Effects of mannan-oligosaccharides and xylo-oligosaccharides on the chicken gut microbiota

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Abstract

The emergence and spread of antibiotic resistance in pathogens have led to a restriction on the use of antibiotic growth promoters (AGPs) in chicken production. In the absence of AGPs, viable alternatives are required to improve chicken health and maintain efficiency of production and safety of poultry products. Given the interest in using prebiotics as one type of alternatives to AGPs and the importance of gut microbiota in regulating metabolic and immune functions, we investigated the effects of two potential prebiotics, mannan-oligosaccharides (MOS) and xylo-oligosaccharides (XOS), on the chicken gut microbiota. In our first study, a higher bacterial diversity was observed in the cecum of MOS-fed chickens that were raised under sub-optimal conditions. MOS changed the cecal microbiota in favor of the *Firmicutes* members but not the *Bacteroidetes*. In addition, MOS increased villus height and goblet cell numbers in the ileum and jejunum. In the second experiment, two levels of XOS, 0.1% and 0.2%, were evaluated in broilers under normal experimental conditions. Addition of 0.2% XOS to the feed increased the proportion of *Lactobacillus* genus in the cecum, and the increase was associated with an increase in the cecal concentration of acetate. In the third experiment, young chickens (1 wk) were challenged with *Salmonella* Enteritidis in the presence or absence of XOS or MOS. Treatment with either 0.2% XOS or 0.1% MOS differentially altered the relative abundance of certain bacteria, but the overall microbial diversity remained unchanged. The genera *Clostridium*, *Lactobacillus*, and *Roseburia* were increased in response to XOS, whereas MOS significantly enriched *Coprococcus*, *Ruminococcus* and *Enterococcus*. The number of *S.* Enteritidis recovered from the cecum was significantly lower in the MOS and to a less extent in the XOS-fed birds. Moreover, XOS and MOS differentially regulated production of inflammatory related cytokines upon *S.* Enteritidis infection.

Résumé

L'émergence et la propagation de la résistance aux antibiotiques chez les bactéries pathogènes ont conduit à une restriction sur l'utilisation des antibiotiques facteurs de croissance (AFCs) de la production de poulet. En l'absence de AFCs, des alternatives viables sont nécessaires pour améliorer la santé de poulet, et de maintenir l'efficacité de la production et de la sécurité des produits de volaille. Compte tenu de l'intérêt d'utiliser les prébiotiques, comme un type d'alternatives à l'AGP, et l'importance de la flore intestinale dans la régulation des fonctions métaboliques et immunitaires, nous avons étudié les effets de deux prébiotiques potentiels, mannan-oligosaccharides (MOS) et xylo-oligosaccharides (XOS) sur le microbiote intestinal de poulet. Dans notre première étude, une diversité bactérienne élevée a été observée dans le caecum de poulets MOS nourris qui ont été soulevées dans des conditions sous-optimales. MOS changé le microbiote caecum en faveur des membres *Firmicutes* mais pas le *Bacteroidetes*. En outre, augmenté MOS hauteur des villosités et le nombre de cellules caliciformes dans l'iléon et le jéjunum. Dans la deuxième expérience, deux niveaux de XOS, 0,1% et 0.2%, ont été évalués dans les poulets dans des conditions expérimentales normales. L'addition de 0.2% XOS à la charge a augmenté la proportion de *Lactobacillus* genre dans le caecum, et l'augmentation a été associée à une augmentation de la concentration d'acétate caecale. Dans la troisième expérience, les jeunes poulets (une semaine) ont été provoqués avec *Salmonella* Enteritidis en présence ou en l'absence de XOS ou MOS. Le traitement avec soit 0.2% XOS ou 0.1% MOS différentielle modifié l'abondance relative de certaines bactéries, mais la diversité microbienne globale est restée inchangée. Les genres *Clostridium*, *Lactobacillus*, et *Roseburia* ont été augmentées en réponse à XOS, alors MOS enrichi de manière significative *Coprococcus*, *Ruminococcus*, et *Enterococcus*. Le nombre de *S.* Enteritidis dans le caecum était plus faible dans le MOS, et dans une moindre

mesure dans les oiseaux nourris XOS. En outre, XOS et MOS régulés différemment la production de cytokines inflammatoires connexes après l'infection *S.* Enteritidis.

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Contributions of authors

Four co-authored manuscripts are included in this thesis.

Chapter 2: M. Pourabedin and X. Zhao

M. Pourabedin wrote a review manuscript and made the illustrations. X. Zhao reviewed and edited the manuscript. Published in part in FEMS Microbiology Letters. 2015. Prebiotics and gut microbiota in chicken. 362(15), fnv122

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M. Pourabedin, E. Chevaux and X. Zhao designed the study. M. Pourabedin carried out the experiments, analyzed, and interpreted the results. Z. Xu assisted in qPCR analysis. B. Baurhoo formulated the feed and helped to take samples. M. Pourabedin wrote the manuscript. X. Zhao reviewed and edited the manuscript. Published in Canadian journal of microbiology. 2014. Effects of mannan oligosaccharide and virginiamycin on the cecal microbial community and intestinal morphology of chickens raised under suboptimal conditions. 60(5), 255-266.

Chapter 4: M. Pourabedin, L.Guan and X. Zhao

M. Pourabedin and X. Zhao designed the study. M. Pourabedin carried out the experiments, analyzed, and interpreted the results. L. Guan re-analyzed the sequencing data and verified the results. M. Pourabedin wrote the manuscript. X. Zhao reviewed and edited the manuscript. Published in Microbiome. 2015. Xylo-oligosaccharides and virginiamycin differentially modulate gut microbial composition in chickens. 3(1), 15.

Chapter 4: M. Pourabedin, Q. Chen, M. Yang and X. Zhao

M. Pourabedin, M.Yang and X. Zhao designed the study. M. Pourabedin carried out the experiments, analyzed, and interpreted the results. Q. Chen and M. Yang prepared the *Salmonella* strain, helped to take samples, and performed qPCR assay. M. Pourabedin wrote the manuscript. X. Zhao reviewed and edited the manuscript.

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List of abbreviations

AGP	Antibiotic growth promoter
ANOSIM	Analysis of similarities
BM	Body mass
bp	Base pairs
CFU	Colony forming unit
DGGE	Denaturing gradient gel electrophoresis
DP	Degree of polymerization
FCR	Feed conversion ratio
FI	Feed intake
FOS	Fructo-oligosaccharides
GI	Gastrointestinal
HTS	High throughput sequencing
INF	Interferon
IL	Interleukin
Kg	Kilogram
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
MOS	Mannan-oligosaccharides
OTU	Operational taxonomic unit
PCoA	Principal component analysis
PCR	Polymerase chain reaction
PD	Phylogenetic diversity

QD	Quinupristin-Dalfopristin
qPCR	Quantitative PCR
RDP	Ribosomal Database Project
rRNA	Ribosomal ribonucleic acid
SCFA	Short chain fatty acids
STD	Standard deviation
wk	Week
U.S	United States
TLR	Toll-like receptor
XOS	Xylo-oligosaccharides

Chapter 1. General introduction

The poultry industry is an important and growing agricultural sector in Canada, comprising of more than 2600 regulated producers and a large number of related businesses. In 2013, Canada produced 1.04 billion kilograms (kg) of chicken, 60% of which was produced in Quebec and Ontario (Agriculture and Agri-Food Canada, 2015). This provides a significant boost to the economy by contributing about $2.4 billion (Agriculture and Agri-Food Canada, 2015). According to the Animal Industry Division of the Agriculture and Agri-Food Canada, domestic consumption of chicken meat increased from 16.9 kg per person in 1980 to 30.1 kg in 2013, whereas beef and pork consumption dropped over the same period by 29% and 34%, respectively (Animal Industry Division-AAFC, 2015). A shift toward higher poultry consumption has been principally attributed to favouring poultry over red meats due to increasing consumer health knowledge (Yen *et al.*, 2008).

Despite the fact that poultry production has been steadily increasing, there is a growing public concern over transmission of foodborne pathogens and antibiotic resistance genes from livestock into the food supply. Over the past 50 years, antibiotics have been widely used in animal production for different purposes including 1) therapeutic use to treat illness, 2) prophylactic use to prevent disease, and 3) sub-therapeutic use to improve feed efficiency and growth performance. However, using antibiotic growth promoters (AGPs) in food producing animals has recently been heavily criticized due to emergence of antibiotic resistance and its potential spread to pathogens (Marshall & Levy, 2011). Thus, use of all AGPs in animal feed has been banned in the European Union since 2006, while they are being prudently used in Canada and the United States (U.S.). The U.S. Food and Drug Administration proposed a voluntary initiative in December 2013 to phase out the use of AGPs in animal feed. In April 2014, Health Canada implemented a similar

plan to reduce medically important antibiotics in food animal production. To help alleviate some of the potential problems that may occur with the removal of AGPs, researchers are working on to find effective alternatives such as prebiotics to maintain and promote the health of animals. A prebiotic is defined as "a nondigestible compound that selectively stimulate growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota and confer(s) health benefits to the host" (Roberfroid *et al.*, 2010). Prebiotics act through diverse mechanisms, such as providing nutrients, preventing pathogen adhesion to host cells, interacting with host immune systems and affecting gut morphological structure, all presumably through modulation of intestinal microbiota. A wide variety of dietary compounds may fulfil the prebiotic criteria. However, most of them have not been sufficiently studied to be classified as such. To date, most promising dietary fibers with prebiotic functions are non-digestible oligosaccharides containing 3 to 9 sugar monomers. In fact, some of prebiotics such as mannan-oligosaccharides (MOS) have been adopted in the poultry industry, while others such as xylo-oligosaccharides (XOS) are still at an early development stage. The majority of studies regarding prebiotic impacts have focused on chicken production parameters or relied on culture-dependent methods to assess microbial composition. These methods, however, inevitably underestimate gut microbial diversity as less than 40% of the gut microbial members are cultivable (Suau *et al.*, 1999).

Development of molecular techniques such as high throughput sequencing (HST) has provided researchers new innovative tools to study microbial communities. While microbial communities in mice and humans have been intensively studied, there have been few comprehensive evaluations of the prebiotic impacts on the chicken gut microbiota. In addition, the effects are variable, depending on the type of prebiotics, dosage, and experimental conditions. Therefore, the overall goal of this study is to evaluate effects of MOS and XOS on the gut

microbiota of broiler chickens during normal physiological, environmental stress and *Salmonella* challenged conditions. The gut microbial changes in response to prebiotics supplementation would then be correlated to the host production performance and intestinal morphological parameters as well as immune responses. Our research would help us better understand how the gut microbiota contributes to poultry health and productivity and support the development of new prebiotic products as an alternative to in-feed antibiotics.

1.1 Objectives

- To evaluate cecal microbiota of broilers that have been grown under suboptimal conditions and given either subtherapeutic level of virginiamycin (VIRG) or MOS over the entire production cycle. The cecal microbial community was analysed using gradient gel electrophoresis (DGGE) and species-specific real-time quantitative PCR (qPCR) in combination with classical culture-based bacterial detection. In addition, growth rate, feed conversion efficiency, and changes in intestinal morphological parameters in response to VIRG and MOS were studied.
- To identify changes in the broiler ileal and cecal microbiota as a results of subtherapeutic concentration of VIRG or two levels of XOS supplementation using 454 pyrosequencing of 16S rRNA gene. Growth performance as well as ileal and cecal concentrations of lactate and short chain fatty acids (SCFAs) were also measured.
- To investigate the impact of MOS and XOS supplementations on cecal microbiota and inflammatory cytokine changes in chickens challenged with pathogenic *Salmonella Enteritidis* on day 5 of age. Cecal microbial population and cytokine gene expression were studied using 16S rRNA gene pyrosequencing and qPCR, respectively.

1.2 References

Agriculture and Agri-Food Canada (2015) Poultry and Egg Market Information - Canadian Industry, Retrieved June 23, 2015, from http://www.agr.gc.ca/poultry/index_eng.htm.

Animal Industry Division-AAFC (2015) Per capita consumption-Poultry and other animal protein sources comparison, Source: Statistics Canada - CANSIM Tables 002-0010 and 051-0001, Calculations done by Animal Industry Division-Market Information Section.

Marshall BM & Levy SB (2011) Food animals and antimicrobials: impacts on human health. *Clinical microbiology reviews* **24**: 718-733.

Roberfroid M, Gibson GR, Hoyles L, McCartne AL, Rastall R, Rowland I, ... & Meheust A (2010). Prebiotic effects: metabolic and health benefits. *British Journal of Nutrition*, **104**(S2), S1-S63.

Suau A, Bonnet R, Sutren M, Godon J-J, Gibson GR, Collins MD & Doré J (1999) Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Applied and environmental microbiology* **65**: 4799-4807.

Yen ST, Lin B-H & Davis CG (2008) Consumer knowledge and meat consumption at home and away from home. *Food Policy* **33**: 631-639.

Chapter 2. Literature review

2.1 Chicken gut microbiota

The chicken gastrointestinal (GI) tract is home to a diverse population of bacteria, with over 600 species from more than 100 bacterial genera (Torok *et al.*, 2011a). The dynamic interactions between a host and its indigenous microbial communities are shaped by a long mutual co-evolution that confers numerous benefits on the host (Ley *et al.*, 2008). The GI microbes have essential roles in metabolic and protective functions, such as helping the host to digest nutrients, develop immune system and improve intestinal epithelium (Oakley *et al.*, 2014, Stanley *et al.*, 2014a). It is therefore important to identify the gut microbial composition and diversity to improve chicken health and productivity.

In general, the most abundant phylum in the chicken GI tract microbiota is *Firmicutes* followed by two minor phyla, *Proteobacteria* and *Bacteroidetes.* In addition*,* members of phyla *Actinobacteria*, *Tenericutes (*Waite & Taylor, 2014*), Cyanobacteria* and *Fusobacteria (*Qu et al., 2008*)* can be found in very low abundance. Bacterial communities vary considerably by locations along the GI tract of chickens. Crop, gizzard and duodenum share similar microbiota, dominated by the genus *Lactobacillus,* as high as 99% in some birds (Gong *et al.*, 2007, Sekelja *et al.*, 2012). The highest diversity of *Lactobacillus* was observed in the crop (Gong *et al.*, 2007). The jejunum is also dominated by *Lactobacillus* species, mainly *Lactobacillus salivarius* and *Lactobacillus aviarius* (Gong *et al.*, 2007, Feng *et al.*, 2010). The microbial composition of the ileum is more diverse and less stable compared with the duodenum and the jejunum. The ileum is dominated by *Lactobacillus*, *Candidatus Arthromitus, Enterococcus*, *Escherichia_Shigella* and *Clostridium_XI* (Asrore *et al.*, 2015).

The cecum is by far the most densely colonized microbial habitat in chickens and its bacterial diversity is much higher than those in the upper GI tract (Oakley *et al.*, 2014). The most detailed information regarding chicken gut microbiota is available for the cecum (Stanley *et al.*, 2014a). The cecum is a key region for bacterial fermentation of non-digestible carbohydrates and a main site for colonization by pathogens. Chickens have two paired ceca, both harbouring similar bacterial communities (Stanley *et al.*, 2015). In a study by Gong *et al.* (2007), the cecum was mainly occupied by the *Clostridia* genus followed by genera *Lactobacillus* and *Ruminococcus*. The majority of *Clostridia* detected in the cecum fall primarily into three main families, *Clostridiaceae*, *Lachnospiraceae* and *Ruminococcaceae* (Danzeisen *et al.*, 2011). *Enterococcaceae*, *Enterobacteriaceae* and *Bacteroidaceae* are other reported abundant families in the cecal microbiota (Yin *et al.*, 2010). The cecum is also rich in unknown and unclassified bacterial residents (Stanley *et al.*, 2013b). At the species level, *Bacteroides fragilis, Lactobacillus crispatus, Lactobacillus johnsonii, Lactobacillus salivarius* and *Lactobacillus reuteri* comprised more than 40% of cecal microbiota (Stanley *et al.*, 2015).

To study GI microbiota, fecal samples are often used because of easy sampling. The composition of fecal microbiota highly fluctuates depending on varying contributions of microbiota from different GI segments (Sekelja *et al.*, 2012). *Lactobacillaceae*, *Peptostreptococcaceae*, *Streptococcaceae*, *Clostridiaceae* and *Enterobacteriaceae* were identified as common families of the fecal microbiota (Videnska *et al.*, 2014). The fecal microbiota of laying hens are generally more complex than the fecal microbiota of broilers (Videnska *et al.*, 2014). Recently, Stanley *et al.* (2015) indicated that about 88% of all operational taxonomic units (OTUs), comprising 99.25% of sequences, were shared between cecal and fecal samples in broiler chickens.

The GI microbiota of chickens could be separated into four potential robust clusters, referred to as enterotypes (Kaakoush *et al.*, 2014), similar to the presence of three enterotypes in human gut microbiome (Arumugam *et al.*, 2011). Enterotypes are in fact distinct bacterial communities, each dominated by different bacteria genera (Arumugam *et al.*, 2011). Enterotypes in humans are correlated with long-term dietary patterns but independent of host phenotypes such as gender, age or body mass index (Wu *et al.*, 2011). However, whether such associations exist in chickens are not studied. Despite existence of such enterotypes, there is a strong individual variation among chickens of a same breed, on a same diet and even under highly controlled experimental conditions (Nordentoft *et al.*, 2011, Sekelja *et al.*, 2012, Stanley *et al.*, 2013a). This variation could be explained by the fact that in the modern industrial poultry production, chickens are hatched in highly hygiene incubators and reared without exposure to maternally derived bacteria. The random colonization by surrounding environmental bacteria is assumed to be a key reason for a high variation in the intestinal microbiota (Stanley *et al.*, 2013a).

The chicken gut microbiota is affected by diet (Torok *et al.*, 2008), gender (Lumpkins *et al.*, 2008), background genotype (Zhao *et al.*, 2013), housing condition (Nordentoft *et al.*, 2011), floor litter (Torok *et al.*, 2009, Cressman *et al.*, 2010), feed restriction (Callaway *et al.*, 2009) and stocking density (Guardia *et al.*, 2011). Furthermore, as a bird ages, the microbiome complexity increases (Yin *et al.*, 2010, Crhanova *et al.*, 2011, Danzeisen *et al.*, 2011, Sekelja *et al.*, 2012). Certain bacteria may disappear or emerge over time in the intestinal microbiota of older chickens while others remain stable throughout the life. *Firmicutes* species are dominant in young chickens while the representatives of *Bacteroidetes* are most common in adult birds (older than 7 months) (Callaway *et al.*, 2009, Videnska *et al.*, 2014). In layers, four different profiles of cecal microbiota were identified from the day of hatching until 60 weeks of age (Videnska *et al.*, 2014). However,

temporal characterization of gut microbiota in poultry varies among studies and needs more frequent sampling and robust sequencing and analyses.

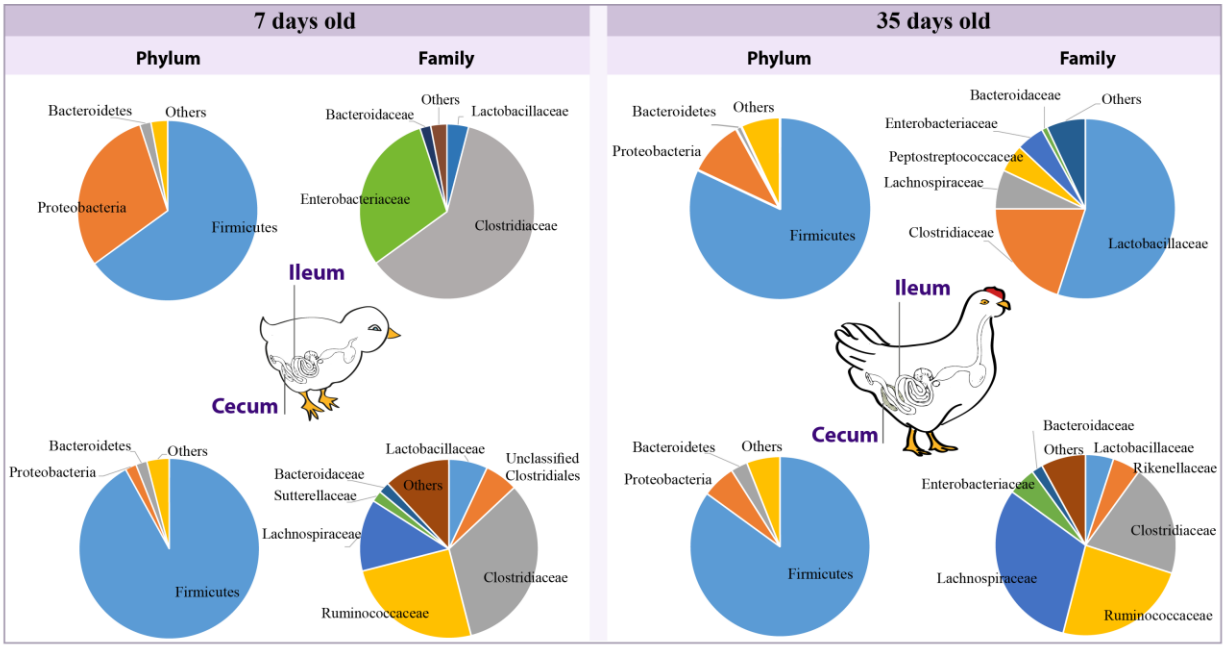

Figure 2.1. The chicken gut microbiome.

The graphs provide an overview of the relative abundance of dominant bacterial phyla and families of the broiler chicken ileal (top level) and cecal (bottom level) microbiota in two different ages, 7 and 35 days. Data are compiled from three studies: (Asrore *et al.*, 2015) for ileum on day 7, (Corrigan *et al.*, 2015) for cecum on day 7, and (Pourabedin *et al.*, 2015) for ileum and cecum on day 35. This figure is reproduced from Pourabedin & Zhao (2015)

2.1.1 Gut microbiota and chicken health

The significant role of microbiota in maturation of the gut immune system (Brisbin *et al.*, 2008, Mwangi *et al.*, 2010, Crhanova *et al.*, 2011) and intestinal mucosa (Forder *et al.*, 2007) has been evident in chickens. In addition, the gut microbiota is one of the main defense components in the GI tract against enteric pathogens. Possible mechanisms by which the gut microbiota inhibits pathogens include (i) competition for available nutrients, (ii) competition for attachment sites on the intestinal epithelium, (iii) production of toxins and antimicrobials such as bacteriocins, (iv) production of SCFA which can suppress the growth of pH-sensitive pathogens, and (v) stimulation of the immune system. These are certainly not mutually exclusive and some bacteria may use several mechanisms (Patterson & Burkholder, 2003). Disturbance of the gut microbiota-host interaction with an increase of pathogens and a decrease of health-promoting bacteria plays a crucial role in development of intestinal disorders. An unbalanced microbiota, referred as dysbiosis, has been shown to be associated with pathogenesis of many intestinal disorders such as necrotic Enteritidis causing by *Clostridium perifingens* (Van Immerseel *et al.*, 2009). Dysbacteriosis has been defined as "the presence of a qualitatively and/or quantitatively abnormal microbiota in proximal parts of the small intestine, inducing a cascade of reactions in the gastro-intestinal tract such as reduced nutrient digestibility and impaired intestinal barrier function and increasing the risk of bacterial translocation and inflammatory responses"(Teirlynck *et al.*, 2011). Intestinal dysbacteriosis can be caused by a variety of infectious and non-infectious factors. Main infectious agents are enteric pathogens, whereas non-infectious factors include environmental stressors, dietary changes, antibiotic therapy, nutritional imbalance, mycotoxins and management disorders (Teirlynck *et al.*, 2011).

Previous studies have indicated that pathogens can induce structural and compositional changes in the animal intestinal microbiota (Stecher *et al.*, 2007, Barman *et al.*, 2008, Bearson *et al.*, 2013). Significant changes in cecal microbiota have been evident in chickens infected with *Clostridium perfringens* (Feng *et al.*, 2010, Stanley *et al.*, 2012, Skraban *et al.*, 2013), *Eimeria* species (Perez *et al.*, 2011, Stanley *et al.*, 2014b, Wu *et al.*, 2014) and *Salmonella* Enteritidis (Nordentoft *et al.*, 2011, Juricova *et al.*, 2013, Videnska *et al.*, 2013). It has been shown that lack of normal gut microbiota in newly hatched chickens makes them highly vulnerable to colonization by pathogens (Juricova *et al.*, 2013). Furthermore, pathogen-mediated inflammation in the intestine of older birds was more restricted than in the young chicks (Withanage *et al.*, 2005, Crhanova *et al.*, 2011). Using a germ-free chicken model, Mwangi *et al.* (2010) indicated that gut microbiota remarkably influenced T cell receptor repertoire profile in both the gut and the spleen, suggesting that gut microbial change in chickens may greatly affect systemic immune responses. In addition, the chicken gut microbiota has been shown to modulate intestinal gene expression (Yin *et al.*, 2010), and accelerate gut immune system maturation (Crhanova *et al.*, 2011).

The gut microbiota and its metabolites are able to affect positively the integrity of the intestinal barrier. Accordingly, loss of barrier integrity causes a progressive increase in intestinal permeability, leading to a switch from "physiological" to "pathological" intestinal mucosal inflammation (Lambert, 2009). Conventionally reared broilers have a different intestinal mucus composition and a higher number of goblet cells compared with chickens hatched and reared in a sterile brooding isolator (Forder *et al.*, 2007). Goblet cells synthesize and secret glycoproteins known as mucins onto the mucosal surface, which serve as a defensive barrier (Deplancke & Gaskins, 2001). In an *in vitro* study, intestinal mucus of poultry origin, but not human mucus, was shown to attenuate *Campylobacter jejuni* virulence (Byrne *et al.*, 2007). Thus, *Campylobacter*

jejuni, an important foodborne bacterial pathogen for humans, is tolerated in avian hosts without causing clinical infection (Young *et al.*, 2007).

2.1.2 Gut microbiota and chicken growth

Microorganisms in the gut interact with each other as well as with the host, influencing many physiological functions within the host. It has been indicated that the balance between two phyla, *Firmicutes* and *Bacteroidetes*, affects the amount of energy extracted from the diet and this proportion associated with adiposity in human (Ley *et al.*, 2006, Turnbaugh *et al.*, 2009), mice (Turnbaugh *et al.*, 2006) and pigs (Guo *et al.*, 2008). A significant increase in the body weight in relation to an increase in *Firmicutes* to *Bacteroidetes* ratio has also been observed in chickens treated with penicillin (Singh *et al.*, 2013). In addition, several bacterial phylotypes, more specifically within genera *Lactobacillus*, *Ruminococcus* and *Clostridium,* have been shown to be associated with performance enhancement in chicken (Torok *et al.*, 2008, Stanley *et al.*, 2013b).

In the chicken cecum, *Clostridium* species, particularly certain species in clusters IV and XIVa (these two clusters are predominant in the chicken cecal microbiota), are significant butyrate-producers that contribute to growth (Eeckhaut *et al.*, 2011). Butyrate is not only an important energy source for cecal epithelial cells but also inhibits inflammatory responses by acting on pro-inflammatory cytokines (Eeckhaut *et al.*, 2011). In a metagenomic analysis of cecal microbiota, over 200 non-starch polysaccharide-degrading enzymes and several pathways associated with production of short chain fatty acids (SCFAs) were detected (Sergeant *et al.*, 2014). These SCFAs not only provide energy for the chickens but also indirectly benefit them by lowering cecal pH, which prevents growth of pathogens, and enhance mineral absorption. SCFAs can also act as signal transduction molecules via host SCFA receptors and regulate various host metabolic and immune response pathways (Kasubuchi *et al.*, 2015).

2.1.3 Environmental effects on the gut microbiota

There are a variety of production-related environments in poultry industries that can affect overall chicken physiology, health, and productivity (Humphrey, 2006). These include overcrowding, temperature fluctuations, feed withdrawal, and transportation. Through the gut-brain axis, the GI tract is particularly responsive to environmental factors, which can cause changes in the gut microbiota (Burkholder *et al.*, 2008, Bercik *et al.*, 2012). However, very little is known about the impact of environmental stressors on the chicken GI microbiota. In a study by Burkholder *et al.* (2008), laying hens were stressed by feed deprivation and high temperature (30°C) for one day. Compared with controls, the stressed chickens showed altered microbial community structure in the ileum and cecum. In the same study, attachment of *S.* Enteritidis to ileal tissue was increased as a result of the acute stress (Burkholder *et al.*, 2008). Another study examined high stocking density as a stressor in broiler farm production, and showed that intensively reared broilers had different bacterial community in the gut, with the highest modifications observed in the crop and ceca (Guardia *et al.*, 2011). Lactobacilli and bifidobacteria appear to be sensitive to environmental factors, and their populations tend to decrease in stressed birds (Patterson & Burkholder, 2003).

Exposure to environmental stressors can modulate gut epithelial cell differentiation and decrease expression of tight junction proteins, leading to increase in epithelial permeability and susceptibility to infection (Meddings & Swain, 2000, Lambert, 2009). For example, after prolonged heat exposures, compromised mucosal immunity and integrity of intestinal epithelium were observed in broilers (Quinteiro-Filho *et al.*, 2010) and layers (Deng *et al.*, 2012). Increased susceptibility to *Salmonella* and *Campylobacter* was reported in chickens when they were in a poor environment and/or under physical or psychological stress (Humphrey, 2006, Rostagno, 2009). The higher circulating levels of stress hormones in stressed chickens may be associated

with an increase in colonization and systemic spread of *Salmonella enterica* (Cheng *et al.*, 2002, Methner *et al.*, 2008). Recently, Gomes *et al.* (2014) reported that overcrowding reduced macrophage activity and increased *S.* Enteritidis invasion in broiler chickens.

2.1.4 Culture-independent methods for the analysis of gut microbiota

In the past, determination of microbiota changes and corresponding functions in response to dietary treatments has been a challenge, largely due to the limitation of conventional microbiological methods. In fact, it has been estimated that only 20-40% of the gut microbiota can be cultivated (Suau *et al.*, 1999). The traditional culture-based methods are extremely laborious, may impose stress on the microorganisms, and it can be difficult to simulate the interactions of bacteria with other microbes and host cells. In addition, the growth requirements of many bacteria are unknown (Zoetendal *et al.*, 2004).

To overcome these challenges associated with selective growth media and isolation of bacteria from environmental samples, culture-independent methods have become fundamental tools in studying bacterial communities (Rastogi & Sani, 2011). Most of these molecular methods rely on the sequence analysis of the 16S ribosomal RNA (rRNA) gene for identification, quantification, and classification of bacteria. The 16S rRNA is a subunit of the 30S small compartment of prokaryotic ribosomes and was introduced in the 1980s as a new standard for identifying bacteria (Woese *et al.*, 1985, Woese, 1987). The 16S rRNA gene is about 1,550 base pairs (bp) long and is composed of both variable and conserved regions among different species of bacteria (Figure **2.2**). Universal primers are often used to amplify the conserved regions, whereas the sequence of the variable regions in between is used for the comparative taxonomy (Greisen *et al.*, 1994). The 16S rRNA has nine hypervariable regions, V1 to V9, which shows a considerable sequence diversity (Lane *et al.*, 1985).

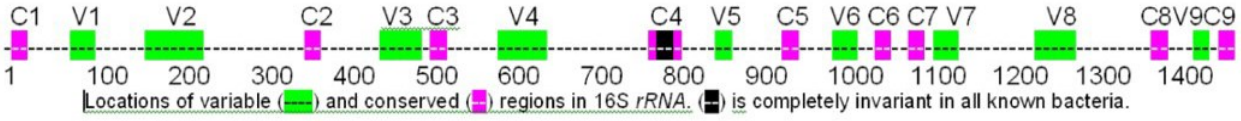

Figure 2.2. Location of conserved and hypervariable regions in the 16S rRNA gene. Numbers below dotted line refer to base pair position in *E. coli*. The figure is reproduced from Ram *et al.* (2011).

Common culture-independent methods for profiling microbial communities are genetic finger printing techniques such as denaturing- or temperature-gradient gel electrophoresis (DGGE and TGGE), real-time PCR and more recently, whole community analysis approaches such as high throughput sequencing (HTS) platforms (Rastogi & Sani, 2011). DGGE was introduced into microbial ecology by Muyzer *et al.* (1993). This technique examines microbial diversity based upon PCR-amplified 16S rRNA fragments electrophoresed on a polyacrylamide gel containing a linear gradient of DNA denaturant such as a mixture of urea and formamide (Muyzer *et al.*, 1993). Different bacterial species have different sequences within the variable regions of the 16S rDNA. Thus, each bacterial species theoretically yields a different DGGE profile after amplification of variable regions of the 16S rRNA (Ercolini, 2004). The DGGE method has been shown to be very useful for detecting shifts in the chicken gut microbial community structure (Hume *et al.*, 2003, Zhou *et al.*, 2007, Rehman *et al.*, 2008). Among nine hypervariable regions of 16S rRNA genes, amplification of the V3 region has been shown to produce the best DGGE profile of GI tract microbiomes (Yu & Morrison, 2004).

DGGE has the drawback that it is typically not quantitative. Instead, real-time PCR is a quantitative approach to measure the abundance of total or specific strain bacteria by counting the number of target gene copies in DNA directly extracted from an environmental sample (Bustin *et al.*, 2005, Smith & Osborn, 2009). Several sets of 16S rRNA gene primers have been designed and

used in chickens for quantitative detection of important bacterial groups such as *Lactobacillus*, *Bifidobacterium* (Wise & Siragusa, 2007), *Campylobacter* spp. (Lund *et al.*, 2004) and *C. perfringens* (Wise & Siragusa, 2005).

In the recent years, the Sanger sequencing method, which was introduced in 1977 (Sanger *et al.*, 1977), has been partially replaced by HTS technologies. HTS platforms such as 454 pyrosequencing (Roche, Basel, Switzerland), SOLiD (Applied Biosystems, MA, USA) and Hi/MiSeq systems (Illumina, CA, USA), have enabled us to perform ultra-deep sequencing projects for studying complex microbial populations (Shendure & Ji, 2008). The 454 pyrosequencing and Illumina systems are the most common platform used for the analysis of microbial communities (Andersson *et al.*, 2008, Diaz-Sanchez *et al.*, 2013). To date, there have been only a few studies to investigate the poultry GI tract microbiota using HTS of the 16S rRNA.

2.2 Antibiotic growth promoters in poultry production

Soon after the discovery of antibiotics to control bacterial infections, the growth promoting activity of these products was introduced in chickens given dried mycelia of *Streptomyces aureofaciens* containing chlortetracycline residues (Jukes & Williams, 1953). Ever since, antibiotics have been widely used in poultry production as growth promoters (Castanon, 2007, Diarra & Malouin, 2014). In general, therapeutic use of antibiotics involves treating sick birds over a short period, whereas antibiotics used for growth-promoting purposes are administered at sub-therapeutic levels to the entire flock over an extended period (Barton, 2000). The recognition that antibiotics can enhance animal growth efficiency has coincided with industrial poultry production that involves intensive chicken rearing. In U.S. alone, it has been reported that poultry producers use about 10.5 million pounds of antibiotics annually for nontherapeutic purposes (Oliver *et al.*, 2011).

It has been estimated that the use of AGPs in animal feed improves weight gain by 4 to 8% and feed utilization efficiency by 2 to 5% (Butaye *et al.*, 2003). The majority of these antimicrobials are given to birds with their feed. The exact mechanisms by which AGPs promote growth are not clearly understood, but findings that AGPs have no growth-promoting effects in germ-free chickens suggest that AGPs mainly act on the intestinal microbiota (Feighner & Dashkevicz, 1987). Several hypotheses have been proposed to explain how antibiotics improve growth performance. These include: (i) an increase in efficiency of nutrient absorption due to a thinner intestinal epithelium in antibiotics-treated animals, (ii) reduction or elimination of gut pathogen load and subclinical infections, (iii) an increase in nutrient availability due to a reduced microbial destruction of nutrients and (iv) reduction of toxins and growth-depressing metabolites produced by bacteria (Feighner & Dashkevicz, 1987, Butaye *et al.*, 2003). Growth promoting antibiotics are tended to be less effective in animals when used under hygienic and controlled experimental conditions, suggesting a reduction or inhibition of subclinical infections as the most probable mechanism for their action (Brüssow, 2015). Further to their antimicrobial effect, it has been proposed that AGPs have anti-inflammatory effects on intestinal phagocytic cells by inhibiting the production and release of catabolic mediators (Niewold, 2007).

In Canada, 27 antibiotics are approved for use in chicken production (Diarra & Malouin, 2014). Among them, arsanilic acid, bacitracin, bambermycin, chlortetracycline, virginiamycin, roxarsone and penicillin are approved for growth promotion (Diarra & Malouin, 2014). All antibiotics used by Canadian livestock producers require a veterinary prescription. One of the most commonly used AGPs in Canadian broiler production is virginiamycin.

2.2.1 Virginiamycin

A streptogramin antibiotic virginiamycin is produced by incubation of bacterium *Streptomyces virginiae* (Yamada *et al.*, 1987), and composed of two antibiotic molecules, pristinamycin IIA (virginiamycin M1) and virginiamycin S1 (Figure 2.3). They exhibit a narrow spectrum of activities mainly against gram-positive bacteria by binding to two separate loci on the 23S rRNA of the 50S sub-unit of bacterial ribosome and inhibiting protein synthesis (Yonath, 2005). Virginiamycin has been used for decades in livestock production as a growth promoter and therapeutic agent in many countries (Acar *et al.*, 2000).

Virginiamycin, at a subtherapeutic level of 20 mg/kg, has been reported to increase body weight and improve feed efficiency from day 0 to 15, but induce no significant improvement in the growth performance for the remainder of the study (Dumonceaux *et al.*, 2006). In Canada, virginiamycin has been approved to use in broiler feed at a dose of 11 to 22 mg/kg of feed for growth promotion or as an aid in the prevention of necrotic enteritidis.

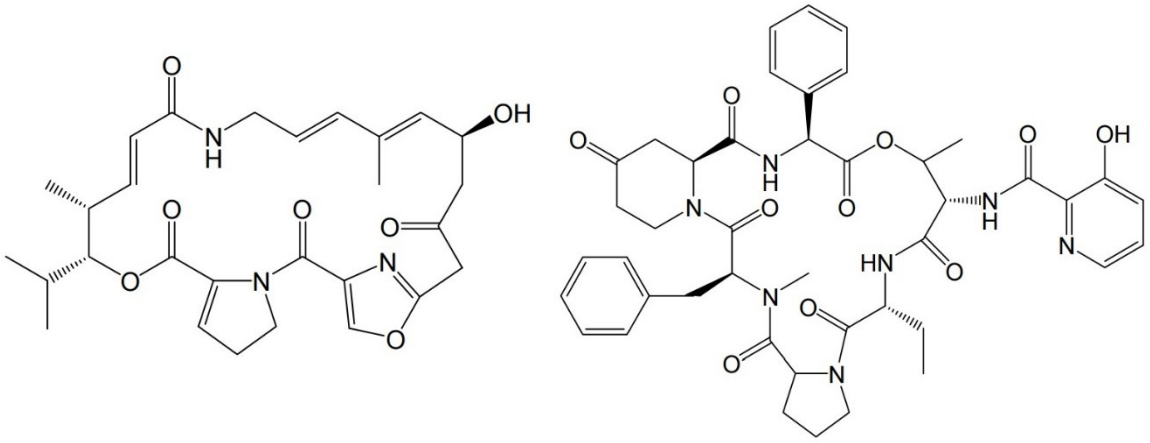

Figure 2.3. Structure of virginiamycin M1 and virginiamycin S1.

2.2.2 Effects of antimicrobials on the chicken gut microbiota

Numerous early studies, reviewed by Dibner & Richards (2005), examined the effect of antibiotics on the intestinal microbiota of chickens using culture-dependant methods. Most of these studies have focused on population of specific bacterial strains mainly pathogenic bacteria cultivated from intestinal or fecal samples. Engberg *et al.* (2000) reported that the number of *C. perfringens* and lactobacilli were reduced in the ileum of broilers treated with subtherapeutic levels of salinomycin in combination with zinc bacitracin. Similar results were found when broilers were fed diets supplemented with subtherapeutic levels of salinomycin and avilamycin (Knarreborg *et al.*, 2002). In another study, Baurhoo *et al.* (2009) treated broiler chickens with 16.5 mg/kg virginiamycin or 55 mg/kg bacitracin and found that treated chickens had a significantly lower population of *E. coli* and *Campylobacter* in the cecum. Over the past decade, studies evaluating antibiotic effects on the chicken gut microbiota using molecular methods have revealed conflicting results. Based on the DGGE analysis, inclusion of bacitracin, avilamycin, or enramycin in the diets had no significant effect on the species richness and evenness, but changed the composition of the chicken small intestinal microbiota (Pedroso *et al.*, 2006, Gong *et al.*, 2008). Using qPCR, Dumonceaux *et al.* (2006) indicated that subtherapeutic virginiamycin affected bacterial abundance mainly in the proximal GI tract (duodenal loop to proximal ileum) with fewer changes toward the distal end (ileocecal junction and cecum). The authors found that *Lactobacillus crispatus, Lactobacillus aviarius, Lactobacillus johnsonii* and *Lactobacillus vaginalis* were more prevalent in the duodenal loop of the antibiotic treated birds (Dumonceaux *et al.*, 2006). Meanwhile, Lin *et al.* (2013) reported that tylosin-containing feed had no effect on ileal bacterial biomass, but reduced *Lactobacillus* spp. populations in the ileum. A differential effect of antibiotics on lactobacilli has been reported to be dependent on the bacterial species, GI tact

location and the type of antibiotic used (Torok *et al.*, 2011b). In addition, the impact of antibiotics on microbial composition appeared to be dose and age dependent (Zhou *et al.*, 2007). Using 454 sequencing, Danzeisen *et al.* (2011) found that a diet supplemented with anticoccidial monensin in combination with virginiamycin led to a decrease in cecal abundance of *Firmicutes,* most of which belonged to the family *Lachnospiraceae*. The authors also observed an increase in the abundance of *E. coli* in response to the antibiotics, which was in contrast with previous culture-dependent studies (Danzeisen *et al.*, 2011).

2.2.3 Resistance to antibiotics

Antimicrobial products kill susceptible microorganisms or inhibit their growth while those bacteria with the ability to resist antibiotics survive and propagate (Davies & Davies, 2010). A bacterium can acquire resistance to an antibiotic either by a genetic mutation within the organism or by accepting existing resistance genes from other bacteria. Resistance gene determinants can be transferred between bacteria by several mobile genetic elements including plasmids, transposons, integrons, and bacteriophages (Ochman *et al.*, 2000). The transfer of these genetic elements is facilitated among bacteria by horizontal gene transfer, which may occur via three main mechanisms: conjugation, transduction, and transformation (Ochman *et al.*, 2000). Nowadays, antimicrobial resistance is an emerging global concern that significantly challenges public health.

Despite all the benefits of antibiotics for the poultry industry, their increased use has given rise to a fear of the development of resistant pathogenic bacterial strains and residual contamination of the food chain with antibiotics. Many studies have reported that multiple antibiotic-resistance genes can be found in foodborne pathogens such as *E. coli* and *Salmonella* spp. isolated from Canadian broiler chicken farms (Diarrassouba *et al.*, 2007, Forgetta *et al.*, 2012, Diarra *et al.*, 2014). In addition, several lines of evidence link human infections to antibiotic resistance in

foodborne pathogens of poultry origin. For instance, two studies in Canada suggest chicken meat as the most probable reservoir of antimicrobial resistant extra-intestinal pathogenic *E. coli*, causing urinary tract infections in humans (Manges *et al.*, 2007, Bergeron *et al.*, 2012). Moreover, a strong correlation has been shown between ceftiofur-resistant *Salmonella* strains isolated from retail chicken and incidence of ceftiofur-resistant *Salmonella* infection in humans across Canada (Dutil *et al.*, 2010).

The most controversial use of antibiotics in chicken production involves using antimicrobial drugs to promote growth. Use of AGPs such as salinomycin or bacitracin in broiler feed may modulate the phenotype and the distribution of resistance genes in pathogens (Diarra *et al.*, 2007, Bonnet *et al.*, 2009). The streptogramin resistance gene, vatD, was found to be more abundant in *Enterococcus* spp. isolated from birds treated with virginiamycin, and raised in a commercial broiler chicken farm in Quebec (Thibodeau *et al.*, 2008). The chemical structure of virginiamycin has been modified to make therapeutic drugs such as Quinupristin-Dalfopristin (QD), marketed as Synercid™ (Moellering *et al.*, 1999). In human, QD is used to treat infections caused by vancomycin-resistant *Enterococcus faecium* (Moellering *et al.*, 1999, Manzella, 2001). QD-resistant *E. faecium* has been commonly reported in chickens, raising concerns that overuse of virginiamycin in poultry production might compromise the effectiveness of QD in treating human infections (McDonald *et al.*, 2001, Cox & Popken, 2004).

2.2.4 Alternatives to antibiotic growth promoters

The public concern over spread of antibiotic resistant bacteria has drawn attention to the use of AGPs in agriculture (Allen *et al.*, 2013, Diarra & Malouin, 2014). However, eliminating all in-feed antibiotics in livestock production may not be an ideal approach because it may increases disease occurrences and reduces production efficiencies as reported in Europe after all AGPs were

banned (Ferber, 2003). Therefore, there is now an urgency to find and develop viable alternatives to maintain efficiency of animal production and decrease disease rates. An effective alternative will preferably mimic the beneficial effects of antibiotics on growth performance without increasing the dissemination of antibiotic resistance (Verstegen & Williams, 2002). To date, several alternatives with varying degrees of efficacies have been proposed. Indeed, there has been extensive researches on certain feed additives such as prebiotics, probiotics, organic acids, essential oils and egg yolk antibodies (Verstegen & Williams, 2002, Allen *et al.*, 2013). Among them, prebiotics and probiotics are gaining in popularity due to their beneficial effects on the gut microbiota and disease suppression (Patterson & Burkholder, 2003, Gaggìa *et al.*, 2010). An advantage of prebiotics over probiotics is that they stimulate commensal bacteria that already exist in the GI tract of that individual animal and therefore adapted to that environment. A combination of probiotics and prebiotics, known as synbiotics, has also been looked at (Awad *et al.*, 2009, Dibaji *et al.*, 2014, Mookiah *et al.*, 2014). Currently, the mixing proportions to combine prebiotic and probiotic are unknown, in most cases result in non-additive effect (Cheng *et al.*, 2014). In addition, a synergic mechanism of probiotics and prebiotics has not been studied, hence, the synbiotics application is still faraway (Cheng *et al.*, 2014).

2.3 Prebiotics

A prebiotic is defined as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Roberfroid *et al.*, 2010). This definition was recently refined to shift the focus from selective targets to microbial ecological functions within the gut. The new definition of a prebiotic is "a nondigestible compound that, through its metabolization by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial

physiological effect on the host" (Bindels *et al.*, 2015). To be classified as a prebiotic compound, a dietary ingredient has to be 1) neither digestible nor absorbable through the GI tract, 2) metabolised by one or limited number of gut commensal bacteria and 3) able to induce health benefits for the host (Roberfroid *et al.*, 2010).

The prebiotic approach does not have a long history of use in broiler chickens compared to the prebiotics use in human and pet food (Yang *et al.*, 2008). At this stage, oligosaccharides including fructo-oligosaccharides (FOS) (Kim *et al.*, 2011, Swiatkiewicz *et al.*, 2011), mannan-oligosaccharides (MOS) (Baurhoo *et al.*, 2009, Xiao *et al.*, 2012), xylo-oligosaccharides (XOS) (Courtin *et al.*, 2008, Sun *et al.*, 2013), galacto-oligosaccharides (Faber *et al.*, 2012) and soybean meal oligosaccharides (Lan *et al.*, 2007) are the most commonly studied prebiotics in chicken production. However, the results of these studies regarding changes in the gut microbiota are conflicting. In fact, some of early culture-based studies have revealed the ability of prebiotics to increase the abundance of beneficial bacteria (i.e., *Bifidobacterium* and *Lactobacillus*), and reduce potential pathogens (i.e., *clostridia* and *E. coli*) (Kim *et al.*, 2011, Peinado *et al.*, 2013, Shanmugasundaram *et al.*, 2013). At the same time, other studies have showed little or no significant effect (Zhang *et al.*, 2003, Jiang *et al.*, 2006, Biggs *et al.*, 2007). This discrepancy may reflect differences in laboratory methods, experimental conditions, and variation in the gut microbiota of individual animals.

Dietary supplementation with prebiotic oligosaccharides have been reported to reduce intestinal *Salmonella* colonization (Eeckhaut *et al.*, 2008), modulate immune cell parameters (Shanmugasundaram & Selvaraj, 2012), and ameliorate inflammation response (Shanmugasundaram *et al.*, 2013) and severity of lesions during intestinal infection (Lensing *et al.*, 2012). Various potential mechanisms have been proposed for health benefits of prebiotics

(Figure 2.4). These include: 1) providing a substrate for the gut commensal microbiota, and will affect their growth and metabolic activities, 2) preventing adhesion of certain bacterial species by occupying carbohydrate-binding sites in bacteria and host cells, and 3) an increase in SCFA production, and will affect immunomodulation and host metabolism (Saulnier *et al.*, 2009, Roberfroid *et al.*, 2010). In addition, the presence of SCFA in the intestines contributes to a lower pH, a better bioavailability of calcium and magnesium, and inhibition of potentially harmful bacteria (Teitelbaum & Walker, 2002, Wong *et al.*, 2006). Among SCFA, butyrate as a preferred source of energy for colonocytes, stimulates colon increasing the absorptive capacity of the epithelium, and inhibits the growth of colonic carcinoma cells, both in vitro and in vivo (Van Craeyveld et al., 2008). The cancer-suppressing properties of dietary fibers appear to correlate with their ability to generate butyrate upon colonic fermentation (Perrin et al., 2001). The selective stimulation by prebiotics of certain colonic bacteria, such as bifidobacteria is in some cases paralleled by suppression of protein fermentation in the colon (De Preter et al., 2004, Geboes et al., 2006). Reduced protein fermentation in the colon is a desired outcome, as the amino acid degradation pathways in bacteria result in the production of potentially toxic catabolites such as ammonia, amines and phenols, some of which have been implicated in bowel cancer and in exacerbation of diseases such as ulcerative colitis (Van Craeyveld et al., 2008).

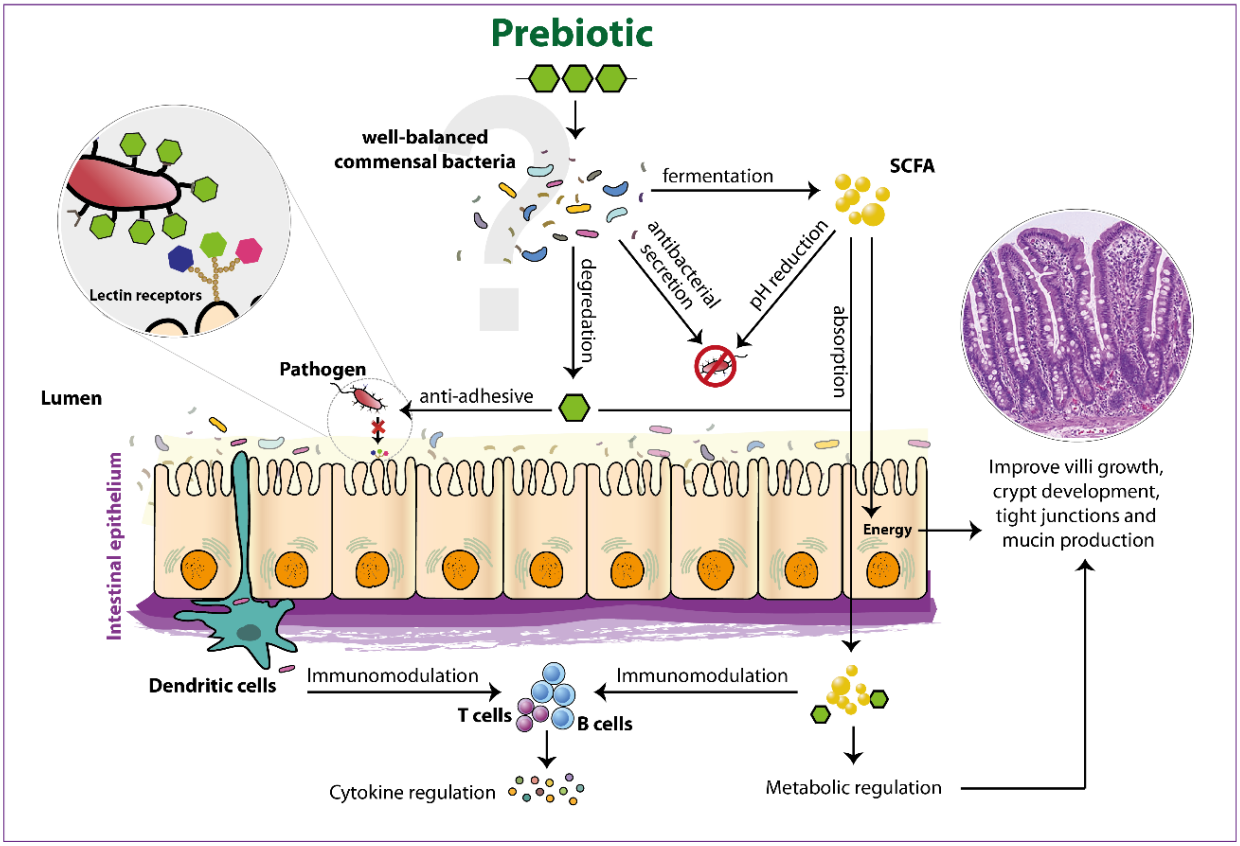

Figure 2.4. Potential mechanisms of action of prebiotics.

Prebiotics are metabolized by the gut commensal microbiota. The gut microbiota can ferment prebiotics into short-chain fatty acids (SFCA), mainly acetate, propionate, and butyrate. SCFA lower the luminal pH, provide energy sources for epithelial cells, and have profound effects on inflammation modulators and metabolic regulations. A well-balanced bacterial community can also improve intestinal mucosal structure. Some bacterial strains produce antimicrobial factors or stimulate the immune system by signaling dentric cells. Oligosaccharides and monosaccharides can reduce pathogen colonization by blocking the receptor sites used by pathogens for attachment to the epithelial cell surface. This figure is reproduced from Pourabedin & Zhao (2015).

2.3.1 Mannan Oligosaccharides (MOS)

MOS are mannose-based oligomers linked together by β-1,4 glycosidic bonds. They are naturally found in certain plants, beans and the mannoprotein portion of the cell wall of the yeast *Saccharomyces cerevisiae* (Figure 2.5). Because birds do not have enzymes to break down the mannan backbone, this oligosaccharide reaches the lower GI tract undigested. Culture based studies have indicated that various bacterial strains within the genera *Bacteroides*, *Bacillus* and *Clostridium* produce mannanases as endohydrolases with an ability to cleave β-1,4 mannopyranoside in mannan products (Dhawan & Kaur, 2007). However, it should be noted that polysaccharide utilization is a multistep process facilitated by synergistic interactions between widespread members of the gut microbiota (Rakoff-Nahoum *et al.*, 2014).

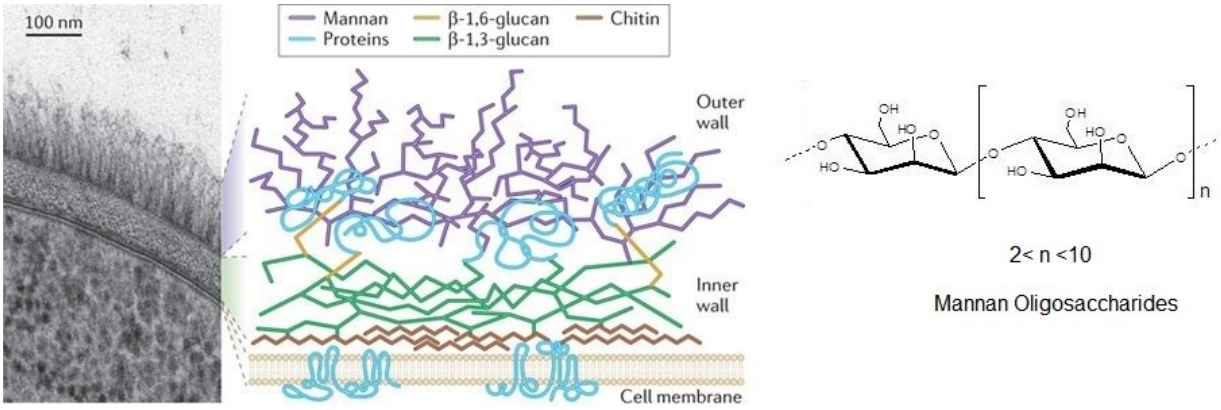

Figure 2.5. Structure of the yeast cell wall and mannan oligosaccharides.
The left image is reproduced from Gow *et al.* (2012),

Several bacterial culture-based studies have shown the ability of MOS to prevent colonization by undesirable organisms, promote the growth of beneficial bacteria, improve gastrointestinal maturation and increase feed energy utilization (Agunos *et al.*, 2007, Baurhoo *et*

al., 2007a, Baurhoo *et al.*, 2007b, Brzoska *et al.*, 2007, Benites *et al.*, 2008, Yang *et al.*, 2008, Baurhoo *et al.*, 2009).

In a study by Corrigan *et al.* (2011), MOS significantly altered bacterial community structure and composition in broiler chickens as revealed by automated ribosomal intergenic spacer technique coupled with 16S rRNA gene clone library analysis. Kim *et al.* (2011) investigated the impact of 0.025% and 0.05% of MOS on the ileocecal microbiota of broilers using DGGE and qPCR methods. They showed that 0.05% MOS reduced *Clostridium perfringens* and *E. coli*, and increased the relative population of *Lactobacillus*. More recently, bacterial 16s rRNA pyrosequencing was used to study phylogenetic alterations of cecal microbiota in response to MOS supplementation in broilers (Corrigan *et al.*, 2015). MOS consistently and reproducibly modified the cecal microbial composition. More specifically, MOS resulted in an increase in the number of species within the phylum *Bacteroidetes*, particularly after 35 days (Corrigan *et al.*, 2015).

The dietary MOS may have greater influences in birds subjected to pathogens or environmental stresses. In *E. coli*-challenged, transport-stressed turkey poults, yeast extracts supplemented (1 g/kg) diet increased the number and oxidative burst activity of heterophils, and enhanced disease resistance (Huff *et al.*, 2010). Mannose-containing carbohydrates such as MOS may bind with pathogen lectins and prevent its attachment to the epithelial surface. Mannose-bound pathogens therefore pass through the GI tract without colonization. Whole yeast cell wall supplementation (2 g/kg) also decreased a coccidial infection-induced increase in the cecal *E. coli* and *Salmonella* colonization (Shanmugasundaram *et al.*, 2013). Dietary supplementation with a whole yeast cell product modulated chicken immune response by increasing the IFNγ and reducing IL-10 cytokines mRNA expression of the cecal tonsil. In broilers challenged with *Clostridium perfringens*, MOS (2 g/kg) resulted in an up-regulation of ileal toll-like receptor (TLR)2b, TLR4,

IL-12 and IFN-γ, whereas it down-regulated cecal tonsil TLR2b expression (Yitbarek *et al.*, 2012). In layers, supplementation of *Saccharomyces cerevisiae* fermentation products (0.75g/kg) reduced incidence and severity of lesions caused by *Eimeria maxima (Lensing et al., 2012).* The exact reason for these health benefits is unknown and could be due to the indirect effects of prebiotic on the immune system and intestinal epithelial integrity.

In addition to MOS, innate immune-modulatory activities of mannobiose supplementation have been reported in broilers (Ibuki *et al.*, 2010). Mannobiose could change expression of genes related to the host defense, increase IgA production, and improve *S.* Enteritidis clearance (Ibuki *et al.*, 2011). In an *in vitro* model, phagocytic and *Salmonella*-killing activity of chicken macrophage cell line (MQ-NCSU) were increased following treatment with prebiotic β1-4 mannobiose, and the increase was associated with enhanced production of hydrogen peroxide and nitric oxide as well as up-regulation of genes involved in innate immunity (Ibuki *et al.*, 2011). The underlying mechanisms by which MOS modulate immune responses may be through the cell surface mannose receptor that recognizes both host glycoproteins and microbial glycans, or via mannose binding lectins that trigger and propagate an inflammatory response by initiating a cascade of cytokine expression.

2.3.2 Xylo-oligosaccharides (XOS)

XOS are chains of β-1,4-linked D-xylopyranoside units, appear naturally in bamboo shoots, fruits, vegetables, milk and honey (Vazquez *et al.*, 2000). The structure of XOS differs in degree of polymerization (DP: number of xylose units in their backbone) and degree of substitution (arabinose to xylose ratio), depending on the type of xylan sources and hydrolysis method used for XOS production. The DP can vary from 2 to 10, and they are known as xylobiose, xylotriose, and so on (Figure **2.6** 2.6) (Aachary & Prapulla, 2011). They are produced at an industrial scale by

partial hydrolytic degradation of lignocellulosic materials, commonly arabinoxylans, which are found in abundance in the cereal grains (Carvalho *et al.*, 2013).

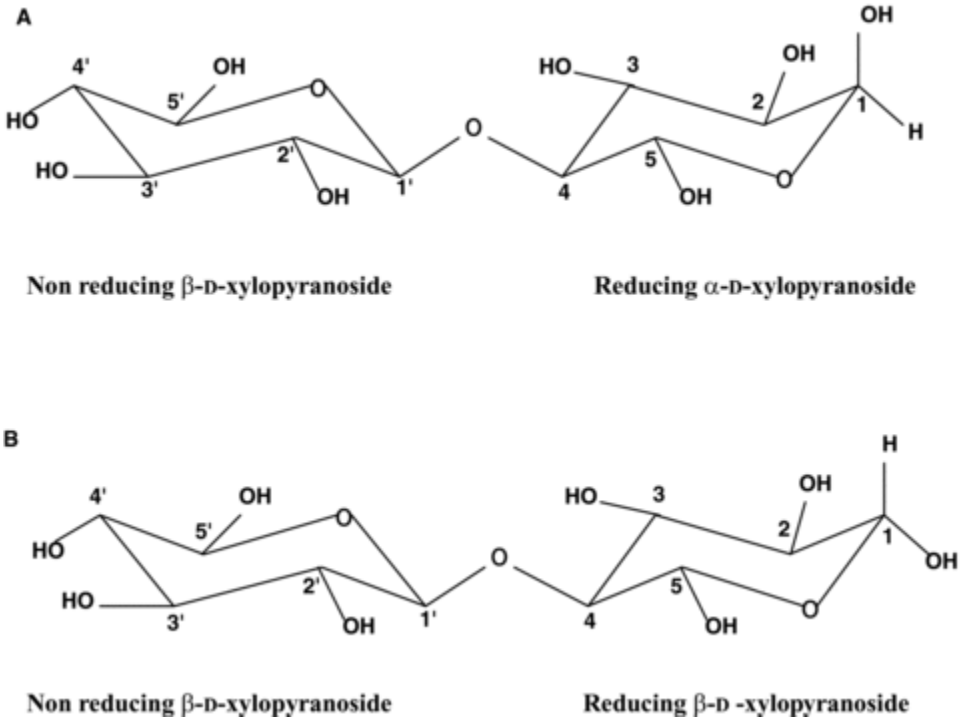

Figure 2.6. Structure of xylobiose:
(A) β-D-xylopyranosyl-(1→4)-α-D-xylanopyranos, **(B)** β-D-xylopyranosyl-(1→4)- β-D-xylanopyranos. Source: Aachary & Prapulla (2011)

XOS show advantages over other oligosaccharides such as FOS in terms of resistance to heat and a wide range of pH (2.5–8.0) (Vazquez *et al.*, 2000). XOS remain stable in gastric acidic condition and resist hydrolysis by GI tract enzymes (Aachary & Prapulla, 2011). Chickens lack enzymes required to degrade the glycoside link between xylose monomers, therefore XOS reach the lower intestinal tract and cecum, where they are metabolized by xylanolytic microorganisms. *In vitro* fermentation assays of XOS with faecal batch and semi-continuous mixed-culture have

indicated that XOS are efficiently utilized by various bacterial strains and modulate the microbial metabolism (Moura *et al.*, 2008, Chapla *et al.*, 2012). Microbial endo-xylanases depolymerize long-chain oligosaccharides into short-chain oligomers, which are transported into cells and hydrolyzed by the periplasmic exo-xylanase. Short-chain xylo-oligomers are further hydrolyzed into xylose by the intracellular β-xylosidase (Juturu & Wu, 2014). Structural features of xylan-rich compounds strongly affect their intestinal fermentation, probiotic effects (Damen *et al.*, 2011) and antioxidant properties (Snelders *et al.*, 2014).

In a pure culture study, XOS were utilized with a high specificity by *Bifidobacterium lactis* (Mäkeläinen *et al.*, 2010). *In vitro* fermentation test of XOS with bifidobacteria strains indicated that 77% of XOS were consumed by *B. adolescentis* after 24 h which was higher than the ones determined for *B. longum, B. infantis,* and *B. breve* (Gullón *et al.*, 2008). In the same study, xylotriose had the highest percentage of utilization by *B. adolescentis* (90%) followed by xylobiose (84%), xylotetraose (83%), and xylopentaose (71%). *B. adolescentis* produce three known enzymes that convert prebiotic XOS to xylose: RexA, a xylose-releasing exo-oligoxylanase, and two β-xylosidases, XylB and XylC, with different substrate specificities (Lagaert *et al.*, 2011). Other studies have showed the ability of *Lactobacillus brevis, L. fermentum* and *L. acidophilus* to grew and utilize XOS as carbon and energy sources (Moura *et al.*, 2007, Chapla *et al.*, 2012). An *in vitro* study showed that incubation of epithelial colorectal cells (Caco-2) with XOS significantly decreased the ability of *Listeria monocytogenes* strains to adhere to the cells (Ebersbach *et al.*, 2012). In this study, XOS reduced expression of the adhesion-relevant genes, inlA and lap, involved in the attachment of *L. monocytogenes* to intestinal cells.

Several studies in human and laboratory animals have proposed health related effects of XOS including growth of health-promoting bacteria, increase of colonic or cecal SCFA

production, improvements of immune functions, reduction in colonic protein fermentation, exerts of anti-oxidant and anti-inflammatory activity, and modulation of lipid and glucose metabolism (Broekaert *et al.*, 2011, Carvalho *et al.*, 2013, Hansen *et al.*, 2013). However, compared to MOS, much less attention has been paid to the potential prebiotic effects of XOS in chickens. In a study by Courtin *et al.* (2008), qPCR analysis revealed a bifidogenic effect of wheat-bran-derived arabino-XOS (AXOS) at a 0.25% dosage in the ceca of the chickens. Acetate was found to be the major SCFA produced as the end-product of xylo-oligosaccharides fermentation by lactic acid bacteria and bifidobacteria (Damen *et al.*, 2011, Madhukumar & Muralikrishna, 2012).

2.3.3 Alleviation of environmental stress by prebiotics

Environmental stress can change the activity and/or composition of the chicken GI microbiota, and lead to delayed growth and various infectious disease (Lan *et al.*, 2004). Because of the positive effects of prebiotics on the gut microbiota, it seems possible that prebiotics ameliorate stress-induced gut dysbiosis in chickens. However, only a few studies have attempted to evaluate the effectiveness of prebiotic supplementation in birds under stressful conditions (Ghareeb *et al.*, 2008, Houshmand *et al.*, 2012, Sohail *et al.*, 2012). Sohail *et al.* (2010) investigated the effects of 0.5% MOS, either alone or in combination with *Lactobacillus*-based probiotic, on stress biological markers in heat stressed broilers and reported a decrease in the serum cortisol and cholesterol concentrations, and an increase in the thyroxin level after 42 days of dietary supplementations. In another study, Sohail *et al.* (2012) treated heat stressed broilers with 0.5% MOS and found that treated chickens had higher body weight gain and lower feed conversion ratio compared with control group, however, no significant change was reported in term of ileal morphology. Song *et al.* (2013) observed an increased in jejunal villus height and villus height to crypt depth ratio, as well as a decrease in jejunal permeability in heat stressed chickens

supplemented with a plant cellulose based oligosaccharide. Hooge *et al.* (2003) indicated that MOS enhanced growth performance of broilers at relatively high stocking density. In contrast, supplementation with MOS was found to have no significant effect on performance, immunity, and stress indicators of broilers raised at two levels of stocking density, 10 birds/m^2 as the normal density and 16 birds/m^2 as the high density (Houshmand *et al.*, 2012).

2.4 Enteric pathogens in chickens

Enteric pathogens infect chickens through colonization of the GI tract and lead to poor growth performance and livability of a flock (Porter, 1998). They are considered as a major source of economic loss to the poultry industry (Williams, 1999, Steiner, 2010). Moreover, these pathogens are often carried asymptomatically in the GI tract and can be transmitted to humans via the food chain and cause foodborne diseases. *Salmonella* spp., *E. coli, C. perfringens* and *Campylobacter* spp. are common enteric pathogens in chickens, and are associated with most human foodborne illnesses. In Canada, it has been estimated that roughly 4 million cases of foodborne diseases occur annually (Thomas *et al.*, 2013). In particular, *Salmonella* is the most prevalent cause of human salmonellosis with about 5000 cases reported each year in Canada (Nesbitt *et al.*, 2012). The genus *Salmonella* currently contains two species, *S. enterica* and *S. bongorii* (Brenner *et al.*, 2000). *S. bongorii* subspecies are mostly associated with cold-blooded animals, whereas *S. enterica* predominantly infect humans and other warm-blooded animals (Fookes *et al.*, 2011). *S. enterica* species are further subdivided into more than 2,500 serovars, and include both typhoidal and non-typhoidal *Salmonella* strains (Fookes *et al.*, 2011). *S. enterica* serovar Enteritidis has been ranked in the top three non-typhoidal serovars, causing infections in humans (Nesbitt *et al.*, 2012). In Canada, the proportion of human salmonellosis caused by *S.* Enteritidis increased from 13% in 2003 to 38% in 2010 (Ogunremi *et al.*, 2014). The majority of

S. Enteritidis outbreaks are associated with consumption of contaminated poultry products. In addition, detected antibiotic resistance in *Salmonella* spp. isolated from poultry farms increases both human and animal health concerns (Diarrassouba *et al.*, 2007, Diarra *et al.*, 2014).

2.4.1 Intestinal pathogenesis of *Salmonella* Enteritidis

The majority of *S. enterica* serovars, notably *S.* Enteritidis and *S.* Typhimurium, are able to colonize the lower GI tract and induce a low-level systemic infection in chickens, particularly within the first days of their life (Beal *et al.*, 2005). *Salmonella* colonization may persist in the GI tract for several weeks without causing any clinical sign of disease. The pathogenesis process of *S. enterica* serovars mainly includes adhesion, colonization, invasion, and intracellular replication. Efficient adhesion to the epithelial layer is the first required step in colonization and persistence in the GI tract. *S. enterica* serovars predominantly colonize the cecum but their translocation to internal organs are often limited, especially in adult birds (Van Immerseel *et al.*, 2003, Beal *et al.*, 2005). *S. enterica* serovars have different adhesion systems belonging to various classes of fimbrial and non-fimbrial adhesions (Wagner & Hensel, 2011). In addition, many surface components such as flagella and lipopolysaccharide contribute in part to *Salmonella* attachment to the intestinal wall. FimH is a member of the type 1 fimbriae, and the most studied fimbria in *S. enterica* serovars. FimH is highly specific for mannose residues and binds to mannose-containing glycoprotein receptors on intestinal epithelial cells (Wagner & Hensel, 2011). Among epithelial cells, M cells are preferred sites for *Salmonella* invasion (Neutra *et al.*, 1996), although invasion of enterocytes also occurs. This is mainly because that M cells lack the rigid brush border and have reduced mucosal layer thickness at the apical surface (Neutra *et al.*, 1996).

Subsequent to successful adhesion, a specialized protein secretion system named “type III” injects pathogenic agents such as SipA into the host cell through a needle-like structure.

Intracellular replication of the pathogen then occurs in membrane-bound compartments called *Salmonella*-containing vacuoles (Salcedo & Holden, 2003). *S. enterica* can also pass through the epithelial layer by being engulfed by resident macrophages or dendritic cells (Mastroeni *et al.*, 2009). Dendritic cells open the tight junctions between adjacent epithelial cells, and sample bacteria residing in the mucosal surface (Rescigno *et al.*, 2001). If local immune response was unable to suppress the infection, the pathogen could be disseminated to internal organs through the blood circulation and cause systemic infections (Mastroeni *et al.*, 2009).

2.4.2 Chicken immune response to *Salmonella* infection

Intestinal epithelial cells not only provide a physical barrier against pathogen invasion but also exhibit several physiological and immunological responses to prevent infections. *Salmonella* colonization and invasion of the GI tact activate inflammatory responses characterized by the release of many cytokines and chemokines (Wigley, 2014). Previous studies have reported that infection with *S. enterica* serovars changed expression levels of cytokines such as interleukin (IL)-1β, IL6, IL10, IL12, IL17, IL18, IL22, IL23 and interferon gamma (IFNγ) mainly in the cecum and cecal tonsils (Berndt *et al.*, 2007, Haghighi *et al.*, 2008, Crhanova *et al.*, 2011). The cecal tonsils, located at the ileo-cecal junction, are the major gut-associated lymphoid tissue in the chicken GI tract. They are one of the important sites for T- and B-cells differentiation with essential roles in antibody production and cell-mediated immune functions (Bar-Shira *et al.*, 2003).

The released cytokines and other proinflammatory mediators trigger a cascade of events that lead to increased local blood flow and rapid recruitment of heterophils, macrophages and T- and B-lymphocytes to the infection site (Wigley, 2014). Heterophils are phagocytes, and have the same function as neutrophils in mammals. They possess a various types of toll-like receptors to recognize pathogens through conserved structural features on the surface of pathogens known as

pathogen-associated molecular patterns. Heterophils can also produce antimicrobial peptides (Evans *et al.*, 1994) and extracellular traps (Chuammitri *et al.*, 2009) to bind and kill pathogens. An increase in heterophils to the infection site has been shown to contribute to increased resistance against systemic *S.* Enteritidis infections in young chickens (Swaggerty *et al.*, 2005). Although the innate immune system is considered as the early line of host defense against pathogens, cell-mediated immunity has been shown to be much more important for clearance of *Salmonella* infection in poultry (Beal *et al.*, 2006, Penha Filho *et al.*, 2012). Among *S. enterica* serovars, *S.* Enteritidis is a serotype, which induces strong inflammatory cytokines production (Setta *et al.*, 2012). It has been shown that *S.* Enteritidis can induce inflammatory response as early as 1 day post-challenge (Van Immerseel *et al.*, 2002).

2.4.3 Prebiotics for *Salmonella* control in poultry

The control of *Salmonella* in poultry production has been a high priority for the poultry industry. In addition to regular hygienic and biosecurity measures, several preventive strategies have been proposed to reduce the incidence of *Salmonella* colonization at the farm level (Vandeplas *et al.*, 2010). Vaccination and feed additives such as organic acids, antibiotics, prebiotics and probiotics are the most widely used control methods that have been investigated in poultry production (Vandeplas *et al.*, 2010). A number of studies have suggested prebiotics as efficient and cost-effective feed additives to limit intestinal colonization by enteric pathogens such as *Salmonella*. However, very limited studies have been performed to understand the mechanism of action of prebiotics against enteric diseases. MOS are the most extensively studied oligosaccharides with respect to their activity against *Salmonella.* Spring *et al.* (2000) investigated the effects of 4000 ppm MOS in chickens that were orally challenge with *S.* Typhimurium or *S.* Dublin at 3 days of age. At 7 days after challenge, a significant decrease in cecal *Salmonella*

concentration was observed when MOS was part of diet (Spring *et al.*, 2000). Similar results were reported when chickens were challenged with *S.* Enteritidis (Fernandez *et al.*, 2002). In a study on broilers challenged with lipopolysaccharide derived from *Salmonella*, MOS (2 g/kg) resulted in a mild immune response that terminated the systemic inflammation earlier than sub-therapeutic virginiamycin (Baurhoo *et al.* 2012). Feed supplementation with a specific prebiotic mixture consisting of galactoglucomannan oligosaccharides and arabinoxylan did not limit intestinal colonization of *Salmonella* in broilers challenged with *S.* Typhimurium on day 10 post-hatch (Faber *et al.*, 2012). More recently, MOS increased CD4+ and CD8+ lymphocyte counts in the ileum and cecum, and reduced fecal shedding of Salmonella in chickens challenged with *S.* Enteritidis (Lourenço *et al.*, 2015). Other prebiotics have also been studied. Feed supplementation with a specific prebiotic mixture consisting of galactoglucomannan oligosaccharides and arabinoxylan did not limit intestinal colonization of *Salmonella* in broilers challenged with *S.* Typhimurium on day 10 post-hatch (Faber *et al.*, 2012). When FOS was fed to chickens at the 0.375% or 0.75% levels, little effects on *Salmonella* incidence was observed, however, when birds were stressed by feed and water withdrawal, *Salmonella* level was reduced about four folds in the cecum of 0.75% FOS treated birds (Bailey *et al.*, 1991). Reduced *Salmonella* colonization was also observed in low-dose (0.1%) feeding of FOS (Fukata *et al.*, 1999). In an *in vitro* study, Babu *et al.* (2012) investigated the influence of FOS-inulin on the ability of the chicken macrophage-like HD11 cell line to phagocytose and kill *Salmonella* Enteritidis. They found that prebiotic treated cells had significantly fewer viable intracellular *S.* Enteritidis than the untreated cells, and this effect was linked to reduced IL-1β- associated macrophage cell death. Eeckhaut *et al.* (2008) evaluated administration of two different doses (0.2% and 0.4%) and chain length (average DP of 3 and 9) of AXOS for 5 weeks on *Salmonella* colonization in chickens experimentally infected by

S. Enteritidis at 14 days post-hatch. In their study, AXOS significantly reduced cecal colonization and translocation of *S.* Enteritidis to the spleen at 3 and 7 d post-infection.

2.5 Summary

The chicken GI tract harbours a diverse population of bacteria, which provide important protective effects against pathogens, and carry out many metabolic functions including digestion of plant fibers that are otherwise indigestible by host enzymes. Over the past decades, much effort has gone into optimizing the gut microbiota of chickens using dietary interventions. Among them, use of antibiotics at sub-therapeutic levels has been the most popular and probably most effective strategy to enhance feed efficiency and to keep animals healthy. However, such a practice has been heavily criticized due to emergence of antibiotic resistance and its potential spread to human pathogens. Instead, there is an interest in using prebiotics such as MOS or XOS, as one of possible ways to enhance the host's natural defense through modulation of the gut microbiota. While effects of prebiotics on poultry production parameters have been well studied (Patterson & Burkholder, 2003, Barry et al., 2009, Gaggìa et al., 2010), much less has been investigated concerning their impact on microbial communities in the gut. In addition, little is known about the effect of prebiotics on birds under sub-optimal environmental conditions or during pathogen colonization. Furthermore, most previous studies have relied on *in vitro* observations or microbial culture methods that fail to provide accurate taxonomic composition and community structure. Recent advances in HTS technologies have provided a more in-depth insight into bacterial diversity, and allowed the study of microbiota–host interactions. In order to develop prebiotics as viable alternatives to AGPs, it important to understand the mechanism(s) by which prebiotics contribute to the health and growth of chickens.

2.6 References

Aachary AA & Prapulla SG (2011) Xylooligosaccharides (XOS) as an emerging prebiotic: microbial synthesis, utilization, structural characterization, bioactive properties, and applications. *Comprehensive Reviews in Food Science and Food Safety* **10**: 2-16.

Acar J, Casewell M, Freeman J, Friis C & Goossens H (2000) Avoparcin and virginiamycin as animal growth promoters: a plea for science in decision-making. *Clinical Microbiology and Infection* **6**: 477-482.

Agunos A, Ibuki M, Yokomizo F & Mine Y (2007) Effect of dietary beta 1-4 mannobiose in the prevention of Salmonella enteritidis infection in broilers. *British Poultry Science* **48**: 331-341.

Allen HK, Levine UY, Looft T, Bandrick M & Casey TA (2013) Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. *Trends in Microbiology* **21**: 114-119.

Andersson AF, Lindberg M, Jakobsson H, Backhed F, Nyrén P & Engstrand L (2008) Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PloS One* **3**: e2836-e2836.

Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T & Batto J-M (2011) Enterotypes of the human gut microbiome. *Nature* **473**: 174-180.

Asrore SMM, Sieo CC, Chong CW, Gan HM, Ho YW & Sunway B (2015) Deciphering chicken gut microbial dynamics based on high-throughput 16S rRNA metagenomics analyses. *Gut Pathogens* **7**: 4.

Awad W, Ghareeb K, Abdel-Raheem S & Böhm J (2009) Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. *Poultry Science* **88**: 49-56.

Babu US, Sommers K, Harrison LM & Balan KV (2012) Effects of fructooligosaccharide-inulin on Salmonella-killing and inflammatory gene expression in chicken macrophages. *Veterinary Immunology and Immunopathology* **149**: 92-96.

Bailey J, Blankenship L & Cox N (1991) Effect of fructooligosaccharide on Salmonella colonization of the chicken intestine. *Poultry Science* **70**: 2433-2438.

Bar-Shira E, Sklan D & Friedman A (2003) Establishment of immune competence in the avian GALT during the immediate post-hatch period. *Developmental & Comparative Immunology* **27**: 147-157.

Barman M, Unold D, Shifley K, Amir E, Hung K, Bos N & Salzman N (2008) Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. *Infection and Immunity* **76**: 907-915.

Barry KA, Vester BM & Fahey Jr GC (2009) Prebiotics in companion and livestock animal nutrition. *Prebiotics and Probiotics Science and Technology*. 353-463. Springer, New York, USA.

Baurhoo B, Ferket PR & Zhao X (2009) Effects of diets containing different concentrations of mannanoligosaccharide or antibiotics on growth performance, intestinal development, cecal and litter microbial populations, and carcass parameters of broilers. *Poultry Science* **88**: 2262-2272.

Baurhoo B, Letellier A, Zhao X & Ruiz-Feria CA (2007b) Cecal Populations of lactobacilli and bifidobacteria and Escherichia coli Populations after in vivo Escherichia coli challenge in birds fed diets with purified lignin or mannanoligosaccharides. *Poultry Science* **86**: 2509-2516.

Baurhoo B, Phillip L & Ruiz-Feria CA (2007a) Effects of purified lignin and mannan oligosaccharides on intestinal integrity and microbial populations in the ceca and litter of broiler chickens. *Poultry Science* **86**: 1070-1078.

Beal RK, Powers C, Davison TF, Barrow PA & Smith AL (2006) Clearance of enteric Salmonella enterica serovar Typhimurium in chickens is independent of B-cell function. *Infection and Immunity* **74**: 1442-1444.

Beal RK, Powers C, Wigley P, Barrow PA, Kaiser P & Smith AL (2005) A strong antigen-specific T-cell response is associated with age and genetically dependent resistance to avian enteric salmonellosis. *Infection and Immunity* **73**: 7509-7516.

Bearson S, Allen HK, Bearson BL, Looft T, Brunelle BW, Kich JD, Tuggle CK, Bayles DO, Alt D & Levine UY (2013) Profiling the gastrointestinal microbiota in response to *Salmonella*: Low versus high Salmonella shedding in the natural porcine host. *Infection, Genetics and Evolution* **16**: 330-340.

Benites V, Gilharry R, Gernat AG & Murillo JG (2008) Effect of dietary mannan oligosaccharide from Bio-Mos or SAF-mannan on live performance of broiler chickens. *Journal of Applied Poultry Research* **17**: 471-475.

Bercik P, Collins S & Verdu E (2012) Microbes and the gut-brain axis. *Neurogastroenterology & Motility* **24**: 405-413.

Bergeron CR, Prussing C, Boerlin P, Daignault D, Dutil L, Reid-Smith RJ, Zhanel GG & Manges AR (2012) Chicken as reservoir for extraintestinal pathogenic Escherichia coli in humans, Canada. *Emerging Infectious Diseases* **18**: 415-421.

Berndt A, Wilhelm A, Jugert C, Pieper J, Sachse K & Methner U (2007) Chicken cecum immune response to Salmonella enterica serovars of different levels of invasiveness. *Infection and Immunity* **75**: 5993-6007.

Biggs P, Parsons CM & Fahey GC (2007) The effects of several oligosaccharides on growth performance, nutrient digestibilities, and Cecal microbial Populations in young chicks. *Poultry Science* **86**: 2327-2336.

Bindels LB, Delzenne NM, Cani PD & Walter J (2015) Towards a more comprehensive concept for prebiotics. *Nature Reviews Gastroenterology & Hepatology* **12**: 303-310.

Bonnet C, Diarrassouba F, Brousseau R, Masson L, Topp E & Diarra MS (2009) Pathotype and antibiotic resistance gene distributions of Escherichia coli isolates from broiler chickens raised on antimicrobial-supplemented diets. *Applied and Environmental Microbiology* **75**: 6955-6962.

Brenner F, Villar R, Angulo F, Tauxe R & Swaminathan B (2000) Salmonella nomenclature. *Journal of Clinical Microbiology* **38**: 2465-2467.

Brisbin JT, Gong J & Sharif S (2008) Interactions between commensal bacteria and the gut-associated immune system of the chicken. *Animal Health Research Reviews* **9**: 101-110.

Broekaert WF, Courtin CM, Verbeke K, Van de Wiele T, Verstraete W & Delcour JA (2011) Prebiotic and other health-related effects of cereal-derived arabinoxylans, arabinoxylan-oligosaccharides, and xylooligosaccharides. *Critical Reviews in Food Science and Nutrition* **51**: 178-194.

Brzoska F, Buluchevskij S, Stecka K & Sliwinski B (2007) The effects of lactic acid bacteria and mannan oligosaccharide, with or without fumaric acid, on chicken performance, slaughter yield and digestive tract microflora. *Journal of Animal and Feed Sciences* **16**: 241-251.

Burkholder KM, Thompson KL, Einstein ME, Applegate TJ & Patterson JA (2008) Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to *Salmonella Enteritidis* colonization in broilers. *Poultry Science* **87**: 1734-1741.

Bustin S, Benes V, Nolan T & Pfaffl M (2005) Quantitative real-time RT-PCR–a perspective. *Journal of Molecular Endocrinology* **34**: 597-601.

Butaye P, Devriese LA & Haesebrouck F (2003) Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on gram-positive bacteria. *Clinical Microbiology Reviews* **16**: 175-188.

Byrne CM, Clyne M & Bourke B (2007) Campylobacter jejuni adhere to and invade chicken intestinal epithelial cells in vitro. *Microbiology* **153**: 561-569.

Callaway T, Dowd S, Wolcott R, Sun Y, McReynolds J, Edrington T, Byrd J, Anderson R, Krueger N & Nisbet D (2009) Evaluation of the bacterial diversity in cecal contents of laying hens fed various molting diets by using bacterial tag-encoded FLX amplicon pyrosequencing. *Poultry Science* **88**: 298-302.

Carvalho AFA, Neto PdO, Da Silva DF & Pastore GM (2013) Xylo-oligosaccharides from lignocellulosic materials: Chemical structure, health benefits and production by chemical and enzymatic hydrolysis. *Food Research International* **51**: 75-85.

Castanon J (2007) History of the use of antibiotic as growth promoters in European poultry feeds. *Poultry Science* **86**: 2466-2471.

Chapla D, Pandit P & Shah A (2012) Production of xylooligosaccharides from corncob xylan by fungal xylanase and their utilization by probiotics. *Bioresource technology* **115**: 215-221.

Cheng G, Hao H, Xie S, Wang X, Dai M, Huang L & Yuan Z (2014) Antibiotic alternatives: the substitution of antibiotics in animal husbandry?. *Frontiers in Microbiology* **5**: 1-15

Cheng HW, Singleton P & Muir W (2002) Social stress in laying hens: differential dopamine and corticosterone responses after intermingling different genetic strains of chickens. *Poultry Science* **81**: 1265-1272.

Chuammitri P, Ostojić J, Andreasen CB, Redmond SB, Lamont SJ & Palić D (2009) Chicken heterophil extracellular traps (HETs): novel defense mechanism of chicken heterophils. *Veterinary Immunology and Immunopathology* **129**: 126-131.

Corrigan A, Horgan K, Clipson N & Murphy R (2011) Effect of dietary supplementation with a Saccharomyces cerevisiae mannan oligosaccharide on the bacterial community structure of broiler cecal contents. *Applied and Environmental Microbiology* **77**: 6653-6662.

Corrigan A, de Leeuw M, Penaud-Frézet S, Dimova D & Murphy R (2015) Phylogenetic and functional alterations of bacterial community composition in the broiler caecum as a result of mannan oligosaccharide supplementation. *Applied and Environmental Microbiology* **81**: 3460-3470.

Courtin CM, Swennen K, Broekaert WF, Swennen Q, Buyse J, Decuypere E, Michiels CW, Ketelaere BD & Delcour JA (2008) Effects of dietary inclusion of xylooligosaccharides, arabinoxylooligoscaccharides and soluble arabinoxylan on the microbial composition of caecal contents of chickens. *Journal of the Science of Food and Agriculture* **88**: 2517-2522.

Courtin CM, Broekaert WF, Swennen K, Lescroart O, Onagbesan O, Buyse J, Decuypere E, Van de Wiele T, Marzorati M & Verstraete W (2008) Dietary inclusion of wheat bran arabinoxylooligosaccharides induces beneficial nutritional effects in chickens. *Cereal Chemistry* **85**: 607-613.

Cox LA & Popken DA (2004) Quantifying human health risks from virginiamycin used in chickens. *Risk Analysis* **24**: 271-288.

Cressman MD, Zhongtang Y, Nelson MC, Moeller SJ, Lilburn MS & Zerby HN (2010) Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. *Applied and Environmental Microbiology* **76**: 6572-6582.

Crhanova M, Hradecka H, Faldynova M, Matulova M, Havlickova H, Sisak F & Rychlik I (2011) Immune response of chicken gut to natural colonization by gut microflora and to Salmonella enterica serovar Enteritidis infection. *Infection and Immunity* **79**: 2755-2763.

Damen B, Verspreet J, Pollet A, Broekaert WF, Delcour JA & Courtin CM (2011) Prebiotic effects and intestinal fermentation of cereal arabinoxylans and arabinoxylan oligosaccharides in rats depend strongly on their structural properties and joint presence. *Molecular Nutrition & Food Research* **55**: 1862-1874.

Danzeisen JL, Kim HB, Isaacson RE, Tu ZJ & Johnson TJ (2011) Modulations of the chicken cecal microbiome and metagenome in response to anticoccidial and growth promoter treatment. *PLoS One* **6**: e27949.

Davies J & Davies D (2010) Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews* **74**: 417-433.

De Preter V, Geboes K, Verbrugghe K, De Vuyst L, Vanhoutte T, Huys G, Swings J, Pot B & Verbeke K (2004) The in vivo use of the stable isotope-labelled biomarkers lactose- N-15 ureide and H-2(4) tyrosine to assess the effects of pro- and prebiotics on the intestinal flora of healthy human volunteers. *British Journal of Nutrition* **92**: 439-446.

Deng W, Dong XF, Tong JM & Zhang Q (2012) The probiotic Bacillus licheniformis ameliorates heat stress-induced impairment of egg production, gut morphology, and intestinal mucosal immunity in laying hens. *Poultry Science* **91**: 575-582.

Deplancke B & Gaskins HR (2001) Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. *The American Journal of Clinical Nutrition* **73**: 1131-1141.

Dhawan S & Kaur J (2007) Microbial mannanases: an overview of production and applications. *Critical Reviews in Biotechnology* **27**: 197-216.

Diarra MS & Malouin F (2014) Antibiotics in Canadian poultry productions and anticipated alternatives. *Frontiers in Microbiology* **5**: 1-15

Diarra MS, Delaquis P, Rempel H, Bach S, Harlton C, Aslam M, Pritchard J & Topp E (2014) Antibiotic resistance and diversity of Salmonella enterica serovars associated with broiler chickens. *Journal of Food Protection.* **77**: 40-49.

Diarra MS, Silversides FG, Diarrassouba F, Pritchard J, Masson L, Brousseau R, Bonnet C, Delaquis P, Bach S & Skura BJ (2007) Impact of feed supplementation with antimicrobial agents on growth performance of broiler chickens, Clostridium perfringens and Enterococcus counts, and antibiotic resistance phenotypes and distribution of antimicrobial resistance determinants in Escherichia coli isolates. *Applied and Environmental Microbiology* **73**: 6566-6576.

Diarrassouba F, Diarra MS, Bach S, Delaquis P, Pritchard J, Topp E & Skura BJ (2007) Antibiotic resistance and virulence genes in commensal Escherichia coli and Salmonella isolates from commercial broiler chicken farms. *Journal of Food Protection.* **70**: 1316-1327.

Diaz-Sanchez S, Hanning I, Pendleton S & D'Souza D (2013) Next-generation sequencing: the future of molecular genetics in poultry production and food safety. *Poultry Science* **92**: 562-572.

Dibaji SM, Seidavi A, Asadpour L & da Silva FM (2014) Effect of a synbiotic on the intestinal microflora of chickens. *The Journal of Applied Poultry Research* **23**: 1-6.

Dibner JJ & Richards JD (2005) Antibiotic growth promoters in agriculture: History and mode of action. *Poultry Science* **84**: 634-643.

Dumonceaux TJ, Hill JE, Hemmingsen SM & Van Kessel AG (2006) Characterization of intestinal microbiota and response to dietary virginiamycin supplementation in the broiler chicken. *Applied and Environmental Microbiology* **72**: 2815-2823.

Dutil L, Irwin R, Finley R, Ng LK, Avery B, Boerlin P, Bourgault A-M, Cole L, Daignault D & Desruisseau A (2010) Ceftiofur resistance in *Salmonella enterica* serovar Heidelberg from chicken meat and humans, Canada. *Emerging Infectious Diseases* **16**: 48.

Ebersbach T, Andersen JB, Bergström A, Hutkins RW & Licht TR (2012) Xylo-oligosaccharides inhibit pathogen adhesion to enterocytes in vitro. *Research in Microbiology* **163**: 22-27.

Eeckhaut V, Van Immerseel F, Croubels S, De Baere S, Haesebrouck F, Ducatelle R, Louis P & Vandamme P (2011) Butyrate production in phylogenetically diverse Firmicutes isolated from the chicken caecum. *Microbial Biotechnology* **4**: 503-512.

Eeckhaut V, Van Immerseel F, Dewulf J, Pasmans F, Haesebrouck F, Ducatelle R, Courtin C, Delcour J & Broekaert W (2008) Arabinoxylooligosaccharides from wheat bran inhibit Salmonella colonization in broiler chickens. *Poultry Science* **87**: 2329-2334.

Engberg RM, Hedemann MS, Leser T & Jensen BB (2000) Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. *Poultry Science* **79**: 1311-1319.

Ercolini D (2004) PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods* **56**: 297-314.

Evans EW, Beach GG, Wunderlich J & Harmon BG (1994) Isolation of antimicrobial peptides from avian heterophils. *Journal of Leukocyte Biology* **56**: 661-665.

Faber TA, Dilger RN, Iakiviak M, Hopkins AC, Price NP & Fahey GC, Jr. (2012) Ingestion of a novel galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex affected growth performance and fermentative and immunological characteristics of broiler chicks challenged with Salmonella typhimurium. *Poultry Science* **91**: 2241-2254.

Feighner SD & Dashkevicz MP (1987) Subtherapeutic levels of antibiotics in poultry feeds and their effects on weight gain, feed efficiency, and bacterial cholyltaurine hydrolase activity. *Applied and Environmental Microbiology* **53**: 331-336.

Feng Y, Gong J, Yu H, Jin Y, Zhu J & Han Y (2010) Identification of changes in the composition of ileal bacterial microbiota of broiler chickens infected with Clostridium perfringens. *Veterinary Microbiology* **140**: 116-121.

Ferber D (2003) WHO advises kicking the livestock antibiotic habit. *Science* **301**: 1027-1027.

Fernandez F, Hinton M & Gils BV (2002) Dietary mannan-oligosaccharides and their effect on chicken caecal microflora in relation to Salmonella Enteritidis colonization. *Avian Pathology* **31**: 49-58.

Fookes M, Schroeder GN, Langridge GC, Blondel CJ, Mammina C, Connor TR, Seth-Smith H, Vernikos GS, Robinson KS & Sanders M (2011) Salmonella bongori provides insights into the evolution of the Salmonellae. *PLOS Pathogens* **7**: e1002191.

Forder REA, Howarth GS, Tivey DR & Hughes RJ (2007) Bacterial Modulation of Small Intestinal Goblet Cells and Mucin Composition During Early Posthatch Development of Poultry1. *Poultry Science* **86**: 2396-2403.

Forgetta V, Rempel H, Malouin F, Vaillancourt R, Topp E, Dewar K & Diarra M (2012) Pathogenic and multidrug-resistant Escherichia fergusonii from broiler chicken. *Poultry Science* **91**: 512-525.

Fukata T, Sasai K, Miyamoto T & Baba E (1999) Inhibitory effects of competitive exclusion and fructooligosaccharide, singly and in combination, on Salmonella colonization of chicks. *Journal of Food Protection* **62**: 229-233.

Gaggìa F, Mattarelli P & Biavati B (2010) Probiotics and prebiotics in animal feeding for safe food production. *International Journal of Food Microbiology* **141**: S15-S28.

Geboes KP, De Hertogh G, De Preter V, Luypaerts A, Bammens B, Evenepoel P, Ghoos Y, Geboes K, Rutgeerts P & Verbeke K (2006) The influence of inulin on the absorption of nitrogen and the production of metabolites of protein fermentation in the colon. *British Journal of Nutrition* **96**: 1078-1086.

Ghareeb K, AwacT W, Nitsclf S, Abdel-Raheem S & Böhm J (2008) Effects of transportation on stress and fear responses of growing broilers supplemented with prebiotic or probiotic. *International Journal of Poultry Science* **7**: 678-685.

Gomes A, Quinteiro-Filho W, Ribeiro A, Ferraz-de-Paula V, Pinheiro M, Baskeville E, Akamine A, Astolfi-Ferreira C, Ferreira A & Palermo-Neto J (2014) Overcrowding stress decreases macrophage activity and increases Salmonella Enteritidis invasion in broiler chickens. *Avian Pathology* **43**: 82-90.

Gong J, Yu H, Liu T, Gill J, Chambers J, Wheatcroft R & Sabour P (2008) Effects of zinc bacitracin, bird age and access to range on bacterial microbiota in the ileum and caeca of broiler chickens. *Journal of Applied Microbiology* **104**: 1372-1382.

Gong J, Si W, Forster RJ, Huang R, Yu H, Yin Y, Yang C & Han Y (2007) 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca. *FEMS Microbiology Ecology* **59**: 147-157.

Gow NA, van de Veerdonk FL, Brown AJ & Netea MG (2012) Candida albicans morphogenesis and host defence: discriminating invasion from colonization. *Nature Reviews Microbiology* **10**: 112-122.

Greisen K, Loeffelholz M, Purohit A, & Leong D (1994) PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. *Journal of clinical microbiology* **32**: 335-351.

Guardia S, Konsak B, Combes S, Levenez F, Cauquil L, Guillot J-F, Moreau-Vauzelle C, Lessire M, Juin H & Gabriel I (2011) Effects of stocking density on the growth performance and digestive microbiota of broiler chickens. *Poultry Science* **90**: 1878-1889.

Gullón P, Moura P, Esteves MaP, Girio FM, Domínguez H & Parajó JC (2008) Assessment on the fermentability of xylooligosaccharides from rice husks by probiotic bacteria. *Journal of Agricultural and Food Chemistry* **56**: 7482-7487.

Guo X, Xia X, Tang R, Zhou J, Zhao H & Wang K (2008) Development of a real-time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs. *Letters in Applied Microbiology* **47**: 367-373.

Haghighi HR, Abdul-Careem MF, Dara RA, Chambers JR & Sharif S (2008) Cytokine gene expression in chicken cecal tonsils following treatment with probiotics and Salmonella infection. *Veterinary Microbiology* **126**: 225-233.

Hansen CH, Frøkiær H, Christensen AG, Bergström A, Licht TR, Hansen AK & Metzdorff SB (2013) Dietary xylooligosaccharide downregulates IFN-γ and the low-grade inflammatory cytokine IL-1β systemically in mice. *The Journal of Nutrition* **143**: 533-540.

Hooge D, Sims M, Sefton A, Connolly A & Spring P (2003) Effect of dietary mannan oligosaccharide, with or without bacitracin or virginiamycin, on live performance of broiler chickens at relatively high stocking density on new litter. *The Journal of Applied Poultry Research* **12**: 461-467.

Houshmand M, Azhar K, Zulkifli I, Bejo MH & Kamyab A (2012) Effects of prebiotic, protein level, and stocking density on performance, immunity, and stress indicators of broilers. *Poultry Science* **91**: 393-401.

Huff G, Huff W, Farnell M, Rath N, de Los Santos FS & Donoghue A (2010) Bacterial clearance, heterophil function, and hematological parameters of transport-stressed turkey poults supplemented with dietary yeast extract. *Poultry Science* **89**: 447-456.

Hume M, Kubena L, Edrington T, Donskey C, Moore R, Ricke S & Nisbet D (2003) Poultry digestive microflora biodiversity as indicated by denaturing gradient gel electrophoresis. *Poultry Science* **82**: 1100-1107.

Humphrey T (2006) Are happy chickens safer chickens? Poultry welfare and disease susceptibility 1. *British poultry science* **47**: 379-391.

Ibuki M, Kovacs-Nolan J, Fukui K, Kanatani H & Mine Y (2010) Analysis of gut immune-modulating activity of β-1, 4-mannobiose using microarray and real-time reverse transcription polymerase chain reaction. *Poultry Science* **89**: 1894-1904.

Ibuki M, Kovacs-Nolan J, Fukui K, Kanatani H & Mine Y (2011) β 1-4 mannobiose enhances Salmonella-killing activity and activates innate immune responses in chicken macrophages. *Veterinary Immunology and Immunopathology* **139**: 289-295.

Jiang H, Gong L, Ma Y, He Y, Li D & Zhai H (2006) Effect of stachyose supplementation on growth performance, nutrient digestibility and caecal fermentation characteristics in broilers. *British Poultry Science* **47**: 516-522.

Jukes TH & Williams WL (1953) Nutritional effects of antibiotics. *Pharmacological Reviews* **5**: 381-420.

Juricova H, Videnska P, Lukac M, Faldynova M, Babak V, Havlickova H, Sisak F & Rychlik I (2013) Influence of Salmonella enterica serovar Enteritidis infection on the development of the cecum microbiota in newly hatched chicks. *Applied and Environmental Microbiology* **79**: 745-747.

Juturu V & Wu JC (2014) Microbial Exo-xylanases: A Mini Review. *Applied Biochemistry and Biotechnology* **174**:1-12.

Kaakoush NO, Sodhi N, Chenu JW, Cox JM, Riordan SM & Mitchell HM (2014) The interplay between Campylobacter and Helicobacter species and other gastrointestinal microbiota of commercial broiler chickens. *Gut Pathogens* **6**: 18-18.

Kasubuchi M, Hasegawa S, Hiramatsu T, Ichimura A & Kimura I (2015) Dietary Gut Microbial Metabolites, Short-chain Fatty Acids, and Host Metabolic Regulation. *Nutrients* **7**: 2839-2849.

Kim GB, Seo YM, Kim CH & Paik IK (2011) Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. *Poultry Science* **90**: 75-82.

Knarreborg A, Simon MA, Engberg RM, Jensen BB & Tannock GW (2002) Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. *Applied and Environmental Microbiology* **68**: 5918-5924.

Lagaert S, Pollet A, Delcour JA, Lavigne R, Courtin CM & Volckaert G (2011) Characterization of two β-xylosidases from Bifidobacterium adolescentis and their contribution to the hydrolysis of prebiotic xylooligosaccharides. *Applied Microbiology and Biotechnology* **92**: 1179-1185.

Lambert G (2009) Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. *Journal of Animal Science* **87**: 101-108.

Lan PTN, Sakamoto M & Benno Y (2004) Effects of two probiotic Lactobacillus strains on jejunal and cecal microbiota of broiler chicken under acute heat stress condition as revealed by molecular analysis of 16S rRNA genes. *Microbiology and Immunology* **48**: 917-929.

Lan Y, Williams B, Verstegen M, Patterson R & Tamminga S (2007) Soy oligosaccharides in vitro fermentation characteristics and its effect on caecal microorganisms of young broiler chickens. *Animal Feed Science and Technology* **133**: 286-297.

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML & Pace NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences* **82**: 6955-6959.

Lensing M, van der Klis J, Yoon I & Moore D (2012) Efficacy of Saccharomyces cerevisiae fermentation product on intestinal health and productivity of coccidian-challenged laying hens. *Poultry Science* **91**: 1590-1597.

Ley RE, Turnbaugh PJ, Klein S & Gordon JI (2006) Human gut microbes associated with obesity. *Nature* **444**: 1022-1023.

Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD & Knight R (2008) Evolution of mammals and their gut microbes. *Science* **320**: 1647-1651.

Lin J, Hunkapiller AA, Layton AC, Chang Y-J & Robbins KR (2013) Response of intestinal microbiota to antibiotic growth promoters in chickens. *Foodborne pathogens and disease* **10**: 331-337.

Lourenço M, Kuritza L, Hayashi R, Miglino L, Durau J, Pickler L & Santin E (2015) Effect of a mannanoligosaccharide-supplemented diet on intestinal mucosa T lymphocyte populations in chickens challenged with Salmonella Enteritidis. *The Journal of Applied Poultry Research* **24**: 15-22.

Lumpkins B, Batal A & Lee M (2008) The effect of gender on the bacterial community in the gastrointestinal tract of broilers. *Poultry Science* **87**: 964-967.

Lund M, Nordentoft S, Pedersen K & Madsen M (2004) Detection of Campylobacter spp. in chicken fecal samples by real-time PCR. *Journal of Clinical Microbiology* **42**: 5125-5132.

Madhukumar M & Muralikrishna G (2012) Fermentation of xylo-oligosaccharides obtained from wheat bran and Bengal gram husk by lactic acid bacteria and bifidobacteria. *Journal of Food Science and Technology* **49**: 745-752.

Mäkeläinen H, Saarinen M, Stowell J, Rautonen N & Ouwehand A (2010) Xylo-oligosaccharides and lactitol promote the growth of Bifidobacterium lactis and Lactobacillus species in pure cultures. *Beneficial Microbes* **1**: 139-148.

Manges AR, Smith SP, Lau BJ, Nuval CJ, Eisenberg JN, Dietrich PS & Riley LW (2007) Retail meat consumption and the acquisition of antimicrobial resistant Escherichia coli causing urinary tract infections: a case-control study. *Foodborne Pathogens and Disease* **4**: 419-431.

Manzella JP (2001) Quinupristin-dalfopristin: a new antibiotic for severe gram-positive infections. *American Family Physician* **64**: 1863-1866.

Mastroeni P, Grant A, Restif O & Maskell D (2009) A dynamic view of the spread and intracellular distribution of Salmonella enterica. *Nature Reviews Microbiology* **7**: 73-80.

McDonald LC, Rossiter S, Mackinson C, Wang YY, Johnson S, Sullivan M, Sokolow R, DeBess E, Gilbert L & Benson JA (2001) Quinupristin-dalfopristin–resistant Enterococcus faecium on chicken and in human stool specimens. *New England Journal of Medicine* **345**: 1155-1160.

Meddings JB & Swain M (2000) Environmental stress–induced gastrointestinal permeability is mediated by endogenous glucocorticoids in the rat. *Gastroenterology* **119**: 1019-1028.

Methner U, Rabsch W, Reissbrodt R & Williams PH (2008) Effect of norepinephrine on colonisation and systemic spread of Salmonella enterica in infected animals: role of catecholate siderophore precursors and degradation products. *International Journal of Medical Microbiology* **298**: 429-439.

Moellering R, Linden P, Reinhardt J, Blumberg E, Bompart F & Talbot G (1999) The efficacy and safety of quinupristin/dalfopristin for the treatment of infections caused by vancomycin-resistant Enterococcus faecium. *Journal of Antimicrobial Chemotherapy* **44**: 251-261.

Mookiah S, Sieo CC, Ramasamy K, Abdullah N & Ho YW (2014) Effects of dietary prebiotics, probiotic and synbiotics on performance, caecal bacterial populations and caecal fermentation concentrations of broiler chickens. *Journal of the Science of Food and Agriculture* **94**: 341-348.

Moura P, Barata R, Carvalheiro F, Gírio F, Loureiro-Dias MC & Esteves MP (2007) In vitro fermentation of xylo-oligosaccharides from corn cobs autohydrolysis by Bifidobacterium and Lactobacillus strains. *LWT-Food Science and Technology* **40**: 963-972.

Moura P, Cabanas S, Lourenço P, Gírio F, Loureiro-Dias MC & Esteves MP (2008) In vitro fermentation of selected xylo-oligosaccharides by piglet intestinal microbiota. *LWT-Food Science and Technology* **41**: 1952-1961.

Muyzer G, Dewaal EC & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16s ribosomal RNA. *Applied and Environmental Microbiology* **59**: 695-700.

Mwangi WN, Beal RK, Powers C, Wu X, Humphrey T, Watson M, Bailey M, Friedman A & Smith AL (2010) Regional and global changes in TCRαβ T cell repertoires in the gut are dependent upon the complexity of the enteric microflora. *Developmental & Comparative Immunology* **34**: 406-417.

Nesbitt A, Ravel A, Murray R, McCormick R, Savelli C, Finley R, Parmley J, Agunos A, Majowicz S & Gilmour M (2012) Integrated surveillance and potential sources of Salmonella Enteritidis in human cases in Canada from 2003 to 2009. *Epidemiology and Infection* **140**: 1757-1772.

Neutra MR, Frey A & Kraehenbuhl J-P (1996) Epithelial M cells: gateways for mucosal infection and immunization. *Cell* **86**: 345-348.

Niewold T (2007) The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. *Poultry Science* **86**: 605-609.

Nordentoft S, Mølbak L, Bjerrum L, De Vylder J, Van Immerseel F & Pedersen K (2011) The influence of the cage system and colonisation of Salmonella Enteritidis on the microbial gut flora of laying hens studied by T-RFLP and 454 pyrosequencing. *BMC Microbiology* **11**: 1-11

Oakley BB, Lillehoj HS, Kogut MH, Kim WK, Maurer JJ, Pedroso A, Lee MD, Collett SR, Johnson TJ & Cox NA (2014) The chicken gastrointestinal microbiome. *FEMS Microbiology Letters* **360**: 100-112.

Ochman H, Lawrence JG & Groisman EA (2000) Lateral gene transfer and the nature of bacterial innovation. *Nature* **405**: 299-304.

Ogunremi D, Devenish J, Amoako K, Kelly H, Dupras AA, Belanger S & Wang LR (2014) High resolution assembly and characterization of genomes of Canadian isolates of Salmonella Enteritidis. *BMC Genomics* **15**: 1-13

Oliver SP, Murinda SE & Jayarao BM (2011) Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: a comprehensive review. *Foodborne Pathogens and Disease* **8**: 337-355.

Patterson J & Burkholder K (2003) Application of prebiotics and probiotics in poultry production. *Poultry Science* **82**: 627-631.

Patterson JA & Burkholder KM (2003) Application of prebiotics and probiotics in poultry production. *Poultry Science* **82**: 627-631.

Pedroso AA, Menten JFM, Lambais MR, Racanicci AMC, Longo FA & Sorbara JOB (2006) Intestinal bacterial community and growth performance of chickens fed diets containing antibiotics. *Poultry Science* **85**: 747-752.

Peinado MJ, Echavarri A, Ruiz R, Suarez-Pereira E, Ortiz Mellet C, Garcia Fernandez JM & Rubio LA (2013) Effects of inulin and di-D-fructose dianhydride-enriched caramels on intestinal microbiota composition and performance of broiler chickens. *Animal* **7**: 1779-1788.

Penha Filho RAC, Moura BS, de Almeida AM, Montassier HJ, Barrow PA & Junior AB (2012) Humoral and cellular immune response generated by different vaccine programs before and after Salmonella Enteritidis challenge in chickens. *Vaccine* **30**: 7637-7643.

Perez V, Jacobs C, Barnes J, Jenkins M, Kuhlenschmidt M, Fahey G, Parsons C & Pettigrew J (2011) Effect of corn distillers dried grains with solubles and Eimeria acervulina infection on growth performance and the intestinal microbiota of young chicks. *Poultry Science* **90**: 958-964.

Perrin P, Pierre F, Patry Y, Champ M, Berreur M, Pradal G, Bornet F, Meflah K & Menanteau J (2001) Only fibres promoting a stable butyrate producing colonic ecosystem decrease the rate of aberrant crypt foci in rats. *Gut* **48**: 53-61.

Porter RE (1998) Bacterial enteritides of poultry. *Poultry Science* **77**: 1159-1165.

Pourabedin M & Zhao X (2015) Prebiotics and gut microbiota in chickens. *FEMS Microbiology Letters* **15**:fnv122.

Pourabedin M, Guan L & Zhao X (2015) Xylo-oligosaccharides and virginiamycin differentially modulate gut microbial composition in chickens. *Microbiome* **3**: 1-12.

Qu A, Brulc JM, Wilson MK, Law BF, Theoret JR, Joens LA, Konkel ME, Angly F, Dinsdale EA & Edwards RA (2008) Comparative metagenomics reveals host specific metavirulomes and horizontal gene transfer elements in the chicken cecum microbiome. *PLoS One* **3**: e2945.

Quinteiro-Filho W, Ribeiro A, Ferraz-de-Paula V, Pinheiro M, Sakai M, Sá L, Ferreira A & Palermo-Neto J (2010) Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens. *Poultry Science* **89**: 1905-1914.

Rakoff-Nahoum S, Coyne MJ & Comstock LE (2014) An ecological network of polysaccharide utilization among human intestinal symbionts. *Current Biology* **24**: 40-49.

Ram JL, Karim AS, Sendler ED & Kato I (2011) Strategy for microbiome analysis using 16S rRNA gene sequence analysis on the Illumina sequencing platform. *Systems Biology in Reproductive Medicine* **57**: 162-170.

Rastogi G & Sani RK (2011) Molecular techniques to assess microbial community structure, function, and dynamics in the environment. *Microbes and Microbial Technology*. 29-57. Springer, New York, USA.

Rehman H, Hellweg P, Taras D & Zentek J (2008) Effects of dietary inulin on the intestinal short chain fatty acids and microbial ecology in broiler chickens as revealed by denaturing gradient gel electrophoresis. *Poultry Science* **87**: 783-789.

Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl J-P & Ricciardi-Castagnoli P (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature Immunology* **2**: 361-367.

Roberfroid M, Gibson GR, Hoyles L, McCartney AL, Rastall R, Rowland I, Wolvers D, Watzl B, Szajewska H & Stahl B (2010) Prebiotic effects: metabolic and health benefits. *British Journal of Nutrition* **104**: 1-63.

Rostagno MH (2009) Can stress in farm animals increase food safety risk? *Foodborne Pathogens and Disease* **6**: 767-776.

Salcedo SP & Holden DW (2003) SseG, a virulence protein that targets Salmonella to the Golgi network. *The EMBO Journal* **22**: 5003-5014.

Sanger F, Nicklen S & Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences* **74**: 5463-5467.

Saulnier DM, Spinler JK, Gibson GR & Versalovic J (2009) Mechanisms of probiosis and prebiosis: considerations for enhanced functional foods. *Current Opinion in Biotechnology* **20**: 135-141.

Sekelja M, Rud I, Knutsen S, Denstadli V, Westereng B, Næs T & Rudi K (2012) Abrupt temporal fluctuations in the chicken fecal microbiota are explained by its gastrointestinal origin. *Applied and Environmental Microbiology* **78**: 2941-2948.

Sergeant MJ, Constantinidou C, Cogan TA, Bedford MR, Penn CW & Pallen MJ (2014) Extensive microbial and functional diversity within the chicken cecal microbiome. *PloS One* **9**: e91941.

Setta A, Barrow P, Kaiser P & Jones M (2012) Early immune dynamics following infection with Salmonella enterica serovars Enteritidis, Infantis, Pullorum and Gallinarum: cytokine and chemokine gene expression profile and cellular changes of chicken cecal tonsils. *Comparative Immunology, Microbiology and Infectious Diseases* **35**: 397-410.

Shanmugasundaram R & Selvaraj RK (2012) Effect of killed whole yeast cell prebiotic supplementation on broiler performance and intestinal immune cell parameters. *Poultry Science* **91**: 107-111.

Shanmugasundaram R, Sifri M & Selvaraj RK (2013) Effect of yeast cell product supplementation on broiler cecal microflora species and immune responses during an experimental coccidial infection. *Poultry Science* **92**: 1195-1201.

Shendure J & Ji H (2008) Next-generation DNA sequencing. *Nature Biotechnology* **26**: 1135-1145.

Singh P, Karimi A, Devendra K, Waldroup PW, Cho KK & Kwon YM (2013) Influence of penicillin on microbial diversity of the cecal microbiota in broiler chickens. *Poultry Science* **92**: 272-276.

Skraban J, Dzeroski S, Zenko B, Tusar L & Rupnik M (2013) Changes of poultry faecal microbiota associated with Clostridium difficile colonisation. *Veterinary Microbiology* **165**: 416-424.

Smith CJ & Osborn AM (2009) Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiology Ecology* **67**: 6-20.

Snelders J, Olaerts H, Dornez E, Van de Wiele T, Aura A-M, Vanhaecke L, Delcour JA & Courtin CM (2014) Structural features and feruloylation modulate the fermentability and evolution of antioxidant properties of arabinoxylanoligosaccharides during in vitro fermentation by human gut derived microbiota. *Journal of Functional Foods* **10**: 1-12.

Sohail M, Hume M, Byrd J, Nisbet D, Ijaz A, Sohail A, Shabbir M & Rehman H (2012) Effect of supplementation of prebiotic mannan-oligosaccharides and probiotic mixture on growth performance of broilers subjected to chronic heat stress. *Poultry Science* **91**: 2235-2240.

Sohail MU, Ijaz A, Yousaf MS, Ashraf K, Zaneb H, Aleem M & Rehman H (2010) Alleviation of cyclic heat stress in broilers by dietary supplementation of mannan-oligosaccharide and Lactobacillus-based probiotic: Dynamics of cortisol, thyroid hormones, cholesterol, C-reactive protein, and humoral immunity. *Poultry Science* **89**: 1934-1938.

Song J, Jiao L, Xiao K, Luan Z, Hu C, Shi B & Zhan X (2013) Cello-oligosaccharide ameliorates heat stress-induced impairment of intestinal microflora, morphology and barrier integrity in broilers. *Animal Feed Science and Technology* **185**: 175-181.

Spring P, Wenk C, Dawson K & Newman K (2000) The effects of dietary mannaoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of salmonella-challenged broiler chicks. *Poultry Science* **79**: 205-211.

Stanley D, Keyburn AL, Denman SE & Moore RJ (2012) Changes in the caecal microflora of chickens following *Clostridium perfringens* challenge to induce necrotic enteritis. *Veterinary microbiology* **159**: 155-162.

Stanley D, Geier MS, Hughes RJ, Denman SE & Moore RJ (2013a) Highly Variable Microbiota Development in the Chicken Gastrointestinal Tract. *Plos One* **8**.

Stanley D, Geier MS, Denman SE, Haring VR, Crowley TM, Hughes RJ & Moore RJ (2013b) Identification of chicken intestinal microbiota correlated with the efficiency of energy extraction from feed. *Veterinary Microbiology* **164**: 85-92.

Stanley D, Hughes RJ & Moore RJ (2014a) Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. *Applied Microbiology and Biotechnology* **98**: 4301-4310.

Stanley D, Wu S-B, Rodgers N, Swick RA & Moore RJ (2014b) Differential Responses of Cecal Microbiota to Fishmeal, *Eimeria* and *Clostridium perfringens* in a Necrotic Enteritis Challenge Model in Chickens. *PloS One* **9**: e104739.

Stanley D, Geier MS, Chen H, Hughes RJ & Moore RJ (2015) Comparison of fecal and cecal microbiotas reveals qualitative similarities but quantitative differences. *BMC Microbiology* **15**: 51.

Stecher B, Robbiani R, Walker AW, Westendorf AM, Barthel M, Kremer M, Chaffron S, Macpherson AJ, Buer J & Parkhill J (2007) Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota. *PLoS Biology* **5**: e244.

Steiner T (2010) *Phytogenics in animal nutrition: natural concepts to optimize gut health and performance*. Nottingham University Press, Nottingham, UK.

Suau A, Bonnet R, Sutren M, Godon J-J, Gibson GR, Collins MD & Doré J (1999) Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Applied and Environmental Microbiology* **65**: 4799-4807.

Sun Z, Wenting L, Ruikui Y, Jia L, Honghong L, Wei S, Zhongmie W, Jingpan L, Zhe S & Yuling Q (2013) Effect of a straw-derived xylooligosaccharide on broiler growth performance, endocrine metabolism, and immune response. Canadian Journal of Veterinary Research 77: 105109

Swaggerty CL, Ferro PJ, Pevzner IY & Kogut MH (2005) Heterophils are associated with resistance to systemic Salmonella enteritidis infections in genetically distinct chicken lines. *FEMS Immunology and Medical Microbiology* **43**: 149-154.

Swiatkiewicz S, Koreleski J & Arczewska-Wlosek A (2011) Effect of inulin and oligofructose on performance and bone characteristics of broiler chickens fed on diets with different concentrations of calcium and phosphorus. *British Poultry Science* **52**: 483-491.

Teirlynck E, Gussem M, Dewulf J, Haesebrouck F, Ducatelle R & Van Immerseel F (2011) Morphometric evaluation of "dysbacteriosis" in broilers. *Avian Pathology* **40**: 139-144.

Teitelbaum JE & Walker WA (2002) Nutritional impact of pre- and probiotics as protective gastrointestinal organisms. *Annual Review of Nutrition* **22**: 107-138.

Thibodeau A, Quessy S, Guévremont E, Houde A, Topp E, Diarra MS & Letellier A (2008) Antibiotic resistance in Escherichia coli and Enterococcus spp. isolates from commercial broiler chickens receiving growth-promoting doses of bacitracin or virginiamycin. *Canadian Journal of Veterinary Research* **72**: 129.

Thomas MK, Murray R, Flockhart L, Pintar K, Pollari F, Fazil A, Nesbitt A & Marshall B (2013) Estimates of the burden of foodborne illness in Canada for 30 specified pathogens and unspecified agents, circa 2006. *Foodborne Pathogens and Disease* **10**: 639-648.

Torok VA, Ophel-Keller K, Loo M & Hughes RJ (2008) Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism. *Applied and Environmental Microbiology* **74**: 783-791.

Torok VA, Hughes RJ, Ophel-Keller K, Ali M & MacAlpine R (2009) Influence of different litter materials on cecal microbiota colonization in broiler chickens. *Poultry Science* **88**: 2474-2481.

Torok VA, Hughes RJ, Mikkelsen LL, Perez-Maldonado R, Balding K, MacAlpine R, Percy NJ & Ophel-Keller K (2011a) Identification and characterization of potential performance-related gut microbiotas in broiler chickens across various feeding trials. *Applied and Environmental Microbiology* **77**: 5868-5878.

Torok VA, Allison GE, Percy NJ, Ophel-Keller K & Hughes RJ (2011b) Influence of antimicrobial feed additives on broiler commensal posthatch gut microbiota development and performance. *Applied and Environmental Microbiology* **77**: 3380-3390.

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER & Gordon JI (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**: 1027-1131.

Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA & Affourtit JP (2009) A core gut microbiome in obese and lean twins. *Nature* **457**: 480-484.

Van Craeyveld V, Swennen K, Dornez E*, et al.* (2008) Structurally Different Wheat-Derived Arabinoxylooligosaccharides Have Different Prebiotic and Fermentation Properties in Rats. *Journal of Nutrition* **138**: 2348-2355.

Van Immerseel F, Rood JI, Moore RJ & Titball RW (2009) Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. *Trends in Microbiology* **17**: 32-36.

Van Immerseel F, De Buck J, De Smet I, Mast J, Haesebrouck F & Ducatelle R (2002) Dynamics of immune cell infiltration in the caecal lamina propria of chickens after neonatal infection with a Salmonella enteritidis strain. *Developmental & Comparative Immunology* **26**: 355-364.

Van Immerseel F, De Buck J, Pasmans F, Velge P, Bottreau E, Fievez V, Haesebrouck F & Ducatelle R (2003) Invasion of Salmonella enteritidis in avian intestinal epithelial cells in vitro is influenced by short-chain fatty acids. *International Journal of Food Microbiology* **85**: 237-248.

Vandeplas S, Dubois Dauphin R, Beckers Y, Thonart P & Thewis A (2010) Salmonella in chicken: current and developing strategies to reduce contamination at farm level. *Journal of Food Protection* **73**: 774-785.

Vazquez M, Alonso J, Domınguez H & Parajo J (2000) Xylooligosaccharides: manufacture and applications. *Trends in Food Science & Technology* **11**: 387-393.

Verstegen MW & Williams BA (2002) Alternatives to the use of antibiotics as growth promoters for monogastric animals. *Animal Biotechnology* **13**: 113-127.

Videnska P, Faldynova M, Juricova H, Babak V, Sisak F, Havlickova H & Rychlik I (2013) Chicken faecal microbiota and disturbances induced by single or repeated therapy with tetracycline and streptomycin. *BMC Veterinary Research* **9**:1-9

Videnska P, Sedlar K, Lukac M, Faldynova M, Gerzova L, Cejkova D, Sisak F & Rychlik I (2014) Succession and replacement of bacterial populations in the caecum of egg laying hens over their whole life. *PLoS One* **9**: e115142.

Videnska P, Rahman MM, Faldynova M, Babak V, Matulova ME, Prukner-Radovcic E, Krizek I, Smole-Mozina S, Kovac J & Szmolka A (2014) Characterization of egg laying hen and froiler fecal microbiota in poultry farms in croatia, czech republic, hungary and slovenia. *PloS One* **9**: e110076.

Wagner C & Hensel M (2011) Adhesive mechanisms of Salmonella enterica. *Bacterial Adhesion,* 17-34. Springer, New York, USA

Waite DW & Taylor MW (2014) Characterizing the avian gut microbiota: membership, driving influences, and potential function. *Frontiers in Microbiology* **5**.223

Wigley P (2014) Salmonella enterica in the chicken: how it has helped our understanding of immunology in a non-biomedical model species. *Frontiers in Immunology* **5**.482

Williams R (1999) A compartmentalised model for the estimation of the cost of coccidiosis to the world's chicken production industry. *International Journal for Parasitology* **29**: 1209-1229.

Wise MG & Siragusa GR (2005) Quantitative detection of Clostridium perfringens in the broiler fowl gastrointestinal tract by real-time PCR. *Applied and Environmental Microbiology* **71**: 3911-3916.

Wise MG & Siragusa GR (2007) Quantitative analysis of the intestinal bacterial community in one- to three-week-old commercially reared broiler chickens fed conventional or antibiotic-free vegetable-based diets. *Journal of Applied Microbiology* **102**: 1138-1149.

Withanage G, Wigley P, Kaiser P, Mastroeni P, Brooks H, Powers C, Beal R, Barrow P, Maskell D & McConnell I (2005) Cytokine and chemokine responses associated with clearance of a primary Salmonella enterica serovar Typhimurium infection in the chicken and in protective immunity to rechallenge. *Infection and Immunity* **73**: 5173-5182.

Woese C, Stackebrandt E, Macke T & Fox G (1985) A phylogenetic definition of the major eubacterial taxa. *Systematic and Applied Microbiology* **6**: 143-151.

Woese CR (1987) Bacterial evolution. *Microbiological Reviews* **51**: 221.

Wong JMW, de Souza R, Kendall CWC, Emam A & Jenkins DJA (2006) Colonic health: Fermentation and short chain fatty acids. *Journal of Clinical Gastroenterology* **40**: 235-243.

Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, Keilbaugh SA, Bewtra M, Knights D, Walters WA & Knight R (2011) Linking long-term dietary patterns with gut microbial enterotypes. *Science* **334**: 105-108.

Wu S-B, Stanley D, Rodgers N, Swick RA & Moore RJ (2014) Two necrotic enteritis predisposing factors, dietary fishmeal and Eimeria infection, induce large changes in the caecal microbiota of broiler chickens. *Veterinary Microbiology* **169**: 188-197.

Xiao R, Power RF, Mallonee D, Routt K, Spangler L, Pescatore AJ, Cantor AH, Ao T, Pierce JL & Dawson KA (2012) Effects of yeast cell wall-derived mannan-oligosaccharides on jejunal gene expression in young broiler chickens. *Poultry Science* **91**: 1660-1669.

Yamada Y, Sugamura K, Kondo K, Yanagimoto M & Okada H (1987) The structure of inducing factors for virginiamycin production in Streptomyces virginiae. *The Journal of Antibiotics* **40**: 496-504.

Yang Y, Iji PA, Kocher A, Mikkelsen LL & Choct M (2008) Effects of mannanoligosaccharide and fructooligosaccharide on the response of broilers to pathogenic Escherichia coli challenge. *British Poultry Science* **49**: 550-559.

Yang Y, Iji P, Kocher A, Thomson E, Mikkelsen L & Choct M (2008) Effects of mannanoligosaccharide in broiler chicken diets on growth performance, energy utilisation, nutrient digestibility and intestinal microflora. *British Poultry Science* **49**: 186-194.

Yin Y, Lei F, Zhu L, Li S, Wu Z, Zhang R, Gao GF, Zhu B & Wang X (2010) Exposure of different bacterial inocula to newborn chicken affects gut microbiota development and ileum gene expression. *ISME Journal* **4**: 367-376.

Yitbarek A, Echeverry H, Brady J, Hernandez-Doria J, Camelo-Jaimes G, Sharif S, Guenter W, House JD & Rodriguez-Lecompte JC (2012) Innate immune response to yeast-derived carbohydrates in broiler chickens fed organic diets and challenged with Clostridium perfringens. *Poultry Science* **91**: 1105-1112.

Yonath A (2005) Antibiotics targeting ribosomes: resistance, selectivity, synergism, and cellular regulation. *Annual Review of Biochemistry* **74**: 649-679.

Young KT, Davis LM & DiRita VJ (2007) Campylobacter jejuni: molecular biology and pathogenesis. *Nature Reviews Microbiology* **5**: 665-679.

Yu Z & Morrison M (2004) Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology* **70**: 4800-4806.

Zhang W, Li D, Lu W & Yi G (2003) Effects of isomalto-oligosaccharides on broiler performance and intestinal microflora. *Poultry Science* **82**: 657-663.

Zhao L, Wang G, Siegel P, He C, Wang H, Zhao W, Zhai Z, Tian F, Zhao J & Zhang H (2013) Quantitative genetic background of the host influences gut microbiomes in chickens. *Scientific Reports* **3**.1163

Zhou H, Gong J, Brisbin J, Yu H, Sanei B, Sabour P & Sharif S (2007) Appropriate chicken sample size for identifying the composition of broiler intestinal microbiota affected by dietary antibiotics, using the polymerase chain reaction-denaturing gradient gel electrophoresis technique. *Poultry Science* **86**: 2541-2549.

Zoetendal EG, Collier CT, Koike S, Mackie RI & Gaskins HR (2004) Molecular ecological analysis of the gastrointestinal microbiota: a review. *Journal of Nutrition* **134**: 465-472.

Chapter 3. Effects of Mannan-oligosaccharides and Virginiamycin on the Cecal Microbial Community and Intestinal Morphology of Chickens Raised under Sub-optimal Conditions

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3.1 Abstract

There is an increasing movement against use of antibiotic growth promoters (AGP) in animal feed. Prebiotic supplementation is a potential alternative to enhance the host's natural defense through modulation of gut microbiota. In the present study, the effect of mannan oligosaccharide (MOS) and virginiamycin (VIRG) on cecal microbial ecology and intestinal morphology of broiler chickens raised under sub-optimal conditions was evaluated. MOS and VIRG induced different bacterial community structures as revealed by denaturing gradient gel electrophoresis (DGGE) of 16S rDNA. The antibiotic treatment reduced cecal microbial diversity while the community equitability increased. A higher bacterial diversity was observed in the cecum of MOS supplemented birds. Quantitative PCR results indicated that MOS changed the cecal microbiota in favor of the *Firmicutes* but not the *Bacteroidetes* population. No difference was observed in total bacterial counts among treatments. MOS promoted the growth of *Lactobacillus* sp. and bifidobacteria in the cecum and increased villus height and goblet cell numbers in the ileum and jejunum. These results provide a deeper insight into the microbial ecological changes after supplementation of MOS prebiotic in poultry diets.

3.2 Introduction

Antibiotics have been used in poultry feeds at sub therapeutic levels for more than 50 years to improve growth performance, feed efficiency and reduce intestinal pathogens. Due to the potential linkage between the use of AGP in animals and the prevalence of antibiotic resistance pathogens in humans, serious attempts have been made to reduce the massive use of sub therapeutic antibiotics in animal production (Silbergeld et al., 2008). The European Union took the lead and banned all AGP in animal feed on January 1, 2006. Although a similar action has not yet been initiated in North America, the US Food and Drug Administration asked farmers to voluntarily phase out AGP from livestock production in April 2012 (FDA, 2012).

In the absence of AGP, prebiotics have been suggested as one of possible ways to enhance the host's natural defense through modulation of bacterial population in the gut. Prebiotics are nondigestible ingredients that promote health benefits by increasing the growth or activity of selected gut microbiota (Gibson *et al.*, 2004). Gut microbiota can have both favorable and unfavorable effects on the intestinal health of the host and its susceptibility to diseases. In chickens, the gastrointestinal tract becomes rapidly colonized by bacteria, with the maximum bacterial densities being reached within the first 5 days after hatching (Apajalahti *et al.*, 2004). The exact mechanism by which AGP promote animal growth is not clearly understood, but the finding that AGP have no growth-promoting effects in germ-free animals (Dibner & Richards, 2005) suggests that AGP mainly act on the intestinal microbiome. Altering the microbial ecology towards the healthy status by increasing the number of beneficial species has been shown to improve host immune responses and well-being (Sekirov et al., 2010).

MOS is one of promising prebiotics as poultry feed additive. It is extracted from the cell wall of the yeast, Saccharomyces cerevisiae, and has been reported to have several health benefits

by competing with pathogens for attachment sites (Baurhoo *et al.*, 2012), increasing the population of commensal bacteria and improving intestinal morphology (Baurhoo *et al.*, 2009).

Little is known about the effect of MOS on birds under sub optimal environmental conditions, since most of the previous studies in chickens have been carried out under experimentally controlled and clean environmental conditions. Broiler chickens growing in industrial production systems are often exposed to a wide range of stressors such as overcrowding, changes in temperature and poor feeding that may alter the balanced population of gut microbiota (Sohail *et al.*, 2011). High stocking density and cold temperatures are common environmental factors that affect broiler growth and gut physiology on Canadian broiler farms. In addition, prebiotic effects on the gastrointestinal tract and immune system could be more obvious at times of stress (Baurhoo *et al.*, 2007).

The intestinal microbiota comprises a diverse collection of microbial species in broiler chickens. For many years, researchers have investigated microbial alternation using culturing techniques. While plating can quantify some specific bacteria, there are several drawbacks to its use. To address the problems associated with plating, molecular methods have been developed to analyze the intestinal microbiota more in-depth. Among them, polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) is a very useful method to understand the makeup of gut microbiota and to evaluate the diversity and richness of the bacterial community. Thus, the main objective of this study was to use PCR-DGGE and species specific quantitative PCR (qPCR) in combination with classical culture-based bacterial detection to compare the effects of virginiamycin (VIRG) and MOS on cecal microbiota of chickens grown up under sub-optimal conditions. In addition, intestinal morphological parameters were studied following dietary treatments.

3.3 Material and Methods

3.3.1 Birds, Diet and Experimental Design

A total of 1344 male broiler chickens, each 1 day old and of the same breed line (Cobb 500) were obtained from a local commercial hatchery (Couvoir Simetin, Mirabel, Québec, Canada). Birds were randomly assigned to 1 of 4 dietary treatments (6 pen replicates; 56 broilers/pen), and grown over a 35-d experimental period. Dietary treatments included: 1) a typical standard diet (CTL); 2) MOS (diet 1 + 1g/kg MOS); 3) VIRG (diet 1 + 16.5 mg/kg virginiamycin); 4) MAV (diet 1 + 1g/kg MOS + 16.5 mg/kg virginiamycin). All diets (Belisle Solution and Nutrition Inc, QC, Canada) composed of corn, soy bean meal, soybean oil, guar gum (5g/kg), amino acids supplements, vitamin and mineral premix, and formulated to meet NRC nutrient requirements (NRC, 1994). Feed and water were provided *ad libitum*. A two-phase feeding program was used with a starter diet (23% protein and 3029 kcal/kg metabolisable energy) from day 1 to day 17, followed by a grower diet (20% protein and 3154 kcal/kg ME) from day 17 to day 35. The MOS was supplied as AgriMOS (Lallemand Inc., Montreal, Canada).

To induce sub-optimal growing conditions, broilers were raised at a high stocking density of 16 birds/m^2 (Houshmand *et al.*, 2012) and mild cold temperature of 20°C starting at day 10 of age, i.e. 4°C lower than the optimal temperature. In addition, 0.5% guar gum was added into all diets to increase viscosity of intestinal digesta in order to facilitate growth of pathogenic bacteria (Silbergeld et al., 2008). The lighting program was 20 h light and 4 h darkness throughout the study. Body weight, feed intake and feed conversion rate (FCR) were recorded weekly for each pen. The Animal Care Committee of McGill University approved all experimental conditions and animal care protocol.

3.3.2 Bacterial Culture Analyses and Intestinal Morphology

At day 16 and 26, one bird per pen (6 per treatment) were selected randomly and euthanized to collect intestinal samples for histological and microbiological assays. For each sample, about 1 g of cecal content was immediately frozen in liquid nitrogen and stored at -80°C for isolation of genomic DNA. One gram of fresh cecal content was homogenized in 9 ml buffer peptone water and serially 10 fold diluted in 0.85% sterile saline solution. Diluted contents were plated in duplicate and the average values of colony-forming units (CFU) were used for the statistical analysis. *Lactobacillus* counts were determined using MRS agar (BD, Mississauga, ON, Canada) after 48 h anaerobic incubation at 37°C. Bifidobacteria were detected on a selective medium containing Wilkins-Chalgren agar (Oxoid, Nepean, ON, Canada), glacial acetic acid and mupirocin (Oxoid), according to a method previously described (Rada *et al.*, 1999). Plates were incubated at 37°C for 3 d under anaerobic condition. *E coli* colonies were grown on the RAPID E. coli 2 selective medium (Bio-Rad, Mississauga, ON, Canada) and incubated at 37°C for 1 d under aerobic condition. Colonies were counted after the incubation period and values are presented as CFU per gram of cecal content.

Approximately 2 cm of jejunum (proximal to Meckel's diverticulum) and ileum (proximal to cecal tonsils) were collected from 1 bird per replicate (6 per treatment) on day 16 and 26. Tissue samples were fixed in 10% buffered formalin for at least 48 hours, rinsed in phosphate buffer, dehydrated through a series of graded ethanol and finally embedded in paraffin wax. Trimmed sections (5 μm) were deparaffinised in Histoclear (Electron Microscopy Science, Hatfield, PA, USA) followed by staining with hematoxylin and eosin. A leica microscope integrated with leica imaging software (Leica Application Suite, Leica, New Jersey, USA) was used to measure villus

height and count goblet cell numbers. Villus length was measured from villus tip to the villus-crypt junction. A mean value of 10 measurements for each sample was used for statistical analysis.

3.3.3 DNA extraction

Total genomic DNA was extracted from the cecal digesta by using an UltraClean fecal DNA extraction kit (MoBio Labs, Carlsbad, CA, USA). Briefly, 250 mg of cecal content was added to the 2 ml bead beating tube containing beads and lysis solutions. Tubes were vortexed for 10 minutes at maximum speed, and samples were transferred onto silica spin filter units. Bound DNA was washed and eluted from the spin filters according to the manufacture's protocol. DNA concentration and purity were determined by measuring the absorbance at A260 and A280 using an ND-2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

3.3.4 DGGE Analyses of 16S rDNA

DNA samples (n = 6 per treatment) extracted from cecal contents were used as templates. The V3 region of the 16S rDNA gene was amplified using universal primers 341F (5′-CCTACGGGAGGCAGCAG-3′) with a GC clamp at the 5' end, and 534R (5′-ATTACCGCGGCTGCTGG -3′) (Muyzer *et al.*, 1993). The amplicons were visualized using 1.5% agarose gel electrophoresis, and reaction products were purified into a final volume of 30 µl using the QIAquick PCR purification kit (Qiagen). The D-Code DGGE system (Bio-rad) was used to perform DGGE of 16S rDNA genes. Normalized concentrations of the purified PCR amplicons were pooled pairwise to obtain 3 replicates for each treatment. Equal amounts of samples were loaded on to a linear gradient polyacrylamid gel ranging with a denaturant mixture ranging from 30% at the top of the gel to 70% at the bottom (100% denaturant mixture corresponds to 7 M urea and 40% deionized formamide). The gel was run in 1X Tris-acetate-EDTA buffer at 60°C at a constant voltage of 100V for 16 h. Gels were stained in ethidium bromide solution for

30 min, rinsed with Milli-Q water and photographed under UV light using Red AlphaImager (Proteinsimple, Santa Clara, CA, USA) with Alphaview software (Version 3.3).

3.3.5 Real-time PCR Analysis

Real-time PCR was carried out using the Brilliant III Ultra-Fast SYBR Green Master Mix (Agilent Technologies, Mississauga, ON, Canada) and a Stratagene Mx3005P qPCR system. 16S rDNA specific primers (Table 3.1) were used to quantify total bacteria, *E. coli* species, *Lactobacillus* spp and bifidobacteria as well as *Firmicutes* and *Bacteroidetes* phylum. The 16S rDNA gene of *E. coli* (DH5-alpha), *Lactobacillus salivarius* (ATCC 11741), *Bifidobacterium longum* (ATCC 55814) and *Bacteroides thetaiotaomicron* (ATCC 29741) were amplified and gel purified to construct standard curves with a 10-fold dilution series. To calculate the DNA copy number of each standard solution, the concentration (ng/µl) of the template DNA was converted to copy numbers per µl using the following formula: number of copies = (DNA concentration * 6.022×10^{23}) / (amplification size * 10^9 * 650). The thermal profile included an initial DNA denaturation for 3 min at 95°C, followed by 40 cycles of 20s at 95°C and 20 s at 60°C. To analyze the melting curve, the temperature was increased 0.5°C every 20 s from 55°C to 95°C as an additional cycle. All the real time reactions were performed in triplicate in a 96 well plate.

Table 3.1. Primers used to quantify 16S rDNA in real-time qPCR reactions

Target	Primer	Sequence (5′-3′)	Reference
All bacteria	UniF UniR	ACTCCTACGGGAGGCAGCAGT ATTACCGCGGCTGCTGGC	(Barman *et al.*, 2008)
Escherichia coli	EcoliF EcoliR	GTTAATACCTTTGCTCATTGA ACCAGGGTATCTAATCCTGTT	(Malinen *et al.*, 2003)
Lactobacillus spp.	LacF LacR	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	(Rinttilä *et al.*, 2004)
Bifidobacterium spp.	BifidoF BifidoR	GATTCTGGCTCAGGATGAACGC CTGATAGGACGCGACCCCAT	(Gueimonde *et al.*, 2004)
Firmicutes	FirmF FirmR	GGAGYATGTGGTTTAATTCGAAGCA AGCTGACGACAACCATGCAC	(Guo *et al.*, 2008)
Bacteroidetes	BacF BacR	AGCTGACGACAACCATGCAG GGARCATGTGGTTTAATTCGATGAT	(Guo *et al.*, 2008)

3.3.6 Data Analyses

Effects of MOS (0 g or 1 g/kg) and VIRG (0 mg or 16.5 mg/kg) were evaluated in a 2 × 2 factorial design. Data were analyzed by ANOVA using the MIXIED procedure of SAS (2003). For growth parameters, a pen was considered as the experimental unit whereas a nested model design was used for microbiological and histological parameters with pens nested within treatments and birds as the sub-samples. Significant differences among treatment means were determined by Scheffe's Multiple Comparison test and the difference was declared significant at $P<0.05$. Principal component analyses (PCA) and DGGE banding patterns were processed using the BioNumerics software package (Version 6.6, Applied Maths, Austin, TX). An unweighted pairwise grouping method with mathematical averages (unweighted pair group method with arithmetic averages) dendrogram was generated based on similarity matrix created using dice

similarity coefficients. Diversity indices of cecal microbial population was evaluated by: (i) the Shannon diversity index, $H = -\sum p_i \ln p_i$, and (ii) the evenness, $e = H/\ln S$, where p_i represents the intensity of the i-th band relative to intensity of all bands, and **S** is the total number of bands.

3.4 Results

3.4.1 DGGE and diversity indices

To evaluate bacterial diversity and community structure, DGGE analyses of bacterial 16S rDNA were performed for cecal samples. The DGGE banding pattern was slightly affected by treatments on day 16 with a similarity index of between 62.3% and 93.3% (Figure 3.1A). On day 26, a different bacterial pattern was observed among treatments with a similarity index ranged from 47.4% to 87.4%, with the mean value of 68% (Figure 3.1B). In general, the similarity index within each treatment was higher on day 16 than on day 26. As shown in Figure 1b, CTL, MOS and VIRG diets formed three separate clusters. Two of 3 replicates of MAV clustered together and 1 replicate clustered with MOS (Figure 3.1B). Within-group dice similarity coefficients of MOS, CTL and VIRG were 77.8%, 68.6% and 65.1% respectively on day 26 (Figure 3.1B). At the same day, DGGE banding profile of cecal microbiota of chickens in MOS group showed 57.9% and 50.3% similarity with those in the CTL and VIRG groups, respectively (Figure 3.1B). Diversity indices did not significantly change by any treatments on day 16; however, on day 26, the Shannon's diversity index (H') was greater in the MOS group and lower in VIRG compared to that of the CTL group, indicating a reduced bacterial diversity in antibiotic-treated birds (Table 3.2). Cecal microbial evenness declined in MOS-fed chickens compared to that for chickens in the CTL or VIRG groups on day 26, suggesting that the bacterial species were not equally distributed and the microbial community was dominated by some specific species in the MOS group (Table 3.2).

According to the PCA ordination, on day 16, the cecal microbial community of all chickens formed a close cluster (Figure 3.2). In contrast, on day 26, the cecal communities of the MOS group were most dissimilar from other treatments. The second principal component (PC2), which explained 10.1% of the total variation, separated the bacterial community of VIRG from that of the other treatment samples.

Table 3.2. Diversity indices calculated from the denaturing gradient gel electrophoresis banding patterns.

Time	Diet*	Shannon's index (H')	Evenness (e)
Day 16	CTL	2.45 ±0.05	0.89 ±0.02
	VIRG	2.49 ±0.04	0.91 ±0.03
	MOS	3.45 ±0.04	0.87 ±0.02
	MAV	2.47 ±0.05	0.90 ±0.02
Day 26	CTL	2.87 ±0.17 [b]	0.96 ±0.03 [a]
	VIRG	2.71 ±0.09 [d]	0.94 ±0.03 [a]
	MOS	3.02 ±0.11 [a]	0.88 ±0.02 [b]
	MAV	2.80 ±0.11 [c]	0.81 ±0.03 [c]

Note: Means followed by different letters are significantly different at $P < 0.05$.

*CTL, control diet without mannan oligosaccharide or virginiamycin; VIRG, control diet supplemented with 16.5 mg virginiamycin/kg; MOS, control diet supplemented with 1 g mannan oligosaccharide/kg; MAV, control diet supplemented with 1 g mannan oligosaccharide/kg and 16.5 mg virginiamycin/kg.

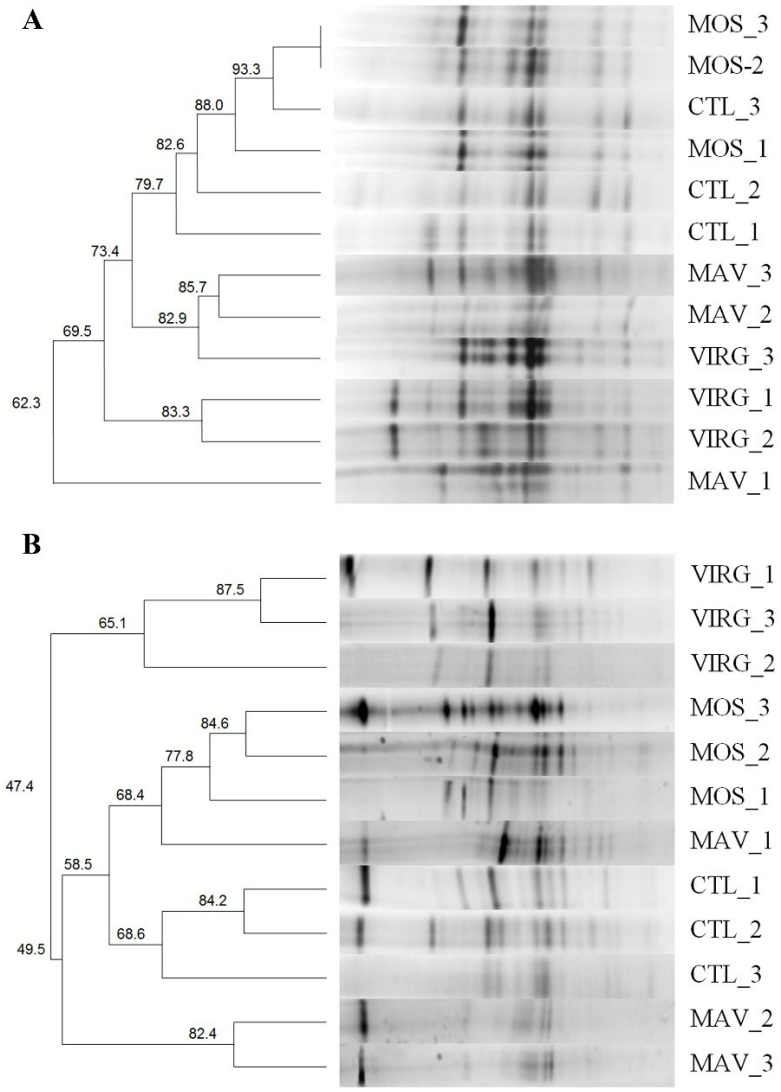

Figure 3.1. Denaturing gradient gel electrophoresis (DGGE) profiles

The dendrogram illustrating the bacterial diversity of chicken's cecal microbiota, collected on (A) day 16 and (B) day 26. DNA was extracted from 250 mg of cecal content collected from 1 bird per pen. Equal concentrations of 6 DGGE–PCR amplicons were pooled pairwise to obtain 3 replicates for each treatment. Dendrogram, based on the Dice similarity coefficient, was generated from DGGE profiles. CTL= control diet; VIRG= control diet + 16.5 mg virginiamycin/kg; MOS= control diet + 1 g mannan oligosaccharides/kg; and MAV= control diet + 16.5 mg virginiamycin/kg + 1 g mannan oligosaccharides/kg.; MOS, control diet+1g mannan oligosaccharide/kg; and MAV, control diet + 16.5 mg virginiamycin/kg+1gmannan oligosaccharide/kg.

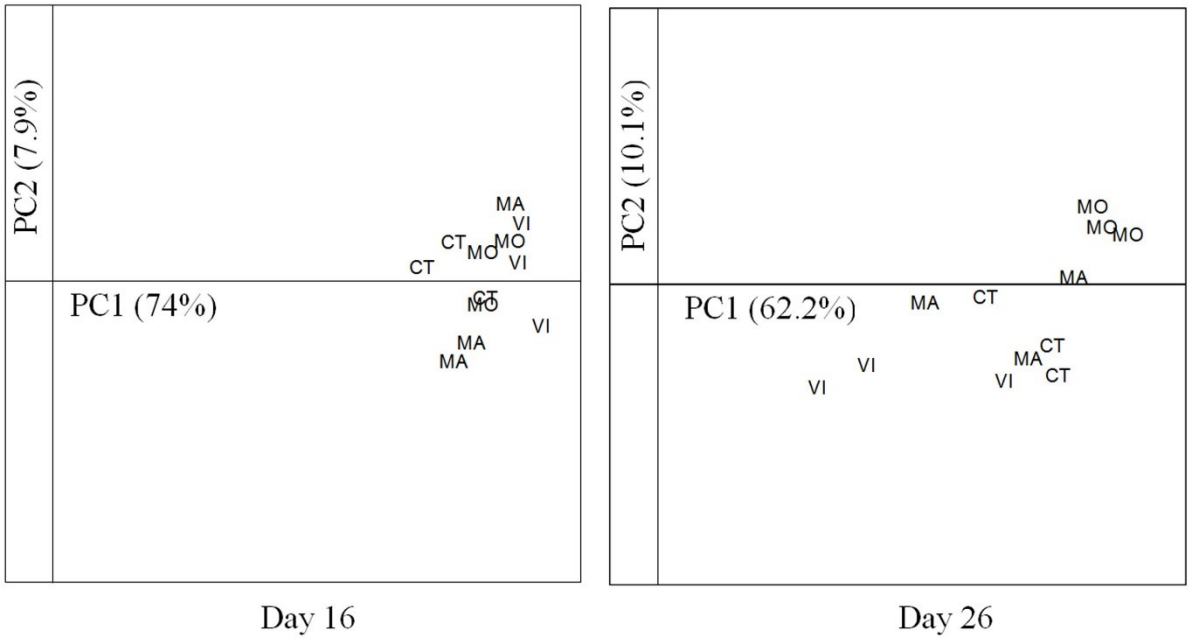

Figure 3.2. Principal component analysis.
Two first components represented 81.9% of total variation on day 16 and 72.3% on day 26. CT, control diet (a typical standard diet); VI, control diet + 16.5 mg virginiamycin/kg; MO, control diet+1g mannan oligosaccharide/kg; MA, control diet + 16.5 mg virginiamycin/kg+1g mannan oligosaccharide/kg/kg; MOS, control diet+1g mannan oligosaccharide/kg; and MAV, control diet + 16.5 mg virginiamycin/kg+1gmannan oligosaccharide/kg.

3.4.2 Bacterial Enumeration

Fresh cecal samples were plated on selective media to measure the population of three commensal bacteria, *Lactobacillus*, bifidobacteria and *E. coli*. There was a decrease ($P < 0.05$) in cecal *Lactobacilli* populations in broilers fed VIRG diet in comparison with those fed CTL or MOS diet (Figure 3.3A). The mean count of bifidobacteria in the MOS group was significantly higher than other treatments at the two time points of sampling (Figure 3.3B). No significant differences were noted for bifidobacteria populations of the VIRG and MAV groups. In comparison with the control group, VIRG reduced bifidobacteria counts on both day 16 and day

26, while MAV only decreased bifidobacteria counts on day 16. No significant difference was observed for the *E. coli* populations in cecal samples of chickens fed different dietary treatments (Figure 3.3C).

To further confirm the culture results, qPCR analysis was performed and results were presented in Figure 3.4. The relative gene copy numbers of 16S rDNA specific for the *E. coli* species were unaffected by dietary treatments (Figure 3.4A). The relative abundance of *lactobacillus* species had the highest median value in MOS group on day 16 and 26 (0.34 and 0.40, respectively), followed by CTL (0.31 and 0.37), MAV (0.19 and 0.18) and VIRG group (0.15 and 0.18) (Figure 3.4B). Relative bifidobacteria population was less than 0.03 in all the groups, with the highest copy number in the MOS group (0.027) on day 26 compared with CTL (0.015), VIRG (0.014) and MAV (0.013) (Figure 3.4C). In contrast to our cultural results, there was no difference in bifidobacteria on day 16. To further assess the cecal microbiota of chickens, two main bacterial phyla, *Firmicutes* and *Bacteroidetes*, were quantified by qPCR. No significant changes in the *Firmicutes*/*Bacteroidetes* populations were noted on day 16 (Figure 3.5). *Firmicutes* increased ($P<0.05$) in the MOS group compared to VIRG on day 26, whereas no significant differences were observed in the *Bacteroidetes* population (Figure 3.5).

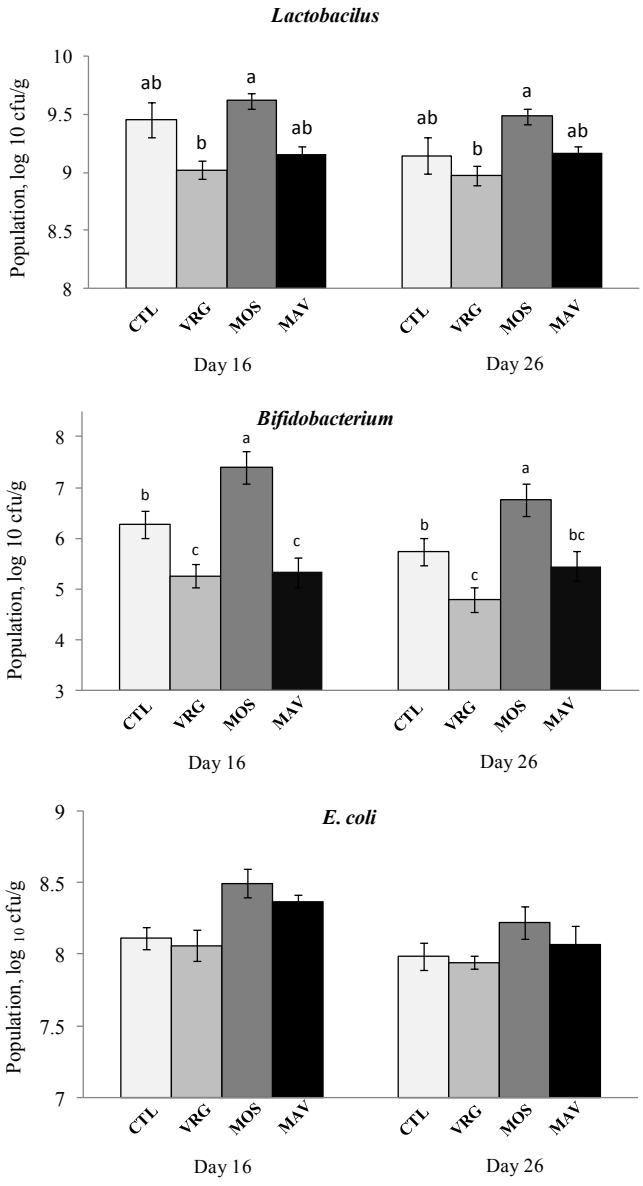

Figure 3.3. Effects of dietary treatments on the population of *Lactobacillus*, *Bifidobacterium*, and *Escherichia coli*

Samples were collected from 1 bird per pen (n = 6/treatment) and plated in duplicate. Mean values (±SE) are plotted. CTL, control diet (a typical standard diet); VIRG, control diet + 16.5 mg virginiamycin/kg; MOS, control diet+1g mannan oligosaccharide/kg; MAV, control diet + 16.5 mg virginiamycin/kg+1g mannan oligosaccharide/kg. The bars with different letters indicate statistical differences among treatments (Scheffé's t test, $P < 0.05$).

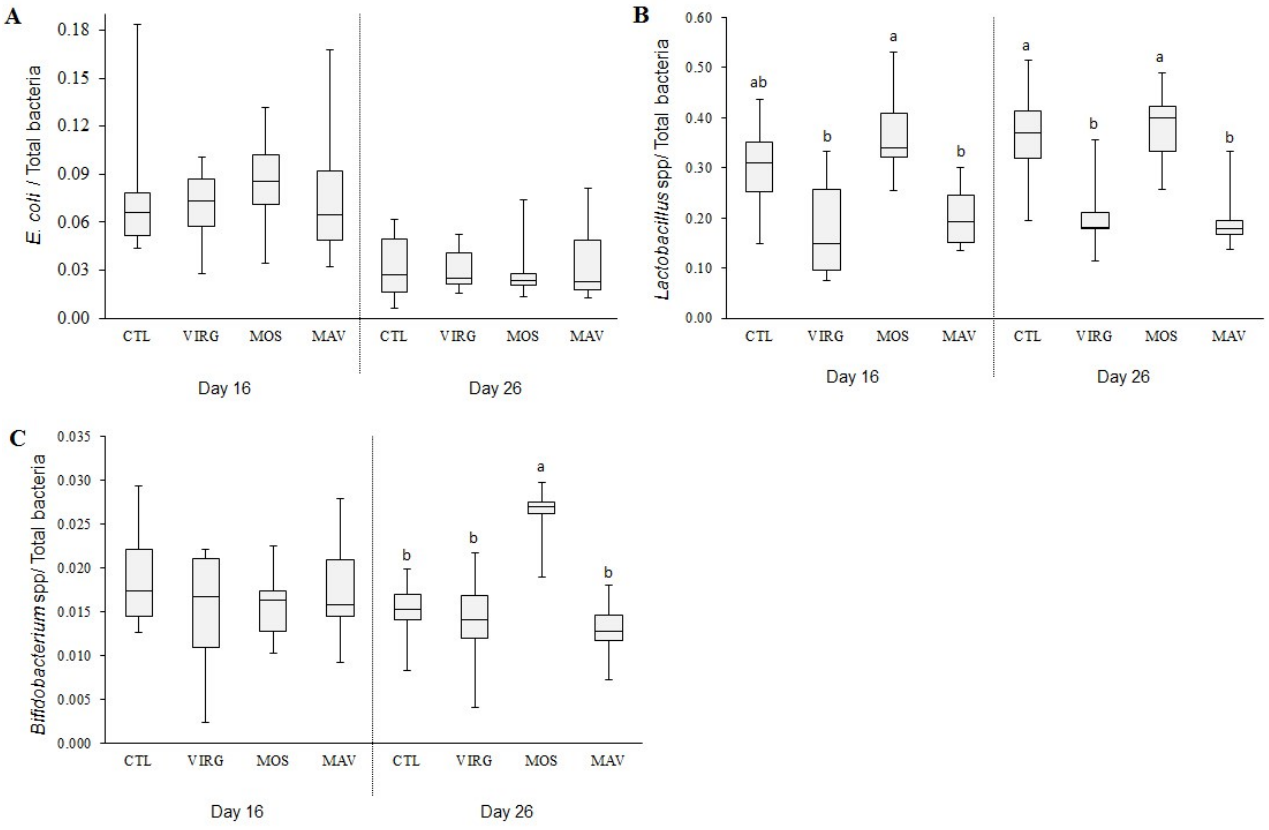

Figure 3.4. Real-time quantification of bacteria

The relative abundance of **(A)** *Escherichia coli*, **(B)** *Lactobacillus spp*., and **(C)** *Bifidobacterium* spp. in 1 g of cecum content from chickens fed different treatments (n = 6/treatment). The box extends from the 25th percentile to 75th percentile, with a line at the median (50th percentile); the whiskers extending above and below the box show the highest and lowest values, respectively, for the targeted genomic DNA. CTL, control diet (a typical standard diet); VIRG, control diet + 16.5 mg virginiamycin/kg; MOS, control diet+1g mannan oligosaccharide/kg; MAV, control diet + 16.5 mg virginiamycin/kg+1g mannan oligosaccharide/kg. Different letters indicate statistical differences among treatments (Scheffé's t test, $P < 0.05$).

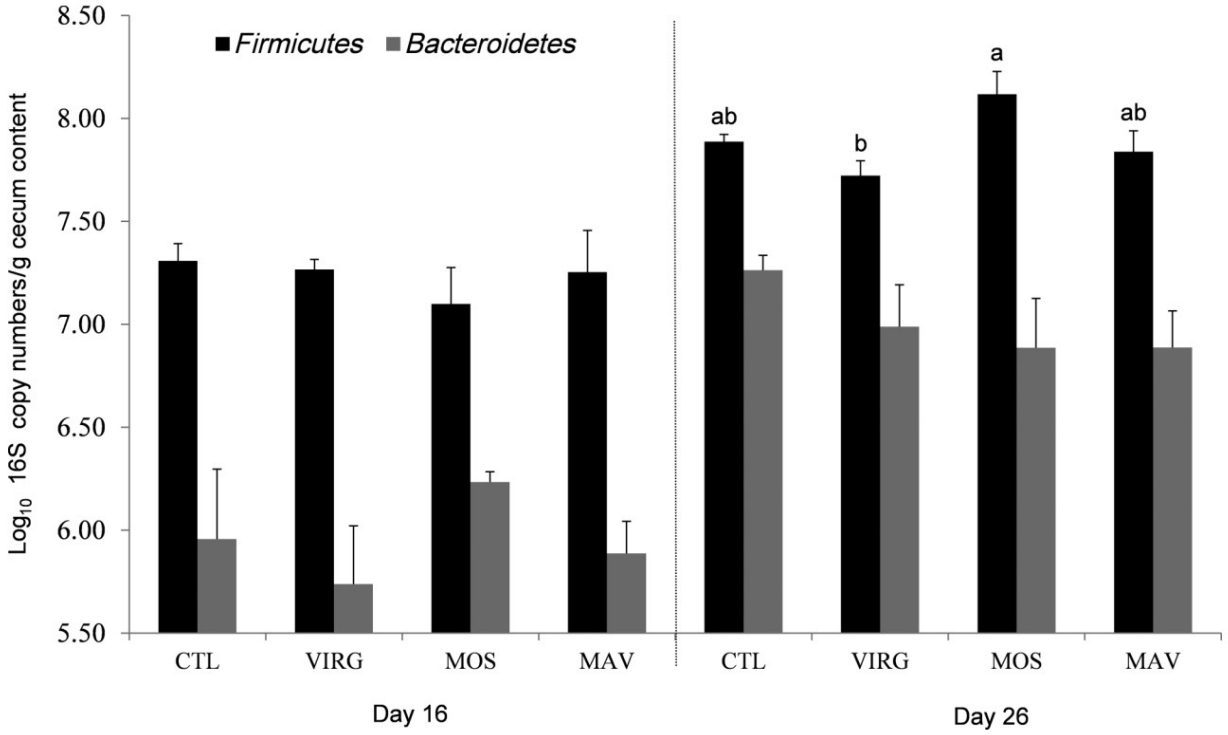

Figure 3.5. Abundance of phyla *Firmicutes* and *Bacteroidetes* in the cecal microbiota of chickens. Absolute quantification was performed using phylum-specific primers and data were expressed as $\log_{10}$ 16S copy numbers per 1 g of cecum content. CTL, control diet (a typical standard diet); VIRG, control diet + 16.5 mg virginiamycin/kg; MOS, control diet+1g mannan oligosaccharide/kg; MAV, control diet + 16.5 mg virginiamycin/kg+1g mannan oligosaccharide/kg. Bars with different letters indicate statistical differences (Scheffé's t test, $P < 0.05$).

3.4.3 Intestinal Morphology

To determine whether microbial changes affect intestinal morphological parameters, jejunum and ileum segments were examined for villus length and goblet cell numbers in each villus (Figure 3.6A). No statistical differences were observed among dietary treatments at day 16 (Figure 6B). MOS supplement increased ($P<0.05$) the jejunum and ileum villus height compared with those of CTL or antibiotic-treated chickens at day 26. Birds given the diet containing MOS alone

or in combination with antibiotic (MAV) had higher (P<0.01) numbers of goblet cells in the villi of the jejunum and ileum compared with those from the CTL or VIRG groups (Figure 3.6C).

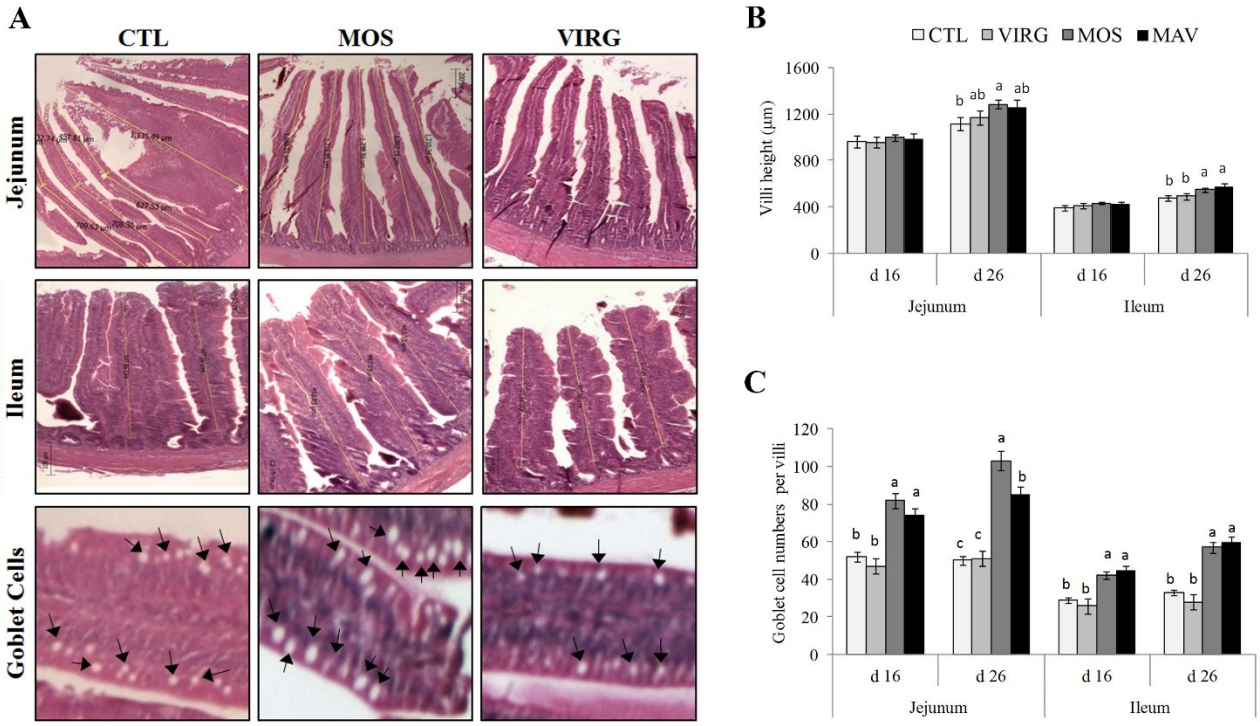

Figure 3.6. Effect of treatments on morphological parameters in jejunum and ileum of chickens.

Tissue samples were taken from 1 bird per pen (n = 6 per treatment), and 10 measurements were performed in each sample. Part **A** shows Hematoxylin and eosin stained jejunum and ileum of chickens. Arrows show goblet cells in villus. Part **B** shows villus length (m) in jejunum and ileum. Bars with different letters indicate statistical differences (Scheffé's t test, P < 0.05). Part **C** shows Goblet cell numbers per villus. Bars with different letters indicate statistical differences (Scheffé's t test, P < 0.05). CTL, control diet (a typical standard diet); VIRG, control diet + 16.5 mg virginiamycin/kg; MOS, control diet+1g mannan oligosaccharide/kg; MAV, control diet + 16.5 mg virginiamycin/kg+1g mannan oligosaccharide/kg.

3.4.4 Growth performance

The effect of feeding experimental diets on body weight (BW), feed intake (FI) feed conversion ratio (FCR) and mortality are shown in Table 3. For FI, FCR and mortality rate, no significant changes were seen among dietary treatments at any point of the experiment. In the starter phase (0 – 17 d), the main effect of VIRG was not significant on BW ($P>0.05$) but chickens fed diets supplemented with MOS showed a decrease in BW ($P<0.05$) in comparison with those with no MOS supplementation. On d 35, no significant effect of MOS on BW was noted, but an increase was evident in chickens receiving VIRG compared to those fed antibiotic-free diet ($P<0.01$). An interaction between MOS and VIRG supplementation (MAV) was only observed on d 21. The mortality rate ranged from 5.8 to 6.5%, and the treatments did not affect it during the 35-days experimental period.

Table 3.3. Effect of dietary treatments on body mass (BM), feed intake (FI), feed conversion ratio (FCR), and mortality of broiler chickens

		BM (g)			FI (g)			FCR			Mortality (%)
		Day			Day			Day			Day
	Diet*	7	21	35	0-7	0-21	0-35	0-7	0-21	0-35	0-35
Main effect											
Prebiotic	None	152	887[a]	2079	96	1240	3486	0.88	1.47	1.73	6.0
	MOS	153	855[b]	2040	87	1228	3501	0.85	1.52	1.76	6.5
	SEM#	1.11	7.83	18.1	5.0	39.7	48.7	0.03	0.02	0.02	0.2
Antibiotic	None	154	864	2008[b]	92	1229	3432	0.87	1.50	1.75	6.3
	VIRG	151	878	2110[a]	91	1239	3554	0.86	1.49	1.73	6.2
	SEM	1.1	7.8	18.1	5.0	39.7	48.7	0.03	0.02	0.02	0.2
Interaction effect											
Prebiotic× Antibiotic	CTL	155	900[a]	2040	104	1281	3482	0.92	1.50	1.75	6.3
	VIRG	149	875[a]	2117	88	1199	3491	0.86	1.45	1.70	5.8
	MOS	153	829[b]	1976	83	1176	3384	0.83	1.50	1.75	6.3
	MAV	153	880[a]	2103	94	1280	3618	0.87	1.53	1.76	6.6
	SEM	1.6	11.0	25.6	7.0	56.2	68.9	0.05	0.04	0.02	0.3
P											
Prebiotic		0.62	0.008	0.14	0.22	0.83	0.83	0.44	0.19	0.24	0.14
Antibiotic		0.15	0.24	0.007	0.85	0.85	0.09	0.80	0.87	0.44	0.80
Prebiotic×Antibiotic		0.06	0.002	0.34	0.06	0.11	0.11	0.30	0.12	0.19	0.15

Note: Means followed by different letters are significantly different at $P < 0.05$.

* CTL, control diet without mannan oligosaccharide or virginiamycin; VIRG, control diet supplemented with 16.5 mg virginiamycin/kg; MOS, control diet supplemented with 1 g mannan oligosaccharide/kg; MAV, control diet supplemented with 1 g mannan oligosaccharide/kg and 16.5 mg virginiamycin/kg.

SEM, standard error of the mean

3.5 Discussion

To our knowledge, this is the first study that evaluated effects of MOS prebiotic and VIRG antibiotic on gut microbiota and intestinal morphological parameters of chickens reared under sub-optimal environmental and dietary conditions. It has been suggested that responses of animals to the prebiotics could be different under stress or pathogen challenge conditions.

Cecal microbial diversity was studied by DGGE analyses of V3 region of bacterial 16S rDNA. Compared to the other hyper variable regions of 16S rDNA gene, V3 region alone was found to produce most informative DGGE profiles of gastrointestinal microbiota (Yu & Morrison, 2004). In the current study, the similarity index for each treatment was higher on day 16 than on day 26. This is in agreement with the observation of Weielen *et al*. (2002) that the dominant bacterial community became more complex when broiler chickens grew older. In addition, MOS and VIRG clearly induced a different clustering on day 26 as revealed by the dendrogram based on Dice similarity coefficients. The Dice's coefficient is a statistical index for measuring the similarity of two samples. It is used for analyzing the DGGE image based on presence/absence of bands in each lane of the gel. Based on the Shannon-Wiener index and evenness score, MOS supplementation resulted in a higher bacterial diversity, while the antibiotic treatment reduced cecal microbial diversity and increased the community equitability. Similarly, Collier *et al*. (2003) indicated that AGPs reduced total bacteria and inter-individual variation in porcine ileal microbiota.

The MOS treatment increased the number of *Firmicutes* but had no effect on the *Bacteroidetes* population. *Firmicutes* and *Bacteroidetes* are the two most abundant bacterial phyla in the cecum of broilers (Threlfall *et al.*, 2000) and the importance of these two phyla in host metabolism has been highlighted. An increased ratio of *Firmicutes*/*Bacteroidetes* has been shown

to be associated with obesity in human and mice; and this is due to the increased energy harvesting capacity of bacterial species in the *Firmicutes* phylum (Turnbaugh *et al.*, 2006, Turnbaugh *et al.*, 2008). The bacterial culture results as well as the data obtained from the qPCR showed that MOS favorably increased the numbers of bacteria from two major families, *Lactobacillus* and *Bifidobacterium*. This shift in the gut microbiota is possibly linked with intestinal health and overall well-being (Rastall *et al.*, 2005). Although the mechanisms through which beneficial bacteria promote health remain unclear, several experiments have confirmed their positive effects on the host immune response and reduced risk of gastrointestinal disease. Recognition of commensal bacteria by toll like receptors and dendritic cells leads to differentiation of regulatory T cells (Bron *et al.*, 2011). In a study performed by Sohail *et al*. (2010), *Lactobacillus* species improved humoral immunity of chickens exposed to heat stress. Acetate produced by bifidobacteria can protect intestinal epithelial cells against enteropathogenic infection (Fukuda *et al.*, 2011). Khailova *et al*. (2009) observed that in cold stressed rats, bifidobacteria might maintain intestinal integrity by stimulating mucin production and modulating tight junction proteins. Although qPCR results generally agreed with those obtained by the culture method, a difference was found in relative population of bifidobacteria, particularly on day 16. Discrepancy between results may be explained by the lack of colony-forming ability of some *Bifidobacterium* species or the bias selection by the culture medium. In contrast, quantification of bifidobacteria using qPCR generates more sensitive and reliable results (Matsuki *et al.*, 2004).

Neither MOS nor VIRG affected the population of *E. coli* in the cecal content of chickens as revealed by quantitative analyses of 16S rDNA as well as culturing on selective medium. Similar results were reported for healthy chickens (Yang *et al.*, 2007), turkeys (Sims *et al.*, 2004), pigs (White *et al.*, 2002), and dogs (Middelbos *et al.*, 2007). *E. coli* strains are commensal inhabitants

of gastrointestinal tracts and only certain strains cause an infection. MOS has been shown to reduce *E. coli* (Baurhoo *et al.*, 2007) and *Salmonella* concentrations in cecal contents of experimentally infected chickens (Fernandez *et al.*, 2002). This reduction may be due to the ability of mannose in MOS to bind to mannose-binding lectins of gram-negative bacteria expressing type 1 fimbriae, and consequently, reduce bacterial attachment to the intestinal epithelial cells (Ganner & Schatzmayr, 2012). To obtain a more in-depth view of the chicken gut microbiota, next generation sequencing and metagenomic studies would be of great interest.

Gut microbial composition can directly or indirectly affect intestinal morphological structure. Bifidobacteria have been reported to increase the proliferation of intestinal epithelial cells along the length of the villus and improve intestinal function (Yang *et al.*, 2009). Increased dietary fiber shifts microbial population towards acetic acid and butyrate producing bacteria (Liu *et al.*, 2012) which are associated with increased villi height in the jejunum and ileum (Kuzmuk *et al.*, 2005). The current study demonstrated that in birds under mild environmental stress, MOS increased villi heights and goblet cell numbers when compared with those from birds exposed to the antibiotic. More goblet cells in the MOS group can potentially produce more mucin, a high molecular weight glycoprotein and main component of mucus, and help the birds maintain intestinal health during challenging conditions. The intestinal mucus layer mainly acts as a defensive barrier and limits attachment of enteric pathogens to epithelial cells (Bergstrom *et al.*, 2008). Altering the microbial community may disrupt intestinal integrity and lead to a decrease in mucus thickness and an increase in the risk of colonization by pathogens (Burkholder *et al.*, 2008). VIRG did not affect goblet cell numbers. Antibiotics are reported to reduce the weight of the gastrointestinal tract, mainly by thinning the intestinal wall (Miles *et al.*, 2006). Although a thinner

and lighter intestinal tract has been linked to the lower maintenance energy and higher nutrient absorption, it may increase intestinal susceptibility to enteric pathogens.

The microbiota of the gut can affect the nutrient utilization of birds positively or negatively. Large numbers of microbes need and consume energy, some of which would otherwise be available for the chicken. On the other hand, the microbiota can benefit the host by producing energy in the form of short chain fatty acids. Under the condition of the present study, MOS supplementation did not show a significant effect on body weight or FCR by the end of the experiment. Similar results were observed with birds fed MOS and grown under experimentally controlled conditions (Sims *et al.*, 2004, Baurhoo *et al.*, 2009) or a high stocking density (Houshmand *et al.*, 2012). This observation, however, stands in contrast to previous reports that MOS supplementation led to higher BW and feed efficiency in broiler chickens (Yang *et al.*, 2008, Kim *et al.*, 2011). VIRG increased body weight and the main effect was significant on day 35. How the change of microbiota affected the body weight is not clear at this time. Taken together, the inconsistency in reported findings about the effects of prebiotics and antibiotic on chicken body weight is expected as growth performance is highly influenced by other factors such as diet formulation, feed quality, management, environment, and disease control.

3.6 Conclusion

The results of this experiment indicate that under mild environmental and dietary challenge conditions, MOS and VIRG altered the cecal bacterial community differently. A decrease in bacterial diversity, *Lactobacillus* and *Bifidobacterium* population was noted in the cecum of VIRG treated chickens. Intestinal morphology was not affected by antibiotic treatment. MOS supplementation possibly conferred intestinal health benefits to the broilers by promoting growth of *Lactobacillus* and *Bifidobacterium* species in the cecum and by increasing villi height and goblet

cell numbers of the ileum and jejunum. Therefore, dietary supplementation of MOS as an alternative for antibiotic growth promoters may alleviate detrimental effects of sub-optimal growing conditions on broiler productivity and health.

3.7 Acknowledgements

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3.8 References

Apajalahti J, Kettunen A & Graham H (2004) Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. *Worlds Poultry Science Journal* **60**: 223-232.

Barman M, Unold D, Shifley K, Amir E, Hung K, Bos N & Salzman N (2008) Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. *Infection and Immunity* **76**: 907-915.

Baurhoo B, Phillip L & Ruiz-Feria CA (2007) Effects of purified lignin and mannan oligosaccharides on intestinal integrity and microbial populations in the ceca and litter of broiler chickens. *Poultry Science* **86**: 1070-1078.

Baurhoo B, Ferket PR & Zhao X (2009) Effects of diets containing different concentrations of mannanoligosaccharide or antibiotics on growth performance, intestinal development, cecal and litter microbial populations, and carcass parameters of broilers. *Poultry Science* **88**: 2262-2272.

Baurhoo B, Ferket P, Ashwell CM, de Oliviera J & Zhao X (2012) Cell walls of saccharomyces cerevisiae differentially modulated innate immunity and glucose metabolism during late systemic inflammation. *PloS One* **7**: e30323.

Bergstrom KSB, Guttman JA, Rumi M, Ma C, Bouzari S, Khan MA, Gibson DL, Vogl AW & Vallance BA (2008) Modulation of intestinal goblet cell function during infection by an attaching and effacing bacterial pathogen. *Infection and Immunity* **76**: 796-811.

Bron PA, van Baarlen P & Kleerebezem M (2011) Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. *Nature Reviews Microbiology*. **10**: 66-78

Burkholder KM, Thompson KL, Einstein ME, Applegate TJ & Patterson JA (2008) Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to Salmonella Enteritidis colonization in broilers. *Poultry Science* **87**: 1734-1741.

Collier C, Smiricky-Tjardes M, Albin D, Wubben J, Gabert V, Deplancke B, Bane D, Anderson D & Gaskins H (2003) Molecular ecological analysis of porcine ileal microbiota responses to antimicrobial growth promoters. *Journal of Animal Science* **81**: 3035-3045.

Dibner JJ & Richards JD (2005) Antibiotic growth promoters in agriculture: History and mode of action. *Poultry Science* **84**: 634-643.

Food and Drug Administration (2012) The judicious use of medically important antimicrobial drugs in food producing animals. Food and Drug Administration, Rockland, Md., USA.

Fernandez F, Hinton M & Van Gils B (2002) Dietary mannan-oligosaccharides and their effect on chicken caecal microflora in relation to Salmonella Enteritidis colonization. *Avian Pathology* **31**: 49-58.

Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K, Tobe T, Clarke JM, Topping DL & Suzuki T (2011) Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature* **469**: 543-547.

Ganner A & Schatzmayr G (2012) Capability of yeast derivatives to adhere enteropathogenic bacteria and to modulate cells of the innate immune system. *Applied Microbiology and Biotechnology* 1-9.

Gibson GR, Probert HM, Van Loo J, Rastall RA & Roberfroid MB (2004) Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition Research Reviews* **17**: 259-275.

Gueimonde M, Tölkkö S, Korpimäki T & Salminen S (2004) New real-time quantitative PCR procedure for quantification of bifidobacteria in human fecal samples. *Applied and Environmental Microbiology* **70**: 4165-4169.

Guo X, Xia X, Tang R, Zhou J, Zhao H & Wang K (2008) Development of a real-time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs. *Letters in Applied Microbiology* **47**: 367-373.

Houshmand M, Azhar K, Zulkifli I, Bejo MH & Kamyab A (2012) Effects of prebiotic, protein level, and stocking density on performance, immunity, and stress indicators of broilers. *Poultry Science* **91**: 393-401.

SAS (2003) SAS user's guide. Version 9.1. SAS Institute Inc., Cary, N.C., USA.

Khailova L, Dvorak K, Arganbright KM, Halpern MD, Kinouchi T, Yajima M & Dvorak B (2009) Bifidobacterium bifidum improves intestinal integrity in a rat model of necrotizing enterocolitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **297**: 940-949.

Kim GB, Seo YM, Kim CH & Paik IK (2011) Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. *Poultry Science* **90**: 75-82.

Kuzmuk KN, Swanson KS, Tappenden KA, Schook LB & Fahey Jr GC (2005) Diet and age affect intestinal morphology and large bowel fermentative end-product concentrations in senior and young adult dogs. *The Journal of Nutrition* **135**: 1940-1945.

Liu HY, Ivarsson E, Dicksved J, Lundh T & Lindberg JE (2012) Inclusion of chicory (cichorium intybus L.) in pigs' diets affects the intestinal microenvironment and the gut microbiota. *Applied and Environmental Microbiology* **78**: 4102-4109.

Malinen E, Kassinen A, Rinttilä T & Palva A (2003) Comparison of real-time PCR with SYBR Green I or 5′-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria. *Microbiology* **149**: 269-277.

Matsuki T, Watanabe K, Fujimoto J, Kado Y, Takada T, Matsumoto K & Tanaka R (2004) Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for analysis of human intestinal bifidobacteria. *Applied and Environmental Microbiology* **70**: 167-173.

Middelbos IS, Godoy MR, Fastinger ND & Fahey GC, Jr. (2007) A dose-response evaluation of spray-dried yeast cell wall supplementation of diets fed to adult dogs: Effects on nutrient digestibility, immune indices, and fecal microbial populations. *Journal of Animal Science* **85**: 3022-3032.

Miles R, Butcher G, Henry P & Littell R (2006) Effect of antibiotic growth promoters on broiler performance, intestinal growth parameters, and quantitative morphology. *Poultry Science* **85**: 476-485.

Muyzer G, Dewaal EC & Uitterlinden AG (1993) Profiling of Complex Microbial-Populations by Denaturing Gradient Gel-Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes-Coding for 16s Ribosomal-Rna. *Applied and Environmental Microbiology* **59**: 695-700.

National Research Council (1994) Nutrient requirements of poultry. 9th ed. National Academy Press, Washington, D.C., USA

Rada V, Sirotek K & Petr J (1999) Evaluation of selective media for bifidobacteria in poultry and rabbit caecal samples. *Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health* **46**: 369-373.

Rastall RA, Gibson GR, Gill HS, Guarner F, Klaenhammer TR, Pot B, Reid G, Rowland IR & Sanders ME (2005) Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: An overview of enabling science and potential applications. *FEMS Microbiology Ecology* **52**: 145-152.

Rinttilä T, Kassinen A, Malinen E, Krogius L & Palva A (2004) Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *Journal of applied microbiology* **97**: 1166-1177.

Sekirov I, Russell SL, Antunes LCM & Finlay BB (2010) Gut microbiota in health and disease. *Physiological reviews* **90**: 859-904.

Silbergeld EK, Graham J & Price LB (2008) Industrial food animal production, antimicrobial resistance, and human health. *Annual Review of Public Healtth* **29**: 151-169.

Sims MD, Dawson KA, Newman KE, Spring P & Hooge DM (2004) Effects of dietary mannan oligosaccharide, bacitracin methylene disalicylate, or both on the live performance and intestinal microbiology of turkeys. *Poultry Science* **83**: 1148-1154.

Sohail MU, Ijaz A, Yousaf MS, Ashraf K, Zaneb H, Aleem M & Rehman H (2010) Alleviation of cyclic heat stress in broilers by dietary supplementation of mannan-oligosaccharide and Lactobacillus-based probiotic: Dynamics of cortisol, thyroid hormones, cholesterol, C-reactive protein, and humoral immunity. *Poultry Science* **89**: 1934-1938.

Sohail MU, Rahman ZU, Ijaz A, Yousaf MS, Ashraf K, Yaqub T, Zaneb H, Anwar H & Rehman H (2011) Single or combined effects of mannan-oligosaccharides and probiotic supplements on the total oxidants, total antioxidants, enzymatic antioxidants, liver enzymes, and serum trace minerals in cyclic heat-stressed broilers. *Poultry Science* **90**: 2573-2577.

Threlfall EJ, Ward LR, Frost JA & Willshaw CA (2000) The emergence and spread of antibiotic resistance in food-borne bacteria. *International Journal of Food Microbiology* **62**: 1-5.

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER & Gordon JI (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**: 1027-1131.

Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA & Affourtit JP (2008) A core gut microbiome in obese and lean twins. *Nature* **457**: 480-484.

van der Wielen P, Keuzenkamp DA, Lipman LJA, van Knapen F & Biesterveld S (2002) Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. *Microbial Ecology* **44**: 286-293.

White LA, Newman MC, Cromwell GL & Lindemann MD (2002) Brewers dried yeast as a source of mannan oligosaccharides for weanling pigs. *Journal of Animal Science* **80**: 2619-2628.

Yang HY, Liu AP, Zhang M, Ibrahim SA, Pang ZH, Leng XJ & Ren FZ (2009) Oral administration of live bifidobacterium substrains isolated from centenarians enhances intestinal function in mice. *Current Microbiolgy* **59**: 439-445.

Yang Y, Iji P, Kocher A, Mikkelsen L & Choct M (2007) Effects of mannanoligosaccharide on growth performance, the development of gut microflora, and gut function of broiler chickens raised on new litter. *The Journal of Applied Poultry Research* **16**: 280-288.

Yang Y, Iji PA, Kocher A, Thomson E, Mikkelsen LL & Choct M (2008) Effects of mannanoligosaccharide in broiler chicken diets on growth performance, energy utilisation, nutrient digestibility and intestinal microflora. *British Poultry Science* **49**: 186-194.

Yu Z & Morrison M (2004) Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology* **70**: 4800-4806.

Connecting text

In Chapter 3, we evaluated the effects of MOS prebiotic and VIRG antibiotic on the cecal microbiota and intestinal morphological parameters of chickens reared under sub-optimal environmental and dietary conditions. We observed that MOS and VIRG altered the cecal bacterial community differently. DGGE profiles indicated that antibiotic treatment reduced cecal microbial diversity while the community equitability increased. A higher bacterial diversity was observed in the cecum of MOS supplemented chickens. Neither MOS nor VIRG changed *E. coli* cecal counts as identified by qPCR as well as culturing on selective medium. We also observed that MOS promoted the growth of lactobacilli and bifidobacteria in the cecum, and increased villus height and goblet cell numbers in the ileum and jejunum.

In Chapter 4, we would like to investigate the effects of another prebiotic candidate, XOS, on broiler chickens over a 5 weeks production cycle. To understand prebiotics mode of action, it is important to identify microbial diversity and composition in a greater depth. We therefore used 454 pyrosequencing of the 16S rRNA gene to expand our ability to analyse complex microbial communities in the ileum and cecum.

Chapter 4. Xylo-oligosaccharides and Virginiamycin Differentially Modulate Gut Microbial Composition in Chickens.

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4.1 Abstract

The emergence and spread of antibiotic resistance in pathogens have led to a restriction on the use of antibiotic growth promoters (AGPs) in animal feed in some countries. The potential negative after-effects of a ban on AGPs could be mitigated by improving animal intestinal health with prebiotic dietary fibers such as xylo-oligosaccharides (XOS). However, the mechanism(s) by which an antibiotic or prebiotic contributes to the health and growth of animals are not well understood. Here, we evaluated XOS and virginiamycin (VIRG)-mediated changes in gut microbiota of broiler chickens using pyrosequencing of the 16S rRNA gene. There was a significant change in the relative abundance of certain bacteria, but the overall microbial diversity was not affected by treatment with either XOS or VIRG. Supplementation of HXOS (2g XOS/kg diet) increased the proportion of *Lactobacillus* genus in the cecum, whereas *Propionibacterium* and *Corynebacterium* genera were enriched in the ileum of VIRG (16 mg/kg) treated birds. Furthermore, an increase in the cecal concentrations of acetate and propionate was observed in HXOS and VIRG fed chickens, respectively. These two groups of birds had better feed conversion efficiencies in comparison with the control group from day 7 to 21. In addition, temporal variations in the gut microbiota were evident in the chickens of different ages. Treatments with XOS or VIRG modified the relative abundance but not the presence or absence of specific microbial genus. The increase in both *Lactobacillus* spp. and acetate production in the cecum of HXOS treated chickens may promote intestinal health.

4.2 Introduction

Antibiotic growth promoters (AGPs) have been widely used in poultry production to improve growth performance, feed efficiency and overall health (Castanon, 2007). However, this practice has been discontinued in the European Union since 2006 due to increasing concern over spread of antibiotic-resistant genes to human and animal pathogens (Maron *et al.*, 2013). The withdrawal of AGP from poultry feed may increase bird disease rates, causing a rise in veterinary use of antibiotics for therapeutic purpose (Casewell *et al.*, 2003). Therefore, there is the need to find effective alternatives to AGPs that improve chicken health and maintain efficiency of production and safety of poultry products.

Although it is still unclear how AGPs enhance animal performance, it is believed that they mainly act on gut microbiota (Dibner & Richards, 2005). The chicken gastrointestinal microbiota harbors dynamic and complex bacterial communities with an important role in metabolic activity and immune development (Oakley *et al.*, 2014). In addition, some gut microbes produce a variety of enzymes that ferment complex polysaccharides into short chain fatty acids (SCFA), mainly acetate, propionate and butyrate. SCFA are major end-products of bacterial fermentation of dietary fiber and provide several health benefits to the host including regulation of intestinal inflammation (Arpaia *et al.*, 2013, Smith *et al.*, 2013). We have previously shown that supplementation of certain indigestible but fermentable dietary fibers, such as mannan oligosaccharides, promoted the growth of bacterial species with potential health benefits (Pourabedin *et al.*, 2014). Accumulating evidence has suggested that xylo-oligosaccharides (XOS) are another promising prebiotic candidate (Aachary & Prapulla, 2011, Broekaert *et al.*, 2011). While XOS are not degraded by recognized enteric pathogens such as *Staphylococcus aureus, Clostridium difficile, Salmonella enterica* and *Campylobacter jejuni,* probiotic strains such as *Lactobacillus* spp. and *Bifidobacterium* spp. are

able to utilize XOS (Moura *et al.*, 2007, Kondepudi *et al.*, 2012). However, the effect of XOS on the gut microbiota remains unclear as previous studies have often relied on *in vitro* observation (Gullón *et al.*, 2008, Mäkeläinen *et al.*, 2010, Madhukumar & Muralikrishna, 2012) or microbial culture methods (Courtin *et al.*, 2008) that fail to provide accurate taxonomic composition and community structure.

Previous analysis of chicken intestinal microbiota using 16S rRNA clone library sequencing method has indicated that dietary supplementation with sub-therapeutic level of tylosin (Lin *et al.*, 2013) or virginiamycin (Dumonceaux *et al.*, 2006) influence the population of specific bacterial species in the small intestine. A pyrosequencing analysis of the 16S V3 region has also revealed a number of significant changes in the cecal microbiota of chickens treated with monensin in the presence or absence of tylosin or virginiamycin (Danzeisen *et al.*, 2011). However, their study did not elucidate how virginiamycin alone could affect microbial communities in the cecum and other gastrointestinal tract locations. Far less is known about the prebiotic mediated changes in the chicken microbiome.

We hypothesized that, in chickens, the mode of action of virginiamycin or XOS occurs through the gut microbiota. Therefore, it is necessary to better understand how gastrointestinal bacterial communities react to these feed additives. In this study, we used 454 pyrosequencing of the V1-V3 region of 16S rRNA gene to assess the ileal and cecal microbiota in male broiler chickens fed either a commercial diet free of antibiotics and prebiotics (CTL), the same basal diet supplemented with a sub-therapeutic level (16.5 g/ton diet) of virginiamycin (VIRG) or the basal diet supplemented with 1 g/kg (LXOS) or 2 g/kg (HXOS) of XOS. Ileal and cecal concentrations of lactate and SCFA were also measured.

4.3 Materials and Methods

4.3.1 Birds, diet and experimental design

One hundred and twenty male one-day-old broiler chickens (Ross × Ross) were obtained from a local commercial hatchery and grown over a 35 day experimental period at the Macdonald Campus Poultry Complex, McGill University. Birds were randomly assigned to 1 of 4 dietary treatments (6 cage replicates; 5 birds/cage) which included 1) CTL: a commercial and typical broiler diet without any supplements, 2) VIRG: diet 1 supplemented with sub-therapeutic levels of virginiamycin (16.5 g/ton diet), 3) LXOS: diet 1 + 1g XOS/kg and 4) HXOS: diet 1 + 2g XOS/kg. The main ingredients of the diets (one phase feeding program) were corn and soybean meal, formulated according to the NRC requirement (Table 4.1). Chickens had free access to feed and water. Birds were raised under controlled environmental conditions with an 18 hour lighting cycle and a temperature of 32°C at day 1 which was gradually reduced and maintained at 24 °C on day 10. Body weight (BW) and feed intake were recorded on a cage-by-cage basis on day 7, 21 and 35. Feed conversion ratio (FCR) was calculated as feed intake (kg) divided by body mass gain (kg). The animal use protocol was according to the guidelines of the Canadian Council on Animal Care and approved by the Animal Ethics Committee of McGill University (Protocol no. 2012-6073).

Table 4.1. Ingredient composition (g/kg) of experimental diets

Ingredient	CTL	VIRG	LXOS	HXOS
Corn	511.28	511.28	511.28	511.28
Soya	300.36	300.36	300.36	300.36
Soybean meal, 48% CP	141.64	141.64	141.64	141.64
Phosphore	17.22	17.22	17.22	17.22
Calcium	13.88	13.88	13.88	13.88
micro debut	5	5	5	5
NaCL	2.17	2.17	2.17	2.17
Lysine	2.16	2.16	2.16	2.16
Filler	2	1.62	1	0
Methionine	1.3	1.3	1.3	1.3
Choline chloride	1	1	1	1
Sodium carbonate	1	1	1	1
XOS	0	0	1	2
Coban®	0.5	0.5	0.5	0.5
Threonine	0.48	0.48	0.48	0.48
Virginiamycin	0	0.38	0	0
Vitamin Mixture 10%	0.01	0.01	0.01	0.01
Calculated analysis				
ME, kcal/kg	3029	3029	3029	3029
Protein, g/kg	23	23	23	23
Lysine, g/kg	1.43	1.43	1.43	1.43
Methionine, g/kg	0.51	0.51	0.51	0.51
Crude Fat, g/kg	4.45	4.45	4.45	4.45

Note: CTL: Control diet without any antibiotic or prebiotic; VIRG: Control diet supplemented with 16.5 mg virginiamycin; LXOS: Control diet supplemented with 1g xylo-oligosaccharides/kg feed; HXOS: Control diet supplemented with 2g xylo-oligosaccharides/kg fee

4.3.2 Sample collection and DNA extraction

Six birds per treatment were randomly chosen at 3 different time points, 15, 25 and 35 days of age, and euthanized by electrical stunning and carotid artery bleeding. The ileum (about 2 cm proximal to cecal tonsils) and cecum were collected within 5 minutes of euthanasia, immediately placed in cryogenic vials, snap-frozen in liquid nitrogen, delivered to the laboratory and stored at

−80°C until DNA extraction. Total genomic DNA was isolated from 220 mg of frozen ileal and cecal contents using the QIAamp DNA Stool Mini Kit (Qiagen, ON, Canada). The DNA concentration and purity was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA).

4.3.3 Pyrosequencing

The normalized concentration (20 ng/µl) of purified genomic DNA was used as a template to analyze the microbial communities. The V1-V3 region of the 16S rRNA gene was amplified using universal eubacterial primers (27F: AGRGTTTGATCMTGGCTCAG and 519R: GTNTTACNGCGGCKGCTG) (Kim *et al.*, 2011). Unique 8 nucleotide sample-specific barcodes and Roche 454 A-adapters were fused to the 5′ end of the forward primer while the B-adapters were added to the 5′ end of the reverse primer. PCR reactions were performed by initial denaturation at 94°C for 3 minutes and then 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, followed by a final elongation step at 72°C for 5 minutes. PCR products were purified using the MinElute kit (Qiagen, ON, Canada). Amplicon pyrosequencing was performed at the MR DNA sequencing center (Shallowater, TX, USA) using 454 GS FLX technology.

4.3.4 Data processing

Sequence reads were analyzed by the quantitative insights into microbial ecology (QIIME) v.1.8.0 software package (Caporaso *et al.*, 2010). Briefly, sequences were demultiplexed and assigned to individual samples according to the specific barcode of each sample. Barcodes and primers were trimmed, where maximum 2 base differences in barcodes and no primer mismatches were permitted. Sequences were excluded if they were not meeting the default QIIME quality criteria. Sequences with an average quality score less than 25 in a sliding window of 50 nucleotides

were also discarded. The sequence data were denoised using the *denoise_wrapper.py* command (Reeder & Knight, 2010) within QIIME. The chimeras were identified using the UCHIME method (Edgar *et al.*, 2011) against the GOLD database and removed from further analyses. The remaining quality-filtered reads were clustered *de novo* (97% similarity threshold) into OTUs using the CD-HIT method (Fu *et al.*, 2012), and the most abundant sequence was selected as the OTU representative. The sequence alignment was performed against the Greengenes core set using the PyNAST method (Caporaso *et al.*, 2010). OTUs were taxonomically categorized using the naïve Bayesian RDP classifier (Wang *et al.*, 2007) trained on the Greengenes database with a minimum confidence score of 0.8. For downstream analysis, the OTU table was filtered by discarding OTUs that comprised less than 0.005% of all sequences (Bokulich *et al.*, 2012).

4.3.5 Cecal SCFA and lactate concentrations

For determination of cecal SCFA and lactate concentrations, 0.5 g of fresh cecal contents were diluted in 1 ml of 10% perchloric acid, homogenized, and centrifuged at 15,000 × g for 10 min at 4 °C. The supernatant was filtered through syringe filters with 25 mm diameter membrane and stored at -20°C. The samples (20 µl) were injected into a high performance liquid chromatograph (HPLC) system equipped with a Varian ProStar AutoSampler (Hamilton, NV, USA), a UV detector (210 nm) and an ion-exclusion Aminex HPX-87H 300 × 7.8 mm column (Bio-Rad, Hercules, CA, USA). The column was maintained at room temperature with 0.013 M sulfuric acid as the eluent (0.6 ml/min flow rate). Lactate, acetate, propionate and butyrate in the samples were quantified using external calibration curves.

4.3.6 Statistical measurements

To compare microbial community structure, unweighted UniFrac distance matrices were computed using the OTU table and phylogenetic tree information to serve as input to plot PCoA

using QIIME. Analysis of similarities (ANOSIM) with 999 permutations was used to detect statistical significances between microbial communities in different groups. This test measures a value of R, normally scaled from 0 to 1, which is based on the average rank similarity among groups and replicates within each group (Clarke, 1993). R=0 indicates that two groups are similar whereas R=1 shows a perfect separation between groups. Differentially abundant taxa were identified using the LEfSe method (Segata *et al.*, 2011). The LEfSe algorithm uses the non-parametric factorial Kruskal-Wallis test (α=0.05) to analyze differences between classes (treatments) and the pairwise Wilcoxon test (α=0.05) to check differences among subclasses (time points) within different classes. To evaluate the α-diversity in samples, the rarefaction curves of PD and number of observed OTUs were computed using QIIME. To normalize the sequencing depth, the lowest counts among samples were randomly subsampled in each library 1000 times and average values were used to measure diversity indices. The differences between the mean values were identified by analyses of variance (ANOVA) and Scheffe's multiple comparison test using SAS v9.1 software. PROC CORR was used to analyze the Pearson correlation between bacterial genera and SCFA concentrations. The differences of growth performance parameters among treatments were analyzed by 1-way ANOVA and each cage was considered as an experimental unit.

4.4 Results

4.4.1 Sequence analysis and quality filtering

A total of 2,063,514 pyrosequencing reads were obtained from 96 ileal and cecal samples. After removing 280,537 low quality and chimeric sequences, the average number of reads generated per chicken was 17570 (±8459 STD) from ileal samples and 19444 (±4273 STD) from cecal samples, with the median read length of 402 (±93 STD) bases in all samples. In total, 6544

distinct Operational Taxonomic Units (OTUs) at the 97% identity level were obtained from all samples. After rare OTUs (<0.005% of total OTUs) were removed a total of 3248 OTUs remained for downstream analyses.

4.4.2 Effects of dietary treatments on the ileal microbiota

To assess the within-community (α) diversity, the number of observed OTUs (at the 97% level) and phylogenetic diversity (PD) were calculated using QIIME. None of the dietary treatments had a significant effect on the α-diversity indices of the ileal bacterial community (P>0.05; Figure 4.1A-B, Table 4.2). Rarefaction curves for the observed OTUs (Figure 4.1A) and PD values (Figure 4.1B) approach a plateau, indicating that sequencing depth was sufficient for the coverage of all OTUs present in ileal samples. Although the number of observed OTUs and the PD values were higher in chickens fed HXOS, these differences did not reach a statistical significance (P > 0.05; Table 4.2). PD values differed most between the HXOS and control group (P=0.06). To determine similarities between pairs of microbial communities (β-diversity), a principal component analysis (PCoA) was performed using unweighted UniFrac distance matrices. Because of high inter-individual variation, no distinguishable clustering of the samples was evident based on the dietary treatments (ANOSIM: R=0.09, P=0.006; Figure 4.1C). These results demonstrated that chickens shared a core set of microbiota in the ileum regardless of dietary supplementation.

OTUs were taxonomically assigned with the Ribosomal Database Project (RDP) classifier at 80% confidence threshold. The relative abundance of OTUs was analyzed at different ranking levels from phylum to genus. At the phylum level, ileal microbiota was mainly composed of *Firmicutes* (>85%) followed by *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*. We used the linear discriminant analysis (LDA) effect size (LEfSe) method and identified 5 taxonomic

biomarkers (LDA>2) in the ileal microbial community of VIRG treated birds (Figure 4.1D). The relative abundance of two genera, *Propionibacterium* (Figure 4.1E) and *Corynebacterium* (Figure 4.1F) in *Actinobacteria* phylum was significantly (LDA>2) higher in the VIRG group compared with other dietary groups.

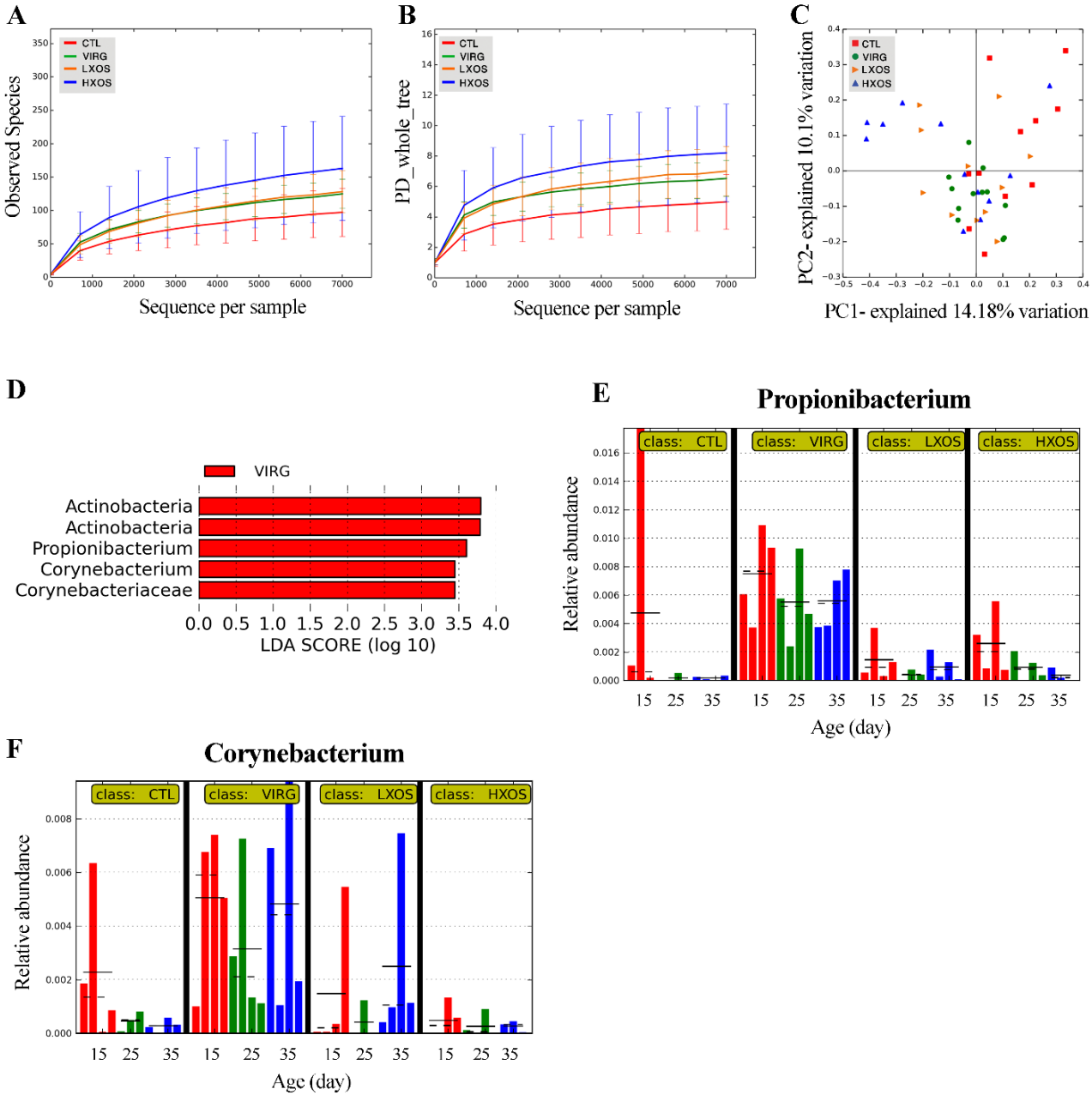

Figure 4.1. Treatment effects on ileal microbiota diversity and composition.

Rarefaction curves, calculated at the lowest subsample size of 7,000 sequences per sample, show the effects of sequencing efforts on the observed number of OTUs at 97% sequence similarity **(A)** and phylogenetic diversity (whole tree) **(B)**. Data are calculated at 3% distance. Error bars show standard deviation for each category. Principal component analysis (PCoA) of unweighted UniFrac distances from 24 ileal samples shows no difference in the community phylotype structure among treatments **(C)**. Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify specific phylotypes that significantly associated with treatments. LDA more than 2 reflects significant difference between groups. LEfSe analysis provided the list of phylotypes that are differential among dietary supplementations with statistical and biological significance **(D)**. The histograms indicated the increased relative abundance of the genera *Propionibacterium* **(E)** and *Corynebacterium* **(F)** in the ileal microbiota of chickens fed VIRG diet compared with other treatments. Each bar represents the relative abundance of the taxa in a sample at the age of 15 (red line), 25 (green line), and 35 days (blue line). The mean and median relative abundance are indicated with solid and dashed lines, respectively. CTL: control diet without any antibiotic or prebiotic; VIRG: control diet supplemented with 16.5 mg virginiamycin; LXOS: control diet supplemented with 1 g/kg xylo-oligosaccharides; HXOS: control diet supplemented with 2 g/kg xylo-oligosaccharides.

4.4.3 Effects of dietary treatments on the cecal microbiota

Rarefaction curves of 9000 subsampled reads in the cecum showed comparable numbers of OTUs (at the 97 % identity level) for each dietary treatment (Fig 4.2A and Table 4.2). Similarly, there was no apparent difference ($P>0.05$) in rarefaction curves for the PD values (Fig 4.2B and Table 4.2). The microbial community structure between dietary treatments (β-diversity) was compared using PCoA of the unweighted UniFrac distances. These PCoA plots showed that microbial communities from XOS and VIRG supplemented birds did not clearly separate from those of the non-supplemented birds. ($R= 0.02$, $P= 0.78$). The first axis of the PCoA explained

15.3% of the variation in bacterial diversity while the second axis explained 5.8% (Figure 4.2C). More than 99% of the sequences were assigned to bacterial phyla with the RDP classifier. LEfSe detected a marked increase (LDA score > 4) in the relative abundance of the *Lactobacillus* genus in chickens fed HXOS compared to other treatment groups (Fig 4.2D). Figure 4.2E shows the histogram of the relative abundance of *Lactobacillus* in each treatment at different time points. At the phylum level, the cecal microbiota was dominated by *Firmicutes* (>80%), followed by *Proteobacteria* and *Bacteroidetes* (Figure 4.3).

Table 4.2. Statistical comparison of alpha diversities between treatments in the ileal and cecal microbiota. A nonparametric t-test was run in QIIME to compare the alpha diversities using the default number of Monte Carlo permutations (999) and the greatest rarefaction depth. Cells shaded with light blue (upper right section) shows the p-values for the number of observed species while those shaded with light yellow (lower left section) shows the p-values of phylogenetic diversity comparison.

Observed Species								
	Ileum				Cecum			
Treatments	CTL	VIRG	LXOS	HXSO	CTL	VIRG	LXOS	HXOS
CTL	1	0.24	0.33	0.08	1	1	1	1
VIRG	0.16	1	1	0.87	1	1	1	1
LXOS	0.15	1	1	1	1	1	1	1
HXOS	0.06	0.79	1	1	1	1	1	1
Phylogenetic Diversity								

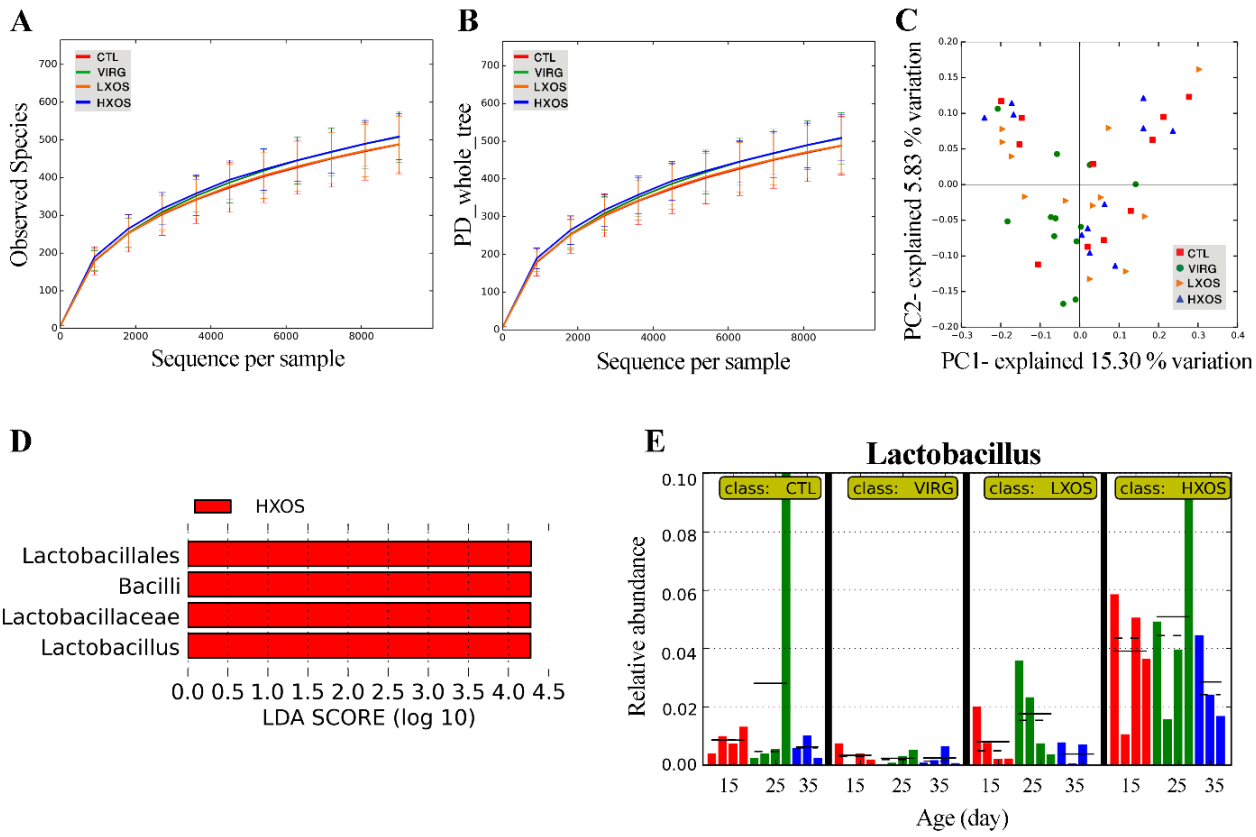

Figure 4.2. Treatment effects on diversity and composition of cecal microbiota.

Alpha diversity indices were calculated on rarefied samples at the lowest subsample size of 9,000 sequences per sample. There was no significant ($P > 0.05$) effect of prebiotic or antibiotic on the observed number of OTUs **(A)** and phylogenetic diversity (whole tree) **(B)**. Error bars show standard deviation for each category. Unweighted UniFrac PCoA plot shows no separation of bacterial communities between dietary groups **(C)**. Key phylotypes of cecal microbiota responding to dietary treatments were identified using LEfSe algorithm **(D)**. The histogram shows the increased abundance of the genus Lactobacillus in the cecal microbiota of chickens fed HXOS diet compared with other treatments **(E)**. Each bar represents the relative abundance of the taxa in a sample at the age of 15 (red line), 25 (green line), and 35 days (blue line). The mean and median relative abundance are indicated with solid and dashed lines, respectively.

4.4.4 Comparison between the ileal and the cecal microbiota

Lactobacillaceae and *Clostridiaceae* were the dominant families in the ileum while the cecum was inhabited mostly by the *Lachnospiraceae* and *Ruminococcaceae* families (Figure 4.3). The most dominant genera in the cecum were *Ruminococcus* and *Oscillospira* accounting for greater than 35% of all observed sequences. The number of observed OTUs and PD values were higher ($p<0.001$) in the cecal samples than those in corresponding ileal samples (Figure 4.4A and 4.4B), indicating that the cecal microbiota was more diversified than the ileal microbiota. The PCoA of OTUs from the ileum and cecum (Figure 4.4C) also demonstrated that the bacterial community structure differed significantly according to sampling site ($R=0.94$, $P=0.001$). In addition, the phylogenetic composition of the microbiota was noticeably different between ileum and cecum samples. LEfSe results showed that 46 bacterial clades at all taxonomic levels were differentially abundant (LDA score >2.0) between the ileal and cecal microbiota (Fig 4.4D).

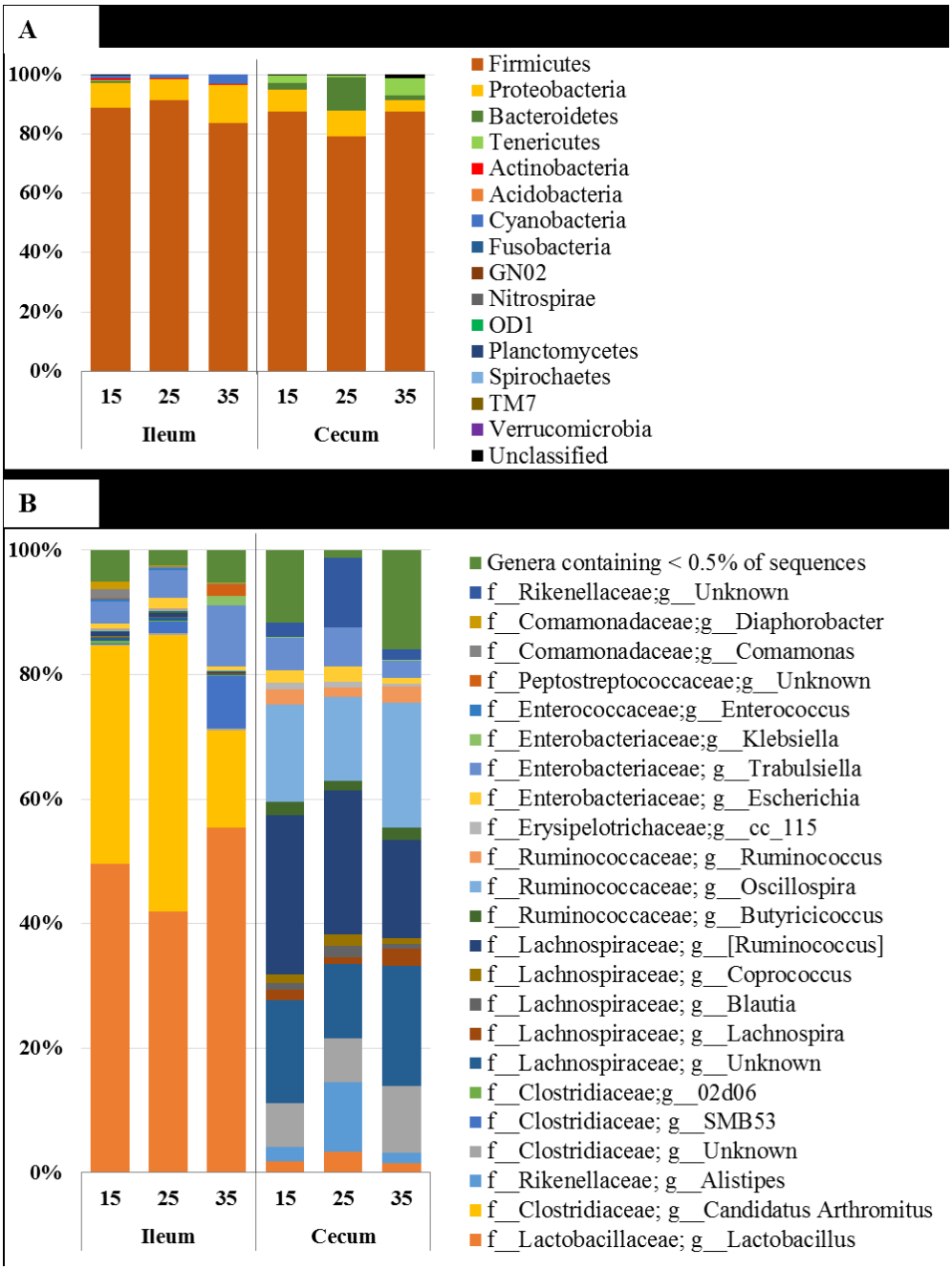

Figure 4.3. The ileal and cecal microbial composition.

The bar charts indicate the relative abundance (%) of bacterial phyla (**A**) and the dominant (>0.5% of sequences) bacterial families and genera (**B**) in the ileum and cecal microbiota of chickens. For each habitant, ileal and cecal contents of 16 birds were collected at 15, 25 and 35 days old.

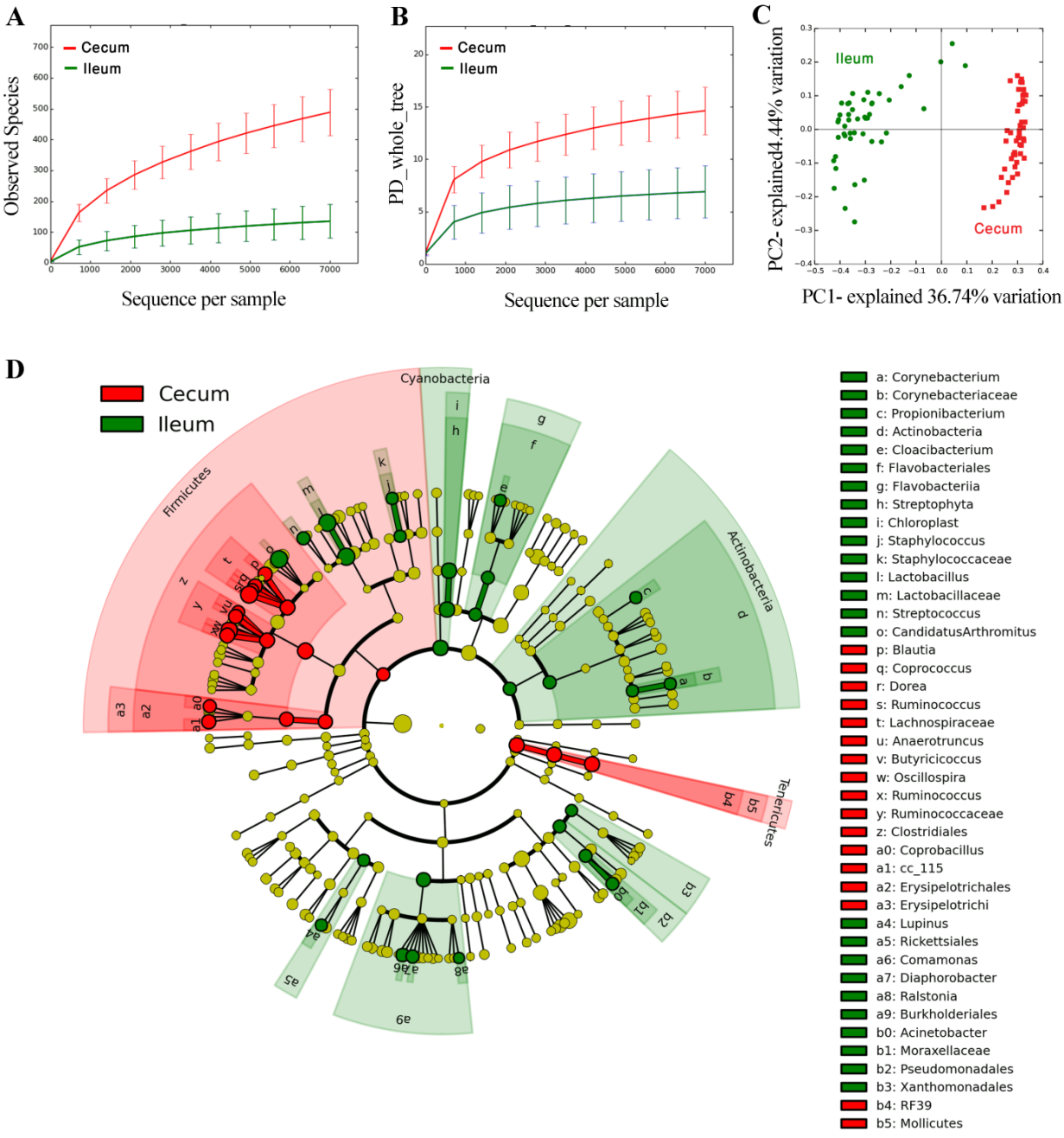
A
Cecum
Ileum
Observed Species
Sequence per sample
B
Cecum
Ileum
PD_whole_tree
Sequence per sample
C
Ileum
Cecum
PC2- explained4.44% variation
PC1- explained 36.74% variation
D
Cecum
Ileum
Cyanobacteria
Firmicutes
Actinobacteria
Tenericutes
a: Corynebacterium
b: Corynebacteriaceae
c: Propionibacterium
d: Actinobacteria
e: Cloacibacterium
f: Flavobacteriales
g: Flavobacteriia
h: Streptophyta
i: Chloroplast
j: Staphylococcus
k: Staphylococcaceae
l: Lactobacillus
m: Lactobacillaceae
n: Streptococcus
o: CandidatusArthromitus
p: Blautia
q: Coprococcus
r: Dorea
s: Ruminococcus
t: Lachnospiraceae
u: Anaerotruncus
v: Butyricicoccus
w: Oscillospira
x: Ruminococcus
y: Ruminococcaceae
z: Clostridiales
a0: Coprobacillus
a1: cc_115
a2: Erysipelotrichales
a3: Erysipelotrichi
a4: Lupinus
a5: Rickettsiales
a6: Comamonas
a7: Diaphorobacter
a8: Ralstonia
a9: Burkholderiales
b0: Acinetobacter
b1: Moraxellaceae
b2: Pseudomonadales
b3: Xanthomonadales
b4: RF39
b5: Mollicutes

Figure 4.4. Differences between ileal and cecal microbiota of broiler chickens.
Rarefaction curves for ileal and cecal bacteria, for the observed OTUs (**A**) and phylogenetic diversity (**B**) indices. Significant differences were seen between the alpha diversity indices of ileal samples and samples taken from cecum (nonparametric t-test, $P < 0.01$). PCoA analysis of OTUs (**C**) indicates that the bacterial profile differed strongly according to sampling site ($R = 094$, $P = 0.001$). A five-level circular cladogram based on the RDP taxonomy reporting taxa detected using LEfSe showing statistically and biologically consistent differences between ileal communities (green) and cecal communities (red) (D). Each circle's diameter is proportional to the taxon's abundance. The cutoff value of linear discriminant analysis (LDA) was 2.0 or higher. Biomarker taxa are heighted by colored circles and shaded areas. Each circle's diameter is relative to abundance of taxa in community

4.4.5 Temporal changes in the ileal and the cecal microbiota

To determine whether the age of the birds affected the gut microbiota, the ileal and cecal microbiota of chickens at different ages, 15, 25 and 35 days old, in each treatment group were compared. Rarefaction plots indicated no significant ($P>0.05$) changes in α-diversity metrics of the ileal samples at three different time points (Figure 4.5A, 4.5B and Table 4.3). Unweighted Unifrac PCoA revealed the statistical significant effect of age on the ileal samples ($P=0.01$) but the R-value was relatively small ($R=0.1$) and therefore the difference was probably not biologically significant (Figure 4.5C). In the cecum, a marked increase ($P<0.01$) in observed OTUs and the PD values occurred on day 35 compared with cecal samples from day 15 and 25 (Fig 4.5D, Fig 4.5E and Table 4.3). The PCoA plot of unweighted UniFrac distances indicated a clear separation between samples from day 35 and samples taken at days 15 and 25 ($R = 0.42$, $P<0.01$; Fig 4.5F). Bacteria that were differentially abundant between sampling times in the ileum and cecum were detected using LEfSe (Fig 4.6 and 4.7). For example, in the ileum, the order *Burkholderiales* and the candidate genus SMB53 of *Clostridiaceae* were the most differentially abundant taxa at days

15 and 35, respectively (Fig 4.6). The most differentially overrepresented taxa in the cecum at 15, 25 and 35 days of age were the genus *Enterococcus*, family *Rikenellaceae* and genus *Oscillospira*, respectively (Fig 4.7).

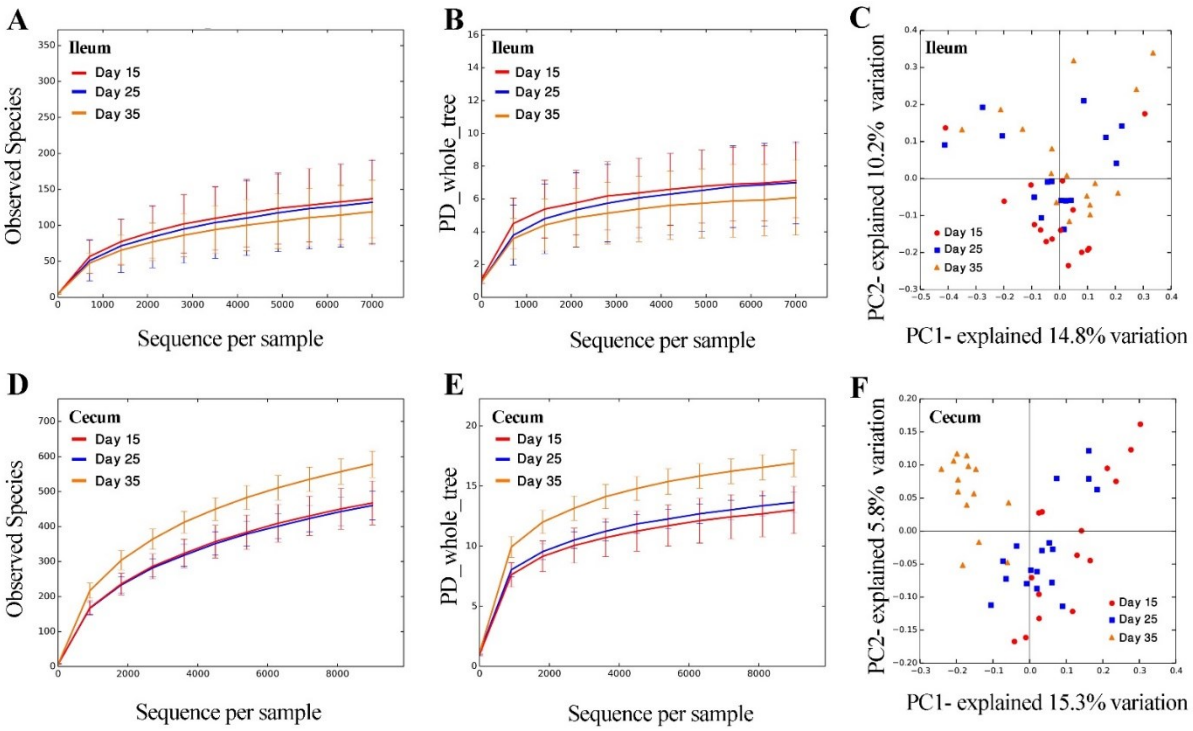

Figure 4.5. Ileal and cecal microbiota of broiler chickens at different ages.

Observed OTUs **(A)** and phylogenetic diversity **(B)** rarefactions of ileal samples collected on days 15, 25, and 35. Unweighted Unifrac PCoA shows a statistically, but not biologically, significant effect of age on the ileal microbiota ($R = 0.1$, $P = 0.01$) **(C)**. Alpha rarefaction analysis of cecal samples **(D, E)** shows that observed OTUs and phylogenetic diversity both increased on day 35 compared with those samples collected on day 15 and 25 ($P < 0.01$). A separate clustering of cecal microbiota was observed on day 35 ($R = 0.42$, $P < 0.01$) **(F)**.

Table 4.3. Statistical comparison of alpha diversities between sampling time in the ileal and cecal microbiota. A nonparametric two sample t-test was run in QIIME to compare the alpha diversities using the default number of Monte Carlo permutations (999) and the greatest rarefaction depth. Cells shaded with light blue (upper right section) shows the p-values for the number of observed species while those shaded with light yellow (lower left section) shows the p-values of phylogenetic diversity comparison. Significant p-values are bolded.

Observed Species						
	Ileum			Cecum		
Age	Day 15	Day 25	Day 35	Day 15	Day 25	Day 35
Day 15	1	1	1	1	1	**0.003**
Day 25	1	1	1	0.73	1	**0.003**
Day 35	0.66	1	1	**0.003**	0.003	1
Phylogenetic Diversity						

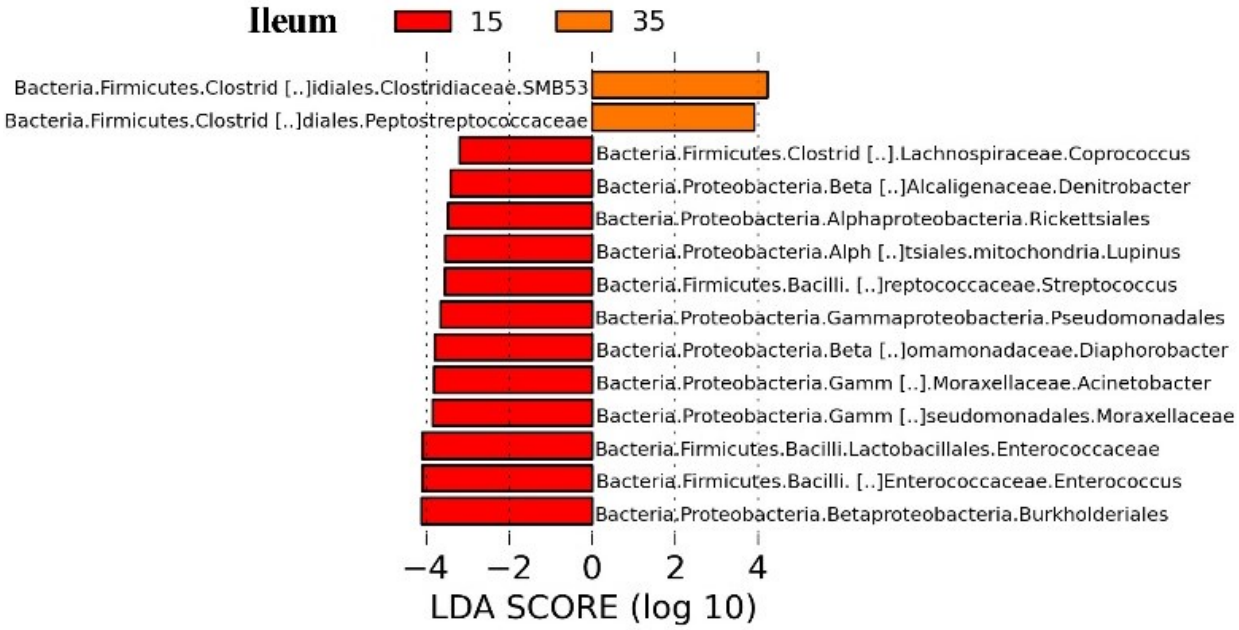

Figure 4.6. Bacteria that were differentially abundant between sampling times in the ileum. The linear discriminant analysis (LDA) effect size (LEfSe) provided the list differentially abundant taxa between ages in the ileum. The cutoff value of LDA analysis was 2.0 or higher

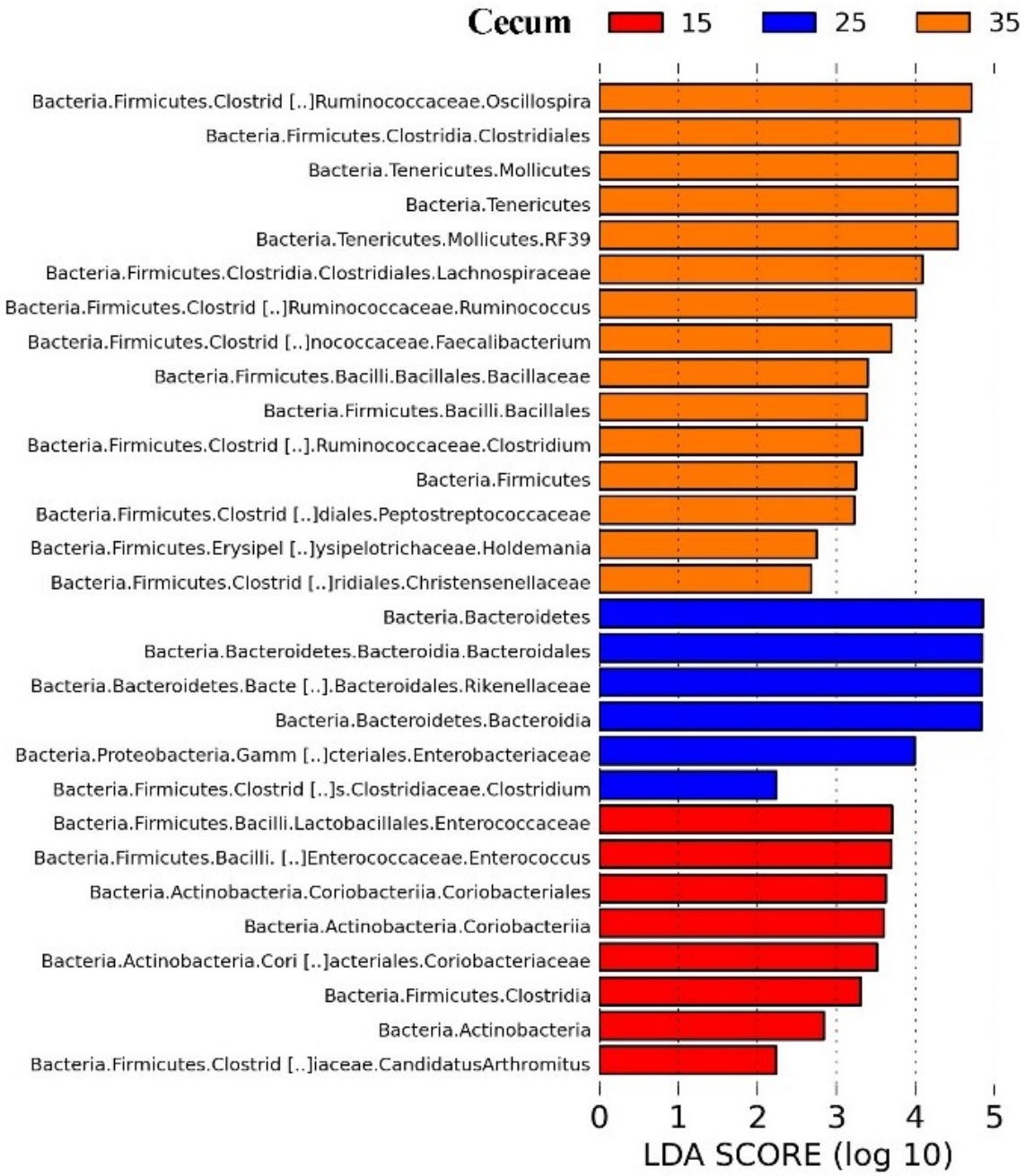

Figure 4.7. Bacteria that were differentially abundant between sampling times in the cecum. The linear discriminant analysis (LDA) effect size (LEfSe) provided the list differentially abundant taxa between ages in the cecum. The cutoff value of LDA analysis was 2.0 or higher

4.4.6 SCFAs in the ileum and cecum

To further identify whether the observed microbial changes due to dietary treatment also affected the gut function, SCFA and lactate concentrations were measured. In the ileum, the acetate concentration ranged from 6.2 to 7.1 μmol/g digesta and was not affected by dietary treatments, while lactate, propionate and butyrate were not detected (data not shown). The cecal propionate concentration in the VIRG group was significantly higher ($P < 0.05$) than LXOS and HXOS groups on day 15 (Fig 4.8A) and also significantly higher than the control, LXOS and HXOS groups on day 35 (Fig 4.8C). The cecal acetate concentration was significantly higher in the HXOS group than in the VIRG group on days 25 and 35 ($P < 0.05$; Fig 4.8B and Fig 4.8C) and the LXOS group on day 25 ($P < 0.01$; Fig 4.8B). Correlation analyses showed that the relative abundance of the *Lactobacillus* genus in the cecum was positively correlated with cecal acetate production ($r = 0.57$, $P < 0.05$), whereas ileal *Propionibacterium* relative abundance was positively correlated with cecal propionate concentrations ($r = 0.51$, $P < 0.05$).

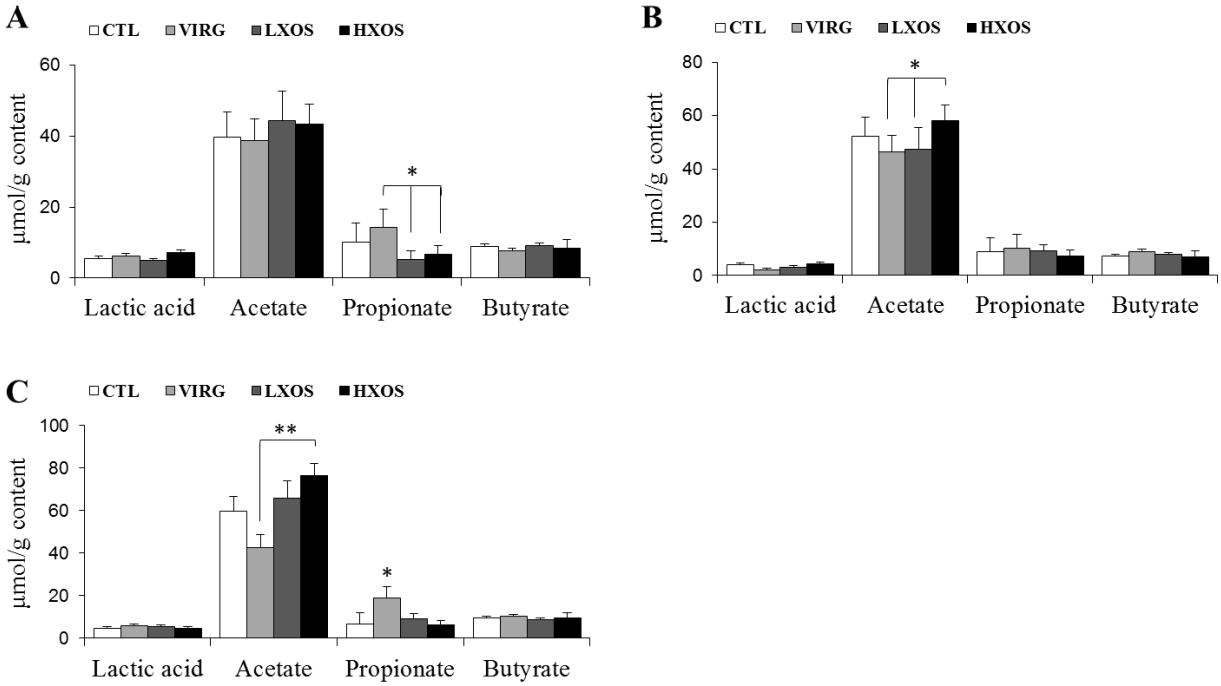

Figure 4.8. SCFA and lactate concentrations in the cecum.

Lactate, acetate, propionate, and butyrate concentrations (µmol/g content) of 24 cecal samples collected on 15 (**A**), 25 (**B**), and 35 (**C**) days of age. Significant differences (*P < 0.05 and **P < 0.01) were detected using the Scheffe's multiple comparison test by ANOVA. CTL: control diet without any antibiotic or prebiotic; VIRG: control diet supplemented with 16.5 g/ton virginiamycin; LXOS: control diet supplemented with 1 g xylo-oligosaccharides/kg; HXOS: control diet supplemented with 2 g xylo-oligosaccharides/kg

4.4.7 Growth Performance

Results for production traits of broilers through the experimental period are shown in Table 4.4. The average body weight of chickens did not differ among treatments. The feed conversion ratio (FCR) in broilers fed VIRG and HXOS diets was lower ($P<0.05$) than those fed CTL or LXOS between days 7 to 21. No difference was observed in FCR during the first week and last 15 days of experiment. The mortality rate of chickens was not affected by dietary treatments and was lower than 7% in all groups.

Table 4.4. Growth performance of broilers in different treatment groups.

Parameter	Treatments[1]				SEM[2]
	CTL	VIRG	LXOS	HXOS	
BW (g)					
Day 0	38.9	38.5	40.0	39.6	4.2
Day 7	261.5	254.4	269.7	259.0	18.3
Day 21	733.6	753.0	728.2	741.5	29.1
Day 35	1892.0	1909.0	1891.2	1914.7	26.7
FCR					
Day 0 to 7	0.95	0.97	1.00	1.02	0.081
Day 7 to 21	1.77[a]	1.61[b]	1.75[a]	1.62[b]	0.044
Day 21 to 35	2.14	2.09	2.17	2.13	0.078
Survival (%)	93.3	93.3	96.6	93.3	1.0

[1] CTL: Control diet without any antibiotic or prebiotic; VIRG: Control diet supplemented with 16.5 g/ton virginiamycin; LXOS: Control diet supplemented with 1g/kg xylo-oligosaccharides; HXOS: Control diet supplemented with 2g/kg xylo-oligosaccharides

[2] Standard Error of the Mean. Each mean represents 6 replicate cages with 5 broilers per cage

[a,b], Means in the same row with different superscripts differ ($P < 0.05$).

4.5 Discussion

HTS of 16S rRNA gene amplicons has been used more recently to identify functional diversity (Sergeant *et al.*, 2014) or variability (Stanley *et al.*, 2013) of the microbiome in the gut of broiler chickens. However, on the subject of dietary supplementation with XOS or VIRG, previous studies have used either low-resolution bacterial detection techniques (Dumonceaux *et al.*, 2006, Courtin *et al.*, 2008, Lin *et al.*, 2013) or considered an antibiotic mixture, rather than VIRG alone (Danzeisen *et al.*, 2011). In the present study, we used 454 pyrosequencing of the V1-V3 region of the 16s rRNA gene to monitor the ileal and cecal microbiota of a large number of individual chickens fed either a sub-therapeutic level of VIRG or one of two levels of XOS over a 5 weeks production cycle. VIRG is one of the most commonly used in-feed antibiotics in the poultry industry for disease prevention and growth promotion. Our results indicate that VIRG and XOS differentially modified the proportion of specific OTUs and these changes were associated with cecal acetate and propionate production.

Based on the phylogenetic diversity of bacterial communities and number of observed OTUs, we concluded that the VIRG inclusion (16.5 g/ton) did not change the chicken ileal and cecal bacterial community membership. Similar results have been previously reported in chickens (Gong *et al.*, 2008, Danzeisen *et al.*, 2011) and swine gut microbiota following treatments with in-feed antibiotics (Kim *et al.*, 2012, Holman & Chénier, 2014, Looft *et al.*, 2014). However, VIRG treatment significantly altered relative abundance of certain taxa in the ileum whereas no effect was observed on the cecal microbial composition. This observation is in accordance with the study of Dumonceaux *et al.* (2006) who reported that virginiamycin addition (20 g/ton) altered the chicken gut microbiota most significantly in the upper intestinal tract. In contrast, Danzeisen *et al.* (2011) described a number of changes in the proportion of taxa including a reduction in lactobacilli

and an increase in *Escherichia coli* in the cecal contents of chickens exposed to a mixture of monensin (110 g/ton) with virginiamycin (15 g/ton) or tylosin (20 g/ton). This discrepancy is likely due to the higher dose of antibiotic used in their study. Interestingly, lactobacilli was not identified as a biomarker of VIRG treatment in our study, although it is generally considered to be reduced with antibiotics (Guban *et al.*, 2006, Danzeisen *et al.*, 2011, Lin *et al.*, 2013). The relatively low levels of antibiotics used in Canadian poultry industry and in the present study, may be responsible for the lack of significant changes in the gut bacterial community membership or the cecal microbial composition in the current study.

We identified two genera of bacteria as being linked to VIRG treatment, namely *Corynebacterium* and *Propionibacterium*. Dumonceaux *et al.* (2006) also observed an increase in *Corynebacterium glutamicum* in the proximal intestinal of virginiamycin-treated chickens, using quantitative PCR method. Under anaerobic conditions, *Corynebacterium glutamicum* catabolizes different carbohydrates and produces organic acids such as lactate and succinate (Okino *et al.*, 2005). The genus *Propionibacterium* is a gram-positive bacterium with a unique ability to produce propionate. In our study, the propionate concentration was lower than the limit of detection in the ileum. However, a marked increase in cecal propionate concentration was observed as a result of the VIRG treatment and was positively correlated with the change in the relative abundance of *Propionibacterium* in the ileum. The immunomodulatory effects of selected strains of *Propionibacterium* such as *P. freudenreichii* and *P. acidipropionici* have been established in humans and animals (Jan *et al.*, 2002, Kekkonen *et al.*, 2008, Foligné *et al.*, 2010, Cousin *et al.*, 2012). *Propionibacterium* species are also able to bind to aflatoxin B1 and reduce its intestinal absorption in chickens (El-Nezami *et al.*, 2000). Aflatoxin B1 is a major food contaminant in poultry production that depresses growth performance. At this point, it is unclear whether the

improved feed efficiency in VIRG fed chickens is related to the increased relative abundance of *Corynebacterium* and *Propionibacterium* or not. We hypothesize that these genera may contribute to reported growth promoting functions of antibiotic.

While XOS are not digestible by gastrointestinal digestive enzymes, they can be fermented by the gut microbiota, producing SCFA and lactate (Kabel *et al.*, 2002). Previous studies on humans (Chung *et al.*, 2007), rats (Campbell *et al.*, 1997, Hsu *et al.*, 2004) and chickens (Courtin *et al.*, 2008) have analyzed cultivable members of the fecal and cecal microbiota and found that XOS is effective in promoting intestinal health by encouraging the growth of beneficial bacterial species. Our results demonstrate that the cecal microbiota of HXOS-fed chickens contained significantly higher proportions of the genus *Lactobacillus* than the other dietary treatments. Several strains of *Lactobacillus* have been identified as functional probiotics with associated anti-inflammatory and antimicrobial activities. *In vitro* fermentation of XOS by *Lactobacillus brevis* and *L. fermentum* has been previously reported (Moura *et al.*, 2007). Lactate produced by *Lactobacillus* species, is rarely accumulated and is mostly converted to butyrate and acetate as shown by *in vitro* studies (Elferink *et al.*, 2001, Duncan *et al.*, 2004). This was substantiated in the current study by the increased production of acetate after HXOS supplementation in comparison with the VIRG and LXOS groups.

The average number of high-quality sequencing reads obtained per sample in this study was higher than previously published studies of the chicken gut microbiota (Danzeisen *et al.*, 2011, Stanley *et al.*, 2013, Sergeant *et al.*, 2014). This has enabled us to provide a more comprehensive view about the ileal and cecal microbiota composition and structure. The ileal microbiota was mainly composed of *Firmicutes* (>85%) and within this phylum the majority belonged to the *Lactobacillus* genus, a finding that is consistent with previous 16S rRNA gene based studies (Gong

et al., 2007, Sekelja *et al.*, 2012). In addition to *Lactobacillus*, an unknown genus in the *Clostridiaceae* family was reported to be dominant in the ileum (Sekelja *et al.*, 2012). This was identified as the genus *Candidatus Arthromitus* in our study. At the genus level, most of the OTUs were classified as, *Oscillospira* and unknown genera of the *Lachnospiraceae* and *Clostridiaceae* families, which was in accordance with the earlier pyrosequencing-based studies (Danzeisen *et al.*, 2011, Stanley *et al.*, 2013).

Furthermore, we found that cecal bacterial diversity increased over time, similar to what has been previously observed in chickens (Wielen *et al.*, 2002, Gong *et al.*, 2008, Danzeisen *et al.*, 2011) and wild bird species (van Dongen *et al.*, 2013). In addition, the relative abundance of certain bacterial families or genera was altered over time in the ileum and cecum of the chickens. For example, the relative abundance of the genus *Enterococcus* declined whereas *Faecalibacterium* and *Clostridium* increased in the cecum with increasing age. It was also noted that *Enterobacteriaceae*, a family that comprises many known pathogens such as *Salmonella*, *Shigella* and *E. coli*, was more abundant in the ileum and cecum of the young birds. Wise and Siragusa (2007) obtained similar results in a qPCR-based study using group-specific 16S rDNA primers. They reported that members of *Enterobacteriaceae* are the most abundant in the cecum at day 7 but being replaced by obligate anaerobe sequences by day 14 and 21. The ileal bacterial community appeared to be more stable than the cecum, a finding that was similar to the observation of Lu *et al.* (2003). Bird age, as evident from this study and others (Gong *et al.*, 2008, Danzeisen *et al.*, 2011), had a higher impact on gut microbiota as compared to dietary treatments.

Taken together, this study indicates that dietary prebiotic or sub-therapeutic antibiotic supplementation modulated the relative abundance of specific bacteria without changing the overall microbial structure. We showed that bacterial community clustering was mainly due to the

sample location and the age of the birds rather than dietary supplementation. Increased population levels of lactate-producing bacteria and elevated cecal acetate concentrations in chickens fed HXOS might be an intestinal health-promoting attribute and may contribute to improved feed efficiency during the growth period.

4.6 Acknowledgements

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4.7 References

Aachary AA & Prapulla SG (2011) Xylooligosaccharides (XOS) as an emerging prebiotic: microbial synthesis, utilization, structural characterization, bioactive properties, and applications. *Comprehensive Reviews in Food Science and Food Safety* **10**: 2-16.

Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, Liu H, Cross JR, Pfeffer K, Coffer PJ & Rudensky AY (2013) Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* **504**: 451-455.

Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA & Caporaso JG (2012) Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nature Methods* **10**: 57-59.

Broekaert WF, Courtin CM, Verbeke K, Van de Wiele T, Verstraete W & Delcour JA (2011) Prebiotic and other health-related effects of cereal-derived arabinoxylans, arabinoxylan-oligosaccharides, and xylooligosaccharides. *Critical Reviews in Food Science and Nutrition* **51**: 178-194.

Campbell JM, Fahey GC & Wolf BW (1997) Selected indigestible oligosaccharides affect large bowel mass, cecal and fecal short-chain fatty acids, pH and microflora in rats. *The Journal of Nutrition* **127**: 130-136.

Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL & Knight R (2010) PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* **26**: 266-267.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK & Gordon JI (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**: 335-336.

Casewell M, Friis C, Marco E, McMullin P & Phillips I (2003) The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *Journal of Antimicrobial Chemotherapy* **52**: 159-161.

Castanon J (2007) History of the use of antibiotic as growth promoters in European poultry feeds. *Poultry Science* **86**: 2466-2471.

Chung Y-C, Hsu C-K, Ko C-Y & Chan Y-C (2007) Dietary intake of xylooligosaccharides improves the intestinal microbiota, fecal moisture, and pH value in the elderly. *Nutrition Research* **27**: 756-761.

Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. *Australian Journal of Ecology* **18**: 117-143.

Courtin CM, Swennen K, Broekaert WF, Swennen Q, Buyse J, Decuypere E, Michiels CW, Ketelaere BD & Delcour JA (2008) Effects of dietary inclusion of xylooligosaccharides, arabinoxylooligoscaccharides and soluble arabinoxylan on the microbial composition of caecal contents of chickens. *Journal of the Science of Food and Agriculture* **88**: 2517-2522.

Cousin FJ, Jouan-Lanhouet S, Dimanche-Boitrel M-T, Corcos L & Jan G (2012) Milk fermented by Propionibacterium freudenreichii induces apoptosis of HGT-1 human gastric cancer cells. *PloS One* **7**: e31892.

Danzeisen JL, Kim HB, Isaacson RE, Tu ZJ & Johnson TJ (2011) Modulations of the chicken cecal microbiome and metagenome in response to anticoccidial and growth promoter treatment. *PLoS One* **6**: e27949.

Dibner J & Richards J (2005) Antibiotic growth promoters in agriculture: history and mode of action. *Poultry Science* **84**: 634-643.

Dumonceaux TJ, Hill JE, Hemmingsen SM & Van Kessel AG (2006) Characterization of intestinal microbiota and response to dietary virginiamycin supplementation in the broiler chicken. *Applied and Environmental Microbiology* **72**: 2815-2823.

Duncan SH, Louis P & Flint HJ (2004) Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. *Applied and Environmental Microbiology* **70**: 5810-5817.

Edgar RC, Haas BJ, Clemente JC, Quince C & Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**: 2194-2200.

El-Nezami H, Mykkanen H, Kankaanpaa P, Salminen S & Ahokas J (2000) Ability of Lactobacillus and Propionibacterium strains to remove aflatoxin B1 from the chicken duodenum. *Journal of Food Protection* **63**: 549-552.

Elferink SJO, Krooneman J, Gottschal JC, Spoelstra SF, Faber F & Driehuis F (2001) Anaerobic conversion of lactic acid to acetic acid and 1, 2-propanediol by Lactobacillus buchneri. *Applied and Environmental Microbiology* **67**: 125-132.

Foligné B, Deutsch S-M, Breton J, Cousin FJ, Dewulf J, Samson M, Pot B & Jan G (2010) Promising immunomodulatory effects of selected strains of dairy propionibacteria as evidenced in vitro and in vivo. *Applied and Environmental Microbiology* **76**: 8259-8264.

Fu L, Niu B, Zhu Z, Wu S & Li W (2012) CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics* **28**: 3150-3152.

Gong J, Yu H, Liu T, Gill J, Chambers J, Wheatcroft R & Sabour P (2008) Effects of zinc bacitracin, bird age and access to range on bacterial microbiota in the ileum and caeca of broiler chickens. *Journal of Applied Microbiology* **104**: 1372-1382.

Gong J, Si W, Forster RJ, Huang R, Yu H, Yin Y, Yang C & Han Y (2007) 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca. *FEMS Microbiology Ecology* **59**: 147-157.

Guban J, Korver D, Allison G & Tannock G (2006) Relationship of dietary antimicrobial drug administration with broiler performance, decreased population levels of Lactobacillus salivarius, and reduced bile salt deconjugation in the ileum of broiler chickens. *Poultry Science* **85**: 2186-2194.

Gullón P, Moura P, Esteves MaP, Girio FM, Domínguez H & Parajó JC (2008) Assessment on the fermentability of xylooligosaccharides from rice husks by probiotic bacteria. *Journal of Agricultural and Food Chemistry* **56**: 7482-7487.

Holman DB & Chénier MR (2014) Temporal changes and the effect of subtherapeutic concentrations of antibiotics in the gut microbiota of swine. *FEMS Microbiology Ecology* **90**: 599-608.

Hsu C-K, Liao J-W, Chung Y-C, Hsieh C-P & Chan Y-C (2004) Xylooligosaccharides and fructooligosaccharides affect the intestinal microbiota and precancerous colonic lesion development in rats. *The Journal of Nutrition* **134**: 1523-1528.

Jan G, Belzacq A, Haouzi D, Rouault A, Metivier D, Kroemer G & Brenner C (2002) Propionibacteria induce apoptosis of colorectal carcinoma cells via short-chain fatty acids acting on mitochondria. *Cell Death and Differentiation* **9**: 179-188.

Kabel MA, Kortenoeven L, Schols HA & Voragen AG (2002) In vitro fermentability of differently substituted xylo-oligosaccharides. *Journal of Agricultural and Food Chemistry* **50**: 6205-6210.

Kekkonen RA, Lummela N, Karjalainen H, Latvala S, Tynkkynen S, Järvenpää S, Kautiainen H, Julkunen I, Vapaatalo H & Korpela R (2008) Probiotic intervention has strain-specific anti-inflammatory effects in healthy adults. *World Journal of Gastroenterology* **14**: 2029- 2036.

Kim HB, Borewicz K, White BA, Singer RS, Sreevatsan S, Tu ZJ & Isaacson RE (2012) Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin. *Proceedings of the National Academy of Sciences* **109**: 15485-15490.

Kim M, Morrison M & Yu Z (2011) Evaluation of different partial 16S rRNA gene sequence regions for phylogenetic analysis of microbiomes. *Journal of Microbiological Methods* **84**: 81-87.

Kondepudi KK, Ambalam P, Nilsson I, Wadström T & Ljungh Å (2012) Prebiotic-non-digestible oligosaccharides preference of probiotic bifidobacteria and antimicrobial activity against Clostridium difficile. *Anaerobe* **18**: 489-497.

Lin J, Hunkapiller AA, Layton AC, Chang Y-J & Robbins KR (2013) Response of intestinal microbiota to antibiotic growth promoters in chickens. *Foodborne Pathogens and Disease* **10**: 331-337.

Looft T, Allen HK, Casey TA, Alt DP & Stanton TB (2014) Carbadox has both temporary and lasting effects on the swine gut microbiota. *Antimicrobials, Resistance and Chemotherapy* **5**: 276.

Lu J, Idris U, Harmon B, Hofacre C, Maurer JJ & Lee MD (2003) Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Applied and Environmental Microbiology* **69**: 6816-6824.

Madhukumar M & Muralikrishna G (2012) Fermentation of xylo-oligosaccharides obtained from wheat bran and Bengal gram husk by lactic acid bacteria and bifidobacteria. *Journal of Food Science and Technology* **49**: 745-752.

Mäkeläinen H, Saarinen M, Stowell J, Rautonen N & Ouwehand A (2010) Xylo-oligosaccharides and lactitol promote the growth of Bifidobacterium lactis and Lactobacillus species in pure cultures. *Beneficial Microbes* **1**: 139-148.

Maron DF, Smith TJ & Nachman KE (2013) Restrictions on antimicrobial use in food animal production: an international regulatory and economic survey. *Global Health* **9**: 48.

Moura P, Barata R, Carvalheiro F, Gírio F, Loureiro-Dias MC & Esteves MP (2007) In vitro fermentation of xylo-oligosaccharides from corn cobs autohydrolysis by Bifidobacterium and Lactobacillus strains. *LWT-Food Science and Technology* **40**: 963-972.

Oakley BB, Lillehoj HS, Kogut MH, Kim WK, Maurer JJ, Pedroso A, Lee MD, Collett SR, Johnson TJ & Cox NA (2014) The chicken gastrointestinal microbiome. *FEMS Microbiology Letters* **360**: 100-112.

Okino S, Inui M & Yukawa H (2005) Production of organic acids by Corynebacterium glutamicum under oxygen deprivation. *Applied Microbiology and Biotechnology* **68**: 475-480.

Pourabedin M, Xu Z, Baurhoo B, Chevaux E & Zhao X (2014) Effects of mannan oligosaccharide and virginiamycin on the cecal microbial community and intestinal morphology of chickens raised under suboptimal conditions. *Canadian Journal of Microbiology* **60**: 255-266.

Reeder J & Knight R (2010) Rapid denoising of pyrosequencing amplicon data: exploiting the rank-abundance distribution. *Nature Methods* **7**: 668.

Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS & Huttenhower C (2011) Metagenomic biomarker discovery and explanation. *Genome Biology* **12**: R60.

Sekelja M, Rud I, Knutsen S, Denstadli V, Westereng B, Næs T & Rudi K (2012) Abrupt temporal fluctuations in the chicken fecal microbiota are explained by its gastrointestinal origin. *Applied and Environmental Microbiology* **78**: 2941-2948.

Sergeant MJ, Constantinidou C, Cogan TA, Bedford MR, Penn CW & Pallen MJ (2014) Extensive microbial and functional diversity within the chicken cecal microbiome. *PloS One* **9**: e91941.

Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN & Garrett WS (2013) The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* **341**: 569-573.

Stanley D, Geier MS, Hughes RJ, Denman SE & Moore RJ (2013) Highly variable microbiota development in the chicken gastrointestinal tract. *Plos One* **8**: e84290.

van Dongen WF, White J, Brandl HB, Moodley Y, Merkling T, Leclaire S, Blanchard P, Danchin É, Hatch SA & Wagner RH (2013) Age-related differences in the cloacal microbiota of a wild bird species. *BMC Ecology* **13**: 11.

Wang Q, Garrity GM, Tiedje JM & Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73**: 5261-5267.

Wielen P, Keuzenkamp D, Lipman L, Knapen F & Biesterveld S (2002) Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. *Microbial Ecology* **44**: 286-293.

Wise M & Siragusa G (2007) Quantitative analysis of the intestinal bacterial community in one- to three-week-old commercially reared broiler chickens fed conventional or antibiotic-free vegetable-based diets. *Journal of Applied Microbiology* **102**: 1138-1149.

Connecting Text

In Chapters 3 and 4, we identified changes in the chicken gut microbiota following treatments with MOS or XOS, respectively. We noted that microbial alteration was associated with changes in intestinal morphological structure, and cecal SCFA production. In Chapter 5, we would like to compare the effectiveness of these two prebiotics against *Salmonella* Enteritidis colonization. We were also interested to evaluate whether and how MOS and XOS alter inflammation responses in young chickens (1 wk) after infection. We used 454 pyrosequencing to assess changes in the cecal microbiota of chickens challenged with *S.* Enteritidis at 5 days of age, in the presence or absence of MOS or XOS. Relative expression levels of several cytokines were measured using qPCR.

Chapter 5. Mannan-oligosaccharides and Xylo-oligosaccharides modulated cecal microbiota and expression of inflammatory related cytokines and reduced cecal *Salmonella* Enteritidis colonization in young chickens

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5.1 Abstract

Salmonella Enteritidis is a foodborne pathogen, which causes enteritis in human, mostly due to consumption of contaminated poultry products. This pathogen infects chickens, particularly at an early age, and often persists long after without showing detrimental effects. This study was designed to address the impact of two potential prebiotics, mannan-oligosaccharide (MOS) and xylo-oligosaccharide (XOS), and virginiamycin (VIRG) as an antibiotic growth promoter, on the cecal microbiota and inflammatory related cytokines in chickens infected with *S.* Enteritidis at 5 days of age. Newly hatched chicks (n=150) were randomly assigned to 1 of 5 groups: (1) uninfected control; (2) infected control; (3) infected + XOS; (4) infected + MOS; and (5) infected + VIRG. The number of *S.* Enteritidis recovered from the cecum was significantly lower, by 1.6 log, in the MOS, and to a less extent (1.0 log) in the XOS-fed birds compared to the infected control. Using 16S rRNA gene pyrosequencing, we found that genera *Clostridium*, *Lactobacillus*, and *Roseburia* were increased in response to XOS, whereas MOS significantly enriched *Coprococcus*, *Ruminococcus*, and *Enterococcus*. VIRG did not alter cecal counts of *S.* Enteritidis or any other microbial abundance. Real-time qPCR data showed that MOS, but not XOS, lessened the increase of IFN-γ and TNF-α in cecal tonsils post challenge. Thus, MOS and XOS differently changed the relative abundance of specific microbial genera and the immune response during infection, and these changes were reflected in their abilities to reduce *S.* Enteritidis colonization.

5.2 Introduction

Salmonella species cause over 90 million human cases of gastroenteritis globally each year; about 85% of those are foodborne-related (Majowicz *et al.*, 2010). This imposes a substantial health and economic burden. In the United States alone, for example, the total annual cost of foodborne salmonellosis was approximately $3.6 billion in 2013 (U.S. Department of Agriculture-Economic Research Service, 2014). An analysis of the worldwide foodborne outbreaks occurred between 1988 and 2007 indicated that *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) was the most frequent *Salmonella* serovar that causes human diseases (Greig & Ravel, 2009). The majority of *S.* Enteritidis related outbreaks have been associated with consumption of contaminated poultry products. *S.* Enteritidis infect chickens, particularly at an early age, and often persist long after without clinical sign. The pathogen, thereby, can be passed to humans through contaminated eggs and meat. Despite implementation of several biosecurity guidelines and control programs in poultry industries, chickens are still predominant *Salmonella* reservoirs for human infection.

During the last several decades, antibiotics have been used in poultry production to prevent, control, and treat diseases. Furthermore, sub-therapeutic antibiotics are routinely added to chicken feed to promote growth performance (Castanon, 2007). The massive antibiotic use, however, contributes to development of antibiotic resistance in bacteria. Acquisition of resistance genes by foodborne pathogens from other bacteria has become an important threat to public health (Marshall & Levy, 2011). This concern has led the European Union to ban the use of all antibiotic growth promoters (AGPs) in animal feed (Castanon, 2007). Recently, Health Canada and the United States Food and Drug Administration have also asked for voluntary withdrawal of AGPs in food-

producing animals (Kuehn, 2014). In the absence of AGPs, viable alternatives may be required to maintain animal health and improve efficiency of production.

The chicken gastrointestinal tract is colonized by trillions of microorganism, constituting a dynamic ecosystem with a significant impact on metabolism and immune responses (Oakley *et al.*, 2014, Stanley *et al.*, 2014a). In addition, some of these microbes produce a broad range of polysaccharide- and oligosaccharide-degrading enzymes that utilize plant fibers that are otherwise indigestible by host enzymes, resulting in the production of short chain fatty acids (SCFA) as fermentation end-products (Sergeant *et al.*, 2014). These beneficial effects have led to use certain oligosaccharides as prebiotic compounds. A prebiotic is "a nondigestible compound that selectively stimulate growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota and confer(s) health benefits to the host" (Roberfroid et al., 2010). We have previously shown that mannan-oligosaccharides (MOS) (Pourabedin *et al.*, 2014) and xylo-oligosaccharides (XOS) (Pourabedin *et al.*, 2015) were able to induce changes in the gut microbial composition, intestinal morphological structure and cecal SCFA production in broilers. However, whether and how such oligosaccharides reduce Salmonella load in young chicks are not clear. A number of studies used microbial culture methods to evaluate the effects of MOS (Spring *et al.*, 2000, Fernandez *et al.*, 2002, Santos *et al.*, 2013), and XOS (Eeckhaut *et al.*, 2008) on the gut microbiota in *Salmonella*-challenged chickens, but none have used high-throughput sequencing to analyse the gut microbiota in a greater depth. Here, we addressed this issue by using 454 pyrosequencing and studied the impact of dietary supplementation with MOS, XOS or virginiamycin on the cecal microbiota of chickens infected with *S.* Enteritidis at 5 days of age. Furthermore, given the importance of inflammatory responses during *Salmonella* infection,

we also investigated several pro- and anti-inflammatory cytokines following infection and dietary supplementations.

5.3 Material and Methods

5.3.1 Birds, diet and experimental groups

One hundred fifty newly hatched commercial male broiler chickens (Arbor Acres) were obtained from a local hatchery in Shaanxi province of China. Birds were randomly housed in 30 cages at the isolation unit of College of Animal Science and Technology, Northwest Agriculture and Forestry (A&F) University (Yangling, China). Each cage was randomly assigned to 1 of 5 treatments, 1) negative control **(NC**): non-infected chickens fed a typical standard diet; 2) positive control (**PC**): infected chickens fed diet 1; 3) **MOS**: infected chickens fed diet 1 supplemented with 1g MOS/kg; 4) **XOS**: infected chickens fed diet 1 supplemented with 2g XOS/kg; and 5) **VIRG**: infected chickens fed diet 1 supplemented with sub-therapeutic levels of virginiamycin (16.5 g/ton diet). Each treatment had 6 cages with 5 birds in one cage. Chickens had free access to feed and water during the experiment. The main ingredients of the diets were corn and soybean meal, formulated according to the NRC requirement (23% protein and 3029 kcal/kg metabolisable energy). The MOS was supplied as AgriMOS from Lallemand Inc. (Montreal, Canada), whereas XOS was purchased as XOS 95P from Shandong Longlive Bio-Technology Co. (Shandong, China). On a dry weight basis, XOS 95P contains 95.5% (w/w) XOS with degree of polymerization (DP) of 2 to 7, and 70.9% XOS with DP of 2 to 4. All experimental protocols used in this experiment were in accordance with those approved by the Northwest A&F University Institutional Animal Care and Use Committee.

5.3.2 *Salmonella* Enteritidis challenge

A poultry isolate of *Salmonella enterica* serovar Enteritidis, having novobiocin resistance obtained from the China Veterinary Culture Collection Center, was used in this study to infect chickens. *S.* Enteritidis was cultured at 37°C in Luria Bertani (LB) containing 25 μg of novobiocin/ml for 16h with shaking. At 5 days of age, the NC group was moved to a separate isolated room, whereas all chickens in other groups were orally gavaged with 0.2 ml of *S.* Enteritidis (1×10^8 CFU/0.2 ml). Chickens in the NC group were orally received 0.2 mL sterilized phosphate-buffered saline (PBS, pH 7.2) as a placebo.

5.3.3 Sample collection

Six birds per treatment were euthanized by cervical dislocation at 1, 3, and 5 days after inoculation. From each bird, one of the cecum was collected immediately, placed in cryogenic vials, snap-frozen in liquid nitrogen, and stored at −80°C until DNA extraction. Another cecum was placed in a 2 ml tube containing 1 ml of sterile 0.1% peptone water, weighted and homogenized by bead beating. Total genomic DNA was isolated from 220 mg of frozen cecal contents using the QIAamp DNA Stool Mini Kit (QIAGEN, Shanghai, China). The DNA concentration and purity was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA). Approximately 1 cm of ileocecal junction (cecal tonsils) was collected from each bird, placed in RNAlater RNA Stabilization Reagent (QIAGEN, Shanghai, China) and store at −80°C until RNA extraction.

5.3.4 Detection of cecal *Salmonella* colonization

On the same day of sample collection, homogenized cecal samples were serially diluted in sterile PBS, and then plated in triplicate on Brilliant Green Sulfa Agar (BD Diagnostics, Sparks, MD) containing 25 μg of novobiocin/mL. Plates were incubated for at least 24 h at 37°C. The

number of *Salmonella* colony-forming units (CFU) per gram was log-transformed and the average values were used for the statistical analysis.

5.3.5 Quantitative PCR

Total RNA was extracted from cecal tonsil tissues using the TRIzol reagent (TaKaRa, Dalian, China) according to the manufacturer's instruction. The RNA quantity was measured using an ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA). The cDNA was synthesised using 1 μg of total RNA, oligo (dT) primers, and PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer's protocol. The cDNA was used as a PCR template for real-time qPCR quantification of mRNA expression. The qPCR was performed using a Bio-Rad CFX 96™ Real Time Detection System (Bio- Rad Laboratories, USA) and SYBR Green PCR Master Mix (TaKaRa, Dalian, China) in a final volume of 20 μl. Primer sequences can be found in table 5.1. The thermal cycle profile included an initial activation step at 95°C for 30 s, followed by 40 cycles of 95°C for 10 sec (denaturation), 60°C for 20 sec (annealing) and 72°C for 10 sec (extension). All reactions were carried out in triplicate. The expression levels of cytokine genes were normalized to GAPDH expression, which was used as an internal reference gene. Fold change of target genes was determined using the $2^{-\Delta\Delta CT}$ method.

Table 5.1. Primers used for QPCR analyses

Target	Primer	Sequence (5′-3′)
IL-6	F	ATCCCTCCTCGCCAATCT
	R	GGCACTGAAACTCCTGGTCT
IL-10	F	AGCAGATCAAGGAGACGTTC
	R	ATCAGCAGGTACTCCTCGAT
IFN-γ	F	ATCATACTGAGCCAGATTGTTTC
	R	CGCCATCAGGAAGGTTGT
TNF-α	F	TACCCTGTCCCACAACCTG
	R	TGAACTGGGCGGTCATAGA
TGF-β	F	CGGCCGACGATGAGTGGCTC
	R	CGGGGCCCATCTCACAGGGA
GAPDH	F	TGGAGAAACCAGCCAAGTAT
	R	GCATCAAAGGTGGAGGAAT

5.3.6 454 Pyrosequencing

Purified genomic DNA (20 ng/μl) was used as a template to analyze the microbial communities. The V1-V3 region of the 16S rRNA gene was amplified using universal eubacterial primers (27F: AGAGTTTGATCCTGGCTCAG and 533R: TTACCGCGGCTGCTGGCAC). Unique 8 nucleotide sample-specific barcodes and Roche 454 A-adapters were fused to the 5′ end of the forward primer while the B-adapters were added to the 5′ end of the reverse primer. PCR reactions were performed in triplicate by initial denaturation at 94°C for 4 minutes and then 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, followed by a final elongation step at 72°C for 10 minutes. Before sequencing, the DNA concentration of each PCR product was detected using a Quant-iT PicoGreen double-stranded DNA assay (Invitrogen, Germany), and their quality was controlled on an Agilent 2100 bioanalyzer (Agilent, USA). Amplicon pyrosequencing was performed on a Roche Genome Sequencer GS FLX Titanium platform at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

5.3.7 Data processing

Raw sequence data were analyzed using the quantitative insights into microbial ecology (QIIME) v.1.9.0 software package (Caporaso *et al.*, 2010). For assigning sequences to individual samples, sequence reads were demultiplexed according to the specific barcode of each sample. Barcodes and primers were detected and removed, while sequences with barcode and primer mismatches were rejected. Sequences were also excluded if they were not meeting the default QIIME quality criteria. Sequences with an average quality score less than 25 in a sliding window of 50 nucleotides were also discarded. The sequence data were denoised using the *denoise_wrapper.py* command (Reeder & Knight, 2010) within QIIME. The chimeras were identified using the UCHIME method (Edgar *et al.*, 2011) against the GOLD database and removed from further analyses. The remaining quality-filtered reads were clustered *de novo* (97% similarity threshold) into OTUs using the CD-HIT method (Fu *et al.*, 2012), and the most abundant sequence was selected as the OTU representative. The sequence alignment was performed against the Greengenes core set using the PyNAST method (Caporaso *et al.*, 2010). OTUs were taxonomically categorized using the naïve Bayesian RDP classifier (Wang *et al.*, 2007), which was trained on the Greengenes database with a minimum confidence score of 0.8. For downstream analysis, the OTU table was filtered by discarding OTUs that comprised less than 0.005% of all sequences (Bokulich *et al.*, 2012).

Statistical measurements

To compare microbial community structure, weighted and unweighted UniFrac distance matrices were computed using the OTU table and phylogenetic tree information using QIIME. The distance matrices were then served as inputs to plot PCoA. Analysis of similarities (ANOSIM) with 999 permutations was used to detect statistical significances between microbial communities

in different groups. This test measures a value of R, normally scaled from 0 to 1, which is based on the average rank similarity among groups and replicates within each group (Clarke, 1993). R=0 indicates that two groups are similar whereas R=1 shows a perfect separation between groups. Differentially abundant taxa were identified using the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata *et al.*, 2011). The LEfSe algorithm uses the non-parametric factorial Kruskal-Wallis test (α=0.05) to analyze differences between classes (treatments) and the pairwise Wilcoxon test (α=0.05) to check differences among subclasses (time points) within different classes. To evaluate the α-diversity in samples, the rarefaction curves of phylogenetic diversity (PD), Chao1 index and number of observed OTUs were computed using QIIME. To normalize the sequencing depth, the lowest counts among samples were randomly subsampled in each library 1000 times and average values were used to measure diversity indices. Diversity indexes were compared between all groups by a nonparametric t-test using Monte Carlo permutations. *Salmonella* counts between the infected groups were compared using the Dunnett's multiple comparisons test using the GraphPad Prism v6 (La Jolla, CA, USA). The $P < 0.05$ was considered statistically significant.

5.4 Results

5.4.1 Cecal *Salmonella* Enteritidis counts after oral infection

From each bird, one cecum with content was homogenized and plated on the selective media to measure the population number of inoculated *S.* Enteritidis. No S. Enteritidis colony was recovered from the cecum of the uninfected group. One-day after infection, there was no significant difference among dietary treatments. However, at 3 days after infection, *S.* Enteritidis counts were reduced by 1.25 log in chickens fed the MOS diet in comparison with those in the PC group (Figure 5.1). Five days post challenge, the mean count of *S.* Enteritidis in both the MOS and

XOS groups were significantly lower than that in the PC group by 1.62 and 1.01 log, respectively (Figure 5.1). VIRG treatment did not significantly affect colonization of *S.* Enteritidis at any sampling time.

Figure 5.1. Effect of dietary supplementations on cecal *Salmonella* Enteritidis counts
Samples were collected from 1 bird per cage (n = 6/treatment) and plated in triplicate. PC = infected control; XOS: infected + 2 g xylo-oligosaccharides/kg; MOS: infected + 1 g mannan-oligosaccharides/kg; VIRG: infected + 16.5 mg virginiamycin/kg. Asterisks (* $P < 0.05$, ** $P < 0.01$) indicate statistical difference between the treatment group and the PC.

5.4.2 Cecal microbial community analysis

A total of 955,877 sequences of the V1-V3 region of the 16S rRNA gene were obtained from a total of 90 cecal samples. After trimming and removing low quality and chimeric sequences, 843,947 sequences were remained for downstream analyses. The average number of reads retained per sample was 9,377 (±3094 STD), with the median read length of 482 (±86 STD) bases in all samples. After removing low-abundance OTUs (<0.005% of total OTUs), a total of 4,742 OTUs remained for further analyses. The alpha-diversity of a subsampled OTU table was evaluated by calculating the Chao1 index (Figure 5.2), phylogenetic diversity (PD whole tree; Figure 5.3), and the number of observed OTUs (Figure 5.4). Neither the colonization by S. Enteritidis nor dietary supplements significantly changed the alpha-diversity indices of cecal microbiota (Table 5.2).

To estimate β-diversity between cecal microbial communities, weighted and unweighted UniFrac distance matrices were used. Analysis of similarities (ANOSIM) of unweighted UniFrac demonstrated that there was no clear clustering associated with dietary treatments or *S.* Enteritidis colonization (Table 5.2; Figure 5.5). In contrast, when the relative abundance of taxa (weighted UniFrac distance matrix) was take into account, the differences became more distinct; however, the R-value was less than 0.5, indicating the low degree of separation between the groups (Table 5.2). A recognizable β-diversity clustering of the samples at 3 and 5 days after infection was visually confirmed by PCoA analysis based on weighted UniFrac distance matrices (Figure 5.6). The LEfSe method was used to detect the most differentially affected taxa (LDA>2) between the groups. The relative abundance of *Ruminococcus*, *Coprococcus* and *Lachnospiraceae* were higher in the uninfected group (NC) compared to non-supplemented infected group (PC) (Figure 5.7A). When all the infected groups were compared, LEFSe indicated the enrichment of genera *Lactobacillus*, *Roseburia*, and *Clostridium* in birds fed XOS, and increases in *Ruminococcus*,

Coprococcus, and *Enterococcus* species in the MOS-treated group (Figure 5.7B). VIRG treatment did not affect the relative abundance of bacterial taxa in the cecum.

Table 5.2. Cecal microbial diversity and abundance estimates (means ± standard deviation) across chicken groups at each sampling time

Group	Chao1	Observed species	PD* whole tree
1 day after inoculation			
NC	1575.1 ± 233	530.2 ± 77	28.7 ± 4.0
PC	1861.0 ± 278	604.6 ± 80	34.9 ± 3.2
XOS	1771.8 ± 332	597.6 ± 107	34.1 ± 4.8
MOS	1633.1 ± 668	543.0 ± 198	29.9 ± 10.4
VIRG	1713.2 ± 285	579.3 ± 90	33.0 ± 5.8
3 days after inoculation			
NC	1359.0 ± 254	543.3 ± 82.4	29.1 ± 4.1
PC	1207.5 ± 362	480.0 ± 73.2	26.5 ± 2.9
XOS	1635.7 ± 436	603.1 ± 112.7	34.2 ± 5.7
MOS	1548.8 ± 359	536.5 ± 87.2	32.6 ± 4.8
VIRG	1474.8 ± 213	557.1 ± 74.9	34.1 ± 4.0
5 days after inoculation			
NC	1389.6 ± 465	512.1 ± 101	31.5 ± 6.3
PC	1153.5 ± 676	435.2 ± 232	29.3 ± 13.2
XOS	2081.5 ± 282	726.7 ± 55	45.0 ± 7.3
MOS	1541.4 ± 654	533.5 ± 181	34.8 ± 10.4
VIRG	1793.8 ± 590	616.1 ± 175	39.2 ± 8.5

Note: Indices were calculated at 7000 sequences per sample. The Chao 1 index estimates species richness, Observed OTUs is the number of unique OUT within a sample. "Phylogenetic Diversity (PD) whole tree" is the sum of the branch length connecting all taxa present in the cecal microbiota. No significant differences ($p>0.05$) were noted between any groups. NC= uninfected control; PC = infected control; XOS = infected + 2 g xylo-oligosaccharides/kg; MOS = infected + 1 g mannan-oligosaccharides/kg; VIRG = infected + 16.5 mg virginiamycin/kg.

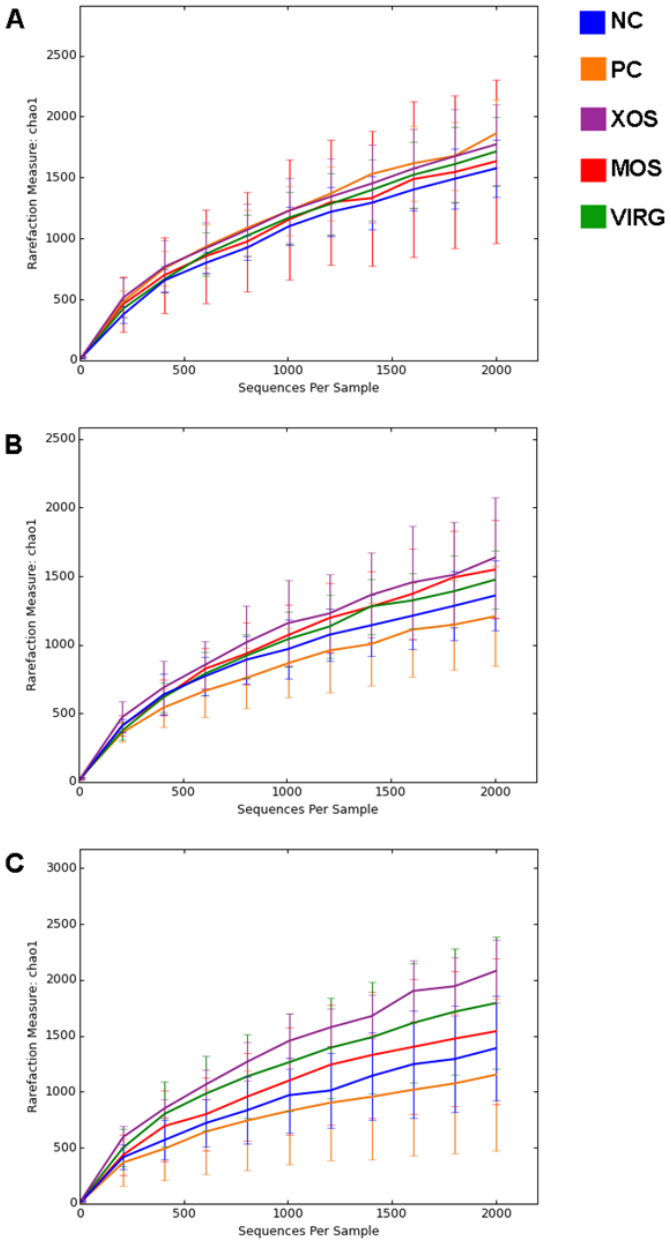

Figure 5.2. Rarefaction curves for Chao1 diversity index

Samples were collected from 1 bird per cage (*n* = 6/treatment) at 1 (**A**), 3 (**B**) and 5 (**C**) days after inoculation. Rarefaction curves calculated at the lowest subsample size of 2,000 sequences per sample. Error bars show standard deviation for each category. NC= uninfected control; PC= infected control; XOS= infected + Xylo-oligosaccharides; MOS= infected + Mannan-oligosaccharides; and VIRG= infected + virginiamycin

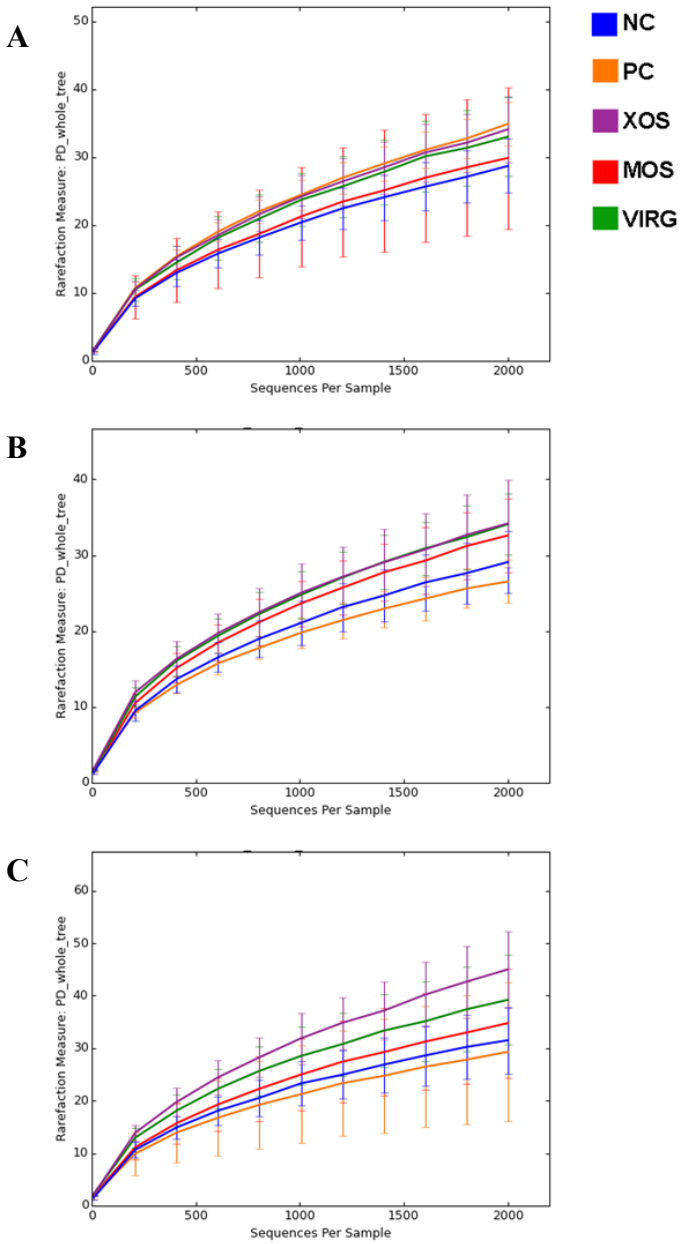

Figure 5.3. Rarefaction curves for phylogenetic diversity (PD)

Samples were collected from 1 bird per cage (n = 6/treatment) at 1 (**A**), 3 (**B**) and 5 (**C**) days after inoculation. Rarefaction curves calculated at the lowest subsample size of 2,000 sequences per sample. Error bars show standard deviation for each category. NC= uninfected control; PC= infected control; XOS= infected + Xylo-oligosaccharides; MOS= infected + Mannan-oligosaccharides; and VIRG= infected + virginiamycin

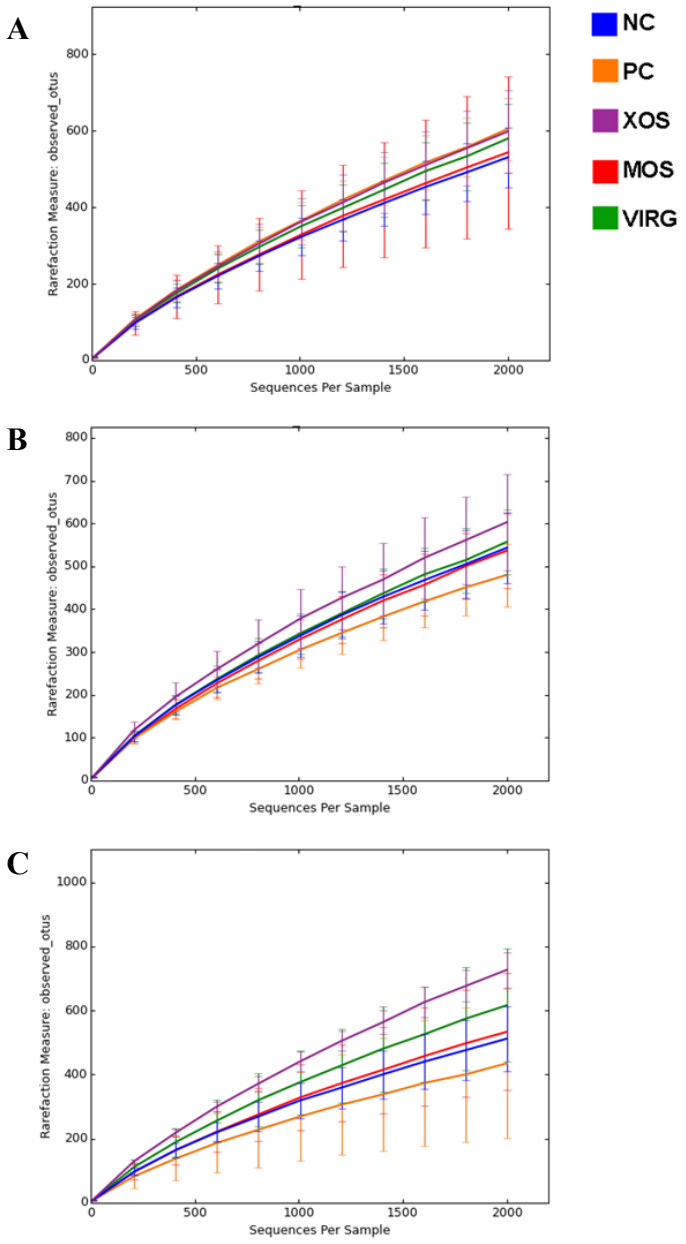

Figure 5.4. Rarefaction curves for the number of observed OTUs

Samples were collected from 1 bird per cage (*n* = 6/treatment) at 1 (**A**), 3 (**B**) and 5 (**C**) days after inoculation. Rarefaction curves calculated at the lowest subsample size of 2,000 sequences per sample. Error bars show standard deviation for each category. NC= uninfected control; PC= infected control; XOS= infected + Xylo-oligosaccharides; MOS= infected + Mannan-oligosaccharides; and VIRG= infected + virginiamycin

Table 5.3. Analysis of similarities (ANOSIM) of weighted and unweighted UniFrac distances

Sampling time	Unweighted UniFrac distance		Weighted UniFrac distance	
	R-value	*P*-value	R-value	*P*-value
1 day post infection	0.086	0.067	0. 08	0.751
3 days post infection	0.087	0.075	0.295	**0.006**
5 days post infection	0.002	0.441	0.271	**0.005**

Note: ANOSIM measures a value of R, normally scaled from 0 to 1, which is based on the average rank similarity among groups and replicates within each group. R=0 indicates that two groups are similar whereas R=1 shows a perfect separation between groups. Statistically significant *P*-values are in bold

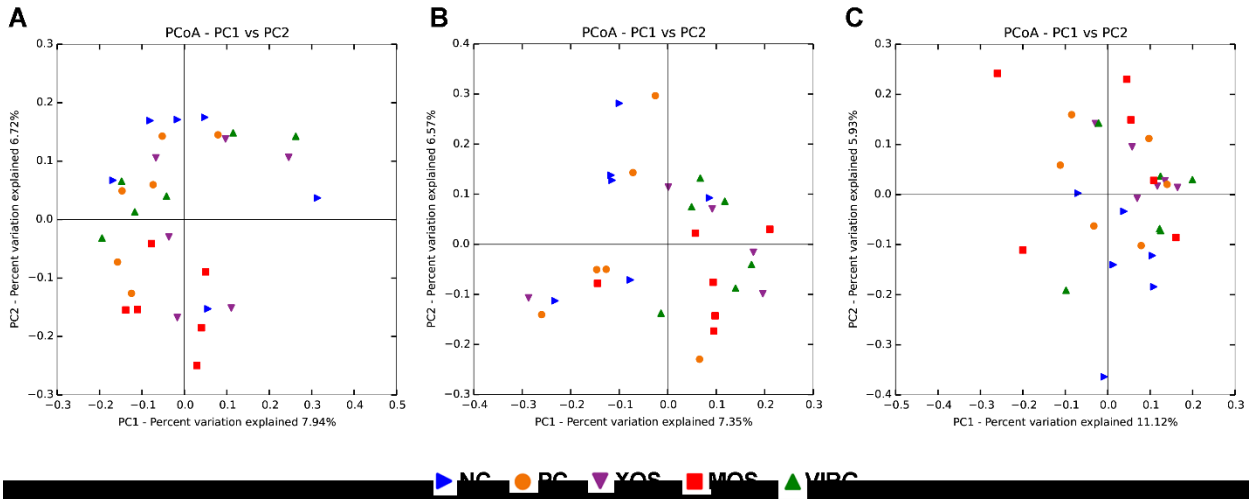

Figure 5.5. Principal Component Analysis of the cecal microbiota based on unweighted Unifrac distance.

Each data point shows a cecal sample collected at 1 (**A**), 3 (**B**) and 5 (**C**) days post inoculation with PBS or *S.* Enteritidis. NC: non-infected chickens fed control diet; PC: infected chickens fed control diet; MOS: infected chickens fed control diet supplemented with 1g MOS/kg; XOS: infected chickens fed control diet supplemented with 2g XOS/kg; VIRG: infected chickens fed control diet supplemented with 16.5 g virginiamycin /ton diet.

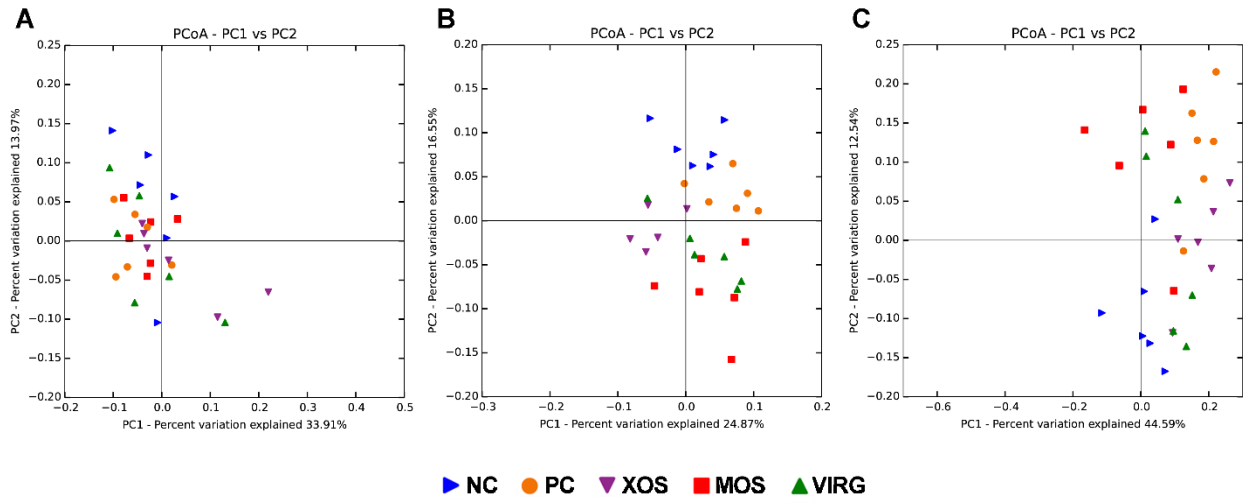

Figure 5.6. Principal Component Analysis of the cecal microbiota based on weighted Unifrac distance.

Each data point shows a cecal sample collected at 1 (**A**), 3 (**B**) and 5 (**C**) days post inoculation with PBS or *S.* Enteritidis. NC: non-infected chickens fed control diet; PC: infected chickens fed control diet; MOS: infected chickens fed control diet supplemented with 1g MOS/kg; XOS: infected chickens fed control diet supplemented with 2g XOS/kg; VIRG: infected chickens fed control diet supplemented with 16.5 g virginiamycin /ton diet.

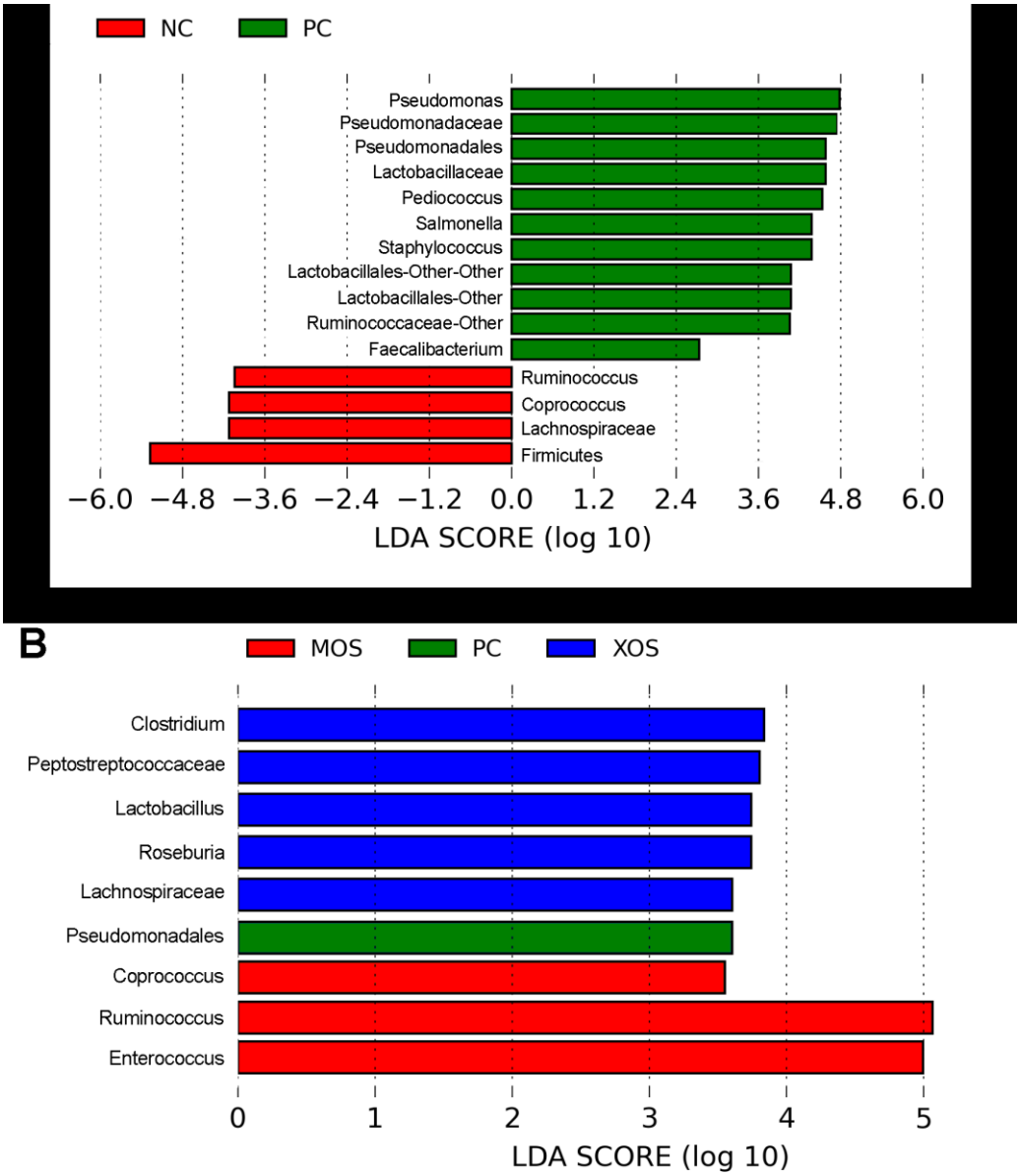

Figure 5.7. Linear discriminant analysis (LDA) effect size (LEfSe).

Panel **A** shows phylotypes that were differentially abundant comparing the cecal microbiome of birds fed the control diet and inoculated with either PBS (NC) or Salmonella Enteritidis (PC). Panel **B** shows bacterial taxa of cecal microbiome responding to dietary supplementation with either MOS or XOS in the S. Enteritidis infected birds. The cut-off value of LDA analysis was 2.0 or higher.

5.4.3 Cytokines gene expression in cecal tonsils

To address whether intestinal immune function was altered by *S.* Enteritidis infection or dietary treatments, we measured the gene expression level of various inflammatory associated cytokines using qPCR method (Figure 5.8). One day after inoculation with either PBS or *S.* Enteritidis, the relative expression of IL-6, tumor necrosis factor (TNF)-α, interferon (IFN)-γ and transforming growth factor (TGF)-β were significantly lowered in the uninfected control compared to that for the infected control (Figure 5.8A). Dietary treatments with XOS, MOS, or VIRG significantly reduced expression of pro-inflammatory cytokines, IL-6 and tumor necrosis factor (TNF)-α compared to that in the PC group at 1 day post challenge (Figure 5.8A). At the same time point, IL-10 expression was significantly increased in the VIRG, while it was reduced in the XOS group compared to the infected non-treated control. The MOS treatment, but not XOS, significantly decreased IFN-γ expression compared to the PC group at 1 and 3 days after infection (Figure 5.8A-B). At day 3 post infection, similar to IFN-γ, the relative expression level of TNF-α was reduced in the MOS, but not in the XOS group (Figure 5.8B). At 3 and 5 days post S. Enteritidis challenge, no significant change was observed in the level of the IL-6, IL-10 and TGF-β expression between the dietary supplemented groups and the PC (Figure 5.8B-C).

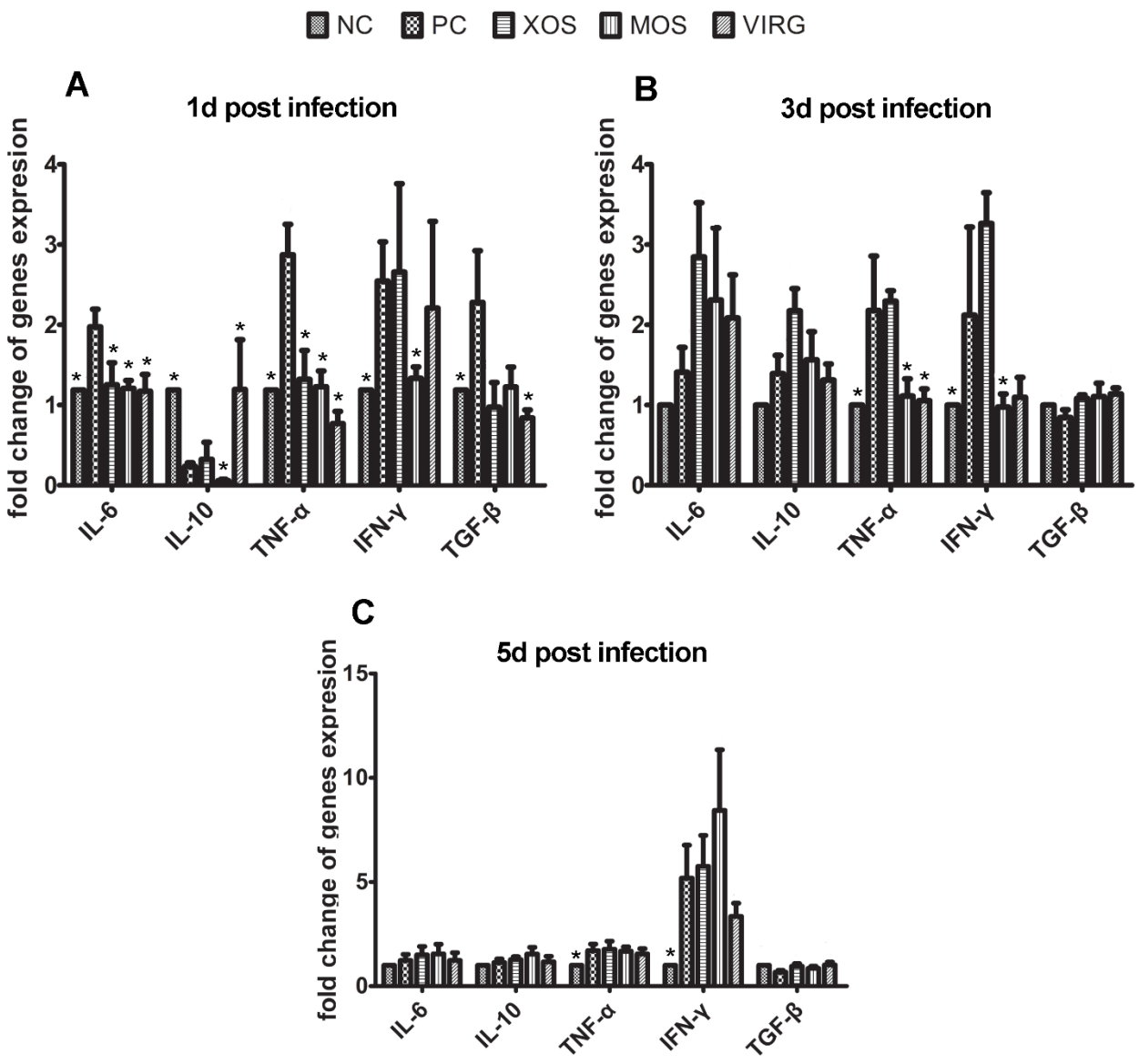

Figure 5.8. Relative expression of cytokines in cecal tonsils

Samples were collected from 1 bird per cage (n = 6/treatment) at 1 (**A**), 3 (**B**) and 5 (**C**) days after oral gavage with PBS and fed non-supplemented diet (NC), or after infection with *Salmonella* Enteritidis and fed either non-supplemented diet (PC), or diets supplemented with xylo-oligosaccharides (XOS), mannan oligosaccharides (MOS) or virginiamycin (VIRG). An asterisk indicates a significant difference ($P < 0.05$) compared to the PC group.

5.5 Discussion

The changes in chicken gut microbial diversity and composition induced after *S.* Enteritidis infection are poorly understood. In addition, whether and by which mechanisms antibiotic or prebiotic feed additives ameliorate *Salmonella* colonization are unclear and sometimes controversial. In this study, we used 454 pyrosequencing of V1-V3 region of the 16S rRNA gene and found differences in the relative abundance of certain bacterial taxa in the cecal microbiota of chickens infected with *S.* Enteritidis, and fed diets supplemented with XOS, MOS or sub-therapeutic level of VIRG antibiotic. We found that the number of *S.* Enteritidis recovered from the cecum of chickens was significantly lower by 1.6 log in the MOS-treated group, and to a less extent in the XOS-fed birds compared to the infected group but not treated with prebiotics. The α-diversity indices did not differentiate among all the five groups, indicating that the cecal microbial diversity remained relatively stable even after *S.* Enteritidis colonization. Nevertheless, when comparing the relative abundance of taxa between the uninfected and the infected controls, the genera *Lachnospiraceae*, *Ruminococcus*, and *Coprococcus* were enriched in the uninfected birds and that *Staphylococcus* species, *Lactobacillaceae*, *Pseudomonadales* and *Salmonella* were overrepresented in the infected control. We further indicated that inoculation with *S.* Enteritidis rapidly induced production of both anti- and pro-inflammatory cytokines, which were differentially regulated by dietary treatments.

Comparing the cecal microbiota as a community, we revealed that neither *S.* Enteritidis colonization nor dietary treatments were cable of altering the PD, the Chao1 index (richness) and the number of observed OTUs across all time points. Cross-community comparisons, considering the presence or absence of taxa (unweighted Unifrac method) also indicated similarities between the groups. These findings suggest that MOS, XOS or VIRG antibiotic as well as colonization with

S. Enteritidis may only marginally affect microbial community membership in the cecum. This is consistent with other studies that has shown similar effects in chickens fed different feed additives including AGP (Danzeisen *et al.*, 2011), prebiotic (Pourabedin *et al.*, 2015) or organic acids (Thibodeau *et al.*, 2015), or challenged with *Campylobacter jejuni* (Thibodeau et al., 2015), *S.* Enteritidis (Videnska *et al.*, 2013) or *C. perfringens* (Stanley *et al.*, 2014b). However, considering relative abundance of each OUT within the community, we found significant changes among the groups. These results highlight the importance of not looking at just one parameters of the bacterial community but rather collection of metrics to identify changes in population that can be associated with dietary supplementation or disease state.

Previous works performed studying the effects of prebiotics on the gut microbiota have shown variable results suggesting that different prebiotics may have different modes of action (Pourabedin & Zhao, 2015). Here, we revealed that the genera *Clostridium*, *Lactobacillus,* and *Roseburia* were increased in response to XOS supplementation, whereas MOS significantly enriched sequences classified as *Coprococcus*, *Ruminococcus*, and *Enterococcus*. These results confirm those of recent studies, which have reported that XOS stimulated the growth of *Lactobacillus* (Pourabedin *et al.*, 2015) and butyrate-producing *Clostridium* cluster XIV (De Maesschalck *et al.*, 2015) in 2 to 5 weeks old broilers. Lactate, produced by lactobacilli during XOS fermentation, can be utilized by the butyrate-producing *Anaerostipes* species (De Maesschalck *et al.*, 2015). *Roseburia* species, belong to *Clostridium* cluster XIV, are also important butyrate-producing bacteria, utilizing a variety of polysaccharide substrates of the diet (Duncan *et al.*, 2007, Scott *et al.*, 2011). *Roseburia* species have been found in both human and chicken intestine (Lei *et al.*, 2012). Butyrate provides energy for the epithelial cells, exerts anti-inflammatory properties by inhibiting nuclear factor kappa B (NF-κB) transcriptional activity

(Segain *et al.*, 2000), and demonstrates inhibitory activities against *Salmonella* species in chickens (Van Immerseel *et al.*, 2005). In addition, butyric acid increases production of mucin (Barcelo *et al.*, 2000) and antimicrobial peptides (Sunkara *et al.*, 2011), improving host intestinal defence barrier. The genera *Coprococcus* and *Ruminococcus*, enriched by MOS, are other members of *Clostridium* cluster XIV that contribute to butyrate production. An increase in *Coprococcus* genus was previously observed in the cecum of broilers in response to anticoccidial and growth promoters (Danzeisen *et al.*, 2011). Another abundant taxa enriched by MOS was the genus *Enterococcus*, containing highly adaptable lactic acid bacteria, and commensals of the animal and human GI tract (Gilmore *et al.*, 2014). Many enterococci species such as *E. faecium* produce bacteriocins, generally called enterocins, which has been associated with growth inhibition of food-borne pathogens in the gut (Franz *et al.*, 2007). While we cannot confirm in this study, it is possible that increases in the relative abundance of above-mentioned commensals in MOS or XOS treated chickens reduced *Salmonella* colonization in the cecum or simply contributed to intestinal health.

Our study generally indicated quick and significant changes in expression of various cytokines upon *S.* Enteritidis infection in the cecal tonsils, which was in line with observations of previous authors (Haghighi *et al.*, 2008, Setta *et al.*, 2012). Cecal tonsils were chosen because they are major gut-associated lymphoid tissues in chickens, and responsible for inducing immune responses against enteric pathogens (Bar-Shira *et al.*, 2003). They are located at the proximal ends of the ceca. Similar to Peyer's patches in mammals, the chicken cecal tonsils contains T and B lymphocyte populations, and therefore appears to be critical for maintaining intestinal immune homeostasis (Bar-Shira *et al.*, 2003). Investigating expression of pro- and anti-inflammatory cytokines following treatment with AGP or prebiotic is of great interest to understand the

relationship between microbial modulation and intestinal cell-mediated immunity. In this study, 1day after infection, level of IL-10 expression was enhanced in the antibiotic-treated birds while it was downregulated in MOS-fed chickens compared with the infected control group. The IL-10 is an immunosuppressive cytokine, and its increased level in pathogen challenged chickens has been associated with increased susceptibility to infection (Rothwell *et al.*, 2004). In addition, MOS prevented the development of IFN-γ and TNF-α pro-inflammatory cytokines, which were both highly overexpressed in the infected control group. IFN-γ and TNF-α play an essential role in the early phase of *Salmonella* infection (Nauciel & Espinasse-Maes, 1992). TNF-α, mainly produced by macrophages, regulates innate immune responses, particularly, the maturation dendritic cells (Trevejo *et al.*, 2001). IFN-γ is actively produced by T helper cells and natural killer cells and regarded as a major immune stimulator driving protection against *Salmonella* infection in birds (Crome *et al.*, 2013). A general mechanism by which IFN-γ reduces infection is that it activates macrophages, inducing them to produce nitric oxide (Ma *et al.*, 2003) and major histocompatibility complex class II molecules (Kaspers *et al.*, 1994). In this study, increased mRNA expression of IFN-γ in the cecal tonsils of chickens following *S.* Enteritidis infection confirmed its important functions in defense against *Salmonella*, as shown by other authors (Withanage *et al.*, 2005, Berndt *et al.*, 2007). It has been also demonstrated that treatment of chickens with probiotics decreased IFN-γ expression in cecal tonsils of chickens infected with *S.* Typhimurium compared to the *Salmonella*-infected birds not treated with probiotics (Haghighi *et al.*, 2008). It is likely that the lower cecal colonization by *S.* Enteritidis in MOS-fed birds compared to the infected control triggered less secretion of IFN-γ and TNF-α at 1 and 3 days post challenge. Additionally, we observed that mRNA expression of IL-6 and TNF-α in the XOS group was decreased 1 day after infection compared to that in infected control. Whether this is related to the changes in the relative

abundance of some bacterial taxa such as lactobacilli in the XOS-fed group remains unclear. However, there is reported evidence that different species of *Lactobacillus* differentially activate dendritic cells (Christensen *et al.*, 2002), and modulate cytokine production (Brisbin *et al.*, 2010), regulating innate and adaptive immune responses.

In conclusion, we indicated that MOS and XOS prebiotics provided a protective effect against *S.* Enteritidis in young chickens since their removal in the infected group increased cecal colonization of the pathogen and exaggerated production of inflammatory cytokines. Noticeably, we found that MOS and XOS were differentially capable of changing the relative abundance of specific bacterial taxa in the cecal microbiota. Moreover, the current work indicated that MOS and XOS induced different cytokine expression patterns upon *S.* Enteritidis infection, and the difference was reflected in their abilities to reduce S. Enteritidis colonization. The potential link between changes in microbial abundance and cytokine expression must be further investigated to define underlying mechanisms by which MOS and XOS reduce *S.* Enteritidis colonization in the chicken cecum.

5.6 Acknowledgements

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5.7 References

Bar-Shira E, Sklan D & Friedman A (2003) Establishment of immune competence in the avian GALT during the immediate post-hatch period. *Developmental & Comparative Immunology* **27**: 147-157.

Barcelo A, Claustre J, Moro F, Chayvialle J, Cuber J & Plaisancie P (2000) Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon. *Gut* **46**: 218-224.

Berndt A, Wilhelm A, Jugert C, Pieper J, Sachse K & Methner U (2007) Chicken cecum immune response to Salmonella enterica serovars of different levels of invasiveness. *Infection and Immunity* **75**: 5993-6007.

Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA & Caporaso JG (2012) Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nature Methods* **10**: 57-59.

Brisbin JT, Gong J, Parvizi P & Sharif S (2010) Effects of lactobacilli on cytokine expression by chicken spleen and cecal tonsil cells. *Clinical and Vaccine Immunology* **17**: 1337-1343.

Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL & Knight R (2010) PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* **26**: 266-267.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK & Gordon JI (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**: 335-336.

Castanon J (2007) History of the use of antibiotic as growth promoters in European poultry feeds. *Poultry Science* **86**: 2466-2471.

Christensen HR, Frøkiær H & Pestka JJ (2002) Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. *The Journal of Immunology* **168**: 171-178.

Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. *Australian Journal of Ecology* **18**: 117-143.

Crome SQ, Lang PA, Lang KS & Ohashi PS (2013) Natural killer cells regulate diverse T cell responses. *Trends in Immunology* **34**: 342-349.

Danzeisen JL, Kim HB, Isaacson RE, Tu ZJ & Johnson TJ (2011) Modulations of the chicken cecal microbiome and metagenome in response to anticoccidial and growth promoter treatment. *PLoS One* **6**: e27949.

De Maesschalck C, Eeckhaut V, Maertens L, De Lange L, Marchal L, Nezer C, De Baere S, Croubels S, Daube G & Dewulf J (2015) The effects of xylo-oligosaccharides on performance and microbiota in broiler chickens. *Applied and environmental microbiology* AEM. 01616-01615.

Duncan SH, Belenguer A, Holtrop G, Johnstone AM, Flint HJ & Lobley GE (2007) Reduced dietary intake of carbohydrates by obese subjects results in decreased concentrations of butyrate and butyrate-producing bacteria in feces. *Applied and Environmental Microbiology* **73**: 1073-1078.

Edgar RC, Haas BJ, Clemente JC, Quince C & Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**: 2194-2200.

Eeckhaut V, Van Immerseel F, Dewulf J, Pasmans F, Haesebrouck F, Ducatelle R, Courtin C, Delcour J & Broekaert W (2008) Arabinoxylooligosaccharides from wheat bran inhibit Salmonella colonization in broiler chickens. *Poultry Science* **87**: 2329-2334.

Fernandez F, Hinton M & Gils BV (2002) Dietary mannan-oligosaccharides and their effect on chicken caecal microflora in relation to Salmonella Enteritidis colonization. *Avian Pathology* **31**: 49-58.

Franz CM, Van Belkum MJ, Holzapfel WH, Abriouel H & Gálvez A (2007) Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. *FEMS Microbiology Reviews* **31**: 293-310.

Fu L, Niu B, Zhu Z, Wu S & Li W (2012) CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics* **28**: 3150-3152.

Gilmore MS, Clewell DB, Ike Y, Shankar N, Lebreton F, Willems RJ & Gilmore MS (2014) Enterococcus diversity, origins in nature, and gut colonization. The Massachusetts Eye and Ear Infirmary, Boston, Massachusetts, USA.

Greig J & Ravel A (2009) Analysis of foodborne outbreak data reported internationally for source attribution. *International Journal of Food Microbiology* **130**: 77-87.

Haghighi HR, Abdul-Careem MF, Dara RA, Chambers JR & Sharif S (2008) Cytokine gene expression in chicken cecal tonsils following treatment with probiotics and Salmonella infection. *Veterinary Microbiology* **126**: 225-233.

Kaspers B, Lillehoj H, Jenkins M & Pharr G (1994) Chicken interferon-mediated induction of major histocompatibility complex class II antigens on peripheral blood monocytes. *Veterinary Immunology and Immunopathology* **44**: 71-84.

Kuehn BM (2014) FDA moves to curb antibiotic use in livestock. *JAMA* **311**: 347-348.

Lei F, Yin Y, Wang Y, Deng B, Yu HD, Li L, Xiang C, Wang S, Zhu B & Wang X (2012) Higher-Level Production of Volatile Fatty Acids In vitro by chicken gut microbiotas than by

human gut microbiotas as determined by functional analyses. *Applied and Environmental Microbiology* **78**: 5763-5772.

Ma J, Chen T, Mandelin J, Ceponis A, Miller N, Hukkanen M, Ma G & Konttinen Y (2003) Regulation of macrophage activation. *Cellular and Molecular Life Sciences CMLS* **60**: 2334-2346.

Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A & Hoekstra RM (2010) The global burden of nontyphoidal Salmonella gastroenteritis. *Clinical Infectious Diseases* **50**: 882-889.

Marshall BM & Levy SB (2011) Food animals and antimicrobials: impacts on human health. *Clinical Microbiology Reviews* **24**: 718-733.

Nauciel C & Espinasse-Maes F (1992) Role of gamma interferon and tumor necrosis factor alpha in resistance to Salmonella typhimurium infection. *Infection and Immunity* **60**: 450-454.

Oakley BB, Lillehoj HS, Kogut MH, Kim WK, Maurer JJ, Pedroso A, Lee MD, Collett SR, Johnson TJ & Cox NA (2014) The chicken gastrointestinal microbiome. *FEMS Microbiology Letters* **360**: 100-112.

Pourabedin M & Zhao X (2015) Prebiotics and gut microbiota in chickens. *FEMS Microbiology Letters* **15**: fnv122.

Pourabedin M, Guan L & Zhao X (2015) Xylo-oligosaccharides and virginiamycin differentially modulate gut microbial composition in chickens. *Microbiome* **3**: 1-12.

Pourabedin M, Xu Z, Baurhoo B, Chevaux E & Zhao X (2014) Effects of mannan oligosaccharide and virginiamycin on the cecal microbial community and intestinal morphology of chickens raised under suboptimal conditions. *Canadian Journal of Microbiology* **60**: 255-266.

Reeder J & Knight R (2010) Rapid denoising of pyrosequencing amplicon data: exploiting the rank-abundance distribution. *Nature Methods* **7**: 668.

Roberfroid M, Gibson GR, Hoyles L, McCartney AL, Rastall R, Rowland I, Wolvers D, Watzl B, Szajewska H & Stahl B (2010) Prebiotic effects: metabolic and health benefits. British Journal of Nutrition 104: 1-63.

Rothwell L, Young JR, Zoorob R, Whittaker CA, Hesketh P, Archer A, Smith AL & Kaiser P (2004) Cloning and characterization of chicken IL-10 and its role in the immune response to Eimeria maxima. *The Journal of Immunology* **173**: 2675-2682.

Santos E, Costa F, Silva J, Martins T, Figueiredo-Lima D, Macari M, Oliveira C & Givisiez P (2013) Protective effect of mannan oligosaccharides against early colonization by Salmonella Enteritidis in chicks is improved by higher dietary threonine levels. *Journal of Applied Microbiology* **114**: 1158-1165.

Scott KP, Martin JC, Chassard C, Clerget M, Potrykus J, Campbell G, Mayer C-D, Young P, Rucklidge G & Ramsay AG (2011) Substrate-driven gene expression in Roseburia inulinivorans: importance of inducible enzymes in the utilization of inulin and starch. *Proceedings of the National Academy of Sciences* **108**: 4672-4679.

Segain J, De La Blétiere DR, Bourreille A, Leray V, Gervois N, Rosales C, Ferrier L, Bonnet C, Blottiere H & Galmiche J (2000) Butyrate inhibits inflammatory responses through NFκB inhibition: implications for Crohn's disease. *Gut* **47**: 397-403.

Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS & Huttenhower C (2011) Metagenomic biomarker discovery and explanation. *Genome Biology* **12**: R60.

Sergeant MJ, Constantinidou C, Cogan TA, Bedford MR, Penn CW & Pallen MJ (2014) Extensive microbial and functional diversity within the chicken cecal microbiome. *PloS One* **9**: e91941.

Setta A, Barrow P, Kaiser P & Jones M (2012) Early immune dynamics following infection with Salmonella enterica serovars Enteritidis, Infantis, Pullorum and Gallinarum: cytokine and chemokine gene expression profile and cellular changes of chicken cecal tonsils. *Comparative Immunology, Microbiology and Infectious Diseases* **35**: 397-410.

Spring P, Wenk C, Dawson K & Newman K (2000) The effects of dietary mannaoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of salmonella-challenged broiler chicks. *Poultry Science* **79**: 205-211.

Stanley D, Hughes RJ & Moore RJ (2014a) Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. *Applied Microbiology and Biotechnology* **98**: 4301-4310.

Stanley D, Wu S-B, Rodgers N, Swick RA & Moore RJ (2014b) Differential responses of cecal microbiota to fishmeal, *eimeria* and *Clostridium perfringens* in a necrotic enteritis challenge model in chickens. *PloS One* **9**: e104739.

Sunkara LT, Achanta M, Schreiber NB, Bommineni YR, Dai G, Jiang W, Lamont S, Lillehoj HS, Beker A & Teeter RG (2011) Butyrate enhances disease resistance of chickens by inducing antimicrobial host defense peptide gene expression. *PLoS One* **6**: e27225.

Thibodeau A, Fravalo P, Yergeau É, Arsenault J, Lahaye L & Letellier A (2015) Chicken Caecal Microbiome Modifications Induced by Campylobacter jejuni Colonization and by a Non-Antibiotic Feed Additive. *PloS One* **10**: e0131978.

Trevejo JM, Marino MW, Philpott N, Josien R, Richards EC, Elkon KB & Falck-Pedersen E (2001) TNF-α-dependent maturation of local dendritic cells is critical for activating the adaptive immune response to virus infection. *Proceedings of the National Academy of Sciences* **98**: 12162-12167.

U.S. Department of Agriculture-Economic Research Service (2014) Cost Estimates of Foodborne Illnesses. http://ers.usda.gov/data-products/cost-estimates-of-foodborne-illnesses.aspx.

Van Immerseel F, Boyen F, Gantois I, Timbermont L, Bohez L, Pasmans F, Haesebrouck F & Ducatelle R (2005) Supplementation of coated butyric acid in the feed reduces colonization and shedding of Salmonella in poultry. *Poultry Science* **84**: 1851-1856.

Videnska P, Sisak F, Havlickova H, Faldynova M & Rychlik I (2013) Influence of Salmonella enterica serovar Enteritidis infection on the composition of chicken cecal microbiota. *Bmc Veterinary Research* **9**.

Wang Q, Garrity GM, Tiedje JM & Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73**: 5261-5267.

Withanage G, Wigley P, Kaiser P, Mastroeni P, Brooks H, Powers C, Beal R, Barrow P, Maskell D & McConnell I (2005) Cytokine and chemokine responses associated with clearance of a primary Salmonella enterica serovar Typhimurium infection in the chicken and in protective immunity to rechallenge. *Infection and Immunity* **73**: 5173-5182.

Chapter 6. General Discussion and Conclusions

Since the 1950s, antibiotics at subtherapeutic levels have been added in chicken feed to promote growth and prevent disease. At the same time, antibiotic resistance has been on the rise. Due to concerns over the spread of resistance genes to human pathogens, antibiotic use in animal agriculture is now under much scrutiny. To mitigate the negative consequences of antibiotic removal in poultry industry, viable and cost effective alternatives are needed. Among alternative approaches, use of prebiotics has been received increasing attention in poultry production. Although the exact mechanism(s) by which prebiotics promote health and growth is not clear, it is believed that they are likely influencing the gut microbiota. Thus, it is important to measure the abundance and enormous diversity of the gut microbiota following dietary supplementation with prebiotics. Here, we used DGGE, qPCR and 454 pyrosequencing as well as classical culture-based method to evaluate the impact of MOS and XOS, in comparison with an antibiotic growth promoter, VIRG, or a control non-supplemented diet on the chicken gut microbiota.

In our first study, we evaluated the effect of MOS on the gut microbiota of broilers kept under sub-optimal conditions. DGGE analysis of the cecal microbiota indicated that the bacterial diversity in MOS-fed birds was greater than the control group as measured by the Shannon index. MOS supplementation increased the proportion of *Firmicutes* members, while level of *Bacteroidetes* remained stable. This was coincided with an increase in the length of villi and the number of goblet cells in the small intestine of MOS-fed birds. The chicken intestinal *Firmicutes* consist primarily of members of the *Clostridium* and *Lactobacillus,* which are capable of metabolizing carbohydrates via fermentation to produce SCFA (Pourabedin & Zhao, 2015). The observed improvement in the intestinal morphological structures may be related to the enhanced bacterial fermentation products such as butyrate that can regulate epithelial cell proliferation and

differentiation (Hamer *et al.*, 2008). Further studies are required to define the mechanisms involved in the development of intestinal epithelium.

In our second study, we investigated the effect of two different concentrations of XOS, as a new prebiotic candidate, on the chicken gut microbiota during a 5 weeks production cycle. Intestinal samples were taken from the ileum and cecum at 15, 25 and 35 days of age. The genus *Lactobacillus* was the only differentially abundant taxa that was consistently increased in response to supplementation with 0.2% XOS, but not 0.1%. The microbial communities tended to cluster together by the sampling location and time rather than by dietary supplementations. When we analyzed the impact of XOS on SCFA production, we found that acetate concentration was increased in the cecum of broilers fed the 0.2% XOS supplemented diet.

In our third study, young chickens were infected with *S.* Enteritidis, and were given a diet supplemented with either 0.1% MOS or 0.2% XOS. In contrast to the results observed in the first study, MOS did not cause significant changes in the bacterial diversity indices. This is likely because of increased sampling depth and improved bacterial resolution obtained by pyrosequencing method vs. DGGE. *S.* Enteritidis challenged chickens fed MOS or XOS supplemented diets were found to have distinct cytokine patterns as well as different microbial community structures in terms of OTU abundance. Similar to our findings in the first study, MOS increased the relative abundance of *Firmicutes* members, mainly *Ruminococcus*, *Coprococcus*, and *Enterococcus* species. Although the significance of these species is unclear in the chicken gut microbiota, their important role in fermentation of non-digestible carbohydrates suggests their benefits for the host (Flint *et al.*, 2008). XOS effect on the cecal microbiota was more profound in young challenged chickens compared to the samples that were taken from 3 to 5 weeks old birds in the second study. This may be explained by the increasing stability of the intestinal microbiota

as the birds aged (Kohl, 2012). The genera *Clostridium*, *Lactobacillus*, and *Roseburia* were significantly enriched in XOS-fed chickens. MOS, and to a less extent XOS, were able to reduce *S.* Enteritidis counts in the cecum upon the pathogen inoculation. A reduction in expression of proinflammatory cytokines in MOS- and XOS-fed chickens may also be related to the reduced *S.* Enteritidis colonization that was observed in those groups. An increase in the relative abundance of butyrate-producing bacteria in the cecum of MOS- and XOS-fed chickens could provide colonization resistance to *Salmonella* (Gantois *et al.*, 2006).

6.1 Future direction

The research on responses of the intestinal microbiota to prebiotic supplementation is still limited in terms of extent and depth. More research is required to provide mechanistic insights into health effects of prebiotics. One possible approach to establish the cause-effect relationship would be to compare effects of potential prebiotics in germ-free and conventionalized animals. However, this method may not be appropriate given the fact that lack of gut microbiota in germ-free animals is associated with immature immune responses and altered physiological functions. Another elegant approach would be transfer of gut microbiota from a prebiotic treated animal to a non-prebiotic treated animal. In addition, most associations between observed changes of microbiota and probiotic supplementation remain at levels of phyla and genera. To find out causative changes of microorganisms in the gut, we have to study the microbiota at the species level. At this time, most studies have focused on populations of bifidobacteria and lactobacilli as beneficial bacteria for prebiotics. However, it seems rather simplistic to consider only these genera as beneficial or others such as *Clostridium* and *E.coli* as detrimental. In fact, different strains belonging to other numerous genera may have more profound implications for health than the genera commonly used as probiotics. For instance, *Clostridium butyricum* (Yang et al., 2012) and *Faecalibacterium*

prausnitzii have been identified to be dominant in the gut, (Torok et al., 2011, Oakley et al., 2013) metabolically active and highly beneficial in models of intestinal disorders (Martín et al., 2014, Zhang et al., 2014). Furthermore, various species within the genus *Lactobacillus* may induce different functional changes in immune responses, metabolic activities or epithelial barrier integrity (Kleerebezem & Vaughan, 2009, Wells, 2011). Therefore, it is important for future studies to apply HTS techniques and provide a community-wide analysis of the gut microbiota at different levels of the phylogenetic classification following prebiotic supplementation.

Dietary fibers including prebiotics are metabolized by the gut microbiota to yield an enormous range of metabolites besides SCFA with significant physiological functions. Thus, those other microbial metabolites such as bile acids and polyamines are worthy to be investigated. Furthermore, considering a high variability in gut microbiota of chickens, it is essential in future studies to analyze larger numbers of samples across different populations, from different regions of the gastrointestinal tract and from different geological locations. Use of metagenomics approaches together with metabolite profiling would advance our understanding of the gut microbiota-driven pathways and the role played by prebiotics. These will provide new opportunities for improving gut health and preventing disorders associated with gut microbiota. Finally, how to translate research results to field operation for poultry farms remains nontrivial due to existence of many key differences between poultry research facilities and poultry farms.

6.2 References

Flint HJ, Bayer EA, Rincon MT, Lamed R & White BA (2008) Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nature Reviews Microbiology* **6**: 121-131.

Gantois I, Ducatelle R, Pasmans F, Haesebrouck F, Hautefort I, Thompson A, Hinton J & Van Immerseel F (2006) Butyrate specifically down-regulates Salmonella pathogenicity island 1 gene expression. *Applied and Environmental Microbiology* **72**: 946-949.

Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost F & BRUMMER RJ (2008) Review article: the role of butyrate on colonic function. *Alimentary Pharmacology & Therapeutics* **27**: 104-119.

Kleerebezem M & Vaughan EE (2009) Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity. *Annual Review of Microbiology* **63**: 269-290.

Kohl KD (2012) Diversity and function of the avian gut microbiota. *Journal of Comparative Physiology B* **182**: 591-602.

Martín R, Chain F, Miquel S, Lu J, Gratadoux J-J, Sokol H, Verdu EF, Bercik P, Bermúdez-Humarán LG & Langella P (2014) The commensal bacterium Faecalibacterium prausnitzii is protective in DNBS-induced chronic moderate and severe colitis models. *Inflammatory Bowel Diseases* **20**: 417-430.

Oakley BB, Morales CA, Line J, Berrang ME, Meinersmann RJ, Tillman GE, Wise MG, Siragusa GR, Hiett KL & Seal BS (2013) The poultry-associated microbiome: network analysis and farm-to-fork characterizations. *PloS One* **8**: e57190.

Pourabedin M & Zhao X (2015) Prebiotics and gut microbiota in chickens. *FEMS Microbiology Letters* **15**:fnv122.

Torok VA, Allison GE, Percy NJ, Ophel-Keller K & Hughes RJ (2011) Influence of Antimicrobial Feed Additives on Broiler Commensal Posthatch Gut Microbiota Development and Performance. *Applied and Environmental Microbiology* **77**: 3380-3390.

Wells JM (2011) Immunomodulatory mechanisms of lactobacilli. *Microbial Cell Factories* **10**: S17.

Yang C, Cao G, Ferket P, Liu T, Zhou L, Zhang L, Xiao Y & Chen A (2012) Effects of probiotic, Clostridium butyricum, on growth performance, immune function, and cecal microflora in broiler chickens. *Poultry Science* **91**: 2121-2129.

Zhang L, Cao G, Zeng X, Zhou L, Ferket P, Xiao Y, Chen A & Yang C (2014) Effects of Clostridium butyricum on growth performance, immune function, and cecal microflora in broiler chickens challenged with Escherichia coli K88. *Poultry Science* **93**: 46-53.