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Strategies to reduce the use of antibiotics in commercial broiler chickens
Impacts on growth performance, intestinal health and microbiota

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Résumé

Il y a actuellement une pression mondiale pour revoir les pratiques d'utilisation des antimicrobiens (UAM) en production animale afin de limiter la propagation de bactéries résistantes aux antibiotiques. Conséquemment, les Producteurs de Poulet du Canada examinent la possibilité de réduire leur UAM en supprimant les antibiotiques médicalement importants en médecine humaine (AIM) des programmes préventifs avec la mise en place de leur stratégie de réduction de l'UAM. Cependant, les informations sont rares sur les conséquences de telles approches dans un contexte canadien. L'objectif de cette thèse était d'étudier les impacts sur les performances zootechniques, le contrôle des maladies intestinales et le microbiote cécal de deux stratégies de réduction de l'UAM dans des troupeaux commerciaux de poulets de chair par rapport à une UAM conventionnelle. Sur sept fermes commerciales de poulets de chair, un poulailler a été attribué aux traitements de réduction des antibiotiques pour six troupeaux consécutifs, tandis qu'un poulailler similaire sur le même site a été alloué à l'UAM conventionnelle (CONV) pour six troupeaux consécutifs ($n = 84$). Les stratégies de réduction des antibiotiques consistaient en l'utilisation continue d'ionophores dans l'alimentation sans (TX1) ou avec de l'acide butyrique (TX2). Aucune différence statistique ($p > 0.05$) n'a été notée entre TX1, TX2 et CONV sur les performances zootechniques et la santé intestinale. Les comptes d'oocystes d'*Eimeria* spp. étaient significativement ($p < 0.05$) inférieurs entre 22 et 34 jours d'âge dans les troupeaux CONV comparé aux TX1 et TX2. Le type de programme antibiotique a eu un impact relativement mineur (valeur R = 0.039), mais statistiquement significatif ($p =$ 0.002), sur le microbiote cécal, tandis que les facteurs environnementaux ont montré les corrélations significatives ($p = 0.001$) les plus fortes avec le microbiote. Parmi les composantes du microbiote cécal associées à la croissance, le gain quotidien moyen (GMQ) était significativement associé à la Richesse bactérienne ($p < 0.05$). L'abondance relative de la famille bactérienne *Lachnospiraceae* fut la mesure la plus fortement corrélée à un GMQ augmenté, tandis que l'abondance relative de nombreuses familles bactériennes, incluant les *Porphyromonadaceae*, les *Planococcaceae* et les *Veillonellaceae*, fut corrélée à un faible GMQ. Ces taxons défavorables formaient un vaste réseau de corrélations positives entre elles, et négativement corrélées aux *Lachnospiraceae*. En conclusion, ces travaux ont contribué à améliorer la résilience de l'industrie avicole en fournissant des

stratégies alternatives aux AIM pour prévenir les maladies intestinales. Des connaissances importantes sur le microbiote cécal des poulets de chair furent générées et pourront considérablement influencer les directions futures de la manipulation du microbiote pour favoriser la croissance. Par exemple, un paradigme important a été remis en question en illustrant que les additifs médicamenteux dans l'alimentation n'influencent que marginalement le microbiote cécal et que ce sont plutôt des facteurs environnementaux qui sont fortement impliqués dans la formation des communautés bactériennes cécales. La clé pour développer un microbiote cécal idéal chez les poulets de chair pourrait résider dans la capacité d'influencer ces facteurs, plus particulièrement l'exposition précoce à des communautés bactériennes bénéfiques et le contrôle de la flore résidente spécifique à la ferme.

Mots-clés : poulet, antibiotique, santé intestinale, performance, microbiote, 16S rRNA, coccidiose, prévention de maladie, système gastro-intestinal, production animale.

Abstract

There is a global pressure to review current antimicrobial use (AMU) practices in animal production and limit large-scale propagation of antibiotic resistant microorganisms. Consequently, the Chicken Farmers of Canada are examining the possibility to responsibly reduce AMU by discontinuing medically important antibiotics (MIAs) for humans from disease prevention programs of broiler chicken flocks through the implementation of their *Antimicrobial Use Reduction Strategy*. However, information is sparse on the consequences of such approaches in a Canadian commercial poultry production context. The general objective of this thesis was to investigate the impacts of two strategies reducing AMU in commercial broiler chicken flocks on zootechnical performance, control of intestinal diseases and the cecal microbiota compared to conventional AMU. On seven commercial broiler chicken farms, a house was allocated to the antibiotic reduction treatments for six consecutive flocks, while a similar house on the same premises was assigned to the conventional AMU (CONV) for six consecutive flocks ($n = 84$). The antibiotic reduction strategies consisted of continuous in-feed ionophores without (TX1) or with butyric acid (TX2). There were no statistical differences ($p > 0.05$) between TX1, TX2 and CONV for zootechnical performance and intestinal health. Predicted *Eimeria* spp. oocysts were significantly lower ($p < 0.05$) between 22 to 34 days of age in CONV flocks compared to TX1 and TX2. The type of antibiotic program had a relatively minor impact (R-value = 0.039), but statistically significant ($p = 0.002$), on the cecal microbiota composition, while environmental factors such as the farm and flock cycle showed the strongest statistically significant ($p = 0.001$) correlations with the microbiota composition (R-values of 0.239 and 0.374, respectively). Amongst the cecal microbiota components associated with weight gain, the average daily gain (ADG) was significantly associated with bacterial Richness ($p < 0.05$). The relative abundance of the bacterial family *Lachnospiraceae* was the most important measure correlated with ADG, while the relative abundance of numerous bacterial families, including *Porphyromonadaceae, Planococcaceae* and *Veillonellaceae,* were correlated with decreased growth rate. These unfavourable taxa formed a large network of positive correlations, indicating potential co-occurring synergies between these undesirable taxa. This network was also negatively correlated to *Lachnospiraceae*. In conclusion, the findings in this work contributed to

improve the sustainability of the modern poultry industry by providing feasible alternatives to the practice of using MIAs for the prevention of intestinal diseases in broiler chickens. This project also generated important knowledge on the cecal microbiota of broiler chickens that could considerably influence future directions of microbiota manipulation in a perspective of improving zootechnical performance. For instance, an important paradigm was challenged by the indication that in-feed antibiotics and prebiotics may only influence marginally the microbiota during grow-out. Rather, this work suggests environmental factors are strongly involved in shaping the bacterial communities residing in the ceca of broiler chickens. Hence, the key to successfully develop an ideal cecal microbiota in broiler chickens may reside in the ability to influence such factors, more specifically the early exposure to beneficial bacterial communities and the control of farm-specific resident flora.

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List of Acronyms and Abbreviations

ABF	Antibiotic-free
AGP	Antibiotic growth promoter
AIM	Antibiotique médicalement important en médecine humaine
AMR	Antimicrobial resistance
AMU	Antimicrobial use
ARG	Antimicrobial resistance gene
ASV	Amplicon sequence variant
BWG	Body weight gain
CARD	Comprehensive Antibiotic Resistance Database
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
CPH	*Clostridium perfringens*-related hepatitis
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
dPCR	Digital PCR
PCR	Polymerase chain reaction
EU	European Union
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
FB	Fumonisin
FCR	Feed conversion ratio

GIT	Gastro-intestinal tract
GRE	Glycopeptide-resistant *Enterococcus faecium*
H&E	Hematoxylin and eosin
HIV	Human immunodeficiency virus
IBD	Infectious bursal disease
ITS-1	Internal transcribed spacer 1
LA-MRSA	Livestock-Associated Methicillin Resistant *Staphylococcus aureus*
LEfSe	Linear discriminant analysis Effect Size
LPS	Lipopolysaccharide
MD	Marek's disease
MHC	Major histocompatibility complex
MGE	Mobile genetic element
MIA	Medically important antibiotic
MLS	Macrolides-lincosamides-streptogramins-pleuromutilins
MOS	Manno-oligosaccharides
NAE	No antibiotic ever
NGS	Next-generation sequencing
NE	Necrotic enteritis
NMDS	Nonmetric multidimensional scaling
OPG	Oocysts per gram
OTU	Operational taxonomic unit
PBP	Protein binding protein
PCR	Polymerase chain reaction

PI	Post infection
RDA	Redundancy analysis
RDP	Ribosomal Database Project
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RWA	Raised without antibiotics
SCAR	Sequence characterized amplified region
SE	*Salmonella* Enteritidis
TE	Transposable element
T-RFLP	Terminal restriction fragment length polymorphism
UAM	Utilisation des antimicrobiens
VRE	Vancomycin-resistant enterococci

Dedication

I want to dedicate this thesis to all young scientists who pursue excellence through their graduate studies. You are the next generation of researchers, professionals and decision makers leading innovation in our society. Enjoy every moment spent in your program, you can truly define and establish your self identity during this period. In the countless hours required to complete your current work, take the time to take additional courses, attend conferences, and nurture relationships both within and outside your network; every extra-curriculum activity will be rewarded and bring success to your career.

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Chapter 1. Introduction

The judicious use of antibiotics in animal production is a contemporary issue. In broiler chicken production, consumer demand for chicken products raised without antibiotics and public health concerns about the impact of antibiotic use on antimicrobial resistance have challenged the current use of antibiotics. To adapt to this new situation, various efforts and trials were put in place to increase the knowledge on the opportunities and challenges related to chickens raised without antibiotics.

The withdrawal of antibiotics in poultry production for treatment and preventive use was shown to adversely affect chickens' health and limit the potential for control of diseases during grow-out. A previous study at the Chair in Poultry Research of the Université de Montréal on the viability of drug-free commercial broiler chicken production has shown significant performance losses related to a higher incidence of enteric diseases outbreaks in antibiotic-free flocks compared to conventional flocks (Gaucher et al., 2015). These bacterial and parasitic infections remained untreated with antibiotics for maintaining their "antibiotic-free" status in retail stores, as required by the Canadian Food Inspection Agency (Canadian Food Inspection Agency, 2019). However, according to the American Association of Avian Pathologists' *White Paper on Poultry Welfare and Careful Use of Antibiotics*, such practices should not be supported (American Association of Avian Pathologists, 2016):

> Antibiotic treatment remains an important tool for poultry veterinarians to protect the health and well-being of flocks and should not be sacrificed in the name of marketing of an antibiotic-free product. Veterinarians should use their scientific knowledge to carefully consider all treatment options to protect both animal and human health.

The Canadian poultry industry used for many years various antibiotics important for human medicine to prevent diseases in poultry flocks (Agunos et al., 2017) and a change in the current practices may put at risk the broiler chicken production system regarding the control and prevention of these diseases. Still, international entities such as the World

Health Organization have described antimicrobial resistance as one of the biggest threats to global health. These organisations emphasize the urge of reviewing current antimicrobial use practices in animal production to limit the large-scale propagation of antibiotic resistant microorganisms (Tangcharoensathien et al., 2017). For this reason, the Chicken Farmers of Canada are currently examining the possibility to responsibly reduce antimicrobial use by discontinuing antibiotics considered important for human medicine in disease prevention programs of broiler chicken flocks through the implementation of the so-called *Antimicrobial Use Reduction Strategy* (https://www.chickenfarmers.ca/the-antimicrobial-use-reduction-strategy/). If an infectious disease outbreak occurs during grow-out, this strategy would allow the use of antibiotics important for human medicine for treatment purposes. Hence, this approach aims to promote a judicious use of antibiotics in the Canadian poultry industry, while preserving high animal welfare standards. Understanding and analyzing the positive and negative impacts of the Chicken Farmers of Canada's *Antimicrobial Use Reduction Strategy* will be of the foremost importance for the long-term success of reducing antibiotic use in Canadian poultry flocks. However, information is sparse on such approaches in a Canadian commercial poultry production context, and a feasibility study is needed to assess potential health and zootechnical performance consequences prior to applying these industry-driven policies to a larger extent. Furthermore, recent technological advances in nucleic acid sequencing and computational capabilities now allow scientists to characterize microbial populations to unreachable extents in the past. Indeed, the study of the microbiota is a rapid growing discipline exposing the numerous interactions between microbial communities with their hosts and environment. In the advent of this new field of study, there is an expanding interest to understand key factors driving the development of beneficial or detrimental communities impacting the chickens raised for meat consumption. For instance, identifying these components are considered critical for the advancement of poultry science and improving our current methods for raising chickens. These approaches, relying on understanding and modulating the microbiota of broiler chickens, are highly valued for their potential to improve the sustainability of an industry constantly looking to limit its carbon footprint.

The project described in this thesis took place in a worldwide context of reducing antibiotic use in animal production with the intention to decrease the likelihood of

developing and spreading bacteria resistant to antimicrobials. The principal objective of this study was to investigate the impacts of two strategies reducing antibiotic use in Canadian commercial broiler chicken flocks compared to the conventional use of antibiotics. More specifically, zootechnical performances, control of intestinal diseases and the gastro-intestinal microbiota were compared to assess the impacts of these strategies and potentially assess the success of future policies reducing antibiotic use in broiler chickens. The gastro-intestinal microbiota was further described to study its association with growth rate in broiler chickens, as it could unveil pivotal knowledge to develop microbiota-based strategies aiming to improve performance in commercial broiler chicken production, decrease our reliance on antibiotics and improve the poultry sector's sustainability.

Chapter 2. Literature review

2.1. Antimicrobial use and resistance

2.1.1 Antibiotic use in poultry production

Antibiotics are widely used in the Canadian broiler chicken industry. A study reported that 91% (339/373) of commercial flocks monitored between 2013 and 2015 were exposed to in-feed antimicrobials while 13% of these flocks used antimicrobial products in the drinking water (Agunos et al., 2017). In-feed antibiotics were mostly used for the prevention of necrotic enteritis and coccidiosis, respectively caused by *Clostridium perfringens* and *Eimeria* spp. The combination of trimethoprim and sulfadiazine was also used in the feed for a large proportion of flocks (13%), but its purpose was for the treatment of systemic or localized infections related to *Escherichia coli.* The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) reported in its 2016 Annual Report (Government of Canada, 2018) that 6% of the broiler chicken population sampled in Canada (10/136 flocks) was classified as Raised without antibiotics (RWA), while the remaining flocks received antibiotics with an average of 124 to 130 mg/PCU (population correction unit). From these flocks, 23% (31/136) reported hatchery medication (gentamicin or lincomycin-spectinomycin), a proportion that decreased by 39% in 2016 compared to 2015. Bacitracin was the most common antibiotic used (61%, 82/136 flocks) via in-feed administration in 2016, followed by avilamycin (35%), virginiamycin (28%), penicillin G procaine (9%), trimethoprim-sulfadiazine (8%), tylosin (7%) and bambermycin (1%). Water medication included amoxycillin (1%), penicillin G potassium (3%), penicillin-streptomycin (1%), sulfamethazine (1%), sulfaquinoxaline (2%), sulfaquinoxaline-pyrimethamine (1%) and tetracycline-neomycin (1%). Overall use of ionophores remained stable from 2013 to 2016, with respectively 91%, 88%, 86% and 89% of all flocks using this type of medication via the feed.

The Chicken Farmers of Canada, regrouping 2 800 Canadian farmers under the supply management policy, deployed their *Antimicrobial Use Reduction Strategy* to

ultimately eliminate the preventive use of antimicrobials of human importance. Health Canada categorizes each antibiotic class according to their importance in human medicine, based on two criteria: (1) Preferred option for treatment of serious human infections and (2) No or limited alternatives available (Table 1). These categories are listed from I to IV, where category I, II and III antibiotics are considered important to human health, while category IV antibiotics are not used in human medicine and mostly used in veterinary medicine. In May 2014, the Chicken Farmers of Canada banned prophylactic use of category I antibiotics, which include antibiotics of very high importance for human health (Table 2). In January 2019, the Canadian producers removed category II antibiotics from prevention programs and ultimately, their goal is to eliminate the preventive use of category III antibiotics (date to be determined). Moreover, the Government of Canada revised as of December 1st, 2018, all medically important antimicrobials (MIAs) for veterinary use (Government of Canada, 2019b). More specifically, the Government reworked the legislation by adding all MIAs previously sold over the counter to the Prescription Drug List (Government of Canada, 2019a), but also by removing all growth promotion claims from MIAs and adding labels for all in-feed and soluble MIAs with responsible use statements.

Table 1. Application of criteria for antimicrobial categorization

Category	Preferred option for treatment of serious human infections*	No or limited alternatives available
I- Very High Importance	Yes	Yes
II- High Importance	Yes	No
III- Medium Importance	No	No/Yes
IV- Low Importance	Not Applicable	Not Applicable

*Serious infections are considered those which if left untreated would lead to significant morbidity requiring emergency care including hospitalization and/or mortality.

Table 2. Categorization of principal antibiotic classes used in broiler chicken production, based on their importance in human medicine

Category	Antimicrobial class
I- Very High Importance[1]	Cephalosporins 3rd generation (Ceftiofur)
	Fluoroquinolones (Enrofloxacin)
II- High Importance[2]	Aminoglycosides (Spectinomycin, gentamicin, neomycin, streptomycin)
	Lincosamides (Lincomycin)
	Macrolides (Tylosin, erythromycin)
	Penicillins (Penicillin G, amoxycillin)
	Streptogramins (Virginiamycin)
	Trimethoprim – sulfadiazine (combination)
III- Medium Importance	Bacitracin
	Sulphonamides (Sulfamethazine, sulfaquinoxaline)
	Tetracyclines
IV- Low Importance	Ionophores (Salinomycin, narasin, monensin, lasalocid, maduramycin)
	Flavophospholipols (Bambermycin)
Uncategorized	Orthosomycin (Avilamycin[3])

Unclassified: Chemical anticoccidials

[1] Voluntary ban for preventive use by the Chicken Farmers of Canada in 2014

[2] Voluntary ban for preventive use by the Chicken Farmers of Canada in 2019

[3] Not categorized by Health Canada, but identified by the World Health Organization as not being used in humans (World Health Organization, 2017)

In a US study evaluating antibiotic use (AMU) in broiler chickens farms (7.5 billion chickens slaughtered) and hatcheries between 2013 from 2017, a constant decrease in AMU was reported in hatcheries (Singer et al., 2020). Indeed, 93% of all chicks placed

received antimicrobials by injection in 2013; from 2014 to 2017, respectively 64%, 40%, 31% and 17% of the chicks placed were administered injectable antibiotics. In the same time frame, the authors reported a significant decrease of MIAs such as aminoglycosides, lincosamides, macrolides, sulfonamides and tetracyclines administered in the feed or drinking water of broiler chicken flocks. These antibiotics were reported to target several diseases such as necrotic enteritis, colibacillosis and gangrenous dermatitis. Rennier Associates Inc. also reported the same trend in US broilers health programs from 2014 to 2018, where the proportion of programs including MIAs constantly decreased during that time frame and the proportion of programs using no MIAs (Reduce Use or Ionophores Only) or no antibiotics (No Antibiotics Ever) increased during the same period (The Poultry Site, 2019).

The European Medicines Agency reported that antimicrobial sales decreased by 32.5% across all food-producing animals from 2011 to 2017 in the 25 countries reporting sales to the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC), even if the animal population remained relatively stable during the same period (European Medicines Agency, 2019). However, large variations were observed between the 25 countries monitored; while 19 countries reported a decrease of more than 5% (range -7.7% to -57.9%), an AMU increase of more than 5% (range +29.8% to +42.9%) was recorded in three other countries. Overall, the use of tetracyclines, penicillins and sulfonamides, the top selling antibiotics during that period, decreased by 44%, 18% and 46%, respectively.

2.1.2 Antimicrobial resistance

2.1.2.1 Definition of antimicrobial resistance

The World Health Organization defines antimicrobial resistance (AMR) as:

> Antimicrobial resistance occurs when microorganisms such as bacteria, viruses, fungi and parasites change in ways that render the medications used to cure the infections they cause ineffective. When the microorganisms become resistant to most antimicrobials they are often referred to as "superbugs". This is a major concern because a resistant infection may kill, can spread to others, and imposes huge costs to individuals and society (World Health Organization, 2017).

AMR in bacteria can be either intrinsic or extrinsic. Bacteria with intrinsic resistance possess the innate ability to resist the action of an antibiotic, mostly through structural or biochemical characteristics (Giguère et al., 2013). For example, penicillins are inherently ineffective against *Mycoplasma* spp. as these bacteria lack a cell wall around their membrane, which is the primary target of this antibiotic class. Another example relates to anaerobic bacteria being resistant to aminoglycosides as these large molecules require oxygen-dependant active transport systems to cross cell walls and reach the cytoplasmic 30S subunit of ribosomes. Extrinsic resistance occurs when bacteria respond to changing environmental conditions by acquiring characteristic traits, altering the functionality of its genome or by acquiring competent genome from other species (Sagar et al., 2019).

2.1.2.2 Genetic basis of antimicrobial resistance acquisition in bacteria

Bacteria may develop resistance to antimicrobials by the mutation of genes involved in physiological processes and cellular structures, by the acquisition of foreign resistance genes or by a combination of both mechanisms (Giguère et al., 2013). Mutations in the bacterial genome may occur sporadically and lead to random mutants to antibiotics. Under certain stressful circumstances, such as exposition to antibiotics, these mutation rates can increase. For example, mutation frequencies for *Escherichia coli*, enterococci and coagulase-negative staphylococci were significantly increased by 3, 1.8 and 1.5-fold, respectively, in human patients with high antibiotic use compared to patients receiving no antibiotics (Gustafsson et al., 2003). Increased mutation rates have also been observed in *E. coli* with increasing norfloxacin concentrations, and this response has been associated with enhanced expression of error-prone DNA polymerases (Long et al., 2016).

The acquisition of foreign resistance genes is a method more frequently used by bacteria to develop phenotypic resistance against antibiotics (Porse et al., 2020), which can be attained by the transduction, transformation or conjugation of antimicrobial resistance genes (ARGs). Evolving resistance by acquisition of plasmids tends to carry a smaller fitness cost compared to evolving resistance *de novo* by chromosomal mutation (Vogwill & MacLean, 2015). Since a lower fitness cost is related to better competitive abilities in an environment, it may explain that bacteria developing phenotypic resistance to

antimicrobials by acquiring foreign resistance genes is the predominant mechanism of AMR. In transduction, bacteriophages infect bacteria and incorporate their genome in the host, potentially including genetic material giving resistance to one or more antibiotics to the infected bacterium (Yang et al., 2020). For example, environmental bacteriophages were shown to harbor the same resistance genes as bacterial DNA fractions recovered from the same sampling location (Colomer-Lluch et al., 2011; Marti et al., 2014). In addition, ampicillin resistance genes recovered from bacteriophages induced phenotypic resistance to ampicillin in host bacteria following electroporation *in vitro*, showing that ARGs in bacteriophages can incorporate bacterial genomes and that phages are potential sources of ARGs for bacteria (Colomer-Lluch et al., 2011). Transformation occurs when bacteria acquire extracellular DNA, for example ARGs, directly from their environment (Lu et al., 2020), or other genes possibly giving an ecological advantage to bacteria such as resistance to mercury (Kothari et al., 2019). Conjugation is a mechanism involving the transfer of mobile genetic elements (MGEs) between bacteria through a direct cell to cell contact, transforming recipient cells by receiving a copy of the genetic material present in the donor bacterium. Plasmids are a common type of MGEs (Siefert, 2009), which are self-replicating extrachromosomal DNA molecules within a bacterium that contain genetic material possibly advantageous for the host. For example, plasmids are known to carry one or more genes coding for various functions such as resistance to antibiotics (Adams et al., 2018), resistance to heavy metals (Ferreira et al., 2019; Hernandez-Ramirez et al., 2018) or virulence factors (Bannam et al., 2011; Freedman et al., 2015; Hughes et al., 2007; Lepp et al., 2010; Li et al., 2013). Transposable elements (TEs), also called transposon or "jumping genes", are another type of MGEs involved in the transfer of ARGs and other genes between bacteria conferring additional survival abilities. TEs work by an excision and insertion mechanism, as this mobile element possesses genes allowing to cleave the TE and integrate it in a new location (Munoz-Lopez & Garcia-Perez, 2010). ARGs can also be present in integrons, specialized DNA elements embedding one or more genes in a structure called gene cassette (Giguère et al., 2013). For example, a study showed that most resistance genes isolated from various food animal sources were located on plasmids of multiple isolates of *Salmonella enterica*, and one to five ARGs could be identified on six different cassettes (McMillan et al., 2019).

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2.1.2.3 Mechanisms of antimicrobial resistance

Bacteria have developed a plethora of strategies encoded in their genetic material to resist the lethal action of antibiotics or to reduce their efficacy. For example, the database CARD (Comprehensive Antibiotic Resistance Database: https://card.mcmaster.ca/home) contains resistance mechanisms from 2 923 AMR gene determinants with 1 304 resistance variant mutations from 82 pathogens and 100 000 genomes (Alcock et al., 2020). Several strategies involve antibiotic inactivation by degradation or modification. For example, bacteria such as *Staphylococcus aureus* and *E. coli* can deactivate the beta-lactam ring in penicillins and cephalosporins by producing β-lactamases. These bacterial enzymes cause a hydrolytic deactivation of penicilloic acid and deny its binding to Protein Binding Proteins (PBPs), protecting the cell wall synthesis by bacteria (Bush, 2018). Another mechanism decreases bacterial permeability to antibiotics, such as various Gram-negative bacteria reducing antibiotics penetration through their outer membrane by modulating the function and frequency of porins or lipopolysaccharides (LPS) assisting the transport of various antibiotics across bacterial membranes. Consequently, the amount of antibiotic molecules reaching their intracellular target decreases, allowing bacteria to survive in the presence of these antimicrobials (Delcour, 2009). Bacteria can also show phenotypic resistance to antibiotics by producing active transporters known as efflux pumps that force the expulsion of one or more antibiotics outside the cell before they can exert their effects (Blanco et al., 2016). Finally, the target site can be changed, either by being provided with protection or by modifying its own structure. For example, the *mcr-1* gene causes plasmid-mediated colistin resistance by transferring a phosphoethanolamine residue on the lipid A, a component of the LPS on Gram-negative bacteria, and hence decreasing its affinity for colistin (Liu et al., 2016).

2.1.2.4 Distribution of antimicrobial resistance in Canadian poultry

The CIPARS surveillance systems is actively monitoring AMR in the Canadian poultry industry by evaluating *Salmonella*, *E. coli* and *Campylobacter* isolates recovered from retail meat, abattoirs, broiler chicken farms, turkey farms, animal clinical isolates and feed and feed ingredients by testing their sensitivity to antimicrobials (Government of Canada, 2018). In 2016, 72 of the 183 (39.3%) *Salmonella* isolates, 235/310 (75.8%) *E. coli* isolates and 86/176 (48.9%) *Campylobacter* isolates recovered from Canadian

chicken farms were resistant to at least one antibiotic class. Resistance patterns from 2012 to 2016 showed large variations between bacterial species, sampling year, antibiotic classes and provinces. In chicken clinical isolates of 2016, 50/227 (22.2%) *Salmonella* isolates were resistant to at least one antibiotic class. Feed and feed ingredients contained 6/46 (13.0%) *Salmonella* isolates resistant to at least one antibiotic class, although the analysis was not specific for chicken feed and ingredients as the data was aggregated for all species. In a study using CIPARS data from 2003 to 2008, it has been observed that ceftiofur resistance in *Salmonella* serovar Heidelberg from human clinical and chicken retail meat isolates varied between regions and years (Dutil et al., 2010). For example, early years (2003-2004) in Quebec were characterized by 30% to 60% resistance to ceftiofur, while later years (2005-2008) were characterized by a prevalence lower than 30% in both human clinical and chicken retail isolates. Large differences were also observed between provinces, especially in early years where ceftiofur resistance in Quebec isolates was higher than the province of Ontario. From a study evaluating CIPARS data from 2013 to 2017 in the province of British Columbia (Agunos et al., 2019), it was observed that resistance to tetracycline, ceftriaxone, ampicillin and streptomycin in *Salmonella* isolates from pooled feces recovered from broiler chicken farms at the end of grow-out was between 10% and 50%, while resistance to gentamicin and trimethoprim-sulfamethoxazole remained close to 0%. Additionally, a significant proportion of these isolates were resistant to two or more antibiotic classes with a multiclass resistance proportion varying between 16% and 48% of all isolates.

In addition to active surveillance systems, various research projects evaluated the prevalence of AMR in bacteria recovered from poultry and poultry meat. For example, a study evaluated antimicrobial susceptibilities for 17 antibiotics of *Enterococcus faecalis* and *Enterococcus faecium*, bacterial species not evaluated by the CIPARS (Tremblay et al., 2011). In 270 *E. faecalis* and 117 *E. faecium* isolates recovered from turkey and chicken cecal contents at five processing plants in Quebec (statistical unit = individual isolate), high levels of AMR were observed for bacitracin (88.1% and 94% for *E. faecalis* and *E. faecium*, respectively), erythromycin (72.6% and 80.3%), lincomycin (100% and 94%), quinupristin-dalfopristin (98.5% and 89.7%), tetracycline (95.6% and 89.7%) and tylosin (73% and 75.2%), which many of these molecules belong to antibiotic classes routinely

used in commercial poultry (Government of Canada, 2018). Low AMR frequencies were observed for ciprofloxacin (0.7% and 14.5%), flavomycin (3.7% and 41%), gentamicin (9.6% and 4.3%), kanamycin (25.2% and 17.1%), streptomycin (46.7% and 38.5%), nitrofurantoin (2.6% and 20.5%), penicillin (3% and 27.4%) and salinomycin (7% and 12.8%). In another study evaluating *E. coli* and *Enterococcus* spp. isolates in Quebec, Canada, high levels of flock-level (statistical unit = flock) resistance to tetracycline (98% in chicken flocks, 100% in turkey flocks), streptomycin (94.7% in chicken flocks, 92.8% in turkey flocks) and sulfisoxasole (94.7% in chicken flocks, 96.4 % in turkey flocks) was identified in *E. coli* isolates recovered from flocks slaughtered in Canadian federal processing plants in the province of Quebec, Canada from April 2003 to February 2004 (Boulianne et al., 2016). In *Enterococcus* spp., AMR was detected in more than 90% of the broiler and turkey flocks for bacitracin, erythromycin, lincomycin, quinupristin-dalfopristin and tetracycline. In Avian pathogenic *E. coli* (APEC) isolates recovered from clinical isolates of colibacillosis from broilers and broiler breeders in Ontario (statistical unit = individual chicken), resistance to tetracycline (57%), gentamicin (50%), spectinomycin (46%) and ampicillin (44%) was commonly observed, while lower AMR prevalence was observed for trimethoprim-sulfamethoxazole (18%), ceftiofur (15%), kanamycin (11%) and apramycin (3%) (Varga et al., 2018). From 170 *E. coli* isolates harvested from non-viable chicken embryos in three Western Canada hatcheries (statistical unit = individual bacterial isolate), resistance was observed for tetracycline (52.9% of the isolates), ampicillin (50.6%), amoxycillin-clavulanic acid (40.0%), triple sulpha (31.8%), ceftiofur (29.4%), gentamicin (29.4%), and spectinomycin (21.8%) (Karunarathna et al., 2020). In 256 *E. faecalis* isolates from the same study, resistance was observed for tetracycline (61.9%), ceftiofur (46.2%), bacitracin (43.9%), erythromycin (31.4%) and tylosin (27.4%). Resistance to various antibiotics was observed in 206 *E. coli* isolates recovered from chicken meat in retail stores in Alberta (statistical unit = individual meat sample), Canada: amoxicillin/clavulanic acid (31.1%), ceftiofur (22.3%), ceftriaxone (28.5%), ampicillin (43.0 %), cefoxitin (30.1%), gentamicin (4.1%), kanamycin (16.4%), nalidixic acid (4.1%), streptomycin (23.8%), trimethoprim/sulfamethoxazole (4.1%), sulfisoxazole (21.2%), chloramphenicol (4.1%), tetracycline (57.0%), while no resistance was observed in chicken meat samples against ciprofloxacin and amikacin (Sheikh et al.,

2012). In retail chicken meat from Quebec, Canada, Livestock-Associated Methicillin Resistant *Staphylococcus aureus* (LA-MRSA) was observed in 1.3% (4/309) of the chicken meat samples (statistical unit = individual meat sample), while no LA-MRSA isolates were recovered from nasal swabs and cecal samples collected from 200 live broiler chickens at the processing plant before slaughter (Bernier-Lachance et al., 2020).

2.1.2.5 Risk factors associated with antimicrobial resistance in poultry

Antibiotic use is a major factor contributing to increase antimicrobial resistance in poultry production. In the presence of antibiotics, bacteria possessing ARGs have a significant survivability advantage over bacteria without ARGs. However, it has been identified that carrying resistance genes reduces the competitive ability and survivability of bacteria in the absence of antibiotics (Vogwill & MacLean, 2015). Increased AMU selects for bacteria resistant to antibiotics and lead to increased AMR, while using less antibiotics provides an advantageous environment for bacteria susceptible to antibiotics and consequently decrease AMR. For example, it has been recently observed that acquired resistance gene pools (resistome) in 178 broiler chicken farms across nine European countries were associated with livestock antibiotic sales for each country, where more ARGs were observed in countries selling higher quantities of antibiotics than countries relying on less antibiotics for animal production (Munk et al., 2018). Additionally, it was observed from the same project that the type of ARG was associated with the antibiotic classes used on farm (Luiken et al., 2019). More specifically, the increased abundance of genes coding for resistance to tetracycline, macrolides-lincosamides-streptogramins-pleuromutilins (MLS), trimethoprim and aminoglycoside was positively associated with each corresponding antibiotic product purchased and used for treatment in sampled flock, but there were no associations between the presence of ARGs coding against an antibiotic class and other antibiotic classes. Similarly, the ban of antimicrobials for growth promotion has been associated with decreased AMR in *E. faecalis* and *E. faecium* isolates from poultry in Denmark (Aarestrup et al., 2001). For instance, the proportion of glycopeptide-resistant *E. faecium* (GRE) isolates recovered from cloacal swabs in broiler chicken flocks (one swab and one isolate per flock) decreased following the ban of avoparcin in 1999, an antibiotic that was largely used in Europe for growth promotion. Indeed, while GRE

corresponded to 72.5% of all *E. faecium* isolates from broiler chickens in 1995, this proportion decreased to 5.8% in 2000.

It has been observed from the CIPARS data that temporal prevalence of ceftiofur resistance in *Salmonella* Heidelberg from humans and retail chickens and *E. coli* isolates from retail chickens were closely associated with ceftiofur use in Quebec hatcheries from 2003 to 2008 (Dutil et al., 2010). In 2003 and 2004, the industry was extensively using extra-label *in ovo* ceftiofur administration for the prevention of colibacillosis in broiler chickens, but the high incidence of ceftiofur-resistant *Salmonella* Heidelberg isolates from retail chickens pressured the local industry to voluntarily ban this antibiotic for extra-label use between 2005 and 2006. In 2007, ceftiofur use was partially reintroduced by Quebec hatcheries. Following the ban of ceftiofur in 2005, the proportion of resistant isolates considerably decreased to reach lowest levels in 2006 and 2007. Then, resistance to ceftiofur increased after its partial reintroduction for *in ovo* injections in hatcheries, indicating that ceftiofur use in hatcheries could have been an important factor leading to ceftiofur resistance in *Salmonella* Heidelberg and *E. coli* from retail chickens and humans. Hence, it would be expected that chicks receiving ceftiofur via *in ovo* administration host more bacteria resistant to ceftiofur, as AMU of specific antibiotics is closely related to AMR for the same antibiotics. Since ceftiofur resistant genes were shown to persist in fecal *E. coli* isolates throughout broiler chickens grow-out (Baron et al., 2014), it would also be expected to identify a larger proportion of bacterial isolates resistant to ceftiofur in retail poultry meat and humans, as observed by Dutil et al. (2010). The Quebec broiler chicken industry reinstated the ban of *in ovo* ceftiofur in 2015, and a study demonstrated that the proportion of cephalosporin-resistant *E. coli* decreased after the cessation of ceftiofur use (Verrette et al., 2019), strengthening previous observations that ceftiofur use in broiler chickens is linked to ceftiofur resistance in bacteria.

A study used an epidemiological approach to identify farm variables associated with ceftiofur resistance in 32 Belgian broiler chicken farms, where the proportion of ceftiofur resistance in *E. coli* isolates on each farm ranged from 8% to 62% (Persoons et al., 2011). Co-resistances with other antibiotic classes and other factors were identified, as significant associations ($p < 0.01$) were observed when considering farm clustering in the

models. For instance, amoxicillin-clavulanic acid and trimethoprim–sulfamethoxazole co-resistances, hygienic condition of the medicinal treatment reservoir, acidification of drinking water, more than three feed changes per cycle, hatchery, breed, litter material and amoxicillin treatment were statistically associated with ceftiofur resistance.

2.1.3 Impacts of reducing antibiotic use on performance and health in poultry

Restrictions on AMU in animal production were implemented in various countries to counteract the global threat of AMR for human and animal health (Maron et al., 2013), possibly impacting zootechnical performance in broiler chicken flocks as antibiotics have been known since the 1940's to possess growth promoting effects in broilers (Castanon, 2007; Dibner & Richards, 2005). For example, a meta-analysis of 174 articles published between 1998 and 2018 on antibiotic growth promoters (AGPs) in pen trials identified a significant advantage of using AGPs compared to AGP-free diets on weight gain and feed conversion ratio (FCR), where an improvement of 3.84% and 3.48% for each indicator between 1 and 42 days of age was observed, respectively (Maria Cardinal et al., 2019). However, feed intake was not reported to be significantly modified by the use of AGPs. Avilamycin, flavomycin and virginiamycin all significantly improved FCR, while only virginiamycin was significantly associated with improved weight gain. A simulation of the economical impact of AGPs withdrawal in a Brazilian context estimated losses to 3 cents per chicken based on 2017 data (Maria Cardinal et al., 2019). However, benefits of AGPs on zootechnical performance are less marked in conventional and commercial farms as farms with low and high AMU can attain high technical performance, possibly indicating that the real economic value of AMU related to its growth promoting effects may be overestimated (Roskam et al., 2020).

Although the effects of AMU on performance in conventional and commercial broiler chicken farms are reported to be marginal (Roskam et al., 2020), antimicrobials are still widely used for prophylactic use in poultry (Agunos et al., 2017; Chauvin et al., 2005). Indeed, broiler chicken flocks are at risk of developing intestinal diseases in the absence of antibiotics, causing performance losses in affected flocks. For example, 76% of the antibiotic-free (ABF) flocks were affected by clinical necrotic enteritis (NE) or subclinical enteritis in a study comparing ABF flocks to flocks receiving antibiotics, while none of the

flocks raised with antibiotics showed enteric health issues during the study. Additionally, the drug-free treatment significantly impaired body weight at slaughter, average daily gain, feed conversion ratio and kilograms of meat produced per square meter compared to flocks raised with antibiotics (Gaucher et al., 2015). Historical data in Norway has also shown that NE was more frequent after the withdrawal of in-feed avoparcin, which resulted in high incidence of *Clostridium perfringens*-related hepatitis (CPH) at slaughter and 25 to 43% lower margin in flocks affected by the condition compared to healthy flocks (Lovland & Kaldhusdal, 2001). Monetary losses in diseased flocks were estimated to be caused by impaired feed conversion ratio, high mortalities, increased condemnations at slaughter mostly due to CPH and other causes of condemnations. Drug-free production was also shown to be problematic in the USA, where a large integrator suffered heavy losses after the implementation of an ABF program in commercial farms (Smith, 2011). For instance, the company reported early challenges due increased gut health issues, particularly NE, and production losses. Despite testing many solutions, such as alternatives to antibiotics, vaccines and better-quality management practices, improvements were modest and results were lower than those obtained before the transition to ABF production. Because of these issues, reducing prophylactic use of antibiotics has been linked to increased AMU due to an increased number of broiler chicken flocks being administered antibiotics for the treatment of bacterial infections (Jensen & Hayes, 2014; Smith, 2011). In a US study comparing NAE (no antibiotic), antibiotic-reduced (use of nonmedically important antibiotic) and conventional (use of medically important antibiotics) broiler chicken flocks in a commercial context, NAE flocks showed higher odds of eyes burns (i.e., corneal erosion or ulceration), footpad lesions and airsacculitis compared to the 2 other groups using antibiotics (Karavolias et al., 2018). When flocks receiving nonmedically important antibiotics were compared to flocks receiving medically important antibiotics, only footpad lesions showed slightly higher odds, while eyes burn had similar odds and airsacculitis lesions had lower odds.

2.2. Gastro-intestinal diseases in broiler chicken production

2.2.1 Coccidiosis

2.2.1.1 Etiology

Coccidiosis in chickens is caused by various protozoal species belonging to the phylum *Apicomplexa* and genus *Eimeria*. Among the 1200 *Eimeria* spp. identified (Chapman et al., 2013), seven are known to infect chicken intestinal cells: *E. acervulina*, *E. maxima*, *E. tenella*, *E. necatrix*, *E. brunetti*, *E. praecox* and *E. mitis* (Mattiello et al., 2000; Reid et al., 2014). However, the exact number of *Eimeria* spp. infecting chickens is still a debate in the scientific community as nine species have also been reported to infect chickens, with the addition of *E. hagani* and *E. mivati* (Levine, 1938; Norton & Joyner, 1980;). A recent Australian study analysed the mitochondrial genome of 25 chicken *Eimeria* isolates and identified three additional *Eimeria* species to the seven described by Reid et al. (2014), called OTU-X, OTU-Y and OTU-Z (Morgan & Godwin, 2017), hence indicating that modern molecular approaches could identify more coccidia species able to infect chickens.

2.2.1.2 Life cycle

The life cycle of *Eimeria* spp. is complex, involving phases outside and inside the host, as well as sexual and asexual reproductive stages. In the external environment, coccidia are found as oocysts, ovoid structures protecting the protozoan from adverse conditions. Once shed from the chicken in feces, oocysts are unsporulated, non infective for the host and must become infective by sporulating with adequate oxygen, moisture and temperature in the environment. The sporulation time varies between species; it has been observed that 216 h was required for the sporulation of 90% of *E. maxima* oocysts while 96 h were required to sporulate 90% of *E. necatrix* and *E. tenella* oocysts with a room temperature ranging between 32 to 39°C, a relative humidity from 65 to 75% and constant aeration (Venkateswara Rao et al., 2015). The sporulation process can be faster with a lower moisture content, as the sporulation of *E. maxima* was shown to be most efficient under 16% moisture content in wood shavings compared to 42% and 62% of moisture content (Waldenstedt et al., 2001). Temperature also influences sporulation as the process

is faster at 33°C compared to 21°C for *E. acervulina* (Graat et al., 1994). However, large discrepancies, most likely associated with different environmental conditions, are reported for *Eimeria* spp. sporulating time, as Levine (Levine, 1985) defined the sporulation time for *E. acervulina* at 24 h, while 48 h was required for the sporulation of *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix* and *E. tenella*.

The sporogony phase involves the sporulation of the oocyst by transforming four intracellular sporoblasts into four sporocysts, each containing two sporozoites (Mehlhorn, 2016). Once ingested by a chicken, sporozoites are released from the oocyst by excystation in the gastro-intestinal tract by the action of bile salts, pepsin, trypsin and HCl (Chapman, 1978; Kowalik & Zahner, 1999). Sporozoites then infect intestinal cells to begin the schizogony phase. Schizonts are formed in the cell cytoplasm and produce merozoites, which may initiate the production of new sporozoites that will infect other intestinal cells. This cycle can be repeated for a few generations; for example, *E. maxima* can repeat more than three generations of schizonts in its life cycle before beginning the sexual reproductive stage, also called gametogony or gamogony (Dubey & Jenkins, 2018). After these generations, merozoites transform into gametocytes to enter the sexual reproductive stage. Macrogametes and microgametes are then produced in macrogametocytes and microgametocytes, respectively. A motile microgamete will locate and fertilize a macrogamete to produce a zygote and will be shed in feces as an unsporulated oocyst to complete the life cycle.

Life cycle length varies between *Eimeria* species as observed by different prepatent periods, defined as the time between infection with an infective oocyst and the recovery of a new unsporulated oocyst in the feces (Table 3).

Table 3. Prepatent period (hours) of the seven *Eimeria* species in chickens.

Eimeria species	Minimum prepatent period (hours)	Source
E. acervulina	97	Cha et al., 2018
E. maxima	120	Brito Lda et al., 2014; Cha et al., 2018
E. tenella	121	Cha et al., 2018
E. necatrix	138	Shirley & Bellatti, 1984
E. brunetti	120	Johnson et al., 1986; Shirley et al., 1986
E. praecox	90	Mattiello et al., 2000
E. mitis	95	Mattiello et al., 2000

2.2.1.3 Incidence and distribution

Coccidia are highly prevalent in the worldwide poultry industry and nearly all flocks are thought to be infected at a young age (McDougald & Fitz-Coy, 2013). However, due to the exposition at a young age and the development of a long-lasting immunity, these parasites are rarely seen in older flocks such as layers and breeders. Broiler chickens are likely to be infected at a very young age as infective oocysts of *Eimeria acervulina*, *E. maxima*, *E. praecox* and/or *E. tenella* were identified in the litter of nearly 100% of broiler chicken houses using anticoccidial drug or *Eimeria* spp. live vaccine programs (Jenkins et al., 2019). The prevalence may vary between geographies, as two studies reported that coccidia were identified in 30% (95/310) of broiler flocks sampled in Iran and 31.5% (126/400) of the flocks sampled in the province of Zhejiang, China (Hamidinejat et al., 2010; Lan et al., 2017), while another study reported that more than 1000 oocysts were identified in 44% (238/545) of broiler farms sampled in differences provinces of China (Zhang et al., 2013). However, these studies didn't report flocks' age, which may be an important bias for classifying a flock as positive or negative for the presence of *Eimeria* spp. oocysts. Indeed, oocysts numbers in litter or feces of broiler chicken flocks vary with flocks' age; low numbers or no oocysts are usually seen at younger ages while higher numbers are observed around 3-4 weeks of age, depending on the prevention program used

(Chapman et al., 2016; Parent et al., 2018; Williams et al., 1999; Williams & Gobbi, 2002). Consequently, a study evaluating the prevalence of *Eimeria* spp. in 251 Brazilian broiler flocks between 28 and 48 days of age identified 96% of the flocks positive for either *E. acervulina* (individual prevalence of 63.3%), *E. maxima* (63.7%), *E. tenella* (54.6%), *E. mitis* (38.6%), *E. praecox* (25.1%), *E. necatrix* (24.3%) or *E. brunetti* (13.1%) (Moraes et al., 2015).

2.2.1.4 Modes of transmission

Coccidia in chickens are host specific as *Eimeria* spp. are not reported to infect multiple animal species. This host specificity removes other animal species as potential sources of infections for chickens. Since coccidia are shed in feces, the mode of transmission is fecal-oral through the ingestion of sporulated oocysts (McDougald & Fitz-Coy, 2013). Due to the resistant nature of the oocyst found outside the host, coccidia may survive for extended periods of time in a poultry farm environment. Indeed, oocysts are resistant to a wide range of temperature, desiccation and to most common disinfectants used in poultry production (Jenkins et al., 2013; McDougald & Fitz-Coy, 2013). For example, oocysts are reported to survive 2-3 days at 37°C, but exposure over 55°C or freezing temperatures can kill them quickly. Mechanical vectors, for example personnel, equipment, wild animals and other fomites may be important carriers of oocysts and ease the transmission of coccidia between farms. Litter is known to harbour significant amounts of infective oocysts, even at chicks placement (Jenkins et al., 2019), but oocysts are normally starting to deteriorate after 24 h and most are destroyed in 5 days in litter (Williams, 1995). However, viable oocysts may still survive in litter up to 54 days at 25-28°C with a moisture content between 31.0% and 62.1%.

2.2.1.5 Predisposing factors

The presence of concurrent immunosuppressive diseases, such as Marek's disease (MD) or Infectious bursal disease (IBD), are reported to exacerbate lesions and mortality related to coccidiosis and to impair the development of immunity against the disease (Anderson et al., 1977; Biggs et al., 1968; McDougald et al., 1979). Other immunosuppressive conditions, such as the presence of subclinical doses of deoxynivalenol (DON) and fumonisin (FB) in the feed were reported to increase the presence and intensity of intestinal lesions and the number of oocysts in the jejunal mucosa

6 days after the inoculation of coccidia to broiler chickens, but without impacting growth rate further than the coccidiosis challenge alone (Grenier et al., 2016).

2.2.1.6 Clinical signs

Among the seven most recognized *Eimeria* spp. in chickens, four are reported to be pathogenic in commercial production: *E. acervulina*, *E. maxima*, *E. tenella* and *E. necatrix*. A prominent manifestation of coccidiosis is the negative impact on zootechnical performance. Indeed, *in vivo* challenges have shown a consistent increase in the feed conversion ratio (FCR) and decrease of body weight gain (BWG) in chickens infected with *E. acervulina*, *E. maxima*, *E. tenella* (Barrios et al., 2017; Conway et al., 1993; Rochell et al., 2016). A meta-analysis of 69 scientific publications evaluating the effects of coccidia on performance in broiler chickens determined that groups challenged by *E. acervulina*, *E. maxima*, *E. tenella* or a mix of these species showed decreased BWG, even if feed intake remained similar to uninfected controls (Kipper et al., 2013). Moreover, the association between *Eimeria* spp. infection and loss in BWG was dose-dependant, where the BWG gets lower as the inoculated dose of *E. acervulina*, *E. maxima*, *E. necatrix* and *E. brunetti* or the severity of intestinal lesions increase (Barrios et al., 2017; Hein, 1971, 1974; Rochell et al., 2016). High mortalities are also reported in severe *E. tenella* infections, while other coccidia such as *E. acervulina* and *E. mitis* are not reported to cause deaths even with a heavy infection (Tyzzer, 1929). When large doses of *E. tenella* are given to chickens, mortality may appear on the fifth-, sixth- or seventh-day post-infection and blood in the feces is normally observed. *E. necatrix* is also reported to cause increased morbidity and mortality with bloody droppings (Hein, 1971), but the condition is normally diagnosed in older chicken flocks such as breeder pullets (McDougald et al., 1990). Due to significant blood losses, these two species may also cause anemia and birds may appear pale. Given a dose of 20 000 or more *E. necatrix* sporulated oocysts without other concurrent *Eimeria* spp. infection, clinical signs with high mortalities can be reproduced in an experimental context (Hein, 1971). Because of their lethality, *E. tenella* and *E. necatrix* are often classified as the most pathogenic coccidia in chickens. Watery and mucoid droppings, ruffled feathers and depression are also reported as possible clinical signs caused by coccidiosis (Fitz-Coy & Edgar, 1992; McDougald & Fitz-Coy, 2013). Due to decreased

absorption of pigments such as carotenoids, pale skin color may be observed with *E. acervulina* and *E. mitis* (Fitz-Coy & Edgar, 1992; Rochell et al., 2016).

2.2.1.7 Gross lesions and histopathology

Eimeria spp. cause damage to the gastro-intestinal mucosa, more specifically enterocytes and the lamina propria during schizogony and gametogony phases (Dubey & Jenkins, 2018). *Eimeria* spp. in chickens are site specific, i.e. each species invades a different section of the gastro-intestinal tract. Various findings may be observed on post-mortem examination and histopathology, depending on the species causing the infection.

E. acervulina invades the upper half of the small intestine. The parasites concentrate in the duodenum and proximal jejunum and are more scattered throughout the lower intestinal tract and rarely found in the ceca (Tyzzer, 1929). Parasites have the tendency to mass at the apex of villi, macroscopically appearing as elongated small white plaques on post-mortem examination (Figure 2.1) (McDougald & Fitz-Coy, 2013). As the inoculation dose gets higher, the number of plaques increases and they can coalesce with each other (Johnson & Reid, 1970). The intestinal wall is thickened, and intestinal content is watery. On histopathology, these lesions contain high numbers of various developmental stages of *E. acervulina* such as schizonts, gametocytes and developing oocysts in the mucosal cells, mostly located at the apex of villi (Williams et al., 2009). Villi length may be shortened compared to uninfected chickens (Williams et al., 2009).

E. maxima is found in the mid-small intestine. It mostly parasitizes the jejunal section (Dubey & Jenkins, 2018), but infection may be located throughout the small intestine in severe cases (McDougald & Fitz-Coy, 2013; Tyzzer, 1929). On post-mortem examination, the intestine is flaccid, and the luminal content is often watery with yellow to orange mucoid material (Johnson & Reid, 1970). Small hemorrhages and reddish foci may be observed on the mucosa and through the serosa (Figure 2.2). A small amount of blood clots may be observed in more severe infections, but massive hemorrhages similar to *E. tenella* infections are not reported. On histopathology, asexual stages mostly occur superficially in epithelial cells, but damage related to edema, cellular infiltration and thickening of the mucosa is observed during the later sexual stage occurring deeper in the mucosa. Microscopic hemorrhages may also be present near the tips of the villi.

E. tenella causes a spectacular hemorrhagic typhlitis related to massive hemorrhages in the ceca (Figure 2.3). The peri-cloacal area and posterior feathers may be tainted red due to the intestinal discharge of bright red blood (Tyzzer, 1929). Ceca are often filled and distended by the presence of blood in the lumen. Multiple pinpoints of hemorrhages can be observed on the mucosa (Johnson & Reid, 1970). If chickens survive for more than seven- or eight-days post-infection, cecal walls may be severely thickened and the lumen may contain whitish caseous cores. On histopathology, various developmental stages of *E. tenella* can be identified in enterocytes and the lamina propria of the ceca, and mature oocysts may be visible in the lumen in association with numerous necrotic debris and/or red blood cells. Hemorrhages from multiple vessels in deeper sections of the cecal mucosa may start 24 h before the release of second generation merozoites (Tyzzer, 1929). Heterophils in the mucosa and submucosa are normally present in large numbers during *E. tenella* infections.

E. necatrix causes a hemorrhagic enteritis mostly in the jejunal section of the small intestine, the same location as *E. maxima* (Hein, 1971; McDougald & Fitz-Coy, 2013). Hemorrhages can be identified in the mucosa of the small intestine four- and five-days post-infection. The intestinal tract may appear distended, and blood admixed with necrotic debris may be present in the small intestine lumen during heavier infections (Johnson & Reid, 1970; Mattiello et al., 2000). Red petechia, representing mucosal hemorrhages, and white circular lesions, representing colonies of schizonts, can be observed on the serosal surface of the small intestine, giving a characteristic "salt and pepper" appearance (Figure 2.4). On histopathology, schizonts are deeply located in the mucosa and often invade the submucosa and cause damage to the layers of smooth muscles and destroy blood vessels (McDougald & Fitz-Coy, 2013). Oocysts are never associated with lesions in the small intestine, as the sexual stage occurs in the cecal epithelium. Consequently, developing oocysts in enterocytes and mature oocysts in the lumen are observed in the ceca.

E. brunetti is considered less pathogenic than the previous species, where low mortality and decreased BWG have been reported only if chickens are challenged with a high inoculum of 10^5 oocysts per chicken (Kawahara et al., 2014). Few gross lesions are usually seen with low doses administered to chickens (Shirley et al., 1986). Lesions of

hemorrhagic enteritis located in the lower part of the small intestine and rectum (Hein, 1974; Mattiello et al., 2000) only appear with high doses and consist of a blood-tinged catarrhal exudate on the mucosal surface or extensive coagulation necrosis in more severe cases (Johnson & Reid, 1970). Various developmental stages such as schizonts, gamonts and oocysts can be observed in the distal intestine and lower halves of villi (Shirley et al., 1986).

E. praecox is not recognized as pathogenic in commercial production. Experimental challenges may result in the absence of gross lesions, but the inoculation of high doses can result in poorer BWG and FCR (Johnson & Reid, 1970; Reperant et al., 2012). This species invades the duodenal epithelium and thickened intestinal walls with watery to mucoid luminal content may be observed on post-mortem examinations if chickens are administrated 10^6 oocysts (Williams et al., 2009). Microscopically, villi may be severely eroded, leaving ghosts of the villi normal architecture.

E. mitis invades epithelial cells mainly in the ileum, but can also be found in the jejunum, cecal pouches, cloaca and bursa of Fabricius (Novilla et al., 1987). Although heavy infections may cause watery diarrhea, this species is not reported to be associated with gross lesions, morbidity or mortality (Fitz-Coy & Edgar, 1992; Novilla et al., 1987). On histopathology, the sporogony phase can be observed mostly in the lower half of villi, but sporozoites and merozoites may also be present in the upper half. Between 80 h and 96 h post-infection, necrosis and sloughing of mucosal enterocytes are prominent and villi length is decreased (Novilla et al., 1987).
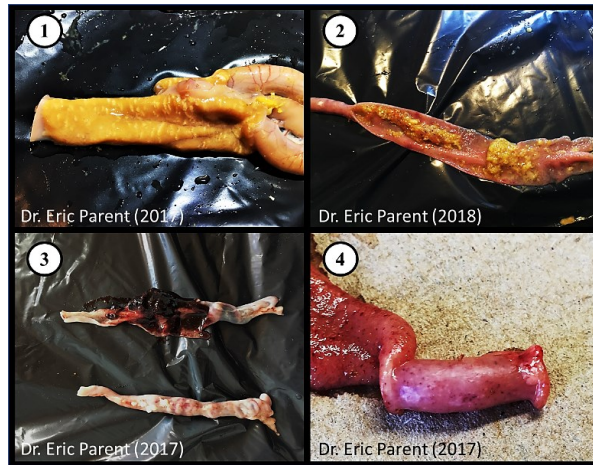
Figure 1. Gross lesions caused by (1) *E. acervulina* in the duodenum, (2) *E. maxima* in the jejunum, (3) *E. tenella* in the ceca and (4) *E. necatrix* in the ileum. *E. acervulina, E. maxima* and *E. tenella lesions* were observed in clinically normal broiler chickens naturally infected in commercial farms of the province of Quebec, Canada. *E. necatrix* lesions were observed in a 9 weeks-old broiler breeder pullet naturally infected and showing impaired growth, ruffled feathers and pasty vent in the province of Quebec, Canada.

2.2.1.8 Diagnosis

Due to characteristic gross lesions caused by various *Eimeria* spp., it is possible to make an accurate preliminary diagnosis based on post-mortem examination. Indeed, location and appearance of lesions can be used to identify *E. acervulina*, *E. maxima*, *E. tenella* and *E. necatrix*. Since *E. brunetti*, *E. praecox* and *E. mitis* produce unspecific or hardly visible lesions, post-mortem examination is less accurate to diagnose these species (McDougald & Fitz-Coy, 2013). The severity of lesions is proportional to the number of oocysts ingested where higher quantities of ingested oocysts are associated with higher lesion scores. Lesion scoring systems, such as the well-known Johnson and Reid lesion scoring system (Johnson & Reid, 1970), are widely used in research and commercial production to assess the presence and severity of coccidiosis in chickens. Johnson and Reid developed a scoring system to evaluate *E. acervulina*, *E. maxima*, *E. tenella, E. necatrix* and *E. brunetti* infection severity with a 0+ to 4+ scale, where each additional level corresponds to more severe lesions. Scores for *E. praecox* and *E. mitis* were not developed as pure colonies of these species were unavailable for their experiments at the time. In a

field context, mixed species infections are often present and may cause problems in the identification of coccidia present in a flock (Johnson & Reid, 1970). For this reason, it is preferable to confirm a diagnosis of coccidiosis by evaluating mucosal scrapings diluted with saline on a microscope slide. Indeed, developing schizonts, gametocytes and oocysts can be observed in smears and oocysts size and shape can complement lesions location and appearance to make a final diagnosis (Table 4). Since oocysts size and shape overlaps between species, microscopy cannot be used alone for a final identification but is a complement to other diagnostic methods. The number of oocysts on a slide from a mucosal scrapping can be used to evaluate the intensity of the infection and it can be occasionally present in coccidiosis scoring systems (Elanco Animal Health, 2010). Moreover, the sole presence of *Eimeria* spp. oocysts in scrapings, feces or litter should not be considered confirmatory for coccidiosis as the pathogen can be identified in chicken flocks potentially unaffected by coccidiosis (Parent et al., 2018).

Table 4. *Eimeria* spp. oocysts size and shape (Mattiello et al., 2000; McDougald & Fitz-Coy, 2013; Mehlhorn, 2016).

Eimeria species	Oocyst size			Oocyst Shape
	Length x Width	Length range	Width range	
E. acervulina	18.3 × 14.6	17.7 – 20.2	13.7 – 16.3	Ovoid
E. maxima	30.5 × 20.7	21.5 – 42.5	16.5 – 29.8	Ovoid
E. tenella	22.0 × 19.0	19.5–26.0	16.5–22.8	Ovoid
E. necatrix	20.4 × 17.2	13.2–22.7	11.3–18.3	Oblong ovoid
E. brunetti	24.6 × 18.8	20.7–30.3	18.1–24.2	Ovoid
E. praecox	21.3 × 17.1	19.8–24.7	15.7–19.8	Ovoid
E. mitis	15.6 × 14.2	11.7 – 18.7	11.0 – 18.0	Subspherical

Histopathology is a common procedure to diagnose coccidiosis in chickens. Various developmental stages can be observed with standard hematoxylin and eosin (H&E) stains from fixed gastro-intestinal tissues. Coccidia location can be helpful in determining each species (Dubey & Jenkins, 2018; McDougald & Fitz-Coy, 2013). For example, *E. acervulina* is identified by the presence of numerous developmental stages along the upper

one-third of the villi in duodenal sections, while *E. necatrix* can be identified by the presence of clusters of meronts destroying and replacing intestinal crypts and extending into the *muscularis* layer of the small intestine (Fletcher & Abdul-Aziz, 2008). *E. maxima* is recognized by the presence of various developmental stages destroying and replacing enterocytes predominantly in the jejunum, but higher magnifications also show characteristic prominent eosinophilic peripheral wall-forming granules in the parasites. *E. tenella* causes a characteristic diffuse infection of enterocytes by its intracellular stages, where destruction of villi, marked cellular infiltration in the lamina propria and edema of the papillary connective tissue can be observed simultaneously in the ceca. Oocysts can be observed in intestinal sections where the coccidia is causing infection, except for *E. necatrix* since its asexual stage occurs in the small intestine while its sexual reproduction is located in the ceca.

Molecular identification of *Eimeria* spp. is possible by using species-specific primers for amplification by PCR. Indeed, Internal Transcribed Spacer 1 (ITS-1) have been used to identify *E. acervulina*, *E. maxima*, *E. tenella*, *E. necatrix*, *E. mitis*, *E. praecox*, *E. brunetti* from field samples (Hamidinejat et al., 2010; Haug et al., 2007; Jenkins et al., 2017). However, ITS-1 regions present a high degree of polymorphism within a same species, which may result in decreased sensitivity (Lew et al., 2003; Morgan et al., 2009). Besides, the presence of multiple copies of the ITS-1 gene can be problematic for the quantification of *Eimeria* spp. Other regions such as the Sequence Characterized Amplified Regions (SCARs) may prove more useful in the identification and quantification of the seven *Eimeria* species (Peek et al., 2017; Vrba et al., 2010).

E. praecox has a significantly earlier prepatent period of 90 hours compared to the other six species. Since other diagnostic methods are less reliable for this species due to the lack of species-specific intestinal lesions and overlaps in oocysts shape and size, the prepatent period may be used to diagnose *E. praecox* by isolating oocysts from clinical samples and administering sporulated oocysts to chickens susceptible to coccidiosis, i.e., no prior infection (Mattiello et al., 2000). By determining the time required to produce oocysts following the inoculation, *E. praecox* will be accurately identified if oocysts appear in the fecal content 90 hours post-infection.

2.2.2 Necrotic enteritis

2.2.2.1 Etiology

The etiologic agent of necrotic enteritis (NE) is *Clostridium perfringens*, an anaerobic, spore-forming, rod-shaped and Gram-positive bacterium (Opengart & Songer, 2013). *C. perfringens* is further divided in seven toxinotypes (A to G) based on the production of six major toxins: α-toxin, β-toxin, ε-toxin, ι-toxin, CPE and NetB (Table 5) (Rood et al., 2018). This new nomenclature was updated from an older classification (Petit et al., 1999) of five toxinotypes (A to E) based on the production of four major toxins (α-toxin, β-toxin, ε-toxin, ι-toxin), to consider newly discovered toxins involved in various diseases. For instance, toxinotype F has been added to include isolates causing food poisoning and antibiotic associated diarrhea in humans. Toxinotype G was added to include isolates producing the NetB toxin associated with NE in chickens.

It was reported that *C. perfringens* recovered from healthy chickens or other animal species can't trigger NE when inoculated to a susceptible population of chickens (Cooper et al., 2010; Lee et al., 2011). For example, *C. perfringens* strains isolated from chickens affected by NE could reproduce the disease in 11.11% to 55.56% of challenged chickens, while strains recovered from healthy chickens or calves affected by hemorrhagic enteritis failed to reproduce NE in chickens (Timbermont, Lanckriet, Gholamiandehkordi, et al., 2009). The ability to cause NE is reported to be associated with the carriage of the *netB* gene in the genome of pathogenic *C. perfringens* strains. This plasmid encoded gene was shown to be essential in the reproduction of NE by producing the NetB toxin, a pore-forming toxin cytotoxic to avian cells (Keyburn, Bannam, et al., 2010; Keyburn et al., 2008). The importance of *netB* was questioned as a few studies identified mildly virulent *C. perfringens* strains harboring *netB* (Cooper & Songer, 2010; Smyth & Martin, 2010) or pathogenic *C. perfringens* strains without the *netB* gene (Parent et al., 2017), but the recent general consensus of the scientific community is that *netB* is an important factor involved for triggering NE in chickens (Prescott, Parreira, et al., 2016; Prescott, Smyth, et al., 2016; Rood et al., 2016; Smyth, 2016). The *netB* gene being conjugative, it has shown the ability of being transferred from pathogenic to commensal *C. perfringens* strains in the chicken gut environment and potentially converting non-pathogenic strains to pathogenic strains (Lacey et al., 2017). Additionally, the conversion of *C. perfringens* from a commensal to

NE-producing phenotype by acquiring the *netB* gene from the environment strengthens the evidence that NetB is a critical factor in the pathogenesis of NE.

Table 5. Classification of *Clostridium perfringens* by toxinotype, based on the most recent typing scheme (Rood et al., 2018).

Toxinotype	α-toxin (*plc* or *cpa*)*	β-toxin (*cpb*)*	ε-toxin (*etx*)*	ι-toxin (*iap* and *ibp*)*	CPE (*cpe*)*	NetB (*netB*)*
A	+	-	-	-	-	-
B	+	+	+	-	-	-
C	+	+	-	-	+/-	-
D	+	-	+	+	+/-	-
E	+	-	-	-	+/-	-
F	+	-	-	-	+	-
G	+	-	-	-	-	+

*Structural genes in parentheses

2.2.2.2 Epidemiology

Necrotic enteritis outbreaks caused by *C. perfringens* in commercial broiler chicken flocks have been globally reported for decades (Gardiner, 1967; Long, 1973; Parish, 1961). NE has been well controlled in poultry flocks with antibiotics (Brennan, Bagg, et al., 2001; Brennan, Moore, et al., 2001; Paradis et al., 2016), but changes in antibiotic use led to a resurgence of the disease in recent years (Gaucher et al., 2015; Lovland & Kaldhusdal, 1999, 2001; Smith, 2011).

C. perfringens is a normal inhabitant of the gastro-intestinal tract of chickens and may be recovered from fecal material of any poultry farms, although its recovery rate is higher in flocks affected by NE compared to healthy flocks (Parent et al., 2017). Because of its high resistance to adverse environmental conditions and ubiquitous nature, it can be found throughout the broiler chicken supply chain. For example, the same *C. perfringens* ribogroup has been identified in a breeder farm, hatchery, broiler farms and processing plant within an integrated poultry system, suggesting a transmission of the bacterium

between facilities (Craven et al., 2003). In hatcheries, *C. perfringens* can be found in fluff, on eggshells after hatching and on chick pads sent in poultry farms (Craven, Cox, et al., 2001). In broiler chicken operations, the bacterium can be isolated from carcasses of healthy chickens, walls, fans, fly strips, workers' boots, boot socks and dirt outside of the farm entrance (Craven, Stern, et al., 2001). At the processing plant, it can be recovered from scalding or chilling water and carcasses after chilling (Craven, 2001; Craven, Stern, et al., 2001). Additionally, it was shown that crates used for transporting broiler chickens from farms to processing plants were already contaminated with *C. perfringens*, i.e. before putting chickens in the crates (Craven, Stern, et al., 2001).

In clinical outbreaks of NE, *C. perfringens* isolates are reported to be clonal (Gholamiandekhordi et al., 2006; Nauerby et al., 2003), even between consecutive flocks of a same farm with recurring NE issues (Parent et al., 2017) or after the cleaning and disinfection process (Engstrom et al., 2012). Conversely, *C. perfringens* genomic diversity is reported to be higher in flocks showing no sign of NE (Engstrom et al., 2003; Nauerby et al., 2003; Profeta et al., 2020). Pathogenic *C. perfringens* strains are reported to have the ability to displace non-pathogenic strains from the gut (Barbara et al., 2008) and could be related to the production of bacteriocins by pathogenic strains, for example Perfrin, inhibiting the growth of non-pathogenic strains (Timbermont et al., 2014; Timbermont, Lanckriet, Pasmans, et al., 2009; Timbermont et al., 2012). In addition, *C. perfringens* isolates recovered from flocks with clinical NE are reported to carry the *netB* gene more frequently than isolates recovered from healthy flocks (Chalmers et al., 2008; Engstrom et al., 2012; Keyburn, Yan, et al., 2010; Parent et al., 2017; Valgaeren et al., 2013). However, the relatively high prevalence of *netB* reported in some healthy flocks (Abildgaard et al., 2010; Martin & Smyth, 2009) may suggest that additional factors may be required to trigger NE, since these strains are more likely to be pathogenic compared to *netB*-negative strains.

2.2.2.3 Predisposing factors

The reproduction of NE is typically unsuccessful without additional factors helping the colonization and multiplication of pathogenic *C. perfringens* strains in the intestines of broiler chickens, indicating that risk factors are important in the pathogenesis of NE.

Coccidiosis, caused by various species of *Eimeria*, is one of the most important factors predisposing chickens to NE. Field cases of NE are often associated with the presence of coccidiosis (Broussard et al., 1986; Hermans & Morgan, 2007) and coccidiosis preceding NE challenges is reported to increase the severity of the disease (Al-Sheikhly & Al-Saieg, 1980; Hofacre et al., 2018). Furthermore, the increased abundance of taxa associated with *C. perfringens* during an *E. tenella* infection has been suggested as a possible mechanism involved in the interaction between coccidiosis and NE (Macdonald et al., 2017). Coccidial infections cause epithelial damage inducing mucogenesis and may provide nutrients to *C. perfringens* required for its growth in the intestinal lumen (Collier et al., 2008). Exposing the underlying tissue following the epithelial barrier disruption may also put *C. perfringens* adhesins in close contact with collagen types IV and V and gelatin (Wade et al., 2016; Wade et al., 2015). This event may precede the production of lethal toxins, since toxin regulation systems are upregulated when *C. perfringens* binds to eukaryotic cells (Vidal et al., 2009). Various studies take advantage of the dynamic between coccidiosis and NE to reproduce the disease. For instance, coccidiosis is frequently used to trigger NE and to increase mortality or lesions severity caused by NE (Al-Sheikhly & Al-Saieg, 1980; Eeckhaut et al., 2016; Liu et al., 2019; Williams et al., 2003). The coccidial inoculum, which must be administered close before to the *C. perfringens* challenge to increase the odds of causing NE lesions (Rodgers et al., 2015; Van Waeyenberghe et al., 2016), may be constituted of individual species recovered from field cases or from a mixture of various species such as live vaccines. In the latter situation, studies typically use high vaccine doses to cause sufficient intestinal lesions to trigger NE (McReynolds et al., 2004).

Manipulation of feed ingredients is also a common tool used by researchers to reproduce NE in chickens (Chalmers et al., 2007; Keyburn et al., 2008; Van Waeyenberghe et al., 2016; Williams et al., 2003), as various ingredients are recognized as risk factors for NE. For example, wheat, rye, barley or oat groats have been associated with increased NE mortalities and lesions severity compared to corn-based diets (Branton et al., 1997; Branton et al., 1987; Kaldhusdal & Hofshagen, 1992; Riddell & Kong, 1992). In Norway, historic field data has shown that two important epidemics of NE were closely associated to the use of barley and wheat. Conversely, the use of higher levels of maize in diets was considered

a protective factor against NE (Kaldhusdal & Skjerve, 1996). *In vitro* experiments showed that proliferation of *C. perfringens* is higher in digested wheat and barley diets compared to a digested corn diet (Annett et al., 2002), which may contribute to increase *C. perfringens* counts in the gastro-intestinal tract. For instance, *in vivo* experiments reported that *C. perfringens* counts are higher in the intestine of chickens fed a rye-based diet compared to a corn-based diet (Craven, 2000). Additionally, protein source and levels of crude protein in diets may impact performance and *C. perfringens* counts in the gastro-intestinal tract (GIT) (Drew et al., 2004). More specifically, the use of various levels of fishmeal as a protein source would decrease body weight gain and increase ileal and cecal counts of *C. perfringens* compared to diets using soy as the protein source.

The presence of mycotoxins in the feed can predispose chickens to NE. For example, NE lesions were more severe during a *C. perfringens* challenge in chickens fed deoxynivalenol (DON) or fumonisins (FB) contaminated feed compared to diets without these mycotoxins (Antonissen et al., 2015; Antonissen et al., 2014). DON-contaminated diet was associated with decreased duodenal villi height, reduced transepithelial electrical resistance in the duodenum and increased protein concentrations in the duodenal lumen, indicating that DON may damage epithelial cells. Chickens consuming FB-contaminated feed showed decreased villi length and altered sphingolipid metabolism compared to control diets. These events may promote *C. perfringens* overgrowth in the GIT by providing nutrients to the bacterium, as shown with higher ileal *C. perfringens* counts in chickens fed FB. The contamination of feed by mycotoxins is a serious concern since various studies reported regular contamination of feed and feed ingredients by a wide variety of mycotoxins, including DON and FB levels above the concentrations evaluated by Antonissen et al. (Al-Jaal et al., 2019; Streit et al., 2012). Mycotoxins are also well known for their immunosuppressive effects (Bondy & Pestka, 2000; Oswald et al., 2005), but a direct connection between mycotoxin-related immunosuppression and NE would still needs to be demonstrated.

Immunosuppressive diseases are also thought to play a role in predisposing chickens to NE. In a study, 10x of the recommended dose of a commercial live Infectious bursal disease (IBD) vaccine was compared to the administration of 24x of the

recommended dose of a live coccidial vaccine for triggering NE in a challenge model (McReynolds et al., 2004). In addition to causing regressed bursa of Fabricius, NE lesions scores and *C. perfringens* counts were similar between groups receiving either the IBD or coccidial vaccine. Moreover, both groups showed a significant increase of both outcomes compared to chickens challenged with *C. perfringens* only.

Environmental factors are also reported to increase chickens' susceptibility to NE. For example, it has been shown that wet litter, stocking density and cold stress can increase the occurrence of NE, the severity of intestinal lesions and *C. perfringens* cecal counts (Hermans & Morgan, 2007; Tsiouris et al., 2015a, 2015b).

2.2.2.4 Clinical signs

Broiler chickens affected by NE are usually depressed, reluctant to move and often unable to reach food or water (Brennan, Moore, et al., 2001; Opengart & Songer, 2013). A study reported that no clinical signs were observed before 8 hours post-infection (PI), but birds could be depressed, moribund, recumbent and unable to stand between 8 and 12 hours post-infection (Al-Sheikhly & Truscott, 1977). Whitish to greenish diarrhea may be occasionally observed in chickens infected with pathogenic *C. perfringens* strains (Al-Sheikhly & Al-Saieg, 1980). Mortality may occur within a few hours to a few days following the infection with *C. perfringens*. In peracute cases, clinical signs may be absent before death, carcasses may be in good body condition and feed may be present in their digestive tract, suggesting that chickens were in normal condition before death (Al-Sheikhly & Al-Saieg, 1980).

Poor zootechnical performance has been reported in chickens affected by NE. For example, chickens challenged with *C. perfringens* in research facilities may have impaired feed intake, average daily gain, feed conversion ratio and increased mortality compared to negative controls (Brennan, Moore, et al., 2001; Paradis et al., 2016). Still, different strains may cause different impacts on growth performance parameters and mortality (Chalmers et al., 2007). In commercial farms, diseases associated with *C. perfringens* have been associated with impaired mean live weight at slaughter, mean daily weight gain, feed conversion ratio and increased condemnations (Gaucher et al., 2015; Lovland & Kaldhusdal, 2001).

2.2.2.5 Macroscopic lesions

C. perfringens mainly damages the proximal part of the jejunum, but the entire small intestine (duodenum, jejunum and ileum), cecum and/or colorectum may be affected (Olkowski et al., 2006; Smyth, 2016). Lesions may vary in severity and may be focal, multifocal, coalescent or diffuse. The intestine is often flaccid, friable and distended by the accumulation of gas and/or brownish fluid (Figure 3.1). While opening the intestinal tract, a foul odor may be experienced due to gas leaking from the gut. The intestinal mucosa may become thickened because of fibrin and necrotic debris accumulating on the mucosa and in more severe cases, the mucosa may be covered by a "Turkish towel" (Broussard et al., 1986) appearance consisting of a diphteric pseudomembrane partly or entirely covering the small intestine (Figure 3.2). In milder cases, ulcers and re-epithelialized craters (erosions) may be observed randomly and multifocally throughout the small intestine. Occasionally, ulcers may be visible from the serosal surface (Olkowski et al., 2006). Mesenteric vessels may be engorged with blood (Olkowski et al., 2006). Cholangiohepatitis is a complication of the intestinal disease if *C. perfringens* reaches the liver through bile ducts. In that case, thickened gall bladder walls may be seen in addition to congested livers (Figure 3.1) or multiple 1-2 mm necrotic foci in the liver parenchyma (Al-Sheikhly & Truscott, 1977; Frame & Bickford, 1986; Lovland & Kaldhusdal, 1999; Sasaki et al., 2000).

Figure 2. Gross lesions of necrotic enteritis in a naturally infected chicken found dead in a broiler chicken flock of approximately four weeks of age in the province of Quebec, Canada. In (1), a flaccid intestine distended by gas, fluid and necrotic debris is a prominent

presentation of NE caused by *C. perfringens*. Dark, small and congested livers, as displayed in (1), are occasionally found during post-mortem examination of field cases. In (2), a striking "Turkish towel" is visible on the mucosa of the jejunum, which consists of a diphteric pseudomembrane covering a large portion of the small intestine.

The appearance of NE lesions in chickens is time dependant (Al-Sheikhly & Truscott, 1977). No lesions may be observed 1 hour PI, but the duodenum and jejunum may become grayish and the mucosa thickened 3 hours PI. Marked necrosis of the intestinal mucosa, typical of field cases, may be observed as early as 5 hours PI. Intestines are usually severely necrotic 8 to 12 hours PI and grossly hemorrhagic intestines with blood-stained fluid content in the lumen may be occasionally observed. Congested livers may also be observed after 8 hours PI.

2.2.2.6 Microscopic lesions

Diffuse and severe fibrinous to fibrinonecrotic enteritis and desquamation of the intestinal epithelium with the infiltration of numerous mononuclear cells in the lamina propria is a typical presentation of NE field cases (Figure 4.1) (Broussard et al., 1986; Frame & Bickford, 1986). The resulting coagulation necrosis preserves the architecture of tissues and create "ghost villi" because of the absence of viable cells even if villi can be recognized by their structure (Figure 4.1) (Al-Sheikhly & Al-Saieg, 1980; Olkowski et al., 2006). Lesions may be localized to the villi tips in mild cases, but they may extend to the entire villi, crypts, lamina propria, submucosa and muscularis mucosa in more severe cases. A demarcation line including a high density of heterophils between necrotic and viable tissues might be visible (Figure 4.2) (Nairn & Bamford, 1967). Large clusters of Gram-positive rod-shaped bacteria are normally localized on the necrotic villi and amongst necrotic material in the lumen (Broussard et al., 1986; Gholamiandehkordi et al., 2007; Parent et al., 2017; Williams et al., 2003). These rods have been confirmed to be *C. perfringens* by immunochemistry (Kaldhusdal et al., 1995). The lamina propria can be infiltrated by a variable number of heterophils and blood vessels may be congested (Al-Sheikhly & Al-Saieg, 1980; Gholamiandehkordi et al., 2007; Parent et al., 2017; Smyth, 2016). Histopathological findings related to cholangiohepatitis may include proliferation of bile ductules, granulomas, extramedullary hematopoiesis, lymphocytic accumulation

and clusters of Gram-positive bacilli in the affected areas (Sasaki et al., 2000). However, the only major lesion found in chronic cases might be fibrinoid necrosis, as observed in livers examined histologically from chickens at slaughter (Lovland & Kaldhusdal, 1999).

Similar to gross lesions, microscopic lesions caused by *C. perfringens* are time dependant (Al-Sheikhly & Truscott, 1977). One hour following the infusion of a broth culture of *C. perfringens* in the duodenum of male broiler chicks, mild lesions can be observed: slight edema and dilatation of vessels in the lamina propria with some sloughed epithelial cells in the intestinal lumen. Mononuclear cells may also be present in the lamina propria. Large numbers of Gram-positive rods can be observed in luminal debris and on the epithelial surface of the mucosa. Three hours PI, there is marked edema and the epithelial layer is detaching from the lamina propria, mostly at the apex of villi, and early stages of degeneration of villi tips may be observed. Infiltration of mononuclear cells in the lamina propria is more marked than 1-hour PI and heterophils can be occasionally identified. Five hours PI, there is a marked coagulation necrosis of the villi tips epithelial layer. Intestinal blood vessels are severely congested and hyaline thrombi may be occasionally present. Numerous bacteria can be identified at the apex of the exposed lamina propria and in necrotic debris. Inflammatory response is similar to 3 hours PI. At 8- and 12-hours PI, severe necrosis of the villi occasionally extending to the crypts of Lieberkühn may be observed. No significant histological changes were observed in other tissues for all time points recorded, which extended to 12 hours PI. However, frequent congestion and marked changes to erythrocytes morphology could be identified throughout the experiment.
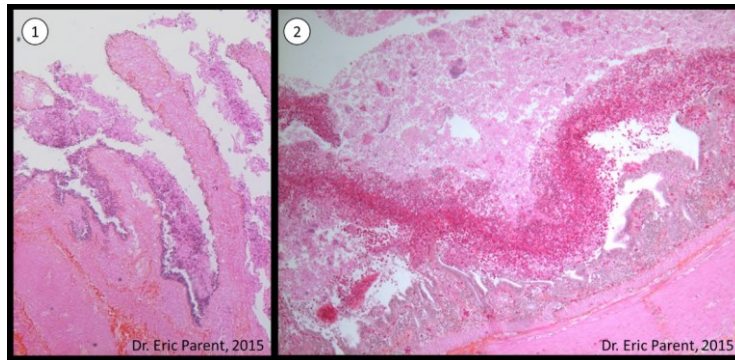
Figure 3. Microscopic intestinal lesions of necrotic enteritis in leghorn chickens of 10 weeks of age experimentally infected with a pathogenic *C. perfringens* strain. In (1), resembling typical field cases of NE, "ghost villi" can be identified by an extensive necrotic to fibrinonecrotic enteritis with large numbers of Gram-positive rod-shaped bacteria in the necrotic debris. In (2), a sharp line of demarcation between viable and necrotic tissue is clearly visible and represents an area densely populated by heterophils.

2.2.2.7 Diagnosis

The diagnosis of NE caused by *C. perfringens* relies on the combination of multiple criteria since *C. perfringens* can be recovered from healthy chickens (Smyth, 2016). Clinical signs, macroscopic findings and microscopic lesions are highly suggestive of NE in chickens. However, *Clostridium colinum*, *Clostridium sordellii*, *Clostridium difficile* and numerous other potential pathogens have been described to cause one or more signs similar to those found in chickens affected by NE (Cooper et al., 2013; Uzal et al., 2016). Intestinal *C. perfringens* counts are most likely of low value to diagnose NE in chickens found dead since the bacterium multiplies in high numbers after death. For instance, it was reported that enterotoxemia in calves, caused by *C. perfringens*, could not be diagnosed by intestinal clostridial counts since mean *C. perfringens* counts were similar between cases of enterotoxemia and controls, even when sampled within 3 hours after death (Valgaeren et al., 2013). Molecular identification of the *cpa* gene of *C. perfringens* is not a good indicator of NE as it can be detected from healthy chickens (Profeta et al., 2020) and its presence was reported non-essential to trigger the disease (Keyburn et al., 2006). The gene *netB* has been shown to be a crucial factor to reproduce NE in chickens (Keyburn et al., 2008), but its presence cannot be used alone for a final diagnosis since it can be present from isolates

recovered from healthy flocks showing no sign of NE (Keyburn, Yan, et al., 2010). Rapid onset of mortality, macroscopic lesions and microscopic findings combined with the isolation of *netB*-positive *C. perfringens* from intestines of suspected birds should provides enough evidence to confirm a diagnosis of NE in broiler chickens.

2.2.3 Prevention of intestinal diseases in commercial broiler chickens

The ubiquitous nature of common intestinal pathogens such as *Eimeria* spp. and *C. perfringens* in broiler chickens and their high resistance to various environmental conditions make it virtually impossible to eliminate these pathogens or to avoid the contamination of commercial broiler chicken during grow-out. Hence, the prevention of intestinal diseases in a commercial context primarily focuses on controlling rather than eliminating the exposition to these pathogens. Since coccidiosis and NE are synergetic, it is also of the foremost importance to elaborate a prevention plan integrating both diseases to successfully control intestinal disorders in broiler chicken flocks. Maintaining a healthy gut and controlling risk factors for both diseases have been crucial for the contemporary commercial broiler chicken production and different approaches are used to control these intestinal diseases.

A turning point in the control of coccidiosis has been the discovery of synthetic anticoccidial compounds, commonly grouped as "chemicals" with a broad spectrum of action against multiple *Eimeria* species (Chapman, 2014; Peek & Landman, 2011). These products possess specific modes of actions targeting the metabolism of the parasite (Noack et al., 2019) such as inhibition of parasite mitochondrial respiration (decoquinate, clopidol, toltrazuril) (Fry & Williams, 1984; Harder & Haberkorn, 1989; Williams, 1997), inhibition of the folic pathway (sulfonamides) (McCullough & Maren, 1974) or the competitive inhibition of thiamine uptake (Amprolium) (James, 1980). There are still modes of action that have not been elucidated for some chemicals, for example diclazuril, halofuginone, nicarbazin and robenidine (Noack et al., 2019). Several chemicals administered via drinking water or feed are licensed in Canada for the prevention or control of coccidiosis in broiler chickens (Table 6) and anticoccidial prevention programs in Canada typically include chemicals during the first stages of grow-out in broiler chicken production (Parent et al., 2018).

Ionophores are a type of anticoccidials produced by the *Streptomycetaceae* family and commonly used in broiler chicken production to prevent coccidiosis. For instance, it has been reported that Canadian broiler chicken flocks monitored between 2013 and 2015 ($n = 373$) were administered either lasalocid (4%), maduramycin (3%), monensin (30%), narasin (20%), narasin-nicarbazin (31%) or salinomycin (38%) for the prevention of coccidiosis (Agunos et al., 2017). Indeed, these molecules are listed as veterinary drugs approved for the prevention of coccidiosis in broiler chickens (Table 6). Ionophores, meaning ion carriers, catalyse the transport of ions between the intra- and extracellular compartments of coccidia by binding to cations such as K^+, Na^+, Ca^{2+} or Mg^{2+} (Jaenicke, 1983) and leading to disbalanced cation balance in the cell. For example, *in vitro* exposure of free *E. tenella* sporozoites to monensin caused structural changes such as swelling, vacuolation and a distinctive bleb on scanning electron microscopy (Smith et al., 1981). This experience also led to the conclusion that monensin, and potentially other ionophores, can exert their effects outside the host cells. It further indicated that ionophores could have an anticoccidial activity in the gastro-intestinal lumen. Because ionophores do not fully kill the parasites before the zygotes infect intestinal cells, hosts can normally develop an immunity against coccidia during the administration of ionophores in the medicated feed and after a first exposure to this pathogen.

Table 6. List of veterinary drugs approved in Canada for the prevention of coccidiosis in broiler chickens (Compendium of veterinary products - Canada edition, 2020).

Route of administration	Dosage	Veterinary drug	Concentration	Withdrawal time in meat (days)
	Chemicals			
In feed	Feed continuously as the sole	Amprolium	0.0125% or 0.025%	0
		Clopitol	0.0125%	0

	ration from day-old to slaughter	Decoquinate	30 mg/kg of feed	0
		Diclazuril	0.0001%	0
	Feed continuously as the sole ration from day-old to 4 days prior to slaughter	Nicarbazin	0.01% to 0.015%	4
	Feed continuously as the sole ration from day-old to 6 days prior to slaughter	Robenidine	33 mg/kg of feed	6
Drinking water	Administer for 2 consecutive days out of 5 from day old until 6 to 10 weeks	Sulfaquinoxaline 19.2%	0.132%	12
	First 2 days at 0.15% followed by 3 days without medication; repeat until all dangers of infection are gone.	Sulfaquinoxaline and Pyrimethamine	0.15%	4

Ionophores

In feed	Feed continuously as the sole ration from day-old to slaughter	Lasalocid	105 mg/kg of feed	0
		Monensin	100 mg/kg of feed	0
		Narasin	70 mg/kg of feed	0

		Salinomycin	60 mg/kg of feed	0
	Feed continuously as the sole ration from day-old to 5 days prior to slaughter	Maduramycin	5 mg/kg of feed	5
	Potentiated ionophores			
In feed	Feed continuously as the sole ration from day-old to slaughter	Narasin and Nicarbazin	40 to 50 mg/kg of feed for each product	0

One of the major drawbacks of using anticoccidials is the ability of coccidia to develop resistance to these drugs and survive or multiply despite the administration and absorption of doses usually effective against them, even if the development of resistance against ionophores is generally considered slow (Chapman, 1984). Still, decreased sensitivity to ionophores has been described as a potential problem related to the long-term use of these products in commercial farms. For instance, this decreased effectiveness may increase the coccidiosis challenge during grow-out, as reported by an anecdotal investigation showing very high oocysts per gram (OPG) counts in one commercial broiler chicken facility using an anticoccidial control program relying on ionophores (Snyder et al., 2021). Managing resistance through products rotations between flocks and the use of successional products within a same grow-out (commonly named shuttle programs) have been two pivotal strategies used by poultry health professionals to ensure the sustainability of prophylactic chemotherapy strategies to prevent coccidiosis (Chapman, 2014). More specifically, drugs with different mode of actions are used in a shuttle program within a same flock and drugs are rotated after a few flocks to avoid the development of resistance or a decrease in the effectiveness of anticoccidials. Chemical anticoccidials or a mixture of a chemical and an ionophore are often used in the front end of the shuttle while ionophores

are more commonly used in the end of the program (Chapman, 2001; Parent et al., 2018), but flocks may also be exclusively administered ionophores during the entire grow-out for the prevention of coccidiosis (Chapman, 2001). A more recent type of anticoccidial program, called bio-shuttle, involves the use of a live *Eimeria* spp. vaccine at day-old, followed by the use of chemicals or ionophores during grow-out (Kimminau & Duong, 2019). It has been implemented in commercial farms to decrease the development of anticoccidial resistance, but the exact mechanism by which sensitivity to anticoccidials is restored or the success of such strategy is currently undefined. Since the species included in live vaccines are drug-sensitive, it is thought that inoculating chicks with the vaccine constantly seeds broiler chicken farms with drug-sensitive vaccine oocysts. Live coccidial vaccines may also be administered to broiler chicks without the use of anticoccidials, as approved for use in Canada (Table 7). Current registered vaccines in Canada may contain attenuated or non-attenuated strains of various live *Eimeria* species less virulent than field strains, and these products aim to immunize the chickens against coccidiosis before entering in contact with potentially more virulent resident coccidia (Ahmad et al., 2016; Peek & Landman, 2011; Witcombe & Smith, 2014). However, the large delay between the exposition to the vaccine and the development of an effective immunity against coccidiosis may pose a risk for the chickens to develop clinical signs of coccidiosis, since their exposition to environmental strains of coccidia is most likely occurring shortly after placement on the farm because viable *Eimeria* oocysts can be present in poultry house litter at the time of chicks placement (Jenkins et al., 2019). The choice of an anticoccidial program will provoke large changes in the dynamics of *Eimeria* spp. populations identified in broiler chicken farms. Indeed, it was shown that using chemotherapy or vaccination to prevent coccidiosis will change the excretion of *Eimeria* spp. oocysts present in litter or feces during grow-out (Jenkins et al., 2017; Parent et al., 2018; Snyder et al., 2021).

Table 7. List of vaccines approved in Canada for the immunization against coccidiosis in chickens (Compendium of veterinary products - Canada edition, 2020).

Vaccine	Type of vaccine	*Eimeria* species	Route of administration	Age of administration (days)	Withdrawal time in meat (days)
Coccivac®-B52 *(Merck Animal Health)*	Non-attenuated	*E. acervulina* *E. maxima* *E. maxima MFP* *E. mivati* *E. tenella*	Spray cabinet	1	21
Coccivac®-D2 *(Merck Animal Health)*	Non-attenuated	*E. acervulina* *E. brunetti* *E. maxima* *E. mivati* *E. necatrix* *E. tenella*	1) Spray cabinet 2) On the feed	1) 1 2) 4	21
Hatchpak™ Cocci III *(Boehringer Ingelheim)*	Attenuated	*E. acervulina* *E. maxima* *E. tenella*	Spray cabinet	1	21
Immucox® For Chickens I *(Ceva Animal Health)*	Non-attenuated	*E. acervulina* *E. maxima* *E. necatrix* *E. tenella*	Drinking water	Up to 5 day-old	21
Immucox® For Chickens II	Non-attenuated	*E. acervulina*	Drinking water	Up to 5 day-old	21

(Ceva Animal Health)	*E. brunetti*
	E. maxima
	E. necatrix
	E. tenella

The prevention of NE in broiler chickens has been traditionally done by using low doses of antibiotics effective against *C. perfringens* in the feed of broiler chickens. For instance, three antibiotic products are currently registered in Canada for the prevention of necrotic enteritis by administering the medication for 21 continuous days or from day-old to slaughter (Table 8). Many studies showed the efficacy of various antibiotics to prevent NE, including tylosin, amoxicillin, bacitracin, avoparcin, virginiamycin and avilamycin (George et al., 1982; Martel et al., 2004; Paradis et al., 2016; Prescott, 1979; Prescott et al., 1978; Wicker et al., 1977). In addition to prevent mortality caused by *C. perfringens* in challenge models compared to chickens receiving no antibiotics, the use of these antibiotics is also shown to decrease the severity of intestinal lesions associated with necrotic enteritis and to improve growth parameters such as BWG and FCR. Ionophores, in addition to their anticoccidial effects, were shown to exhibit significant antibacterial effects on *C. perfringens*. For instance, various ionophores, such as monensin, lasalocid, maduramycin, narasin and salinomycin, showed low *in vitro* MICs against *C. perfringens* categorizing these strains as susceptible to these ionophores (Lanckriet et al., 2010; Martel et al., 2004). Similar to other antibiotics, these products are also reported to prevent necrotic enteritis lesions *in vivo* and associated mortality in broiler chickens in *C. perfringens* challenge models compared to non-medicated infected groups (Lanckriet et al., 2010). At the commercial level, it is thought that ionophores may play an important role to prevent clinical NE in poultry. For example, an upsurge in the consumption of antibiotics has been observed in Norway following the ban of avoparcin in 1995, but the low antibiotic treatment frequency of NE in the following years could be at least partially explained by the introduction of narasin as a feed additive for use in broiler chickens (Grave et al., 2004).

Table 8. List of veterinary drugs approved in Canada for the prevention of necrotic enteritis in broiler chickens (Compendium of veterinary products - Canada edition, 2020).

Route of administration	Dosage	Veterinary drug	Concentration	Withdrawal time in meat (days)
In feed	Feed continuously as the sole ration for 21 days during the necrotic enteritis risk period	Avilamycin	15 to 30 mg/kg of feed	0
	Feed continuously as the sole ration from day-old to slaughter	Bacitracin	55 mg/kg of feed	0
		Virginiamycin	22 mg/kg of feed	0

Many non-antibiotic products, for example probiotics, mannan oligosaccharides and similar yeast derivatives; botanicals such as yucca, oregano, and cinnamon derivatives; and organic and mineral acid mixtures, are often used by the industry for the prevention of NE (Smith, 2011), but none of these products are currently registered for this indication in Canada. While these compounds are reported to decrease the severity of the disease and counts of intestinal *C. perfringens* in research settings (Diaz Carrasco et al., 2016; Eeckhaut et al., 2016; Engberg et al., 2012; Pham et al., 2020), commercial operations report the use of these products as hardly justifiable due to the lack of economic return and the absence of improvement regarding the incidence of clinical NE compared to negative control groups without antibiotic or non-antibiotic products (Smith, 2011).

2.2.4 Treatment of intestinal diseases in broiler chickens

In the event of a clinical outbreak of coccidiosis or NE causing mortality and/or significant clinical signs associated with these diseases, treatments using various products specifically targeting the causal agent can be implemented to mitigate the impacts of the disease in a flock. Anticoccidial drugs and antibiotics have been used successfully to treat

coccidiosis or necrotic enteritis. For instance, various veterinary drugs are licensed in Canada to treat or mitigate mortality and clinical signs associated with coccidiosis or necrotic enteritis in chickens (Table 9) (Compendium of veterinary products - Canada edition, 2020).

Table 9. List of veterinary drugs approved in Canada for the treatment of coccidiosis or necrotic enteritis in broiler chickens (Compendium of veterinary products - Canada edition, 2020).

Route of administration	Dosage	Veterinary drug	Concentration	Withdrawal time in meat (days)
Coccidiosis				
	First 5 to 7 days	Amprolium	0.024%	0
	Additional 1-2 weeks		0.006%	
	First 2 days at 0.4%, then no medication for 4 days, treat at 0.4% for 1 day, stop for 4 days and treat at	Sulfamethazine 25%	0.40%	12
Drinking water	0.4% for 1 more day.			
	First 2 days at 0.198%, then no medication for 3 days, treat next 2 days at 0.132%. Repeat once if bloody droppings appear.	Sulfaquinoxaline 19.2%	0.198% and 0.132%	12

	First 2 days at 0.15% followed by 3 days without medication; repeat until all clinical signs are resolved.	Sulfaquinoxaline and Pyrimethamine	0.15%	4

Necrotic enteritis

Drinking water	Administer continuously for 7 days	Lincomycin	16 mg /L	0
	Administer continuously for 5 days	Penicillin G Potassium	297 000 I.U. /L	1
	Administer for 1 to 5 days	Tylosin	100 to 150 mg/L	0
In feed	Feed continuously for 7 days	Bacitracin	110 mg/kg of feed	0
	Feed continuously for 7 days	Tylosin	200 mg / kg of feed	0

2.3 The gastro-intestinal microbiota of broiler chickens

2.3.1 Characterization of the microbiota

The microbiota refers to all microorganisms found in a specific environment, which may include viruses, bacteria, archaea, protozoa and fungi (Morgan & Huttenhower, 2012). Current techniques explore the bacterial microbiota composition by analyzing the genes catalog of these microbes, defined as the microbiome, with culture-independent methods. These methods have the advantage of identifying taxa difficult to detect by culture-dependant techniques traditionally used in microbiology (Morgan & Huttenhower, 2012; Pace et al., 1986). These culture-independent techniques are based on extracting and

sequencing DNA directly from a biological sample without culturing microbes, and then analyzing genomic characteristics describing these microbial communities. Various techniques based on 16S ribosomal RNA (rRNA) gene amplification, for example denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP), have been initially described as culture-independent methods for analyzing the diversity and composition of complex microbial populations (Liu et al., 1997; Muyzer et al., 1993). In recent years, next-generation sequencing (NGS) technologies established themselves as reliable and powerful tools to classify bacterial communities because of their capability to accurately sequence DNA and RNA at an affordable price (Park & Kim, 2016). These platforms generate large quantities of data, an outcome harder to attain with older sequencing platforms such as Sanger sequencing (Mardis, 2011). For example, the Human Genome Project required almost 15 years to sequence approximately 6 gigabases with the Sanger platform, while the Genome Sequencer FLX instrument achieved this task in two months (Wheeler et al., 2008). Hence, access to cheaper, more efficient new generation sequencing methods was followed by an exponential increase in the number of publications on chicken microbiota in recent years; from a few publications per year in the early 2000's to more than 150 publications per year in 2017, 2018 and 2019 (Figure 5).
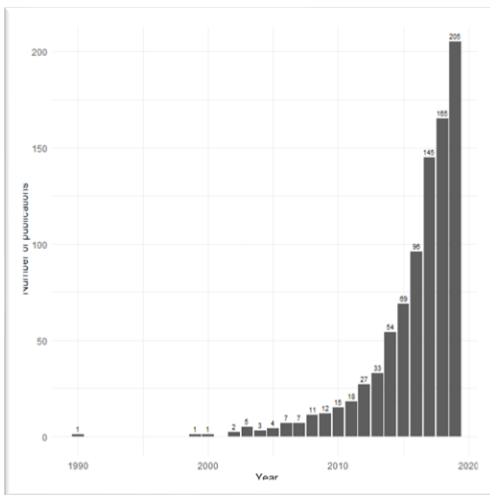
Figure 4. Number of publications on chicken microbiota from 1990 to 2019 (20 years), based on a PubMed search by keywords "chicken" and "microbiota" (Updated January 20, 2020).

The 16S rRNA is part of the prokaryotic 30S small subunit ribosome present in every bacterium and archaea. Its related gene (16S rRNA gene) is of particular interest for phylogenetic studies as it has been highly conserved among bacterial and archaeal species, which is a consequence of its slow evolution. This feature allows the use of universal primers for the amplification of the target gene from every bacterium and archaea in a biological sample. Between these conserved regions, nine hypervariable regions (V1 to V9) have been identified in the 16S rRNA gene structure and these segments were shown to greatly vary between bacterial species (Chakravorty et al., 2007; Lane et al., 1985). The 16S rRNA gene of every bacterial and archaeal species can be sequenced by using universal primers targeting highly conserved regions, but DNA sequences of amplified products will differ depending on the hypervariable regions of each bacterial species. Consequently, the classification of the 16S rRNA genes can be done by associating specific nucleic acid sequences of a hypervariable region to its corresponding bacterial species. Due to the 16S rRNA gene length exceeding sequencing capabilities of the most widely used sequencing platforms, full-length sequencing of the gene has often been replaced by partial sequencing of a few hypervariable regions (Johnson et al., 2019). For example, the approximately 1500 base pairs (bp) composing the full 16S rRNA gene exceed the Illumina range, which produces short sequences ($\leq$ 300 bp). The choice of a hypervariable region for sequencing is important as significantly different bacterial community structures may be generated by choosing different hypervariable regions, even within a same biological sample (Bukin et al., 2019; Chakravorty et al., 2007; Graspeuntner et al., 2018; Johnson et al., 2019; Yang et al., 2016). Additionally, it has been reported that sequencing of a few hypervariable regions would be less accurate than full-length sequencing of the 16S rRNA gene (Johnson et al., 2019). Nonetheless, it has been proposed to combine sequences of multiple hypervariable regions to improve the accuracy of the results (Fuks et al., 2018).

In 16S rRNA analysis pipelines, sequences are clustered and assigned to "operational taxonomic units (OTU)" based on their similarity (Nguyen et al., 2016), an operation called "binning". A similarity threshold is frequently established arbitrarily at 97%, as this level of similarity is considered acceptable to define bacterial strains belonging to a species (Konstantinidis & Tiedje, 2005; Nguyen et al., 2016; Stackebrandt & Goebel, 1994). For this reason, the OTU is seldom used interchangeably with the bacterial species.

However, this threshold has been recently challenged; an analysis showed that 97% similarity would be too low and thresholds of approximately 99% and 100% respectively for the V4 hypervariable region and full length 16S rRNA gene would be required for clustering all sequences of a bacterial species within an OTU (Edgar, 2018). New methods using the amplicon sequence variants (ASVs) have been proposed to replace the arbitrary clustering of similar sequences in OTUs as they give a higher resolution to the level of single-nucleotide differences in the amplified region (Callahan et al., 2017). In a study comparing the performance of two OTU clustering algorithms and 3 ASV methods to correctly identify mock community composition from 16S rRNA sequence reads, it has been shown that no method could identify perfectly true community sequences (Caruso et al., 2019). However, ASV methods proved to be more accurate when compared to OTU clustering algorithms. Most popular methods for resolving ASVs include DADA2 (Callahan et al., 2015), Deblur (Amir et al., 2017), MED (Eren et al., 2015) and UNOISE2 (Edgar, 2016).

A reference database containing the information on bacterial 16S rRNA sequences can be used after binning to assign each OTU to a taxon. GreenGenes (DeSantis et al., 2006), SILVA (Glockner et al., 2017) and Ribosomal Database Project (RDP) (Cole et al., 2014) are among the most used public reference databases in microbiota studies based on the analysis of the 16S rRNA gene. GreenGenes provides chimera screening, standard alignment and taxonomic classification for Bacteria and Archaea using multiple taxonomies. Its database has been used in a multitude of scientific articles, but the relative long time since the last update (between 2012 and 2013) created doubts on its current usability compared to databases updated more frequently. SILVA is a comprehensive web-based resource providing services for quality checked and aligned rRNA sequence data for small (16S/18S) and large subunit (23S/28S) rRNA sequences for Bacteria, Archaea and Eukarya. The first SILVA release was in 2007 and its database is regularly updated. RDP, firstly released in 1992 (Olsen et al., 1992) and last updated in 2016, offers quality controlled, aligned and annotated bacterial and archaeal 16S rRNA sequences, fungal 28S rRNA sequences and various analysis tools available for use by the scientific community. It has been shown that these databases share many taxonomic units and compare well to the daily updated NCBI sequences database (Balvociute & Huson, 2017), and there is no

clear choice at the moment on the best database available for microbial communities assessment. A study showed that GreenGenes, SILVA and RDP databases contain between 0.2% to 2.5% of mislabelled sequences in their taxonomy (Kozlov et al., 2016), which could be empirically considered as a low percentage of errors. Another study estimated annotation errors to approximately 10% in RDP and 17% in GreenGenes and SILVA (Edgar, 2018). However, it is important to consider these 2 studies did not use the same databases versions. Indeed, Kozlov, et al (2016) used GreenGenes 13.8, SILVA v123 and RDP v11 while Edgar (2018) used GreenGenes v13.5, SILVA v128 and RDP v16. These differences may be an undetermined source of variation between the two studies and modify the level of agreement between databases.

Microbiota studies are often based on descriptive or comparative analyses of OTU formed with bacterial 16S rRNA amplicons sequenced from one or more groups of biological samples. Describing these complex bacterial communities relies on concepts developed in the field of community ecology (Costa & Weese, 2019). Firstly, a microbial community can be characterized by the alpha diversity, which corresponds to the diversity of OTU contained within an environment. It includes Richness, i.e. the number of different OTU present in the community, and Evenness, which describes the distribution and uniformity of these OTU in the community (Costa & Weese, 2019; Kim et al., 2017; Morgan & Huttenhower, 2012). Several diversity indices have been developed to evaluate the alpha diversity in biological samples, such as the Shannon diversity index, Simpson's index, ACE and Chao1. Depending on the formula used for each estimator, a diversity index will put more weight on Richness or Evenness (Kim et al., 2017). There is no actual consensus on the most relevant index for evaluating alpha diversity and a combination of multiple indices is frequently presented in microbiota analyses (Morgan & Huttenhower, 2012). Diversity index values were shown to increase with sample size as the number of unique OTU identified will be higher as more individual sequences are sampled (Gotelli & Colwell, 2001; Lemos et al., 2011). In other terms, comparing the Richness from samples with different number of reads could be caused by a real Richness difference between samples, but it could also lead to misleading conclusions as a high number of sequence reads is expected to cause an increased Richness in samples. Normalizing the number of

reads per sample obtained from sequencing by rarefying the results is critical to avoid bias when comparing alpha diversity indices between groups.

A second community ecology concept used to evaluate the microbiota is beta diversity, which compares differences in community membership and structure between individuals or groups (Costa & Weese, 2019). Beta diversity is evaluated by using multivariate explanatory, interpretive or discriminatory methods accounting for the presence and relative abundance of taxa in different samples or groups (Paliy & Shankar, 2016). The results of these ordination analyses are mostly presented graphically, where unconstrained or constrained techniques are used to respectively reflect the overall variance in the data or explain the species abundance associated with specified explanatory variables (Paliy & Shankar, 2016). For example, various ordination techniques and multivariate statistical methods, such as Principal Component Analysis (PCA), Principal Coordinate Analysis (PCoA), UniFrac, Nonmetric Multidimensional Scaling (NMDS) and Redundancy Analysis (RDA) have been used to describe the variation in various bacterial communities. With the advancement of machine learning techniques applied to microbiota analysis, machine learning can be used to predict host characteristics based on the composition of their microbiota (Qu et al., 2019; Zhou & Gallins, 2019). For example, it has been possible to determine the human deficiency virus (HIV) status (infected vs. non infected) based on its microbiota composition with a 4.33% ± 0.821% error rate using a Random Forest classifying algorithm, compared to a 45.83% error rate by random guessing (Lozupone et al., 2013).

2.3.2 Roles of the gastro-intestinal microbiota in chickens

2.3.2.1 Involvement in the intestinal metabolism and energy production

Bacteria contained within the gastro-intestinal tract microbiota contribute to host homeostasis through various physiological functions. The Human Microbiome Project Consortium reconstructed microbial metabolic profiles of a large human population and determined that several pathways, for example ribosome and translational machinery, nucleotide charging and ATP synthesis, and glycolysis, are omnipresent among humans and body habitats (The Human Microbiome Project, 2012). In the gastro-intestinal tract, these functions can be helpful in the metabolism and production of nutrients vital to the host. For instance, it has been estimated that between 1 and 20% of the circulating plasma

lysine, urinary lysine and body protein of adult humans originate from microbial sources (Metges, 2000). Also, more than 200 bacterial genomes from the human colon, for instance *Lachnospiraceae* and *Ruminococcaceae* belonging to the phylum *Firmicutes*, have been identified to possess butyrate-producing pathways important for maintaining a healthy colon (Vital et al., 2014).

In chickens, it has been observed that *Faecalibacterium*, *Butyrivibrio*, *Megasphaera*, *Subdoligranulum*, *Oscillibacter*, *Anaerostipes* and *Anaerotruncus*, members of the phylum *Firmicutes*, expressed enzymes required for butyrate production while all members identified from the *Bacteroidetes* phylum expressed enzymes for propionate production pathways (Polansky et al., 2015). In another study evaluating sixteen butyrate-producing bacteria recovered from chicken ceca, it was observed that these isolates were among four clostridial clusters: IV, XIVa, XIVb and XVI (Eeckhaut et al., 2011). Acetate and propionate production, short-chain fatty acids formed by fermentation in the ceca, were also identified from pathways involving kinase/phosphotransferase sequences or methylmalonyl-CoA decarboxylase and methylmalonyl-CoA epimerase in the chicken cecal metagenome (Sergeant et al., 2014). The chicken microbiota possesses many other important enzymes involved in nutrients transformation. For example, sequences of over two hundred different non-starch polysaccharide-degrading enzymes have been identified from bacterial metagenomes in ceca (Sergeant et al., 2014). Enzymes such as alkaline phosphatase show a greater activity in chickens colonized by bacteria compared to germ-free chickens (Palmer & Rolls, 1983), indicating that intestinal bacteria contribute to the host metabolic functions. The intestinal microbiota may also be involved in the intestinal absorption of nutrients, as the regulation of dietary fat absorption by the intestinal epithelium was shown to be affected by the microbiota composition in zebrafishes (Semova et al., 2012).

2.3.2.2 Impacts on zootechnical performances

The gastro-intestinal microbiota is thought to be an important factor modulating chickens' growth rate by improving nutrient metabolism (Broom, 2017; Diaz Carrasco et al., 2019). Small bacterial microbiota changes have been observed between individual chickens showing different feed conversion ratios (FCR) in a highly controlled experiment

(Stanley et al., 2012). More specifically, individual subjects with higher and lower feed conversion ratios showed clustered beta diversity, where groups could be identified based on their high or low efficiency to convert feed into weight gain. This clustering was possibly associated with different bacterial memberships, where 24 OTU were differentially expressed between high and low FCR chickens. Moreover, 7 of these OTU were absent from the cecal microbiota of the low FCR chickens, but present in many high FCR chickens. In a field study evaluating the impacts of the cecal microbiota of antibiotic-free chickens on growth performances, correlations between the microbiota structure and individual bodyweight were identified at 7, 14, 21, 28, 35 and 42 days of age (Johnson et al., 2018). Fifty-seven genus-level taxa in the cecal and ileal microbiota were positively or negatively correlated with broilers weight. Interestingly, taxa including known pathogenic bacteria such as *Clostridium*, *Enterococcus* and unclassified *Enterobacteriaceae* were negatively associated with growth performance. Also, the taxon *Lactobacillus*, which many bacterial species of this genus are used in probiotic products, was negatively correlated with chickens' bodyweights. However, these performance-related microbiota features may be dependant of other factors, such as host lineage (Diaz-Sanchez et al., 2019). Indeed, most of the top contributing OTU features associated with FCR differed between two pedigree lineages, and only two of the 51 reported taxa were common to both genetic lines. These two taxa were identified as the family *Clostridiales* and the genus *Lactobacillus*.

In a review of the chicken microbiota and productivity, it has been reported that various taxa are associated with performances (Diaz Carrasco et al., 2019). However, most studies cited are not reporting the same results. For example, Torok, et al. (Torok et al., 2011) reported that *Faecalibacterium prausnitzii*, *Clostridium lactatifermentans* and *Ruminococcus torques* were associated with improved FCR, while *Bacteroides vulgatus* and *Alistipes finegoldii* were linked to higher FCR. On the other hand, Stanley, et al (Stanley et al., 2012) reported *that Bacterioides fragilis* was associated with improved FCR, while *Ruminococcus*, *Lactobacillus crispatus* and *Clostridiales* were associated with higher FCR. These results show that specific microbiota characteristics in broiler chickens can be associated with improved or decreased zootechnical performances, but the lack of consistency within and between studies emphasizes the need to clarify the functional role of the microbiota in modulating performances via improved metabolism.

2.3.2.3 Resistance against pathogens and diseases

Little is known on the influence of the microbiota in triggering or protecting chickens against diseases. In humans, *Clostridium difficile* is a normal inhabitant of the intestinal microbiota that can become harmful to humans under certain conditions such as antibiotic therapy (Deshpande et al., 2015), which could be associated with an altered microbiota promoting the overgrowth of *C. difficile*. Thus, it could be hypothesized that the existing gastro-intestinal microbiota may be a protective or risk factor for enteric disorders in chickens. For instance, it has been reported that various predisposing factors such as fishmeal diet and exposure to *Eimeria* spp. decrease the relative abundance of segmented filamentous, lactic acid and butyrate-producing bacteria in the gastro-intestinal tract (GIT) microbiota of chickens (Antonissen et al., 2016). Since these bacterial groups are reported to improve intestinal health, a decreased abundance could be related with an increased risk of NE outbreaks while an increased relative abundance could be beneficial to the host. However, in a controlled challenge experiment reproducing NE in broiler chickens, it has been reported that the pre-existing microbiota did not influence the outcome of the disease (Lacey et al., 2018). Indeed, it has been noted that the pre-existing (pre-challenge) microbiota was highly similar between chickens showing intestinal lesions and those without intestinal lesions post-challenge, where both groups were showing identical alpha and beta diversity and relative abundance of numerous bacterial taxa before the challenge.

The resident microbiota has been shown to be protective against other bacterial pathogens such as *Salmonella* Enteritidis (SE). More specifically, a series of experiments have reported that by inoculating various bacterial mixtures isolated from the GIT of adult chickens to newly hatched chicks before a challenge with SE, it was possible to prevent SE colonization in these chicks. The authors reported these results were most likely obtained due to the competitive exclusion of SE by the existing microbiota following its adherence to the cecal wall (Gleeson et al., 1989; Stavric et al., 1985, 1987). However, results were variable, and protection was not shown to be uniform between chicks.

2.3.3 Factors influencing the gastro-intestinal microbiota composition in chickens

Due to variations in the bacterial microbiota, many efforts were made to define the healthy gastro-intestinal microbiota compositions as the establishment of a normal baseline is crucial for differentiating healthy and diseased individuals related to host-microbiota interactions (Integrative HMP (iHMP) Research Network Consortium, 2019). For example, The Human Microbiome Project Consortium described a normal human microbiota by characterizing the bacterial diversity of 18 body sites from 242 occidental humans clinically screened for the absence of diseases (The Human Microbiome Project, 2012). Subsequently, these results have been used to reveal abnormal features in the microbiota composition of subjects affected by pregnancy with preterm birth, inflammatory bowel disease and prediabetes compared to healthy individuals (Lloyd-Price et al., 2019; Serrano et al., 2019; Zhou et al., 2019). Likewise, the chicken gastro-intestinal microbiota has been studied to characterize its normal composition. *Firmicutes*, *Proteobacteria* and *Bacteroidetes* were shown to be the most abundant phyla in various sections of the GIT, where more than 90% of the bacterial population could be represented by these taxa in the ileum and cecum (Han et al., 2016; Huang et al., 2018). However, microbiota composition is reported to largely vary between individual chickens, even from a same batch (Stanley et al., 2013). It is important to consider the GIT microbiota as a dynamic population of microorganisms being affected by a large number of variables as numerous factors are involved in shaping the structure and membership of bacterial communities found in chickens GIT.

2.3.3.1 Early exposure to bacteria and development of the GIT microbiota

As opposed to mammals, which are mostly viviparous, birds are oviparous and lay hard-shelled eggs. Embryos are incubated in this structure and commercial broiler chicks will hatch and grow without parental contact. Consequently, early life microbiota is developed differently than mammalian species such as humans, where the GIT microbiota development is influenced *in utero*, at birth and also by early feeding modes such as breastfeeding (Zhuang et al., 2019). For instance, this limited early exposure to bacteria at hatch may be related to the low observed Richness and Evenness during the early life of broiler chickens (Ballou et al., 2016; Donaldson et al., 2017; Jurburg et al., 2019; Oakley, Buhr, et al., 2014; Richards et al., 2019). Indeed, alpha diversity was shown to gradually

increase with birds' age until it stabilizes at approximately two weeks post-hatch (Ballou et al., 2016; Crhanova et al., 2011; Donaldson et al., 2017; Jurburg et al., 2019; Oakley, Buhr, et al., 2014; Oakley & Kogut, 2016). The cecal microbiota at hatch was mostly represented by *Proteobacteria*, which is highly different from the microbiota composition in later ages mainly associated with *Firmicutes* (Ballou et al., 2016). The increased microbiota complexity in later ages has been hypothesized as important feature for intestinal pathogen resistance, where older chickens with increased Richness and Evenness showed better developed immune systems and decreased colonization of spleens and livers by *Salmonella enterica* serovar Enteritidis compared to post-hatch chicks (Crhanova et al., 2011).

2.3.3.2 Environment

The low microbial abundance at hatch predisposes the gastro-intestinal tract to be largely colonized by bacteria present in the environment. For example, various mixed bacterial inocula given to chicks post-hatch significantly impacted the structure and membership of the microbiota later in life (Yin et al., 2010). The type of housing can significantly impact the gastro-intestinal microbiota as the abundance and membership of bacteria may vary according to the type and cleanliness of the environment. Indeed, in a study comparing the cecal microbiota of broiler chickens raised in a standard grow-out feed trial facility, a facility with floor pens for small-scale experiment and isolators, the cecal microbiota structure and functionality significantly changed between housing types (Kers et al., 2019). The environmental load of bacteria was lower in the isolators compared to the other housing systems, which might have been related to the lower observed alpha diversity in the GIT of chickens raised in isolators compared to other housing environments harbouring higher loads of bacteria.

Contemporary commercial broiler chickens are raised on litter and its microbial composition is an important factor contributing to the GIT colonization by bacteria. For instance, bedding materials have been associated with different microbial communities in the ceca of broiler chickens (Torok et al., 2009). A few countries are re-using the litter for multiple flock cycles in commercial broiler chickens and when comparing reused wood shaving litter to fresh wood shaving bedding, it has been observed that bacteria membership

was different as more bacteria of intestinal origin were identified in the reused litter groups while more environment-related bacteria were found in the ceca of chickens raised on fresh material (Cressman et al., 2010). As a consequence, microbiota communities within the GIT of broiler chickens showed different beta diversity and relative abundance of *Firmicutes* and *Proteobacteria*, while the alpha diversity remained stable between reused and fresh litter at multiple time points during grow-out (Cressman et al., 2010; Wang et al., 2016).

2.3.3.3 Host genetics and immune system

Host genotype impacts on the gastro-intestinal microbiota have been studied by evaluating the bacterial communities present in various chicken lines, where many evidences indicate that chicken intestinal microbiota composition and structure can be influenced by host genetics (Diaz-Sanchez et al., 2019; Mon et al., 2015; Richards et al., 2019; Schokker et al., 2015; Zhao et al., 2013). For example, genotype-specific microbiota has been associated with the Major Histocompatibility Complex (MHC) class II (Bolnick et al., 2014; Pearce et al., 2017; Toivanen et al., 2001), which is a group of surface proteins on macrophages, dendritic cells and lymphocytes helping the adaptative immune system to recognize foreign substances such as bacteria from the GIT microbiota. The resulting differential immune response mediated by the MHC-class II against bacterial antigens was shown to modulate the microbiota composition. In return, the intestinal microbiota revealed to influence immunoglobulin production. For example, immunoglobulin genes were not expressed in ceca of germ-free chickens or chickens inoculated post-hatch with a heat killed microbiota at 56 days of age, while inocula ranging from individual bacterial suspensions to complex conventional microbiota induced various levels of immunoglobulin expression (Volf et al., 2017). Specific taxa are also reported to modulate the immune response, where an increased relative abundance of *Proteobacteria* and a decreased relative abundance of *Firmicutes* was positively correlated with pro-inflammatory responses (Oakley & Kogut, 2016).

2.3.3.4 Diet and nutrition

Diet is an important factor affecting the GIT microbiota in chickens as microorganisms are using this source of energy and carbon for growth. For example, *in*

vitro proliferation of *Clostridium perfringens* is higher in wheat and barley diets compared to corn-based diets (Annett et al., 2002). Real-time PCR bacterial quantification in chickens GIT showed that *in vivo* proliferation of the Bacteria domain was higher with a sorghum diet compared to a maize-based feed formulation, which was also associated with higher counts of the *Enterobacteriaceae* family (Lunedo et al., 2014). Complex communities found in the intestinal microbiota are altered by different diets, where each bacterial species will be affected differently depending on the type of diet. Type of feed, for example corn-based, sorghum-based, wheat-based or with the inclusion of fishmeal, was shown to impact community structure and membership in chickens GIT by modulating the relative abundance of various bacterial genera such as *Clostridium* and *Lactobacillus* (Crisol-Martinez et al., 2017a; Fagundes et al., 2017; Ludvigsen et al., 2016; Stanley, Wu, et al., 2014). Many other feed components are reported to alter the intestinal microbiota, for example vitamins (Luo et al., 2013), enzymes (Borda-Molina et al., 2019) and protein source and feed particle size (Vermeulen et al., 2018). The influence of the diet on the GIT microbiota may be explained by the distinctive microbiota found in diverse poultry feed ingredients (Haberecht et al., 2020). For example, bloodmeal, meat and bone meal, limestone and poultry oil were reported to exhibit the most distinct microbial communities, while barley, canola, corn, millrun, oats, sorghum, soybean meal and wheat showed lower differentiation in their microbiota composition. Hence, feeding these ingredients to chickens may seed their GIT and influence the development and maturation of the microbiota.

2.3.3.5 Antimicrobials

A common concept largely promoted by researchers is that antibiotics alter the composition of the intestinal bacterial microbiota and provide beneficial effects such as improved weight gain and feed conversion efficiency (Dibner & Richards, 2005). With the most recent progresses in sequencing and computing capabilities, these effects are now being studied to understand the specific GIT microbiota changes provoked by in-feed antibiotics. Different antibiotic classes were shown to induce specific changes to the cecal microbiota membership of broiler chickens even if performance outcomes such as weight gain and feed conversion were unchanged by antibiotics compared to non-medicated diets (Costa et al., 2017). In the study from Costa et al. (2017), the use of antibiotics in the diet

of broiler chickens had no significant impact on the cecal microbiota structure, but the use of antibiotics such as enramycin or bacitracin was associated with differential abundance of minor phyla in the cecal microbiota. In a trial comparing the effects of zinc bacitracin and avilamycin on growth performances and cecal microbiota in broiler chickens reared in individual cages between 13 and 25 days of age, it has been shown that zinc bacitracin significantly lowered the feed conversion ratio whereas avilamycin had no significant impact on growth performances compared to non-medicated control chickens (Crisol-Martinez et al., 2017b). Alpha and beta diversity were significantly impacted by the use of zinc bacitracin, but avilamycin had minimal effect on microbial diversity measures. Zinc bacitracin significantly altered the relative abundance of eight taxa: *Lactobacillus*, *Faecalibacterium*, *Ruminococcus torques* phylotype, *Lactobacillales* and *Clostridiales*. On the other hand, avilamycin was shown to alter the relative abundance of two taxa: *Catabacteriaceae* and *Clostridium spiroforme* phylotype. Except for *Faecalibacterium*, the taxa identified in the study from Crisol-Martínez, et al. (2017) are different from those identified in the study from Costa et al. (2017). This inconsistency may indicate that the intestinal microbiota alteration by antibiotics might be dependent of other factors such as early exposure to different bacteria, environment, housing system, diet composition and host genetic lineage as the microbiota already present in the GIT at the beginning of these trials was most likely different.

2.3.3.6 Prebiotics and probiotics

Various prebiotic and probiotic products, also referred as alternatives to antibiotics, have been shown to impact the GIT microbiota composition. For example, in-feed mannan-oligosaccharides (MOS) at a concentration of 1 g MOS per kg of feed has been reported to increase the Shannon's alpha diversity index and to increase *Lactobacillus*, *Bifidobacterium* and *E. coli* counts compared to control and virginiamycin-complemented diets fed to broiler chickens (Pourabedin et al., 2014). The cecal microbiota diversity was shown to be significantly impacted by the use of sodium butyrates, which could be associated with a decreased relative abundance of *Lactobacillaceae* (Zou et al., 2019). In a study from Bortoluzzi et al. (2017), the altered microbiota composition by butyrates showed improved carbohydrate and lipid pathways as analysed by predicted functional composition (PICRUSt analysis from 16S rRNA sequencing). This finding was also

observed in a study evaluating the effects of administering a commercial mixture of butyrate glycerides to broiler chickens, where lipid serum concentrations were significantly higher in the group receiving butyrate glycerides (Yang et al., 2018). Other serum metabolites, for instance LDL/VLDL and lactates were significantly increased following the administration of butyrate glycerides. Although alpha diversity remained unchanged by the treatment, it has been observed that butyrates glycerides would alter the microbiota composition by affecting the relative abundance of 39 OTU in the cecal microbiota.

Many probiotics, defined as live microorganisms providing health benefits, are also broiler chicken feed additives reported to impact the GIT microbiota. For example, *Bacillus subtilis* has been associated with decreased alpha diversity and altered microbiota composition by altering the relative abundance of various taxa such as *Firmicutes* and *Bacteroidetes* in broiler chickens (Pereira et al., 2019) and Shaver Whites, a layer chicken breed (Neijat et al., 2019). A mixture of *B. subtilis* was shown to significantly decrease total aerobic bacteria, *Salmonella* spp. and *Escherichia coli* (Gao et al., 2017). However, *B. subtilis* may cause variable changes to the GIT microbiota function, as few changes have also been reported on metabolic pathways of the predicted functional metagenome by the administration of *B. subtilis* even if the probiotic caused increased *Firmicutes* and decreased *Bacteroidetes* abundance in the ceca of broiler chickens (Ma et al., 2018). *Lactobacillus* spp. have also been reported to cause changes to broiler chickens GIT microbiota. For example, an inoculum mixture composed of equal numbers of *L. ingluviei*, *L. agilis* and *L. reuteri* administered at hatch was associated with a different microbiota composition in the feces at 14- and 28-days post inoculation (Baldwin et al., 2018). Although the mix resulted in the colonisation of only *L. ingluviei*, the probiotic product caused long term changes in the abundance of various taxa, for instance *Eubacterium*, *Arthrobacter*, *Blautia schinkii*, *Ruminococcus lactaris*, *Alistipes onderdonkii* and *Bacteroides uniformis*.

2.3.3.7 Gastro-intestinal pathogens and diseases

Increased pathogen growth may be observed in a disease outbreak. For example, it has been observed that the recovery rate of *C. perfringens* isolates can be ten times higher in broiler chicken flocks experiencing necrotic enteritis compared to healthy flocks (Parent

et al., 2017). The resulting microbiota composition is most likely to be altered as the relative abundance and taxa diversity can be affected by this microbial shift. In a study comparing chickens challenged with *Eimeria* spp. and *C. perfringens* to negative control groups to reproduce necrotic enteritis in an experimental facility, challenged birds showed a decreased Richness and Evenness in the ileal and cecal microbiota compared to healthy individuals (Bortoluzzi et al., 2019). The relative abundance of *Firmicutes* and *Bacteroidetes* was also significantly affected by the challenge model. On the opposite, the cecal microbiota may remain unchanged following the inoculation of large number of bacteria to chickens if no clinical signs are observed, as reported during challenges with *C. perfringens* (Stanley et al., 2014) and *Campylobacter jejuni* (Thibodeau et al., 2015). However, the absence of clinical signs following the inoculation of intestinal pathogens is not indicative of an unchanged microbiota, since it was observed that broiler chickens inoculated with *Salmonella* Enteritidis (SE) showed a different cecal microbiota compositions between 7 and 21 days post-inoculation compared to non-challenged chickens (Mon et al., 2020). SE is a bacterium commonly found in ceca of commercial chickens and rarely cause clinical signs (Gast, 2013), but its inoculation to chickens has been reported to alter the composition of the GIT microbiota (Liu et al., 2018; Videnska et al., 2013). Nonetheless, no differences in the GIT microbiota of SE-positive and SE-negative hens were observed in a challenge model causing subclinical infections (Nordentoft et al., 2011), possibly indicating that subclinical infections may cause inconsistent modifications to the GIT microbiota. These irregular microbiota changes in subclinical infections contrast with clinical infections, where more consistent alterations to the microbiota composition are reported.

The inoculation of intestinal parasites, such as *Eimeria tenella* may also alter the microbiota. Indeed, it has been observed that the alpha and beta diversity can be decreased following a challenge with *E. tenella*, which was also associated with a decrease in the relative abundance of *Lactobacillus* and *Faecalibacterium* and increase of *Clostridium*, *Lysinibacillus*, and *Escherichia* (Huang et al., 2018). Still, another study evaluating microbiota modifications induced by *E. tenella* reported no change in the taxa diversity, while the beta diversity was different between infected and non-infected groups (Macdonald et al., 2017). In this study, the relative abundance of *Enterobacteriaceae* and

Clostridiales was increased, while taxa from *Bacillales* and *Lactobacillales* were decreased by the coccidiosis challenge. The increase of taxa associated with *C. perfringens* during an *E. tenella* infection may be an important mechanism explaining the interaction between coccidiosis and necrotic enteritis, where the former is considered as an important risk factor for the latter (Moore, 2016).

Chapter 3. Articles

Article 1. Impacts of antibiotic reduction strategies on zootechnical performances, health control and *Eimeria* spp. excretion compared to conventional antibiotic programs in commercial broiler chicken flocks

Published

Authors' contributions

Conceptualization, MB, EP, RM and MA; methodology, MB, EP, RM and MA; data collection, EP; data analysis, EP; resources, MB; writing-original draft preparation, MB, EP, RM and MA; writing-review and editing, EP, RM, MB and MA; supervision, MB, EP, RM and MA; project administration, MB; funding acquisition, MB.

ANTIBIOTIC REDUCTION IN BROILER CHICKENS

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ABSTRACT

Increasing efforts have been made in recent years to reduce antimicrobial use in animal production. The objective of this prospective study was to evaluate, in commercial broiler chicken farms, two antibiotic reduction strategies that eliminated the use of antibiotics that are important for human medicine, in comparison to the conventional use of antibiotics. On seven broiler chicken farms, a house was allocated to the antibiotic reduction treatments for 6 consecutive flocks, while a similar house on the same premises was assigned to the conventional use of antibiotics (CONV) for 6 consecutive flocks. The antibiotic reduction strategies consisted of continuous in-feed use of ionophores (TX1) and continuous in-feed use of ionophores with butyric acid (TX2). In the 84 flocks, zootechnical performance was recorded, lesion scoring at 21 and 28 days of age was performed, and fecal samples were recovered during growout for *Eimeria* spp. oocysts counts. There was no statistical difference between TX1, TX2 and CONV for weights at slaughter, feed conversion ratios, average daily gains, age at slaughter, total mortalities and condemnations. The probability of identifying oocysts in the fecal samples significantly increased with the age of the flock, but there was no significant treatment effect between 7 and 16 days of age. At 19 days of age, the probability of a sample containing oocysts was higher in TX1 compared to CONV, but TX2 was not statistically different from TX1 and CONV. Predicted oocysts per gram in CONV flocks were significantly lower between 22 to 34 days of age compared to TX1 and TX2, while there were no significant differences between TX1 and TX2 for all ages. Lesion scoring of the gastro-intestinal system showed no differences for coccidiosis scores between TX1, TX2 and CONV. No lesions of necrotic enteritis were observed. In conclusion, it was possible to adequately control intestinal diseases and maintain zootechnical performances by relying exclusively on ionophores, when compared to broiler chicken flocks using standard shuttle programs with antibiotic growth promoters.

Key words: Antibiotic reduction, broiler, intestinal health, zootechnical performance, *Eimeria* spp.

INTRODUCTION

Awareness about the growing incidence of antimicrobial resistance (**AMR**) of important bacterial pathogens and its impacts on human and animal health has increased in recent years. The World Health Organization (**WHO**) describes this phenomenon as one of the biggest threats to global health, which could cause 10 million human deaths each year by 2050 if no action is taken (Tangcharoensathien et al., 2017). To help meliorate this issue, the international agency is calling for a ''One Health'' approach, which includes various recommendations such as urgently phasing out the use of critically important antimicrobials for growth promotion and prevention of diseases in agriculture. Indeed, the extensive use of antibiotics has been shown to be associated with an increased abundance of resistance genes to many antibiotics. For instance, metagenomics analyses of broiler chickens and slaughter pigs' fecal samples in European countries showed a positive association between the abundance of resistance genes and antimicrobial use (**AMU**) (Munk et al., 2018). This emphasizes the importance of reducing antimicrobial use in animal production to decrease the selective pressure on antibiotic-resistant bacteria and the prevalence of AMR gene.

In Europe, concerns about AMR have led to the ban of antibiotics used for growth promotion in farm animals (Castanon, 2007; Cogliani, 2011). However, the transition has been associated with increased *Clostridium perfringens*-associated enteritis and cholangiohepatitis infections (Van Immerseel et al., 2004). Substantial losses were observed in broiler chicken flocks affected by these conditions, leading to poor zootechnical performance and increased condemnations at slaughter (Lovland and Kaldhusdal, 1999, 2001). In the United States of America (**USA**), consumer demand has recently driven the broiler chicken industry to reduce AMU (Karavolias et al., 2018). Three categories of broiler production can be identified based on AMU: (1) Flocks using antibiotics considered medically important for human medicine, reported as conventional flocks in the present article; (2) flocks using exclusively antibiotics nonmedically important for human medicine, reported as antibiotic-reduced flocks in the present article, and (3) no antibiotics ever (**NAE**), also named ''raised without the use of antibiotics (**RWA**)'' or ''antibiotic-free'' flocks. Between 2013 and 2017 in the USA, a substantial decrease has

been recorded for the use of most of the antibiotic classes administered to broilers for diseases treatment or prevention (Singer and Porter, 2019). Most importantly, these authors also reported a shift from antimicrobial drugs medically important to humans towards antibiotic classes considered not important for human medicine. In Canada, where there is no antibiotic ban, the conventional use of antibiotics is comparable to the use of medically important antibiotics in the USA. The Canadian federal authorities labeled a type of production called RWA (CFIA, 2016) similar to the NAE programs in the USA. In a study comparing drug-free to conventional broiler chicken flocks, significant production losses were associated with the RWA flocks (Gaucher et al., 2015) and many farms experienced recurring outbreaks of necrotic enteritis NE caused by pathogenic and clonal *C. perfringens* strains carrying the *netB* gene (Gaucher et al., 2017; Parent et al., 2017). Thus, there is a global trend for AMU reduction in broiler chicken production, but it has often been associated with increased intestinal health disorders and production losses. Controlling intestinal diseases such as NE has become critical to successfully raise healthy broiler chicken flocks with antibiotic reduction programs.

The epidemiology of NE has been extensively studied and risk factors, for instance coccidiosis, play a significant role in the pathogenesis of the disease (Lee et al., 2011; Moore, 2016; Prescott et al., 2016; Shojadoost et al., 2012; Van Waeyenberghe et al., 2016). Indeed, coccidiosis has been shown to exacerbate NE in challenge models (Al-Sheikhly and Al-Saieg, 1980; Rodgers et al., 2015) and to impair zootechnical performance of broiler chickens (Rochell et al., 2016). Various products, including chemical anticoccidials, ionophores and vaccines are currently used in commercial farms to control this protozoal disease. Each product has a different effect on the excretion and cycling of *Eimeria* spp., the causative agent of coccidiosis (Chapman et al., 2016; Jenkins et al., 2017; Parent et al., 2018; Williams and Gobbi, 2002). Various alternatives to antibiotics such as herbal products or organic acids have also been reviewed as compounds with prophylactic/therapeutic potential for coccidiosis control (Ali et al., 2014; Muthamilselvan et al., 2016).

The use of nonmedically important antibiotics may provide an adequate control of diseases, most likely equivalent to the use of medically important antibiotics in broiler

chickens (Karavolias et al., 2018). The hypothesis of this study is that antibiotic reduction strategies using nonmedically important antibiotics in commercial broiler chicken flock prevention programs can provide similar zootechnical performance and offer similar health control to the prevention programs using medically important antibiotics for human medicine. The objective of the study was to evaluate, in commercial broiler chicken farms, two antibiotic reduction strategies eliminating the use of antibiotics considered critically important for human medicine, in comparison to the conventional use of antibiotics. More specifically, production performance, flock health and *Eimeria* spp. excretion were compared between antibiotic reduction and conventional strategies.

MATERIALS AND METHODS

Study Design

   Care and Use of Animals. The committee on animal care in research (Comité d'éthique pour l'utilisation des animaux) of the Faculté de médecine vétérinaire of the Université de Montréal approved the study and protocols involving animal use with the project number 16-Rech-1850.

   Farm Eligibility Criteria and Description. The prospective study was conducted in commercial broiler chicken farms owned by chicken farmers in the province of Quebec, Canada. Producers from the Poultry Farmers Association of Quebec ("Éleveurs de volailles du Québec"), who owned at least two houses on the same farm, were contacted to participate in this year-long study. Seven broiler chicken farms were included on a voluntary basis. Each farm was visited prior to the beginning of the study to inspect the facilities. The premise was required to have 2 broiler chicken houses, with similar stocking densities, surface areas, feeding systems, water equipment and ventilation systems. A summary of the 7 broiler chicken farms characteristics is presented in Table 1. The houses capacity ranged from 9,800 to 22,000 broiler chickens per flock. Three hatcheries provided chicks to the farms, 4 feed mills prepared the feed during the study and 3 processing plants slaughtered the chickens at market weight. A premise was required to keep the same hatchery, feed mill and slaughterhouse for the duration of the study. All flocks raised were male. The average downtime between flocks on a farm ranged from 11.4 to 20.0 days for the duration of the study.

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Rearing and Housing Conditions. On each farm, a broiler house was randomly allocated to the antibiotic reduction treatments for 6 consecutive flocks, while the other house was allocated to the conventional treatment for 6 consecutive flocks. Flocks were raised simultaneously in the 2 houses at each farm, i.e. the flocks in both houses of a same farm were always placed on the same day with chicks originating from the same hatchery and breeder flocks. As more than one breeder flock contributed to the chicks placed in each house, the same proportion of chicks from each breeder flock were placed in both houses. A specific breeder flock age was not required for the study, but the placements in each paired broiler houses needed to be identical to control for chick quality. Chicks were vaccinated against Marek's disease and received lincomycin-spectinomycin *in ovo*. The brooding method described in Chick Champs was used (Chicken Farmers of Canada, 2015). Management on each farm followed rearing standards in the broiler chicken flock industry, which can be found in Aviagen® and Cobb® broiler management guides (Aviagen, 2018; Cobb-Vantress, 2018). On a farm, daily care of chickens and management of both houses were performed by the same employees. Shipping to processing plants was individually determined for each flock to meet target average bodyweight. For this reason, the slaughtering day was allowed to vary between 2 paired flocks. More precisely, the processing of a flock would be pre-empted or delayed to reach a target weight depending on its weight a few days prior to slaughter, a common practice within the Quebec industry to standardize carcass weights at processing. After each flock, as per Chicken Farmers of Canada On-Farm Food Safety Assurance Program manual (Chicken Farmers of Canada, 2014), litter was removed from broiler houses and a dry cleaning (dust removal) was performed. Fresh pine wood shavings were used as bedding material by the 7 participating farms for each lot. The drinking water of all flocks was acidified with an inorganic acid (Phosphoric acid H_3PO_4 17%) at an inclusion rate targeting an end-of-line pH between 5 and 6. Water lines were flushed daily for the first 7 days, then weekly until shipping to the processing plant. Producers washed and disinfected lines as per the standard operating procedures on each farm.

Antibiotic Reduction Treatments. Two antibiotic reduction strategies were randomly allocated to the 6 consecutive flocks of the first broiler house of each farm, for a total of 3 repetitions per farm for each strategy. The first strategy consisted of the

continuous use of monovalent ionophores from placement to shipping to the processing plant, accordingly with the Veterinary Drugs Directorate of the Health Canada's Health Products and Food Branch (Government of Canada, 2018). The second strategy used the same monovalent ionophores from placement to shipping and an inorganic acid (butyric acid 65%) was also added as a feed additive at the concentration of 0.7 kg of premix per ton of feed. No antibiotics other than ionophores were used in the prevention programs of this broiler house for each farm. A summary of medication programs used in each flock is presented in Supplemental table 1.

Conventional Treatment. The conventional treatment consisted of the normal use of shuttle anticoccidial programs with antibiotics considered critically important for human medicine in the feed for the 6 consecutive flocks of the second broiler house in each farm. The programs used in the study were prepared by the referring veterinarian and were not modified by the research team. Briefly, veterinarians used chemical anticoccidial until 3 weeks of age, followed by a monovalent ionophore until shipping to slaughter. From placement to shipping, antibiotics were included in the feed as a common practice to prevent necrotic enteritis during rearing. Product rotations were performed every 2 flocks as a common practice within the industry to prevent the development of pathogen resistance against anticoccidials and antibiotics. The antimicrobials and rotations used in the conventional programs were considered as the best practices to maximize production performances and health control in broiler chicken flocks. Details of medication programs are included in Supplemental table 1.

Feed and Nutritional Guidelines. Nutritional guidelines were provided to each participating feed mill (Supplemental table 2). Feed formulation and ingredient inclusion rates were identical between 2 flocks raised simultaneously on a premise, but it was allowed to vary within the nutritional guidelines for flocks on different premises.

Zootechnical Performances

For the 84 flocks, bodyweights at slaughter (kg) and total condemnations (%) were retrieved from the slaughterhouse data. Age at slaughter (days) and total mortality (%) were recovered from farm data. Feed conversion ratio (**FCR**) was calculated with the formula: FCR = Total feed consumed (kg) / Total chickens' weight at slaughter (kg). The average

daily gain (**ADG**) was calculated with the formula: ADG (g/day) = Mean bodyweight at slaughter (g) / Age at slaughter (days).

Flock Health

Eimeria spp. Oocysts Fecal Counts. Fresh feces from all flocks were sampled every 3 days, starting at 7 d of age until the end of the grow-out period. One pooled sample of fecal content was taken per time point in each flock, consisting of 20 to 25 fresh fecal droppings evenly distributed in the house. A total of 84 samples per sampling day, 21 from the ionophores group, 21 from the ionophores with butyric acid group and 42 from conventional flocks, were planned for collection. Fecal droppings were recovered in a Whirlpak bag and stored immediately at 4℃ until processing for oocyst counts. In accordance with the procedures of the Faculty of Veterinary Medicine parasitology diagnostic laboratory, a slightly modified McMaster technique was used to count total oocysts per gram of fecal content (**OPG**) in each sample. After homogenization of the pooled sample, 10 g of fecal content were weighed and mixed with 100 mL of water. The mixture was stored at 4℃ for 24 h, then filtered with a sieve. The filtrate (15 mL) was transferred in a Falcon tube to be centrifuged at 1500 rpm for 10 min. The pellet was resuspended in 5 mL of 35.5% NaCl solution, vortexed, and the solution transferred to a 50 mL beaker. Two additional rinses with 5 mL of 35.5% salt solution were performed to recover all oocysts in the tube. A Pasteur pipette was used to fill a McMaster chamber (Partnar Animal Health, Ilderton, Ontario, Canada) with the homogenized solution. Readings were performed 1 min after filling the chamber. All oocysts in the limits of each chamber were recorded. To express the results in OPG, the following formula was used: OPG = (Oocyst counts in chamber 1 + Oocyst counts in chamber 2)/2 × 66.6.

Lesion Scoring. Post-mortem sessions, based on the lesion scores in the Elanco Animal Health's Health Tracking System (**HTSi®**) described in the Broiler Disease Reference Guide (Elanco Animal Health, 2010; Kasab-Bachi et al., 2017), were conducted at 21 and 28 days of age to evaluate the health condition of all flocks. Twelve live chickens per flock at each time point were randomly selected across the house to represent the flock. Chickens were humanely euthanized by a standard cervical dislocation (American Veterinary Medical Association, 2013) and weighed. The same observer then performed

the HTSi® scoring method to identify and score each lesion or condition described in the aforementioned guide for the 2016 individual chickens selected across the 84 flocks.

Fecal and Litter Humidity. In each flock, fecal and litter samples were recovered at 21 days of age, 28 days of age and before shipping to slaughter. Approximately 20 g of fresh fecal droppings and litter were sampled across the broiler chicken house and put in separate hermetic Whirlpak® bags. Then, samples were refrigerated at 4°C until processing at the lab. From each sample, 5 g of feces or litter were put in a moisture analyzer (Denver IR120 Moisture Balance, Laboratory Instrument Specialists, CA, USA) for the determination of moisture content by infrared radiation and measuring the weight loss on drying.

Statistical Analysis

Zootechnical Performance, Lesion Scoring, Fecal Humidity and Litter Humidity. The flock was considered the experimental unit for all analyses. All statistical analyses were performed with R statistical software (R Core Team, 2017) using the lme4 package (Bates et al., 2015) and the function lmer to fit linear mixed-effect models with the restricted maximum likelihood (**REML**) approach for coefficients estimation. Six different models were built for the zootechnical performances outcomes: bodyweight at slaughter (kg), feed conversion ratio, average daily gain (g/day), age at slaughter (days), total mortality (%), percentage of condemnations at slaughter (%). Data from the percentage of condemnations at slaughter was log-transformed to improve model fit. Results were back-transformed to their original scale for presentation of results. For the lesion scoring, the mean lesion scores were calculated from the 12 chickens evaluated at 21 or 28 days of age in each flock by adding each individual score and by dividing the total by 12. For the litter and fecal humidity analyses, models were built for each sampling time point at 21 days of age, 28 days of age and before slaughter. For all models, treatment was included as a fixed effect and the farm was used as a random intercept. Coefficients with a p-value ≤ 0.05 were considered significant. Models validity were assessed by the visual inspection of quantile–quantile plots for normality and by scatter plots of the standardized residuals as a function of the adjusted outcome values for homoscedasticity.

Oocysts Excretion Modeling. Two different statistical models were built to model the dynamics of *Eimeria* spp. oocysts excretion during growout for the 3 treatments. A mixed multivariable logistic regression model for the first 5 sampling ages (7, 10, 13, 16 and 19 days of age) and a mixed multivariable linear regression model for the next 5 time points (22, 25, 28, 31 and 34 days of age) were built as the data distribution differed between early and late flocks' ages. Samples at 37 and 40 days of age were not considered in the analyses due to most flocks being slaughtered before these ages. A small number of samples (33/840) were missing between 7 and 34 days of age due to sampling omission, and these were not considered in the analyses since missing samples were evenly distributed across the 7 farms and 3 treatments for all ages. In the early growout, many samples contained no *Eimeria* spp. oocysts, while most of the later ages' samples did contain oocysts. Due to many zero values in the early ages, linear regression models did not fit the data. Hence, the oocysts per gram of fecal content were dichotomized for the presence (≥ 1 oocyst) or absence (0 oocyst) of *Eimeria* spp. in each sample. Using the glmer function from the lme4 package in the R statistical software (Bates et al., 2015), a mixed logistic regression model was fitted with the inclusion of the treatment and age as fixed effects and the farm as random intercept. The outcome was the presence or absence of *Eimeria* spp. oocysts in the samples. The logistic regression model performance was assessed by determining the goodness of fit with the Homer-Lemeshow test and by evaluating the accuracy of the model to correctly predict the outcome. In later ages, a mixed linear regression model with the REML approach for coefficients estimation was built to model the excretion of *Eimeria* spp. oocysts. OPG values were log-transformed to improve model fit. Treatments and flocks' age were considered as fixed effects and the farm was included as a random intercept. Due to the curvilinear relationship between the log10 OPG values and flocks' age, a quadratic variable of age (age^2) was added to improve the final model fit. Coefficients with a *p*-value ≤ 0.05 were considered significant. The mixed linear regression model validity was assessed by the visual inspection of quantile–quantile plots for normality and by scatter plots of the residuals as a function of the adjusted outcome values for homoscedasticity. Predicted probabilities of identifying *Eimeria* spp. oocysts and predicted log10 OPG in the droppings were computed respectively from the logistic

and regression models and then plotted against flocks' age to display differences between treatments for the excretion of oocysts in each treatment during growout.

Pre-study Power Analysis. Prestudy statistical power analyses on sample size were performed while devising the study to determine the number of flocks required to adequately evaluate group differences. For example, the ability to detect significant differences between groups for the feed conversion ratio was based on standard errors of 0.05 and means of 1.65. A sample size per group of 16 was determined to be sufficient to detect a difference between groups with a significance threshold of 0.05.

RESULTS

Influence of the Treatments on Zootechnical Performance

There were no significant differences between treatments ($p > 0.05$) for the bodyweight at slaughter, feed conversion ratio, average daily gain, age at slaughter, total mortality and total condemnations (Table 2).

Influence of the Treatments on Flocks' Health

Eimeria Excretion. Predicted probabilities of a fecal sample containing *Eimeria* spp. oocysts from 7 to 19 days of age for each treatment are presented in Figure 1 and the model results are presented in Supplemental table 3. The mixed multivariable logistic regression model showed an accuracy of 79.4% to correctly identify the presence or absence of oocysts in a fecal sample. The probability of identifying oocysts in the fecal samples significantly increased with the age of the flock, but there was no significant effect of the treatment between 7 and 16 days of age based on the 95% confidence intervals. At 19 days of age, the probability of having a sample containing oocysts was higher in the ionophores group compared to the conventional flocks, but the flocks receiving ionophores and butyric acids were not statistically different from the two other groups. Predicted log10 OPG values from 22 to 34 days of age for each treatment are displayed in Figure 2 and results of the mixed multivariable linear regression model are shown in Supplemental table 3. Predicted OPG in the conventional flocks were significantly lower for all ages compared to the two other groups, while there was no significant difference between the two

antibiotic-reduced groups for all ages. Predicted OPGs increased from 22 to 28 days of age for all treatments, then decreased at 31 and 34 days old.

      Lesion Scoring per Treatment at 21 Days of Age. Average bodyweights ranged from 952.3 to 964.9 g with no significant differences between groups ($p > 0.05$) (Table 3). For the gastro-intestinal system, mean scores for the Intestinal Integrity (I^2) Index, *Eimeria acervulina*, *E. maxima*, *E. tenella*, microscopic *E. maxima*, NE and gizzard erosions were not statistically different between groups ($p > 0.05$). For the evaluation of the integumentary and skeletal systems, scores of burned feet (pododermatitis), femoral head necrosis and tibial dyschondroplasia were recorded. For all mean scores, there was no difference between treatments ($p > 0.05$). Burned feet lesions were present in nearly all flocks, but low scores on the 0 to 3 scale were mostly recorded. The presence of femoral head necrosis and tibial dyschondroplasia was infrequent; hence the mean scores close to 0 for all groups. The bursal diameter was evaluated to evaluate the immune system. The mean diameter ranged from 1.82 to 2.05 cm in the 3 groups, and no statistical difference was noted ($p > 0.05$). Finally, the respiratory system was evaluated by scoring the presence of airsacculitis and tracheal lesions. No significant difference was observed between the two antibiotic reduction groups and the conventional group ($p > 0.05$).

      Lesion Scoring per Treatment at 28 Days of Age. Average bodyweights in all groups ranged from 1596.4 to 1617.3 g, with no statistical differences between groups ($p > 0.05$) (Table 3). Scores in the gastro-intestinal and respiratory systems were not statistically different between all groups ($p > 0.05$). In the integumentary and skeletal systems, the mean score of pododermatitis was significantly higher in the ionophores with butyric acid group compared to the ionophores only and conventional groups ($p = 0.05$). Femoral head necrosis and tibial dyschondroplasia lesions were sporadic, resulting in mean scores close to 0 for all groups. There was no statistical difference between groups for these two parameters ($p > 0.05$). The average bursal diameter of 2.02 cm in the ionophores only group was significantly higher than the mean diameter of 1.79 cm in the ionophores plus butyric acid group ($p = 0.05$). The conventional group was not statistically different ($p > 0.05$) from the other groups with a bursal mean diameter of 1.86 cm.

Litter and Fecal Humidity. There was no significant difference between the 3 treatments for the humidity in the litter and fecal samples at 21 days old, 28 days old and before slaughter (p > 0.05 for all comparisons) (Supplemental table 4).

DISCUSSION

This study aimed to evaluate the impacts of reducing AMU in a commercial context of broiler chicken production by removing medically important in-feed antibiotics from disease prevention programs. The seven selected farms followed management and vaccination procedures commonly used within the industry for the six paired consecutive flocks and feed formulation did not differ from current industry standards. Hence, the results obtained from this trial can be extrapolated across the current broiler chicken production system. Our data supports the concept of replacing medically important antibiotics by nonmedically important antibiotics in the feed without impacting performance in commercial broiler chicken flocks. However, the removal of lincomycin and spectinomycin from *in ovo* injection would need to be separately evaluated; it was logistically impossible to remove it from the present study. Indeed, this antimicrobial product was used as a standard procedure by the hatcheries to prevent early mortalities caused by *Escherichia coli* septicemia and omphalitis. Our results contrast with a previous study in a similar context, where RWA broiler chicken flocks showed significantly decreased zootechnical performances compared to conventionally raised flocks (Gaucher et al., 2015). The major difference between the two studies was the use of a live coccidial vaccine for the prevention of coccidiosis in the RWA flocks from Gaucher et al. (2015), while the present study used ionophores, considered nonmedically important antibiotics (World Health Organization, 2017). No antibiotic growth promoters were used in the antibiotic-free or antibiotic-reduced flocks in both studies. However, preventive programs used in the present study are not consistent with Canadian RWA or USA NAE standards since ionophores are antibiotics. Compared to Gaucher et al. (2015), it can be hypothesized that ionophores have a critical role in the maintenance of zootechnical performance. More specifically, the results of this study suggest the shuttle programs using antibiotic growth promoters for NE prevention provide similar zootechnical performances to programs

relying exclusively on ionophores. This could also represent an economic advantage to the industry by decreasing antibiotic use.

The advantages of using antibiotics as growth promoters have been extensively studied and reviewed (Dibner and Richards, 2005; Jones and Ricke, 2003). Although the exact mechanism of action still needs to be elucidated, the administration of sub-therapeutic doses of antibiotics in chickens' diet is believed to alter the intestinal microbiota to improve feed efficiency (Broom, 2017). Since various antibiotics will have different effects on the bacterial membership in the ceca (Costa et al., 2017), the exact microbiota composition which leads to increased performances with the use of antibiotic growth promoters is still unclear. More investigations would be needed to clarify the influence of the chickens' intestinal microbiota on growth performances in chickens as dissimilar intestinal microbiota compositions can result in similar growth performances (Stanley et al., 2016).

Numerous prebiotic products, for instance essential oils and organic acids, have been reviewed as alternatives to antibiotic growth promoters in broiler chicken production due to their positive effect on growth performances (Ducatelle et al., 2015; Khan and Iqbal, 2016; Zeng et al., 2015). For example, butyric acids were shown to improve zootechnical performances such as body weight gain and feed conversion ratio without a disease challenge (Bortoluzzi et al., 2017; Kaczmarek et al., 2016). Although not completely understood, the growth promoting mechanism of these products is thought to be related to the specific changes induced in the intestinal microbiota composition and function (Ducatelle et al., 2015). For instance, the cecal diversity was shown to be significantly impacted by the use of sodium butyrates, which could be associated with a decreased relative abundance of *Lactobacillaceae* (Zou et al., 2019). In a study from Bortoluzzi et al. (2017), the altered microbiota composition by butyrates showed improved carbohydrate and lipid pathways as analysed by predicted functional composition (PICRUSt analysis from 16S rRNA sequencing). However, butyrates seems to be less effective when facing a disease, as shown in studies evaluating the efficacy of butyrates to improve growth performances with a NE challenge (Liu et al., 2017; Liu et al., 2019). In the present study, clinical enteric diseases were absent at the flock level based on the post-mortem intestinal evaluations at 21 and 28 days of age in the three groups, where no NE lesions and low coccidiosis scores were recorded. Based on the aforementioned studies, it would have been

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expected to observe improved growth performances in the antibiotic-reduced flocks receiving butyric acids compared to the antibiotic-reduced flocks without these organic acids. However, no beneficial effect was observed; zootechnical performances were similar between the two groups. From our results, it could be assumed that butyric acids would not be required in broiler chicken flocks receiving exclusively ionophores in their prevention program.

The antibiotic-reduction strategies were associated with a higher excretion of *Eimeria* spp. oocysts after 22 days of age compared to the conventional strategy. Indeed, the statistical model showed constantly higher OPG counts through the different timepoints compared to the conventional flocks. The peak of excretion, which is thought to be related to the immunization of the flocks against coccidiosis (Chapman et al., 2016), occurred at 28 days of age in all treatments. This observation was also reported from previous studies, where the quantity of oocysts in the feces or litter is maximal around 4 weeks of age when using anticoccidials (Chapman et al., 2016; Parent et al., 2018). However, it has also been reported that OPG in the litter of flocks treated with anticoccidial drugs were not seen until 34 days of age (Williams and Gobbi, 2002). This observation might have been explained by the environment, since the studies from Chapman, et al. (2014) and Parent, et al. (2018) took place in commercial farms, while the study from Williams, et al. (2002) was conducted in a research facility. Indeed, a commercial environment is known to harbour viable *Eimeria* spp. oocysts at placement (Jenkins et al., 2019), which may contribute to an earlier infection compared to a clean environment such as a research facility. However, the higher excretion of oocysts in the fecal content is most likely not affecting the flocks, as zootechnical performances and post-mortem examinations were not affected by the antibiotic-reduction treatments.

The reduction and elimination of antibiotic use in poultry production has often been associated with health disorders. For example, a large US poultry company experienced important technico-economical losses associated with the transition to a drug-free program (Smith, 2011). These problems associated with NE were also reported in another study, where 27.45% of the RWA flocks experienced outbreaks of clinical NE and 49.02% of these flocks were affected by subclinical NE, which was significantly different from

conventional flocks using antibiotics as none of these flocks experienced issues with clinical or subclinical NE (Gaucher et al., 2015). In contrast, none of these conditions were observed during the present study. This observation can be supported by the absence of generalized clinical signs of enteritis in the three groups, but also by normal mortality rates, standard growth rates and low intestinal lesion scores. In a US study comparing NAE (no antibiotic), antibiotic-reduced (use of nonmedically important antibiotic) and conventional (use of medically important antibiotics) broiler chicken flocks in a commercial context, NAE flocks showed higher odds of eyes burns (i.e., corneal erosion or ulceration), footpad lesions and airsacculitis compared to the 2 other groups using antibiotics (Karavolias et al., 2018). When flocks receiving nonmedically important antibiotics were compared to flocks receiving medically important antibiotics, only footpad lesions showed slightly higher odds, while eyes burns had similar odds and airsacculitis lesions had lower odds. Similarly to the results from Karavolias et al. (2018), there were no significant differences in the incidence of eye burns between flocks using nonmedically and medically important antibiotics. The slightly higher odds of having footpad lesions were partially seen in our results; the antibiotic-reduced group receiving butyrates showed higher pododermatitis mean scores at 28 days of age. This condition could be an indicator of an impaired gastro-intestinal system, since any disease that induce watery droppings and diarrhea can cause wet litter problems (Dunlop et al., 2016). The presence of wet litter can be an important factor contributing to pododermatitis (Tullo et al., 2017), thus the importance of monitoring foot pad lesions during grow-out to assess the presence of intestinal disorders. However, the litter and fecal humidity in our study were similar between the three groups for the three time points recorded. Hence, this hypothesis would not be consistent with pododermatitis caused by intestinal diseases and wet litter in the group receiving butyrates. The slightly lower odds of identifying airsacculitis lesions in the flocks receiving nonmedically important antibiotics compared to the flocks receiving medically important antibiotics from Karavolias et al. (2018) was not observed in our study. Bacterial airsacculitis lesions being mostly related to an infection by the Gram-negative bacterium *E. coli* (El-Sukhon et al., 2002), the prevention programs for coccidiosis and NE are most likely not influencing this condition since most antibiotics in these programs are active against Gram-positive bacteria (Agunos et al., 2017). Bursal diameter is a variable rarely evaluated in studies evaluating

the impact of antibiotic use on performance and health even if its size can be related to immunosuppression caused by Chicken Infectious Anemia Virus (CIAV) (Haridy et al., 2012), Infectious Bursal Disease virus (IBDv) (Withers et al., 2005), Marek's Disease Virus (MDV) (Chang et al., 2011), reovirus (Wang et al., 2007) or mycotoxin contamination in the feed (Peng et al., 2014), all potentially decreasing bursal size and impacting health and performance. At 21 days of age, bursal diameter was similar between the three groups, but a statistical difference has been identified at 28 days of age between the group receiving ionophores only and the group receiving ionophores and butyrates. Although significant, the 0.23 cm difference is most likely marginal due to the absence of differences between the two groups on zootechnical performance or the severity of lesions evaluated in other systems.

This study presented a disease prevention program relying on the continuous use of ionophores as a potential replacement of current conventional shuttle programs using antibiotics medically important for human medicine. Discontinuing the use of medically important antibiotics is a crucial step to decrease the selective pressure on bacteria harbouring resistance genes to antibiotics and decrease the likelihood of animal production transferring antibiotic resistant bacteria to the human population. The results obtained during this study in a commercial context do not support the extensive use of medically important antibiotics in disease prevention programs of broiler chicken flocks. Indeed, zootechnical performance and control of intestinal diseases were shown to be similar between conventional shuttle programs using medically important antibiotics and antibiotic reduction strategies using ionophores continuously. The addition of in-feed butyric acids as a replacement of antibiotic growth promoters did not result in zootechnical performance improvement or better health control compared to flocks receiving exclusively ionophores in their prevention program. In a "One Health" perspective, this study provides to the broiler chicken industry a successful strategy to decrease the impacts of agriculture on antimicrobial resistance by using nonmedically important antibiotics in broiler chicken production.

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Table 1. Summary of the participating farms

Farm ID	Houses capacity (# chickens)	Hatchery ID	Feed mill ID	Processor ID	Average downtime between flocks (days)
1	12 000	1	1	1	11.4
2	22 000	2	2	2	17.4
3	15 000	2	3	3	14.4
4	13 500	2	2	2	18.2
5	19 000	3	3	2	20.0
6	13 500	2	2	2	18.0
7	9 800	3	4	2	19.4

Table 2. Impacts of treatments on zootechnical performances

Zootechnical parameter	Ionophores (n = 21)		Ionophores and butyric acids (n = 21)		Conventional (n = 42)		
	Mean	S.E.	Mean	S.E.	Mean	S.E.	*p*-value
Slaughter weight (kg)	2.44	0.07	2.41	0.04	2.43	0.03	0.40
Feed conversion ratio	1.62	0.04	1.64	0.02	1.64	0.02	0.23
Average daily gain (g/day)	66.7	1.1	65.7	1.1	66.1	0.9	0.34
Age at slaughter (days)	36.7	1.0	36.7	0.3	36.6	0.3	0.66
Mortality (%)	2.92	0.58	3.03	0.38	3.18	0.33	0.43
Total condemnations (%)	1.74	1.17	1.62	1.17	1.70	1.15	0.64

Table 3. Results of the lesion scoring by treatment at 21 and 28 days of age.

Lesion score		Ionophores		Ionophores and butyric acids		Conventional		p-value
		Mean	S.E.	Mean	S.E.	Mean	S.E.	
Bodyweight (g)	21 days	952.3	19.9	964.9	22.4	953.8	19.4	0.57
	28 days	1605.4	38.2	1596.4	35.2	1617.3	30.5	0.70
Gastro-intestinal system								
Intestinal Integrity Index (I2)	21 days	93.9	0.8	93.9	0.8	93.9	0.7	0.95
	28 days	92.5	0.9	91.8	1.1	92.7	1.0	0.52
Eimeria acervulina	21 days	0.30	0.09	0.21	0.12	0.25	0.11	0.48
	28 days	0.44	0.10	0.36	0.13	0.44	0.12	0.52
Eimeria maxima	21 days	0.03	0.01	0.02	0.01	0.01	0.01	0.06
	28 days	0.02	0.02	0.05	0.03	0.03	0.02	0.12
Eimeria tenella	21 days	0.04	0.03	0.04	0.03	0.01	0.03	0.2
	28 days	0.01	0.03	0.07	0.05	0,00	0.04	0.13
Microscopic *E. maxima*	21 days	0.21	0.09	0.18	0.11	0.15	0.09	0.52
	28 days	0.5	0.13	0.49	0.16	0.35	0.14	0.27
Necrotic enteritis	21 days	0.0	0.0	0.0	0.0	0.0	0.0	1.00
	28 days	0.0	0.0	0.0	0.0	0.0	0.0	1.00
Gizzard erosions	21 days	0.08	0.06	0.06	0.05	0.08	0.04	0.59
	28 days	0.07	0.06	0.15	0.05	0.14	0.05	0.14
Intertegumentory and skeletal systems								
Burned feet (pododermatitis)	21 days	0.64	0.18	0.80	0.12	0.61	0.11	0.21
	28 days	0.84[a]	0.20	1.11[b]	0.14	0.83[a]	0.12	0.05

Femoral head necrosis	21 days	0.02	0.01	0.01	0.01	0.01	0.01	0.11
	28 days	0.003	0.010	0.002	0.010	0.013	0.09	0.15
Tibial dyschondroplasia	21 days	0.11	0.04	0.09	0.03	0.08	0.03	0.25
	28 days	0.11	0.06	0.08	0.03	0.08	0.03	0.36
Immune system								
Bursal diameter (cm)	21 days	2.05	0.13	1.82	0.19	1.88	0.16	0.22
	28 days	2.02[a]	0.08	1.79[b]	0.10	1.86[ab]	0.08	0.05
Respiratory system								
Airsacculitis	21 days	0.13	0.11	0.34	0.13	0.23	0.11	0.09
	28 days	0.17	0.11	0.26	0.11	0.34	0.10	0.08
Tracheal mucosa reddening	21 days	0.63	0.08	0.75	0.11	0.70	0.09	0.29
	28 days	0.75	0.11	0.72	0.11	0.69	0.09	0.52
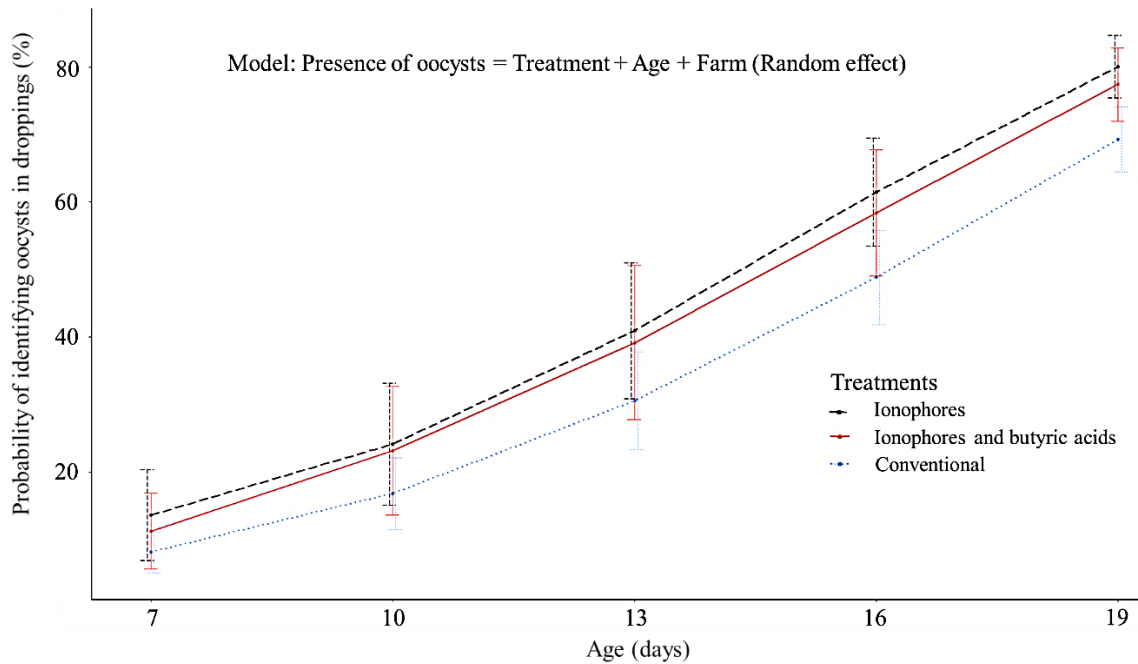
Figure 1. Predicted probabilities of a fecal sample containing *Eimeria* spp. oocysts from 7 to 19 days old flocks for each treatment based on the mixed multivariable logistic regression model ($n = 412$). Error bars are corresponding to the 95% confidence intervals computed for each predicted probability by the model.
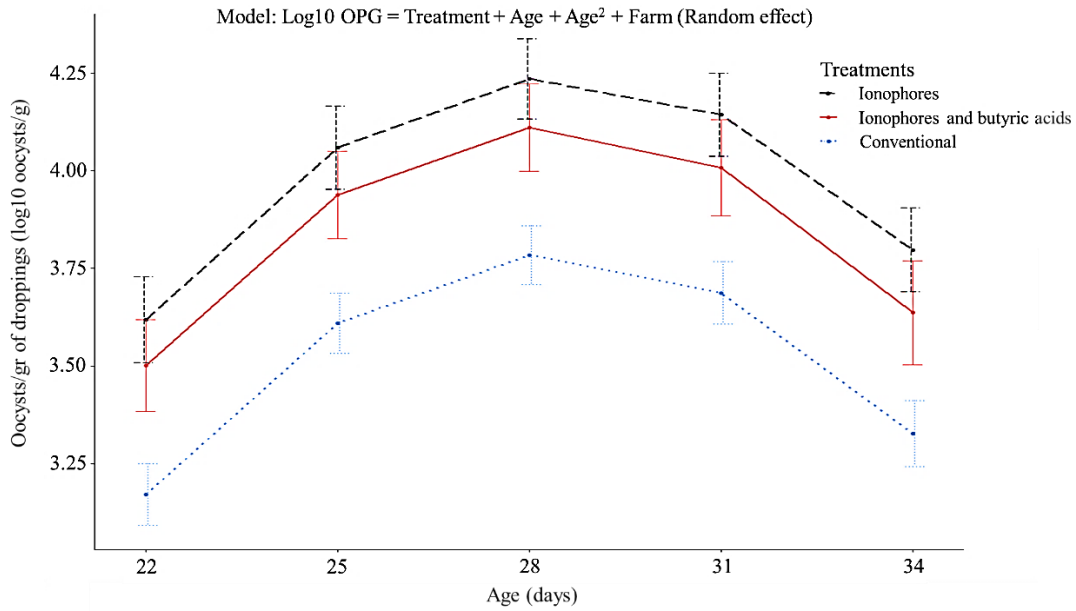
Figure 2. Predicted log10 OPG values from 22 to 34 days old flocks for each treatment based on the mixed multivariable linear regression model ($n = 395$). The error bars are corresponding to the 95% confidence intervals computed for each predicted OPG value by the model.

Supplemental table 1. Medication programs used in all flocks

Supplemental table 1. Medication programs used in all flocks.

Farm	House	1 Starter	1 Grower	1 Finisher	2 Starter	2 Grower	2 Finisher	3 Starter	3 Grower	3 Finisher	4 Starter	4 Grower	4 Finisher	5 Starter	5 Grower	5 Finisher	6 Starter	6 Grower	6 Finisher
1	Antibiotic reduction	Sal	Sal	Sal	Sal / But	Sal / But	Sal / But	Mo / But	Mo / But	Mo / But	Mo	Mo	Mo	Nar / But	Nar / But	Nar / But	Nar	Nar	Nar
	Conventional	Nar + Nic / Pen	Nar + Nic / Pen	Mo / Virg	Nar + Nic / Pen Nar + Nic / Virg	Sal / But	Mo / Virg	Zo / Pen	Zo / Tyl	Nar / Avi	Zo / Pen	Clo / Tyl	Nar / Avi	Clo / Fla	Clo / Fla	Mo / Fla	Nic / Fla	Nic / Fla	Mo / Fla
2	Antibiotic reduction	Mo	Mo	Mo	Sal / But	Sal / But	Sal / But	Nar	Nar	Nar	Mo / But	Mo / But	Mo / But	Nar / But	Nar / But	Nar / But	Sal	Sal	Sal
	Conventional	Nic / Virg	Nic + Nar / Tyl	Sal / Avi	Nic + Nar / Tyl Nic + Nar / Tyl	Sal / But	Sal / Avi	Nic + Nar / Tyl Nic + Nar / Tyl	Nic + Nar / Tyl Nic + Nar / Tyl	Sal / Avi	Nic + Nar / Tyl Nic + Nar / Tyl	Mo / But	Sal / Avi	Nic + Nar / Bac Nic + Nar / Bac	Nic + Nar / Bac	Mo / Bac	Nic / Bac	Mo / Bac	Mo / Bac
3	Antibiotic reduction	Nar	Nar	Nar	Sal / But	Sal / But	Sal / But	Sal	Sal	Sal	Nar / But	Nar / But	Nar / But	Mo	Mo	Mo	Mo / But	Mo / But	Mo / But
	Conventional	Nic / Virg	Nic / Virg	Mo / Virg	Ro / Bac	Ro / Bac	Sal / Bac	Ro / Bac	Ro / Bac	Sal / Bac	Nic + Nar / Avi Nic + Nar / Avi	Sal	Sal	Nic + Nar / Avi Nic + Nar / Avi	Mo / But	Mo / But	Nic + Nar / Virg Nic + Nar / Virg	Sal / But	Sal / But
4	Antibiotic reduction	Mo	Mo	Mo	Nar / But	Nar / But	Nar / But	Nar	Nar	Nar	Sal	Sal	Sal	Mo / But	Mo / But	Mo / But	Sal / But	Sal / But	Sal / But
	Conventional	Nic / Virg	Nar / Virg	Nar / Virg	Nic / Virg	Nic + Nar / Tyl	Sal / Avi	Nic + Nar / Tyl Nic + Nar / Tyl	Nic + Nar / Tyl Nic + Nar / Tyl	Sal / Avi	Nic + Nar / Tyl Nic + Nar / Tyl	Sal	Sal / Avi	Nic + Nar / Avi Nic + Nar / Avi	Mo / But	Mo / But	Nic / Virg	Nic / Virg	Mo / Virg
5	Antibiotic reduction	Mo	Mo	Mo	Nar / But	Nar / But	Nar / But	Nar	Nar	Nar	Sal	Sal	Sal	Mo / But	Mo / But	Mo / But	Sal / But	Sal / But	Sal / But
	Conventional	Ro / Bac	Ro / Bac	Sal / Bac	Ro / Bac	Ro / Bac	Sal / Bac	Sal	Sal	Nar	Mo / But	Mo / But	Nar / Bac	Nar	Nar	Nar	Sal / But	Sal / But	Sal / But
6	Antibiotic reduction	Mo	Mo	Mo	Nar / But	Nar / But	Nar / But	Nar / But	Nar / But	Nar / But	Nic + Nar / Tyl Nic + Nar / Avi	Mo / But	Mo / But	Nic + Nar / Bac Nic + Nar / Bac	Nic + Nar / Bac Nic + Nar / Bac	Mo / Bac	Nic / Bac	Mo / Bac	Mo / Bac
	Conventional	Nic / Virg	Nar / Virg	Nar / Virg	Nic + Nar / Tyl Nic + Nar / Avi	Nic + Nar / Tyl Nic + Nar / Avi	Nar / But	Nar / But	Nar / But	Sal / Avi	Nic + Nar / Tyl Nic + Nar / Avi	Mo / But	Mo / But	Nic + Nar / Bac Nic + Nar / Bac	Sal / But	Mo / Bac	Nic / Bac	Mo / Bac	Mo / Bac
7	Antibiotic reduction	Nar	Nar	Sal / Fla	Mo	Mo	Mo	Nar / But	Nar / But	Mo	Mo / But	Mo / But	Mo / But	Sal / But	Sal / But	Sal / But	Sal	Sal	Sal
	Conventional	Dec / Avi	Dec / Avi	Sal / Fla	Dec / Avi	Dec / Avi	Sal / Fla	Nar + Nic / Virg Nar + Nic / Virg	Nar + Nic / Virg Nar + Nic / Virg	Nar / Virg	Nar + Nic / Virg Nar + Nic / Virg	Nar / Virg	Nar / Virg	Nar + Nic / Tyl Nar + Nic / Avi	Nar + Nic / Tyl Nar + Nic / Avi	Sal / Avi	Nar + Nic / Tyl Nar + Nic / Avi	Sal / Avi	Sal / Avi

Anticoccidials: Clo: Clopitol / Dec: Decoquinate / Mo: Monensin / Nar: Narasin / Nic: Nicarbazin / Nar + Nic: Mixture of narasin and nicarbazin / Ro: Robenidine / Sal: Salinomycin / Zo: Zoalene
Antibiotics: Avi: Avilamycin / Bac: Bacitracin / Fla: Flavomycin / Pen: Penicillin Procaine / Tyl: Tylosin / Virg: Virginiamycin
Prebiotic: But: Butyric acids

Supplemental table 2. Ingredients inclusion rates guidelines for the feed formulation of all participating farms.

Ingredients	Inclusion rate
Barley	0%
Dried distillers' grain	7% maximum
Shorts	5% maximum
Meat meal	2 à 10%
Canola	5% maximum
Wheat	15% maximum
Fine gluten	5% maximum
Coarse gluten	5% maximum
Feather meal	3% maximum
Fat	0 to 4%
Bakery by-products	12.5% maximum
Vitamins and minerals	Within the guidelines provided in Table 4: Range of Nutrient Guarantees for Complete Feeds for Use in the Exemption of Feeds from Registration[1]
Enzymes	Allowed
Prebiotic/Probiotic	Not allowed

[1]https://laws-lois.justice.gc.ca/PDF/SOR-83-593.pdf

(Last date consulted: January 27, 2020)

Supplemental table 3. Logistic and linear multivariable regression models fixed effect results with the farm as a random intercept.

Fixed effects	Category	Coefficient	*p*-value	95% CIs
Early ages[*]	*Mixed multivariable logistic regression (n = 412)*			
Outcome	*Presence or absence of Eimeria spp. oocysts*			
Treatment	Ionophores	Referent		
	Ionophores and butyric acids	-0.226	0.529	(-0.936, 0.478)
	Conventional	-0.663	0.033	(-1.278, -0.058)
Age		0.344	< 0.001	(0.272, 0.424)
Late ages[#]	*Mixed multivariable linear regression (n = 395)*			
Outcome	*Log10 OPG in droppings*			
Treatment	Ionophores	Referent		
	Ionophores and butyric acids	-0.117	0.488	(-0.446, 0.211)
	Conventional	-0.447	0.002	(-0.726, -0.167)
Age		0.817	< 0.001	(0.385, 1.252)
Age^2		-0.014	< 0.001	(-0.022, -0.007)

[*]Early ages: 7, 10, 13, 16 and 19 days of age

[#]Late ages: 22, 25, 28, 31 and 34 days of age

Supplemental table 4. Impacts of the treatments on litter and fecal humidity.

Type of sample	Ionophores (n = 21)		Ionophores and butyric acids (n = 21)		Conventional (n = 42)		
	Mean	S.E.	Mean	S.E.	Mean	S.E.	*p*-value
Litter (% humidity)							
21 days of age	30.8	2.2	33.0	2.1	32.3	1.8	0.31
28 days of age	33.2	2.0	33.9	1.9	31.8	1.7	0.40
Before shipping to slaughter	32.1	2.1	31.4	1.7	30.7	1.5	0.32
Feces (% humidity)							
21 days of age	80.4	0.9	79.0	1.1	80.0	0.9	0.20
28 days of age	78.8	0.6	79.9	0.7	79.6	0.6	0.08
Before shipping to slaughter	79.4	0.7	79.9	0.8	79.1	0.7	0.58

Article 2. Environmental factors outweigh the impact of antibiotic growth promoters on cecal microbiota composition of commercial broiler chickens

Unpublished manuscript. Ready for submission to Applied and Environmental Microbiology

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Running head: Gastro-intestinal microbiota in broiler chickens

Abstract

The objective of the study was to investigate the association of various flock-level factors on the composition of the cecal microbiota in commercial broiler chicken at the end of grow-out. The cecal content chickens in 84 broiler chicken flocks was recovered at the end of grow-out (n = 1002) and total DNA was extracted to amplify and sequence the V3-V4 region of 16S rRNA to assess the composition of the microbiota. All factors evaluated significantly impacted the structure of the microbiota (p < 0.002). The farm (R-value = 0.239) and flock cycle (R-value = 0.374) showed the highest association with the microbiota composition, the feed provider (R-value = 0.118), type of antibiotic program (R-value = 0.039) and chick provider (R-value = 0.035) were considerably smaller. There were important microbiota differences between farms as taxa identified by LEfSe were highly variable between farms and determinant microbiota features in one farm could be partially to totally different from other farms. Numerous significant differences were identified between farms for Richness and Evenness as five significant pairwise comparisons were identified (p < 0.05) between the seven farms. No significant alpha diversity variation was detected between antibiotic programs (p > 0.05). In conclusion, this study highlights the importance of environmental factors possibly influencing the microbiota in broiler chickens, and future strategies aimed at modulating the GIT microbiota of broiler chickens should involve a holistic approach by considering the multifactorial aspects of the microbiota development in commercial broiler chickens.

Importance

The cecal microbiota in broiler chickens is an important determinant of growth performance as it assists intestinal physiological functions such as metabolism, nutrient release, and energy production. It is believed that modulating the cecal microbiota is pivotal to improve poultry production sustainability by improving feed utilization efficiency. However, the cecal microbiota can be influenced by numerous concurrent factors in commercial operations and understanding these complex interactions may be critical in developing innovative strategies to modulate its composition. In this study, it has been observed that changing the type of antibiotic programs in commercial farms was not

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associated in a shift of microbial communities, which is against a widespread paradigm that antibiotics promote growth by modulating the intestinal microbiota. Conversely, it was observed that environmental factors were more strongly associated with the cecal microbiota, indicating that microbiota manipulation methods should emphasize on these factors to improve growth performance.

Introduction

The bacterial microbiota in the gastro-intestinal tract (GIT) of broiler chickens is a highly diverse population predominantly composed of phyla including *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* (1-4). These communities benefit the host by assisting physiological functions such as intestinal metabolism, nutrient release, and energy production. For instance, numerous members of the GIT microbiota have a central role in butyrate, propionate, and acetate production (5-7); substrates oxidized by GIT cells as a source of energy (8). Hence, the GIT microbiota of chickens is seen as an important contributor to growth performance (9, 10). *Lachnospiraceae* and *Ruminococcaceae*, abundant bacterial families of the cecal microbiota in broiler chickens (2), have been correlated with improved growth rate and feed efficiency in controlled experiments (4, 11-13), but numerous other taxa of the GIT have since been correlated to improved or decreased growth rate in a commercial context (14).

The GIT microbiota is a dynamic population of microorganisms affected by numerous factors shaping the structure and membership of bacterial communities through chickens' life. Its composition is reported to exhibit considerable variation between individual chickens, even within the same flock (15). In contrast to mammals, which are mostly viviparous, birds are oviparous and lay hard-shelled eggs. Embryos are incubated in this structure and commercial broiler chicks hatch and grow without parental contact. This limited early exposure to bacteria at hatch might be related to the low bacterial diversity observed during the early life of broiler chickens (16-20) and most likely

predisposes the GIT to be largely colonized by bacteria present in the environment rather than the bacterial species that newly hatched chicks would be exposed to within a nest in a natural wild setting. Besides, contemporary commercial broiler chickens are typically raised on litter and its microbial composition is an important factor contributing to the GIT colonization by bacteria. For instance, bedding materials and housing type have been associated with different microbial communities in the ceca of broiler chickens (21, 22). The GIT microbiota may also be affected by chickens' diet, both via direct seeding of bacteria and because environmentally derived bacteria use the diet components as sources of energy, carbon, and other nutrients, for growth (23). The type of feed, for example corn-based, sorghum-based, wheat-based or with the inclusion of fishmeal, has been shown to impact microbiota community structure and membership in chickens' GIT by modulating the relative abundance of various bacterial genera, such as *Clostridium* and *Lactobacillus* (24-27). Feed additives, such as sodium butyrates, may also alter the cecal microbiota diversity as it was shown to significantly decrease the relative abundance of *Lactobacillaceae* (28). Many other feed components and characteristics are reported to impact the intestinal microbiota, for example vitamins (29), enzymes (30), protein source and feed particle size (31). A common and largely promoted concept in animal production is that antibiotics alter the composition of the intestinal bacterial microbiota and provide beneficial effects such as improved weight gain and feed conversion efficiency (32). However, the use of antibiotics in the diet of broiler chickens may have no or limited impact on the core cecal microbiota structure but may alter the differential abundance of minor phyla (33, 34).

Commercial chickens are exposed to a large number of these factors reported to modify the microbiota and the resulting microbial communities found in the GIT are most likely the consequence of a combination of these factors. However, assessing the individual effects of these variables may not accurately reflect the relative importance of each factor in shaping the microbiota in commercial populations. Indeed, each component, such as early exposure to bacteria, diet and antibiotic use, may have variable effects on the development of microbial communities depending on timing and combinatorial effects. Understanding these complex interactions in a commercial context may be critical in developing new and innovative strategies to modulate the GIT microbiota of commercial

chickens. Hence, the objective of the study was to investigate the association of various flock-level factors on the composition of the cecal microbiota in commercial broiler chicken at the end of grow-out. More precisely, we determined the strength of association and significance of the farm environment, feed provider, chick provider, flock cycle and type of antibiotic program on the diversity and composition of the cecal microbiota of broiler chickens raised in commercial farms.

Results

Microbiota composition of commercial broiler chickens is predominantly influenced by the farm and flock cycle, while the type of antibiotic program used during grow-out has a relatively minor impact on microbial communities.

Using the ANOSIM output (Table 1), we evaluated the statistical significance and effect size of various factors to assess their relative correlation with cecal microbiota communities in broiler chickens. The farm (p-value = 0.001), feed provider (p-value = 0.001), chick provider (p-value = 0.017), flock cycle (p-value = 0.001) and type of antibiotic program (p-value = 0.002) were all significantly associated with the microbiota composition. R-values were then assessed to evaluate the strength of association of these factors with the structure of the microbiota and identify the principal variables associated with the microbiota composition. R-values close to 1.0 suggests a relatively higher dissimilarity between groups than within groups, while R-values close to 0 suggests an even distribution of high and low ranks within and between groups (35). R-values close to -1.0 would be associated with relatively higher dissimilarity within groups than between groups. The farm (R-value = 0.239) and flock cycle (R-value = 0.374) showed the highest dissimilarities between groups, while the impacts of other factors such as the feed provider (R-value = 0.118), type of antibiotic program (R-value = 0.039) and chick provider (R-value = 0.035) were considerably smaller with low R-values, suggesting more even microbiota compositions between the groups of these variables. Redundancy analysis (RDA) plots were used to display the degree of microbiota dissimilarity for each factor (Figures 1 and 2). Visually more variation in the microbiota composition could be seen between farms and flock cycles compared to the variation that could be attributed to feed providers, chick providers or type of antibiotic programs. Farms F2 and F7 were shown to

be the most dissimilar compared to other farms. Microbiota composition between feed providers was mostly similar, but chickens fed by the feed mill 2 showed the highest dissimilarity compared to chickens fed by other feed providers. Low dissimilarity could be observed between chickens originating from different hatcheries, but chickens provided by the hatchery B were the most dissimilar compared to other hatcheries. RDA plots indicated that microbiota composition of chickens exposed to different types of antibiotic programs were highly similar and low variation could be observed between groups. Microbiota composition dissimilarities between flock cycles was variable between farms; F6 and F7 exhibited highly dissimilar microbiota composition between flock cycles, while other farms such as F1, F2, F3, F4 and F5 displayed more even microbiota composition through successive flocks.

Table 1. Effect size and significance of farm-level factors with the microbiota composition evaluated by the ANOSIM ($n = 1002$).

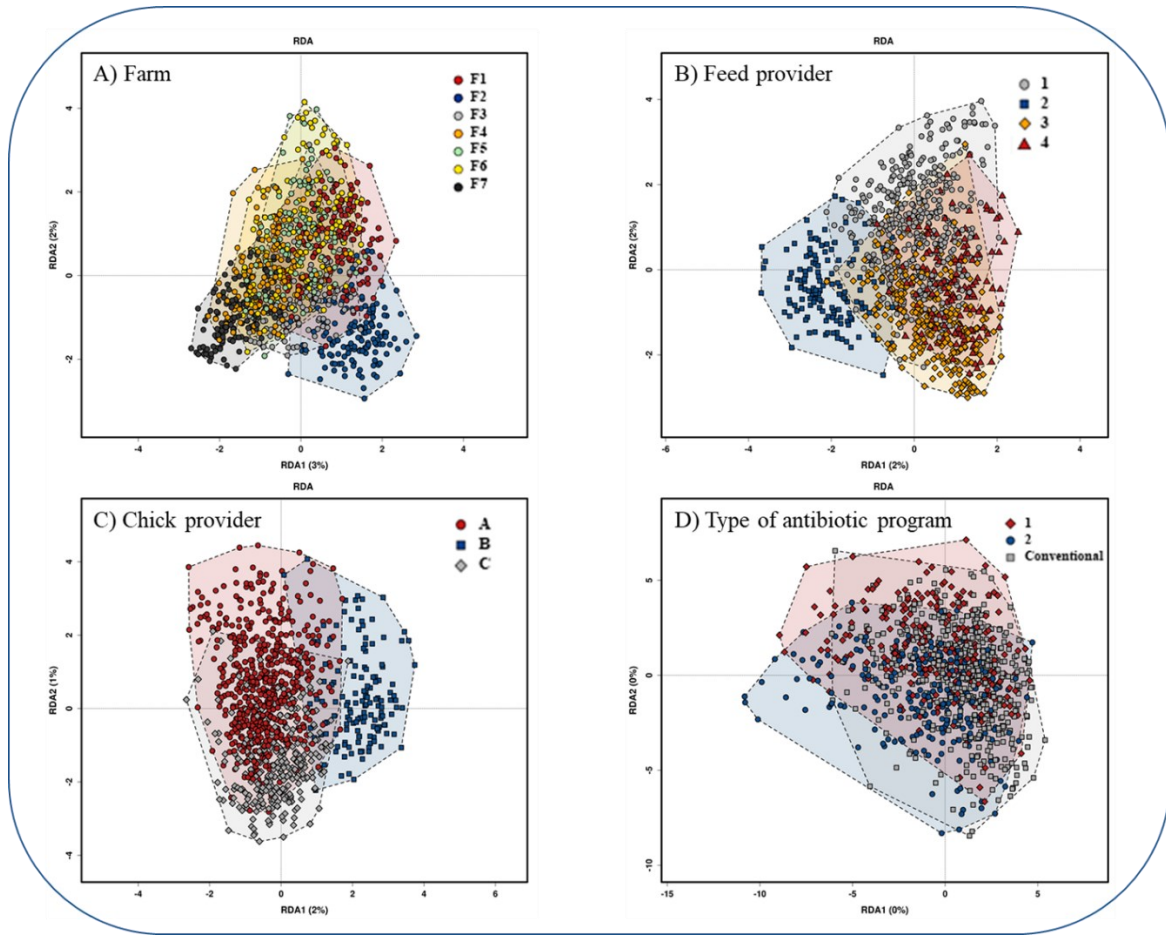|	Microbiota	
Factor	R-value[†]	p-value
Flock cycle	0.374	0.001
Farm	0.239	0.001
Feed provider	0.118	0.001
Type of antibiotic program	0.039	0.002
Chicks provider	0.035	0.017

Figure 1. Redundancy analysis (RDA) plots showing the variation of the cecal microbiota composition that can be explained by the farm (A), the feed provider (B), the chick provider (C) and the type of antibiotic program (D). Each dot represents the cecal sample recovered from one chicken ($n = 1002$).
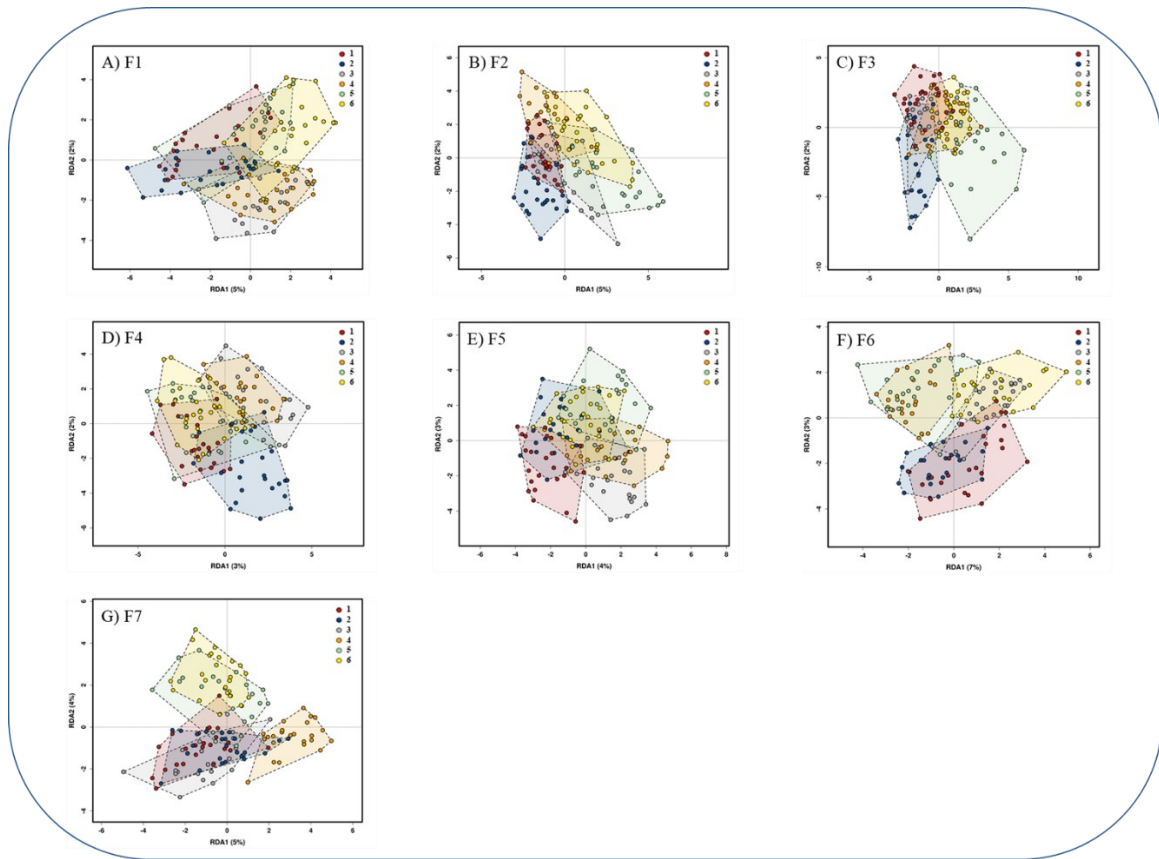
Figure 2. Redundancy analysis (RDA) plots showing the variation of the cecal microbiota beta diversity in each farm that can be explained by the flock cycle. A total of six consecutive flock cycles were done on each of the seven farms. Each dot represents the cecal sample recovered from one chicken ($n = 1002$).

A few taxa of the cecal microbiota explain compositional differences between farms

Since the composition of the cecal microbiota was largely impacted by each farm, linear discriminant analysis effect size (LEfSe) were conducted to identify bacterial phyla and families explaining these significant differences identified by the ANOSIM. Seven phyla and 27 families were identified by the LEfSe as important taxa to discriminate microbial communities between the seven farms (Figure 3). The analysis showed that *Tenericutes*, *Firmicutes*, *Cyanobacteria*, *Actinobacteria*, *Bacteroidetes*, *Proteobacteria* and *Verrucomicrobia* were important phyla of the cecal microbiota to distinguish six of the

seven farms included in the study, while no specific phylum could be attributed to the seventh farm. A single phylum was associated with the differentiation of each specific farm, excluding F2 as *Bacteroidetes* and *Proteobacteria* were both important to discriminate this farm. F3, the farm without a phylum to represent its dissimilarity from other farms, was the only farm with only one family, *Alcaligenaceae*, explaining microbiota differences from other farms. For other farms, between two and seven families were identified as explaining microbiota differences between groups. For example, *Lactobacillaceae* and *Streptococcaceae* were important families to discriminate F6 from other farms, while *Unclassified Bacteroidales*, *Rikenellaceae*, *Helicobacteraceae*, *Flavobacteriaceae*, *Unclassified SHA98*, *Veillonellaceae*, and *Clostridiales Family XIII IncertaeSedis* were important taxa to explain microbiota differences between F2 and other farms.
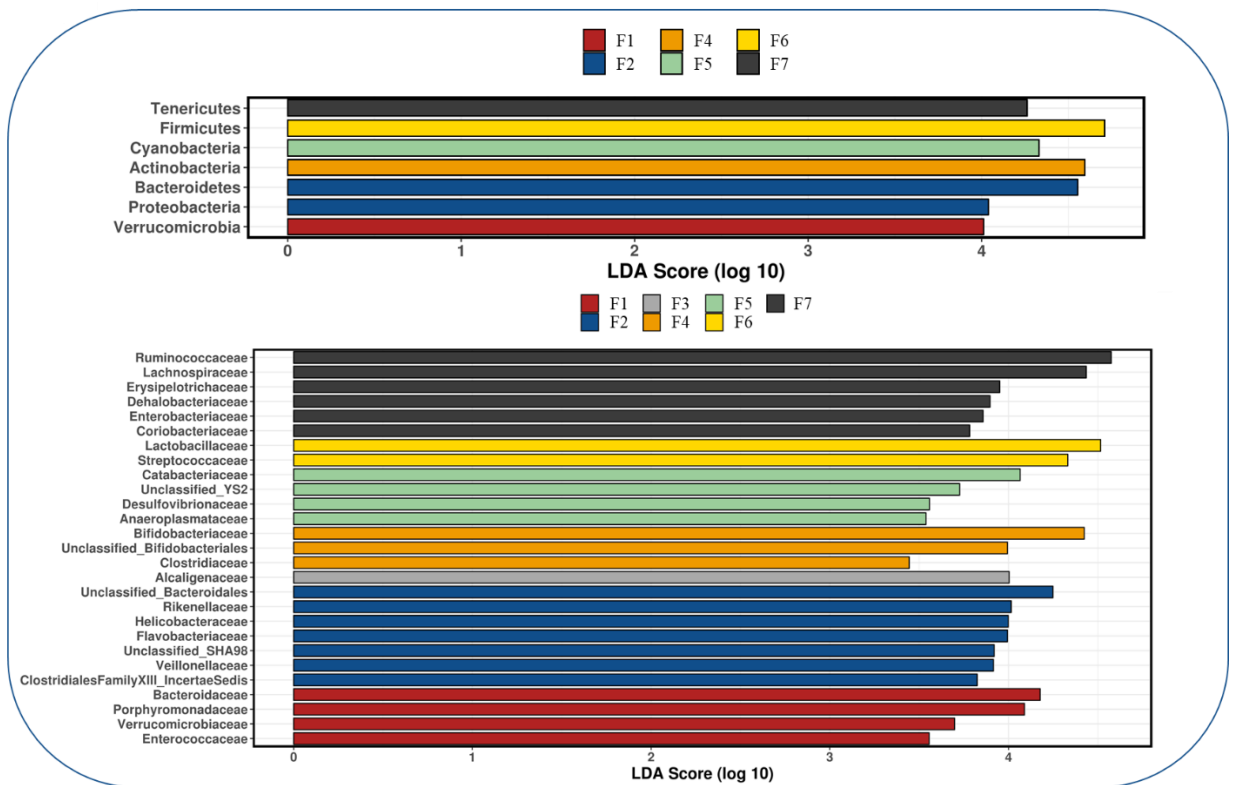

Figure 3. Linear discriminant analysis effect size (LEfSe) identifying bacterial phyla and families involved in determining microbiota differences between farms.

Microbiota composition differences between flock cycles are farm dependant and bacterial families are more important than phyla to explain changes between flock cycles on each farm.

As the highest R-value by the ANOSIM was attributed to the flock cycle, a LEfSe per flock cycle on each farm was conducted to identify phyla (Figure 4) and families (Figure 5) explaining cecal microbiota differences found between cycles on each farm. Since the microbiota composition was also influenced by farm clustering, the data was separated by farm to better discriminate individual variations between flock cycles on each farm. A minority of flock cycles on each farm could be differentiated with the LEfSe based on the relative abundance of various phyla, while the microbiota of most flock cycles on each farm could be differentiated by evaluating bacterial families. In farm F3 for example, flock cycle 6 was the only flock cycle to be discriminated by LEfSe, where only the relative abundance of *Actinobacteria* was identified to explain microbiota differences between flocks. However, when conducting the same analysis at the family level, the distinctive microbiota in five of the six flock cycles could be discriminated using 13 different families: *Bifidobacteriaceae*, *Unclassified Bifidobacteriales*, *Porphyromonadaceae*, *Clostridiaceae*, *Ruminococcaceae*, *Erysipelotrichaceae*, *Lachnospiraceae*, *Unclassified Clostridiales*, *Flavobacteriaceae*, *Coriobacteriaceae*, *Lactobacillaceae*, *Alcaligenaceae* and *Veillonellaceae*. However, these phyla and families identified by LEfSe were highly variable between farms, as determinant microbiota features in one farm could be partially to totally different from results obtained in other farms. For instance, flock cycle 2 in farm F4 could be differentiated from other flock cycles based on the abundance of the phylum *Verrucomicrobia*. At the family level, the microbiota composition in five of the six flocks could be differentiated by the relative abundance of *Anaeroplasmataceae*, *Desulfovibrionaceae*, *Flavobacteriaceae*, *Unclassified Bacteroidales*, *Porphyromonadaceae* and *Erysipelotrichaceae*.
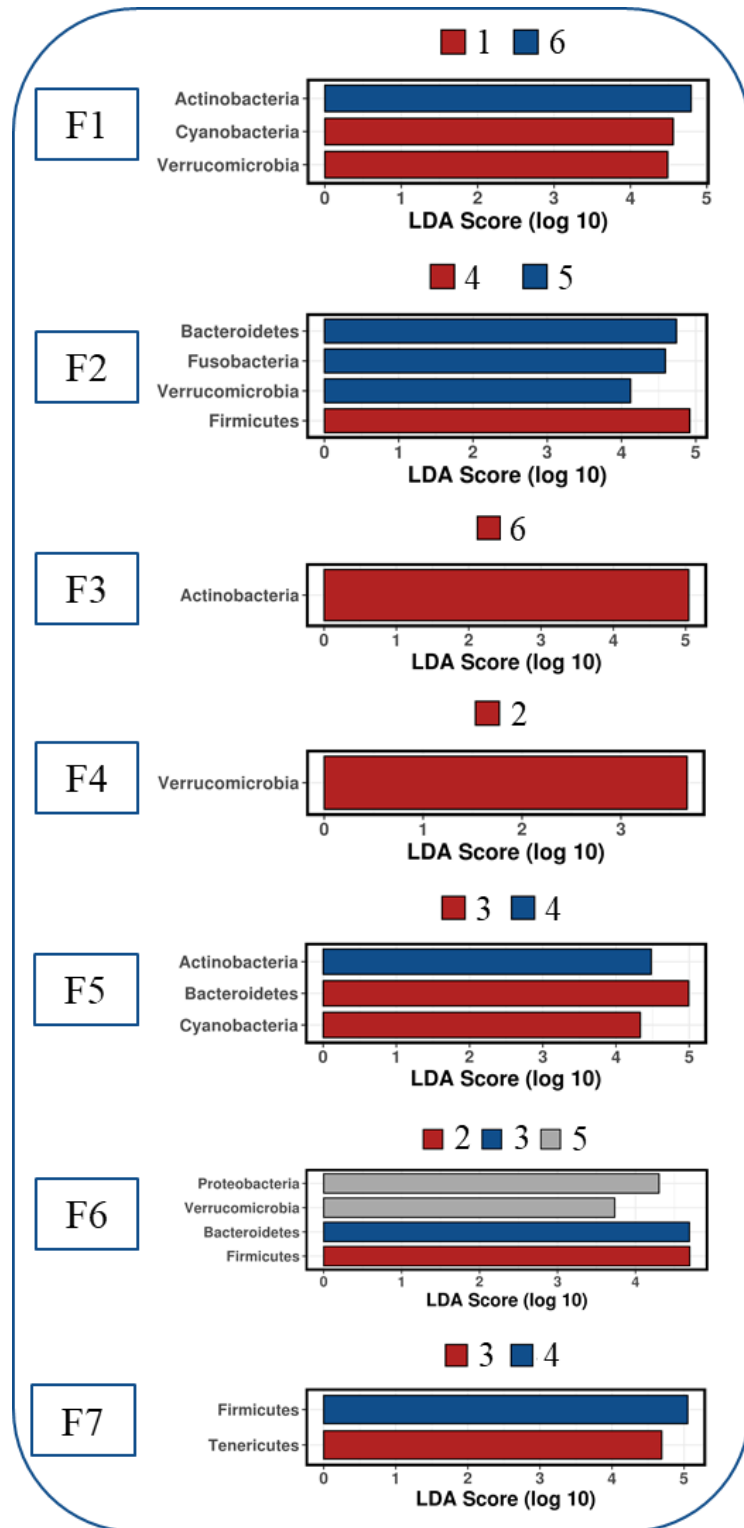
Figure 4. Linear discriminant analysis effect size (LEfSe) identifying the bacterial phyla involved in determining microbiota differences between flock cycles on each farm.
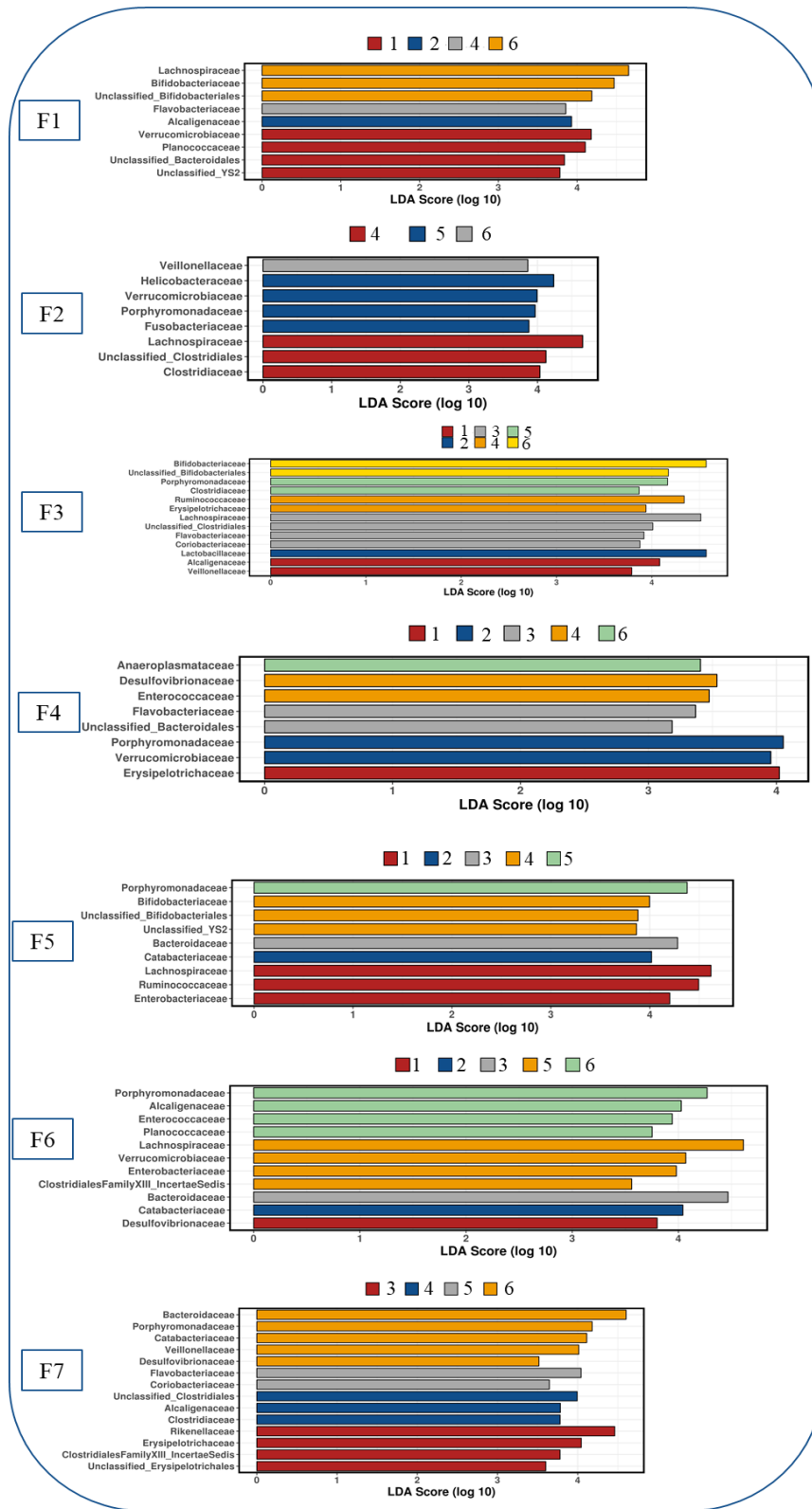
Figure 5. Linear discriminant analysis effect size (LEfSe) identifying the bacterial families involved in determining microbiota differences between flock cycles on each farm.

The type of antibiotic program does not impact alpha diversity, but significant variations were observed between farms, feed providers and chick providers.

Numerous significant differences were identified between farms for the two alpha diversity indicators measured (Richness and Evenness) as five significant pairwise comparisons were identified ($p < 0.05$) between the seven farms for each indice (Figure 5). Farm F7 showed significantly lower Richness compared to all other farms, while the six other farms showed a variety of dissimilarities and similarities for both alpha diversity indices. In general, both alpha diversity indices were correlated in a same sample, e.g. high Richness values were positively and significantly correlated to high Evenness values while low Richness was correlated to low Evenness (Supplemental Table A1). Chicken flocks fed by the feed provider 2 showed significantly increased Richness and Evenness compared to chickens fed by feed mills 1, 3 and 4 ($p < 0.05$). Alpha diversity indices in flocks receiving chicks from hatchery B were also significantly higher compared to flocks receiving chicks from hatcheries A and C. Evenly distributed Richness and Evenness was observed between the three types of antibiotic programs and no significant differences were detected between groups ($p > 0.05$).
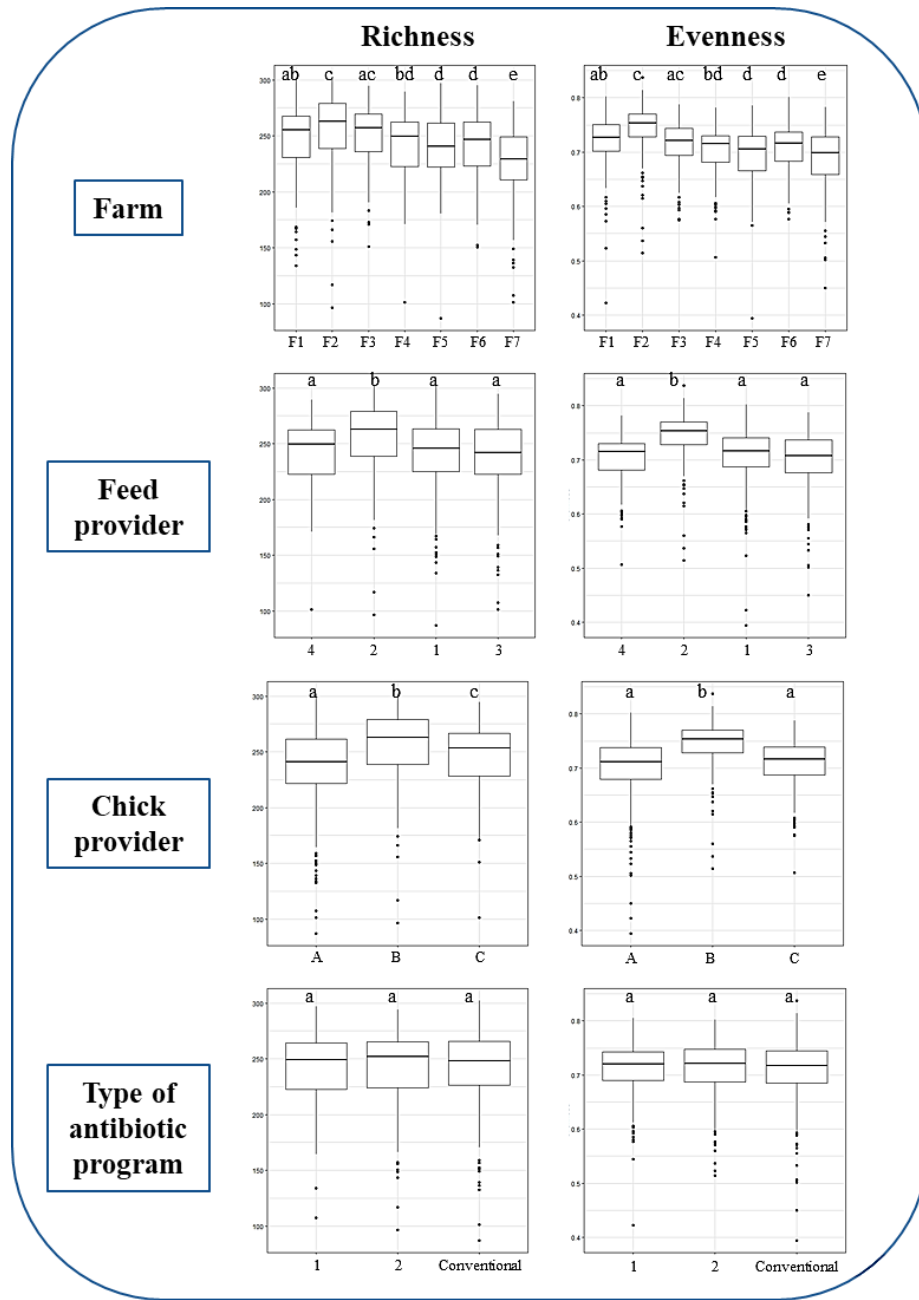
Figure 6. Distribution of the cecal Richness and Evenness between farms, feed providers, chick providers and type of antibiotic prevention programs in commercial chicken flocks. Groups sharing a same letter are not significantly different (p-value > 0.05).

Richness fluctuates more between flock cycles compared to Evenness

Significant variation ($p < 0.05$) of either cecal Richness or Evenness was observed between the six flock cycles raised for the majority of the seven farms (Figure 6). For

Richness, significant differences (p-value < 0.05) between flock cycles were observed in five of the seven farms, while no significant variation (p-value > 0.05) were observed for farms F4 and F6 by the Kruskal-Wallis test and Dunn's post-hoc test. For Evenness, there were significant variations between one or more flock cycles for three of the seven farms, while no significant differences were observed in four farms.
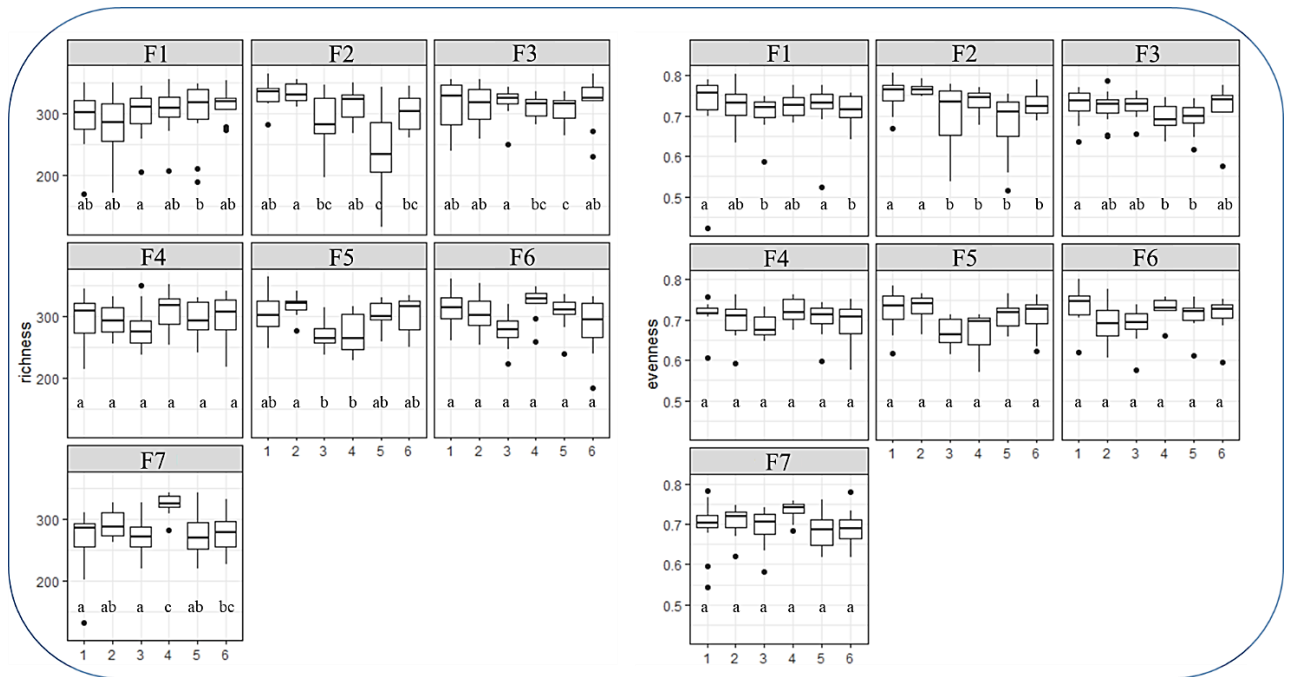
Figure 7. Evolution of the cecal Richness and Evenness from the first to last flock cycle in each farm. Groups sharing a same letter are not significantly different (p-value > 0.05).

Discussion

The aim of the study was to evaluate the association of various factors with the cecal microbiota of broiler chickens raised in commercial settings. The main findings indicated that environmental exposures during a flock life cycle are pivotal in shaping the composition of bacterial communities found in the cecal microbiota, since there was a higher strength of association of the farms and flock cycles with the cecal microbiota at the end of grow-out compared to the antimicrobial programs, chick providers and feed providers. On the opposite, a study previously suggested that diet may have greater impacts on the microbiota than the physical environment (25), but it must be noted that experimental rooms were identical and decontaminated with the same procedures before

the placement of chickens, which may be poorly representative of the variability inherent to commercial operations. Indeed, poultry houses are not systematically decontaminated between each flock cycle and cleaning and disinfection procedures are not likely to remove all bacterial species residing in commercial farms. For instance, fresh and reused broiler litter can hold 2 to 6 log10 CFU/g of *Enterobacteriaceae* and 8 to 10 log10 CFU/g of aerobic mesophilic bacteria (36) that may influence the early GIT colonization of newly arrived chicks on farm and impact the successional development of a flock's microbiota in its lifecycle (37). In consequence, housing conditions are reported to better explain the cecal microbiota variation compared to the effect size of nutritional interventions when different types of environments are considered (22). In this case, it was shown that chickens raised in grow-out feed trial facilities had more diverse and complex GIT microbiota compared to floor pens facilities for small scale experiments and isolators, where the highest bacterial loads could be observed in the first type of environment that was more comparable to commercial environments. Evidences are showing that significant differences in the GIT microbiota of chickens are observed between farms (38, 39), and the results of the present study strengthen these previous findings by showing that the farm bacterial microbiota is an important contributor to chickens' GIT microbiota. Furthermore, the relative abundance of numerous bacterial phyla and families in the cecal microbiota of broiler chickens fluctuated between farms, indicating that the collection of taxa in the environmental microbiota is potentially farm specific.

This farm specificity couldn't however explain all the variation in the composition of the cecal microbiota in chickens sampled, since considerable variation was observed in the relative abundance of taxa identified in the six consecutive flock cycles monitored on each farm. This inconsistency between flocks may be a consequence of the lack of early exposure to maternally derived bacteria (15). Indeed, modern commercial hatcheries use thorough cleaning and disinfection procedures to reduce the microbial loads (40, 41), which may limit the impact of this stage of production on the microbiota by offering low numbers of bacteria to colonize day-old chicks before shipping on farms. Hence, the marginal influence of the chicks providers on the cecal microbiota in the present study might be related to heavily sanitized hatchery facilities between segregated hatches. The distinct microbiota between flock cycles may also be related to on-farm cleaning and disinfection

procedures not monitored during the study, as Canadian broiler chicken producers are required to replace used litter by fresh bedding material between flocks. Sanitation with proper washing and disinfection may also be performed, although not a requirement between each flock, and might have been a source of variation between flocks in each poultry house (42). On the opposite, recycling litter from previous flocks can lead to the colonization of intestinal bacteria from previous flocks (37) and could prove to be an effective method to uniformize the microbiota between flocks in the same house. Still, reused litter is often treated between flocks to reduce its microbial load (36, 43) and may be insufficient in practice to carry intact bacterial communities between flocks.

Antimicrobials have been used for decades to treat or prevent bacterial infections in commercial poultry production. In broiler chicken production, most flocks are exposed to antimicrobials via in-feed administration for the prevention of intestinal diseases such as coccidiosis and necrotic enteritis (44), but they are also thought to alter the composition of the intestinal bacterial microbiota to improve feed efficiency and growth rate (32). Diets using antibiotics have been associated in research facilities with the modification of the cecal microbiota when compared to non-medicated diets (33, 34, 45, 46). However, with a coefficient of dissimilarity (R-value) close to 0 attributed to antibiotic programs, the results in the present study suggest that the differences in the microbiota composition between prevention programs are not more important than variations within each group, and that the different antibiotics used during grow-out are not key factors involved in modulating the cecal microbial communities. The addition of butyric acid as a feed additive has also been reported to modify the GIT microbiota (28, 47), but the results of this study are not concordant with previous works. Based on the extent of changes observed by the inclusion of butyrate as feed additives, it could be proposed that this product may have limited impacts when used in commercial operations with complex and diverse microbial communities in the environment. Hence, in a commercial context, the prevention programs using antibiotics or butyric acid may have limited influence on the cecal microbiota of broiler chickens at the end of grow-out. Since the microbiota plays an important role in modulating chicken flocks' growth performance and feed utilization efficiency, it could be hypothesized that the absence of significant variation between antibiotic and butyric acid programs for all key production indicators in the present study (48) could be related to the

ineffectiveness of these molecules to trigger profound changes in the composition and functionality of the cecal microbiota of broilers raised in commercial settings with already well established bacterial communities in their physical environment. Hence, the mechanistic action of antibiotics and alternatives for growth promotion via the improvement of the GIT microbiota and its functionalities should be reconsidered since their real impacts in commercial diets appear to be limited compared to other factors impacting the microbiota.

In conclusion, the cecal microbiota of commercial broiler chickens is highly diverse, and its complexity is most likely the result of successional and concurrent exposures shaping its composition through the lifetime of a flock. Nonetheless, the results of this study indicate that some expositions might have a greater importance in determining the structure and composition of the GIT microbiota. More specifically, largely promoted concepts, such as the role of antibiotics and butyric acid in modulating the intestinal microbiota, were shown to be marginal in commercial settings. Rather, environmental exposures such as the physical farm environment and flock cycle showed higher correlations to the cecal microbiota. However, the microbiota was highly dissimilar in different flock cycles of a same farm and specific changes caused by environmental variations could not be identified due to the observational nature of the study. Also, the interpretation of these effects is limited as different variables could not be individually separated. For instance, farm F2 might have been highly different because of its feed provider and chick provider not found in other farms. On the opposite, farms F1, F5 and F6 shared the same feed supplier and hatchery, which might have influenced the development of the microbiota in these flocks to be more similar between each other compared to the cecal microbiota communities in farm F2. It would have been ideal to switch these effects randomly between farms to assess the impacts of different feed and chick providers on the same farm. However, commercial farms cannot easily change providers as they are often part of the same integration or bound to contracts and it would have not been feasible in the current study. Still, this study highlights the large microbiota variabilities inherent to raising chickens in different environments. Hence, it could be anticipated that interventions designed at improving phenotypic traits via microbiota manipulations, for example improved growth rate and resilience to diseases, may interact

with resident microbial communities colonizing chickens during the entire grow-out and consequently cause inconsistent results. Future strategies aimed at modulating the GIT microbiota of broiler chickens should involve a holistic approach by considering the multifactorial aspects of the microbiota development in commercial broiler chickens.

Materials and Methods

Ethical statement. The committee on animal care in research (Comité d'éthique pour l'utilisation des animaux) of the Faculté de médecine vétérinaire of the Université de Montréal approved the study and protocols involving animal use with the project number 16-Rech-1850.

Farms. The farms described in this study participated in a project on the impacts of eliminating medically important antibiotics (MIAs) from disease prevention programs in broiler chickens (48). Seven chicken producers, located in geographically distinct areas of the province of Quebec, Canada, voluntarily joined this prospective study conducted in 2017. The project involved over 1.2 million chickens raised in 84 flocks from these seven broiler chicken farms, for six consecutive flock cycles. The terminology 'flock cycle' refers to two paired flocks placed simultaneously in two adjacent chicken houses on each farm and raised under the same environmental conditions for assessing the impacts of various types of antibiotic programs in controlled settings. The two houses on each farm were located on the same premise and were managed by the same caregivers during grow-out. These paired flocks also received chicks from the same hatch in a commercial hatchery. Placed chicks were evenly divided in each house to minimize chick quality variation by matching parent flocks for both houses. A total of three hatcheries located in the province of Quebec, Canada provided chicks to the seven farms during the study. Details on chick providers for each farm are described in Table 2. Each producer was supplied from the same hatchery for the six consecutive flock cycles during the study. A total of four feed mills provided feed to the seven farms during the study. Each feed provider managed its own feed formulation within guidelines established by the research team at the beginning of the study (48). Like the chick supplier, producers used the same feed provider during the six consecutive flock cycles (Table 1). Feed formulation was identical between paired

flocks in a same flock cycle, apart from the addition of antibiotics for one flock in each pair, for comparison of conventional antibiotic programs using a combination of anticoccidials and MIAs (shuttle programs) to two alternative prevention programs relying on antibiotics categorized as unimportant for human medicine in Canada, namely ionophores (49), and butyric acids (Table 3). Typically, disease prevention programs in poultry use products, such as anticoccidials, antibiotics, alternatives to antibiotics, or a combination of these molecules, for the prevention of coccidiosis and necrotic enteritis during grow-out in broiler chickens. None of these products were administered through drinking water to reflect current industry practices, since the principal method of administration is via the feed (44). All producers followed industry standards related to housing conditions and flock management, which can be found in Aviagen® and Cobb® management guides (50, 51).

Table 2. Description of chick and feed providers of the seven farms participating in the study.

Farm	Houses capacity	Chick provider	Feed provider
F1	13 500	A	1
F2	12 000	B	2
F3	19 000	C	3
F4	9 800	C	4
F5	22 000	A	1
F6	13 500	A	1
F7	15 000	A	3

Table 3. Description of in-feed antibiotic programs implemented in broiler chicken farms.

Antibiotic programs	Products	Starter phase	Grower phase	Finisher phase
1	Anticoccidials	Ionophore	Ionophore	Ionophore
	Antibiotics	No	No	No
	Butyric acids	No	No	No
2	Anticoccidials	Ionophore	Ionophore	Ionophore
	Antibiotics	No	No	No
	Butyric acids	Yes	Yes	Yes
Conventional	Anticoccidials	Chemicals /Ionophore	Chemicals /Ionophore	Ionophore
	Antibiotics	Yes	Yes	Yes
	Butyric acids	No	No	No

Samples collection. Twelve chickens per flock (n = 1008 chickens in 84 flocks) were randomly selected at the end of grow-out and euthanized on farm by cervical dislocation as described by the American Veterinary Medical Association (52). The cecal content of each chicken was recovered in 2.0 mL cryogenic tubes and samples were kept at -80°C until further processing.

DNA extraction, amplification and sequencing. Total DNA was extracted using the Bioline ISOLATE faecal DNA kit according to the manufacturer's instructions. Forward primer 5′ACTCCTACGGGAGGCAGCAG3′ and reverse primer 5′GGACTACHVGGGTWTCTAAT3′, which also incorporated barcode sequences and capture sequences for MiSeq sequencing (Illumina MiSeq; 2 × 300 bp), were used to amplify and sequence the V3-V4 region of 16S rRNA by the variable spacer, dual barcoding method of Fadrosh et al. (53). Sequence data was analyzed using QIIME version 1.9.1 (54) using default parameters and a Phred quality threshold of > 20. Operational Taxonomic Units (OTUs) were picked using the UCLUST algorithm (55) at 97% sequence identity. Chimeric sequences were inspected using Pintail (56). BLAST was used to assign

taxonomy against the GreenGenes database (57) and QIIME version 1.9.1 defaults. Additional assignment of taxonomy was performed using a command line version of BLASTn (58) against the NCBI 16S microbial database. At the end of the sequencing process, three samples with low DNA yield or poor amplicon generation could not be sequenced and were excluded from the study.

Statistical analysis. All analyses were performed in R (59) and Calypso, a web-server interface designed for analysis of 16S rRNA gene amplicon datasets (60). Data was filtered in Calypso to exclude samples with less than 1000 sequence reads and taxa with less than 0.01% relative abundance across all samples. Count data was transformed using the square root combined with the Total Sum Scaling (TSS) method (61). Three samples with less than 1000 reads were removed from the analysis. The final dataset included 1002 cecal samples from the 84 flocks monitored. Analysis of similarities (ANOSIM) was used as a non-parametric statistical test to evaluate the strength of association and statistical significance between the microbiota composition in the ceca and multiple explanatory variables, namely the type antibiotic program, the farm, the feed provider, the chicks provider and the flock cycle. Redundancy Analysis (RDA) plots based on Bray-Curtis dissimilarity metrics were generated to visualize the variance in community composition explained by each of these explanatory variables. Following the identification of the most impactful explanatory variables on the microbiota composition with the ANOSIM, the Linear discriminant analysis effect size (LEfSe) method was used to determine bacterial phyla and families most likely to explain differences between groups by emphasizing on statistical significance, biological consistency, and effect relevance (62). Two rarefied alpha diversity indices, Richness and Evenness were extracted from Calypso and imported into R to evaluate statistical differences between groups for each explanatory variable with the Kruskal-Wallis rank sum test and Dunn's post-hoc test. Statistical significance for all tests was set to p-value < 0.05. Calculations for Richness, Evenness, ANOSIM and RDA used OTUs, while the LEfSe used the relative abundance of either the phylum or family.

Acknowledgments

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Article 3. Weight gain in commercial broiler chickens is correlated to *Lachnospiraceae* abundance and bacterial species diversity in the cecal microbiota

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Abstract

Background

The gastrointestinal microbiota is thought to play an important role in the growth rate of broiler chickens and improving performance is central to improving the sustainability of the poultry industry. We aimed to investigate components of the cecal microbiota of commercial broiler chickens associated with weight gain. The V3-V4 region of 16S rRNA genes was sequenced from the cecal content of broiler chickens ($n = 1002$) from 84 commercial broiler chicken flocks. We analyzed microbiota diversity, microbiota composition and correlation networks between taxa to reveal community relationships shaping chickens' microbiota in association with their growth performance.

Results

The average daily gain (ADG) was significantly associated with the bacterial Richness of the cecal microbiota ($p < 0.05$), but not Evenness and Shannon index ($p > 0.05$). *Lachnospiraceae* were shown to be the most important bacterial family significantly correlated ($p < 0.05$) with ADG, while *Porphyromonadaceae*, *Planococcaceae*, *Veillonellaceae*, *Enterococcaceae*, *Unclassified.YS2*, *Clostridiales Family XIII Incertae Sedis*, *Rikenellaceae* and *Bacteroidaceae*, were significantly correlated ($p < 0.05$) to decreased growth rate. Furthermore, these unfavourable taxa formed a large network of positive and significant ($p < 0.05$) correlations, indicating potential co-occurring synergies between these undesirable taxa. This network was also negatively correlated ($p < 0.05$) to *Lachnospiraceae*, which may indicate that decreasing the abundance of bacteria within this cluster could be a strategy to increase the relative abundance of *Lachnospiraceae* and subsequently improve performance of broiler chickens.

Conclusions

These findings show that multiple features of the cecal microbiota need to be considered to modulate the microbiota and improve weight gain in commercial broiler chickens. Hence, this study provides significant knowledge on the cecal microbiota composition of commercial broiler chickens linked to growth rate. For instance, these results suggest that improving cecal Richness and the relative abundance of *Lachnospiraceae* may lead to improved growth rate, while controlling harmful networks of bacteria would be critical for successfully manipulating the microbiota to improve growth rate in broiler chickens.

Keywords

Chicken, microbiota, gastro-intestinal tract, growth performance, 16S rRNA gene, alpha diversity, correlation analysis, network analysis.

Background

Poultry is one of the most consumed meat globally with an estimated worldwide consumption of 120,487 kilotons of ready to cook equivalent (kt rtc) in 2018 (OECD, 2019). The Organisation for Economic Co-operation and Development (OECD) predicts that consumption will increase by a further 16.4% by 2028 and global consumption of poultry meat will reach 140 253 kt rtc. This projection would place poultry meat as the most consumed meat worldwide, ahead of other meat products such as pig meat, beef and veal. As consumption rapidly grows, the sustainability of chicken production, a source of greenhouse gas emissions and a user of agricultural and water resources, is an important concern (MacLeod et al., 2013) and hence opportunities to improve its carbon and ecological footprints must be pursued. Decreasing the time required to reach market weight is therefore highly desirable and can be achieved by improving the average daily gain (ADG) of body mass.

Weight gain efficiency has been improved constantly over the years through various approaches, such as genetic selection (Mebratie et al., 2019; Mebratie et al., 2017; Moreira et al., 2019), nutrition (Niemann et al., 2011), management practices (Dawkins, Donnelly, & Jones, 2004) and disease control (Lovland & Kaldhusdal, 1999, 2001). Many feed or drinking water additives, for example antibiotics, probiotics, and prebiotics have also been used as growth promoters (Maria Cardinal et al., 2019; Pereira et al., 2019; Salaheen et al., 2017). One possible mechanism of action of dietary antibiotics is to favor a gut microbiota that improves nutrient production and digestibility, although the exact mechanism is not fully understood (Gadde et al., 2018). Indeed, the gastrointestinal microbiota of broiler chickens possesses metabolic pathways helpful for chickens' development and products that modulate these pathways may improve growth rate. For example, it has been observed that many members of the phylum *Firmicutes* can degrade complex carbohydrates, to release nutrients and produce butyrate, while members from the *Bacteroidetes* phylum express enzymes for propionate production pathways (Polansky et al., 2015). Chickens can benefit from these bacterial by-products as short-chain fatty acids (SCFA), in particular butyrate, are energy sources that can be readily assimilated by gut cells (Fleming et al., 1991).

Consequently, the gastrointestinal microbiota is thought to play a central role in the growth rate of broiler chickens (Broom, 2017; Diaz Carrasco et al., 2019). For example, changes in the relative abundance of various taxa have been observed between individual chickens showing different feed conversion ratios (FCR) in a highly controlled experiment (Stanley et al., 2012). In another controlled experiment, it was shown that the relative abundance of various bacterial taxa was correlated with broiler chicks' weight at 17 days of age (Han et al., 2016). Although these experimental trials provided some insight into the relationship between the chicken gastrointestinal microbiota and growth performance, they were performed in battery cages, which are environmental conditions that contrast with the normal commercial conditions under which broilers are generally raised. Contemporary commercial broiler chickens are raised on litter and given their natural foraging behavior, litter microbial composition is recognized as an important factor contributing to the gastrointestinal tract colonization by bacteria (Torok et al., 2009). This characteristic is possibly an important limitation for translating results from experimental cage-based studies to commercial chicken populations, as the cecal microbiota structure and functionality was shown to be significantly altered by housing type (Kers et al., 2019). Such limitations highlight the need to evaluate the gastrointestinal microbiota of large number of chickens, raised in commercial settings, to bring a comprehensive understanding of the gastrointestinal microbiota features that may modulate growth rate and propose effective microbiota-based strategies which will ultimately improve poultry production sustainability.

The work described here used an experimental design including chickens from multiple farms, flocks, and antibiotic treatment regimens, to provide a large and mixed population representing the gastrointestinal microbiota found in commercial chickens raised for meat consumption. We aimed to investigate components of the cecal microbiota composition and structure associated with weight gain and analyze networks built from significant interactions between taxa to reveal community relationships shaping chickens' microbiota in correlation with their growth performance at the end of grow-out.

Results

Descriptive analysis of growth rates of the chickens sampled

The average daily gain (ADG) of the 1002 chickens sampled showed a bell-shaped curve approximating normally distributed data, with a mean of 68.55 g/day and standard deviation of 7.42 g/day (Figure 1).
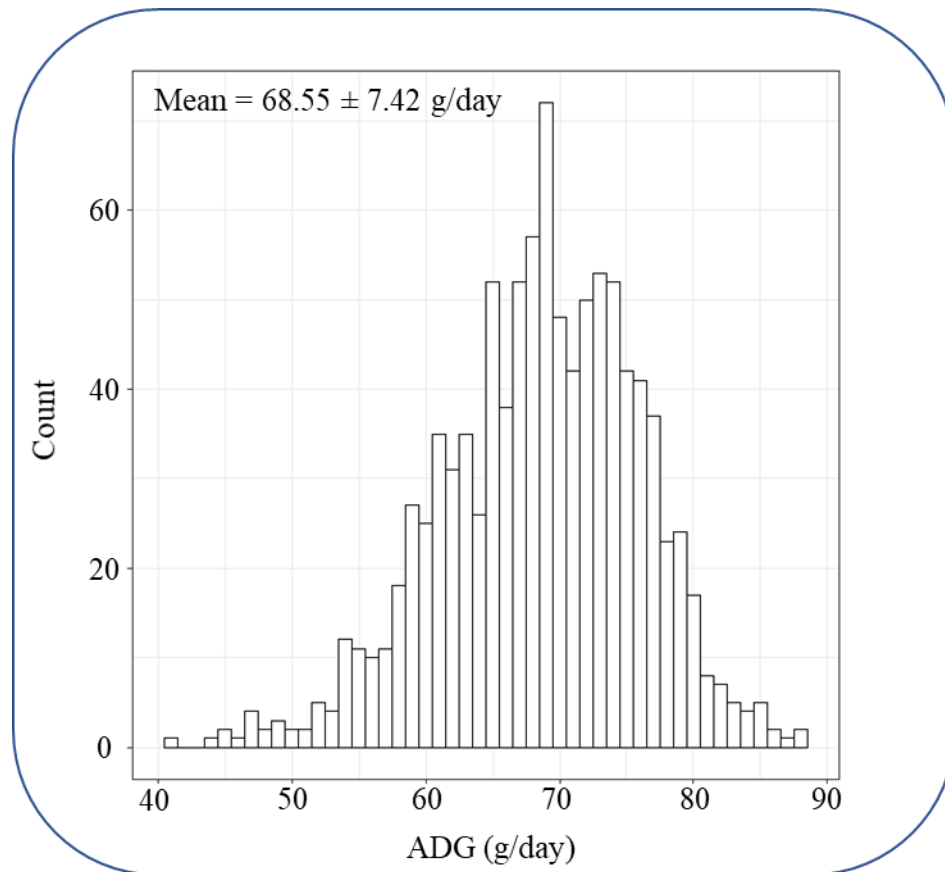
Figure 1. Distribution of ADG (g/day) from all chickens included in the study ($n = 1002$). ADG was normally distributed with a mean of 68.55 g/day and standard deviation of 7.42 g/day.

A significant association explain the relationship between Richness and growth performance

We firstly constructed 3-level linear regression models to explore the relationship between alpha diversity indices and ADG (Additional file 2: Table S1). Bacterial Richness, represented by the total number of different OTUs in a sample, showed a significant and curvilinear association with ADG ($p = 0.008$) (Figure 2). ADG increased constantly as

Richness increased until approximately 225 different OTUs were present. Then, an inflexion point was detected and ADG stabilized even if Richness increased further than 225 different OTUs. The marginal R^2 value considering only the fixed effects (Nakagawa et al., 2013), i.e. bacterial Richness and its quadratic term added to fit the curvilinear relationship, was 0.0085. The conditional R^2 value, also considering the random effects (Flock cycle and Farm) in the model, was 0.338. Evenness, representing the distribution of all OTUs within a cecal sample, was not significantly associated with ADG ($p = 0.219$) (Figure 2). No curvilinear association was detected by adding a quadratic term of Evenness to the final model. The marginal R^2 value was 0.0012 and the conditional R^2 value was 0.339. Shannon diversity index, an estimator combining Richness and Evenness, was not significantly associated with ADG ($p = 0.0927$) (Figure 2). The marginal R^2 value was 0.0022 and the conditional R^2 value was 0.34. The association between alpha diversity indicators and ADG was also explored graphically for each farm to assess these associations in individual farms (Additional file 1: Figure S1). Overall, the trend was evident for most individual farms, but the correlation was stronger for some farms (e.g., farms B and D) and less obvious for others (e.g., farms A and E).
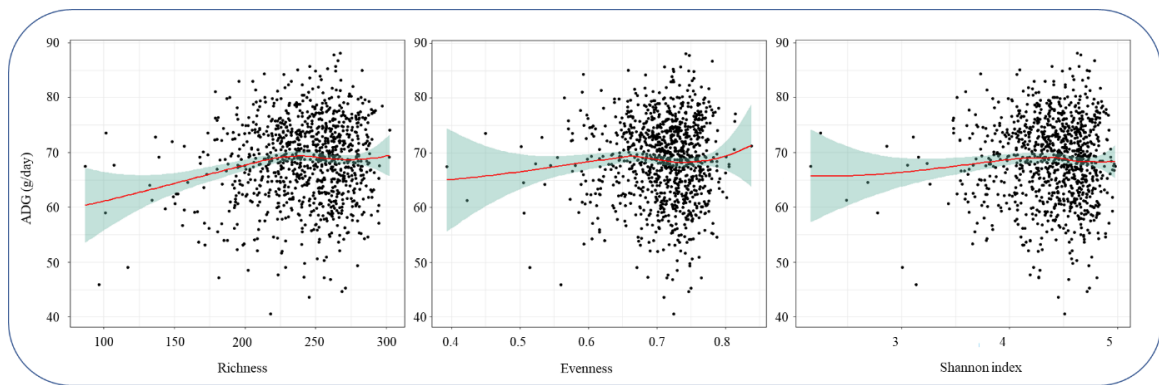
Figure 2. Associations between ADG and Richness, Evenness, and Shannon indices. Each dot corresponds to a sample ($n = 1002$). LOESS (locally weighted smoothing) curves with corresponding standard errors were used to create smoothing lines in the scatter plots to display the relationship between alpha diversity indicators and ADG. Richness showed a significant and curvilinear association with ADG ($p < 0.05$), while no significant association was identified for Evenness and Shannon index ($p > 0.05$).

Beta diversity reveals significant bacterial community shifts associated with weight gain variation

Principal coordinate analysis (PCoA) based on Bray-Curtis distances showed superposition of a large cluster of samples, but a few samples with lower ADG separated from the main group in the bottom right of the PCoA plot (Figure 3). A significant relationship between the cecal microbiota composition and ADG was observed by analysis of similarities (ANOSIM) (p = 0.008). The correlation between community composition and ADG was low, as the R value from the ANOSIM was 0.024.
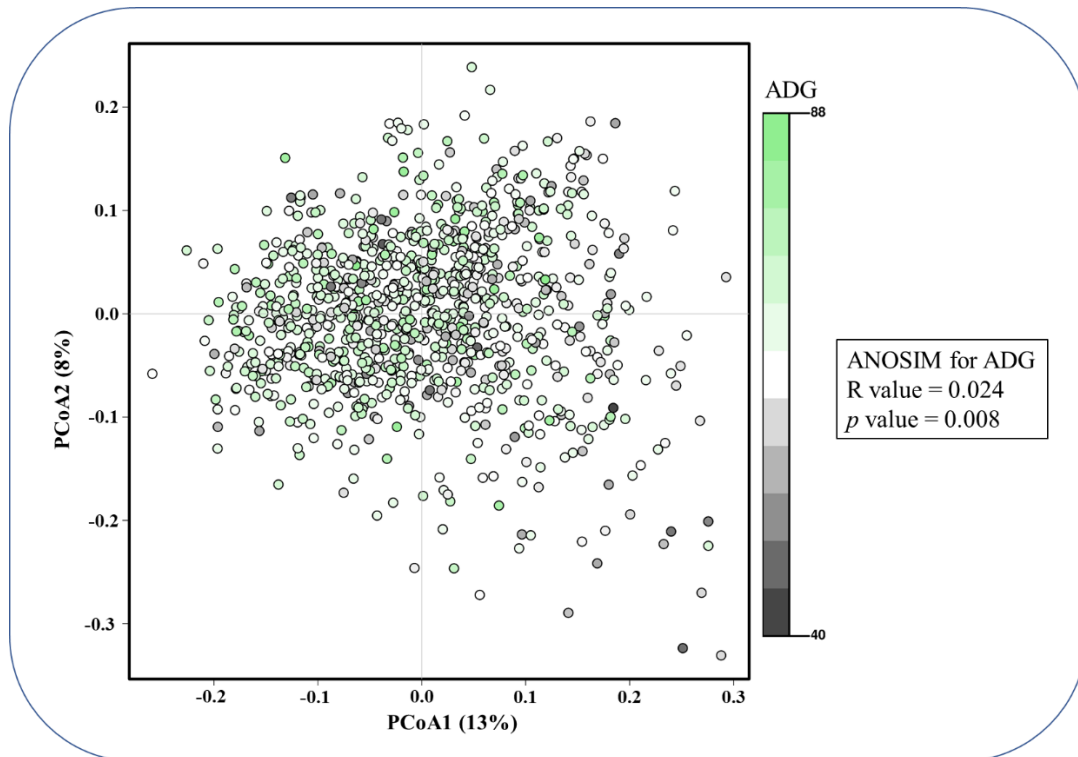
Figure 3. Principal coordinate analysis (PCoA) plot based on Bray-Curtis distances evaluating the relationships between the cecal microbiota composition and the ADG in all samples analyzed (n = 1002 chickens). Analysis of similarities (ANOSIM) showed a significant association (p = 0.008) between the microbiota composition and ADG.

Analysis of the relative abundance identifies individual taxa correlated to growth rate

Spearman's rank correlation coefficients were computed to assess the strength of the monotonic relationship between individual taxa and growth rate. At the phylum level, there was a significant positive correlation between the relative abundance of *Firmicutes* (r = 0.15, p = 1.5e-05) and ADG. In contrast, there were significant negative correlations

between ADG and the relative abundance of *Bacteroidetes* (r = -0.17, p = 5.0e-07) and *Cyanobacteria* (r = -0.1, p = 0.0075). At the family level, the relative abundance of *Lachnospiraceae* (*Firmicutes*) was positively correlated with higher ADG (r = 0.13, p = 0.00057), while *Porphyromonadaceae* (*Bacteroidetes*) (r = -0.25, p = 1.1e-14), *Planococcaceae* (*Firmicutes*) (r = -0.15, p = 4.0e-05), *Veillonellaceae* (*Firmicutes*) (r = -0.16, p = 1.1e-05), *Enterococcaceae* (*Firmicutes*) (r = -0.14, p = 0.00016), *Unclassified.YS2* (*Cyanobacteria*) (r = -0.1, p = 0.03), *Clostridiales Family XIII Incertae Sedis* (*Firmicutes*) (r = -0.1, p = 0.023), *Rikenellaceae* (*Bacteroidetes*) (r = -0.12, p = 0.0029) and *Bacteroidaceae* (*Bacteroidetes*) (r = -0.15, p = 6.2e-05) were negatively correlated with ADG. These correlations for some bacteria were also consistent across farms, despite the reduction in statistical power due to lower number of samples in each analysis, hence these trends could be observed in multiple farms.

Table 1. Analysis of the correlation of individual phyla and families with ADG. Taxa negatively correlated with ADG (p < 0.05) by the Spearman's rank correlation analysis are colored in red and taxa positively correlated with ADG (p < 0.05) are in green. Taxa without colored box were not significantly correlated to ADG (p > 0.05).

Taxa	Mean relative abundance (%)	Number of positive samples	r (spearman)	*p*-value
Phylum				
Bacteroidetes	2.43	1001	-0.17	5.0e-07
Firmicutes	9.34	1002	0.15	1.5e-05
Cyanobacteria	0.26	830	-0.1	0.0075
Verrucomicrobia	0.072	180	-0.079	0.064
Proteobacteria	0.88	1000	-0.063	0.18
Actinobacteria	0.99	1000	0.043	0.53
Tenericutes	1.08	1002	-0.0093	1.00

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Fusobacteria	0.0051	13	0.0076	1.00
Family				
Porphyromonadaceae	0.59	746	-0.25	1.1e-14
Veillonellaceae	0.1	362	-0.16	1.1e-05
Bacteroidaceae	1.45	970	-0.15	6.2e-05
Planococcaceae	0.18	682	-0.15	4.0e-05
Enterococcaceae	0.18	602	-0.14	0.0002
Lachnospiraceae	6.56	1002	0.13	0.0006
Rikenellaceae	1.58	999	-0.12	0.0029
ClostridialesFamilyXIII. IncertaeSedis	0.27	979	-0.1	0.023
Unclassified.YS2	0.26	830	-0.1	0.03
Unclassified. Bacteroidales	0.23	363	-0.089	0.11
Verrucomicrobiaceae	0.072	180	-0.079	0.28
Alcaligenaceae	0.43	760	-0.07	0.58
Ruminococcaceae	5.01	1002	0.067	0.66
Unclassified.SHA98	0.17	745	-0.067	0.66
Lactobacillaceae	2.74	1002	-0.062	0.87
Dehalobacteriaceae	0.19	867	0.054	1.00
Unclassified. Erysipelotrichales	0.15	607	0.054	1.00
Unclassified. Bifidobacteriales	0.23	479	0.043	1.00
Desulfovibrionaceae	0.32	955	-0.037	1.00
Coriobacteriaceae	0.45	998	0.032	1.00
Helicobacteraceae	0.12	243	-0.027	1.00
Flavobacteriaceae	0.075	294	-0.021	1.00
Erysipelotrichaceae	1	1002	-0.019	1.00
Anaeroplasmataceae	0.16	624	0.017	1.00

Unclassified.RF39	0.18	808	0.015	1.00
Enterobacteriaceae	0.45	864	0.011	1.00
Fusobacteriaceae	0.0051	13	0.0076	1.00
Catabacteriaceae	1.48	1002	-0.0046	1.00
Bifidobacteriaceae	0.66	633	0.0024	1.00

A large bacterial network connects bacterial families associated with decreased growth rate while *Lachnospiraceae* negatively correlate to that network

A network of all bacterial families was built to further illustrate the complexity of the interactions between bacterial taxa identified in the cecal microbiota (Figure 4). Edges (lines) represent significant positive (red line) or negative (blue line) correlations (Spearman's rank correlation coefficient, $p < 0.05$ after adjustment for multiple comparisons using the Holmes method) between two nodes corresponding to two different bacterial families. Taxa relative abundance is represented by the node size, where a larger node size is indicative of a higher relative abundance in the samples. For visualizing the connections between phyla in the network, each node was coloured to represent its corresponding phylum. A large network of positively correlated families can be identified in Figure 4. This network positively connected all bacterial families significantly and negatively correlated with ADG, i.e. *Porphyromonadaceae*, *Planococcaceae*, *Veillonellaceae*, *Enterococcaceae*, *Unclassified.YS2*, *Clostridiales Family XIII Incertae Sedis*, *Rikenellaceae* and *Bacteroidaceae*, but also families in the phyla *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Tenericutes* and *Verrucomicrobia* not significantly correlated with ADG. *Lachnospiraceae* (*Firmicutes*), the only family positively and significantly correlated with ADG, was negatively correlated with bacterial families forming the large network related to lower ADG. *Lachnospiraceae* was also positively correlated to *Unclassified.Bifidobacteriales* and *Coriobacteriaceae*, while it was negatively connected to *Enterococcaceae* and *Ruminococcaceae*.
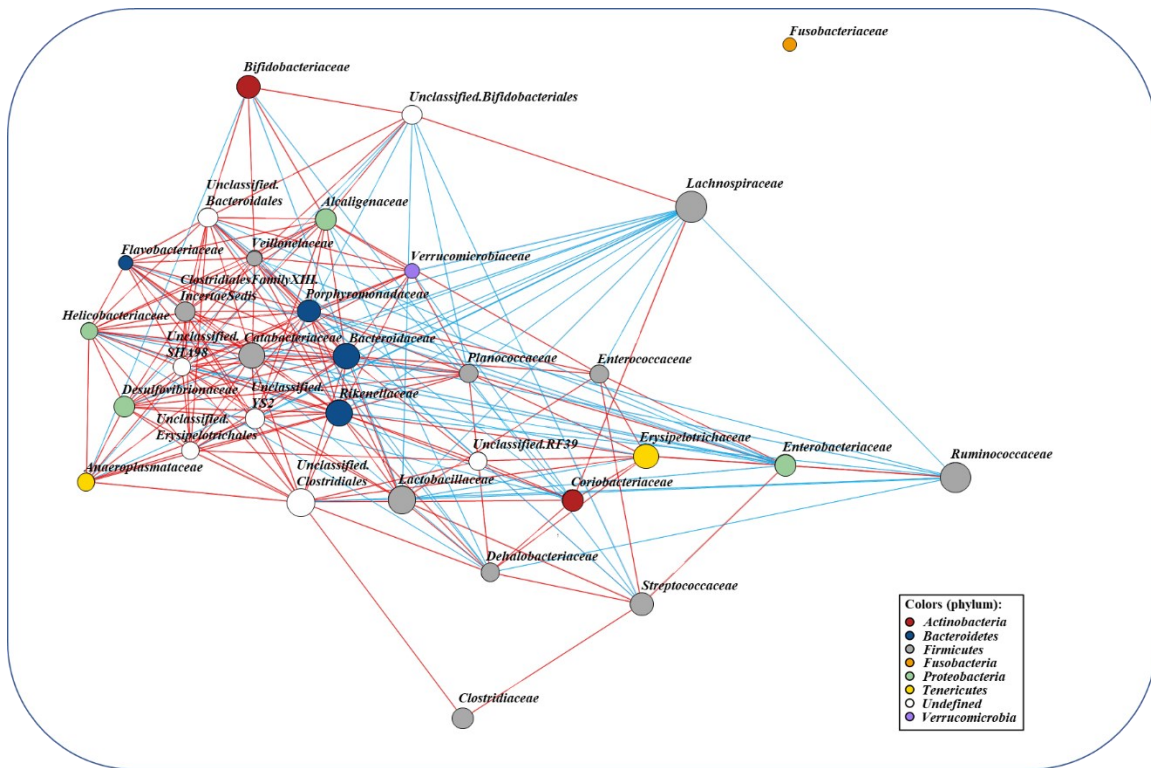
Figure 4. Network analysis of the associations between bacterial families in all samples (*n*= 1002). Edges (lines) represent positive (red) or negative (blue) significant correlations (Spearman's rank correlation coefficient, *p* < 0.05 corrected for multiple comparisons with the Holmes method) between nodes (bacterial families). Nodes are coloured by phylum to show the connections between phyla and node size represents its relative abundance in the samples.

Discussion

The study aimed to identify characteristics of the cecal microbiota correlated with growth rate in commercial broiler chickens, which could lead to novel microbiota-based advances to improve performance. Since the environment in which chickens are raised is recognized as a key factor shaping their gastrointestinal microbiota (Kers et al., 2019), the experimental design involving multiple commercial flocks provides a comprehensive and representative analysis of microbiota features associated with weight gain. There is an increasing number of publications linking microbiota and performance in broiler chickens, but most were performed in research facilities with battery cage housing systems that may not represent the microbiota composition found in commercial settings. The study reported

here has investigated correlations between microbiota composition and broiler performance in a real-world commercial setting.

The assessment of alpha diversity showed that Richness in individual chickens was significantly associated with improved body weight gain, where chickens with increased Richness in their cecal lumen microbiota were more likely to gain weight faster during grow-out. More diverse microbiotas have been associated with more efficient microbial communities by consuming relatively less energy (Larsen & Claassen, 2018), possibly helping chickens extracting more energy from their diet for growth. Alternatively, more diverse communities may provide greater metabolic potential and hence an expanded ability to break down and release nutrients from complex feeds. The litterature is however not clear about the influence of Richness on weight gain as residual feed intake (RFI), a measure of feed efficiency in production animals (Berry & Crowley, 2012), has been reported to be variable in chickens with high or low cecal Richness (Siegerstetter et al., 2017). A study also reported that Richness in the crop and ileum was positively correlated with heavier chickens, but the cecal Richness was negatively correlated with increasing body weight (Han et al., 2016). By using a large number of samples from a representative population of commercial chickens, we determined that Richness, but not Evenness or Shannon index, showed a degree of correlation with body weight gain. Two studies determined various fecal microbiota biomarkers predictive of performance in chickens, but Shannon index was not correlated to feed efficiency (Diaz-Sanchez et al., 2019; Yan et al., 2017). There is a knowledge gap about Evenness, where its importance for improved growth performance in broiler chickens is currently unknown. These results suggest that strategies to modulate chickens' microbiota to improve performance should prioritize increasing the complexity of cecal bacterial populations. However, cecal Richness mostly impacted chickens possessing less than 225 different OTUs in the ceca as no weight gain improvement was visually observed after this cut point. Hence, increasing Richness may be valuable to improve weight gain in broiler chickens, but the beneficial impact of adding new OTUs in the ceca may reach a maximal value after which no more advantage on the average daily gain may be observed passed this limit.

Compositional changes to the microbial communities in the cecal lumen were identified by the association between beta diversity and weight gain variation. Bacterial

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taxa that contributed to the correlation between beta diversity and growth rate were also identified. Improved weight gain was correlated to increased *Firmicutes*, decreased *Bacteroidetes* and decreased *Cyanobacteria*. In comparison, pediatric obesity in humans has been associated with increased *Firmicutes*, depleted *Bacteroidetes* and more SCFA in stools (Riva et al., 2017). This outcome, considered harmful for human health, can be highly desirable in poultry production as increased weight gain is considered beneficial. *Lachnospiraceae*, an important member of *Firmicutes* due to its high relative proportion within this phylum in chicken ceca (Oakley et al., 2014), was significantly correlated with improved weight gain. Considering the current litterature and the results from this study, these findings strongly suggest that species within the *Lachnospiraceae* family are important members of the cecal microbiota to improve weight gain in chickens. This family includes strictly anaerobic bacteria of the *Firmicutes* phylum which are amongst the taxa that degrade complex carbohydrates to produce SCFA, mainly acetate, butyrate and propionate (Biddle et al., 2013). Butyrate can improve gut health and growth performance in animal production when given as a feed additive (Bedford & Gong, 2018) and enhancing its endogenous production by bacteria such as *Lachnospiraceae* or *Ruminococcaceae* in the cecal microbiota has been proposed as an approach to improve growth performance and pathogen control in the gut (Onrust et al., 2015). However, SCFA production is not a universal feature of *Lachnospiraceae* as it was previously reported that only 40% of sequenced organisms (12/30 isolates) may contain genes for butyrate production pathways (Meehan & Beiko, 2014), indicating that further investigations would be required to identify more precisely which bacterial species are associated with improved zootechnical performance in the current study. Riva et al. (2017) also reported that SCFA production was closely related to an increased abundance of *Ruminococcaceae*. Similar to *Lachnospiraceae*, bacteria within this family can degrade complex polysaccharides to produce SCFA and could potentially improve growth rate. In this study, this family showed no association with weight gain from the correlation analyses. Based on the results across multiple farms and flocks, the role of *Ruminococcaceae* in improving growth rate appear to be minimal, but it remains possible that specific species within this family may impact the average daily gain. *Ruminococcaceae* and *Lachnospiraceae* are abundant members of the cecal microbiota in chickens (Oakley et al., 2014) and many species within both

families are recognized as important SCFA producers. An increased relative abundance of these families has been foreseen to be correlated with improved growth rate and feed efficiency in various *in vivo* experiments involving broiler chickens (Han et al., 2016; Siegerstetter et al., 2017; Singh et al., 2012; Stanley et al., 2013; Torok et al., 2011). Hence, the findings on taxa improving growth performance in commercially reared chickens is concordant with experiments conducted in research facilities regarding the role of *Lachnospiraceae*, but further work is required to understand the possible involvement of *Ruminococcaceae* in the improvement of growth rate in broiler chickens.

Conversely, taxa associated with low performing birds were more numerous and most of these bacterial families were intercorrelated in a network of co-existing micro-organisms. For instance, *Porphyromonadaceae*, *Veillonellaceae*, *Bacteroidaceae*, *Planococcaceae*, *Enterococcaceae*, *Rikenellaceae*, *Clostridiales Family XIII. IncertaeSedis* and *Unclassified YS2*, the families showing significant negative correlations with ADG, were all found in a coordinated network but also in correlation with several other families that were not identified as impactful for growth rate. This analysis indicates that providing a full systems-level understanding of the microbiota reveals complex poly-microbial interactions possibly impacting the host. For example, it has been recently reported that co-occurrence networks were linked to obesity in African-origin women and patterns could be identified based on the structure and robustness of the microbiota (Dugas et al., 2018). Similarly, the abundance of this large network of bacteria in the current study was associated with low performing birds and could be a signature of harmful cecal microbiotas for broiler chickens. Consequently, developing strategies to control this network could prove to be effective for improving weight gain in broiler chickens, but this hypothesis would require further investigations. This network was also negatively correlated to *Lachnospiraceae*, which indicates that decreasing the abundance of bacteria within this cluster might be a strategy to increase the relative abundance of *Lachnospiraceae* and subsequently improve performance in broiler chickens. The scale of the study from multiple farms and flocks strengthens the link between this network and low performing chickens, but these findings would still need to be evaluated in other independent studies to confirm the real implication of this network in broiler

chickens. Elucidating how to favour beneficial bacteria over harmful networks could lead to reliable and successful microbiota manipulation strategies to improve weight gain.

In summary, various indicators of weight gain were revealed by analysing the cecal microbiota of commercial broiler chickens across multiple farms. Richness was positively associated with weight gain. The increased relative abundance of *Firmicutes* and *Lachnospiraceae* was correlated with increased growth rate, while the relative abundance of *Bacteroidetes*, *Cyanobacteria* and various bacterial families such as *Porphyromonadaceae*, *Planococcaceae* and *Veillonellaceae* were correlated with decreased growth rate. Finally, a large co-occurring network of bacterial families negatively correlated with weight gain and abundance of *Lachnospiraceae* was identified. All these findings show that multiple features of the cecal microbiota need to be considered when trying to modulate the microbiota to improve weight gain in commercial broiler chickens. As the microbiota results from complex microbe-microbe and microbe-host interactions, improving the microbiota structure must rely on a comprehensive and systematic understanding of these connections to cause persistent and beneficial modifications to the microbiota. Hence, this study provides significant knowledge on the cecal microbiota composition of commercial broiler chickens linked with growth rate and these findings could pave the way for future successful microbiota manipulations.

Methods

Study design. Chickens sampled were raised in commercial farms owned by chicken farmers participating in a large-scale longitudinal study that evaluated the impacts of antibiotic use on performance and gut health in the province of Quebec, Canada in 2017 and 2018 (Parent et al., 2020). Seven farms were located on geographically different sites within the province. Six consecutive flock cycles were sampled in each of the two adjacent and identical poultry houses on each farm (Figure 6). In total, approximately 1.2 million chickens were raised in 84 flocks from the 14 poultry houses located on the 7 farms. Each farm placed male broiler chicks provided by commercial hatcheries and broiler chickens were slaughtered at market weight in processing plants based on target average flock weights varying from 2.4 kg to 2.8 kg per chicken. Housing conditions and flock management followed general industry standards, which can be found in Aviagen® and Cobb® management guides (Aviagen, 2018; Cobb-Vantress, 2018).

Antibiotic/anticoccidial programs preventing coccidiosis and necrotic enteritis, two major diseases in broiler chickens, were used in the feed of all flocks. Briefly, these prevention programs used shuttle programs, a combination of anticoccidials and antibiotics, or ionophore only programs rotating between flocks as a common procedure to avoid pathogens resistance against these in-feed products (Peek & Landman, 2011). Since statistical analyses showed no significant difference between the various antibiotic treatments on zootechnical performance and intestinal disorders at the flock level, these prevention programs were not further considered for their implication in the microbiota composition as they did not influence the outcome of interest (average daily gain).
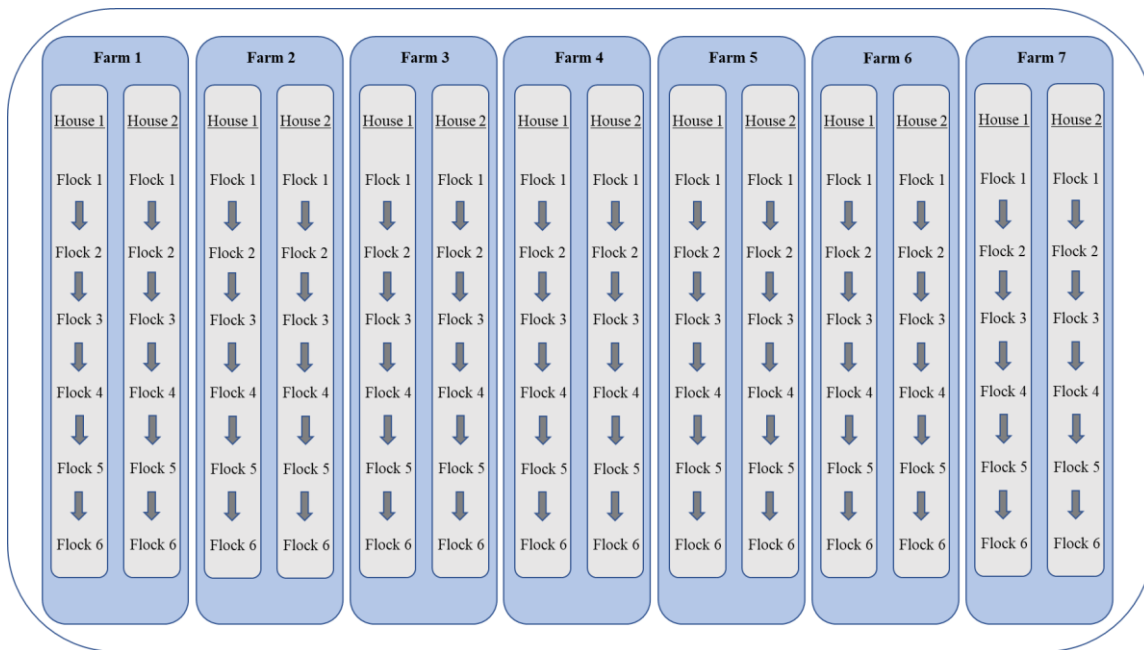
Figure 5. Schematic representation of the sampling design. The cecal content of twelve randomly selected broiler chickens per flock was recovered from two broiler chicken houses on seven farms for six consecutive flock cycles, for a total of 1008 samples from 84 flocks. Each farm was located on a geographically different site. Houses on the same farm were adjacent and had identical husbandry.

Samples collection. Before a flock was shipped to the processing plant, twelve chickens in each flock ($n = 1008$ chickens) were randomly selected and euthanized on the farm by cervical dislocation as described by the American Veterinary Medical Association (AVMA, 2020). The content of both ceca in each chicken was rapidly recovered in 2.0 mL

sterile freezing tubes and labelled to associate each individual cecal sample with its corresponding metadata. Samples were kept at -80°C until further processing. The same sampling protocol was followed during the study for recovering the 1008 samples.

Growth performance data. Each chicken was individually weighed, and body weight was recorded before recovering the cecal content from both ceca. The average daily gain (ADG) was calculated individually with the formula: ADG (g/day) = Body weight (g) / Age (days).

DNA extraction, amplification and sequencing. Total DNA was extracted using the Bioline ISOLATE fecal DNA kit according to the manufacturer's specifications. Forward primer 5′ACTCCTACGGGAGGCAGCAG3′ and reverse primer 5′GGACTACHVGGGTWTCTAAT3′, which also incorporated barcode sequences and capture sequences for MiSeq sequencing (Illumina MiSeq; 2 × 300 bp), were used to amplify and sequence the V3-V4 region of 16S rRNA by the variable spacer, dual barcoding method of Fadrosh et al. (Fadrosh et al., 2014). Operational Taxonomic Units (OTUs) were picked using the UCLUST algorithm (Edgar, 2010) at 97% sequence identity. Chimeric sequences were inspected using Pintail (Ashelford et al., 2005). BLAST was used to assign taxonomy against the GreenGenes database (DeSantis et al., 2006). Additional assignment of taxonomy was performed using a command line version of BLASTn (Altschul et al., 1997) against the NCBI 16S microbial database. Three samples had low DNA yield or poor amplicon generation and could not be sequenced and were excluded from the study. The adequacy of the sampling depth was assessed with a rarefaction curve generated in Calypso (Additional file 2: Figure S2.1). The number of reads per sample distribution were visually inspected by a histogram generated in Calypso (Additional file 2: Figure S2.2).

Statistical analysis. All analyses were performed in R, version 4.0.0 (R Core Team, 2019) and Calypso, a user-friendly web-server interface designed for analyzing 16S rRNA datasets (Zakrzewski et al., 2017). Calypso source code can be found at https://zenodo.org/record/50931. Data was filtered in Calypso to exclude samples with less than 1000 sequence reads and taxa that have less than 0.01% relative abundance across all samples. Count data was transformed using the square root combined with the Total Sum Scaling (TSS) method (Legendre & Gallagher, 2001). Three samples with less than 1000

reads were removed from the analysis. The final dataset included 1002 cecal samples from the 84 flocks monitored. After rarefaction, three alpha diversity indices, Richness, Evenness, and Shannon diversity index were extracted from Calypso and imported into R to build multilevel linear regression models to evaluate the association between these indices and ADG (outcome). Richness measures the number of present OTUs in a sample (Kim et al., 2017). Community richness is estimated by rarefaction analysis to account for differences in sample sizes. Evenness measures how evenly abundant the present OTUs are in a sample. The Shannon index is an estimator of species Richness and Evenness and it has more weight on Richness than Evenness. The lm function implemented in R and the lmer function in the lme4 package (Bates et al., 2015) were used to build models with a forward stepwise regression approach. Briefly, a first model was built for each alpha diversity indice considering only the fixed effect (Richness, Evenness or Shannon index). To find the best fit model reflecting the data, additional parameters were added with a stepwise approach. Firstly, the flock cycle (six flock cycles per farm), then the farm was added as a random effect allowing for independent regression intercepts, and a random slope was added to each model. Models with quadratic terms were also built to assess the presence of a curvilinear association between each alpha diversity parameter and ADG. Each new model improvement was compared by likelihood ratio test with simpler ones. The final models, corresponding to the best fit, consisted of 3-level models with random intercepts including the alpha diversity indicator as a fixed effect, the flock cycle as a second-level random effect, and the farm as a third-level random effect. A quadratic term of Richness was added to the final model of this indicator since a significant inflexion point ($p < 0.05$) was detected by adding the quadratic term to the model (Dohoo et al., 2014). No curvilinear associations were identified for Evenness and Shannon index models. Statistical significance was set to p-value < 0.05 for all tests. Validity of the final models were assessed by the visual inspection of quantile–quantile plots for normality and by scatter plots of the residuals as a function of the adjusted outcome values for homoscedasticity. Marginal and conditional R^2 from each final model (Nakagawa et al., 2013) were computed using the r.squaredGLMM() function implemented in R. Multivariate analysis of the influence of the microbiota community composition on ADG was assessed by constructing in Calypso a Principal Coordinates Analysis (PCoA) plot

based on Bray-Curtis dissimilarity metrics. Analysis of similarities (ANOSIM) was used as a non-parametric statistical test to evaluate significance between microbiota composition in the ceca and ADG. The statistical significance was set to p-value < 0.05. ANOSIM requiring categorical variables, Calypso will automatically categorize numeric values using the "cluster" method of the discretitize() function implemented in the R package arules. The function assigns values to categorize by k-means clustering. Taxa significantly correlated with ADG were determined by calculating the Spearman's rank correlation coefficient in Calypso and significance was set to p-value < 0.05, adjusted for multiple comparisons with the Bonferroni correction. A network identifying co-occurring clusters of bacteria was built in Calypso based on significant correlations ($p < 0.05$) between taxa using Spearman's rank correlation coefficient to connect nodes, with p-values corrected for multiple comparisons by using the Holmes method.

List of abbreviations

ADG: average daily gain

FCR: Feed conversion ratio

OTU: Operational taxonomic unit

RFI: Residual feed intake

SCFA: Short-chain fatty acid

Declarations

Ethics approval and consent to participate

The Committee on Animal Care in Research (Comité d'éthique pour l'utilisation des animaux) of the Faculté de médecine vétérinaire of the Université de Montréal approved the study and protocols involving animal use with the project number 16-Rech-1850.

Consent for publication

An agreement form was signed by each broiler chicken producer for voluntarily participating to the study. The consent form included the use of the data recovered from their farms, with the exception of nominative information, for preparing, presenting and publishing scientific material.

Availability of data and material

The dataset supporting the conclusions of this article is available in the MG-RAST repository, under the QuebecCecalMicrobiota dataset name, https://www.mg-rast.org/mgmain.html?mgpage=overview&metagenome=mgm4919437.3.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization, MB, EP, RM and MA; methodology, EP, MB and RM; data collection, EP; data analysis, EP, TTHV and RM; resources, MB and RM; writing-original draft preparation, EP, RM and MB; writing-review and editing, EP, RM, MB and MA; supervision, MB and RM; project administration, MB; funding acquisition, MB.

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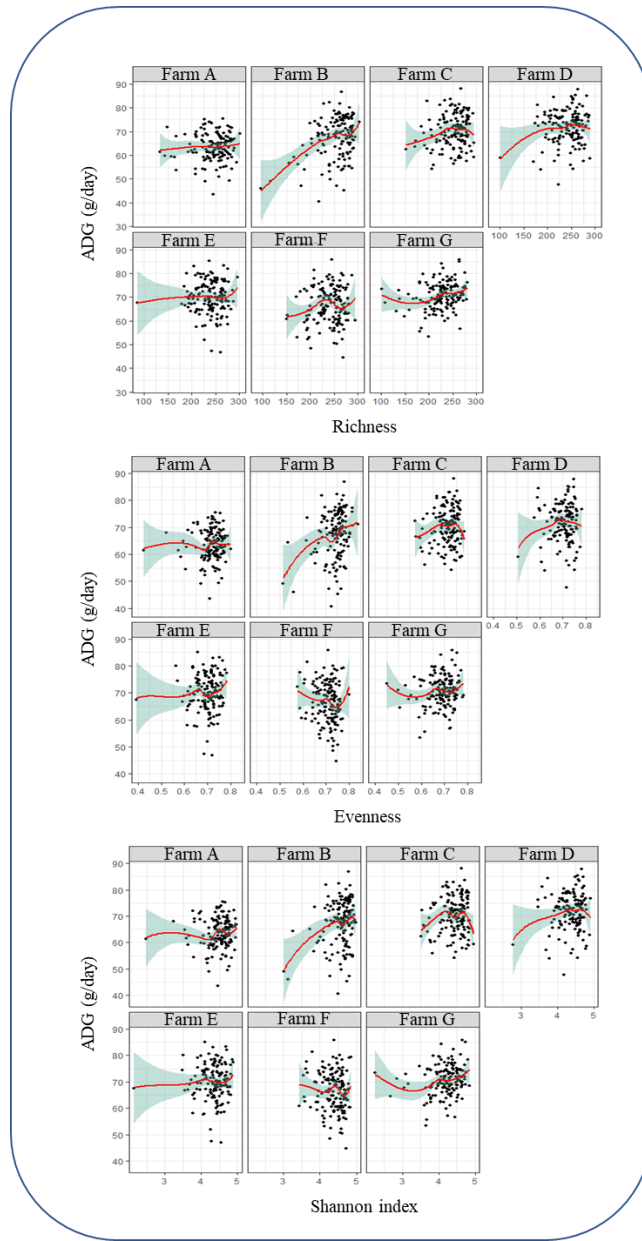
Figure S1. Association between Richness, Evenness or Shannon index and ADG for each farm. Each dot corresponds to a sample (*n* = 1002). LOESS (locally weighted smoothing) curves with corresponding standard errors were used to create smoothing lines through scatter plots displaying relationships between alpha diversity indicators and ADG. The trend of increased diversity associated with ADG was evident for most individual farms, but also variable between farms. For example, strong associations were observed for farms B and D, but less evident associations were observed for farms A and E.
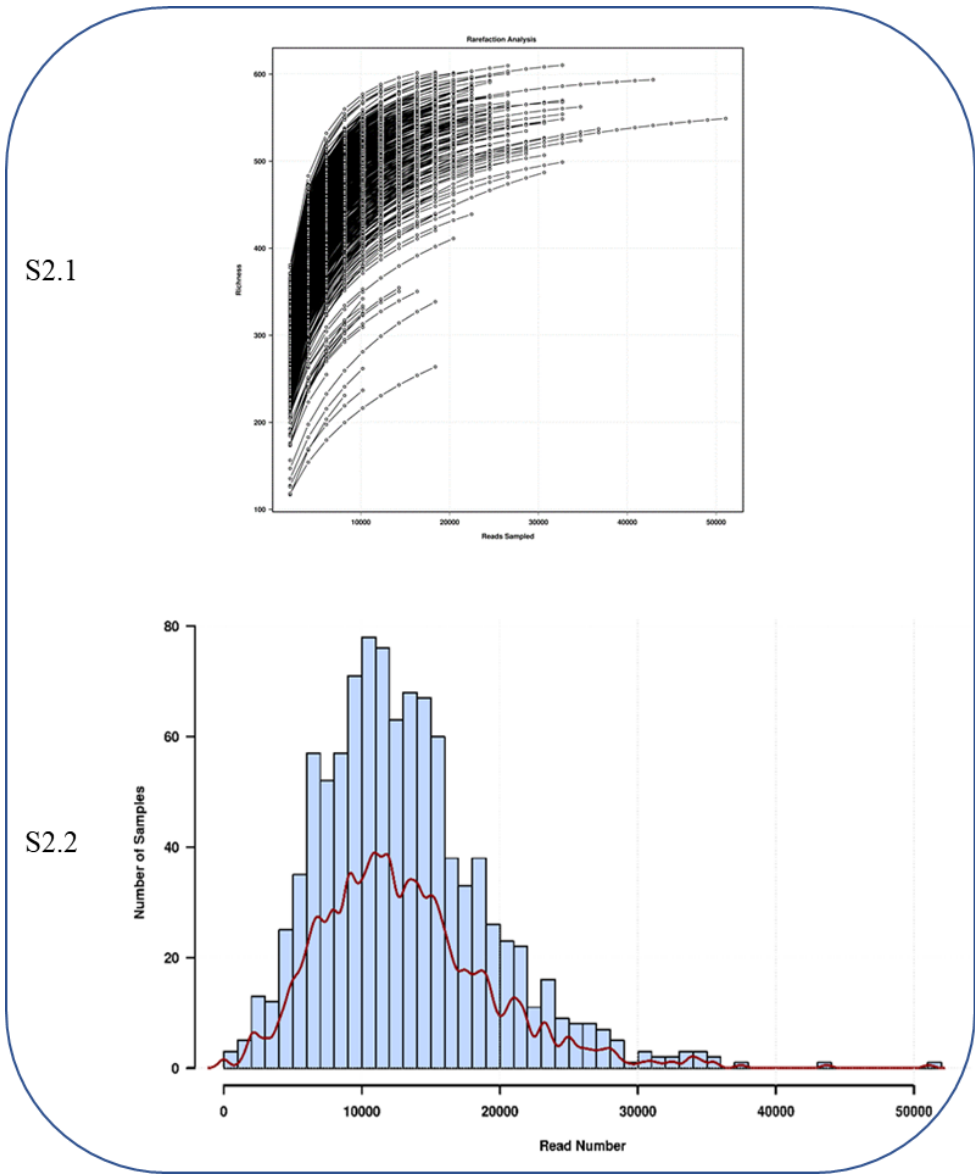
Figure S2. Rarefaction analysis at the OTU level (S2.1) and histogram of the distribution of the number of reads per sample (S2.2)

Table S1. Results of the final statistical models of the association between Richness, Evenness and Shannon Index with ADG.

Variable	Coefficient*	Standard error	Variance	*p*-value
Richness				
Richness	0.14	0.0500	0.003	0.008
Richness^2	-0.00028	0.00012	1.4×10^{-8}	0.017
Flock cycle (2nd level)		0.11	12.074	
Farm (3rd level)		0.079	6.301	
Evenness				
Evenness	5.02	4.087	16.706	0.219
Flock cycle (2nd level)		0.112	12.667	
Farm (3rd level)		0.08	6.364	
Shannon Index				
Shannon Index	0.97	0.579	0.335	0.093
Flock cycle (2nd level)		0.112	12.620	
Farm (3rd level)		0.08	6.456	

*ADG variation for a 1-unit increase of the coefficient

Chapter 4. Discussion

Being more aware of the impacts of antimicrobial use (AMU) on antimicrobial resistance (AMR) and public health, the Canadian broiler chicken industry underwent major changes in recent years to decrease its usage of medically important antibiotics (MIAs). From an industry relying for decades on numerous MIAs for the prevention of diseases during grow-out, such as necrotic enteritis, broiler chicken farmers transformed the landscape of AMU in Canada via the elaboration of their *Antimicrobial Use Reduction Strategy* in 2013 (https://www.chickenfarmers.ca/the-antimicrobial-use-reduction-strategy/). In May 2014, the Canadian poultry industry voluntarily removed the preventive use of Category I antibiotics, which mainly impacted the *in ovo* administration of ceftiofur (Verrette et al., 2019) routinely used to prevent early chick mortality caused by avian pathogenic *Escherichia coli* (APEC). While the proportion of broiler flocks in provenance of commercial Canadian hatcheries using ceftiofur was reported at 31.3% in 2013, none of the flocks monitored by the Canadian Integrated Program for Antimicrobial Resistance System (CIPARS) in 2015 were administered that antibiotic (Agunos et al., 2017). In comparison, none of the antimicrobials used in the feed of broiler chickens monitored between 2013 and 2015 showed important variation, except for proportionally higher avilamycin use (uncategorized antibiotic) and a decrease in virginiamycin use (category II antibiotic). Avilamycin arrived in the Canadian market in March 2014, and most likely partially replaced virginiamycin as both antibiotics are registered to prevent necrotic enteritis in broiler chickens (Compendium of veterinary products - Canada edition, 2020). Ceftiofur use stopped following the implementation of the *Antimicrobial Use Reduction Strategy*, but led hatcheries to either use no medication, lincomycin-spectinomycin or gentamicin. Following this industry intervention, CIPARS reported an increase of gentamicin resistance in sentinel farms (Agunos et al., 2017), showing undesired collateral effects of the policy implemented in 2014. Even if the withdrawal of ceftiofur has been followed by a decrease in the prevalence of ceftiofur resistance genes identified in poultry, the use of lincomycin-spectinomycin was followed by an increase in multidrug resistant

E. coli (Verrette et al., 2019). Hence, removing the preventive use antibiotics must be supported with proper antibiotic stewardship, otherwise future efforts to decrease AMR may be mitigated by inappropriate AMU following these new policies.

In-feed MIAs used for prevention were a significant proportion of all antibiotics used in commercial broiler chickens at the end of 2015. As these antibiotics were mainly used to prevent necrotic enteritis caused by *Clostridium perfringens* (Agunos et al., 2017), removing this practice was seen as a possible source of intestinal health issues. For example, it has been reported in a commercial study that a quarter of the broiler chicken flocks raised without antibiotic (RWA) were affected by clinical necrotic enteritis, while half of the flocks showed signs of non-specific enteritis and the remaining flocks were healthy (Gaucher et al., 2015). On the opposite, intestinal health was adequately controlled in 100% of the conventional flocks using antibiotics for the prevention of necrotic enteritis. That study, using no antibiotics (including category IV), showed that RWA production was less sustainable than conventional production as antibiotic-free (ABF) flocks experienced significant production losses. It also showed the limits of such type of production as no antibiotics could be used to prevent or treat diseases causing significant illness in broiler chickens, and thus being a practice conflicting with the position of poultry veterinarians; the American Association of Poultry Veterinarians stated in 2016 that marketing purposes should not compromise the health of poultry flocks (American Association of Avian Pathologists, 2016). Additionally, the low adoption rate of this type of production in Canada, estimated at less than 10% (personal communication with the Chicken Farmers of Canada, 2020) is likely associated to the increased incidence of enteric diseases in RWA flocks, which causes poorer zootechnical performance and significant economical losses. Hence, the acceptance and adoption of a new AMU policy such as the one elaborated by the Chicken Farmers of Canada may be driven by its success to control intestinal diseases and maintain zootechnical performance equivalent to conventional production. However, no information on the evaluation of this policy was available in commercial operations and a feasibility study was deemed necessary to properly assess the future impacts of such changes before the implementation at a national level.

Since the objective of this project was to determine the possible impacts of an AMU policy removing the use of MIAs in prevention (category I, II and III antibiotics), in-feed medication programs were designed to allow category IV antibiotics for prevention. Ionophores were selected from this category for their dual spectrum of action against coccidia (Jaenicke, 1983; Smith et al., 1981) and *C. perfringens* (Lanckriet et al., 2010; Martel et al., 2004), two major infectious pathogens causing significant economical losses to broiler chicken production globally (Blake et al., 2020; Wade & Keyburn, 2015). To homogenize programs across farms, it was decided to use the same ionophores during grow-out, because the switch from starter to grower and finisher phases were occurring at different ages across the four feed mills providing feed to the seven participating broiler chicken producers. Additionally, in-feed prevention programs relying on the continuous use of ionophores have been reported to perform equally to shuttle programs (chemical followed by ionophore) during a 5-year study evaluating the use of anticoccidial drugs in the USA between 1995 and 1999 (Chapman, 2001). Indeed, no significant differences were observed between continuous and shuttle programs on feed conversion ratios (FCR) and the number of days to produce a 2.27 kg broiler chicken, while mortality was significantly lower in flocks with a continuous ionophore program (4.78% ± 0.14) compared to shuttle programs (5.21% ± 0.14). The simultaneous use of MIAs and anticoccidials was however not described by Chapman et al. (2001), and it wasn't possible to determine probable impacts of removing MIAs before conducting the current study. A recent meta-analysis of 174 articles published between 1998 and 2018 on the growth promoting effects of in-feed antibiotics in broiler chicken estimated that broilers fed diets with antibiotics growth promoters (AGP) showed improved FCR and weight gain compared to chickens fed AGP-free diets, resulting in significant projected economical gains for producers (Maria Cardinal et al., 2019). Still, the control of diseases between conventional flocks and flocks receiving no MIAs in commercial operations was shown to be equivalent (Karavolias et al., 2018). Hence, it could be hypothesized that zootechnical performance in commercial farms using no MIAs may be maintained if diseases are adequately controlled.

Antibiotic use being reduced or removed in animal production across many countries, effective alternative products to antibiotics were developed to improve performance and control intestinal diseases in broiler chickens. For instance, prebiotic

products including benzoic acid, butyric acid or various other blends of essential oils and plant extracts were reported to significantly improve growth rate and feed efficiency of broiler chickens compared to negative controls, and often providing performance equivalent to antibiotics such as avilamycin (Aristimunha et al., 2016; Bortoluzzi et al., 2017; Kaczmarek et al., 2016; Liu et al., 2017). Antimicrobial properties of many alternatives to AGPs have also been extensively reviewed and are often shown to inhibit *C. perfringens* growth (Diaz Carrasco et al., 2016), hence their large potential to replace MIAs in necrotic enteritis prevention programs of broiler chickens. However, the positive impact of such products is still disputed as the absence of significant improvement by using these products in commercial operations (Smith, 2011) question the real efficacy of numerous alternatives to antibiotics. Because of their uncertain potential for commercial use in replacement of MIAs, it was decided that the design of the current study would benefit from adding an experimental group including a commercially available in-feed prebiotic.

The first hypothesis of this project was that strategies removing the use of MIAs in disease prevention programs of broiler chickens would provide zootechnical performance and control of diseases similar to conventional programs using MIAs if *Eimeria* and *C. perfringens* were controlled. The objective was to evaluate, in commercial broiler chicken farms, two antibiotic reduction strategies eliminating the use of MIAs, in comparison of the conventional use of antibiotics. More specifically, production performance, flock health, and *Eimeria* spp. excretion were compared between antibiotic reduction and conventional strategies. At the beginning of this study in 2016, only category I antibiotics used for prevention were banned by the Chicken Farmers of Canada, meaning that numerous MIAs were still used in conventional flocks during the on-farm trial phase. Indeed, 38/42 (90%) of the conventional flocks were administered at least one of these following MIAs during grow-out: bacitracin, penicillin procaine, tylosin or virginiamycin (Parent et al., 2020). Bambermycin and/or avilamycin, respectively category IV and uncategorized antibiotics, were used in combination with in-feed anticoccidials in the other four flocks, meaning that 10% of the conventional flocks were already in compliance with the final objective of the *Antimicrobial Use Reduction Strategy* to remove the use category I to III antibiotics for prevention. Nonetheless, as the industry relied on category II to IV

antibiotics for the prevention of intestinal diseases during grow-out, control flocks using MIAs were considered representative of prevention practices in place before the implementation of the AMU policy. The comparison of zootechnical results between treatments confirmed the first part of the hypothesis that antibiotic reduction strategies did not impact flocks performance during grow-out and at the processing plant. Indeed, feed conversion ratio (FCR), slaughter weight, average daily weight gain (ADG), age at slaughter, mortality and condemnations were not statistically different between the three treatments. Considering the total number of flocks evaluated ($n = 84$) and the standard errors obtained for each parameter, it can be hypothesized that implementing these antibiotic reduction strategies to the national level would most likely result in zootechnical results equivalent to those observed before the implementation of the policy. These results contrast with the observations made by Gaucher et al. (2015), where significantly lower zootechnical performance was reported in RWA broiler chicken flocks compared to conventional production. The main difference between the experimental groups of the two studies being the use of a commercial live non-attenuated *Eimeria* spp. vaccine in the previous study and ionophores in the current study, it can be suggested that ionophores performed significantly better in this commercial trial compared to the coccidial vaccine in the other trial. This finding may be closely related to the different efficacy to control intestinal diseases in both types of program (RWA vs. no MIA for prevention). Indeed, the RWA study showed that three-quarter of the flocks administered the coccidial vaccine suffered from intestinal diseases, which are strongly associated with decreased performance (Blake et al., 2020; Kaldhusdal et al., 2016; Kasab-Bachi et al., 2017; Lovland & Kaldhusdal, 2001). Although the microscopic analysis of digestive tracts recovered from 270 randomly selected chickens in Gaucher, et al. (2015) showed no significant differences between drug-free and conventional programs, the clinical presentation of these flocks affected by enteritis outbreaks was unambiguous with increased mortality, presence of moribund birds, bloody droppings on the floor and/or necropsied chickens presenting typical macroscopic necrotic enteritis lesions. On the opposite, no apparent clinical presentation suggestive of an enteric disease outbreak was observed in the present study. This finding was also reinforced by the post-mortem macroscopic evaluation of 2016 randomly selected chickens at 21 and 28 days of age, which showed that macroscopic

lesion scores were similar between antibiotic reduction and conventional programs. A possible explanation to these differences between the two studies might be related to the different dynamic and control of *Eimeria* spp. populations in flocks administered anticoccidials or a live non-attenuated *Eimeria* spp. vaccine. Indeed, the excretion of *Eimeria* spp. oocysts has been evaluated in both trials and while minimal differences were observed between antibiotic reduction and conventional groups in this study, large variations were observed between RWA and conventional flocks (Parent et al., 2018). More specifically, fecal counts of *Eimeria* spp. oocysts (OPG) in RWA flocks increased and reached their maximal levels earlier than conventional flocks and might have negatively impacted the process of immunization against coccidiosis. Coccidiosis being an important intestinal disease predisposing to necrotic enteritis (Al-Sheikhly & Al-Saieg, 1980; Eckert et al., 2021; Hofacre et al., 2018; Moore, 2016; Prescott et al., 2016), it can be suggested that inadequate *Eimeria* spp. cycling in RWA flocks using a coccidiosis vaccine negatively impacted the overall control of intestinal diseases. Still, it must be stated that ionophores possess significant antimicrobial properties against *C. perfringens* (Lanckriet et al., 2010; Martel et al., 2004), and most likely contributed to the control of pathogenic *C. perfringens* populations while the vaccine does not provide protection against this bacterium.

The use of in-feed butyric acids in the continuous ionophore program was unsuccessful as it did not prove any additional benefit to ionophores alone. Indeed, performance, macroscopic lesion scores and *Eimeria* spp. excretion were not significantly different between the flocks receiving ionophores and those administered the same ionophores with the addition of butyric acid. Being an additional cost to the diet without improving performance, the use of this product could not be justified in the present study. It could also be proposed that continuous ionophore programs are not likely to benefit from the inclusion of butyrates in the feed. Intestinal diseases such as coccidiosis and necrotic enteritis being well controlled by ionophores alone, the addition of butyric acid to the diet in diseased flocks could however not be assessed in the present study. It may still be hypothesized that these alternatives to antibiotics might only reveal their additional benefits in broiler chicken flocks at risk of developing severe intestinal diseases such as necrotic enteritis. For instance, it cannot be excluded that their antimicrobial properties against

C. perfringens may decrease the incidence or the severity of necrotic enteritis in broiler flocks at risk, i.e. RWA flocks using an *Eimeria* spp. vaccine.

The experimental design in the present study involved commercial operations and has the advantage of evaluating flocks highly representative of contemporary broiler chicken production with management, environmental conditions, feed formulation and breeds currently used by chicken farmers. However, it must also be considered that seven producers represent a small proportion of the 2800 producers forming the Chicken Farmers of Canada, with many of these producers owning more than one production site and multiple houses per site. Hence, it highlights an important limitation of the study that the seven farms selected are not likely to represent the entirety of the broiler chicken farms in Canada. Indeed, different variables, for example regional disease challenges, feed ingredients and general weather conditions, may limit the representativity of the broiler chicken population. It would have been ideal to randomly select seven chicken farmers and production sites amongst the total population eligible, i.e. on premises including at least two highly similar houses for the implementation of the conventional and antibiotic reduction strategies. However, this path was not realistically feasible due to human resources factors, where many farmers fulfilling the original criterion of possessing two similar houses on a premise were not interested, mostly by a lack of time and commitment to actively participate in a year-long study involving multiple samples recovered by the farmers and their employees. Hence, it was decided that possibly missing many samples and critical information (ex. zootechnical results) would be more detrimental to the study than the selection bias caused by choosing seven broiler chicken producers. It must also be considered that chicken farmers are owners of their operations and we couldn't implement this project in their farms without consent and cooperation, hence the impossibility to do a random farm selection. The intensive sampling schedule also created an unconscious observer bias related to the amount of lesion scoring sessions and the lack of blinded evaluators. Indeed, the rigorous need of a qualified evaluator could only be filled by the internal research group, which was also well-informed of the study design. Observer bias may occur when there are systematic differences in the way information is collected between groups and may happen when the person scoring lesions in chickens is knowledgeable about the experimental design and the treatments implemented in each

group evaluated. For example, lesion scoring may be subjective due to the scorer deciding the grade of each lesion in a chicken and/or altering the scores to favor or disadvantage a treatment group to the detriment of other groups. However, lesion scores are highly standardized and observer bias may be limited by following a comprehensive document clearly describing the grades for each score (Elanco Animal Health, 2010). In addition, the objective information recovered from flock data, which cannot be altered by an observer, could be used to cross-check lesion scoring data. For instance, concerns would have been raised if discordant information was identified between lesion scores and growth performance, but it was not the case in this study.

Still, the data generated in this study strongly supports the removal of MIAs from in-feed prevention programs without the addition of butyric acid, as performance and disease control in flocks relying on non-medically important antibiotics (category IV) were equivalent to flocks raised with the conventional use of antibiotics. The strategy proposed in this study has been sustained for six consecutive flock cycles, corresponding to approximately one year of production, with production parameters showing no trend of decline over time (data not shown) that would be indicative of *Eimeria* spp. resistance against ionophores. Still, the long-term sustainability (> 1 year) of such programs remains unknown and should be carefully monitored if producers adopt this strategy in the future. Indeed, assessing the effectiveness of ionophores against *Eimeria* spp. would be critical as decreased coccidiosis control would lead to significant technico-economical losses in the industry. Given the design of the study and limited sample size, inherent to large-scale controlled trials in commercial poultry operations, it was however impossible to test additional strategies to control coccidiosis and necrotic enteritis without loosing statistical power and compromising the accuracy of the results obtained. In the future, other initiatives to develop and test strategies to control coccidiosis and necrotic enteritis without the use of MIAs should be promoted to propose suitable substitutions to continuous ionophore programs and avoid exclusively relying on a single type of program for the control of intestinal diseases. For instance, alternating the vaccination of broiler chicken flocks with live *Eimeria* spp. vaccines with anticoccidial-based programs has been shown to improve the level of *Eimeria* sensitivity to diclazuril, monensin and salinomycin (Chapman, 1994; Chapman & Jeffers, 2015; Jenkins et al., 2010; Mathis & Broussard, 2006; Peek &

Landman, 2006) and could increase the longevity and efficacy of coccidiosis prevention programs using chemotherapy by constantly re-introducing drug-sensitive strains in poultry farms. Indeed, the *Eimeria* spp. strains in live vaccine were recovered decades ago, before the introduction of chemicals and ionophores in poultry production and are thought to be sensitive to most anticoccidial products used in the modern poultry industry (Chapman & Jeffers, 2014). More recently, the concept of combining *Eimeria* vaccination and chemotherapy was extended to the administration of both types of products within a same flock, described as bio-shuttle programs (Kimminau & Duong, 2019). These programs have been proposed to increase the sustainability of coccidiosis prevention programs by introducing *Eimeria* vaccine strains that are still sensitive to in-feed anticoccidials in each new grow-out, but the long-term impacts of such strategies remain yet to be evaluated in contemporary poultry operations.

The Chicken Farmers of Canada's AMU strategy being voluntarily implemented, its acceptance by the industry in the future might be closely connected to its long-term technico-economical viability. Nevertheless, the final accomplishment of such strategy should be evaluated by its capability to decrease AMR in poultry and humans, as stated by the farmers' association (https://www.chickenfarmers.ca/the-antimicrobial-use-reduction-strategy/, 2021):

> Ultimately, the goal of the AMU strategy is to ensure the continued
> effectiveness of antibiotics for both humans and animals.

Consequently, monitoring AMR in poultry and human-related bacteria should be prioritized after the complete implementation of the AMU policy. For example, the success of this policy may be determined by a well-established governmental surveillance system such as the CIPARS (Agunos et al., 2019), developed to monitor trends in AMU and AMR in selected bacterial organisms from human, animal and food sources across Canada. For instance, removing the use of antibiotic classes such as bacitracin, virginiamycin, tylosin and penicillin in Canadian broiler chickens should be followed by a decrease in total AMU and cause a decrease of AMR prevalence in broiler chickens over time. For example, a study recently showed that AMU and AMR were closely associated in pig and poultry farms (Munk et al., 2018) and the researchers demonstrated that among nine European

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countries, those with higher overall antimicrobial usage in livestock were associated with a higher prevalence of antimicrobial resistance genes (ARGs) in its resistome. Additionally, this project showed that the presence of ARGs related to specific antibiotics was positively associated with each corresponding antibiotic (Luiken et al., 2019), strengthening the idea that removing the use of MIAs for prevention in broiler chickens should decrease the selection pressure of these critical antibiotics on AMR. Hence, the new AMU policy would be expected to decrease the incidence of AMR related to MIAs in bacteria and "ensure the continued effectiveness of antibiotics for both humans and animals" (https://www.chickenfarmers.ca/the-antimicrobial-use-reduction-strategy/, 2021).

The long-term effectiveness of strategies relying on ionophores to decrease AMR, and especially AMR related to MIAs, is yet to be determined as it was not evaluated in the present study. Indeed, the possibility of cross-resistance in bacteria with reduced susceptibility to ionophores could compromise the success of the *Antimicrobial Use Reduction Strategy*. For example, avoparcin was banned from animal production the European Union (EU) due to concerns regarding the dissemination of vancomycin-resistant enterococci (VRE), but the prevalence of VRE in Norway persisted even after its ban in 1995 (Borgen et al., 2001). Later, it was reported that VRE from poultry also showed decreased susceptibility to narasin, a monovalent polyether ionophore (Simm et al., 2019; Sorum et al., 2004). In 2016, the Norwegian broiler chicken industry banned the use of narasin and surveillance data showed a significant reduction of VRE in broiler chickens, from 91% in 2014 to 24.7% in 2018 (Simm et al., 2019). As vancomycin resistance was reported to be co-transferred with decreased susceptibility to narasin in enterococci (Nilsson et al., 2012), relying on ionophores for coccidiosis and necrotic enteritis prevention after the removal of MIAs in Canada may not necessarily lead to the expected outcome of reducing AMR against critical antibiotic classes. Furthermore, questions still remain about the impacts of allowing the therapeutic use of MIAs, as the use of these antibiotic classes could select for AMR related to the same antibiotic classes considered critical for human medicine (Luiken et al., 2019). For instance, Denmark's ban of AGPs led to a reduction in the total consumption of antibiotics in animal production but has also been followed by an increase of the therapeutic use of antibiotics and resistance in

important zoonotic bacteria persisted (Jensen & Hayes, 2014). In the present study, therapeutic use of MIAs was also permitted to follow the *Antimicrobial Use Reduction Strategy*; a flock was consequently treated with antibiotics, regardless of their categorization, if a bacterial disease was diagnosed during grow-out. Among the 84 flocks, a total of 10 flocks (11.9%) were treated with a combination of trimethoprim and sulfadiazine (Uniprim©, Bio Agri Mix, Mitchell, Canada) because of increased daily mortalities related to *E. coli* during grow-out. This antibiotic product, commonly used in Canadian broiler chickens for this indication (Agunos et al., 2017), is categorized as highly important (Category II) in Canada (Government of Canada, 2009) and continuing this practice may mitigate the positive impacts of the removal of MIAs for prevention. Because the proportion of treated flocks in groups receiving ionophores only (1/21 (4.8%) flocks treated with Uniprim©) or ionophores with butyric acid (3/21 (14.3%) flocks treated with Uniprim©) did not significantly differ ($p > 0.05$, data not shown) from the conventional group (6/42 (14.3%) flocks treated with Uniprim©), the antibiotic reduction strategies tested in this study are not expected to increase the therapeutic use of this product compared to prevention programs using MIAs. Additionally, due to logistic issues of removing the *in ovo* injection of lincomycin and spectinomycin (Linco-Spectin® Sterile Solution, Zoetis, Kirkland, Canada) potentially compromising the normal workflow, commercial hatcheries providing chicks to each participating farmer did not stop this practice in the antibiotic reduction groups. Hence, this limitation must be considered when interpreting the results of this project, as early chick mortality may be impacted with the ban of this practice post-implementation of the *Antimicrobial Use Reduction Strategy* addressing the preventive use of category II antimicrobials. It is therefore possible that a higher number of flocks could be treated with Uniprim©, or other antibiotics, if early chick mortality caused by *E. coli* increases. Nonetheless, the therapeutic use of antimicrobials currently still remains a small proportion of all antibiotics used in broiler chickens (Government of Canada, 2018) and the prevalence of AMR at the national level may not be impacted by this type of AMU. For instance, a systematic review of 127 studies evaluated different antibiotic reduction approaches on AMR and reported that interventions allowing the therapeutic use of antibiotics appeared similarly effective at reducing AMR prevalence compared to more restrictive AMU approaches (Tang et al., 2019). More specifically, ABF practices were

associated with a 15% (95% CI: -18% to -12%) reduction in AMR, but other interventions such as "all non-therapeutic use restriction" (-10%, 95% CI: -13% to -8%) and "growth promoter restriction" (-30%, 95% CI: -42% to -17%) were statistically similar to ABF production on AMR reduction. On the opposite, the interventions restricting only one antibiotic or a single antibiotic class were not associated with AMR reduction ($p > 0.05$), hence the need of a broad AMU reduction policy such as the one elaborated by the Chicken Farmers of Canada compared to narrower restrictions.

Antibiotics are well known for their toxic effects on pathogenic bacteria by various mechanisms, including mode of actions targeting the bacterial cell wall synthesis, protein synthesis, cell membrane stability and nucleic acid synthesis (Kapoor et al., 2017). The administration of an antibiotic is not without consequences for other bacteria living in the same environment; any bacteria susceptible to that antibiotic is subject to be inhibited or killed if minimal inhibitory or bactericidal concentrations are reached, resulting in a pressure of selection that can alter the composition of these bacterial communities. On the other hand, the study of the microbiota in poultry is a relatively new field of research characterizing features of these bacterial populations assembled in a defined environment such as the gastro-intestinal tract (GIT) of broiler chickens. These living organisms perform numerous metabolic functions and are well recognized as important contributors to chickens' global health and performance (Diaz Carrasco et al., 2019; Stanley et al., 2014). For instance, growth promoting effects of antibiotics have been described (Dibner & Richards, 2005; Jones & Ricke, 2003) and are believed to modify the intestinal microbiota by improving metabolic pathways leading to energy production (Eeckhaut et al., 2011; Polansky et al., 2015; Sergeant et al., 2014). Although antibiotics specifically influence bacterial growth, the real impacts of in-feed antibiotics used to prevent necrotic enteritis on bacterial communities in chickens' GIT are still misunderstood as common antibiotics, for example bacitracin, enramycin and avilamycin, may not strongly impact the structure of the microbiota (Costa et al., 2017; Crisol-Martinez et al., 2017b). Since this project aimed to modify AMU in commercial broiler chicken operations, there was a unique opportunity to describe in a controlled design the impacts of such interventions on the GIT bacterial microbiota of chickens reared with or without in-feed MIAs. Since no significant differences on intestinal health and performance were observed between the three

experimental groups during the on-farm trial, the second hypothesis of this project was that antibiotic reduction strategies marginally impacted the cecal microbiota composition of broiler chickens at the end of rearing. Besides, the GIT microbiota of broiler chickens is a complex collection of microorganisms influenced by various factors, including the early establishment of bacterial species in the GIT, diet composition, environment, and several other exposures interacting between each other and the host during grow-out (Kers et al., 2018). It was hypothesized, at the end of the on-farm trial, that other factors such as the early exposure to bacteria and the farm environment were more important than AMU interventions due to the absence of performance and intestinal health differences between experimental groups. Hence, the objective of this section was to investigate the association of various flock-level factors on the composition of the cecal microbiota in commercial broiler chicken at the end of grow-out. More precisely, the strength of association and significance of the farm environment, feed provider, chick provider, flock cycle and the type of antibiotic program on the diversity and composition of the cecal microbiota of broiler chickens raised in commercial farms were determined.

The GIT of broiler chickens is made of different compartments, which includes in the aboral direction: mouth, oesophagus, crop, proventriculus, gizzard, intestines, and cloaca. The intestinal tract can be subsequently divided in the small intestine (duodenum, jejunum, ileum), the ceca and the colon-rectum. Each section of the GIT possessing its own microbiota composition and diversity (Han et al., 2016; Oakley et al., 2014; Yeoman et al., 2012), the results obtained from studying the GIT microbiota may greatly vary depending on these spatial differences. The ceca, being the primary location of bacterial fermentation in the avian GIT and an important site transforming undigested carbohydrates in short-chain fatty acids (SCFA), are believed to produce 8% of the energy utilized by chickens via these SCFA (Jozefiak et al., 2004). Hence, understanding the characteristics of the cecal microbiota associated with increased SCFA production is considered critical to improve gut health and growth performance in broiler chickens (Onrust et al., 2015). For instance, *Firmicutes* are reported to degrade complex carbohydrates to release nutrients and produce butyrate, while members from the *Bacteroidetes* phylum express enzymes for propionate production pathways (Polansky et al., 2015). Based on the importance of the cecal

microbiota in chickens and its potential to substantially improve broiler chicken production, microbial populations residing within this structure were investigated.

The structure and composition of the cecal microbiota is also subject to important successional changes in the short lifespan of broiler chickens from hatch to market weight. Indeed, from a poorly diversified microbiota after hatch, the broiler's GIT is rapidly colonized by various bacteria found in the environment and then modified through its lifetime by different factors (Jurburg et al., 2019; Oakley et al., 2014). Sampling chickens at early times may be inadequate for a proper evaluation of the cecal microbiota, as the temporal development of the microbiota can create greater inter- and intra-individual variability (Moore & Stanley, 2016) that could have interfered with the assessment of the factors of interest in the present study. Among the 4032 cecal samples recovered at 0, 21, 28 days of age and before shipping to the processing plant (1008 samples per time point), the subset of samples before slaughter was considered the most relevant to decrease this temporal variation and evaluate the microbiota resulting from various exposures that shaped its composition during grow-out.

The marginal influence of the three AMU strategies on the diversity and composition of the cecal microbiota in broiler chickens at the end of grow-out was a major finding of this study. Indeed, the data showed that in-feed antibiotic programs had a relatively low strength of association with the microbiota structure, as represented by a low R-value of 0.04, even if statistically significant (p-value = 0.002). As R-values close to 1.0 or indicate relatively more important microbiota dissimilarities between groups than within groups while R-values close to 0 suggest an even distribution of high and low ranks within and between groups, the R-value of 0.04 suggests that microbial communities between the three antibiotic reduction strategies are uniform. This finding was also supported by the important superposition between samples from all groups in the Redundancy analysis (RDA) plot on the two principal axes explaining the variance. Additionally, the supplementation of butyrates in the diet did not result in major changes in the structure of the microbiota. Still, the Linear discriminant analysis Effect Size (LEfSe), showed that *Rikenellaceae*, *Erysipelotrichaceae*, *Flavobacteriaceae*, *Coriobacteriaceae* and *Unclassified Bacteroidales* were bacterial families differentiating the flocks receiving

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butyrates from the other two groups (data not shown). These observations differ from other studies, where it has been reported that sodium butyrates could significantly decrease the relative abundance of *Lactobacillaceae* (Zou et al., 2019) or significantly increase the relative abundance of *Bifidobacterium* (Yang et al., 2018). Although the addition of butyrate has been reported to select for microbiota compositions improving carbohydrate and lipid pathways, as analysed by predicted functional composition (PICRUSt analysis from 16S rRNA sequencing) (Bortoluzzi et al., 2017), it can be suggested that the alteration of the microbiota caused by this feed additive in the present study did not influence the functionality of the microbiota to the point of providing significantly more energy that would have resulted in quantifiable improved growth performance. However, this affirmation would need to be confirmed by analysing the predicted functional composition of the microbiota from the samples recovered in this study. Also, it must be considered that both ionophores and butyrates possess spectrum of activities against Gram-positive bacteria such as *C. perfringens* (Diaz Carrasco et al., 2016; Lanckriet et al., 2010; Martel et al., 2004), resembling the spectrum of MIAs used in the control group. Consequently, the cecal microbiota might have been importantly altered by the inclusion of these products in each group's diet, but without measurable variation between them, because all groups were administered products with comparable spectrum of actions. Hence, it cannot be excluded that other antibiotic reduction strategies may induce more prominent structural and compositional changes in the cecal microbiota of broiler chickens compared to the conventional use of antibiotics, possibly with different consequences on the functionality of these bacterial communities.

The inability of the AMU interventions to induce major changes in the cecal microbiota may also be related to the presence of more influential factors in commercial broiler chicken operations; it could be suggested that in-feed antibiotics or butyrates in commercial settings may have limited impacts on the cecal microbiota if already complex and diverse microbial communities are established in the environment. Indeed, the type of housing conditions being associated with environmental loads of bacteria (Kers et al., 2019) and large number of bacteria living in reused and new litter (Vaz et al., 2017), there are dynamic exchanges of bacteria occurring between the GIT microbiota and the environment during grow-out (Cressman et al., 2010; Torok et al., 2009) that possibly

reduce the impacts of feed additives. Combined with the possibility that different microbial populations are likely to reside in each farm, this process may explain the large microbiota dissimilarities observed between farms even if in-feed prevention programs were repeated across premises. Also, it supports previous findings that significant differences in the GIT microbiota of chickens can be observed between farms (Rothrock & Locatelli, 2019). Still, the significant variation of the cecal microbiota between flocks raised uninterruptedly on a same farm implies that either the farm-specific microbiota evolves overtime or that other factors contribute to the cecal microbiota compositional variation between flocks. For instance, the farm environment is expected to vary between flocks since litter and dust are removed after each grow-out and replaced with fresh wood shavings before the placement of a new flock. As different microbial communities reside in these types of bedding material (Torok et al., 2009; Wang et al., 2016), the chickens are likely to be exposed to different environmental bacteria from flock to flock. Also, sanitation being a procedure by which broiler houses are cleaned with water and disinfected between flock cycles, large shifts in the environmental microbiota may occur (Jiang et al., 2018) and influence the colonization of chickens' GIT in subsequent flocks. Wet cleaning and disinfection are not necessarily performed after each grow-out as Canadian broiler chicken producers are only required to fully clean the environment at least once a year. Since this practice was not actively monitored during the project, this variable must be considered as an important limitation in explaining compositional microbiota shifts observed in consecutive flock cycles on the same farm. It must also be considered that commercial broiler chicks are hatched in heavily sanitized environments (Kim & Kim, 2010; Samberg & Meroz, 1995), most likely leading to poorly colonized GITs at hatch (Ballou et al., 2016; Donaldson et al., 2017). This poor colonization may cause important changes in the microbiota of broiler chickens at the end of grow-out, as new flocks would constantly be exposed to different environmental bacteria on farms, with potentially important consequences such as variable growth performance related to heterogeneous cecal microbiota compositions. The application of cecal microbiota content on the surface of incubating eggs has been linked to decreased bird-to-bird fecal microbiota variation later in the life of broiler chickens (Donaldson et al., 2017) and could be an effective method to colonize chicks GIT with beneficial bacterial communities before being exposed to variable communities on farms.

Furthermore, it could be suggested that uniformizing the environmental microbiota between farms combined with controlling early exposition to beneficial bacterial communities in the hatchery, preferably at hatch or *in ovo*, could lead to standardized microbiota compositions and more constant zootechnical performance between farms and successional flocks. Still, other factors such as the diet may be difficult to control as feed cost strongly influences the choice of ingredients included in the final feed composition. Since the cecal microbiota composition can be simultaneously impacted by the choice of ingredients (Crisol-Martinez et al., 2017a; Ludvigsen et al., 2016; Lunedo et al., 2014; Stanley, Wu, et al., 2014) and that feed carries rich microbial communities colonizing chickens GIT (Haberecht et al., 2020), the homogenization of broilers' cecal microbiota may be limited by the diet.

It must be considered that the data from the present study is limited to the observational evaluation of most factors analysed. Except for the three antibiotic interventions implemented on each farm with an original experimentation designed to evaluate transversal and longitudinal effects of these treatments, none of the other exposures (feed mill, hatchery, farm environment) were controlled or substituted between farms in a cross-sectional design. Hence, it was not possible to assess the individual impact of each factor on the cecal microbiota of broiler chickens. The project carried out in a commercial environment would not have permitted to make such changes, as it would have compromised the production (hatchery or feed mill switch) or been impossible to swap between farms (farm environment). Still, the data generated from this study can provide significant knowledge on the relative importance of each factor in commercial broiler chicken operations, and it can pave the way to develop strategies targeting to modulate the GIT microbiota of broiler chickens in a perspective of improving performance and disease control. In the future, it would certainly be important to evaluate interventional approaches focused on improving the cecal microbiota composition of broiler chickens that should emphasize on the early development of the GIT microbiota and the control of the farm-specific environmental microbiota, rather than strategies using in-feed products such as antibiotics or butyric acid. This type of comprehensive approach should produce more reliable and repeatable results, compared to targeted strategies, as the microbiota may show

greater uniformization between flocks by considering the multifactorial aspects involved in its development in commercial poultry operations.

The manipulation of the chicken GIT microbiota is a concept by which the malleability of the bacterial population allows to positively influence desirable outcomes, such as improved growth rate, feed efficiency or resilience to diseases by purposely altering the composition of the microbiota (Kogut, 2019). The presence of beneficial populations although implies that other microbial communities may negatively impact the host. For instance, dysbiosis, i.e. the disruption of the normal GIT microbiota composition leading to intestinal inflammation, is described in chickens as a microbial imbalance between protective and harmful bacteria leading to the malabsorption of nutrients, wet droppings, enteritis and impaired growth performance (Teirlynck et al., 2009; Teirlynck et al., 2011). Hence, there is a large potential to take advantage of the GIT microbiota malleability and manipulate its composition to improve nutrient uptake efficiency. However, since most previous studies were performed in research facilities with microbial environments significantly dissimilar from field conditions (Kers et al., 2019), there was a need to validate the current knowledge on the characteristics of the cecal microbiota of broiler chickens associated with enhanced growth performance. This project gave a substantial opportunity to cover this knowledge gap, since numerous cecal samples were recovered before slaughter with each corresponding chicken weight. Hence, components of the cecal microbiota composition and structure associated with weight gain were investigated and networks built from significant interactions between taxa were analyzed to reveal community relationships shaping chickens' microbiota in association with their growth performance at the end of grow-out.

Bacterial communities can be firstly described by the number of species identified in a sample, or operational taxonomic units (OTU) in 16S rRNA microbiota studies, and their numerical composition (Kim et al., 2017). These metrics, respectively named Richness and Evenness, can subsequently be included in formulas to generate a variety of diversity indices, such as the Shannon diversity index. Higher indices, i.e. greater bacterial diversity in the samples, are generally considered beneficial for the host as it has been associated with more efficient microbial communities consuming less energy (Larsen &

Claassen, 2018). However, the link between growth rate and alpha diversity is not well defined in broiler chickens as different studies report inconsistent (Diaz-Sanchez et al., 2019; Liu et al., 2021; Siegerstetter et al., 2017; Yan et al., 2017) or negative associations between alpha diversity parameters and weight gain (Han et al., 2016). However, it must be noted that Han et al. (2016) evaluated a low number of chickens ($n = 20$) and that the accuracy of the linear regressions built from the association between observed OTU and weight are questionable, as the regressions were poorly representing the association between the two variables. Hence, there was certainly a need to evaluate a greater number of samples to accurately model this association. For instance, the results generated from the 1002 cecal samples analysed revealed that Richness was positively associated with weight gain, indicating that zootechnical gains should be expected by improving Richness in the ceca of broiler chickens. Still, the benefits of increasing Richness were maximized at 225 OTU, possibly indicating the metabolic potential and ability to break down and release nutrients from complex feed is not further improved once a certain number of different bacterial species are established in the ceca. Hence, this finding may explain the inconsistencies reported between studies as the range of OTU in each study could significantly impact their respective results and interpretations. Also, it must be considered that these studies evaluated different zootechnical parameters, i.e. residual feed efficiency, feed conversion ratio (FCR), final body weight and weight gain during grow-out, and it remains conceivable that alpha diversity would impact each of these parameters differently even if those are closely connected. It is habitually desirable to evaluate FCR in broiler chickens as feed cost is an important factor related to profitability. Improving FCR means that a lower amount of feed is required to grow a chicken to target weight (FCR = Total feed consumed / Total weight gain) and since feed is one of the most important variable expenses in broiler chicken production (Willems et al., 2019), even small improvements can generally provide positive economical returns on the investments. However, this metric could not be measured at the bird level due to the commercial settings of this study as the individual feed consumption could not be quantified. Still, the average daily weight gain (ADG = Body weight before slaughter / Age) would provide the most accurate measure of growth for broiler chickens and was deemed a reliable metric of growth performance in this study.

Microbial communities can be further described by their beta diversity, i.e. the variation of their composition by focusing on the difference in taxonomic relative abundance between samples. The significant Analysis of Similarities (ANOSIM) firstly indicated that potential taxa of the cecal microbiota were associated with ADG, although the Principal Coordinates Analysis (PCoA) showed less evidence, most likely related to the low R-value of 0.024 returned by the ANOSIM. The differential taxa abundance in the cecal microbiota was analysed to quantitatively describe the impacts of its compositional variation on growth rate. *Firmicutes* were identified as the most important phylum associated with improved weight gain. Within *Firmicutes*, the *Lachnospiraceae* was the only family positively correlated to ADG, which is possibly related to its implication in improving the cecal metabolism (Onrust et al., 2015). Still, not all members of the *Firmicutes* were beneficial, as families such as *Planococcaceae* and *Veillonellaceae* were both significantly and negatively correlated to ADG. Combined with the possibility that not all *Lachnospiraceae* possess the same metabolic pathways leading to SFCA production (Meehan & Beiko, 2014), it shows that studying the taxonomic abundance at higher ranks may only provide general guidance on the structure and composition of the microbiota. Hence, including large number of heterogenous bacterial species in the same group could remain an important limitation to microbiota studies. Furthermore, *Ruminococcaceae* were not quantitatively correlated to improved ADG. This observation may be related to the important heterogeneity of the members included within this family, as possibly only a small proportion of these members may enhance energy production in the ceca. Hence, there would be a need to study the bacterial microbiota at lower taxonomic ranks to correctly identify the various impacts of various bacterial species on the host metabolism. With novel high throughput sequencing capabilities providing increased taxonomic resolution to identify members of bacterial communities at the species and strain level (Johnson et al., 2019), this path should definitively be explored in the field of poultry science to improve the accuracy of studies evaluating approaches to successfully modify the GIT microbiota.

The analysis of co-occurrence networks revealed the connections between microbial communities in the ceca of broiler chickens. Important patterns associated with a detrimental network in the community structures were identified and potentially provided

significant evidence on the interactions driving metabolic processes and leading to energy production in the ceca. For instance, the three families with the largest negative correlation with ADG (*Porphyromonadaceae*, *Planococcaceae*, and *Veillonellaceae*) were all interconnected in a complex network that also included various families in the *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Tenericutes* and *Verrucomicrobia*. On the opposite, there was a clear negative correlation between *Lachnospiraceae* and this network, which could be explained by different hypotheses. The mechanisms underlying the composition of the intestinal microbiota are still poorly understood, but species-species interaction may suggest that redundant functionalities are present in these communities and possibly complementing each other (The Human Microbiome Project Consortium, 2012). For instance, it is possible that the bacterial species making the detrimental network may possess complementary metabolic pathways. On the opposite, the survival of some bacterial species may depend on inhibiting the presence of other bacteria in the same environment that competes for the same resources. For example, bacteriocins are well-known antibacterial peptides produced by bacteria that inhibit the growth of other bacteria (Nishie et al., 2012) and it remains possible that either bacteria within the detrimental network or species within the *Lachnospiraceae* family produce bacteriocins inhibiting the opposite side. Based on the nature of relative abundance analysis in 16S rRNA gene profiling, the increase in a taxon's abundance is however inherently related to an equivalent decrease across other taxa (Barlow et al., 2020). Consequently, the negative relative abundance correlation between the detrimental network and *Lachnospiraceae* could have been caused by an absolute abundance variation of either the taxa in the detrimental network or *Lachnospiraceae*, or even both processes simultaneously. As the total numbers of these taxa were not assessed in the study, there is no clear indication of which mechanism associated with the modulation of ADG in broiler chickens, but further works evaluating the absolute abundances of taxa could reveal additional information on strategies to enhance the composition of the microbiota. For instance, methods using digital PCR (dPCR) could provide quantitative differential taxon analysis on the absolute abundance of taxa when combined to 16S rRNA gene amplicon sequencing (Barlow et al., 2020) that could help differentiate if the improvement of ADG in broiler chickens via microbiota-

based strategies would be more successful by focusing on increasing beneficial bacteria or on the opposite, targeting to control harmful bacterial communities.

The field of bioinformatics is a rapidly growing field and newer methods have been developed to mitigate the weaknesses of older methods. For instance, the OTU clustering method at the 97% and 99% threshold is known for its low resolution and its dependence on reference databases to infer taxonomic profiles of 16S rRNA genes sequences (Callahan et al., 2017). Instead, the ASV methods can differentiate sequence variants at the single nucleotide level and may prove more useful in future microbiota studies. Indeed, associating the growth rate of chickens with specific bacterial species would most likely yield more precise results, rather than identifying heterogenous taxonomic groups such as the bacterial family and phylum presented in this work. Older downstream analyses were used, and it is a limitation to the conclusions drawn in the present study as no specific beneficial or detrimental bacterial species could be identified. Future microbiota-based projects would benefit from adapting to most recent bioinformatics pipelines using ASVs to identify the bacterial species involved in improved metabolic processes. Additionally, evaluating microbial communities by their taxonomic composition, as it was performed in the present work, is considered inferior to study the functional profile of these communities (Doolittle et al., 2017). Indeed, it cannot be excluded that metabolic pathways may be comparable between different bacteria and that even if their taxonomic phyla, family or species are different, the function of these groups of microorganisms may be similar. For instance, it may explain the low correlation of the beta diversity with ADG (R-value of 0.024) as different taxonomic profiles may result in highly similar metabolic functions related to energy production and weight gain. Predictive methods using large reference genome databases, such as PICRUSt, could be a valuable complement to the compositional evaluation of bacterial communities by sequencing the 16S rRNA gene. However, the analyses can be dependant of the genomes included in these databases and although it is well adapted to human microbiota datasets (Sun et al., 2020), its applicability to chicken microbiota profiles may be questionable.

Chapter 5. Conclusion

The long-term impacts of the *Antimicrobial Use Reduction Strategy* in Canada are yet to be determined and only time will tell if the Chicken Farmers of Canada took the right decisions towards the reduction of AMR in broiler chickens. Nonetheless, the findings in this work contributed to improve the sustainability of the modern poultry industry by providing feasible alternatives to the practice of using of medically important antibiotics to prevent intestinal diseases in broiler chickens. For instance, this project showed that medically important antibiotics are not necessarily associated with improved performance or better control of intestinal diseases, as they were removed from in-feed prevention programs without measurable negative consequences. Rather, high antibiotic use in poultry flocks may be associated with poor environmental conditions and could be mitigated by targeted veterinary interventions (Roskam et al., 2019; Roskam et al., 2020). Hence, this project opened the eyes of the Canadian poultry industry on successful methods adapted to the worldwide context of antibiotic reduction, and producers should foresee a viable transition from an industry structure relying on medically important antibiotics to a new era focusing on the responsible use of antimicrobials for the long-term effectiveness of these products in human and animal health. There are conceivably numerous forthcoming roadblocks that the industry will face in a constantly evolving poultry production system, but the progresses made by the completion of this thesis will provide substantial assistance for the future development of poultry science.

This project also generated important new knowledge on the cecal microbiota of broiler chickens that could considerably influence future directions of microbiota manipulation in a perspective of improving zootechnical performance. Firstly, contributions of different factors influencing the cecal microbiota were described, and an important paradigm was challenged by the indication that in-feed antibiotics and prebiotics may not be the most important factors affecting the microbiota during grow-out. Rather, this work suggests the farm environment and early exposure to bacteria are prominent factors involved in shaping the composition and structure of the bacterial communities residing in the ceca of broiler chickens. Hence, considering these factors should be

prioritized in the future to increase the success and reliability of cecal microbiota modifications in a perspective of improving zootechnical performance. Indeed, important features of the cecal microbiota, such as Richness, beta diversity, taxonomic composition and co-occurrence patterns were significantly associated and correlated to greater weight gain in broiler chickens. The key to develop an ideal cecal microbiota successfully and constantly may reside in the ability to influence such environmental factors; more specifically the early exposure to beneficial bacterial communities and the control of farm-specific resident flora.

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