenotypes are conferred by either mutations of chromosomal genes or by the acquisition of new genes carried by plasmids or transposons (Cocito et al. 1997).

Target-site modification is achieved by methylation or mutation that prevents the binding of MLS_B to their ribosomal target site. The alteration of the binding site results from the action of an adenine-specific N-methyltransferase (methylase). This enzyme is designated *erm* (erythromycin ribosome methylation) gene. Ribosomal methylation is the most widespread mechanism of resistance to MLS_B antibiotics (Cocito et al. 1997; Rice and Bonomo 2005).

The efflux of the antibiotic out of the bacteria is realized via active transport pumps spanning bacteria cell walls. The active efflux of antibiotics was discovered over 25 years ago, during the study of the mechanism of tetracycline resistance in *Enterobacteria* (Levy 1992). Efflux pumps can selectively extrude specific antibiotics as well as structurally different natural or synthetic antimicrobial agents (Lomovskaya et al. 2002). The latter are called multiple antibiotic resistance (MDR) pumps. Based on the energy source used to extrude substances and the sequence similarity, five families composing the MDR pump group, have been recognized by Lomovskaya et al.: ATP-binding cassette (ABC) Superfamily and the four subfamilies of the proton motive force-dependant family (PMF); Small Multiple antibiotic Resistant family (SMR); Major Facilitator Superfamily (MFS); Multiple antibiotic and Toxic Compounds Extrusion family (MATE) and Resistance/Nodulation/Cell Division family (RND) (Lomovskaya et

al. 2002). In Gram negative bacteria, the efflux pump is encoded by the chromosome resulting in their natural resistance to some MLS_B hydrophobic compounds. For Gram positive bacteria, the efflux is the consequence of the acquisition of the ABC and the MFS families pumps (Leclercq 2002). This results in an impaired permeability of the antibiotic inside the bacteria cell (Cocito et al. 1997).

The last mechanism is the enzymatic inactivation of MLS_B antibiotics. This mechanism is highly antibiotic-specific (Lomovskaya et al. 2002). Resistance to type-A streptogramins is conferred by acetyl- transferases (*vatA*, *vatB*, and *vatC*) while type-B is inactivated by hydrolases such as lactonases capable of cleaving the macrocyclic lactone ring structure (Cocito et al. 1997; Lomovskaya et al. 2002). However, the synergy of the combination of the two types of streptogramins is achieved by the fact that a similar individual inhibitory element, such as methylase for MLS_B antibiotics or acetyl-transferases for type-A, does not inhibit both compounds together. Thus, bacteria should be resistant to both streptogramins A and B to cause clinically significant resistance.

I.4.5.2.3 Glycolipid: Bambermycin

I.4.5.2.3.1 Mode of action

Bambermycin, also named moenomycin, flavophospholipol, or flavomycin, is an antibiotic complex obtained from *Streptomyces* species, including *S. bambergiensis*, *S. ghanaensis*, *S. geysirensis*, and *S. ederensis* (Huber et al. 1965). Bambermycin's main

component is moenomycins A, which is a phosphorus-containing glycolipid. The lipid moiety is a C-25 compound called moenocinol (van den Bogaard et al. 2002).

Bambermycin inhibits the synthesis of bacterial peptidoglycan, the principal constituent of Gram positive bacteria cell walls (Huber and Neesemann 1968). This is achieved by blocking Penicillin Binding Proteins (PBP) in their transglycosylates activities, resulting in inhibition of peptidoglycan polymerases (Huber et al. 1965; Huber and Neesemann 1968; van den Bogaard et al. 2002). Gram positive bacteria such as staphylococci and streptococci are more susceptible to bambermycin than Gram negative bacteria; this is due to the impermeability of most Gram negative outer membranes to this antibiotic (van den Bogaard et al. 2002). It is used as feed additives and growth promoters for poultry, swine and cattle (Huber et al. 1965; van den Bogaard et al. 2002).

In poultry production, bambermycin is also used as a coccidiostat. Bambermycin is compatible with monensin sodium and salinomycin sodium to increase the rate of weight gain and improve feed efficiency (CFIA 2008b). The regulatory level of bambermycin per kg of complete feed for broiler chickens is 2mg (0.0002%) granular meal or pellets, as the sole ration from day 1 to market weight (CFIA 2008b).

I.4.5.2.3.2 Mechanism of resistance

So far, there is no report confirming acquired resistance case to bambamycin (Butaye et al. 2003). *Enterococcus faecium* resistance to bambamycin has been attributed to natural occurring resistance or intrinsic resistance (Butaye et al. 2003). Butaye et al. (2003) suggested bambamycin could selectively inhibit the growth of bacteria harbouring certain plasmids such as R plasmids which carry genes responsible for antibiotic resistance among bacteria.

I.4.5.2.4 Beta-lactam: Penicillin

I.4.5.2.4.1 Mode of action

Penicillin was discovered in the 1940s and was applied in clinics in the 1950s (Gordon et al. 2001). Enzymes involved in the construction and maintenance of the bacteria cell wall constitute the target of this antibiotic family (Gordon et al. 2001). These enzymes, called penicillin binding proteins (PBP), are structurally similar to the beta-lactam antibiotics. PBP are D-alanyl-D-alanine carboxypeptidase/transpeptidases. They catalyze the final step of bacteria cell wall synthesis allowing the formation of the peptidoglycan also named murein. The peptidoglycan is present in both Gram-positive and Gram-negative bacteria cell walls and is composed of linear glycan chains that are interlinked by short peptides (van Heijenoort 2001). The glycan chains are composed of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid. The most frequent

peptide chains are L-alanyl- γ -D-glutamyl-diaminopimelyl and L-lysyl-D-alanyl-D-alanine in nascent peptidoglycan and which subsequently lose one or both D-alanine residues in mature peptidoglycan (van Heijenoort 2001).

The peptidoglycan is the exoskeleton that is necessary to maintain the bacteria's shape, rigidity and osmotic stability and to ensure the availability of nutrients. In addition, bacteria peptidoglycan is involved in the cell division process (Gordon et al. 2001; van Heijenoort 2001). By inhibiting the murein synthesis, betalactam antibiotics seriously impair the integrity of the bacteria. Penicillin is used in meal or pellet feed for chickens for growth promotion at a dose of 2.2 mg/kg (0.00022%) of complete feed. Chickens at starter, grower and finisher stages are fed the medicated feed as the sole ration (CFIA 2008b).

I.4.5.2.4.2 Mechanics of resistance

Bacteria developed different pathways for penicillin resistance. One of the resistance mechanisms involves the secretion of beta-lactamases which hydrolyze the beta-lactam core. These enzymes belong to the same family as PBPs and they include in their formula an active-site serine residue, responsible for the cleavage of the beta-lactam ring (Gordon et al. 2001). It results in modification of the permeability of bacteria's outer membrane (Gordon et al. 2001). The target can also be modified by mutation or the decrease of affinity for the target by production of novel PBPs. Efflux pumps have been

identified in Gram-negative bacteria. This resistance mechanism involves changes in the outer membrane permeability and appearance of efflux pumps (Gordon et al. 2001).

Structurally modified β -lactams such as cephalosporins, which are less susceptible to hydrolysis by β -lactamases, have been synthesized to overcome the ever increasing and widespread resistance to penicillin (Barlow and Hall 2002). In response to the clinical use of cephalosporins, resistance (extended-spectrum β -lactamases (ESBL)) has rapidly evolved under the form of AmpC β -lactamases that are cephalosporinases mediating resistance to these antibiotics (Nordmann 1998). Over 20 plasmid-borne ampC genes have been identified among at least 50 different ampC genes described in the literature (Nordmann 1998). The presence of ampC on plasmids allows the wide distribution of this resistance gene among different bacteria species via horizontal transmission. CMY-2 is the third plasmid-borne ampC gene, after CMY-1 and MIR-1 (Bauernfeind et al. 1989; Papanicolaou et al. 1990; Daniels et al. 2007).

Plasmid borne ampC CMY-2 β -lactamases (bla_{CMY-2}) have been identified in both *Salmonella* and *E. coli* (Daniels et al. 2007). Furthermore, evidence suggests that commensal bacteria may serve as a reservoir of plasmid-borne antimicrobial resistance genes for pathogens and that plasmid transfer occurs readily between *E. coli* and *S. enterica* (Poppe et al. 2005; Daniels et al. 2007). *Salmonella* infections are a major concern in broiler chicken production and Extended-Spectrum Cephalosporins (ESC) such as ceftriaxone and ceftiofur are important therapeutic agents and are often used for invasive *Salmonella* infections (Bryan and Scheld 1992). In Canada, ceftiofur is also used

to prevent colibacillosis (which causes early poult mortality) associated with *E. coli* infection in day-old poults (Canadian Animal Health Institute 2001). Cephalosporin resistant *E. coli* and *Salmonella* have been recovered from retail chicken meat and given that these antibiotics are first-line therapy in pediatric salmonellosis, *bla*_{CMY-2}-bearing plasmids are therefore an important resistance mechanism that needs more attention (Fey et al. 2000; Forward et al. 2004; Zhao et al. 2006). This stresses once again the importance of studying the relationship between the use of antibiotics in food-producing animals and their effects on resistance in commensal and pathogenic bacteria.

I.4.5.2.5 Peptide: Bacitracin

I.4.5.2.5.1 Mechanism of action

Bacitracin belongs to the family of peptides antibiotics and is constituted by mixture of high-molecular-weight polypeptides produced by the organism *Bacillus licheniformis* (Manson et al. 2004). These polypeptides are non-ribosomally synthesized by multi-enzyme complexes called peptide synthetases (Zhang and Hancock 2001). Bacitracin is a Gram-positive specific antibiotic that works by inhibiting the synthesis of peptidoglycan by tightly binding to the complex undecaprenyl diphosphate (C₅₅ PP) and a metal cation (Zhang and Hancock 2001). The C₅₅ PP is a carrier for N-acetylmuramyl pentapeptide that is intermediate in the synthesis of peptidoglycan and it is released at the end of each cycle. C₅₅ PP is recycled by dephosphorylation by a phosphatase, and then it starts the new cycle again by accepting a murine precursor UDP-acetylmuramyl

pentapeptide. The inhibition of C₅₅ PP results in disruption of bacteria cell wall synthesis followed by inhibition of bacterial growth (Zhang and Hancock 2001).

Bacitracin methylene disalicylate is used in poultry and swine production to improve feed efficiency and the rate of weight gain. It is applied at 4.4 mg/kg (0.00044%) of complete feed and is compatible with nicarbazin, a urea derivative coccidiostat, in feed for chickens (CFIA 2008b).

I.4.5.2.5.2 Mechanism of resistance

At least three resistance mechanisms to bacitracin have been recognized in bacteria. The first one involves active efflux pumps from the ABC family of transport proteins that pumps bacitracin out of the cell (Manson et al. 2004). Decreased bacitracin concentration inside the target bacteria would certainly reduce the lethal effects of the antibiotic. Further studies suggested the overproduction of bacitracin binding-enzymes, surpassing the capacity of bacitracin in sequestering these enzymes (Cain et al. 1993). Finally, the increased resistance to bacitracin could be due to competitive inhibition with another membrane protein (YwoA) for the same substrate C₅₅ PP, given that *ywoA* gene also encodes for resistance to bacitracin (Bernard et al. 2003).

I.4.5.2.6 Chlortetracycline

I.4.5.2.6.1 Mechanism of action

Tetracyclines are broad-spectrum antibiotics discovered in 1948 after isolation of chlortetracycline from fermentation products of *Streptococcus aureofaciens* (Chopra 1994). This class of antibiotics inhibits protein synthesis by binding to the 7S site of the 30S subunit of the ribosome, inhibiting bacterial growth (Goldman et al. 1983; Speer et al. 1992). Chlortetracycline hydrochloride is used as an aid in stimulating appetite and maintaining weight gain in broiler chickens during periods of stress caused by moving, chilling, vaccination and extreme temperatures (CFIA 2008b). It is used at a dose of 110 mg/kg (0.011%) of complete feed and has a seven day minimum withdrawal period (CFIA 2008b).

I.4.5.2.6.2 Mechanism of resistance

Three resistance mechanisms have been identified in both Gram-positive and Gram-negative bacteria. Gram-negative bacteria usually resist tetracycline by protecting the ribosome with specific proteins such as *tet(M)*, *tet(O)*, *tet(Q)*, *tet(S)* (Lomovskaya et al. 2002; Rice and Bonomo 2005). Specific and multi-resistant active efflux pumps were also identified in both Gram-positive (*tet(A)*, *tet(B)*, *tet(C)*, *tet(D)*, *tet(E)*) and negative bacteria (*tet(K)*, *tet(L)*, *tet(P)*). These pumps are energy-dependent and inserted into bacteria cytoplasmic membrane (Lomovskaya et al. 2002; Rice and Bonomo 2005;

Sapunaric et al. 2005). Finally, enzymatic alterations and inactivation of tetracycline makes the antibiotic inactive in inhibiting the protein synthesis (Rice and Bonomo 2005). The resistance genes are inserted on the plasmid or transposons and result mostly from the acquisition of *tet* determinants rather than gene mutations (Rice and Bonomo 2005).

I.4.6 Mechanisms of dissemination of resistance

Although factors contributing to the emergence of antibiotic resistance are varied, they basically remain the same for all antimicrobial classes. Although the presence of the resistance gene, its characteristics in addition to the characteristics of the host and selective pressure of the antibiotic are a prerequisite for the dissemination of antibiotic resistance, these criteria are considered to be the most important. Undoubtedly, the extensive use of antimicrobials in the agricultural and animal-food producing industry have largely contributed to the bacteria to bacteria, animal to animal and finally animal to human dissemination of antibiotic resistance genes (Levesque et al. 1995; Gold and Moellering 1996; Clark et al. 2003; Salyers et al. 2005; Poppe et a. 2006). Many authors agree that the concern is not in the transmission of the pathogenic bacteria themselves, but to the transfer of resistance genes, especially multiple antibiotic resistance genes (Khachatourians 1998; Wegener et al. 1999; Salyers et al. 2005). From farm to fork, there are many possible points for entry of resistant bacteria into the food chain and dissemination into the environment. The farm workers could easily be either contaminated or the source of contamination and spread the resistant microorganisms in the rearing environment or bring it home where they could silently pass it to family members, other

environments or friends. Insufficient and improper personal hygiene practice is an important means of dissemination of antibiotic resistant bacteria into the environment (Turabelidze et al. 2006).

Food producing animals could enter the slaughter house along with the resistant bacteria and if not detected at entrance, the bacteria could proliferate and find ideal niches to persist in the environment. Even if controlled at the farm and slaughter levels, bacterial contamination could occur at various further processing stages allowing many entry points for antibiotic resistant pathogens. Processing environments, equipment, packaging rooms, washrooms and warehouses are examples of locations within processing plants where pathogens could persist before detection. Once again, employee personal hygiene practices such as proper clothing, proper hand washing and food handling in processing plants and food service outlets are of paramount importance in reducing the risk of food contamination. Any degree of carelessness could lead to major food safety issues, resulting in product recall and total ban on products leading to high economic cost and legal fees. Most importantly, the consumer will lose confidence in the product and the reputation of the producer.

Consumers also bear responsibilities. Negligence in respecting the four basic principles for consuming safe food could result in fatal issues: (i) cleaning of hands and surfaces; (ii) separation of raw and cooked products; (iii) cooking at proper temperature and time; and (iv) quickly refrigerating foods to avoid proliferation of bacteria. Moreover, increased international trade takes away virtual boundaries and sometimes resistant

bacteria are not detected by monitoring systems (Motarjemi and Adams 2006). Antimicrobial resistance genes are therefore freely transported in luggage, in human or animal GI tracts, on the surface of their bodies and on clothing and are ready to adapt to the future conditions (Takashima et al. 2004).

I.4.7 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing relies on the determination of the breakpoints for bacteria (Wilker and Ambrose 2005). The breakpoint represents the concentration of an antibiotic that separates populations of susceptible and resistant micro-organisms (Wilker and Ambrose 2005). It has three components. Firstly, the microbiological breakpoint provides valuable information to clinicians for an optimal therapy choice. Secondly, the epidemiological breakpoint detects the emergence of resistance. Finally, the clinical breakpoint is the most varying and differs from country to country (Wilker and Ambrose 2005).

This thesis will only consider the microbiological breakpoint which provides data on the minimum inhibitory concentration (MIC) of bacteria. MIC is defined as the concentration of antibiotic giving inhibition of visible bacterial growth in liquid medium or radically decreased growth on solid medium (Burman and Olsson 2001).

I.5 Public health issues

I.5.1 Benefits of antimicrobial use

Early warnings have been made since the 1940s regarding the subtherapeutic use of antibiotics that might expose microbes to non-lethal quantities of antibiotics, making them resistant (Fleming 1945). The food-animal industry is ostensibly ignoring this admonition by daily use of sub-therapeutical antimicrobial doses in animal feeds. Although this common practice is increasingly controversial, it is hard to disregard the potential benefits of antibiotic use in food-animal production, for both animal and human health. Antimicrobial prophylaxis prevents diseases and decreases microbial competition for nutrients in the animals' GI tract. Furthermore, sick animals are treated with adequate medication, reducing their microbial load and thus, enhancing their growth performances and general health status. This allows better economic returns to producers and increases consumers' confidence in consuming food products produced from those animals. On the other hand, antibiotics significantly reduce the threat to human health related to foodborne pathogens such as *Campylobacter*, *Salmonella*, hemorrhagic *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Clostridium* (NRC 1999).

Use of antibiotics to eliminate transferable bacteria from food producing animals is a measure of food security that protects consumers from exposure to life threatening diseases, thus preserving their good health status. Most importantly, this measure also could reduce infiltration of pathogenic bacteria into the environment, mainly through manure application. Runoff water, especially after heavy rains, could contaminate wells

and other community water systems as happened in Walkerton in 2000. This deadly waterborne outbreak resulted from entry of *Escherichia coli* O157:H7 and *Campylobacter* spp. from neighbouring farms into the town water supply (Clark et al. 2003). Crops, fruit trees and other commodities are commonly sprayed with antimicrobials to prevent microbial spoilage, thus contributing in controlling environmental contamination (Khachatourians 1998).

The benefits for animal health and welfare and for human health are undeniable. However, there are concerns arising about the appropriateness of the use of antibiotics in food animal production. Some of these concerns include antibiotic residues and the emergence of antibiotic resistant pathogens (Khachatourians 1998; Conly 2002; Bywater 2005).

1.5.2 Disadvantages of antimicrobial use

Any drug has a connotation of toxicity, especially when it and/or its metabolites could be absorbed from the intestines. Antimicrobials used in food-animal production are not an exception to this rule. Residues from drugs used in food animal production could be ingested either directly through animal tissues or products, or indirectly through the environment (Samanidou and Evaggelopoulou 2008). Antibiotic residue consumption can induce adverse effects such as toxicities, allergies and infection by disease-causing bacteria that are antibiotic-resistant. Antimicrobials and their metabolites can concentrate in animal tissue leading to toxicities that could be manifested by teratogenic or

carcinogenic effects. Consequently, the US FDA has strict regulations: “no proven carcinogen should be considered suitable for use as a food additive in any amount” (Committee on Drug Use in Food Animals Panel on Animal Health, Food Safety, and Public Health, 1999); Canada also follows similar regulations (Health Canada 2002). Therefore antimicrobials, that are systemic and thus absorbed from intestines in significant amounts (e.g. tetracycline, penicillin, erythromycin and lincomycin), supplied to animals through feed or water, fall under regulatory withdrawal periods. Non systemic antimicrobials, which are not absorbed or very slightly absorbed from the intestine, do not require any removal period and can be administered to animals until slaughter (e.g. bacitracin, virginiamycin, bambamycin, neomycin, tylosin, novobiocin and streptomycin) (Health Canada 2002).

Allergic reactions are less frequently reported in the literature (Health Canada 2002). Few cases are related to the consumption of milk containing penicillin residues although there is not enough evidence to prove that antimicrobials used in animal food provoke allergic reactions in humans (Borrie and Barret 1961; Wicher et al. 1969; Barton 2000). Heat treatment during further processing could degrade the residue epitopes and reduce the potential for allergic reactions (NRC 1999). Although strict control exerted by regulatory agencies strongly contributed to limit the risks of food-animal tainting by antimicrobial residues, antibiotic resistant bacteria still could contaminate animal carcasses and enter the food chain.

Numerous studies have eliminated any doubt that antibiotic-resistant bacteria could be transferred from food animals to humans (FSIS 1997; Wegener et al. 1999; Poppe et al. 2006). Commensal pathogens such as *Salmonella*, entero-hemorrhagic *E. coli* could exchange or receive multiple antibiotic resistance genes from non pathogenic carriers such as generic *E. coli*. (Schwarz et al. 2006; Zhao et al. 2006). Young children, elderly and immuno-compromised people are the most at risk during infection by such pathogens. They pay for what they are usually not directly responsible for and sometimes, they have to pay with their own lives. Seven pathogens were recognized by the Center for Disease Control and Prevention (CDC) as the most common cause of foodborne illnesses. *Campylobacter* is the most frequently isolated foodborne bacterium (49.4%), followed by *Salmonella* (27.4%), *Shigella* (15.7%), *E. coli* O157:H7 (4.2%), *Yersinia* (1.7%), *Listeria* (1%) and *Vibrio* (0.6%). They constitute a serious health risk due to the fact that they are easily transferable, thus making them difficult to control (FSIS 1997).

Table I.1. Antibiotics and growth promoters approved for use in chickens in Canada^a

Compound	Growth and feed efficiency	Various infections
Bambermycin	Yes	No
Bacitracin	Yes	Yes
Chlortetracycline	Yes	Yes
Erythromycin	No	Yes
Lincomycin	Yes	Yes
Piperazine ^d	No	Yes
Morantel ^d	No	Yes
Hygromycine B ^d	No	Yes
Pyrantel ^d	No	Yes
Dichlorvos ^d	No	Yes
Fenbendazole ^d	No	Yes
Ivermectin ^d	No	Yes
Levamisole ^d	No	Yes
Neomycin	No	Yes
Novobiocin	No	Yes
Oxytetracycline	Yes	Yes
Penicillin	Yes	Yes
Spectinomycin ^b	No	Yes
Tylosin	No	Yes
Virginiamycin	Yes	Yes
Melengestrol acetate	Yes	No
Sulfamethazine ^c	Yes	No
Tiamulin	No	Yes
Tilmicosin	No	Yes
Ractopamine	Yes	No

^a CMIB, Compendium of Medicating Ingredient Brochures (CFIA 2008b)

^b Used in combination with lincomycin

^c Used in combination with chlortetracycline;

^d For the treatment of infections and infestations due to the following parasites such as gastrointestinal roundworms (e.g. *Ascaris suum*), whipworm (*Trichuris suis*)

Table I.2. Antimicrobial Growth promoter used in broiler chicken production in Canada

Compound	Level in complete feed (mg/kg)	Claim (CMIB)^a
Arsanilic acid	99	growth, f.c.
Chlortetracycline HCl	5.5	growth, f.e.
Bacitracin (Zn or MD)	4.4	gain, f.e.
Lincomycin HCl	2.2	growth, f.u.
Procaine Penicillin	2.2	growth
Virginiamycin	11	gain, f.e.
Bambermycins	2	gain, f.e.
Zinc Bacitracin and Procaine Penicillin	3.3/1.1	gain, f.e.
3-nitro-4-hydroxy-phenylarsonic acid	50	gain, f.e.
Oxytetracycline	110	Growth promotion/f.e.

NB: Growth and increased rate of gain are taken to be synonymous. Feed conversion (f.c.), feed efficiency (f.e.) and feed utilization (f.u.) are taken to be synonymous and are generally defined as feed intake per unit of live weight gain.

^a CMIB, Compendium of Medicating Ingredient Brochures (CFIA 2008b)

Chapter II. Antibiotic resistance and virulence genes in commensal *Escherichia coli* and *Salmonella* isolates from commercial broiler chicken farms

II.1 General objective

It is known that antibiotic growth promoters used in chicken production can modify chicken intestinal flora by creating a selective pressure favoring the development of antibiotic resistant bacteria. The focus of this chapter was to evaluate the distribution of various virulence and antibiotic resistance determinants in sorbitol negative *E. coli* and *Salmonella* isolates recovered from commercial broiler chicken farms in the Fraser Valley of British Columbia.

II.2 Specific objectives

- a) Isolate commensal *E. coli* and *Salmonella* from commercial broiler chickens fed different growth promoting antibiotics
- b) Determine the susceptibility of the isolates to antibiotics of clinical importance in chicken production
- c) Determine virulence and antibiotic resistance genes present in resistant isolates

II.3 Synopsis of major results

Commensal *E. coli* and *Salmonella* were isolated from broiler chickens fecal materials collected from nine commercial farms in the Fraser Valley of British Columbia. The farms were supplied by three different feed companies using different antimicrobial growth promoter formulations.

- (a) Multiple antibiotic resistant commensal *E. coli* and *Salmonella* were isolated from fecal materials.
- (b) Some isolates harboured antibiotic resistant [*bla*_{CMY-2}, *tet(A)*, *te(tB)*] genes, class 1 integron (*qacEΔ1-SulI*) and virulence (*iss*, *tsh*, *traT*, *iucC eaeA*, *inv*, *spv*) genes.
- (c) Virulence genes were detected in some multiple-antibiotic resistant *E. coli* and *Salmonella* isolates.
- (d) There was a significant difference ($P < 0.01$) between the feed companies regarding the presence of *tet(B)* which was significantly more prevalent in *E. coli* isolates from farms supplied by feed company B.

II. 4 Introduction

Escherichia coli form a part of the bacterial population of the gastrointestinal tract of animals and humans (Gross 1994). Some strains of this bacterium are agents of colibacillosis, a primary cause of morbidity, mortality and carcass condemnation in broilers, resulting in significant economic losses to the poultry industry (Gross 1994; Yogaratnam 1995; White 2005). This disease is characterized by an upper respiratory tract infection that can lead to pericarditis, perihepatitis, peritonitis and salpingitis (Vandemaele et al. 2003). Antibiotics are now widely used to control avian colibacillosis (Amara et al. 1995; Blanco et al. 1997). As a result, multi-resistant *E. coli* strains may emerge and become a cause of concern.

In humans, *Salmonella* is one of the most common causes of bacterial gastroenteritis. *Salmonella* infection has been associated with many different foods including poultry products (Altekruse et al. 1997; Aarestrup 2000). The cecum has been considered as one of the primary sites of *Salmonella* colonization in chicken (Corrier et al. 1999). Therefore, broiler chickens may carry this organism undetected into an abattoir at the time of slaughter. Given the association of certain poultry *Salmonella* serotypes with foodborne illness and the likelihood that some of the isolates may be resistant to multiple antibiotics (Adesiyun et al. 2007), it is important to understand the ecology of these pathogens as well as their antibiotic susceptibility profile.

Antibiotics are used in poultry production at sub-therapeutic levels as growth promoters to prevent and control sub-clinical infections and to improve food conversion and body-weight gain (Aarestrup et al. 1998; Butaye et al. 2003). This practice may modify the intestinal flora by creating selective pressure favoring the development of antibiotic resistant bacteria strains (Aarestrup et al. 2001). Many resistance genes have been identified on mobile genetic elements such as plasmids, transposons and integrons, allowing for the dissemination of resistance genes among bacteria. For example, *E. coli* resistance to extended-spectrum cephalosporin may be due to the production of extended-spectrum beta-lactamase encoded by genes (*bla_{CMY}*) located on transferable plasmids (Zhao et al. 2001; Poppe et al. 2005). Extended-spectrum cephalosporins are used in food-producing animal (Zhao et al. 2001). It was reported that *S. enterica* isolates from domestic animal species including turkey exhibited resistance to ceftiofur and ceftriaxone, and possessed the *bla_{CMY}* gene (Gray et al. 2004). The development of such extended spectrum resistance in *Salmonella* may have serious public health implications (Gupta et al. 2003).

Numerous studies reported tetracycline resistance in avian *E. coli* (Smith and Lowell 1981; Chopra and Roberts 2001; Maynard et al. 2003; Maynard et al. 2004). However, tetracyclines are inexpensive antibiotics and have been used extensively in the prophylaxis and therapy of human and animal infections as well as at subtherapeutic levels in animal feed as growth promoters. The presence of tetracycline-resistant pathogens limits the use of these agents in treatment of disease (Chopra and Roberts

2001). Little attention has been paid to the relationship between resistance and virulence genes in specific chicken bacterial isolates.

Interestingly, little is known about the distribution and dissemination of antibiotic resistance genes in food production animal, especially in broiler chicken production. One of the mechanisms of resistance dissemination is DNA-mediating resistance gene integration into a site-specific recombination (Bass et al. 1999; Maynard et al. 2004). This DNA element is called integron and class 1 is the predominant class among the four identified classes (Partridge et al. 2001). The increase of antibiotic resistance and the emergence of foodborne pathogens are both serious public health concerns. The objective of this study was to determine the antibiotic resistance profiles in *E. coli* and *Salmonella* recovered from different commercial broiler chicken farms that used antimicrobial agents in feed for growth promotion. The focus was to evaluate the distribution of various virulence and antibiotic resistance determinants in sorbitol negative *E. coli* and in *Salmonella* isolates, as very little data exists on the characterization of such isolates from poultry.

II. 5 Materials and Methods

II.5.1 Feed companies, farms and sampling

Sample collection for the study was carried between May 2004 and February 2005. Nine commercial broiler farms were selected in the Fraser Valley of British Columbia for this study. Three independent feed companies provided feed to the farms. The feed companies were designated A, B and C while the nine farms were designated a, b, c, d, e, f, g, j and i (Table II.1). The antibiotics and dosages used in the feed at different growth phases as described by the feed companies are presented in Table II.1. Twenty-six fecal and two litter samples were collected at days 8-10 and days 25-28 respectively, from each farm. At slaughter (days 35-40), the cecal contents were obtained from five birds from each farm (Figure II.1). The samples (fecal, cecal and litter) were collected aseptically and transferred to pre-weighed tubes containing Carry-Blair transport medium (Quelab, Montréal, QC, Canada) and kept on ice until laboratory analysis, which was performed on the same day. The sample weight in Carry-Blair containing-tube was estimated by subtracting the weight of the tube without sample from the weight of the tube containing samples.

II.5.2 Bacteriological cultures

The samples (5 to 6 g) in Carry-Blair were vigorously vortexed for 1 minute then 10-fold serial dilutions were prepared in sterile saline. Counts of generic *E. coli* were obtained from individual sample from each farm by plating 1 ml of the appropriate dilution on *E. coli* and Coliform Petrifilms (3M, St. Paul, MN, USA). After 24h incubation at 37°C, blue gas producing colonies were counted as generic *E. coli*. Results were expressed as numbers per gram of material (Lefebvre et al. 2006).

Sorbitol negative *E. coli* were also isolated as described by Lefebvre et al. (Lefebvre et al. 2005). Briefly, after homogenization of the individual Carry-Blair containing fecal, litter or cecal samples, 20 µl of samples were transferred to 20 ml of Tryptic Soy Broth (TSB, Becton Dickinson, Sparks, MD, USA) containing 10 µg/ml of cefsulodin (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), 40 µg/ml of vancomycin (Sigma-Aldrich) and 50 ng/ml of cefixime (Fisher Scientific Ltd., Nepean, ON, Canada). After 6 h of incubation at 37°C with agitation (150 rpm) in TSB-VCC, 10 µl of the enriched samples were streaked onto Sorbitol-MacConkey agar (Becton Dickinson) and incubated at 37°C for 18 to 24 h. Colorless colonies were considered as non-fermenting sorbitol isolates. A minimum of five non-sorbitol fermenting colonies were purified from each analyzed sample and stored at -80°C in 25% glycerol in TSB. API20E strips (BioMérieux, St-Laurent, QC, Canada) were used to confirm *E. coli* identity according to the manufacturer's specifications. All sorbitol negative *E. coli* isolates were assayed for the O157 antigen detection using the *E. coli* O157 latex agglutination test kit (Oxoid).

The isolation of *Salmonella* also was performed on fecal, cecal and litter samples collected from each farm at each sampling time. Diluted Carry Blair samples were pre-enriched in Nutrient Broth (Becton Dickinson) overnight at 35°C. Cultures from these pre-enrichments (200µl) were used to inoculate Tetrathionate Brilliant Green broth (TBG, Becton Dickinson) for an additional overnight incubation at 35°C. One loopfull of the TBG culture was spread directly onto Brilliant Green Sulfa agar (BGS, Becton Dickinson) and incubated at 35°C for 24. Lactose negative colonies were confirmed by latex polyclonal agglutination test (Oxoid) and by API 20E (BioMérieux). Serological identification of *Salmonella* isolates was performed at the Reference Laboratory for Salmonellosis (Public Health Agency of Canada, Guelph, Ontario, Canada). The O or somatic antigens of the *Salmonella* isolates were determined by slide agglutination (Ewing 1986). The H or flagellar antigens were identified using a microtechnique (Shipp and Rowe 1980) that employs microtitre plates. The nomenclature of Le Minor and Popoff (Le Minor and Popoff 2001) was used to name the serovars.

II.5.3 Antimicrobial susceptibility testing

The antibiotic minimal inhibitory concentrations (MIC) for all sorbitol negative *E. coli* and *Salmonella* isolates were determined at the British Columbia Ministry of Agriculture and Lands Animal Health Laboratory (Abbotsford, BC, Canada) using the Sensititre automated system (Trek Diagnostic Systems), according to the Clinical Laboratory Standard Institute's guidelines (Chopra and Roberts 2001). *E. coli* ATCC 25922 was used as control (NCCLS 1999). The following 18 antimicrobials were tested:

amoxicillin, penicillin, ceftiofur, erythromycin, tylosin, clindamycin, spectinomycin, spectromycin, gentamicin, neomycin, oxytetracycline, tetracycline, enrofloxacin, trimethoprim/sulphadimetoxazole.

II.5.4 Detection of antibiotic resistance genes by PCR

Detection of antibiotic resistance genes was performed according to the antimicrobial susceptibility profile of the study isolates. Fifty-five *E. coli* isolates that were phenotypically resistant to amoxicillin and/or ceftiofur and eight additional *E. coli* isolates that were susceptible to these antibiotics but resistant to penicillin were analyzed for the presence of the extended-spectrum beta-lactamase encoding genes *bla_{CMY-2}* and *qacEΔ1-Sul1* (resistance to quaternary ammonium containing antiseptics and sulfamide, class 1 integron 3' conserved segment). The presence of the tetracycline resistance genes *tet(A)*, *tet(B)*, *tet(C)* and *tet(D)* was determined only in tetracycline resistant *E. coli*. The detection of the target genes were performed using specific primers (Table II.2) as previously described (Maynard et al. 2003; Gray et al. 2004; Poppe et al. 2005). All isolates containing the *qacEΔ1-Sul1* gene were further investigated by a second PCR amplification of a variable region of the class 1 integron using the primers described by Maynard et al. (Maynard et al. 2003) (Table II.2). The protocol for DNA isolation was adapted from Lefebvre et al. (Lefebvre et al. 2005). *Salmonella* isolates that were also resistant to both amoxicillin and ceftiofur were analyzed for the presence of the above mentioned genes. *E. coli* strain MPS57 and *Salmonella* serovar NEWPORT 02-6203 (C. Poppe, Guelph, Ontario) were used as *bla_{CMY-2}* positive and negative controls respectively.

Integron and *tet* (*A*, *B*, *C* and *D*) positive *E. coli* strains provided by J. Harel (Groupe de Recherche sur les Maladies Infectieuses du Porc, St-Hyacinth Quebec) were also used as controls.

II.5.5 Virulence gene detection

The presence of enterohemorrhagic *E. coli* (EHEC) genes *stx1* and *stx2* (Shiga-like toxins 1 and 2), *eaeA* (intimin) and *e-hlyA* (enterohemolysin) were determined in all sorbitol negative *E. coli* isolates with specific primers (Table II.2), using multiplex PCR assays as previously described (Paton and Paton 1998; Lefebvre et al. 2005). In addition, the genes encoding increased serum survival (*iss*), temperature-sensitive hemagglutinin (*tsh*), aerobactin operon *iucC* and the outer membrane protein conferring resistance to complement (*traT*) were detected by PCR according to Johnson et al. (Johnson et al. 2004). For the *Salmonella* isolates, the presence of the invasin gene (*inv*) and the *Salmonella* plasmid virulent gene (*spv*) were determined using the method and the primer set (Table II.2) described by Chiu and Ou (Chiu and Ou 1996).

II.5.6 DNA sequencing

The sequencing was performed to confirm the identity of the targeted genes [(*bla*_{CMY-2} in *E. coli* and *Salmonella*), *qacEΔ1-SulI* (in *E. coli*)]. For each target gene, amplicons from three *E. coli* (1/Feed Company) isolates and one *Salmonella* isolate (from

Feed company B) were used for sequencing. Variable regions of class 1 integron were also sequenced to determine the different antibiotic resistance genes inserted in those regions. Two *E. coli* isolates, for which the 1 kb amplicon size was found, were used for sequencing. Agarose gel fragments of the amplicons were cut and purified using QIAEX gel extraction kit (QIAGEN Inc., Mississauga, Ontario, Canada). They were re-amplified and PCR products were purified using the PCR Product extraction kit (QIAGEN Inc., Mississauga, Ontario, Canada) prior to DNA sequencing. Sequencing was performed on an ABI 3100 16-capillary automated genetic analyzer at the Centre for Molecular Medicine and Therapeutics (University of British Columbia, Vancouver, BC Canada). Sequences were analyzed with the GenBank database using the BLASTN (www.ncbi.nih.gov/BLASTN/).

II.5.7 Statistical methods

Bacterial counts were log-transformed and analyzed using the GLM procedure of Statistical Analysis System (SAS 2000). The statistical significance was set at a *P* value of $P < 0.05$. The association test of Cochran-Mantel-Haenszel and logistic analysis (Proportional Odds Model) was used to determine the relationship between feed companies and the presence of *Salmonella* and the target genes using the FREQ procedures of the SAS Institute (SAS 2000).

II.6 Results

II.6.1 Bacteriological analysis

The generic *E. coli* counts obtained from chicken farms supplied by the three feed companies (A, B and C) using Petrifilm™ as well as the mean number of presumptive sorbitol negative *E. coli* and *Salmonella* isolates are presented on Table II.3. Some variations among farms supplied by the same feed company were observed, however, no significant difference ($P > 0.05$) was noticed between feed companies regarding the number of both types of *E. coli*. The mean \log_{10} *E. coli* colony forming units (CFU) per gram of sample recovered were 8.9, 8.7 and 6.8/g of cecal, fecal and litter samples respectively. Seventy-four presumptive sorbitol negative *E. coli* were isolated from feed companies A (20), B (23) and C (31) after enrichment in TSB-VCC. All isolates were confirmed to be non-O157 by latex agglutination therefore the H type was not determined.

Sixty-two *Salmonella* were isolated and confirmed by both biochemical and serotyping representing 11.2% of the total number of cecal, fecal and litter samples. The majority (47) of these 62 *Salmonella* positive samples were from farms b (25) and f (22) both supplied by feed company C (Table II.3). The remaining *Salmonella* positive samples were from farms supplied by feed companies A (10) and B (5). No significant association ($P > 0.05$) was observed between the feed companies and the prevalence of *Salmonella*.

In total, six different serovars of *Salmonella enterica* were isolated (Table II.4). Serovars Typhimurium (40.3%), Kentucky (24.2%) and Heidelberg (16.1%) were the most frequently isolated in this study. Serovar Enteritidis (11.3%) which is often associated with egg contamination was found in farms supplied by both feed companies A and C. Serovars Brandenburg (6.5%) and Heidelberg were isolated only from farms supplied by feed company C (Table II.4). Serovar Kentucky was found on farms supplied by all three companies. The rare Tokoin serovar was isolated from a farm receiving feed from company C, while Typhimurium was detected in samples of chickens from farms supplied by feed company B.

II.5.2 Antimicrobial susceptibility testing

The susceptibility to 18 antibiotics was determined by Sensititre technique in the 74 sorbitol negative *E. coli* and the 62 *Salmonella* isolates. In general, no significant difference ($P > 0.05$) in individual antibiotic resistance was observed between farms supplied by the different feed companies. The MIC values and the levels of resistance of *E. coli* and *Salmonella* isolates to the different antimicrobials are presented in Table II.5. Both *E. coli* and *Salmonella* isolates were resistant to penicillin, erythromycin, tylosin, clindamycin, and novobiocin and exhibited different resistance levels to other antibiotics (Figure II.2 A and B). All the *E. coli* strains were susceptible to enrofloxacin. Two *E. coli* isolates (ECD-227 and ECB-175-2) were intermediately susceptible to gentamycin. Tetracycline resistance was found in 56 of the 74 *E. coli* isolates. More than 20% of *E.*

coli isolates were multi-resistant to 12 antibiotics among which one (isolate ECD 227 from farm b) was resistant to 15 of the 18 antibiotics tested (Figure II.3A).

All the *Salmonella* isolates were susceptible to gentamycin and enrofloxacin and only one isolate presented decreased susceptibility to sarafloxacin. About ninety percent (90.3%) of *Salmonella* isolates were resistant to spectinomycin whereas their resistance levels to β -lactam amoxicillin and ceftiofur were 41.9% and 43.6%, respectively (Figure II.2B). More than 90% of the *Salmonella* were multi-resistant to six antibiotics with one isolate resistant to 13 of the 18 antibiotics tested (Figure II.3B).

II.6.3 Virulence genes

Seventy four *E. coli* strains were tested for the presence of the four specific virulence genes including *eaeA*, *hlyA*, *stx1* and *stx2* of EHEC. Among these genes, only the intimin gene *eaeA* was detected in six (8.1%) isolates. In addition, the 74 *E. coli* isolates were tested for the presence of four virulence genes implicated in avian colibacillosis. Twenty-five (33.8%) isolates had at least one virulence gene involved in colibacillosis. The aerobactin operon (*iucC*) gene was found in nine (12.2 %) *E. coli* isolates. The increased serum survival (*iss*) gene was also present in nine (12.2 %) of tested isolates. Seven (9.46%) *E. coli* were positive for temperature-sensitive hemagglutinin (*tsh*) gene whereas *traT* gene was detected in only four (5.40%) isolates. Associations of virulence genes within the same isolate also were observed. Two isolates were positive for *iss* and *iucC*, and one of those, was also positive for a third virulence

gene (*tsh*). Only one *E. coli* isolate was positive for *iucC* and *traT*. There was no significant difference ($P > 0.05$) between the farms supplied by specific feed companies for the prevalence of a given virulence gene.

Salmonella invasin (*inv*) and virulence (*spv*) genes were found in 61 (98.4%) and 26 (42%) isolates, respectively. Except for one Heidelberg isolate, all *Salmonella* serovars were positive for *inv* gene (Table II.4). The *spv* gene was detected in only serovars Typhimurium (73.10%) and Enteritidis (27.0%) as shown in Table II.4.

II.6.4 PCR detection of antibiotic resistance genes

Among the 74 *E. coli* isolates studied, 55 (74.3%) were resistant to amoxicillin and 47 (63.5%) were resistant (38) or presented a decreased resistance (9) to ceftiofur. These 55 isolates and eight additional *E. coli* isolates that were resistant to penicillin only were selected for the detection of the extended-spectrum-cephalosporin resistance gene (*bla_{CMY-2}*), as well as the *qacEΔ1* and *SulI* genes in order to investigate the presence and distribution of these resistance determinants among the isolates. Positive isolates for the class 1 integron 3' conserved segment were further amplified by PCR to obtain the variable region that contains resistance gene cassettes.

Among the 55 amoxicillin and ceftiofur resistant *E. coli* isolates, 44 (80.0%) were positive for *bla_{CMY-2}*. None of the eight penicillin resistant (susceptible to amoxicillin and

ceftiofur) isolates possessed *bla*_{CMY-2}. No difference ($P > 0.05$) was observed between the feed companies regarding the presence of the *bla*_{CMY-2} gene (Figure II.4).

Of the 63 *E. coli* analyzed, 27 (42.8%) isolates were positive for the *qacEΔ1-Sull* gene. Of these 27 isolates, the variable region of the class 1 integron was detected in 23 (22 amoxicillin-ceftiofur and one penicillin resistant). The phenotype and genotype of these 23 class 1 (*qacEΔ1-Sull* gene and variable) positive *E. coli* isolates are presented in Table II.6. All 23 isolates were multi-resistant to several of the antibiotics tested. Except for one isolate (ECD 227), all the other integron-bearing isolates were susceptible to enrofloxacin and sarafloxacin. Amplicons of 1.5 kb and 1 kb were detected in 6 and 17 isolates, respectively. Amplicons of 1 kb of two *E. coli* isolates (ECD-227 resistant to ceftiofur and ECB 175-2 sensitive to ceftiofur) were sequenced.

Fifty six tetracycline resistant *E. coli* strains were analyzed for the presence of the four tetracycline resistance genes, *tet(A)*, *tet(B)*, *tet(C)* and *tet(D)*. Only two of those genes, *tet(A)* and *tet(B)*, were detected in 12 (21.4%) and 24 (42.9%) of the isolates, respectively. There was a significant difference ($P < 0.01$) between the feed companies regarding the presence of *tet(B)* which was significantly more prevalent in *E. coli* isolates from farms supplied by feed company B (Figure II.4).

Seven of the integron positive *E. coli* also were positive for at least one virulence determinant, *bla*_{CMY-2}, *tet(A)* or *te(B)* genes. Interestingly, isolate ECD 93-2 carried the *eae* gene of EHEC (Table II.6).

Twenty seven (41.9%) *Salmonella* isolates were resistant to ceftiofur; among them 22 (81.5%) possessed *bla*_{CMY-2} (10 Heidelberg, 11 Typhimurium and one Brandenburg) as presented in Table II.4. No difference ($P > 0.05$) was observed between the feed companies for the presence of the *bla*_{CMY-2} gene. None of the *Salmonella* isolates were positive to *qacEΔ1-Sul1* genes of class 1 integron. No further variable region detection was performed on these isolates. All *Salmonella* isolates were susceptible to tetracycline and were not analyzed for the presence of tetracycline resistance genes.

II.6.5 DNA Sequencing

There was 99% identity between our query sequences (*bla*_{CMY-2} and *qacEΔ1-Sul1*) and the reference sequences deposited in the data base (GeneBank accession no. AB212086 and U37105, respectively). Analysis of the 1-kb amplification products of the variable region revealed that they contained the *aadA1* coding for aminoglycoside adenylyltransferase. There was 99% identity with *aadA* gene of *E. coli* (GeneBank accession no. DQ517526.1). In addition, 97% identity was found between the 74 bp at 3' side of the 1-kb amplification product with the quaternary ammonium compound resistance protein described on the plasmid pAPEC-O1-R of *E. coli* strain O1:K1 (no. DQ517526.1).

II.7 Discussion

It is well known that the microbial ecosystem of the chicken is inextricably connected to management practices including feeding (Knarreborg et al. 2002). The growth of *E. coli* depends on the gut environment and there is a growing interest in commensal components of the gut micro flora associated with food-producing animals (Langhout et al. 1999; Durso et al. 2004; Fairchild et al. 2005; White 2005). However, little is known about non-O157 sorbitol negative *E. coli* (atypical) from the chicken gut. In this study, sorbitol negative *E. coli* strains were isolated from nine commercial broiler chicken farms that used three different antimicrobial containing feed formulations. When compared to data reported by Gabriel et al. (Gabriel et al. 2005), high *E. coli* numbers were recovered in the entire broiler chicken production environment and no difference was found between feed companies for fecal, cecal and litter samples over time in the present study. The data indicates that antibiotic resistant *E. coli* can be isolated from broiler fecal samples and litter, suggesting the potential to contaminate the environment if litter is applied to soil as fertilizer.

Salmonella infections in chicken are recognized as an important contributor to human salmonellosis. The present study confirms the sub-clinical *Salmonella* infection in chicken as 62 *Salmonella* strains with different serotypes were isolated from six of the nine (66.6%) farms studied. These results show that despite control strategies developed by chicken producers, *Salmonella* eradication has not been achieved. This may be in part due to the presence of the invasin gene *inv* that enables this bacterium to colonize the

intestinal tissues of their host (Chiu and Ou 1996). Except for one strain all *Salmonella* isolates assayed were positive for *inv*. It also may be important to establish the current status of *Salmonella* prevalence in large studies of Canadian broiler chicken farms.

In addition to a role in disease processes, virulence factors presumably enable the pathogens to exploit hosts in ways unavailable to commensal strains, and thus to spread and persist in the bacterial community (Donnenberg and Whittam 2001). In this study, *E. coli* isolates harboring some virulence genes were found in healthy broiler chickens. The pathogenicity of these *E. coli* isolates was not determined. Intimin is an outer membrane protein involved in the intimate attachment of the *E. coli* to the human intestine and is encoded by the *eae* gene, which is part of the locus of enterocyte effacement (LEE). The LEE is required for formation of the attachment and effacing lesions in the large intestine, and regarded as a crucial pathogenicity factor (Frankel et al. 1998). In the present study, *eae* was detected in *E. coli* isolates from broiler chicken fecal material. The genetic variants of *eae* have not been identified in this work. This finding underlines the importance of the determination of virulence factors to assess the potential pathogenicity of chicken *E. coli* isolates for humans. Although chickens may harbor many different *E. coli* strains in their gastrointestinal tracts, only a restricted number of strains can cause colibacillosis in poultry. The present study shows that some sorbitol negative *E. coli* possessed one or more genes involved in this disease indicating that *E. coli* harboring virulence genes can colonize the gastrointestinal tracts of healthy broiler chickens, potentially representing a reservoir for the contamination of the environment and/or the food chain.

The monitoring of antimicrobial resistance in bacteria isolates from poultry is imperative due to food and occupational safety concerns (O'Brien 2002). The development of antibiotic resistance in *E. coli* isolated from broiler chicken farms is a well known phenomenon in many nations around the world (Nazer 1980; Asai et al. 2005; White 2005). All the *E. coli* strains isolated on the farms in this present study were resistant to penicillin, erythromycin, tylosin, clindamycin, and novobiocin. Multiple-antibiotic resistance phenotypes were also reported in *E. coli* isolated from food, animals and humans (Sáenz et al. 2004; Zhao et al. 2005). In the present study, *E. coli* isolates were susceptible to both fluoroquinolone, and trimethoprim-sulfamethoxazole. In Canada, tetracycline is not used as a growth promoter in poultry but interestingly, more than 74% of the *E. coli* isolated tested were tetracycline resistant. Zhao et al. (2005) reported that 87% of avian pathogenic *E. coli* were tetracycline resistant. Tetracycline is one of the limited choices of antibiotics to treat *E. coli* airsacculitis which may result in the selection of *E. coli* resistant to this antibiotic in commercial poultry production (Singer and Hofacre 2006). The current study showed the presence of multi-resistant commensal *E. coli* isolates in broiler chicken production in the Fraser Valley of British Columbia.

In agreement with Scalzo et al. (Scalzo et al. 2004) there was no difference between farms supplied by feed companies that use supplementation of the diet with antibiotics on the antibiotic resistance or the level of excretion of *Salmonella*. In this study, all Enteritidis serovars isolated were resistant to about five antibiotics. One of the Heidelberg serovars exhibited resistance to 13 antimicrobials including amoxicillin, ceftiofur, spectinomycin, streptomycin, oxytetracycline, tetracycline, sulphathiazole and

trimethoprim/sulfamethoxazole. The present study also revealed the presence of the rare Tokoin serovar that was resistant to seven antibiotics including spectinomycin and sulphadimethoxime suggesting a possible presence of multiple antibiotic resistant *Salmonella* in healthy broiler chicken production in the Fraser Valley of British Columbia.

The gene *bla*_{CMY-2}, an AmpC-like β -lactamase gene, was detected on plasmids in several multiple antibiotic resistant *E. coli* and *Salmonella* isolates from humans and animals (Poppe et al. 2005; Adesiyun et al. 2007). The *bla*_{CMY-2} β -lactamase mediates resistance to expanded-spectrum cephalosporins including ceftiofur. In this study, *bla*_{CMY-2} gene was found in 80.0% and 81.5% of amoxicillin and ceftiofur resistant *E. coli* and *Salmonella* isolates, respectively. Generally, invasive *Salmonella* infections require antimicrobial treatment with ampicillin, chloramphenicol, or trimethoprim-sulfamethoxazole (Falkow and Mekalanos 1990). But the increasing number of *Salmonella* strains resistant to these antibiotics has led to a decrease in their efficacy against this pathogen. Thus, the broad-spectrum cephalosporins including ceftiofur are used for treatment of *Salmonella* infection because of the low number of *Salmonella* isolates showing resistance to these antibiotics (Angulo et al. 2000). The present study showed the presence of *Salmonella* isolates carrying *bla*_{CMY-2} gene in broiler chicken farms. Interestingly, all the 22 *bla*_{CMY-2} positive *Salmonella* isolates of this study were resistant to at least eight antibiotics each, including amoxicillin and ceftiofur. Given that *E. coli* strains were isolated from the same farms as were *Salmonella* isolates, a potential transfer of the plasmid between these two bacterial species may have occurred. In fact, Poppe et al. (Poppe et al. 2005), demonstrated the acquisition of resistance to extended-

spectrum cephalosporins by *Salmonella enterica* serovar Newport and other *E. coli* strains by conjugation in poultry intestinal tracts that may represent a serious health issue (Gupta et al. 2003; Gray et al. 2004).

In *E. coli*, tetracycline resistance is frequently regulated by efflux genes that are normally associated with large plasmids which often carry other antibiotic resistance genes, heavy metal resistance genes and/or other pathogenic factors such as toxins, hence the selection for any of these factors selects for the plasmid (Chopra and Roberts 2001). Tetracycline resistance was found in 56 of the *E. coli* isolates studied among which, 43 (67.8%) were also resistant to streptomycin. Little is known about the distribution of tetracycline resistance genes among commensal *E. coli* from broiler chickens. The *tet(C)* and *tet(D)* genes were not found in any of the isolates while *tet(A)* and *tet(B)* were detected in 12 and 24 isolates, respectively. Similar results pertaining to the presence of *tet(A)* and *tet(B)* but not *tet(C)* or *tet(D)* were found by Fairchild et al. (Fairchild et al. 2005). The remaining tetracycline-resistant isolates, in which none of the four genes was detected, suggests the possible presence of other tetracycline resistance genes. Interestingly, the present study found that there was a higher incidence of *tet(B)* positive *E. coli* in the chickens from farms supplied by feed company B that added salinomycin to the grower and finisher feeds despite that phenotypically, no difference in tetracycline resistance was observed among farms supplied by those feed companies. Similar observations were made in pigs by Delsol et al. (Delsol et al. 2005) found that the *tet(B)* gene dominated the tetracycline resistant *E. coli* population isolated from pigs treated with avilamycin when compared to the control, untreated pigs. It remains to be established

whether salinomycin or other growth promoting antibiotics can select for a given antibiotic resistance gene like *tet(B)*. The results of the present study and those from other reports suggest the possibility that *E. coli* from poultry may be a reservoir for tetracycline resistance (Miles et al. 2006).

Integrans contribute to the spread of antimicrobial resistance by gene transfer in a variety of enteric bacteria including *E. coli* and *Salmonella* (Bass et al. 1999; Maynard et al. 2004). The presence of integrans in enteric bacteria from poultry was previously reported (Bass et al. 1999; Nandi et al. 2004; Nógrády et al. 2006). However, there are limited studies focused on the presence of integron among avian commensal *E. coli* (White 2005). The results of this study show the presence of the class 1 integron gene within 23 (31%) of *E. coli* isolates from healthy chickens. This prevalence was similar to that reported by Nógrády et al. (Nógrády et al. 2006) who showed that 32.6% of *E. coli* isolates from dead birds contained the class 1 integron. The 1.5-kb amplification product of the variable region was not analyzed in the present study however, the nucleotide sequences of 1-kb of this region found in 17 of class 1 integron-bearing *E. coli* contained the spectinomycin-streptomycin resistance gene *aadA1*. Decreased susceptibility (intermediate susceptibility) and resistance to spectinomycin were found in 12 (70.5%) and four (23.5%) of these 17 *E. coli* isolates, respectively. Eleven (64.7%) of the *E. coli* isolates were resistant to streptomycin. The *aadA* gene cassette in class 1 integrans is conserved among avian *E. coli* isolates (Bass et al. 1999) suggesting a possible selective mechanism for this cassette in enteric bacteria in the chicken gut.

Further characterization of these integron positive strains revealed that several of them had at least one known gene implicated in avian colibacillosis. Whether these virulence genes are physically linked to the integron was not determined in this study. Johnson et al. (Johnson et al. 2004) reported possible relationships between virulence and antimicrobial resistance genes in avian *E. coli* suggesting that use of antimicrobial agents in poultry production could lead to the selection and spread of pathogenic antibiotic resistant *E. coli*. Thus, sorbitol negative *E. coli* may play important roles in bacterial evolution and the transmission of antimicrobial resistance and virulence genes in the chicken gut.

The data from the present study confirms that the gastrointestinal tract of broiler chickens can be colonized by atypical *E. coli* and *Salmonella* strains harboring virulence and antibiotic resistance genes. The presence of integron in *E. coli* indicates a potential for lateral antibiotic resistance gene transfer between this bacterium and other chicken gut bacteria. These bacteria have the potential to spread in the environment through manure, and subsequently to farm workers and throughout the processing plant.

Table II.1. Feed antimicrobial composition per feed company

Feed (farm)^a	Antimicrobial agents in diet (mg/kg of feed)		
	Starter: Days 0-20	Grower: Days 21-27	Finisher: Days 28-36
A (a, c, i)	Narasin (40)	Narasin (70)	
	Nicarbazin (40)	BMD (55)	Virginiamycin (22)
	BMD (110) ^b		
B (d, e, j)		Narasin (40)	
	Narasin (40)	Nicarbazin (40)	Salinomycin (60)
	Nicarbazin (40)	Salinomycin (60)	BMD 55
C (b, f, g)		BMD (55)	
	Narasin (40)	Narasin (40)	Diclazuril (1)
	Nicarbazin (40)	Nicarbazin (40)	Virginiamycin (22)
	BMD (110)	BMD (55)	

^aFeed companies are in capital; farms are in lower case in brackets.

^bBacitracin Methylene Disalicylate

Table II.2. Primers used for PCR in this study

Primer	Sequence (5' to 3')	Amplicon size (bp)	Reference
PCR for antibiotic resistance genes			
Tet A-F	GTGAAACCCAACATACCCC	888	Maynard et al. 2003
Tet A-R	GAAGGCAAGCAGGATGTAG		
Tet B-F	CCTTATCATGCCAGTCTTGC	774	
Tet B-R	ACTGCCGTTTTTTTCGCC		
Tet C-F	ACTTGGAGCCACTATCGAC	881	
Tet C-R	CTACAATCCATGCCAACCC		
Tet D-F	TGGGCAGATGGTCAGATAAG	827	
Tet D-R	CAGCACACCCTGTAGTTTTC		
bla _{CMY-2} F	ATAACCACCCAGTCACGC	631	Poppe et al. 2005
bla _{CMY-2} R	CAGTAGCGAGACTGCGCA		
PCR for Class 1 integron			
qacEΔ1&SulI-F	ATC GCA ATA GTT GGC GAA GT	797	Maynard et al. 2003
qacEΔ1&SulI-R	GCAAGG CGG AAA CCC GCG CC		
5' CS-F	GGCATCCAAGCACAAGC	Variable	
3' CS-R	AAGCAGACTTGACTG AT		
PCR for <i>E. coli</i> O157 genes			
EaeA-F	GACCCGGCACAAGCATAAGC	384	Paton and Paton 1998
EaeA-R	CCACCTGCAGCAACAAGAGG		
hlyA-F	GCATCATCAAGCGTACGTTCC	534	
hlyA-R	AATGAGCCAAGCTGGTTAAGCT		
STX1-F	ATAAATCGCCATTCGTTGACTAC	180	
STX1-R	AGAACGCCCACTGAGATCATC		
STX2-F	GGCACTGTCTGAAACTGCTCC	255	
STX2-R	TCGCCAGTTATCTGACATTCTG		
PCR for Colibacillosis genes			
Iss-F	GTGGCGAAAAGTAGTAAAACAGC	760	Johnson et al. 2004
Iss-R	CGCCTCGGGGTGGATAA		
TraT-F	ATCCGATAAGATAAAAAGGAGAT	416	
TraT-R	TAGACAATCCACCAAGAAGAAATA		
IucC-F	GTCGCCGTGGTGGGGTAAAGAG	429	
IucC-R	TCCCCCGGTAGCATG AGG TGTTGT		
Tsh-F	GGG AAATGACCTGAATGCTGG	420	
Tsh-R	CCG CTC ATC AGT CAG TAC CAC		
PCR for <i>Salmonella</i> genes			
InvA-F	ACAGTGCTCGTTTACGACCTGAAT	244	Chiu and Ou 1996
InvA-R	AGACGACTGGTACTGATCGATAAT		
SpvC-F	ACTCCTTGACAACCAAATGCGGA	571	
SpvC-R	TGTCTTCTGCATTTGCCACCATCA		

Table II.3. Mean *E. coli* number per g of material and mean of PSN^b *E. coli* and *Salmonella* isolates

Feed: Farm	Bacteria				
	Total generic <i>E. coli</i> number (Log ₁₀ ± SEM ^a)			Number (mean ± SEM) of isolates	
	Fecal	Cecal	Litter	PSN ^b <i>E. coli</i>	<i>E. coli</i> <i>Salmonella</i>
A: a, c, i	8.6 ± 0.35	8.8 ± 0.32	6.4 ± 1.31	6.6 ± 2.28	3.3 ± 7.71
B: d, e, j	9.0 ± 0.35	8.9 ± 0.32	7.4 ± 1.31	7.6 ± 2.28	1.6 ± 4.71
C: b, f, g	8.6 ± 0.35	9.2 ± 0.32	6.7 ± 1.31	10.3 ± 2.28	15.6 ± 4.71

^aStandard error of the mean n = 3 farms/feed company

^bPresumptive Sorbitol Negative

Table II.4. *Salmonella* isolates serotyping and virulence genes detection

Serotypes	Number (%)	<i>Bla</i> _{CMY-2} (n=22)	Invasine genes	
			<i>Inv</i> (n=61)	<i>Spv</i> (n=26)
Heidelberg	10 (16.1) ^a	10 (45.5) ^b	9 (14.8) ^c	0 (0.0) ^c
Brandenburg	4 (6.5)	1 (4.5)	4 (6.6)	0 (0.0)
Kentucky	15 (24.2)	n/a	15 (24.6)	0 (0.0)
Typhimurium	25 (40.3)	11 (50.0)	25 (41.0)	19 (73.1)
Enteritis	7 (11.3)	n/d ^d	7 (11.5)	7 (27.0)
Tokoin	1 (1.6)	n/d	1 (1.6)	0 (0.0)
Total	62 (100)	22 (100)	61 (100)	26 (100)

^aNumbers in brackets represent the percentage of serotypes in the collection of *Salmonella*

^bPercentage of isolates with *bla*_{CMY-2} gene in the different serovars groups.

^cPercentage of isolates with Invasine genes in the serovars population.

^dIsolates that were susceptible to amoxicillin and ceftiofur and were not screened for *bla*_{CMY-2}.

Table II.5. Antimicrobial resistance phenotypes of *E. coli* and *Salmonella* isolates

Antibiotic	Breakpoint ^a											
	<i>E. coli</i> (n = 74)						<i>Salmonella</i> (n=62)					
	Compound	(µg/ml)	MIC ^b ₅₀	MIC ^c ₉₀	Range	% Resistant	MIC ^b ₅₀	MIC ^c ₉₀	Range	% Resistant	MIC ^b ₅₀	% Resistant
Beta-Lactam	Amoxicillin	≥32	> 16	> 16	2 - > 16	74.0	2	> 16	1 - > 16	> 16	41.9	
	Ceftiofur	≥4	> 4	> 4	0.25 - > 4	51.4	2	> 4	0.5 - > 4	> 4	43.55	
Aminocyclitol	Spectinomycin	≥16	16	> 64	≤ 8 - > 64	33.8 ^d	32	64	16 - 64	64	90.3	
Aminoglycoside	Streptomycin	≥64	64	512	8 - > 512	59.5	≤ 32	≤ 32	16 - 128	≤ 32	1.6	
	Gentamicin	≥16	≤ 0.5	2	≤ 0.5 - > 8	0.0	≤ 0.5	1	≤ 0.5 - 1	1	0.00	
	Neomycin	≥16	≤ 2	≤ 2	≤ 2 - > 32	5.4	≤ 2	≤ 2	≤ 2 - 32	≤ 2	1.6	
Quinolone	Sarafloxacin	≥0.25	≤ 0.03	≤ 0.03	≤ 0.03 - > 0.25	1.4	≤ 0.03	≤ 0.06	≤ 0.03 - 0.12	≤ 0.03	0.0	
Tetracycline	Oxytetracycline	≥16	> 8	> 8	1 - > 8	75.7	1	2	1 - > 8	2	1.6	
	Tetracycline	≥16	> 8	> 8	1 - > 8	74.3	≤ 4	≤ 4	1 - > 8	≤ 4	1.6	
Sulfamide	Sulphadimethoxime	≥512	128	> 256	64 - > 256	48.6	128	256	64 - > 256	256	9.7	
	Sulphathiazole	≥512	64	> 256	≤ 32 - > 256	48.6	64	64	≤ 32 - > 256	64	3.2 ^e	
	Trimethoprim/Sulphadimethoxime	≥4/76	≤ 0.5	≤ 0.5	≤ 0.5 - > 2	9.5	≤ 0.5	≤ 0.5	≤ 0.5 - > 2	≤ 0.5	1.6	

^aValues are based on National Institute of Clinical Standards. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls.

^bMIC₅₀, MIC at which 50% of isolates were inhibited.

^cMIC₉₀, MIC at which 90% of isolates were inhibited.

^d4 (5.4%) and 45 (60.8%) *E. coli* isolates were susceptible and of intermediate resistance respectively to this antibiotic.

^eThe remaining 60 *Salmonella* isolates had a decreased susceptibility to this antibiotic.

Table II.6. Minimal inhibitory concentration (MIC) and genotype of 23 integron positive *E. coli*

Strain	Feed company	Antibiotic MIC ($\mu\text{g/ml}$) ^a										Genotype (gene)	
		Amo	Cef	Spec	Strep	Gen	Neo	Suldtx	Sulthz	Virulence	<i>bla</i> _{CMY-2}	<i>Tet(A)</i> or (B)	
ECA-137-3	A	>16 R	4 I	16 I	128 R	1 S	≤2 S	64 S	64 I	-	-	-	B
ECA-137-5	A	2 S	≤0.25 S	16 I	128 R	1 S	≤2 S	64 S	≤32 I	-	-	-	-
ECA-137-8	A	2 S	0.50 S	32 R	128 R	1 S	≤2 S	64 S	64 I	-	-	-	-
ECA-137-1	A	2 S	≤0.25 S	32 R	128 R	1 S	≤2 S	64 S	64 I	<i>iucC</i>	-	-	-
ECA-137-2	A	2 S	≤0.25 S	16 I	128 R	1 S	≤2 S	64 S	64 I	<i>Tsh</i>	-	-	-
ECD 93-2	B	>16 R	>4 R	16 I	≤8 S	≤0.5 S	≤2 S	>256 R	>256 R	<i>eaeA</i>	+	+	B
ECD 93-3	B	>16 R	>4 R	16 I	≤8 S	1 S	≤2 S	>256 R	>256 R	<i>iucC, eaeA</i>	+	+	B
ECD 93-4	B	>16 R	>4 R	16 I	≤8 S	≤0.5 S	≤2 S	>256 R	>256 R	-	+	+	B
ECD 93-5	B	>16 R	>4 R	≤8 S	128 R	1 S	≤2 S	>256 R	>256 R	-	+	+	B
ECD 99-2	B	>16 R	>4 R	32 R	≤8 S	≤0.5 S	≤2 S	>256 R	>256 R	-	+	+	B
ECD 99-3	B	>16 R	>4 R	16 I	≤8 S	≤0.50 S	≤2 S	>256 R	>256 R	-	+	+	B
ECD 99-4	B	>16 R	>4 R	16 I	≤8 S	≤0.5 S	≤2 S	>256 R	>256 R	-	+	+	B
ECD 227*	B	>16 R	>4 R	>64 R	64 R	8 I	32 R	>256 R	>256 R	<i>iucC, traT</i>	-	-	A and B
ECE 2-Cec	B	>16 R	1 S	32 R	128 R	≤0.5 S	≤2 S	64 S	64 I	<i>iucC</i>	-	-	B
ECB-47-2	C	>16 R	4 I	16 I	≤8 S	≤0.5 S	≤2 S	>256 R	>256 R	-	+	+	-
ECB 175-2	C	>16 R	1 S	>64 R	64 R	8 I	≤2 S	>256 R	>256 R	<i>traT</i>	-	-	A
ECF 264-2	C	>16 R	>4 R	16 I	128 R	1 S	≤2 S	64 S	64 I	-	+	+	B
F-264-3	C	>16 R	>4 R	16 I	128 R	1 S	≤2 S	128 S	64 I	<i>iss, iucC</i>	+	+	-
ECF 266-2	C	>16 R	>4 R	16 I	128 R	1 S	≤2 S	64 S	64 I	-	+	+	-
ECF 270-1	C	>16 R	>4 R	16 I	64 R	1 S	≤2 S	64 S	64 I	-	+	+	-
ECF 276-2	C	>16 R	>4 R	16 I	128 R	≤0.5 S	≤2 S	64 S	64 I	-	+	+	-
ECF 276-3	C	>16 R	≤0.25 S	16 I	128 R	1 S	≤2 S	64 S	128 R	-	+	+	-
ECF 302	C	>16 R	1 S	16 I	64 R	≤0.50 S	16 R	64 S	64 I	-	+	+	-

All the isolates were resistant to penicillin (MIC >8 $\mu\text{g/ml}$), erythromycin (MIC >4 $\mu\text{g/ml}$), tylosin (MIC >20 $\mu\text{g/ml}$), clindamycin (MIC >4 $\mu\text{g/ml}$), oxytetracycline (MIC >8 $\mu\text{g/ml}$) and novobiocin (MIC >4 $\mu\text{g/ml}$). Except strain ECD 227, all the isolates were susceptible to trimethoprim/sulpha (MIC ≤0.50 $\mu\text{g/ml}$), enrofloxacin (MIC ≤0.12 $\mu\text{g/ml}$) and sarafloxacin (MIC ≤0.03 $\mu\text{g/ml}$).

^aMICs values with phenotype: S= susceptible, I = decreased (intermediate) susceptibility and R = resistant

*MIC of enrofloxacin and sarafloxacin against ECD 227 were 1 $\mu\text{g/ml}$ (intermediate) and >0.25 $\mu\text{g/ml}$ (resistant) respectively.

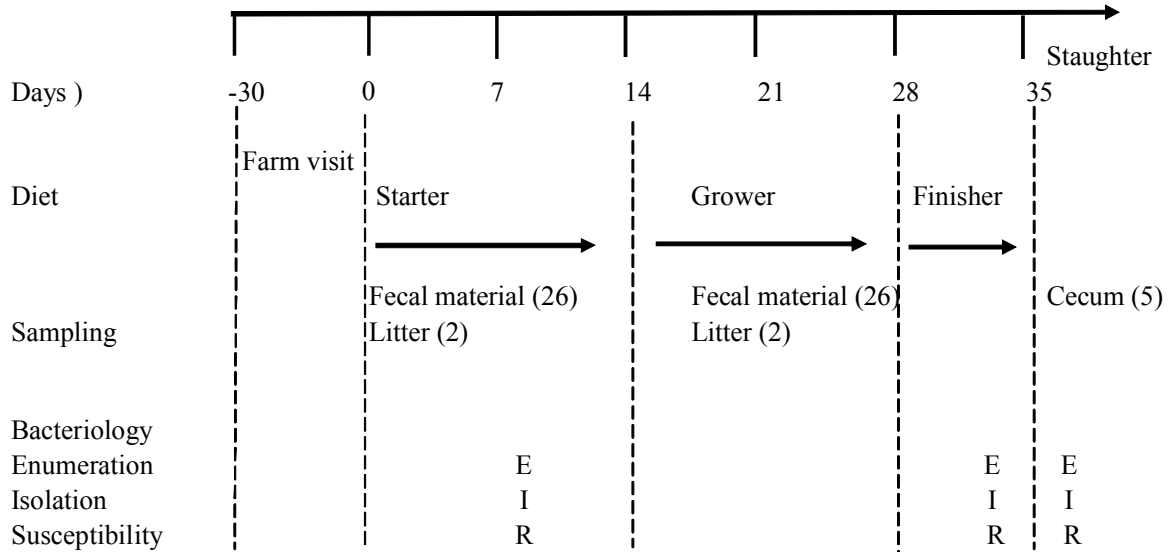


Figure II.1. Study time line

Farm visit: interview about farm management practices, diet sources and sanitation procedures; diet modification; E: Determination of total CFU *E. coli*/gram of sample; I: isolation of bacterial strains (sorbitol negative *E. coli* and *Salmonella*); R: determination of antimicrobial susceptibility. In total 549 samples (52 fecal material samples, 4 litter samples and 5 cecal samples) were collected from each broiler farm.

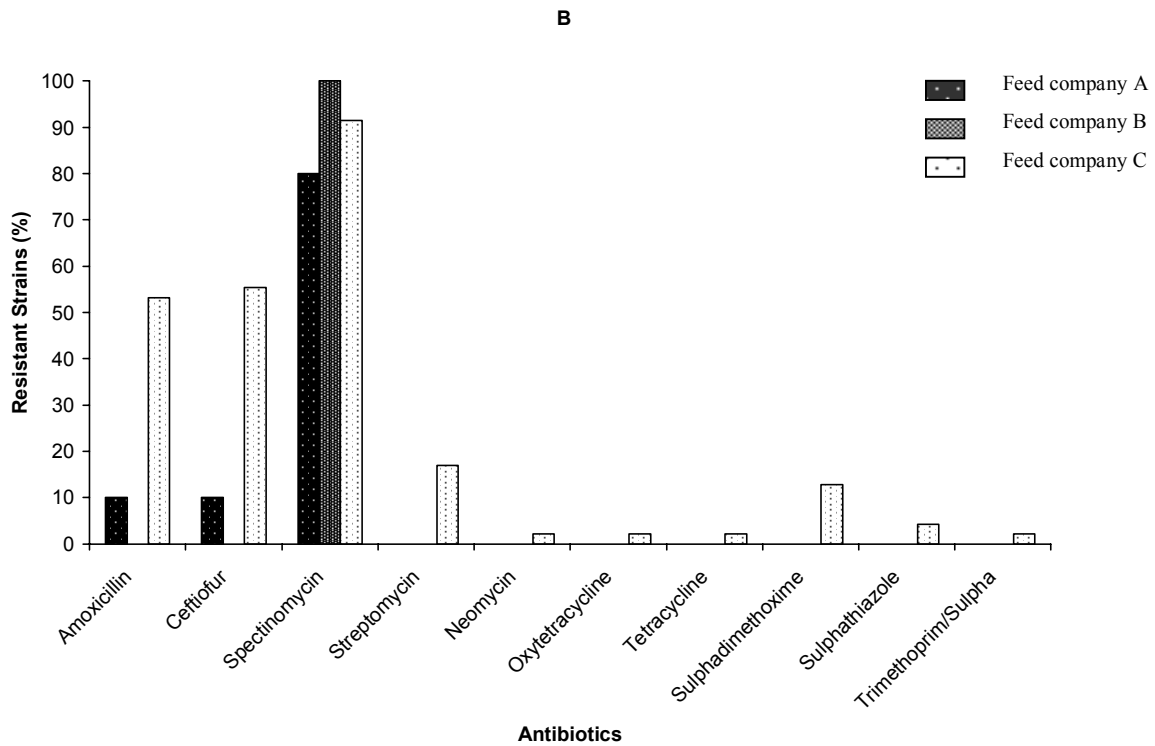
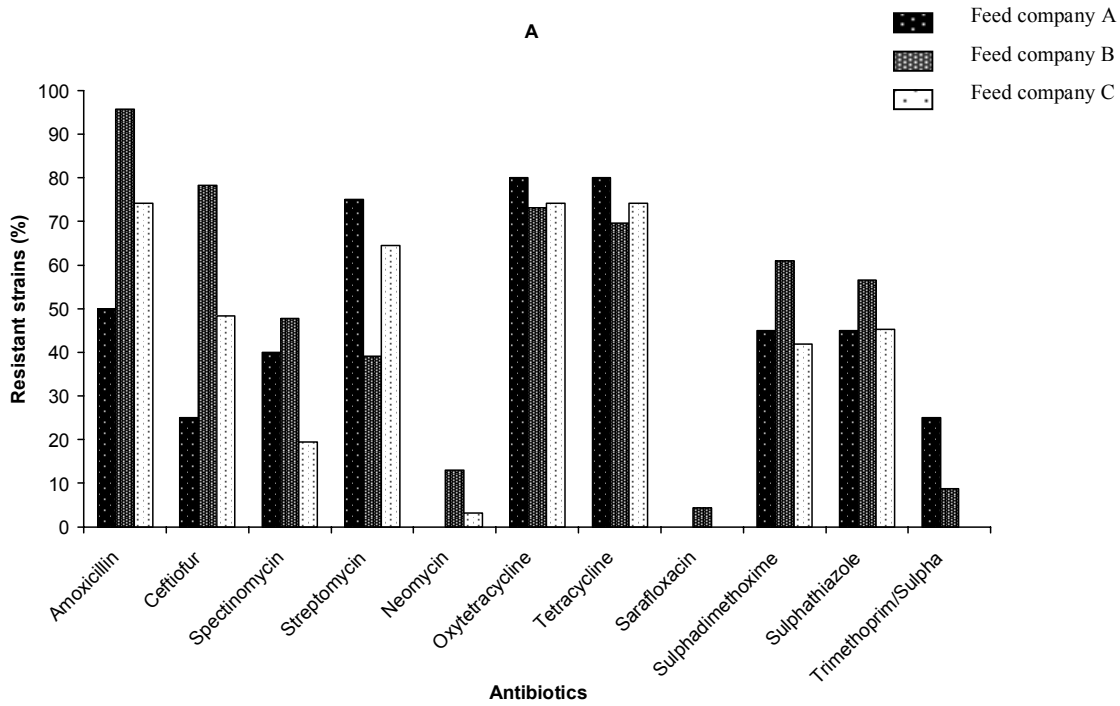
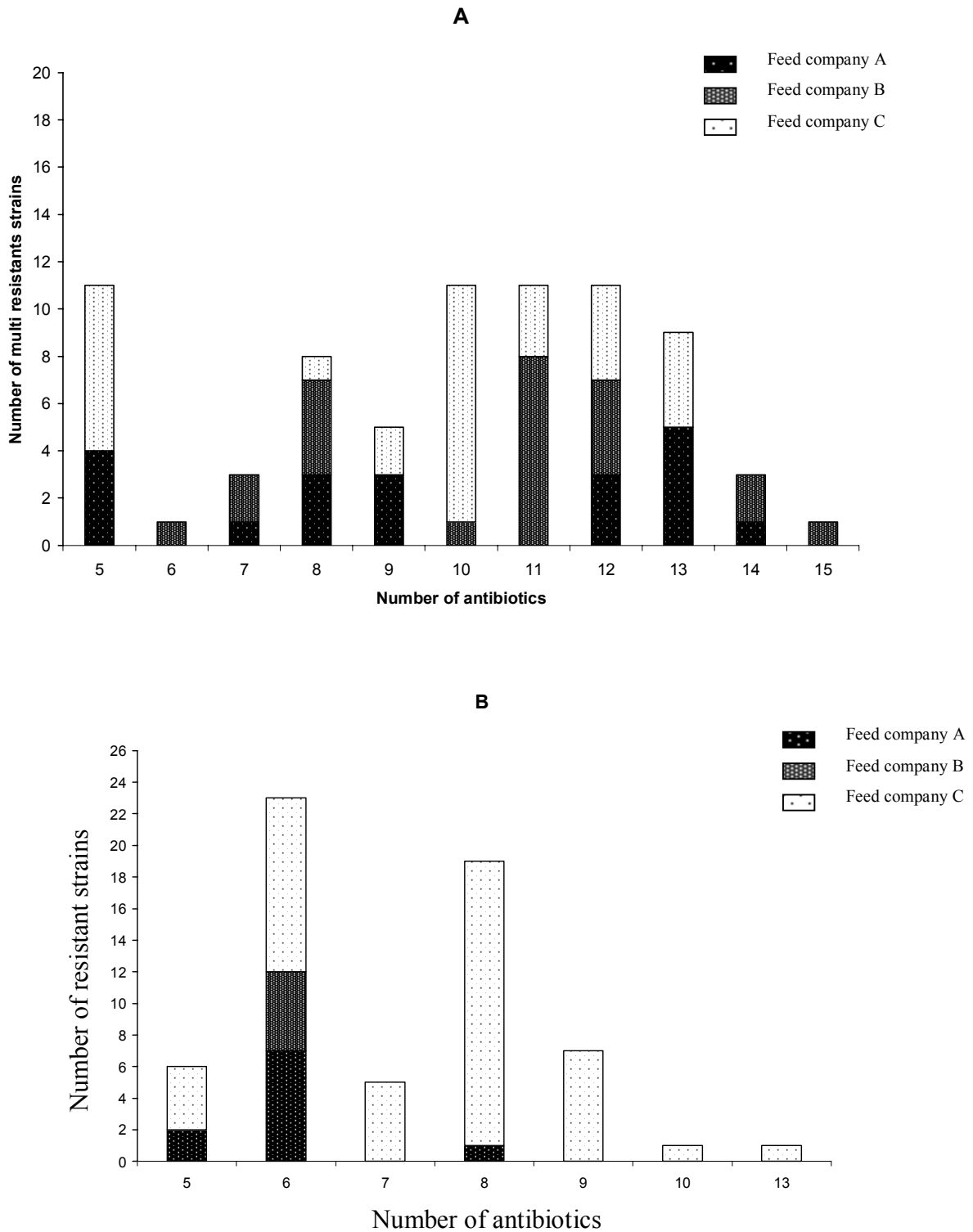


Figure II.2. Antibiotic resistance levels in *E. coli* (A) and *Salmonella* (B).

Resistance level against antibiotics of interest in 74 *E. coli* (20, 23 and 31 for feed company A, B and C, respectively) (A) and 62 *Salmonella* (10, 5 and 47 for feed company A, B and C, respectively) (B) isolates from nine chicken farms.



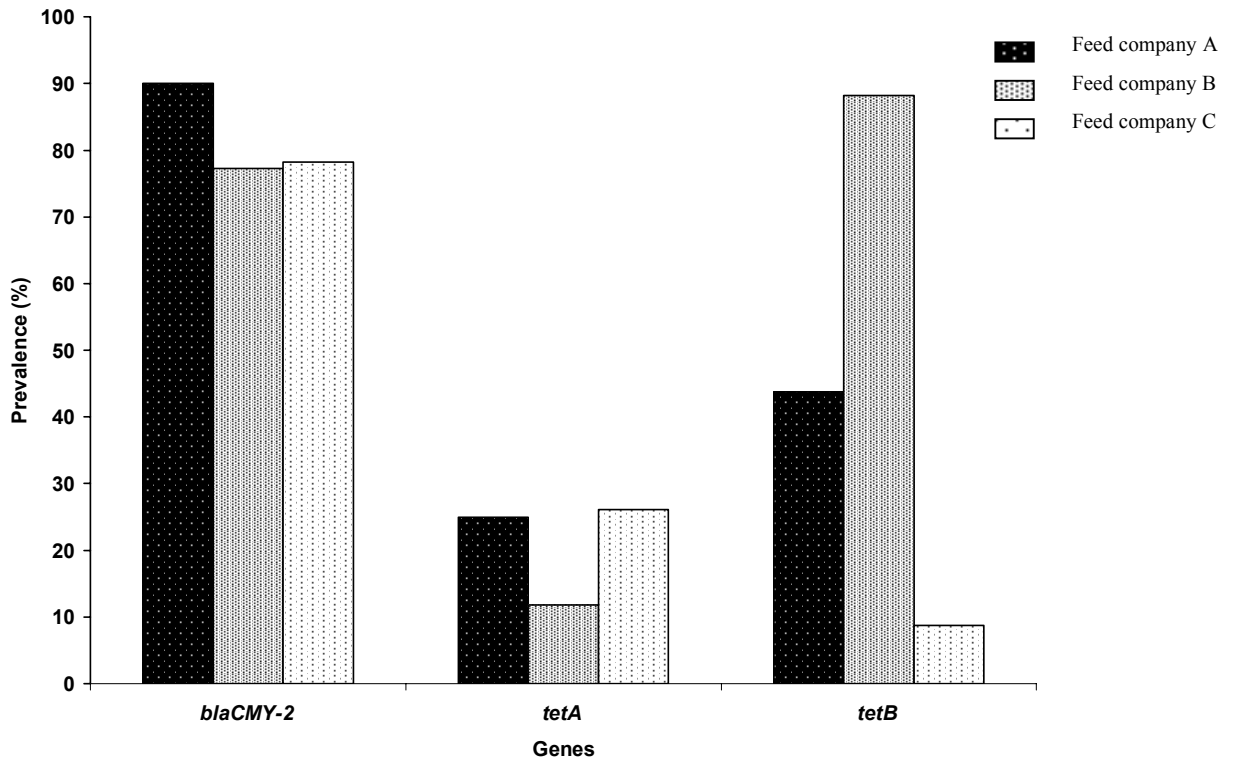


Figure II.4. Prevalence of resistance gene in *E. coli* isolates

The presence of *bla_{CMY-2}*, was detected in 55 isolates (10, 22 and 23 for feed company A, B and C, respectively) that were resistant to amoxicillin and ceftiofur. Tetracycline resistance genes (*tet(A)* and *tet(B)*) were detected in 56 (16, 17 and 23 for feed company A, B and C, respectively) tetracycline resistant isolates.

Chapter III. Evaluation of changes in antibiotic resistance levels of commensal *E. coli* isolated from healthy broiler chickens fed individual antimicrobial growth promoters

III.1 General objective

Antimicrobial growth promoters used in poultry production can prevent and control sub-clinical infections and improve food conversion and body-weight gain. This practice may modify the intestinal flora by creating selective pressure favoring the development of antibiotic resistant bacteria. The objective of the present study was to study the impact of individual growth promoting antibiotic on broiler chicken performance and on the incidence of antibiotic resistance in commensal *E. coli*.

III. 2 Specific objectives

- a) Evaluate the effects of individual antimicrobial growth promoters on broiler chickens performance
- b) Quantify *E. coli* populations in chickens fed diets with individual antimicrobial growth promoters
- c) Determine antibiotic resistance susceptibility of commensal *E. coli* to antibiotics of veterinary importance (avian)

III. 3 Synopsis of major results

The effects of feed supplementation with approved antimicrobial agents including bambarmycin, penicillin, salinomycin, bacitracin, combination of salinomycin + bacitracin, chlortetracycline, virginimycin, monensin and narasin on chickens growth performance, commensal *E. coli* counts, and the incidence of antibiotic resistance in *E. coli* isolated from broiler chickens raised in controlled environment were evaluated.

- (a) Under high bio-safety procedures, antimicrobial growth promoters used individually, did not significantly improve broiler chickens performance.
- (b) In spite of chloramphenicol not being used in poultry production since the 1980s, chloramphenicol resistant isolates were detected at high frequency.
- (c) Multi-antibiotic resistant commensal *E. coli* were found in broiler chickens fed the medicated and non medicated feeds.
- (d) There was significant effect of antimicrobial growth promoters, taken individually, on the incidence of antibiotic resistance in commensal *E. coli*.

III.4 Introduction

The beneficial effects of antimicrobial growth promoters (AGP) in animal production were barely hampered by evidence suggesting their possible contribution to the emergence of resistance in bacteria isolated from food animals (Swann 1969). In fact, the prophylactic use of antimicrobials in food animal production may transform food animals into a reservoir for antibiotic resistant bacteria (OTA 1995; Aarestrup et al. 2001). Further studies established that subtherapeutic use of antimicrobials in animal feeds could promote the transmission of antibiotic resistance genes from food animals to humans (Bates et al. 1993; Greko 2001; Roe and Pillai 2003). Consequently, numerous European countries instituted the ban on AGP use in agricultural animal production as a precautionary measure (Dibner and Richards 2005; Phillips 2007). Denmark remains the worldwide pioneer in monitoring antimicrobial resistance in food producing animals (Aarestrup 1995). However, the Danish Integrated Antimicrobial Resistance Monitoring and Research Program failed to bring proof of an existing association between the resistance in bacteria from food animals and infection in humans (Dibner and Richards 2005). On the contrary, the therapeutic use of antibiotics increased, boosting again the debate on the beneficial effects of the ban of AGP (Dawe 2004, Phillips et al. 2004, Vaughn and Copeland 2004).

The World Health Organization recommends management changes to diminish therapeutic and prophylactic use of antimicrobials (WHO 2000). Despite this recommendation, AGP are still in abundant use in North America (Dibner and Richards

2005). Antimicrobials such as virginiamycin and bacitracin, banned since 1999 in the European Union countries, are routinely used in the Canadian broiler chicken industry (CFIA 2008b). Others such as chlortetracycline and penicillin, belonging to antibiotic classes used for human therapy, are also used for growth promotion, feed efficiency and treatment of various sub-clinical infections in broiler chickens (CFIA 2008b). Ionophore anticoccidials such as monensin, narasin and salinomycin are used against *Clostridium perfringens* as well (CFIA 2008b). The lack of a convincing argument against the use of AGP favors their continuing utilization in food animal production. However, the globalization of the market, internalization of the food source and consumer pressure may force producers to modify their agricultural practices to be able to export their products, especially to the European markets (Dibner and Richards 2005). Meanwhile, well designed studies are necessary to accurately determine the impact of routinely used AGP on bird performance and their role in the emergence and spread of antibiotic resistance in both pathogenic and commensal bacteria isolated from chickens.

Commensal *Escherichia coli* may carry antibiotic resistant genes that may be transferred to coexistent pathogenic strains in the GI tract of food producing animals (Poppe et al. 2005). It is well established that aerobic and facultative anaerobes such as environmental *E. coli* and streptococci exist in the environment and are among the first ones to colonize birds's GI tracts immediately after hatch (Mackie et al. 1999). These bacterial populations can be spread into the environment through manure and runoff water (Lefebvre et al. 2006). It is therefore of public health importance to determine the impact of AGP on commensal bacteria. This study was initiated to evaluate the effects of

ten approved AGP in Canadian broiler chicken production on commensal *E. coli* and bird performance including body weight, feed intake, feed efficiency and mortality rate. It is to the author's knowledge the first time that such a study has been undertaken.

III.5 Materials and Methods

III.5.1 Experimental broiler chicken model

Two different experimental feeding trials were performed using a total of 1800 day-old chicks to evaluate the use of AGP on the emergence of resistant bacterial strains in poultry barns and on bird performance. Day old chicks were obtained from the same source and were all vaccinated for Marek's disease (into the egg at transfer time) and bronchitis (sprayed on after hatch). In each feeding trial, 900 chicks were assigned to 18 pens (50 birds per pen) under controlled conditions. Pens were assigned at random to ten different growth promoting antibiotics approved for use in poultry production in Canada and a non-medicated diet (Table III.1). Three pens were allotted to each treatment in both trials. The two feeding trials were constructed as a randomized complete block design, containing two blocks, each block consisting of 9 contiguous pens facing each other in the same barn and under the same environmental conditions (Figure III.1). Heat was provided through gas-fired brooders; water was offered through nipple drinkers and feed through tube feeders *ad libitum*. The clean and disinfected concrete floor was covered with approximately 3 inches of clean softwood shavings and the bird density was approximately 0.75 square feet per bird, which is industry standard. Airflow was

provided by negative pressure with fans. All experimental procedures were performed according to the Canadian Council of Animal Care guidelines (CCAC 1993).

III.5.2 Feed composition

Starter, grower, and finisher diets that were formulated with wheat, barley and corn as the principal cereals and soybean and canola meals as protein concentrates to meet the National Research Council nutrient requirements (NRC 1994) for broiler chickens (Table III.2). The first experimental study consisted of: a control, and in parts per million (ppm) in diet: bambarmycin (2), penicillin (2.2), salinomycin (60), bacitracin (55) and salinomycin + bacitracin (60/55). The second experimental trial included in addition to the control: chlortetracycline (110), virginiamycin (11), virginiamycin (22), monensin (99) and narasin (70). The determination of dry matter, crude protein, soluble carbohydrates, volatiles fatty acids, and some of the most common minerals were performed at the Centre de Recherche en Sciences Animales de Deschambault (CRSAD) Deschambault, Quebec, Canada with standard laboratory analysis methods (Table III.2). Diets contained no antimicrobial other than those prescribed in the experimental design. Antimicrobials were included in the diet for each treatment group throughout the growing period, except for regulated withdrawal periods.

III.5.3 Data collection and animal performance

At day 0, 50 chicks were weighed in bulk and randomly assigned to each pen. Performance traits including body weight, weight gain, feed consumption and feed efficiency were evaluated weekly on day 7, 14, 21, 28 and 35. Weekly feed intake for the six treatment groups was estimated by calculating weekly feed consumed minus feed left over by pen for each treatment group. Feed efficiency is the feed intake for a given period divided by the total gain of bulk body weight including mortality weights for the same period.

Birds with kinky legs or obvious light weight were culled by cervical dislocation. Birds found dead and laying on their backs were considered dead by heart attack, called sudden death syndrome. All deaths were recorded on the mortality record sheet located in each pen. Necropsy on all birds was performed for further investigation.

III.5.4 Fecal sample collection

Ten chicks (day 0) and two birds per pen were killed by cervical dislocation at each sampling time (7, 14, 21, 28 and 35 day of age). Cecal contents and cloacal samples from the two sacrificed birds were aseptically collected and transferred to peptone buffer in test tubes and sterile “whirl-pack” plastic bags, respectively. The samples were placed onto ice and transported to the microbiology laboratory for bacteriological analysis that was carried out the same day. Sample weights were estimated by subtracting the weight of the container without sample from the weight with the samples.

III.5.5 Bacteriological analysis

For each feeding trial, a total of 90 cloacal and 90 cecal samples, in addition to 10 gut samples obtained from day old chicks, were analyzed for enumeration of *E. coli*.

E. coli counts were obtained using 3M *E. coli* and coliform petrifilms (St. Paul, Minn., USA), as previously described (Diarrassouba et al. 2007). After incubation for 24 h at 37°C, blue gas producing colonies were counted as *E. coli*. Results were expressed as colony forming units (cfu) per gram of material. At each sampling time, six blue colonies with gas from each treatment group (2 colonies/pen) were further purified on Sorbitol-MacConkey agar (Becton Dickinson) and stored at -80°C in 25% glycerol.

III.5.6 Antimicrobial susceptibility testing

Antibiotic minimal inhibitory concentration (MIC) of 360 *E. coli* isolates from cecal contents and cloacal samples was determined by Sensititre (Trek Diagnostic Systems) as previously described (Diarrassouba et al. 2007) according to the Clinical Laboratory Standard Institute's (CLSI) guidelines. *E. coli* ATCC 25922 was used as control (NCCLS 1999).

III.5.7 Statistics

Data on the performance and bacterial numbers were analyzed according to a randomized complete block design using the General Linear Model procedure of SAS and the pen as the experimental unit (three pens per treatment group) (SAS, 2000). Statistics for antimicrobial susceptibility testing were performed using the Pearson Chi-Square test and confirmed by both the Cochran and Mantel-Haenszel statistics of SPSS version 12.0. Significance level was fixed at $P < 0.05$.

III.6 Results

III.6.1 Broiler chicken performance

The cumulated effects of feed supplementation with test compounds in feeding trials one and two are presented in Tables III.3 and III.4, respectively. In both feeding trials, there was no significant difference between the treatment groups for body weight and feed intake ($P > 0.05$). In the first feeding trial, a slight increase in body weight was observed during week 4 in birds fed PEN, but this difference was not statistically significant ($P = 0.09$). Nevertheless, there was no difference between treatments groups including the control at the end of the feeding trial (Table III.3). Birds fed PEN consumed less feed to reach the same weight as those fed the other antimicrobials (Table III.3). Although this improvement of the feed efficiency was not significant ($P = 0.09$ for week 2 and $P = 0.06$ for week 4) at the end of the feeding trial, it became significant when the cumulated feed efficiency was considered ($P = 0.04$). The least improvement of feed efficiency was observed in birds fed the control, SAL+BAC and BBM, with a difference of 0.06 to 0.07 less than birds fed PEN. The inclusion of the combination SAL+BAC did not significantly improve bird performance compared to SAL and BAC taken alone ($P > 0.05$). No statistically significant difference was observed in mortality rates during the first feeding trial ($P > 0.05$).

During the second feeding trial, chickens consuming VIR11 tended to have increased body weight at weeks 3 and 4 (Table III.4). Contrary to the first feeding trial, this difference persisted until the end of the trial although no significant difference was

observed compared to the other treatment groups ($P > 0.05$). A significant improvement of the feed efficiency was observed but only at week 2 of the second feeding trial (Table III.4). Interestingly, birds fed the control and VIR11 converted feed more efficiently compared to the other treatment and control groups, with a difference varying from 0.06 (MON) to 0.14 (CHLORT) ($P = 0.04$). However, at the end of the second feeding trial, no significant difference was noticed between the treatment groups regarding feed efficiency ($P > 0.05$). VIR22 (22ppm) did not significantly improve bird performance compared to VIR11 (11ppm) ($P > 0.05$). During the first week of the second trial, the mortality rate was significantly higher in birds fed CHLOT than in the other treatment and control groups ($P = 0.02$). After this first week, no significant difference was noticed in the mortality rates during the remaining period of the feeding trial (Table III.4).

III.6.2 Bacteriological analysis

E. coli was not isolated from feed and litter samples collected before the two feeding trials. Chicks used in the two feeding trials arrived in our experimental barn free of *E. coli* (Figures III.2 and III.3).

Regardless of the antimicrobials used in the first feeding trial, *E. coli* numbers were statistically higher in the cecum than in the cloaca ($P < 0.001$). This effect was not attributable to any antibiotics used in the trial ($P > 0.05$). *E. coli* numbers in the cecum ranged from 7.5 to 10.5 \log_{10} CFU/g of sample while in cloacal samples, the number ranged from 5.2 to 7.9 \log_{10} CFU/g of sample (Figure III.2). It was only on day 21 that a

significant effect of AGP on *E. coli* numbers was noticed ($P = 0.04$). The lowest counts were observed in birds fed BAC and BBM ($7.5 \log_{10}$ CFU/g in the cecum) and the highest counts were found in birds fed PEN ($9.6 \log_{10}$ CFU/g in the cecum). There was no significant difference between *E. coli* numbers in birds fed SAL and BAC alone or in combination SAL+BAC ($P > 0.05$). After five weeks of rearing, *E. coli* populations stabilized between 8.2 and $9.1 \log_{10}$ CFU/g of fecal material (Figure III.2). At the end of the first feeding trial, no significant difference was noticed between the six treatment groups, including the control ($P > 0.05$).

In the second feeding trial, the highest numbers of *E. coli* ($10.3 \log_{10}$ CFU/g) were observed in the cecum of birds fed VIR22 and the control ($9.9 \log_{10}$ CFU/g) on day 7 (Figure III.3). *E. coli* numbers were significantly higher in the cecum than in the cloaca at the beginning of the feeding trial (days 7 and 14) and at the end of the trial (day 35), regardless of the AGP used ($P > 0.001$). On days 21 and 28, *E. coli* numbers were not significantly different in the cecum and cloaca for any treatment group ($P > 0.05$). After the five week rearing period, the lowest numbers of *E. coli* were in the cloaca of birds fed the control diet ($5.0 \log_{10}$ CFU/g) and the highest numbers were observed in the cecum of birds fed MO ($8.9 \log_{10}$ CFU/g) and VIR22 ($5.8 \log_{10}$ CFU/g).

III.6.3 Antimicrobial susceptibility testing

The susceptibility of *E. coli* to 18 antibiotics was performed to determine a potential association between the presence of AGP in feed and the emergence of antibiotic resistance in the targeted commensals. In each feeding trial, 180 *E. coli* isolates, 30 per treatment group, were tested. Regardless of the AGP used in both feeding trials, all the tested *E. coli* isolates were susceptible to neomycin and resistant to five antibiotics including penicillin, erythromycin, tylosin, clindamycin, novobiocin. Therefore, no association could be made between the resistance of *E. coli* to those antibiotics and the inclusion of AGP in the feed. The susceptibility level of *E. coli* isolates to the 12 remaining antibiotics varied according to the tested antibiotic and AGP used in feeds. Chloramphenicol resistant isolates were detected at high frequency (over 20% in the first feeding trial and almost 100% in the second feeding trial).

In the first feeding trial, at least 6 (20.0%), 1 (3.3%) and 3 (10.0%) *E. coli* in all treatment groups, including the control, were resistant to five, nine and 14 antibiotics, respectively (Figure III.4). Interestingly, one *E. coli* resistant to 15 antibiotics was isolated from birds fed the control and BAC while two were isolated from birds fed SAL (Figure III.4). An isolate multi-resistant to 16 antibiotics was found in the fecal material of birds fed SAL+BAC. In the second feeding trial, at least three (10.0%), one (3.3%) and one (3.3%) *E. coli* in all treatment groups, including the control, were resistant to five, 10 and 11 antibiotics, respectively (Figure III.5). Two *E. coli* isolates obtained from birds fed VIR22 were resistant to 12 antibiotics while one *E. coli* resistant to 15 antibiotics was

isolated from birds fed VIR11. One *E. coli* isolated from birds fed the non-medicated (control) diet was multi-resistant to 16 antibiotics (Figure III.5).

The 12 remaining antibiotics were divided into six classes, including β lactam (amoxicillin and ceftiofur), aminocyclitol (spectinomycin), aminoglycoside (streptomycin and gentamicin), tetracycline (oxytetracycline and tetracycline), quinolone (enrofloxacin and sarafloxacin) and sulfamide (sulfadimethoxime, sulfathiazole and trimethoprim/sulfadimethoxime). In the first feeding trial, *E. coli* isolates were resistant to amoxicillin, ceftiofur, oxytetracycline and tetracycline, independently of the AGP included in the diet (Table III.5). *E. coli* isolated from birds fed SAL seemed to be more resistant to spectinomycin, streptomycin, sulfadimethoxime and sulfathiazole than those isolated from birds included in the other medicated groups although no significant difference was observed between treatment groups ($P > 0.05$). Overall, in the first feeding trial, no significant difference was noticed between the *E. coli* isolates obtained from birds fed the control and those included in the medicated groups regarding antibiotics included in the 6 antibiotic classes ($P > 0.05$).

In the second feeding trial, *E. coli* isolated from birds fed virginiamycin had a higher resistance level to amoxicillin than *E. coli* isolated from birds included in the non-medicated group, but a significant difference was noticed only for isolates from birds fed VIR22 ($P < 0.05$, Table III.5). *E. coli* isolated from birds fed the control were significantly more resistant to spectinomycin and gentamicin than isolates obtained from birds fed CHLOT ($P < 0.05$, Table III.5). Surprisingly, *E. coli* isolated from birds fed the

control were significantly more resistant to tetracycline than those obtained from birds fed CHLOT, VIR11, MON and NAR ($P < 0.05$, Table III.5). The resistance level to tetracycline of isolates obtained from birds included in the control group was comparable to the resistance level of isolates from birds fed VIR22 (Table III.5). Although the resistance level to quinolone of *E. coli* isolated from birds included in the control group was higher than the other treatment groups, no significant difference was noticed ($P > 0.05$). However, the resistance level to sulfadimethoxime and sulfathiazole of isolates obtained from birds fed the control was significantly higher than those isolated from CHLOT ($P < 0.05$). The resistance level to sulfadimethoxime was also higher in the control group than the isolates obtained from birds fed MON ($P < 0.05$). Although the resistance of *E. coli* isolated from birds fed VIR11 seemed higher than those isolated from the control group, no significant difference was noticed ($P > 0.05$, Table III.5). Interestingly, in the second feeding trial, the resistance level to antibiotics of *E. coli* isolated from birds fed the different AGP was lower than isolates obtained from birds included in the control group, except for VIR22 where the resistance level was higher for a few antibiotics (Table III.5).

III.7. Discussion

Antimicrobial growth promoters (AGP), used at subtherapeutic dose throughout the entire growing period may select for antibiotic resistant strains that could be transmitted to humans (OTA 1995). As a precautionary measure, many countries decided to ban the use of AGP despite their recognized beneficial effects on animal performance (Dibner and Richards 2005). In North America, especially in Canada, AGP are still in use in food animal production. According to Boulianne, the ban of antibiotics in food animals will not solve the problem of antibiotic resistance and would have serious consequences for the economics of the food industry and animal welfare (Boulianne 2006). This study was initiated to evaluate the effects of routinely used AGP in Canadian broiler chicken production on growth performance and most importantly, to assess their impact on commensal *E. coli* and role in the emergence of resistance to antibiotics.

The effects of ten AGP on broiler chicken growth performance including the body weight, feed intake, feed efficiency and mortality rate were evaluated. Overall, AGP did not significantly improve birds' performance compared to the control. This result could be attributable to the highly sanitary conditions in which the birds were raised throughout the two feeding trials, as observed by Jukes and William (Jukes and William 1953). The results of this current study are also in accordance with the findings of Eyssen and De Somer who did not observe any improvement of growth and feed efficiency when broiler chickens fed virginiamycin (20ppm) were placed in disinfected rooms in new quarters (Eyssen and De Somer 1963). Therefore, the present study supports the assertion that

chicks kept in a new or clean environment may not benefit from dietary antibiotics (Coates et al. 1963). Promoting biosecurity measures and improving environmental quality noticeably decreases the incidence of pathogenic bacteria (Doyle 2001). As an example of the effectiveness of good hygiene, *Salmonella* were not detected in fecal and litter samples during the first feeding trial (data not shown). Therefore, *Salmonella* detection was not conducted during the second feeding trial, since both trials were carried out in the same environmental conditions. However, this study was performed on a smaller scale, thus using a small barn where biosafety measures and good management practice were easily carried out. This condition certainly contributed to the similarity between the control and the ten different treatments regarding the measured parameters. In commercial barns where hundred of thousands of birds are raised, good management practices and biosafety measures may be more difficult to achieve and monitor. For instance, many serotypes of *Salmonella* were isolated in commercial broiler chickens in an earlier study (Diarrassouba et al. 2007).

Presently, the effects of antimicrobial growth promoters on animal performance can not be clearly explained and their benefits to the gut microflora remain controversial (Doyle 2001). This may be due to the complex relationship between the gut microflora, the different metabolites excreted and the gut physiological composition and structure (Dibner and Richards 2005). A combination of numerous factors that contribute to maintain equilibrium in the gut allows promotion of growth and feed efficiency. The germ-free animal model could certainly help in solving the enigma based on the fact that the effect of AGP is not by promoting growth performance but rather by altering certain

bacterial species in the gut (Dibner and Richards 2005). Well designed experiments in addition to molecular biology and quantitative amplification techniques such as PCR-DGGE (denaturing gradient gel electrophoresis) and Real Time PCR will surely improve our understanding of the effects of AGP on complex microbial communities such as food animal gut microflora (Muyzer et al. 1993; Collier et al. 2003). This may contribute to open a clearer path for selecting the most beneficial microflora that will allow efficient growth at less cost for producers raising food animals and later for the consumer.

Controversies also exist around the fact that AGP may promote the profusion of harmful Gram negative bacteria by eliminating competitive Gram positive bacteria. In fact, most AGP target Gram positive bacteria that may cause disease in food animals and may be detrimental to the producers and food industry. The debate is usually focused on Gram negative bacteria such as *E. coli* 0157:H7 which cause life-threatening diseases in humans but are harmless for food animals (Karmali 1989). Nonpathogenic Gram negative bacteria mostly are excluded from monitoring systems. For this reason, there was interest in determining the effects of routinely used AGP on commensal *E. coli* isolated from fecal materials collected from healthy broiler chickens in the present study.

In the two feeding trials, *E. coli* was not isolated from any 0-day old chicks that were tested. The current study confirmed findings that newly hatched chicks may be free from any bacterial infection (Dibner and Richards 2005). This result also excludes any potential contamination source before chicks arrived at the barn, regarding the hatchery where the chicks came from and during their transportation to the barn. However, from

day 7 to 35, high numbers of *E. coli* were recovered from all birds tested, regardless of the treatment and control groups. During the second feeding trial, on day 21, *E. coli* numbers significantly dropped in the cecum contents of chickens fed the non-medicated diet and then were similar to the other diets containing AGP on days 28 and 35. Although the low count of *E. coli* on day 21 could be explained by sampling errors, other factors such as high water content or changes in the pH of the fecal sample may have contributed as well. In future studies, it would be advisable to measure the water content and pH of feces in the cecum. *E. coli* numbers, ranging from 5.0 to 10.5 log₁₀ CFU/g of fecal material, were higher than those observed by Shapiro and Sarles who found that normal population of *E. coli* in the cecal contents of healthy chickens was below 9.0 log₁₀ CFU/g of fecal material (Shapiro and Sarles 1949). However, similar to those authors, the present study revealed an unstable *E. coli* population resulting in significant differences within the weeks during the first four weeks of growth. This phenomenon is well described in the literature and evidence suggests that the normal gut population may stabilize after five weeks of growth (Lu et al. 2003). The current study did not go beyond this growing period; therefore it is not possible to provide any further discussion on this subject. Since the sampled litter (sampled before the chicks were placed inside the pens) and feed were also free from any *E. coli* contamination, also taking into account the good management practice, the current study could not really identify any contamination source.

Overall, there was no treatment effect on *E. coli* numbers obtained in the two feeding trials, which was not surprising since most AGP used in poultry production are

inefficient against Gram negative bacteria. The results of the current study are in accordance with the findings of Edrington and coworkers who demonstrated that ionophores such as monensin had no effect on Gram-negative bacteria such as pathogenic *E. coli* O157:H7 and *Salmonella* (Edrington et al. 2003). Although those authors used a ruminant model and carried out their experiments *in vitro*, evidence abounds to support the lack of effect of ionophores on Gram-negative bacteria isolated from the food animal gastrointestinal tract (Dealy and Moeller 1977; Losinger et al. 1997). However, during the current study *Enterococcus spp.* numbers were also elevated, reaching 9.5 log₁₀ CFU/g of fecal material in cecal contents (data not shown). Thus, more research is needed to provide a better knowledge on the spectrum of ionophores and their role in the emergence of foodborne pathogens.

It is well recognized that antibiotic use increases the emergence of antibiotic-resistant bacteria by creating a selective pressure that promotes the proliferation of both resistant and multi-resistant strains (OTA 1995; Committee on Drug Use in Food Animals Panel on Animal Health, Food Safety, and Public Health, 1998). Multi-resistant commensal *E. coli* were isolated from birds independently of the AGP used in the feed in the current study. Surprisingly, *E. coli* isolates obtained from birds included in the non-medicated (control) group were multi-resistant to 15 and 16 antibiotics in the first and second feeding trial, respectively. The chickens in our study did not receive any anticoccidial or antibacterial agents, other than those used in the experimental design. Care was taken to avoid contamination and clean pens and fresh wood shavings were used. The present study agrees with data reported by Smith et al. (2007) who showed a

high prevalence of resistance to antimicrobials that are not commonly used in broiler chicken production.

In the first feeding trial, the resistance level to antibiotics of *E. coli* isolated from birds included in the control was comparable to the resistance level of those isolated from the medicated groups. This study proves that normal intestinal microflora of broiler chickens can constitute an important reservoir for antimicrobial resistant bacteria that could be later transmitted to pathogenic bacteria cohabiting the birds' gastrointestinal tract. It is well known that genetic transfers of resistant genes can occur between bacteria species and from bacteria to humans (Poppe et al. 2005). Therefore antimicrobial resistance in commensal isolates should be considered a major concern.

Important efforts must be made to reinforce bio-safety measures and improve management practices at any level of the food production chain, as suggested by Doyle (Doyle 2001). As well, these measures may not address the issue of the increasing presence of antimicrobial resistance. In fact, despite compliance with good management practices, there was no significant difference in the antibiotic susceptibility level of *E. coli* isolated from birds fed the control diet compared to some medicated groups in the current study. This implies that even in the absence of any selection pressure of resistance, birds were colonized with resistant commensal isolates. This constitutes an important concern that merits more study. Thus, commensal isolates from healthy broiler chicken present an underlying threat to human health since most of the time, only strains

pathogenic to both the birds and humans are considered in the national surveillance systems and the food industry (Smith et al. 2007).

Although, the ban on AGP in European countries as a preventive measure did not reach all expectations and resulted in an increase in the veterinary antibiotic use (e.g. penicillins and macrolides), alternatives to antimicrobial use (e.g. probiotics, enzymes, acidifiers) should be considered to significantly reduce the use of antibiotics in animal husbandry (Aarestrup 1995; Bywater 2005; Dibner and Richards 2005). Mead and Doyle reviewed different alternatives currently available for antimicrobial use (Mead 2002; Doyle 2001). A combination of strict measures including a reduced pressure selection for antimicrobial resistance, the improvement of management practices and bio-safety complemented with an ongoing antimicrobial monitoring system should be implemented in any commercial barn and throughout the food system. This is an important condition for insuring the production of high quality food products. Advancement in molecular biology techniques with the development of accurate identification methods may considerably improve our understanding of complex microflora populating intestinal tracts of food producing animals and allow manipulation in favour of beneficial microflora. The spreading of resistant bacteria and emerging foodborne pathogens, encouraged by the globalization of the food and ingredients supplies and the international movement of populations and genetic evolution, may be preventable by a global collaboration between countries. Some authors predicted that we may be entering an era that resembles the pre-antibiotic period (OTA 1995). Without going that far, there is a

need for more studies, management changes, improvement of bio-security measures and the use of alternatives to AGP.

Table III.1. Antimicrobial compounds used in feed during the experimental feeding trials

Antimicrobials	Brand names	Dosage in feed (mg/kg feed)	Activity in complete diet (mg/kg feed)	Antimicrobial Class	Major Claims
Bambermycin	Flavomycin [®]	4	2	Glycolipid	Growth and feed efficiency
Procaine Penicillin	APA Pen-P 110 [®]	66.2	2.2	Beta-lactam	- Growth and feed efficiency - Various infections
Salinomycin	Coxistac [®]	60	60	Ionophore	Coccidiostat
Bacitracin Methylene Disalicylate	BMD 110 [®]	110	55	Polypeptide	- Growth and feed efficiency - Various infections
Chlortetracycline	Aureomycin 110 [®]	110	110	Tetracycline	- Growth and feed efficiency - Various infections
Virginiamycin	Stafac 44 [®]	44	11 & 22	Streptogramin	- Growth and feed efficiency (11mg/kg) - Various infections, necrotic enteritis (22 mg/kg)
Monensin	Coban [®]	200	99	Ionophore	Coccidiostat
Narasin	Monteban [®]	70	70	Ionophore	Coccidiostat

NB: Two concentrations of virginiamycin (11 and 22 mg/kg of feed) were used. Salinomycin (60 mg/kg of feed) and Bacitracin Methylene Disalicylate (55 mg/kg of feed) were used in combination in another diet bringing the total number of AGP in the study to ten.

Table III.2. Composition of the diet base used in the study

Ingredients	Inclusion (%)		
	Starter (d 0 to d 14)	Grower (day 15 - 28)	Finisher (d 29 – 35)
Wheat	34.96	35.03	40.79
Soya	23	0	0.51
Barley	10	0	0
Canola	9	22	18
Canola Oil	8.6	7	7
Corn	7	25	25
Corn Gluten	2.3	6	4
Limestone	1.6	1.3	1.2
Biofos	1.6	1.5	1.4
Lysine	0.4	0.71	0.63
Iodized Salt	0.3	0.32	0.32
DL-Methionine	0.18	0.09	0.1
Avizyme 1302	0.05	0.05	0.05
Nutrient ^z			
Dry Matter	89	88.7	88.7
Ash	6.41	5.74	5.74
Crude Protein	24.8	21.3	21.3
Fat	10.6	10.4	10.4
Glucose	19.3	17.3	18.6
Fructose	22.0	22.7	24.4
^y Ca	1.0	1.0	1.0
^y Mg	0.2	0.2	0.2
^y K	0.89	0.55	0.55
^y P	0.80	0.81	0.81
^y Na	0.22	0.26	0.26
^y Fe	0.04	0.04	0.04
^y Zn	0.02	0.02	0.02
^y Mn	0.02	0.02	0.02

^z On a dry matter basis obtained by standard laboratory analysis at CRESAD

^y Minerals: Ca, calcium; Mg, magnesium; K, potassium; P, phosphorus; Na, sodium; Fe, iron; Zn, zinc; Mn, manganese.

Table III.3. Birds performance observed in broiler chickens fed diets containing bambermycin, penicillin, salinomycin, bacitracin and salinomycin-bacitracin combination ^z

Trait / Week	Control	BBM	PEN	SAL	BAC	SAL+BAC	SEM ^x	P
Body weight (g/week)^y								
0	40.69	40.76	40.64	40.71	40.67	40.40	0.232	0.91
1	163.80	167.24	169.75	165.31	168.64	168.99	2.052	0.34
2	457.31	459.89	462.87	441.32	451.67	458.77	7.224	0.38
3	735.78	749.54	730.57	706.27	704.34	716.47	12.277	0.13
4	1252.02	1265.29	1267.10	1209.64	1224.63	1255.29	14.605	0.09
5	1815.09	1811.42	1805.12	1783.69	1776.75	1825.11	27.805	0.80
Feed intake (g)^y								
1	135.93	139.74	136.28	135.39	136.57	138.56	2.447	0.79
2	365.90	373.21	363.39	361.82	360.72	368.93	6.272	0.72
3	528.75	537.73	492.13	496.78	493.41	501.85	12.479	0.09
4	896.23	906.58	868.70	861.47	870.33	901.47	15.969	0.27
5	1288.22	1280.62	1223.70	1263.36	1264.54	1329.18	23.142	0.12
Total	3215.03	3237.87	3084.20	3118.83	3125.57	3239.98	47.916	0.14
Feed efficiency (gain/feed efficiency)^y								
2	1.21	1.22	1.17	1.24	1.21	1.20	0.014	0.09
4	1.78	1.77	1.69	1.76	1.76	1.77	0.018	0.06
5	2.29	2.35	2.28	2.17	2.30	2.34	0.060	0.37
Total	1.78	1.79	1.72	1.75	1.77	1.78	0.015	0.04 ^w
Mortality (%)^y								
1	0.0	0.0	0.0	0.0	0.7	0.0	0.277	0.45
2	0.0	1.4	0.7	0.7	0.7	0.7	0.636	0.78
3	1.5	1.5	0.7	0.0	0.8	0.7	0.845	0.82
4	1.6	3.1	2.3	6.1	2.3	3.0	1.738	0.52
5	2.5	6.7	3.4	6.0	3.2	0.0	1.804	0.1
Total	5.5	12.6	7.1	12.8	7.6	4.5	2.682	0.26

^z Bambermycin (BBM), penicillin (PEN), salinomycin (SAL), bacitracin (BAC) and salinomycin-bacitracin combination (SAL+BAC).

^y Values are the least square means of weekly cumulative effects of diets containing AGP.

^x SEM, Standard Error of the Means. n=3 pens per treatment, 50 birds per pen.

^w Values in this row are statistically different ($P < 0.05$)

Table III.4. Birds performance observed in broiler chickens fed diets containing chlortetracycline, virginiamycin 11 ppm, virginiamycin 22 ppm^w, monensin and Narasin^z

Trail / Week	Control	CHLOT	VIR11	VIR22	MON	NAR	SEM ^x	P
Body weight (g) ^y								
0	37.54	37.04	37.36	37.58	37.43	37.85	0.286	0.53
1	135.52	138.92	139.92	137.11	141.40	140.62	1.997	0.35
2	423.53	400.54	436.13	416.09	427.78	403.87	9.461	0.13
3	661.83	645.43	695.28	669.15	681.64	616.83	17.468	0.09
4	1054.10	1036.54	1119.50	1069.53	1092.32	979.49	29.230	0.07
5	1509.89	1459.17	1583.74	1510.83	1542.40	1409.02	39.371	0.10
Feed intake (g) ^y								
1	116.08	119.11	124.44	115.50	122.95	122.23	4.916	0.72
2	369.21	392.17	379.13	399.92	393.74	386.99	9.835	0.34
3	420.61	452.60	455.85	477.37	457.64	443.98	16.730	0.35
4	697.22	705.76	709.66	754.68	745.11	730.14	28.797	0.67
5	892.17	870.51	852.78	931.47	882.98	853.49	25.248	0.31
Total	2495.29	2540.15	2521.87	2678.93	2602.42	2536.83	67.876	0.47
Feed efficiency (gain/feed efficiency) ^y								
2	1.26	1.40	1.26	1.36	1.32	1.39	0.034	0.04 ^v
4	1.77	1.82	1.72	1.90	1.82	2.04	0.076	0.13
5	1.95	2.06	1.83	2.12	1.97	1.99	0.081	0.27
Total	1.67	1.77	1.62	1.80	1.71	1.83	0.053	0.10
Mortality (%) ^y								
1	0.0	1.4	0.0	0.0	0.0	0.0	0.3	0.02 ^v
2	0.0	0.0	0.0	1.4	0.7	0.0	0.4	0.13
3	5.8	0.7	2.2	1.5	2.2	1.4	1.9	0.78
4	1.6	3.1	3.9	2.3	2.4	3.1	1.8	0.99
5	0.0	1.8	0.8	0.0	0.0	0.9	0.6	0.3
Total	7.4	6.9	6.9	5.1	5.3	5.4	3.1	0.22

^z Chlortetracycline (CHLOT), virginiamycin 11 ppm (VIR11), virginiamycin 22 ppm (VIR22), monensin (MON) and narasin (NAR).

^y Values are the least square means of weekly cumulative effects of diets containing AGP.

^x SEM, Standard Error of the Means. n=3 pens per treatment, 50 birds per pen.

^w ppm, parts per million. These two different dosages of virginiamycin, used in broiler chicken feed formulation in Canada, were selected for the second experimental study.

^v Values in this row are statistically different ($P < 0.05$)

Table III.5. Resistance levels (%) of *E. coli* isolates obtained from fecal material from healthy broiler chickens ^z

Antibiotics ^y	Treatments ^x													
	First feeding trial							Second feeding trial						
	C	BM	PN	SA	BA	SABA	C	CHLOT	VIR11	VIR22	MON	NAR		
Amo	63.3	56.7	60.0	76.7	56.7	50.0	63.3	50.0	66.7	86.7 ^w	40.0	46.7		
Cef	50.0	43.3	46.7	73.3	46.7	50.0	33.3	23.3	40.0	46.7	23.3	26.7		
Spe	36.7	23.3	30.0	53.3	43.3	23.3	16.7	3.3 ^w	16.7	10.0	10.0	6.7		
Str	46.7	50.0	46.7	63.3	50.0	30.0	30.0	10.0	30.0	40.0	23.3	23.3		
Gen	23.3	20.0	16.7	40.0	36.7	16.7	13.3	0.0 ^w	6.7	3.3	6.7	6.7		
Oxy	70.0	56.7	60.0	76.7	70.0	66.7	83.3	40.0 ^w	33.3 ^w	66.7	56.7 ^w	56.7 ^w		
Tet	70.0	56.7	60.0	76.7	70.0	66.7	86.7	40.0 ^w	33.3 ^w	66.7	53.3 ^w	56.7 ^w		
Enro	0.0	3.3	6.7	0.0	0.0	0.0	3.3	0.0	3.3	0.0	0.0	0.0		
Sara	6.7	10.0	10.0	10.0	6.7	10.0	6.7	0.0	3.3	0.0	0.0	0.0		
Slx	43.3	36.7	40.0	60.0	43.3	30.0	36.7	13.3 ^w	33.3	40.0	13.3	20.0		
Slz	43.3	36.7	40.0	60.0	43.3	23.3	16.7	0.0 ^w	16.7	13.3	6.7	10.0		
Tr/Slx	6.7	6.7	0.0	6.7	3.3	0.0	0.0	0.0	10.0	6.7	0.0	0.0		
Chloramp	24.2	36.3	36.3	39.3	33.3	31.2	100	96.6	100	100	100	100		

^z In total 180 *E. coli* isolates were tested for antibiotic susceptibility testing with a battery of 18 antibiotics, 90 from the ceca and 90 from the cloaca. For each treatment group, 30 (6 / week) isolates each week during 5 weeks.

^y Amoxicillin (Amo), ceftiofur (Cef), spectinomycin (Spe), streptomycin (Str), gentamicin (Gen), oxytetracycline (Oxy), tetracycline (Tet), enrofloxacin (Enro), sarafloxacin (Sara), sulfadimethoxime (Slx), sulfathiazole (Slz) trimethoprim/sulfadimethoxazole (Tr/Slx) and chloramphenicol (Chloramp).

Note: 0% resistance to neomycin and 100% resistance to penicillin, erythromycin, tylosin, clindamycin, novobiocin: data not shown.

^x Bambermycin (BBM), penicillin (PEN), salinomycin (SAL), bacitracin (BAC) and salinomycin-bacitracin combination (SAL+BAC), chlortetracycline (CHLOT), virginiamycin 11 ppm (VIR11), virginiamycin 22 ppm (VIR22), monensin (MON) and narasin (NAR).

^w Pearson Chi-Square test was performed and confirmed by both the Cochran and Mantel-Haenszel statistics. Values obtained for each treatment were compared to the control (non-medicated). Values with same letter (^w) are significantly different from the control. Significance level was fixed at $P < 0.05$.

Pen 1	Pen 3	Pen 5	Pen 7	Pen 9	Pen 11	Pen 13	Pen 15	Pen 17
T2	T4	T2	T5	T3	T5	T6	T3	T1



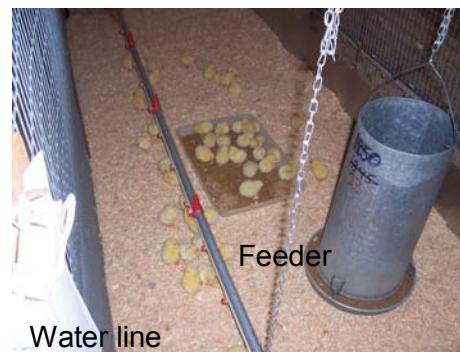
T1	T4	T3	T6	T1	T6	T4	T5	T2
Pen 2	Pen 4	Pen 6	Pen 8	Pen 10	Pen 12	Pen 14	Pen 16	Pen 18



Partition and preparation of pens (18): 9'x16'x30"
 Flore: three inches (7.6 cm) of clean softwood shavings



Double counting of male chicks
 Vaccinated: Mareck & bronchitis



50 chicks/pen
 Density: approximately 0.75 square feet (0.07 m²/bird)

Figure III.1. Configuration of the barn used in the experimental feeding trial

The barn was divided in two blocks, each one with 9 contiguous pens (Pen 1 to 18). Diets supplemented with antimicrobials were randomly distributed. The arrow represents the entrance of the barn while the coded treatments are T1, T2, T3, T4, T5 and T6.

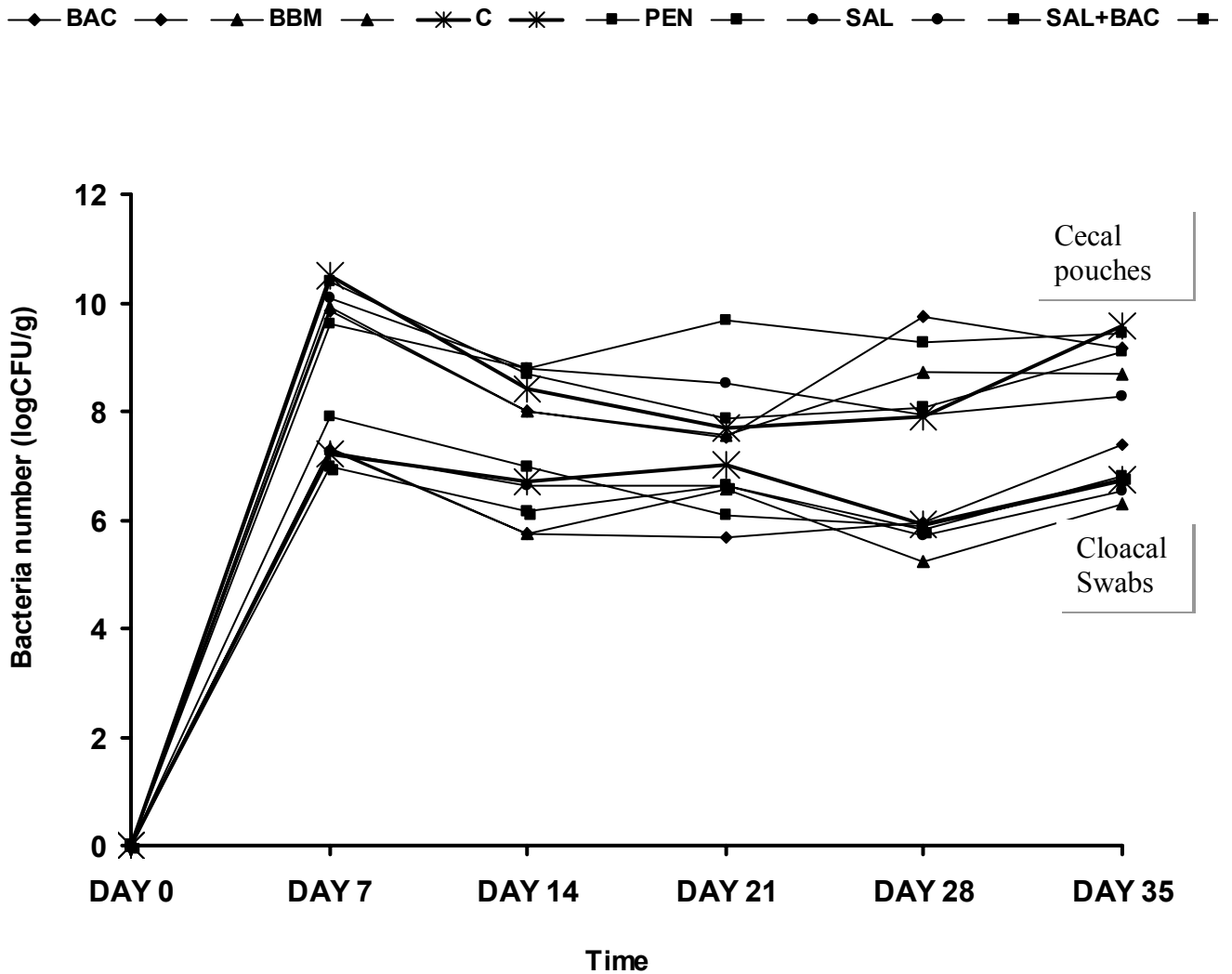


Figure III.2. *E. coli* populations in log₁₀ CFU/g from the cecal pouches and cloacal swabs of broiler chickens used for the first feeding trials.

Antimicrobials growth promoters added in feed included bambarmycin (BBM), penicillin (PEN), salinomycin (SAL), bacitracin (BAC) and salinomycin-bacitracin combination (SAL+BAC). Birds included in the control (C) group were fed diet with no antimicrobial supplementation. *E. coli* numbers were statistically higher in the cecum than in the cloaca, whatever the antibacterial used ($P < 0.001$). There was no *E. coli* in the 0-day old chicks at the beginning of the feeding trial. *E. coli* numbers in the cecum ranged from 7.5 to 10.5 log₁₀ CFU/g of fecal material while in the cloacal swabs, the number ranged from 5.25 to 7.9 log₁₀ CFU/g of fecal material. There was no significant difference between *E. coli* number in birds fed SAL and BAC alone and the combination SAL+BAC ($P > 0.05$).

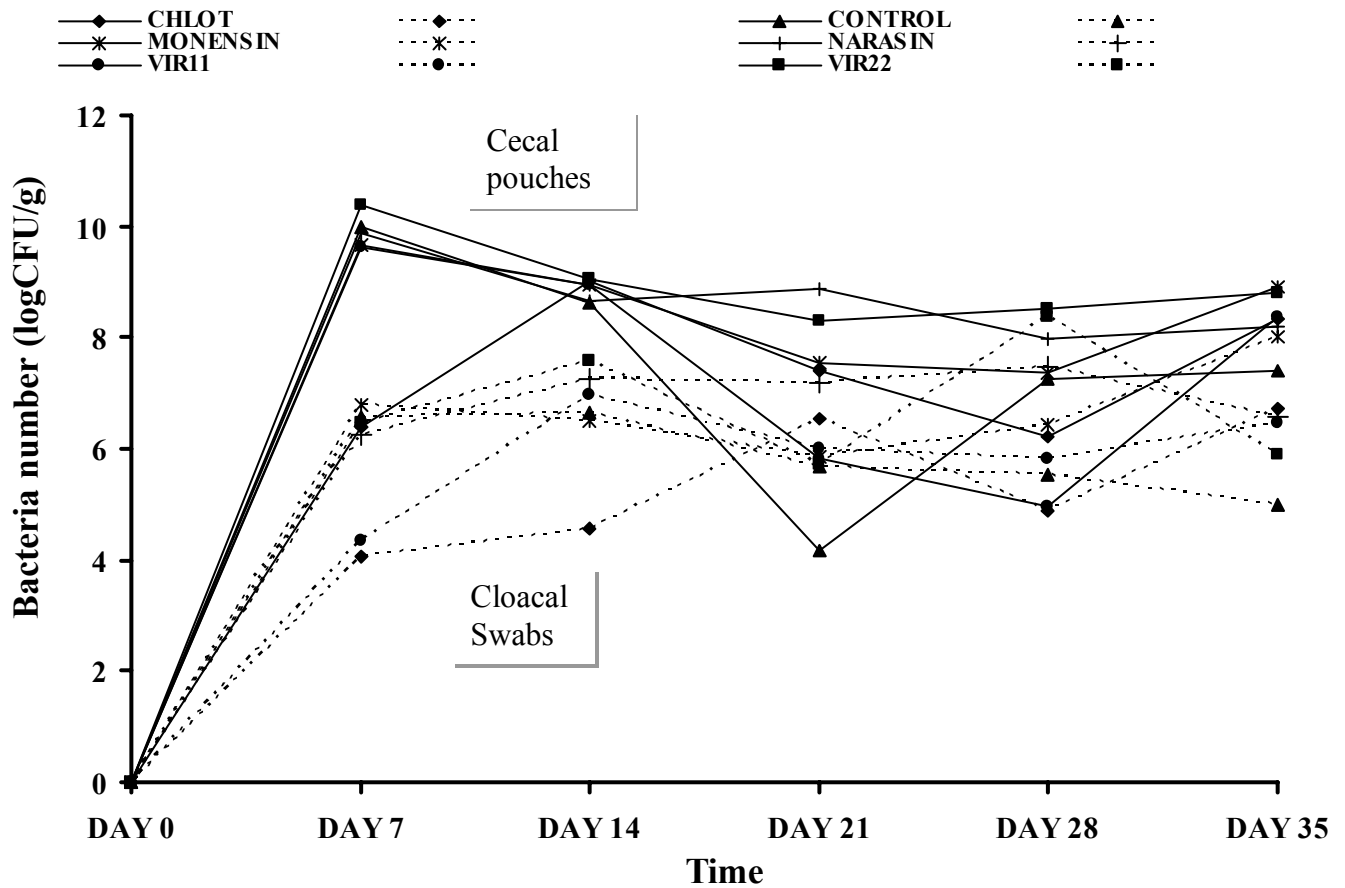


Figure III.3. *E. coli* populations in log₁₀ CFU/g from the cecal pouches and cloacal swabs of broiler chickens used for the second feeding trials.

Antimicrobial growth promoters added in feed included chlortetracycline (CHLOT), virginiamycin 11 ppm (VIR11), virginiamycin 22 ppm (VIR22), monensin (MON) and narasin (NAR). Birds included in the control (C) group were fed diet with no antimicrobial supplementation. On days 7, 14 and 35, *E. coli* numbers were significantly higher in the cecum than in the cloaca regardless of the antimicrobial used ($P < 0.001$) although this difference was not due to the effect of AGP ($P > 0.05$). No significant difference was noticed on days 21 and 28 ($P > 0.05$).

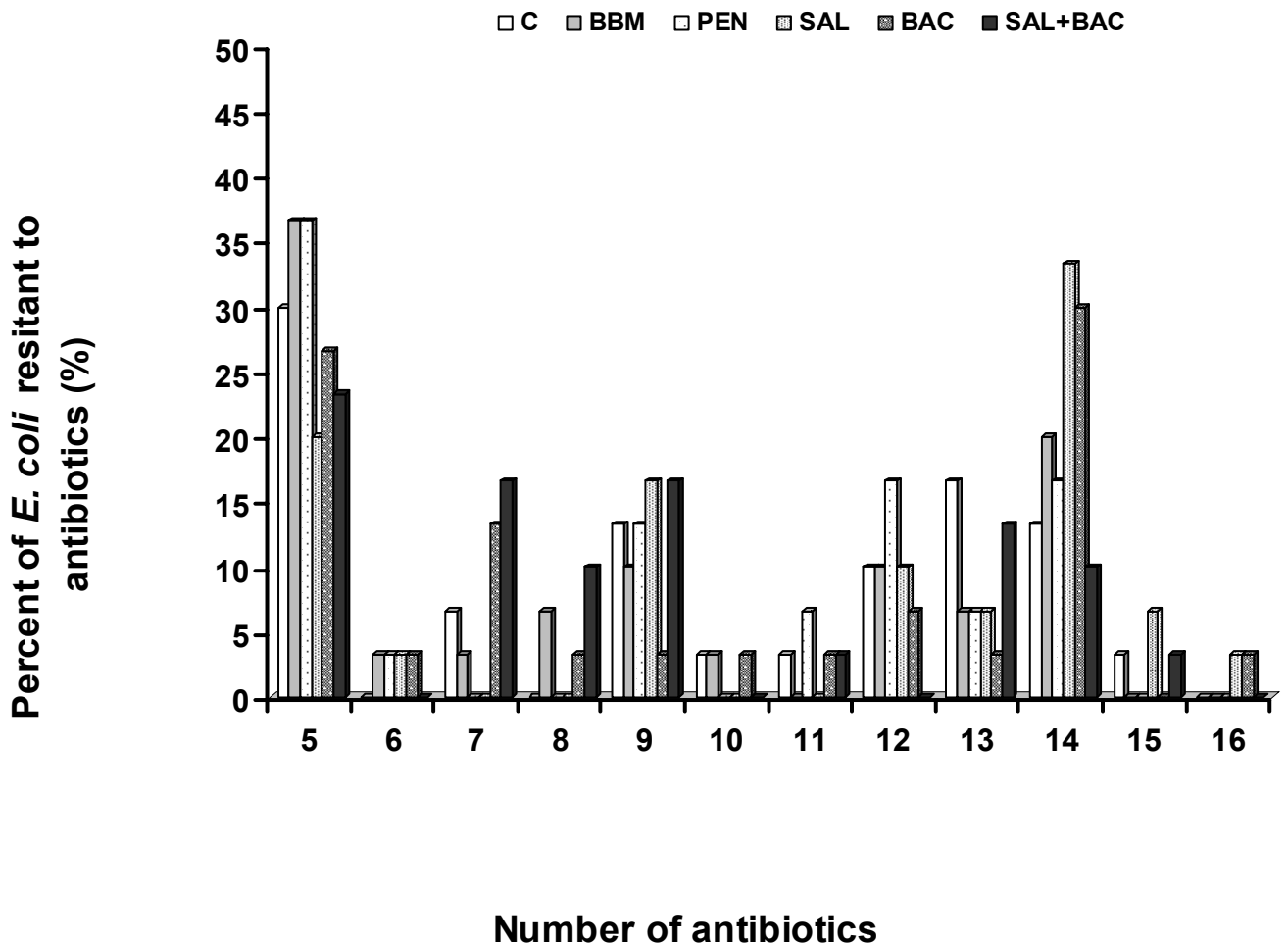


Figure III.4. Percent of *E. coli* multiple antibiotic resistant to 5 and more antibiotics in first feeding trial.

E. coli was isolated from fecal material of broiler chickens fed a control (non-medicated), bambermycin (BBM), penicillin (PEN), salinomycin (SAL), bacitracin (BAC) and salinomycin-bacitracin combination (SAL+BAC). The antimicrobial susceptibility test of 30 *E. coli* per treatment group to 18 antibiotics was determined. At least 6 (20.0%), 1 (3.3%) and 3 (10.0%) *E. coli* in all treatment groups, including the control, were resistant to 5, 9 and 14 antibiotics, respectively. One *E. coli* multi-resistant to 15 antibiotics was isolated from birds fed the control diet and BAC diet while two were isolated from birds fed SAL. One *E. coli* (3.3%) from the fecal materials of birds fed SAL and BAC was multi-resistant to 16 antibiotics.

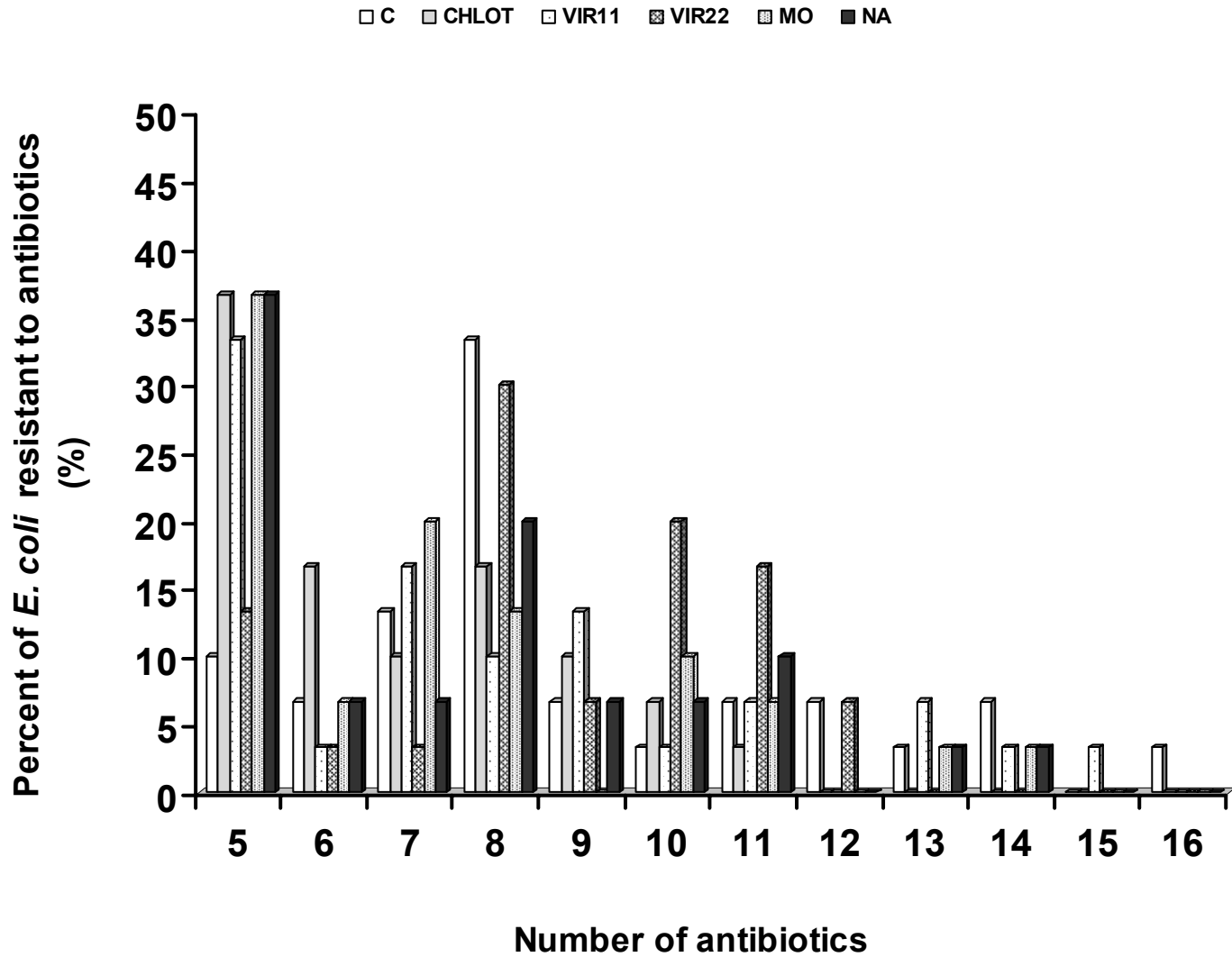


Figure III.5. Percent of *E. coli* multiple antibiotic resistant to 5 and more antibiotics in second feeding trial.

E. coli was isolated from fecal material of broiler chickens fed a control (non-medicated), chlortetracycline (CHLOT), virginiamycin 11 ppm (VIR11), virginiamycin 22 ppm (VIR22), monensin (MON) and narasin (NAR). The antimicrobial susceptibility test of 30 *E. coli* per treatment group to 18 antibiotics was determined. At least 3 (10.0%), 1 (3.3%) and 1 (3.3%) *E. coli* in all treatment groups, including the control, were resistant to 5, 10 and 11 antibiotics, respectively. Two *E. coli* isolates obtained from birds fed VIR22 were resistant to 12 antibiotics while one *E. coli* resistant to 15 antibiotics was isolated from birds fed VIR11. One *E. coli* isolated from birds fed the non-medicated (control) diet was multi-resistant to 16 antibiotics.

Chapter IV. Conclusions and Recommendations

IV.1 Conclusion

The spread of pathogenic and resistant bacteria in chickens and rearing environments causes significant economic losses to the chicken industry. These resistant disease-causing bacteria could further contaminate chicken carcasses at the slaughter house, reach the retail market and cause millions of deaths globally each year. The Canadian Integrated Program of Antimicrobial Resistance Surveillance (CIPARS) was initiated in 2002 to provide antimicrobial resistance trends among some enteric organisms from humans, animals and animal-derived food sources across Canada. This program includes pathogens such as *Salmonella*, which are developing resistance to cephalosporins such as ceftiofur, belonging to the category 1 of very important antibiotics for human health. On the other hand, evidence suggests that commensal *E. coli* which are part of the normal microflora of chicken gut may harbour antibiotic resistance genetic determinants that might be transferred to pathogenic bacteria such as *Salmonella*. They could also receive virulence genes from other pathogens and thus, become a threat for both chicken and human health.

Education of the public, producers, processors and food service facilities employees, in addition to efficient quality assurance and control systems in conjunction with accurate detection methods, are extremely important in controlling and reducing the

emergence of antibiotic resistant pathogens. Most importantly, consumers would be more confident to buy safe and high quality chicken products. To reach this objective there is an increasing need for more reliable data for evaluating the current situation of the impact of the use of antimicrobials in broiler chicken production on the emergence of antibiotic resistant bacteria. This thesis aimed at studying the effects of antibiotics used as growth promoters on commensal *E. coli* and *Salmonella* isolated from two different environmental sources: commercial broiler chicken farms and a broiler barn under controlled environmental conditions.

At the end of the study, it was observed that multi-antibiotic resistant commensal *E. coli* are present in healthy broiler chickens raised in the Fraser Valley of B.C. Furthermore, multi-antibiotic resistant commensal *E. coli* were found in broiler chicken intestines independently of any antibiotic growth promoter utilization. Furthermore, chloramphenicol resistant *E. coli* were detected during the experimental feeding trials although this antibiotic has not been used in chicken production since the 1980s. Therefore, this study suggests that commensal *E. coli* may constitute an important reservoir for antimicrobial resistance genetic determinants in the intestinal tracts of commercial broiler chickens and contribute to the dissemination of antibiotic resistance genetic determinants in the environment and the food production chain.

The detection of commensal *E. coli* harboring virulence genes such as *aea*, encoding for intimin commonly found in the pathogenic strain *E. coli* 0157:H7 is of particular interest. Intimin allows pathogenic bacteria to attach to the human intestines, an

important step for the gastrointestinal tract colonization and disease generation. Furthermore, collibaciliosis determining virulence genes were detected in healthy commercial broiler chickens. This finding represents a serious alert for producers in the Fraser Valley of British Columbia, because collibaciliosis can be responsible of carcass condemnation and important economic losses.

The present study confirms the presence of sub-clinical *Salmonella* infection in chickens raised on commercial farms in the Fraser Valley of British Columbia. *Salmonella* infections in chickens are an important contributor to human salmonellosis, counting among the most frequent foodborne diseases. The findings of this study indicate that important effort must be deployed to improve control strategies aiming at eradicating *Salmonella* from broiler chicken husbandries. The current study also detected the presence of multi-antibiotic resistant *Salmonella* harboring *bla*_{CMY-2}, resistance gene to extended-spectrum cephalosporins which are used as first line treatment of human salmonellosis and are of high importance for human health. Most importantly, virulence genes *inv*, that enables *Salmonella* to colonize the intestinal tissues of the host, and *spv*, that is significantly associated with extraintestinal and severe infections in humans, were detected in some resistant serovars such as Typhimurium and Enteritidis. The findings of this study indicate that antibiotic resistant *Salmonella* carrying virulence genes can be isolated from healthy commercial broiler chickens fecal samples and litter, suggesting the potential to contaminate the environment including crops and runoff water, if litter is applied to soil as fertilizer, and to enter the slaughter house on infected chicken carcasses.

The present study suggests that horizontal transfer of mobile genetic elements such as AmpC plasmid encoding for resistance to extended-spectrum cephalosporins and integron class 1 may occur between commensal *E. coli* and *Salmonella*. Therefore, commensal bacteria may develop pathogenic characteristics and thus become a serious health concern and an important economic burden. Each year, millions of illnesses and thousands of deaths are caused by foodborne diseases and the costs in terms of pain, suffering, reduced productivity and medical costs are estimated to range from millions to several tens of billions.

The present study found a significant difference between the feed companies regarding the presence of tetracycline resistance gene *tet(B)* which was significantly more prevalent in *E. coli* isolates from commercial farms supplied by the feed company using salinomycin in the chicken finisher diet. On the other hand, antimicrobial growth promoters used individually, did not significantly improve broiler chickens performance compared to the non-medicated diet. Therefore, the current study suggests that maintaining proper bio-safety procedures on the farm and inside the barn, in addition to implementing appropriate manure management measures are key factors in minimizing flock contamination and mortality rate. Consequently, this will significantly reduce contamination at the slaughter house and throughout the food chain to the retail market, which represents a reduced risk for consumer health.

Producing and consuming high quality and safe foods constitutes an important challenge. It should be viewed by all parties as an integrated and continued fight against

invisible but life threatening enemies that must absolutely be defeated. Country-wide and provincial monitoring systems are the leading institutions, but any single individual should participate in the reduction and eventually the elimination of harmful bacteria from the food supply. Some authors predicted that we may be entering an era similar to the pre-antibiotic period. Although this point of view seems pessimistic, evidence suggests antibiotics are definitively less effective than 60 years ago

V.2 Recommendations for further research

More studies need to be carried out to identify the source of resistant commensal *E. coli* and risk factors for foodborne pathogens infection in broiler chickens. Furthermore, research should focus on finding alternatives to antibiotic growth promoters that do not confer antimicrobial resistance to intestinal bacteria. Probiotic bacteria such as *Lactobacillus* and *Bifidobacteria* in addition to the concept of competitive exclusion, used to reduce sub-clinical infections are examples of alternatives to antibiotics. Enzymes such as phytase (improves digestion of phosphorus in vegetable matters) and carbohydrate-degrading enzymes (amylase, glucanase, glucoamylase) were proven to improve feed conversion in food producing animals.

Intrinsic factors such as the pH and the water content of fecal samples should be carefully monitored during bacteria enumeration in fecal materials from food producing animals. These parameters may affect commensal bacteria such as *E. coli* counts and

therefore bias the evaluation of carcass microbiological quality, since *E. coli* are often used as indicator bacteria.

Bio-safety procedures should be reinforced and management practices improved throughout the food production chain, starting at the farm level, in addition to carefully evaluating the economic viability of these measures.

Global collaboration between countries should be encouraged in order to reduce the spread of foodborne diseases and to trace back food pathogens since globalization of food supplies and international movement of people facilitate cross-border dissemination of food pathogens.

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Co-Authorship Statements

A version of the work presented in Chapter II of this thesis was published in “*Journal of Food Protection*” (2007), **70**(6):1316–1327, entitled “Antibiotic Resistance and Virulence Genes in Commensal *Escherichia coli* and *Salmonella* Isolates from Commercial Broiler Chicken Farms”. Fatoumata Diarrassouba, the thesis author, was the principal author and Dr Moussa Sory Diarra, Fatoumata Diarrassouba’s co-supervisor, Dr Susan Bach, Dr Pascal Delaquis (member of Fatoumata Diarrassouba supervisory committee), Dr Jane Pritchard, Dr Edward Topp, and Dr Brent J. Skura (Fatoumata Diarrassouba’s co-supervisor) were the co-authors.

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The six posters, cited below, were presented at diverse conferences. Fatoumata Diarrassouba, the thesis author, was the principal author for two of them and co-author in the others.

1. Diarra, M. S., **F. Diarrassouba**, H. Rempel, R. Brousseau, L. Masson, C. Bonnet, J. Pritchard, P. Delaquis, S. Bach, E. Topp and B. Skura. 2008. DNA-microarray analyses of antimicrobial resistance and virulence genes of *Escherichia coli* O7 isolates from healthy broiler chickens. Joint 48th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) of American Society for Microbiology / 46th Annual Meeting of the Infectious Diseases Society of America (IDSA), October 25-28, Washington DC, USA.
2. Diarra, M. S., H. Rempel, **F. Diarrassouba**, T. Forge, T. Kannangara, S. Bittman, P. Delaquis, S. Bach, E. Topp, J. Pritchard, K. Stanich, M. Jaeckel. 2008. Persistence of *Escherichia coli* in soil fertilised with chicken manure. 108th General Meeting of American Society for Microbiology, June 1-5, Boston, Massachusetts.
3. Diarra M. S., **Diarrassouba F.**, S. Garcia, J. Pritchard, B. Lefebvre and F. Malouin. 2006. Hypermutability, antibiotic resistance and virulence genes in commensal broiler chicken *Escherichia coli* isolates. Abstract book p 135. 46th ICAAC of American Society for Microbiology, September 27 -30, San Francisco, CA, USA.

4. Diarra, M. S., **F. Diarrassouba**, W. Luk, J. Pritchard, H. Rempel, R. Yu, C. Harlton, S. Bach, P. Delaquis, and E Topp. 2006. Multiple antibiotic resistances of *Escherichia coli* isolated from commercial broiler chicken farms. 93rd Annual Meeting of International Association for Food Protection, August 13-16, Calgary, Alberta, Canada

5. **Diarrassouba F.**, H. Rempel, J. Takizawa, G. Leusink, J. Pritchard, P. Delaquis, S. Bach, E. Topp and M. S. Diarra. 2005. Antibiotic susceptibility profiles of *Salmonella* and *Enterococcus* from broiler chicken farms. The 2005 Federal Food Safety and Nutrition Research Meeting. “Protecting the Canadian Food Supply” October 19 - 21, Ottawa ON Canada.

6. **Diarrassouba F.**, W. Luk, J. Pritchard, H. Rempel, S. Bach, P. Delaquis, E. Topp and M. S. Diarra. 2005. Antibiotic resistance and virulence genes of *Escherichia coli* from broiler chicken farms. The 2005 Federal Food Safety and Nutrition Research Meeting: “Protecting the Canadian Food Supply” October 19 - 21, Ottawa ON Canada.