Effects of *Bacillus* probiotics and transfer of fecal microbiota on production, health, and gut microbiota of broiler chickens

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Abstract

The rise and spread of antibiotic resistance in pathogens have led to ban on the use of antibiotic growth promoters (AGPs) in chickens. In the absence of AGPs, alternatives are needed to enhance chicken's ability against diseases and maintain efficacy of production. Given the interest in using probiotics, as a traditional means, and fecal microbiota transfer, as an emerging means of intervention, as alternatives to AGPs, we investigated the effects of two potential probiotics, *Bacillus pumilus* and *Bacillus subtilis*, and transfer of fecal microbiota from donor birds of different ages on the chicken's production, gut microbiota and health parameters. In the first part of our study, it was observed that both probiotics, *Bacillus pumilus* and *Bacillus subtilis*, significantly improved production parameters, gut health and immunity in broiler chickens. The gut health and immune responses in broiler chickens were prominent at day 14 in both probiotic groups but became insignificant before day 42 in *Bacillus pumilus* and *Bacillus subtilis* groups in comparison with the control group. Further, effects of *Bacillus pumilus* and *Bacillus subtilis* were evaluated on cecal microbiota through analysis of diversity and composition at days 7, 14, 28 and 42 of broiler's life. Overall, microbial alpha diversity of both probiotic groups was improved in comparison with the control and antibiotic groups in the first two weeks of life. The microbial composition in both probiotic groups at day 14 was dominated by members of family Ruminococcaceae, which is considered a hallmark of mature microbiota, but later at day 42 differences in diversity of microbiota in treatment groups became non-significant. In the second part of the study, fecal microbiota from 14- and 42-days old broiler chickens was transferred to day-old chicks through cohousing and impact of intervention on production, immunity, and bone health of the day-old broiler chickens was evaluated at days 14 and 42 of age during their growing period. The intervention improved tibial bone length, bone mineral content and bone porosity in

both treatment groups while the response of T cells was skewed to the anti-inflammatory arm in the group that was cohoused with 42 days old birds. These studies have demonstrated the potential of *Bacillus pumilus* and *Bacillus subtilis* and fecal microbiota transfer for improvement of poultry performance and health.

Résumé

L'augmentation et la propagation de la résistance aux antibiotiques chez les agents pathogènes ont conduit à un frein à l'utilisation d'antibiotiques promoteurs de croissance (AGP) chez les poulets. En l'absence d'AGP, des alternatives viables sont nécessaires pour améliorer la santé des poulets contre les maladies et maintenir l'efficacité de la production. Compte tenu de l'intérêt d'utiliser les probiotiques, en tant que transfert traditionnel du microbiote fécal, comme moyen d'intervention émergent, comme alternatives aux AGP, nous avons étudié les effets de deux probiotiques potentiels, *Bacillus pumilus* et *Bacillus subtilis*, et le transfert du microbiote fécal des oiseaux donneurs de différents âges sur la production du poulet, le microbiote intestinal et les paramètres de santé. Dans la première partie de notre étude, il a été observé que les deux probiotiques, *Bacillus pumilus* et *Bacillus subtilis*, amélioraient considérablement les paramètres de production, la santé intestinale et l'immunité chez les poulets de chair. La santé intestinale et les réponses immunitaires chez les poulets à griller étaient importantes au jour 14 dans les deux groupes probiotiques, mais sont devenues non significatives avant le jour 42 dans les groupes *Bacillus pumilus* par rapport aux groupes *Bacillus subtilis*. De plus, les effets de *Bacillus pumilus* et *Bacillus subtilis* ont été évalués sur le microbiote caecal par l'analyse de la diversité et de la composition aux jours 7, 14, 28 et 42 de la vie. Dans l'ensemble, des tendances à l'amélioration ont été observées dans la diversité alpha microbienne des deux groupes probiotiques par rapport aux groupes témoins et antibiotiques au cours des deux premières semaines de vie. La composition microbienne dans les deux groupes de probiotiques au jour 14 présentait une domination des membres de la famille des Ruminococcaceae, qui est considérée comme une caractéristique du microbiote mature, mais plus tard au jour 42, les différences d'abondance sont devenues non significatives. Dans la deuxième partie de notre étude, nous avons transféré le microbiote fécal de

poulets de chair âgés de 14 et 42 jours à des poussins d'un jour par cohabitation et évalué l'impact de l'intervention sur la production, l'immunité et la santé osseuse des poulets de chair aux jours 14 et 42. L'intervention a amélioré la longueur de l'os tibial, le contenu minéral osseux et la porosité osseuse dans les deux groupes de traitement, tandis que la réponse des lymphocytes T était biaisée par rapport au bras anti-inflammatoire dans le groupe co-hébergé avec des oiseaux âgés de 42 jours. Ces études reflètent le potentiel de *Bacillus pumilus* et *Bacillus subtilis* et le transfert du microbiote fécal à utiliser pour améliorer les performances et la santé des volailles.

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Contributions to knowledge

This thesis introduced two novel strains of *Bacillus* probiotics, *Bacillus pumilus* and *Bacillus subtilis*, and a novel methodology of transferring fecal microbiota from different age donor birds through cohousing and studied effects of these interventions on production, gut microbiota and different health parameters of broiler chickens. The main contributions to knowledge resulting from this research are summarized below:

1. We generated a broad range of in vivo data for two novel strains of *Bacillus pumilus* and *Bacillus subtilis* as potential probiotic products.

A broad range of data were generated with these *Bacillus* strains in terms of production and gut homeostasis. In particular, *Bacillus pumilus* was evaluated for the first time in broiler chickens for production and health benefits. It was also the first time to study the effects of Bacillus probiotics on two types of T regulatory cells ($CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$) in broiler chickens as a part of gut immune homeostasis.

2. Probiotics improved diversity of gut microbiota and helped to achieve a mature gut microbial configuration early in broiler life.

We found that *Bacillus* probiotics improved diversity of cecal microbiota of broiler chickens, which is a hallmark of good gut health, and prevented an age-related drop in the alpha diversity mainly through the improvement in richness in younger birds. In addition, *Bacillus* probiotics could help chickens to achieve early mature configuration of microbiota with higher abundance of family Ruminococcaceae, which is observed in high abundance in old age birds, and decrease in abundance of family Enterobacteriaceae that include potential pathogenic bacterial population.

3. Cohousing young chicks with aged birds improved bone health and promoted anti-inflammation in gut of broiler chickens.

Our study with transferring fecal microbiota from old donor chickens to day-old chickens through cohousing reported improvement in tibial bone length and cortical bone mineralization and porosity. The transfer of fecal microbiota through cohousing inhibited proliferation of pro-inflammatory CD4+ cells and promoted T regulatory cells in broiler chickens toward anti-inflammatory state.

Contributions of authors

Three co-authored manuscripts are included in this thesis.

Chapter 2: M. Bilal and X. Zhao

M. Bilal wrote this chapter while X. Zhao edited and reviewed this chapter.

Chapter 3: M. Bilal, W. Si, F. Barbe, E. Chevaux, O. Sienkiewicz and X. Zhao

M. Bilal, F. Barbe, E. Chevaux and X. Zhao designed the study. M. Bilal carried out the experiments, analyzed, and interpreted the results. W. Si helped in flow cytometry while O. Sienkiewicz assisted in samples collection. F. Barbe, E. Chevaux formulated the feed and helped in editing the manuscript. X. Zhao reviewed and edited the manuscript. Published in Poultry Science. 2021. Effects of novel probiotic strains of *Bacillus pumilus* and *Bacillus subtilis* on production, gut health and immunity of broiler chickens raised under sub-optimal conditions. 100:100871.

Chapter 4: M. Bilal, C. Achard, F. Barbe, E. Chevaux, J. Ronholm and X. Zhao

M. Bilal, F. Barbe, E. Chevaux and X. Zhao designed the study. M. Bilal carried out the experiments, analyzed, and interpreted the results. C. Achard helped in interpretation of results. J. Ronholm helped in sequencing of samples. X. Zhao and J. Ronholm reviewed and edited the manuscript. Published in Microorganisms. 2021. *Bacillus pumilus* and *Bacillus subtilis* Promote Early Maturation of Cecal Microbiota in Broiler Chickens. 9: 1899.

Chapter 5: M. Bilal, W. Si, I. Vitienes, G. Jia, H. Lin, B.M Willie and X. Zhao

M. Bilal and X. Zhao designed the study. M. Bilal carried out the experiments, analyzed, and interpreted the results. W. Si helped in flow cytometry while I. Vitienes assisted in CT scanning of bones. G. Jia and H. Lin helped in carrying out trial and samples collection. X. Zhao and BM Willie reviewed and edited the manuscript.

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List of abbreviations

Ab	Antibiotic
ADG	Average daily gain
AGP	Antibiotic growth promoter
PERMANOVA	Permutational multivariate analysis of variance
ANOVA	Analysis of variance
B-actin	Beta-actin
BM	Body mass
bp	Base pairs
BPH	*Bacillus pumilus* high dose
BPL	*Bacillus pumilus* low dose
BSH	*Bacillus subtilis* high dose
BSL	*Bacillus subtilis* low dose
CD	Cluster of differentiation
CFU	Colony forming unit
Con	Control
CRD	Completely randomized design
CTAn	Computed tomography analyzer
E. coli	Escherichia coli
EDTA	Ethylenediamine tetra acetic acid
FCR	Feed conversion ratio
FDR	False discovery rate
FI	Feed intake
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
INF	Interferon
Ig	Immunoglobulin
IL	Interleukin
JAM2	Junctional adhesion molecule 2
Kg	Kilogram
MUC2	Mucin 2
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCoA	Principal component analysis
PCR	Polymerase chain reaction
QIIME2	Quantitative Insights Into Microbial Ecology 2
RPM	Revolutions per minute
SCFA	Short chain fatty acids
SEM	Standard error of mean
SPSS	Statistical Package for the Social Sciences
Th	T helper
TJ	Tight junction
Tregs	T regulatory cells
VOI	Vertical volume of interest
ZO1	Zona Occludens 1

Chapter 1. General introduction

The demand for food is increasing day by day with increase in human population, which will be increased to 9.7 billion (United Nations., 2015) by 2050. Poultry is one of the main contributors in the livestock sector. In Canada, poultry is a growing agriculture sector and comprised of more than 4700 regulated producers and many related businesses. In 2020, Canada produced 1.27 billion kilograms (kg) of chicken, 60% of which was produced in Quebec and Ontario (Agriculture and Agri-Food Canada, 2020).

Meeting higher production goals is not possible without maintenance of a good health in poultry. In the past, farmers used antibiotics 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 (Barton., 2000). However, use of antibiotic growth promoters (AGPs) in food producing animals has been condemned due to emergence of antibiotic resistance and its potential spread to pathogens (Marshall & Levy, 2011). Thus, different countries started to impose restrictions on use of AGPs. In Canada, zero use of antibiotic growth promoters was recorded in broilers since 2014 (Public Health Agency of Canada., 2020). To help lessen some of the potential complications that may accompany the removal of AGPs, investigators are working on different effective alternatives including probiotics and transfer of fecal microbiota to maintain and promote the health and production of animals.

Probiotics are live microorganisms that induce beneficial effects on host health when administered adequately (FAO., 2002). Probiotics act through diverse mechanisms to promote health benefits, such as release of antimicrobial peptides, reducing pH, competitive exclusion, adhesion to the gut epithelial layer and induction of anti-inflammatory cytokines. Probiotics used in livestock are mainly based on species related with *Lactobacillus*, *Enterococcus* and *Bacillus* (Mingmongkolchai and Panbangred., 2018). *Bacillus* probiotics are different from lactic acid

probiotics because of their spore forming capability that confer them higher resistance to external and internal factors and better viability in the host (Popov et al., 2021). In some studies, *Bacillus* probiotics granted production (Upadhaya et al., 2019) and health benefits (Luan et al., 2019) in chickens. However, in other studies opposite results have been observed in terms of production (Oladokun et al., 2021) and health (Gadde et al., 2017). These inconsistencies in results justify for investigation of novel candidates of *Bacillus* probiotics with potential of inducing broader beneficial effects on production as well as health parameters.

The gut microbiota plays an important role in host health and production. 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). Gut microorganisms play an important role in nutrient digestion, pathogen inhibition and interaction with the gut-associated immune system (Borda-Molina et al., 2018). In chickens, gut microbiota matures with time and becomes mature and attains stable microbial composition around 42 days of life (Ocejo et al., 2019), which provides a window of opportunity to transfer mature microbiota from donor birds to day-old chicks through cohousing for early exposure and interaction of young chicks with mature and diverse microbiota for better production and health benefits. Cohousing is a technique of sharing microbiota among experimental animals and has been used previously in mice (Ridaura et al., 2013) and chickens (Lee et al., 2018) for transferring microbiota. However, impact of co-housing on day-old chick's performance, immunity and bone health after transferring microbiota from donor birds of different ages is not available. Therefore, the overall goals of this study are to evaluate the effects of two novel strains of *Bacillus* probiotics, *Bacillus pumilus* and *Bacillus subtilis*, and transfer of fecal microbiota from different age of broiler chickens through cohousing on production and health of broiler chickens. Our research would help us evaluate the potential of using fecal microbiota transfer and new probiotic products as an alternative to in-feed antibiotics.

1.1 Objectives

- To study whether novel strains of *Bacillus* probiotics (*Bacillus pumilus* and *Bacillus subtilis*) have comparable effects as an antibiotic growth promotor (Virginiamycin) in terms of changes in gut health, immunity, and growth parameters over the entire production cycle. The growth rate, feed conversion efficiency, changes in expression levels of gut integrity and function related genes, and responses of immune related T regulatory cells and their cytokines were studied.
- To evaluate effects of novel strains of *Bacillus* probiotics (*Bacillus pumilus* and *Bacillus subtilis*) on development and maturity of cecal microbiota at different stages of life. The 16S rRNA gene of cecal bacterial population was sequenced and their alpha and beta diversity and relative abundance at phylum, family and genus levels at different stages of life were determined.
- To investigate the impact of transferring fecal microbiota through cohousing, from 14- and 42-days old donor broiler chickens, on production, immunity and bone health of day-old chicks. The body weight, feed intake, feed conversion efficiency, ratios of $CD4^+$, $CD8^+$ and $CD4^+CD25^+$ T cells through flow cytometry and bone health related parameters through computed tomography scanning were studied.

1.2 References

Agriculture and Agri-Food Canada (2020) Poultry and Egg Market Information - Canadian Industry, Retrieved September 23, 2021, from https://agriculture.canada.ca/en/canadas-agriculture-sectors/animal-industry/poultry-and-egg-market-information/industry-profile.

Barton MD (2000). Antibiotic use in animal feed and its impact on human health. *Nutrition Research Reviews*. **13:** 279-299.

Borda-Molina D, Seifert J, & Camarinha-Silva A (2018) Current perspectives of the chicken gastrointestinal tract and its microbiome. *Computational and Structural Biotechnology Journal*. **16:** 131–139.

FAO (2002) Agriculture Organization of the United Nation and World Health Organization. Joint FAO/WHO working group report on drafting guidelines for the evaluation of probiotics in food.[online] http://www. who. int/foodsafety/fs_management/en/probiotic_guidelines. pdf.

Gadde U, Oh ST, Lee YS, Davis E, Zimmerman N, Rehberger T & Hyun SL (2017) The effects of direct-fed microbial supplementation, as an alternative to antibiotics, on growth performance, intestinal immune status, and epithelial barrier gene expression in broiler chickens. *Probiotics & Antimicrobial Proteins*. **9:** 397–405.

Lee IK, Gu MJ, Ko KH, Bae S, Kim G, Jin G, Kim EB, Kong Y, Park TS, Park B, Jung HJ, Han SH & Yun C (2018) Regulation of CD4+CD8−CD25+and CD4+CD8+CD25+ T cells by gut microbiota in chicken. *Scientific Reports*. **8:** 8627.

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.

Luan SJ, Sun YB, Wang Y, Sa RN & Zhang HF (2019) *Bacillus* amyloliquefaciens spray improves the growth performance, immune status, and respiratory mucosal barrier in broiler chickens. *Poultry Science*. **98:** 1403–1409.

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

Mingmongkolchai S, & Panbangred W (2018) *Bacillus* probiotics: an alternative to antibiotics for livestock production. *Journal of Applied Microbiology*. **124:** 1334—1346.

Ocejo M, Oporto B, & Hurtado A (2019) 16S rRNA amplicon sequencing characterization of caecal microbiome composition of broilers and free-range slow growing chickens throughout their productive lifespan. *Scientific Reports*. **9:** 2506.

Oladokun S, Koehler A, MacIsaac J, Ibeagha-Awemu EM & Adewole DI (2021) *Bacillus* subtilis delivery route: effect on growth performance, intestinal morphology, cecal short-chain

fatty acid concentration, and cecal microbiota in broiler chickens. *Poultry Science*. **100:**100809.

Popov IV, Algburi A, Prazdnova EV, Mazanko MS, Elisashvili V, Bren AB, Chistyakov VA, Tkacheva EV, Trukhachev VI & Donnik IM (2021). A review of the effects and production of spore-forming probiotics for poultry. *Animals.* **11:** 1941.

Public Health Agency of Canada. 2020. Canadian antimicrobial resistance surveillance system report. Cat. #: HP37-21E-PDF: 190498.

Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE & Kau AL (2013). Cultured gut microbiota from twins discordant for obesity modulate adiposity and metabolic phenotypes in mice. *Science*. **341:** 6150.

Upadhaya SD, Rudeaux F & Kim IH (2019). Effects of inclusion of *Bacillus* subtilis (Gallipro) to energy- and protein-reduced diet on growth performance, nutrient digestibility, and meat quality and gas emission in broilers. *Poultry Science*. **98:** 2169–2178.

United Nations (2015) World population prospects: The 2015 revision. *United Nations Economic and Social Affairs*. **33:** 1-66.

Chapter 2. Literature review

2.1 Chicken gut microbiota

Microbiome refers to the collective genomes of all microorganisms inhabiting an environment. While isolating and culturing each individual species is an intractable task, a cutting-edge method of sequence analysis, metagenomics, has enabled the reconstruction of microbial species and their function from the collective nucleotide contents contained in a stool sample (Hills et al., 2019). This metagenome is often called the third main mammalian genome, in addition to the nuclear and mitochondrial genomes (Markowiak-Kopec and Slizewska., 2020). Gut microbiota usually refers to all microorganisms that are found within the gut. The chicken gastrointestinal (GI) tract carries a diverse population of bacteria, with over 600 species from more than 100 bacterial genera (Torok *et al.*, 2011). It provides a habitat to these microbial communities that are mainly determined by the host, interspecies microbial competition and diet (Qu et al., 2008).

The intestinal microbiota starts to inhabit chicken gut in the embryonic life (day 16 of incubation) (Pedrosa., 2009). It is plausible that the intestinal microbiota is vertically transferred from the breeder to chicken embryos during egg formation or it is transmitted through the eggshell during the laying posture and incubation process. After hatch, feed intake and environment become main sources of microbiota that populate the chicken gut. The ingested microorganisms pass through the stressful acidic environment in the proventriculus and gizzard that significantly decreases the bacterial number. The stress is further elevated when microbiota is exposed to bile acids, enzymes and rapid shifts in pH in duodenum that leads to lower numbers of microbiota in the upper gut of chickens. As the intestinal digesta goes down, bile acids start to be diluted and the gut environment becomes more favorable to microorganisms, leading to higher numbers of microorganisms down the gut (Bedford, 2000). The microbial diversity and maturity changes with

age. It is considered immature before day 3 followed by a developing stage before day 14 and it finally attains a mature configuration at day 42 (Ocejo et al., 2019).

In general, microbiota in the chicken GI tract mainly belongs to the phylum Firmicutes followed by Proteobacteria and Bacteroidetes. The Firmicutes and Proteobacteria can be seen during a whole productive lifespan of broiler chickens while Bacteroidetes is seen only in the second half of their lifespan (Ocejo et al., 2019). Further, members of phyla Actinobacteria, Tenericutes *(*Waite & Taylor, 2014*)*, Cyanobacteria and Fusobacteria *(*Qu et al., 2008*)* can be found in low abundance. Bacterial communities vary considerably by locations along the GI tract of chickens. Crop, gizzard and duodenum share similar microbiota profiles (Rychlik., 2020). The jejunum is dominated by *Lactobacillus* species (Gong *et al.*, 2007, Feng *et al.*, 2010), while ileum is occupied with species of *Lactobacillus, Candidatus Arthromitus, Enterococcus*, *Escherichia, Shigella* and *Clostridium XI* (Asrore *et al.*, 2015).

Chickens have paired ceca, harbouring similar bacterial communities, and providing main ground for bacterial fermentation of non-digestible carbohydrates (Stanley et al., 2015). The cecal microbiota is the best documented and have the most abundant (10^{10} cfu/gram of digesta) microflora compared to other regions of the gut. These mainly belong to two Gram-positive (Firmicutes and Actinobacteria) and two Gram-negative (Bacteroidetes and Proteobacteria) bacterial phyla (Rychlik., 2020). At the family level, most cecal microbiota falls into Clostridiaceae, Lachnospiraceae, and Ruminococcaceae (Danzeisen et al., 2011). Other families like Enterococcaceae, Enterobacteriaceae, and Bacteroidaceae are also abundant in the cecal microbiota (Yin et al., 2010). At the genus level, different genera like *Ruminococcus, Clostridium, Eubacterium, Faecalibacterium, Blautia, Butyrivibrio*, *Lactobacillus, Roseburia, Ethanoligenens, Hespillia, Megamonas, Veillonella* and *Anaerostipes* groups have been reported to cover a substantial part of cecal microbial population (Wei et al., 2013). For gut microbiota studies, the

cecal samples are considered as gold standard and are preferred over faecal samples in terms of studying microbial diversity (Stanley et al., 2015).

The chicken gut microbiota is affected by different factors like 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). The sex may play a role in shaping composition of microbiota. For example, increased abundance of *Oscillospira* and *Bacteroides* in ceca are reported in female and male birds, respectively (Lee et al., 2017). Furthermore, feed form (pellet or mash) and composition, use of antimicrobials for preventive, therapeutic or growth promotion purposes, farm environment and management practices and feed additives like probiotics, organic acids, enzymes, yeast, and other additives can affect the diversity and composition of intestinal microbiota (Feye et al., 2020).

2.1.1 Gut microbiota and chicken health

The gut microbiota plays an important role in host health and production. 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). Intestinal microbiota has a direct or indirect role in modulation and maturity of host immunity, gut integrity, production and prevention from pathogens. It is therefore important to identify the gut microbial composition and diversity to improve chicken health and productivity.

The host gut provides a nutrient rich habitat to microflora. In return, microflora degrades undigested carbohydrates to produce short chain fatty acids (SCFA) (Topping & Clifton., 2001), synthesize vitamins (Kau et al., 2011) and develop and regulate the immune system (Bäckhed et al., 2005). Gut associated lymphoid tissues (GALT) are lymphoid aggregates that are present on the gut mucosa. These include the epithelial lining with subepithelial T-cells zone and

dendritic cells (Newberry and Lorenz, 2005). Specialized M cells in epithelial layer are responsible for antigen uptake and their exposure to underlying immune cells (Kraehenbuhl & Neutra, 2000). The intestinal epithelial cells can produce mucus (Johansson et al., 2008) and antimicrobial compounds (Zheng et al., 2008) to inhibit pathogens.

In comparison with conventional mice, germ-free mice have immature lymphoid tissues (Bouskra et al., 2008), a lower level of antimicrobial peptides (Hooper, 2004) and a thinner mucus layer (Petersson et al., 2010). However, when germ-free (GF) mice are exposed to normal microflora, they regain their mucosal immune functions (Hooper, 2004; Petersson et al., 2010). Comparison of GF with conventional animals provides the most fundamental model to explore the influence of the gut microbiota on immune system development, while gnotobiotic and antibiotic treatment models allow investigation of the effects of more defined microbiota (Broom and Kogut., 2018). The intestinal microbiota affects the development of both innate and adaptive immune responses. IgA and host defensive proteins are both important antimicrobial components of intestinal defense. In GF chickens, IgAs were not detected in the intestine (or serum) up to 4 weeks of age, whereas IgA concentrations in conventional birds essentially increased from 1-week post hatch (Kaspers et al., 2015). Similarly, GF birds at 7d of age had lower neutral and acidic goblet cell numbers and MUC2 expression (Cheled-Shoval et al., 2014). Kaspers et al. (2015) demonstrated that the GF effects could be partially 'corrected' through mono- (*Escherichia coli* Nissle) or tetra-colonisation (a strain of each of *E. coli*, *Lactobacillus*, *Enterococcus*, *Clostridium*), with the better result in the latter. These GF studies reflect that the gut microbiome has a significant role in the development of the immune system and thus strongly influence optimal health and productivity.

The short chain fatty acids, produced by intestinal microbiota, can cross the epithelial lining and bind to G-protein-coupled receptor 43 (GPR43) on antigen presenting cells to modulate

inflammation (Böhmig et al., 1997; Maslowski et al., 2009). The intestinal microflora such as clostridium clusters IV and XIVa can induce T regulatory cells that are important to keep a check on abnormal expansion of CD4 positive T cells (Atarashi et al., 2011) that avoid inappropriate inflammation. Dendritic cells in the GALT plays an important role in directing the immune response toward tolerance or inflammation. The conventional dendritic cells activate the T regulatory cells and prevail tolerance towards microflora while plasmacytoid dendritic cells activate B cells that release secretory Immunoglobulin A that control the gut commensals as well as pathogens (Iwasaki, 2007). In human, a subtype of T lymphocytes (T regulatory cells) identified by the expression of CD4, CD25, and FOXP3 has been shown to be a major player in the maintenance of immune tolerance and homeostasis (Sakaguchi et al., 2010). Defective T regulatory cells function leads to development of inflammatory bowel disease (Fontenot et al., 2003) and type 1 diabetes (Mahne et al., 2015). In mice, immunological or genetic inhibition of Tregs function in a sepsis model of bacterial infection leads to exaggerated production of pro-inflammatory cytokines including IL-1b, IL-6, IL-12, TNF, and CCL2 and condition was reversed when transferred with Tregs from wild-type mice (Okeke et al., 2013). Regulatory T cells can be defined based on their potential to suppress other immune cells and thereby limit or suppress immune responses. Interleukin-10 (IL-10) is the main cytokine produced by these cells and is required for their function (Ouyang et al., 2011). IL-10 is viewed as an anti-inflammatory cytokine and can improve intestinal barrier by decreasing inflammation. IL-10 knockout mice, which are widely used as a model of spontaneous colitis, show increased intestinal permeability with elevated pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 expression (Madsen et al., 1999) and IL-10 treatment can lead to reversal of increased intestinal permeability and decreased levels of tight junction proteins (Sun et al., 2008). In chicken, CD4+CD25+ T cells are considered the T regulatory cells (Lee et al., 2017) as the key Foxp3 equivalent gene is not described in poultry yet

except in one report that provided evidence of Foxp3 presence in peregrine falcon and saker falcon (Denyer, et al., 2016). Recently, the relationship between gut microbiota and Tregs (CD4+CD25+ T cells) has been established in chickens and documented alterations in gut microbiota under influence of antibiotic cocktail alters anti-inflammatory response of CD4+CD8−CD25+ and CD4+CD8+CD25+ T cells (Lee et al., 2018). These findings provided clues regarding cross talk of intestinal microbiota with T regulatory cells and influence on generation of controlled response to inflammatory signals originated from gut environment. Probiotics, like antibiotics as AGP's, remodel the diversity and richness of intestinal microbiota and may have direct or indirect influence on regulation of T cells to check the inflammatory mechanisms. Recently, it is reported that chickens that received lactobacilli via in ovo route followed by weekly oral administration showed an increase in the percentage of CD4+CD25+ T cells in the spleen (Alizadeh et al., 2021). Little is established from the available literature about the effects of *Bacillus* probiotics on T regulatory cells and their anti-inflammatory response in chickens at the gut level, which provides a strong basis for investigations.

The gut microbiota provides protection in the GI tract against enteric pathogens. Different potential mechanisms can be used by gut microbiota to exclude pathogens, which include competition for nutrients, occupying attachment sites gut epithelium, releasing toxins and antimicrobials like bacteriocins, decreasing pH to inhibit pH-sensitive pathogens, and activation of immune response (Patterson & Burkholder, 2003). An unbalanced microbiota, referred as dysbiosis, has been reported to be involved in pathogenesis of diseases such as necrotic enteritis causing by *Clostridium perfringens* (Van Immerseel et al., 2009). Disturbances in intestinal microbiota can be caused by many factors including dietary changes, environmental stressors, antibiotic therapy, infections, mycotoxins, nutritional imbalances, and management disorders (Teirlynck et al., 2011).

The gut microbiota and its metabolites are able to affect the integrity and function of the intestinal barrier. The loss of barrier integrity leads to a gradual increase in intestinal permeability, which cause a shift from "physiological" to "pathological" intestinal mucosal inflammation (Lambert, 2009). The junctional complexes in intestinal epithelial cells ensure the intestinal integrity and regulate paracellular permeability. The junction complexes are composed of tight junctions, gap junctions, adherens junctions, and desmosomes. Tight junctions (TJ) include four integral transmembrane proteins (occludin, claudin, junctional adhesion molecule, and tricellulin) that interact with cytosolic scaffold proteins zonula occludens (ZO), which in turn bind to the actin cytoskeleton. TJ proteins are dynamic in nature and are subject to change and remodel in response to external stimuli in the gut lumen such as food/nutrients, commensal and pathogenic bacteria (Ulluwishewa et al., 2011). The intestinal microbiota or their components activate different sub-mucosal immune cells including Th-17 cells that secrete different cytokines such as IL-17A, IL-17F and IL-22. These cytokines activate epithelial cells to increase expression of TJ proteins (Weaver et al., 2013). The Th17 cells, with the help of their key cytokines, IL-17A, IL-17F and IL-22, can stimulate the production of antimicrobial proteins by intestinal epithelial cells, formation of tight junctions between these cells, recruitment of granulocytes and mediation in transportation of IgA across mucosa (Honda and Littman., 2016; Weaver et al., 2013). These cells are concentrated more in barrier sites like intestine than systemic sites (Weaver et al., 2013). Certain bacteria such as segmented filamentous bacteria from family Clostridiaceae have been reported to have a direct link with stimulation of Th17 cells (Ohnmacht et al., 2011). Further, mucins, a major component of mucus, are large glycoproteins with a highly polymeric protein backbone structure and can be either gel-forming (secretory) or membrane bound. MUC2, the major secretory mucin, plays a vital role in keeping the architecture of the mucus layer on the intestinal surface and in preventing microorganisms from approaching the innermost mucus layer

(Jiang et al., 2013). Alterations in intestinal microbiota can lead to changes in bacterial fermentation products like short chain fatty acids (SCFA) (Pan and Yu., 2014). These SCFA, especially butyrate, are considered to regulate the mucin production locally (Tellez et al., 2006). Other reports describe the role of IL-22 from Th-17 and other cells to induce goblet cells to secrete mucins in response to antigenic challenges (Sugimoto et al., 2008).

The gut microbiota has also beneficial effects on bone health of the bird. It is clear that Calcium availability plays an important role in influencing bone mass of an animal. Microbiota improves Calcium bioavailability through different mechanisms that include use of phytase enzyme to catalyze the hydrolysis of phytic acid in plants to release bound minerals like Ca and P (Cho et al., 2011, Tamayo-Ramos et al., 2012), lowering intestinal pH that favors Ca absorption (Suvarna and Boby, 2005) and reduction in inflammation by reduction in pro-inflammatory cytokines, which in turn reduce osteoclast activity of bone resorption (Humphrey and Nakamura, 2015, Zhu et al., 2020).

2.1.2 Gut microbiota and chicken growth

Microorganisms in the intestine interact with each other as well as with the host and thus affect many physiological functions in the host. It has been observed that the ratio between two phyla, Firmicutes and Bacteroidetes, are linked with the amount of energy to be extracted from the diet. In chickens, a significant improvement in the body weight in relation to an increase in Firmicutes to Bacteroidetes ratio has been observed when treated with penicillin (Singh et al., 2013). It has been observed that birds with higher body weight displayed a higher cecal microbial alpha diversity, a higher microbiome uniformity (i.e., lower beta diversity within the group of Big birds), higher levels of SCFA-producing and health-associated bacterial taxa such as family Lachnospiraceae, order Christensenellales and genera *Faecalibacterium* and *Butyricicoccus* and lower levels of *Akkermansia muciniphila* and *Escherichia coli* species as compared to birds with

lower body weight (Lundberg et al., 2021). Similarly, Lee et al. (2017) investigated 12 male and 12 female broiler chickens by 16S rRNA sequencing and observed the genus of *Faecalibacterium* to be enriched in male chickens with the highest body weights. Many members of families Lachnospiraceae and Ruminococcaceae are linked with high chicken productivity (Lundberg et al., 2021, Carrasco et al., 2019), possibly because of having capacity to produce short chain fatty acids (SCFA), which are linked with anti-inflammatory effects (Biddle et al., 2013). Butyrate is one of such SCFA that not only act as an energy source for cecal epithelial cells but also induce anti-inflammatory responses by acting on pro-inflammatory cytokines (Eeckhaut et al., 2011). Conversely, Enterobacteriaceae is considered as a pro-inflammatory marker of dysbiosis in intestinal microbiota of poultry (Ducatelle et al., 2018) and its genus *Escherichia* is often correlated with low productivity and diseases (Carrasco et al., 2019). Further, Han et al. (2016) negatively correlated cecal abundance of *Streptococcus* and *Akkermansia* with chicken's body weight while *Bifidobacterium* and *Lactococcus* was positively correlated.

2.1.3 Environmental effects on the gut microbiota

There are a range of production-related local climates that can affect the intestinal microbiota of poultry (Kers et al., 2018). These include overcrowding, heat stress, feed withdrawal, and transportation. Through the gut-brain axis, the intestinal tract is receptive to environmental factors, which can subsequently cause changes in the intestinal microbiota (Burkholder et al., 2008, Bercik et al., 2012). In one study, a decline in performance and cecal bacterial composition was observed in a flock kept at a stocking density of 17 birds per m^2 in comparison with a flock kept at a stocking density of 12 birds per m^2 (Guardia et al., 2011). Other studies described large effects of heat stress on performance and changes in microbial composition of broilers (Lan et al., 2004; Sohail et al., 2015). Similarly, Burkholder et al. (2008) reported that exposure of birds to higher temperatures for 24 hours, induced greater changes in the ileal microbiota compared to cecal

samples, suggesting higher sensitivity of ileal microbiota to stress factors. Other studies observed that stress factors may increase abundance and colonization of potentially pathogenic bacteria such as *E. coli* (Laudadio et al., 2012) and *Salmonella* (Burkholder et al., 2008; Soliman et al., 2009). Environment related stress factors can also impact the integrity and function of intestinal epithelial cells (Lambert, 2009). It is observed that prolonged exposures to heat stress can compromise intestinal integrity and mucosal immunity in broilers (Quinteiro-Filho et al., 2010) and layers (Deng et al., 2012).

2.1.4 Culture-independent methods for the analysis of gut microbiota

The chicken intestinal microbiota has been evaluated by two approaches; 1. The traditional culture-based techniques and 2. modern culture independent techniques. The former has a limited sensitivity, since only 20-40% of bacterial species could be cultured (Oakley et al., 2014). On the other hand, the later approach was established after technological development of sequencing (Sanger et al., 1977), 16s rRNA gene analysis (Woese and Fox., 1977) and polymerase chain reaction (Mullis et al., 1987) techniques. Most of these molecular methods depend on the sequence analysis of the 16S ribosomal RNA (rRNA) gene for identification, classification, and quantification of bacteria. The 16S rRNA gene is about 1,550 base pairs (bp) long and is composed of conserved and variable (V1-V9) regions. Different sequencing platforms have been used for sequencing the 16S rRNA gene. Usually, the sequencing platform is selected based on different consideration such as quality of sequence data, cost of sequencing, length of generated reads and number of samples analysed per sequencing run. Similarly, different bioinformatics tools such as QIIME, MG-RAST, UPARSE and Mothur are available to process and analyse 16S rRNA gene sequencing data. The choice of choosing an analysis platform depends on user's level of experience in bioinformatics, availability of resources at the user's end, documentation and time needed for analysis (Pollock et al., 2018).

2.2 Use of antibiotics in poultry production

Antibiotics have been used for therapeutic and growth promoting prophylactic purposes in animals since the 1940s (Gustafson and Bowen., 1997). Ever since, antibiotics have been 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 over an extended period. The recognition that antibiotics can improve efficiency of animal growth has matched with industrial poultry production that comprises intensive chicken rearing. In Canada, one million kilograms of antimicrobial active ingredient were distributed for use in animals between 2017 and 2018, which represented 79% of the total antimicrobial active ingredient used in Canada (Public Health Agency of Canada., 2020). These antimicrobial active ingredients were used for prevention and therapy, not for growth promotions, in animals including broiler chicken and turkey flocks.

It has been estimated that the use of antibiotic growth promoters (AGPs) in animal feed can improve weight gain by 4 to 8% and feed utilization efficiency by 2 to 5% (Butaye *et al.*, 2003). The exact pathway by which AGPs improve growth are not clearly known, but several propositions have been proposed to explain probable pathways that antibiotics choose to improve growth performance (Feighner & Dashkevicz, 1987, Butaye *et al.*, 2003). These include: (i) an increase in efficiency of nutrient intake due to a thinner intestinal epithelium in antibiotics-treated animals (Boyd et al., 1967), (ii) reduction or elimination of gut pathogen load and subclinical infections (Barnes et al., 1978), (iii) an increase in nutrient availability due to a reduced microbial destruction of nutrients (Eyssen, 1962) and (iv) reduction of toxins and growth-depressing metabolites produced by bacteria (Dang et al., 1960). Growth promoting antibiotics appear less effective in animals when used under good hygienic and controlled experimental conditions,

suggesting a reduction or inhibition of subclinical infections as the probable mechanism for their action (Brüssow, 2015). For the host, antibiotics have been shown to increase the nutrient availability in the gut, which lead to decrease in amino acid catabolism and bile salts breakdown in the gut leading to improvement in the digestibility of dietary protein. Further, the efficient absorption of nutrients and its utilization increases because of the thinner epithelium and decreased microbial utilization of nutrients, and thus, more nutrients are available to reach the host's tissues (Niewold, 2007). In addition to direct impact of AGPs on intestinal microbiota, it has been observed that AGPs have anti-inflammatory effects as they have down regulated production and release of intestinal pro-inflammatory mediators therefore saving energy to be used in growth (Oh et al., 2019, Niewold, 2007). It seems that antibiotic growth promoters work by manipulating both gut microbiota as well as physiological processes (Low et al., 2021).

Previously, different antimicrobials were used in Canada for growth promotion purposes including bambermycin, penicillin, salinomycin, bacitracin, salinomycin-bacitracin, virginiamycin, chlortetracycline, monensin, and narasin. The penicillin and virginiamycin have been observed to enhance feed efficiency (Diarra & Malouin, 2014). Virginiamycin is a streptogramin antibiotic produced by incubation of bacterium *Streptomyces virginiae* (Yamada *et al.*, 1987), and consist of two antibiotic molecules, pristinamycin IIA (virginiamycin M1) and virginiamycin S1. It has a narrow spectrum activity against gram-positive bacteria. It interacts with two separate loci of bacterial ribosome and interfere with protein synthesis (Yonath, 2005). Baurhoo *et al.* (2009) treated broiler chickens with virginiamycin (16.5 mg/kg) and bacitracin (55 mg/kg) and observed that chickens had a considerably lower abundance of *E. coli* and *Campylobacter* in the cecum. Similarly, Danzeisen *et al.* (2011) reported that diet supplemented with monensin, anticoccidial, in combination with virginiamycin led to decrease in cecal abundance of family Lachnospiraceae. They also observed an increase in the abundance of *E. coli*

in response to the antibiotics, which was different from previous studies. Furthermore, xylooligosaccharides and virginiamycin fed chickens were observed to improve feed conversion efficiency and concentrations of acetate and propionate (Pourabedin et al., 2015).

In Canada, while keeping in view the emerging trends of antimicrobial resistance, the use of growth promoters reduced to zero in broilers and turkeys since 2014 (Public Health Agency of Canada., 2020). The antimicrobials are classified into four different categories (medically important antimicrobials) based on criteria of being essential for the treatment of serious bacterial infections and limited or no availability of alternative antimicrobials for effective treatment in case of emergence of resistance to these agents (Government of Canada., 2009). The labels of these medically important antimicrobials (MIA) claiming growth promotion and direction for its usage have been removed (Government of Canada., 2018). Further, Chicken Farmers of Canada have voluntarily restricted the preventive use of category 1 of MIA in 2014, category 2 of MIA in 2018 and now planning to restrict the preventive use of category 3 of MIA in future. However, the preventive use of category 4 MIA and therapeutic use of all antimicrobials will remain in use in the Canadian poultry industry (Chicken Farmers of Canada., 2021).

2.2.1 Resistance to antibiotics

Despite all the benefits of antibiotics for the poultry industry, improper use of these antimicrobials has led to the advancement in bacterial resistance. Antimicrobial resistance is considered a main hazard to human and animal health globally with considerable economic repercussions (O'Neill., 2016, World Health Organization., 2015). Antimicrobial resistance is the ability of microorganisms, such as bacteria, to become increasingly resistant to an antimicrobial to which they were previously susceptible, and consequently, become persistent and spread infection to others (World Health Organization., 2015, European Commission., 2017). Antimicrobial resistance can be gained through mutations in genes or by horizontal gene transfer between

bacteria. The later process is the key cause of antimicrobial resistance (Boerlin and Reid-Smith., 2008). Antimicrobial resistance is a natural phenomenon, but the improper use of antimicrobials can speed up this process (D'Costa et al., 2011) by increasing selection pressure and mobilizing genes between bacteria (Aarestrup., 2015). The use of antimicrobials in large quantities can lead to the development of antimicrobial resistance in animals (Chantziaras., 2013, ECDC., 2017), which can be transferred to humans either directly through animals at farms, handling and consumption of raw food and consumption of under cooked food or indirectly through the environment (Boerlin and Reid-Smith., 2008, Smith et al., 2013). Use of different antimicrobials like cefotaxime, avoparcin, tylosin, virginiamycin, avilamycin and ceftiofur have been associated with antimicrobial resistance in *E. coli*, *E. faecium* and *Salmonella enterica* serovar Heidelberg isolated from broiler chickens (Bennani et al., 2020).

2.2.2 Alternatives to antibiotic growth promoters

The withdrawal of AGP's as preventive use of antibiotics in animal production is vital to avoid antibiotic resistances, but this may compromise productivity and health of animals due to restoration of microbial composition to pre-AGP's state, increase in gut inflammation and host susceptibility to pathogens (Tarradas et al., 2020). The ban on antibiotics at sub therapeutic level in animal feed led to decrease in animal production (Cheng et al., 2014) because of higher rates of infections and increase in the risk of food-borne infections in consumers (Hao et al., 2014). Therefore, in the post AGP's era there is now an urgency to find and develop alternatives to tackle the consequences of AGP's withdrawal. A best alternative to antibiotics should have antibiotic like beneficial effects on the host, and hence, comparable modes of action on GIT, microbiota, and immune system (Gadde et al., 2017). Further, the alternative should guarantee optimum animal performance and nutrients availability (Seal et al., 2013).

To date, several types of alternatives have been proposed. These include probiotics, prebiotics, synbiotics, organic acids, enzymes, phytogenics, antimicrobial peptides, hyperimmune egg antibodies, bacteriophages, clay, metals (Gadde et al., 2017), phage derived peptidoglycan degrading enzymes, quorum quenching molecules (Low et al., 2021), paraprobiotics, postbiotics (Abd El-Ghany., 2020), cecal and fecal microbial transfer (Thomas et al., 2019, Volf et al., 2016) and competitive exclusion products (Rychlik., 2020). Among them probiotics have been extensively researched and gained popularity due to their beneficial effects on the host performance, gut microbiota, and disease suppression.

2.3 Probiotics

Probiotics are live microorganisms that induce beneficial effects on host health when administered adequately (FAO., 2002). The use of probiotics in livestock and poultry has long been practiced even before ban on sub-therapeutic use of antibiotics (Vanbelle et al., 1990). The probiotics should have the capability to induce beneficial health effects in the host, being not pathogenic and toxic in the host, have the ability to survive in the gut environment and show resistance to the field and storage adversaries (Fuller., 1989). In poultry, different probiotics showed their effects in growth performance, intestinal morphology, intestinal microbiota, meat quality and immune response **(**Alagawany et al., 2018, Patel et al., 2015, Bai et al., 2013, Gao et al., 2008, Samanya and Yamauchi, 2002). The growth in poultry may be stimulated by higher production of short chain fatty acids produced by probiotics, especially butyrate that alters the insulin receptors in muscles (Matis et al., 2015). Supplementation of the probiotics like lactic acid bacteria and *Saccharomyces cerevisae* showed higher growth performance and T cell function in broilers (Bai et al., 2013). Similarly, probiotics in chickens demonstrated a higher growth and improved intestinal morphology (Samanya and Yamauchi, 2002). The fungi as probiotics also showed good effects on growth performance and nutrient digestibility of chickens (Sugiharto.,

2019, Huang et al., 2004). The probiotics can inhibit the growth of intestinal pathogens like *Campylobacter*, *Salmonella*, *Clostridium* and *Eimeria* in the poultry gut (Smialek et al., 2021**,** Higgins et al., 2010, Jayaraman et al., 2013, Mohsin et al., 2021). Probiotics can induce their health effects through the following several ways and in most cases by a blend of mechanisms.

(1): Probiotics can release bacteriocins and antimicrobial peptides that can damage cell walls of Gram-positive bacteria or interfere with DNA, RNA and protein of Gram-negative bacteria (Cotter et al., 2013). For example, Corr et al. (2007) demonstrated that the mutant strain of *Lactobacillus salivarius* lacking the ability to produce bacteriocin failed to stop *Listeria monocytogenes* infection in mice as compared to bacteriocin producing *Lactobacillus salivarius.* In addition to bactericidal effects, the immunomodulatory effect of bacteriocins has been reported. Bacteriocins released by probiotics can also activate the host immune system, enhancing the bactericidal effect, particularly during infections (Hernández-González et al., 2021). (2): Certain probiotics can inhibit pathogens by production of organic acids and hydrogen peroxide that lower the pH and cause oxidative damage to pathogens (Nair et al., 2017). (3): Competition for nutrients and adhesion to the gut epithelial layer is another way that probiotics use for interference with pathogens (Smith et al., 2014). For example, the *Escherichia coli* strain Nissle 1917 can compete for iron with *Salmonella enterica* serovar Typhimurium pathogenic strain and inhibit *Salmonella* strain when administered together in mice (Deriu et al., 2013). (4): Probiotics with high affinity to the epithelial cells can reduce the chances of pathogens to adhere to mucosal layer of intestine (Guglielmetti et al., 2010) and interacts with immune cell, like dendritic cells, and stimulate their ability of bacterial endocytosis (Taverniti et al., 2019). In addition, probiotics can induce mucin binding proteins to interfere with the pathogens attachment and translocation (Nair et al., 2017). Attachment to the gut epithelial cells is a key step to modulate the host immune system for both probiotics and pathogens (Collado et al., 2006; Wall et al., 2008). (5): Certain probiotics induce

anti-inflammatory cytokines that reduces the induction of inflammation (Vanderpool et al., 2008), while others alter the diversity of microbiota that promotes anti-inflammatory state in the gut (Yin et al., 2018; Carasi et al., 2017). Probiotics can modulate inflammatory pathway activation by interacting with the intestinal epithelial and immune cells. These cells, with help of Toll-like receptors (TLR), recognize both antigens derived from the microbiota and antigens from invading pathogens and either maintain immune tolerance to the communities of resident commensal bacteria or mount robust immune responses against pathogens. Further, probiotics can aid in fermentation of dietary fibers that generate short chain fatty acids (SCFA), which can be utilized by gut epithelial cells as energy source and also play a role in reduction of pro-inflammatory cytokines (Tarradas et al., 2020). (6): Probiotics can modulate the innate and acquired immunity. For example, *Lactobacillus fermentum* and *Saccharomyces cerevisiae* in broiler chickens induced T cell immunity (Bai et al., 2013) while *Lactobacillus* strains in mice activated the T regulatory cell that helps in suppression of inflammation (Petersen et al., 2011).

The probiotics use in livestock feeds has increased significantly in recent years due to their associations with decrease in animal diseases and improvement in performance. Most probiotics are *Lactobacillus, Enterococcus* and *Bacillus* (Mingmongkolchai and Panbangred., 2018). *Bacillus* species have the capability to produce spores in harsh conditions (Sanders et al., 2003; Hong et al., 2005). The main feature that separates spore-forming probiotics from the more common lactic acid probiotics is their high resistance to external and internal factors, resulting in higher viability in the host and correspondingly, greater efficiency (Popov et al., 2021). Further, *Bacillus* species show antagonistic properties against pathogens through release of antimicrobial compounds (Mongkolthanaruk., 2012) and competitive exclusion, preventing *Salmonella Enteritidis* and enterotoxin *Escherichia coli* attachment to the surface of intestinal epithelial cells (Thirabunyanon and Thongwittaya 2012; Ye et al. 2013), which make it an ideal substitute for antibiotics (Popov

et al., 2021). *Bacillus* species are also involved in activation of host immunity (Novak et al., 2012) and are able to neutralize the negative effects of aflatoxin B1 on performance and health parameters of broiler chickens (Solis-Cruz et al., 2019).

2.3.1 *Bacillus subtilis*

Bacillus subtilis is a Gram-positive, rod-shaped spore-forming bacterium and is used in different poultry trials as a probiotic. Supplementation of *Bacillus subtilis* in broilers showed beneficial effects on production in terms of body weight, feed intake and feed conversion ratio (FCR) (Upadhaya et al., 2019, Ma et al., 2018, Zaghari et al., 2017, Harrington et al., 2016, Bai et al., 2016, Sen et al., 2012). However, not all the studies have reported positive results on production parameters after supplementation of *Bacillus* probiotics. For example, supplementation of *Bacillus* species to chickens did not show any effect over the body weight and FCR (Oladokun et al., 2021, Brzoska et al., 2012, Lee et al., 2010). Similarly, Froebel et al. (2020) and Gadde et al. (2017) found significant changes earlier (day 14) in body weight and FCR of broilers in response to *B. subtilis* strains while Jacquier et al. (2019) reported no change in broiler performance between groups up to 21 days of age in response to *Bacillus subtilis* strain (29784) but later FCR and body weight were improved by day 35 and 42 of age, respectively. The discrepancy is also seen in other parameters. Aliakbarpour et al. (2013) and Luan et al. (2019) reported increase in mucin mRNA expression in the intestine upon supplementation with *Bacillus subtilis* and *Bacillus amyloliquefaciens*, respectively but in contrast Gadde et al. (2017) observed no differences in the expression of mucin in any of the probiotic (*Bacillus subtilis* strains) or antibiotic-fed broilers groups. In the same manner, Gadde et al. (2017) found that *B. subtilis* strain 1104 + strain 747 (PB2) in broilers increased gene expression of pro-inflammatory cytokines (IL-6, IL-8 and TNFSF15) as well as anti-inflammatory cytokines (IL-10). However, Ma et al. (2018) did not find any significant increase in pro-inflammatory (IL-1B, TNF-alpha, IFN-g and IL-6) as well as anti-

inflammatory (IL-10) cytokines while using *Bacillus subtilis* DSM 32315 strain. These strain specific effects show the need for investigation of novel strains with potential of inducing broader beneficial effects on different parameters of broiler production and health.

2.3.2 *Bacillus pumilus*

Bacillus Pumilus is also a Gram positive, rod shape and spore forming bacterium (Handtke et al., 2014). Use of *Bacillus Pumilus* in Holstein cows as a probiotic demonstrated beneficial results on milk composition (Luan et al., 2015). In poultry, Bonos et al. (2021) recently reported beneficial effects of *Bacillus pumilus* on growth, gut health and meat oxidative stability of broiler chickens while Jagadeesan et al. (2020) and Reddy et al. (2017) previously reported keratinase activity of *B. Pumilus* for bioconversion of poultry feathers waste. The *Bacillus Pumilus* spores were reported to have inhibitory effects on abundance of aerobic bacteria, Enterobacteriaceae, and coagulase positive staphylococci in poultry litter and used as cleaning strategy on the microbial decontamination of reused litters (De Cesare et al., 2019). Kapoor & Kuhad. (2007) used the xylanase activity of *B. Pumilus* for degradation of xylan, a major component in plant cell wall, for production of xylooligosaccharides, which can be used as a prebiotic in poultry. Despite the reported benefits of *Bacillus Pumilus*, effects of *Bacillus Pumilus* as a probiotic on beneficial bacterial population, intestinal integrity and immune functions of chickens have been poorly investigated, especially when this study was initiated in 2018.

2.4 Cohousing and transfer of mature microbiota

Cohousing can be a powerful technique of sharing microbiota among experimental animals. Ridaura et al. (2013) demonstrated that cohousing of obese and lean mice prevented increases in adiposity, reduced weight in obese mice and transformed the metabolic profile of obese mice to resemble that of lean mice, an effect driven by the transfer of specific bacteria from

lean to the obese microbiota. Similarly, Lee et al. (2018) reported that broiler chickens treated with an antibiotic cocktail, starting at hatching for 7 days, led to significant decline in phylum Firmicutes microbiota but when co-housed with control chickens it regained its original population in 5 days. Similarly, Kubasova et al. (2019) cohoused day-old chicks with 45- and 34-weeks old hen and found that a mere 24-hour-long contact between a hen and newly hatched chickens was long enough for transfer of hen gut microbiota to chicks.

In broiler chickens, the indigenous microbiota evolves with time. The one-day-old broiler chicks already carry a community of microorganisms in their intestinal tract that are acquired directly from mother oviduct or environment. The microbiota of growing chicks develops rapidly from days 1–3, and the microbiota is primarily Enterobacteriaceae, but Firmicutes increase in abundance and taxonomic diversity starts around day 7 and increases with time (Ballou et al., 2016). Ocejo et al. (2019) described the development of microbiota in broilers in 3 stages. The first stage, represented by 3-day-old broilers, showed a clearly immature microbiota dominated by Proteobacteria and Firmicutes. The second and third stage represented by 14-day-old and 42-day-old broilers, respectively and the abundance of core microbiota shifted from phylum Firmicutes (around 90%) at day 14 with main representation of families Lachnospiraceae and Ruminococcaceae to phylum Bacteroidetes at day 42 with main representation of Bacteroides. The microbial community of broilers at 29 days old could be considered a transition between stages 2 and 3, when some of the birds still had similar microbiota composition to the previous age-group while others were already diverging towards a more mature microbial structure. This suggests that a different set of microbiotas dominate the course of life in broiler birds and hence would have different interactions with hosts.

The idea to use indigenous mature microbiota in day-old chicks to speed up their gut and immune system maturity has the potential to induce beneficial impacts on overall health and

production of birds. This concept has been proofed in mice. McCafferty et al. (2013) gavaged mature microbiota to young mice and compared with naturally cage acquired microbiota and found higher microbial abundance in the gavage group with long-lasting effects. In commercial poultry management system, the chicks are hatched in a high hygienic hatchery environment and are transported to farm without having chance to contact with the hen. The initial colonized microbiota of these chicks originates primarily from surrounding hatchery environment and depends on the likelihood of chicken exposure to a particular microbiota member (Rychlik et al., 2020). Kubasova et al. (2019) compared the abundance of different microbial genera in chicks raised with or without a contact hen and observed that 45 genera were more abundant in the cecal microbiota of contact chicks in comparison to control chicks, describing a big chunk of microbiota became underrepresented due to poultry management practices in hatcheries and farms. In the same study abundance of 68 genera were similar in both control and contact groups while abundance of 8 genera including *E. coli*, Proteus and Salmonella were high in control group, depicting that these microbial genera became suppressed in contact group and presenting importance of transferring mature microbiota to young birds in disease prevention.

Previously, different studies reported impact of transferring microbiota on chicken's gut microbiota and disease prevention. Gong et al. (2019) used fermentation broth from broiler cecal content on the colonization and development of the gut microbiota in newly hatched broiler chicks and observed increase in the relative abundance of the genus Bacteroides on days 1, 3, and 7, and the family Ruminococcaceae on days 1, 3, and 28. Similarly, Metzler-Zebeli et al. (2019) used fecal microbiota transplant (FMT) from highly feed efficient donor chicken (30^{th} day post hatch) in 1, 6 and 9 days old chicks and reported that modulating the early microbial colonization results in long lasting changes in bacterial taxonomic and metabolite composition as well as in host intestinal development. Varmuzova et al. (2016) reported oral inoculation of cecal extracts from

old donor birds (3, 16, 28 and 42 weeks) was protective against *S. Enteritidis* challenge at 8th day of age. Similarly, competitive exclusion products have been reported to have inhibitory effect on to *Salmonella* in chickens (Jiratitipat et al., 2019, Milbradt et al., 2017, Milbradt et al., 2014). These clearly indicate the beneficial aspects of transferring mature microbiota to young chicken in terms of intestinal microbiota and disease prevention. However, a detailed study is still missing to demonstrate the effects of mature microbiota with different microbial diversity through cohousing, on production and health parameters of broiler chickens.

2.5 Response of broiler chickens in mild suboptimal conditions

Poultry raised in industrial production systems is exposed to different types of stresses such as fluctuation in temperature, overcrowding, poor diet quality and other environmental conditions (Sohail et al., 2011). The bird's production responses raised in good sanitation conditions may be different and compromised when raised in industrial conditions. It is appropriate to raise experimental birds in mild stressed conditions to simulate the industrial conditions and evaluate the treatment responses. Pourabedin et al., (2014) evaluated the effects of prebiotic (mannan oligosaccharide) on chicken microbial community and intestinal morphology under suboptimal conditions like higher stocking density, colder temperature and higher feed viscosity. Similarly, chicken responses to probiotics under suboptimal conditions would be close to industry conditions and more obvious in mild stressed environment.

2.6 Summary

The chicken intestinal tract carries a diverse population of microbiota that offer protection against different pathogens, execute many metabolic tasks including fermentation of indigestible fibers in feed that are otherwise not possible for host enzymes to digest and have beneficial effects on health and production of the host. Over the past decades, much effort has been

made for enhancing the gut microbiota of chickens using dietary interventions for coping with surging poultry meat demands. Among them, use of antibiotics at sub-therapeutic levels as growth promoters has been the most popular and probably most effective strategy to enhance production and to keep animals healthy. However, such practices lead to antimicrobial resistance and its potential spread to human pathogens. Thus, we need to search for alternatives to antibiotics. Different classical and new strategies such as probiotics and transfer of mature microbiota are under investigation. The effects of *Bacillus* probiotics on production, intestinal microbiota and health parameters of host are not consistent, which emphasize the need for searching novel strains that have comparable production and health benefits as of antibiotic growth promoters. In addition, little is known about the effect of probiotics on birds under sub-optimal environmental conditions. Other new strategies like transfer of mature microbiota can also enhance the host defense against pathogens through competitive exclusion. However, much less has been investigated concerning its impact on production and health parameters of the host. To develop practical alternatives to AGPs, it is important to try novel candidates and understand their impact on diverse parameters related with host microbiota, production and health.

2.7 References

Aarestrup FM (2015) The livestock reservoir for antimicrobial resistance: A personal view on changing patterns of risks, effects of interventions and the way forward. *Philosophical Transactions of the Royal Society B*. **370**: 20140085.

Abd El-Ghany WA (2020) Paraprobiotics and postbiotics: Contemporary and promising natural antibiotics alternatives and their applications in the poultry field. *Open Veterinary Journal*. **10**: 323–330.

Alagawany M, Abd El-Hack ME & Farag MR (2018) The use of probiotics as eco-friendly alternatives for antibiotics in poultry nutrition. *Environmental Science and Pollution Research*. **25:** 10611–10618.

Alexandratos N & Bruinsma J (2012) World agriculture towards 2030/2050: the 2012 revision. In.: ESA Working paper FAO, Rome.

Aliakbarpour HR, Chamani M, Rahimi G, Sadeghi AA & Qujeq D (2012) The *Bacillus* subtilis and lactic acid bacteria probiotics influences intestinal mucin gene expression, histomorphology and growth performance in broilers. *Asian-Australasian Journal of Animal Sciences.* **25:** 1285-1293.

Alizadeh M, Bavananthasivam J, Shojadoost B, Astill J, Taha-Abdelaziz K, Alqazlan N, Boodhoo N, Shoja DJ & Sharif S (2021) In ovo and oral administration of probiotic Lactobacilli modulate cell- and antibody-mediated immune responses in newly hatched chicks. *Frontiers in Immunology.* **12:** 664387.

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.

Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y & Honda K (2011) Induction of colonic regulatory T cells by indigenous Clostridium species. *Science.* **331:** 337-341.

Awad WA, Mann E, Dzieciol M, Hess C, Schmitz-Esser S, Wagner M & Hess M (2016) Age-related differences in the luminal and mucosa-associated gut microbiome of broiler chickens and shifts associated with Campylobacter jejuni infection. *Frontiers in Cellular and Infection Microbiology*. **6**:154.

Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA & Gordon JI (2005) Host-bacterial mutualism in the human intestine. *Science.* **307:** 1915-1920.

Bai K., Huang Q, Zhang J, He J, Zhang L & Wang T (2016) Supplemental effects of probiotic *Bacillus* subtilis fmbJ on growth performance, antioxidant capacity, and meat quality of broiler chickens. *Poultry Science*. **96**: 74–82.

Bai S, Wu A, Ding X, Lei Y, Bai J, Zhang K & Chio J (2013) Effects of probiotic-supplemented diets on growth performance and intestinal immune characteristics of broiler chickens. *Poultry Science.* **92:** 663-670.

Ballou AL, Ali RA, Mendoza MA, Ellis JC, Hassan HM & Croom WJ (2016) Development of the chick microbiome: how early exposure influences future microbial diversity. *Frontier in Veterinary Science*. **3**:2.

Barnes, EM., Mead GC, Impey CS, & Adams BW (1978). The effect of dietary bacitracin on the incidence of Streptococcus faecalis subspecies liquefaciens and related streptococci in the intestines of young chicks. Br. Poultry Science. 19:713-723.

Barton MD (2000) Antibiotic use in animal feed and its impact on human health. *Nutrition Research Reviews.* **13:** 279-299.

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.

Bedford MR (2000) Exogenous enzymes in monogastric nutrition, their current value and future benefits. *Animal Feed Science and Technology*. **86:** 1-13.

Bennani H, Mateus A, Mays N, Eastmure E, Stärk KDC & Häsler B (2020). Overview of evidence of antimicrobial use and antimicrobial resistance in the food chain. *Antibiotics*. **9**: 49.

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

Biddle A, Stewart L, Blanchard J & Leschine S (2013) Untangling the genetic basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. *Diversity*. **5:** 627-40.

Boerlin, P & Reid-Smith RJ (2008) Antimicrobial resistance: Its emergence and transmission. *Animal Health Research Reviews.* **9:** 115-126.

Böhmig GA, Krieger PM, Säemann MD, Wenhardt C, Pohanka E, Zlabinger GJ (1997) n-butyrate downregulates the stimulatory function of peripheral blood-derived antigen-presenting cells: a potential mechanism for modulating T-cell responses by short-chain fatty acids. *Immunology*. **92:** 234-243.

Bouskra D, Brézillon C, Bérard M, Werts C, Varona R, Boneca IG & Eberl G (2008) Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. *Nature*. **456:** 507.

Boyd, FM, and Edwards, JHR (1967) Fat absorption by germ-free chicks. Poult. Sci. 46:1481-1483.

Broom JL & Kogut HM (2018) The role of the gut microbiome in shaping the immune system of chickens. *Veterinary Immunology and Immunopathology*. **204:** 5144-5145.

Brown K, Uwiera RR, Kalmokoff ML, Brooks SP & Inglis GD (2017) Antimicrobial growth promoter use in livestock: a requirement to understand their modes of action to develop effective alternatives. *International Journal of Antimicrobial Agents.* **49:** 12-24.

Brüssow H (2015) Growth promotion and gut microbiota: insights from antibiotic use. *Environmental Microbiology.* **17:** 2216–2227.

Brzoska F, Śliwiński B & Stecka K (2012) Effect of Lactococcus lactis vs. Lacto*Bacillus* Spp. bacteria on chicken body weight, mortality, feed conversion and carcass quality. *Annals of Animal Science.* **12:** 549-559.

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.

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.

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.

Carasi P, Racedo SM, Jacquot C, Elie AM, Serradell MdlÁ & Urdaci MC (2017) Enterococcus durans EP1 a promising anti-inflammatory probiotic able to stimulate sIgA and to increase Faecalibacterium prausnitzii abundance. *Frontiers in Immunology*. **8:** 88.

Carrasco JMD, Casanova NA & Miyakawa MEF (2019) Microbiota, gut health and chicken productivity: what is the connection? *Microorganisms.* **7:** 374.

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

Chantziaras I, Boyen F, Callens B & Dewulf J (2013) Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: A report on seven countries. *Journal of Antimicrobial Chemotherapy.* **69:** 827–834.

Cheled-Shoval SL, Withana Gamage NS, Amit-Romach E, Forder R, Marshal J, Van Kessel A, Uni Z (2014) Differences in intestinal mucin dynamics between germ-free and conventionally reared chickens after mannan-oligosaccharide supplementation. *Poultry Science.* **93:** 636-644.

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:** 69-83.

Chicken Farmers of Canada (2021) https://www.chickenfarmers.ca/faq/. Last accessed in August 2021.

Cho EA, Kim EJ & Pan JG (2011). Adsorption immobilization of Escherichia coli phytase on probiotic *Bacillus* polyfermenticus spores. *Enzyme and Microbial Technology*. **49:** 66-71.

Collado MC, Gueimonde M, Sanz Y & Salminen S (2006) Adhesion properties and competitive pathogen exclusion ability of bifidobacteria with acquired acid resistance. *Journal of Food Protection.* **69:** 1675-1679.

Corr SC, Li Y, Riedel CU, O'Toole PW, Hill C, & Gahan CG (2007) Bacteriocin production as a mechanism for the antiinfective activity of Lacto*Bacillus* salivarius UCC118. *Proceedings of the National Academy of Sciences.* **104:** 7617-7621.

Cotter PD, Ross RP & Hill C (2013) Bacteriocins—a viable alternative to antibiotics*? Nature Reviews Microbiology.***11:** 95.

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.

D'Costa VM, King CE, Kalan L, Morar M, Sung WWL, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R, Golding GB, Poinar HN & Wright GD (2011) Antibiotic resistance is ancient. *Nature.* **477:** 457-461.

Dang, HC., and Visek WJ (1960) Effects of urease injection on body weights of growing rats and chicks. Proc. Soc. Exp. Biol. Med. 105:164-167.

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. *Public Library of Science One.* **6:** e27949.

De Cesare A, Caselli E, Lucchi A, Sala C, Parisi A, Manfreda G & Mazzacane S (2019) Impact of a probiotic-based cleaning product on the microbiological profile of broiler litters and chicken caeca microbiota. Poultry Science. 98: 9- 3602-3610.

De Oliveira JE, Van der Hoeven-Hangoor E, Van de Linde IB, Montijn RC & Van der Vossen JMBM (2014) In ovo inoculation of chicken embryos with probiotic bacteria and its effect on posthatch Salmonella susceptibility. *Poultry Science.* **93:** 818-829.

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.

Denyer MP, Pinheiro DY, Garden OA & Shepherd AJ (2016) Missed, Not Missing: Phylogenomic Evidence for the Existence of Avian FoxP3. *Public Library of Science One.* **11:** e0150988.

Deriu E, Liu JZ, Pezeshki M, Edwards RA, Ochoa RJ, Contreras H & Raffatellu M (2013) Probiotic bacteria reduce *Salmonella typhimurium* intestinal colonization by competing for iron. *Cell Host and Microbe.* **14:** 26-37.

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

Ducatelle R, Goossens E, De Meyer F, Eeckhaut V, Antonissen G, Haesebrouck F & Van Immerseel F (2018) Biomarkers for monitoring intestinal health in poultry: Present status and future perspectives. *Veterinary Research.* **49:** 43.

ECDC/EFSA/EMA (2017) Second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals: Joint interagency antimicrobial consumption and resistance analysis (jiacra) report. *EFSA Journal.* **15:** 43.

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.

European Commission (2017) A European One Health Action Plan Against Antimicrobial Resistance (amr). https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52017DC0339&from=EN. Last accessed on 11 August 2021.

Eyssen, H. (1962) The additive effects of nucleic acids and antibiotics as individual growth promotants for chicks. Poultry Science. 41:1822-1828.

FAO (2002) Agriculture Organization of the United Nation and World Health Organization. Joint FAO/WHO working group report on drafting guidelines for the evaluation of probiotics in food.[online] http://www. who. int/foodsafety/fs_management/en/probiotic_guidelines. pdf.

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.

Feye KM, Baxter MFA, Tellez-Isaias G, Kogut MH & Ricke SC (2020) Symposium: Current and future directions for next generation sequencing of poultry microbiomes. Influential factors

on the composition of the conventionally raised broiler gastrointestinal microbiomes. *Poultry Science.* **99:** 653–659.

Fontenot JD, Gavin MA & Rudensky AY (2003) Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nature Immunology.* **4:** 330-336.

Froebel LK, Jalukar S, Lavergne TA, Coufal CD & Duong T (2020) Administration of direct-fed *Bacillus* cultures and refined functional carbohydrates to broiler chickens improves growth performance and promotes positive shifts in gastrointestinal microbiota. *Journal of Applied Poultry Research.* **29:** 4-765-774.

Fuller R (1989) Probiotic in man and animals. *Journal of Applied Bacteriology.* **66:** 131-139.

Gadde U, Kim WH, Oh ST & Lillehoj HS (2017) Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: A review. *Animal Health Research*. **18**: 26–45.

Gadde U, Oh ST, Lee YS, Davis E, Zimmerman N, Rehberger T & Hyun SL (2017) The effects of direct-fed microbial supplementation, as an alternative to antibiotics, on growth performance, intestinal immune status, and epithelial barrier gene expression in broiler chickens. *Probiotics & Antimicrobial Proteins*. **9:** 397–405.

Gao J, Zhang H, Yu S, Wu S, Yoon I, Quigley J & Qi G (2008) Effects of yeast culture in broiler diets on performance and immunomodulatory functions. *Poultry Science.* **87:** 1377-1384.

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.

Gong Y, Yang H, Wang X, Xia W, Lv W, Xiao Y & Zou X (2019) Early intervention with cecal fermentation broth regulates the colonization and development of gut microbiota in broiler chickens. *Frontiers in Microbiology.* **10:** 1422.

Government of Canada (2009) Categorization of Antimicrobial Drugs Based on Importance in Human Medicine. https://www.canada.ca/en/health-canada/services/drugs-health-products/veterinary-drugs/antimicrobial-resistance/categorization-antimicrobial-drugs-based-importance-human-medicine.html. Last accessed in March 2022.

Government of Canada (2018) https://www.canada.ca/en/public-health/services/antibiotic-antimicrobial-resistance/animals/actions/responsible-use-antimicrobials.html. Last accessed in August 2021.

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.

Guglielmetti S, Taverniti V, Minuzzo M, Arioli S, Stuknyte M, Karp M & Mora D (2010) Oral bacteria as potential probiotics for the pharyngeal mucosa. *Applied and Environmental Microbiology.* **76:** 3948-3958.

Gustafson RH & Bowen RE (1997) Antibiotic use in animal agriculture. *Journal of Applied Microbiology.* **83:** 531–41.

Han GG, Kim EB, Lee J, Lee J-Y, Jin G, Park J, Huh C-S, Kwon L-K, Kil DY, Choi Y-J & Kong C (2016) Relationship between the microbiota in different sections of the gastrointestinal tract, and the body weight of broiler chickens. *Springerplus.* **5:** 911.

Handtke S, Schroeter R, Jürgen B, Methling K, Schlüter R, Albrecht D & Lalk M (2014) *Bacillus pumilus* reveals a remarkably high resistance to hydrogen peroxide provoked oxidative stress. *Public Library of Science One.* **9:** e85625.

Hao H, Cheng G, Iqbal Z, Ai X, Hussain HI, Huang L, Dai M, Wang Y, Liu Z & Yuan Z (2014) Benefits and risks of antimicrobial use in food-producing animals. *Frontiers in Microbiology.* **5:** 288.

Harrington D, Sims M & Kehlet AB (2016) Effect of *Bacillus* subtilis supplementation in low energy diets on broiler performance. *Journal of Applied Poultry Research.* **25:** 29-39.

Hernández-González JC, Martínez-Tapia A, Lazcano-Hernández G, García-Pérez BE & Castrejón-Jiménez NS (2021) Bacteriocins from Lactic Acid Bacteria. A Powerful Alternative as Antimicrobials, Probiotics, and Immunomodulators in Veterinary Medicine. *Animals.* **11:** 979.

Higgins J, Higgins S, Wolfenden A, Henderson S, Torres-Rodriguez A, Vicente J & Tellez G (2010) Effect of lactic acid bacteria probiotic culture treatment timing on *Salmonella enteritidis* in neonatal broilers. *Poultry Science.* **89:** 243-247.

Hills RD, Pontefract BA, Mishcon HR, Black CA, Sutton SC & Theberge CR (2019) Gut microbiome: profound implications for diet and disease. *Nutrients*. **11:** 1613.

Honda K & Littman DR (2016) The microbiota in adaptive immune homeostasis and disease. *Nature*. **535:** 75-84.

Hong HA, Duc LH & Cutting SM (2005) The use of bacterial spore formers as probiotics. *FEMS Microbiology Reviews.* **29:** 813-835.

Hooper LV (2004) Bacterial contributions to mammalian gut development. *Trends in Microbiology.* **12:** 129-134.

Huang M, Choi Y, Houde R, Lee JW, Lee B & Zhao X (2004) Effects of Lactobacilli and an acidophilic fungus on the production performance and immune responses in broiler chickens. *Poultry Science.* **83:** 788-795.

Hume M (2011) Food safety symposium: potential impact of reduced antibiotic use and the roles of prebiotics, probiotics, and other alternatives in antibiotic-free broiler production. *Poultry Science.* **90:** 2663-2669.

Humphrey MB & Nakamura MC (2015) A comprehensive review of immunoreceptor regulation of osteoclasts. *Clinical Reviews in Allergy & Immunology.* **51:** 48-58.

Iwasaki A (2007) Mucosal dendritic cells. *Annual Review of Immunology.* **25:** 381-418.

Jacquier V, Nelson A, Jlali M, Rhayat L, Brinch KS & Devillard E (2019) *Bacillus subtilis* 29784 induces a shift in broiler gut microbiome toward butyrate producing bacteria and improves intestinal histomorphology and animal performance. *Poultry Science.* **98:** 2548–2554.

Jagadeesan Y, Meenakshisundaram S, Saravanan V & Balaiah A (2020) Sustainable production, biochemical and molecular characterization of thermo-and-solvent stable alkaline serine keratinase from novel *Bacillus pumilus* AR57 for promising poultry solid waste management. *International Journal of Biological Macromolecules.* **163:** 135-146.

Jayaraman S, Thangavel G, Kurian H, Mani R, Mukkalil R & Chirakkal H (2013) *Bacillus subtilis* PB6 improves intestinal health of broiler chickens challenged with Clostridium perfringens-induced necrotic enteritis. *Poultry Science.* **92:** 370-374.

Jiang Z, Applegate TJ & Lossie AC (2013) Cloning, annotation and developmental expression of the chicken intestinal MUC2 gene. *Public Library of Science One.* **8:** e53781.

Jiratitipat N, Srikhong P & Chansiripornchai N (2019) Efficacy of competitive exclusion to reduce Salmonella in broiler chickens. *Thai Journal of Veterinary Medicine.* **49:** 385-391.

Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L & Hansson GC (2008) The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proceedings of the National Academy of Sciences*. **105:** 15064-15069.

Kapoor M & Kuhad RC (2007) Immobilization of xylanase from *Bacillus* pumilus strain MK001 and its application in production of xylo-oligosaccharides. *Applied Biochemistry and Biotechnology.* **142:** 125-138.

Kaspers B, Lettmann S & Roell S (2015) Development of the gut associated immune system. 20th european symposium on poultry nutrition, Prague, Czech Republic.

Kau AL, Ahern PP, Griffin NW, Goodman AL & Gordon JI (2011) Human nutrition, the gut microbiome and the immune system. *Nature.* **474:** 327.

Kers JG, Velkers FC, Fischer EAJ, Hermes GDA, Stegeman JA & Smidt H (2018) Host and environmental factors affecting the intestinal microbiota in chickens. *Frontiers in Microbiology.* **9:** 235.

Kraehenbuhl JP & Neutra MR (2000) Epithelial M cells: differentiation and function. *Annual Review of Cell and Developmental Biology.* **16:** 301-332.

Kubasova T, Kollarcikova M, Crhanova M, Karasova D, Cejkova D & Sebkova A (2019) Contact with adult hen affects development of caecal microbiota in newly hatched chicks. *Public Library of Science One*. **14:** e0212446.

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 Lacto*Bacillus* 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.

Laudadio V, Dambrosio A, Normanno G, Khan RU, Naz S, Rowghani E & Tufarelli V (2012). Effect of reducing dietary protein level on performance responses and some microbiological aspects of broiler chickens under summer environmental conditions. *Avian Biology Research.* **5:** 88-92.

Lee IK, Bae S, Gu MJ, You SJ, Kim G, Park S, Jeung W, Ko KH, Cho JK, Kang JS & Yun C (2017) H9N2-specific IgG and CD4+CD25+ T cells in broilers fed a diet supplemented with organic acids. *Poultry Science.* **96:** 1063-1070.

Lee IK, Gu MJ, Ko KH, Bae S, Kim G, Jin G, Kim EB, Kong Y, Park TS, Park B, Jung HJ, Han SH & Yun C (2018) Regulation of CD4+CD8−CD25+and CD4+CD8+CD25+ T cells by gut microbiota in chicken. *Scientific Reports*. **8:** 8627.

Lee K, Lee S, Lillehoj H, Li G, Jang S, Babu U & Neumann A (2010) Effects of direct-fed microbials on growth performance, gut morphometry, and immune characteristics in broiler chickens. *Poultry Science.* **89:** 203-216.

Lee KC, Kil DY & Sul WJ (2017) Cecal microbiome divergence of broiler chickens by sex and body weight. *Journal of Microbiology.* **55:** 939-45.

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.

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

Low CX, Tan LT-H, Ab Mutalib N-S, Pusparajah P, Goh B-H, Chan K-G, Letchumanan, V & Lee L-H (2021) Unveiling the impact of antibiotics and alternative methods for animal husbandry: A Review. *Antibiotics.* **10:** 578.

Luan S, Duersteler M, Galbraith E & Cardoso F (2015) Effects of direct-fed *Bacillus pumilus* 8G-134 on feed intake, milk yield, milk composition, feed conversion, and health condition of pre-and postpartum Holstein cows. *Journal of Dairy Science.* **98:** 6423-6432.

Luan SJ, Sun YB, Wang Y, Sa RN & Zhang HF (2019) *Bacillus* amyloliquefaciens spray improves the growth performance, immune status, and respiratory mucosal barrier in broiler chickens. *Poultry Science*. **98:** 1403–1409.

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.

Lundberg R, Scharch C & Sandvang D (2021) The link between broiler flock heterogeneity and cecal microbiome composition. *Animal Microbiome.* **3:** 54.

Ma Y, Wang W, Zhang H, Wang J, Zhang W, Gao J, Wu S & Qi G (2018) Supplemental *Bacillus subtilis* DSM 32315 manipulates intestinal structure and microbial composition in broiler chickens. *Scientific Reports.* **8:** 15358.

Madej J, Stefaniak T & Bednarczyk M (2015) Effect of in ovo-delivered prebiotics and synbiotics on lymphoid-organs' morphology in chickens. *Poultry Science.* **94:** 1209-1219.

Madsen KL, Malfair D, Gray D, Doyle JS, Jewell LD & Fedorak RN (1999) Interleukin-10 gene-deficient mice develop a primary intestinal permeability defect in response to enteric microflora. *Inflammatory Bowel Disease.* **5:** 262-270.

Mahne AE, Klementowicz JE, Chou A, Nguyen V & Tang Q (2015) Therapeutic regulatory T cells subvert effector T cell function in inflamed islets to halt autoimmune diabetes. *The Journal of Immunology*. **194:** 3147-3155.

Markowiak-Kopeć P & Śliżewska K (2020) The effect of probiotics on the production of short-chain fatty acids by human intestinal microbiome. *Nutrients.* 12: 1107.

Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Di Y & Mackay CR (2009) Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature.* **461:** 1282-1286.

Matis G, Kulcsar A, Turowski V, Febel H, Neogrády Z & Huber K (2015) Effects of oral butyrate application on insulin signaling in various tissues of chickens. *Domestic Animal Endocrinology.* **50:** 26-31.

McCafferty J, Mühlbauer M, Gharaibeh RZ, Arthur JC, Perez-Chanona E, Sha W, Jobin C & Fodor AA (2013) Stochastic changes over time and not founder effects drive cage effects in microbial community assembly in a mouse model. *ISME Journal.* **7:** 2116-2125.

Metzler-Zebeli BU, Siegerstetter SC, Magowan E, Lawlor PG, O'Connell NE & Zebeli Q (2019) Fecal microbiota transplant from highly feed efficient donors affects cecal physiology and microbiota in low and high-feed efficient chickens. *Frontiers in Microbiology*. **10**:1576.

Milbradt EL, AS Okamoto, CR Padovani, VB Fascina, TM Silva, R Altarúgio, A Hataka, EMS Schmidt and RL Andreatti Filho (2017) Use of organic acids and a competitive exclusion product as growth promoter and *Salmonella enteritidis* control in commercial turkeys. *Brazilian Journal of Poultry Science.* **19:** 551-558.

Milbradt EL, Zamae JR, Araujo Junior JP, Mazza P, Padovani CR, Carvalho VR, Sanfelice C, Rodrigues DM, Okamoto AS, Andreatti Filho RL (2014) Control of *Salmonella enteritidis* in turkeys using organic acids and competitive exclusion product. *Journal of Applied Microbiology.* **117:** 554-563.

Mingmongkolchai S & Panbangred W (2018) *Bacillus* probiotics: an alternative to antibiotics for livestock production. *Journal of Applied Microbiology*. **124:** 1334-1346.

Mohsin M, Abbas RZ, Yin G, Sindhu Z, Abbas A, Huang Z, Aleem MT, Saeed Z, Afzal MZ, Ejaz A & Shoaib M (2021) Probiotics as therapeutic, antioxidant and immunomodulatory agents against poultry coccidiosis. *World's Poultry Science Journal.* **77:** 2.

Mongkolthanaruk W (2012) Classification of *Bacillus* beneficial substances related to plants, humans and animals. *Journal of Microbiology and Biotechnology*. **22:** 1597-1604.

Mullis K, Erlich H, Arnheim N, Horn G, Saiki R & Scharf S (1987) One of the first Polymerase Chain Reaction (PCR) patents. *Google Patents*.

Nair MS, Amalaradjou M & Venkitanarayanan K (2017) Antivirulence properties of probiotics in combating microbial pathogenesis. *Advances in Applied Microbiology.* **98:** 1-29.

Newberry RD & Lorenz RG (2005) Organizing a mucosal defense. *Immunological Reviews.* **206:** 6-21.

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.

O'Neill J (2016) Tackling dRug-Resistant Infections Globally: Final Report and Recommendations. 2016. https://amr-review.org/sites/default/files/160525_Final%20paper_with%20cover.pdf. Last accessed on 11 August 2021.

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

Ocejo M, Oporto B, & Hurtado A (2019) 16S rRNA amplicon sequencing characterization of caecal microbiome composition of broilers and free-range slow growing chickens throughout their productive lifespan. *Scientific Reports*. **9**: 2506.

Oh S, Lillehoj HS, Lee Y, Bravo D & Lillehoj EP (2019) Dietary antibiotic growth promoters down-regulate intestinal inflammatory cytokine expression in chickens challenged with LPS or co-infected with Eimeria maxima and Clostridium perfringens. *Frontier in Veterinary Science* **6:** 420.

Ohnmacht C, Marques R, Presley L, Sawa S, Lochner M & Eberl G (2011) Intestinal microbiota, evolution of the immune system and the bad reputation of pro-inflammatory immunity. *Cellular Microbiology.* **13:** 653-659.

Okeke EB, Okwor I, Mou Z, Jia P & Uzonna JE (2013) CD4+CD25+ regulatory T cells attenuate lipopolysaccharide-induced systemic inflammatory responses and promotes survival in murine Escherichia coli infection. *Shock.* **40:** 65-73.

Oladokun S, Koehler A, MacIsaac J, Ibeagha-Awemu EM & Adewole DI (2021) *Bacillus subtilis* delivery route: effect on growth performance, intestinal morphology, cecal short-chain fatty acid concentration, and cecal microbiota in broiler chickens. *Poultry Science.* **100:** 100809.

Ouyang W, Rutz S, Crellin NK, Valdez PA & Hymowitz SG (2011) Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annual Review of Immunology*. **29:** 71-109.

Pan D & Yu Z (2014) Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes.* **5:** 108-119.

Park JH & Kim IH (2014) Supplemental effect of probiotic *Bacillus* subtilis B2A on productivity, organ weight, intestinal Salmonella microflora, and breast meat quality of growing broiler chicks. *Poultry Science*. **93:** 2054-2059.

Patel SG, Raval AP, Bhagwat SR, Sadrasaniya DA, Patel AP & Joshi SS (2015) Effects of probiotics supplementation on growth performance, feed conversion ratio and economics of broilers. *Journal of Animal Research.* **5:** 155.

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

Pedrosa AA. Which came first: the egg or its microbiota? Poult Inform Prof (2009):1–5. Available from:https://www.researchgate.net/publication/257367431_Which_came_first_the_egg_ or its_microbiota.

Petersen ER, Claesson MH, Schmidt EGW, Jensen SS, Ravn P, Olsen J & Kristensen NN (2011) Consumption of probiotics increases the effect of regulatory T cells in transfer colitis. *Inflammatory Bowel Diseases.* **18:** 131-142.

Petersson J, Schreiber O, Hansson GC, Gendler SJ, Velcich A, Lundberg JO & Phillipson M (2010) Importance and regulation of the colonic mucus barrier in a mouse model of colitis. *American Journal of Gastrointestinal and Liver Physiology.* **300:** 327-333.

Pollock J, Glendinning L, Wisedchanwet T & Watson M (2018) The madness of microbiome: Attempting to find consensus "Best practice" for 16S microbiome studies. *Applied and Environmental Microbiology.* **84:** e02627-17.

Popov IV, Algburi A, Prazdnova EV, Mazanko MS, Elisashvili V, Bren AB, Chistyakov VA, Tkacheva EV, Trukhachev VI & Donnik IM (2021). A review of the effects and production of spore-forming probiotics for poultry. *Animals.* **11:** 1941.

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.

Public Health Agency of Canada (2020) Canadian antimicrobial resistance surveillance system report. Cat. #: HP37-21E-PDF: 190498.

Qu A, Brulc JM, Wilson MK, Law BF, Theoret JR, Joens LA & Edwards RA (2008) Comparative metagenomics reveals host specific metavirulomes and horizontal gene transfer elements in the chicken cecum microbiome. *Public Library of Science One.* **3:** e2945.

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. *Public Library of Science 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.

Reddy MR, Reddy KS, Chouhan YR, Bee H & Reddy G (2017) Effective feather degradation and keratinase production by *Bacillus* pumilus GRK for its application as bio-detergent additive. *Bioresource Technology.* **243:** 254-263.

Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE & Kau AL (2013) Cultured gut microbiota from twins discordant for obesity modulate adiposity and metabolic phenotypes in mice. *Science*. **341:** 6150.

Rychlik I (2020) Composition and Function of Chicken Gut Microbiota. *Animals*. **10**: 103.

Sakaguchi S, Miyara M, Costantino CM & Hafler DA (2010) FOXP3+ regulatory T cells in the human immune system. *Nature Reviews Immunology.* **10:** 490-500.

Samanya M & Yamauchi K (2002) Histological alterations of intestinal villi in chickens fed dried *Bacillus subtilis* var. natto. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology.* **133:** 95-104.

Sanders M, Morelli L & Tompkins T (2003) Sporeformers as human probiotics: *Bacillus*, Sporolacto*Bacillus*, and Brevi*Bacillus*. *Comprehensive Reviews in Food Science and Food Safety.* **2:** 101-110.

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

Seal BS, Lillehoj HS, Donovan DM & Gay CG (2013) Alternatives to antibiotics: A symposium on the challenges and solutions for animal production. *Animal Health Research. Reviews.* **14:** 78-87.

Sen S, Ingale S, Kim Y, Kim J, Kim K, Lohakare J & Kwon I (2012) Effect of supplementation of *Bacillus* subtilis LS 1-2 to broiler diets on growth performance, nutrient retention, caecal microbiology and small intestinal morphology. *Research in Veterinary Science.* **93:** 264-268.

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.

Śmiałek M, Kowalczyk J & Koncicki A (2021) The use of probiotics in the reduction of campylobacter spp. prevalence in poultry. *Animals.* **11:** 1355.

Smith AL, Powers C & Beal RK (2014) Chapter 13—The avian enteric immune system in health and disease. Avian Immunology, 2nd ed.; Academic Press: Boston, MA, USA. pp.227–250.

Smith TC, Gebreyes WA, Abley MJ, Harper AL, Forshey BM, Male MJ, Martin HW, Molla BZ, Sreevatsan S, Thakur S, Thiruvengadam M & Davies PR (2013) Methicillin-resistant Staphylococcus aureus in pigs and farm workers on conventional and antibiotic-free swine farms in the USA. *Public Library of Science One.* **8:** e63704.

Sohail MU, Hume ME, Byrd JA, Nisbet DJ, Shabbir MZ, Ijaz A, Rehman A (2015). Molecular analysis of the caecal and tracheal microbiome of heatstressed broilers supplemented with prebiotic and probiotic. *Avian Pathology.* **44:** 67-74.

Sohail MU, Rahman ZU, Ijaz A, Yousaf MS, Ashraf K & Yaqub T (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.

Soliman ES, Taha E, Infante KD, Laboy K, Sobieh MA & Reddy PG (2009) Stressors influence on Salmonella enterica serovar enteritidis colonization in broilers. *American Journal of Animal and Veterinary Sciences.* **4:** 42-48.

Solis-Cruz B, Hernandez-Patlan D, Petrone VM, Pontin KP, Latorre JD, Beyssac E, Hernandez-Velasco X, Merino-Guzman R, Arreguin MA, Hargis BM, Lopez-Arellano R & Tellez-Isaias G (2019) Evaluation of a *Bacillus*-Based Direct-Fed Microbial on Aflatoxin B1 Toxic Effects, Performance, Immunologic Status, and Serum Biochemical Parameters in Broiler Chickens. *Avian Diseases.* **63:** 659-669.

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.

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

Sugiharto S (2019) A review offilamentous fungi inbroiler production. *Annals of Agricultural Sciences.* **64:** 1-8.

Sugimoto K, Ogawa A, Mizoguchi E, Shimomura Y & Andoh A (2008) IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *Journal of Clinical Investigation.* **118:** 534-544.

Sun X, Yang H & Nose K (2008) Decline in intestinal mucosal IL-10 expression and decreased intestinal barrier function in a mouse model of total parenteral nutrition. *American Journal Physiology-Gastrointestinal and Liver Physiology.* **294:** 139-147.

Suvarna V & Boby V (2005) Probiotics in human health: A current assessment. *Current science.* **88:** 1744-1748.

Tamayo-Ramos JA, Sanz-Penella JM, Yebra MJ, Monedero V & Haros M (2012) Novel phytases from *Bifidobacterium pseudocatenulatum* ATCC 27919 and *Bifidobacterium longum* subsp. *infantis* ATCC 15697. *Applied and Environmental Microbiology.* **78:** 5013-5015.

Taverniti V, Marengo M, Fuglsang E, Skovsted HM, Arioli S, Mantegazza G, Gargari G, Iametti S, Bonomi F, Guglielmetti S, Frøkiær H (2019) Surface layer of *Lactobacillus helveticus* MIMLh5 promotes endocytosis by dendritic cells. *Applied and Environmental Microbiology.* **85:** e00138-19.

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.

Tellez G, Higgins SE, Donoghue AM & Hargis BM (2006) Digestive physiology and the role of microorganisms. *Journal of Applied Poultry Research.* **15:** 136-144.

Thirabunyanon M & Thongwittaya N (2012) Protection activity of a novel probiotic strain of *Bacillus* subtilis against Salmonella Enteritidis infection. *Research in Veterinary Science.* **93:** 74-81.

Thomas M, Wongkuna S, Ghimire S, Kumar R, Antony L, Doerner KC, Singery A, Nelson E, Woyengo T & Chankhamhaengdecha, S (2019) Gut microbial dynamics during conventionalization of germfree chicken. *mSphere*. **4**: e00035-19.

Topping DL & Clifton PM (2001) Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiological Reviews.* **81:** 1031-1064.

Torok VA, Hughes RJ, Mikkelsen LL, Perez-Maldonado R, Balding K, MacAlpine R, Percy NJ & Ophel-Keller K (2011) 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, 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, 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.

Ulluwishewa D, Anderson AC, McNabb WC, Moughan PJ, Well JM & Roy NC (2011) Regulation of tight junction permeability by intestinal bacteria and dietary components. *Journal of Nutrition*. **141:** 769-776.

United Nations (2015) World population prospects: The 2015 revision. *United Nations Economic and Social Affairs.* **33:** 1-66.

Upadhaya SD, Rudeaux F & Kim IH (2019) Effects of inclusion of *Bacillus* subtilis (Gallipro) to energy- and protein-reduced diet on growth performance, nutrient digestibility, and meat quality and gas emission in broilers. *Poultry Science.* **98:** 2169-2178.

Valin H, Sands RD, van der Mensbrugghe D, Nelson GC, Ahammad H, Blanc E, Bodirsky B, Fujimori S, Hasegawa T & Havlik P (2014) The future of food demand: understanding differences in global economic models. *Agricultural Economics.* **45:** 51-67.

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.

Vanbelle M, Teller E & Focant M (1990) Probiotics in animal nutrition: a review. *Archives of Animal Nutrition.* **40:** 543-567.

Vanderpool C, Yan F & Polk BD (2008) Mechanisms of probiotic action: implications for therapeutic applications in inflammatory bowel diseases. *Inflammatory Bowel Diseases.* **14:** 1585-1596.

Varmuzova K, Kubasova T, Davidova-Gerzova L, Sisak F, Havlickova H, Sebkova A, Faldynova M & Rychlik I (2016) Composition of gut microbiota influences resistance of newly hatched chickens to Salmonella enteritidis infection. *Frontiers in Microbiology.* **7:** 957.

Volf J, Polansky O, Varmuzova K, Gerzova L, Sekelova Z, Faldynova M, Babak V, Medvecky M, Smith AL, Kaspers B, Velge P & Rychlik I (2016) Transient and prolonged response of chicken cecum mucosa to colonization with different gut microbiota. *Public Library of Science One.* **11:** e0163932.

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

Wall R, Hussey SG, Ryan CA, O'neill M, Fitzgerald G, Stanton C & Ross RP (2008) Presence of two Lacto*Bacillus* and Bifidobacterium probiotic strains in the neonatal ileum. *The ISME Journal.* **2:** 83.

Weaver CT, Elson CO, Fouser LA & Kolls JK (2013) The Th17 pathway and inflammatory diseases of the intestines, lungs, and skin. *Annual Review of Pathology.* **8:** 477-512.

Wei S, Morrison M & Yu Z (2013) Bacterial census of poultry intestinal microbiome. *Poultry Science.* **92:** 671-683.

Woese CR & Fox GE (1977) Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proceedings of the National Academy of Sciences*. **74:** 5088-5090.

World Health Organization. Global Action Plan on Antimicrobial Resistance. 2015. Available online:https://apps.who.int/iris/bitstream/handle/10665/193736/9789241509763_eng.pdf?sequence=1&isAllowed=y. Last accessed on 11 August 2021.

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 P & Choct M (2009) Dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. *World's Poultry Science Journal.* **65:** 97-114.

Ye X, Li P, Yu Q &Yang Q (2013) *Bacillus subtilis* inhibition of enterotoxic *Escherichia coli*-induced activation of MAPK signaling pathways in Caco-2 cells. *Annals of Microbiology.* **63:** 577-581.

Yin X, Heeney D, Srisengfa Y, Golomb B, Griffey S & Marco M (2018) Bacteriocin biosynthesis contributes to the anti-inflammatory capacities of probiotic *Lactobacillus plantarum*. *Beneficial microbes.* **9:** 333-344.

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.

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

Zaghari M, Derakhshani-Diba M, Moravej H & Zahroojian N (2017) Estimation of metabolizable energy equivalency of *Bacillus subtilis* spore in male broiler chickens. *Journal of Livestock Science and Technologies.* **5:** 9-18.

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.

Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q & De Sauvage FJ (2008) Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nature Medicine.* **14:** 282.

Zhu L, Hua F, Ding W, Ding K, Zhang Y & Xu C (2020) The correlation between the Th17/Treg cell balance and bone health. *Immunity & Ageing*. **17**: 30.

Chapter 3. Effects of novel probiotic strains of *Bacillus pumilus* and *Bacillus subtilis* on production, gut health and immunity of broiler chickens raised under sub-optimal conditions

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3.1 Abstract

Probiotics are being developed as alternatives to antibiotic growth promoters. The aim of the study was to investigate the effects of two novel strains of *Bacillus pumilus* and *Bacillus subtilis* on production, intestinal microbiota, gut health, and immunity of broilers raised under suboptimal conditions. Day-old chicks (Cobb 500, n = 2,073) were randomly assigned into 6 groups: Con group (group fed with basal diet), Ab group (group treated with virginiamycin), groups treated with 2 levels of *B. pumilus* (low dose: 3×10^8 cfu/kg of feed [BPL] and high dose: 1×10^9 cfu/kg [BPH]), and groups treated with 2 levels of *B. subtilis* (low dose: 3×10^8 cfu/kg [BSL] and high dose: 1×10^9 cfu/kg [BSH]). Production parameters were recorded weekly. Cecal tonsils and content as well as ileum samples were collected on day 14 and day 42. Cecal tonsils were used to sort T-regulatory cells (CD4+CD8-CD25+ and CD4+CD8+CD25+) to study expression of IL-10 and interferon gamma, whereas cecal content was used for bacterial culture. Ileum samples were used to measure gene expression of tight junction proteins, mucin, and cytokines. BW and feed intake increased in the Ab, BPL, BSL, and BSH groups compared with the Con group between day 35 and day 42. The CD4+CD8-CD25+ cells expressed high levels of IL-10 in the BSH group on day 14 and in the BPL, BSL, and BSH groups on day 42 and high levels of interferon gamma in the BPL, BSL, and BSH groups on day 14 and in the BSL and BSH groups on day 42. The expression of IL-10 and interferon gamma in CD4+CD8+CD25+ cells was higher only in the BSH group on day 14 and day 42. Cecal bacterial populations of genera, Lacto*Bacillus* (day 14 and day 42) and Clostridium (day 14), were higher in the BSH group. Expression of tight junction protein increased significantly in the ileum on day 14 in the BPL (occludin, zona occludens 1 [ZO-1]), BSL (occludin, ZO-1), and BSH (occludin, ZO-1, junctional adhesion molecule 2 [JAM-2]) groups compared with that in the Con group and declined in all

groups except in the BSH group (occludin, ZO-1, JAM-2) on day 42. Expression of MUC2 and IL-17F increased in all groups on day 14 and remained high on day 42 in the BSL and BSH groups. Taken together, both *Bacillus* probiotics altered the intestinal and immune activities, particularly on day 14, suggesting beneficial influence of probiotics.

3.2 Introduction

To meet the growing demand for animal protein, world poultry meat production soared from 9 to 122 million tonnes between 1961 and 2017 (FAO, 2020). It is expected to continue increasing annually by 2.4% between 2015 and 2030 (FAO, 2015). To help broilers to maintain good health after the ban of subtherapeutic antibiotics as growth promoters, many different classes of alternatives are being developed, including probiotics, prebiotics, synbiotics, organic acids, phytogenics, antimicrobial peptides, and bacteriophages (Gadde et al., 2017a). Probiotics represent a nutritional approach to enhance production- and health-related parameters in broiler chickens (Grant et al., 2018). Probiotics also help in disease prevention and recovery from infections. Our laboratory has previously shown that *Lactobacillus plantarum* reversed *Salmonella typhimurium*–induced negative effects in terms of inflammation (Chen et al., 2017) and disrupted intestinal permeability (Wang et al., 2018). *Bacillus*-based probiotics used in recent studies showed strain-specific effects on the host, based on production and health parameters measured. Gadde et al. (2017b) and Jacquier et al. (2019) reported improvement in different growth parameters including feed conversion rate (FCR), using different *Bacillus subtilis* strains as probiotics. However, other authors reported no significant changes in FCR while using different *Bacillus* strains (Teo and Tan, 2007; Ma et al., 2018; Luan et al., 2019). Similar to growth parameters, the host immune and gut health responses to probiotics also appeared to be strain specific. Ma et al. (2018) found no significant immune response to a *B.*

subtilis strain, whereas others reported strong activation of immune-related components by different *Bacillus* strains (Teo and Tan, 2007; Gadde et al., 2017b; Luan et al., 2019). In addition, while Aliakbarpour et al. (2012) and Luan et al. (2019) observed significant increases in mucin production in response to *Bacillus*-based probiotics, Gadde et al. (2017b) reported no difference in mucin production in response to different *Bacillus* strains. The strain-specific effects were also reported for gut integrity (Gadde et al., 2017b; Rhayat et al., 2019). Currently, there is no clear explanation for strain- and dose-specific effects of *Bacillus* probiotics. Different groups are still developing and testing novel strains of *Bacillus*-based probiotics that could have potential to influence hosts with broader beneficial effects.

Probiotics may induce beneficial effects through different mechanisms including modulation of intestinal microbiota, which is closely linked with maturation of the immune system (Broom and Kogut, 2018). The composition of gut microbiota in broilers is age dependent, and 2 distinct diversified sets of microbiota are present on day 14 and on day 42 during the broiler life cycle (Ocejo et al., 2019). Furthermore, commensal microbes affect various immune cells, including regulatory T cells (Treg), dendritic cells, and IgA-secreting B cells, leading to suppression of unnecessary inflammation in a mouse model (Chu and Mazmanian, 2013). Regulatory T cells are a subtype of CD4+ T cells and play an important role in keeping gut immune homeostasis as the intestinal barrier is constantly exposed to microbial antigens with a potential to induce inflammation (Sun et al., 2008). In chickens, CD4+CD25+ T cells are considered as the Treg (Lee et al., 2017), as the key Foxp3 equivalent gene, the master transcription factor for Treg, is not described in poultry yet except in peregrine falcons and saker falcons (Denyer et al., 2016). Recently, a relationship between Treg (CD4+CD25+ T cells) and gut microbiota in chicken was studied in antibiotic-treated chickens through administration of an

antibiotic cocktail consisting of ampicillin, gentamycin, neomycin, metronidazole, and vancomycin in water for 7 d (Lee et al., 2018). Both CD4+CD8−CD25+ and CD4+CD8+CD25+ T cells in cecal tonsils were significantly decreased by antibiotic treatment, and gram-positive bacteria, especially *Clostridia*, were responsible for the changes in CD4+CD8−CD25+ or CD4+CD8+CD25+ T cells in cecal tonsils (Lee et al., 2018). These findings provided clues for potential cross talk between intestinal microbiota and Treg and influence on generation of controlled response to inflammatory signals originating from the gut environment. Probiotics, like antibiotics used as antibiotic growth promoters, remodel the diversity and richness of intestinal microbiota and may have direct or indirect influence on regulation of T cells to check the inflammatory mechanisms. However, the effects of probiotics on Treg and their anti-inflammatory response in chickens have not been investigated so far.

The present study, therefore, was designed to evaluate the effects of novel strains of *Bacillus pumilus* and *B. subtilis* on production, intestinal microbiota, gut health, and immunity (Treg) of broiler chickens raised under suboptimal conditions.

3.3 Material and Methods

3.3.1 Birds, Diet, and Experimental Design

A total of 2,073 one-day old male broiler chicks (Cobb 500) were obtained from a local hatchery (Grains Natures, Tonton Falls, Quebec Canada) and randomly divided into 36 pens (6 pens/treatment). These birds were assigned to 6 treatments and grown until 35 days (6 pens/treatment) or until 42 days (3 pens/treatment). The dietary treatments included (1) a standard basal diet (Con); (2) a basal diet with antibiotic (Virginiamycin @ 16.5mg/kg of feed) (Ab); (3) a basal diet with a low dose of *Bacillus pumilus* (3×10^8 CFU/kg of feed) (BPL); (4) a basal diet with a high dose of *Bacillus pumilus* (1×10^9 CFU/kg of feed) (BPH); (5) a basal diet with a low

dose of *Bacillus subtilis* (3 x 10^8 CFU/kg of feed) (BSL); and (6) a basal diet with a high dose of *Bacillus subtilis* (1 x 10^9 CFU/kg of feed) (BSH). The basal diet was composed of corn, soybean meal, soybean oil, amino acids supplements, monensin as anticoccidial, vitamins and mineral premix, and mixed as per the standard of National Research Council (National Research Council, 1994) (Table 1). The chickens were fed with a starter feed (23% protein and 2,977 kcal metabolizable energy/kg) from day 1 to day 14 and a grower feed (20% protein and 3,056 kcal metabolizable energy/kg) from day 15 to day 42 (Table 1). The feed and water supplied *ad libitum*. The probiotics (*Bacillus pumilus* and *Bacillus subtilis*) were provided by Lallemand SAS, France.

The experiment conducted in sub-optimal conditions to simulate industrial conditions and evaluate treatment responses, as previously described (Pourabedin et al., 2014). In brief, birds were reared at a higher density (16 birds/m^2) than normal density (12 birds/ m^2), colder temperature starting at day 8^{th} (4 °C lower than stipulated code of practice) and higher intestinal viscosity by adding 0.5% guar gum in the feed (Silbergeld et al., 2008). The lighting regimen was 23-hour light and 1-hour darkness by day 5 of placement and darkness gradually increased to 4 hours for the rest of the study. The feed intake (FI), body weight (BW) and feed conversion rate (FCR) calculated on a weekly basis and mortality checked daily for each pen. The birds were sacrificed for sampling purposes through cervical dislocation at days 14 and 42. The study protocol was approved by the Animal Care Committee of McGill University (Ref # 2018-8002/150930269).

3.3.2 Sorting of Immune Cells through Flow Sorter for RNA Extraction

Cecal tonsils (longitudinally cut) from 3 sacrificed birds/treatment group at day 14 and day 42 obtained, washed, and crushed with the flat end of a 3-ml syringe plunger in 1mM EDTA solution. The solution passed through a 40-µm cell strainer (BD Biosciences, NJ, USA) into a 50-

ml conical tube. The cells centrifuged for 8 minutes at 400 RCF (~1200 RPM) and washed with cold PBS twice. Cells re-suspended to a concentration of 1 x 10^6 cells/mL in the Flow Staining Buffer. The viability dye (FVD eFluor 780) (eBioscience, CA, USA) added to cells at a concentration of 1 µl/ml and the cells incubated for 30 minutes on ice and in a dark place. For examination of T cell subsets, the cells were stained with anti-chicken CD3-Dylight 405 (clone PC3/188A), TCR gamma/delta-PerCP (clone TCR1) (from Novus Biologicals, CO, USA), CD4-FITC (clone CT-4) and CD8a-PE (clone CT-8) (from Southern Biotech, Alabama, USA) and CD25-Alexa Fluor 647 (clone-AbD13504) (from Bio-Rad, QC, Canada). The cells fixed through fixative solution (1-step Fix/Lyse Solution-10X) (eBioscience, CA, USA). Different controls such as unstained, single stained for each antibody, fluorescence minus 1 for each fluorophore and viability dye were included in the experiment. T cell sub-populations ($CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$) were sorted through a BD (NJ, USA) FACSAriaTM Fusion cell sorter and stored in TRIzol (Invitrogen, MA, USA) solution at -20°C for RNA extraction to determine expression levels of IL-10 and IFN-γ.

3.3.3 RNA Isolation and Measurement of mRNA Levels of Immunity and Gut Integrity related Genes

Ileum tissue samples (3 cm) collected from 3 sacrificed birds/group at day 14 and day 42. These samples were stored at -20°C in the TRIzol solution (Invitrogen, MA, USA) before RNA extraction. The ileum tissues and immune cells in the TRIzol solution were homogenized and centrifuged at 12000 * g for 10 minutes. The supernatant collected mixed with chloroform (257 µl/ml) following the manufacturer's recommendations and centrifuged at 12000 * g for 15 minutes at 4°C to achieve phase separation. The RNA in supernatant was mixed with equal quantity of 70% ethanol and passed through the membrane cartridges. The samples were treated

with the DNAase enzyme (Invitrogen, MA, USA) and after washings the RNA was eluted in RNase free water. The RNA quantity assessed with a nanodrop spectrophotometer by measuring absorbance at 260 nm and the RNA purity was determined by the optical density ratios at 260/280 and 260/230. The eluted RNA was stored at -80°C. Total RNA (1 ug) was reverse transcribed to complementary DNA, following the manufacturer's instructions (cDNA kit from Applied Biosystems, MA, USA). The cDNA samples were stored at -20°C. Expression levels of genes related to immunity (IL-10 and IFN-γ), tight junction (Junctional Adhesion Molecule 2, Occludin and Zona Occludens 1), mucin (MUC2) and pro-inflammatory cytokine (IL-17 F) were determined through specific primers (Table 2) by real time PCR (Biorad, QC, Canada). SYBR Green PCR master mix (Biorad, QC, Canada) were used as per manufacturer instructions for real time PCR. Expression levels of target genes normalized by β-actin and GAPDH and relative quantification was determined through the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Each sample was analyzed in triplicate and no template controls were used to assess the non-specific primer amplification.

3.3.4 Bacterial Culture analyses of Cecal Content Samples

Six and three birds/group randomly collected and euthanized through cervical dislocation on days 14 and 42, respectively. The fresh cecal contents of birds were collected and transferred to the laboratory in sterile tubes having peptone water (1 g/9ml). The contents were serially diluted 10-fold in 0.85% sterile saline solution. Diluted contents plated in duplicate on sterile petri dishes having different selective agar and mean values of colony forming units were recorded for the statistical analysis. *Lactobacillus* were detected through the de Man-Rogosa-Sharpe agar (BD, Mississauga, Ontario, Canada) after 48-hours anaerobic incubation at 37°C, whereas *Clostridium* were detected on the Reinforced Clostridial Agar (Sigma-Aldrich) after 48-

hours of incubation in anaerobic conditions at 35°C. *Escherichia coli* was detected after 24-hours aerobic incubation on the RAPID *E. coli* 2 selective medium (Bio-Rad, Mississauga, Ontario, Canada) at 37°C. The colonies counted after the incubation periods as colony forming units per gram of cecal contents.

3.3.5 Statistical Analysis

A completely randomized design (CRD) used for different parameters in the study. The pen was considered as experimental unit and Shapiro–Wilk test applied to ascertain normality of the data. The data analyzed using one-way ANOVA through SPSS software (version 24). The data presented as Least Squares Means ± SEM for each treatment. The differences were considered significant with a P value ≤ 0.05. When the main effect was significant, differences between means analyzed using Duncan's multiple range test.

Statistical model for CRD was: $Yij = \mu + TRTi + eij$

where Yij represents the observation for the dependent variables at j^{th} replicate in the i^{th} treatment (i = 1 to 6); μ is the overall mean; TRTi is the fixed effect of treatments (i = 1 to 6); eij is the random residual error. The mortality was estimated through the Kaplan-Meier estimation method.

Table 3.1. Composition (%) of the basal diet.

Ingredients	Starter %	Grower%
Corn	54.15	52.70
Soybean meal, 48% CP	38.55	30.84
Soybean Oil	2.16	2.25
Phosphorus	1.74	0.93
Calcium	1.54	1.62
Vitamin-mineral premix (Starter)	0.50	0.40
Salt	0.27	0.36
Lysine HCL	0.13	0
Methionine	0.14	0.12
Threonine	0.03	0
Choline chloride	0.10	0.10
Sodium carbonate	0.10	0.10
Anticoccidial (Monensin)	0.05	0.05
Wheat	0	10.00
ME, kCal/kg	2,977	3,056
Crude protein, %	23.00	20
Lysine total, %	1.43	1.11
Methionine total, %	0.51	0.44
Crude fat, %	4.45	4.6
Calcium, %	1.05	0.92
Phosphorus total, %	0.75	0.56

Table 3.2. Primers used for quantitative real-time PCR.

Gene[1]	Primer sequence	
IL-10	3'-AGCTGACGGTGGACCTATTATT-5'	Forward
	3'-GGCTTTGCGCTGGATTC-5'	Reverse
IFN- γ	3'-CGGGAGCTGAGGGTGAA-5'	Forward
	3'-GTGAAGAAGCGGTGACAGC-5'	Reverse
IL17F	5-TGAAGACTGCCTGAACCA-3	Forward
	5-AGAGACCGATTCCTGATGT-3	Reverse
Occludin	5-GAGCCCAGACTACCAAAGCAA-3	Forward
	5-GCTTGATGTGGAAGAGCTTGTTG-3	Reverse
ZO1	5-CCGCAGTCGTTCACGATCT-3	Forward
	5-GGAGAATGTCTGGAATGGTCTGA-3	Reverse
JAM2	5-AGCCTCAAATGGGATTGGATT-3	Forward
	5-CATCAACTTGCATTCGCTTCA-3	Reverse
MUC2	5-GCCTGCCCAGGAAATCAAG-3	Forward
	5-CGACAAGTTTGCTGGCACAT-3	Reverse
B-actin	3'-CAACACAGTGCTGTCTGGTGGTA-5'	Forward
	3'-ATCGTACTCCTGCTTGCTGATCC-5'	Reverse
GAPDH	5-GGTGGTGCTAAGCGTGTTAT-3	Forward
	5-ACCTCTGCCATCTCTCCACA-3	Reverse

Abbreviations: [1]IL-10 = Interleukin 10; IFN-γ = Interferon gamma; IL-17F = Interleukin 17F; ZO1 = Zona Occludens 1; JAM2 = Junctional Adhesion Molecule 2; MUC2 = Mucin 2; B-actin = Beta-actin; GAPDH = Glyceraldehyde-3-phosphate dehydrogenase.

3.4 Results

3.4.1 Effects of *Bacillus pumilus* and *Bacillus subtilis* Probiotics on Production parameters of Broilers

In order to evaluate the effects of *Bacillus* probiotics on production parameters of broiler chickens, body weight (BW), average daily gain (ADG), feed intake (FI), feed conversion ratio (FCR) and mortality were monitored (Tables 3-7). The effects of *Bacillus pumilus* and *Bacillus subtilis* on BW, ADG, FI and FCR between d1 and d35 were not statistically different from the Con group, except that the BPL and BSH groups that had the highest and lowest ADG

during d14-d21, respectively (Table 6). Mortality in the BSH group was higher than other groups (Table 3). At the end of the d35-d42 period, the body weights in the Ab, BPL, BSL and BSH groups were higher than the Con group (Table 4), whereas the feed intake was greater in the Ab and BSH groups and lower in the BPH group compared to Con group (Table 5). Feed conversion was not affected by dietary treatments.

Table 3.3. Effects of dietary treatments on percent mortality of broilers.

Treatments[1]	**% mortality**
Con	2.03 [b]
Ab	3.47 [ab]
BPL	2.56 [b]
BPH	2.32 [b]
BSL	2.03 [b]
BSH	5.56 [a]
P-value	0.050

Means with different superscripts in the same column differ (P < 0.05). Abbreviations: "[1]Con = Control; Ab = antibiotic (Virginiamycin); BPL = *Bacillus pumilus* low dose; BPH = *B. pumilus* high dose; BSL = *B. subtilis* low dose (BSL); BSH = *B. subtilis* high dose" n= at least 342 birds/group.

Table 3.4. Effects of dietary treatments on body weight (g) of broilers.

Trt[1]	Day1	Day7	Day14	Day21	Day28	Day35	Day42
Con	38.3	128	376	801	1376	2055	2780 [b]
Ab	38.4	128	375	818	1380	2084	3010 [a]
BPL	38.0	126	373	831	1413	2130	3033 [a]
BPH	38.5	125	363	801	1369	2087	2862 [ab]
BSL	38.1	131	364	790	1366	2045	2973 [a]
BSH	38.5	129	368	777	1376	2105	3052 [a]
SEM	0.47	2.0	6.8	13.0	19	27	55
P-value	0.959	0.436	0.635	0.086	0.597	0.285	0.034

Means with different superscripts in the same column differ (P < 0.05). Abbreviations: "[1]Con = Control; Ab = antibiotic (Virginiamycin); BPL = *Bacillus pumilus* low dose; BPH = *B. pumilus* high dose; BSL = *B. subtilis* low dose (BSL); BSH = *B. subtilis* high dose. n= 6 pens/group, at least 57 birds/pen".

Table 3.5. Effects of dietary treatments on average daily gain (g) of broilers.

Trt[1]	Day 1-7	Day 7-14	Day 14-21	Day 21-28	Day 28-35	Day 35-42
Con	12.8	35.4	60.7 [bc]	82.1	97.0	97
Ab	12.8	35.3	63.2 [ab]	80.4	100.5	125
BPL	12.6	35.3	65.4 [a]	83.1	102.4	129
BPH	12.3	34.0	62.5 [abc]	81.2	102.6	114
BSL	13.2	33.4	60.9 [bc]	82.3	97.0	126
BSH	12.9	34.1	58.6 [c]	85.5	104.1	132
SEM	0.28	0.83	1.4	2.6	2.7	8.8
P-value	0.366	0.392	0.027	0.785	0.324	0.152

Means with different superscripts in the same column differ (P < 0.05). Abbreviations: "[1]Con = Control; Ab = antibiotic (Virginiamycin); BPL = *Bacillus pumilus* low dose; BPH = *B. pumilus* high dose; BSL = *B. subtilis* low dose (BSL); BSH = *B. subtilis* high dose. n= 6 pens/group, at least 57 birds/pen".

Table 3.6. Effects of dietary treatments on weekly feed intake (g) of broilers.

Trt[1]	Day 1-7	Day 7-14	Day 14-21	Day 21-28	Day 28-35	Day 35-42
Con	22.5	58.2	113	149	170	206 [d]
Ab	22.2	56.5	117	149	185	248 [a]
BPL	22.9	55.0	118	143	162	214 [c]
BPH	21.2	53.6	111	170	170	201 [e]
BSL	22.1	57.5	114	155	161	239 [b]
BSH	22.3	56.9	114	162	187	250 [a]
SEM	1.2	2.2	8.9	10.4	12.9	0.86
P-value	0.943	0.697	0.994	0.509	0.591	< 0.001

Means with different superscripts in the same column differ (P < 0.05). Abbreviations: "[1]Con = Control; Ab antibiotic (Virginiamycin); BPL = *Bacillus pumilus* low dose; BPH = *B. pumilus* high dose; BSL = *B. subtili* low dose (BSL); BSH = *B. subtilis* high dose. n= 6 pens/group, at least 57 birds/pen".

Table 3.7. Effects of dietary treatments on weekly feed conversion ratio of broilers.

Trt[1]	Day 1-7	Day 7-14	Day 14-21	Day 21-28	Day 28-35	Day 35-42
Con	1.77	1.66	1.94	1.83	1.83	2.16
Ab	1.79	1.61	1.93	1.87	1.94	1.99
BPL	1.84	1.59	1.91	1.75	1.63	1.67
BPH	1.78	1.61	1.82	2.13	1.77	1.79
BSL	1.71	1.73	1.94	1.91	1.72	1.95
BSH	1.77	1.75	1.98	1.95	1.86	1.91
SEM	0.11	0.08	0.17	0.14	0.15	0.14
P-value	0.980	0.665	0.989	0.488	0.759	0.318

Means with different superscripts in the same column differ (P < 0.05). Abbreviations: "[1]Con = Control; Ab = antibiotic (Virginiamycin); BPL = *Bacillus pumilus* low dose; BPH = *B. pumilus* high dose; BSL = *B. subtilis* low dose (BSL); BSH = *B. subtilis* high dose. n= 6 pens/group, at least 57 birds/pen".

3.4.2 Effects of *Bacillus pumilus* and *Bacillus subtilis* Probiotics on Intestinal Tight Junction and Mucin Protein's Expression in Ileum

To determine whether *Bacillus pumilus* and *Bacillus subtilis* affect intestinal integrity of broiler chickens, expression of the selected tight junction genes (Occludin, ZO1 and JAM2) and mucin gene (MUC2) was determined through RT-PCR. The expression of occludin (Figure 1a), ZO1 (Figure 1b) and JAM2 (Figure 1c) was increased in the ileum at day 14 of age in the BPL (Occludin and ZO1), BSL (Occludin and ZO1) and BSH (Occludin, ZO1 and JAM2) groups. However, expression of these genes in all groups except the BSH (Occludin, ZO1 and JAM2) group became non-significant at day 42 in comparison with the Con group. Expression of occludin was different between the two levels of each probiotic on day 14 and between the BSL and BSH groups at day 42 ($P < 0.05$). Expression of ZO1 at day 14 was significantly different between the two levels of each probiotic (Figure 1b). There were also significant differences in expression of ZO1 and JAM2 between the BSL and BSH groups at day 42. Expression of mucin (MUC2) gene was significantly higher (Figure 1d) in all groups compared to the Con group at day 14 and remained significantly higher than the Con group at day 42 for BSL and BSH groups. The expression of mucin gene was significantly different between BSL and BSH groups on day 14 ($P<0.05$), but not on day 42. There were no differences in mucin expression between BPL and BPH groups on both day 14 and day 42.

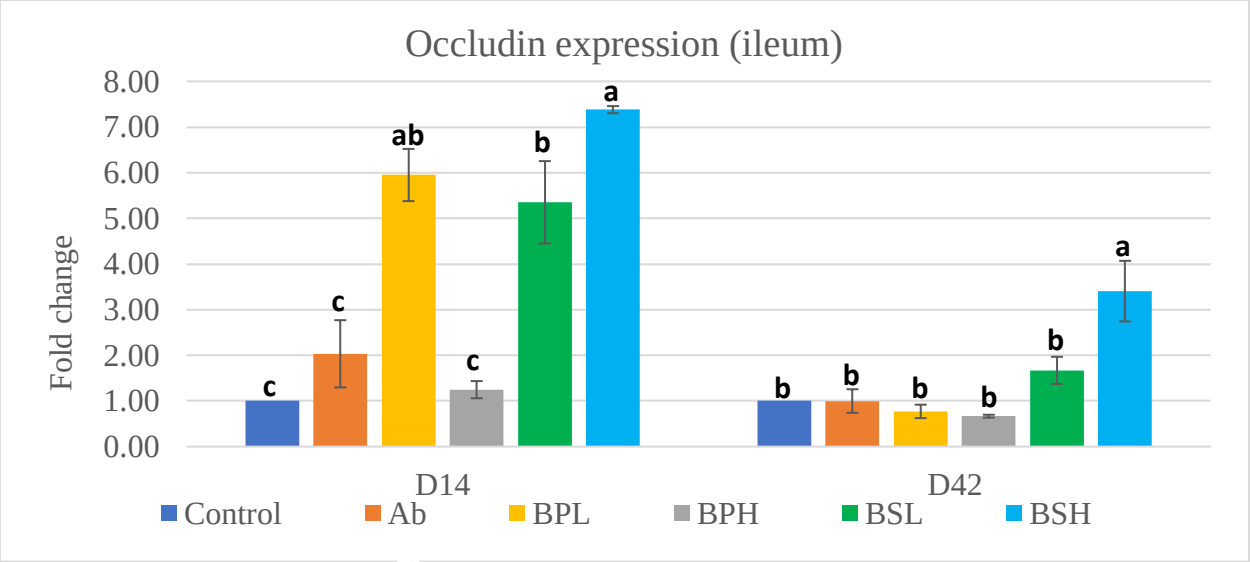

Figure 3.1a. Expression of *Occludin* mRNA in broiler ileum samples at Day 14 and Day 42 of age. Chickens were fed basal diets (Con), diets supplemented with antibiotic (Ab), or various strains of *Bacillus pumilus* low dose (BPL) and *B. pumilus* high dose (BPH) and *B. subtilis* low dose (BSL) and *B. subtilis* high dose (BSH). [abc]Different letters mean significant differences between groups (P<0.05) and bars represent Least Squares Means +/- SEM (n=3).

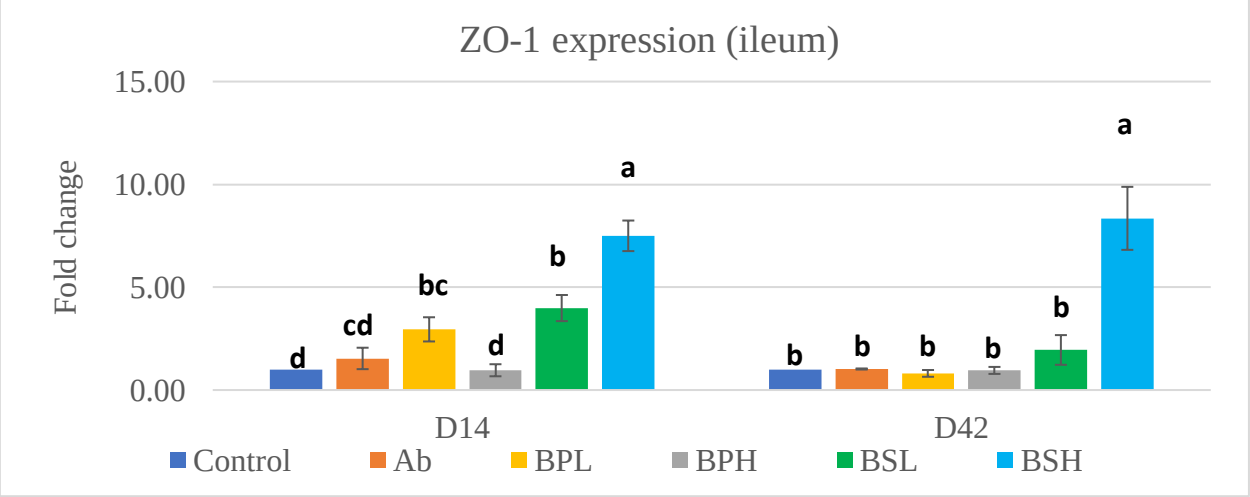

Figure 3.1b. Expression of *ZO-1* mRNA in broiler Ileum samples at Day 14 and Day 42 of age. Chickens were fed basal diets (Con), diets supplemented with antibiotic (Ab), or various strains of *Bacillus pumilus* low dose (BPL) and *B. pumilus* high dose (BPH) and *B. subtilis* low dose (BSL) and *B. subtilis* high dose (BSH). [abcd]Different letters mean significant differences between groups (P<0.05) and bars represent Least Squares Means +/- SEM (n=3).

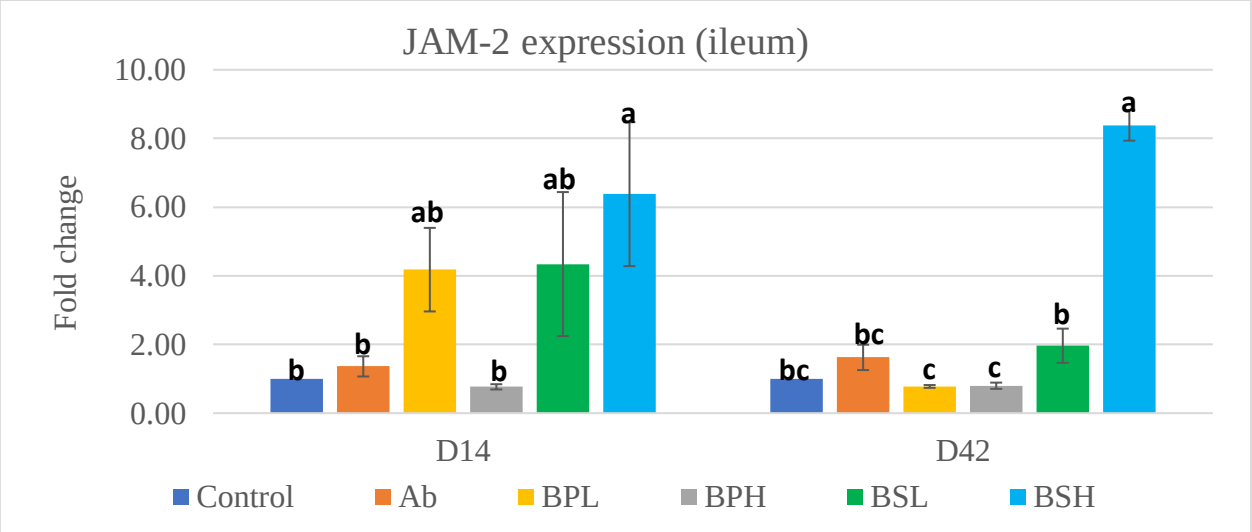

Figure 3.1c. Expression of *JAM-2* mRNA in broiler Ileum samples at Day 14 and Day 42 of age. Chickens were fed basal diets (Con), diets supplemented with antibiotic (Ab), or various strains of *Bacillus pumilus* low dose (BPL) and *B. pumilus* high dose (BPH) and *B. subtilis* low dose (BSL) and *B. subtilis* high dose (BSH). [abc]Different letters mean significant differences between groups (P<0.05) and bars represent Least Squares Means +/- SEM (n=3).

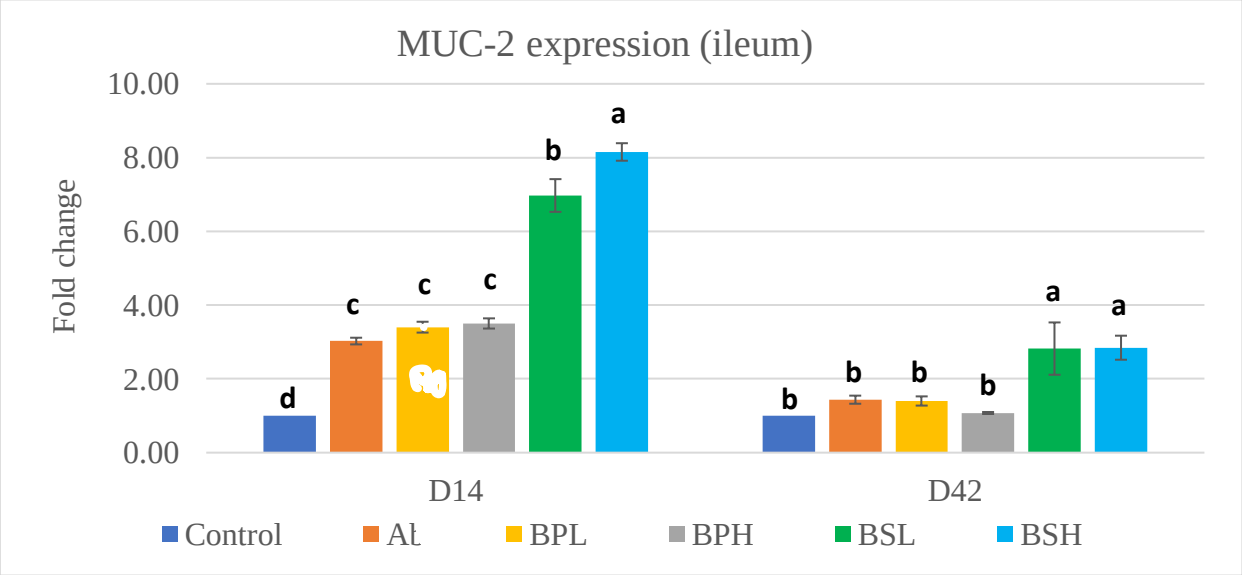

Figure 3.1d. Expression of *MUC-2* mRNA in broiler Ileum samples at Day 14 and Day 42 of age. Chickens were fed basal diets (Con), diets supplemented with antibiotic (Ab), or various strains of *Bacillus pumilus* low dose (BPL) and *B. pumilus* high dose (BPH) and *B. subtilis* low dose (BSL) and *B. subtilis* high dose (BSH). [abcd]Different letters mean significant differences between groups (P<0.05) and bars represent Least Squares Means +/- SEM (n=3).

3.4.3 Effects of *Bacillus pumilus* and *Bacillus subtilis* Probiotics on Cytokines (IL 10 and IFN-γ) secreted by $CD4^{+}CD8^{-}CD25^{+}$ And $CD4^{+}CD8^{+}CD25^{+}$ T Cells in Cecal Tonsils

In order to investigate how the *Bacillus* probiotics affect immune regulation, CD4+CD8−CD25+ T cells and CD4+CD8+CD25+ T cells were sorted from cecal tonsils and expression of IL-10 and IFN-γ genes in these cells was evaluated through RT-PCR. Expression of IFN-γ (Figure 2a) and IL-10 (Figure 2b) in CD4+CD8−CD25+ T cells was increased in response to the BPL (IFN-γ), BSL (IFN-γ) and BSH (IL-10 and IFN-γ) groups at day 14 in comparison with the Con group. Expression of IL-10 in the BPL, BSL and BSH groups and IFN-γ in the BSL and BSH group remained high by day 42 and expression in other groups became non-significant compared to Con group. Expression levels of IFN-γ and IL-10 between the two levels of each probiotic on day 42 were significantly different. Expression of IFN-γ (Figure 2c) and IL-10 (Figure 2d) in CD4+CD8+CD25+ T cells was significantly higher only in the BSH group on day 14 and day 42. The significant differences in expression of IFN-γ and IL-10 seen at both days 14 and 42 between the BSL and BSH groups ($P<0.050$). The results of the study demonstrated that three probiotic groups (BPL, BSL and BSH) potentially stimulated CD4+CD8+CD25+ and CD4+CD8-CD25+ T cells and influenced the expression of IL-10 and IFN-γ.

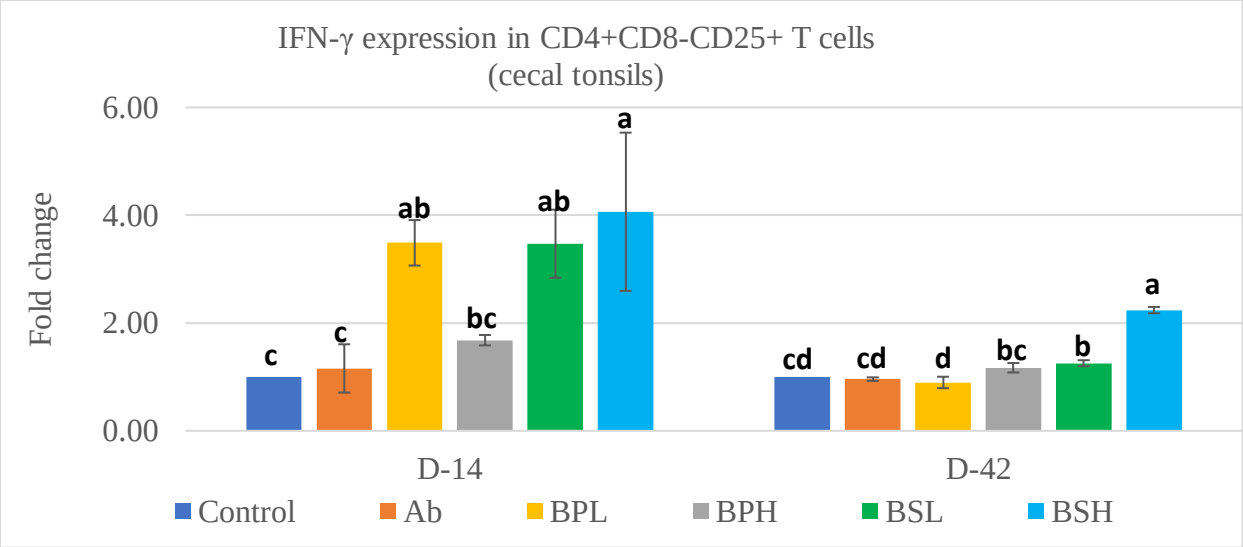

Figure 3.2a. Expression of *IFN-γ* mRNA in CD4+CD8-CD25+ T cells in broiler cecal tonsil samples at Day 14 and Day 42 of age. Chickens were fed basal diets (Con), diets supplemented with antibiotic (Ab), or various strains of *Bacillus pumilus* low dose (BPL) and *B. pumilus* high dose (BPH) and *B. subtilis* low dose (BSL) and *B. subtilis* high dose (BSH). [abcd]Different letters mean significant differences between groups (P<0.05) and bars represent Least Squares Means +/- SEM (n=3).

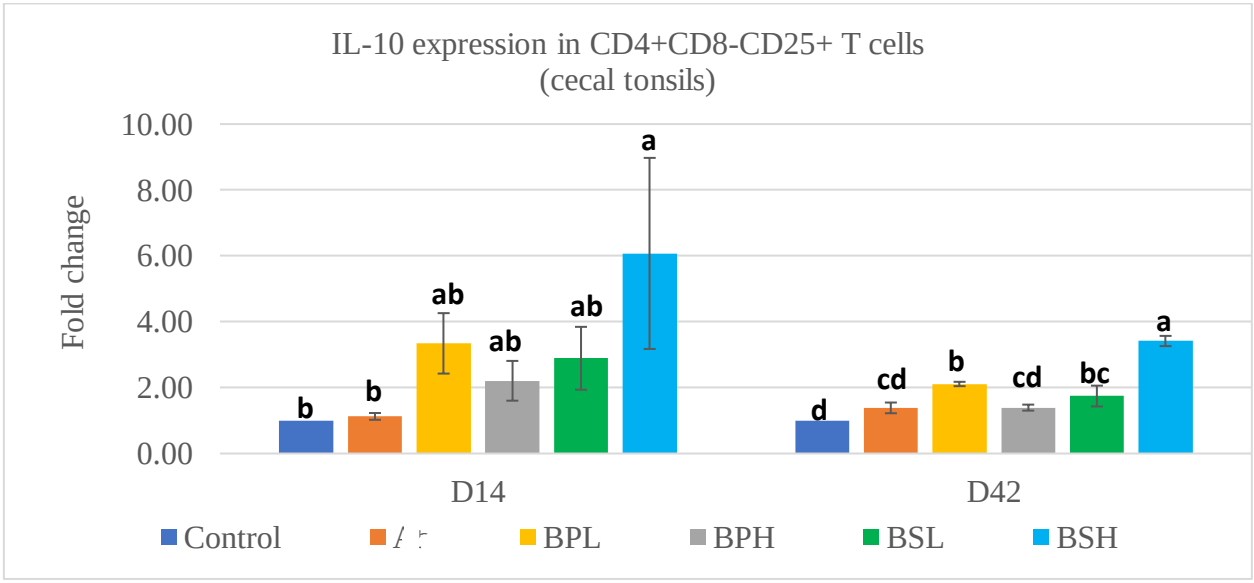

Figure 3.2b. Expression of *IL-10* mRNA in CD4+CD8-CD25+ T cells in broiler cecal tonsil samples at Day 14 and Day 42 of age. Chickens were fed basal diets (Con), diets supplemented with antibiotic (Ab), or various strains of *Bacillus pumilus* low dose (BPL) and *B. pumilus* high dose (BPH) and *B. subtilis* low dose (BSL) and *B. subtilis* high dose (BSH). [abcd]Different letters mean significant differences between groups (P<0.05) and bars represent Least Squares Means +/- SEM (n=3).

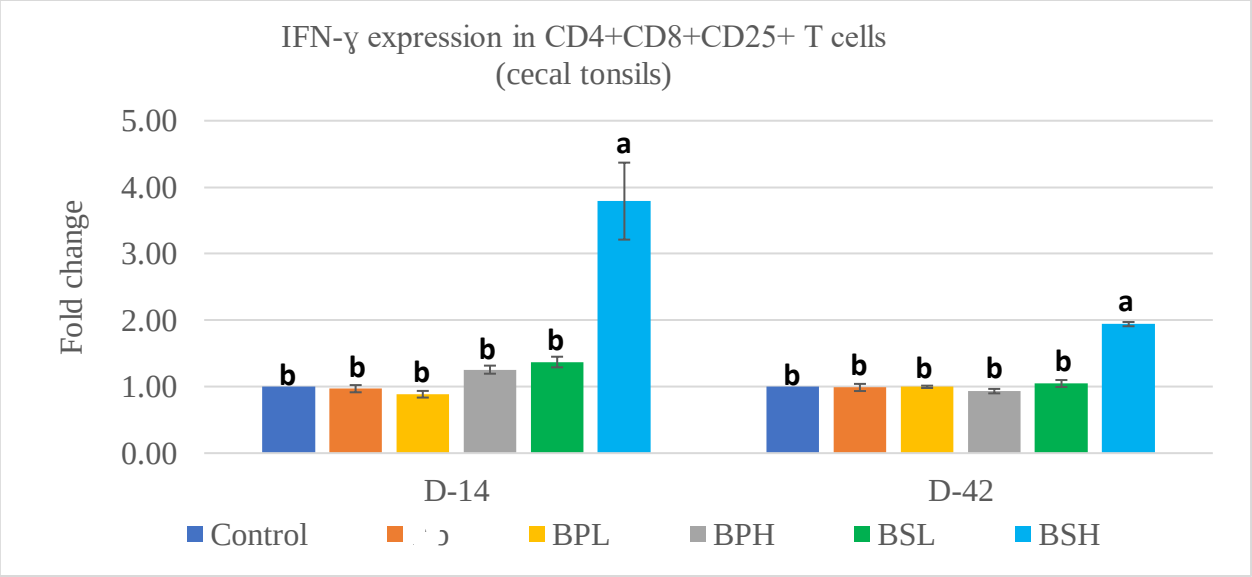

Figure 3.2c. Expression of *IFN-* γ mRNA in CD4+CD8+CD25+ T cells in broiler cecal tonsil samples at Day 14 and Day 42 of age. Chickens were fed basal diets (Con), diets supplemented with antibiotic (Ab), or various strains of *Bacillus pumilus* low dose (BPL) and *B. pumilus* high dose (BPH) and *B. subtilis* low dose (BSL) and *B. subtilis* high dose (BSH). [ab]Different letters mean significant differences between groups (P<0.05) and bars represent Least Squares Means +/- SEM (n=3).

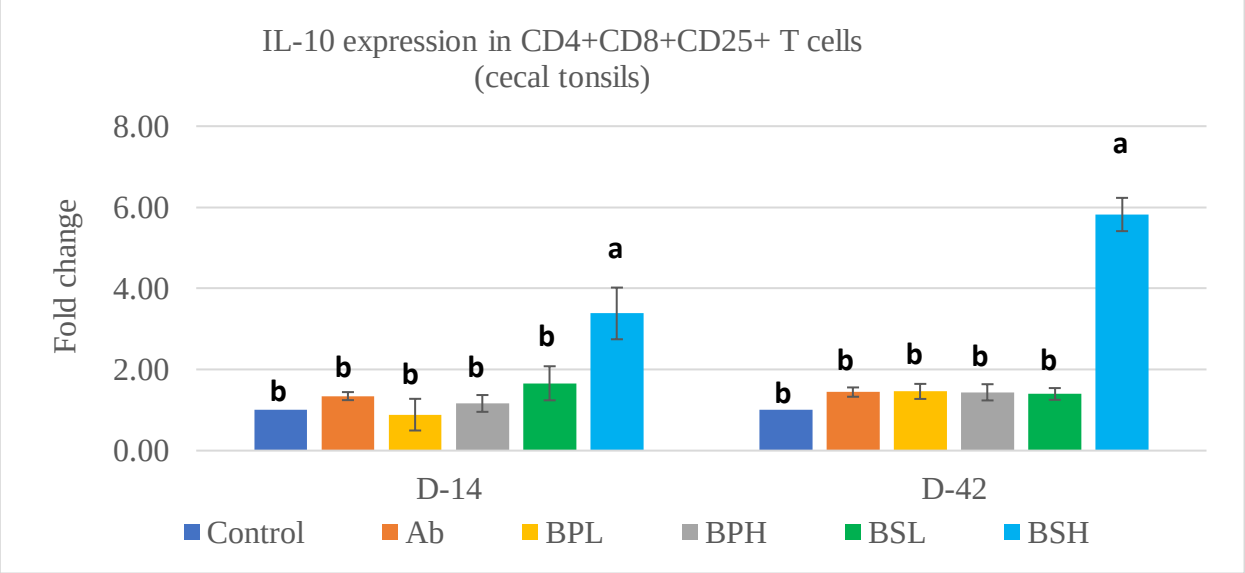

Figure 3.2d. Expression of *IL-10* mRNA in CD4+CD8+CD25+ T cells in broiler cecal tonsil samples at Day 14 and Day 42 of age. Chickens were fed basal diets (Con), diets supplemented with antibiotic (Ab), or various strains of *Bacillus pumilus* low dose (BPL) and *B. pumilus* high dose (BPH) and *B. subtilis* low dose (BSL) and *B. subtilis* high dose (BSH). [ab]Different letters mean significant differences between groups (P<0.05) and bars represent Least Squares Means +/- SEM (n=3).

3.4.4 Effects of *Bacillus pumilus* and *Bacillus subtilis* Probiotics on IL 17F Cytokine Expression in Ileum

IL17F is a pro-inflammatory cytokine, secreted by T cells including Th17 cells, and plays a role in immune homeostasis and regulation of gut integrity and function. In order to investigate whether the *Bacillus* probiotics affected the expression of IL-17F gene, its expression in ileum was measured through RT-PCR. Expression levels of IL17F (Figure 2e) significantly elevated in all groups compared with the Con group by day 14 and remained higher at day 42 in the BSL and BSH groups only. Expression of IL-17F in the BSH group was seen significantly higher than in the BSL group (P< 0.050) on both day 14 and day 42.

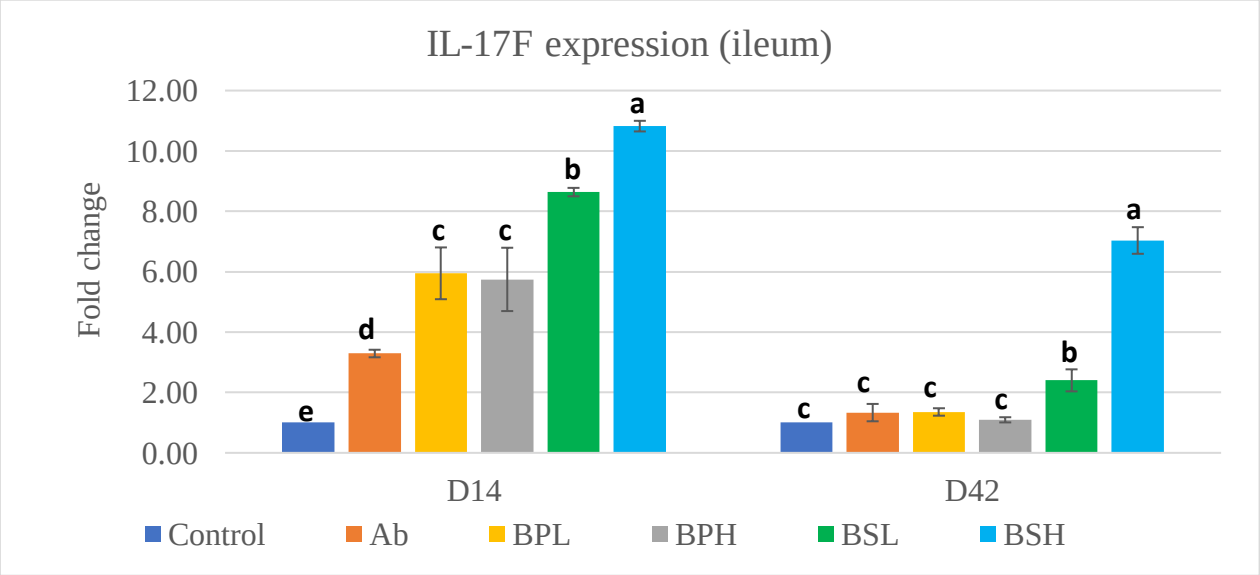

Figure 3.2e. Expression of *IL-17F* mRNA in broiler Ileum samples at Day 14 and Day 42 of age. Chickens were fed basal diets (Con), diets supplemented with antibiotic (Ab), or various strains of *Bacillus pumilus* low dose (BPL) and *B. pumilus* high dose (BPH) and *B. subtilis* low dose (BSL) and *B. subtilis* high dose (BSH). [abcde]Different letters mean significant differences between groups (P<0.05) and bars represent Least Squares Means +/- SEM (n=3).

3.4.5 Effects of *Bacillus pumilus* and *Bacillus subtilis* Probiotics on Cecal Bacterial Populations

To determine whether *Bacillus pumilus* and *Bacillus subtilis* have effects on the intestinal bacterial populations of broiler chicken, the selected genera (*Lactobacillus* and *Clostridium*) and species (*Escherichia coli*) were determined through bacterial culturing. As shown in Table 8, the bacterial counts of *Lactobacillus* were significantly higher on day 14 in the BSH group and remained higher on day 42 in comparison with the Con group. In contrast, *Lactobacillus* counts in the BPL and BSL groups were significantly lower at day 14 in comparison with the Con group. The *Lactobacillus* population was higher in the BSH group than in the BSL group both at day 14 and day 42 ($P<0.05$) and higher in the BPH group than in the BPL group at day 14 only ($P<0.050$). The Clostridium count among different groups was not statistically different both at days 14 and 42 except in the BSH group where it was higher than Con, Ab and BSL groups at day 14 and the Ab group at day 42. There was a decrease in cecal *E. coli* population in broilers fed with the BPH diet in comparison with those fed with the Con diet both at days 14 and 42 and in the BSL group at day 14. On the other hand, *E. coli* population in other diet groups was not statistically different from the Con groups both at days 14 and 42. The *E. coli* population between BPL and BPH groups at days 14 and 42 and between BSL and BSH at day 14 ($P<0.050$) was significantly different from each other.

Table 3.8: Effects of dietary treatments on cecal bacterial populations (log10 cfu/g).

	***Lactobacillus* sp.**		***Clostridium* sp.**		**E. coli**	
Trt[1]	Day 14	Day 42	Day 14	Day 42	Day 14	Day 42
Con	9.12 ^{b}	8.53 ^{cd}	9.04 ^{bc}	8.81 ^{ab}	8.51 ^{a}	8.39 ^{a}
Ab	8.97 ^{c}	8.37 ^{cd}	8.84 ^{c}	8.60 ^{b}	8.42 ^{ab}	8.12 ^{a}
BPL	8.82 ^{d}	9.09 ^{ab}	9.10 ^{abc}	8.77 ^{ab}	8.37 ^{ab}	8.16 ^{a}
BPH	9.01 ^{bc}	8.73 ^{bc}	9.28 ^{ab}	8.73 ^{ab}	7.75 ^{c}	7.49 ^{b}
BSL	8.79 ^{d}	8.23 ^{d}	8.94 ^{bc}	8.73 ^{ab}	8.34 ^{b}	8.37 ^{a}
BSH	9.55 ^{a}	9.36 ^{a}	9.45 ^{a}	9.01 ^{a}	8.53 ^{a}	8.51 ^{a}
SEM	0.04	0.13	0.05	0.04	0.05	0.08
P-value	<0.001	<0.001	0.010	0.131	<0.001	<0.001

Means with different superscripts in the same column differ (P < 0.05). Abbreviations: "[1]Con = Control; Ab = antibiotic (Virginiamycin); BPL = *Bacillus pumilus* low dose; BPH = *B. pumilus* high dose; BSL = *B. subtilis* low dose (BSL); BSH = *B. subtilis* high dose. n= 6/group at day 14 and n=3/group at day 42".

3.5 Discussion

In this study, we investigated the effects of *Bacillus pumilus* and *Bacillus subtilis* on performance of broilers in sub-optimal conditions. While effects of *Bacillus subtilis* strains in broilers have been widely investigated (Grant et al., 2018), the effect of *Bacillus pumilus* on broilers is rarely reported. During the first five weeks of age (day 1-35), there was no significant effect of *B. pumilus* and *B. subtilis* strains on growth performance. However, looking at the period of day 35 to day 42, the body weight and feed intake were significantly higher in the Ab, BPL, BSL and BSH groups at day 42 comparing with the Con group whereas feed intake in the BSH group was significantly lower at day 42 than the Con group. Other authors also reported this delayed response of probiotics on growth performance. Jacquier et al. (2019) reported no change in broiler performance up to 21 days of age in response to *Bacillus subtilis* strains, but later FCR and body weight improved by day 35 and 42 of age, respectively. In contrary, Gadde et al. (2017b) saw significant changes at day 14 in body weight and FCR of broilers in response to *B. subtilis*

strain 1781. The *Bacillus pumilus* was also reported to have beneficial effects on the body weight of giant freshwater prawns (Zhao et al., 2019) and striped catfish (Thy et al., 2017). These dissimilarities in results could be attributed to the differences in strains used, probiotic dose, diet composition and rearing conditions.

Effects of *B. pumilus* and *B. subtilis* probiotics on expression of various intestinal TJ proteins were also investigated. These junctional proteins maintain the integrity of the epithelial barrier and regulate paracellular permeability. The junction complexes are composed of tight junctions, gap junctions, adherens junctions, and desmosomes. Tight junctions include four integral transmembrane proteins (occludin, claudin, JAM, and tricellulin) that interact with cytosolic scaffold proteins (ZO), which in turn bind to the actin cytoskeleton (Ulluwishewa et al., 2011). Therefore, to better understand how *B. pumilus* and *B. subtilis* affected tight junctions, changes in the gene expression of Occludin, JAM2 and ZO-1 at the mRNA level were determined in the ileum. Significant up-regulation of expression of TJ proteins were seen in response to *Bacillus* treatment groups, except BPH, on day 14 that became non-significant in all groups except the BSH group on day 42. This increase in the first two weeks of life and then decline in TJ protein expression in later weeks of life, especially in the *B. pumilus* probiotic groups, may be attributed to the bacterial species specificity and interactions of these probiotic strains with changing populations of indigenous intestinal microbiota. The intestinal microbiota or their components activate different sub-mucosal immune cells including Th-17 cells that secrete different cytokines such IL-17A, IL-17F and IL-22. These cytokines activate epithelial cells to increase expression of TJ proteins (Weaver et al., 2013). TJ proteins are dynamic in nature and are subject to change and remodel in response to external stimuli in the gut lumen such as food/nutrients, commensal and pathogenic bacteria (Ulluwishewa et al., 2011). Thus, when antigenic signals from intestinal

lumen declines, their expression also decreases as per conditions. Our results are in agreement with those by Gadde et al. (2017b), who used *B. subtilis* strain 1781 (PB1), a combination of *B. subtilis* strain 1104 + strain 747 (PB2), or *B. subtilis* strain 1781 + strain 747 (PB3) and found that these *Bacillus* strains significantly increased expression of TJ proteins JAM2, ZO1 (PB2, PB3), and occludin (PB1, PB2) on day 14 in broilers. Rhayat et al. (2019) reported that *Bacillus subtilis* strain Bs 29784 improved expression of TJ proteins (occludin, claudin-1 and ZO-1) and transepithelial electrical resistance in CACO-2 cells in-vitro. Jacquier et al. (2019) also reported significant increase in intestinal microvilli length (+18% in ileum and +17% in cecum) in the broiler group fed with a *Bacillus*-strain. Improvement in TJ proteins at day 14 would be beneficial for young chickens as higher expression of TJ proteins will reduce the intestinal permeability and leakage of feed originated toxins and contaminants across epithelial lining. It will reduce inflammation and more energy will be available for host production. Peng et al. (2019) used *Bacillus subtilis* CW14 strain as probiotic to mitigate the tight junction injury by improving TJ proteins expression, and reduced apoptosis that induced by Ochratoxin A. Similarly, Emami et al. (2019) used a cocktail of probiotics to alleviate losses induced by *Clostridium perfringens* to production and TJ proteins. Thus, higher expression of TJ proteins in response to *B. pumilus* and *B. subtilis* at day 14 can be interpreted as an improvement of intestinal integrity.

Mucins, a major component of mucus, are large glycoproteins with a highly polymeric protein backbone structure and can be either gel-forming (secretory) or membrane bound. MUC2, the major secretory mucin, plays a vital role in keeping the architecture of the mucus layer on the intestinal surface and in preventing microorganisms from approaching the innermost mucus layer (Jiang et al., 2013). In this study, MUC2 expression increased in all treatment groups on day 14 and remained high in *Bacillus subtilis* groups at day 42. Aliakbarpour et al. (2012) reported similar

increase in mucin mRNA expression in the intestine upon supplementation with *Bacillus* probiotics. Similarly, Luan et al. (2019) reported increases in total goblet cells and expression of mucin-2 in broiler tracheal samples in response to the *Bacillus amyloliquefaciens* probiotic. In contrast, Gadde et al. (2017b) observed no difference in the expression of MUC2 in any of the probiotic or antibiotic-fed broilers at day 14 despite significant increases in body weight and FCR. Probiotics can bring changes in intestinal microbiota that lead to changes in bacterial fermentation products like alterations in short chain fatty acids (SCFA) profile (Pan and Yu., 2014). These SCFA, especially butyrate, considered to regulate the mucin production locally (Tellez et al., 2006). The SCFA producing bacterial populations like genera of *Lactobacillus* and *Clostridium* were higher in our study (BPL and BSH groups), which may contribute to higher expression of mucin. Other reports describe the role of IL-22 from Th-17 and other cells to induce goblet cells to secrete mucin in response to antigenic challenges (Sugimoto et al., 2008). A high production of mucin is a beneficial protective measure to cope with emerging intestinal challenges by invading pathogens. The high expression levels of mucins after supplementation of *Bacillus* probiotics during the 42 days of the life cycle could be helpful to the chickens.

One sub-type of CD4 positive T cells in human, mice and poultry expresses an added receptor, CD25. CD4+CD25+ T cells in chicken have been reported as Tregs (Shanmugasundaram and Selvaraj, 2011). CD4+CD25+ can be divided into CD4+CD8−CD25+ and CD4+CD8+CD25+, even though their functional differences are unknown. It has been reported that reduction of gut microbiota reduced mRNA expression of both IL-10 and IFN-γ in CD4+CD8−CD25+ T cells, but not in CD4+CD8+CD25+ T cells, from cecal tonsils in chickens, suggesting existence of potential functional differences between these two populations of cells (Lee et al., 2018). These CD4+CD25+ cells can regulate immune homeostasis with a key anti-

inflammatory cytokine, IL-10. Lee et al. (2018) reported that the percentages of CD4+CD8-CD25+ and CD4+CD8+CD25+ cells were decreased when chickens were treated with an antibiotic cocktail and regained the normal percentage when co-housed with untreated birds, indicating a link between T regulatory cells and intestinal microbiota. In our study, we investigated the impact of probiotics (*Bacillus pumilus* and *Bacillus subtilis*) on cytokines (IL-10 and IFN-γ) of CD4+CD8-CD25+ and CD4+CD8+CD25+ T cells in cecal tonsils of chickens. We saw high expression of IL-10 and IFN-γ (co-expression) in CD4+CD8−CD25+ and CD4+CD8+CD25+ T cells in cecal tonsils in response to probiotics, particularly in the case of the BSH group. The bacterial species that belong to *Clostridium* and *Lactobacillus* in the intestine can produce short chain fatty acids and activate the T regulatory cells through GPR43 (G-protein coupled receptor 43) receptors and elicit their regulatory functions to maintain intestinal homeostasis (Honda and Littman., 2016; Lee et al., 2018). Our study observed increase in cecal population of Clostridium and *Lactobacillus* species in the BSH group that may provide an explanation for higher expression of IL-10 and IFN-γ in CD4+CD8−CD25+ and CD4+CD8+CD25+ T cells in this group. The co-production of IL-10 and IFN-γ by CD4+CD8−CD25+ and CD4+CD8+CD25+ T cells may work like Th1 cells in chickens, as suggested by Lee et al. (2018), to use IL-10 to suppress and tolerate the immune responses.

We also observed that expression of IL-17F in ileum of chickens was increased in response to all four probiotics groups (BPL, BPH, BSL & BSH) on day 14 and remained significantly high in the BSL and BSH groups, but not in the BPL and BPH groups on day 42. The Th17 cells, with the help of their key cytokines, IL-17A, IL-17F and IL-22, can stimulate the production of antimicrobial proteins by intestinal epithelial cells, formation of tight junctions between these cells, recruitment of granulocytes and mediation in transportation of IgA across mucosa (Honda

and Littman., 2016; Weaver et al., 2013). These cells are concentrated more in barrier sites like intestine than systemic sites (Weaver et al., 2013). Certain bacteria such as segmented filamentous bacteria from family Clostridiaceae were directly linked with stimulation of Th17 cells (Ohnmacht et al., 2011). A recent study in broilers reported elevated expression of IL-17 in response to a mix of *Lactobacillus*, *Bifidobacterium* and *Enterococcus* based probiotic product (Emami et al., 2019), suggesting a potential role of IL-17 in alleviation of the damages to TJ proteins and intestinal epithelial cells due to pathogenic infection. Despite protective role of Th17 cells, they may play a role in pathological consequences if overwhelmed by large microbial intestinal breaches (Ohnmacht et al., 2011). Whether there is a link between the high mortality rate in early weeks of age and sustained higher expression of IL-17F in the BSH group is intriguing. In addition, why expression of IL-17F was reduced on d 42 in the *B. pumilus* groups needs further investigation.

The *Bacillus* probiotics performed better than the Ab group in several aspects. Expression of many genes in the Ab group was significantly lower than the BPL, BSL and BSH groups for occludin and ZO-1, the BSH group for JAM-2 and the BSL and BSH groups for MUC-2 on day 14, and the BSH group for occludin, ZO-1 and JAM-2, and the BSL and BSH groups for MUC-2 on day 42. Similarly, the effect of BSH on CD4+CD8-CD25+ and CD4+CD8+CD25+ T cells was significantly higher than in the Ab group in terms of IFN- γ and IL-10 secretions on days 14 and 42. The BPL and BSL groups at day 14 and BSL group at day 42 were also significantly better than in the Ab group in terms of their effects on IFN- γ secretion from CD4+CD8-CD25+ cells. Similarly, IL-10 secreted by CD4+CD8-CD25+ cells at day 42 was higher in the BPL group than in the Ab group. The expression of IFN- γ and IL-10 from CD4+CD8+CD25+ cells was not significantly different among Ab, BPL, BPH and BSL groups at days 14 and 42. These results are in agreement with those reported by Gadde et al. (2017b), who found that *Bacillus* probiotics

generated better results than the antibiotic (Bacitracin methylene disalicylate) group in terms of immunity and tight junction proteins expression.

3.6 Conclusion

Taken together, this study documented the effects of *B. pumilus* and *B. subtilis* strains on growth performance, intestinal microbiota, immunity and gut health. We observed that *B. pumilus* and *B. subtilis* supplementation conferred intestinal health benefits to the broilers by promoting gut integrity and function coupled with activation of T regulatory cells of the immune system. These effects were strain, dose and age sensitive and were different for *B. pumilus* and *B. subtilis*.

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3.8 References

Aliakbarpour HR, Chamani M, Rahimi G, Sadeghi AA, & Qujeq D (2012) The *Bacillus subtilis* and lactic acid bacteria probiotics influences intestinal mucin gene expression, histomorphology and growth performance in broilers. *Asian-Australasian Journal of Animal Sciences.* **25:** 1285-1293.

Broom JL & Kogut MH (2018) Gut immunity: its development and reasons and opportunities for modulation in monogastric production animals. *Animal Health Research Reviews.* **19:** 46-52.

Chen Q, Tong C, Ma S, Zhou L & X. Zhao (2017) Involvement of microRNAs in probiotics-induced reduction of the cecal inflammation by *Salmonella typhimurium*. *Frontiers in Immunology*. **8:** 704.

Chu H & Mazmanian SK (2013) Innate immune recognition of the microbiota promotes host-microbial symbiosis. *Nature Immunology.* **14:** 668–675.

Denyer MP, Pinheiro DY, Garden OA & Shepherd AJ (2016) Missed, not missing: phylogenomic evidence for the existence of avian FoxP3. *Public Library of Science One.* **11:** e0150988.

Emami NK, Calik A, White MB,Young M & Dalloul RA (2019) Necrotic enteritis in broiler chickens: the role of tight junctions and mucosal immune responses in alleviating the effect of the disease. *Microorganisms.* **7:** 231.

Food and Agriculture Organization (2015) http://www.fao.org/3/y4252e/y4252e07.htm.

Food and Agriculture Organization of the United Nations (2020) Gateway to poultry production and products. http://www.fao.org/poultry-production-products/production/en/.

Gadde U, Kim WH, Oh ST & Lillehoj HS (2017_a) Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: a review. *Animal Health Research Reviews.* **18:** 26-45.

Gadde U, Oh ST, Lee YS, Davis E, Zimmerman N, Rehberger T & Hyun ST (2017_b) The effects of direct-fed microbial supplementation, as an alternative to antibiotics, on growth performance, intestinal immune status, and epithelial barrier gene expression in broiler chickens. *Probiotics and Antimicrobial Proteins.* **9:** 397-405.

Grant A, Gay CG & Lillehoj HS (2018) *Bacillus* spp. as directed microbial antibiotic alternatives to enhance growth, immunity, and gut health in poultry. *Avian Pathology.* **47:** 339-351.

Honda K & Littman DR (2016) The microbiota in adaptive immune homeostasis and disease. *Nature.* **535:** 75–84.

Jacquier V, Nelson A, Jlali M, Rhayat L, Brinch KS & Devillard E (2019) *Bacillus subtilis* 29784 induces a shift in broiler gut microbiome toward butyrate producing bacteria and improves intestinal histomorphology and animal performance. *Poultry Science.* **98:** 2548–2554.

Jiang Z, Applegate TJ & Lossie AC (2013) Cloning, annotation and developmental expression of the chicken intestinal MUC2 gene. *Public Library of Science One*. **8:** e53781.

Lee IK, Bae S, Gu MJ, You SJ, Kim G, Park S, Jeung W, Ko KH, Cho JK, Kang JS & Yun C (2017) H9N2-specific IgG and CD4+CD25+ T cells in broilers fed a diet supplemented with organic acids. *Poultry Science*. **96:** 1063–1070.

Lee IK, Gu MJ, Ko KH, Bae S, Kim G, Jin G, Kim EB, Kong Y, Park TS, Park B, Jung HJ, Han SH & Yun C (2018) Regulation of CD4+CD8−CD25+and CD4+CD8+CD25+ T cells by gut microbiota in chicken. *Scientific Reports.* **8:** 8627.

Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2 (- Delta Delta (CT)) method. *Methods.* **25:** 402-408.

Luan SJ, Sun YB, Wang Y, Sa RN & Zhang HF (2019) *Bacillus amyloliquefaciens* spray improves the growth performance, immune status, and respiratory mucosal barrier in broiler chickens. *Poultry Science.* **98:** 1403–1409.

Ma Y, Wang W, Zhang H, Wang J, Zhang W, Gao J, Wu S, & Qi G (2018) Supplemental *Bacillus subtilis* DSM 32315 manipulates intestinal structure and microbial composition in broiler chickens. *Scientific Reports.* **8:** 15358.

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

Ocejo M, Oporto B & Hurtado A (2019) 16S rRNA amplicon sequencing characterization of caecal microbiome composition of broilers and free-range slow growing chickens throughout their productive lifespan. *Scientific Reports.* **9:** 2506.

Ohnmacht C, Marques R, Presley L, Sawa S, Lochner M & Eberl G (2011) Intestinal microbiota, evolution of the immune system and the bad reputation of pro-inflammatory immunity. *Cellular Microbiology.* **13:** 653-659.

Pan D & Yu Z (2014) Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes.* **5:** 108-119.

Peng M, Liu J & Liang Z (2019) Probiotic *Bacillus subtilis* CW14 reduces disruption of the epithelial barrier and toxicity of ochratoxin A to Caco-2 cells. *Food and Chemical Toxicology*. **126:** 25-33.

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.

Rhayat L, Maresca M, Nicoletti C, Perrier J, Brinch KS, Christian S, Devillard E & Eckhardt E (2019) Effect of *Bacillus subtilis* strains on intestinal barrier function and inflammatory response. *Frontiers in Immunology*. **10:** 564.

Shanmugasundaram R & Selvaraj RK (2011) Regulatory T cell properties of chicken CD4+CD25+ cells. *Journal of Immunology*. **186:** 1997-2002.

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

Sugimoto K, Ogawa A, Mizoguchi E, Shimomura Y & Andoh A (2008) IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *Journal of Clinical Investigation*. **118:** 534-544.

Sun X, Yang H & Nose K (2008) Decline in intestinal mucosal IL-10 expression and decreased intestinal barrier function in a mouse model of total parenteral nutrition. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. **294:** 139-147.

Tellez G, Higgins SE, Donoghue AM & Hargis BM (2006) Digestive physiology and the role of microorganisms. *Journal of Applied Poultry Research*. **15:** 136-144.

Teo AY & Tan HM (2007) Evaluation of the performance and intestinal gut microflora of broilers fed on corn-soy diets supplemented with *Bacillus subtilis* PB6 (CloSTAT). *Journal of Applied Poultry Research*. **16:** 296–303.

Thy HTT, Tri NN, Quy OM, Fotedar R, Kannika K, Unajak S & Areechon N (2017) Effects of the dietary supplementation of mixed probiotic spores of *Bacillus amyloliquefaciens* 54A, and *Bacillus pumilus* 47B on growth, innate immunity and stress responses of striped catfish (Pangasianodon hypothalamus). *Fish and Shellfish Immunology*. **60:** 391-399.

Ulluwishewa D, Anderson AC, McNabb WC, Moughan PJ, Well JM & Roy NC 2011. Regulation of tight junction permeability by intestinal bacteria and dietary components. *Journal of Nutrition*. **141:** 769–776.

Wang L, Li L, Lv Y, Chen Q, Feng J & Zhao X (2018) *Lactobacillus plantarum* restores intestinal permeability disrupted by Salmonella infection in newly hatched chicks. *Scientific Reports*. **8:** 2229.

Weaver CT, Elson CO, Fouser LA & Kolls JK (2013) The Th17 pathway and inflammatory diseases of the intestines, lungs, and skin. *Annual Review of Pathology*. **8:** 477–512.

Zhao C, Zhu J, Hu J, Dong X, Sun L, Zhang X & Miao M (2019) Effects of dietary *Bacillus pumilus* on growth performance, innate immunity and digestive enzymes of giant freshwater prawns (Macrobrachium rosenbergii). *Aquaculture Nutrition.* **25:** 712-720.

Connecting text

In Chapter 3, we evaluated the effects of two *Bacillus* probiotics, *Bacillus pumilus* and *Bacillus subtilis*, on production, gut health and immunity of broiler chickens reared under sub-optimal environmental conditions. It was notable that probiotic effects on gut health and immunity were prominent at day 14 but started to fade with passage of time. Since probiotics induced the effects mainly through alteration in intestinal microbiota, it became a matter of interest to investigate effects of these probiotics on intestinal microbiota at different time points.

In Chapter 4, we investigated the effects of *Bacillus* probiotics on cecal microbiota of broiler chickens at different time points of production cycle (Days 7, 14, 28 and 42). To understand impact of probiotics on cecal microbiota, it is important to identify microbial diversity and composition in a greater depth. We therefore used sequencing of the 16S rRNA gene to analyse complex microbial communities in the cecum.

Chapter 4. *Bacillus pumilus* and *Bacillus subtilis* Promote and Modulate Early Maturation of Cecal Microbiota in Broiler Chickens

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4.1 Abstract

Mature and stable intestinal microbiota in chickens is essential for health and production. Slow development of microbiota in young chickens prolongs the precarious period before reaching mature configuration. Whether probiotics can play a role in the early maturation of intestinal microbiota is unknown. To address this, day-old chicks were assigned into six groups: NC (basal diet), PC (virginiamycin), low (BPL) and high-dose (BPH) of *Bacillus pumilus*, and low (BSL) and high-dose (BSH) of *Bacillus subtilis*. Cecal contents at days 7, 14, 28 and 42 were used to analyze the treatment and time effects on the diversity and composition of microbiota. Overall, the alpha diversity was significantly decreased in the NC group between days 7 and 14, while this decline was prevented in the *Bacillus subtilis* probiotic (BSL and BSH) and even reversed in the BPH group. The beta-diversity showed significant responses of microbial communities to probiotics in first two weeks of life. Analyses of the abundance of microbiota reflected that members of the family Ruminococcaceae (*Ruminnococcus, Oscillospira, Faecalibacterium, Butyricicoccus*, and *Subdoligranulum*), which were dominant in mature microbiota, were significantly higher in abundance at day 14 in the probiotic groups. Conversely, the abundance of genera within the family Lachnospiraceae (*Ruminococcus, Blautia, and Coprococcus*) was dominant in early dynamic microbiota but was significantly lower in the probiotic groups at day 14. The *Lactobacillus* and *Bifidobacterium* abundance was higher, while the Enterobacteriaceae abundance was lower in the probiotic groups. In summary, the probiotics efficiently helped the cecal microbiota reach mature configuration earlier in life. These results could be used for the future manipulation of microbiota from the perspective of improving poultry performance.

4.2 Introduction

Poultry is a growing contributor to human dietary protein intake and is an important contributor to feeding a growing human population. Poultry production increased from 9 to 132 million tons between 1961 and 2019 (FAO, 2020). The poultry sector is estimated to grow at an annual rate of 2–3% between 2015 and 2030, the highest growth rate in the livestock sector (FAO, 2015). The tremendous advances in the poultry production system during the last 50 years has been achieved through improvements in genetics, management, and nutrition. Among the improvement of nutrition, the use of feed additives has increased and has contributed to the success in current broiler production. Probiotics are among the most researched feed additives and show promising results for production and health parameters (Grant et al., 2018). Probiotics produce their effects through different mechanisms. Our laboratory has previously demonstrated the role of probiotics in the alleviation of pathogen-associated inflammation (Chen et al., 2017) and disrupted intestinal permeability (Wang et al., 2018).

The intestinal microbiota plays a key role in immune development, and its homeostatic interactions with the host are now well established (Marcolla et al., 2019). Intestinal microbiota can be influenced by both environmental- and host-related factors. Host-related factors such as age, sex, breed (Kers et al., 2018), and immune system (Pabst et al., 2016) influence the structure and composition of microbiota. For example, the immune system has the ability to change the configuration of the microbiota by determining which bacteria are allowed to colonize the gut and which will be excluded via secreted antibodies (Sterlin et al., 2020). The age of the host also affects the diversity and stability of the microbiota. In broiler chickens, the intestinal microbiota is dynamic during the first few weeks of life, which is followed by a mature and stable microbiota (Ocejo et al., 2019). Hartog et al. (2016) observed a marked decrease in the microbial diversity in

the early weeks of layer chickens followed by a stable microbiota after 42 days of life. They associated this early life decrease followed by stability in microbial diversity with host immune response, which gradually matures and stabilizes with passage of time. We recently observed that immune and gut health responses to the probiotic groups in broilers were different at different stages of life, which were shown to be significant at day 14 and insignificant at day 42 (Bilal et al., 2021). Intestinal perturbation in microbiota can be induced by exogenous factors such as antibiotics. Probiotics have been used to prevent antibiotic-induced dysbiosis (Pereira et al., 2019). However, whether age-related low microbial diversity in early life can be improved with probiotics is less studied in broiler chickens.

The maturation of microbiota is important for optimal host metabolism (Turnbaugh et al., 2006) and immune development (Olszak et al., 2012). A mature microbiota has higher resilience to different stress factors (Gasparrini et al., 2019). The phylum Firmicutes is the main and dominant group in chicken intestine. Lachnospiraceae and Ruminococcaceae are two main families in the phylum Firmicutes that can be found in chickens (Ocejo et al., 2019). Members of Lachnospiraceae are considered biomarkers of an early and immature microbiota (Ocejo et al., 2019, Jurburg et al., 2019), while members of the family Ruminococcaceae are in higher abundance in mature stable microbiota (Richards et al., 2019, Oakley et al., 2014). It is worth noting that for more than half of the production period, the microbiota of broiler chickens is developing and is vulnerable to external stressors. Dietary interventions in the microbiota are likely to be more successful if they can promote the early maturation of the microbiota, particularly with respect to members of Lachnospiraceae and Ruminococcaceae. Recently, contact with adult hens (Kubasova et al., 2019) and inoculation with adult-derived microbiota (Meijerink et al., 2020) showed an acceleration in the maturation of intestinal microbiota. Though several studies have

evaluated the effect of probiotics and antibiotics on microbiota (Marcolla et al., 2019), investigations of whether these interventions influence the maturity of microbiota is still relatively scarce.

The main objective of this study was to evaluate the impact of *B. pumilus,* and *B. subtilis* on microbial diversity and maturity in terms of changes in the composition of the families Lachnospiraceae and Ruminococcaceae in cecal microbiota at different stages of life in broiler chickens. *Bacillus*-based probiotics were used in this study since they have an advantage over other probiotics due to their ability to form spores, which increases their survivability in feed processing and in the gastrointestinal tract.

4.3 Material and Methods

4.3.1 Birds, Diet and Experimental Design

A total of 2,073 one-day old male Cobb 500 chicks were obtained from a local hatchery (Grains Natures, Roxton Falls, QC Canada) and were randomly divided into 36 pens (6 pens/treatment). These broilers were assigned to 6 treatments and were grown for 42 days. The dietary treatments included a standard basal diet as a negative control (NC), a basal diet with antibiotic growth promoter as a positive control (PC) (Virginiamycin at 16.5 mg/kg of feed), a basal diet with a low-dose of *B. pumilus* (3×10^8 CFU/kg of feed) (BPL), a basal diet with a high-dose of *B. pumilus* (1×10^9 CFU/kg of feed) (BPH), a basal diet with a low-dose of *B. subtilis* (3×10^8 CFU/kg of feed) (BSL), and a basal diet with a high-dose of *B. subtilis* (1×10^9 CFU/kg of feed) (BSH). The management of broilers including the composition of the basal diet with monensin as the anticoccidial agent was described previously (Bilal et al., 2021). The probiotics were provided by Lallemand SAS, France. The study protocol was approved by the Animal Care Committee of McGill University (Ref # 2018-8002).

4.3.2 Sample Collection and DNA Extraction

The baseline data at day one was not collected since the cecal content is minimal at this age. Instead, the first sampling was conducted on day 7. In addition, we took measures to avoid initial biases for all of the groups, such as all of the chicks being from the same source of hatchery, all of the chicks being of the same sex (male broilers) of equal average weight, the randomized allocation of the chicks to the pens, and the randomized allocation of the pens to the treatments. At days 7, 14, 28, and 42, one bird per pen (n = 6/group) was randomly selected and was euthanized by cervical dislocation, and both ceca were removed to obtain the cecal contents. The cecal contents were collected in cryovials, snap-frozen in liquid nitrogen, and stored at −80 °C. DNA from these samples were isolated through a DNeasy PowerSoil Pro Kit (QIAGEN, Montreal, Canada) with a bead-beating mechanical lysis step to increase the DNA yield. After quality checks through a spectrophotometer, DNA was stored at −80 °C for further use.

4.3.3 Sequencing and Data Analysis of Cecal Microbial Community

Illumina MiSeq (Illumina, San Diego, CA, USA) paired-end sequencing was performed to determine the bacterial community composition of each sample using the 548F and 806R primers for the V4 region of the 16S rRNA gene amplicon library preparation. The MiSeq sequencing was performed according to standard Illumina protocol using a dual-indexing strategy for multiplexed sequencing (Kozich et al., 2013). Raw sequencing data were received as the 250-bp length of each pair of the reads in FASTQ format for further processing. The Illumina data were analyzed with QIIME2 software version 2019.10.0 (Bolyen et al., 2018). The reads were checked for quality and were subjected to a denoising method for the removal of low-quality reads and chimeras and for the correction of sequencing errors through the DADA2 plugin with default parameters. The phylogenetic tree was constructed through the q2-phylogeny plugin and the

taxonomy was assigned using the q2-feature-classifier plugin (Bokulich et al., 2018) through a pre-trained Naïve Bayes classifier based on the Greengenes v. 13_8 database. Alpha and beta diversity analyses were performed at a sequencing depth of 12,850 using the QIIME2 alpha and beta diversity plugins. The quality checks and normalization of data resulted in removal of some samples that reduced number of replicates to 4 or 5 in different treatment groups. Different metrics, such as observed operational taxonomic units (OTU), Pielou, and Shannon, were used to assess alpha diversity. The comparison of the alpha diversity metric among the treatments was made through the two-way ANOVA test with the groups, time, and their interaction, which was followed by the Sidak test as a post hoc test for multiple comparisons. Beta diversity, a metric used for the comparison of microbial diversity between samples, was calculated with a weighted UniFrac metric. The results were tested at each time point with a PERMANOVA test, and the multiple-test correction was completed with the Benjamini–Hochberg FDR method (q-values < 0.05) in order to assess the community differences between the groups. Taxa plots were generated using the q2-taxa plugin (https://github.com/qiime2/q2-taxa, accessed 15 May 2021) to visualize the differences in the treatment groups at the phylum level. The relative abundance of microbiota between NC and other the treatments at the family and genus levels at days 7, 14, 28, and 42 was generated and ranked through Songbird software (Morton et al., 2019). These ranked differentials were used to pick suitable reference frames based on their presence across the most samples as the denominator for the log-ratio test using Qurro software, version v0.5.0, Knight lab, University of California, San Diego, CA USA (Fedarko et al., 2020). The log ratios were calculated between the observed features and the taxon used as a reference to avoid bias associated with the analysis of compositional or relative abundance data. This method provides the opportunity to reveal microbial changes without the need to estimate the total microbial load (Engelbrektson et al.,

2009). In this study, the Ruminococcaceae was used as a reference frame for the features of all of the other microbial families, while Lachnospiraceae was used as a reference frame for the features of family Ruminococcaceae, unless different reference frames were indicated. Qurro-generated log ratios at days 7, 14, 28, and 42 were further analyzed through one-way ANOVA followed by Duncan's test as a post hoc test for multiple comparisons.

4.4 Results

4.4.1 Sequencing Data

From the 144 samples, a total of 15,772,526 sequences were obtained, with mean of 109,531 sequences per sample. After DADA2 quality control processes, 9,317,770 sequences with a mean of 64,707 sequences per sample were retained. The samples were rarefied at 12,850 sequences per sample for even depth of analysis. The low read samples were removed, and the remaining 108 samples that reached the saturation plateau of the rarefaction curve were included for further analyses.

4.4.2 Probiotics Improve the Cecal Microbial Alpha Diversity in Young Chickens

In order to investigate the effects of supplementing probiotics on gut microbiota, the microbial richness, evenness, and diversity were examined for the cecal microbiota on days 7, 14, 28, and 42. The interaction terms (Groups * Time) for richness, evenness, and diversity were significant ($p < 0.05$) and were included in the analyses. The microbial richness was not different among treatments at days 7, 28, and 42, but significant improvement ($p < 0.05$) was observed in all of the probiotic groups at day 14 when compared to the PC and NC groups (Table 1a). Looking at the different time points (Table 1a), microbial richness significantly increased earlier in the BPL and BSH groups between days 7 and 14 and reached their peaks at day 28, while microbial richness

in the BSL and BPH groups significantly increased between days 14 and 42 and reached their highest values at days 28 (BPH) and 42 (BSL). A significant increase in the species richness of the NC and PC groups also happened between days 14 and day 42.

The effects of the treatments on the evenness of the microbiota were evaluated through the Pielou index (Table 1b). The evenness of the microbiota in all of the probiotic groups at day 7 was comparable to the NC group but was significantly greater than the PC group, except for the BPH group. A significant difference was also observed between the BPH and BSH groups and between the NC and PC groups at day 7. At day 14, the evenness of the microbiota in all of the probiotic groups were comparable to the NC group, except for the BPL group, where the microbiota was significantly lower than it was in the NC and BPH groups. The evenness at days 28 and 42 was comparable among all of the groups except for that the evenness in the BSH group was significantly lower than it was in the PC group at day 28. While comparing the treatment groups at different time points (Table 1b), the microbial evenness significantly decreased in the NC and BPL groups between days 7 and 14 ($p < 0.05$), but later it was improved in the BPL group by day 28. The evenness in the BPH and BSL groups remained the same between days 7 and 14 and later significantly decreased between days 14 and 28 (BPH) and days 28 and 42 (BSL). The evenness of the BSH and PC groups were similar between days 7 and 14 but significantly increased in the PC group on day 28, while the evenness was decreased in the BSH group on days 28 and 42.

The Shannon index, which combines the effects of richness and evenness, was used to assess the changes in the alpha diversity of the microbiota in the different groups (Table 1c). The diversity of all of the probiotic groups at day 7 was equivalent to that of the NC group but was significantly greater (BPL, BSL and BSH) than that of the PC group. At day 14, the diversity in

the probiotic groups was significantly higher than the NC (BPH) and PC (BPH and BSH) groups. At day 28, the microbial diversity in all of the probiotic groups became equivalent to the NC and PC groups, and this trend continued until day 42. While comparing the microbial diversity of the treatment groups among different time points (Table 1c), a significant decline was seen in the NC and BPL groups ($p \leq 0.05$) between days 7 and 14, which significantly increased by day 28 and remained the same during rest of the study period. However, this decline was prevented in the *B. subtilis* probiotic groups (BSL and BSH) between days 7 and 14 and remained unaffected through rest of the study period. The BPH group showed a significant increase in diversity between day 7 and 14 and remained stable until day 42. The PC group remained unchanged until day 14, showed a significant increase in diversity between days 14 and 28, but the diversity significantly declined in the period between days 28 and 42 ($p < 0.05$).

Table 4.1. Effects of dietary treatments on microbial richness (a), evenness (b), and diversity (c) in broiler chickens at days 7, 14, 28, and 42 of age.

a. Effects of dietary treatments on microbial richness (observed operational taxonomic units).

Groups/Time	Mean Day-7	SEM	Mean Day-14	SEM	Mean Day-28	SEM	Mean Day-42	SEM
NC	110 ^{3}	11.9	100 ^{b,3}	13.3	188 ^{2}	11.9	241 ^{1}	13.3
PC	92 ^{2}	11.9	109 ^{b,2}	13.3	188 ^{1}	11.9	202 ^{1}	13.3
BPL	116 ^{3}	13.3	172 ^{a,2}	13.3	231 ^{1}	13.3	228 ^{1}	13.3
BPH	118 ^{2}	11.9	163 ^{a,12}	11.9	202 ^{1}	11.9	198 ^{1}	13.3
BSL	135 ^{2}	11.9	172 ^{a,2}	13.3	234 ^{1}	11.9	249 ^{1}	11.9
BSH	106 ^{2}	11.9	177 ^{a,1}	13.3	221 ^{1}	11.9	199 ^{1}	13.3

b. Effects of dietary treatments on microbial evenness (Pielou index) at days 7, 14, 28, and 42.

Groups/Time	Mean Day-7	SEM	Mean Day-14	SEM	Mean Day-28	SEM	Mean Day-42	SEM
NC	0.77 ^{ab,1}	0.025	0.66 ^{ab,2}	0.029	0.75 ^{ab,12}	0.025	0.69 ^{12}	0.029
PC	0.65 ^{c,2}	0.025	0.63 ^{bc,2}	0.029	0.77 ^{a,1}	0.025	0.61 ^{2}	0.029
BPL	0.77 ^{ab,1}	0.029	0.52 ^{c,2}	0.029	0.74 ^{ab,1}	0.029	0.73 ^{1}	0.029
BPH	0.68 ^{bc,12}	0.025	0.77 ^{a,1}	0.025	0.68 ^{ab,2}	0.025	0.64 ^{2}	0.029
BSL	0.76 ^{ab,1}	0.025	0.68 ^{ab,12}	0.029	0.71 ^{ab,12}	0.025	0.63 ^{2}	0.025
BSH	0.80 ^{a,1}	0.025	0.72 ^{ab,12}	0.029	0.66 ^{b,23}	0.025	0.61 ^{3}	0.029

c. Effects of dietary treatments on microbial diversity (Shannon index) at days 7, 14, 28, and 42.

Groups/Time	Mean Day-7	SEM	Mean Day-14	SEM	Mean Day-28	SEM	Mean Day-42	SEM
NC	5.30 ^{a,1}	0.218	4.39 ^{bcd,2}	0.244	5.67 ^{1}	0.218	5.46 ^{ab,1}	0.244
PC	4.24 ^{b,2}	0.218	4.25 ^{cd,2}	0.244	5.77 ^{1}	0.218	4.69 ^{ab,2}	0.244
BPL	5.25 ^{a,1}	0.244	3.88 ^{d,2}	0.244	5.81 ^{1}	0.244	5.69 ^{a,1}	0.244
BPH	4.67 ^{ab,2}	0.218	5.66 ^{a,1}	0.218	5.17 ^{12}	0.218	4.89 ^{ab,12}	0.244
BSL	5.37 ^{a}	0.218	5.04 ^{abc}	0.244	5.59	0.218	5.02 ^{ab}	0.218
BSH	5.39 ^{a}	0.218	5.36 ^{ab}	0.244	5.11	0.218	4.65 ^{b}	0.244

Chickens were fed a basal diet (NC), a basal diet with antibiotic as a positive control (PC), a basal diet with a low-dose of *B. pumilus* (BPL), a basal diet with a high-dose of *B. pumilus* (BPH), a basal diet with a low-dose of *B. subtilis* (BSL), and a basal diet with a high-dose of *B. subtilis* (BSH). $^{a–d}$ Different letters in superscript mean significant differences between treatment groups in columns, while $^{1–3}$ different numbers in superscript mean significant differences within groups at different time points (days 7, 14, 28, and 42) in rows ($p < 0.05$) ($n = 4$ or 5).

4.4.3 Probiotics Affect the Cecal Microbial Beta Diversity in Young Chickens

To study the changes in microbial responses to probiotics at different stages of life, a weighted UniFrac metric was used to analyze the beta diversity of the microbial communities across treatments and at different time points. The beta diversity of the BPH and BSH groups at

day 7 was significantly different from other groups (Table 2a), while at day 14, all of the treatment groups were significantly different from each other, except for the BPH and BSH groups, which were not statistically different from each other (Table 2b). At day 28, differences among the microbiota from the different treatment groups became less prominent, except between the NC group and the BPL, BSL, and BSH groups. The BSL and BSH groups were also different from the BPL group (Table 2c). The samples from the different treatment groups overlapped at day 42 (Table 2d), and significant differences were only seen between the PC group and the NC and BPL groups. While comparing the microbial beta diversity of the treatment groups among time points (Table 2e), it was observed that the microbiota in the probiotic groups BPL, BPH, and BSH was significantly different at day 14 than it was at day 7 and at day 28 than it was at day 14, but no significant difference seen between microbiota at days 28 and 42. The NC and BSL groups were different at all time points (days 14, 28, and 42) when compared to the previous time points. The PC group showed significant differences in the microbiota between days 7 and 14 followed by insignificant changes in the microbiota between days 14 and 28, with a significantly different set of microbiota being presented again at day 42. These results again showed that changes in the cecal microbiota for the probiotic groups were visible in the early weeks of life, which were the most prominent at day 14, and these became insignificant after day 28.

Table 4.2. Pairwise microbial community dissimilarity (beta diversity) in response to dietary treatments between groups at days 7 (a), 14 (b), 28 (c), and 42 (d) and within groups between different timepoints (e).

a. Pairwise microbial dissimilarity between groups at day 7.

Treatment Groups	**NC**	**PC**	**BPL**	**BPH**	**BSL**	**BSH**
NC	1	0.04	0.04	0.03	0.04	0.04
PC		1	0.04	0.03	0.04	0.04
BPL			1	0.03	0.04	0.04
BPH				1	0.03	0.06
BSL					1	0.04
BSH						1

b. Pairwise microbial dissimilarity between groups at day 14.

Treatment Groups	**NC**	**PC**	**BPL**	**BPH**	**BSL**	**BSH**
NC	1	0.07	0.07	0.04	0.07	0.03
PC		1	0.06	0.03	0.08	0.04
BPL			1	0.03	0.06	0.03
BPH				1	0.03	0.03
BSL					1	0.03
BSH						1

c. Pairwise microbial dissimilarity between groups at day 28

Treatment Groups	**NC**	**PC**	**BPL**	**BPH**	**BSL**	**BSH**
NC	1	0.24	0.03	0.06	0.03	0.03
PC		1	0.18	0.14	0.11	0.14
BPL			1	0.24	0.04	0.04
BPH				1	0.24	0.79
BSL					1	0.09
BSH						1

d. Pairwise microbial dissimilarity between groups at day 42.

Treatment Groups	**NC**	**PC**	**BPL**	**BPH**	**BSL**	**BSH**
NC	1	0.04	0.29	0.13	0.13	0.14
PC		1	0.04	0.22	0.17	0.64
BPL			1	0.56	0.13	0.41
BPH				1	0.28	0.56
BSL					1	0.78
BSH						1

e. Pairwise microbial dissimilarity within groups between days 7 and 14, 14 and 28, and 28 and 42.

Groups/Time Points	**Day 7 vs. Day 14**	**Day 14 vs. Day 28**	**Day 28 vs. Day 42**
NC	0.04	0.03	0.03
PC	0.03	0.14	0.03
BPL	0.05	0.04	0.27
BPH	0.04	0.03	0.52
BSL	0.03	0.03	0.03
BSH	0.03	0.05	0.53

The data of weighted UniFrac distance matrix were analyzed through a PERMANOVA test, and the results were corrected for significance through the Benjamini–Hochberg FDR method (q-values). q-values equal to or less than 0.05 were considered statistically significant (n = 4 or 5). Chickens were fed dietary treatments as described in Table 1.

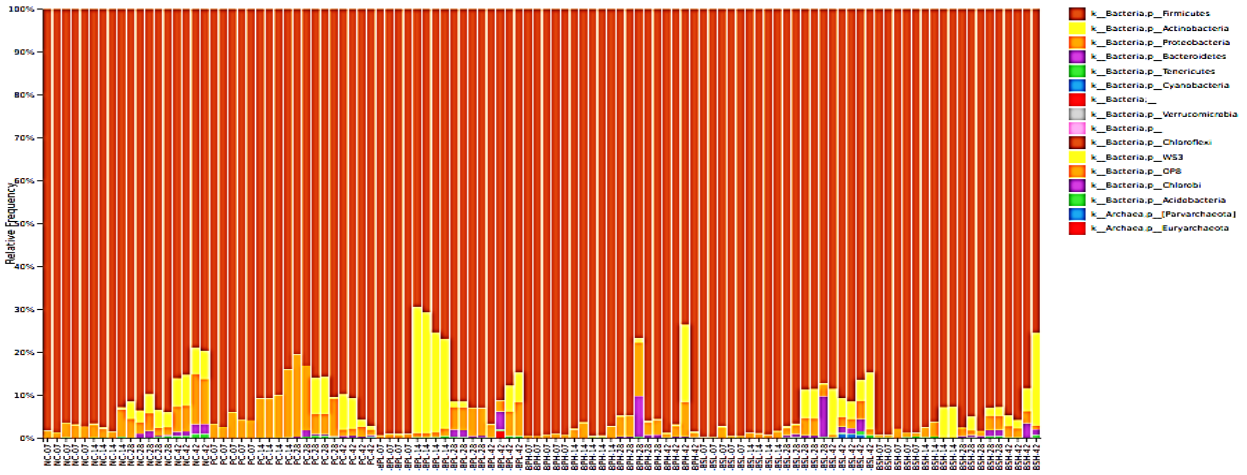
4.4.4 Probiotics Change Composition of Cecal Microbiota in Broiler Chickens

To study changes of microbial taxa by probiotics, the abundance of microbiota was examined at days 7, 14, 28, and 42 of the broiler's life due to the importance of these time points in the development of stable microbiota. From the cecal samples from all of the treatments and time points, Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes were the dominant phyla, accounting for a major part of the total sequence reads (Figure 1a). The families of Lachnospiraceae and Ruminococcaceae from the Phylum Firmicutes accounted for around 70% of the microbiota and showed higher differences among the treatment groups at day 14 than they did at the other timepoints (Figure 1b, c). The microbial abundance of Ruminococcaceae and Lachnospiraceae was not significantly different among different treatments at day 7, except for the PC group, which reduced the abundance of the Ruminococcaceae compared to the NC group. The family of Lachnospiraceae showed the highest abundance in the NC and PC groups at day 14 followed by a comparative decline in abundance at days 28 and 42 (Figure 1b). In contrast, the Ruminococcaceae family had the lowest abundance at day 14 in the NC and PC groups followed by increase in abundance at days 28 and 42 (Figure 1c). Conversely, the probiotic groups had a significantly ($p < 0.05$) lower abundance of Lachnospiraceae (Figure 1b) and a higher abundance of Ruminococcaceae (Figure 1c) at day 14 compared to the NC group, which became comparable to the NC group at days 28 and 42 in all of the probiotic groups except for in the BPH group at day 28, which had a higher abundance of Ruminococcaceae than the NC group and the BPL group

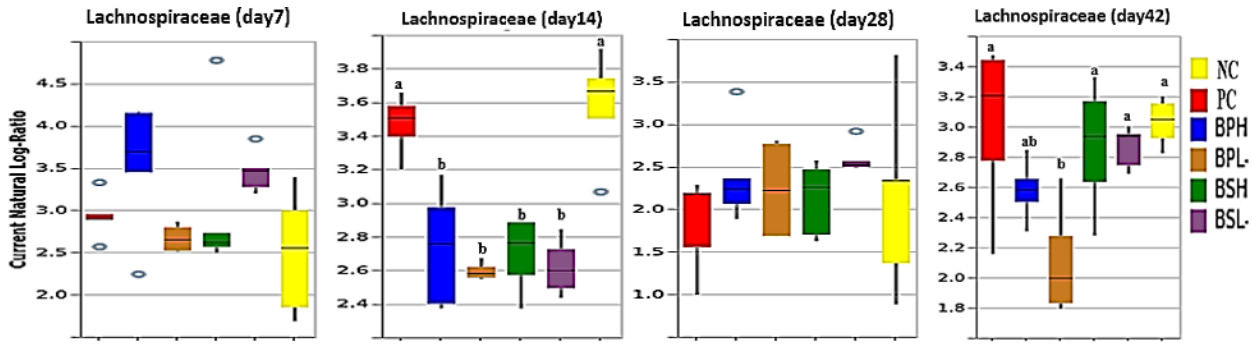
at day 42, which had a lower abundance of Lachnospiraceae than the NC group. The abundance of Ruminococcaceae was also significantly higher ($p < 0.05$) in the PC group at day 14 compared to the NC group, which became insignificant at day 28 and onward in comparison with the NC group (Figure 1c). These changes were further analyzed in the detected genera that covered at least 2% of the features. Differences in the composition of the microbiota among the treatment groups at the genus level were presented in Table 3. As shown in Table 3a, at day 7, the abundance of *Oscillospira* (BPL and BSL), *Faecalibacterium* (BPH), and *Butyricicoccus* (BSH) was significantly high, while the abundance of *Blautia* (BPH and BPL) and Enterobacteriaceae (BPH and BSL) was substantially lower in the probiotic groups when compared to the NC group. The PC group showed a significant increase in the abundance of Enterobacteriaceae and a decline in *Oscillospira* in comparison with the NC group at day 7. The abundance of different genera from family Ruminococcaceae, such as *Ruminococcus* (BPL, BPH, BSL, and BSH), *Oscillospira* (BPL, BSL, and BSH), *Faecalibacterium* (PC, BPL, BPH, BSL, and BSH), *Butyricicoccus* (BSL and BSH), and *Subdoligranulum* (BPL), was significantly higher at day 14 (Table 3a) compared to the NC group but became non-significant in many groups by days 28 and 42 (Table 3b), with the exception of *Ruminococcus* (BPL), *Oscillospira* (PC, BPL, BPH, BSL, and BSH), and *Faecalibacterium* (BPH and BSH) at day 28 and *Ruminococcus* (BPL) and *Subdoligranulum* (BSH) at day 42, where the abundance of these groups remained high. The abundance of *Subdoligranulum* and *Butyricicoccus* was lower in the PC group than in the NC group at day 14 and was similar to the NC group at days 28 and 42. In contrast, the abundance of genera from the family Lachnospiraceae, such as *Ruminococcus* (PC, BPL, BPH, BSL, and BSH), Blautia (PC, BPL, and BPH), *Coprococcus* (PC, BPL, BPH, BSL, and BSH), and *Dorea* (PC), was significantly lower than it was in the NC group ($p < 0.05$) at day 14 but became comparable to the NC group at

days 28 and 42 in all of the groups, except Blautia (for BPH and BSH) at day 28 and Coprococcus (PC, BPH and BSL) at days 28 and 42, where the abundance of these groups remained lower than it was in the NC group. The abundance of *Dorea* (PC and BSL) and *Blautia* (BPL) was higher than it was in the NC group at day 42. The abundance of *Dorea* in the BPL and BPH groups was significantly higher ($p < 0.05$) at day 14 and became insignificant at days 28 and 42. The abundance of other genera such as *Lactobacillus*, a member of the Lactobacillaceae family, and Bifidobacterium, a member of the family Bifidobacteriaceae, was significantly greater ($p < 0.05$) in the BPH, BSH, and BPL groups at day 14 but was not different from the NC group at days 28 and 42. The abundance of *Enterococcus* (BPL and BSL), *Sutterella* (BSH and BSL), and Erysipelotrichaceae (PC and BPL) was lower at day 14 in different groups and became equivalent to the NC group at days 28 and 42, except in case of *Sutterella* (PC, BPH, BSH and BSL) at days 28 and 42 and Enterococcus (BPL) at day 42. The abundance of Enterococcus was high in the PC group at day 14, which was detected to be equivalent to the NC group at days 28 and 42. Interestingly, the abundance of the Enterobacteriaceae family was significantly lower in the probiotic groups but was significantly higher in the PC group at days 7, 14, and 28. At day 42, all of the groups became insignificant in comparison with the NC group.

a.



b.



c.

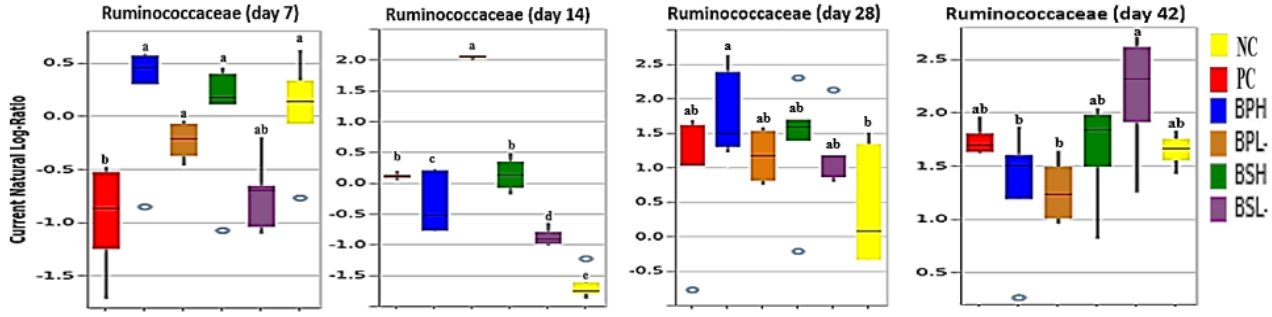


Figure 4.1. Plots showing relative frequency of the microbial taxonomic composition of different phyla (**a**) and the microbial relative abundance of the family Lachnospiraceae (**b**) and the family Ruminococcaceae (**c**) in response to treatments at 7, 14, 28, and 42 days of age. The abundance of Lachnospiraceae and Ruminococcaceae was calculated as the natural log-ratios. The Ruminococcus was used as a reference frame in the log ratio test. Chickens were fed dietary

treatments as described in Table 1. Boxes in panels b and c show the medians/quartiles of treatment samples, and the error bars extend to the most extreme values within the 1.5 interquartile ranges (n = 4 or 5). [a–e] Different letters mean significant differences among groups ($p < 0.05$).

Table 4.3. Effects of treatments on the relative abundance of cecal microbiota at days 7, 14, 28 and 42.

a. Effects of dietary treatments on the relative abundance of cecal microbiota at days 7 and 14.

		Treatments (Day-7) [1]						Treatments (Day-14) [1]					
Family	**Genus**	**PC**	**BPH**	**BPL**	**BSH**	**BSL**	**NC**	**PC**	**BPH**	**BPL**	**BSH**	**BSL**	**NC**
Ruminococcaceae	*Ruminococcus*	-2.9	-3.5	-2.7	-3.0	-3.5	-2.5	-3.5 [b]	**-2.7 [a]**	**-2.6 [a]**	**-2.7 [a]**	**-2.6 [a]**	-3.6 [b]
	Oscillospira	**-4.3 [d]**	-3.8 [c]	**-2.9 [a]**	-3.4 [b]	**-2.8 [a]**	-3.5 [bc]	-3.9 [bc]	-3.5 [abc]	**-2.9 [a]**	**-2.8 [a]**	**-3.2 [ab]**	-4.1 [c]
	Faecalibacterium	-6.4 [b]	**-1.2 [a]**	-6.0 [b]	-6.9 [b]	-6.5 [b]	-6.3 [b]	**-0.7 [a]**	**-3.7 [cd]**	**-2.9 [b]**	**-3.6 [c]**	**-4.0 [d]**	-6.2 [e]
	Butyricicoccus	-4.2 [b]	-4.8 [b]	*	**-2.2 [a]**	-4.6 [b]	-4.4 [b]	**-6.5 [c]**	-4.2 [ab]	-4.4 [ab]	**-3.7 [a]**	**-4.0 [a]**	-5.1 [b]
	Subdoligranulum	*	*	*	*	*	*	**-7.0 [c]**	-3.3 [b]	**0.28 [a]**	-2.7 [b]	-4.2 [b]	-3.4 [b]
Lachnospiraceae	*Ruminococcus*	0.15	-0.21	0.24	-0.01	0.29	-0.05	**-0.1 [d]**	**0.3 [c]**	**-2.0 [e]**	**-0.1 [d]**	**0.9 [b]**	1.7 [a]
	Blautia	-0.9 [b]	**-1.6 [a]**	**-1.4 [a]**	-0.4 [bc]	-0.3 [c]	-0.7 [bc]	**-2.7 [c]**	**-1.2 [b]**	**-2.7 [c]**	-0.7 [ab]	0.1 [a]	-0.1 [a]
	Coprococcus	-0.6 [b]	-2.5 [b]	-1.3 [b]	-0.9 [b]	-0.7 [b]	-1.6 [ab]	**-2.6 [c]**	**-1.0 [b]**	**-3.7 [d]**	**-1.5 [b]**	**-1.1 [b]**	-0.4 [a]
	Dorea	-3.4	-4.3	-4.1	-3.4	-3.6	-4.3	**-5.2 [d]**	**-3.4 [b]**	**-2.6 [a]**	-3.8 [bc]	-4.4 [c]	-4.1 [c]
	Clostridium	-5.4	-6.1	-5.4	-5.9	-5.7	-5.4	-5.3 [a]	-6.1 [ab]	**-7.3 [b]**	-4.8 [a]	-6.5 [ab]	-5.2 [a]
Lactobacillaceae	*Lactobacillus*	-1.01	-1.81	-0.04	-1.18	-0.74	-1.08	*	**-2.7 [b]**	-4.8 [c]	**-0.9 [a]**	-5.1 [c]	-5.2 [c]
[2] Erysipelotrichaceae	-	-2.6	-3.2	-2.3	-1.9	-2.0	-2.6	**-4.0 [b]**	-2.5 [a]	**-4.4 [b]**	-2.7 [a]	-2.6 [a]	-2.8 [a]
Enterococcaceae	*Enterococcus*	-0.4 [ab]	-1.8 [abc]	0.2 [a]	-1.8 [bc]	-3.0 [c]	-1.2 [a–c]	**-3.3 [a]**	-4.5 [b]	**-5.2 [c]**	-4.4 [b]	**-6.0 [d]**	-4.0 [b]
Bifidobacteriaceae	*Bifidobacterium*	*	*	*	*	*	*	-6.7 [b]	-6.6 [b]	**-0.6 [a]**	-3.9 [b]	-5.6 [b]	-4.6 [b]
Alcaligenaceae	*Sutterella*	*	*	*	*	*	*	-2.2 [ab]	-4.0 [ab]	-4.0 [ab]	**-5.0 [bc]**	**-7.2 [c]**	-1.0 [a]
[2] Enterobacteriaceae	-	**-0.7 [a]**	**-3.7 [c]**	-2.8 [bc]	-2.9 [bc]	**-3.7 [c]**	-1.9 [b]	**-1.6 [a]**	**-5.3 [c]**	**-5.0 [c]**	**-5.6 [c]**	-2.9 [b]	-2.5 [b]

b. Effects of dietary treatments on relative abundance of cecal microbiota at days 28 and 42.

		Treatments (Day-28) [1]						Treatments (Day-42) [1]					
Family	**Genus**	**PC**	**BPH**	**BPL**	**BSH**	**BSL**	**NC**	**PC**	**BPH**	**BPL**	**BSH**	**BSL**	**NC**
Ruminococcaceae	*Ruminococcus*	-1.8 [ab]	-1.8 [ab]	**-1.1 [a]**	-2.1 [b]	-2.6 [b]	-2.3 [b]	-3.0 [b]	-2.6 [ab]	**-2.1 [a]**	-2.9 [b]	-2.8 [b]	-3.0 [b]
	Oscillospira	**-2.7 [a]**	**-2.6 [a]**	**-2.4 [a]**	**-2.5 [a]**	**-2.8 [a]**	-3.4 [b]	-2.6	-2.9	-2.6	-3.2	-2.6	-2.8
	Faecalibacterium	-1.66 [b]	**0.03 [a]**	-0.68 [ab]	**-0.07 [a]**	-0.46 [ab]	-1.7 [b]	0.6 [a]	0.2 [ab]	-0.4 [b]	0.3 [ab]	0.5 [ab]	0.01 [ab]
	Butyricicoccus	-4.0	-3.8	-4.5	-4.5	-3.6	-4.5	-4.9 [ab]	-4.3 [ab]	-3.6 [a]	-5.1 [b]	-4.6 [ab]	-3.9 [ab]
	Subdoligranulum	-4.1	-4.8	-4.5	-4.6	-6.3	-4.9	-4.1 [a b]	-5.7 [b]	-5.1 [b]	**-2.8 [a]**	-5.3 [b]	-5.2 [b]
Lachnospiraceae	*Ruminococcus*	-0.9	-1.7	-1.2	-1.3	-1.2	-0.5	-1.7 [ab]	-1.3 [a]	-1.3 [a]	-1.6 [ab]	-2.1 [b]	-1.6 [ab]
	Blautia	-1.5 [ab]	**-2.9 [c]**	-1.2 [ab]	**-2.5 [bc]**	-1.4 [ab]	-1.0 [a]	-2.9 [ab]	-2.4 [ab]	**-2.0 [a]**	-2.5 [ab]	-2.6 [ab]	-3.2 [b]
	Coprococcus	**-2.6 [bc]**	**-3.3 [c]**	-2.1 [ab]	-2.2 [ab]	**-2.5 [b]**	-1.7 [a]	**-3.5 [b]**	**-3.4 [b]**	-2.4 [ab]	-3.0 [ab]	**-3.5 [b]**	-2.3 [a]
	Dorea	-3.8	-3.6	-3.6	-3.7	-3.3	-3.6	**-3.1 [a]**	-4.7 [b]	-3.8 [ab]	-4.4 [b]	**-3.1 [a]**	-4.3 [b]
	Clostridium	-3.9	-4.5	-4.2	-4.5	-4.2	-4.1	-4.3	-3.8	-3.6	-4.2	-3.5	-3.5
Lactobacillaceae	*Lactobacillus*	-4.5	-3.5	-4.1	-4.6	-3.9	-3.4	-4.4 [b]	-2.2 [a]	-2.3 [a]	-4.0 [ab]	-4.5 [b]	-3.6 [ab]
[2] Erysipelotrichaceae	-	-3.1	-3.9	-3.8	-3.6	-3.2	-3.5	-4.6	-3.1	-3.2	-4.1	-3.8	-3.9
Enterococcaceae	*Enterococcus*	-4.1	-5.6	-5.3	-5.6	-5.2	-3.9	-5.5 [ab]	-5.7 [ab]	**-4.5 [a]**	-6.8 [b]	-6.1 [ab]	-6.4 [b]
Bifidobacteriaceae	*Bifidobacterium*	-2.4	-6.0	-5.4	-5.0	-4.1	-3.1	-3.0	-5.1	-3.0	-2.4	-2.1	-1.9
Alcaligenaceae	*Sutterella*	**-3.7 [c]**	**-3.7 [c]**	-1.9 [ab]	**-3.4 [c]**	**-2.8 [bc]**	-1.4 [a]	**-4.5 [c]**	**-3.4 [bc]**	-2.4 [ab]	**-3.8 [c]**	**-3.8 [c]**	-1.7 [a]
[2] Enterobacteriaceae	-	**-1.2 [a]**	-3.0 [b]	-4.2 [cd]	**-5.1 [d]**	-4.3 [cd]	-3.8 [bc]	-4.5	-5.6	-3.8	-4.3	-4.7	-5.1

The relative abundance of microbiota was calculated as the natural log-ratios at days 7 and 14 (a) and days 28 and 42 (b). The Ruminococcaceae was used as a reference frame for the features of all of the other microbial families, while Lachnospiraceae was used as a reference frame for the features of the family Ruminococcaceae. [1] Chickens were fed dietary treatments as described in

Table 1. Numbers in green, red, and black represents high, low, and no significant difference in abundance from the NC group, respectively. [2] Genera not detected. * Values not detected. [a–d] Different letters mean significant differences among groups ($p < 0.05$). Significance level was adjusted for multiple comparison through Duncan test (n = 4 or 5).

4.5 Discussion

Our results revealed that probiotics alleviated an age-related (compared to the NC group) and antibiotic induced (compared to the PC group) drop in the alpha diversity mainly through the improvement in richness in younger birds before day 14. Our results are in agreement with earlier studies that used probiotics to alleviate dysbiosis caused by antibiotics. The study of Engelbrektson et al. (2009) reported a lessening in antibiotic-induced dysbiosis using a probiotic preparation carrying bacterial populations of *Bifidobacteria* and *Lactobacilli* in humans. In chickens, Pereira et al. (2019) reported that *B. subtilis*-based probiotics prevented an antibiotic-induced reduction in microbial richness and diversity. In another study, Oh et al. (2016) reported improvements in the functional parameters of microbiota following probiotic supplementation with antibiotic therapy. Our study results also displayed that improving bacterial diversity in younger birds (before d14) was dependent on the strain and the dose of the probiotic. The *B. subtilis* probiotics (BSL and BSH) maintained the diversity between days 7 and day 14, while the BPL group exhibited a reduction in the alpha diversity of the microbiota at day 14 compared to the BPH group, where it increased significantly at day 14. Nevertheless, BPL induced a reduction in the alpha-diversity on day 14, which was quickly recovered at day 28. A similar drop in the microbial diversity in response to probiotics in broiler chickens was also observed by Trela et al. (2020), who reported a decrease in the biodiversity indices, Shannon and Simpson, of crop and jejunum microbiota in response to the *B. licheniformis* probiotic. These observations highlight that

probiotics can help in the prevention of a decline in the microbial diversity in cecal microbiota, in strain- and dose-dependent manner. In addition, beta diversity analyses support the notion that changes in the cecal microbiota due to probiotic groups were visible in the early weeks of life and were most prominent at day 14, and these changes became insignificant after day 28.

The maturation of microbiota can be reflected in alpha diversity, beta diversity, changes in composition of microbiota, and functional genes. Our results also demonstrated that probiotics help cecal microbiota achieve early maturation at day 14 through an increase in the abundance of the core members of the family Ruminococcaceae such as *Ruminnococcus*, *Oscillospira*, *Faecalibacterium*, and *Butyricicoccus*. Growth in the family Ruminococcaceae happens at the cost of members of the family Lachnospiraceae such as *Ruminococcus*, *Blautia*, and *Coprococcus*. The bacterial fermentation of indigestible polysaccharides into short chain fatty acids (SCFA) is one of main functions in the cecum. SCFA are utilized by intestinal epithelial cells. The bacterial populations that are active in fermentation belong to certain families of Firmicutes such as Lachnospiraceae or Ruminococcaceae (Richards et al., 2019). After day 21, the intestinal microbota become stable and mature as variations in its structure and composition become lessened (Richards et al., 2019, Feye et al., 2020). The members of the family Lachnospiraceae, *Blautia* and *Ruminococcus*, are reported as the dominant bacterial population in the dynamic microbiota at the early days of life (Jurburg et al., 2019, Oakley et al., 2014), while members of the family Ruminococcaceae, *Faecalibacterium*, dominate in mature microbiota at day 21 and onward (Richards et al., 2019, Oakley et al., 2014). Our results reflected that *Bacillus subtilis* and *Bacillus pumilus* improved the strictly anaerobic population of the family Ruminococcaceae in early weeks of life, which is considered a major part of mature microbiota and have beneficial effects on the host physiology.

The early maturation of microbiota is beneficial to host's immune functions, as we reported previously (Bilal et al., 2021), in which the improvement in intestinal integrity and in the function and activation of anti-inflammatory T regulatory cells were observed in response to *B. subtilis* and *B. pumilus* probiotics at day 14 of broiler life. Our results are also supported by results from others. The *Faecalibacterium prausnitzii* is thought to have an anti-inflammatory effect (Sokol et al., 2008) and improved intestinal barrier function in a mouse IBD model (Carlsson et al., 2013). Massacci et al. (2019) reported an increased abundance of *Faecalibacterium prausnitzii* in response to *Saccharomyces cerevisiae boulardii* , which also potentially improved gut health and reduced *Campylobacter jejuni* excretion in broiler birds.

The bacterial population related to the family of Enterobacteriaceae was significantly higher in the PC group but was substantially lower in the probiotic groups in the current study. This bacterial family is important in poultry production, as it contains many pathogens with antimicrobial resistance such as extended-spectrum beta-lactamase genes (Saliu et al., 2017). Byndloss (2020) reported that antibiotics decreased the butyrate producing obligate anaerobic bacterial population, such as *Clostridia* (Ruminococcaceae and Lachnospiraceae), which are responsible for the maintenance of physiologic hypoxia on intestinal epithelial surfaces. A reduction in hypoxic conditions ease oxygen tolerant facultative anaerobes, such as Enterobacteriaceae, to grow fast and to overgrow other microbial populations. Thus, the probiotics (BPL, BPH and BSH) in this study may improve hypoxic conditions at the epithelial level by supporting the growth of short chain fatty acid producing (butyrate) bacteria such as members of the family Ruminococcaceae, which prevent the colonization of the Enterobacteriaceae population containing disease-causing bacteria. These results highlight that *Bacillus* probiotics favor the health promoting microbial population and play a protective role for the host.

The bacterial populations of *Bifidobacterium* (BPL) and *Lactobacillus* (BPH and BSH) were higher in specific probiotic groups at day 14. *Bifidobacteria* provides a substrate for bacteria that constitute the mature configuration of stable microbiota. Earlier studies reported that certain strains of *Bifidobacteria* secrete exopolysaccharides, a complex carbohydrate that acts as a substrate for mature microbiota such as *Faecalibacterium prausnitzii* (Salazar et al., 2008) and *Bacteroides fragilis* (Rios-Covian et al., 2016). In addition to promoting the growth of mature cecal microbiota, *Bifidobacteria* are reported to have a role in the intestinal barrier functions (Ling et al., 2016) and in the maturation and balancing of immune cells (Lopez et al., 2011). This suggests that an early rise in the population of strains of Bifidobacterium could help in the development and the activation of mature microbiota in hosts. *Lactobacillus* bacteria were higher in the BPH and BSH groups. *Lactobacillus* species are considered to be beneficial to the host in terms of their potential roles in decreasing intestinal pathogens through competitive exclusion, the production of bacteriocins, and antagonistic activities (Lan et al., 2005).

In this study the Gram-positive bacterial populations such as *Lactobacillus* may be underrepresented due to the presence of monensin in the feed as the anticoccidial in all treatment groups. By nature, monensin belongs to monovalent carboxylic ionophore group of anticoccidials and is naturally produced by the fermentation of Streptomyces species. It interacts with the sporozoite stage of coccidial parasites in the intestinal lumen and interferes with ion transportation across the coccidial membrane, which leads to the death of the parasite (Kadykalo et al., 2018). The effect of monensin on intestinal microbiota is not very clear. It is generally considered that Gram-negative bacteria are intrinsically resistant to monensin, while Gram-positive bacteria show susceptibility to monensin (Simjee et al., 2012). In an in vitro study, the *Lactobacillus* population decreased in response to monensin (Dec et al., 2020). However, in in vivo studies, either no

significant effect of monensin on intestinal microbiota was seen when comparing the monensin control with the negative control (Vieira et al., 2020) or some Gram-positive microbial genera decreased in abundance, while others were significantly enriched (Danzeisen et al., 2011). Considering that monensin was applied across all of the treatment groups in this study, the effects of the probiotics could be minimally compromised. However, the effect of monensin on the action of probiotics on poultry intestinal microorganisms requires further investigation.

4.6 Conclusion

In summary, the probiotic groups efficiently promoted the earlier maturation of cecal microbiota. The effects were strain and dose specific. *B. pumilus* and *B. subtilis* improved health promoting microorganisms such as members of Ruminococcaceae, *Lactobacillus,* and *Bifidobacterium* while virginiamycin increased the abundance of Enterobacteriaceae, which is linked with entero-pathogens. These results will set the stage for the design of microbiota-based interventions to promote production and health or to prevent diseases in chickens.

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4.8 References

Bilal M, Si W, Barbe F, Chevaux E, Sienkiewicz O & Zhao, X (2021) Effects of novel probiotic strains of *Bacillus pumilus* and *Bacillus subtilis* on production, gut health and immunity of broiler chickens raised under sub-optimal conditions. *Poultry Science*. **100:** 100871.

Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E & Knight R (2018) Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome*. **6:** 90.

Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet C & Al-Ghalith GA (2018) QIIME 2: reproducible, interactive, scalable, and extensible microbiome data science. PeerJ. **6:** e27295v2.

Byndloss XM (2020) Microbial management: Colonocyte metabolism plays an essential role in balancing the gut microbiota. *Science*. **369:** 6500.

Carlsson AH, Yakymenko O, Olivier I, Håkansson F, Postma E, Keita AV (2013) *Faecalibacterium prausnitzii* supernatant improves intestinal barrier function in mice DSS colitis. *Scandinavian Journal of Gastroenterology*. **48:** 1136–44.

Chen Q, Tong C, Ma S, Zhou L & Zhao X (2017) Involvement of microRNAs in probiotics-induced reduction of the cecal inflammation by salmonella typhimurium. *Frontiers in Immunology*. **8:** 704.

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. 2011. *Public Library of Science One*. **6:** e27949.

Dec M, Puchalski A, Stępień-Pyśniak D, Marek A & Urban-Chmiel R (2020) Susceptibility of chicken Lacto*Bacillus* bacteria to coccidiostats. *Journal of Veterinary Medical Science*. **24:** 333-336.

Engelbrektson A, Korzenik JR, Pittler A, Sanders ME, Klaenhammer TR & Leyer G (2009) Probiotics to minimize the disruption of faecal microbiota in healthy subjects undergoing antibiotic therapy. *Journal of Medical Microbiology*. **58:** 663–670.

Fedarko MW, Martino C, Morton JT, Gonz´alez A, Rahman G, Marotz CA, Minich JJ, Allen EE & Knight R (2020) Visualizing 'omic feature rankings and log-ratios using Qurro. *NAR Genomics and Bioinformatics*. **2:** 2.

Feye KM, Baxter FMA, Tellez-Isaias G, Kogut MH & Ricke SC (2020) Influential factors on the composition of the conventionally raised broiler gastrointestinal microbiomes. *Poultry Science*. **99:** 653–659.

Food and Agriculture Organization (2015). From http://www.fao.org/3/y4252e/y4252e07.htm. Accessed on 1-04-2021.

Food and Agriculture Organization of the United Nations (2020) Gateway to poultry production and product. From http://www.fao.org/poultry-production-products/production/en/. Accessed on 1-04-2021.

Gasparrini AJ, Wang B, Sun X, Kennedy EA, Hernandez-Leyva A & Ndao IM (2019) Persistent metagenomic signatures of early-life hospitalization and antibiotic treatment in the infant gut microbiota and resistome. *Nature Microbiology*. **4:** 2285–97.

Grant A, Gay CG & Lillehoj HS (2018) *Bacillus* spp. as directed microbial antibiotic alternatives to enhance growth, immunity, and gut health in poultry. *Avian Pathology*. **47:** 339-351.

Hartog DG, Vries-Reilingh GD, Wehrmaker AM & Savelkoul HFJ (2016) Parmentier, H.K. Lammers A. Intestinal immune maturation is accompanied by temporal changes in the composition of the microbiota. *Beneficial Microbes*. **7:** 677-685.

Jurburg SD, Brouwer MSM, Ceccarelli D, Goot J, Jansman AJM & Bossers A (2019) Patterns of community assembly in the developing chicken microbiome reveal rapid primary succession. *Microbiology Open*. **8:** e821.

Kadykalo S, Roberts T, Thompson M, Wilson W, Lang M & Espeisse O (2018) The value of anticoccidials for sustainable global poultry production. *International Journal of Antimicrobial Agents*. **51:** 304–310.

Kers JG, Velkers FC, Fischer EAJ, Hermes GDA, Stegeman JA & Smidt H (2018) Host and environmental factors affecting the intestinal microbiota in chickens. *Frontiers in Microbiology*. **9:** 235.

Kozich JJ, Westcott SL, Baxter NT, Highlander SK & Schloss PD (2013) Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and Environmental Microbiology*. **79:** 5112-5120.

Kubasova T, Kollarcikova M, Crhanova M, Karasova D, Cejkova D & Sebkova A (2019) Contact with adult hen affects development of caecal microbiota in newly hatched chicks. *Public Library of Science One*. **14:** e0212446.

Lan Y, Verstegen MWA, Tamminga S & Williams BA (2005) The role of the commensal gut microbial community in broiler chickens. *World's Poultry Science Journal*. **61:** 95–104.

Ling X, Linglong P, Weixia D & Hong W (2016) Protective effects of Bifidobacterium on intestinal barrier function in LPS-induced enterocyte barrier injury of Caco-2 monolayers and in a rat NEC model. *Public Library of Science One*. **11:** e0161635.

Lopez P, Gonzalez-Rodriguez I, Gueimonde M, Margolles A & Suarez A (2011) Immune response to *Bifidobacterium bifidum* strains support Treg/Th17 plasticity. *Public Library of Science One*. **6:** e24776.

Marcolla CS, Alvarado CS & Willing BP (2019) Early life microbial exposure shapes subsequent animal health. *Canadian Journal of Animal Science*. **99:** 661–677.

Massacci FR, Lovito C, Tofani S, Tentellini M, Genovese DA, De Leo A, Papa P, Magistrali CF, Manuali E, Trabalza-Marinucci M, Moscati L & Forte C (2019) Dietary *Saccharomyces cerevisiae boulardii* CNCM I-1079 positively affects performance and intestinal ecosystem in broilers during a *Campylobacter jejuni* Infection. *Microorganisms*. **7:** 596.

Meijerink N, Kers JG, Velkers FC, Van-Haarlem DA, Lamot DM, de Oliveira JE, Smidt H, Stegeman JA, Rutten VPMG & Jansen CA (2020) Early life inoculation with adult-derived microbiota accelerates maturation of intestinal microbiota and enhances NK cell activation in broiler chickens. *Frontiers in Veterinary Science*. **7:** 584561.

Morton TJ, Marotz C, Washburne A, Silverman J, Zaramela LS, Edlund A, Zengler K & Knight R (2019) Establishing microbial composition measurement standards with reference frames. *Nature Communications*. **10:** 2719.

Oakley BB, Buhr R, Ritz CW, Kiepper BH, Berrang ME & Seal BS (2014) Successional changes in the chicken cecal microbiome during 42 days of growth are independent of organic acid feed additives. *BMC Veterinary Research*. **360:** 282.

Ocejo M, Oporto B & Hurtado A (2019) 16S rRNA amplicon sequencing characterization of caecal microbiome composition of broilers and free-range slow growing chickens throughout their productive lifespan. *Scientific Reports*. **9:** 2506.

Oh B, Kim JW & Kim B (2016) Changes in the functional potential of the gut microbiome following probiotic supplementation during helicobacter pylori treatment. John Wiley & Sons Ltd. Helicobacter. **21:** 493–503.

Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, Glickman JN, Siebert R, Baron RM, Kasper DL & Blumberg RS (2012) Microbial exposure during early life has persistent effects on natural killer T cell function. *Science*. **336:** 489–493.

Pabst O, Cerovic V & Hornef M (2016) Secretory IgA in the coordination of establishment and maintenance of the microbiota. *Trends in Immunology*. **37:** 5.

Pereira R, Bortoluzzi C, Durrer A, Fagundes NS, Pedroso AA, Rafael JM, Perim JE, Zavarize KC, Napty GS, Andreote FD, Costa DP & Menten JFM (2019) Performance and intestinal microbiota of chickens receiving probiotic in the feed and submitted to antibiotic therapy. *Journal of Animal Physiology and Animal Nutrition*. **103:** 72–86.

Richards P, Fothergill J, Bernardeau M & Wigley P (2019) Development of the caecal microbiota in three broiler breeds. *Frontiers in Veterinary Science*. **6:** 201.

Rios-Covian D, Cuesta I, Alvarez-Buylla JR, Ruas-Madiedo P, Gueimonde M & Reyes-Gavilán CG (2016) Bacteroides fragilis metabolises exopolysaccharides produced by Bifidobacteria. *BMC Microbiology*. **16:** 150.

Salazar N, Gueimonde M, Hernandez-Barranco AM, Ruas-Madiedo P & de los Reyes-Gavila CG (2008) Exopolysaccharides produced by intestinal Bifidobacterium strains act as fermentable substrates for human intestinal bacteria. *Applied and Environmental Microbiology*. **74:** 4737–45.

Saliu EM, Vahjen W & Zentek J (2017) Types and prevalence of extended spectrum beta lactamase producing Enterobacteriaceae in poultry. *Animal Health Research Reviews*. **18:** 46–57.

Simjee S, Heffron A, Pridmore A & Shryock TR (2012) Reversible monensin adaptation in *Enterococcus faecium*, *Enterococcus faecalis* and *Clostridium perfringens* of cattle origin: potential impact on human food safety. *Journal of Antimicrobial Chemotherapy*. **67:** 2388–2395.

Sokol H, Watterlot L, Lakhdari O, Bermu LG, Bridonneau C & Furet JP (2008) Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patient. *Proceedings of the National Academy of Sciences*. **105:** 16731–6.

Sterlin D, Fadlallah J, Slack E & Gorochov G (2020) The antibody/microbiota interface in health and disease. *Mucosal Immunology*. **13:** 3–11.

Trela J, Kieronczyk B, Hautekiet V & Józefiak D (2020) Combination of *Bacillus licheniformis* and salinomycin: Effect on the growth performance and GIT microbial populations of broiler chickens. *Animals*. **10:** 889.

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:** 1027–1031.

Vieira AM, Soratto TAT, Cardinal KM, Wagner G, Hauptli L & Lima ALF (2020) Modulation of the intestinal microbiota of broilers supplemented with monensin or functional oils in response to challenge by Eimeria spp. *Public Library of Science One*. **15:** e0237118.

Wang L, Li L, Lv Y, Chen Q, Feng J & Zhao, X (2018) Lacto*Bacillus* plantarum restores intestinal permeability disrupted by Salmonella infection in newly hatched chicks. *Scientific Reports*. **8:** 2229.

Connecting Text

In Chapter 3, we observed that effects of *Bacillus pumilus* and *Bacillus subtilis* on chicken's gut health and immunity were prominent at day 14. Similarly, in Chapter 4, significant alterations in cecal microbial diversity and composition were observed in the first two weeks of life in response to probiotics, which became non-significant on day 42. Furthermore, probiotics efficiently helped the cecal microbiota to reach mature configuration earlier in life, suggesting that interventions with capability to speed up gut microbiota to attain mature configuration earlier in life may confer health and production benefits to the host. Following this hypothesis, in Chapter 5, we transferred fecal microbiota from donor birds with different ages (days 14 and 42) to day-old chicks through cohousing and assessed the effects of intervention on production, gut immunity and bone health.

Chapter 5. Cohousing-mediated microbiota transfer promotes bone health and modulate gut immunity in young broiler chickens

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5.1 Abstract

The intestine is a complex ecosystem harboring a dense and diverse microbial community called the gut microbiota, which is co-evolved with the host to develop a mutualistic relationship. Microbiota before day 14 is immature and develop slowly in young chickens that prolongs the precarious period before reaching a mature configuration. Supplementation of probiotics may speed up the maturation. However, whether cohousing with older broiler chickens could speed up the maturation of microbiota in day-old chicks and subsequently improve production, immunity and bone health is unknown. In this study day-old broiler chickens were cohoused with 14 (A14) or 42 (B42) days old birds while the control day-old chickens did not cohouse with old chickens. These chickens were raised for 42 days and tibial bone and cecal tonsil samples were collected from sacrificed birds at days 14 and 42 for bone traits and T cells subsets ($CD4^+$, $CD8^+$ and $CD4^+CD25^+$ T cells) analyses. The production related parameters were recorded weekly while mortality was recorded daily. Both treatment groups (A14 and B42) significantly improved the tibial bone length, cortical bone volume and cortical bone mineral content and notably decreased tibial bone pore volume and diameter. These significant effects were observed earlier at day 14 in the B42 group in comparison with A14 group. The percentages of T regulatory cells ($CD4^+CD25^+$ T cells) and $CD4^+$ T cells were significantly higher and lower, respectively, in the B42 group. Further, no significant changes in $CD8^+$ T cells were observed among treatment groups both at days 14 and 42. The cohousing also did not significantly affect production and mortality. In short, cohousing with older chickens significantly improved tibial bone health and skewed the immune system toward anti-inflammatory state. These effects could be used for future manipulation of microbiota to improve poultry bone health.

5.2 Introduction

The gut microbiota plays an important role in host health and production. 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 microbial communities inhabiting the gastrointestinal tract (GIT) of chickens play an important role in nutrient digestion, pathogen inhibition and interaction with the gut-associated immune system (Borda-Molina et al., 2018). The interactions of the gut microbiome with the host may be altered by dysbiosis, which is defined as adverse changes in bacterial composition, diversity, and functions (Ibanez et al., 2019). Different interventions have been used to influence the gut microbiota for improved health and production, which included probiotics, prebiotics (Bilal et al., 2021a, Gadde et al., 2017) and transfer of fecal microbiota (Thomas et al., 2019). In addition, fecal microbiota transplantation has been widely studied in mice (Yan et al., 2016) and humans (Cammarota et al., 2014) as therapy for different inflammatory diseases while recent reports have shown its beneficial effects in chickens (Metzler-Zebeli et al., 2019).

In broiler chickens, the indigenous microbiota evolves with time. One-day-old broiler chicks already carry a community of microorganisms in their intestinal tract that are acquired directly from mother's oviduct or environment. The microbiota of growing chicks develops rapidly from day 1 to day 3, and the microbiota is primarily Enterobacteriaceae. Firmicutes increase in abundance and increasing taxonomic diversity starts around day 7 and is more obvious with time (Ballou et al., 2016). Ocejo et al. (2019) described that microbiota in broilers develops in 3 main stages. The first stage, represented by 3-day-old broilers, showed a clearly immature microbiota dominated by Proteobacteria and Firmicutes. The second and third stage represented by 14-day-

old and 42-day-old broilers, respectively, where abundance of the core microbiota at day 14 shifts from phylum Firmicutes to phylum Bacteroidetes at day 42. We previously observed that cecal microbiota in broiler chickens at days 14 and 42 responded differently to probiotics (Bilal et al., 2021b). Similarly, the host responses in terms of immunity and gut health to probiotics were also different at different stages (days 14 and 42) of production cycle (Bilal et al., 2021a). These studies show that chickens possess different sets of microbiotas at different stages of life and emphasize the significance of speeding up the maturation of gut microbiota.

Besides a direct role in maturation and modulation of immune system, gut microbiota may have indirect influence on bone homeostasis. Recently, a new interdisciplinary field bridging the study of gut microbiome and bone biology, known as 'osteomicrobiology' has emerged (Cooney et al., 2021). It has been observed in a germ-free mouse model that there was delay in most of the main growth parameters including shorter femurs compared to conventionally raised controls (Schwarzer et al., 2016). In another study, the axenic mice restored its skeletal growth after 4 weeks when transplanted with gut microbiota from normal mice (Yan et al., 2016). In the poultry sector, growth rate of broiler chickens has increased by over 300% (from 25 g per day to 100 g per day) in the last 50 years due to intense genetic selection (Knowles et al., 2008). However, this quick growth led to decrease in mobility, changes in bird gait and increase in leg disorders (Phibbs et al., 2021). Several studies have found that 14% to 50% of broilers suffer from lameness (Granquist et al., 2019), which is a major welfare issue and causes economic losses (Kittelsen et al., 2017). Different methods like environmental enrichment have been applied in different forms to improve lameness but their results are conflicting (Phibbs et al., 2021) and emphasize to search for novel interventions.

Intestinal inflammation also plays a key role in bone resorption and formation. The pro-inflammatory cytokines such as IL-1 beta, IL-6, IL-17, TNF-alpha, interferon (IFN)-gamma, and activator of nuclear factor kappa-B ligand (RANKL) activate osteoclasts and promote bone resorption (Amin et al., 2020) while anti-inflammatory components such as T regulatory cells and IL-4 and IL-10 cytokines activate osteoblasts and help bone formation (Zhu et al., 2020). In this regard, short chain fatty acids like butyrate from intestinal microbiota have been reported to activate T regulatory cells through GPR-43 (Singh et al., 2014) and GPR-109A (Haase et al., 2018) receptors and play a role in inhibition of inflammation and subsequent promotion of bone health. Bone disorders during different diseases like inflammatory bowel disease has been associated with gut dysbiosis related inflammation (Sgambato et al., 2019). Previously, fecal microbiota transplantation has shown an anti-inflammatory potential and has been used successfully in humans to treat inflammatory diseases like *Clostridioides difficile* infection (CDI) and inflammatory bowel disease (Wargo, 2020). Here, we hypothesize that transfer of fecal microbiota through cohousing would modulate intestinal microbiota and exert anti-inflammatory effects on immune system and thus enhancing the bone health and thus improve bird's welfare. Very little information on this aspect is available in poultry.

Keeping in view these facts, the following study was designed to evaluate the effects of fecal microbiota transfer, from donor chickens at different ages (day 14 and 42), on production, T cells immunity and bone health in broiler chickens.

5.3 Materials and methods

5.3.1 Birds and experimental design

A total of 180 day-old male broiler chicks (Cobb 500) were obtained from a local hatchery (Grains Natures, Roxton Falls, Quebec Canada). These day-old chicks were acquired in 3 batches. The first two batches of day-old chicks (n=18 chicks in each) acquired 42 and 14 days ahead of the experiment and raised separately for 42 days and 14 days, respectively. These birds were raised on the basal diet through a two-phase, starter and grower, program (Table 5.1). These birds were used as donors for cohousing and transfer of fecal microbiota. The third batch of day-old chicks (n=144) were divided randomly into three groups, two cohoused (day 14 and day 42) and one control group, with 8 replicates per group (6 birds/cage). The 14- and 42-days-old birds from donor groups (1 bird/cage (2 square feet)) were transferred to respective cohoused groups along with 100 grams of fecal material, which was spread in the cage. These day-old chicks were raised for 42 days. The donor birds were removed after one week of co-housing. The basal diet was provided to all groups and was composed of corn, soybean meal, soybean oil, amino acids supplements, vitamins and mineral premix, and were mixed as per standard of National Research Council (National Research Council., 1994). The feed was provided through a two-phase program, a starter feed (23% protein and 2977 kcal metabolizable energy/kg) was served from day 1 to day 14, while a grower feed (20% protein and 3056 kcal metabolizable energy/kg) was provided from day 15 to day 42 of the trial (Table 5.1). The feed and water were provided ad libitum. Feed intake (FI), body weight (BW) and feed conversion ratio (FCR) were recorded on weekly basis for each cage, while morbidity and mortality were recorded daily. The study protocol was approved by the Animal Care Committee of McGill University (Ref # 2018-8002).

5.3.2 Sample Collection

The birds from all three groups were sacrificed at two time-points (day 14 and 42) for sampling purposes. These birds were sacrificed (8 birds/ group) at each time-point through cervical dislocation.

5.3.3 Flow cytometry and T cells population

In order to observe T cells response to treatments, cecal tonsils (longitudinally cut) from birds were obtained, washed and crushed with flat end of 3-ml syringe plunger in 1mM EDTA solution. The solution was passed through a 40-μm cell strainer (BD Biosciences, NJ, USA) into a 50-ml conical tube. The cells were centrifuged for 8 minutes at 400 RCF (~1200 RPM) and washed with cold PBS twice. Cells were re-suspended to a concentration of 1 x 10^6 cells/ml in the Flow Staining Buffer. Viability dye (FVD eFluor 780) (eBioscience, CA, USA) was added to the cells at the concentration of 1ul/ml and the cells were incubated for 30 minutes on ice and in dark place. For examination of T cell subsets, the cells were stained with anti-chicken CD4-FITC (clone CT-4) and CD8a-PE (clone CT-8) (from Southern Biotech, Alabama, USA) and CD25-Alexa Fluor 647 (clone-AbD13504) (from Bio-Rad, QC, Canada). The cells were fixed through fixative solution (1-step Fix/Lyse Solution-10X) (eBioscience, CA, USA). Different controls such as unstained, single stained for each antibody, fluorescence minus 1 for each fluorophore and viability dye were included. The cytometry data were obtained and analyzed through FlowJo software for percentages of immune cells (Bilal et al., 2021a).

5.3.4 Micro-CT for tibial cortical bone tissue mineral content and morphology analysis

The muscles and other tissues around tibial bone were removed through a scalpel and right tibial bone harvested and preserved at -20C for later analysis. These bones were soaked in 70% (vol/vol) ethanol and scanned through Bruker (MA, USA) MicroCT computed tomography analyzer (Version: 1.16.1.0). Phantoms (standards with a known density) were used to calibrate

the gray values in the image to the density of hydroxyapatite and were imaged at the same time as the bone samples in the study were imaged. Using these phantoms, the calibration coefficients were calculated for tibial bone scans. Further, the vertical volume of interest (VOI) was determined for midshaft bone that were based on slices equivalent to 5% of the bone's length and thresholds of two VOI per group were used to calculate the global threshold that was further used for all bones in analysis. The 2D and 3D microstructural properties and tissue mineral content of bones were calculated using a CTAn software supplied by the manufacturer (CTAn user's guide, 2021). Bone length was measured using the scout view feature.

5.3.5 Latency to lie test

Latency to lie test is a method commonly used to assess lameness in broilers and is a sign of a broiler's ability to stand during uncomfortable conditions. Eight broilers from each group at days 14 and 42 of age were used to perform the latency to lie test following the procedure of Berg and Sanotra. (2003). Briefly, each bird was put into warm water (28° C) in a tub filled up to 3 cm. The time (seconds) the birds took to sit and touch the water was recorded and the flew away birds were excluded from the data set. If the broilers were still standing after 600 seconds, the test was stopped.

5.3.6 Data analysis

A completely randomized design (CRD) was used for different parameters in the study. The cage was considered as experimental unit and Shapiro–Wilk test applied to ascertain normality of the data. The data were analyzed using one-way ANOVA through SPSS software. The data are presented as means ± SEM for each treatment. The differences were considered significant with a P value ≤0.05. When the main effect was significant, differences between means were analyzed using Duncan's multiple range test. The statistical model was: $Y_{ij} = \mu + TRT_i + e_{ij}$,

where Yij represents the observation for the dependent variables at jth replicate in the ith treatment; μ is the overall mean; TRTi is the fixed effect of treatments (i = 1 to 3); eij is the random residual error.

Table 5.1: Composition (%) of the basal diet

Ingredients	Starter %	Grower%
Corn	54.74	53.29
Wheat	0	10.00
Soybean meal, 48% CP	38.55	30.84
Soybean Oil	2.16	2.25
Phosphorus	1.74	0.93
Calcium	1.54	1.62
Vitamin-mineral premix (Starter)	0.50	0
Vitamin-mineral premix (Grower)	0.00	0.40
Salt	0.27	0.36
LYSINE HCL	0.13	0.00
METHIONINE	0.14	0.12
THREONINE	0.03	0.00
Choline chloride	0.10	0.10
Sodium carbonate	0.10	0.10
ME, kCal/kg	2,977	3,056
Crude protein, %	23.00	20.1147
Lysine Total, %	1.43	1.11
Methionine Total, %	0.51	0.44
Crude Fat, %	4.45	4.60
Calcium, %	1.05	0.92
Phosphorus Total, %	0.75	0.56

5.4 Results

5.4.1 Effects of cohousing mediated fecal microbiota transfer on production parameters of broiler chickens

In order to evaluate the effects of cohousing mediated fecal microbiota transfer on production parameters of broiler chickens, body weight (BW), feed intake (FI) and feed

conversion ratio (FCR) were monitored (Figure 5.1 A-C). The effects of fecal microbiota transfer from A14 and B42 groups on weekly BW, FI and FCR of broiler chickens were not statistically different from the Control group. No mortality was observed in different treatment groups except that one bird was found squished in the B42 group during the first week of life.

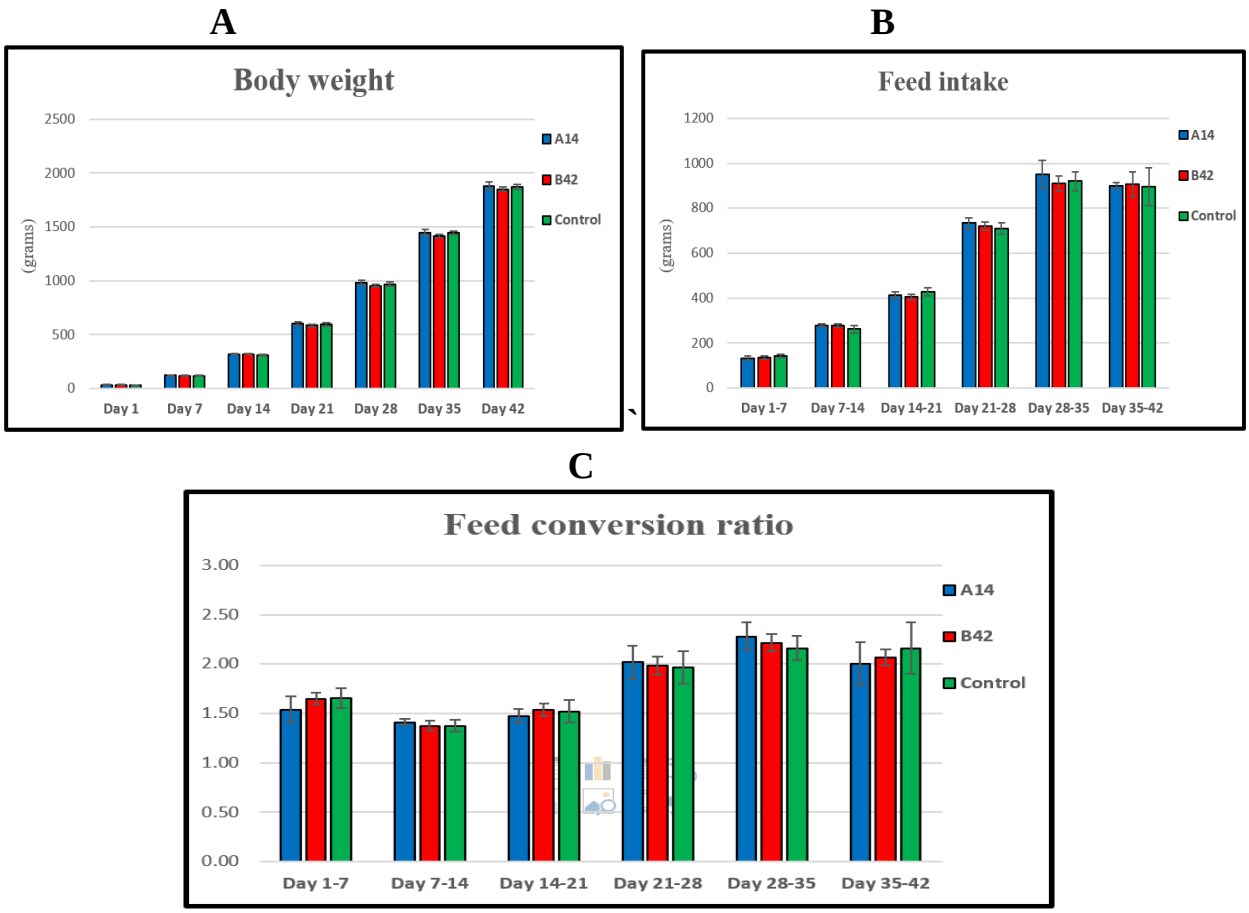

Figure 5.1. Effects of cohousing with day 14 and day 42 broilers on body weight (A), feed intake (B) and feed conversion ratio (C) of broiler chickens raised for 42 days. The cages of day-old-chicks received 100 g of fecal material/day and one bird per cage, cohoused for one week, from donor birds of ages 14 (A14) and 42 (B42) while control chicks (Control) neither received fecal material nor cohoused with birds. The bars represent mean values +/- SEM (n=8).

5.4.2 Cohousing mediated fecal microbiota transfer promotes tibial bone health of broilers

To assess the effect of cohousing mediated fecal microbiota transfer on skeletal health of the broiler birds, tibial bone length, mineral contents and micro-structures were analyzed and bird's tolerance to unfavorable conditions were examined through a latency to lie test. In general, the tibial bone length at day 14 was comparable among treatment groups but significantly increased in A14 and B42 groups in comparison with the Control group at day 42 (Figure 5.2). The tibial bone mineral content was significantly higher in the B42 group than A14 and Control groups at day 14 but became comparable with other two groups at day 42. In contrast, the mineral content in tibial bones of A14 group at day 14 was not different from the Control group but later at day 42, it was considerably higher in comparison with the Control group (Figure 5.3). Regarding morphology of bone, the tibial cortical bone area at day 14 was substantially higher in the A14 and B42 groups, however, differences among treatments became non-significant at day 42 (Figure 5.4A). Further, the tibial cortical bone pore volume was significantly lower in the B42 group at day 14 and in the A14 group at day 42 in comparison with the Control groups on the respective days (Figure 5.4B). The tibial cortical bone pore diameter was also significantly lower in the A14 and B42 groups at day 42 in comparison with the Control group (Figure 5.4C). The time to tolerate unfavorable conditions (latency to lie test) was improved in A14 and B42 treatment groups but did not reach a statistical significance level both at days 14 and 42 (Figure 5.5). These results suggested that cohoused mediated transfer of fecal microbiota from birds of different ages improved the tibial bone health parameters of broiler chickens during the trial period and the improved parameters appeared earlier in the B42 group than the A14 group.

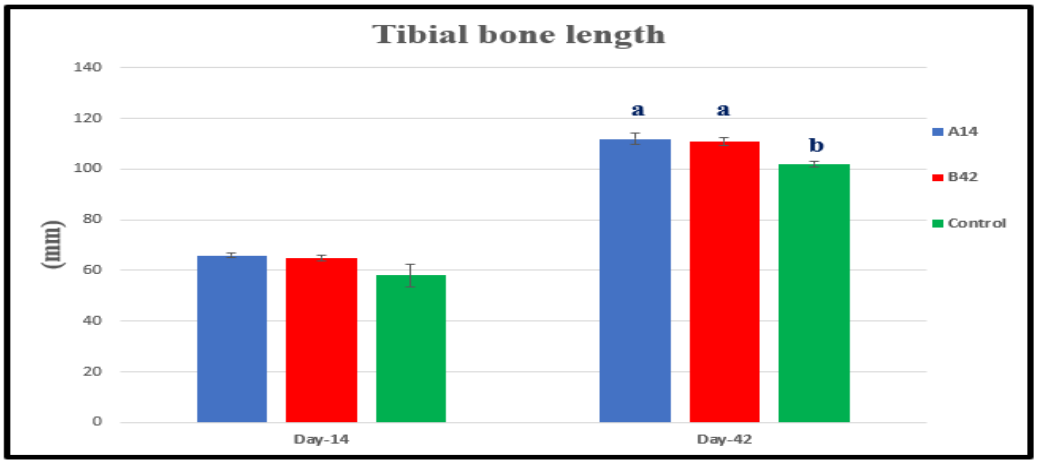

Figure 5.2. Effects of cohousing with day 14 and day 42 broilers on tibial bone length of broiler chickens at day-14 and day-42 of age. The cages of day-old-chicks received 100 g of fecal material/day and one bird per cage, cohoused for one week, from donor birds of ages 14 (A14) and 42 (B42) while control chicks (Control) neither received fecal material nor cohoused with birds. [ab]Different letters mean significant differences among groups (P<0.05, Duncan test) at days 14 and 42 and bars represent mean +/- SEM (n=8).

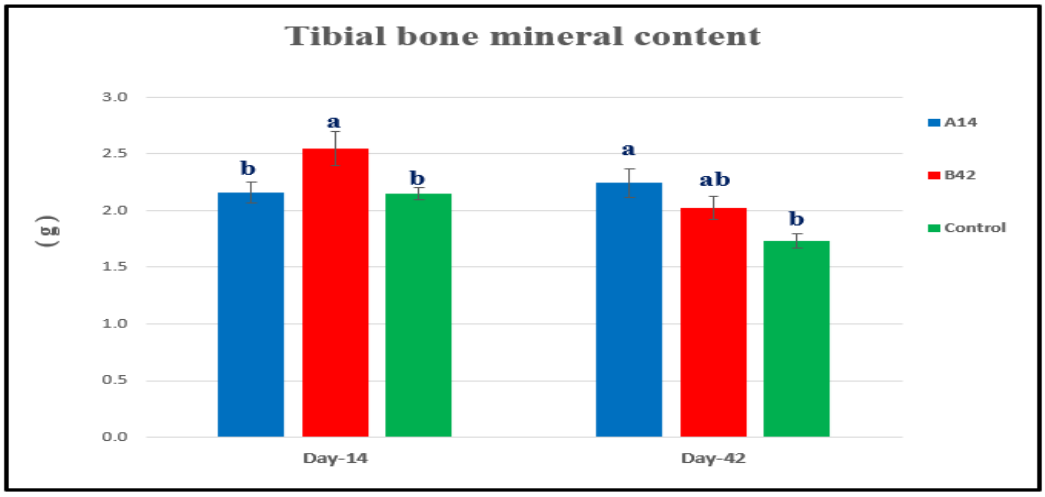

Figure 5.3. Effects of cohousing with day 14 and day 42 broilers on tibial bone mineral content of broiler chickens at day-14 and day-42 of age. The cages of day-old-chicks received 100 g of fecal material/day and one bird per cage, cohoused for one week, from donor birds of ages 14 (A14) and 42 (B42) while control chicks (Control) neither received fecal material nor cohoused with birds. [ab]Different letters mean significant differences among groups (P<0.05, Duncan test) at days 14 and 42 and bars represent mean +/- SEM (n=8).

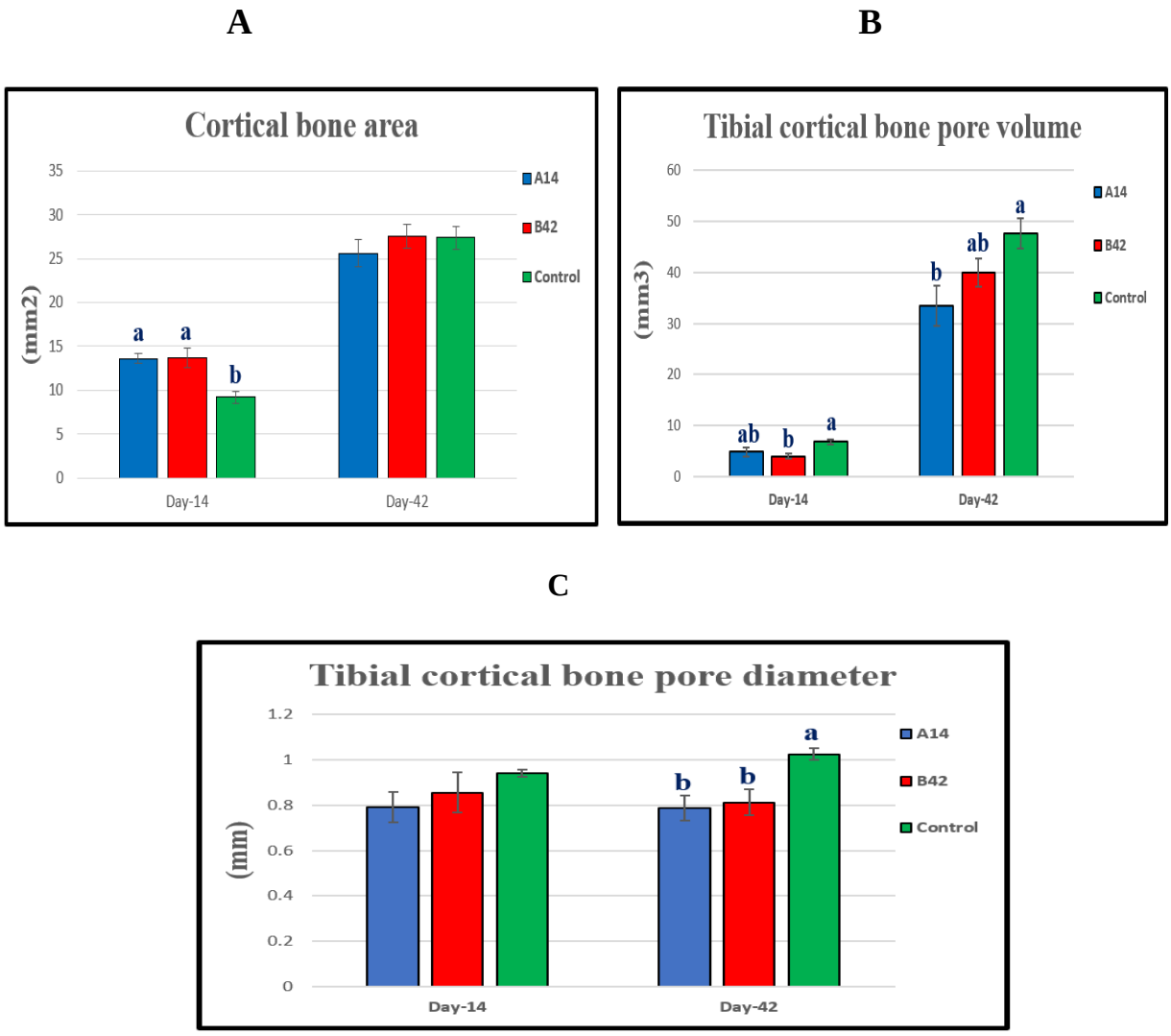

Figure 5.4. Effects of cohousing with day 14 and day 42 broilers on tibial cortical bone area (A), pore volume (B) and pore diameter (C) of broiler chickens at day-14 and day-42 of age. The cages of day-old-chicks received 100 g of fecal material/day and one bird per cage, cohoused for one week, from donor birds of ages 14 (A14) and 42 (B42) while control chicks (Control) neither received fecal material nor cohoused with birds. [ab]Different letters mean significant differences among groups ($P<0.05$, Duncan test) at days 14 and 42 and bars represent mean +/- SEM (n=8).

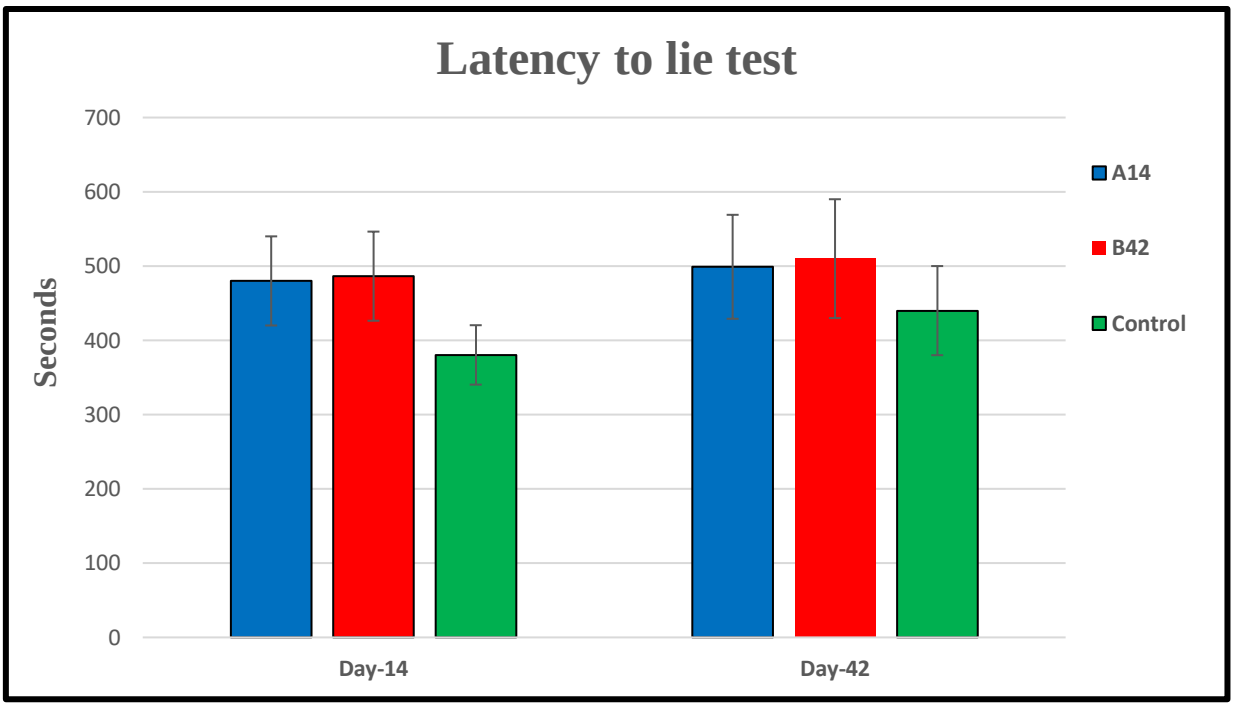

Figure 5.5. Effects of cohousing with day 14 and day 42 broilers on broiler's ability to stand in warm water (latency to lie test) at day-14 and day-42 of age. The cages of day-old-chicks received 100 g of fecal material/day and one bird per cage, cohoused for one week, from donor birds of ages 14 (A14) and 42 (B42) while control chicks (Control) neither received fecal material nor cohoused with birds. The bars represent mean values +/- SEM (n=8).

5.4.3 Cohousing mediated fecal microbiota transfer modulates cecal T cell response

In order to investigate how the cohoused mediated transfer of fecal microbiota from birds of different ages affect the intestinal immune regulation through subsets of cecal T cells, the response of $CD4^+$, $CD8^+$ and $CD4^+CD25^+$ T cells to treatments were evaluated at days 14 and 42 of broiler life. The number of $CD4^+$ cells was not statistically different among treatment groups at day 14 but later at day 42 the percentage of CD4 positive cells was substantially low in the B42 group in comparison with A14 and Control groups (Figure 5.3A). Similarly, $CD4^+CD25^+$ T cells were not significantly different among treatments at day 14 but at day 42 a significant increase in the B42 group was observed in comparison with A14 and Control groups (Figure 5.3C). Further,

the percentages of $CD8^+$ T cells were not different among treatment groups both at days 14 and 42 of chicken life (Figure 5.3B). These results suggested that cohoused mediated transfer of fecal microbiota can impact intestinal T cell responses and the B42 group has potentially shifted the immune regulation toward anti-inflammatory side in later weeks of broiler life.

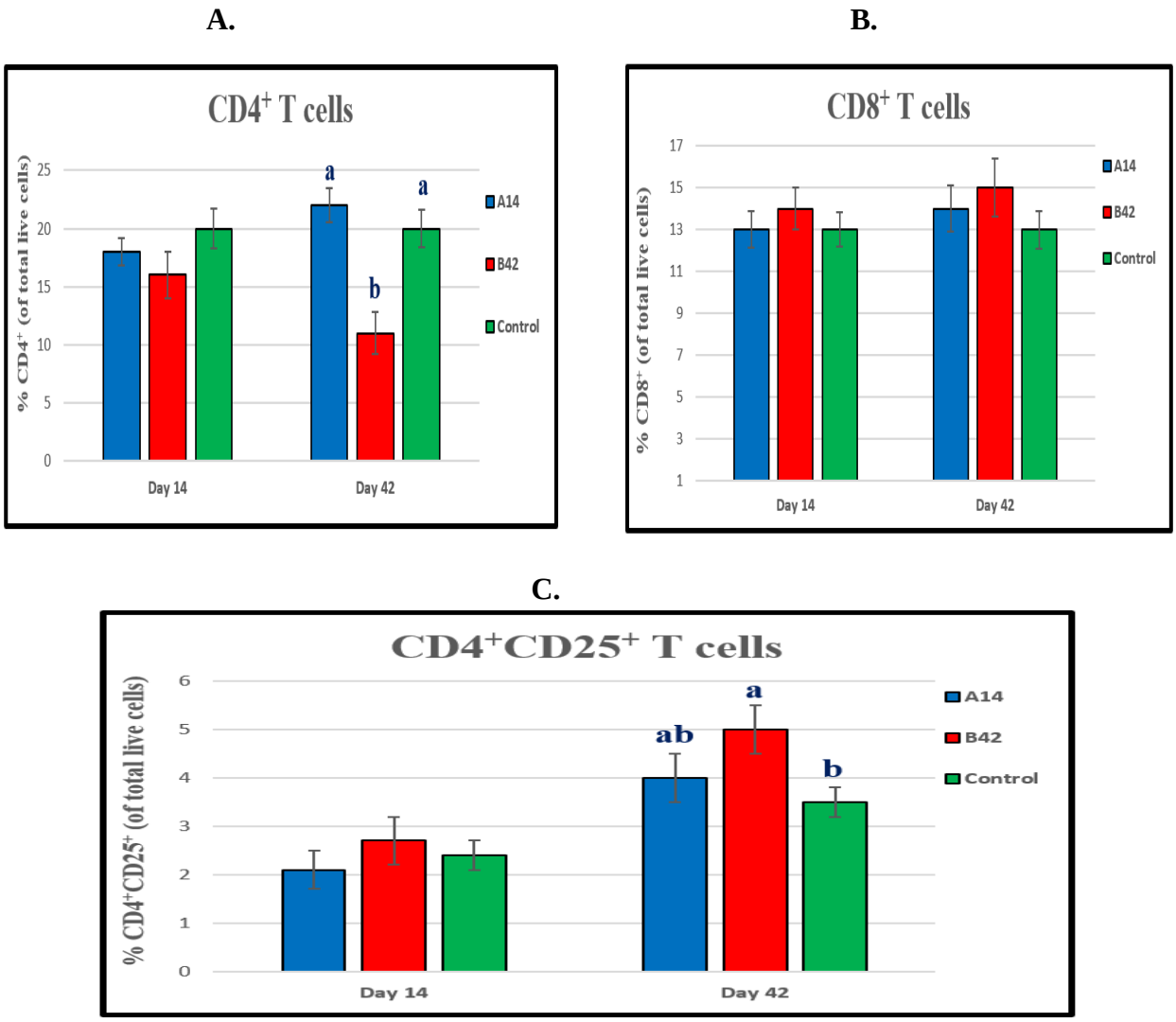

Figure 5.6. Effects of cohousing with day 14 and day 42 broilers on cecal tonsil's $CD4^+$ (A), $CD8^+$ (B) and $CD4^+CD25^+$ (C) T cells of broiler chickens raised for 42 days. The cages of day-old-chicks received 100 g of fecal material/day and one bird per cage, cohoused for one week, from donor birds of ages 14 (A14) and 42 (B42) while control chicks (Control) neither received fecal material nor cohoused with birds. ^{ab}Different letters mean significant differences among groups ($P<0.05$, Duncan test) at days 14 and 42 and bars represent mean +/- SEM (n=8).

5.5 Discussion

Broilers are among the most efficient feed converting livestock in the world, thanks to the genetic selection of broilers over the past 60 years. However, the selection programs have made broilers more susceptible to leg abnormalities that lead to welfare and economic issues. Various infectious and non-infectious factors contribute to lameness in broiler chickens. The lame birds face a lot of pain, less access to feed and water, reduced mobility and ultimately mortality or culling from the flock (Kieronczyk et al., 2017). Different interventions to improve bird's leg health have been carried out, including environmental enrichment (Phibbs et al., 2021), genetic selection (Hartcher and Lum., 2020), improving nutrition (Waldenstedt, 2006), exposure to light (Hassanzadeh et al., 2019), stocking density and use of antibiotics (Knowles et al., 2008). Here, we have adopted a new approach, transferring fecal microbiota from day 14 (A14) and day 42 (B42) chickens to young day-old-chicks by cohousing, to improve bone health of broiler birds. Previously, many studies supported the hypothesis that a healthy gut microbiota is linked with good performance of different host systems including the skeleton system (Cooney et al., 2021). Thus, we have evaluated the effect of cohousing on production, bone health and host immunity.

In this study, cohousing improved bone health but did not compromise the production parameters like BW, FI and FCR and mortality of broiler chickens. Previously, several interventions were used to reduce growth rate in order to improve leg and metabolic conditions, such as adopting extended dark periods and using slow growing birds. Knowles et al (2008) reported a 0.079 improvement in flock gait score for every 1 hour increase in the daily period of darkness across the range of 0 to 8.5 hours. Similarly, Wilhelmsson. (2019) demonstrated that the slower-growing Rowan Range chickens had a significantly better ability to walk and a lower percentage of culls than a fast-growing commercial Ross strain. However, a method to

compromise on the growth rate for better bone health is not going to be adopted by the industry. Other studies also used environmental enrichment as a tool to improve leg health, with inconsistent results. Yildirim and Taskin. (2017) described that access to perches has improved gait scores and duration of latency to lie in meat chickens. However other studies have reported negative physiological consequences of perches including decreased levels of bone mineralisation (Nielsen 2004; Karaarslan and Nazlıgül 2018).

Both treatment groups in this study, A14 and B42, showed improved bone health in terms of increase in tibial bone length, cortical bone area and cortical bone mineral contents and decrease in pore volume and diameter. In a recent study, fecal microbiota transplant from melatonin treated mice were used to treat osteolysis, an inflammatory degenerative bone condition, with very good results (Wu et al., 2021a). The authors attributed the beneficial effects of the fecal microbiota transplant to elevated relative abundance of some short chain fatty acid (SCFA) producing bacteria in melatonin-treated mice (Wu et al., 2021a). Our study results are also in agreement with results of other interventions, like probiotics, that were used for improvement of bone health. Mohammed et al. (2021) and Ciurescu et al. (2020) used *Bacillus subtilis* based probiotics and reported improvement in tibial bone traits like tibial length, weight, strength and bone phosphorus concentration and longer standing of broiler birds in latency-to-lie test in comparison with control broiler chickens. We also observed that effect of cohousing mediated fecal microbiota transfer on bone mineral content and pore volume, which was seen earlier in the B42 group at day 14 compared to the A14 group. These effects may be due to age and composition of fecal microbiota of donor birds as described by Ocejo et al. (2019). These authors reported that broiler birds at days 14 and 42 possess two different sets of intestinal microbiota. Similar observations were previously reported by our laboratory that gut microbiota at days 14 and 42 responded differently to probiotics

and consequently had different effects on host systems at days 14 and 42 (Bilal et al., 2021a and Bilal et al., 2021b).

Different mechanisms have been proposed in literature regarding modulation of bone health through gut microbiota. These mechanisms include help in nutrients acquisition, changes in hormones, stimulation of neurotransmitters and modulation of the immune system (Ibánez et al., 2019). Here, we investigated the response of subsets of T cell ($CD4^+$, $CD8^+$ and $CD4^+CD25^+$) to cohousing mediated transfer of fecal microbiota. In chickens, $CD4^+CD25^+$ T cells have been reported as regulatory cells (Shanmugasundaram and Selvaraj, 2011) with a main role in anti-inflammatory response and are associated with changes in gut microbiota (Lee et al., 2018). In this study, cohousing mediated transfer of fecal microbiota from mature birds (B42) significantly increased the number of $CD4^+CD25^+$ T cells in cecal tonsils at day 42 while $CD4^+$ T cells were significantly lower in the same group, reflecting a shift of balance of immune homeostasis toward the anti-inflammatory arm. The number of $CD4^+CD25^+$ cells in A14 group were also higher than the Control group, though statistically insignificant, reflecting a delayed developing response in this group. It is proven that gut microbiota synthesizes anti-inflammatory factors that helps in reducing local and systemic inflammatory responses (Wenjie et al., 2019). T regulatory cells play a main role in maintenance of anti-inflammatory state and are considered as a promotor of bone formation through inhibiting differentiation of osteoclasts that are required for bone resorption (Zhu et al., 2020). Metzler-Zebeli et al. (2019) also reported enhanced cecal expression of the anti-inflammatory cytokine IL10 in low residual feed intake chickens in response to fecal microbiota transplant. Similarly, Wu et al. (2021b) used fecal microbiota transplant from polyphenol-dosed mice to induce anti-inflammatory effects and alleviate the colitis condition. Round and Mazmanian. (2010) observed that *Bacteroides fragilis* as a gut commensal played a role in

adaptation of $CD4^+$ T cells to become T regulatory cells that produce IL-10. Further, Dar et al. (2018) used *Bacillus clausii* in mice with postmenopausal osteoporosis to increase the levels of anti-inflammatory cytokines and thereby enhanced bone health. Thus, these studies reflect that different types of interventions including fecal microbiota transfer can skew host immune homeostasis toward the anti-inflammatory arm, which may act as one of key mechanisms to stimulate osteoblast activity and inhibit osteoclast cells to improve bone health.

5.6 Conclusion

In summary, this study documented the effects of cohousing mediated fecal microbiota transfer from days 14 and 42 donor broiler birds on growth performance, tibial bone health and intestinal immunity of broiler chickens at different stages of life. The interventions conferred bone health benefits and modulated intestinal T cells immunity. These effects were donor age dependent and were prominent and earlier in the B42 group.

5.7 References

Amin N, Boccardi V, Taghizadeh M & Jafarnejad S (2020) Probiotics and bone disorders: The role of RANKL/RANK/OPG pathway. *Aging Clinical and Experimental Research*. **32:** 363–371.

Ballou AL, Ali RA, Mendoza MA, Ellis JC, Hassan HM & Croom WJ (2016) Development of the chick microbiome: how early exposure influences future microbial diversity. *Frontier in Veterinary Science*. **3**:2.

Berg C. & Sanotra G (2003) Can a modified latency-to-lie test be used to validate gait-scoring results in commercial broiler flocks? *Animal Welfare* **12:** 655-659.

Bilal M, Si W, Barbe F, Chevaux E, Sienkiewicz O & Zhao X (2021a) Effects of novel probiotic strains of *Bacillus pumilus* and *Bacillus subtilis* on production, gut health and immunity of broiler chickens raised under sub-optimal conditions. *Poultry Science*. **100 (3)**: 100871.

Bilal M, Achard C, Barbe F, Chevaux E, Ronholm J & Zhao X (2021b) *Bacillus pumilus* and *Bacillus subtilis* promote early maturation of cecal microbiota in broiler chickens. *Microorganisms*. **9:** 1899.

Borda-Molina D, Seifert J, & Camarinha-Silva A (2018) Current perspectives of the chicken gastrointestinal tract and its microbiome. *Computational and Structural Biotechnology Journal*. **16**. 131–139.

Cammarota G, Ianiro G, & Gasbarrini A (2014) Fecal microbiota transplantation for the treatment of *Clostridium difficile* infection: a systematic review. *Journal of Clinical Gastroenterology*. **48**: 693–702.

Cheng H, Guan X, Chen D & Ma W (2019) The Th17/Treg Cell Balance: A gut microbiota-modulated story. *Microorganisms*. **7:** 583.

Ciurescu G, Dumitru M, Gheorghe A, Untea AE & Draghici R (2020) Effect of *Bacillus subtilis* on growth performance, bone mineralization, and bacterial population of broilers fed with different protein sources. *Poultry Science*. **99:** 5960–5971.

Cooney OD, Nagareddy PR, Murphy AJ & Lee MKS (2021) Healthy gut, healthy bones: targeting the gut microbiome to promote bone health. *Frontier in Endocrinology*. **11**: 620466.

CTAn user's guide (2021) https://docplayer.net/38407-The-user-s-guide-ct-analyser-version-1-13-contents.html. Last accessed September 16, 2021.

Dar HY, Pal S, Shukla P, Mishra PK, Tomar GB & Chattopadhyay N (2018) *Bacillus clausii* inhibits bone loss by skewing Treg-Th17 cell equilibrium in postmenopausal osteoporotic mice model. *Nutrition*. **54:**118–28.

Gadde U, Kim WH, Oh ST & Lillehoj HS (2017) Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: A review. *Animal Health Research*. **18**: 26–45.

Granquist EG, Vasdal G,de Jong IC & Moe RO (2019) Lameness and its relationship with health and production measures in broiler chickens. *Animal*. **13:** 2365–2372.

Haase S, Haghikia A, Wilck N, Muller DN & Linker RA (2018) Impacts of microbiome metabolites on immune regulation and autoimmunity. *Immunology*. **154**: 230–8.

Hartcher KM & Lum HK (2020) Genetic selection of broilers and welfare consequences: a review. *World's Poultry Science Journal*. **76:** 154-167.

Hassanzadeh M, Decuypere E, Lamberigts C & Buyse J (2019) Light as exogenous factor can control metabolic disorders and impact embryonic development in broiler chickens- a review. *European Poultry Science*. **83:** 2019.

Ibanez L, Rouleau M, Wakkacha A & Blin-Wakkach C (2019) Gut microbiome and bone. *Joint Bone Spine*. **86**: 43–47.

Jiang S, Yan FF, Hu JY, Mohammed A, & Cheng HW (2021) *Bacillus subtilis*-based probiotic improves skeletal health and immunity in broiler chickens exposed to heat stress. *Animals*. **11**: 1494.

Karaarslan S & Nazlıgül A (2018) Effects of lighting, stocking density, and access to perches on leg health variables as welfare indicators in broiler chickens. *Livestock Science*. **218:** 31–36.

Kierończyk B, Mateusz R, Damian J & Sylwester S (2017) "Infectious and non-infectious factors associated with leg disorders in poultry -a review". *Annals of Animal Science*. **17:** 645-669.

Kittelsen KE, David B, Moe RO, Poulsen HD, Young JF & Granquist EG (2017) Associations among gait score, production data, abattoir registrations,and postmortem tibia measurements in broiler chickens. *Poultry Science.* **6:** 1033–1040.

Knowles TG, Kestin SC, Haslam SM, Brown SN, Green LE, Butterworth A, Pope SJ, Pfeiffer D & Nicol CJ (2008) Leg disorders in broiler chickens: prevalence, risk factors and prevention. *Public Library of Science One*. **3:** e1545.

Lee IK, Gu MJ, Ko KH, Bae S, Kim G, Jin G, Kim EB, Kong Y, Park TS, Park B, Jung HJ, Han SH & Yun C (2018) Regulation of CD4+CD8−CD25+and CD4+CD8+CD25+ T cells by gut microbiota in chicken. *Scientific Report*. **8:**8627.

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.

Metzler-Zebeli BU, Siegerstetter SC, Magowan E, Lawlor PG, O'Connell NE & Zebeli Q (2019) Fecal microbiota transplant from highly feed efficient donors affects cecal physiology and microbiota in low and high-feed efficient chickens. *Frontier in Microbiology*. **10**:1576.

Mohammed AA, Zaki RS, Negm EA, Mahmoud MA & Cheng HW (2021) Effects of dietary supplementation of a probiotic (*Bacillus* subtilis) on bone mass and meat quality of broiler chickens. *Poultry Science*. **2:** 100.

National Research Council (1994) Nutrient requirements of poultry. 9th ed. National Academy 451 Press, Washington, D.C., USA.

Nielsen BL (2004) Breast blisters in groups of slow-growing broilers in relation to strain and the availability and use of perches. *British Poultry Science*. **45:** 306–315.

Ocejo M, Oporto B, & Hurtado A (2019) 16S rRNA amplicon sequencing characterization of caecal microbiome composition of broilers and free-range slow growing chickens throughout their productive lifespan. *Scientific Reports*. **9**:2506.

Phibbs DV, Groves PJ, & Muir WI (2021) Leg health of meat chickens: impact on welfare, consumer behaviour, and the role of environmental enrichment. *Animal Production Science*. **61:** 1203–1212.

Round JL & Mazmanian SK (2010) Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proceedings of National Academy of Sciences* USA. **107:** 12204–12209.

Schwarzer M, Makki K & Storelli G (2016) *Lactobacillus plantarum* strain maintains growth of infant mice during chronic undernutrition. *Science*. **351**: 854–7.

Sgambato D, Gimigliano F, De Musis C, Moretti A, Toro G, Ferrante E, Miranda A, De Mauro D, Romano L & Iolascon G (2019) Bone alterations in inflammatory bowel diseases. *World Journal of Clinical Cases*. **7:** 1908–1925.

Shanmugasundaram R & Selvaraj RK (2011) Regulatory T cell properties of chicken CD4+CD25+ cells. *Journal of Immunology*. **186:** 1997-2002.

Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R & Shi H (2014) Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity*. **40**: 128–39.

Thomas M, Wongkuna S, Ghimire S, Kumar R, Antony L, Doerner KC, Singery A, Nelson E, Woyengo T & Chankhamhaengdecha, S (2019) Gut microbial dynamics during conventionalization of germfree chicken. *mSphere*. **4**: e00035-19.

Waldenstedt L (2006) Nutritional factors of importance for optimal leg health in broilers: A review. *Animal Feed Science and Technology*. **126:** 291–307.

Wargo JA (2020) Modulating gut microbes. Fecal microbiota transplant and modulation of microbial species show therapeutic promise. *Science.* **369**: 6509.

Wenjie Z, Jie S, Bo T, Peng L, Xu H, Nasser MI, Zhuang Q & Zhao M (2019) Cutting Edge: probiotics and fecal microbiota transplantation in Immunomodulation. *Journal of Immunology Research*. **2:** 1603758.

Wilhelmsson S, Yngvesson J, Jönsson L, Gunnarsson S & Wallenbeck A (2019) "Welfare quality® assessment of a fast-growing and a slower-growing broiler hybrid, reared until 10 weeks and fed a low-protein, high-protein or mussel-meal diet". *Livestock Science* **219:** 71-79.

Wu Y, He F & Zhang C (2021a) Melatonin alleviates titanium nanoparticles induced osteolysis via activation of butyrate/GPR109A signaling pathway. *Journal of Nanobiotechnology*. **19:** 170.

Wu Z, Huang S, & Li T (2021b). Gut microbiota from green tea polyphenol-dosed mice improves intestinal epithelial homeostasis and ameliorates experimental colitis. *Microbiome*. **9:** 184.

Yan J, Herzog JW & Tsang K (2016) Gut microbiota induce IGF-1 and promote bone formation and growth. *Proceedings of the National Academy of Sciences*. **113**: E7554–63.

Yildirim M & Taskin A (2017) The effects of environmental enrichment on some physiological and behavioural parameters of broiler chicks. *Brazilian Journal of Poultry Science*. **19:** 355–362.

Zhu L, Hua F, Ding W, Ding K, Zhang Y & Xu C (2020) The correlation between the Th17/Treg cell balance and bone health. *Immunity & Ageing*. **17**:30.

Chapter 6. General Discussion and Conclusions

Sub-therapeutic levels of antibiotics as growth promoters (AGP) have been used in chicken's feed for the purpose of disease prevention and growth promotion. Meanwhile, antibiotic resistance started to rise. Due to risk of spreading resistance genes to human pathogens, antibiotic use as growth promoters in animal agriculture was either put under much scrutiny or stopped in different countries. In the post AGP era, negative consequences like decrease in performance and surge in diseases have been seen in chickens. To alleviate these negative effects of antibiotic removal in poultry industry, workable and cost-effective replacements are needed. Among alternatives, use of probiotics is a relatively old approach, while transfer of fecal microbiota is an emerging approach for poultry research. Although probiotics are much researched but inconsistencies in their outcomes in terms of production and health parameters have called for expansion of the search net for novel probiotics strains. On the other hand, fecal microbiota transfer which has received good results in human medicine has not been extensively employed in poultry and has great potential in poultry production. Thus, I evaluated effects of two novel *Bacillus* probiotic strains, *B. pumilus* and *B. subtilis*, and transfer of fecal microbiota through cohousing on production, gut microbiota and health parameters of broiler chickens in this thesis.

In our first study, we evaluated the effects of *B. pumilus* and *B. subtilis* strains on growth performance, intestinal health and immunity of broiler chickens. It has been observed that under the influence of the *Bacillus* probiotics, the gut health and immunity significantly improved earlier at day 14 and such effects became insignificant to the control group at day 42, depending on strain and dosage of *Bacillus* probiotics. The significant improvement in production parameter observed in later weeks of life may be linked with availability of additional energy sources that were previously utilized in fast development of gut, immune and other health related factors in early

weeks of life. The same observations were also reported by Jacquier et al. (2019), who reported improvement in production parameters in later weeks of trial. In short, *B. pumilus* and *B. subtilis* showed beneficial effects on production and health of broiler chickens.

Both *Bacillus* probiotic strains efficiently stimulated gut immune system and a significant increase in pro-inflammatory cytokines, gamma interferon and IL17, indicative of immune preparedness for pathogenic incursions and immune maturity. The role of IL-17 is important in stimulation of tight junction and mucin proteins that bolster gut integrity and function (Weaver et al., 2013). However, exaggerated inflammation is not helpful and counter-productive. In this study, anti-inflammatory responses were also activated and the main cytokine IL-10 was released to keep a check over activated pro-inflammatory effects and bring back immune homeostasis to normal. In addition, CD4+CD25+ T regulatory cells were observed more efficient in responses to probiotics compared with CD4+CD8+CD25+ cells, suggesting that double positive cells are the main regulatory cells than the triple positive cells in broiler chickens. These results indicate that both *Bacillus* probiotic strains activated pro- as well as anti-inflammatory arms of gut immunity and thus kept balance in gut immune homeostasis.

We observed significant changes in different genes related with intestinal integrity and function. All genes like occluding, ZO1 and JAM-2 related with tight junction were over-expressed and they confer gut integrity and regulate paracellular transportation. Similarly, the mucin production that play an important role in defences against microbial invasion was significantly higher in response to probiotics. These results reflect that both probiotic strains improved the gut integrity and function and bolstered the host defences against potential microbial incursions.

In our second study, we evaluated the effects of *B. pumilus* and *B. subtilis* on cecal microbiota of broiler chickens at different time points (Days 7, 14, 28 and 42). In agreement with our earlier study, effects of both probiotics were prominent at day 14 and became levelled by day 42. The alpha diversity of cecal microbiota was improved in response to Bacillus probiotics in early weeks of chickens while a significant decrease in alpha diversity was observed in response to the antibiotics. The high gut microbial diversity is an indicator of good gut health and resistance to endogenous and exogenous changes. Our probiotics prevented an age associated drop in alpha diversity (Hartog et al., 2016). It was also observed that significant changes in composition of cecal microbiota in response to probiotics started to appear around day 7 but prominent differences in composition were observed at the second week of life but the differences disappeared later.

During development of microbiota, the members of family Lachnospiraceae are seen in higher abundance in early life of broiler chickens while members of family Ruminococcaceae are observed in higher abundance in mature microbiota in later weeks of life (Ocejo et al., 2019). However, in our study, probiotics significantly increased the abundance of family Ruminococcaceae and reduced the abundance of Lachnospiraceae in early weeks of chicken's life, suggesting that *Bacillus* probiotics speeded up the development of microbiota and helped chickens to achieve mature microbial composition early in life. These results indicate that *Bacillus* probiotics may be helpful to promote early acquirement of mature and resilient microbial configuration when the birds are most susceptible to diseases. Further, it was observed that abundance of family Enterobacteriaceae, having many potential antibiotic resistant pathogens, were significantly lower in the probiotic groups, reflecting protective role of *Bacillus* probiotics against pathogenic microbiota.

In light of our previous observations that at days 14 and 42 of broiler chicken's life, the intestine has two separate configurations of microbiota, based on their composition and maturity, and their distinct effects on the host systems at different stages of life, we transferred fecal microbiota from days 14 and 42 donor broiler birds through cohousing to day old chickens and evaluated the effects of intervention on production, immunity and bone health of broiler birds at days 14 and 42 of life cycle. The intervention showed significant effects on gut immunity and bone health of broiler birds. These effects were seen earlier in chickens that received microbiota from mature donor birds, which may be due to early maturation of microbial configuration and consequent improvement in host's immune and skeletal system responses. Here, we observed that transfer of fecal microbiota improved overall tibial bone length and cortical bone mineral content and pore volume, which are indicative of stronger and resilient bones that may be helpful to avoid leg deformities and improve welfare of broiler chickens. Further, the gut immune homeostasis was skewed toward the anti-inflammatory arm in terms of significant increase in T regulatory (CD4+CD25+) cells and decrease in CD4+ cells number, especially in recipient birds that received fecal microbiota from 42 days old birds. The anti-inflammatory response may be a contributory factor that aided in bone health (Ibánez et al., 2019). These results may be helpful to explain the beneficial effects of re-used litters in poultry setups that prefer to use single litter for multiple production cycles.

6.1 Future direction

Intestinal microbiota plays important roles in bird's productions and health, and it is the target of many interventions to induce beneficial effects. However, there are other contributory factors like host and hatchery that need to be exploited in future studies. Intestinal microbiota of different broiler breeds present different composition of microbiota (Kers et al., 2018) during a

life cycle, which reflect their genetic influence over development of microbiota and thus different interaction and responses to probiotic strains and consequent distinct health and production outcome to the host. The probiotic strains should be evaluated separately for each different chicken commercial breed to have comprehensive understanding of the different host responses to probiotics. Moreover, in future this may be of interest to investigate whether the probiotics added in a starter feed would be enough to have beneficial effects in broiler chickens or probiotics have to be supplied during a full life cycle.

The gut microbiota in chickens matures with passage of time and become resilient to different challenges. The mature microbial configuration, consisting of beneficial microbiota like obligate anaerobes, competitively exclude facultative anaerobes like members of Enterobacteriaceae and thus promote health and prevent diseases. Similarly, the maturity of intestinal epithelial cells set up physiological hypoxia at mucosal surface that favors beneficial microbiota and inhibits facultative anaerobes including enteric pathogens (Lopez et al., 2016). In future studies, speeding up maturity of microbiota as well as gut epithelial lining will be lucrative targets for interventions to achieve growth and health goals and prevent diseases.

Transfer of fecal microbiota has shown potential health benefits to the host. It would be an elegant approach to transfer microbiota from probiotic treated birds to non-probiotic treated birds. Further, as we have observed improvement in bone health of broiler birds in event of exposure to fecal microbiota, how to manipulate microbiota in layer birds in order to reduce osteoporosis and improve eggshell quality could be a very interesting research topic. Considering the fact that a mixture of bacteria may be more efficient than a single bacterium, we will aim to identify a refined form of fecal microbiota, which could be used for health and production benefits in the future.

6.2 References

Hartog DG, Vries-Reilingh GD, Wehrmaker AM & Savelkoul HFJ (2016) Parmentier, H.K. Lammers A. Intestinal immune maturation is accompanied by temporal changes in the composition of the microbiota. *Beneficial Microbes*. 7: 677-685.

Ibanez L, Rouleau M, Wakkacha A & Blin-Wakkach C (2019) Gut microbiome and bone. *Joint Bone Spine*. **86:** 43–47.

Jacquier V, Nelson A, Jlali M, Rhayat L, Brinch KS & Devillard E (2019) *Bacillus subtilis* 29784 induces a shift in broiler gut microbiome toward butyrate producing bacteria and improves intestinal histomorphology and animal performance. *Poultry Science*. **98:** 2548–2554.

Kers JG, Velkers FC, Fischer EAJ, Hermes GDA, Stegeman JA, & Smidt H (2018) Host and environmental factors affecting the intestinal microbiota in chickens. *Frontiers in Microbiol*. **9:** 235.

Lopez CA, Miller BM, Rivera-Chávez F, Velazquez E, Byndloss MX, Chávez-Arroyo A, Lokken KL, Tsolis RM, Winter SE, Bäumler AJ (2016) Virulence factors enhance *Citrobacter rodentium* expansion through aerobic respiration. *Science*. **353:** 1249–1253.

Ocejo M, Oporto B, & Hurtado A (2019) 16S rRNA amplicon sequencing characterization of caecal microbiome composition of broilers and free-range slow growing chickens throughout their productive lifespan. *Scientific Reports*. **9:** 2506.

Weaver CT, Elson CO, Fouser LA & Kolls JK (2013) The Th17 pathway and inflammatory diseases of the intestines, lungs, and skin. *Annual Review of Pathology*. **8:** 477-512.