EFFECTS OF FRUCTOOLIGOSACCHARIDE ALONE OR IN COMBINATION WITH PHYTASE ON GROWTH PERFORMANCE, NUTRIENT UTILIZATION, IMMUNE RESPONSE AND GUT DEVELOPMENT OF BROILER CHICKENS

By

Yue Shang

A Thesis

Submitted to the Faculty of Graduate Studies of

University of Manitoba

In Partial Fulfillment of the Requirement

For the Degree of

MASTER OF SCIENCE

Department of Animal Science

University of Manitoba

Winnipeg, Manitoba

Canada. R3T 2N2

ABSTRACT

Two experiments were conducted to evaluate the effects of supplementing fructooligosaccharides (FOS) in broiler rations. In experiment 1, the effect of dietary FOS supplementation alone or in combination with phytase on growth performance, bone quality, and phosphorus utilization was evaluated in broiler chickens fed standard or low calcium (Ca) and phosphorus (P) diets. The phytase supplementation in low Ca and P diets improved growth performance, bone quality and P utilization. The combination of phytase and FOS increased BW gain and P retention. However, the application of dietary FOS alone had a negative effect on broiler bone quality. The second experiment investigated the effect of dietary FOS supplementation as an alternative to antibiotics on growth performance, immune response, intestinal morphology and ileal microbiota with or without *Salmonella* Enteritidis lipopolysaccharide (LPS) challenge in broiler chickens. The results showed that feed intake, BW gain, feed conversion ratio, mortality and relative lymphoid organ weight did not exhibit significant difference ($P > 0.05$) among the treatments. Villus height, crypt depth and total mucosal thickness were significantly increased ($P < 0.05$) in the ileum of broiler chickens that supplemented FOS. No significant difference on α-, ß- diversity and bacterial phyla in ileal microbiota was observed between mucosa and digesta or between the three dietary treatments. However, partial least square discriminant analysis and Venn analysis showed that unique bacterial genera were associated with different ileal sites or diets. The immunological challenge demonstrated significant difference on relative heterophils and lymphocytes concentrations, and on serum immunoglobulin (Ig) G and IgA levels. Diet × challenge interaction was observed in IgG measurements ($P < 0.001$). Natural IgG and IgA, and specific IgG levels were elevated in FOS supplemented group under the LPS challenge. Supplemental FOS has also up-regulated ileal IL-1ß, -2, -10, -18, TLR-4, IFN-γ and splenic IL-18, IL-1ß expressions ($P < 0.05$), and these

gene exhibited immunological challenge effects upone LPS challenge ($P < 0.05$). These results indicate that fructooligosaccharide may play a protective role on gut development and immunity of broiler chickens.

DEDICATION

I dedicate this thesis to my parents: Lian Shang and Hui Zhu Jiang for their forever love and unlimited support during my entire life. The thesis is also dedicated to my homestay landlady Jessica Holbrow and her beloved family, without meeting them in Winnipeg Manitoba, I would have not been able to archieve this far.

ACKNOWLEDGEMENTS

I am extremely grateful and indebted to many people who contributed in various ways for the successful completion of this thesis. First, my sincere appreciation goes to my supervisor, Dr. Woo Kyun Kim, whose enthusiasm, motivation and financial support enabled me to accomplish a comprehensive M. Sc. research program using both classical and cutting-edge technologies. His guidance and encouragement assisted me to become an independent researcher. It is also a great honor to have Dr. Bogdan Slominski as my co-supervisor, whose knowledge, advice and support has added tremendous value to my program. I am very grateful to Dr. Ehsan Khafipour for the knowledge, resources and expertise he shared with me, and for the help, training and advice he provided as being my committee member. I would like to extend my gratitude to Dr. Peter Eck for serving on my advisory committee, and thanks to Drs. Gary Crow and Loreen Onischuk for statistical analysis advices.

Special thanks go to all of my colleagues in the Department of Animal Science for their encouragement, friendship and support throughout the years. I am very thankful to Dr. Anna Rogiewicz for her professional help and guidance during my poultry trials. Thanks to Dr. Alemu Hunde, Mohammed Alizadeh and Hooman Derakhshani for their ideas and laboratory training. Many thanks to Bishwo Bandhu Pokharel and Dorothy Moseti for being my lab partners and offering help during my trials. I greatly appreciate the assistance received from Harry Muc, Thomas Davie, Navjot Nandha, Attanas Karamanov, Shusheng Zhao, Karen Lim, Prakash Sharma and support staffs of Poultry Research Unit and T.K. Cheung Centre, as well as all the administrative staffs and fellow graduate students of the animal science department.

I would like to acknowledge Natural Sciences and Engineering Research Council of Canada, Ingredion Inc., and Canadian Bio-Systems Inc. for financial support of the present research, and also the University of Manitoba for providing University of Manitoba Graduate Student Travel Awards, Graduate Studies Special Awards and Miriam Green Ellis Bursaries. I also appreciate several short-term jobs offered by individuals, the Department of Animal Science and the Faculty of Agricultural and Food Sciences.

Finally and most importantly, I would like to express my deep gratitude to my family and friends. Special thanks to my parents Lian Shang and Hui Zhu Jiang, for their selfless love and ultimate support on my well-being and career development. All the happiness and difficulties we have been through deepened my understanding of life. I would like to thank Jessica Holbrow and her family for providing me a warm and lovely place to stay in Winnipeg since my first arrival. Ms. Jessica Holbrow is not only my homestay landlady, but rather a mentor for my life. I would also like to thank many friends that I have met in Canada, especially Asagi Funahashi and Zakia Akhter, for their long-term friendship, encouragement and spiritual support. To them I dedicate this thesis.

FOREWORD

This thesis was prepared in a manuscript format and is composed of two manuscripts. Manuscript I was presented at the 11th World Conference on Animal Production, Beijing, China (October, 2013) and it was formatted to meet the guidelines of Poultry Science. Manuscript II was partially presented at the Poultry Science Annual Meeting in Corpus Christi, TX (July, 2014). Manuscript II was formatted to meet the guidelines of Poultry Science.

TABLE OF CONTENTS

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGEMENTS v

FOREWORD vii

TABLE OF CONTENTS viii

LIST OF TABLES xi

LIST OF FIGURES xiii

LIST OF ABBREVIATIONS xvi

1. GENERAL INTRODUCTION 1

2. LITERATURE REVIEW 6

2.1 Prebiotics 6

2.1.1 An overview of prebiotics 6

2.2 Fructooligosaccharide (FOS) 7

2.2.1 An overview of FOS 7

2.2.2 The applications of FOS supplementation in broiler chickens 8

2.3 Phytase 11

2.3.1 An overview of phytase 11

2.3.2 Effects of phytase on phosphrous utilization and bone mineralization in broiler chickens……..12

2.4 An overview of chicken gut and gastrointestinal microbiology 14

2.4.1 Chicken gut and its function 14

2.4.2 The role of chicken gastrointestinal microbiota 16

2.4.3 The diversity of chicken gut microbiota 20

2.4.4 Effects of dietary prebiotics on gut microbiota 22

2.4.5 Discovery of chicken gut microbiome by molecular approaches 24

2.5 An overview of chicken immunology 31

2.5.1 Chicken immune system 31

2.5.2 Chicken gut immunity 34

2.6 Antibiotic growth promoters and antibiotic alternatives in poultry industry 35

2.6.1 Antibiotic growth promoters (AGPs) 35

2.6.2 Antibiotic alternatives 37

2.7 Summary 40

3. MANUSCRIPT I 42

The effect of phytase and fructooligosaccharide supplementation on growth performance, bone quality and phosphorus utilization in broiler chickens 42

3.1 Abstract 43

3.2 Introduction 44

3.3 Materials and methods 46

3.4 Results 53

3.5 Discussion 62

3.6 Acknowledgements 68

4. MANUSCRIPT II 69

The effect of dietary fructooligosaccharide supplementation on growth performance, immune response, intestinal morphology and ileal microbiota with or without *Salmonella* Enteritidis lipopolysaccharide challenge in broiler chickens 69

4.1 Abstract 70

4.2 Introduction 71

4.3 Materials and methods 73

4.4 Results 86

4.5 Discussion 133

4.6 Acknowledgements 148

5. GENERAL DISCUSSION 149

6. CONCLUSIONS 151

7. FUTURE DIRECTIONS 153

8. REFERENCES 153

LIST OF TABLES

Table 1. 16S rRNA-based molecular approaches for studying microbial ecology 25

Table 2. The comparison of characteristics between the 454 GS Junior and Illumina MiSeq benchtop sequencing instruments 30

Table 3. Current methods for the replacement of antibiotic growth promoters in poultry diets .. 39

Table 4. Composition and analysis of experimental diets (as-fed basis) 49

Table 5. Effect of phytase and fructooligosaccharides (FOS) supplementation on growth performance of broiler chickens from 5 to 14 days of age 55

Table 6. Effect of phytase and fructooligosaccharides (FOS) supplementation on growth performance of broiler chickens during 15 to 21 days of age 56

Table 7. Effect of phytase and fructooligosaccharides (FOS) supplementation on growth performance of broiler chickens during the entire experimental period (5-21 days of age) 57

Table 8. Effect of phytase and fructooligosaccharides (FOS) supplementation on femur and tibia bone mineral density (BMD), bone mineral content (BMC) and bone area (BA) of broiler chickens 59

Table 9. Effect of phytase and fructooligosaccharides (FOS) supplementation on phosphorus utilization in broiler chickens at 21 d of age 61

Table 10. Composition of experimental diets (as-fed basis) 75

Table 11. Chicken cytokine primer sequences. 81

Table 12. Feed intake, body weight gain, feed conversion ratio (FCR), and mortality of broiler chickens fed experimental diets for 21 days 87

Table 13. Relative lymphoid organ weight of broiler chickens fed experimental diets at 21 days of age 88

Table 14. Effect of fructooligosaccharide (FOS) as an antibiotic alternative on the small intestine morphology of broiler chickens at 21 days of age 90

Table 15. Bacterial alpha diversity based on the main effects of diet and ileal site of broiler chickens at 21 days of age 92

Table 16. Relative abundance of bacterial phyla based on the main effects of diet and ileal site of broiler chickens at 21 days of age 99

Table 17. Relative abundance of Lachnospiraceae (f), S247 (f), *Helicobacter* and *Desulfovibrio* based on the main effects of diet and ileal site of broiler chickens at 21 days of age 100

Table 18. Main effects of diet and challenge on relative percentage of white blood cell differential of broiler chickens at 21 days of age 118

LIST OF FIGURES

Figure 1. The structure and enzymatic preparation of fructooligosaccharides 8

Figure 2. Measurement of villus height, crypt depth and total mucosal thickness of ileal segment of broiler chicken 16

Figure 3. The next generation sequencing technologies. A. the 454 Pyrosequencing technology, and b. the Illumina SOLEXA technology. 28

Figure 4. Rarefaction analysis on Chao1 estimates of species richness at an even sequencing depth of 18,585, in ileal mucosa samples of broiler chickens that fed PC, NC and NC + FOS diets 93

Figure 5. Rarefaction analysis on Chao1 estimates of species richness at an even sequencing depth of 20,820, in ileal digesta samples of broiler chickens that fed PC, NC and NC + FOS diets 94

Figure 6. Principal coordinates analysis (PcoA) of A) unweighted Unifrac and B) weighted Unifrac distance of ileal mucosa bacterial community between the chickens that fed PC, NC and NC + FOS diets 95

Figure 7. Principal coordinates analysis (PcoA) of A) unweighted Unifrac and B) weighted Unifrac distance of ileal mucosa bacterial community between the chickens that fed PC, NC and NC + FOS diets. 96

Figure 8. Venn diagram of shared and unique bacterial taxa in the ileal mucosa of broiler chickens fed PC, NC and NC + FOS diets. 102

Figure 9. Partial least square discriminant analysis (PLS-DA) score scatter plot of the ileal mucosa microbiota between broiler chickens that fed PC and NC + FOS diets 103

Figure 10. Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundance of bacterial taxa in the ileal mucosa of broiler chickens that fed PC or NC + FOS diets 104

Figure 11. Partial least square discriminant analysis (PLS-DA) score scatter plot of the ileal mucosa microbiota between broiler chickens that fed NC and NC + FOS diets.. 106

Figure 12. Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundance of bacterial taxa in the ileal mucosa of broiler chickens that fed NC and NC + FOS diets 107

Figure 13. Venn diagram of shared and unique bacterial taxa in ileal digesta of broiler chickens fed PC, NC and NC + FOS diet 110

Figure 14. Partial least square discriminant analysis (PLS-DA) score scatter plot of the ileal digesta microbiota between broiler chickens that fed PC and NC + FOS diets 111

Figure 15. Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundance of bacterial taxa in the ileal digesta of broiler chickens that fed PC and NC + FOS diets 112

Figure 16. Partial least square discriminant analysis (PLS-DA) score scatter plot of the ileal digesta microbiota between broiler chickens that fed NC and NC + FOS diets 114

Figure 17. Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundance of bacterial taxa in the ileal digesta of broiler chickens that fed NC and NC + FOS diets. 115

Figure 18. a) Natural serum immunoglobulin (Ig)G concentration (µg/ml) in broiler chickens that fed PC, NC, and NC + FOS diets under phosphate buffered saline (PBS) injection as control or *Salmonella* lipopolysaccharide (LPS) challenge conditions. B) Specific serum IgG level in

response to *salmonella* LPS (expressed as optical density) of broiler chickens fed PC, NC and NC + FOS diet under PBS injection as control or *Salmonella* LPS challenge conditions. 120

Figure 19. a) Natural serum immunoglobulin (Ig)A concentration (µg/ml) in broiler chickens that fed PC, NC, and NC + FOS diets under phosphate buffered saline (PBS) injection as control or Salmonella lipopolysaccharide (LPS) challenge conditions. B) Specific serum IgA level in response to salmonella LPS (expressed as optical density) of broiler chickens fed PC, NC and NC + FOS diet under PBS injection as control or Salmonella LPS challenge conditions. 123

Figure 20. Ileal gene expressions of a) toll-like receptor (TLR) -4, b) interleukin (IL) -1ß, c) IL-2, d) IL-6, e), IL-10, f) IL- 18, and g) interferon (IFN) –Ɣ of chickens fed PC, NC, and NC + FOS diets, under phosphate buffered saline (PBS) injection as control or *Salmonella* lipopolysaccharide (LPS) challenge conditions. 128

Figure 21. Splenic gene expressions of a) toll-like receptor (TLR) -4, b) interleukin (IL) -1ß, c) IL-2, d) IL-6, e), IL-10, f) IL- 18, and g) interferon (IFN) –Ɣ of chickens fed PC, NC, and NC + FOS diets, under phosphate buffered saline (PBS) injection as control or *Salmonella* lipopolysaccharide (LPS) challenge conditions 132

LIST OF ABBREVIATIONS

16S rRNA	16S ribosomal ribonucleic acid
AGPs	Antibiotic growth promoters
ANOVA	Analysis of variance
APD	Apparent P digestibility
APS	Adenosine 5’ phosphosulfate
ATP	Adenosine triphosphates
BA	Bone area
BMC	Bone mineral content
BMD	Bone mineral density
BWG	Body weight gain
Ca	Calcium
CCAC	Canadian Council on Animal Care
cDNA	Complementary deoxyribonucleic acid
cfu	Colony-forming unit
DEXA	Dural energy x-ray absorptiometry
DGGE	Denaturing gradient gel electrophoresis
DM	Dry matter
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
ELISA	Enzyme-linked immunosorbent assay

EU	European union
FCR	Feed conversion ratio
FDA	Food and drug administration
FI	Feed intake
FISH	Fluorescence in situ hybridization
FOS	Fructooligosaccharides
GALT	Gut-associated lymphoid tissue
Gb	Gigabyte
GI	Gastrointestinal
GLM	General linear models
GOS	Glucooligosaccharides
H: L	Heterophils to lymphocytes ratio
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
K_2EDTA	Potassium ethylenediaminetetra-acetic acid
LPS	Lipopolysaccharides
MAFRI	Manitoba Agriculture, Food and Rural Initiatives
Mb	Megabyte
MHC	Major histocompatibility complex
MOS	Mannooligosaccharides
NC	Negative control

NCBI	National Centre for Biotechnology Information
NGS	Next generation sequencing
NK	Natural killer
NRC	National Research Council
NS	Not significant
OTUs	Operational taxonomic units
P	Phosphorous
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PC	Positive control
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PI	Production index
PLS-DA	Partial least square discriminant analysis
PPi	Inorganic pyrophosphate
PRR	Pattern recognition receptors
QIIME	Quantitative insights into microbial ecology
qRT-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
SE	*Salmonella* Enteritidis
SCFA	Short-chain fatty acids
SEM	Standard error of the mean

SSCP	Single strand conformation
Tc	T cytotoxic
TGGE	Temperature gradient gel electrophoresis
Th	T helper
TiO_2	Titanium dioxide
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TOS	Transgalactooligosaccharides
T-RFLP	Terminal restriction fragment length polymorphism
TTGE	Temporal temperature gradient gel electrophoresis
VH: CD	Villus height to crypt depth
VIP	Variable influence on projection value
WBC	White blood cell
WHO	World Health Organization

1. GENERAL INTRODUCTION

"A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/ or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson and Roberfroid, 1995). It has been shown that dietary prebiotics supplementation improves gut health by changing gut microbial community and modulating gut immunity (Wang and Gibson, 1993; Bogusławska-Tryk et al., 2012). Furthermore, prebiotics could also increase gut fermentation activity and reduce gastrointestinal pH (Cummings et al., 2001). Fructooligosaccharides (FOS) are one of the common prebiotics, consisting of short-chain and non-digestible carbohydrates (Williams et al., 2008; Świątkiewicz and Arczewska-wlosek, 2012). Because of the ß (2, 1) glycosidic linkage between fructose molecules, FOS cannot be broken down by the endogenous digestive enzymes of monogastric animals and is available for fermentation by intestinal microbiota, resulting in increased *lactobacillus* and *bifidobacteria* population, lowered gut pH, elevated production of short-chain fatty acids (SCFA) and suppression of putrefactive substances (Hidaka and Hirayama, 1991; Swiatkiewicz et al., 2011; Bogusławska-Tryk et al., 2012). Fructooligosaccharides also have the potential to increase mineral bioavailability due to enhanced bacterial fermentation and mineral transpoter activities (Gudiel-Urabano and Goni, 2002; Zafar et al., 2004; Ohta, 2006). Studies conducted on mice have demonstrated that FOS counteracted the deleterious effects of phytic acid and increased the absorption of calcium, iron and magnesium in the intestine (Ohta et al., 1995; Lopez et al., 2000; Scholz-Ahrens et al., 2001; Wang et al., 2010).

Previous studies with broiler chickens have shown that dietary supplementation of FOS has the ability to improve growth performance (Ammerman et al., 1988; Bailey et al., 1991; Yusrizal

and Chen, 2003), enhance immune response (Khodambashi Emami et al., 2012), improve intestinal mucosa structures (Xu et al., 2003) and shift the gut microbiota (Bailey et al., 1991; Yusrizal and Chen, 2003; Xu et al., 2003; Kim et al., 2011). Dietary FOS could stimulate gut fermentation of beneficial bacteria such as *bifidobacteria* and *lactobacilli* and limit the colonization of pathogenic bacteria such as *Salmonella spp.* and *Escherichia coli*, thus improving the overall health of the birds (Bogusławska-Tryk et al., 2012). *Salmonella spp.* is one of the major foodborne bacteria associated with human illness and can be found in the poultry meat. Dietary FOS supplementation has the potential to elevate the anti-*salmonella* activity, which is mainly due to the shift of intestinal microbiota and the production of SCFA (Van Immerseel et al., 2009). FOS also has indirect effects toward the immune system of chickens by promoting the growth of lactic acid producing bacteria (Xu et al., 2003). In general, supplementing dietary FOS may result in improved immunity and reduced susceptibility to pathogen colonization in broiler chickens.

In the poultry industry, supplementation of exogenous phytase has been proven to improve the hydrolysis of phytate-P, increase P digestibility and lower the cost of inorganic phosphate addition (Nahm, 2002; Knowlton et al., 2004; Coppedge et al., 2011; Powell et al., 2011). It is generally recognized that a 0.1% reduction of the available P content can be achieved with phytase supplementation. Moreover, promising results have been observed on the growth performance of broiler chickens by supplementing phytase. For example, Simons et al. (1990) reported that the use of phytase increased bird performance and improved bone mineralization, while El-Sherbiny et al. (2010) reported that the addition of 500 U/kg phytase improved body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) of the birds from 23 to 40 d of age. An acidic gut pH is favourable for mineral solubility as well as for phytase activity

(Wyss et al., 1999; Selle et al., 2009; Naves et al., 2012). Therefore, combining dietary FOS supplementation with phytase in the broiler rations may have additive effects to improve growth performance, bone quality and P utilization of the broiler chickens.

Antibiotic growth promoters (AGPs) have been supplemented in poultry feed for subtherapeutic use to improve growth performance, feed efficiency and production uniformity in the past 60 years. Evidence showed that AGPs could interact with intestinal microbiota of the animals and act by modifying the gut bacterial composition (Dibner and Richards, 2005). Favourable and direct effects of AGPs are accredited to antimicrobial activities that suppress the competition between the host and its intestinal microbes (Dibner and Richards, 2005). However, heavy indiscriminate use of antibiotics may lead to the emergence of antibiotic resistant mutants and these genes will further transfer into humans, causing foodborne illness (Van Immerseel et al., 2009; Gaggìa et al., 2010). Finding out antibiotic alternatives would be beneficial and essential for broiler production.

Salmonella is a major cause of foodborne illness of the bacterial origin and is very common in broiler production. It colonizes in the crop, ceca and intestinal tract of the chicken and can cause asymptomatic intestinal infections in birds (Babu and Raybourne, 2008; Dunkley et al., 2009). *Salmonella* infection can activate both innate and acquired responses in chicken. Protective role of immunoglobulins (Ig) including IgG, IgM and IgA, increased number of CD4+ and CD8+ cells have been detected in the serum, intestinal tract, spleen and other tissues from birds challenged with various *Salmonella* serotypes (Babu and Raybourne, 2008). Cheeseman et al. (2007) observed higher mRNA expressions on interferon-Ɣ (IFN-Ɣ), interleukin (IL) -1ß, -6, -10, -12, -18 and inducible nitric oxide synthase (iNOS) in the spleen and ceca of day old chicks infected with *Salmonella* Enteritidis. The immune responses and infection resistance specific to

Salmonella can be evaluated by the number of macrophages, heterophil: lymphocyte ratio (H: L); and the exprssions of cytokines and enzyme genes in the intestine, spleen and cecal tonsil of the broiler chickens (Babu and Raybourne, 2008).

The gastrointestinal microbiota plays important roles on nutrition, immunity and physiological systems of the chickens. Changes on the gut microbiota may affect feed efficiency along with the health and disease status of the birds (Gaskins et al., 2002; Jeurissen et al., 2002; Kohl, 2012). The living microbes can be classified into health-promoting (or probiotic) and pathogenic groups, which interact with the intestinal wall and bring either beneficial or deleterious effects to the host (Gaskins et al., 2002; Jeurissen et al., 2002). Health-promoting bacteria such as *Lactobacillus* and *Bifidobacteria* are mostly gram-positive facultative anaerobic bacteria and dominate by attaching to the enterocytes, thereby reducing the opportunity of pathogenic bacteria for establishment in the GI tract (Gaskins et al., 2002). Pathogenic microbes such as *Salmonella*, *Escherichia coli* and *Campylobacter* may increase localized or systemic infections, encourage intestinal putrefaction, form toxins and produce mutagenic and carcinogenic substances (Jeurissen et al., 2002). The gastrointestinal microbiota can also be divided into the luminal microbiota and the mucosal-attached microbiota. The composition of luminal microbiota is determined by the available nutrients, the feed passage rate and the effects of antimicrobial substances, while the composition of mucosal-attached microbiota is determined by the expression of specific adhesion sites on the enterocyte membrane, the surrounding luminal microorganisms, the mucus production rate and the immunoglobulin secretion intensity (Jeurissen et al., 2002). Several selective, culture-based techniques have been used to characterize the microbial diversity of the avian gut. The discovery of bacterial 16S ribosomal RNA sequences offered molecular tools on understanding the composition and diversity of gut

microbiota. In particular, the 16S rDNA high-throughput next generation sequencing technology is capable of obtaining in-depth information on a larger profile, and is becoming a powerful tool to investigate the biological and ecological roles of gut microbiota (Diaz-Sanchez et al., 2013).

The objectives of study 1 were to investigate the interaction between phytase and FOS on growth performance, P utilization and skeletal integrity in broiler chickens fed standard and moderately low Ca and available P diets, as well as to determine an optimum FOS inclusion rate for broiler diets. The objectives of study 2 were: 1) to evaluate the effects of dietary FOS supplementation on growth performance, intestinal morphology, immune organ health, and gastrointestinal microbiota of broiler chickens, 2) to investigate the changes in immune function indicators such as white blood cell composition, cytokine gene expression and immunoglobulin levels by supplementing FOS and challenging the birds with *Salmonella* Enteritidis lipopolysaccharides (LPS), and 3) to examine the possibility of replacing antibiotic growth promoters with FOS in broiler rations.

2. LITERATURE REVIEW

2.1 Prebiotics

2.1.1 An overview of prebiotics

Prebiotics are defined as non-digestible food ingredients that stimulate the growth of beneficial microorganisms in the intestines in ways claimed to be beneficial to health (Gibson and Roberfroid, 1995). To be classified as a prebiotic the compound has to be 1) neither hydrolysable nor absorbable in the stomach or the small intestine, 2) a selective substrate for beneficial bacteria to be colonized in the large intestine, 3) able to alter the gastrointestinal microbiota in favor of a healthier composition, and 4) able to induce luminal or systemic effects that are beneficial to the host health (Gibson and Roberfroid, 1995; Gaggia et al., 2010). Prebiotic products are predominantly oligosaccharides, which include fructooligosaccharides (FOS, oligofructose, and inulin), mannoligosaccharides (MOS), glucooligosaccharides (GOS), transgalactooligosaccharides (TOS), xylooligosaccharides, soybean galactooligosaccharides and lactulose (Gibson, 1998; Gaggia et al., 2010; Dankowiakowska et al., 2013). Other sources of prebiotics include undigestible polysaccharides, certain proteins, peptides and lipids such as ethers and esters (Gibson and Roberfroid, 1995; Dankowiakowska et al., 2013).

In humans, dietary prebiotic supplementations have demonstrated positive effects on promoting beneficial gut microorganisms (especially on the stimulation of endogenous *bifidobacteria*), modulating lipid metabolism via fermentation and reducing gastrointestinal pH (Gibson and Roberfroid, 1995; Cummings et al., 2001). The use of prebiotics in animal production, as a possible alternative to antibiotic growth promoters (AGPs), has also exhibited the capability of modulating the gut microbial communities. They contribute to the establishment of benefitial microbial community with an increased number of *bifidobacteria* and/or *lactobacilli*

(Gaggia et al., 2010). In poultry, the prebiotics are able to modulate the immune cells in the gut-associated lymphoid tissue (GALT) due to the lactic action that stimulates the innate and adaptive immune activity (Janardhana et al., 2009; Dankowiakowska et al., 2013), It has also been shown that dietary prebiotics supplementation reduced the population of *Clostridium perfringens*, *Escherichia coli* and *Salmonella spp.* in the large intestine and cecum of the chickens (Bogusławska-Tryk et al., 2012).

2.2 Fructooligosaccharide (FOS)

2.2.1 An overview of FOS

The fructooligosaccharides (FOS) are one of the most popular prebiotic supplements available, consisting of several (on average 5) fructosyl residues that are linked by a ß (2, 1) glycosidic bond to a terminal glucose moiety (Hidaka and Hirayama, 1991; Barry et al., 2009; Bogusławska-Tryk et al., 2012). This structure is different from oligofructose, which may only contain fructose molecules. The ß (2, 1) glycosidic bond is resistant to be broken down by endogenous digestive enzymes of the monogastric animals, and thus, becomes available for intestinal microbiota fermentation resulting in increased *bifidobacteria* population, lowered gut pH, production of short-chain fatty acids (SCFA) as well as suppression of putrefactive substances (Hidaka and Hirayama, 1991; Swiatkiewicz et al., 2011; Bogusławska-Tryk et al., 2012). The fermentation of FOS is faster than that of other fructans such as inulin, which has a degree of polymerization (DP) of 10 to 60, whereas FOS's DP ranges from 3 to 7 (Scholz-Ahrens et al, 2001; Nyman, 2002).

Fructooligosaccharides can be naturally extracted from plant sources such as chicory root, onion, asparagus, beet, edible burdock, wheat, bananas and cane sugar (Hidaka and Hirayama, 1991; Williams et al., 2008). They can also be commercially produced from sucrose by the

transfructosylation of *A. niger* enzyme or from inulin by enzymatic hydrolysis (Hidaka and Hirayama, 1991). The FOS compound consists of a glucose monomer (G) linked by a α-1, 2 bound to two or more β-2,1-linked fructosyl units (F), forming 1-kestose (GF_2), nystose (GF_3) and l^F-ß-fructofuranosylnystose (GF_4), as shown in Figure 1 (Hidaka and Hirayama, 1991).

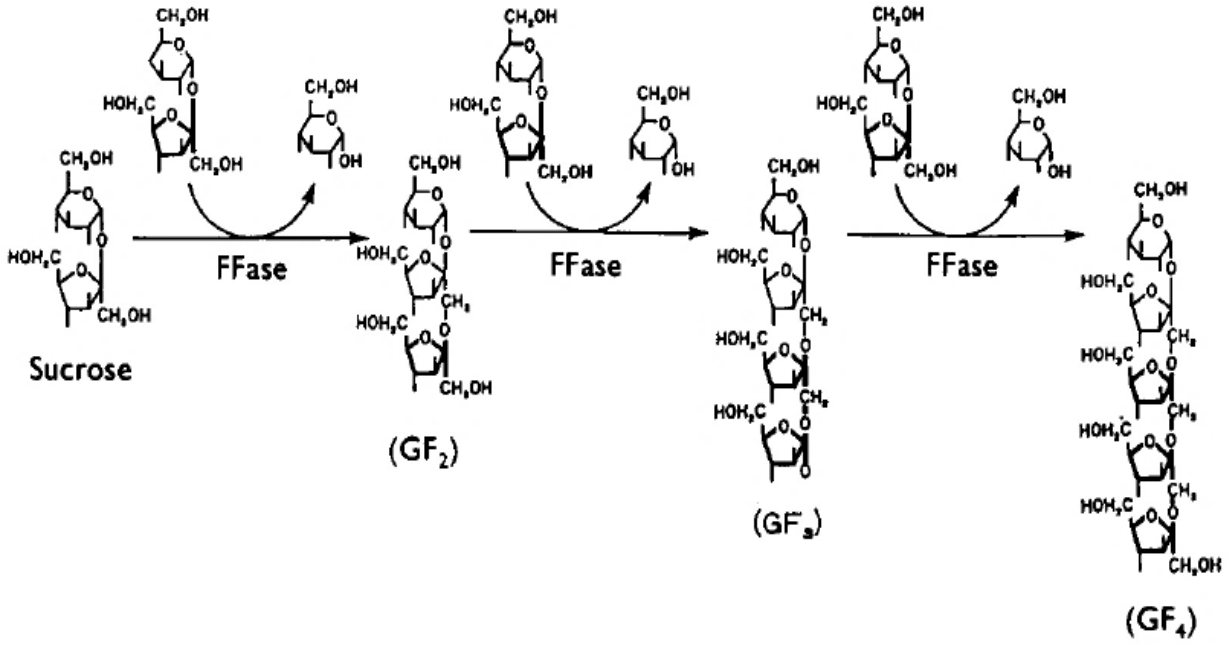


Figure 1. The structure and enzymatic preparation of fructooligosaccharides (Reproduced with permission from Hidemasa Hidaka and Masao Hirayama, (1991), (*Biochemical Society Transactions*), (**19**), (561-565). © the Biochemical Society).

2.2.2 The applications of FOS supplementation in broiler chickens

Several studies have been conducted in previous years to discover the effect of dietary FOS supplementation on growth performance, nutrient utilization, intestinal morphology, gut microbiota, immune response and *Salmonella* immunity in broiler chickens.

Positive effects on growth performance parameters were reported by Yusrizal and Chen (2003) that FOS supplementation has improved body weight gain (BWG), feed conversion and carcass weight of female broiler chickens. Similar results related to increased BWG and improved feed conversion ratio (FCR) were observed by Ammerman et al. (1988), Bailey et al. (1991), and Xu et al. (2003). Variation in FOS inclusion levels may affect the growth rate and performance parameters of the bird (Yang et al., 2009). It has been reported that excessive FOS (1%) may cause diarrhea and generate carbon dioxide and hydrogen gases due to intensive fermentation in the gastrointestinal (GI) tract, thus decreasing the production performance (Cummings et al., 2001; Xu et al. 2003). Ammerman et al. (1988) observed 0.25% to 0.5% FOS significantly improved feed efficiency and had positive effect on BWG in broiler chickens.

The gut morphology is an important indicator on digestive tract health and bird performance. Stress factors in the intestine can result in the changes of intestinal mucosa such as shortening of villus and deepening of crypts (Bogusławska-Tryk et al., 2012). It is commonly believed that increasing in the villus height and decreasing in the crypts depth can positively affect the digestive and absorptive functions of the birds, due to an enlarged absorptive area and reduced tissue turnover rate in the GI tract (Xu et al., 2003; Bogusławska-Tryk et al., 2012). Xu et al. (2003) have reported that FOS exhibited positive effects on intestinal morphology in broilers. A 0.4% of FOS supplementation significantly increased ($P < 0.05$) ileal villus height, jejunal and ileal microvillus height, and villus height to crypt depth ratio, while decreased crypt depth in the jejunum and ileum. The beneficial changes in the intestinal mucosa structures are most likely due to the ability of FOS to create a favorable gut microbial environment (Bogusławska-Tryk et al., 2012).

Recent studies with dietary FOS supplementation have also been shown to improve intestinal microbiota of broiler chickens by stimulating the growth of beneficial bacteria such as bifidobacteria and lactobacilli, while limiting the growth of pathogenic bacteria such as *Salmonella spp*. and *Escherichia coli* (Bogusławska-Tryk et al., 2012; Flickinger et al., 2003; Xu et al., 2003). A number of in vivo studies have demonstrated that the intensive growth of beneficial bacteria suppresses the activities of the potential hazardous bacterial species and reduced the production of toxic substances such as ammonia and phenols, thereby improving the overall health of the animals (Bailey et al., 1991; Yusrizal and Chen, 2003; Xu et al., 2003; Kim et al., 2011; Bogusławska-Tryk et al., 2012). Furthermore, the supplementation of FOS in poultry diet increases gut fermentation, SCFA production and enzymes activities, which results in acidification and reducing pH in the GI tract (Xu et al., 2003; Yang et al., 2009; Bogusławska-Tryk et al., 2012).

Fructooligosaccharide supplementations have demonstrated positive effects toward the immune responses of the chickens by promoting the growth of lactic acid producing bacteria (Xu et al., 2003). Janardhana et al. (2009) supplemented 5 g/ kg of FOS in addition to the basal broiler chicken diet and observed higher titers of plasma immunoglobulin (Ig) M ($P < 0.01$) and Ig G ($P < 0.01$) than the control group. The FOS-treated birds also had reduced number of B cells and depressed mitogen responses of lymphocytes in the cecal tonsil ($P < 0.05$), without detrimental effects on performance, which is likely due to the SCFA fermentation and a combination of toll-like receptor mediated responses through their interaction with the gut microbie and microbial products. Emami et al. (2012) investigated FOS as an alternative to virginiamycin on immune response of male broilers, and discovered that the primary antibody titers against sheep red blood cell were higher in the FOS fed treatment. Kim et al. (2011)

reported that the H: L ratio and the basophil leukocytes were significantly higher in 0.5% FOS groups than treatments with other prebiotics.

Salmonella spp. infection is a major cause of food-borne illness in human. Effective control of salmonellosis in meat-type chicken production is essential to ensure poultry food safety (Chambers and Gong, 2011). The anti-s*almonella* activity of FOS is due to the shift of intestinal microbiota, and the production of short-chain fatty acids (Van Immerseel et al., 2009). Bailey et al. (1991) reported that treatments with FOS showed a fourfold reduction of *Salmonella* in chicken ceca. In general, feeding FOS in broiler diet may result in improved immunity against *Salmonella* and reduced *Salmonella* colonization.

2.3 Phytase

2.3.1 An overview of phytase

Phytases are types of phosphatase enzymes that can be found naturally in plants and microoganisms such as fungi and bacteria (Wyss et al., 1999). Depending on the activity profile and the optimum pH for catalysis, phytase enzymes can be further classified as acid, neutral and alkaline phosphatases (Mullaney and Ullah, 2003). The majority of the phytases (myo-inositol hexakisphosphate phosphohydrolase) are acid phosphatases from fungal sources and belong to a subfamily of the high molecular weight histidine acid phosphatases (HAPs). The HAPs have a two-step mechanism to hydrolyze phosphomonoesters bond from phytic acid (myo-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate) and release phytate phosphorous (P) (Mullaney and Ullah, 2003; Mittal et al., 2011). Some of the commercial HAPs include *Aspergillus terreus, A. fumigatus*, *A. niger*, *A. oryzae*, *Emericella nidulans, Myceliophthora thermophila* and *Saccharomyces cerevisae* (Wyss et al., 1999; Naves et al., 2012). Specific enzymatic activities of these fungal phytase are closely related to the environmental pH and the temperature. The

optimum pH ranges from 2.5 to 7.0, with most of the phytase enzymes achieving their maximum activity at under pH 5.0 (Wyss et al., 1999; Naves et al., 2012). The optimum temperature is ranging from 40 to 60 ºC, with an acceptable temperature at 41 ºC for highest phytase activities (Naves et al., 2012). These conditions are close to the ideal physical condition in the GI tract of the animals, thus dietary supplementation of phytase would result in a high rate for hydrolyzing phytic acid from the animal feeds (Naves et al., 2012).

Monogastric animals such as poultry and swine are unable to utilize phytic acid (phytate) due to minimum phytase activity in the brush broader membrane of their digestive tracts and since phytate P cannot be absorbed (Maenz and Classen, 1998; Wyss et al., 1999; Selle and Ravindran, 2007). However, the majority of poultry and swine feeds are of plant origin, in which around 50% to 80% of total P are presented as phytate-P. Therefore, phytase has been supplemented in animal diets to liberate phytate bond P molecule and prevent the formation of insoluble Ca-phytate complexes (Wyss et al., 1999; Woyengo et al., 2010; Slominski, 2011). Supplementation with phytase has been proven to be an effective method to increase the P availability in seed-based animal feed and also to improve P digestibility in the animals (Wyss, 1999; Nahm, 2002; Selle and Ravindran, 2007). It further reduces the excessive P level from animal waste that may lead to environmental pollution (Boling et al., 2000; Knowlton et al., 2004).

2.3.2 Effects of phytase on phosphrous utilization and bone mineralization in broiler chickens

Similar to other monogastric animals, positive effects such as improvement in hydrolysing phytate-P, increased P digestibility, improved bone mineralization, and reduced P excretion have been observed in poultry with phytase supplementation (Selle and Ravindran, 2007). In the same time, phytase supplementation reduced the addition of inorganic phosphate in poultry rations,

and thus lowered the production costs (Nahm, 2002; Knowlton et al., 2004; Coppedge et al., 2011; Powell et al., 2011).

It is generally recognized that in poultry a 0.1% reduction of the available P content can be achieved with phytase supplementation, although as recently reviewed by Slominski (2011) approximately 0.05% of phytate-P would only originate from poultry diets following phytase supplementation. Moreover, promising results have been observed on the growth performance of broiler chickens by supplementing phytase. For instance, Simons et al. (1990) reported that phytase increased bird performance and improved bone mineralization, while El-Sherbiny et al. (2010) examined broiler diets containing a reduced level of dicalcium phosphate and concluded that the addition of 500 U/kg phytase enhanced BWG, feed intake (FI) and FCR of the birds from 23 to 40 d of age. Phytase supplementation in P standard broiler diets have been shown to generate equivalent growth performance, whereas significantly increased weight gain (7.6%) and feed efficiency (4.7%) have been observed in birds that fed reduced P and calcium (Ca) diet (Selle et al., 1999).

Phytase addition has been shown to have positive effects on bone ash content and bone mineralization in broiler chickens fed low available P diets (Angel et al., 2006; Woyengo et al., 2008; Coppedge et al., 2011). El-Sherbiny et al. (2010) reported that phytase increased dietary Ca and P utilization, reduced Ca and P excretion and improved tibia breaking strength and tibia ash percentage in broiler chickens. Previous studies indicated that the tibia ash percentage and bone breaking strength of birds fed low Ca and available P diet were improved by phytase supplementation, however, the values were not equivalent to that of the control diet (Powell et al., 2008; Woyengo et al., 2008; El-sherbiny et al., 2010). Angel et al. (2006) reported that whole body and tibia bone mineral density (BMD) and bone mineral content (BMC) of birds were

higher in diets with 0.26% available P and 600 U/kg of phytase, although lower than those fed the control diet. Chung et al. (2013) found similar results showing that phytase supplementation improved bird femur and tibia BMD and BMC when compared with birds fed the low-P control diet (available P reduced by 0.1%).

2.4 An overview of chicken gut and gastrointestinal microbiology

2.4.1 Chicken gut and its function

The gastrointestinal (GI) tract of chicken is an alimentary canal that starts at the beak and ends in the cloaca. It consists of several compartments that have specific roles for nutrients digestion and absorption. These compartments are the crop, proventriculus, gizzard, small intestine (duodenum, jejunum and ileum) and large intestine (ceca, colon, rectum and cloaca).

The gut is responsible for converting the consumed feed into nutrients, in order to fulfill the body's needs for maintenance, growth and production. Mechanical and chemical actions, as well as intestinal microorganism fermentation are involved in the feed digestion process. The swallowed feed first enters and stores in the crop as a whole. In the crop, although digestion is limited, the digestion process starts with moistening and fermenting the ingested carbohydrates. *Lactobacillus* (e.g., *L. reuteri*) is the dominant species that colonizes in the crop of the chicken (Abbas Hilmi et al., 2007). It secretes organic acids (e.g., lactic acid), reduces crop pH and thus may improve nutrients absorption (Rehman et al., 2007). The proventriculus is the glandular stomach (as known as the true stomach) of the birds. It secretes hydrochloric acid and produces digestive enzymes from the granular tissues, and maintains a low pH environment. The gizzard, or ventriculus, is the site for mechanical grinding. It is made up of two sets of strong muscles and often contains hard objects such as gravel or grit that could assist in breaking down of the consumed feed. It also sets the passage rate through the GI tract depending on the size and

texture of the feed. The small intestine which includes duodenum, jejunum and ileum, is the most important site that contains a number of enzymes for chemical digestion and nutrients absorption. Digestive enzymes and bile produced by pancreas and liver, respectively, are received in the duodenum, and are involved in the digestion of fat, sugar, protein, lipid and fat-soluble vitamins (i.e., vitamins A, D, E and K). The mucosal epithelium of the small intestine builds a dynamic and functional barrier between the environment and the host. It plays a key role in nutrient digestion, transportation and absorption, as well as in the exclusion of microorganisms and toxins (Mitchell and Moretó, 2006). Villi and microvilli are presented on the surface of the intestinal mucosa. They are long, folded, finger-like tissues that extended to the lumen of the intestine. This structure can dramatically increase the surface area and lead to more efficient absorption of the ingested nutrients. The villus height, crypt depth and villus height to crypt depth ratio are common intestinal morphology measurements. They are good indicators of gastrointestinal health and absorption capability (Figure 2). The large intestine which consists of ceca, colon, rectum and cloaca is relatively short, and is responsible for reabsorption of water and nutrients. A bursa of fabricius is an epithelial and lymphoid organ that is located above the cloaca of young birds, it atrophies after the birds have reached approximately one year old. The ceca harbor a large community of microorganisms. These microbes produce fatty acids and vitamins, which can facilitate the fermentation of indigestible feed materials such as fiber and cellulose. Little nutrients absorption occurs in the colon. The digestive wastes mix with urine wastes (uric acid) after leaving the colon and entering into the rectum and cloaca. The large intestine ends at the front of cloaca and the excreta eventually expels via the vent. The readers are referred to Jacob and Pescatore (2009), Bailey (2013) and PoultryHub (2013) for more detailed descriptions of the poultry GI tract and digestive system.

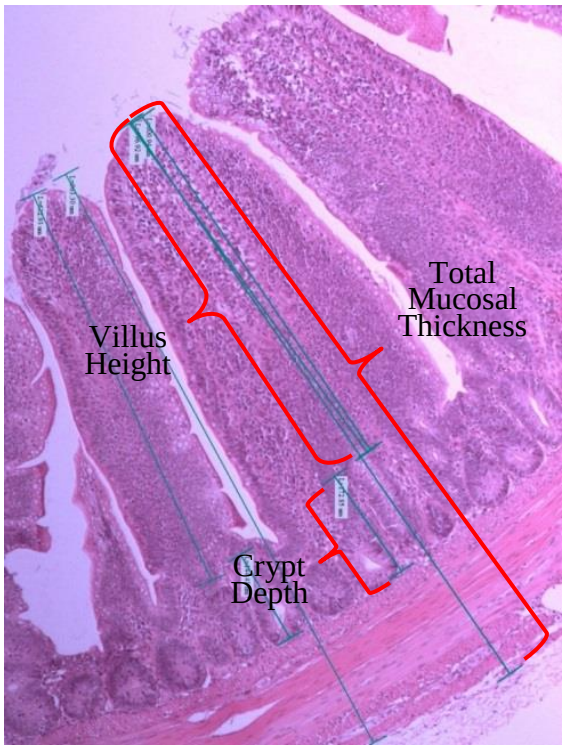


Figure 2. Measurement of villus height, crypt depth and total mucosal thickness of ileal segment of broiler chicken

2.4.2 The role of chicken gastrointestinal microbiota

The integrity, functionality and health of chicken GI tract depend not only on the host itself, but also on the interactions between the environment, the feed materials and the gastrointestinal microbiota (Kohl, 2012). The gastrointestinal compartments described in the previous section are populated with different kinds of microorganisms such as bacteria, fungi and protozoa. These microorganisms, predominately bacteria, make up the gastrointestinal microbiota of the chicken (Yegani and Korver, 2008). The bacteria in the gut microbiota can be further categorized to commensal, opportunistic, pathogenic and benefitial bacteria. The commensal bacteria are those microorganisms that "present on body surfaces covered by epithelial cells and are exposed to the external environment (gastrointestinal and respiratory tract, vagina, skin, etc.)" (Tlaskalova-

Hogenova et al., 2004). Opportunistic bacteria are "normally a commensal or does not harm its host but can cause disease when the host's resistance is low" (von Graevenitz, 1977). Pathogenic bacteria are commonly refered to those bacteria that cause bacterial infection and disease, whereas beneficial bacteria can provide health benefit on the host (i.e., probiotics) (Rijkers et al., 2011).

Studies on chicken gut microbiota and its relationship with the host have been conducted since the 19th century (Shapiro and Sarles, 1949). Results have now been shown that the gastrointestinal microbiota play important roles in nutrition, immunity and other physiological systems of the bird (Kohl, 2012). In general, the chicken GI tract consists of microorganisms that can interact with the intestinal wall and bring beneficial or deleterious effect to the host (Jeurissen et al., 2002). According to the principle of competitive exclusion, beneficial bacteria can form a protective barrier in preventing the colonization of pathogenic bacteria (Hardin, 1960; Yegani and Korver, 2008). Beneficial bacteria such as *Lactobacillus* and *Bifidobacteria* are mostly gram-positive facultative anaerobic bacteria (Gaskins et al., 2002). They dominate by attaching to the epithelial walls of the enterocyte and thus reduce the opportunity for pathogenic bacteria to establish in the GI tract. The probiotic-like bacteria can also produce vitamins (e.g., vitamin K and vitamin B groups), volatile fatty acids (acetic acid, butyric acid and propionic acid), organic acids (e.g., lactic acid) and antimicrobial compounds (e.g., bacteriocins), as well as lower triglyceride and induce non-pathogenic immune responses, which provide both nutrition and protection to the animal, and at the same time inhibit the growth and colonization of pathogenic bacterial species (Jeurissen et al., 2002; Dibner and Richards, 2005; Yegani and Korver, 2008; Bailey, 2013). On the other hand, unfavourable microbes such as *Salmonella*, *Escherichia* and *Campylobacter* may increase localized or systemic infections, encourage

intestinal putrefaction, form toxins and produce mutagenic and carcinogenic substances (Jeurissen et al., 2002). Widespread diseases such as necrotic enteritis causing by *Clostridium perifingens* often occur in unbalanced microbiota and can cause severe damage to the chicken intestine. Outcomes associated with enteritis include diarrhea, dehydration, weakness, decreased digestion, reduced weight gain and increased mortality (Yegani and Korver, 2008; Bailey, 2013). Coccidiosis caused by protozoan *Eimeria* is another enteric disease that leads to mucosal damage and lesions. It is also a precursor for necrotic enteritis (Williams, 2005).

A balanced microbiota is refered to as a commensal microbiota (normal microbiota, indigenous microbiota), which is associated with certain benefits and costs (Gaskins et al., 2002; Dibner and Richards, 2005). As described above, the primary benefit that provided by commensal microbiota is competitive exclusion of pathogens or non-indigenous microbes (Dibner and Richards, 2005). Earlier studies have demonstrated that conventional animals are far less susceptible to pathogens when compared with germ-free animals (Koopman et al., 1984). Another benefit is that the commensal microbiota can stimulate the development of immune system including the mucus layer, the epithelial monolayer, the intestinal immune cells (e.g., cytotoxic and helper T cells, immunoglobulin producing cells and phagocytic cells) and the lamina propria (Dibner and Richards, 2005). These tissues build barriers between the host and the microbes, and combat with undesirable gut microorganisms. In the distal gut (i.e., ceca and colon), microbiota produce energy and nutrients such as vitamins, amino acids and short chain fatty acids (SCFA) from undigested feed, which eventually become available for the host (Gaskins et al., 2002; Dibner and Richards, 2005). Short chain fatty acids, such as acetate, propionate and butyrate, are among the compounds produced by the gut microbiota. These fatty acids have bacteriostatic properties that are capable of eliminating foodborne pathogens, such as

Salmonella spp. (Ricke, 2003). Short chain fatty acids also contribute as a good source of energy to the animals, and can further stimulate gut epithelial cell proliferation and thus increasing the gastrointestinal absorption surface (Dibner and Richards, 2005). On the contrast, commensal microbiota also brings costs to the GI tract. In the proximal gut (gizzard and small intestine), bacterial species compete with the host for energy and protein. In both proximal and distal gut, microbes produce toxic metabolites (e.g., amino acid catabolites) and catabolize bile acids, which depress growth and decrease fat digestibility of the birds, respectively (Gaskins et al., 2002). With the presence of microbiota, gut mucus layer increases mucin secretion and epithelial cell turnover rate, thus, to keep the GI tract lubricated and to prevent the microorganisms from invading into intestinal epithelial cells of the host. The intestinal immune system is also more developed and increased in microbiota-specific IgA secration with the existence of commensal gut microbiota. These processes increase the demand of energy and protein from the host, and therefore influence on the growth performance of the birds (Dibner and Richards, 2005; Yegani and Korver, 2008).

An imbalanced gut microbiota is often refered as dysbiosis. Dysbacteriosis is a specific cause of dysbiosis that result from intestinal disruption (Bailey, 2013). It has been defined as "the presence of a qualitatively and/or quantitatively abnormal microbiota in the the small intestine, inducing a cascade of reactions in the gastro-intestinal tract including reduced nutrient digestibility and impaired intestinal barrier function (e.g., thinning of intestinal wall), increasing the risk of bacterial translocation and inflammatory responses" (Teirlynck et al., 2011). Both non-infectious and infectious stressors can lead to dysbacteriosis. The non-infectious factors include: environmental stressors, nutritional imbalances, dietary changes, mycotoxins, management disorders, enzymatic dysfunction and genetic background (Teirlynck et al., 2011;

Bailey, 2013). The infectious factors include: viral or bacterial challenge, coccidiosis and toxic metabolities produced by harmful microorganisms (Bailey, 2013). Studies on dysbacteriosis indicated that the composition of gut bacterial communities and possible shifts may contribute to this syndrome (Teirlynck et al., 2011).

The gastrointestinal microbiota can also be divided into 2 sub-classes: the luminal microbiota and the mucosal-attached microbiota. The composition of luminal microbiota is determined by the available nutrients, the feed passage rate and the effects of antimicrobial substances. The composition of mucosal-attached microbiota is determined by the host's expression of specific adhesion sites on the enterocyte membrane, the surrounding luminal microorganisms, the mucus production rate and immunoglobulin secretion intensity (Jeurissen et al., 2002). Therefore, it is important to recognize that dietary components could alter both luminal and mucosal-attached microbiota, and the mucosal-attached microbiota could further interact with the host and determine the gut health.

2.4.3 The diversity of chicken gut microbiota

The GI tract of the chicken harbours a diverse bacterial community. Each bacterium specializes on its own function and synergistically lives with other bacterial species in the same community (Apajalathi et al., 2004). The composition of these bacteria varies depending on the age of the birds, the location of the GI organs and the consumed diet (Apajalathi et al., 2004; Rehman et al., 2007; Diarrassouba, 2008).

The age of the birds is one of the factors that influence the bacterial density and composition in the GI tract. Before receiving feed, the newly-hatched chicks only contain bacteria in the cecum (Apajalahti et al., 2004; Zhu et al., 2002). *Streptococci* and *Enterobacteria* are first

colonized in the cecum and spread out in the GI tract within 24 hours (Smith, 1965). After consuming feed and water, the bacterial population significantly increases (Shapiro and Sarles, 1949). The chicken ileal and cecal digesta harbour 10^8 and 10^{10} cfu/g bacteria, respectively, at one day post-hatching. After three days, the bacterial population increase to 10^9 and 10^{11} cfu/g in the ileum and cecum of chicken, respectively, and stay stable in the following 30 days (Apajalahti et al., 2004). The composition of the GI bacterial community is constantly being changed due to the settlement and replacement of more stable and dominate bacterial species as the bird ages (Lu et al., 2003). Lee et al. (2002) and Lu et al. (2003) discovered that the GI tract of chicken at 3 days of age is populated with *L. delbrueckii*, *C. perfringens* and *Campylobactor coli*, whereas from 7 to 21 days of age, it is dominant by *L. acidophilus*, *Enterococcus* and *Streptococcus*. At 28 and 49 days of age, the GI tract is occupied with *L. crispatus*, but the composition is significantly different from other ages.

The bacterial community differs in various GI compartments. The chicken crop harbours 10^8 to 10^9 cfu/g bacteria, which is dominated by gram-positive facultative anaerobic bacteria *Lactobacillus* spp. including *L. salivarius* and *L. aviaries* (Gong et al., 2007; Rehman et al., 2007). Lactobacilli and other species, such as enterobacteria and gram-positive cocci, are also colonized on the crop mucosa (Gong et al., 2007). These bacteria produce SCFA, predominately acetate, in the crop (Rehman et al., 2007). In the gizzard, the concentration of bacteria is similar to that in the crop but the bacterial fermentation activities are low mainly because of the low pH. The majority of bacteria in the gizzard are lactobacilli, enterococci, lactose-negative enterobacteria and coliform bacteria (Rehman et al., 2007). In the small intestine, the bacterial density is low in the duodenum due to short passage time and a dilution of digesta by secreted bile (Shapiro and Sarles, 1949). Duodenal bacterial community mainly consists of clostridia,

streptococci, enterobacteria and lactobacilli (Smith, 1965). Ileum microbiota has been studied the most among small intestine segments. Lu et al. (2003) evaluated the ileal bacterial community by examining 16S rRNA gene sequences and stated that the major species are *Lactobacillus* (70%), *Clostridiaceae* (11%), *Streptococcus* (6.5%) and *Enterococcus* (6.5%). Bacteria colonized on the mucosa and lumen of the ileum has different compositions (Gong et al., 2002). The ceca harbour a more diverse and abundant microbial community, with 65% of *Clostridiaceae,* 14% of *Fusobacterium,* 8% of *Lactobacillus* and 5% of *Bacteroides* (Gong et al., 2002; Lu et al., 2003). Similar studies by Dumonceaux et al. (2006) showed that dominant orders include *Clostridiales* (68%), *Lactobacillales* (25%) and *Bacteroidetes* (6%).

Feed processing methods, feed ingredients and feed additives also influence on the gut microbiota. Engberg et al. (2002) stated that mash feed lowers the number of *Enterococcus* spp. and coliforms, but increases *Lactobacillus* spp. and *C. perfringens* in broiler ileum, when compared to pellet feed. Corn favours low %G + C clostridia, enterococci and lactobacilli, whereas wheat favours higher %G + C *bifidobacteria* (Apajalathi et al., 2004). Feed supplementation, such as fermentable sugars (prebiotics), can also have an impact on the composition and diversity of chicken gut microbiota.

2.4.4 Effects of dietary prebiotics on gut microbiota

The use of prebiotics as dietary modulators has been shown positive effects on the balance of intestinal microbiota (Gaggia et al., 2010). Fructo-oligosaccharides (i.e., FOS, oligofructose and inulin) modify the gut microbiota by competitive exclusion of pathogens and fermentation of beneficial microbes. *In vitro* studies that incubated fecal slurries with oligofructose and inulin exhibited an increase in *Bifidobacteria* population in the human large intestine, whereas potential pathogens such as *Escherichia coli* and *Clostridium spp.* were maintained at lower levels (Wang

and Gibson, 1993). The majority of *Bifidobacteria* strains (e.g., *B. fiagilk*, *B. thetaiotaomicron*, B. *vulgatus*, *B. dktasonk* and *B. ovatus*) except *B. bifidum*, can utilize FOS as a growth and fermentation promoter (Hidaka and Hirayama, 1991; Ferket, 2004). These bacteria secrete ß-fructoside enzyme that can readily degrade and ferment FOS. However, microorganisms such as *E. coli* and *C. perfringens* are not able to exploit FOS as a fermentative carbohydrate source (Ferket, 2004). Rats that fed dietary FOS resulted in a short term elevation of lactic acid-producing bacteria and a long term increase in cecal butyrate (Le Blay et al., 1999). Patterson et al. (1997) evaluated the effects of thermal ketoses oligosaccharides on cecal microbial populations of broiler chickens. The results showed that cecal *bifidobacteria* and *lactobacilli* concentrations were increased 24-fold and 7-fold, respectively, when compared the ketoses supplemented diet to the control. Another type of prebiotics, mannoligosaccharides (MOS), are proposed to have different mechanisms. They can 1) bind to potential pathogenic gram-negative bacteria (e.g., E. *coli* and *Salmonella*) which possesses type-1 fimbriae (mannose-sensitive lectin), to prevent and dislocate the pathogens from attaching to the gut wall, 2) have immune modulatory effects based on the antigenicity features of mannan and glucan components, 3) modulate intestinal morphology, and 4) enhance the expression of mucin and reduce enterocyte turnover rate (Ferket, 2004; Yang et al., 2009).

Gaggia et al. (2010) summarized the functions of prebiotics on both upper (small intestine) and lower (large intestine) GI tract of humans and animals. The effects of prebiotics on upper GI tract include: 1) resistance to digestion, 2) retarded gastric emptying, 3) increased oro-cecal transit time, 4) hyperplasia of the small intestinal epithelium, and 5) stimulation of small intestinal hormonal peptides secretion. The effects of prebiotics on lower GI tract include: 1) presenting as food and fermentation sources for cecal and colonic microbiota, 2) production of

fermentation end products (e.g., SCFAs), 3) stimulation of saccharolytic fermentation, 4) acidification of the large intestine content, 5) hyperplasia of the cecal and colonic epithelium, 6) stimulation of colonic hormonal peptides secretion, and 7) acceleration of ceco-anal transit.

2.4.5 Discovery of chicken gut microbiome by molecular approaches

Classical culture-based methods have been widely used to study chicken gut microbiota in the past. However, these methods are highly selective to cultivable bacteria under specific conditions (Hugenholtz et al., 1998). Many other bacteria may require different and exclusive growth conditions, and as such are non-cultivable yet (Apajalahti et al., 2004). Therefore, the quantity and diversity of intestinal bacteria have been underestimated, and our knowledge towards gut microbiota may be incomplete (Gong et al., 2002).

The development of molecular biotechnology and the application of bacterial 16S ribosomal RNA (16S rRNA) sequences offered new tools in studying the composition, diversity, function and interaction of gut microbiota in different sections of the GI tract. The 16S rRNA molecule is a small subunit that possesses highly conservative regions in all bacteria. These regions can be identical or distinguished across all phylogenetic groups (Weisburg et al., 1991; Flint et al., 2006). To amplify these genes, 16S rDNA (the gene coding for 16S rRNA) is extracted from fecal or digesta samples and universal primers which target the conserved regions are designed for polymerase chain reaction (PCR) (Apajalahti et al., 2004). The amplification of these regions could provide information on taxonomic relationship and composition of bacterial communities (Gong et al., 2006). Ideally, the yield products should contain all the bacterial 16S rRNA coding sequences. However, the integrity of DNA samples, the condition of PCR amplification and the coverage of universal primers may bring pitfalls and bias to the 16S clone library (Flint et al., 2006). For instance, *bifidobacteria* (high G + C content, gram-positive bacteria) have poor

amplification with certain universal primers, because of its low similarity to other microbial genes in the clone libraries (Apajalahti et al., 2004; Flint et al., 2006). Several 16S rRNA-based approaches have been developed for studying the population of microbial communities and their interactions (Diaz-Sanchez et al., 2013). The sample capacity, applications and limitations of each molecular technique are listed in Table 1.

Table 1. 16S rRNA-based molecular approaches for studying microbial ecology (data from Flint et al., 2006; Diaz-Sanchez et al., 2013; Park et al, 2013)

Approach	Sample Capacity	Applications	Limitations
Sequencing analysis of PCR amplicons			
16S rDNA sequencing	Limited	16S rRNA gene sequence, wide range identification of genus/ species/ strain	Bias in DNA extraction, PCR amplification and numbers of clones, costly, laborious
Real-time PCR (RT-PCR)	Limited	Specific gene expression in targeted groups, high in sensitivity	Bias in DNA extraction and RT-PCR, costly
Profiling approaches			
Fingerprinting DGGE[1], TGGE[2], TTGE[3], T-RFLP[4] and SSCP[5]	Good	Amplify common 16S rDNA sequences, diversity profiles within the targeted group, rapid, comparative	Bias in DNA extraction and PCR
Gene quantification			
FISH[6]	Limited	Enumeration of bacterial population	Laborious at species level
DNA microarray technology			
Diversity arrays	High	Diversity profiles, different gene expression levels	Laborious in development, costly
DNA microarrays	High	Transcriptional fingerprint, comparative	Bias in nucleic acids extraction and their labeling, costly

[1]DGGE = denaturing gradient gel electrophoresis

[2]TGGE = temperature gradient gel electrophoresis

[3]TTGE = temporal temperature gradient gel electrophoresis

[4]T-RFLP = terminal restriction fragment length polymorphism

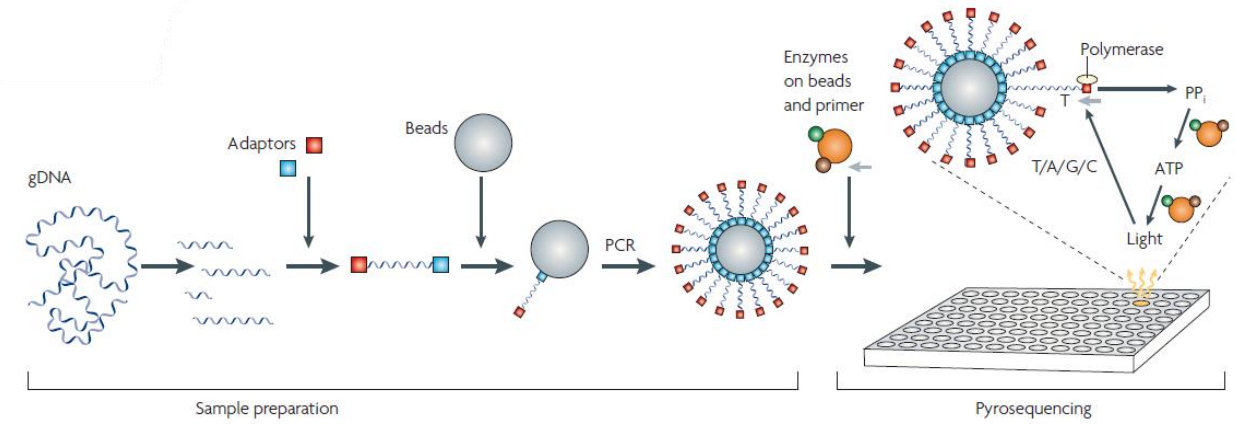
[5]SSCP = single strand conformation

[6]FISH = fluorescence in situ hybridization

Among these molecular approaches, the DNA sequencing technology especially high-throughput next generation sequencing (NGS), is a powerful tool to investigate the biological and ecological role of gut microbiota (Diaz-Sanchez et al., 2013). Sanger et al. (1977) introduced a first generation sequencing technique by using DNA polymerase with chain-terminating inhibitors. This method provides a read length of around 800 base pairs (bp), and it can reach up to 1000 bp (Schadt et al., 2010). In about 20 years, the bioluminescence-based automatic NGS has been developed, and it is becoming a convenient, rapid, accurate and inexpensive method for genomic research (Pettersson et al., 2009; Park et al., 2013). The NGS platforms offer higher throughput, faster turn-around time, lower cost and wider identification range to rare and uncultivable microorganisms (Diaz-Sanchez et al., 2013; Park et al. 2013). Among these platforms, the 454 pyrosequencing (discountinued) and Illumina sequencing are two most frequently used systems in recent research (Luo et al., 2012). The 454 pyrosequencing is an integrated emulsion-based and pyrophosphate-based sequencing method (Figure 3a). It uses luciferase in combination with luciferin and adenosine triphosphates (ATP) (which generated from inorganic pyrophosphate (PPi) and adenosine 5' phosphosulfate (APS)) to detect the light that emitted from incorporated nucleotides of the sample DNA (Margulies et al., 2005; Medini et al., 2008; Park et al., 2013). The average read length of 454 pyrosequencing is approximately 250 bp, and it can reach up to 400 bp (Diaz-Sanchez et al., 2013). The Illumina sequencing technology (i.e., Illumina SOLEXA) is based on sequencing-by-synthesis chemistry (Figure 3b) which uses solid-phase amplification and fluorescent reversible terminators (Medini et al., 2008; Loman et al., 2012). The DNA is first being amplified to create clusters of 1,000 copies of single-stranded DNA on a solid surface using fold-back PCR. Sequencing is then performed using primers, DNA polymerase and 4 fluorescently labelled reversible terminating nucleotides.

An image of the fluorophores is captured after the incorporation of a nucleotide terminator. The first base is recorded and the dye is chemically removed, allowing a repetitive process to record each base. The average read length is around 75-150 bp (Medini et al., 2008; Werner et al., 2012; Diaz-Sanchez et al., 2013).

a. 454 Pyrosequencing technology



b. IlluminaSOLEXA technology

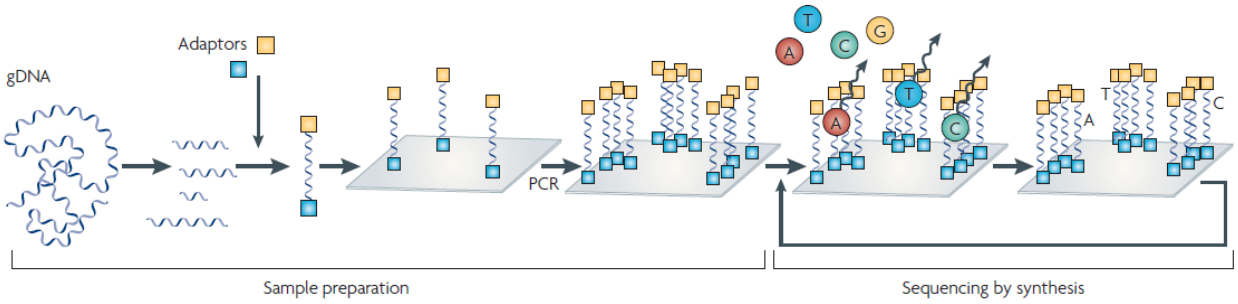


Figure 3. The next generation sequencing technologies. **a**. the 454 Pyrosequencing technology, and **b**. the Illumina SOLEXA technology (adapted from Medini et al., 2008, used with permission of Nature Publishing Group, Aug 20th, 2014 license number: 3453130891517).

When comparing the two NGS sequencing platforms, the 454 produces longer read length than the Illumina (Luo et al., 2012; Werner et al., 2012). However, this read length is still shorter than the average 16S rRNA length (i.e., 1550 bp) or the read length produced by the first generation Sanger sequencing (Clarridge, 2004; Luo et al., 2012). The shorter reads may reduce phylogenetic resolution in picking operational taxonomic units (OTUs) and affect the determination of evolutionary distance. Pair-end approach can be a solution to increase read length. This method sequences from both 5' and 3' ends and doubles the reads, which is now widely used on the Illumina platform (Werner et al., 2012). Regarding to the number of reads per run, the 454 platform produces far less reads when compared with the Illumina system (1 million vs. 1.5 billion per run, respectively, at the same cost). In specific, the Illumina HiSeq2000 platform generates more than 50 Gigabyte (Gb) per day, and 1.6 billion 100 bp pair-end reads in a period of 10.8 days. The Illumina MiSeq produces 1.5 Gb from 5 million 150 bp pair-end reads in a single day experiment (Caporaso et al., 2012). Currently, several 454 and Illumina technology based high-throughput sequencing instruments such as the 454 GS Junior (Roche) and Illumina MiSeq are available. They are laser-printer sized and can generate comprehensive data with modest cost and set-up (Loman et al., 2012). The characteristics between the 454 GS Junior and Illumina MiSeq benchtop instruments are in Table 2.

Table 2. The comparison of characteristics between the 454 GS Junior and Illumina MiSeq benchtop sequencing instruments (data from Medini et al., 2008; schadt et al., 2010; Loman et al., 2012; Diaz-Sanchez et al., 2013)

Item	454 GS Junior[1]	MiSeq[2]	References
Sequencing chemistry	Pyrosequencing	Polymerase-based sequence-by-synthesis	schadt et al., 2010; Diaz-Sanchez et al., 2013
Amplification approach	Emulsion PCR	Solid-phase amplification	Medini et al., 2008; schadt et al., 2010
Minimum throughput (Read length)	35 Mb (400 bases)	1,500 Mb (2 × 150 bases)	Loman et al., 2012
Time per run	8 h	27 h	Loman et al., 2012
Megabyte (Mb) per hour	4.4 Mb	55.5 Mb	Loman et al., 2012
List price, US$	108,000	125,000	Loman et al., 2012
Approximate cost per run, US$	1,100	750	Loman et al., 2012
Approximate cost per Mb, US$	31	0.5	Loman et al., 2012

[1]Roche 454 GS Junior pyrosequencing instrument

[2]Illumina MiSeq sequencing instrument

To date, only a few studies have investigated the poultry gastrointestinal microbiota by using the 16S rRNA high-throughput sequencing technology. Wei et al. (2013) analyzed chicken and turkey intestinal microbiome sequence data (3,184 and 1,345, respectively) from public GenBank and Silva databasese, and discovered 915 and 464 species-equivalent OTUs with 89% and 68% species level diversity coverage in chicken and turkey, respectively. Phylum of Firmicutes, Bacteroidetes and Proteobacteria were among the largest, accounting for more than 90% of the entire sequences in the GI tract of both species. The effects of antibiotic growth promoters: virginiamycin and tylosin, along with the coccidiostat monensin on broiler chicken cecal microbiome were studied by Danzeisen et al. (2011) using 16S rRNA pyrosequencing. The

population of *Roseburia, Lactobacillus* and *Enterococcus* were reduced ($P < 0.05$) while the *Coprococcus* and *Anaerofilum* were increased ($P < 0.05$) in the presence of coccidiostat monensin. An enrichment of *Escherichia coli* was seen in response to treatments that supplemented with monensin in combination of virginiamycin and tylosin. Similarly, Singh et al. (2013) evaluated the influence of penicillin as growth promoter on cecal microbiota of broiler chickens and observed an increased Firmicutes and a decreased Bacteroidetes when compared the microbiome sequence of penicillin supplemented group with the control. Videnska et al. (2013) examined the composition of chicken cecal microbiota by pyrosequencing the V3/ V4 regions of bacterial 16S rRNA. The results showed that cecal microbiota of 4 to 19 d old chickens were dominated by *Enterobacteriaceae, Lachnospiraceae* and *Ruminococcaceae* and *Lactobacillaceae.*

2.5 An overview of chicken immunology

2.5.1 Chicken immune system

Bird and mammals are evolved from a common reptilian ancestor around 200 million years ago and possess many common immunological systems. However, birds have also developed a number of unique features to accommodate immunological tasks (Davison, 2008). Similar to other vertebrates, the chicken immune system consists of innate (non- specific) and adaptive (specific/ acquired) immunity.

The innate immune system of chicken is characterized to have rapid responses without any memory of previous exposures, which is important in the earliest phases of microbial invasion in order to limit the spread of the pathogen (Medzhitov and Janeway, 1997; Juul-Madsen et al., 2008). Physical barriers are the first line to defend the host from pathogen invasion, which include the skin, the mucosal surface of the airways and the GI tract. Innate immune response

can also activate a variety of cells including heterophils, macrophages, dentritic cells, monocytes, mast cells, eosinophils, basophils and natural killer (NK) cells (Juul-Madsen et al., 2008; Munyaka, 2012). Innate immune cells express complement receptors which are referred to as pattern recognition receptors (PRR). The PRR (e.g. toll-like receptors, scavenger receptors and C-type lectin receptors) can recognize the conserved pathogen-associated molecular patterns (PAMP) of infectious microorganisms such as lipopolysaccharides (LPS) of gram-negative bacteria, lipoteichoic acids of gram-positive bacteria, lipoproteins and peptidoglycans of all bacteria (Medzhitov and Janeway, 1997; Juul-Madsen et al., 2008; Echeverry, 2012; Munyaka, 2012). In poultry, the heterophils to lymphocytes (H: L) ratio is a good indicator of stress in the environment. The number of heterophils increases while lymphocyte number decreases in response to stressors (Gross and Siegel, 1983). Heterophils as well as macrophages are capable of eliminating a number of pathogens due to their phagocytosis, chemotaxis and adhesion activities (Munyaka, 2012). Rapid gene expression changes in both pro- and anti- inflammatory cytokines and chemokines can be induced by heterophils and macrophages following receptor mediated phagocytosis (Kogut et al., 2005; Munyaka, 2012). Monocytes constitute 5 - 10% of peripheral blood leukocytes and can move quickly in response to infections and differentiate into macrophages and dendritic cells to assist the innate immune response. Dentritic cells are able to stimulate naïve T cells in antigen-specific immune response, and NK cells can recognize pathogens via Fc receptors that bind with immunoglobulin (Ig) G_1 and G_3 (Beal et al., 2006; Kaspers et al., 2008).

On the contrary, the adaptive immune system takes longer time to activate and is based on the memory of previous exposures. It can be classified to humoral (antibody-mediated) and cell-mediated immune response. Humoral responses are effective against extracellular antigens and

are mediated by B (bursa-derived) cells, whereas cell-mediated immune responses are specialized to remove intracellular antigens and are mediated by T (thymus-derived) cells (Erf, 2004; Scott, 2004). Primary lymphoid organs such as the thymus and the Bursa of the Fabricius are responsible for producing T and B lymphocytes, respectively (Scott, 2004). The Bursa of the Fabricius is only existed in birds and is located in the hind gut where connected to the cloaca. B cells proliferate and differentiate in the Bursa at 10-14 d of embryonic life, and migrate to secondary lymphoid organs such as spleen, cecal tonsil and gut-associated lymphoid organs after d 18 (Reynaud et al., 1992; Scott, 2004; Beal, 2006; Echeverry, 2012). B cells are predominately responsible for the production of antibodies. The activation of B cells depends on specific antigens, and the assistance from $CD4^+$ (helper T) cells via cytokines and co-stimulatory molecules (Toivanen et al., 1972; Beal et al., 2006; Echeverry, 2012). Upon activation, B cells form plasma cells which are capable to secrete antibodies to the surrounding environment (Beal et al., 2006). Both B and T cells require antigens to be presented on cell surface proteins as known as major histocompatibility complex (MHC) class I or II in order to be recognized. T cells play dominant role in adaptive immunity (Erf, 2004). Two different groups of T cells: T cytotoxic (Tc, $CD8^+$) and T helper (Th, $CD4^+$) lymphocytes perform various functions that modulate the immune response. Antigenic peptides presented on MHC class I activate $CD8^+$ cells and further lead to the lysis of infected host cells. Similarly, antigenic peptides presented on MHC class II mature naïve $CD4^+$ and develop these cells into functional effector cells. There are two types of Th cells, Th1 and Th2. Th1 cells are effective to direct toward cell-mediated responses and produce interleukin (IL) -2, interferon (IFN)-ϒ and tumor necrosis factor (TNF) -ß. Th2 cells assist the development of humoral responses and produce IL -4, -5, -6, -10 and -13 (Erf, 2004; Natea et al., 2005; Beal, 2006; Munyaka, 2012).

2.5.2 Chicken gut immunity

The chicken gut serves as a crucial barrier between the internal and external environment. A specialized gut immune system is employed to defend the host from pathogens and is capable of differentiating harmless nutrients apart from pathogen derived antigens (Beal et al., 2006). The gut immune responses include chemical and cellular responses which are involved in both innate and adaptive immune systems (Beal et al., 2006). The gut-associated lymphoid tissues (GALT) which include the Bursa of the Fabricius, cecal tonsils, Meckel's diverticulum, Peyer's patches and sub-epithelial lamina propria, play a major role in producing lymphoid cells and relative products (Beal et al., 2006; Janardhana et al., 2009; Echeverry, 2012). A specialized lymphoepithelium that contains phagocytic M cells transports antigens from the intestinal lumen into GALT (Jeurissen et al., 1999). Different immune cells predominantly T and B lymphocytes, can be found in the mucosa of chicken intestine, along with NK cells, macrophages, plasma cells, dentritic cells, goblet cells and heterophils (Beal et al., 2006; Kogut, 2013). The majority of intestinal antibody-generating plasma cells can produce IgA protecting the intestinal epithelium from pathogens, which makes IgA the most abundant immunoglobulin in the intestine than IgG (Y) (predominant antibody against systemic infection) and IgM (first antibody produced upon infection) (Davison et al., 2006; Dankowiakowska et al., 2013).

The GI microbiota also greatly influences on the development and maintenance of a functional immune system (Gabriel et al., 2006; Lee and Lillehoj, 2011). Luminal bacteria can influence the development of the ultrastructure of the intestinal mucosa as well as the mucosal immune system (Lee and Lillehoj, 2011; Purchiaroni et al., 2013). The gut microbiota can directly interact with exogenous pathogenic bacteria by competitive exclusion or indirectly by stimulating the immune system (Purchiaroni et al., 2013). It can also regulate the production of

mucins from intestinal globlet cells, and directly limit the infection by adhering pathogens and segregate their metabolites (Linden et al., 2008; Purchiaroni et al., 2013). Germ-free mammals have less developed GALT, fewer cellular lymphoid follicles (Peyer's patches), reduced antibody production and reduced lamina propria when compared with conventionally raised animals, and are more susceptible to intestinal infections (Macpherson and Harris, 2004; O' Hara and Shanahan, 2006; Purchiaroni et al., 2013). Germ-free chickens have much lower serum IgG and IgA concentrations and have delayed immunological maturity due to a lack of antibody producing B cells stimulated by the gut microbiota (Parry et al., 1977; Lee and Lillehoj, 2011).

2.6 Antibiotic growth promoters and antibiotic alternatives in poultry industry

2.6.1 Antibiotic growth promoters (AGPs)

Antibiotics are commonly known for controlling bacterial infections. They can work correspondingly with the immune system to prevent bacterial growth and fight against infection. In the poultry industry, antibiotics have been supplemented in feed for subtherapeutic use in the past 60 years (Dibner and Richards, 2005). They act as growth promoters to improve growth performance, feed efficiency and production uniformity of the chickens.

Previous studies have investigated the mode of action and the benefits of antibiotic growth promoters (AGPs). Evidence showed that AGPs could interact with intestinal microbiota of the animals and act by modifying the gut bacterial composition (Dibner and Richards, 2005). The study of Coates et al. (1963) discovered that AGPs has no beneficial effects on the performance of germ-free birds, therefore favourable and direct effects of AGPs are accredited to antimicrobial activities that suppress the competition between the host and its intestinal microbes (Dibner and Richards, 2005). The bacterial population competes with the host for nutrients and

energy, produces toxic amino acid catabolites, increases mucin secretion and intestinal epithelial cell turnover rate, decreases fat digestibility and elevates inflammatory immune responses in the GI tract (Gaskins et al., 2002; Dibner and Richards, 2005). Antibiotic growth promoters, on the other hand, have been proposed to possess several mechanisms that can indirectly alleviate the adverse effects of gut microbiota and enhance performance, which include: (1) reducing microbial utilization of uptake nutrients, (2) decreasing the production of toxins and metabolites such as aromatic phenols, ammonia and bile degradation products by intestinal bacteria, (3) thinning of small intestinal wall and reducing the turnover of gut mucosa for better nutrient absorption, and (4) inhibiting sub-clinical infections of the GI tract (Feighner and Dashkevicz, 1987; Gaskins et al., 2002; Butaye et al., 2003). The targeted bacteria by AGPs are predominately gram-positive organisms, which can produce multiple toxic catabolites and involve in growth depression (Gaskins et al., 2002). In contrast, it is noticeable that some of the gram-positive anaerobes such as *Lactobacillus*, *Streptococcus* and *Enterococcus* are also being used as probiotics in the poultry industry to substitute AGPs (Lutful Kabir, 2009). These organisms may suppress growth in a clean environment due to their negative effects to the GI tract, but promote health and performance through competition with pathogens under undesirable circumstance (Gaskins et al., 2002; Lutful Kabir, 2009).

Although AGPs could bring favourable outcomes to the poultry industry, the emergence of antibiotic resistant mutants in bird's microbiota and the transference of those residual genes into humans has become an arising concern since the past four decades (Dibner and Richards, 2005; Van Immerseel et al., 2009; Gaggìa et al., 2010). More than 150 antibiotics are available for use as either therapeutic medicine for humans or animals (e.g., penicillin, virginiamycin, tylosin, tetracycline, ciprofloxacin, and erythromycin) or exclusively as AGPs for animal production

(e.g., pristinamycin, quinupristin, bambermycin, and avilamycin) (Butaye et al., 2003; Dibner and Richards, 2005; Diarrassouba, 2008). Approximately 90% of these antimicrobial agents are applied for subtherapeutic use to enhance growth performance or prevent disease, whereas only 10% are for clinical use to treat infectious diseases (Khachatourians, 1998). With respect to "survival of the fittest", microorganisms have succeeded in adapting for survival in response to these antibiotics. Foodborne illness caused by pathogens (e.g., *Campylobacter*, *Salmonella*, *E. coli* O157:H7, *Shigella*, *Listeria* and *Clostridium*) has dramatically increased due to the development of resistant genes and the heavy indiscriminate use of antibiotics in animal production (Diarrassouba, 2008). About 70% of bacteria that caused infections in humans have been shown resistance to at least one antibiotic (FDA, 2011). Consequently, antibiotics that were commonly used for therapeutic purpose became ineffective and the discovery of new treatments is facing great challenges. Centers for Disease Control and Prevention (2011) of the United States estimated that nearly 48 million Americans become sick and among those 128,000 are hospitalized and 3,000 die of foodborne diseases annually. In order to control the prevalence of resistant pathogen in the food chain and to improve human health conditions, it would be wise for poultry researchers and producers to discover and practice alternative ways that could replace or reduce antibiotics use in the production.

2.6.2 Antibiotic alternatives

In 1999, the Commission of the European Union (EU) banned AGPs (tylosin, spiramycin, bacitracin and virginiamycin) that are commonly used for treating human diseases in the animal producion. Although anticoccidials (ionophores) is still permitted in Denmark for the poultry prodution, EU has restricted antimicrobials use to only therapeutic doses under prescription (Dibner and Richards, 2005). In North America, the use of AGPs is relatively less regulated.

However, demands from the market and consumers are gradually forcing the American and Canadian poultry producers to phase out the AGPs.

According to WHO (2003), feed efficiency of broiler chickens has decreased by 2.3% after the termination of AGPs in Denmark. Inconsistent diet digestibility and increased variability on bird performance are the main results from the removal of AGPs. To improve this situation, poultry researchers are investigating new methods that could improve feed conversions to provide economic benefits. A number of strategies and substances could influence chicken gut microbiota and serve as antibiotic alternatives. In general, these candidates are capable of altering gastrointestinal pH, enhancing fermentation acids, selection for beneficial gut microbiota and against pathogens, improving nutrient uptake, and elevating the humoral immune response (Ferket, 2004). The category, examples and mode of action on the currently known strategies and substances are summarized in Table 3. However, these methods can only partially compensate for the removal of AGPs other than a complete replacement (Bedford, 2000). Further research is needed to investigate and discover the current and new strategies that can serve as antibiotic alternatives for the poultry industry.

Table 3. Current methods for the replacement of antibiotic growth promoters in poultry diets (partially adapted from Bedford, 2000)

Category	Examples	Hypothesis of mode of action
1. Limit nutrient availability to intestinal bacteria	• High quality ingredients	○ Higher quality of the raw materials provide greater digestibility of the diet and hence less substrate becomes available for bacterial growth. ○ Contain less antinutritional factors such as trypsin inhibitors and lectins, which reduce diet digestibility and damage gut epithelial tissue, respectively.
	• Whole grain cereals	○ Inclusion of whole cereal grains can stimulate gizzard development and can then be ground to smaller size by the gizzard, which result in more efficient digestion. ○ Can alter the patterns of fermentation in the ceca, resulting in higher concentrations of propionic acid and a reduction in *Salmonella* populations.
	• Feed processing	○ Cell wall disruption is a beneficial effect by high temperature and/ or pressure processing (pelleting, expansion or extrusion), which results in a greater exposure of the diet to the digestive enzymes along with the disruption of the structures of the starch and protein.
2. Improve the domination of beneficial bacteria species over pathogens	• Probiotics (live microfloral additives)	○ Populating bird's GI tract early in life with beneficial bacteria could influence the gut microflora, favour the colonization of beneficial rather than detrimental species and reduce the establishment of pathogens.
	• Prebiotics (fermentable sugars)	○ Provide nutrients that encourage the growth of beneficial bacteria, which could compete over pathogenic bacteria, thus, produce benefits on health and performance of the birds.
	• Feed sterilisation	○ By using high temperatures or adding acids to ensure that the bird does not inoculate detrimental bacteria in the feed. Primarily aimed at zoonotic organisms.
	• Lower nitrogen content of the feed	○ By balancing the diet with amino acids to limit excessive protein contents, and thus eliminate the growth of putrefactive organisms which utilize the undigested protein in the lower gut.
3. Improve immunity of the animals	• Vaccines	○ Stimulate the birds' own defences against target organisms to limit their ability to influence performance, health or the ability to transmit zoonotic organisms to humans.

2.7 Summary

Prebiotic fructooligosaccharide is a bioactive substance that may influence on nutrition, immune response, overall health and gut microbiota of the broiler chickens. It has been proven that FOS supplementation stimulates microbial fermentation and produces short-chain fatty acid, and thus acidifies the gastrointestinal tract (Wang et al., 2010; Bogusławska-Tryk et al., 2012). Several studies conducted on mice have demonstrated that FOS counteracted the deleterious effects of phytic acid by improving cecal absorption of minerals and stimulating the hydrolysis of phytate via fermentation by probiotic-like bacteria (Lopez et al., 2000; Wang et al., 2010). Phytase enzyme is commonly applied in the poultry industry to hydrolyze phtate-P and improve P utilization. Results have indicated that an acidic gut pH is favourable for mineral solubility as well as for phytase activity (Wyss et al., 1999; Selle et al. 2009; Naves et al., 2012). Therefore, the combination of FOS and phytase would have additive effects on improving growth performance, bone quality and P utilization of the broiler chickens.

The gastrointestinal microbiota plays important roles on nutrition, immunity and physiological systems of the chickens. Changes on the gut microbiota may affect feed efficiency along with the health and disease status of the birds. The discovery of bacterial 16S ribosomal RNA sequences offered new molecular tools on understanding the composition and diversity of gut microbiota, and also on evaluating the effects of growth promoting substances. Antibiotics have been widely applied in the poultry industry as growth promoters for subtherapeutic use, as they are capable of modifying the gut microbiota by suppressing the competition between the host and its intestinal microbes. The occurrence of resistant bacterial genes and the transference of these products into human food chain has become an increasing food safety concern in recent

decades. Fructooligosaccharide is one of the antibiotics alternative candidates that can shift the gut microbiota and modulate the immune system of the broiler chickens.

The hypothesis of experiment one was that FOS supplementation could increase phytase efficacy and the combination of the two could act additively to improve growth performance, bone quality and total P utilization in broiler chickens. The hypothesis of the second experiment was that the FOS-supplemented birds would maintain similar growth performance while having improved intestinal morphology, enhanced immune response, modulated cytokine gene profile and shifted ileal microbiota when compared to diet that supplemented with or without AGPs, and also in response to a *Salmonella* enteritidis lipopolysaccharides challenge.

3. MANUSCRIPT I

The effect of phytase and fructooligosaccharide supplementation on growth performance, bone quality and phosphorus utilization in broiler chickens

Y. Shang,[*] A. Rogiewicz,[*] R. Patterson,[†] B. A. Slominski,[*] and W. K. Kim[‡,1]

*Department of Animal Science, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada; †Canadian Bio-Systems Inc., Calgary, Alberta T2C 0J7, Canada; ‡Department of Poultry Science, University of Georgia, Athens, Georgia 30602-2772, U.S.A.

(Submitted to Poultry Science on June 18th, 2014)

3.1 Abstract

An experiment was conducted to investigate the effects of phytase and two levels of fructooligosaccharides (FOS) supplementation on growth performance, bone mineralization and phosphorus (P) utilization of broiler chickens. A total of 210-day-old-male broiler chickens (Ross) were randomly placed into 7 dietary treatments, consisting of 6 replicates with 5 birds per pen. The experiment was designed as an augmented 2 × 3 factorial arrangement with 0 or 500 U/kg of phytase and 0, 0.5% or 1% of FOS added to a reduced calcium (Ca) (0.8%) and available P (0.25%) diet (NC). A positive control diet (PC) contained 1% Ca and 0.45% available P was also included. During the entire experimental period, phytase supplementation significantly improved ($P < 0.05$) feed conversion ratio (FCR), BW gain (BWG) or feed intake. The use of phytase and FOS in combination showed a trend ($P < 0.10$) in improved BWG. Birds fed the PC diet showed significantly higher bone mineral density (BMD) and bone mineral content (BMC) in both femur and tibia bones ($P < 0.0001$) than those fed the NC diet. Phytase supplementation increased ($P < 0.05$) femur BMD, whereas FOS decreased femur BMD and BMC ($P < 0.05$). Phosphorus utilization was significantly higher for the NC diet ($P < 0.0001$). Phytase alone and in combination with 0.5% of FOS increased P utilization significantly when compared with other treatments ($P < 0.05$). Fructooligosaccaride, especially at the level of 0.5% increased P retention. In conclusion, phytase supplementation in low Ca and P diets improved growth performance, bone quality and P utilization. The combination of phytase and FOS increased BWG and P retention. However, supplementation of NC diets with phytase and FOS did not result in bone mineralization values comparable with that of the PC diet. The application of dietary FOS alone had a negative effect on broiler bone quality.

Key words: Phytase, fructooligosacchride, bone quality, phosphorus utilization, broiler chicken

3.2 Introduction

Broiler diets based on ingredients of plant origin contain large amounts of unavailable phosphorus (P) in the form of phytate-P, which is poorly hydrolyzed by the endogenous enzymes of monogastric animals (Ravindran et al., 1995). Dietary supplementation of inorganic phosphate is often necessary in order to meet available P requirements for poultry. However, the addition of inorganic P increases the cost of the feed and results in high concentrations of P in the manure. As a consequence, the animal waste that is applied on the soil leads to environmental pollution, allowing excreted P to wash into overland water systems, causing eutrophication (Boling et al., 2000; McGrath et al., 2005). Supplementation of exogenous phytase in broiler chicken rations has been proven to improve the hydrolysis of phytate-P, increase P digestibility, reduce P excretion into the environment and lower the cost of inorganic phosphate addition (Nahm, 2002; Knowlton et al., 2004; Coppedge et al., 2011; Powell et al., 2011). It is generally recognized that a 0.1% reduction of the available P content can be achieved with phytase supplementation, although as recently reviewed by Slominski (2011) approximately 0.05% of phytate P would only originate from poultry diets following phytase supplementation. Moreover, promising results have been observed on the growth performance of broiler chickens by supplementing phytase. For example, Simons et al. (1990) reported that the use of phytase increased bird performance and improved bone mineralization, while El-Sherbiny et al. (2010) examined broiler diets containing a reduced level of dicalcium phosphate and concluded that the addition of 500 U/kg phytase improved body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) of the birds from 23 to 40 d of age.

Fructooligosaccharides (FOS) are short-chain, non-digestible carbohydrates extracted from plant sources (e.g. chicory root, onion, beet and cane sugar), and are considered to have prebiotic

properties (Williams et al., 2008; Kim et al., 2011a). Because of their specific ß (2, 1) glycosidic linkage between fructose molecules, FOS cannot be broken down by the endogenous digestive enzymes of monogastric animals and thus become available for fermentation by intestinal microflora (Swiatkiewicz et al., 2011; Bogusławska-Tryk et al., 2012). Recent studies have shown that FOS supplementation stimulated the growth of beneficial bacteria such as *bifidobacteria* and *lactobacilli* but limited the growth of pathogenic bacteria such as *Salmonella spp.* and *Escherichia coli* in the gut of broiler chickens (Flickinger et al., 2003; Xu et al., 2003; Williams et al., 2008). Several studies have been conducted to evaluate the effect of FOS supplementation on growth performance of broiler chickens. For example, increased BWG and decreased FCR were reported by Ammerman et al. (1988), Bailey et al. (1991), and Xu et al. (2003). Variations in the levels of FOS supplementation may affect the growth rate and performance parameters of the birds (Yang et al., 2009). However, there is no well-defined recommendation for supplementing FOS to poultry diet.

Bone weakness and skeletal disorders such as tibial dyschondroplasia and rickets are existing problems associated with rapid bone growth in broiler chickens, leading to economic losses and animal welfare issues (Fleming, 2008; Kim et al., 2011b). Improved nutrient utilization and mineral absorption have positive influences on bone development and thus reduce the incidence of leg problems in broiler chicken production (Swiatkiewicz and Arczewska-Wlosek, 2012). Phytase addition has been demonstrated to have positive effects on bone ash content and bone mineralization in broilers fed low available P diets (Angel et al., 2006; Woyengo et al., 2008; Coppedge et al., 2011). El-Sherbiny et al. (2010) reported that phytase increased dietary Ca and P utilization, reduced Ca and P excretion and improved tibia breaking strength and tibia ash percentage in broiler chickens. Fructooligosaccharides also have the potential ability to increase

mineral bioavailability due to its effect on bacterial fermentation in the intestine (Gudiel-Urabano and Goni, 2002; Zafar et al., 2004; Ohta, 2006). Xu et al. (2003) indicated that a diet containing 0.4% FOS had positive effects on intestinal morphology in broilers, which may lead to improved mineral absorption. The growth of probiotic-like bacteria stimulated by FOS supplementation produces short-chain fatty acid (SCFA), resulting in acidification of the gastrointestinal tract (Wang et al., 2010; Bogusławska-Tryk et al., 2012). An acidic gastrointestinal pH is favourable for mineral solubility as well as for phytase activity (Selle et al. 2009; Walk et al., 2012). Studies conducted on mice by Lopez et al. (2000) and Wang et al. (2010) have demonstrated that FOS counteracted the deleterious effects of phytic acid by improving cecal absorption of minerals and stimulating the hydrolysis of phytate via fermentation by probiotic-like bacteria. Therefore, dietary FOS could potentially increase phytase efficacy by facilitating phytate hydrolysis and thus improve mineral utilization.

To date, no studies have been conducted examining P utilization and bone mineralization in broiler chickens fed diets supplemented with FOS alone or in combination with phytase. The hypothesis of this study was that FOS supplementation would increase phytase efficacy and the combination of the two would act additively to improve growth performance, bone quality and total P utilization in broiler chickens. The objectives of this study were: 1) to determine the interaction between phytase and FOS on P utilization and skeletal integrity in broiler chickens fed adequate or low Ca and available P (reduced by 0.2 percentage points) diets and 2) to examine the optimum FOS inclusion level in broiler rations.

3.3 Materials and methods

Birds and Housing

One-day-old, male Ross × Ross 308 chicks were obtained from a local commercial hatchery (Carltons Hatchery, Grunthal, Manitoba, Canada). The chicks were housed in electrically heated Jamesway battery brooders (James Mfg. Co., Mount Joy, PA) for the first 4-day pre-experimental period with the temperature maintained at 32ºC. On d 5, birds were individually weighed and sorted into 5 weight classes. Groups of 5 birds, 1 from each weight class were then randomly assigned to 42 battery pens such that the average initial BW was similar across pens. During the experimental period, birds were housed in three electrically heated Alternative Design Super Brooders (Alternative Design Manufacturing & Supply, Inc., Siloam Springs, AR). The temperature was monitored daily and was gradually reduced until a temperature of 24 ºC was reached on day 21. Light was provided for 24h throughout the experimental period. The experimental protocol was approved by the University of Manitoba Animal Care Protocol Management and Review Committee, and birds were handled in accordance with the guidelines established by the Canadian Council on Animal Care (CCAC, 1993).

Dietary Treatments

Seven dietary treatments were randomly assigned to 6 replicate cages of 5 birds each. Composition and analyzed nutrient values of the experiment diets are shown in Table 4. The experiment was designed as an augmented 2 × 3 factorial arrangement with 0 or 500 U/kg Phytase (Bio-Phytase 5000G, Canadian Bio-Systems Inc., Calgary, Alberta, Canada) and 0, 0.5 or 1% of FOS (Nutraflora® P-95, Ingredion, Etobicoke, Ontario, Canada) in a low Ca and available P diet. A positive control (PC) diet contained adequate levels of Ca and available P. The 7 dietary treatments included: PC, wheat-corn-soybean meal-based diet containing 1% Ca and 0.45% of available P; Negative control (NC), wheat-corn-soybean meal-based diet containing 0.8% of Ca and 0.25% of available P; NC + Phytase; NC + 0.5% FOS; NC + Phytase

+ 0.5% FOS; NC + 1% FOS; and NC + Phytase + 1% FOS. A 0.3% of titanium dioxide (Aldrich-248576, Sigma-Aldrich, Oakville, Ontario, Canada) was incorporated into the diets as an indigestible marker. The positive control diet was fed to all the chickens for the first 4-day adaption period, and the experimental diets were provided from d 5 to 21. Water and feed were allowed *ad libitum*. The basal diet was formulated to meet or exceed the National Research Council nutrient requirements for broiler chickens (NRC, 1994).

Table 4. Composition and analysis of experimental diets (as-fed basis)

Item	PC[1]	NC[2]	NC + Phytase	NC + 0.5% FOS	NC + Phytase + 0.5% FOS	NC + 1% FOS	NC + Phytase + 1% FOS
Ingredient (% of diet)							
FOS[3]	-	-	-	0.5	0.5	1	1
Phytase (U/kg)[4]	-	-	500	-	500	-	500
Wheat	35.80	36.00	36.00	35.26	35.26	35.10	35.10
Corn	29.78	31.45	31.45	31.74	31.74	31.28	31.28
Soybean meal	20.46	19.36	19.36	19.58	19.58	19.52	19.52
Canola meal	4.25	5.00	5.00	4.72	4.72	4.70	4.70
Canola Oil	4.50	4.00	4.00	4.00	4.00	4.20	4.20
Limestone	1.38	1.46	1.46	1.46	1.46	1.46	1.46
Dicalcium phosphate	1.76	0.65	0.65	0.65	0.65	0.65	0.65
DL-Metionine	0.10	0.10	0.10	0.10	0.10	0.10	0.10
L-Lysine HCl	0.12	0.14	0.14	0.14	0.14	0.14	0.14
Threoninie	0.04	0.04	0.04	0.04	0.04	0.04	0.04
Mineral premix[5]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix[6]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Titanium dioxide[7]	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Calculated composition[8]							
ME (kcal/kg)	3,111	3,119	3,119	3,105	3,105	3,101	3,101
CP (%)	21.3	21.3	21.3	21.2	21.2	21.1	21.1
Ca (%)	1.00	0.80	0.80	0.80	0.80	0.80	0.80
Available P (%)	0.45	0.25	0.25	0.25	0.25	0.25	0.25
Met + Cys (%)	0.97	0.98	0.98	0.98	0.98	0.97	0.97
Met (%)	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Lys (%)	1.08	1.08	1.08	1.08	1.08	1.08	1.08
Thr (%)	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Analyzed composition							
CP (%)	21.0	20.7	20.7	20.6	20.6	20.8	20.8
Ca (%)	1.24	0.98	0.98	0.95	0.95	0.90	0.90
Total P (%)	0.73	0.54	0.54	0.56	0.56	0.53	0.53
Available P (%)	0.45	0.23	0.23	0.26	0.26	0.23	0.23
Phytase activity (U/kg)	42	65	544	107	545	99	539
DM (%)	90.3	89.3	89.4	89.3	89.3	89.1	89.6

[1]PC: Positive control, wheat-corn-soybean meal-based diet containing adequate Ca and available P (1% Ca and 0.45% available P).

[2]NC: Negative control, wheat-corn-soybean meal-based diet containing low Ca and available P (0.8% Ca and 0.25% available P).

[3]Nutraflora® P-95, Short-Chain Fructooligosaccharides (scFOS®), contains 4.5% of sugar (fructose + glucose + sucrose), 34.2% of GF_2 (glucose + 2 fructose), 48.9% of GF_3 (glucose + 3 fructose) and 12.4% of GF_4 (glucose + 4 fructose) on dry-matter basis. Ingredion, Etobicoke, Ontario, Canada.

[4]Bio-Phytase 5000G, Canadian Bio-Systems Inc., Calgary, Alberta, Canada. Wheat was used as a carrier for 0 or 500 U/kg of Phytase in diets to equal 100%.

[5]Supplied per kilogram of diet: Mn (manganese oxide), 70mg; Zn (zinc oxide), 80mg; Fe (ferrous sulfate), 80mg; Cu (copper sulfate), 10mg; Se (sodium selenite), 0.3mg; I (calcium iodate), 0.5mg; and NaCl (non-iodized white salt), 4.3g.

[6]Supplied per kilogram of diet: vitamin A, 8,250 IU; vitamin D_3, 3,000 IU; vitamin E, 30 IU; vitamin B_{12}, 0.013 mg; vitamin K, 2 mg; riboflavin, 6 mg; pantothenic acid, 11 mg; niacin, 41.6 mg; choline, 1300.8 mg; folic acid, 4 mg; biotin, 0.25mg; pyridoxine, 4 mg; thiamine, 4 mg; endox (anti-ox), 125 mg; dl-methionine, 500 mg; virginiamycin (Stafac-22), 11mg; and monensin sodium (Coban), 99 mg.

[7]Aldrich- 248576, Sigma-Aldrich Co. LLC., Ontario, Canada

[8]Concentrations were calculated based on NRC (1994) guidelines.

Growth Performance and Sample Collection

The BWG and FI for each pen were recorded on d 14 and d 21. The feed conversion ratio was calculated as g feed/g gain. Body weight gain, FI and FCR were corrected for mortality and were calculated for d 5-14, d 15-21 and the entire experimental period. The production index (PI) was calculated for the total experimental period using the following equation (Swiatkiewicz et al., 2011): PI = [body weight (kg) × survival (%)/ age (21d) × FCR (kg)] ×100

On d 21, forty-two birds (1 bird from each pen; 6 birds per treatment) were euthanized by cervical dislocation. Individual BW was recorded from all sacrificed birds. Left femur and tibia bones were collected for the analysis of bone mineralization parameters. The bones were cleaned of the attached tissue, wrapped in 1x phosphate buffered saline soaked cheesecloth and stored at -20ºC. Excreta samples from each pen were collected for 3 hrs and immediately stored at -20ºC. Care was taken during the collection to avoid contamination from feathers, feeds and foreign materials. The excreta samples were then frozen and freeze dried by a VirTis 25LL freeze-dryer (VirTis Co. Inc., Gardiner, NY) for P analysis.

Dual Energy X-Ray Absorptiometry

Bone mineral density (BMD), bone mineral content (BMC) and bone area (BA) of the femur and tibia bones were measured using a dual energy x-ray absorptiometry (pDEXA®, Norland Medical System, Inc. Fort Atkinson, WI). Quality assurance calibration was performed each time before scanning. The femur and tibia bones were placed in a standardized orientation in each scan. The detected BMD was normalized to a two-dimensional bone area instead of a true volume, which represented a combination of bone thickness and density, and was expressed as g/cm^2 (Schreiweis et al., 2005; Kim et al., 2012). All scans were obtained at a scout speed of 40

mm/sec and at a measure speed of 20 mm/sec, with the resolution of 1.0 mm × 1.0 mm. The bone mineralization parameters were corrected by total individual BW as a covariance.

Chemical Analysis

Experimental diets and excreta samples were finely ground and thoroughly mixed using a coffee grinder (CBG5 SmartGrind; Applica Consumer Products Inc. Shelton, CT). Dry matter of the diets and excreta samples was determined using the 934.01 method of AOAC (1990). Crude protein (N × 6.25) levels of the diet samples were determined using a Leco nitrogen analyzer (model NS-2000, Leco Corp., St. Joseph, MI). Samples for the analysis of Ca and total P were ashed at 600ºC for 12 h in a muffle furnace and digested in 1% HNO_3 and 5 N HCl according to AOAC (1990) method 990.08. Calcium and total P concentrations were measured using an inductively coupled plasma optical emission spectrometer (AES Vista, Varian Inc., Palo Alto, CA). Phytate P in the diet was determined as described by Haug and Lantzsch (1983). Available P was calculated as total P minus phytate P. Phytase activity was determined according to Slominski et al. (2007). The measurements of TiO_2 in the diets and excreta samples were carried out according to the method of Lomer et al. (2000), and titanium dioxide (TiO_2) levels were determined using a Varian inductively coupled plasma optical emission spectrometer. Diet and excreta TiO_2 and total P values were used to calculate apparent P digestibility (APD) using the following equation: APD (%) = 100 - [(TiO_{2Diet}/$TiO_{2Excreta}$) × (total $P_{Excreta}$/total P_{Diet}) × 100]. The retained and excreted P (% of diet) was calculated based on APD and expressed as actual total P content of the diet. P excretion was also calculated as g/bird of total P consumed and g/kg of P in excreta (DM basis).

Statistical Analysis

All data were subjected to one-way ANOVA as a completely randomized design using the GLM procedure of SAS (SAS software release 9.1, SAS Institute Inc. Cary, NC). A set of pre-planned orthogonal contrasts were applied to analyze the difference between PC and NC treatments and to determine the main effect of Phytase and FOS as well as their interaction. Treatment means were compared using Duncan's multiple-range test. Differences were considered significant at $P < 0.05$ and trends were considered at $P < 0.10$ (Wilkinson et al., 2014). Least square means were separated using a SAS macro pdglm800 (Saxton, 1998).

3.4 Results

Diets and Growth Performance

The analyzed diet compositions of P, Ca and phytase activity of the seven dietary treatments are listed in Table 1. All values were within acceptable ranges and are in agreement with calculated compositions. Small amounts of endogenous phytase activities were observed in all diets, which is due to the fact that phytase is naturally present in some feedstuffs (e.g., wheat).

During 5 to 14 d of age, no statistically significant effects on BWG and FI were observed for all treatments, which indicated that the two dietary supplements and the application of reduced level of Ca and available P did not make a significant impact on growth and feed consumption of the birds at their early age (Table 5). Negative control diet supplemented with phytase (NC + phytase) showed improved FCR when compared with NC, NC + 0.5% FOS and NC + 1% FOS diet, indicating that phytase had a significant effect ($P < 0.05$) in improving FCR of the birds during this period. From 15 to 21 d of age, phytase supplementation significantly increased BWG ($P < 0.05$) and FI ($P < 0.05$) of birds among the NC treatments (Table 6). The results of the entire experimental period (d 5-21) showed that phytase supplementation increased BWG (*P*

< 0.05) and decreased FCR ($P < 0.05$) of broiler chickens. In addition, treatments supplemented with a combination of phytase and 1% FOS showed an increase in BWG ($P < 0.05$) and FI ($P < 0.05$) especially when compared to the NC diet containing 0.5% FOS and the NC diet, respectively, during the 15-21d period (Table 6) and the entire experiment period (Table 7). The production index (Table 7) calculated for the entire experimental period, and when taking into account mortality, age, BW and FCR values, demonstrated no statistical difference among the treatments. Growth performance of broilers fed NC diets did not differ from that of PC, which in part, may have resulted from the application of both FOS and phytase. Similarly, the two levels of FOS used in this study showed no significant difference in growth performance parameters of the birds. No interaction was detected between FOS and phytase in NC diets on growth performance parameters.

Table 5. Effect of phytase and fructooligosaccharides (FOS) supplementation on growth performance of broiler chickens from 5 to 14 days of age[1]

Item	Body Weight Gain (g/bird)	Feed Intake (g/bird)	Feed Conversion Ratio (g feed/g gain)
Diet			
PC[2]	290.9	380.1	1.31[ab]
NC[3]	272.9	364.7	1.34[a]
NC + Phytase[4]	291.5	369.7	1.27[b]
NC + 0.5% FOS	290.0	389.2	1.34[a]
NC + Phytase + 0.5% FOS	283.1	374.4	1.33[ab]
NC + 1% FOS	277.2	370.5	1.34[a]
NC + Phytase + 1% FOS	294.3	384.2	1.31[ab]
SEM[5]	2.90	3.49	0.008
Contrasts			
PC vs. NC	NS[6]	NS	NS
Phytase + FOS	NS	NS	NS
Effects among NC			
Phytase	NS	NS	0.029
FOS	NS	NS	NS
Phytase × FOS	NS	NS	NS
FOS 0.5% vs. 1%	NS	NS	NS

[1]Six replicates of five birds per each treatment.

[2]PC: Positive control, adequate Ca and available P (1% Ca and 0.45% available P).

[3]NC: Negative control, low Ca and available P (0.8% Ca and 0.25% available P).

[4]Supplemented 500 U/kg of diet.

[5]Standard error of the mean.

[6]NS: Not significant, $P > 0.10$.

[a-b]Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 6. Effect of phytase and fructooligosaccharides (FOS) supplementation on growth performance of broiler chickens during 15 to 21 days of age[1]

Item	Body Weight Gain (g/bird)	Feed Intake (g/bird)	Feed Conversion Ratio (g feed/g gain)
Diet			
PC[2]	391.3[a]	570.1[ab]	1.46
NC[3]	350.1[ab]	518.3[b]	1.48
NC + Phytase[4]	368.4[ab]	552.0[ab]	1.50
NC + 0.5% FOS	323.2[b]	526.6[ab]	1.69
NC + Phytase + 0.5% FOS	377.2[ab]	549.7[ab]	1.46
NC + 1% FOS	347.1[ab]	548.2[ab]	1.62
NC + Phytase + 1% FOS	395.2[a]	580.4[a]	1.47
SEM[5]	7.43	6.97	0.033
Contrasts			
PC vs. NC	NS[6]	NS	NS
Phytase + FOS	0.056	NS	NS
Effects among NC			
Phytase	0.012	0.048	0.090
FOS	NS	NS	NS
Phytase × FOS	NS	NS	NS
FOS 0.5% vs. 1%	NS	NS	NS

[1]Six replicates of five birds per each treatment.

[2]PC: Positive control, adequate Ca and available P (1% Ca and 0.45% available P).

[3]NC: Negative control, low Ca and available P (0.8% Ca and 0.25% available P).

[4]Supplemented 500 U/kg of diet.

[5]Standard error of the mean.

[6]NS: Not significant, $P > 0.10$.

[a-b]Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 7. Effect of phytase and fructooligosaccharides (FOS) supplementation on growth performance of broiler chickens during the entire experimental period[1] (5-21 days of age)

Item	Body Weight Gain (g/bird)	Feed Intake (g/bird)	Feed Conversion Ratio (g feed/g gain)	Production Index[2]
Diet				
PC[3]	682.3[ab]	950.2[ab]	1.39	477.6
NC[4]	623.1[ab]	883.0[b]	1.42	481.1
NC + Phytase[5]	659.9[ab]	921.7[ab]	1.40	498.9
NC + 0.5% FOS	613.2[b]	915.8[ab]	1.51	484.0
NC + Phytase + 0.5% FOS	660.3[ab]	924.1[ab]	1.40	500.3
NC + 1% FOS	624.2[ab]	918.7[ab]	1.49	484.2
NC + Phytase + 1% FOS	689.5[a]	964.6[a]	1.40	484.1
SEM[6]	9.08	9.28	0.016	4.16
Contrasts				
PC vs. NC	NS[7]	NS	NS	NS
Phytase + FOS	0.078	NS	NS	NS
Effects among NC				
Phytase	0.011	NS	0.037	NS
FOS	NS	NS	NS	NS
Phytase × FOS	NS	NS	NS	NS
FOS 0.5% vs. 1%	NS	NS	NS	NS

[1]Six replicates of five birds per each treatment.

[2]Overall mortality of each treatment at 21d: 6.7%, 0%, 0%, 3.3%, 0%, 3.3% and 6.7% (SEM=1.13), respectively.

[3]PC: Positive control, adequate Ca and available P (1% Ca and 0.45% available P).

[4]NC: Negative control, low Ca and available P (0.8% Ca and 0.25% available P).

[5]Supplemented 500 U/kg of diet.

[6]Standard error of the mean.

[7]NS: Not significant, $P > 0.10$.

[a-b]Means with different superscripts within a column differ significantly ($P < 0.05$).

Bone Quality

The results of mineralization parameters of femur and tibia bones showed great differences between PC and NC treatments (Table 8). Birds had significantly higher BMD and BMC ($P < 0.0001$) in the standard Ca and available P diet (PC) when compared with the low Ca and available P NC diets. These results suggest that a 0.2 percentage reduction of Ca and available P than the NRC recommended level was sufficient to reduce the bone quality, but the supplemented diets failed to bring up the bone mineralization to the same values as that of the PC diet. However, the addition of phytase improved femur BMD among the NC diets ($P < 0.05$). The NC diet supplemented with phytase alone exhibited higher femur BMD (0.1210 g/cm^2) compared to the other NC diets; higher femur BMC (0.7010 g) and tibia BMD (0.1165 g/cm^2) compared with the NC diet containing 0.5% of FOS (0.6157 g and 0.1030 g/cm^2, respectively). On the contrary, FOS supplementation exhibited negative effects among the NC treatments on femur BMD ($P < 0.01$) and BMC ($P < 0.05$), which indicates that FOS may not be a suitable supplement in broiler diets for maintaining or improving bone mineralization. The BA did not show much difference after adjusted by individual bird BW as a covariate, except the tibia area of NC diets was bigger than that of PC. No significant effects on bone parameters were observed between diets supplemented with 0.5% and 1% of FOS or on phytase × FOS interactions.

Table 8. Effect of phytase and fructooligosaccharides (FOS) supplementation on femur and tibia bone mineral density (BMD), bone mineral content (BMC) and bone area (BA) of broiler chickens[1]

Item	Femur			Tibia		
	BMD (g/cm^2)	BMC (g)	BA (cm^2)	BMD (g/cm^2)	BMC (g)	BA (cm^2)
Diet						
PC[2]	0.147[a]	0.862[a]	5.8	0.149[a]	1.190[a]	7.9[b]
NC[3]	0.112[bc]	0.674[b]	6.0	0.108[bc]	0.927[b]	8.6[a]
NC + Phytase[4]	0.121[b]	0.701[b]	5.8	0.117[b]	0.938 [b]	8.1[ab]
NC + 0.5% FOS	0.102[d]	0.616[c]	6.0	0.103[c]	0.879[b]	8.5[ab]
NC + Phytase + 0.5% FOS	0.110[cd]	0.655[bc]	6.0	0.107[bc]	0.850[b]	8.0[ab]
NC + 1% FOS	0.109[cd]	0.652[bc]	6.0	0.108[bc]	0.886[b]	8.2[ab]
NC + Phytase + 1% FOS	0.110[cd]	0.660[bc]	6.0	0.109[bc]	0.911[b]	8.4[ab]
SEM	0.0023	0.0131	0.04	0.0026	0.0200	0.09
Contrasts						
PC vs. NC	<0.0001	<0.0001	NS[5]	<0.0001	<0.0001	NS
Effects among NC						
Phytase	0.021	NS	NS	NS	NS	NS
FOS	0.006	0.038	NS	NS	NS	NS
Phytase × FOS	NS	NS	NS	NS	NS	NS
FOS 0.5% vs. 1%	NS	NS	NS	NS	NS	NS
Phytase + FOS vs. others	NS	NS	NS	NS	NS	NS

[1]Bone parameters were adjusted by total individual BW as a covariance of 6 replicates in each treatment, using least square means ± standard error of the mean. Average individual BW of each treatment (n=6): 817.1g, 779.1g, 717.3g, 764.5g, 780.3g, 734.1g, and 838.2g (SEM=16.12), respectively.

[2]PC: Positive control, adequate Ca and available P (1% Ca and 0.45% available P).

[3]NC: Negative control, low Ca and available P (0.8% Ca and 0.25% available P).

[4]Supplemented 500 U/kg of diet.

[5]NS: Not significant, $P > 0.10$.

[a-d]Means with different superscripts within a column differ significantly ($P < 0.05$).

Phosphorus Utilization

The effect of phytase and FOS supplementation on P utilization of broiler chickens is presented in Table 9. The apparent total tract P digestibility of birds significantly increased under low dietary Ca and available P content ($P < 0.0001$), whereas P excretion was significantly reduced ($P < 0.0001$). These results indicate that the PC diet contained total P content in excess of what can be utilized by broiler chickens. Similar to the growth performance and bone integrity results, phytase supplementation had a positive effect on improving APD and P retention of broiler chickens ($P < 0.05$), and significantly ($P < 0.05$) reduced the amount of P excretion (% of diet). Fructooligosaccharide increased P retention (% of diet; $P < 0.05$) among the NC diets, particularly at the inclusion level of 0.5% ($P < 0.05$), while the diet containing a combination of phytase and 0.5% FOS had significantly greater APD value (51.3%) in comparison with the NC diet (40.7%). Treatments with the combination of phytase and FOS (NC + phytase + 0.5% FOS and NC + phytase + 1% FOS) tended to improve (P = 0.08) both APD and P retention (% of diet). No interactions between FOS and phytase supplementation were observed on P utilization parameters.

Table 9. Effect of phytase and fructooligosaccharides (FOS) supplementation on phosphorus utilization in broiler chickens at 21 d of age[1]

Item	Apparent phosphorus digestibility (%)	P retention (% of diet)	P excretion		
			% of diet	g/bird of total P consumed	g/kg of DM excreta
Diet					
PC[2]	29.2[d]	0.213[c]	0.517[a]	4.72[a]	22.16[a]
NC[3]	40.7[c]	0.220[bc]	0.320[b]	2.77[b]	13.47[b]
NC + Phytase[4]	49.1[ab]	0.265[ab]	0.275[b]	2.60[b]	12.58[b]
NC + 0.5% FOS	46.1[abc]	0.258[abc]	0.302[b]	2.74[b]	13.01[b]
NC + Phytase + 0.5% FOS	51.2[a]	0.287[a]	0.273[b]	2.54[b]	12.01[b]
NC + 1% FOS	42.9[bc]	0.228[bc]	0.302[b]	2.79[b]	12.96[b]
NC + Phytase + 1% FOS	46.2[abc]	0.245[abc]	0.285[b]	2.61[b]	11.80[b]
SEM[5]	1.45	0.0066	0.0146	0.133	0.625
Contrasts					
PC vs. NC	<0.0001	0.028	<0.0001	<0.0001	<0.0001
Effects among NC					
Phytase	0.012	0.018	0.018	NS[6]	NS
FOS	NS	0.046	NS	NS	NS
Phytase × FOS	NS	NS	NS	NS	NS
FOS 0.5% vs. 1%	NS	0.021	NS	NS	NS
Phytase + FOS vs. others	0.080	0.080	NS	NS	NS

[1]Six replicates of five birds per each treatment.

[2]PC: Positive control, adequate Ca and available P (1% Ca and 0.45% available P).

[3]NC: Negative control, low Ca and available P (0.8% Ca and 0.25% available P).

[4]Supplemented 500 U/kg of diet.

[5]Standard error of the mean.

[6]NS: Not significant, $P > 0.10$.

[a-d]Means with different superscripts within a column differ significantly ($P < 0.05$).

3.5 Discussion

Effect of Dietary Phytase Supplementation

The results of dietary phytase supplementation in the present study agree with previous findings in that the addition of phytase to low Ca and available P diets is known to enhance growth performance of broilers (Woyengo et al, 2008; Coppedge et al., 2010; El-Sherbiny et al., 2010; Chung et al., 2013). However, Angel et al. (2005) reported that growth performance was not affected when reduced available P content from 0.45% to 0.35% and 0.35% to 0.25% during starter (1 to 18 d) and grower (18 to 32 d) phase, respectively, in phytase-supplemented broiler diets. Similar results were found by Silversides et al. (2004) and Walk et al. (2012) with a 0.1% reduction of available P. However, in the study of Woyengo et al. (2010), the performance of birds fed phytase-supplemented low-P (0.2% lower) diets did not reach the comparable values of the adequate-P diet. Our results showed that 500 U/kg phytase supplementation increased BWG and decreased FCR at 15-21 and 5-14 d of age, respectively, as well as for the entire experimental period. The possible explanation could be that an improvement in P retention and energy utilisation was induced by phytase supplementation. Several studies have shown that phytase improved AME_n values, ileal digestibility of P and the retention of P in broilers fed low Ca and available P diet (Ravindran et al., 2000; Woyengo et al., 2010). Pirgozliev et al. (2011) evaluated the net energy for production in broilers and demonstrated a 15.6% increase with phytase supplementation.

The bone mineralization results from the current study confirmed that low Ca and available P diets were indeed P deficient, which in turn impaired bone quality. Previous studies indicated that the tibia ash percentage and bone breaking strength of birds fed low Ca and available P diet were improved by phytase supplementation, however, the values were not equivalent to that of

the PC diet (Powell et al., 2008; Woyengo et al., 2008; El-sherbiny et al., 2010). Few studies have examined BMD and BMC of broilers fed phytase-supplemented diets by using a DEXA or other bone densitometers. Angel et al. (2006) reported that whole body and tibia BMD and BMC of 49-d-old birds were higher in diets with 0.2% available P and 600 U/kg of phytase, although lower than those fed the PC diet containing 0.3% available P. Chung et al. (2013) found similar results showing that phytase supplementation improved bird femur and tibia BMD and BMC when compared with birds fed the low-P control diet (available P reduced by 0.1%). In the present study, the addition of phytase had a positive effect on femur BMD among NC treatments, indicating that phytase supplementation increased the release of available P for more effective bone mineralization. However, the concentration of dietary available P was still inadequate for normal bone development. Thus, increased phytase efficacy in low Ca and available P diets would be expected to improve bone mineralization further.

Phytase supplementation as a means to improve P utilization is a common practice in poultry nutrition (Woyengo et al., 2008; El-sherbiny et al., 2010). As reviewed by Slominski (2011), numerous studies have elucidated the efficacy of phytase in improving the total tract P digestibility in broiler chickens, which indicate that the utilization of P could be improved with phytase supplementation by preventing the formation of insoluble Ca-phytate complexes in poultry diets (Woyengo et al., 2010). Our results showed that APD significantly increased by 8.4, 10.5 and 5.5 percentage points following phytase addition, when comparing the NC + phytase, NC + phytase + 0.5% FOS, and NC + phytase + 1% FOS diet with the NC diet, respectively. These results are in agreement with the findings of Ravindran et al. (2000) and Woyengo et al. (2010). In the same time, our results showed that phytase-supplemented diets also improved P retention and reduced P excretion when expressed in actual amounts of total P present in the diet,

which is in agreement with the findings of Ravindran et al. (2000) and Powell et al. (2008) who reported that phytase supplementation reduced P excretion.

Effect of Dietary FOS Supplementation

In the current study, FOS supplementation did not exhibit any significant effect on broiler chicken growth performance when fed the low Ca and available P diets. Research findings on the effect of FOS on growth performance parameters are inconsistent. Decreased FI and BWG, and improved FCR were found by Williams et al. (2008) with 0.6% FOS added to a standard broiler ration. Kim et al. (2011a) reported that 0.25% of FOS could be used as an alternative to antibiotic growth promoters to improve productivity in broilers of up to 28 d of age. Altered gastrointestinal fermentation intensity caused by different levels of FOS supplementation may partially explain the variations observed in broiler performance. It has been reported that excessive levels of FOS (1%) may cause diarrhea and generate carbon dioxide and hydrogen gases due to intensive fermentation in the gastrointestinal tract, thus decreasing production performance (Cummings et al., 2001; Xu et al. 2003). Xu et al. (2003) demonstrated that the addition of 0.4% FOS increased average daily gain and FCR in broilers, whereas 0.8% of FOS had no effect on those specific parameters. However, in the present study there was no significant difference in growth performance of birds fed 0.5% or 1% of FOS. Moreover, individual birds may react differently to FOS supplementation, since the composition of gut microflora induced by FOS supplementation varies in different individuals and under different FOS inclusion rates (Williams et al., 2008). Therefore this inherent variability could lead to different rates of SCFA production and different levels of intestinal immune system stimulation, which may eventually result in the performance variations observed in the current study. The reason for the reduced FCR but not FI in FOS-supplemented diets may be attributed to a reduced

BWG caused by FOS ingestion. In a study with rats, it was demonstrated that dietary FOS decreased abdominal fat tissue weight and intestinal mesenteric fat mass, while improving insulin sensitivity (Shinoki and Hara, 2011). Moreover, the SCFA production stimulated by FOS may suppress body fat accumulation, change mesenteric adipocyte property and lower energy intake. Therefore, it would be interesting to investigate the effect of FOS on intestinal fat mass and related gene expressions in future studies.

Dietary FOS supplementation had a negative effect on femur BMD and BMC in birds fed low Ca and available P diets, which indicates that there are no beneficial but rather deleterious effects of FOS on bone mineralization in broiler chickens. Limited literature data is available on the effect of FOS on bone parameters in poultry. Kim et al. (2006) investigated bone breaking strength and mineralization parameters in laying hens fed a FOS-supplemented (0.75%) alfalfa molting diet, and concluded that tibia breaking strength was comparable to control hens. However, DEXA results showed that BMD and BMC of femur and tibia bones for the FOS-supplemented diet did not reach the value of hens fed the control diet. Similar to our findings, Kim et al. (2011b) reported that FOS did not show any beneficial effects on bone growth and skeletal integrity of broilers fed diets adequate in Ca and available P levels and supplemented with 2% or 4% of FOS. In a study with prebiotic fructans added at 0.1% to the deficient Ca and available P diets, Swiatkiewicz et al. (2011) observed that the Ca and available P levels did not affect growth performance but negatively influenced bone biomechanical parameters of broilers at 21 and 42 d of age, and that the supplemental fructans did not improve growth performance or bone quality. These findings are contradicted by studies with rats, which demonstrated increased mineral absorption and bone mineralization by dietary FOS (Lopez et al., 2000; Zafar et al., 2004; Ohta, 2006). Thus, there may be inter-species differences in the beneficial effects of FOS

supplementation (Kim et al., 2011b). Phosphorus utilization data may elucidate the mechanism of mineral utilisation. In our study, FOS showed no significant effect on improving APD or reducing P excretion, however it demonstrated a positive effect when P retention is expressed as actual P content of the diet. These data indicated that FOS supplementation can potentially increase the amount of total P retained in the body. Treatments with FOS alone, especially with 0.5% of FOS demonstrated a moderate increase in P digestibility and retention but reduced growth performance and bone mineralization when compared with the NC diet. It is possible that FOS supplementation increased the composition of gram-positive bacteria (e.g., *Lactobacillus*, *Streptococcus* and *Enterococcus)* via fermentation in the gastrointestinal tract (Lutful Kabir, 2009; Swiatkiewicz et al., 2011). However, these organisms may compete with the host other than with the pathogenic bacteria for nutrients and energy under a clean environment with low Ca and P content (Gaskins et al., 2002). The fermented intestinal microbes may have utilized the uptake nutrients and increased intestinal epithelial cell and bone turnover rate. Thus the ingested minerals and energy were being used for maintenance such as body fat and bone mineral restoration instead of being absorbed for BW gain or bone mineralization.

Additive Effects of Phytase and FOS

The combination of phytase and 1% of FOS showed significant improvement in FI and BWG of birds from 15 to 21 d of age and the entire trial (5-21 d), particularly when compared with the NC and NC + 0.5% FOS diets. When compared with the other treatments, supplemental phytase along with FOS (i.e., NC + phytase + 0.5% FOS and NC + phytase + 1% FOS) exhibited a positive trend in improving BWG during the same periods ($P = 0.056$, $P = 0.078$, respectively). Increased APD and P retention was observed for the diet containing phytase and 0.5% of FOS when compared to the NC diet and the diet containing 1% of FOS. Diets containing both phytase

and FOS also tend to improve APD and P retention (% of diet) (P = 0.080). These results may suggest that FOS and phytase would have additive effects in promoting growth performance and P utilization in broiler chickens. Addition of FOS to the phytase-supplemented diet may further facilitate phytate hydrolysis by prohibiting the formation of Ca-phytate complexes and improving digestive enzyme activities. In the present study, the PC diet had a Ca to available P ratio of 2.8:1, whereas the NC diets had a Ca to available P ratio of 4:1. Wider Ca to available P ratios may lead to formation of insoluble Ca-phytate complexes in the intestine, which could lower the exogenous phytase efficacy and reduce the availability of dietary Ca and P (Selle et al., 2009). The growth performance and APD data for phytase + FOS treatments (i.e., NC + phytase + 0.5% FOS and NC + phytase + 1% FOS) reached or exceeded the values for controls, which revealed that FOS and phytase may have a synergistic effect in alleviating the negative impact of diets with a wide Ca to available P ratio. Furthermore, it has been documented that intestinal bacteria colonization induced by FOS increased amylase, protease and other digestive enzyme activities in broilers (Xu et al. 2003). In addition, phytase has been reported to improve amino acid, fat, protein and starch digestibility (Selle and Ravindran, 2007; Pirgozliev et al., 2011). These functions may act collectively in improving energy utilisation, counteract harmful properties of phytate, and thus contribute to the positive effects observed in the current study. On the contrary, the combination of FOS and phytase did not result in any beneficial effects on bone quality, indicating that although the supplemented diet showed trends in improving weight gain and increasing nutrient retention of birds, the minerals absorbed from the diets were not transformed into bone mineral content. However, the mechanism remains unclear and further research is needed.

In summary, our results confirmed that phytase improved growth performance, bone quality and P utilization in broiler chickens fed diets with 0.2% reduction in Ca and available P contents. Dietary FOS supplementation demonstrated negative effect on bone mineralization, thus it may not be used as a suitable supplement for enhancing bone quality in broilers. Supplementation of phytase and FOS in the low Ca and available P diet failed to improve the bone mineralization parameters to the same level as the adequate Ca and available P diet. The combination of phytase and FOS improved BWG and had a positive effect at improving P utilization. Further research is needed to explain the mechanisms associated with dietary FOS supplementation and the synergetic effect of phytase and FOS in broiler ration.

3.6 Acknowledgements

This project was financially supported by NSERC-Engage grant. The authors thank Dr. G. Crow, H. Muc and T. Davie (University of Manitoba, Winnipeg, MB, Canada) for providing excellent statistical, technical and analytical assistance, respectively. The authors also appreciate the generous support from Canadian Bio-Systems and Ingredion, respectively, for providing phytase and FOS for this research.

4. MANUSCRIPT II

The effect of dietary fructooligosaccharide supplementation on growth performance, immune response, intestinal morphology and ileal microbiota with or without *Salmonella* Enteritidis lipopolysaccharide challenge in broiler chickens

4.1 Abstract

The dietary effects of fructooligosaccharide (FOS) supplementation as an alternative to antibiotic on growth performance, intestinal morphology and ileal microbiota, as well as immunological effects in response to *Salmonella* Enteritidis lipopolysaccharides (LPS) challenge were investigated in broiler chickens (n = 180). The study was based on a 3 × 2 factorial arrangement which included 1) three dietary treatments from d 1 to 21: positive control (PC), wheat-corn-soybean meal based diet contain antibiotics (virginiamycin and monensin); negative control (NC), as a wheat-corn-soybean meal based diet without antibiotics; and NC + FOS, at 0.5%; and 2) immunological challenge on d 21: intraperitoneal injection with 2mg/kg of *Salmonella* Enteritidis (SE) LPS or sterile phosphate buffered saline (PBS). Feed intake, BW gain, feed conversion ratio, mortality and relative lymphoid organ weight did not exhibit significant difference among the treatments. Villus height, crypt depth and total mucosa thickness were significantly increased ($P < 0.05$) in the ileum of broiler chickens that were fed NC + FOS. No significant difference on α-, ß-diversity and bacterial phyla in ileal microbiota was observed between mucosa and digesta or between the three dietary treatments. However, partial least square discriminant analysis and Venn analysis showed that different bacterial genera were associated with different ileal sites or diets. The immunological challenge demonstrated significant difference on relative heterophils and lymphocytes concentrations, as well as serum immunoglobulin (Ig) G and IgA levels. NC + FOS group reduced heterophil but increased monocyte count when compared to NC ($P < 0.05$). Diet × challenge interaction was observed in IgG measurements ($P < 0.001$). Natural IgG and IgA, and specific IgG levels were elevated in NC + FOS group under the LPS challenge. Supplemental FOS has also up-regulated ileal IL-1ß, -2, -10, -18, TLR-4, IFN-γ and splenic IL-18, IL-1ß expressions in PBS groups (P <

0.05), and these gene exhibited immunological challenge effects upone LPS challenge ($P < 0.05$). In conclusion, SE LPS challenge established significant difference towards the immune responses of broiler chickens. Fructooligosaccharides supplementation improved ileal mucosa thickness, shifted ileal microbiota, and also elevated cytokine gene expressions, altered leukocytes composition and serum IgG, and IgA levels in respond to LPS challenge, and it may have a protective role on gut health and immunity of broiler chickens.

Keywords: fructooligosaccharide, *Salmonella* Enteritidis LPS, immune response, gut microbiota, broiler chicken

4.2 Introduction

In the poultry industry, antibiotics have been supplemented in feed for subtherapeutic use in the past 60 years. They act as growth promoters to improve growth performance, feed efficiency and production uniformity of the chickens. Evidence showed that AGPs could interact with intestinal microbiota of the animals and act by modifying the gut bacterial composition (Dibner and Richards, 2005). The study of Coates et al. (1963) discovered that AGPs has no beneficial effects on the performance of germ-free birds, thus, favourable and direct effects of AGPs are accredited to antimicrobial activities that suppress the competition between the host and its intestinal microbes (Dibner and Richards, 2005). However, heavy indiscriminate use of antibiotics may lead to the emergence of antibiotic resistant mutants, and these genes will further transfer into humans, causing foodborne illness (Van Immerseel et al., 2009; Gaggìa et al., 2010). Finding out alternative dietary supplementations that perform the same as antibiotics will be beneficial and essential for the broiler production.

Fructooligosaccharides (FOS) are one of the common types of prebiotics, which consist of short-chain and non-digestible carbohydrates (Świątkiewicz and Arczewska-wlosek, 2012). Previous studies with broiler chickens have shown that dietary supplementation of FOS has the ability to improve growth performance (Ammerman et al., 1988; Bailey et al., 1991; Yusrizal and Chen, 2003), enhance innate and acquired immune response (Khodambashi Emami et al., 2012), improve intestinal mucosa structures (Xu et al., 2003) and positively change the gut microbiota (Bailey et al., 1991; Yusrizal and Chen, 2003; Xu et al., 2003; Kim et al., 2011). Dietary FOS could stimulate gut fermentation of beneficial bacteria such as *bifidobacteria* and *lactobacilli* and limit the colonization of pathogenic bacteria such as *Salmonella spp.* and *Escherichia coli* (Bogusławska-Tryk et al., 2012), thereby improving the overall health of the birds. *Salmonella spp.* is one of the major foodborne bacteria associating with human illness and is commonly present in the broiler production. Dietary FOS supplementation has the potential to elevate the anti-*salmonella* activity, which is mainly due to the shift of intestinal microbiota and the production of short-chain fatty acids (SCFA) (Van Immerseel et al., 2009). Bailey et al. (1991) reported that treatments with FOS showed a four-fold reduction of *Salmonella* in chicken ceca. FOS also has indirect effects toward the immune system of chickens by promoting the growth of lactic acid producing bacteria (Xu et al., 2003). In general, supplementing dietary FOS may result in improved immunity and reduced susceptibility to pathogen colonization in broiler chickens.

The gastrointestinal microbiota plays important roles in nutrition, immunity and physiological systems of the chickens. Changes in the gut microbiota may affect feed efficiency along with the health and disease status of the birds (Gaskins et al., 2002; Jeurissen et al., 2002; Kohl, 2012). The gastrointestinal microbiota can be divided into two sub-classes: the luminal

microbiota and the mucosal-attached microbiota. The composition of luminal microbiota can be influenced by the available nutrients, the feed passage rate and the effects of antimicrobial substances. The composition of mucosal-attached microbiota is determined by the host's expression of specific adhesion sites on the enterocyte membrane, the surrounding luminal microorganisms, the mucus production rate and the immunoglobulin secretion intensity, which are important for pathogen control, immune modulation and nutrients absorption (Gong et al., 2002; Jeurissen et al., 2002). Nowadays, the 16S rDNA high-throughput next generation sequencing technology is able to obtain genus level information of the gut microbiota (Diaz-Sanchez et al. 2013).

To date, only a few studies have been conducted *in vivo* to examine dietary FOS supplementation as an antibiotic alternative on immune responses and its modification of gut microbiota. No previous study has investigated the ileal mucosal-attached and luminal microbiota together with the AGPs and prebiotic effects by using high through-put sequencing. The hypothesis of this study was that the FOS-supplemented birds would maintain similar growth performance while have improved intestinal morphology, enhanced immune response, modulated cytokine gene profile and beneficially shifted ileal microbiota when compared to diets that supplemented with or without AGPs, and also in response to a *Salmonella* enteritidis lipopolysaccharides (LPS) challenge. The objectives were: 1) to investigate the changes in growth performance, intestinal morphology and ileal microbiota of broiler chickens by dietary FOS supplementation, and 2) to evaluate the effects of dietary FOS supplementation and *Salmonella* enteritidis LPS induced immune responses in broiler chickens.

4.3 Materials and methods

Birds and Housing

A total of 180, 1-d-old, male Ross × Ross 308 broiler chicks were obtained from a commercial hatchery (Carltons Hatchery, Grunthal, MB, Canada). The chicks were housed in electrically heated Petersime battery brooders (Petersime Incubator Company, Gettysburg, OH) for 21 days. The temperature was monitored daily and was gradually reduced from 32 ºC to 24 ºC from d 1 to 21. Light was provided for 24h throughout the experimental period. Upon arrival, the birds were individually weighed and sorted into 5 weight classes. Groups of 5 birds, 1 from each weight class were then randomly assigned to 36 battery pens such that the average initial BW was similar across pens. The experimental protocol was approved by the University of Manitoba Animal Care Protocol Management and Review Committee, and birds were handled in accordance with the guidelines established by the Canadian Council on Animal Care (CCAC, 1993).

Experimental Design

Six treatments groups were randomly assigned to 6 replicate cages of 5 birds each. The experiment was designed as a 3 × 2 factorial arrangement based on main factors. The main factors include: 1) Three dietary treatments that were fed from d 1 to 21, which included positive control (PC), wheat-corn-soybean based diet containing antibiotics (virginiamycin and monensin); negative control (NC), as wheat-corn-soybean based diet without antibiotics; and NC + FOS supplemented at 0.5% (Nutraflora® P-95; Ingredion, Etobicoke, ON, Canada), and 2) Immunological challenge on d 21: intraperitoneal injection with 2 mg/kg of *Salmonella* Enteritidis lipopolysaccharides (LPS) (source strain: ATCC 13076; Sigma-Aldrich, St. Louis, MO) or sterile phosphate buffered saline (PBS). The *Salmonella* Enteritidis LPS was dissolved in

PBS at the concentration of 1 mg/ml. Compositions of the dietary treatments are shown in Table 10. Water and feed were allowed *ad libitum*. The basal diet was formulated to meet or exceed the National Research Council nutrient requirements for broiler chickens (NRC, 1994).

Table 10. Composition of experimental diets (as-fed basis)

Item	Diet[1]		
	PC	NC	NC + FOS
Ingredient (% of diet)			
FOS[2]	-	-	0.5
Wheat	35.80	35.80	35.72
Corn	29.80	29.80	29.75
Soybean meal	20.54	20.54	20.42
Canola meal	4.45	4.45	4.20
Canola Oil	4.50	4.50	4.50
Calcium carbonate	1.38	1.38	1.38
Dicalcium phosphate	1.76	1.76	1.76
DL-Metionine	0.10	0.10	0.10
L-Lysine HCl	0.12	0.12	0.12
Threoninie	0.05	0.05	0.05
Mineral premix[3]	0.50	0.50	0.50
Vitamin premix[4]	1.00	1.00	1.00
Calculated composition[5]			
ME (kcal/kg)	3,118	3,118	3,106
CP (%)	21.4	21.4	21.2
Ca (%)	1.00	1.00	1.00
Available P (%)	0.45	0.45	0.45
Met + Cys (%)	0.98	0.98	0.97
Met (%)	0.51	0.51	0.50
Lys (%)	1.08	1.08	1.08
Thr (%)	0.81	0.81	0.80
Analyzed composition			
CP (%)	20.7	20.4	20.2
DM (%)	90.3	89.9	89.9

[1]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix.

Negative control (NC), wheat-corn-soybean meal base-diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

[2]Nutraflora® P-95, Short-chain Fructooligosaccharides (scFOS®), contained 4.5% of sugar (fructose, glucose, sucrose), 34.2% of GF_2 (glucose + 2 molecules of fructose), 48.9% of GF_3 (glucose + 3 molecules of fructose) and 12.4% of GF_4 (glucose + 4 molecules of fructose) on dry-matter basis. Ingredion, Etobicoke, ON, Canada.

[3]Supplied per kilogram of diet: Mn (manganese oxide), 70mg; Zn (zinc oxide), 80mg; Fe (ferrous sulfate), 80mg; Cu (copper sulfate), 10mg; Se (sodium selenite), 0.3mg; I (calcium iodate), 0.5mg; and NaCl (non-iodized white salt), 4.3g.

[4]Supplied per kilogram of diet: vitamin A, 8,250 IU; vitamin D_3, 3,000 IU; vitamin E, 30 IU; vitamin B_{12}, 0.013 mg; vitamin K, 2 mg; riboflavin, 6 mg; pantothenic acid, 11 mg; niacin, 41.6 mg; choline, 1300.8 mg; folic acid, 4 mg; biotin, 0.25mg; pyridoxine, 4 mg; thiamine, 4 mg; endox (anti-ox), 125 mg; and dl-methionine, 500 mg.

[5]Concentrations were calculated based on NRC (1994) guidelines.

Growth Performance and Sample Collection

The body weight gain (BWG) and feed intake (FI) for each pen were recorded on d 7, 14 and 21. The feed conversion ratio (FCR) was calculated as g feed/g gain. Mortality rates were recorded on a daily basis. Body weight gain, FI and FCR were corrected for mortality and were calculated for d 1-7 d 8-14, d 15-21 and the entire experimental period.

On d 21, four hours after the immunological challenge, 36 birds (1 bird from each pen; 6 birds per treatment) were obtained for blood collection. About 6 ml of blood were collected from the wing vein and were divided into 2 aliquots (3 ml each) in 4ml Vacutainer 7.2mg K_2 EDTA and 4 ml Serum tubes (BD, Franklin Lakes, NJ) for the determination of white blood cell (WBC) composition and serum immunoglobulin (Ig) A and G concentrations, respectively. After the blood collection, these birds were euthanized by cervical dislocation, and individual BW was recorded from all sacrificed birds. The lymphoid organs: spleen and bursa of fabricius were excised with the weight recorded. The relative immune organ weights were expressed as a percentage to the individual BW. Spleen and ileum tissues were aseptically excised, immediately frozen in liquid nitrogen and preserved at -80ºC for later determination of cytokine gene expressions by real-time quantitative polymerase chain reaction (qRT-PCR). A 2 cm segment from the median section of duodenum, jejunum and ileum were collected and preserved in buffered formalin (10% neutral buffered formalin; Sigma-Aldrich, St. Louis, MO) for further intestinal morphology observation. Ileal digesta was collected in sterile bags and snap freezed in liquid nitrogen at -80ºC for microbiome analyses by using a Illumina MiSeq platform (Illumina, CA, USA).

Intestinal Morphology Analysis

The formalin preserved duodenum, jejunum and ileum tissues were sectioned and stained with Alcian blue (pH 2.5). All three intestinal segments that came from the same sacrificed bird were presented on each glass slide (n = 36). The stained intestinal sections were used to measure villus height, crypt depth, villus height to crypt depth (VH: CD) ratio and total mucosal thickness. The villus height was measured from the tip of the villus to the top of the lamina propria and the crypt depth was measured from the villus-crypt axis to the tip of the muscularis mucosa (Munyaka et al., 2012). The total mucosal thickness was measured from the tip of the villus to the wall of the intestine, including the length of villus, crypt and muscularis mucosa. The intestinal morphology slides were examined with an Axio Scope A1 microscope (Carl Zeiss Microimaging GmbH, Jena, Germany) coupled with an Infinity 2 digital camera (Lumenera Corporation, Ottawa, ON, Canada). The captured images were analyzed and measured using Infinity Analyze software (Lumenera Corporation, Ottawa, ON, Canada).

White Blood Cell Differential

The blood samples collected in EDTA tubes were kept on ice and were submitted to the Veterinary Diagnostic Services at the Manitoba Agriculture, Food and Rural Initiatives (MAFRI, Winnipeg, MB, Canada) for white blood differential upon sampling. A total of 100 WBCs were counted by light microscopy at 100× magnification from stained blood smears. The relative percentage of heterophils, lymphocytes, monocytes, basophil and eosinophil were determined based on WBC morphology. The heterophils to lymphocytes (H: L) ratio was calculated from the counted cells.

Natural and Specific IgG and IgA Determination

The blood samples collected in serum tubes were clotted at room temperature for approximately 2 h. These samples were then centrifuged at 2,000× g for 15 min at 4 ºC and stored at -20 ºC. Sandwich IgG and IgA ELISA Quantification Set protocols (Bethyl Laboratories, Montgomery, TX) were followed to determine natural immunoglobulin concentrations. The serum samples were diluted to 1:10,000 and 1:1000 for natural IgG and IgA determination, respectively. Indirect ELISA protocols (Abcam, Toronto, ON, Canada) were followed to determine specific IgG and IgA levels in response to LPS. The *Salmonella* Enteritidis LPS (source strain: ATCC 13076, Sigma-Aldrich, St. Louis, MO) that used for immunological challenge was coated on microtiter plates (ELISA Starter Accessory Kit, Bethyl Laboratories, Montgomery, TX) at a final concentration of 50 µg/ml. The serum samples were diluted to 1:100 and 1:10 for specific IgG and IgA determination, respectively. A microplate spectrophotometer (Epoch, BioTek Instruments, Winooski, VT) was used to measure the absorbance at 450 nm. A 4-parameter logistic curve fit and a standard curve with chicken reference serum (Bethyl Laboratories, Montgomery, TX) absorbance was developed using Gen5 Microplate Data Analysis Software (BioTek Instruments, Winooski, VT) for natural IgA and IgG concentration measurements. The specific IgG and IgA levels were determined based on the optical density.

RNA Isolation, cDNA Synthesis and Quantitative Real-Time PCR

Total RNA from the ileum and spleen tissues were extracted using TRIzol reagent (Invitrogen, Life Technologies, Burlington, ON, Canada) according to the manufacturer's instruction. About 100 mg of tissue samples were thawed on ice and added to 1 ml of the TRIzol reagent. The samples were then homogenized in a Mini-BeadBeater-16 homogenizer (Bio Spec Products, Bartlesville, OK) at 3450 oscillations/min for 3 min. After extraction, the RNA pellets

were dissolved in 200 µl nuclease-free water (Ambion, Life Technologies, Burlington, ON, Canada) and total RNA concentrations were determined at an optical density of 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific, DE). All RNA samples were normalized to a concentration of 2 µg/ µl and purity were verified by evaluating the optical density ratio of 260 nm to 280 nm. The normalized total RNA was then reverse transcribed using a High Capacity cDNA synthesis kit (Applied BioSystems, Life Technologies, Burlington, ON, Canada) following the manufacturer's protocol, and the synthesized cDNA were stored at -20 ºC. Pairs of primers for chicken cytokine genes toll-like receptor (TLR)-4, interleukin (IL)-1ß, -2, -6, -8, -10, -18, and interferon (IFN)-Ɣ were designed and checked for target identity using GenBank from the National Centre for Biotechnology Information (NCBI). Quantitative real-time PCR (qRT-PCR) was performed in duplicate reactions including nuclease free water, the forward and reverse primers of each gene, cDNA and SYBR Green (Applied BioSystems, Life Technologies, Burlington, ON, Canada) as a detector on Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, ON, Canada). The data were generated using $\Delta\Delta C_t$ method by normalizing the expression of the target gene to a housekeeping gene (ß-actin) and the values were reported as fold changes of the expression of the target genes in the experimental groups compared with the control group (Regassa and Kim, 2013). Pairs of primers used for q RT-PCR assay and their sequences are presented in Table 11.

Table 11. Chicken cytokine primer sequences[1].

Gene[2]	Forward primer sequence	Reverse primer sequence	GenBank Accession No.	Product length (base pair)	Annealing temperature (ºC)
IL-1ß	CACAGAGATGGCGTTCGTTC	GCAGATTGTGAGCATTGGGC	NM204524	118	58
IL-2	CGTAAGTGGATGGTTTTCCTCT	GGCTAAAGCTCACCTGGGTC	NM204153	161	55
IL-6	TTCGACGAGGCAAGGAACC	AGGTCTGAAAGGCGAACAGG	NM204628	175	59
IL-10	GCTCTCCTTCCACCGAAACC	GGAGCAAAGCCATCAAGCAG	AJ621614	198	58
IL-18	ACTGCCAGAAGAGACATGGTG	CTCTGAGGGGTGTTCTGGTG	NM204608	143	56
IFN-Y	GCATCTCCTCTGAGACTGGC	GCTCTCGGTGTGACCTTTGT	NM205149	159	58
TLR-4	TCCGTGCCTGGAGGTAAGT	TGCCTTGGTAACAGCCTTGA	NM001030693	155	57
ß-actin	CAACACAGTGCTGTCTGGTGGTA	ATCGTACTCCTGCTTGCTGATCC	X00182	560	58

[1]The listed oligonucleotides were used to analyze intestinal gene expression via quantitative real-time PCR.

[2]IL = interleukin; IFN = interferon; TLR = Toll-like receptor.

Microbial Genomic DNA Extraction and Quality Check

Ileal tissue and digesta samples from broiler chickens injected with PBS were used for microbial sequencing (n = 6/treatment). Approximately 200 mg of ileal digesta and 100 mg of ileal mucosa scraped from the ileum wall were used for microbial genomic DNA extraction, using ZR Fecal DNA MiniPrep and ZR Tissue & Insect DNA MiniPrep kits (ZYMO Research, Irvine, CA), respectively. A bead-beating step for the mechanical lysis of the microbial cells was included by using a Mini-BeadBeater-16 homogenizer (Bio Spec Products, Bartlesville, OK) at 3450 oscillations/min for about 5 min. The extracted DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, DE). DNA samples were normalized to 20 ng/µl, and quality was checked by PCR amplification of the 16S rRNA gene using universal primers 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3') as described by Khafipour et al. (2009). Amplicons were verified by agarose gel electrophoresis.

Library Construction and Illumina Sequencing

Library construction and Illumina sequencing were performed as described by Derakhshani et al. (2014). In brief, the V4 region of 16S rRNA gene was targeted for PCR amplification using modified F515/R806 primers (Caporaso et al., 2012). The reverse PCR primer was indexed with 12-base Golay barcodes allowing for multiplexing of samples. PCR reaction for each sample was performed in duplicate and contained 1.0 µl of pre-normalized DNA, 1.0 µl of each forward and reverse primers (10 µM), 12 µl HPLC grade water (Fisher Scientific, ON, Canada) and 10 µl 5 Prime Hot MasterMix® (5 Prime, Inc., Gaithersburg, USA). Reactions consisted of an initial denaturing step at 94°C for 3 min followed by 35 amplification cycles at 94°C for 45 sec, 50°C for 60 sec, and 72°C for 90 sec; finalized by an extension step at 72°C for 10 min in an

Eppendorf Mastercycler® pro (Eppendorf, Hamburg, Germany). PCR products were then purified using ZR-96 DNA Clean-up Kit™ (ZYMO Research, Irvine, CA) to remove primers, dNTPs and reaction components. The V4 library was then generated by pooling 200 ng of each sample and quantified by Picogreen dsDNA (Invitrogen, Life Technologies, Grand Island, NY). This was followed by multiple dilution steps using pre-chilled hybridization buffer (HT1) (Illumina, San Diego, CA) to bring the pooled amplicons to a final concentration of 5 pM, measured by Qubit® 2.0 Fluorometer (Life technologies, Burlington, ON, Canada). Finally, 15% of PhiX control library was spiked into the amplicon pool to improve the unbalanced and biased base composition, a known characteristic of low diversity 16S rRNA libraries. Customized sequencing primers for read 1 (5´- TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3´), read 2 (5´-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3´) and index read (5´-ATTAGAWACCCBDGTAGTCCGGCTGACTGACT-3´) were synthesized and purified by polyacrylamide gel electrophoresis (Integrated DNA Technologies, Coralville, IA) and added to the MiSeq Reagent Kit V2 (300-cycle) (Illumina, San Diego, CA). The 150 paired-end sequencing reaction was performed on a MiSeq platform (Illumina, San Diego, CA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Canada.

Bioinformatics Analyses

Bioinformatic analyses were performed as described by Derakhshani et al. (2014). In brief, the PANDAseq assembler (Masella et al., 2012) was used to merge overlapping paired-end Illumina fastq files. All the sequences with mismatches or ambiguous calls in the overlapping region were discarded. The output fastq file was then analyzed by downstream computational

pipelines of the open source software package Quantitative Insights into Microbial Ecology (QIIME, Caporaso et al., 2010b). Assembled reads were demultiplexed according to the barcode sequences and exposed to additional quality-filters so that reads with more than 3 consecutive bases with quality scores below 1e-5 were truncated, and those with a read length shorter than 75 bases were removed from the downstream analysis. Chimeric reads were filtered using UCHIME (Edgar et al., 2011) and sequences were assigned to Operational Taxonomic Units (OTU) using the QIIME implementation of UCLUST (Edgar, 2010) at 97% pairwise identity threshold. Taxonomies were assigned to the representative sequence of each OTU using RDP classifier (Wang et al., 2007) and aligned with the Greengenes Core reference database (DeSantis, 2006) using PyNAST algorithms (Caporaso et al., 2010a). The OTUs that classified to kingdom Archaea were removed from downstream analysis. Venn diagrams (VENNY; Oliveros, 2007) were produced based on classified and unclassified genera obtained from the Greengenes Core reference database, demonstrating the number of shared and unique genera across the PC, NC and NC + FOS dietary treatments.

Within community diversity (α-diversity) was calculated based on OUT counts using QIIME to evaluate the biodiversity of the bacterial population at the genus level. Alpha rarefaction curve was generated using Chao 1 estimator of species richness (Chao, 1984) with 6 sampling repetitions at each sampling depth. An even depth of 18,585 and 20,820 sequences per sample for ileal mucosa and ileal digesta, respectively, was used for calculation of Shannon and Simpson diversity indices. To compare microbial composition between samples and among different dietary treatments, β-diversity was measured by calculating the weighted and unweighted Unifrac distances (Lozupone and Knight, 2005) using QIIME default scripts. Principal coordinate analysis (PCoA) was applied on resulting distance matrices to generate two-

dimensional plots using PRIMER v6 software (Warwick and Clarke, 2006, PRIMER-E Ltd, Plymouth). Permutational multivariate analysis of variance (PERMANOVA) (Li et al., 2012) was used to calculate *P*-values and test for significant differences of β-diversity among treatment groups.

Statistical Analysis

The dietary effects were analyzed using a one-way analysis of variance (ANOVA) of General Linear Models (GLM) procedure of SAS 9.2 (SAS Inst., 2001). Significant differences between the means of different treatment groups were determined by Duncan's multiple-range test. The effects of diet, immunological challenge and their interaction were subjected to a two-way ANOVA by using the GLM procedure of SAS 9.2 (SAS Inst., 2001). Alpha-biodiversity, major phylum and genus percentage data from the microbiome sequencing were also analyzed using the two-way ANOVA of GLM procedure of SAS 9.2 (SAS Inst., 2001), based on the dietary treatments and the two ileal sites (mucosa and digesta). All phyla were divided into two groups of abundance, above 1% of the population, and low-abundance, below 1% of the population. Differences between groups were considered significant at $P < 0.05$, and trends were considered at $P < 0.10$. Outliers were examined and removed using Grubbs' test at $\alpha < 0.05$ (Grubbs, 1965).

Statistical analyses for microbial sequences were performed as described by Li et al. (2012) and Derakhshani et al. (2014). In brief, partial least square discriminant analysis (PLS-DA; SIMCA P+ 13.0, Umetrics, Umea, Sweden) was performed on bacterial genera to identify the effects of dietary treatments. The PLS-DA is a particular case of partial least square regression analysis in which Y is a set of variables describing the categories of a predictor variable on X

(Pérez-Enciso and Tenenhaus, 2003). In this study, X variables were bacterial taxa and Y variables were observations that belong to different dietary groups (PC, NC or NC + FOS). For this analysis, genera which have a population lower than 0.002% were trimmed and data were scaled using Unit Variance in SIMCA. Cross-validation was performed to determine the number of significant PLS components and a permutation testing was conducted to validate the model. To avoid over parameterization of the model, variable influence on projection value (VIP) was estimated for each genus and genera with VIP < 0.50 were removed from the final model (Pérez-Enciso and Tenenhaus, 2003; Verhulst et al., 2011). R^2 estimate was used to evaluate the goodness of fit and Q^2 estimate was used to evaluate the predictive value of the model. The PLS-regression coefficients were used to identify genera that were most characteristics of each treatment group and the results were visualized by PLS-DA loading scatter plots.

4.4 Results

Dietary Effects

Growth performance and lymphoid organ weight. The three dietary treatments that contained antibiotics virginiamycin and monensin (PC), no antibiotics (NC), and NC diet supplemented with 0.5% of FOS did not exhibit any statistical difference ($P > 0.05$) in growth performance parameters such as FI, BWG, FCR and mortality during 1 to 7 d, 8 to 14 d, 15 to 21 d and the entire experimental period (Table 12). The relative weight of the spleen and the Bursa of Fabricius did not show significant differences ($P > 0.05$) among the dietary treatments (Table 13). The relative weight of the Bursa of Fabricius was above 0.2% of the body weight across all the dietary treatment groups.

Table 12. Feed intake, body weight gain, feed conversion ratio (FCR), and mortality of broiler chickens fed experimental diets for 21 days[1]

Item	Diet[2]				
	PC	NC	NC + FOS	SEM	*P*-value
Feed intake (g/bird)					
1 to 7 d	104.75	106.00	108.78	1.770	0.646
8 to 14 d	343.98	341.15	343.73	4.703	0.966
15 to 21 d	596.56	583.04	579.43	6.808	0.583
1 to 21 d	1045.29	1030.20	1031.93	11.851	0.866
BW gain (g/bird)					
1 to 7 d	80.50	83.73	85.36	1.540	0.446
8 to 14 d	243.74	239.95	241.94	2.974	0.886
15 to 21 d	414.52	406.73	405.76	4.944	0.752
1 to 21 d	738.76	730.41	733.06	8.191	0.923
FCR (feed:gain)					
1 to 7 d	1.303	1.268	1.276	0.0078	0.175
8 to 14 d	1.411	1.421	1.420	0.0066	0.797
15 to 21 d	1.440	1.434	1.428	0.0044	0.562
1 to 21 d	1.415	1.411	1.408	0.0037	0.736
Mortality (%)					
1 to 7 d	0	1.7	0	0.57	NA[3]
8 to 14 d	1.7	0	0	0.57	NA
15 to 21 d	0	0	0	0	NA
1 to 21 d	1.7	1.7	0	0.57	NA

[1]Values are the means of 12 replicates with 5 birds per pen.

[2]Positive control (PC), wheat-corn-soybean meal basal diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal basal diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

[3]NA = Not available.

Table 13. Relative lymphoid organ weights of broiler chickens at 21 days of age[1]

Item	Diet[2]				
	PC	NC	NC + FOS	SEM	*P*-value
Spleen Weight % [3]	0.086	0.093	0.100	0.0029	0.181
Bursa of Fabricius Weight %[4]	0.234	0.259	0.260	0.0087	0.416

[1]Values are the means of 12 birds per diet.

[2]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal base-diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

[3]Expressed as the percentage of spleen weight to individual BW, (spleen weight/individual BW) × 100.

[4]Expressed as the percentage of bursa weight to individual BW, (bursa weight/individual BW) × 100.

Intestinal morphology. The intestinal morphology measurements of villus height were significantly higher in the ileum of broiler chickens fed diet NC + FOS (P = 0.007), when compared with PC and NC (Table 14). Significantly higher crypt depth and total mucosa thickness were also observed in the ileum sections of NC + FOS fed group (P = 0.046 and P = 0.010, respectively), when compared with PC. However, no difference was observed on the VH: CD ratio in the ileum segments. The duodenum and jejunum did not exhibit any difference on villus height, crypt depth, VH: CD ratio or total mucosa thickness among all dietary treatment groups.

Table 14. Effect of fructooligosaccharide (FOS) on the small intestine morphology of broiler chickens at 21 days of age[1]

	Diet[2]				
Site	PC	NC	NC + FOS	SEM	*P*-value
Villus height (µm)					
Duodenum	1972.8	1903.1	2029.2	51.07	0.603
Jejunum	1454.8	1375.4	1363.3	40.64	0.628
Ileum	734.1[b]	787.1[b]	910.0[a]	23.48	0.007
Crypt depth (µm)					
Duodenum	214.3	213.0	226.1	7.07	0.708
Jejunum	202.7	189.4	181.9	5.59	0.362
Ileum	152.0[b]	175.1[ab]	192.3[a]	6.63	0.046
Villus height: crypt depth					
Duodenum	9.4	9.4	9.3	0.39	0.992
Jejunum	7.3	7.5	7.6	0.25	0.912
Ileum	4.9	4.6	4.8	0.14	0.628
Total mucosa thickness[3] (µm)					
Duodenum	2388.2	2322.8	2476.8	50.28	0.452
Jejunum	1876.9	1785.4	1780.0	47.44	0.663
Ileum	1110.5[b]	1223.6[ab]	1362.7[a]	34.19	0.010

[1]Means of one cross-section from each of the three intestinal segments per bird, 12 birds per diet, and 6 measurements of each villus height, crypt depth and mucosa thickness per cross-section for a total of 216 measurements for each of the intestinal segments per dietary treatment.

[2]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal base-diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

[3]Total thickness of villus, crypt and muscularis mucosa.

[a-b]Means with different superscripts within a row differ significantly ($P < 0.05$).

Ileal microbiota sample assessment. A total of 10,930,575 sequences were first generated from all of the ileal mucosa and digesta samples (n = 36), with a median sequence length of 253 bp. After quality filtering and chimeras checking, 1,121,616 reads were used for downstream analyses. The minimum, mean and maximum sequence reads were 18,585, 31,156 and 47,929, respectively, across all the ileal samples. One ileal mucosa sample from each NC and NC + FOS was identified as outlier, and was omitted for downstream microbial diversity and composition analyses.

Ileal microbiota α- and ß- diversity analyses. As shown in Table 15, the bacterial α-diversity of Shannon diversity index, Simpson diversity index, observed species, good coverage and Chao1 were calculated according to three dietary treatments (PC, NC and NC + FOS) and two ileal sample sites (mucosa and digesta). No significant difference ($P > 0.05$) was observed in dietary effects, ileal sites or site × diet interactions among all α-diversity parameters. The richness and diversity did not differ between ileal mucosa and digesta of broiler chickens. Although, the rarefaction plot of Chao 1 (Figure 4) showed that NC + FOS group numerically has higher operational taxonomic units (OTUs) and species richness in the ileal mucosa, followed by PC and NC groups. The rarefaction plot of the ileal digesta (Figure 5) showed that the NC and NC + FOS groups have higher number of OTUs than the PC group. The Principal coordinates analysis (PCoA) plots (Figure 6, A and B; Figure 7, A and B) analyzed by using Permanova for both Unifrac unweighted and weighted ß-diversity demonstrated no significant difference ($P > 0.05$) on ileal mucosa and digesta samples in each dietary group, respectively. However, the unweighted Unifrac analysis of ileal mucosa (Figure 6A) displayed a tendency ($P = 0.09$) of showing the distance on microbial community between PC and NC + FOS groups.

Table 15. Bacterial alpha diversity based on the main effects of diet and ileal site of broiler chickens at 21 days of age[1]

Site (S)	Ileal mucosa			Ileal Digesta				*P*-value			
Diet (D)[2]	PC	NC	NC + FOS	PC	NC	NC + FOS	SEM	S	D	S × D	Model
Item											
Shannon	6.09	5.55	6.95	5.65	6.15	6.64	0.240	0.916	0.223	0.644	0.570
Simpson	0.94	0.89	0.96	0.89	0.91	0.93	0.012	0.421	0.306	0.492	0.499
Observed species	1096	1034	1444	1107	1416	1443	84.317	0.451	0.269	0.593	0.513
Good coverage (%)	97.51	97.80	97.03	97.88	97.05	97.40	0.191	0.988	0.611	0.431	0.744
Chao1	1545.7	1381.5	1901.6	1491.1	1996.9	1864.7	118.222	0.477	0.474	0.452	0.608

[1]Values are the means of 6 replicate samples per treatment per site.

[2]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal base-diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

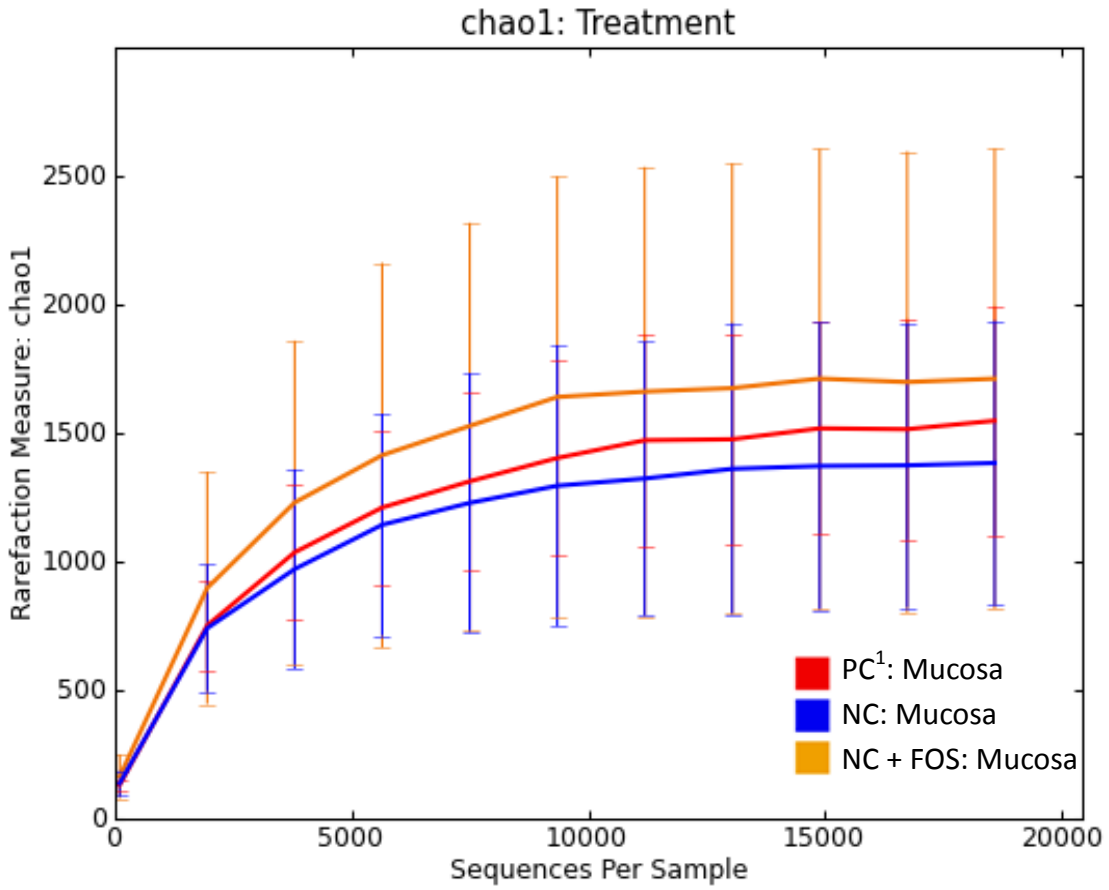


Figure 4. Rarefaction analysis on Chao1 estimates of species richness at an even sequencing depth of 18,585, in ileal mucosa samples of broiler chickens that fed PC, NC and NC + FOS diets[1] (n = 6/treatment).

[1]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal base-diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

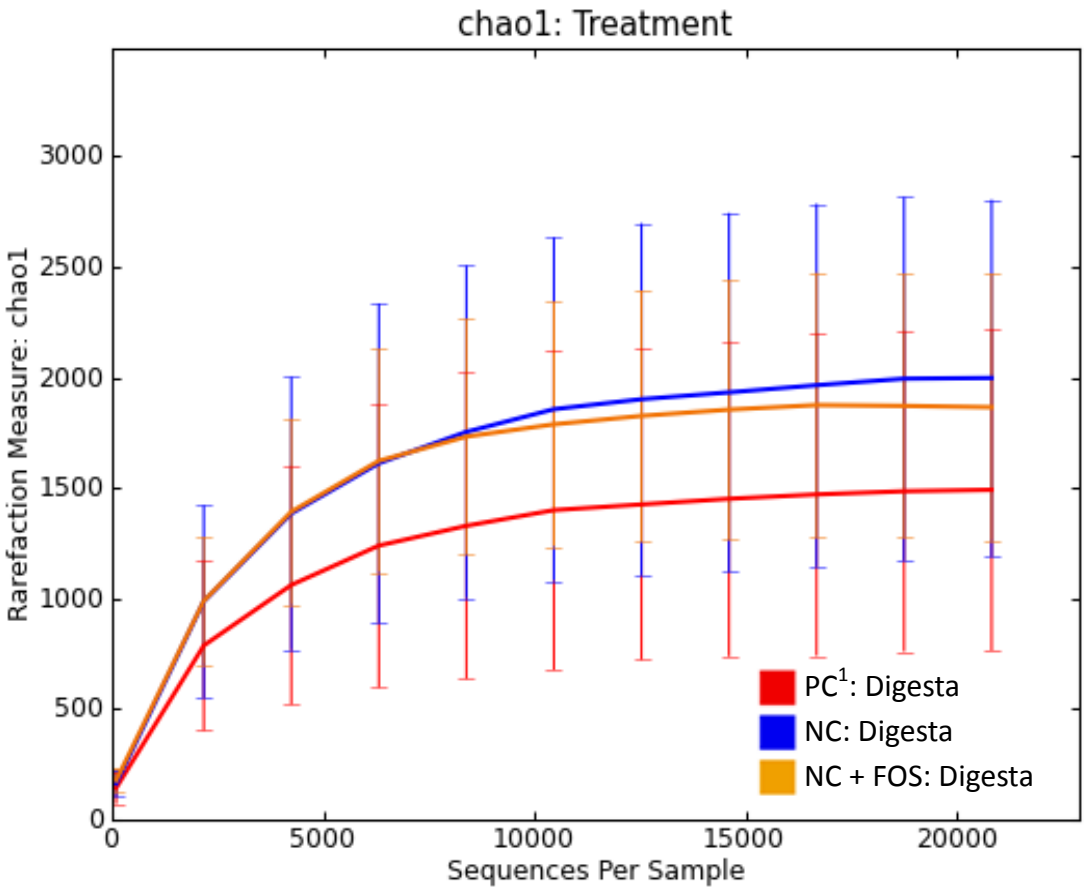


Figure 5. Rarefaction analysis on Chao1 estimates of species richness at an even sequencing depth of 20,820, in ileal digesta samples of broiler chickens that fed PC, NC and NC + FOS diets[1] (n = 6/treatment).

[1]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal base-diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

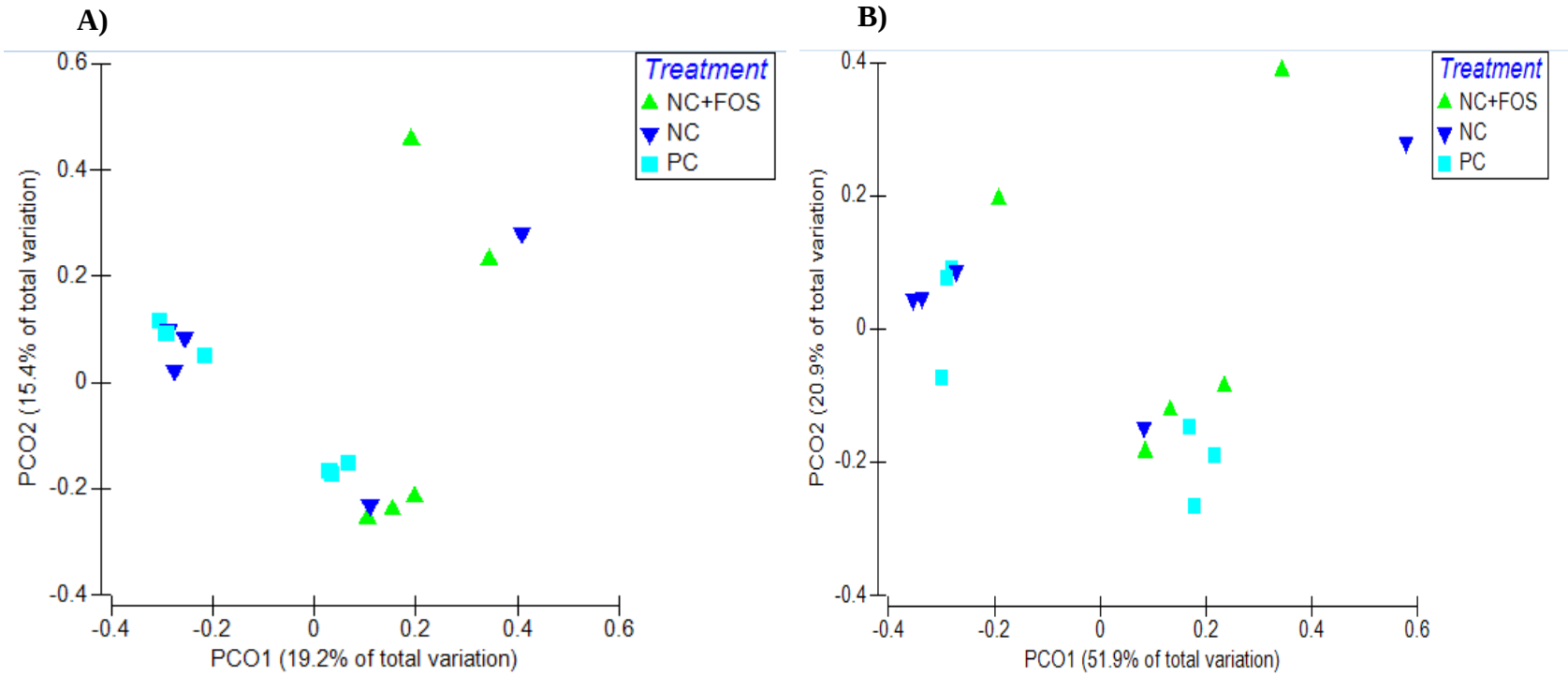


Figure 6. Principal coordinates analysis (PCoA) of A) unweighted Unifrac (P = 0.091) and B) weighted Unifrac (P = 0.431) distance of ileal mucosa bacterial community between the chickens that fed PC, NC and NC + FOS diets[1] (n = 6/treatment).

[1]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal base-diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

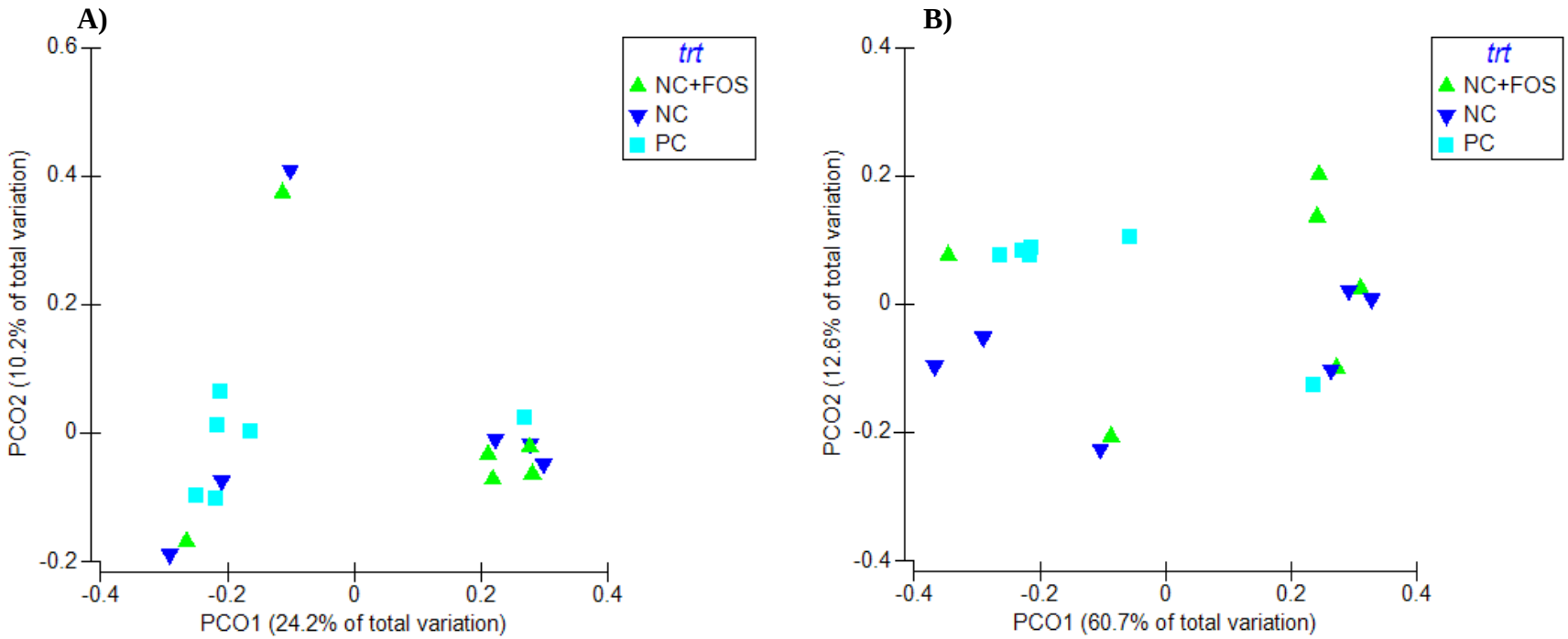


Figure 7. Principal coordinates analysis (PCoA) of A) unweighted Unifrac (P = 0.226) and B) weighted Unifrac (P = 0.371) distance of ileal digesta bacterial community between the chickens that fed PC, NC and NC + FOS diets[1] (n = 6/treatment).

[1]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal base-diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

Bacterial composition in the ileum of broiler chickens. At phylum level, a total of 12 phyla were identified in the ileal mucosa of broiler chickens, which include Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Deferribacteres, Firmicutes, OP8, Proteobacteria, Tenericutes, Thermi, TM7 and Verrucomicrobia. Similar to ileal mucosa, 11 phyla were found in the ileal digesta of broiler chickens except OP8, Thermi and Verrucomicrobia. Instead, Chloroflexi and Spirochaetes were observed in the ileal digesta. Seven phyla that have an over 0.1% population are presented in Table 16. The composition of these bacterial phyla did not exhibit statistical difference ($P > 0.05$) between the ileal tissue and digesta. On average, the ileal mucosa of broiler chickens predominantly consisted 37% of Firmicutes, 35% of Proteobacteria and 25% of Bacteriodetes, whereas 45% of Firmicutes, 27% of Proteobacteria and 25% of Bacteriodetes were found in the ileal digesta (data not shown). Broiler chickens that fed PC diet tended to have ($P = 0.06$) higher percentage of Deferribacteres when compared with NC and NC + FOS groups in both ileal tissue and digesta. However, no dietary or site × diet interactions was seen in other bacterial phyla ($P > 0.05$). At genus level, a total of 179 genera were determined by comparing the Illumina sequences with the Greengenes Core reference database. Some of the novel sequences were only identifiable to certain taxa levels and were assigned to phylum (p), class (c), order (o), family (f) or genus (g), accordingly. In total, 161 bacterial genera were found in the ileal mucosa and 113 bacterial genera were determined in the ileal digesta of broiler chickens. Among them, 95 genera were shared between ileal mucosa and digesta, whereas 66 and 18 were only found in the mucosa or digesta, respectively (data not shown). In all of the ileal mucosa samples, some of the relatively abundant (> 5%) genera included Lachnospiraceae (f) (22.46%), *Helicobacter* (14.70%), *Desulfovibrio* (12.17%) and S24-7 (f) (9.83%), whereas *Helicobacter* (17.24%), *Desulfovibrio* (15.13%), Lachnospiraceae (f) (14.04%), S24-7 (f)

(11.47%), *Oscillospira* (6.83%), *Bacteroides* (6.41%), *Allobaculum* (5.11%) were found abundant in the digesta samples (data not shown). However, the composition of the ileum bacterial community was highly variable. Statistical analysis on the relative abundance of the four major genera Lachnospiraceae (f), S24-7 (f), *Helicobacter* and *Desulfovibrio* was shown in Table 17. The data indicated that Lachnospiraceae (f) was greater in the ileal mucosa than in the digesta ($P = 0.0349$). The dietary treatments tended ($P = 0.0561$) to alter the abundance of *Desulfovibrio*, especially between the chickens that were fed NC and NC + FOS. When comparing the genera in the ileal mucosa alone, NC group had significant greater amount ($P < 0.05$) of *Helicobacter* and *Desulfovibrio* when compared with NC + FOS.

Table 16. Relative abundance of bacterial phyla based on the main effects of diet and ileal site of broiler chickens at 21 days of age[1]

Site (S)	Ileal Mucosa			Ileal Digesta				*P*-value		
Diet (D)[2]	PC	NC	NC + FOS	PC	NC	NC + FOS	SEM	S	D	S × D
Phylum (Relative %)	----------above 1% of population----------									
Bacteroidetes	21.39	23.34	30.44	17.96	29.60	28.21	3.0746	0.9759	0.4570	0.8035
Deferribacteres	2.103	0.735	0.386	2.565	1.071	1.501	0.2811	0.2500	0.0608	0.8253
Firmicutes	46.58	36.96	51.46	30.50	36.40	42.63	3.1582	0.1930	0.3871	0.6102
Proteobacteria	28.79	36.96	15.51	47.72	31.30	25.54	3.7947	0.3025	0.1414	0.3975
	----------below 1% of population----------									
Actinobacteria	0.082	0.128	0.171	0.046	0.238	0.359	0.0504	0.4002	0.2797	0.6599
Cyanobacteria	0.095	0.015	0.843	0.294	0.317	0.252	0.1010	0.8795	0.2394	0.1564
Tenericutes	0.215	1.017	0.294	0.095	0.144	0.428	0.1517	0.3647	0.5363	0.4081
Unclassified	0.738	0.705	0.876	0.826	0.938	1.081	0.0744	0.2666	0.5566	0.9186

[1]Values are the means of 6 replicate samples per treatment per site.

[2]Positive control (PC), wheat-corn-soybean meal based diet supplemented with 5.5 mg virginiamycin (Stafac-44) and 99 mg monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal based diet omitted with Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% fructooligosaccharides (FOS).

Table 17. Relative abundance of Lachnospiraceae (f), S247 (f), *Helicobacter* and *Desulfovibrio* based on the main effects of diet and ileal site of broiler chickens at 21 days of age[1]

Site (S)	Ileal Mucosa			Ileal Digesta				*P*-value		
Diet (D)[2]	PC	NC	NC + FOS	PC	NC	NC + FOS	SEM	S	D	S × D
Genus relative %[3]										
Lachnospiraceae (f)	16.821^{ab}	20.473^{ab}	32.917^{a}	12.942^{b}	12.811^{b}	15.339^{b}	2.2647	0.0349	0.2061	0.4256
S24-7 (f)	12.465	2.345	13.365	6.232	13.096	13.714	1.7234	0.6439	0.4032	0.1451
Helicobacter	15.464^{ab}	22.460$^{ab/*}$	5.781^{b}	27.022^{a}	14.314^{ab}	14.186^{ab}	2.5212	0.4362	0.1779	0.2443
Desulfovibrio	12.403^{ab}	21.356$^{a/*}$	2.633^{b}	19.842^{a}	16.083^{ab}	10.662^{ab}	2.0622	0.4006	0.0561	0.3328

[1]Values are the means of 6 replicate samples per treatment per site.

[2]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal base-diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

[3](f) = family level.

[a-b]Means with different superscripts within a row differ significantly ($P < 0.05$); *Means data differ significantly among PC, NC and NC + FOS dietary groups in the ileal mucosa ($P < 0.05$).

Dietary effects on ileal mucosa microbiota. In the ileal mucosa of broiler chickens, there were 64 bacterial taxa shared across the three dietary treatments (PC, NC and NC + FOS), as shown on the Venn diagram (Figure 8). Fifty-one genera were unique to NC + FOS group (e.g., *Enhydrobacter*, *Erwinia*, *Gordonia*, *Hylemonella*, *Janthinobacterium* and *Shewanella*). Fifteen (e.g., *Alicyclobacillus*, *Butyrivibrio*, *Escherichia*, *Paludibacter*, *Paracoccus*, *Pedobacter*, *Pseudomonas* and *Staphylococcus*) and nine (e.g., *Akkermansia*, *Clostridium*, *Coprococcus*, *Delftia* and *Flavobacterium*) genera were shared with PC or NC group, respectively. The PLS-DA (at cut-off VIP value of 0.5) of the PC and NC + FOS dietary groups has $R^2 = 0.99$ and $Q^2 = 0.80$, based on 3 components (Figure 9), which predicted a distinct distance on ileal mucosa bacteria communities between the two dietary treatments. Furthermore, as shown on the PLS-DA loading plot (Figure 10), the genera *Adlercreutzia*, *Allobaculum*, *Anaerotruncus*, *Heliobacter*, *Lactococcus*, *Mucispirillum*, *Roseburia*, *Ruminococcus*, and Helicobacteraceae (f), Lactobacillaceae (f), Mycoplasmataceae (f), Firmicutes (p), Proteobacteria (p) were positively correlated to the ileal mucosa of broiler chickens that fed PC diet whereas *Akkermansia*, *Rikenella* and Bacteroidetes (p) were correlated to NC + FOS group. Comparison between NC and NC + FOS groups in the ileal mucosa by PLS-DA (Figure 11 and 12) indicated that *Anaerotruncus*, *Dorea*, *Heliobacter*, *Ruminococcus* and Proteobacteria (p), Deltaproteobacteria (c), Peptostreptococcaceae (f) were associated with NC group, whereas *Sutterella*, Bacteroidaceae (f) and Coriobacteriaceae (f) were positively correlated to NC + FOS group ($R^2 = 0.98$ and $Q^2 = 0.59$).

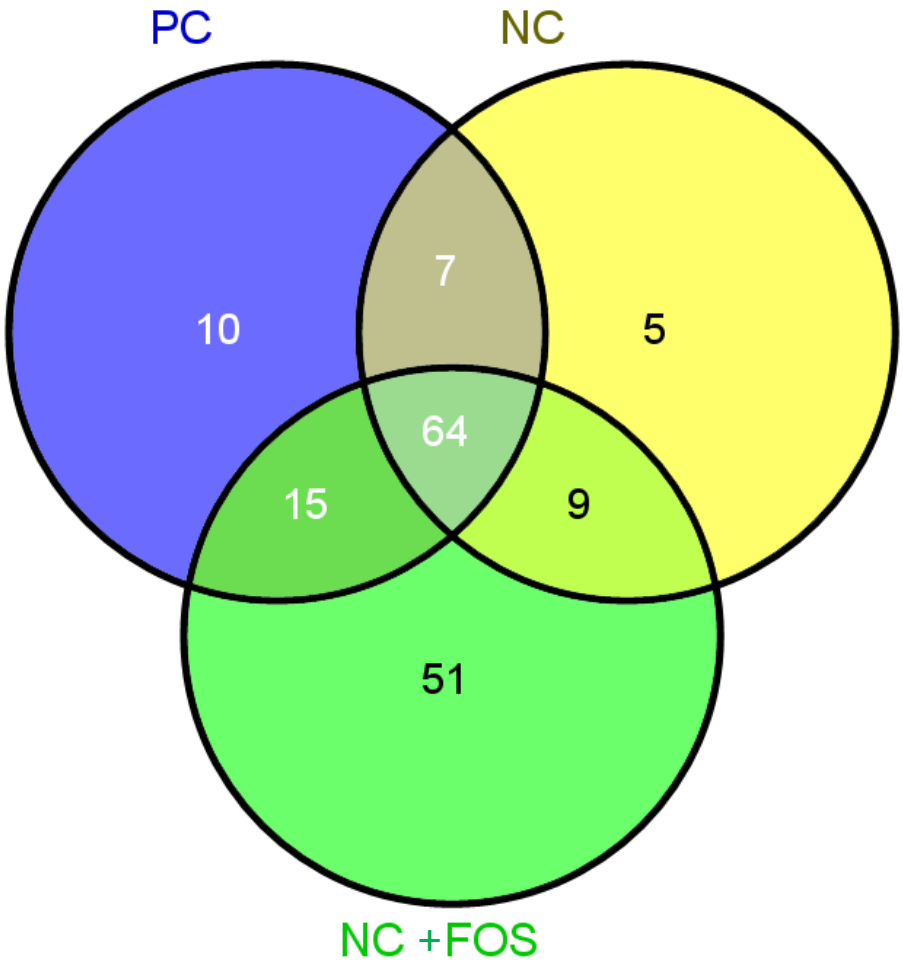


Figure 8. Venn diagram of shared and unique bacterial taxa in the ileal mucosa of broiler chickens fed PC, NC and NC + FOS diets[1] (n = 6/treatment).

[1]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal base-diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

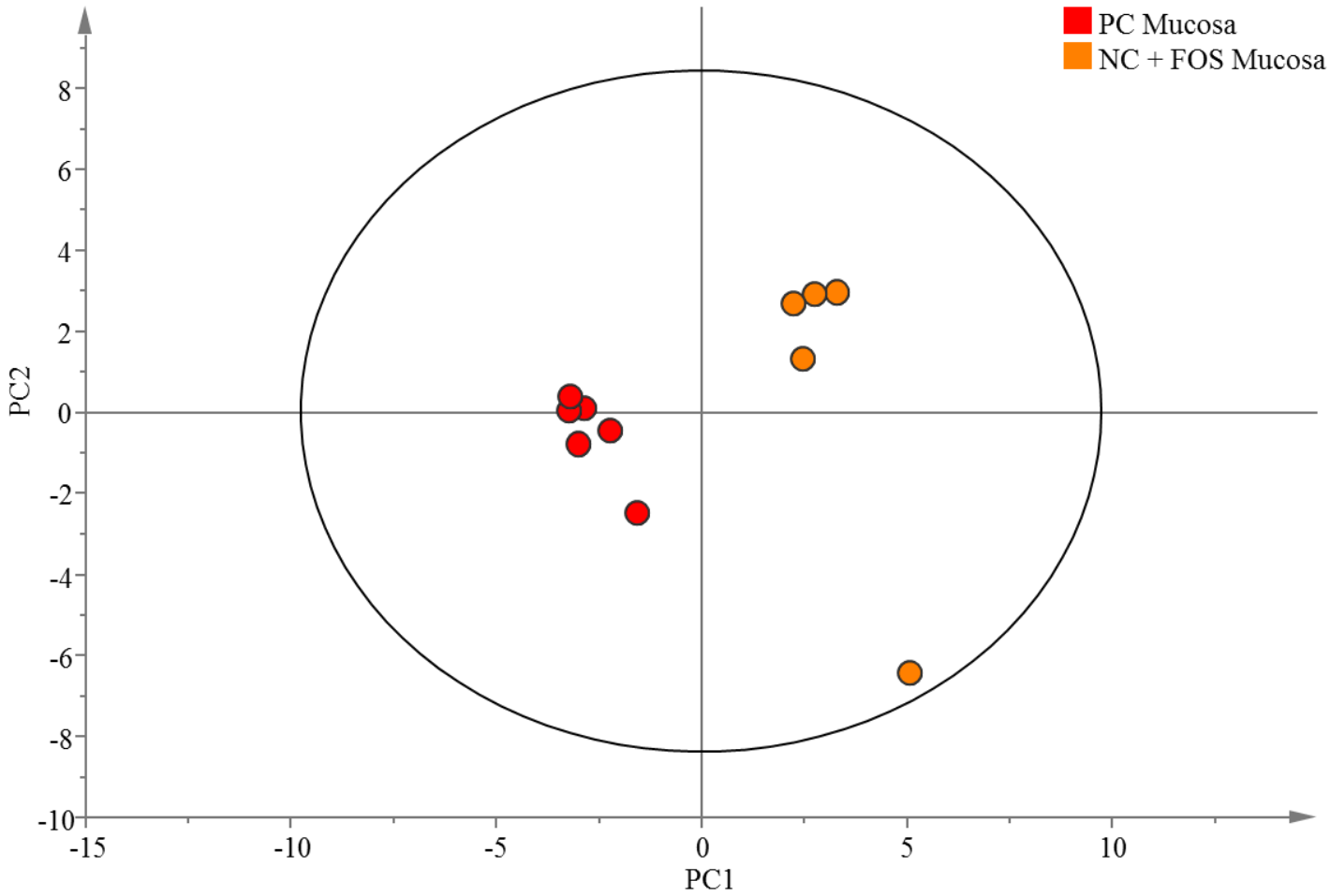


Figure 9. Partial least square discriminant analysis (PLS-DA) score scatter plot of the ileal mucosa microbiota between broiler chickens that fed PC and NC + FOS diets (n = 6/treatment). The horizontal axis represents the first PLS component and the vertical axis represents the second component. The R^2 (= 0.99) and Q^2 (= 0.80) estimates were calculated based on 3 components.

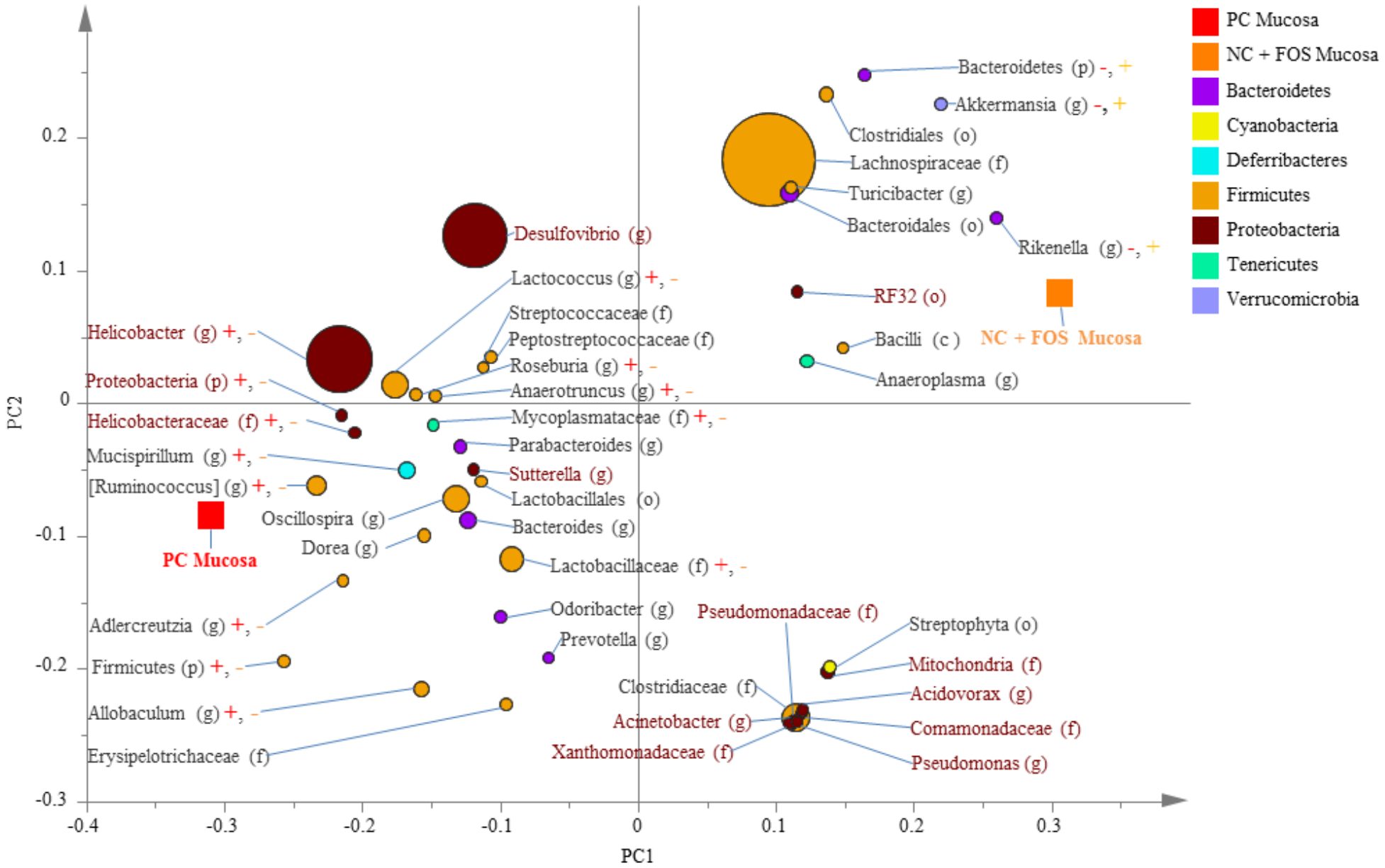


Figure 10. Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundance of bacterial taxa in the

ileal mucosa of broiler chickens that fed PC or NC + FOS diets[1] (n = 6/treatment). The presenting taxa are chosen at variable influence on projection (VIP) value of above 0.5. The size of each circle indicates the abundance of taxa and is colored according to their corresponding phyla. The taxa are presented on phylum (p), class (c), order (o), family (f) or genus (g) levels after comparison of sequences to the Greengenes Core reference database. The coloured "+" and "-" indicates positive or negative correlation of taxa to the same coloured PC or NC + FOS dietary group.

[1]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. NC + FOS, wheat-corn-soybean meal base-diet without Stafac-44 and Coban, and supplemented with 0.5% of fructooligosaccharide (FOS).

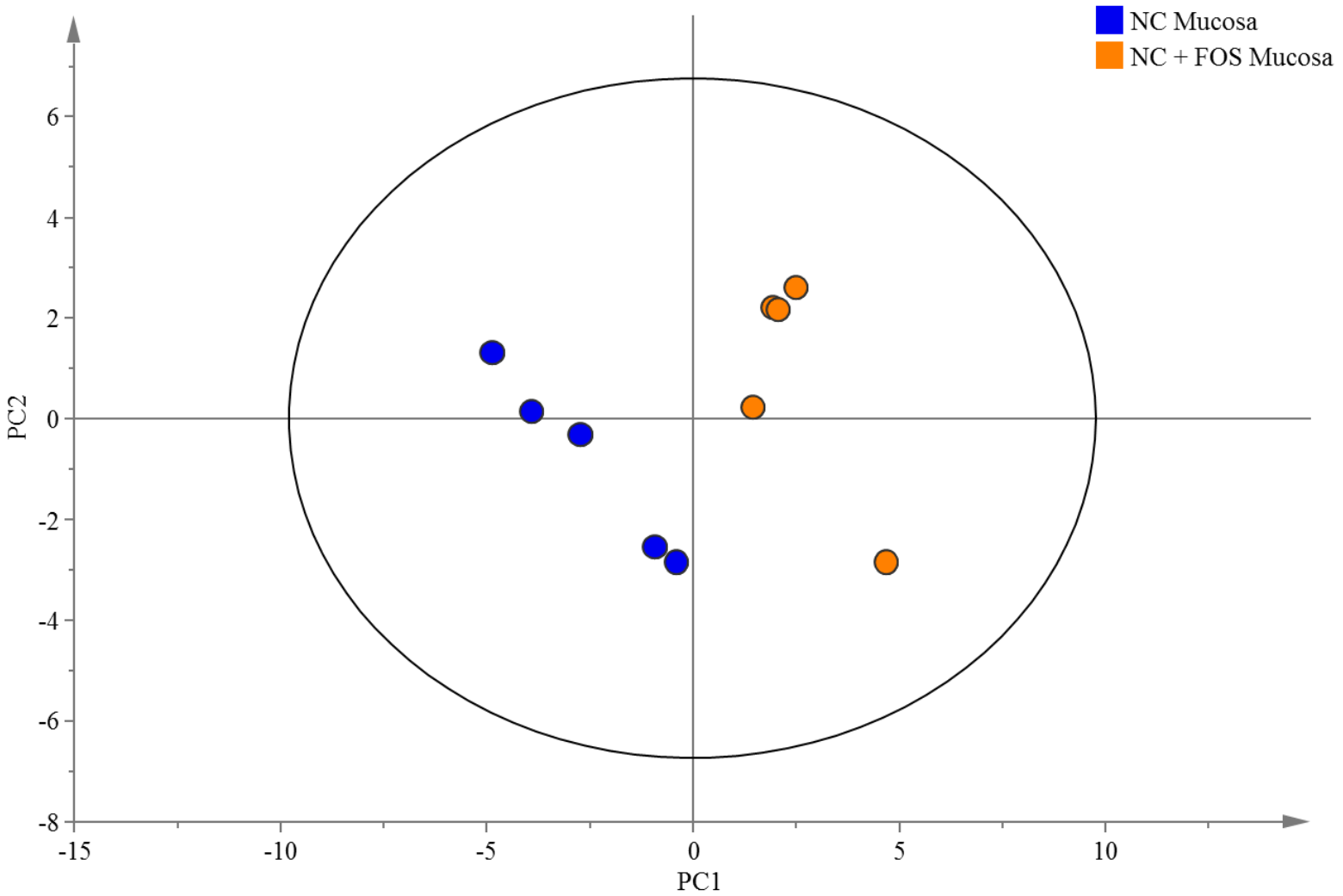


Figure 11. Partial least square discriminant analysis (PLS-DA) score scatter plot of the ileal mucosa microbiota between broiler chickens that fed NC and NC + FOS diets. The R^2 (= 0.98) and Q^2 (= 0.59) estimates were calculated based on 3 components.

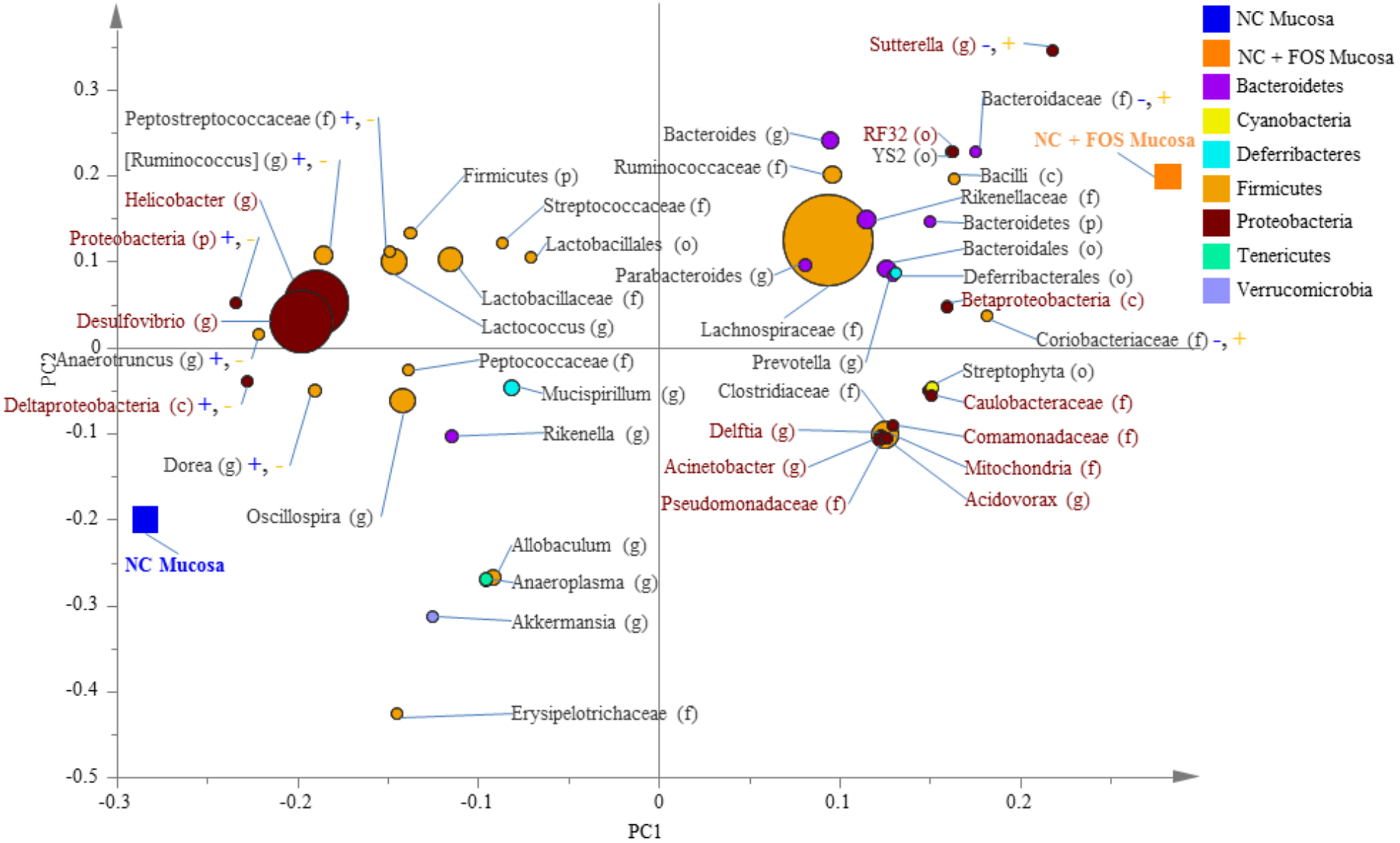


Figure 12. Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundance of bacterial taxa in the

ileal mucosa of broiler chickens that fed NC and NC + FOS diets[1] (n = 6/treatment). The presenting taxa are chosen at variable influence on projection (VIP) value of above 0.5. The size of each circle indicates the abundance of taxa and is colored according to their corresponding phyla. The taxa are presented on phylum (p), class (c), order (o), family (f) or genus (g) levels after comparison of sequences to the Greengenes Core reference database. The coloured "+" and "-" indicates positive or negative correlation of taxa to the same coloured NC or NC + FOS dietary group.

[1]Negative control (NC), wheat-corn-soybean meal based-diet without 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

Dietary effects on ileal digesta microbiota. In the ileal digesta of broiler chickens, 64 bacterial taxa were shared among the three dietary treatments as shown on the Venn diagram (Figure 13). Twelve genera such as *Bacillus*, *Corynebacterium*, *Enhydrobacter*, *Hylemonella*, *Psychrobacter* and *5-7N15* were unique to the NC + FOS group. Five genera were shared with PC and NC + FOS, and 6 genera were shared between NC and NC + FOS. The PLS-DA comparison (at cut-off VIP value of 0.5) between PC and NC + FOS groups in the ileal digesta of broiler chickens (Figure 14 and 15) showed that *Helicobacter*, *Desulfovibrio*, Peptostreptococcaceae (f), Rikenellaceae (f), RF32 (o) and Proteobacteria (p) were positively associated with PC, whereas *Adlercreutzia*, *Paraprevotella*, Coriobacteriaceae (f), Erysipelotrichaceae (f), Ruminococcaceae (f), S24-7 (f), Bacteroides (o), Betaproteobacteria (c) and Bacteroidetes (p) were correlated to NC + FOS group (R^2 = 0.96 and Q^2 = 0.36). Figure 16 and 17 demonstrated the PLS-DA comparison (at cut-off VIP value of 0.5) between NC and NC + FOS diets in the ileal digesta of broiler chickens. *Bacteroides*, *AF12*, Mycoplasmataceae (f) and Rikenellaceae (f) were correlated to NC but negatively correlated with NC + FOS. However, *Oscillospira* and Helicobacteraceae (f) exhibited positive association with NC + FOS (R^2 = 0.96 and Q^2 = 0.16).

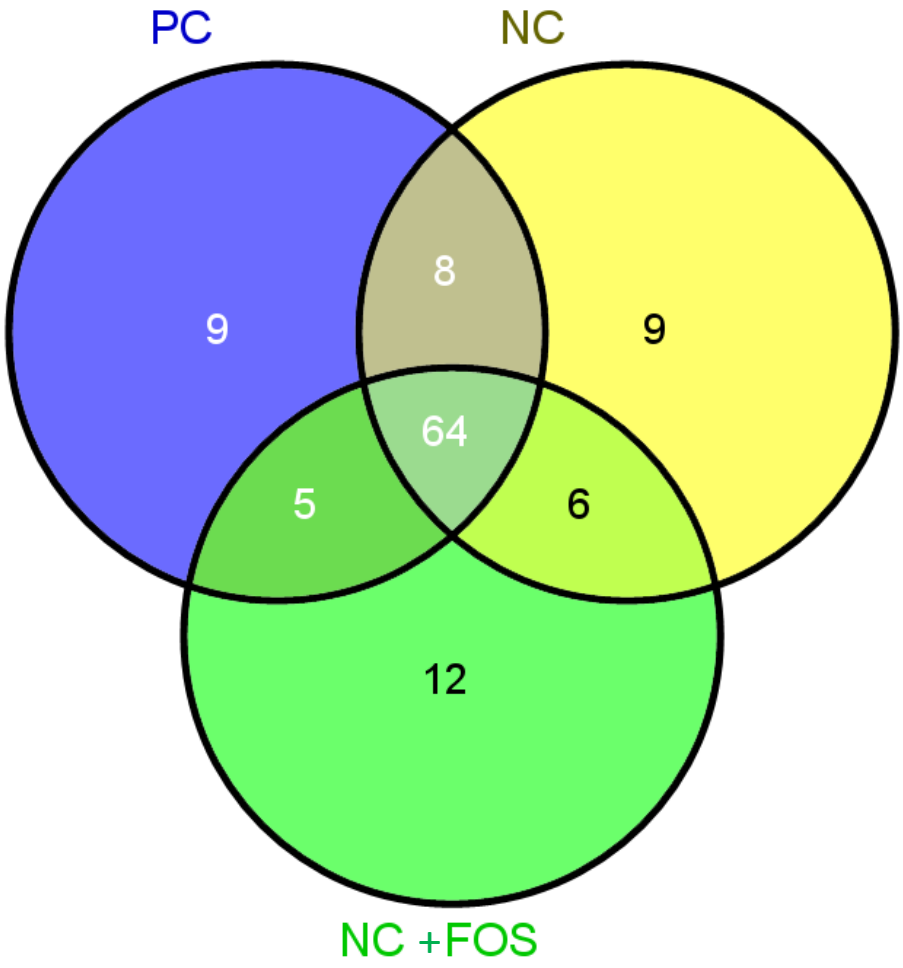


Figure 13. Venn diagram of shared and unique bacterial taxa in ileal digesta of broiler chickens fed PC, NC and NC + FOS diet[1] (n = 6/treatment).

[1]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal base-diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

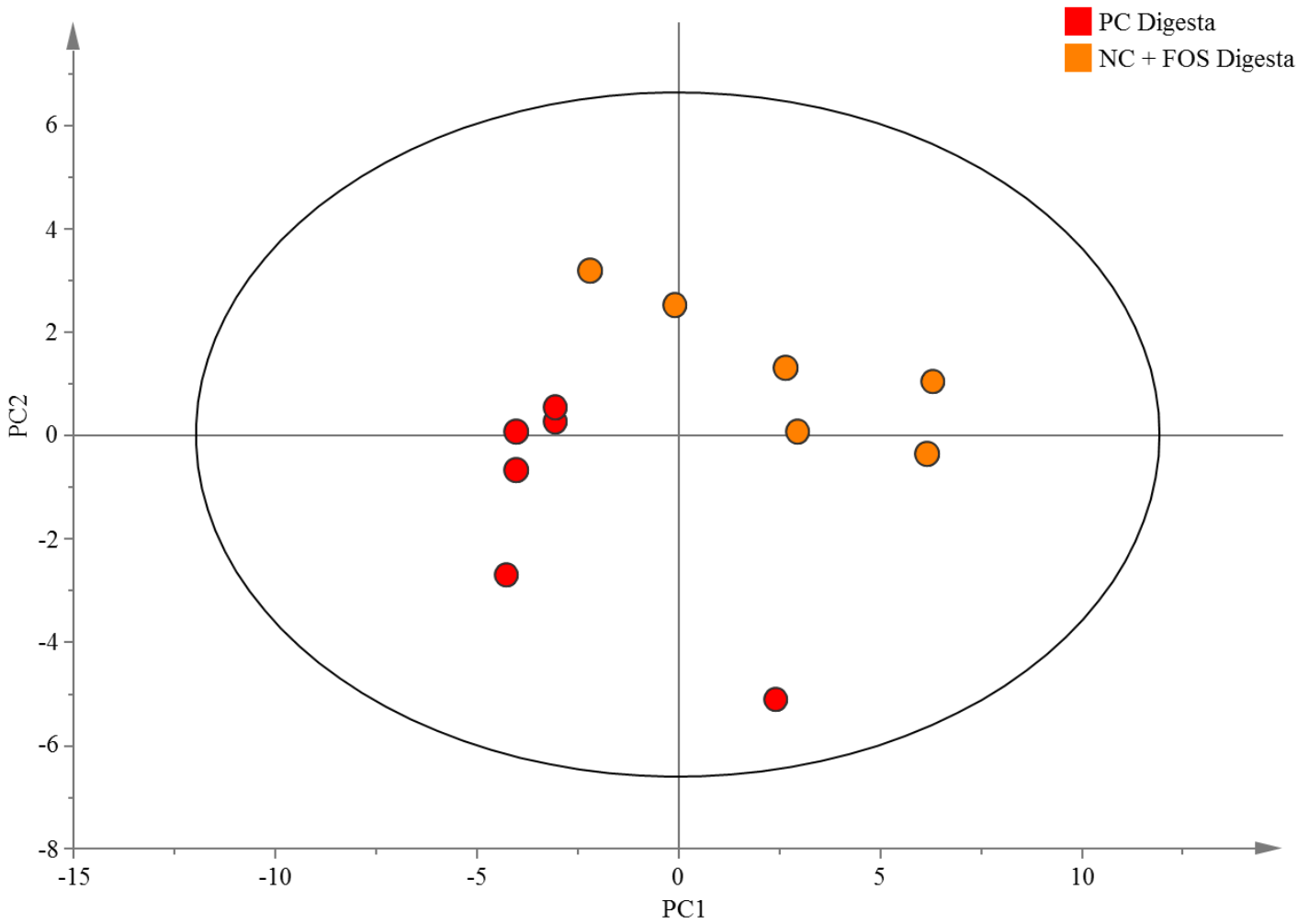


Figure 14. Partial least square discriminant analysis (PLS-DA) score scatter plot of the ileal digesta microbiota between broiler chickens that fed PC and NC + FOS diets (n = 6/treatment). The R^2 (= 0.96) and Q^2 (= 0.36) estimates were calculated based on 3 components.

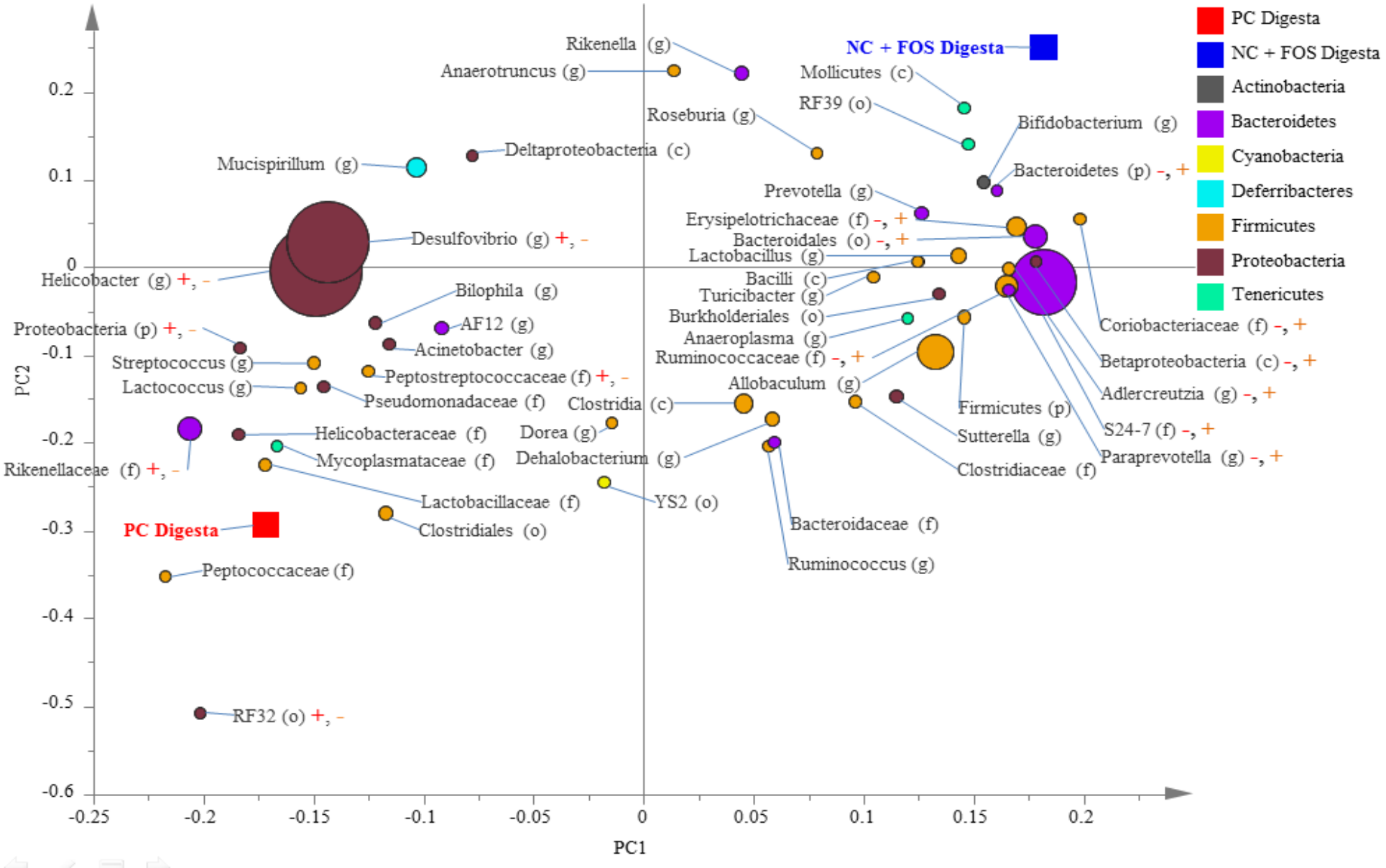


Figure 15. Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundance of bacterial taxa in the

ileal digesta of broiler chickens that fed PC and NC + FOS diets[1] (n = 6/treatment). The presenting taxa are chosen at variable influence on projection (VIP) value of above 0.5. The size of each circle indicates the abundance of taxa and is colored according to their corresponding phyla. The taxa are presented on phylum (p), class (c), order (o), family (f) or genus (g) levels after comparison of sequences to the Greengenes Core reference database. The coloured "+" and "-" indicates positive or negative correlation of taxa to the same coloured PC or NC + FOS dietary group.

[1]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. NC + FOS, wheat-corn-soybean meal based diet without Stafac-44 and Coban, and supplemented with 0.5% of fructooligosaccharides (FOS).

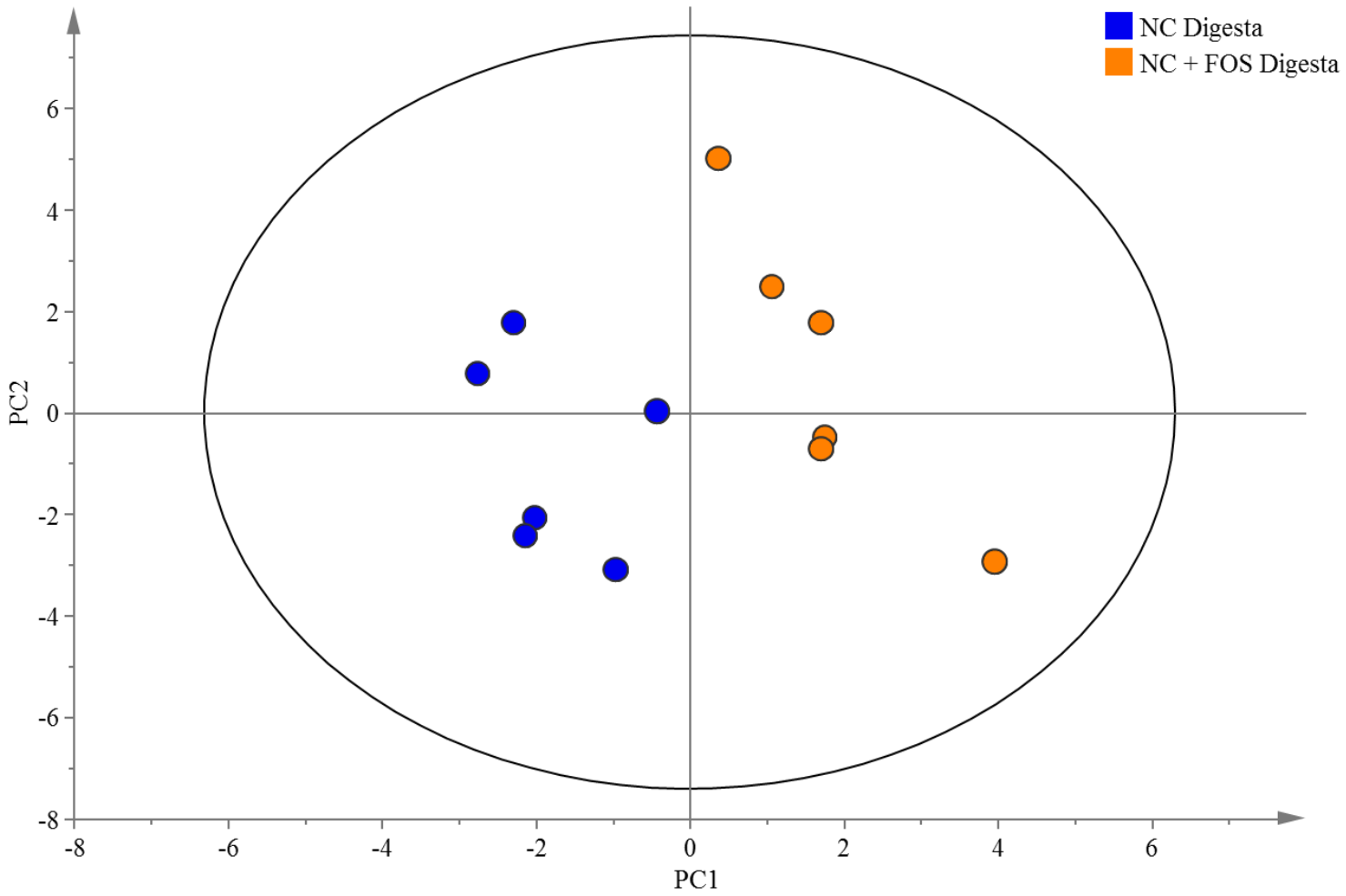


Figure 16. Partial least square discriminant analysis (PLS-DA) score scatter plot of the ileal digesta microbiota between broiler chickens that fed NC and NC + FOS diets (n = 6/treatment). The R^2 (= 0.96) and Q^2 (= 0.16) estimates were calculated based on 3 components.

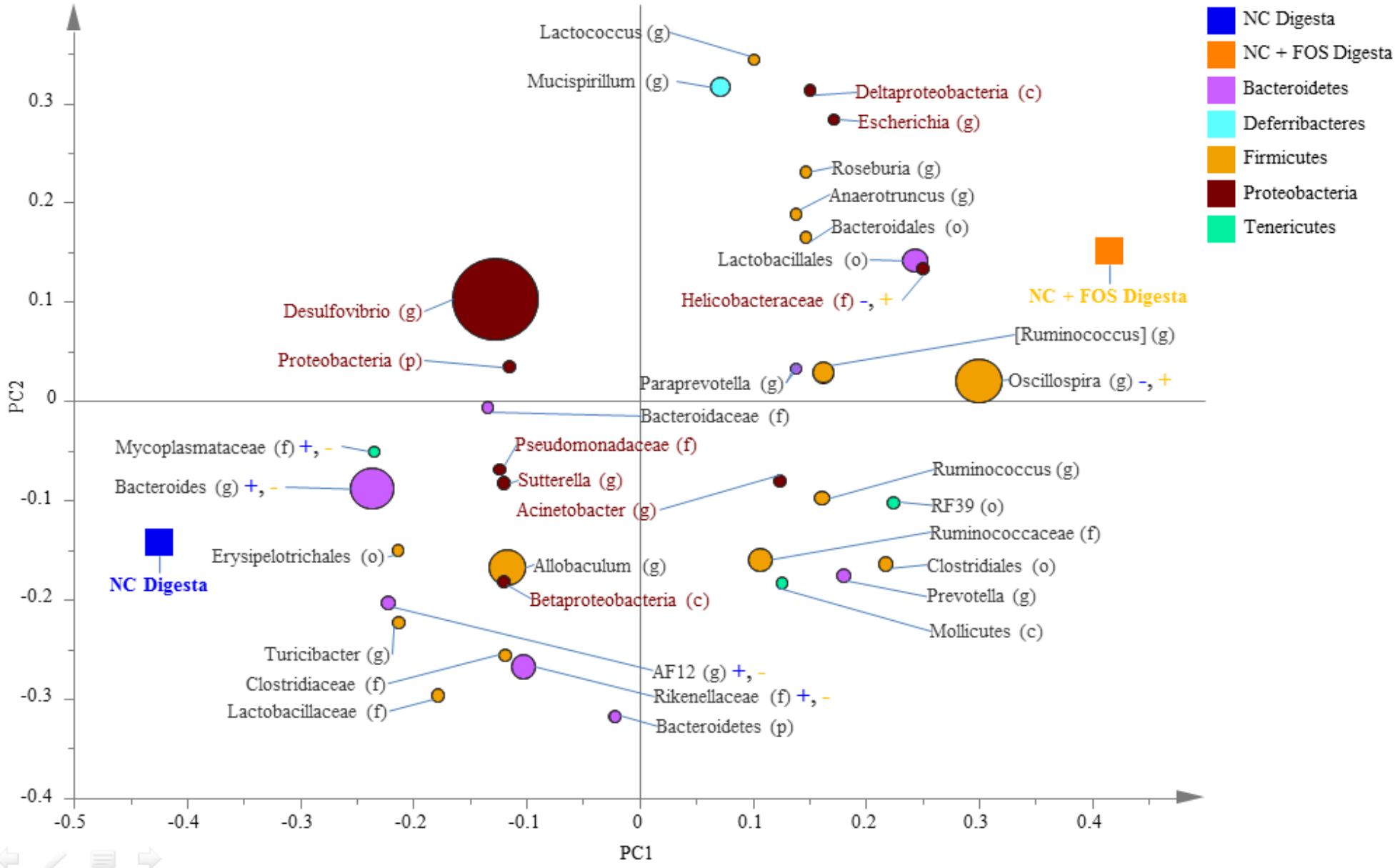


Figure 17. Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundance of bacterial taxa in the

ileal digesta of broiler chickens that fed NC and NC + FOS diets[1] (n = 6/treatment). The presenting taxa are chosen at variable influence on projection (VIP) value of above 0.5. The size of each circle indicates the abundance of taxa and is colored according to their corresponding phyla. The taxa are presented on phylum (p), class (c), order (o), family (f) or genus (g) levels after comparison of sequences to the Greengenes Core reference database. The coloured "+" and "-" indicates positive or negative correlation of taxa to the same coloured NC or NC + FOS dietary group.

[1]Negative control (NC), wheat-corn-soybean meal based-diet without 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

Interaction between Diet and Salmonella LPS Challenge

White blood cells differential. Significant differences in the relative percentage of heterophils and lymphocytes as well as the H: L ratio have been observed between PBS and LPS immunological challenged groups ($P < 0.0001$, $P < 0.0001$ and $P = 0.0004$, respectively) (Table 18). Lipopolysaccharides challenge significantly increased the relative percentage of heterophils and the H: L ratio, whereas decreased the number of lymphocytes. Significant dietary effects ($P = 0.011$ and $P = 0.049$) were observed on the relative percentage of heterophils and monocytes, respectively. Chickens fed NC + FOS diet contained lower heterophils but higher monocytes when compared with NC. The H: L ratio and basophil number was numerically high ($P > 0.05$) in NC + FOS group under LPS challenge, however because of high variations between individual samples, the results failed to have statistical meaning.

Table 18. Main effects of diet and challenge on relative percentage of white blood cell differential of broiler chickens at 21 days of age[1]

Challenge (C) [2]	PBS			LPS				*P*-value		
Diet (D)[3]	PC	NC	NC + FOS	PC	NC	NC + FOS	SEM	C	D	C × D
Relative %										
Heterophils (H)	49.75[b]	46.67[b]	44.75[b]	68.5[a]	71.17[a]	64.20[a]	2.297	< 0.0001	0.0109	0.4179
Lymphocytes (L)	38.25[a]	40.67[a]	40.25[a]	20.75[b]	20.17[b]	20.40[b]	2.353	< 0.0001	0.7691	0.9224
H:L ratio	1.31[b]	1.25[b]	1.12[b]	3.53[ab]	3.64[ab]	4.75[a]	0.404	0.0004	0.6716	0.6293
Monocytes	6.25[ab]	7.00[ab]	9.75[a]	7.25[ab]	4.50[b]	9.80[a]	0.754	0.7526	0.0489	0.6131
Basophil	5.25	4.33	4.75	2.25	3.33	5.20	0.495	0.2824	0.4781	0.3946
Eosinophil	0.50	1.33	0.50	1.25	0.83	0.40	0.160	0.8933	0.3432	0.3176

[1]Values are the means of 6 birds per treatment, in a total of 12 birds per diet and 18 birds per challenge.

[2]Chickens were intraperitoneally injected with either 2ml/kg of BW *Salmonella enteritidis* Lipopolysaccaride (LPS) or sterile phosphate buffered saline (PBS).

[3]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal base-diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

[a-b] Means with different superscripts within a row differ significantly ($P < 0.05$).

Natural and specific IgG levels in serum. The broiler chickens injected with PBS as control did not show significant difference ($P > 0.05$) on natural serum IgG concentration among the three dietary treatments (Figure 18a). The *salmonella* LPS challenge significantly altered the natural serum IgG concentration ($P = 0.026$). NC diet that supplemented with 0.5% of FOS significantly increased the total IgG concentration under LPS challenge condition when compared with the other two dietary groups ($P = 0.001$). There was also a significant diet × challenge interaction among all treatment groups ($P < 0.0001$). The specific IgG level was significantly increased among broiler chickens in response to *Salmonella* LPS challenge ($P = 0.004$) (Figure 18b). Significant dietary effect was also observed among all treatments with $P = 0.033$. The chickens fed NC + FOS significantly increased specific serum IgG level when compared with PC and NC ($P = 0.003$). A trend ($P = 0.075$) was observed on the diet × challenge interaction of specific serum IgG levels.

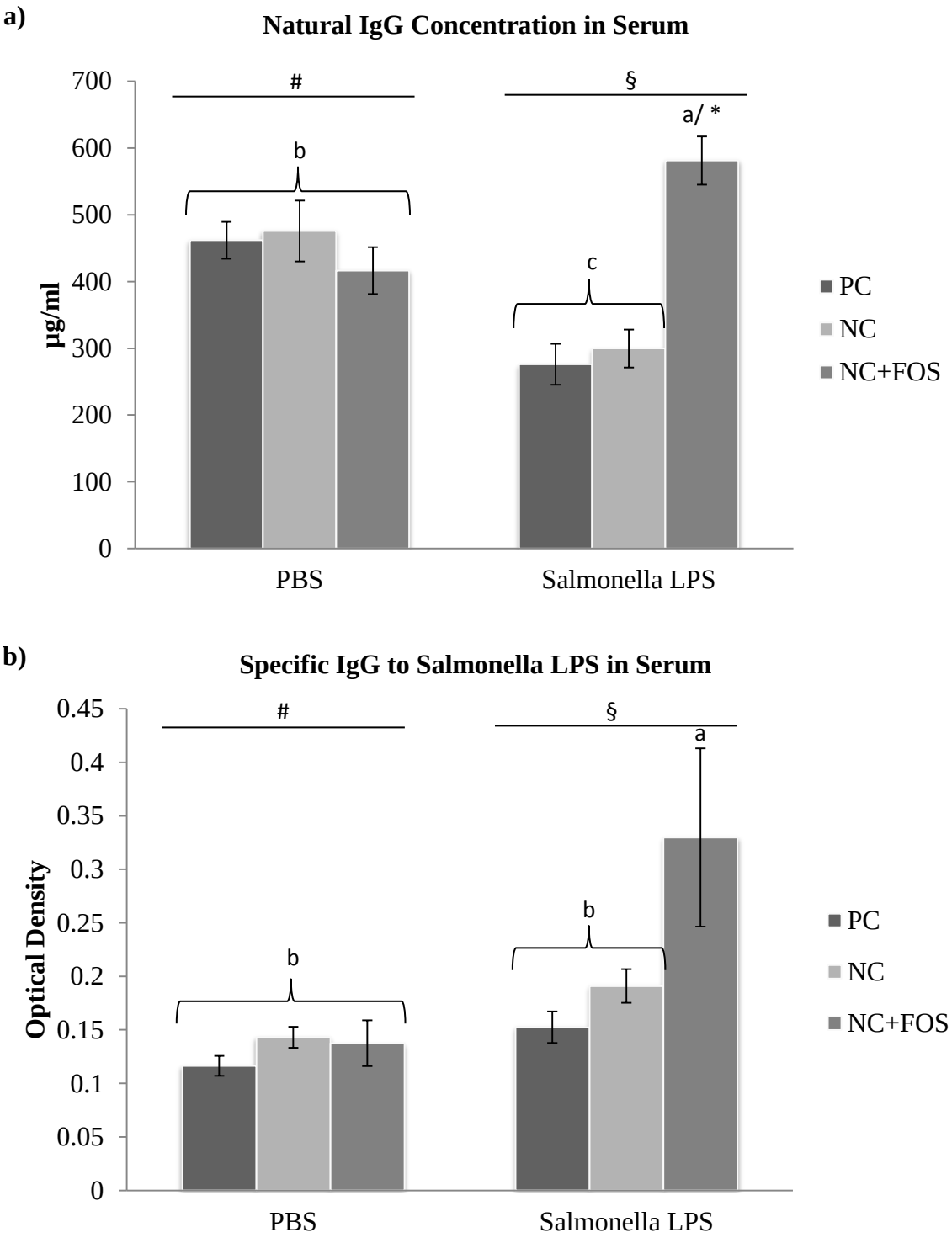


Figure 18. a) Natural serum immunoglobulin (Ig)G concentration (µg/ml) in broiler chickens that fed PC, NC and NC + FOS diet[1], under phosphate buffered saline (PBS) injection as control or *Salmonella* lipopolysaccharide (LPS) challenge conditions. **b)** Specific serum IgG level in

response to *salmonella* LPS (expressed as optical density) of broiler chickens fed PC, NC and NC + FOS diet, under PBS injection as control or *Salmonella* LPS challenge conditions (n = 6/treatment). Error bars represent standard errors and different letters (a to c) represent treatments that differed significantly ($P < 0.05$).

[1]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal base-diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

Natural and specific IgA levels in serum. Similar to IgG, the natural IgA concentration did not exhibit any difference ($P > 0.05$) under PBS condition (Figure 19a). The *Samonella* LPS challenge showed a trend in increasing natural IgA concentration in the serum of broiler chickens ($P = 0.097$), whereas no significant dietary effect was observed ($P > 0.05$). The diet × challenge interaction was significant different on natural IgA concentration, with $P = 0.027$. Chickens that fed NC + FOS diet exhibited significantly increased natural IgA concentration under *Samonella* LPS challenge condition when compared with PC ($P = 0.035$). Relatively low optical density was obtained for specific IgA measurement. The specific IgA level did not show any difference among all the treatments (Figure 19b).

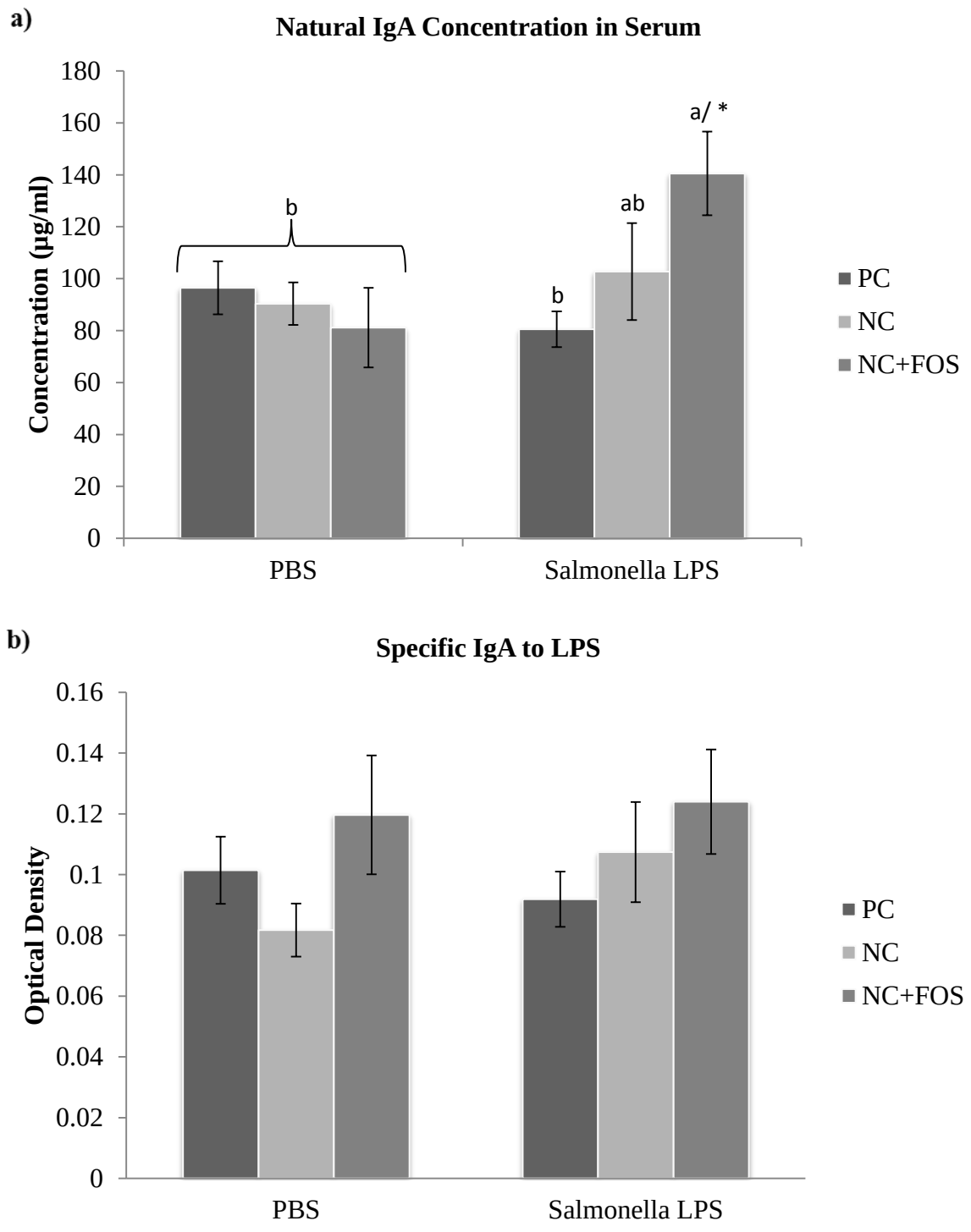
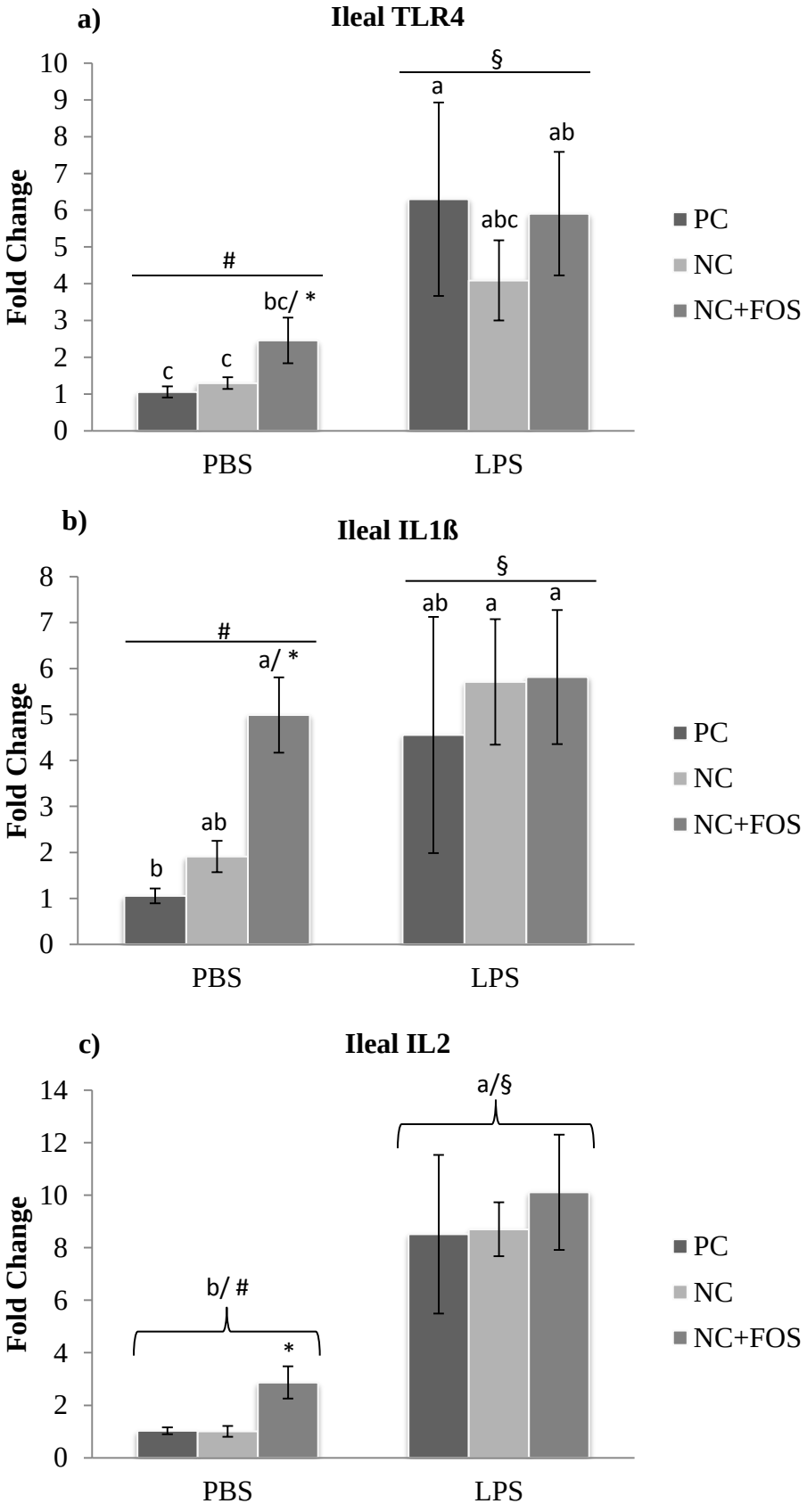


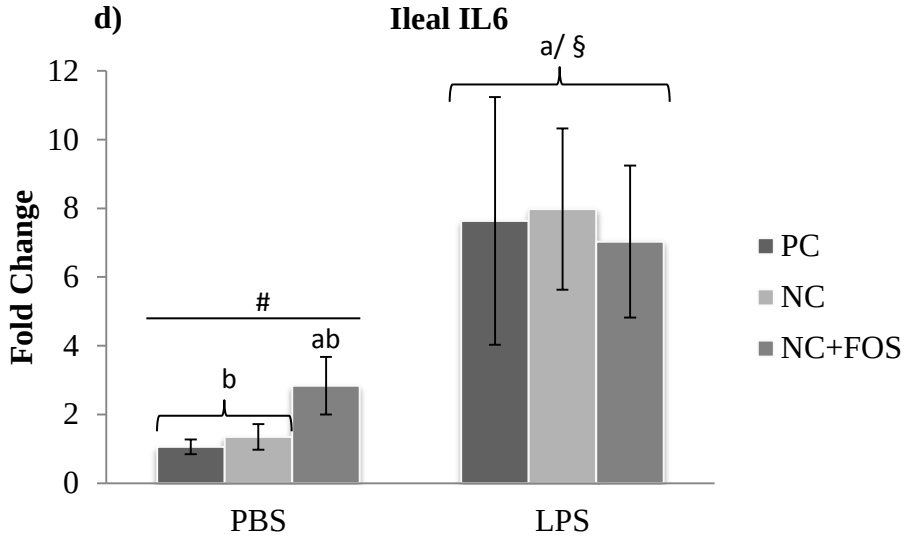
Figure 19. a) Natural serum immunoglobulin (Ig)A concentration (µg/ml) in broiler chickens that fed PC, NC, and NC + FOS diet, under phosphate buffered saline (PBS) injection as control or *Salmonella* lipopolysaccharide (LPS) challenge conditions. **b)** Specific serum IgA level in

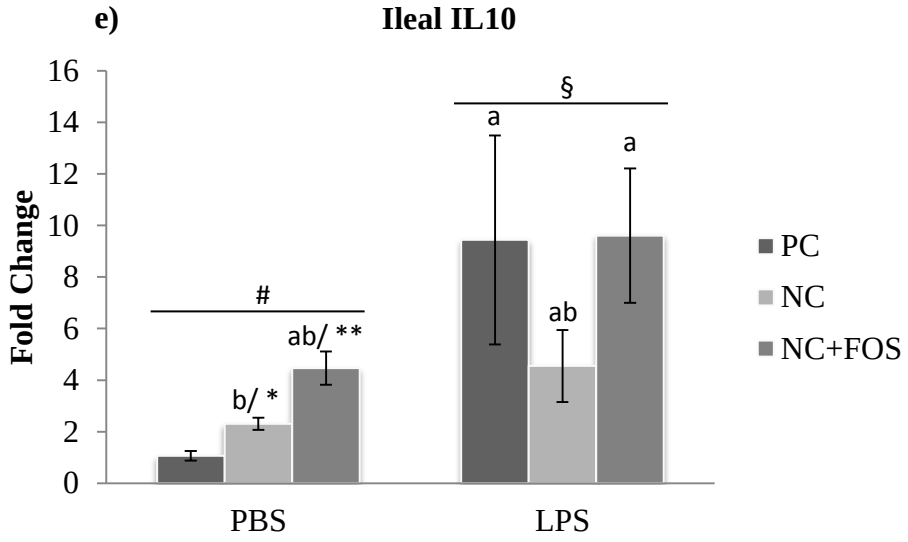
response to *salmonella* LPS (expressed as optical density) of broiler chickens fed PC, NC and NC + FOS diet[1], under PBS injection as control or *Salmonella* LPS challenge conditions (n = 6/treatment). Error bars represent standard errors and different letters (a to b) represent treatments that differed significantly ($P < 0.05$).

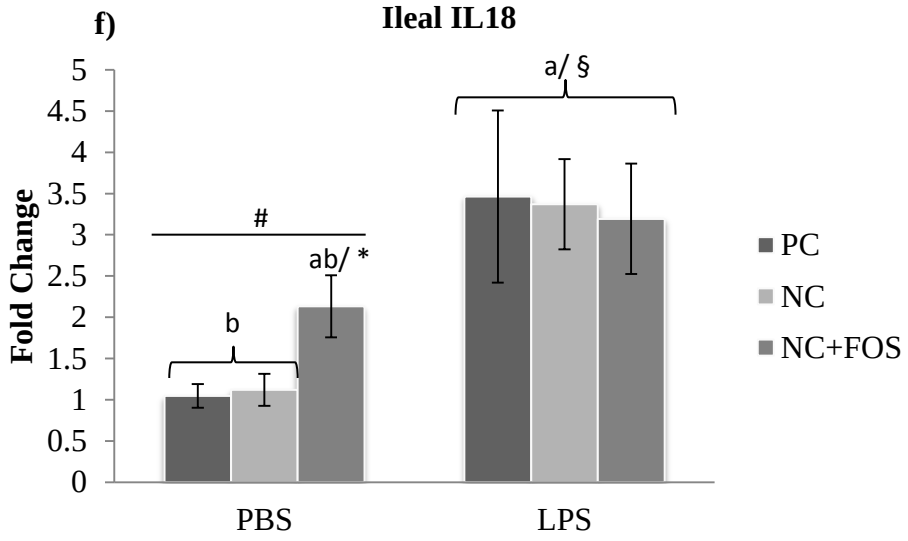
[1]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal base-diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

Cytokine gene expression in the ileum. The *Samonella* LPS challenge had a direct impact on the ileum of the broiler chickens and significantly up-regulated ileal cytokine gene expressions including TLR -4, IL -1ß, -2, -6, -10, -18, and IFN-Ɣ ($P = 0.0003$, $P = 0.0115$, $P < 0.0001$, $P = 0.0006$, $P = 0.0043$, $P = 0.0001$ and $P = 0.0001$, respectively) among all LPS challenged treatments (Figure 20, a to g). The dietary effect showed that NC + FOS treatment significantly up-regulated IFN -Ɣ expressions ($P = 0.0002$) and demonstrated a trend to up-regulate IL -1ß expressions ($P = 0.073$). No significant difference was observed on the interactions between the dietary treatment and the immunological challenge. The three dietary treatments that were challenged with *Salmonella* LPS alone did not show statistical difference on ileal cytokine gene expressions. However, when comparing the three PBS injected groups, the NC + FOS treatment up-regulated TLR -4, IL -1ß, -2, -10, -18, and IFN -Ɣ expressions by 2.5, 5.0, 2.8, 4.5, 2.1 and 4.9 folds, respectively, when compared with PC or NC ($P = 0.043$, $P = 0.0003$, $P = 0.011$, $P = 0.0003$, $P = 0.013$ and $P = 0.002$, respectively).


a)
Ileal TLR4
Fold Change
10
9
8
7
6
5
4
3
2
1
0
§
a
ab
abc
#
bc/ *
c
c
PBS
LPS
PC
NC
NC+FOS
b)
Ileal IL1ß
Fold Change
8
7
6
5
4
3
2
1
0
§
ab
a
a
#
a/ *
ab
b
PBS
LPS
PC
NC
NC+FOS
c)
Ileal IL2
Fold Change
14
12
10
8
6
4
2
0
a/§
b/ #
*
PBS
LPS
PC
NC
NC+FOS


d)
Ileal IL6
Fold Change
12
10
8
6
4
2
0
a/ §
#
ab
b
PBS
LPS
PC
NC
NC+FOS


e)
Ileal IL10
Fold Change
16
14
12
10
8
6
4
2
0
§
a
a
#
ab
ab/ **
b/ *
PBS
LPS
PC
NC
NC+FOS


f)
Ileal IL18
Fold Change
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a/ §
#
ab/ *
b
PBS
LPS
PC
NC
NC+FOS

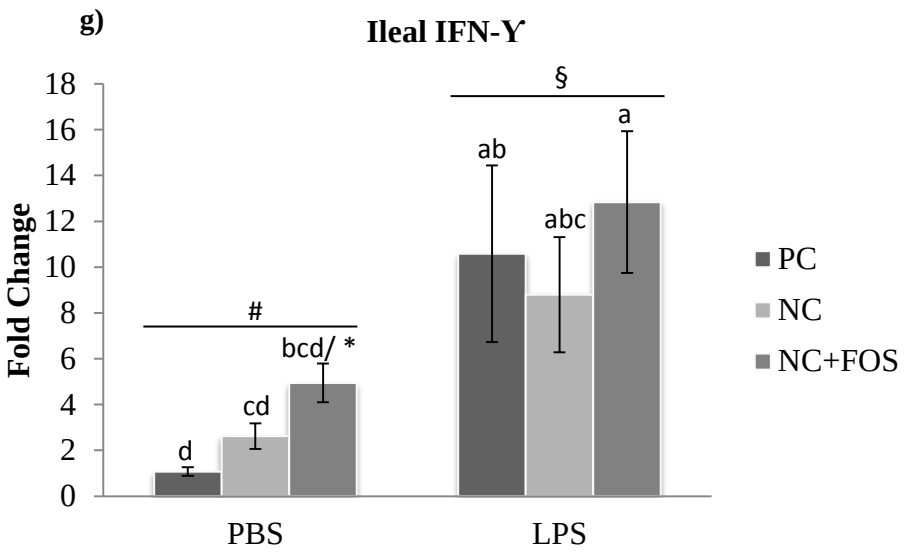
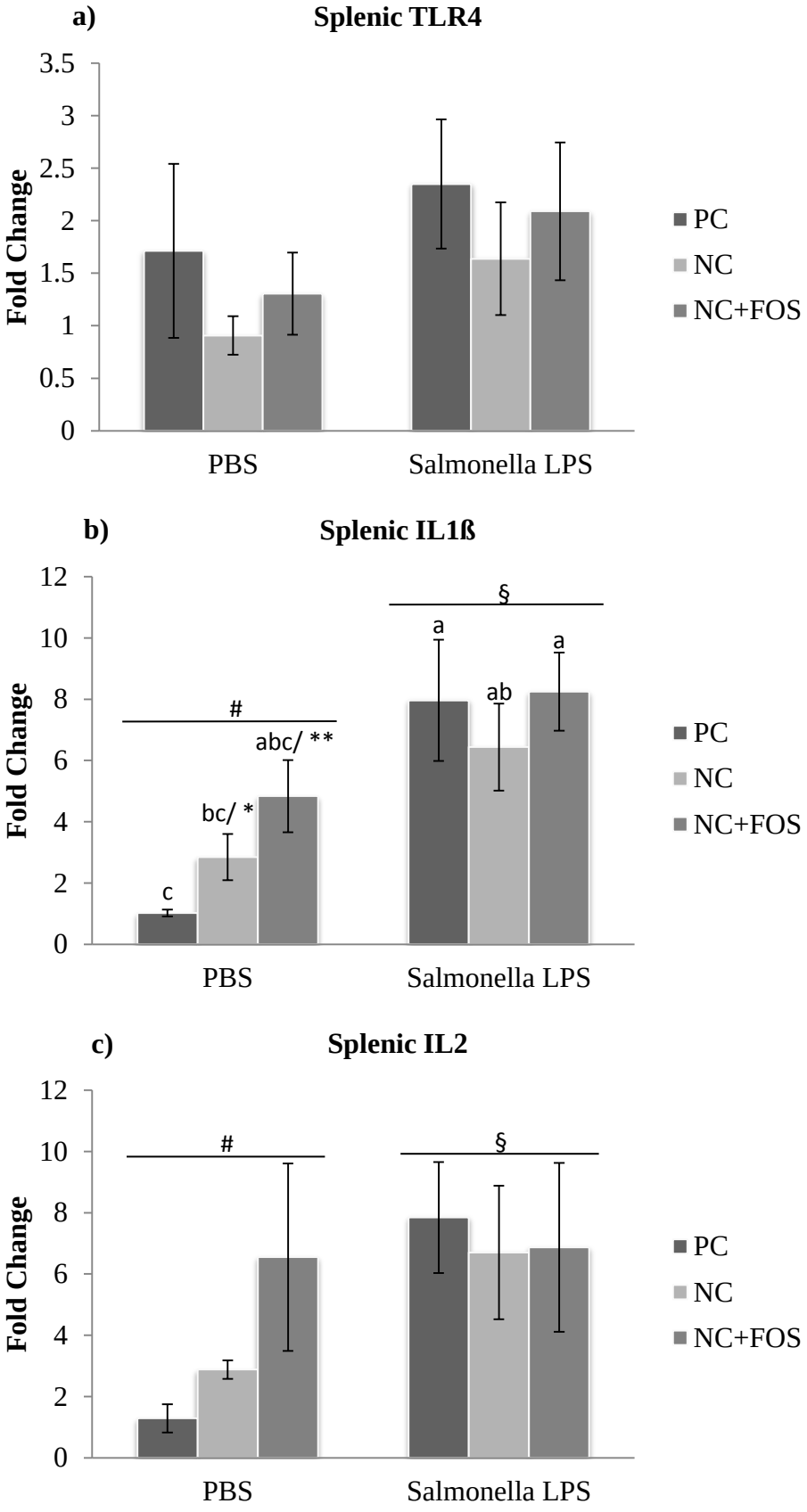
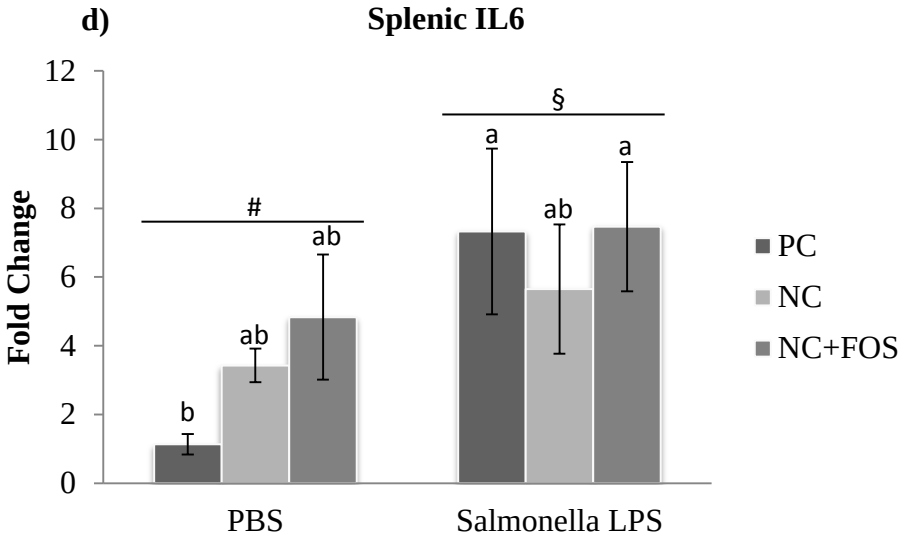


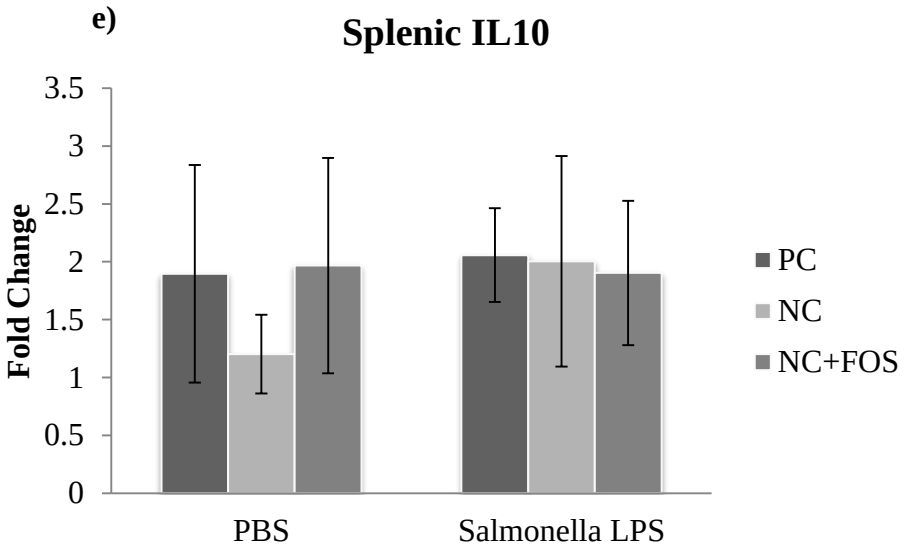
Figure 20. Ileal gene expressions of a) toll-like receptor (TLR) -4, b) interleukin (IL) -1ß, c) IL-2, d) IL-6, e), IL-10, f) IL- 18, and g) interferon (IFN) –Y of chickens fed PC, NC, and NC + FOS diet[1], under phosphate buffered saline (PBS) injection as control or *Salmonella* lipopolysaccharide (LPS) challenge conditions (n = 6/treatment). Gene expressions were calculated relative to the housekeeping gene ß-actin. Error bars represent standard errors. Bars with different letters (a to d) differ significantly across all 6 treatment groups ($P < 0.05$). Bars with asterisks differ significantly among the PBS injected dietary groups ($P < 0.05$).

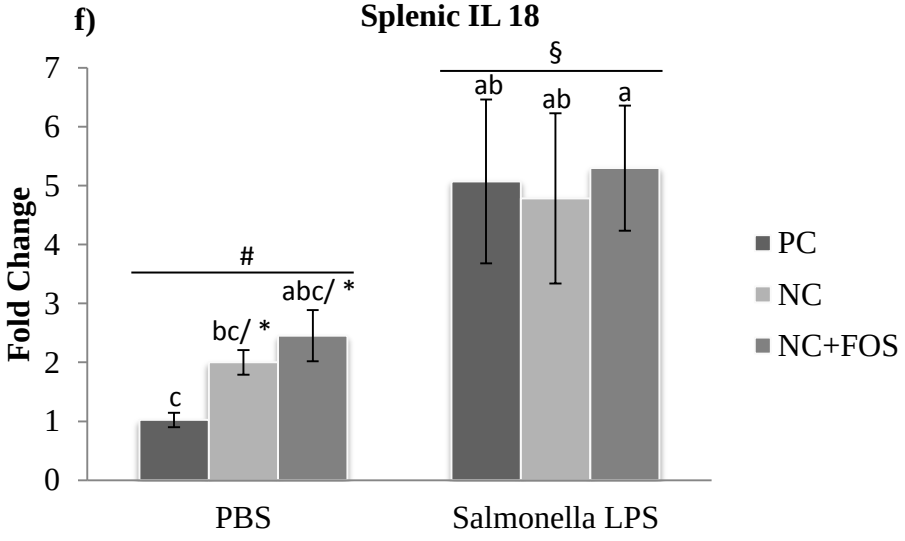
[1]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal base-diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

Cytokine gene expression in the spleen. In the spleen of broiler chickens (Figure 21, a to g), the *Salmonella* LPS challenge also demonstrated significant effects on the expressions of IL -1ß, -2, -6 and -18 ($P = 0.0002$, $P = 0.0477$, $P = 0.0190$ and $P = 0.0006$, respectively), but not on IL -10, TLR -4 or IFN -Y ($P > 0.05$). No dietary or diet × challenge interaction effects were observed among all the treatments and the analyzed cytokines. Similar to the ileum, the three dietary treatment groups challenged with *Salmonella* LPS alone did not exhibit any difference on splenic cytokine gene expressions. However, among the three PBS injected dietary treatments, the expression of splenic IL-18 was 2.4 and 2.0 fold up-regulated in chickens fed NC and NC + FOS, respectively, when compared to PC ($P = 0.022$). The NC + FOS diet also elevated splenic IL-1ß expression by 4.8 fold when compared with PC ($P = 0.031$). However, the expressions of IL -2, -6, -10, TLR -4 and IFN – Y resulted in no statistical differences among the dietary treatments.


a)
Splenic TLR4
Fold Change
3.5
3
2.5
2
1.5
1
0.5
0
PBS
Salmonella LPS
PC
NC
NC+FOS
b)
Splenic IL1ß
Fold Change
12
10
8
6
4
2
0
#
§
a
ab
a
abc/ **
bc/ *
c
PBS
Salmonella LPS
PC
NC
NC+FOS
c)
Splenic IL2
Fold Change
12
10
8
6
4
2
0
#
§
PBS
Salmonella LPS
PC
NC
NC+FOS


d)
Splenic IL6
Fold Change
12
10
8
6
4
2
0
#
§
a
ab
a
ab
ab
b
PBS
Salmonella LPS
PC
NC
NC+FOS


e)
Splenic IL10
Fold Change
3.5
3
2.5
2
1.5
1
0.5
0
PBS
Salmonella LPS
PC
NC
NC+FOS


f)
Splenic IL 18
Fold Change
7
6
5
4
3
2
1
0
#
§
ab
ab
a
abc/ *
bc/ *
c
PBS
Salmonella LPS
PC
NC
NC+FOS

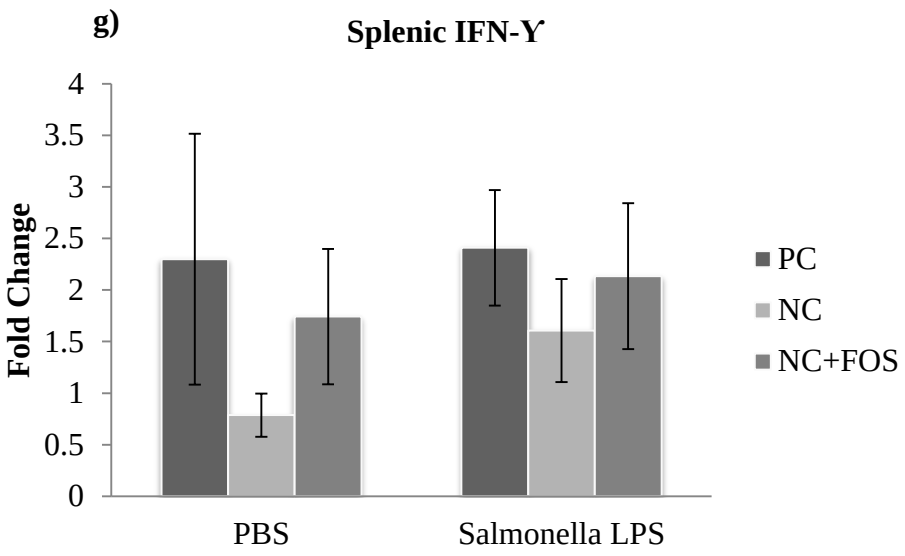


Figure 21. Splenic gene expressions of a) toll-like receptor (TLR) -4, b) interleukin (IL) -1ß, c) IL-2, d) IL-6, e), IL-10, f) IL- 18, and g) interferon (IFN) –Ɣ of chickens fed PC, NC, and NC + FOS diet[1], under phosphate buffered saline (PBS) injection as control or *Salmonella* lipopolysaccharide (LPS) challenge conditions (n = 6/treatment). Gene expressions were calculated relative to the housekeeping gene ß-actin. Error bars represent standard errors. Bars with different letters (a to c) differ significantly across all 6 treatment groups ($P < 0.05$). Bars with asterisks differ significantly among the PBS injected dietary groups ($P < 0.05$).

[1]Positive control (PC), wheat-corn-soybean meal based-diet supplemented with 5.5 mg of virginiamycin (Stafac-44) and 99 mg of monensin sodium (Coban) in the vitamin premix. Negative control (NC), wheat-corn-soybean meal base-diet without Stafac-44 and Coban. NC + FOS, NC diet supplemented with 0.5% of fructooligosaccharide (FOS).

4.5 Discussion

Growth performance and lymphoid organ weight

In the present study, the growth performance and the relative immune organ weight of the broiler chickens did not exhibit any difference between the three dietary groups. Similar findings on growth performance were documented by Kim et al. (2011a), where the BWG, overall feed intake, feed conversion and mortality did not differ between 0.5% FOS-supplemented group and the avilamycin-added control group. Biggs et al. (2007) and Telg and Caldwell (2009) have also observed no growth related difference in broiler chickens fed diets containing 0.4%, 0.8% and 1% of FOS, respectively. In contrast, positive effects on growth performance were described by Yang et al. (2008), that FOS supplementation has significantly improved BWG of broiler chickens at 21 d of age when compared with other antibiotic free groups. Ammerman et al. (1988), Bailey et al. (1991), and Xu et al. (2003) reported similar findings that 0.25% to 0.5% of FOS supplementation improved the FCR and BWG significantly. However, Williams et al. (2008) stated that the daily live weight gain and the FCR in 0.6g/kg FOS-supplemented group were not comparable to that of the avilamycin-added control. This result is also supported in later study by Khodambashi Emami et al. (2012), showing that the FCR was better in the chickens fed the diet supplemented with virginiamycin. Many factors such as the age, sex and health status of the birds, the environmental hygiene, the experiment protocols and the inclusion level of FOS can all affect the growth performance (Yang et al., 2009). Yusrizal and Chen (2003) reported that the BWG and FCR were improved by oligofructose supplementation in female birds but not in the males. Relative lymphoid organ weight that was measured in the current study can further reflect the overall health and immune status of the birds. Generally, an over 0.2% BW of the bursa of fabricius indicates an excellent health of the broiler flock (Bennet, 2002). Although our

results showed that the relative size of the spleen and bursa of fabricius were unaffected by dietary treatments, the relative weight of the bursa was more than 0.2% in all groups, suggesting that no stressors may exist during the raising time (21 d), and the environment did not affect the health and growth performance of broiler chickens that were fed either antibiotic-supplemented or antibiotic-free diets.

Intestinal morphology

The morphology of the intestinal mucosa is an important indicator that reflects the development of the digestive tract and the response of intestine to certain feed substances (Bogusławska-Tryk et al., 2012). It is commonly believed that an increased villus height and a decreased crypts depth is positively correlated to the digestive and absorptive functions in the GI tract of the birds, accounting for an enlarged absorptive area and a reduced tissue turnover rate (Xu et al., 2003; Williams et al., 2008; Bogusławska-Tryk et al., 2012; Munyaka et al., 2012). Xu et al. (2003) have reported that a 0.4% of FOS supplementation significantly increased ($P < 0.05$) ileal villus height, jejunal and ileal microvillus height, and VH: CD ratio, while decreasing crypt depth in the jejunum and ileum. On the contrary, Khodambashi Emami et al. (2012) have observed shorter duodenal crypt depth and higher VH: CD ratio in the antibiotic-added groups when compared with FOS-supplemented group. Our results were partially in agreement with Xu et al. (2003) that the villus height, crypt depth and total mucosal thickness were significantly increased in the ileum of the FOS-supplemented group, which indicated that FOS supplementation may play a positive role in increasing the ileal absorption area, thickening of the ileal mucosa and stimulation of the gut immune system. The characteristics of the ileal microbiota and the immune responses may be able to further explain the changes of the intestinal morphology.

Ileal microbiota

Studies on the relationship of gastrointestinal microbiota with the chickens and the diet ingested by the host have been conducted since the 19th century (Shapiro and Sarles, 1949; Lu et al., 2003). Classical culture-based methods have been widely used to study the chicken microbiota, while these methods are highly selective to cultivable bacteria (1% of all bacteria) under specific conditions (Hugenholtz et al., 1998; Apajalahti et al., 2004). Molecular approaches based on bacterial 16S rDNA sequences have been developed to characterize and identify the gut microbiota (Tannock 2001; Dumonceaux et al., 2006). Among these approaches, high-throughput next generation sequencing (NGS) technology is capable of obtaining in-depth information on a larger profile, and is becoming a powerful tool to investigate the biological and ecological roles of gut microbiota (Diaz-Sanchez et al., 2013).

In the present study, we have applied Illumina MiSeq sequencing to evaluate the mucosal-attached and luminal microbiota in the ileum of broiler chickens and in the same time investigated the effect of FOS supplementation. The most abundant phyla Firmicutes, Proteobacteria and Bacteriodetes that have been observed in our study are in agreement with previous phylogenetic diversity census findings by Wei et al. (2013) and clone library based terminal restriction fragment length polymorphism (T-RFLP) analysis by Torok et al. (2011), where these three phyla were accounted for over 90% of all the sequences. A total of 14 phyla and 179 different bacterial genera were identified in our ileal samples, which exceeded the total of 12 phyla and 117 genera described by Wei et al. (2013), however, the sampling locations were not clearly documented in their census. Gong et al. (2002) first compared the bacterial population in the mucosa and lumen of ileum from 10 broiler chickens by using T-RFLP. Two distinct cloned bacterial sequences with significant level of polymorphism have been observed in the

ileal mucosa, thus differences may exist between the two ileal sites (Gong et al., 2002). In our study, 66 and 18 genera were found unique to ileal mucosa and digesta, respectively. However, no statistical significance ($P > 0.05$) was observed on the diversity indices (Shannon and Simpson), observed species or OTUs (chao1) between the two ileal sites. The diversity indices elucidate both richness and evenness of an ecological community and represent the uncertainty of species identities within a sample. Communities with greater abundance of genera and higher evenness would result in greater uncertainty to estimate the next genera sampled (Hill et al., 2003; Pedroso et al., 2013). It is possible that some rare genera existed in small population or only affiliated to individual samples from our results.

Very few studies have investigated the ileum mucosal-attached microbiota of the chickens. Gong et al. (2002) described that the predominant bacteria in the ileal mucosa of broiler chickens are gram-positive bacteria with low G + C content such as lactobacilli, butyrate-producing and *Enterococcus cecorum*-related bacteria. Our sequencing results showed that a total of 161 bacterial genera were identifiable in the ileal mucosa, in which Lachnospiraceae (f) in Firmicutes, *Helicobacter* in Proteobacteria, S24-7 (f) in Bacteroidetes and *Desulfovibrio* in Proteobacteria were the most abundant bacteria. Furthermore, the relative abundance of Lachnospiraceae (f) was significantly greater in the ileal mucosa than the digesta. The Lachnospiraceae (phylum Firmicutes, class Clostridia) has been found rich in the digestive tract of many animals (Meehan and Beiko, 2014). Members of this family have the ability to produce SCFA such as butyric acid, which can provide energy for the growth of other gut microbes and promote the development of the host epithelial cell (Liu et al., 1999; Yeoman et al., 2012; Meehan and Beiko, 2014). The SCFA butyrate has also been shown to have beneficial effects such as improving growth performance and carcass quality in chickens (Panda et al., 2009). The significantly increased

Lachnospiraceae (f) in the ileal mucosa may indicate that the associated species are more capable of attaching to the luminal wall of the intestine, and may interact closely with the gut of the broiler chickens. *Desulfovibrio* are also ubiquitous microorganisms found in nature (Goldstein et al., 2003). They are a group of sulfate-reducing, anaerobic bacteria with over 30 proposed species, some of which may induce asymptomatic infections in the human gastrointestinal (GI) tract, or may act as opportunistic pathogens (Beerens and Romond, 1977; Goldstein et al., 2003). It is also interesting to observe a relatively high amount of *Helicobacter* from a healthy broiler flock in the present study. The genus *Helicobacter* contains more than 20 species of gram-negative bacteria that share the feature of a sheathed flagellum and has been isolated from intestinal mucos linning or contents of many animals, especially rodent and bird species (Gibson et al., 1999; Robertson et al., 2005). *Helicobacter pullorum* has been reported to be widely found in the intestinal tract of asymptomatic poultry and is susceptible to antibiotics such as penicillin (Young et al., 2000). Previously, *H. pullorum* has been incorrectly classified into atypical *Campolybacter*, so that the true prevalence of *Helicobacter* in poultry may be underestimated (Gibson et al., 1999; Young et al., 2000). Although there is no evidence of showing whether these bacteria are harmful to the host, it may associate with enteritis and vibrionic hepatitis in poultry, and also with diarrhoea, liver and gastric disease in humans (Young et al., 2000; Fox, 2002). The relative abundance cannot truly represent the absolute quantity of each bacterium, since 16S rDNA was amplified by PCR before the sequence reading, the composition and characteristic of *helicobacter* need further quantification. Oakley et al. (2013) obtained microbial sequences from litter (6 wk old), fecal (6 wk old) and carcass (8 wk old) samples in commercial broiler chickens, and documented that pathogenic bacteria (i.e., *Clostridium*, *Campylobacter* and *Shigella*) belong to a core microbiome that is common to all sample types. Poultry processing

can lower the microbial richness of fecal samples and reduce the abundance of *Campylobacter*, whereas it also creates the condition for the growth of relatively unique bacteria (Oakley et al., 2013).

A number of studies have focused on the bacterial community in the ileal content of the chicken. Lu et al. (2003) evaluated the bacterial community in the ileum content by examining the 16S rDNA clone libraries and stated that the major bacteria are *Lactobacillus* (70%), *Clostridiaceae* (11%), *Streptococcus* (6.5%) and *Enterococcus* (6.5%). Similarly, Dumonceaux et al., (2006) observed that the predominant sequences were lactobacilli, in particular *L. crispatus* and *L. salivarious*, from the proximal ileum of broiler chickens. In addition to *Lactobacillus*, Hoeven-Hangoor et al. (2013) also reported that *Streptococcaceae*, *Enterococcaceae*, *Staphylococcaceae*, *Enterobacteriaceae*, *Clostridiaceae*, *Coriobacteriaceae*, *Peptostreptococcaceae* and *Micrococcaceae* were found in lower ratios in the chicken ileum. Our results showed that the relative abundance of *Helicobacter*, *Desulfovibrio*, Lachnospiraceae (f), S24-7 (f), *Oscillospira*, *Bacteroides*, *Allobaculum* were above 5%, Rikenellaceae (f), Bacteroidales (o), Ruminococcaceae (f), *Ruminococcus*, *Mucispirillum*, Clostridia (c), Erysipelotrichaceae (f), *Odoribacter* were above 1%, and *Lactobacillus*, *Parabacteroides*, Clostridiales (o) were above 0.5% in the ileal digesta samples. Although the high throughput sequencing technology was mainly used for microbial qualification and the relative abundance was not comparable to that of the clone libraries, the ileal bacterial composition was quite distinct from previous studies. Many studies have addressed that the chicken small intestinal microbiota is extremely diverse and can be influenced by several factors especially the age of the birds, the diet and the surrounding environment (Knarreborg et al., 2002; Lu et al., 2003; Apajalathi et al., 2004; Rehman et al., 2007; Danzeisen et al., 2011; Torok et al., 2011; Yeoman

et al., 2012). Differences between the breed of chickens, analytical methods, animal research facilities, geographical locations and farm/research personnel can be all accounted for the variations observed from experiment to experiment. Van der Wielen et al. (2002) have also stateded that every chicken has its unique dominant intestinal bacterial community, which supported the high variations observed from our individual samples. The composition of the GI bacterial community is constantly being changed due to the settlement and replacement of more stable and dominate bacterial species as the bird ages (Lu et al., 2003). Lee et al. (2002) and Lu et al. (2003) discovered that the GI tract of chicken at 3 days of age is populated with *L. delbrueckii, C. perfringens* and *Campylobactor coli*, whereas from 7 to 21 days of age, it is dominant by *L. acidophilus, Enterococcus* and *Streptococcus*. Environmental factors such as the type of drinkers (nipple vs. puddle drinker), the water sources, the cleanness of the feeders and the age of litter are also greatly accounted for the bacterial diversity observed in our study. Pedroso et al. (2013) reported that the abundance of pathogenic *Clostridium* spp. increases as the litter ages. In addition, feed processing methods, feed ingredients and feed additives also influence on the gut microbiota (Engberg et al., 2002). For example, corn favours low %G + C clostridia, enterococci and lactobacilli, whereas wheat favours higher %G + C *bifidobacteria* (Apajalathi et al., 2004). It would be necessary to examine and consider the bacterial communities in the surrounding environment as a reference.

In the present study, the SAS analyses have showed that the diets tend to alter the abundance of phylum Deferribacters between PC and NC + FOS groups. Deferribacterales (o) and *Mucispirillum* were found under Deferribacters (p). Deferribacteres (o) are iron-reducing bacteria, which have been isolated from water-based samples and could be involved in detoxification or bioremediation of the minerals in the environment from being utilized as a food

source by the host (Zbinden et al., 2003). *Mucispirillum* is a mucosa-associated spiral-shaped bacterium that has been isolated from mammals (e.g. rodents), and it may behave in a similar way to *Helicobacter* (Robertson et al., 2005). However, little is known about this genus, and our study has documented that it also colonize in the intestine of avian species.

Several biochemistry- and culture-based studies have shown that dietary FOS supplementation has the capability of enhancing gut fermentation, increasing SCFA production and stimulating the growth of beneficial bacteria such as *bifidobacteria* and lactobacilli, while limiting the growth of pathogenic bacteria such as *Salmonella spp*., *Clostridia perfringens* and *Escherichia coli* in broiler chickens (Flickinger et al., 2003; Xu et al., 2003; Kim et al., 2011). However, Fukata et al. (1999) and Biggs et al., (2007) reported that 0.1% and 0.4% of FOS supplementation had no significant effect on cecal *Bifidobacterium*, *Lactobacillius*, *Clostridium perfringens,* or *Escherichia coli* populations in 21-d-old broiler chickens. In this study, the overall abundance of *bifidobacterium* and *lactobacilli* were at lower rates. The reduced abundance of *Helicobacter* and *Desulfovibrio* in the ileal mucosa of NC + FOS fed chickens indicated that other bacteria such as Lachnospiraceae (f), Bacteroidaceae (f), Coriobacteriaceae (f) and *Sutterella* (as shown in the PLS-DA analyses) may have the ability of producing higher level of SCFA and competitive exclusion of unfavourable microorganisms that have pathogenic properties. For instance, Erysipelotrichaceae (f) and Coriobacteriaceae (f) are related to the lipid and cholesterol metabolism of the host, where an altered cholesterol excretion may shift the gut microbiota through antimicrobial actions (Martinez et al., 2013).

The FOS supplemented group also showed an increased number of unique genera (51 vs. 10 and 5, respectively) and a more diverse microbiota (139 vs. 96 and 85, respectively) in the ileal mucosa when compared with PC and NC groups. Relative ($P = 0.091$) distinguished microbial

communities which were measured by unweighted Unifrac analysis may explain the diversity that have been observed between the dietary groups. Unweighted Unifrac algorithm compares the phylogenetic distance between bacterial communities with all OTUs considered at equal abundance, which is useful for examining the occurrence of rare species (Lozupone and Knight, 2005). The colonization of relatively unique bacteria (that specifically found in NC + FOS group) such as *Akkermansia* (mucin-degrading bacteria), *Janthinobacterium* (anti-bacterial and anti-fungus compounds producing bacteria), *Shewanella* (electron-accepting bacteria that can couple the decomposition of organic matter for carbon cycling), *Butyrivibrio* (butyrate producing bacteria), *Coprococcus* (butyrate producing bacteria) and *Paludibacter* (propionate-producing bacteria) may be promoted by FOS supplementation, suggesting that these bacteria exist in small amount on the epithelial wall of the ileum and benefit (e.g. enhance gut immunity and increase mucosal absorption area, as shown in our results) the host and other bacteria (Bryant and Small, 1956; Pryde et al., 2002; Ueki et al., 2006; Fredrickson et al., 2008; Everard et al., 2013; Hornung et al., 2013). Becaue the functions and ecological roles of many gut associated bacteria are still unknown, further investigation is needed for better understanding and characterization of these microbes.

The composition of the intestinal microbiota is also dependent on the antibiotic supplementation (Knarreborg et al., 2002). A T-RFLP analysis by Lu et al. (2008) revealed that antibiotic supplementation (bacitracin/virginiamycin or monensin) reduced ileal bacteria diversity by supressing *lactobacilli* while inducing intestinal Clostridia in all ages of broiler chickens. Knarreborg et al. (2002) also reported that the total *Lactobacillus* and *C. perfringens* population were affected by dietary antibiotic supplementation in the ileal content of broiler chickens. Similary, Danzeisen et al. (2011) investigated the cecal microbiota of chicken in

response to antibiotic growth promoters along with anticoccidial by using pyrosequencing. The result showed that the population of *Roseburia*, *Lactobacillus* and *Enterococcus* were reduced whereas *Coprococcus* and *Anaerofilum* were increased in the presence of monensin, and also an enrichment of *Escherichia coli* was seen in treatments that combined monensin with virginiamycin or tylosin. On the contrary, Singh et al. (2013) evaluated the influence of penicillin as a growth promoter and observed an increased Firmicutes and a decreased Bacteroidetes in chicken ceca, when comparing the penicillin supplemented group with the control. Although no statistical significance on the relative bacterial abundance or diversity indices was obtained in ileal digesta microbiota from the current study, the rarefaction curve showed that numerically the PC group had about 500 OTUs and 300 observed species less than that of NC and NC + FOS, which is in agreement with Gaskins et al. (2002), Dibner and Richards (2005) and Kim et al. (2011) that antibiotic treatment decreased the total microbial population. The PLS-DA diagrams demonstrated shifts in several bacteria between PC, NC and NC + FOS dietary groups. However, it is equally important to understand whether these changes have biological meaning for the modulation of gut microbiota or on the overall health of the host. Thus, further and in-depth analyses such as functional shotgun metagenomic sequencing, as well as multiple experiments under different stressors are warranted to answer these questions.

Effects of diet and immunological challenge on immune responses of broiler chickens

In the previous studies, dietary FOS supplementations have demonstrated positive effects on the modulation of the immune system of broiler chickens. Janardhana et al. (2009) supplemented 0.5% of FOS in addition to the basal broiler chicken diet and observed higher titers of plasma IgM ($P < 0.01$) and IgG ($P < 0.01$) than the control group. The FOS-treated birds have also demonstrated a reduced percentage of B cells and a depressed mitogen responses of lymphocytes

in the cecal tonsil ($P < 0.05$), without exhibiting any detrimental effects on growth performance. Khodambashi Emami et al. (2012) investigated the FOS as alternatives to virginiamycin on immune response of male broilers, and documented that the primary antibody titers against sheep red blood cell were higher in FOS-supplemented treatment. Kim et al. (2011) noticed that the H: L ratio and the basophil counts were higher in antibiotic-free control and 0.5% FOS groups than in the treatments added with antibiotics or other prebiotics, although plasma IgA and IgG concentrations were not significantly different. Pathogen challenged studies have also been conducted to evaluate the effect of FOS supplementation, by feeding or spraying either *Samonella spp.* or *Escherichia coli* to the broiler flock (Bailey et al., 1991; Oyarzabal and Conner, 1996; Yang et al., 2008; Telg and Caldwell, 2009). Results showed that feeding FOS in the diet may reduce the susceptibility to *Salmonella* colonization; however none of those studies have evaluated the immune responses of broiler chickens.

Changes in the number of circulating leukocytes in particular heterophils and lymphocytes are a reliable indicator of stress in poultry. The number of heterophil increases while lymphocyte number decreases in response to stressors (Gross and Siegel, 1983). *Salmonella* Enteritidis and its major pathogen-associated molecular pattern lipopolysaccharides have been well recognized as a stressor to birds (Bailey et al, 1991; Mogut et al., 2005). Increased heterophils count and H: L ratio was observed in our study, confirming that the *Salmonella* LPS dosage and the 4 h post-injection time were very effective to induce stress in broiler chickens. Heterophils are key components in the innate immune system of poultry, and are capable of eliminating a number of pathogens due to their phagocytosis, chemotaxis and adhesion activities (Munyaka et al., 2012; Swaggerty et al., 2014). Lowered level of heterophils may indicate that less pathogenic or pro-inflammatory substances were presented in the birds fed NC + FOS. Monocytes constitute 5 - 10%

of peripheral blood leukocytes. They can move quickly in response to infections, produce cytokines and differentiate into macrophages and dendritic cells to assist the innate immune response (Beal et al., 2006). Therefore, an increased monocytes percentage in the NC + FOS group indicated that FOS supplementation may play a protective role on chicken immunity and is able to alleviate infections. This effect is likely due to the shift in the gut microbiota such as changes to the *Lactobacillus* profile, which exerts different patterns for dendritic cell activation (Christensen et al., 2002; Janardhana et al., 2009).

Natural antibodies are essential components of the innate immune system, which are produced by B type lymphocytes without previous induction such as infection, vaccination or passive immunization (Ochsenbein et al., 1999; Ochsenbein and Zinkernagel, 2000). In the present study, significantly higher natural IgG and IgA concentrations were observed in NC + FOS group under LPS challenge. Chicken IgG is the predominant antibody against systemic infection and IgA is the most abundant antibody in the intestine to protect the epithelium from pathogenic microorganisms (Davison et al., 2006; Dankowiakowska et al., 2013). An elevation of both IgG and IgA could assist in early recognition and clearance of invading pathogens in the whole body and especially at the site of the intestine (Davison et al., 2006). Significant diet × challenge effects indicated that the influence of FOS supplementation was amplified by LPS challenge, which induced higher natural IgG and IgA production in the NC + FOS group. The LPS challenge also exhibited significant difference in natural IgG concentration and a trend to increase IgA concentration under LPS challenge. In the case of natural IgG, it is possible that the PC and NC groups shifted the antibody composition by stimulating the production of specific antibodies instead of natural immunoglobulins. However, the concentration of natural antibodies fluctuates upon receiving antigens, so that there may be a transient effect beyond the current

observations at 4-h post LPS injection (Munyaka, 2012). Specific antibodies are produced by the B lymphocytes in response to extracellular antigens recognized by T cells (Janeway et al., 2001). They consist of the acquired humoral immune system and can respond rapidly to a second exposure of the same antigen. The LPS challenge significantly increased the specific IgG to LPS-antigen production in the broiler chickens that were injected with *Salmonella* Enteritidis LPS, which indicated that the acquired immune response had taken place after 4-h post injection. Lipopolysaccharide challenged NC + FOS group had the highest specific IgG production when compared with the other treatments, which resulted in significant dietary and diet × challenge effects. The specific IgA level did not exhibit any difference between the treatment groups, and it was relatively low (0.08 - 0.12, optical density) when compared with IgG (0.1 - 0.3, optical density) in chicken serum. It is highly possible to be able to observe significantly different levels of specific IgA, if the antibody was measured in the intestinal fluid. In general, our results have demonstrated a significant increase of both natural and LPS specific antibody production, indicating that FOS supplementation may be able to enhance both non-specific and specific immunity of the broiler chicken. It can be speculated that FOS supplementation has increased the SCFA fermentation especially the lactic acid production in the GI tract of the chickens, which further increased the proliferation of macrophages and their phagocytic activity (e.g., cytokine production), and stimulated the antibody production from the B lymphocytes (Dankowiakowska et al., 2013).

Toll-like receptors can recognize the conserved pathogen-associated molecular patterns of the LPS of gram-negative bacteria, and are involved in a chain reaction that stimulates the innate immune response (Aderem and Ulevitch, 2000). In chickens, the TLR-4 has been shown to be linked to resistance to *Salmonella* infection (Leveque et al., 2003). We found in the ileum but not

in the spleen that the LPS challenge significantly up-regulated the TLR-4 expression when comparing the LPS challenged groups with the PBS injected groups, which may indicate that the small intestine is a main site for pathogen control of the gut associated infections. Cytokines are intercellular proteins that associate with both innate and acquired immunity, the expressions of IL-1ß, IL-2, IL-6, IL-10, IL-18 and IFN-Ɣ were measured in this study. Interleukin-1ß is a pro-inflammatory cytokine mainly secreted from monocytes and macrophages. It stimulates the infiltration of T cells and the production of acute phase protein, and it is important for the inhibition of inflammatory responses (Corwin, 2000). Interleukin-2 is a pro-inflammatory cytokine produced by naïve T cells, in response to antigen binding and further proliferation of T cells (Cacalano and Johnston, 1999). Similar to IL-1ß, IL-6 is also produced from monocytes and macrophages, and serves as both pro-inflammatory and anti-inflammatory cytokine, which can secret acute phase protein and in the same time inhibit the production of IL-1 (Corwin, 2000; Waititu et al., 2014). Interleukin-10 is a major anti-inflammatory cytokine which inhibit the cytokine production by T helper 2 cells and down-regulates the expression of major histocompatibility (MHC) antigens in immune cells (Corwin, 2000). Interleukin-18 is another pro-inflammatory cytokine produced by macrophages, and it is able to induce cell-mediated immune response after exposure to a pathogen, stimulating the release of IFN-Ɣ and IL-1 (Corwin, 2000). Interferon-Ɣ is a pro-inflammatory cytokine responsible for increasing the expression of MHC antigens, promoting T helper 1 cell differentiation and stimulating the macrophages activities (Tizard, 2009). Janardhana et al. (2009) found that the expressions of IFN-Ɣ, IL-6 and IL-10 exhibit no difference in the cecal tonsil of broiler chickens between FOS-supplemented group and control. Our results showed that the LPS challenge up-regulated all the measured cytokine gene expression in the ileum and IL-1ß, -2, -6 and -18 in the spleen of the

broiler chickens, which confirmed that the *Salmonella* Enteritidis LPS is a stimulus of pro-inflammatory cytokine production. Interestingly, both ileal pro- and anti- inflammatory cytokines (except IL-6) were elevated around 2 to 5 folds by the FOS supplementation among PBS-injected healthy chickens, indicating that the FOS supplementation promoted both pro- and anti-inflammatory functions and the immune system of the chickens fed FOS supplemented diets may stay more alert under non-stressed circumstance and may react faster when exposed to any infections or stressors. Taking into consideration the leukocytes and ileal microbiota results from our study, the up-regulation of cytokines IL-1ß, IL-10, IL-18 may be due to a significantly increased monocytes count, and the shift in the ileal microbiota especially as seen on the increased species diversity and the reduced *Helicobacter* population of the FOS-supplemented group. An increased pro-inflammatory cytokine and chemokine gene expressions were also reported to be associated with increased resistance to *Salmonella* Enteritidis, including elevated IL-1ß, IL-6, IL-8, IL-18 and CCLi2 in heterophils, monocyte-derived macrophages, the ceca and cecal tonsil (Ferro et al., 2004; Cheeseman et al., 2008; Setta et al. 2012; Swaggerty et al., 2014). Further research is needed to explain the mechanisms and the interaction of these cytokines between the host immune system and the gut microbiota.

In summary, dietary FOS supplementation may serve as a modulator to improve intestinal morphology, elevate pro- and anti-inflammatory immune responses and shift the gut microbiota, especially in the small intestine of the broiler chickens. The microbial composition and diversity between the ileal mucosa and digesta along with the dietary effect of antibiotics and FOS supplementation were described and compared in the current study. The data showed that although the two ileal sites did not exhibit a significant difference, the diets were able to shift the ileal microbiota of the broiler chickens. The *Salmonella* Enteritidis LPS challenge established

significant effects on the immune responses of broiler chickens, in the meantime, the FOS supplementation enhanced innate and humoral immunity of chicken by producing higher levels of natural and specific antibodies when under such a stress. Overall, dietary FOS supplementation played protective roles towards the immune response, intestinal morphology and ileal microbiota of the broiler chickens, without impairing their growth performance. Supplementing FOS in broiler chicken diets may stand as an alternative method to replace AGPs.

4.6 Acknowledgements

This project was financially supported by NSERC-Engage grant. The authors would like to thank Drs. Bogdan A. Slominski and Anna Rogiewicz (University of Manitoba, Winnipeg, MB, Canada) for their insight and guidance throughout the poultry trial. Special thanks to Mohammed Alizadeh for his helpful discussion and technical assistance on immune response related analyses. Thanks to Dr. Alemu Hunde and Navjot Nandha for the demonstration of mRNA and microbial DNA extraction techniques, respectively. The authors also appreciate the generous support from Ingredion Inc. (Etobicoke, ON, Canada) for providing FOS for this research.

5. GENERAL DISCUSSION

The dietary supplementation of prebiotic FOS has been widely studied in poultry and in many other species in the previous years (Bogusławska-Tryk et al., 2012). The present research investigated the effect of dietary FOS supplementation from different pespectives, including nutrition, immunology and microbiology of broiler chicken. The results presented in the current thesis provided in-depth informations to elucidate the FOS-induced mechanisms regarding growth performance, bone mineralization, P utilization, innate and acquired immune responses and gastrointestinal microbiota of broiler chickens, which have progressed our current understanding on supplemental prebiotic FOS in the broiler rations, and also have built a network of knowledge on the metabolic relationships in different physiological systems of poultry.

From the nutrition point of view, the dietary FOS supplementation had no significant effect on growth performance or on P utilization in the broiler chickens, but demonstrated a negative effect on bone mineralization in birds fed low Ca and available P diets, which suggested that supplemental FOS may not impair the growth performance, but it also may not be used as a suitable supplement for enhancing bone quality in broiler chickens. It is possible that the gram-positive commensal microorganisms such as *Lactobacillus*, *Streptococcus* and *Enterococcus* were increased by FOS fermentation in the GI tract and they have competed with the host for nutrients and energy under the low dietary Ca and P content (Gaskins et al., 2002). In contrast, supplemental 0.5% of FOS together with phytase has exhibited significant improvement on BWG, apparent P digestibility and P retention, indicating that FOS and phytase may have additive effects in promoting growth performance and P utilization in broiler chickens. Addition of FOS to the phytase-supplemented diet may further facilitate phytate hydrolysis by prohibiting

the formation of Ca-phytate complexes and improving digestive enzyme activities (Xu et al., 2003).

In terms of the immunology, dietary FOS supplementations have demonstrated positive effects on the modulation of the immune responses of broiler chickens, including altered leukocytes compositions, increased serum natural and specific antibody concentrations upon *Salmonella* LPS challenge and elevated both pro- and anti- inflammatory cytokine gene expressions without signs of impairing the growth performance. Our results indicated that the FOS supplementation may be able to enhance both nonspecific and specific immunity of the broiler chicken. It is possible that FOS supplementation has increased the SCFA production especially lactic acid in the GI tract of the chickens, which further increased the proliferation of macrophages and their phagocytic activity such as increased production of cytokines, and stimulated the antibody production from the B lymphocytes (Dankowiakowska et al., 2013). Incresed cytokine gene expressions may be associated with increased resistance to *Salmonella* Enteritidis infections, which would be beneficial to the health and the intestinal microbial composition of the birds (Swaggerty et al., 2014).

Taking into consideration the gut microbiology, dietary FOS supplementation altered the ileal microbiota with an increased number of unique bacterial genera and a more diverse microbiota in the ileal mucosa of the broiler chickens. The colonization of relatively unique bacteria such as *Akkermansia*, *Janthinobacterium*, *Shewanella*, *Butyrivibrio*, *Coprococcus* and *Paludibacter* may benefit the host in terms of nutrient utilization and synergistically co-exist with other gut bacteria. Although we did not specifically quantify the bacteria of our interests in the present research, it has been reported previously that dietary FOS supplementation has the capability of enhancing gut fermentation, increasing SCFA production and stimulating the

growth of beneficial bacteria such as *bifidobacteria* and lactobacilli, while limiting the growth of pathogenic bacteria such as *Salmonella spp*., *Clostridium perfringens* and *Escherichia coli* in broiler chickens (Flickinger et al., 2003; Xu et al., 2003; Kim et al., 2011a). It is possible that the shifted gut microbiota could alter the nutrient utilization and further enhanced the gut immunity of the broiler chickens.

Overall, dietary FOS supplementation played protective roles on maintaining growth performance, improving intestinal morphology, enhancing immune responses and modifying gut microbiota of the broiler chickens, and it may be used as an alternative substance to replace antibiotics in the broiler rations. However, the mechanisms of FOS induced changes are still unknown, further investigation is needed to better understand the nutritional, immunological and microbiological functions in poultry.

6. CONCLUSIONS

- The two levels (0.5% and 1%) of FOS supplementation in low Ca and available P diets did not exhibit significant difference on performance, bone parameters and total P utilization between the treatments.
- The supplementation of phytase and FOS in low Ca and available P diets was not able to improve the bone mineralization parameters to the same level as to the standard diet; however it showed a trend in increasing BWG at a later age and improved the total P utilization.
- The use of FOS supplementation had negative effect on broiler bone quality in low Ca and available P diets. Further research is necessary to investigate the effect and mechanism of FOS on nutrient absorption in broiler chicks.

- The antibiotic-free diet and FOS-supplemented diet did not impair the growth performance of broiler chicks at different ages.
- The FOS supplementation increased the total mucosa thickness in the ileum including villus height and crypt depth, modified the composition of certain bacterial species in the ileum and up-regulated ileal and splenic cytokine gene expressions of the broiler chicken.
- The *Salmonella* Enteritidis LPS challenge significantly altered the white blood cell composition, upregulated cytokine gene expressions in the ileum and spleen, and demonstrated significant effect towards the immune responses of broiler chicken.
- A 0.5% FOS dietary supplementation significantly increased the natural IgG and IgA concentrations and the specific IgG level in response to *Salmonella* LPS challenge. It also increased monocytes concentration and reduced heterophils concentration upon LPS challenge.
- Fructooligasaccharides supplementation may play protective roles in improving the immunity of broiler chickens.
- Mucosal-attached and luminal microbiota in the ileum of broiler chickens were first compared and characterized by using high-throughput Illumina sequencing in this study. The ileal microbiota is closely related and can be modified by the ingested diets. Shared and unique ileal bacterial taxa to AGPs-supplemented, AGPs-free and FOS supplemented diets were identified in the broiler chicken.

7. FUTURE DIRECTIONS

- Evaluate the function and relationship of associated bacterial species with the dietary treatment by conducting metagenomic functionality test and quantifying interested microorganisms by using qPCR.
- Examine and compare the microbiota of broiler chickens to evaluate tissue-to-tissue and experiment-to-experiment variation.
- Further investigate the mechanism of FOS and other prebiotic products as dietary supplements on nutrient utilization, immunity and gut microflora composition of both broiler and laying hens.
- If possible, conduct future experiments with chickens that are raised in floor pens and oral gavage the experimental birds with live *Salmonella spp.*, in such ways to simulate farm conditions (larger production scale and greater exposure to stressors) and to examine the effectiveness of FOS supplementation on the immunity and gut microbiota of both broiler and layer chickens.
- Combine different feed additives such as probiotics, organic acids together with FOS or other fermentable sugar to evaluate synergistic effects, and their influence on nutrition, immunology and microbiology of poultry.

8. REFERENCES

Abbas Hilmi, H. T., Surakka, A,, Apajalahti, J., Saris, P. E. 2007. Identification of the most abundant lactobacillus species in the crop of 1- and 5-week-old broiler chickens. Appl. Environ. Microbiol. 73:7867–7873.

Aderem, A., and R. J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. Nature 406:782–787.

Ammerman, E., C. Quarles, and P. V. Twining, 1988. Broiler response to the addition of dietary fructooligosaccharides. Poult. Sci. 67(Suppl.1):46. (Abstr.)

Anderson, M. 2005. PERMANOVA: a FORTRAN computer program for permutational multivariate analysis of variance. Department of Statistics, University of Auckland, New Zealand 24.

Angel, R., W. W. Saylor, A. D. Mitchell, W. Powers, and T. J. Applegate. 2006. Effect of dietary phosphorus, phytase, and 25-hydroxycholecalciferol on broiler chicken bone mineralization, litter phosphorus, and processing yields. Poult. Sci. 85: 1200-1211.

Angel, R., W. W. Saylor, A. S. Dhandu, W. Powers, and T. J. Applegate. 2005. Effect of dietary phosphorus, phytase, and 25-hydroxycholecalciferol on performance of broiler chickens grown in floor pens. Poult. Sci. 84:1031–1044.

AOAC International. 1990. Official Methods of Analysis. 15th ed. AOAC Int. Washington, DC.

Apajalahti, J., A. Kettunen, and H. Graham. 2004. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. World's Poult. Sci. J. 60: 223-232.

Babu, U. M. and Raybourne, R. B. 2008. Impact of dietary components on chicken immune system and Salmonella infection. Exp. Rev. Anti-infect. Ther. 6.1: 121.

Bailey, J. S., L.C. Blankenship, and N. A. Cox. 1991. Effect of fructo-oligosaccharide on Salmonella colonization of the chicken intestine. Poult. Sci. 70: 2433-2438.

Bailey, R. A. 2013. Gut health in poultry – the world within. Service bulletin. Aviagen®, U.S.A.

Barry, K. A., B. M. Vester, and G. C. Fahey. 2009. Prebiotics in Companion and Livestock Animal Nutrition. In: Prebiotics and Probiotics Science and Technology. Charalampopoulos, D., and R. A. Rastall, [editor]. Springer Science + Business Media, LLC, New York: 353-457.

Beal, R. K., C. Powers, T. F. Davison and A. L. Smith. 2006. Immunological development of the avian gut. In: Avian Gut Function in Health and Disease. Perry, G. C., [editor]. CABI Publishing, Oxford: 85-103.

Bedford, M. 2000. Removal of antibiotic growth promoters from poultry diets: implications and strategies to minimise subsequent problems. World's Poult. Sci. J. 56: 347-365.

Bennet, C. 2002. How to score bursa size in broiler chicken flocks. Manitoba Agriculture, Food and Rural Initiatives. Winnipeg, Canada.

Biggs, P., C. M. Parsons, and G. C. Fahey. 2007. The effects of several oligosaccharides on growth performance, nutrient digestibilities, and cecal microbial populations in young chicks. Poult. Sci. 86:2327-2336.

Bogusławska-Tryk, M., A. Piotrowska, and K. Burlikowska. 2012. Dietary fructans and their potential beneficial influence on health and performance parametrs in broiler chickens. J. Cent. Euro. Agri. 13:272-291.

Boling, S. D., M. W. Douglas, M. L. Johnson, X. Wang, C. M. Parsons, K. W. Koelkebeck, and R. A. Zimmerman. 2000. The effects of dietary available phosphorus levels and phytase on performance of young and older laying hens. Poult. Sci. 79:224-230.

Bryant, M. P. and N. Small. 1956. The anaerobic monotrichous butyric acid-producing curved rod-shaped bacteria of the rumen. J. Bacteriol. 72:16-21.

Butaye, P., L. A. Devriese, and F. Haesebrouck. 2003. Antimicrobial growth promoters used in animal feeds: effects of less well known antibiotics on Gram-positive bacteria. Clin. Microbiol. Rev. 16:175-188.

Cacalano N. A., J. A. Johnston. 1999. Interleukin-2 signaling and inherited immunodeficiency. Am. J. Hum. Genet. 65:287-93.

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S. M. Owens, J. Betley, L. Fraser, M. Bauer, N. Gormley, J. A. Gilbert, G. Smith, and R. Knight. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 6:1621-1624.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Pena, J. K. Goodrich, and J. I. Gordon. 2010b. QIIME allows analysis of high-throughput community sequencing data. Nat. Meth. 7:335-336.

Caporaso, J. G., K. Bittinger, F. D. Bushman, T. Z. DeSantis, G. L. Andersen, and R. Knight. 2010a. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics. 26:266-267.

CCAC. 1993. Guide to the care and use of experimental animals. 2nd ed. Vol. 1. Can. Counc. Anim. Care, Ottawa, Ontario, Canada.

Centers for Disease Control and Prevention (CDC). [Internet]. 2011 Estimates of Foodborne Illness in the United States. 2011 [modified 2011 Apr 15; cited 2014 Apr 3]. Available from: http://www.cdc.gov/features/dsfoodborneestimates/.

Chambers, J. R. and J. Gong. 2011. The intestinal microbiota and its modulation for Salmonella control in chickens. Food. Res. Int. 44:3149-3159.

Chao, A. 1984. Nonparametric estimation of the number of classes in a population. Scand. J. Statist. 11: 265-270.

Cheeseman J. H., Kaiser, M. G., and Ciraci, C. 2007. Breed effect on early cytokine mRNA expression in spleen and cecum of chickens with and without *Salmonella* enteritidis infection. Dev. Comp. Immunol. 31: 52-60.

Cheeseman, J. H., N. A. Levy, P. Kaiser, H. S. Lillehoj, and S. J. Lamont. 2008. *Salmonella* Enteritidis-induced alteration of inflammatory CXCL chemokine messenger-RNA expression and histologic changes in the ceca of infected chicks. Avian Dis. 52:229–234.

Christensen, H. R., H. Frokiaer, J. J. Pestka. 2002. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J. Immunol. 168:171-8.

Chung, T. K., S. M. Rutherfurd, D. V. Thomas, and P. J. Moughan. 2013. Effect of two microbial phytases on mineral availability and retention and bone mineral density in low-phosphorus diets for broilers. Br. Poult. Sci. 54: 362-373.

Clarridge, J. E., 3rd. 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev. 17: 840-862.

Coates, M. E., R. Fuller, G. F. Harrison, M. Lev, and S. F. Suffolk. 1963. Comparison of the growth of chicks in the Gustafsson germ-free apparatus and in a conventional environment, with and without dietary supplements of penicillin. Br. J. Nutr. 17:141–151.

Coppedge, J. K. Joseph, B. Brown, B. Ratliff, F. Ruch, and J. T. Lee. 2011. Effects of Co-admin Phytase and NSPase on Broiler Performance and Bone Ash. Int. J. Poult. Sci. 10: 933-939.

Corwin, E. J. 2000. Understanding Cytokines Part I: Physiology and Mechanism of Action. Biological Research For Nursing 2:30-40.

Cummings, J. H., G. T. Macfarlane, and H. N., Englyst. 2001. Prebiotic digestion and fermentation. Am. J. Clin. Nutr. 73:415S–20S.

Dankowiakowska, A., I. Kozlowska, and M. Bednarczyk. 2013. Probiotics, Prebiotics and Snybiotics in Poultry - Mode of Action, Limitation, and Achievements. JCEA 14: 467-478.

Danzeisen, J. L., H. B. Kim, R. E. Isaacson, Z. J. Tu, and T. J. Johnson. 2011. Modulations of the chicken cecal microbiome and metagenome in response to anticoccidial and growth promoter treatment. PloS one 6:e27949.

Davison F., K. E. Magor, and B. Kaspers. 2006. Structure and evolution of avian immunoglobulins. In: Avian Immunology. Davison, F., B. Kaspers, and K. A. Schat., [editor]. Elsevier Ltd., London: 107-127.

Davison, F. 2008. The importance of the avian immune system and its unique features. In: Avian Immunology. Davison, F., B. Kaspers, and K. A. Schat., [editor]. Elsevier Ltd., London: 1-11.

Derakhshani, H., S. Alqarni, F. C. Cardoso, H. R. Khazanehei, E. Khafipour, J. C. Plaizier, and J. J. Loor. 2014. The microbiome composition of the rumen is altered during the peripartal period in dairy cattle. PloS One. In press.

DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbio.72:5069-5072.

Diarrassouba, F. 2008. Impacts of antimicrobial growth promoters used in broiler chicken production on the emergence of antibiotic resistance in commensal *E. Coli* and *Salmonella*. M. Sc. Thesis. The University of British Columbia, Canada.

Diaz-Sanchez, S., I. Hanning, S. Pendleton, and D. D'Souza. 2013. Next-generation sequencing: the future of molecular genetics in poultry production and food safety. Poult. Sci. 92: 562-572.

Dibner, J. J., and J. D. Richards. 2005. Antibiotic growth promoters in agriculture history and mode of action. Poult. Sci. 84: 634-643.

Dumonceaux, T. J., J. E. Hill, S. M. Hemmingsen, and A. G. Van Kessel. 2006. Characterization of intestinal microbiota and response to dietary virginiamycin supplementation in the broiler chicken. Appl. Environ. Microbiol. 72: 2815-2823.

Dunkley, K. D., Callaway, T. R., Chalova, V. I., McReynolds, J. L., Hume, M. E., Dunkley, C. S., Kubena, L. F., Nisbet, D. J., and Ricke, S. C. 2009. Foodborne Salmonella ecology in the avian gastrointestinal tract. Anaerobe. 15: 26–35.

Echeverry H. 2012. Effect of different carbohydrate and nucleotide derived from yeast products, including distillers dried grains with soluble (ddgs), on innate immunity using a chicken b cells model: *in vitro studies*. M. Sc. Thesis. The University of Manitoba, Canada.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 26:2460-2461.

Edgar, R. C., B. J. Haas, J. C. Clemente, C. Quince, and R. Knight. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 27:2194-2200.

El-Sherbiny, A. E., H. M. A. Hassan, M. O. Abd-Elsamee, A. Samy, and M. A. Mohamed. 2010. Performance, bone parameters and phosphorus excretion of broiler fed low phosphorus diets supplemented with phytase from 23 to 40 days of age. Int. J. Poult. Sci. 9 (10): 972-977.

Engberg, R. M., M. S. Hedemann, B. B. Jensen. 2002. The influence of grinding and pelleting of feed on the microbial composition and activity in the digestive tract of broiler chickens. Brit. Poult. Sci. 43:569 – 579.

Erf, G. F. 2004. Cell-mediated immunity in poultry. Poult. Sci. 83: 580-590.

Everard, A., C. Belzer, L. Geurts, J. P. Ouwerkerk, C. Druart, L. B. Bindels, Y. Guiot, M. Derrien, G. G. Muccioli, N. M. Delzenne, W. M. de Vos, and P. D. Cani. 2013. Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. PNAS. 110:9066-9071.

Feighner, S. D., and M. P. Dashkevicz. 1987. Subtherapeutic levels of antibiotics in poultry feeds and their effects on weight gain, feed efficiency, and bacterial cholyltaurine hydrolase activity. Appl. Environ. Microbiol. 53:331–336.

Ferket, P. R. 2004. Alternatives to antibiotics in poultry production: responses, practical experience and recommendations. Nutritional biotechnology in the feed and food industries: Proceedings of Alltech's 20th Annual Symposium, Kentucky, USA, 56-67.

Ferro, P. J., C. L. Swaggerty, P. Kaiser, I. Y. Pevzner, and M. H. Kogut. 2004. Heterophils isolated from chickens resistant to extraintestinal Salmonella enteritidis infection express higher levels of pro-inflammatory cytokine mRNA following infection than heterophils from susceptible chickens. Epidemiol. Infect. 132:1029–1037.

Fleming, R. H. 2008. Nutritional factors affecting poultry bone health. Proc. Nutr. Soc. 67: 177-183.

Flickinger, E. A., J. Van Loo, G. C. Jr. Fahey. 2003. Nutritional responses to the presence of inulin and oligofructose in the diets of domesticated animals: a review. Crit. Rev. Food Sci. Nutr. 43: 19-60.

Flint, H. J., E. C. M. Leitch, S. H. Duncan, A. W. Walker, A. J. Patterson, M. T. Rincon, K. P. Scott and P. Louis. 2006. Molecular approaches to the analysis of gastrointestinal microbial ecosystems. In: Avian Gut Function in Health and Disease. Perry, G. C., [editor]. CABI Publishing, Oxford: 107-123.

Fox, J. G. 2002. The non-H pylori *helicobacters*: their expanding role in gastrointestinal and systemic diseases. Gut. 50:273-283.

Fredrickson, J. K., M. F. Romine, A. S. Beliaev, J. M. Auchtung, M. E. Driscoll, T. S. Gardner, K. H. Nealson, A. L. Osterman, G. Pinchuk, J. L. Reed, D. A. Rodionov, J. L. Rodrigues, D. A. Saffarini, M. H. Serres, A. M. Spormann, I. B. Zhulin, and J. M. Tiedje. 2008. Towards environmental systems biology of *Shewanella*. Nat. Rev. Microbiol. 6:592-603.

Fukata, T., K. Sasai, T. Miyamoto, and E. Baba. 1999. Inhibitory effects of competitive exclusion and fructooligosaccharide, singly and in combination, on *Salmonella* colonization of chicks. J. Food Prot. 62:229–233.

Gaggìa, F., P. Mattarelli, and B. Biavati. 2010. Probiotics and prebiotics in animal feeding for safe food production. Int. J Food Microbiol. Suppl 1:S15-28.

Gaskins, H. R., C. T. Collier, and D. B. Anderson. 2002. Antibiotics as growth promotants: Mode of action. Anim. Biotechnol. 13:29–42.

Gibson G. R. and M. B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. 125: 1401–1412.

Gibson, G. R. 1998. Dietary modulation of the human gut microflora using prebiotics. Br. J. Nutr. 80:S209-212.

Gibson, J. R., M. A. Ferrus, D. Woodward, J. Xerry, and R. J. Owen. 1999. Genetic diversity in *Helicobacter pullorum* from human and poultry sources identified by an amplified fragment length polymorphism technique and pulsed-field gel electrophoresis. J. Appl. Microbiol. 87:602-610.

Goldstein, E. J. C., D. M. Citron, V. A. Peraino, and S. A. Cross. 2003. Desulfovibrio desulfuricans Bacteremia and Review of Human Desulfovibrio Infections. J. Clinical. Microbiol. 41:2752-2754.

Gong, J., R. J. Forster, H. Yu, J. R. Chambers, R. Wheatcroft, P. M. Sabour, and S. Chen. 2002. Molecular analysis of bacterial populations in the ileum of broiler chickens and comparison with bacteria in the cecum. FEMS Microbial. Ecol. 41:171-179.

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

Gross, W. B., and H. S. Siegel. 1983. Evaluation of the Heterophil/Lymphocyte Ratio as a Measure of Stress in Chickens. Avian Dis. 27: 972-979.

Grubbs, F. E. 1969. Procedures for detecting outlying observations in samples. Technometrics. 11: 1–21.

Gudiel-Urabano, M., and I. Goni. 2002. Effect of fructooligosaccharides on nutritional parameters and mineral bioavailability in rats. J. Sci. Food Agr. 82:913–917.

Hardin, G. 1960. The Competitive Exclusion Principle. Science. 131: 1292-1297.

Haug, W., and H.-J. Lantzsch. 1983. Sensitive method for the rapid determination of phytate in cereals and cereal products. J. Sci. Food Agr. 34: 1423-1426.

Hidaka, H. and M. Hirayama. 1991. Useful characteristics and commercial applications of fructooligosaccharides. Biochem. Soc. Transact. 19:561- 565.

Hill, T. C., K. A.Walsh, J. A. Harris, and B. F. Moffett. 2003. Using ecological diversity measures with bacterial communities. FEMS Microbiol. Ecol. 43: 1–11.

Hornung, C., A. Poehlein, F. S. Haack, M. Schmidt, K. Dierking, A. Pohlen, H. Schulenburg, M. Blokesch, L. Plener, K. Jung, A. Bonge, I. Krohn-Molt, C. Utpatel, G. Timmermann, E. Spieck, A. Pommerening-Roser, E. Bode, H. B. Bode, R. Daniel, C. Schmeisser, and W. R. Streit. 2013. The *Janthinobacterium* sp. HH01 genome encodes a homologue of the V. cholerae CqsA and L. pneumophila LqsA autoinducer synthases. PloS one 8:e55045.

Hugenholtz, P., B. M. Goebel, and N. R. Pace. 1998. Impact of culture independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180:4765–4774.

Jacob, J. and T. Pescatore. 2009. Chicken anatomy and physiology: digestive system. Cooperative extension service. College of agriculture, University of Kentucky, U.S.A.

Janardhana, V., M. M. Broadway, M. P. Bruce, J. W. Lowenthal, M. S. Geier, R. J. Hughes, and A. G. Bean. 2009. Prebiotics modulate immune responses in the gut-associated lymphoid tissue of chickens. J. Nutr. 139: 1404-1409.

Janeway, C. A. Jr., P. Travers, M. Walport, and Shlomchik, M. J. 2001. Chapter 3, antigen recognition by B-cell and T-cell receptors. In: Immunobiology: The Immune System in Health and Disease. 5th edition. Garland Science, New York.

Jeurissen, S. H. M., F. Wagenaar, and E. M. Janse. 1999. Further characterization of M cells in gut-associated lymphoid tissues of the chicken. Poult. Sci. 78: 965-972.

Jeurissen, S. H., F. Lewis, J. D. van der Klis, Z. Mroz, J. M. Rebel, and A. A. ter Huurne. 2002. Parameters and techniques to determine intestinal health of poultry as constituted by immunity, integrity, and functionality. Curr. Issues Intest. Microbiol. 3:1–14.

Juul-Madsen, H., R. B. Viertlboeck, A. L. Smith, T. W. F. Göbel. 2008. Avian innate immune responses. In: Avian Immunology. Davison, F., B. Kaspers, and K. A. Schat., [editor]. Elsevier Ltd., London: 129-158.

Kaspers, B., S. Kothlow and C. Butter. 2008. Avian antigen presenting cells. In: Avian Immunology. Davison, F., B. Kaspers, and K. A. Schat., [editor]. Elsevier Ltd., London: 183-202.

Khachatourians, G. G. 1998. Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. CMAJ.159:1129-1136.

Khafipour, E., S. Li, J. C. Plaizier, and D. O. Krause. 2009. Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. Appl. Environ. Microbio. 75:7115-7124.

Khodambashi Emami, N., A. Samie, H. R. Rahmani, and C. A. Ruiz-Feria. 2012. The effect of peppermint essential oil and fructooligosaccharides, as alternatives to virginiamycin, on

growth performance, digestibility, gut morphology and immune response of male broilers. Anim. Feed Sci. Tech. 175:57-64.

Kim, G. B., Y. M. Seo, C. H. Kim, and I. K. Paik. 2011a. Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. Poult. Sci. 90: 75-82.

Kim, W. K., L. M. Donalson, A. D. Mitchell, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2006. Effects of Alfalfa and Fructooligosaccharide on Molting Parameters and Bone Qualities Using Dual Energy X-Ray Absorptiometry and Conventional Bone Assays. Poult. Sci. 85: 15-20.

Kim, W. K., S. A. Bloomfield, and S. C. Ricke. 2011b. Effects of age, vitamin D3, and fructooligosaccharides on bone growth and skeletal integrity of broiler chicks. Poult. Sci. 90: 2425-2432.

Kim, W. K., S. A. Bloomfield, T. Sugiyama, and S. C. Ricke. 2012. Concepts and methods for understanding bone metabolism in laying hens. World's Poult. Sci. J. 68: 71-82.

Knarreborg, A., M. A. Simon, R. M. Engberg, B. B. Jensen, and G. W. Tannock. 2002. Effects of Dietary Fat Source and Subtherapeutic Levels of Antibiotic on the Bacterial Community in the Ileum of Broiler Chickens at Various Ages. Appl. Environ. Microbiol. 68:5918-5924.

Knowlton, K. F., J. S. Radcliffe, C. L. Novak, and D. A. Emmerson. 2004. Animal management to reduce phosphorus losses to the environment. J. Anim. Sci. 82:173-195.

Kogut, M. H. 2013. The gut microbiota and host innate immunity: Regulators of host metabolism and metabolic diseases in poultry? J. Appl. Poult. Res. 22: 637-646.

Kogut, M. H., H. He, and P. Kaiser. 2005. Lipopolysaccharide binding protein/CD14/TLR4-dependent recognition of *Salmonella* LPS induces the functional activation of chicken

heterophils and up-regulation of pro-inflammatory cytokine and chemokine gene expression in these cells. Anim. Biotechnol. 16:165-181.

Kohl, K. D. 2012. Diversity and function of the avian gut microbiota. J. Comp. physiol. B. 182: 591-602.

Koopman, J. P., H. M. Kennis, J. W. Mullink, R. A. Prins, A. M. Stadhouders, H. De Boer and M. P. Hectors. 1984. 'Normalization' of germfree mice with anaerobically cultured caecal flora of 'normal' mice. Lab. Anim. 18:188–194.

Le Blay, G., C. Michel, H. M. Blottière, and C. Cherbut. 1999. Prolonged Intake of Fructo-Oligosaccharides Induces a Short-Term Elevation of Lactic Acid-Producing Bacteria and a Persistent Increase in Cecal Butyrate in Rats. J. Nutr. 129: 2231-2235.

Lee, K.-W., and H. S. Lillehoj. 2011. Antimicrobials, Gut Microbiota and Immunity in Chickens. Korean J. Poult. Sci. 38: 155-164.

Lee, M. D., L. Lu, U. Idris, B. Harmon, C. Hofacre, and J. J. Maurer. 2002. Microbial dynamics of the broiler intestinal tract, "Abstract". Proceedings of the Elanco Global Enteritis Symposium. Cambridge, UK, A3-5.

Leveque, G., V. Forgetta, S. Morroll, A. L. Smith, N. Bumstead, P. Barrow, J. C. Loredo-Osti, K. Morgan, and D. Malo. 2003. Allelic Variation in TLR4 Is Linked to Susceptibility to Salmonella enterica Serovar Typhimurium Infection in Chickens. Infection and Immunity 71(3):1116-1124.

Li, R., E. Khafipour, D. O. Krause, M. H. Entz, T. R. de Kievit, and W. D. Fernando. 2012. Pyrosequencing reveals the influence of organic and conventional farming systems on bacterial communities. PloS one. 7:e51897.

Linden S. K., T. H. Florin, M. A. Mcguckin. 2008. Mucin dynamics in intestinal bacterial infection. PloS One. 3: e3952.

Liu Y., D. L. Balkwill, H. C. Aldrich, G. R. Drake, D. R. Boone. 1999. Characterization of the anaerobic propionate-degrading syntrophs *Smithella propionica* gen. nov., sp. nov. and *Syntrophobacter wolinii*. Int. J. Syst. Bacteriol. 49:545–556.

Loman, N. J., R. V. Misra, T. J. Dallman, C. Constantinidou, S. E. Gharbia, J. Wain, and M. J. Pallen. 2012. Performance comparison of benchtop high-throughput sequencing platforms. Nat. Biotech. 30: 434-439.

Lomer, M. C. E., R. P. H. Thompson, J. Commisso, C. L. Keen, and J. J. Powell. 2000. Determination of titanium dioxide in foods using inductively coupled plasma optical emission spectrometry. Analyst. 125: 2339-2343.

Lopez, H. W. C. Coudray, M.-A. Levrat-Verny, C. Feillet-Coudray, C. Demigné, and C. Rémésy. 2000. Fructooligosaccharides enhance mineral apparent absorption and counteract the deleterious effects of phytic acid on mineral homeostasis in rats. J. .Nutr. Biochem. 11: 500-508.

Lozupone, C. and R. Knight. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Appl. Environ. Microbio. 71:8228-8235.

Lu, J., C. Hofacre, F. Smith, and M. D. Lee. 2008. Effects of feed additives on the development on the ileal bacterial community of the broiler chicken. Animal 2:669-676.

Lu, J., U. Idris, B. Harmon, C. Hofacre, J. J. Maurer, and M. D. Lee. 2003. Diversity and Succession of the Intestinal Bacterial Community of the Maturing Broiler Chicken. Appl. Environ. Microbiol. 69:6816-6824.

Luo, C., D. Tsementzi, N. Kyrpides, T. Read, and K. T. Konstantinidis. 2012. Direct comparisons of Illumina vs. Roche 454 sequencing technologies on the same microbial community DNA sample. PloS one 7: e30087.

Lutful Kabir, S. M. 2009. The role of probiotics in the poultry industry. Int. J. Mol. Sci. 10: 3531-3546.

Macpherson A. J., N. L. Harris. 2004. Interactions between commensal intestinal bacteria and the immune system. Nat. Rev. Immunol. 4: 478-485.

Maenz, D. D., and H. L. Classen. 1998. Phytase activity in the small intestinal brush border membrane of the chicken. Poult. Sci. 77: 557-563.

Margulies, M., M. Egholm, W. E. Altman, S. Attiya, J. S. Bader, L. A. Bemben, J. Berka, M. S. Braverman, Y. J. Chen, Z. Chen, S. B. Dewell, L. Du, J. M. Fierro, X. V. Gomes, B. C. Godwin, W. He, S. Helgesen, C. H. Ho, G. P. Irzyk, S. C. Jando, M. L. Alenquer, T. P. Jarvie, K. B. Jirage, J. B. Kim, J. R. Knight, J. R. Lanza, J. H. Leamon, S. M. Lefkowitz, M. Lei, J. Li, K. L. Lohman, H. Lu, V. B. Makhijani, K. E. McDade, M. P. McKenna, E. W. Myers, E. Nickerson, J. R. Nobile, R. Plant, B. P. Puc, M. T. Ronan, G. T. Roth, G. J. Sarkis, J. F. Simons, J. W. Simpson, M Srinivasan, K. R. Tartaro, A. Tomasz, K. A. Vogt, G. A. Volkmer, S. H. Wang, Y. Wang, M P. Weiner, P. Yu, R. F. Begley, and J. M. Rothberg. 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature 437:376–380.

Martinez, I., D. J. Perdicaro, A. W. Brown, S. Hammons, T. J. Carden, T. P. Carr, K. M. Eskridge, and J. Walter. 2013. Diet-induced alterations of host cholesterol metabolism are likely to affect the gut microbiota composition in hamsters. Appl. Environ. Microbiol. 79:516-524.

Masella, A., A. Bartram, J. Truszkowski, D. Brown, and J. Neufeld. 2012. PANDAseq: paired-end assembler for illumina sequences. BMC Bioinformatics. 13:1-7.

McGrath, J. M., J. T. Sims, R. O. Maguire, W. W. Saylor, C. R. Angel, and B. L. Turner. 2005. Broiler Diet Modification and Litter Storage. J. Environ. Qual. 34(5):1896-1909.

Medini, D., D. Serruto, J. Parkhill, D. A. Relman, C. Donati, R. Moxon, S. Falkow, and R. Rappuoli. 2008. Microbiology in the post-genomic era. Nat. Rev. Microbiol. 6:419–430.

Medzhitov, R., and C. A. Janeway Jr. 1997. Innate immunity: Impact of the adaptive immune response. Curr. Opin. Immunol. 9:4–9.

Meehan, C. J. and R. G. Beiko. 2014. A phylogenomic view of ecological specialization in the Lachnospiraceae, a family of digestive tract-associated bacteria. GBE. 6:703-713.

Mitchell, M. A. and M. Moretó. 2006. Absorptive function of the small intestine: adaptations meeting demand. In: Avian Gut Function in Health and Disease. Perry, G. C., [editor]. CABI Publishing, Oxford: 43-63.

Mittal A., G. Singh, V. Goyal, A. Yadav, K. R. Aneja, S. K. Gautam, N. K. Aggarwal. 2011. Isolation and biochemical characterization of acido-thermophilic extracellular phytase producing bacterial strain for potential application in poultry feed. Jundishapur. J. Microbiol. 4: 273-282.

Mullaney E. J., and A. H. Ullah. 2003. The term phytase comprises several different classes of enzymes. Biochem. Biophys. Res. Commun. 312: 179–184.

Munyaka, P. M. 2012. Immunocompetence in young and old laying hens in response to dietary folic acid and escherichia coli lipopolysaccharide challenge. M. Sc. Thesis. The University of Manitoba, Canada.

Munyaka, P. M., H. Echeverry, A. Yitbarek, G. Camelo-Jaimes, S. Sharif, W. Guenter, J. D. House, and J. C. Rodriguez-Lecompte. 2012. Local and systemic innate immunity in broiler chickens supplemented with yeast-derived carbohydrates. Poult Sci 91: 2164-2172.

Nahm, K. H. 2002. Efficient feed nutrient utilization to reduce pollutants in poultry and swine manure. Crit. Rev. Environ. Sci. Technol. 32:1-16.

Natea, M. G., J. W. M. Van der Meer, R. P. Sutmuller, G. J. Adema, and B. J. Kullberg. 2005. From the Th1/Th2 Paradigm towards a Toll-Like Receptor/T-Helper Bias. Antimicrob. Agent. Chemother. 49: 3991–3996.

Naves, L de P, A. D. Corrêa, A. G. Bertechini, E. M. Gomide, and C. D. dos. Santos. 2012. Effect of ph and temperature on the activity of phytase products used in broiler nutrition. Rev. Bras. Cienc. Avic. 14: 181-185.

NRC. 1994. Nutrient requirements of poultry. 9th ed. Natl. Acad. Press, Washington, DC.

Nyman M. 2002. Fermentation and bulking capacity of indigestible carbohydrates: the case of inulin and oligofructose. Brit. J. Nutr. 87:S163–168.

O'Hara A. M., F. Shanahan. 2006. The gut flora as a forgotten organ. EMBO Rep. 7:688-693.

Oakley, B. B., C. A. Morales, J. Line, M. E. Berrang, R. J. Meinersmann, G. E. Tillman, M. G. Wise, G. R. Siragusa, K. L. Hiett, and B. S. Seal. 2013. The poultry-associated microbiome: network analysis and farm-to-fork characterizations. PloS one 8:e57190.

Ochsenbein, A. F., and R. M. Zinkernagel. 2000. Natural antibodies and complement link innate and acquired immunity. Immunol. Today. 21:624-629.

Ochsenbein, A. F., T. Fehr, C. Lutz, M. Suter, F. Brombacher, H. Hengartner, R. M. Zinkernagel. 1999. Control of early viral and bacterial distribution and disease by natural antibodies. Science. 286:2156-2159.

Ohta A, Ohtsuki M, Baba S, Takizawa T, Adachi T, Kimura S. 1995. Effects of fructooligosaccharides on the absorption of iron, calcium and magnesium in iron-deficient anemic rats. J. Nutr. Sci. Vitaminol. 41:281–91.

Ohta, A. Prevention of osteoporosis by foods and dietary supplements. 2006. The effect of fructooligosaccharides (FOS) on the calcium absorption and bone. Clin. Calcium. 16:1639–1645.

Oliveros, J. C. 2007. VENNY. An interactive tool for comparing lists with Venn Diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html.

Oyarzabal, O. A. and D. E. Conner. 1996. Application of Direct-Fed Microbial Bacteria and FOS for Salmonella Control in Broilers During Feed Withdrawal. Poult. Sci. 75:186-190.

Panda A. K., S. V. Rama Rao, M. V. L. N. Raju and G. Shyam Sunder. 2009. Effect of butyric acid on performance, gastrointestinal tract health and carcass characteristics in broiler chickens. Asian. Australs. J. Anim. Sci. 22:1026–1031.

Park, S. H., I. Hanning, A. Perrota, B. J. Bench, E. Alm, and S. C. Ricke. 2013. Modifying the gastrointestinal ecology in alternatively raised poultry and the potential for molecular and metabolomic assessment. Poult. Sci. 92: 546-561.

Parry S. H., W. D. Allen, P. Porter. 1977. Intestinal immune response to E. coli antigens in the germ-free chickens. Immunol. 32:731-741.

Patterson, J. A., J. I. Orban, A. L. Sutton and G. N. Richards. 1997. Selective enrichment of *bifidobacteria* in the intestinal tract of broilers by thermally produced ketoses and effect on broiler performance. Poult. Sci. 76:497-500.

Pedroso, A. A., A. L. Hurley-Bacon, A. S. Zedek, T. W. Kwan, A. P. Jordan, G. Avellaneda, C. L. Hofacre, B. B. Oakley, S. R. Collett, J. J. Maurer, and M. D. Lee. 2013. Can probiotics improve the environmental microbiome and resistome of commercial poultry production? Int. J. Environ. Res. Public. Health. 10:4534-4559.

Pérez-Enciso, M. and M. Tenenhaus. 2003. Prediction of clinical outcome with microarray data: a partial least squares discriminant analysis (PLS-DA) approach. Hum. Genet. 112:581-592.

Pettersson, E., J. Lundeberg, and A. Ahmadian. 2009. Generations of sequencing technologies. Genomics. 93: 105-111.

Pirgozliev, V., M. R. Bedford, T. Acamovic, P. Mares, and M. Allymehr. 2011. The effects of supplementary bacterial phytase on dietary energy and total tract amino acid digestibility when fed to young chickens. Br. Poult. Sci. 52: 245-254.

PoultryHub. [Internet]. The digestive system. 2013 [modified 2013 Nov 11; cited 2014 Apr 11]. Available from: http://www.poultryhub.org/physiology/body-systems/digestive-system/.

Powell, S., S. Johnston, L. Gaston, and L. L. Southern. 2008. The effect of dietary phosphorus level and phytase supplementation on growth performance, bone-breaking strength, and litter phosphorus concentration in broilers. Poult. Sci. 87: 949-957.

Powell, S., T. D. Bidner, and L. L., Southern. 2011. Phytase supplementation improved growth performance and bone characteristics in broilers fed varying levels of dietary calcium. Poult. Sci. 90:604–608.

Pryde S. E., S. H. Duncan, G. L. Hold, C. S. Stewart, H. J. Flint. 2002. The microbiology of butyrate formation in the human colon. FEMS Microbiol. Lett. 217: 133–139.

Purchiaroni, F., A. Tortora, M. Gabrielli, F. Bertucci, G. Gigante, G. Ianiro, V. Ojetti, E. Scarpellini, and A. Gasbarrini. 2013. The role of intestinal microbiota and the immune system. Eur. Rev. Med. Pharmacol. Sci. 17:323-333.

Ravindran, V., S. Cabahug, G. Ravindra, P. H. Selle, and W. L. Bryden. 2000. Response of broiler chickens to microbial phytase supplementation as influenced by dietary phytic acid and non-phytate phosphorous levels. II. Effects on apparent metabolisable energy, nutrient digestibility and nutrient retention. Br. Poult. Sci. 41: 193-200.

Ravindran, V., W. L. Bryden, and E. T., Kornegay. 1995. Phytates: occurrence, bioavailability and implications in poultry nutrition. Poult Avian. Biol. Rev. 6: 125–143.

Regassa, A., and W. K. Kim. 2013. Effects of oleic acid and chicken serum on the expression of adipogenic transcription factors and adipogenic differentiation in hen preadipocytes. Cell Bio. Int. 37: 961-971.

Rehman, H. U., W. Vahjen, W. A. Awad, and J. Zentek. 2007. Indigenous bacteria and bacterial metabolic products in the gastrointestinal tract of broiler chickens. Arch. Anim. Nutr. 61: 319-335.

Reynaud, C. A., B. A. Imhof, V. Anquez, and J. C. Weill. 1992. Emergence of committed B lymphoid progenitors in the developing chicken embrio. EMBO J. 11: 4349 - 4358.

Rijkers, G. T., W. M. de Vos, R. J. Brummer, L. Morelli, G. Corthier, and P. Marteau. 2011. Health benefits and health claims of probiotics: bridging science and marketing. Br. J. Nutr. 106:1291-1296.

Robertson, B. R., J. L. O'Rourke, B. A. Neilan, P. Vandamme, S. L. On, J. G. Fox, and A. Lee. 2005. *Mucispirillum schaedleri* gen. nov., sp. nov., a spiral-shaped bacterium colonizing the mucus layer of the gastrointestinal tract of laboratory rodents. IJSEM 55:1199-1204.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA74:5463–5467.

Saxton, A. M. 1998. A macro for converting mean separation output to letter groupings in Proc Mixed. In Proc. 23rd SAS Users Group Intl., SAS Institute, Cary, NC: pp1243-1246.

Schadt, E. E., S. Turner, and A. Kasarskis. 2010. A window in third-generation sequencing. Hum. Mol. Genet. 19:R227–R240.

Scholz-Ahrens, K. E., G. Schaafsma, E. G. van den Heuvel, and J. Schrezenmeir. 2001. Effects of prebiotics on mineral metabolism. Am. J. Clin. Nutr. 73:459S-464S.

Schreiweis M. A., J. I. O., M. C. Ledur, D. E. Moody, and P. Y. Hester. 2005. Validation of Dual-Energy X-Ray Absorptiometry in Live White Leghorns. Poult. Sci. 84: 91–99.

Scott, T. R. 2004. Our current understanding of humoral immunity of poultry. Poult. Sci. 83: 574-579.

Selle, P. H., A. J. Cowieson, and V. Ravindran. 2009. Consequences of calcium interactions with phytate and phytase for poultry and pigs. Livest. Sci. 124: 126-141.

Selle, P. H., and V. Ravindran. 2007. Microbial phytase in poultry nutrition. Anim. Feed Sci. Technol. 135: 1-41.

Selle, P. H., Ravindran,V., Pittolo, P. H., Bryden,W. L., 1999. An evaluation of microbial phytase in sorghum-based broiler diets. Proc. Aust. Poult. Sci. Symp. 11, 97–100.

Setta, A. M., P. A. Barrow, P. Kaiser, and M. A. Jones. 2012. Early immune dynamics following infection with *Salmonella* enterica serovars Enteritidis, Infantis, Pullorum and Gallinarum: Cytokine and chemokine gene expression profile and cellular changes of chicken cecal tonsils. Comp. Immunol. Microbiol. Infect. Dis. 35:397–410.

Shapiro, S. K. and W. B. Sarles. 1949. Microorganisms in the intestinal tract of normal chickens. J. Bacteriol. 58:531-544.

Shinoki, A., and H. Hara. 2011. Dietary fructo-oligosaccharides improve insulin sensitivity along with the suppression of adipocytokine secretion from mesenteric fat cells in rats. Br. J. Nutr. 106: 1190-1197.

Silversides, F. G., T. A. Scott, and M. R. Bedford. 2004. The Effect of Phytase Enzyme and Level on Nutrient Extraction by Broilers. Poult. Sci. 83:985-989.

Simons, P. C., H. A. J. Versteegh, A. W. Jongbloed and P. A. Kemme. 1990. Improvement of phosphorus availability by microbial phytase in broilers and pigs. Br. J. Nutr. 64:525-540.

Singh, P., A. Karimi, K. Devendra, P. W. Waldroup, K. K. Cho, and Y. M. Kwon. 2013. Influence of penicillin on microbial diversity of the cecal microbiota in broiler chickens. Poult. Sci. 92:272-276.

Slominski, B. A. 2011. Recent advances in research on enzymes for poultry diets. Poult. Sci. 90: 2013-2023.

Slominski, B. A., T. Davie, M. C. Nyachoti, and O. Jones. 2007. Heat stability of endogenous and microbial phytase during feed pelleting. Livest. Sci. 109: 244-246.

Smith, H. W. 1965. The development of the flora of the alimentary tract in young animals. J. Pathol. Bacteriol. 90:495 – 513.

Swaggerty, C. L., I. Y. Pevzner, and M. H. Kogut. 2014. Selection for pro-inflammatory mediators yields chickens with increased resistance against *Salmonella enterica* serovar Enteritidis. Poult. Sci. 93:535-544.

Swiatkiewicz, S. and A. Arczewska-Wlosek. 2012. Prebiotic fructans and organic acids as feed additives improving mineral availability. World Poult. Sci. J. 68(02): 269-279.

Swiatkiewicz, S., J. Koreleski, and A. Arczewska-Wlosek. 2011. Effect of inulin and oligofructose on performance and bone characteristics of broiler chickens fed on diets with different concentrations of calcium and phosphorus. Br. Poult. Sci. 52: 483-491.

Tannock, G. W. 2001. Molecular assessment of intestinal microflora. Am. J. Clin. Nutr. 73:410S–414S.

Teirlynck, E., M. D. Gussem, J. Dewulf, F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. 2011. Morphometric evaluation of "dysbacteriosis" in broilers. Avian Pathol. 40: 139-144.

Telg, B. E. and D. J. Caldwell. 2009. Efficacy testing of a defined competitive exclusion product in combination with fructooligosaccharide for protection against Salmonella Typhimurium challenge in broiler chicks. JAPR 18:521-529.

Tizard, I. R. 2009. Veterinary Immunology: An Introduction. 8th ed. Saunders Elsevier, St. Louis, MO.

Tlaskalova-Hogenova, H., R. Stepankova, T. Hudcovic, L. Tuckova, B. Cukrowska, R. Lodinova-Zadnikova, H. Kozakova, P. Rossmann, J. Bartova, D. Sokol, D. P. Funda, D. Borovska, Z. Rehakova, J. Sinkora, J. Hofman, P. Drastich, and A. Kokesova. 2004. Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. Immunol. Lett. 93:97-108.

Toivanen, P., A. Toivanen and R. A. Good. 1972. Ontogeny of bursal function in chicken. III. Immunocompetent cell for humoral immunity. J. Exp. Med. 136:816-831.

Torok, V. A., G. E. Allison, N. J. Percy, K. Ophel-Keller, and R. J. Hughes. 2011. Influence of antimicrobial feed additives on broiler commensal posthatch gut microbiota development and performance. Appl. Environ. Microbiol. 77:3380-3390.

U.S. Food and Drug Administration (FDA). [Internet]. Battle of the Bugs: Fighting Antibiotic Resistance. 2011 [modified 2011 Aug 17; cited 2014 Apr 3]. Available from: http://www.fda.gov/drugs/resourcesforyou/consumers/ucm143568.htm.

Ueki, A., H. Akasaka, D. Suzuki, and K. Ueki. 2006. *Paludibacter propionicigenes* gen. nov., sp. nov., a novel strictly anaerobic, Gram-negative, propionate-producing bacterium isolated from plant residue in irrigated rice-field soil in Japan. Int. J. Syst. Evol. Micr. 56:39-44.

Van der Wielen, P. W., D. A. Keuzenkamp, L. J. Lipman, F. van Knapen, and S. Biesterveld. 2002. Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. Microb. Ecol. 44:286-293.

Van Immerseel, F., L. De Zutter, K. Houf, F. Pasmans, F. Haesebrouck, and R. Ducatelle. 2009. Strategies to control Salmonella in the broiler production chain. World's Poult. Sci. J. 65:367-392.

Verhulst, N. O., Y. T. Qiu, H. Beijleveld, C. Maliepaard, D. Knights, S. Schulz, D. Berg-Lyons, C. L. Lauber, W. Verduijn, and G. W. Haasnoot. 2011. Composition of human skin microbiota affects attractiveness to malaria mosquitoes. PloS one. 6:e28991.

Videnska, P., F. Sisak, H. Havlickova, M. Faldynova, and I. Rychlik. 2013. Influence of Salmonella enterica serovar Enteritidis infection on the composition of chicken cecal microbiota. BMC Vet. Res. 9: 140.

Von Graevenitz A. 1977. The role of opportunistic bacteria in human disease. Annu. Rev. Microbiol. 31:447–471.

Waititu, S. M., A. Yitbarek, E. Matini, H. Echeverry, E. Kiarie, J. C. Rodriguez-Lecompte, and C. M. Nyachoti. 2014. Effect of supplementing direct-fed microbials on broiler performance, nutrient digestibilities, and immune responses. Poult. Sci. 93:625-635.

Walk, C. L., M. R. Bedford, and A. P. McElroy. 2012. Influence of limestone and phytase on broiler performance, gastrointestinal pH, and apparent ileal nutrient digestibility. Poult. Sci. 91: 1371-1378.

Wang, Q., G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbio. 73:5261-5267.

Wang, X., and G. R. Gibson. 1993. Effects of the in vitro fermentation of oligofructose and inulin by bacteria growing in the human large intestine. J. Appl. Bacteriol. 75: 373–380.

Wang, Y., T. Zeng, S. E. Wang, W. Wang, Q. Wang, H. X. Yu. 2010. Fructo-oligosaccharides enhance the mineral absorption and counteract the adverse effects of phytic acid in mice. Nutr. 26: 305-311.

Warwick, R. and K. Clarke. 2006. PRIMER 6. PRIMER-E Ltd, Plymouth.

Wei, S., M. Morrison, and Z. Yu. 2013. Bacterial census of poultry intestinal microbiome. Poult. Sci. 92:671-683.

Weisburg, W.G., S. M. Barns, D. A. Pelletier, D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173: 697–703.

Werner, J. J., D. Zhou, J. G. Caporaso, R. Knight, and L. T. Angenent. 2012. Comparison of Illumina paired-end and single-direction sequencing for microbial 16S rRNA gene amplicon surveys. ISME. J. 6:1273-1276.

Wilkinson, S. J., E. J. Bradbury, M. R. Bedford, and A. J. Cowieson. 2014. Effect of dietary nonphytate phosphorus and calcium concentration on calcium appetite of broiler chicks. Poult. Sci. 93: 1–9.

Williams, J., S. Mallet, M. Leconte, M. Lessire, and I. Gabriel. 2008. The effects of fructo-oligosaccharides or whole wheat on the performance and digestive tract of broiler chickens. Br. Poult. Sci. 49:329-339.

Williams, R. B. 2005. Intercurrent coccidiosis and necrotic enteritis of chickens: Rational, integrated disease management by maintenance of gut integrity. Avian Pathol. 34:159–180.

World Health Organization (WHO). 2003. Impacts of antimicrobial growth promoter termination in Denmark, The WHO international review panel's evaluation of the termination of the use

of antimicrobial growth promoters in Denmark. Page 6-8 in document WHO/CDS/CPE/ZFK/2003.1. Foulum, Denmark.

Woyengo, T. A., B. A. Slominski, and R. O. Jones. 2010. Growth performance and nutrient utilization of broiler chickens fed diets supplemented with phytase alone or in combination with citric acid and multicarbohydrase. Poult. Sci. 89:2221-2229.

Woyengo, T. A., W. Guenter, J. S. Sands, C. M. Nyachoti, and M. A. Mirza. 2008. Nutrient utilisation and performance responses of broilers fed a wheat-based diet supplemented with phytase and xylanase alone or in combination. Anim. Feed Sci. Technol. 146: 113-123.

Wyss, M., R. Brugger, A. Kronenberger, R. Remy, R. Fimbel, G. Oesterhelt, M. Lehmann, and A. P. van Loon. 1999. Biochemical characterization of fungal phytases (myo-inositol hexakisphosphate phosphohydrolases): catalytic properties. Appl. Environ. Microbiol. 65: 367-373.

Xu, Z. R., C. H. Hu, M. S. Xia, X. A. Zhan, and M. Q. Wang. 2003. Effects of dietary fructooligsaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. Poult. Sci. 82:1030-1036.

Yang, Y., P.A. Iji, and M. Choct. 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 Poult. Sci. J. 65: 97-114.

Yegani, M., and D. R. Korver. 2008. Factors affecting intestinal health in poultry. Poult. Sci. 87: 2052-2063.

Yeoman, C. J., N. Chia, P. Jeraldo, M. Sipos, N. D. Goldenfeld, and B. A. White. 2012. The microbiome of the chicken gastrointestinal tract. Anim. Health. Res. Rev. 13:89-99.

Young, V. B., C. C. Chien, K. A. Knox, N. S. Taylor, D. B. Schauer, and J. G. Fox. 2000. Cytolethal distending toxin in avian and human isolates of Helicobacter pullorum. J. Infect. Dis. 182:620-623.

Yusrizal and T. C. Chen. 2003. Effect of Adding Chicory Fructans in Feed on Fecal and Intestinal Microflora and Excreta Volatile Ammonia. Intl. J. Poult. Sci. 2:188-194.

Zafar, T. A., C. M. Weaver, Y. Zhao, B. R. Martin, and M. E. Wastney. 2004. Nondigestible oligosaccharides increase calcium absorption and suppress bone resorption in ovariectomized rats. J. Nutr. 134: 399-402.

Zbinden, M. and M.-A. Cambon-Bonavita. 2003. Occurrence of Deferribacterales and Entomoplasmatales in the deep-sea Alvinocarid shrimp Rimicaris exoculata gut. FEMS Microbiol. Ecol. 46:23-30.

Zhu, X. Y., T. Zhong, Y. Pandya, and R. D. Joerger. 2002. 16S rRNA-Based Analysis of Microbiota from the Cecum of Broiler Chickens. Appl. Environ. Microbiol. 68:124-137.