IN OVO AND POST-HATCH NUTRITIONAL PROGRAMMING TO IMPROVE BROILER PERFORMANCE AND GUT HEALTH

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI‘I AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
NUTRITION SCIENCES

AUGUST 2019

BY

Amit Kumar Singh

Dissertation committee:

Rajesh Jha, Chairperson
Birendra Mishra
Yong Li
Jenee Odani
Zhiqiang Cheng (University representative)

Keywords: broilers, enzymes, growth performance, gut health, *in ovo* feeding, mannooligosaccharides, prebiotics, xylanase, xylooligosaccharides, resistant starch

ACKNOWLEDGMENTS

First and foremost, I would like to express my deep gratitude to my PhD advisor, Dr. Rajesh Jha (HNFAS, UHM) for his enduring support, academic mentorship and motivation throughout my graduate study at UH. I would also like to extend my sincere thanks to all my committee members: Dr. Birendra Mishra (HNFAS, UHM), Dr. Yong Li (HNFAS, UHM), Dr. Jenee Odani (HNFAS, UHM) and Dr. Zhiqiang Cheng (PEPS, UHM) for their advice, critique and sharing their expertise and knowledge on the research topic. I would also like to appreciate all members of HNFAS department committee for granting departmental assistantship which was utmost necessary for my continuation of the graduate study. Most fervently and sincerely, I would like to thank all my teachers, mentors, seniors, family, friends and colleagues for their unwavering support, guidance, and encouragement that I received throughout my study and research.

It is my pleasure to recognize all the training, help and support that I received from Soccoro Tauyan, our small animal facility manager. I really thank her for the assistance, guidance, encouragement and support that I received during my PhD study and research. I would also like to show gratitude and give special thanks to my colleagues Utsav Prakash Tiwari, Sudhir Yadav, Linge Li and Darcie Inouye for their professional and generous assistance in animal experiments and lab analyses. I would also like to acknowledge Nirvay Sah and Sanjeev Wasti for their support with lab and discussions for my research advancement. My heartfelt thanks also go to Alyssa MacDonald and Rabindra Mandal for their help with bioinformatics works.

With deep respect and highest honor, I would like to dedicate this thesis to my parents for all their painstaking and steadfast efforts in my upbringing and academic journey. It is all due to

your proper parenting and unconditional love and blessings that I have a clear sense of purpose of education and work. I will always remain grateful to my wife Menka, for standing beside me on my academic journey. You gave me love, care and strength that energized me to overcome hurdles and accomplish the task with determination. I would like to take this opportunity to express my earnest thanks to all those involved directly or indirectly in my efforts for this achievement.

ABSTRACT

Feed and gut health management expenses of the flock account for over 70% in poultry production. To reduce the cost of production, nutritionists formulate broiler chickens feed with relatively cheaper alternative feedstuffs. Most of these alternative feedstuffs have high fiber content and low digestibility of nutrients. Thus, it is essential to test different exogenous enzymes to maximize nutrient utilization and enhance feed efficiency of fibrous feedstuffs for profitable broilers production. Better feed utilization also depends on improved gut health and microbial balance in the GIT of the birds. To reduce the load of pathogenic bacteria and maintain gut health for improved productivity, several antibiotic growth promoters (AGP) are used at a subtherapeutic level in poultry feed. However, due to the public health concern and risk of growing antibiotic resistance, the use of AGP has been restricted or regulated in several jurisdictions around the world. This further necessitates the finding of alternatives to AGPs and explore other effective nutritional strategies to modulate the gut health of broilers. Investigation and validation of several additives and functional foods for improving gut health and nutrient utilization are crucial to conveying the mechanistic understanding of these agents for nutritional programming in broilers.

The principal objective of this dissertation research was to evaluate the effects of xylanase enzyme in feed and that of prebiotics application *in ovo* and post-hatch on broiler performance and gut health. To explore the effect of xylanase enzyme on broiler performance, two separate studies were conducted where xylanase was added to a corn-soybean meal (corn-SBM) based diet containing wheat bran in the first study and xylooligosaccharides (XOS) in the second study. Likewise, to examine the effect of prebiotics on gut health and production of

broilers, two more studies were performed where oligosaccharides were applied *in ovo* in the third study, and resistant starch was fed during early post-hatch in the fourth study.

The first study focused on the effect of xylanase supplementation on growth performance and cecal short chain fatty acid (SCFA) production in broilers fed corn-SBM diet containing different levels of fiber incorporated through wheat bran addition. The results from this study indicated that high fiber diets containing more wheat bran are more responsive to xylanase enzyme for improvement in feed efficiency while xylanase can equally improve SCFA production in both corn-based and wheat bran containing diet.

The second study determined the effects of supplemental xylanase and XOS (as prebiotics) on growth performance, cecal SCFA production, and intestinal morphology of broilers fed corn-SBM diet. The results for this study demonstrate that xylanase can improve body weight gain without improving the feed efficiency of corn-SBM diets while both xylanase and XOS can lead to increased cecal fermentation in broilers. Thus, xylanase enzyme could be used in different basal formulations to improve growth performance of broilers, and it can potentially be used along with XOS prebiotics for better cecal fermentation capacity of broilers that might be more useful in a challenged environment.

The third study was conducted to investigate the beneficial role of four different oligosaccharides (differing in chain length) as prebiotics when fed *in ovo* to the embryo of broilers through their amniotic fluid. The gene expression and histomorphometric study demonstrated the potency of xylotriose in stimulating T-cell based adaptive immunity and cecal SCFA compared with controls. The fourth study concentrated on evaluating the effect of resistant starch type 2 and type 4 feeding as prebiotics during early post-hatch in broilers. The results of this study revealed that resistant starch type 4 can be a candidate for

immunomodulation in early post-hatch feeding but may not provide persistent benefit if used for a short duration. The *in ovo* oligosaccharides and early post-hatch resistant starch feeding can support the build-up of immunity in premature birds, but further studies are warranted to ascertain their effects to be used in combination with regular vaccine programs.

Collectively, this dissertation elaborates on the application of xylanase enzyme, oligosaccharides, and resistant starch as alternative resources to improve gut health in broilers, but in the present form, they do not exhibit a greater potency to completely replace the AGPs in feed. However, further research is required to unravel the mechanistic role of existing additives to be used as an alternative to AGP and additional improvement in their potency and efficacy is required to promote their use to obtain the specific health benefits.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADFI	Average daily feed intake
ADG	Average daily gain
AGP	Antibiotic growth promoters
AOAC	Association of Official Analytical Chemists
cDNA	Complementary deoxyribonucleic acid
DM	Dry matter
DNA	Deoxyribonucleic acid
FCR	Feed conversion ratio
GALT	Gut associated lymphoid tissue
GC	Gas chromatography
GE	Gross energy
GIT	Gastrointestinal tract
kg	Kilo gram
MOS	Mannanoligosaccharides
ME	Metabolizable energy
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
QIIME	Quantitative insights into microbial ecology
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RS	Resistant starch
SAS	Statistical analysis system
SCFA	Short chain fatty acids
XOS	Xylooligosaccharides
WB	Wheat bran

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Feed costs have always remained as a major expense for livestock production and the increase in the price of feed ingredients affects the steady growth of the broiler industry. The cost of feed has increased further in the last decade accounting for 70 to 80% of the animal production cost because of use of corn in ethanol production and increase in the demand of grains for feed and fuel (Donohue and Cunningham, 2009; Masey O'Neill et al., 2012). To reduce the cost of broiler feed, nutritionists and poultry producers increased the inclusion level of wheat and alternative ingredients like distiller's dried grain with solubles (DDGS) to the conventional corn-soybean meal-based diets (Adeola and Cowieson, 2011; Lumpkins et al., 2004). The efficiency of nutrients utilization in such alternative ingredients is limited due to the presence of antinutritional factors such as non-starch polysaccharides (NSP; Choct, 2006). These NSP can exert a negative effect on digestion by elevating the viscosity of digesta, reducing the rate of digestion and increasing the microbial activity in the intestine (Bedford, 1995). The improvement in the productivity of broilers is also restricted due to the ban on antibiotics growth promoters (AGP) in several jurisdictions and voluntary discontinuation by farmers in response to consumer concerns (Wegener, 2003). AGP removal has also led to a reduction in feed efficiency, rise in metabolic diseases like subclinical necrotic enteritis and dysbacteriosis leading to wet litter (Dibner and Richards, 2005; Huyghebaert et al., 2011). There has been the use of several feed additives like exogenous enzymes, prebiotics, probiotics organic acids and etheric oils, etc. to improve feed utilization and to replace AGP to a possible extent. The exogenous enzyme phytase has been extensively used as a part of basal diet formulation in broilers because it has consistently demonstrated the potential to alleviate the anti-nutritive effect of phytic acid, improve energy utilization and increase the availability of amino acids (Ravindran et al., 2006).

Besides phytic acid, arabinoxylan is the main component of NSP found in corn DDGS and wheat bran (Widyaratne and Zijlstra, 2007) and is another anti-nutritive feed component, which increases the viscosity and reduces the apparent metabolizable energy (AME) of the diet. NSP degrading enzymes like xylanase can reduce the antinutritive effect of arabinoxylan and provide easily fermentable substrates for the beneficial microbes (Annison and Choct., 1991; De Maesschalck et al., 2015). To overcome the problem associated with the gradual discontinuation of AGPs, the use of prebiotics in poultry diet is gaining attention for its potential to improve intestinal health and diversity of beneficial microbiota (Kim et al., 2011). Non-digestible dietary fibers like oligosaccharides and resistant starch have been used as prebiotic in human to enhance intestinal health and microbial balance (Swennen et al., 2006; Topping et al., 2003). Dietary application of xylooligosaccharides (XOS) and mannooligosaccharides (MOS) is gaining interest for their potential influence on immune stimulation and improvement in microbial diversity in broilers (De Maesschalck et al., 2015; Fernandez et al., 2002). Recently, resistant starch (RS) inclusion in broilers diet is also getting due consideration for being effective as prebiotics due to their positive impact on the improvement of performance, fermentation and gut health of necrotic enteritis challenged birds (M'Sadeq et al., 2015; Regassa and Nyachoti, 2018).

In addition to mixing of prebiotics in different phases of broilers diet, they have been fed to broilers either *in ovo* to the embryo or during the early post-hatch period for improvement in productivity and intestinal health (Jha et al., 2019). This dissertation research focused on the use of xylanase enzyme and prebiotics in the feed as a nutritional strategy to improve growth performance and modulate intestinal health of broilers. The effects of xylanase enzyme were evaluated in diets containing arabinoxylan NSP or XOS prebiotics fed to broilers chicken in different phases. Moreover, the prebiotic effects of different forms of XOS and MOS feeding *in*

ovo and RS feeding during early post-hatch was investigated for improvement in performance and modulation of the gut-health parameter in broilers.

1.1.1 NSP in broiler feed

Crude fiber (CF) is the residue of plant food that remains after its digestion with dilute acid followed by dilute alkali and those residues resistant to hydrolysis by host's digestive enzymes are called dietary fiber (DF, Trowell, 1976). Broadly, DF includes cellulose, hemicellulose, β-glucan, pectins, gums, mucilages and the phenolic compound lignin that is a constituent of the plant cell wall (Davidson and McDonald, 1998; Theander et al., 1989). NSP and RS fractions are also not hydrolyzed by the endogenous host enzymes and are therefore considered as DF from a physiological and functional point of view. In 1982, IUB-IUPAC and Joint Commission on Biochemical Nomenclature defined NSP as 'non-α-glucan polysaccharides containing many monosaccharides (glycose) units connected by glycosidic linkages' (Cummings and Stephen, 2007). NSP consists of various soluble and insoluble polysaccharides like pentoses arabinose and xylose; the hexoses glucose, galactose and mannose; the 6- deoxyhexoses rhamnose and fucose; and the uronic acids glucuronic and galacturonic acids (Bach Knudsen, 2014). Broiler chickens do not produce endogenous enzymes that would help in NSP digestion; rather, they rely on the acidic digestion in the proventriculus and microbial degradation of NSP in large intestine and ceca (Leeson and Summers, 2001). This effect is more prominent in young animals as their GI tract is not matured enough to cope with soluble polysaccharides (Yasar and Forbes, 2000). Arabinoxylan is one of the major components of this NSP, which is the target of some exogenous NSP enzymes is used in broilers feed. Arabinoxylan is a type of NSP that is formed from a linear backbone of (1→4)-β-D-xylopyranosyl residues (X) mainly substituted with α-L-arabinofuranosyl residues (A) to varying degrees at O-2, O-3 or both positions

(Izydorczyk and Biliaderis, 1995; Voragen et al., 1992). Exogenous enzymes that are generally used to enhance nutrient utilization and reduce antinutrient effect are obtained from bacteria or fungi. Exogenous enzymes used in poultry feed are either not produced by the bird or the level of production is not efficient in the host.

1.1.2 Xylanase enzyme in broiler feed

The heterogeneity and complexities of xylan have resulted in several xylanases with varying specificities and they have been classified in several glycosidase families. Xylanases have primarily been classified as glycosyl hydrolase (GH) 10 and 11 based on hydrophobic cluster analysis of the catalytic domains and the similarities in amino acid sequences (Verma and Satyanarayana, 2012). Xylanases that are officially named as endo-1,4-β-xylanase are glycosidases that catalyze the endohydrolysis of 1,4-β-D-xylosidic linkages in hemicellulose xylan and break them into xylose units. Xylanases have been extracted from a range of organisms including bacteria, yeast, marine algae and fungi but the principal commercial source has been filamentous fungi (Polizeli et al., 2005). Xylanase supplementation in wheat-based diet or in a diet containing rye, wheat and SBM can improve the broiler performance (Olukosi et al., 2007; Wu et al., 2004b). Xylanase has also been used in combination with amylase and protease in corn-based diets containing fibers from different ingredient sources and it has been reported to improve growth performance and nutrient digestibility in broilers (Singh et al., 2017; Singh et al., 2019). Xylanase can also increase the digestibility and accretion of N by hydrolysis of encapsulating cell wall (Cowieson et al., 2010; Meng et al., 2005). The xylanase addition in the diet of broilers has not always brought positive response (Olukosi et al., 2007; Olukosi and Adeola, 2008). This can be due to differences in the nutrient density reduction of control and supplemented diets (Adeola and Cowieson, 2011). However, from a nutritional point of view,

partially hydrolyzed arabinoxylan or arabinoxylan oligosaccharides can exert prebiotic properties (Kabel et al., 2002).

1.1.3 Growth performance and feed efficiency

Growth performance of broiler chickens is measured in terms of average daily gain (ADG) of live body weight and total growth. The feed efficiency is determined by the feed conversion ratio (FCR). FCR is the ratio of average daily feed intake (ADFI) to ADG. The lower is the FCR, the better the feed efficiency. The improved feed efficiency conveys that the broiler is consuming less feed to obtain the desired weight gain. However, FCR is not always the benchmark of improved productivity as the broilers may consume more and have a higher ADG. A high ADG implies that the broiler flock can be raised to reach the marketable size earlier than those showing low ADG and can increase the number of flock cycle or total produce in a year.

Growth of broiler chicken is mainly influenced by their genetic makeup and their plane of nutrition besides management and health status. There has been a substantial gain and continuous improvement in the productivity of broilers achieved through traditional quantitative techniques of genetic selection and breeding (Havenstein et al., 1994; Hunton, 2006). The broilers took around 16 weeks to reach marketable weight during 1950 and that was reduced to 6-7 weeks by 1990 (Griffin and Goddard, 1994; Konarzewski et al., 2000). The commercial strains of chickens have demonstrated better growth and feed efficiency compared to heritage chicken breeds, but the nutrient composition of the diet also plays a major role. In a study conducted on four different broiler breeds fed commercial and scavenger diets, a negative effect of scavenger diet on fast-growing broiler breed was observed (Pauwels et al., 2015). This influence was attributed to low feed intake rather than the differences in nutrient digestibility.

1.1.4 Antibiotic growth promoters in broiler feed

The growth promoter effect of antibiotics was noticed in the 1940s when an improvement in the growth of animals fed dried mycelia of *Streptomyces aureofaciens* containing chlortetracycline residue was observed. By the 1960s, the use of antibiotics as animal additives was approved in both the United States and European states (Castanon, 2007; Jones and Ricke, 2003). The mechanism of action of these antibiotics as growth promoters is associated with their ability to influence the population and diversity of intestinal microbiota (Dibner and Richards, 2005). The growth response to such products is often variable and depends on the environment and the composition of feed offered to the birds and understanding of this interaction is necessary to devise a strategy to overcome the losses resulting from their removal from the diet (Bedford, 2000). Some of the common antibiotics that were authorized to be used as additives in poultry feed were bacitracin, neomycin, soframycin, tylosin, erythromycin, lincomycin, tetracyclines, penicillins, oleandomycin, virginiamycin, flavophospholipol, avoparcin and avilamycin etc. (Castanon, 2007). However, there were different lists and directives in place for the use of antibiotics European states and in the United States at national, state and community levels. The risk of antibiotics in edible tissues that can produce allergic or toxic reactions is negligible as the only antibiotics that are authorized to be used as growth promoters in the feed are those that are not absorbed through the GIT (Donoghue, 2003; Castanon, 2007). However, the World Health Organization and European Union concluded that the wider use of antibiotics as feed additives can contribute to the development of resistant bacteria to the drugs used to treat human infections and thus it was a public health issue (Pugh, 2002; World Health Organization, 1997). The increasing restriction on the use of antibiotics demands better farm hygiene and biosecurity to prevent loss in broiler productivity. Moreover, non-antimicrobial substances have been explored as alternatives to antibiotics that can interact with microbiota and includes

enzymes (Bedford, 2000), prebiotics and probiotics (Kocher, 2006), and organic acids (Ricke, 2003) etc.

1.1.5 Alternatives to AGP in broiler feed

Since there is a public health concern associated with the use of antibiotics in animal feed, it would be sensible to incorporate growth-promoting compounds in broiler diets that would have minimal ties with human antimicrobial drugs. More recently, after the increasing consumer concern about the use of AGP, several strategies for the improvement of broiler health, productivity and food security have been explored. The addition of exogenous enzymes is also found to be effective in improving nutrient utilization and reducing the need for AGP (Bedford, 2000; Yang et al., 2008). There are some proteinaceous compounds produced by some bacteria which can be lethal to other strains of bacteria and are often considered more neutral in contrast to antibiotics as they are present in foods that are consumed since ancient times (Cleveland et al., 2001). Bacteriocin-producing *Enterococcus faecium* strain J96 isolated from chicken crop has demonstrated a protective action against chicks infected with *Salmonella pullorum* (Audisio et al., 2000). In a model of the stomach and a small intestine, Gaenzle et al. (1999) demonstrated that *Lactobacillus curvatus*, a producer of bacteriocin curvacin inactivated *E. coli* and caused inhibition of *Listeria innocua*. However, currently, the production of purified bacteriocins and their commercial application is not feasible economically. Joerger (2003) has also reviewed extensively on the use of bacteriophages and their enzymes to target specific bacterial strains but their use at the farm level is uncertain for now but is a potential direction for further research. Organic acids like fumaric, acetic, propionic, butyric, lactic, citric etc. have been used as acidifiers which can also help to improve broilers production in the absence of AGP (Khan and Iqbal, 2016). These organic acids can have the direct effect of reducing the population of

pathogenic bacteria in the GIT and specifically penetrate the cell wall of bacteria in non-dissociated state and then disrupt the normal physiology of such pH-sensitive bacteria. Essentials oils from oregano have also shown potential to modify the gut microbiota and offer an alternative to AGP. It has the phenolic compounds like carvacrol that has shown antimicrobial activity (Akgül. and Kivanc, 1988). Other alternatives are probiotics and direct-fed microbials (DFM) which are the source of live micro-organisms that include bacteria, fungi and yeasts as stated in the US National Food Ingredient Association (Miles and Bootwalla, 1991). A range of microbial species has been used as probiotics, which includes but is not limited to the species of *Bacillus, Bifidobacterium, Enterococcus, Escherichia, Lactobacillus, Lactococcus, Streptococcus,* several yeast species and undefined mixed cultures. *Lactobacillus* and *Bifidobacterium* species are extensively used in humans while *Bacillus, Enterococcus, and Saccharomyces* yeast are commonly used in livestock feeding (Simon et al., 2001). Probiotics are expected to maintain a beneficial microbial population in the host by two basic mechanisms: competitive exclusion and immune modulation. Competitive exclusion comprises competition for substrates and attachment sites, production of antimicrobial metabolites and direct inhibition of pathogens. Probiotics can directly interact with the immune system in the gut and can lead to the modulation of either innate or adaptive or both immune systems (Dugas et al., 1999). In general, probiotics can increase the activities of innate immune cells like macrophages and natural killer cells (McCracken and Gaskins, 1999), improve antibody titer (Huang et al., 2004), and activate and enhance local cell-mediated immunity against the pathogens (Dalloul et al., 2003) but their exact mechanism is largely unknown. Like probiotics, prebiotics, which are non-digestible food ingredients are used in feed to selectively stimulate the growth of beneficial microbes. Compared with probiotics, prebiotics provide the advantage of stimulating the bacteria

that are normally present in the GIT of the host and are more adapted to the environment (Snel et al., 2002).

1.1.6 Probiotics/ direct-fed microbials

Probiotics are defined as live microorganisms that are thought to be beneficial to the host animal by improving their gut microbial balance (Fuller, 1989) or by modifying the properties of the resident microbiota (Havenaar and Huis In'T Veld, 1992). Some examples of probiotics are lactic acid bacteria, bifidobacteria and yeasts. Probiotics are marketed as direct-fed microbials (DFM) which can play a role in meeting the energy requirements of the bird through regulation of fermentation and SCFA synthesis in the hindgut (Caballero-Franco et al.,2007).

There has been a common practice to limit the incidence of enteric pathological or infectious disease by using DFMs or probiotics (Choct, 2009). The direct inhibitory effects of DFMs like *Bacillus* strains isolated from poultry litter, swine lagoons, rumen fluids and other environments were evident on avian pathogenic *Escherichia coli* and *Clostridium perfringens* type A i*n vitro* (Rehberger and Jordan-Parrott, 2009). Apart from the direct inhibitory effects of DFMs on the enteric pathogen, they compete with pathogenic microbes for substrates and attachment sites with concurrent production of antimicrobials which help the host in balancing microbial population and enhance immunomodulation (Yang et al., 2009). In addition to those primary activities, colonic microflora produces short chain fatty acids (SCFA) which is partially responsible for circulation and bowel motility suggesting a form of established mutualism (Kvietys and Granger, 1981). Probiotics can also modulate either innate or adaptive or both immune system (Dugas et al., 1999). The DFM provides benefit through regulation of microbial homeostasis and maintenance of barrier function in the GIT (Salminen et al., 1996). The DFM

supplied via feed can also regulate the hind-gut fermentation and synthesis of short-chain fatty acids and thus, increase the efficiency of the gut microbes to ferment NSP (Sakata, 1987).

Ahmed et al. (2014) reported that *Bacillus amyloliquefaciens* probiotic used as DFM increased ADFI and ADG and improved FCR in an overall experimental period of 35 days. In the same experiment, the DFM inclusion modulated immunity by increasing the level of serum IgG and IgA. Meng et al. (2010) reported a positive effect of *Bacillus subtilis* containing probiotics combination on total tract digestibility of energy and protein, and growth performance of growing-finishing pigs fed low nutrient (energy, protein, and lysine) density diet. Several strains of *Bacillus subtilis* can produce amylase, protease, pectinase and glucanase enzymes during *in vitro* study (Hmani et al., 2017). Inclusion of DFM in broiler diet can promote uptake of glucose, amino acid and minerals across the intestinal epithelium by maintaining the mucosal structural and functional integrity (Li et al., 2008; Wu, 1998).

1.1.7 Prebiotics in broiler feed

In summary, prebiotics is non-digestible feed ingredients, especially the small fragments of carbohydrates which are commercially available as oligosaccharides of galactose, fructose, mannose and xylose. Among food ingredients, some peptides, proteins and certain lipids can also be classified as prebiotics. Likewise, RS is emerging as a major candidate with great potential for prebiotic and colonic food (Sinovec and Marković, 2005; Topping et al., 2003). Many non-digestible carbohydrates or residual fragments of food entering the hindgut can be classified as colonic foods, yet a critical criterion of specific fermentation and metabolic selectivity is expected to be met for them to be considered as prebiotics (Sinovec and Marković, 2005).

The use of prebiotics in poultry has been documented decades back (Iji and Tivey, 1998) and their use is going to be more popular in improving microbiota diversity in the GIT of the

birds (Kermanshahi and Rostami, 2006). In a mice study by Gibson and Roberfroid (1995), it was explained that prebiotics can bring about the bifidogenic effect and shift microbial fermentation from proteolytic to saccharolytic, a more desirable metabolic shift. The definition of prebiotic has been refined recently and the focus is shifted from selective targets to microbial ecological functions within the gut. Prebiotics are now regarded as non-digestible compounds that modulate the composition and/or activity of gut microbiota and through its metabolization confers a beneficial physiological effect on the host (Bindels et al., 2015). The effects of prebiotics on poultry production have been adequately reviewed (Gaggìa et al., 2010; Patterson and Burkholder, 2003), but there is a scarcity of documented information on their effect on the gut microbiota and host immunity (Pourabedin and Zhao, 2015). Prebiotics are reported to increase the number of *Bifidobacteria* and *Lactobacillus* in the gut and thereby aid in the competitive exclusion of pathogens (Alloui et al., 2013; Huyghebaert, 2011). Prebiotics are supposed to increase the production of SCFA leading to an acidic environment in the gut and suppress the proliferation of pathogens (Ganguly, 2013). Prebiotics fed to birds are reported to have caused immunomodulation and enhanced immunity by increasing Ig A secreting cells, IgG and goblet cells in broilers (Teng and Kim, 2018). However, the mechanism of immune interaction and stimulation of immunity by prebiotics is not well established.

1.1.8 Oligosaccharides

Oligosaccharides are a promising alternative to AGP as they can sustain and facilitate the symbiotic interaction between the host and microbiota. The oligosaccharides can be short-chain or long-chain based on the length of the repeating monosaccharides units. Oligosaccharides contain 2-10 monosaccharides unit linked together by glycoside bond between hemiacetal or hemiketal group of one sugar to hydroxy group of second sugar molecule. Physio-chemical

properties of oligosaccharides depend on their chemical structure and composition and most of them are soluble in water or physiological fluids. The most commonly used oligosaccharides as prebiotic fractions are fructooligosaccharides (FOS), galactooligosaccharides (GOS), MOS, XOS, soya oligosaccharides, lactulose and raffinose (Berrocosso et al., 2017; Jung et al., 2008; Vulevic et al., 2004). GOS has also yielded a prebiotic effect by increasing the numbers of *Lactobacilli* and *Bifidobacteria* in the feces of broiler chicken but the potential to modify intestinal microbiota may be dose-dependent (Jung et al., 2008). In a study by Saminathan et al. (2011) using 11 different strains of *Lactobacillus*, it was observed that GOS, and FOS supported the growth of all strains of *Lactobacillus* suggesting that oligosaccharides with a lower degree of polymerization are better fermented. Kaplan and Hutkins (2002) found that *L. plantarum* and *L. rhamnosus* strains were only capable of fermenting trisaccharide and tetrasaccharide fractions of FOS while pentasaccharides were not metabolized. This characteristic suggests that there may be some specific transport systems for these tri- and tetrasaccharides. The longer chain oligosaccharides other than disaccharides and trisaccharides of isomaltose and GOS are only utilized with lower frequency by lactic acid bacteria (Chung and Day, 2004; Gopal et al., 2001). FOS and its longer chain polymer inulin are among the most studied prebiotics. Rehman et al. (2008) did not find the effect of inulin on the microbial community in broilers, while Geier et al. (2009) found a difference in FOS-fed and unfed groups. FOS has been efficient in increasing *Lactobacillus* diversity and restricting the growth of *C. perfringens* (Kim et al., 2011) but there are contrasting evidence that some pathogenic strains of extraintestinal pathogenic *E. coli* contain a gene cluster called ‘fos locus’ that provides it with advantage to metabolize FOS (Porcheron et al., 2011; Schouler et al.2009). XOS is also being used in broiler feed as prebiotic supplements and are chains of β-1,4-linked D-xylopyranoside units, produced by enzymatic

hydrolysis of arabinoxylans found abundantly in cereal grains (Carvalho et al.2013). Broilers lack enzymes to degrade the glycoside bond between xylose monomers in xylan molecule and XOS either supplied in feed or generated by the action of exogenous xylanase can reach the lower intestine and cecum for utilization by xylanolytic microbes. Inclusion of arabino-XOS has revealed a bifidogenic effect and reduced the colonization and translocation of *Salmonella enteritidis* to spleen at d 3 and 7 post-infection (Courtin et al., 2008; Eeckhaut et al., 2008). XOS supplementation also increases the production of bacterial butyrate and f butyryl-CoA:acetate-CoA transferase gene copies in the ceca of chicken (De Maesschalck et al., 2015; Duncan et al., 2004). There is also increase in the abundance of *Lactobacillus* and butyrate-producing bacteria in response to XOS feeding and this effect can be partly explained by the cross-feeding mechanism where acetate and lactate are converted to butyrate (Sato et al., 2008). Recently, there are some reports of antioxidant activity of XOS in mice, where the plasma concentration of catalase and glutathione reductase level was increased in response to dietary XOS inclusion (Gobinath et al., 2010; Wang et al., 2011). XOS supplementation in broilers is also reported to increase the proportion of *Lactobacillus* in the cecum and enhance the concentrations of acetate and propionate (Pourabedin et al., 2015). In laying hen dietary inclusion XOS is reported to increase villus height to crypt depth ratio in the jejunum, increase the number of bifidobacteria in the cecum, enhance the concentration of acetate and butyrate in the cecum, and the contents of IgA, TNF-α, IgM and IL-2 (Ding et al., 2017). Moreover, Ribeiro et al. (2018) found that supplementation of 0.01% of XOS was able to increase body weight gain in broilers and suggested that XOS are acting like ‘pump primers’ and guiding the microbiome to utilize a fermentable substrate more efficiently. MOS is mannose-based oligomers and found naturally in certain plants, beans and cell wall of yeast *Saccharomyces cerevisiae*. Feeding of MOS in

broilers has resulted in the reduction of *C. perfringens* and *E. coli* and an increase in the population of *Lactobacillus* (Corrigan et al., 2011; Kim et al., 2011). Dietary supplementation of whole yeast has been reported to modulate the immune system in chicken by increasing IFN-γ and reducing IL-10 expressions in cecal tonsil and decrease *E. coli* and *Salmonella* colonization in the coccidia-infected flock (Shanmugasundaram et al., 2013). MOS supplementation has been effective in upregulating ileal toll-like receptor (TLR)2b, TLR4, IL-12 and IFN-γ and downregulating TLR2b expression in cecal tonsil (Yitbarek et al., 2012). In a *Salmonella* LPS challenged broiler study, MOS supplementation caused a mild immune response and terminated systemic inflammation earlier than by the AGP virginiamiycin (Baurhoo et al., 2012). The dietary supplementation of MOS is expected to cause greater effect in birds challenged with pathogens or environmental stress. Mannose-containing carbohydrate may bind to the lectins of pathogens and prevent their attachment to the mucosal epithelium and lead to their passage through the GI tract without colonization.

1.1.9 Resistant starch

Chemically, starches are polysaccharides made up of repeating units of monosaccharides or sugar molecules. The sugar molecules are linked together by α-D-(1-4) and/or α-D-(1-6) linkages. The linear molecule consisting essentially glucose molecule linked by α-D-(1-4) linkages are called amylose while those consisting of branched-chain with the addition of α-D-(1-6) linkages are called amylopectin. These starches have been divided into rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) based on their enzymatic interaction (Berry, 1996). Based on their nutritional characteristics, RDS and SDS are considered digestible starch while RS that portion of starch which can resist digestion in the small intestine. RS has been further categorized into 4 distinct types of starch-based on their cause of resistance

(Englyst et al., 1992; Nugent, 2005). Type I RS is physically protected, type II RS is a granular form of starch that limits the accessibility of digestive enzymes, type III RS is a retrograded starch, and type IV RS is chemically modified starch due to crosslinking with chemical reagents. Starch is hydrolyzed by pancreatic amylase in broilers but some inhibitors like tannins, polyphenols and certain legume proteins might affect the digestibility of starch and might lead to undigested portion flowing into the distal GIT. Research interests in the potential of RS as prebiotics have grown in both human and animal studies due to its time-dependent shift in fecal and hind-gut SCFA profiles. Numerous experiments are conducted with high amylose starch to test the prebiotic potential in pigs as they have been a better model of hind-gut fermentation in human (Topping and Clifton, 2001). *Bifidobacterium longum* concentration increases in pigs fed high amylose starch compared with those fed conventional starch (Brown et al., 1997). Several in vitro studies have found that *Bifidobacterium* species physically adhere to both high amylose and chemically modified RS (Brown et al., 1998). The number of *Bifidobacterium* declined slowly after probiotics withdrawal in animals fed RS diet and thus RS could decrease the frequency of probiotics consumption (Topping et al., 2003). Feeding of RS in children has facilitated in recovery from diarrhea (Rabbani et al., 2001) and part of this benefit might have occurred due to the increased fluid absorption in response to greater SCFA production, which stimulates the uptake of water and cations in the proximal colon (Topping et al., 2003). In human subjects, RS consumption caused reduced waste solutes production, which is expected to occur due to improved gut barrier integrity in response to SCFA-producing bacteria (Vaziri et al., 2014). Bermudez-Brito et al. (2015) performed in vitro study with human Caco-2 cells and reported that RS can directly stimulate dendritic cells by interacting with pattern recognition receptors on immune cells and stimulate Th1 production. In case of pigs fed RS, Haenen et al.

(2013a) confirmed that RS feeding increased the relative abundance of several butyrate-producing microbes, positively induced oxidative metabolic pathways like tricarboxylic acid cycle and β-oxidation, while it suppressed the adaptive and innate immune system, as well as cell division. There has been limited information available about the use of RS in poultry, but both acetylated and butylated high-amylose RS is reported to improve the productive performance in necrotic enteritis challenged birds (M'Sadeq et al., 2015). In this study, butylated RS increased jejunal villus to crypt depth ratio and increased butyrate levels in cecum while acetylated RS increased ileal acetate content and decreased cecal pH.

1.1.10 Early nutrition in broilers

Broiler chicken has a relatively shorter grow out period compared to hen or other livestock. Unlike in mammals, the birds can only influence the development of chicks through the composition in the egg. This poses some limitation for the development of the chicks as all the necessary nutrients and requires machinery for the growth needs to be acquired in the fertilized egg. The perinatal period starting from the late-term embryo to few days post-hatch is critical for the development of different organs and systems. There is an unremitting risk of depleting or being deficient in some essential nutrients because of rapidly growing modern chicks as they have a fast-metabolic turnover. Research focusing on early manipulation in broilers have revealed that the first few days are crucial for the developments of organ-systems and desired manipulation must be done at the earliest to achieve long-term effects. The manipulations at this tender age are important to target nutritional efficiency, muscle growth, heat tolerance and immune stimulation (Uni and Ferket, 2004). Early nutrition or feeding is a technique of supplying required nutrients to the chicks either during embryo phase or immediately after hatch until their digestive system becomes fully functional (Uni et al., 2003).

The late incubation phase during pre-hatch is characterized by oral consumption of amnion by the embryo in addition to several physiological processes. One of the major physiological processes during prenatal development in broilers is the glucose homeostasis and the glycogen reserves are withdrawn with the embryo progressing for hatching and is only replenished when the chicks have full access to feed (Moran, 2007). A thorough review of both *in ovo* and early post-hatch feeding in poultry by Jha et al. (2019) summarized different routes of pre-hatch egg injection and various nutritional strategies that are adopted to program the growth and development of broilers. Besides the supply of various essential nutrients, recently, there is an increase in interest of feeding prebiotics and probiotics to the embryo through *in ovo* injection to provide enhanced immunity and improved disease resistance post-hatch (Berrocoso et al., 2017; De Oliveira et al., 2014; Sławinska et al., 2014). Also, early access to acidifiers and oligosaccharides post-hatch has been found to boost the intestinal morphology and stimulate the immune response of chickens against Clostridium perfringens (Ao et al., 2012). Also, feeding of β-glucan as prebiotics can enrich the innate immunity by enhancing the oxidative burst, intensifying phagocytic capacity of heterophils and reducing the level of organ invasion by pathogens (Lowry et al., 2005). However, more research is warranted to understand the mechanism of prebiotics and enzymes influencing the growth and modulation of gut health parameters of broilers during pre-hatch and early post-hatch to obtain a productive and profitable flock.

1.1.11 Immunity in broilers

The immune system acts by two distinct mechanisms in broiler chicken similar to that in other animals. Based on these defense mechanisms, it is categorized into non-specific or innate immunity and antigen-specific or adaptive immunity. The immune system that is mediated by

phagocytes like macrophages, heterophils and dendritic cells, and lymphocytes like Natural Killer (NK) cells constitute an innate defense mechanism. The development of innate immunity is rapid, but it does not have the memory of previous exposure and needs to expend the same process of cellular machinery at all subsequent exposures to pathogens. In contrast, the adaptive immunity that is mediated by T and B-lymphocytes requires a certain period of times ranging from few days to more than a week to protect against specific pathogens on their first exposure. However, due to the production of memory B-cells, adaptive immunity is more effective in subsequent exposure to the same pathogens.

The action of the innate immune system is crucial because they provide the first line of defense against several invading pathogens, but they become ineffective in eliminating some infectious organisms that they do not recognize. The innate immune cells also complement the activity of the adaptive immune system and enhance the immune competence of the host against pathogenic and incompatible tissues. Macrophages and NK cells form a crucial part of natural defense against infections and tumor cells. For the onset of an adaptive immune response, MHC antigens play a major role for both cell-mediated and humoral response. The peptides presented by MHC I originates from intracellular and that presented by MHC II molecules originates from extracellular sources and are also obtained via different pathways (Vyas et al., 2008). However, a cross-presentation also exist between the two pathways as these antigen-presenting cells get manipulated by pathogens through various evolving mechanisms (Crotzer and Blum, 2010; Kurts et al., 2010; Neefjes et al., 2011). Adaptive immunity leads to antigen-specific response and consist of cellular immunity mediated by T-lymphocyte and humoral immunity mediated by antibody circulating in the blood. Both T-helper and cytotoxic T- cells are involved in cellular immune response and act against intracellular pathogens. The antibodies are also known as

immunoglobulins, which are Y shaped protein produced by plasma cells (derived from B-cells) and act mostly against the extracellular antigens. The B-cells are activated to produce antibody either via T-cell dependent activation in response to a foreign protein or through T-cell independent activation in response to foreign polysaccharides and unmethylated CpGDNA (Hoffman et al., 2016; Nutt et al., 2015).

Different forms of T-cell can be distinguished based on their cell surface receptors, but they always express CD3 complexes along with T-cell receptor molecules. The T-helper cells are essentially of two types, namely Th1 and Th2 effector cells. The Th1 cells produce cytokines like interferon-γ (INF- γ), tumor-necrosis factor-α and interleukin (IL)-2 that activates cell-mediated immunity while Th2 cells produce cytokines like IL-4, IL-5, transforming growth factor-β and IL-10, which are essential for activation of humoral response (Abbas et al., 2018). The chB6 (Bu-1) is the marker which is present in B-cells of chicken in all stages of maturation except plasma cells (Houssaint et al., 1989; Igyártó et al., 2008). T helper cell falls into two main subsets, namely Th1 and Th2. Th1 cells secrete IL-2 while Th2 secretes IL-4 cytokine, and these interleukins are also essential for proliferation and differentiation of precursor T helper cells into effector cells (Swain et al., 1990). It has been stated that IL-12 makes a bridge between innate and adaptive immunity. IL-12 is produced by antigen-presenting cells like dendritic cells, B-cells and macrophages etc. in response to antigenic stimulation (Kaliński et al., 1997). It plays an instrumental role in the production and stimulation of Th-1 cells and NK cells (Hsieh et al., 1990; Kobayashi et al., 1989). Likewise, IL-10 is known as anti-inflammatory cytokines which are produced by a myriad of immune effector cells like monocytes, NK cells, Th-2 helper cells, regulatory T-cells, B-cells etc. (Kühn et al., 1993). The cytokine IL-1β is a potent mediator of an inflammatory response and is involved in T-cell dependent antibody production (Nakae et al.,

2001). Toll-like receptor 4 (TLR4) is a transmembrane protein is a member of toll-like receptor family and is a type of pattern recognition receptor (PRR). TLR4 binds to lipopolysaccharides found in the cell wall of gm-ve bacteria (Medzhitov et al., 2001). TLR4 is primarily expressed on the surface of macrophage and it has been used as a marker to study macrophage polarization and peritonitis (Kato et al., 2004; Zhou et al., 2015). Along with TL4, a co-receptor CD14 is also a cell surface marker of macrophage (Wilensky et al., 2015). Like macrophage, the abundance of NK cells is also essential in mapping innate immune response and level of CD56 marker provides a better estimate of its population. CD56 is also called neural cell adhesion molecule (NCAM) and it is a homophilic binding glycoprotein expressed on the surface of lymphoid cells and monocytes in the hematopoietic system (Van Acker et al., 2017). However, it is expressed most stringently on the surface of NK cells and thus serves as a potential marker for NK cells (Cheng et al., 2013). It is essential to note that the immune response can be local or systemic depending on the form and level of immune stimulation. The gene expression of the immune markers from a specific site of the digestive system in chicken can provide information about the immune activation in that particular region of interest.

1.1.12 Intestinal histomorphometry in broilers

The health of the digestive tract in broilers is not only important for the proper utilization of nutrients but it is also essential because it is the largest immunological organ that protects the host from the exogenous pathogen. The small intestine is divided into three sequential segments: upper duodenum, middle jejunum and distal ileum. The duodenum is a hollow tube that continues from the gizzard and forms a distinct duodenal loop that surrounds pancreas. The jejunum is demarcated from duodenum by a duodenojejunal flexure that occurs after the entrance of bile and pancreatic duct in the duodenum. The jejunum continues into ileum and they are

demarcated by a Meckel's diverticulum (yolk stalk). The ileum is the last segment of the small intestine and terminates into the colon at ileocecocolic junction. Histologically all the sections of intestine contain same layers which have mucosa facing the lumen and serosa as the outermost layer. The mucosal epithelium is folded into finger-like projections into the lumen and is called villi, while the invaginations of this epithelium form crypts. The base of these crypts contains stem cells which are continually dividing and provides the sources of epithelial cells for the villus where the cells are dying and shedding regularly.

The villus in the duodenum is short and stubby, while it is tall in the jejunum and of intermediate length in the ileum. The villus also contains tiny little projections on its surface called microvilli which forms a structure called brush border and increases surface area for the absorption of nutrients. Besides intestinal epithelial cells, the adjacent cells linkage by the tight junction (TJ), adherens junction (AJ) and desmosomes also play a major role in providing a protective barrier against pathogens and selective permeability for nutrients and ions (Schneeberger and Lynch, 1992; Steed et al., 2010). The villus height to crypt depth ratio has been accepted as the measure of efficiency because the increase in crypt depth in relation to villus height indicates a greater need of cell proliferation required to maintain a healthy gut barrier integrity (Awad et al., 2011; Uni et al., 1998). Several probiotics and prebiotics are being supplemented in broiler diet to support their intestinal health as there has been a constant increase in public concern against the use of AGPs. Chichlowski et al. (2007) reported that the supplementation of probiotics containing *Lactobacilli*, *Bifidobacterium thermophilum* and *Enterococcus faecium* decreased crypt depth and increased villus height in the jejunum of chicken compared to salinomycin and control. Addition of probiotics has also been reported to increase the ileal villus height in several studies in both layers and broilers (Awad et al., 2006;

Samanya and Yamauchi, 2002). Likewise, Ashraf et al. (2013) observed an increase in villus height in duodenum and ileum of heat-stressed birds when they were fed MOS prebiotics. It is assumed that increased villus height is associated with a concurrent enhancement in the digestive and absorptive function of the intestine because of increased absorptive surface area, increased production of brush border enzymes and higher expression of nutrient transporters (Amat et al., 1996). Improvement in the intestinal mucosal morphology can thus enhance nutrient utilization and reduce the occurrence of enteritis in the gut.

1.1.13 Fermentation and short chain fatty acids

Cereal grains and legumes used in broilers feed also contains a considerable amount of fermentable fibers like RS, arabinoxylan, glucuronoxylan, glucomannan, β-glucans, glucuronic acid and galacturonic acid etc. (Singh et al., 2019; Tiwari et al., 2019). Broiler chickens lack enzymes required for digestion of such NSP and RS and thus these undigested fractions reach the hindgut and provide the substrate for bacterial fermentation. Ceca is the primary site for fermentation of undigested or partially digested carbohydrates and subsequent production of SCFA in broilers (Jamroz et al., 2002). The carbohydrates that get fermented in the GIT of broilers belong to the class of DF. The presence of the high amount of protein without adequate DF in diet can shift the fermentation towards the production of more odorous and toxic nitrogenous compounds like ammonia, indoles and phenols (Jha and Berrocoso, 2016; Macfarlane et al., 1992). The fermentation of DF fractions by microbiota in broilers ceca is less in comparisons to other non-ruminant animals like pigs and rats (Jørgensen et al., 1996; Józefiak et al., 2004). However, the fermentation products yielded in the ceca of broilers are similar to those produced in the rumen, and the colon of human and pigs. Jamroz et al. (2002) reported that in the degradation of total NSP in ceca of chicken, a negative value is obtained for the

degradation of soluble NSPs. The major product of cecal fermentation of RS and NSP is SCFA and it predominantly includes acetate, propionate, butyrate, and lactate (Jozefiak et al., 2007; Meimandipour et al., 2010).

Acetate is either formed from CO_2 via Wood-Ljungdahl pathway or by the hydrolysis of acetyl-CoA; propionate is either formed via electron transfer chain using phosphoenolpyruvate or by the reduction of lactate; butyrate is formed by condensation of two molecules of acetyl-CoA followed by subsequent reduction (Den Besten et al., 2013). The SCFA enters diverse metabolic routes of carbohydrate and lipid metabolism, whereby acetate and butyrate are directed towards lipid biosynthesis, and propionate is incorporated into gluconeogenesis. Based on reviewed information the energy value from NSP fractions is in the following order: butyrate > propionate > acetate (Józefiak et al., 2004). The variation in the production of different SCFA may depend on the availability of microbes and the type and amount of fermentable substrates in the ceca of broilers (Meimandipour et al., 2010; Walugembe et al., 2015). Cecal SCFA are undetectable in broilers on the day of hatch, while acetate could be detected in 3 d old and propionate and butyrate could be detected in 12- 15 d after which all SCFAs reach a higher concentration in broilers (Van der Wielen et al., 2000). Cecal production of SCFA like propionate and butyrate has also been observed to reduce the growth of *Salmonella* in broilers (Meimandipour et al., 2010). It has been reported that in non-ruminant species, 95-99% of the total SCFA produced during fermentation is absorbed by the gut before it reaches rectum (Von Engelhardt et al., 1989). Also, SCFA is reported to stimulate growth and proliferation of enterocyte, affect intestinal immune responses, regulate intestinal blood flow and mucin production. Compared with other SCFA, butyrate is a preferred source of energy for colonocytes and causes a trophic effect on the intestinal mucosa (Campbell et al., 1997). Butyrate provides

the source of energy for the growing epithelial cells of the intestinal mucosa (Toping and Clifton, 2001). It is also involved in the various mechanism that regulates the cellular differentiation, intestinal permeability and gene expression (Mroz et al., 2005). Acetate is produced in a higher amount but contributes to a lower energy value than propionate and butyrate. Therefore, it would be further beneficial to modulate the fermentation process by increasing the amount of desired substrates and abundance of microbes that would enhance the production of butyrate. Increased level of butyrate would not only satisfy energy demand, but it would also reduce cellular apoptosis, enhance gut immunity and support the recycling of inorganic ions (Leng, 1978; Meijer et al., 2010).

1.1.14 Gut microbiota in broilers

The GIT of chicken harbors a diverse community of microbiota that comprises of more than 900 species of bacteria, a low level of protozoa, fungi, yeast and viruses, and this microbiota assist in the breakdown and utilization of consumed feeds (Apajalahti et al., 2004; Wei et al., 2013). Among these vast population of bacteria, estimates suggest that only 10-60% of cecal bacteria can be cultured and only 45% of the intestinal bacteria present in chicken can be assigned to the genus level with much confidence. The phylum Firmicutes dominates the chicken gut microbiota composition and consists of more than two-third while Bacteroidetes constitute 10-50% of the total population (Dumonceaux et al., 2006; Gong et al., 2002; Lu et al., 2003). Most of the sequences found within Firmicutes belongs to the family *Ruminococcaceae* and family *Lachnospiraceae*, and *Clostridium* cluster IV and XIVa (Collins et al., 1994). With the rapid advances in the DNA sequencing technologies and its increasing affordability, amplicon sequencing of 16S rRNA genes has become the preferred method for microbiota profiling in chickens. This technique is still not free of all difficulties and PCR biases, and sequencing depth

is still limited to the resolution of taxa abundance of 0.01 to 0.1% when performed on a set of samples of different studies (Stanley et al., 2014).

The bacterial density in chicken GIT increases rapidly after hatch and becomes more diverse and stable with increasing age. In a study by Apajalahti et al. (2004) in broiler chicks at 1 d post-hatch, the bacterial number in the digesta reached 10^8 and 10^{10} cells/g in proximal and distal intestine respectively. This number increased to 10^9 cells/g and 10^{11} cells/g in ileal and cecal digesta in less than 1 wk. The gut lumen and mucosal surface of ceca are the major sites for colonization by microbes in broilers. Bacteria in the small intestine compete for the same nutrients with the host, but the host can recover the fraction of energy lost to these microbes by absorbing their fermentation metabolites. The population of obligate anaerobes in the distal intestine is considerably higher by several orders of magnitude than those of aerobic and facultative anaerobic microbiota due to the low redox potential in lower GIT (Marteau et al., 2001). Compared to the small intestine, the microbial communities establish later in the cecum and need around 6-7 weeks (Coloe et al., 1984). However, the early period is crucial for the establishment of microbiota in the GIT of broilers. Young birds acquire microorganisms from the outside environment through feed and water and thus diet serves as one of the strongest determinants of the gut microbial community diversity. The genus *Clostridium* is infamous among poultry industry because of one pathogenic species, *Clostridium perfringens*. However, there are many genera and species within order *Clostridiales* which are non-pathogenic and thus should not be inadvertently associated with the poor performance of broiler chickens (Rinttilä and Apajalahti, 2013). There is a growing understanding that most microbe-host relation is commensal and even mutualistic (Dethlefsen et al., 2007). The microbiota present in the cecum of broilers utilizes carbohydrate sources for metabolism but when this primary substrate is

exhausted, they also ferment protein to salvage energy and lead to the generation of toxic products.

The effective ability of pathogenic bacteria like *E. coli*, *Salmonella typhimurium* to adhere to the mucosal lining in the gut is a critical factor in the initiation of infection (Collado et al., 2005). This ability of adherence by pathogenic bacteria can be reduced by the probiotic strain of Bifidobacteria and Lactobacillus through competitive exclusion which is more effective when these probiotic organisms are selected from chicken (Collado et al., 2005; Servin and Coconnier, 2003). The role of commensal microbes in broilers is reviewed by Lan et al. (2005) where their beneficial roles have been summarized. These beneficial bacteria are also associated with the promotion of gut maturation, gut integrity, antagonism against pathogen and immune modulation in the host. The antagonism by beneficial microbes or probiotics against the pathogens can occur either by the release of bacteriocins or due to the competitive exclusion by the occupation of attachment sites and competition for nutrients. The cell wall components like peptidoglycans, polysaccharides present in some lactic acid bacteria in the gut have also shown to have immunostimulatory properties (Takahashi et al., 1993). Also, the diversity of gut microbiota has been reported to affect the complexity of T-cell receptor repertoire in the gut and spleen of broilers (Mwangi et al., 2010). The communication between microbiota and gut immune system is specifically mediated by pattern recognition receptors (PRRs) that are expressed on the various antigen-presenting cells and intestinal epithelial cells, and cause activation of both innate and adaptive immune responses (Sommer and Bäckhed, 2013). Gut microbiota can also affect the intestinal morphology and some probiotic strains have been reported to increase villus height to crypt depth ratio in the ileum of broilers (Barnes et al., 1972). Hence, maintaining the balance of

gut microbiota in broilers by using prebiotics that can support probiotic strains becomes more relevant when there is limited use of AGPs in broilers feed.

1.2 The scope and aim of current research

The broilers are raised on commercial feed that is mainly corn-SBM based and some alternative feed ingredients are regularly incorporated in such diets to reduce the cost of feed production. Most of such alternative ingredients are a rich source of NSPs and thus several exogenous enzymes like xylanase are supplemented in these diets to improve the digestibility of NSP. The addition of xylanase enzyme is not only expected to act on arabinoxylan unit and increase the degradability of NSP in the digesta but also provide a source of readily fermentable substrate for the lower gut microbiota. Gut microbiota also has the ability to utilize different types of substrates ranging from simple to complex carbohydrates and proteins depending on depletion of primary resource. It is expected that the early presence of XOS prebiotic will not only provide an easily fermentable substrate for the lower gut microbiota but will also cause a priming effect. The microbiota that has already started using the available XOS supplement could also gain the ability to better utilize the complex NSP fragments that are generated in response to the action of xylanase enzymes on the digesta matrix. These prebiotics fragments are also reported to cause immune modulation, but the exact mechanism has not been fully understood. It has been suggested that either these prebiotics cause direct stimulation of immune cells or it exerts its effect by boosting the population of microbiota and by enhancing the production of their fermentation metabolites. The prebiotic dietary fibers are also being used in early feeding of broilers based on understanding that it can modulate the colonization of gut microbiota and save the cost of higher inclusion during the later growth period. The prebiotics can be fed to broiler chickens as early as during embryonic stage through *in ovo* injection. The

broilers gut microbiota takes few days to colonize and reach maximum population and then stabilize with increasing age. During early days after hatch, the chicks are dependent on yolk and slowly shift from lipid-based to carbohydrate-based nutrition. The early growth period immediate after hatch provides an opportunity to include prebiotics in the diet of chicks that can provide the substrate for colonization by beneficial microbes. Several studies have been conducted to evaluate the effect of including NSP degrading enzymes and prebiotics on growth performance, fermentation characteristics in the gut and the diversity of gut microbiota in broilers. Some researchers have also investigated the impact of early feeding of prebiotics on growth and microbial composition in broilers. However, there is scanty information on the use of prebiotics along with xylanase enzymes and data on the effect of early feeding of RS and different chain length oligosaccharides on immune stimulation, SCFA production and cecal microbiota diversity is limited. This study focuses on the effect of *in ovo* and post-hatch nutritional modulation through the inclusion of dietary fibers having prebiotic potential on performance, immunity and overall gut health of broilers.

CHAPTER 2: EFFECTS OF XYLANASE ON GROWTH PERFORMANCE, CECAL SHORT CHAIN FATTY ACID PRODUCTION, AND CECAL MICROBIOTA PROFILE IN BROILERS FED DIFFERENT LEVELS OF FIBER

Abstract

This study investigated the effect of xylanase supplementation in the diets with different levels of fiber on growth response and cecal short chain fatty acids (SCFA) production in broiler chickens. A total of 180 day-old chicks (Cobb 500) were randomly and equally distributed over 30 pens (6 birds/pen). Six dietary treatments were tested using a completely randomized design in a 3×2 factorial arrangement (5 pens/treatment). The treatments included 3 levels (0%, 5%, and 10%) of wheat bran (WB) as an additional fiber source, supplemented without or with xylanase (0.01% Econase-XT) and fed in 2 phases (starter, d 0–21 and finisher, d 22–35). All diets were corn and soybean meal-based containing 500 FTU/kg phytase and were offered in mash form ad libitum with free access to water. Xylanase supplementation significantly ($P < 0.05$) increased the average daily gain (ADG) in the finisher phase and over the whole trial and increased ($P < 0.05$) average total gain in broilers (2025 *vs* 1943 g). Broilers fed xylanase also had improved ($P < 0.01$) feed conversion ratio (FCR) in the starter phase. A significant interaction ($P < 0.05$) was found between WB and xylanase on FCR in the finisher phase and total period. The interaction displays that high fiber diets were more responsive to supplemental xylanase and thus showed higher improvement in FCR compared to 0% WB group. Xylanase significantly increased ($P < 0.01$) total cecal SCFA (86.6 *vs.* 63.4 µmol/g) with significant increase ($P < 0.01$) in straight chain SCFA. Besides increasing total SCFA, xylanase also shifted the production of SCFA from acetate to more propionate and butyrate production. Unlike the effect on FCR, xylanase increased total SCFA production independent of diet type, suggesting some differences in the hydrolysis of soluble and insoluble NSP during digestion and cecal fermentation. Amplicon sequencing of V3-V4 region of 16S rRNA gene from d 35 cecal samples revealed that xylanase had a trend for the higher observance of OTUs and exhibited a trend for beta diversity compared to no xylanase group.

Xylanase also increased the abundance of *Lactobacillus salivarius* compared with no xylanase groups ($P < 0.05$). It is concluded that xylanase can enhance productive performance and health of broilers by improving feed efficiency and that some but not all of this response are related to increasing cecal SCFA production in broilers and enhancing the abundance of beneficial microbiota.

Keywords: broilers, enzymes, fiber, growth performance, short chain fatty acids, xylanase

2.1 Introduction

Xylanase has been used in wheat-based broiler diet to remove the antinutritive effect of viscosity caused by soluble NSP like arabinoxylans (Bedford and Classen, 1992). As xylanase breaks fiber into oligosaccharides, entrapped nutrients like starch and protein are also released after cell wall dissolution in both wheat (Zhang et al., 2014) and corn-based diet (Masey O'Neill et al., 2014; Meng and Slominski, 2005; Singh et al., 2019). The released nutrients are utilized by the host in ileal phase and the undigested broken fiber chain like xylooligosaccharides (XOS) work as prebiotic for the cecal microbes (Bedford, 2000; Choct et al., 1996; Courtin et al., 2008). This helps to improve pre-cecal digestibility of major nutrients and provides a resource for cecal production of short chain fatty acids (SCFA) so that the net effect is highly favorable (Jozefiak et al., 2007; Masey O'Neill et al., 2014; Mookiah et al., 2014). SCFA has been shown to increase gut hormone Peptide YY (PYY) production and consequently delay gastric emptying (Cuche et al., 2000; Psichas et al., 2015). Stimulation of PYY release has also been documented in response to xylanase supplementation. These pieces of evidence support multiple pathways of improvement in broilers performance by the inclusion of xylanase enzyme in the diet.

The amount and concentration of SCFA produced in broiler ceca depend on the activity of colonized microbiota (Engberg et al., 2002), the type of feed ingredients and supplemented enzymes (Jamroz et al., 2002; Masey O'neill et al., 2014; Silva and Smithard, 2002). There has been a standard practice of applying phytase enzyme in broiler feed to reduce the need for supplemental phosphorus and to avoid its pollution via fecal wastage (Selle and Ravindran, 2007). Synergism between xylanase and phytase can be expected when applied together where xylanase can break arabinoxylans providing further access of phytase to phytate which can ultimately release the entrapped nutrients and hydrolytic enzymes (Selle et al., 2003b). Exogenous xylanase supplemented in wheat-based broiler diet hydrolyzes NSP in the small

intestine where it reduces digesta viscosity (Bedford and Classen, 1992; Veldman and Vahl, 1994) and decreases the pathogenic microbial load in the lower gut (Engberg et al., 2004; Jozefiak et al., 2010). Xylanase addition to wheat-based diet along with phytase increases AMEn and nitrogen digestibility (Selle et al., 2003a; Wu et al., 2004a) and when diets can achieve similar ME, wheat can completely substitute corn keeping well-maintained broiler performance (Chiang et al., 2005). Besides availability and cost being the reason to replace corn, wheat also possesses higher protein, lysine content and better pellet binding property (Crouch et al., 1997). In addition to powdered and whole wheat, wheat bran (WB), which has been reported to have plasma antioxidant capacity can be also be incorporated in broiler diet at 30% level without any adverse effect (Ali et al., 2008). WB, a co-product of wheat milling accounts for 14-19% of the kernel weight and comprises mainly of the aleurone layer and outer coverings, and is a good source of B-vitamins, folate, protein and lysine (Pomeranz, 1988). It is also a decent source of betaine (Bruce et al., 2010; Zeisel et al., 2003) that has been proven to improve intestinal cell health, osmoregulation and growth performance of chicken (Kidd et al., 1997; Ratriyanto et al., 2009). Xylanase supplementation in wheat-based diet can enhance nutrient utilization at net energy (NE) level and reduce per kg metabolizable body weight ($W^{0.75}$) heat production in broilers along with improving their feed efficiency (Nian et al., 2011).

Inclusion of arabinoxylan oligosaccharides, derived from WB digested with xylanase, in the corn-based diet has revealed similar improvement in feed efficiency of broilers as that of xylanase application in the feed (Courtin et al., 2008). Xylanase supplementation in a wheat-based diet has been reported to alleviate impaired mucosal barrier (Liu et al., 2012) and improve immunity in broilers (Gao et al., 2007). The oligosaccharides produced by the action of xylanase on WB exert prebiotic effect and stimulate beneficial microbiota in the intestine (Broekaert et al.,

2011). These metabolites serve as the fermentable source for gut microbes, thereby prevents protein putrefaction and increase N utilization (Nahm, 2003). However, it is imperative to test various concentrations of exogenous enzymes in diverse feed matrix to ascertain the optimum combination as an excess application of enzymes may render smaller fragments of oligosaccharides that may be ineffective as prebiotics (Zou et al., 2006). This study was conducted to test the hypothesis that xylanase supplementation improves feed utilization and growth of broilers fed WB and enhances cecal short chain fatty acids (SCFA) production. The objectives were set to determine the effects of WB and xylanase on growth, feed intake, cecal SCFA production and cecal microbiota profile in broilers.

2.2 Materials and methods

2.2.1 Experimental design and dietary treatments

A growth performance experiment was conducted at the Small Animal Facility of the University of Hawaii at Manoa (UHM) in accordance with the guidelines described by the Federation for Animal Science Societies (FASS, 2010). All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of UH. The experiment was conducted in completely randomized design in a 2 × 3 factorial arrangement of treatments consisting of two levels of xylanase (0 and 100 mg Econase XT/ kg diet) and three levels of WB (0%, 5% and 10%). Corn and soybean meal-based diets were formulated in two phases: grower (0-21d) and finisher (22-35 d), with supplemental minerals and vitamins to meet or exceed the NRC (1994) recommended levels of nutrients for broilers (Table 2.1). WB was added by replacing an equal amount of corn from the basal diet formulation while xylanase was top dressed. All the diets were in mash form and offered *ad libitum* to broilers with free access to water.

Table 2.1 Ingredient composition and nutrient content of diet fed to the broilers in the study (as-fed basis; % unless otherwise indicated).

	Inclusion level					
	Starter (d 0-21)			Finisher (d 22-35)		
Ingredients, %	**WB 0%**	**WB 5%**	**WB 10%**	**WB 0%**	**WB 5%**	**WB 10%**
Corn	58.86	53.86	48.86	70.23	65.23	60.23
Soybean meal 48	36.93	36.93	36.93	26.51	26.51	26.51
Wheat Bran	0.00	5.00	10.00	0.00	5.00	10.00
Soy oil	0.83	0.83	0.83	0.68	0.68	0.68
Salt	0.40	0.40	0.40	0.37	0.37	0.37
Sodium Bicarbonate	0.04	0.04	0.04	0.00	0.00	0.00
DL Methionine	0.30	0.30	0.30	0.19	0.19	0.19
Lysine HCl	0.08	0.08	0.08	0.17	0.17	0.17
Threonine	0.01	0.01	0.01	0.05	0.05	0.05
L-Tryptophan	0.02	0.02	0.02	0.00	0.00	0.00
Limestone	1.11	1.11	1.11	0.80	0.80	0.80
Mono Ca Phosphorus	0.92	0.92	0.92	0.48	0.48	0.48
Quantum Blue	0.01	0.01	0.01	0.01	0.01	0.01
Vitamin and mineral premix[1]	0.50	0.50	0.50	0.50	0.50	0.50
Calculated composition, %						
Crude protein	22.43	22.85	23.27	18.29	18.71	19.13
ME kcal/kg	3000	2925	2850	3100	3025	2950
Poult ME MJ/kg	12.55	12.24	11.93	12.97	12.66	12.34
Calcium	0.96	0.97	0.97	0.75	0.76	0.76
Phosphorus	0.76	0.79	0.83	0.62	0.66	0.69
Avail Phosphorus	0.47	0.48	0.49	0.36	0.37	0.38
Fat	**3.59**	**3.59**	**3.59**	**3.77**	**3.77**	**3.77**
Fiber	2.67	2.91	3.15	2.59	2.83	3.07
Met	0.65	0.65	0.66	0.48	0.49	0.49
Cys	0.37	0.38	0.39	0.32	0.33	0.33
Me+Cys	1.02	1.03	1.04	0.80	0.81	0.82
Lys	1.31	1.33	1.35	1.09	1.11	1.13
Thr	0.88	0.89	0.90	0.75	0.76	0.77
D Met	0.58	0.59	0.59	0.43	0.44	0.44
D Cys	0.34	0.34	0.35	0.29	0.29	0.30
D Met+Cys	0.92	0.93	0.94	0.72	0.73	0.74
D Lys	1.18	1.20	1.22	0.98	1.00	1.02
Phytate P	0.24	0.26	0.27	0.22	0.24	0.26
Na	0.19	0.19	0.19	0.17	0.17	0.17

Cl	0.30	0.30	0.30	0.30	0.30	0.30
Linoleic acid	1.51	1.51	1.52	1.63	1.64	1.64
Choline (mg/kg)	1373	1414	1455	1159	1200	1241
Analyzed composition, % as-is basis						
DM	87.9	87.8	88.7	87.6	87.7	87.4
Gross energy, kcal/kg	3752	3828	3910	3877	3929	3985
Gross energy, MJ/kg	15.71	16.02	16.37	16.23	16.45	16.68
Crude protein	22.85	23.47	24.07	18.45	19.05	19.77
Crude fat	3.12	3.09	3.07	3.21	3.16	3.14
ADF	3.84	4.22	4.57	3.76	4.12	4.48
NDF	7.08	8.35	9.71	7.03	8.30	9.64
Lignin	0.32	0.45	0.57	0.36	0.48	0.62
Ash	4.85	5.00	5.19	3.83	4.01	4.21
Ca	0.98	1.01	1.03	0.80	0.83	0.85
P	0.68	0.71	0.75	0.57	0.60	0.64

[1]Providing the following (per kg of diet): vitamin A (trans-retinyl acetate), 10,000 IU; vitamin D_3 (cholecalciferol), 3,000 IU; vitamin E (all-*rac*-tocopherol-acetate), 30 mg; vitamin B_1, 2 mg; vitamin B_2, 8 mg; vitamin B_6, 4 mg; vitamin B_{12} (cyanocobalamin), 0.025 mg; vitamin K_3 (bisulphatemenadione complex), 3mg; choline (choline chloride), 250 mg; nicotinic acid, 60 mg; pantothenic acid (D-calcium pantothenate), 15 mg; folic acid, 1.5 mg; betaíne anhydrous, 80 mg; D-biotin, 0.15 mg; zinc (ZnO), 80 mg; manganese (MnO), 70 mg iron ($FeCO_3$), 60 mg; copper ($CuSO_4 \cdot 5H_2O$), 8 mg; iodine (KI), 2 mg; selenium (Na_2SeO_3), 0.2 mg.

2.2.2 Housing and general experimental procedures

A total of 180 day-old broiler chicks (Cobb 500) were obtained from a commercial hatchery and the chicks were weighed individually, wing tagged and placed randomly in one of 30 floor pens (6 birds per pen). The randomization was done by excel based randomization software. All the pens were provided with a litter of wood shavings in environmentally controlled rooms with 24 h of fluorescent lightening. Each of the six dietary treatments was randomly assigned to five replicate pens and the temperature was maintained at 33±1˚C during the first week and gradually reduced to 25˚C by the end of the third week. Bodyweight of the birds was recorded individually, and the feed intake was recorded per pen and then calculated as

average per bird on weekly intervals (0, 7, 14, 21, 28 and 35 d). The mortality of the birds was monitored and recorded every day and the feed intake was adjusted for mortality. Any leftover fed in the feeder was weighed back and recorded weekly. The data generated were used to calculate average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR). After weighing on d 35, all birds were euthanized by CO_2 inhalation. The digesta from both ceca of each bird was collected and pooled in a whirl pack bag with gentle milking and were transferred to -20°C in the freezer until further analysis for short chain fatty acids. Also, another set of cecal digesta was collected at d 35 for DNA extraction, which was snap-frozen in liquid nitrogen and kept at -80°C until further analysis of microbiota.

2.2.3 Chemical analysis

All feed samples were analyzed for their nutrient profile; DM, GE, N (for CP), fat, ADF, NDF, Ca, P and ash according to the standard procedures of the Association of Official Analytical Chemists (AOAC, 2006). In brief, the DM was calculated by placing the sample in a hot air oven at 65ºC for overnight (method 930.15). The GE was determined using an oxygen bomb calorimeter (Parr Bomb Calorimeter 6200, Parr Instrument Co., Moline, IL). The nitrogen (N) content was determined using combustion analysis (LECO, method 990.03) and was used to calculate the crude protein (CP) content (N×6.25). The ether extract was determined by Soxhlet method (method 920.39) and the ash content was measured by burning the sample at 650ºC for overnight (method 942.05). Acid detergent fiber (ADF, method 973.18) and neutral detergent fiber (NDF, method 2002.04) were determined using Ankom^{200} Fiber Analyzer (Ankom Technology, Macedon, NY). Lignin was determined according to method 973.18 (A-D). Samples were digested in concentrated nitric acid and 70% perchloric acid to solubilize Ca and P. The Ca content was determined using flame atomic absorption spectrometry (method

975.03B(b); Varian FS240 AA, Varian, Inc., Palo Alto, CA). The concentration of total P was measured by the gravimetric procedure (method 966.01). Ammonium molybdate was added to the supernatant to form phosphomolybdate, which was then reduced to form a blue phosphomolybdenum complex and the color intensity was read at 620 nm to determine P content.

2.2.4 Short chain fatty acids (SCFA) analysis

The method of GC was set according to the procedure described by Zhao et al. (2006) with some modification. The cecal digesta was analyzed for SCFA profile by gas chromatography using GC (Trace 1300, Thermo Scientific, Waltham, MA) GC equipped with flame ionization detector (FID) and AS 1310 series automatic liquid sampler. A fused silica capillary column with polar free acid phase (Stabilwax-DA, Restek, Restek Corporation, Bellefonte, PA) of 30 m × 0.53 mm i.d. coated with 1 µm film thickness was used. The injector port was fitted with a splitless liner of 4 mm i.d ×78.5 mm length containing wool filter (Thermo Scientific, Waltham, MA). Helium was supplied as a carrier gas at a flow rate of 14.5 ml/min and run time was set for 17.5 min. The initial oven temperature was 100°C held for 0.5 min, then ramped to 180°C at 8°C /min and held for 1 min, then again raised to 200°C at the rate of 20°C /min where it was finally maintained for 5 min. The temperature of the injector port was set at 200°C and that of detector port was set at 240°C. The flow rates of air, hydrogen and makeup gas (nitrogen) were 300, 30 and 20 ml/min respectively. The samples injection volume was kept 0.5 µl in order to reduce the possibility of backflash and a large expansion of water used as a solvent. Isopropyl alcohol and distilled water were used as syringe wash solvent. Data handling and processing were performed on Chromeleon^{TM} 7.2 software (Thermo Scientific, US). The processing of the sample was done after integrating the chromatogram for specific peaks and

then comparing the peak area of each individual SCFA with the respective calibration curve of their standards.

A standard stock of volatile free acid mix containing 10 mM of each short chain fatty acids (formic, acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, hexanoic and n-caproic) in deionized water was procured from Supelco® (Supelco, Bellefonte, PA). Seven levels of concentration (0.1, 0.5, 1, 2, 4, 6 & 8 mM) of the standard solution were prepared from the standard VFA mix and an extra 3 level concentration of the standard solution was prepared for acetic acid. The proportionate volume of the standard stock was mixed with 100 µl of 25% metaphosphoric acid in a 1.8 ml microcentrifuge tube. After this, 50 µl of 97.9 mM trimethylacetic acid (TMA, Pivalic acid from Sigma-Aldrich, St. Louis, MO) in deionized water was added to the above mixture and extra deionized water was added to bring the volume to 1500 µl. The individual SCFA at different concentration were used as external standard and TMA (trimethylacetic acid, final concentration 3.3 mM) was used as an internal standard. This standard SCFA mix was analyzed by GC to get an individual calibration curve for each external standard compound based on their response ratio to the internal standard.

After thawing, approximately 0.02 g cecal content was weighed into a 1.8 ml microcentrifuge tube. The required volume of deionized water was added to bring the volume to 1350 µl and then 50 µl of 97.9 mM TMA and 100 µl 25% metaphosphoric acid was added to bring the final volume to 1500 µl. The suspended cecal content was mixed thoroughly by vortexing for 2-3 minutes. After the samples were mixed uniformly, the samples in microcentrifuge tubes were centrifuged at 13,000 ×g for 10 minutes at 4°C. Following centrifugation, 1000 µl of supernatant was transferred into a GC vial and sealed using septa and screw cap and loaded on the carousel of autosampler for injection.

2.2.5 DNA extraction, 16S rRNA amplicon sequencing and data analysis

2.2.5.1 DNA extraction from cecal digesta

Genomic DNA was extracted from the cecal digesta of broilers by repeated bead beating and column method according to the protocol of Yu and Morrison (2004). Briefly, 0.25 g of cecal digesta was taken into a sterile 2 ml screw-cap tube containing 1 ml Qiagen Cell lysis buffer (Cat No./ID: 158908), and 0.3 g of 0.1 mm and 0.1 g of 0.5 mm pre-weighed zirconia beads. The tube containing a mixture of cecal digesta and beads in cell lysis buffer was homogenized for 3 min at maximum speed on a Mini-Beadbeater™ (BioSpec Products, Bartlesville, OK, USA). After homogenization, the tube was incubated at 70°C for 15 min and was gently shaken every 5 min. Later the tubes were centrifuged 4°C for 5 min at 16,000× g and the supernatant in each tube was transferred to a new 2 ml Eppendorf tube. A 300 µl of fresh cell lysis buffer was added to the lysis tube containing precipitate and the homogenization, centrifugation and collection of supernatant in each tube was repeated as above. Following homogenization and cell lysis, nucleic acid was precipitated using 10 M ammonium acetate and isopropanol. After centrifugation at 4°C for 15 min at 16,000× g, the supernatant was discarded by aspiration and the pellet was gently washed with 70% ethanol. The pellet was dried after removal of ethanol at room temperature for 10 min. The nucleic acid pellet was dissolved in 200 µl of TE buffer. After the dissolution of nucleic acid in TE buffer, removal of RNA and protein was done using the reagent from QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). The quality of the extracted DNA was ascertained by running 2 µl of the aliquot on 1% agarose gel electrophoresis. The staining was done using ethidium bromide and the bands were visualized under UV. The concentration of the DNA in AE buffer was measured on NanoDrop one microvolume UV spectrophotometer (Thermofisher Scientific, NY, US). The

final DNA concentration of all the samples was normalized to 10 ng/µl using nuclease-free water. The normalized samples were store at -20°C and transported on ice to a sequencing core facility at the University of Hawaii at Manoa for Illumina MiSeq sequencing.

2.2.5.2 Amplification of V3 and V4 region of 16S rRNA gene by Next Generation Illumina sequencing

We sequenced V3 and V4 hypervariable regions of the 16S rRNA gene. For 16S library preparation, amplicon PCR was carried out using Platinum® Taq DNA Polymerase High-Fidelity (Invitrogen, Life Technologies Corporation, Grand Island, NY). The specific sequence of 16S rRNA gene was amplified from genomic DNA using forward primer (5′-CCTACGGGNGGCWGCAG-3′) and reverse primer (5′-GACTACHVGGGTATCTAATCC- 3′) according to Illumina MiSeq protocol (Klindworth et al., 2013). The overhang forward (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′) and reverse (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′) adapter sequences were added to 5′ end of the locus-specific primers. The amplicon PCR cycle consisted of initial denaturation step of heating PCR plate at 95°C on a thermal cycler for 3 minutes followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and an extension step at 72°C for 5 minutes, which was then followed by a hold at 4°C. The PCR amplicon was run on 1% agarose gel to ascertain the size of the amplified product to be around 550 bp. The PCR products were then cleaned according to Illumina 16S rRNA PCR Clean-Up protocol with some modifications, where Mag-Bind Total Pure NGS beads (Omega Bio-tek) were used instead of AMPure XP beads and 70% ethanol instead of 80% ethanol. After clean-up of the amplicon PCR product, an index PCR was performed for multiplexing by attaching Nextera XT dual indices and Illumina sequencing adapters to the target amplicon. The PCR conditions used for index PCR were 95°C for 3 minutes; 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C

for 30 seconds; followed by an extension at 72°C for 5 minutes and a final hold at 4°C. The cleaning of the index PCR product was carried similarly as done in the previous clean-up step. After PCR clean-up, the libraries were quantified using the Quant-iT PicoGreen dsDNA Assay Kit, normalized, and pooled. The library pools (0.7 ng) were run on a Bioanalyzer High Sensitivity DNA chip to verify the size of the final library to be around 630 bp. The normalized and pooled amplicons were sequenced on the Illumina MiSeq desktop sequencer (2×300 bp paired-end run) at the University of Hawaii at Manoa Advanced Studies in Genomics, Proteomics and Bioinformatics core facility.

2.2.5.3 Metagenomic sequencing and data analysis

Demultiplexed paired-end R1 and R2 sequencing reads (approximately 300 bp in length) for each sample was generated on the MiSeq platform and downloaded via Illumina BaseSpace® Sequence Hub. The data processing was performed on Quantitative Insights Into Microbial Ecology (QIIME™ version 2.0 release 2019.4; Bolyen et al., 2019). The fastq.gz files for each sample were unzipped and imported using qiime tools import command in Casava 1.8 paired-end demultiplexed fastq format. Denoising, trimming and filtering of phiX reads along with chimeric sequences were done using DADA2 pipeline. The rooted and unrooted tree for phylogenetic diversity analysis was generated using align-to-tree-mafft-fasttree pipeline from qiime phylogeny plugin. Alpha and beta diversity analysis were conducted by applying core-metrics-phylogenetic method from qiime diversity plugin with a sampling depth of 4000 frequency per sample. For alpha diversity, observed OTUs and Shannon Index were determined and for beta diversity, unweighted UniFrac and Bray Curtis metrics were evaluated. For assigning taxonomy, a Naive Bayes classifier pre-trained on the Greengenes 13_8 99% OTUs were applied to the sequences of the present study. We used classify-sklearn command of qiime feature-classifier plugin to

explore the taxonomic composition of the sample. To perform a differential abundance test at the specific taxonomic level, the taxonomy was added to the feature table by collapse method of qiime taxa pipeline. Once the feature table was collapsed at the specific species level, it was then exported to a biom file and later converted to a text file using biom convert. The text file was later used for linear discriminant analysis (LDA) using linear discriminant analysis effect size (LEfSe) tools of Galaxy web application.

2.2.6 Statistical analysis

The growth performance variables (ADG, ADFI and FCR) and cecal SCFA concentration were compared among treatments using MIXED procedure of SAS (SAS Institute Inc., Cary, NC). All the data sets were analyzed for normal distribution by Shapiro-Wilk test using the NORMAL option of the UNIVARIATE procedure and for homogeneity of variances by Levene's test using the HOVTEST option of GLM procedure of SAS. The test model included the main effects of WB and Enzyme along with their interaction. The means were separated by Tukey's method using pdmix macro of SAS and the difference was considered significant at $P \leq 0.05$ and considered a trend at $P < 0.10$. For microbiota data analysis, Kruskal-Wallis pairwise test was used for alpha diversity and pairwise permanova was used for beta diversity in Qiime2.

2.3 Results

2.3.1 Growth performance

The level of WB did not have any significant ($P > 0.05$) effect on ADFI, ADG and average total gain (ATG) throughout the trial period (0-35 d) whereas a trend ($P=0.069$) was observed for FCR (feed: gain) in total period (Table 2.2). A significant interaction ($P < 0.05$) between WB and enzyme supplementation on FCR was registered in the finisher phase (22-35d) and total period. The enzyme supplementation brought improvement in FCR with increasing

level of WB in the diet. Regardless of the level of WB, xylanase supplementation did not affect ($P > 0.05$) ADFI in any phase throughout the trial period (Table 2.2). However, xylanase significantly increased ($P < 0.05$) ADG by 4.2% in the finisher phase and by 4.1% in the total trial period. In the starter phase (0-21 d) xylanase significantly ($P < 0.01$) improved FCR by 2.9% irrespective of the level of wheat bran inclusion. The average daily improvement in the bodyweight of broilers by the xylanase supplementation also resulted in a significantly higher ATG (2025 vs 1943 g, $P < 0.05$) relative to birds not fed xylanase enzyme.

Table 2.2 Effects of enzyme and wheat bran inclusion on growth performance of broilers.

	ADFI (g/day)			ADG (g/day)			FCR			ATG (g)
	0-21 d	22-35 d	0-35 d	0-21 d	22-35 d	0-35 d	0-21 d	22-35 d	0-35 d	0-35 d
Main effects of Wheat bran (WB)										
WB 0%	52.3	138.8	86.9	40	81.9	56.7	1.31	1.69	1.53	1983
WB 5%	53	142.8	88.9	39.8	83	57	1.33	1.72	1.56	1994
WB 10%	52.5	143.9	89.1	39	82.5	56.4	1.35	1.74	1.58	1974
SEM[1] (n=10)	0.92	2.35	1.33	0.61	1.42	0.74	0.014	0.02	0.013	25.6
Main effects of Enzymes										
No xylanase	52.7	142.6	88.7	39	80.8^{b}	55.5^{b}	1.35^{a}	1.77^{a}	1.60^{a}	1943^{b}
Xylanase	52.6	141	88	40.2	84.2^{a}	57.8^{a}	1.31^{b}	1.68^{b}	1.52^{b}	2025^{a}
SEM[2] (n=15)	0.75	1.92	1.08	0.5	1.16	0.6	0.012	0.016	0.01	20.9
Treatments[4]										
No Xylanase										
WB 0%	51.8	135	85.1	39	80	55	1.33	1.69^{abc}	1.54^{bc}	1928
WB 5%	53.4	144.6	89.9	39.3	80.7	55.6	1.36	1.79^{ab}	1.62^{ab}	1946
WB 10%	52.8	148.3	91	38.6	81.9	55.9	1.37	1.81^{a}	1.63^{a}	1956
Xylanase										
WB 0%	52.8	142.5	88.7	41	84.1	58.3	1.29	1.70^{abc}	1.52^{c}	2039
WB 5%	52.6	141	88	40.4	85.3	58.4	1.3	1.65^{c}	1.51^{c}	2042
WB 10%	52.2	139.5	87.2	39.4	83.2	56.9	1.33	1.68^{bc}	1.53^{c}	1993
SEM[3](n=5)	1.3	3.32	1.88	0.87	2.01	1.04	0.02	0.028	0.018	36.2
Sources of variation, P-value										
WB	0.899	0.261	0.412	0.637	0.901	0.843	0.156	0.241	0.069	0.861
Enzyme	1	0.529	0.607	0.087	0.049	0.009	0.01	0.001	<.001	0.011
WB×Enzyme	0.799	0.062	0.126	0.637	0.641	0.603	0.937	0.02	0.037	0.567

[a-c] Within columns in each respective group of factors (the main effect of wheat bran, the main effect of enzymes and [4]treatments with interaction effects), means without a common superscript differ ($P < 0.05$). [1]Pooled SEM (n= 10 replicate cages containing 6 birds per cage). [2]Pooled SEM (n= 15 replicate cages containing 6 birds per cage).[3]Pooled SEM (n= 5 replicate cages containing 6 birds per cage). ADFI: average daily feed intake, ADG: average daily gain, FCR: feed conversion ratio, ATG: average total gain.

2.3.2 Cecal short chain fatty acid production

Wheat bran did not affect major SCFA production and it did not display interaction with xylanase ($P > 0.05$), although it was associated with a decrease ($P < 0.05$) in the production of valerate (Table 2.3). The groups fed supplemental xylanase produced significantly ($P < 0.01$) higher amount of straight SCFA including acetate, propionate, butyrate and total SCFA than those receiving feed without xylanase. A trend ($P = 0.063$) associated with xylanase was observed for valerate production. The ratio of acetate was observed slightly higher to that of propionate and butyrate in high WB incorporated groups in comparison to the corn-soybean group. Moreover, the xylanase fed groups had a lower ratio of acetate and a higher ratio of propionate and butyrate to that of xylanase un-supplemented group.

Table 2.3 Effects of enzyme and wheat bran inclusion on cecal SCFA (µmol/g wet digesta) production in broilers at d 35 post-hatch.

Main effects of Wheat bran (WB)	Acetate	Propionate	Butyrate	Valerate	Isobutyrate	Isovalerate	Straight chain	Branched chain	**Total**
WB 0%	55.83	6.99	8.54	0.80[a]	0.85	0.7	72.16	1.54	**73.7**
WB 5%	59.4	7.03	9.08	0.67[ab]	0.85	0.62	76.17	1.47	**77.64**
WB 10%	56.38	6.98	8.28	0.53[b]	0.87	0.74	72.17	1.62	**73.79**
SEM[1](n=10)	3.105	0.754	0.68	0.08	0.058	0.718	4.276	0.727	**4.229**
Main effects of Enzymes									
No xylanase	48.59[b]	5.76[b]	7.01[b]	0.57	0.85	0.67	61.93[b]	1.52	**63.45[b]**
Xylanase	65.81[a]	8.23[a]	10.26[a]	0.77	0.87	0.7	85.07[a]	1.57	**86.64[a]**
SEM[2](n=15)	2.535	0.615	0.555	0.066	0.047	0.587	3.491	0.594	**3.453**
Treatments[4]									
No Xylanase									
WB 0%	43.67	5.6	6.64	0.64	0.84	0.72	56.56	1.56	**58.12**
WB 5%	54.42	6.18	7.89	0.62	0.84	0.58	69.11	1.42	**70.53**
WB 10%	47.68	5.51	6.5	0.44	0.86	0.72	60.13	1.57	**61.7**
Xylanase									
WB 0%	67.98	8.37	10.44	0.97	0.86	0.67	87.76	1.53	**89.29**
WB 5%	64.37	7.87	10.27	0.72	0.85	0.67	83.23	1.52	**84.75**
WB 10%	65.09	8.46	10.07	0.61	0.89	0.76	84.22	1.66	**85.88**
SEM[3](n=5)	4.391	1.066	0.962	0.114	0.082	1.016	6.047	1.028	**5.981**
Sources of variation, P-value									
WB	0.689	0.991	0.865	**0.045**	0.974	0.64	0.772	0.618	0.775

Enzyme	**<0.001**	0.007	**<0.001**	0.063	0.927	0.182	**<0.001**	0.176	**<0.001**
WB×Enzyme	0.379	0.793	0.926	0.697	0.984	0.63	0.491	0.63	0.489

[a-b] Within columns in each respective group of factors (the main effect of wheat bran, the main effect of enzymes and treatments[4] with interaction effects), means without a common superscript differ (P < 0.05). [1]Pooled SEM (n= 10 replicate cages containing 6 birds per cage). [2]Pooled SEM (n= 15 replicate cages containing 6 birds per cage).[3]Pooled SEM (n= 5 replicate cages containing 6 birds per cage).

2.3.3 Effect on cecal microbiota diversity

Compared with 0% WB without xylanase, 0% WB with supplemental xylanase exhibited a trend (P =0.08) for a higher number of observed operational taxonomic unit (OTU) in cecal contents of 35 d old broilers (Figure 2.1). The other groups containing higher WB inclusion with or without enzymes were not significantly different from 0% WB with supplemental xylanase. However, the Shannon Index remained unchanged among the treatment groups (P >0.05).

For beta diversity comparison of cecal bacteria, a trend (p =0.054) was found for unweighted UniFrac between xylanase and no xylanase group (Figure 2.2, Table 2.4). Bray Curtis metrics of beta diversity displayed a significant difference (P <0.05) between cecal microbiota in 0% WB and 10% WB + xylanase groups. A trend was also observed for Bray Curtis metrics of beta diversity between 0% WB and other groups containing added levels of WB (P <0.01). Moreover, by conducting linear discriminant analysis (LDA), we observed that some enriched cecal bacteria from phylum Firmicutes and Bacteroidetes were differentially abundant (P <0.05) in the groups fed supplemental xylanase compared with those fed without xylanase (Figure 2.3). *Lactobacillus salivarius* of family *Lactobacillaceae* was also significantly enriched in xylanase supplemented groups in comparison with groups fed without xylanase.

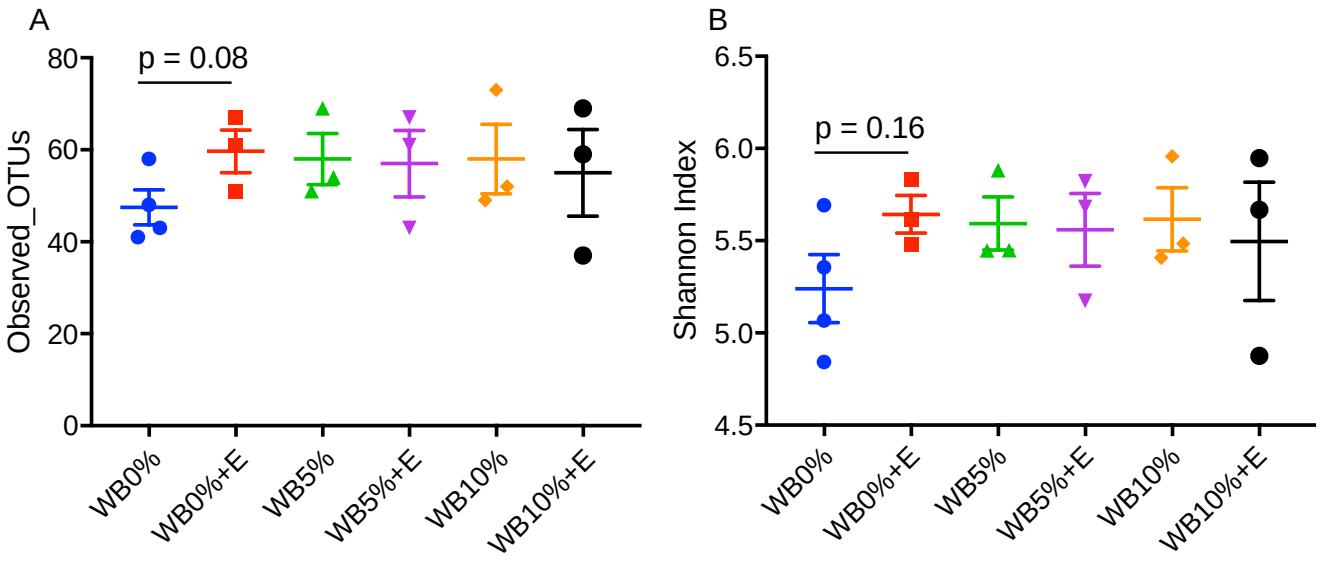

Figure 2.1 Alpha diversity analysis of different treatments at 4000 reads depth per sample of cecal contents from 35 d broilers. A) Observed OTUs B) Shannon Index. Data represent mean ± SE. Statistical analyses were performed using Kruskal-Wallis test. Only the lowest p-value is shown. WB, wheat bran; E, xylanase enzyme.

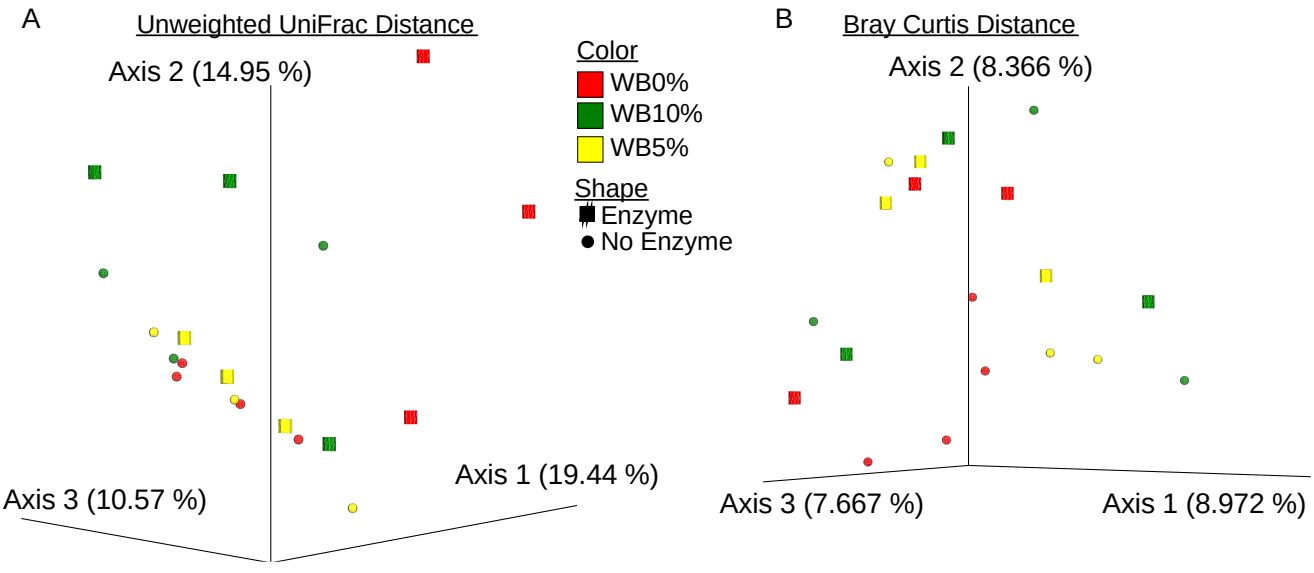

Figure 2.2 Principal coordinate analysis (PCoA) plot shows beta diversity analysis between different treatments at 4000 reads depth per sample of cecal contents from 35 d broilers. A) Unweighted UniFrac distance B) Bray Curtis distance. Statistical analyses are shown in Table 2.4. WB, wheat bran; Enzyme = xylanase.

Table 2. 4 Statistical analysis for beta diversity analysis of cecal contents from 35 d broilers (refer to Figure 2.2).

		Unweighted UniFrac	Bray Curtis
Group1	Group2	p-value	p-value
Enzyme	No Enzyme	0.054*	0.413
WB0%	WB0%+E	0.079*	0.123
WB0%	WB5%	0.636	0.069*
WB0%	WB5%+E	0.339	0.095*
WB0%	WB10%	0.451	0.059*
WB0%	WB10%+E	0.305	0.031**

Statistical analyses were performed using pairwise permanova. *: $p < 0.1$ and **: $p < 0.05$. None of the other comparisons between treatment groups were statistically significant. WB, wheat bran; Enzyme = xylanase.

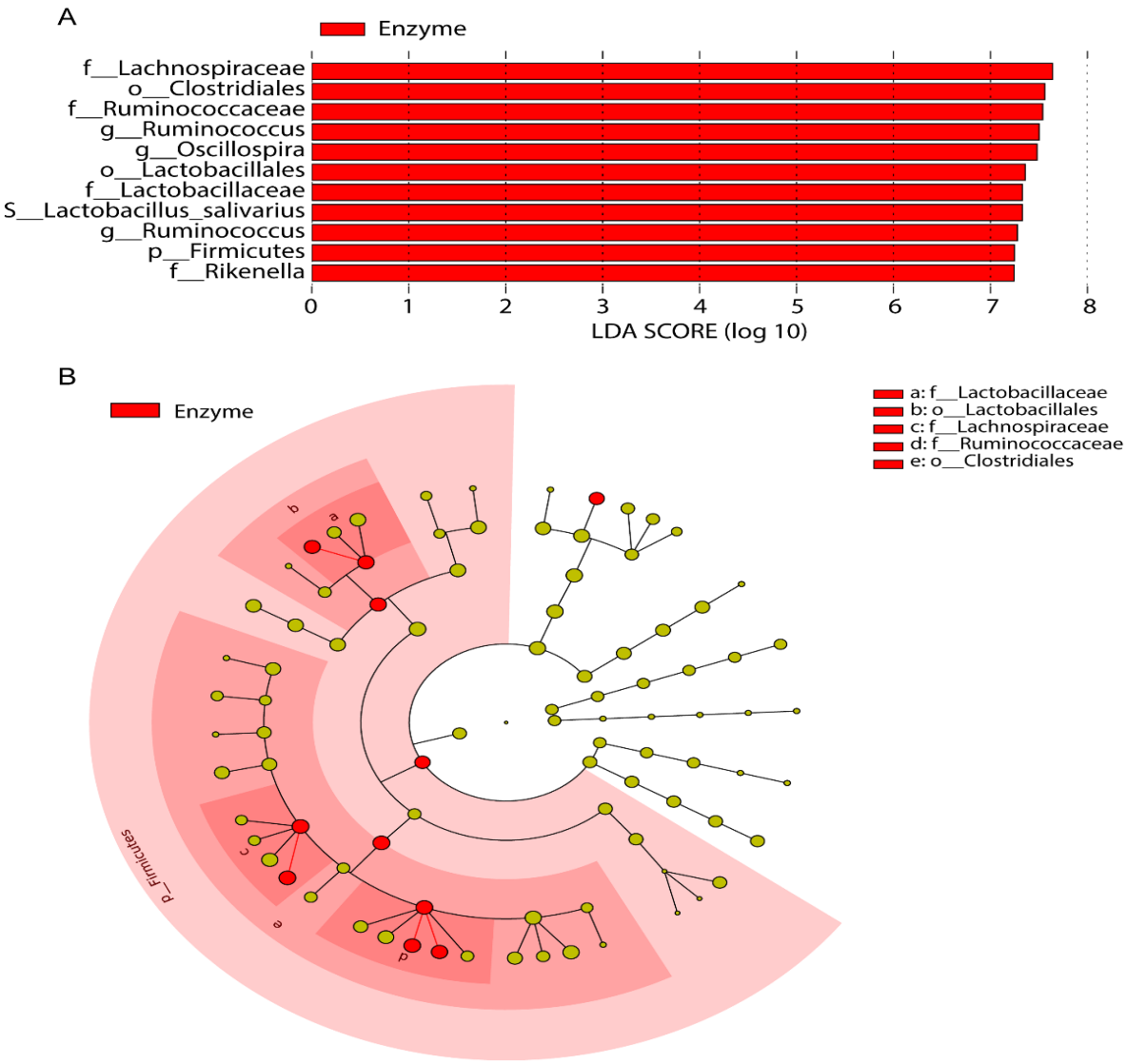

Figure 2.3 A) Histogram shows linear discriminant analysis (LDA) scores of taxa differentially abundant and B) Cladogram shows differentially abundant taxa (highlighted by small circles and by shading) at the various taxonomic level between cecal microbiota of 35 d broilers treated without or with xylanase enzyme. Statistical analyses were performed using linear discriminant analysis effect size (LEfSe). To note, none of the taxa were significantly enriched in chickens that did not receive enzyme treatment.

2.4 Discussion

Exogenous xylanase can improve ileal digestibility by breaking NSP into simpler forms that can either be absorbed directly by the host or it can easily be digested by endogenous

enzymes. If NSP fragments broken down by xylanase, like non-digestible oligosaccharides or some simpler carbohydrates that escape upper GIT, can still provide a suitable substrate for fermentation. The difference in the action of exogenous enzymes on the enhancement of digestion and fermentation can still be expected due to the variation in duration and transit rate of digesta in various segment of broilers GIT (Almirall et al., 1994). The improvement in weight gain and feed efficiency due to xylanase inclusion is in agreement with that reported by Selle et al. (2003a) where they observed increment in body weight gain and improvement in FCR in 6 weeks broilers fed wheat-based diet supplemented with xylanase and phytase. But in the present study, the improvement in FCR was more pronounced in high WB fed groups. In this study, we did not observe a major difference in growth performance in response to varying level of WB, but a trend noticed in FCR in total period indicates a better utilization of corn-based diet by the broilers. Romero et al. (2014) observed the better performance of the birds fed a corn-based diet in comparison with those fed wheat-based diets. This observation cannot be appropriately compared with the present study because the inclusion of wheat in the previous study was in the range of 54 to 65 % while in the present study WB was included only up to 15%. Selle et al. (2003a) found that xylanase along with phytase did not improve bird's performance in finisher phase and this might be due to the vulnerability of younger birds to the antinutrient properties of NSP and phytate when wheat was used in the range of 57 to 64%. In the present study, in both starter and finisher phase, the ADFI was not different across the treatments or WB level compared with that observed in the previous study. This suggests that WB was well tolerated in both phases at this level of inclusion. However, the improvement in the growth performance in xylanase group would have come from the better utilization of the nutrients by the birds owing to their increased ileal digestibility of nitrogen and energy (Cowieson and Masey O'neill, 2013;

Selle et al., 2003a). Similarly, Peng et al. (2003) observed an improvement of 7.3% in the feed efficiency of broilers fed a wheat-based diet with xylanase and phytase supplementation. Likewise, Zyla et al. (1999) found a noticeable improvement in body weight gain (23.2%) and in feed efficiency (16.7%) of broilers fed phosphorus deficient wheat-based diet supplemented with xylanase and phytase. The combination of these enzymes is also reported to have reduced the feed: gain ratio in broilers fed wheat-based diet by 5.4% compared with those receiving un-supplemented basal diet (Wu et al., 2004a). In the same study, it was found that xylanase and phytase combination increased ileal villus height and decreased ileal goblet cell number which supports the possible improvements in absorption and reduction in endogenous nitrogen loss via mucus. This suggests that improvement in growth performance in broilers fed less viscous diet like 0% WB in this study is also feasible because this enzyme combination has potential to generate improvement through other modes of action beyond viscosity reduction.

The improvement in the performance of birds in the present study is consistent with the previous findings (Annison and Choct, 1991; Bedford and Classen, 1992; Steenfeldt and Pettersson, 2001) where birds were also fed wheat-based diet supplemented with xylanase. The feed supplementation of xylanase has been reported to increase body weight gain of broilers fed wheat-based diet (Enerberg et al., 2004) as well as improve feed efficiency of those fed either ground or whole wheat (Wu and Ravindran, 2004). Increase in body weight gain and improvement in feed efficiency in broilers was also observed in several other studies (Gao et al., 2007; Selle et al., 2003a; Wang et al., 2005; Yang et al., 2008). Xylanase supplementation is also very effective in improving FCR of broilers fed energy-deficient diet till marketable age (Cowieson et al., 2010; Williams et al., 2014). In the present study, xylanase addition improved FCR more in high WB groups, which explains that there is some fundamental variation in

response of broilers to xylanase supplementation in corn and wheat-based diet. Luo et al. (2009) did not find any significant effect of xylanase on ADFI in either starter or finisher phase in broilers fed wheat-based diet while FCR was significantly improved in starter phase and total trial period of 1-42 days. In the present study, a similar effect on ADFI was observed while a better FCR was recorded in high WB group compared to a basal corn-based diet which agrees with the above study that xylanase improves feed efficiency in wheat fed broilers. In corn DDGS fed broilers, Liu et al. (2011) found that xylanase increased feed intake without affecting FCR, but it exhibited interaction with corn DDGS on body weight gain by generating greater improvement in high fiber corn DDGS group. In the present study, the higher improvement was observed in FCR of high WB diet and no effect was found on feed intake in response to xylanase supplementation. Also, improvement by xylanase in FCR of broilers fed sorghum distillers' dried grains with solubles during starter phase has been reported but without significant effect on feed intake or body weight (Barekatain et al., 2013). Like the present study, Cowieson and Massey O'Neill (2013) also found that xylanase improved FCR in broilers fed wheat-based diet without any effect on feed intake. The body weight gain was significantly affected by xylanase until 28 days while a trend persisted thereafter. Similarly, Zhang et al. (2014) observed that xylanase supplementation of wheat-based diet in broilers did not affect feed intake but increased body weight gain and improved FCR in a 21d study. Amerah et al. (2017) also found that xylanase addition in corn/soy-based diet of broilers improved FCR in a 6 weeks study without any effect on the feed intake. Such differences in feed intake and utilization due to xylanase among several studies indicate a range of physio-chemical actions of xylanase that can bring variable outcome depending on its type and composition of feed. Peptide YY hormone which acts as 'ileal brake' and slows down digesta transit in the intestine (Taylor, 1993) has been reported to increase by

xylanase in broilers (Singh et al., 2012) and it might be one of the underlying mechanisms in improving feed utilization in the present study.

Xylanase has been shown to improve FCR in broilers receiving an energy-deficient diet containing equivalent or low fat compared to a positive control (Masey O'Neill et al., 2012). This suggests that the slight difference in the crude fat content of the diet of the present study did not make any significant difference in the effect of xylanase supplementation. Masey O'Neill et al. (2012) did not find any difference in feed intake by supplemental xylanase while FCR was improved during 0-21 d and 0-35 d. Similarly, the improvement in weight gain and feed efficiency without influence on feed intake was observed in the present study and the same scenario has been reported in previous studies (Cowieson et al., 2010; Zanella et al., 1999). Xylanase can improve performance of broilers by reducing viscosity induced by soluble NSP, especially by the pentosans (Bedford et al., 1991; Kiarie et al., 2014). NSP like wheat pentosans can also exert its anti-nutrient activity by causing endogenous loss of protein (Angkanaporn et al., 1994) and by chelating ions or bound molecules due to its surface activity (Smits and Annison, 1996). Xylanase can improve AME and total tract amino acid digestibility in broilers fed wheat-based diet (Hew et al., 1998) and produce additional improvement in AME, viscosity reduction and apparent ileal digestibility of amino acids when used in combination with phytase (Selle et al., 2009; Wu et al., 2004a).

There was no major improvement in SCFA production by xylanase with increasing level of WB that may be due to the limited capability of the enzyme in hydrolyzing all-fiber polymers and especially the branched fraction (Veldman and Vahl, 1994). A marginal shift from acetate to butyrate production is noticed in xylanase fed groups compared with no xylanase group, the pattern points out to the increase in the population of anaerobic bacteria in the xylanase group

(Vahjen et al., 1998). Cecal SCFA concentration rises in response to enzyme supplementation due to an increase in fermentation (Choct et al., 1996, 1999). The improvement in the SCFA production by xylanase is in agreement with the study of Massey O'Neill et al. (2014) where cecal acetate, butyrate and total SCFA content were increased in broilers fed either corn or wheat-based diets. In birds fed corn and wheat-based diets, Kiarie et al. (2014) observed an increase in cecal acetate production due to xylanase addition but propionate and butyrate were not affected. The cecal acetate production, on the other hand, was not influenced by xylanase in birds fed sorghum DDGS but butyrate production was increased (Barekatain et al., 2013). In contrast, no effect of xylanase on cecal SCFA other than lactic acid was observed in broilers fed either triticale or rye or wheat-based diets (J´ozefiak et al., 2007). The concentration of SCFA in ceca depends on the type of cereals and enzymes in the feed (J´ozefiak et al., 2007; Silva and Smithard, 2002), and the composition and activity of the resident microbiota (Engberg et al., 2002). Thus, variability in SCFA production due to xylanase addition to a variety of feed formulation can be expected among different studies. The present study shows that WB is well tolerated by both young and finisher age broilers, but further improvement is feasible by supplementation of xylanase. Besides increasing ADG, xylanase interacted with WB for FCR and produced more improvement in high WB groups, whereas it improved SCFA production irrespective of diet type. This difference in the effect of xylanase in feed efficiency and SCFA production of broilers fed corn-based and WB added diet indicates variability in its action in diets containing high and low soluble NSP. The better performance in WB group can be ascribed to the reduction in viscosity by xylanase while enhancement in cecal SCFA production in both diet type can be attributed to the capacity of xylanase to break both soluble and insoluble pentosans into fermentable oligosaccharides.

The number of OTUs tends to be higher in 0% WB supplemented with xylanase but it is not sufficient to produce significant dissimilarity in Shannon Index of alpha diversity. It suggests that there was an increase in the abundance of total cecal bacteria in other treatments compared to control (0% WB), while the species richness and evenness among treatment groups was not much different. The occurrence of trend and significance in beta diversity between the groups supplemented without and with xylanase is indicative of presence selective enrichment of some bacterial species by addition of xylanase in the feed. It is also interesting to note that the addition of WB also tends to influence the species diversity in the cecal contents of broilers. Among those enriched cecal bacteria in xylanase supplemented groups, the *Lactobacillus salivarius* of family *Lactobacillaceae* is of primary importance for its perceived role as a probiotic species. The increase in abundance of phylum Firmicutes in this study resonates well with that observed by Zhang et al. (2018) in the cecum of pigs fed either corn or wheat-based diet. Xylanase has a potential to increase the *Lactobacillus sp.* count in the cecum of chicken and it can produce a complementary effect with *Lactobacillus sp.* for reducing pathogenic strains while improving broiler performance (Nian et al., 2011; Vandeplas et al., 2009).

2.5 Conclusion

This study reveals that xylanase can cause improvement in broiler performance in addition to the benefit obtained from phytase enzyme. The improvements can be attained in both corn and wheat-based diet, although there is more improvement in the wheat-based diet by addition of xylanase. These improvements can be due to the reduction in viscosity and improvement in nutrient availability as reported in previous studies and some part of the benefit can also be attributed to the enhanced production of cecal short chain fatty acids and enhancement in the abundance of beneficial microbiota.

CHAPTER 3: EFFECTS OF XYLANASE ENZYME AND PREBIOTICS SUPPLEMENTATION IN DIETS ON GROWTH PERFORMANCE AND INTESTINAL HEALTH PARAMETERS OF BROILERS

Abstract

The objective of this study was to evaluate the effects of xylanase and xylooligosaccharides (XOS) supplementation in the corn-soybean meal (SBM) based diet on growth performance, cecal short chain fatty acid (SCFA) production and ileal morphology of broilers. A total of 288 day-old chicks (Cobb 500) were equally distributed to 36 pens (8 birds/pen) and the pens were randomly allocated to 9 treatments (n=4) setup in 3×3 factorial arrangement. The treatments included 9 diets: combination of 3 levels of xylanase (0%, 0.005% and 0.01% Econase XT) and 3 levels of prebiotics (0%, 0.005% and 0.01% XOS). All the diets contained same basal composition and were formulated in three phases (starter, d 0-14; grower, d 15-28; finisher, d 29-42). The birds were raised for 42 d under standard husbandry practices and were fed mash feed *ad libitum*. The feed intake and body weight were recorded weekly to determine feed conversion ratio and growth performance. Ileal samples were collected on d 42 for histomorphometric and gene expression analysis. Simultaneously, the cecal digesta were collected for DNA extraction and genomic sequencing, and SCFA determination. Xylanase supplementation significantly ($P < 0.05$) increased the average daily gain (ADG) in the finisher and total period and increased ($P < 0.01$) average total body weight (ATW, 2940 & 2932 vs 2760 g) of broilers, while XOS did not have a significant effect on either ADG or ATW. The effect of 0.005% xylanase on ADG was neither different from 0.01% xylanase nor than that of control. Neither xylanase nor XOS affected average daily feed intake (ADFI) and feed conversion ratio (FCR, $P > 0.05$). Ileal morphology of villus height and crypt depth was not influenced by xylanase and XOS ($P > 0.05$). However, xylanase had a trend ($P = 0.097$) on villus height to crypt depth ratio. The production of acetate was increased by xylanase ($P < 0.01$) and by XOS ($P < 0.05$), but their additive effect was not evident. Xylanase also increased the production of total

SCFA ($P < 0.01$) while XOS exhibited a trend ($P = 0.052$). The treatment containing 0.01% XOS without xylanase also had a higher level of IL-10 (a marker of anti-inflammatory cytokine) and IL-4 (a T-cell differentiation cytokine) genes compared to control ($P < 0.05$). No difference was found in alpha or beta diversity of cecal microbiota among treatments when 16S rRNA amplicon sequencing was performed on cecal contents at d 42 ($P > 0.05$). The results infer that xylanase can improve the growth performance and cecal fermentation in broilers when supplemented in corn-SBM diets along with XOS, but the effects may not always translate into improved feed efficiency or better mucosal absorptive capacity.

Keywords: broilers, growth performance, short chain fatty acids, xylanase, xylooligosaccharides

3.1 Introduction

Several studies report poor growth performance of broiler fed fibrous diets, primarily due to low digestibility of nutrients. However, the use of exogenous enzymes has been shown to enhance the utilization of nutrients, thereby improving the growth performance of chicken. Nevertheless, different enzymes differ in exerting their response depending on the ingredients used in broiler diet. Xylanase enzyme has become one of the most common exogenous enzymes used in wheat-based broiler feed and it is also being included in the corn-based feed to improve NSP degradation in the GIT of broilers. Xylanase enzyme has been reported to increase butyrate production, reduce enterobacteria and gram-positive cocci, and increase *Lactobacillus spp.* counts in the intestinal tissues of broilers (Vahjen et al., 1998). The supplementation of xylanase in the wheat-based diet has also been found to increase the number of goblet cells in the duodenum and decrease crypt depth in the jejunum of broilers (Wu et al., 2004a).

The improvement in the growth performance of broilers is dependent on enhanced digestibility and increased nutrients utilization, which is in turn, relies on good gut health and sound immunity. Enhanced nutrient utilization is ensured by increased degradation of NSP, improved access of digestive enzymes to nutrients in feed matrix and increased absorptive surface area in the intestine of the host. Exogenous enzymes like xylanase also improve absorption of nutrients by reducing the viscosity of intestinal digesta and concurrently provide easily fermentable substrates for the resident gut microbiota. In addition to the effect of xylanase enzyme, prebiotics is also known to modulate and support the growth of beneficial microbiota in the intestine of broiler chicken, which in turn are found to affect growth performance and health of the chicken. Along with the enhancement of colonization of GIT by beneficial microbiota, prebiotics can also stimulate immunity which can either be a direct effect or through the production of fermentation metabolites.

Increased production of fermentation metabolites like short chain fatty acid (SCFA) can also improve gut health by supporting the growth of intestinal villi. Xylooligosaccharides (XOS) are also being studied as a source of potential prebiotics and when added in broiler feed, it stimulates butyrate-producing bacteria by cross-feeding of lactate and thus provides butyrate for intestinal mucosa of broilers (De Maesschalck et al., 2015). XOS has also been found to improve growth performance, enhance endocrine metabolism and improve immune response by maintaining higher antibody titers (Zhenping et al., 2013).

However, more studies are required to define the role of xylanase and XOS in modulating cecal microbiota, enhancing fermentation metabolites and improving immune function in broilers. Thus, it is important to evaluate the interaction effect of xylanase and XOS on broiler growth and gut health before it can be included in a regular feeding program. The objective of this study was to investigate the effect of feeding different levels of xylanase enzyme and XOS prebiotics on growth performance, cecal fermentation characteristics, immune response and cecal microbiota diversity of broiler chicken.

3.2 Materials and methods

3.2.1 Experimental design and dietary treatments

A growth performance study was conducted at the Small Animal Facility (SAF) of the University of Hawaii at Manoa. This study was conducted after approval of the Institutional Animal Care and Use Committee and following the Research Policy of UH.

Corn and soybean meal-based 9 diets combination containing same basal composition (Table 3.1) were formulated in three phases: Stater (d 0-14), grower (d 15-28) and finisher (d 29-42), to meet or exceed the nutrients requirements of broilers (NRC, 1994). The diets were formulated to have 3 levels of xylanase enzyme and three levels of xylooligosaccharides

prebiotic supplemented in a completely randomized design in 3 × 3 factorial arrangement as shown in the following schematic diagram. Also, all the diets were supplemented with phytase enzyme (500 FTU/kg of Quantum blue; AB Vista Feed Ingredients, Marlborough, Wiltshire, UK), with a matrix value of 0.16% Ca and 0.15% P. All the experimental diets were fed in mass form.

Econase XT0			Econase XT8			Econase XT 16		
Prebiotic0	Prebiotic50	Prebiotic100	Prebiotic0	Prebiotic50	Prebiotic100	Prebiotic0	Prebiotic50	Prebiotic100

*Econase XT0- no enzyme; Econase XT8- Econase XT 8,000 BXU/kg (50g/MT); Econase XT16- Econase XT 16,000 BXU/kg (100g/ MT), Prebiotic0- No prebiotic; Prebiotic50- Prebiotic 50g/ MT; Prebiotic100- Prebiotic 100g/ MT.

A total of 292 day-old unsexed broiler (Cobb 500) chicks were used in this study. Birds were raised in a group in floor-pen and were provided standard commercial broiler rearing environment (temperature, humidity, light and built up litters). Four birds out of this flock were removed on d 0 for benchmark sample collection. The remaining 288 day-old chicks were weighed individually, wing tagged and placed randomly in one of 36 pens (8 birds per pen), making 4 replicates of each treatment. Each pen birds were fed with one of 9 diets where diets were the treatment and pens were the experimental units.

All the birds had *ad libitum* access to feed and water. Feed consumption and the bodyweight of the birds were measured on d 0, 7, 14, 21, 28, 35, and 42. Any leftover feed in the feeder was weighed and recorded weekly to adjust the feed intake. The data generated were used to calculate average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR). The mortality of birds was also noted and FCR was adjusted for mortality.

After weighing birds on d 42, two birds from each pen (8 birds per treatment at each time point) were euthanized by CO_2 inhalation. The digesta from both ceca of each bird was collected and pooled separately with gentle milking and transferred to -20°C freezer until further analysis

for short chain fatty acids (SCFA). Also, on d 42 cecal contents of broilers from each pen were collected for DNA extraction, which was snap-frozen and kept at -80°C until further analysis of microbiota. Moreover, on d 42, a section of ilium, approximately 1 cm in length (between 1 cm distal to the Meckel's diverticulum and 1 cm anterior to the ileocecal junction) was collected from all euthanized birds, flushed with physiological saline, fixed in 10% buffered formalin and stored for further histological analysis.

3.2.2 Proximate and chromatographic analysis

All feed samples were analyzed for their nutrient profile: DM, GE, N (for CP), fat, ash, ADF, NDF using standard procedures of Association of Official Agricultural Chemists (AOAC, 2006; Table 3.1). The cecal digesta was analyzed for complete short chain fatty acids (SCFA) profile using gas chromatography as described by Jha et al. (2010) and Zhao et al. (2006). For making calibration curves with external standards, different concentration levels of standard stock volatile free acid mix were prepared ranging from 0 to 8 mM. A final volume of 1.5 ml was made with 100 µl of 25% metaphosphoric acid (MPA), and 100 µl of 48.95 mM trimethylacetic acid (TMA, Pivalic acid from Sigma-Aldrich, St. Louis, MO) in deionized water.

For sample preparation, 1 g of cecal digesta was mixed with 1 ml of distilled water in a centrifuge tube and mixed by vortexing. The mixture was then centrifuged at 10, 000 ×g for 5 minutes at 4°C. After centrifugation, a 400 µl of the supernatant was pipetted into a new 1.8 ml microcentrifuge tube. Next, 100 µl of 25% MPA and 100 µl of 48.95 mM of TMA was added to the microcentrifuge and the final volume was made 1.5 ml by adding distilled water. The solution was again mixed by vortexing and then centrifuged at 13,000 ×g for 10 minutes at 4°C. Following centrifugation, approximately 1000 µl of supernatant was transferred to a GC vial

sealed using septa and screw cap and was then loaded on the carousel of autosampler for injection.

Table 3.1 Ingredient composition and nutrient content of xylanase and xylooligosaccharides diet fed to broilers in different phases (as-fed basis; % unless otherwise indicated).

Ingredients, %	**Inclusion level***		
	Starter (d 0-14)	Grower (d 15-28)	Finisher (d 29-42)
Corn	57.27	61.37	64.15
SBM	36	32	29
Soybean oil	2.8	3.1	3.7
Limestone	1.4	1.4	1.3
Monocalcium phosphate	0.9	0.65	0.5
Lysine	0.35	0.3	0.25
Met	0.25	0.19	0.14
Thr	0.10	0.10	0.10
NaCl	0.30	0.28	0.25
Sodium bicarbonate	0.12	0.1	0.1
Vitamin mineral mix[1]	0.50	0.50	0.50
Phytase	0.01	0.01	0.01
Calculated content, %			
ME, kcal/kg	2988	3064	3148
CP	20.9	19.2	17.9
NDF	9.0	8.9	8.8
CF	3.8	3.6	3.4
dig Lys	1.3	1.1	1.0
digMet	0.54	0.47	0.41
dig Thr	0.71	0.66	0.63
Thr	0.88	0.83	0.78
Trp	0.30	0.27	0.25
Meth+Cysteine	0.98	0.89	0.82
Arg	1.50	1.38	1.29
Val	1.15	1.07	1.02
Ile	0.87	0.81	0.75
Leu	1.79	1.70	1.62
Ca	0.82	0.76	0.69
Total P	0.58	0.52	0.47
npP	0.32	0.27	0.23
Na	0.17	0.15	0.14
Cl	0.22	0.21	0.19

Choline (mg/kg)	1338	1254	1190
Analyzed composition, % as is			
DM	87.8	87.6	88.2
Gross energy, kcal/kg	3787	3848	3916
Crude protein	22.8	21.1	19.3
Crude fat	3.1	3.8	5.3
Crude fiber	2.3	2.2	2.2
ADF	3.4	3.3	4.0
NDF	7.7	7.8	8.6
Ash	5.3	4.8	4.7

[1]Providing the following (per kg of diet): vitamin A (trans-retinyl acetate), 10,000 IU; vitamin D_3 (cholecalciferol), 3,000 IU; vitamin E (all-*rac*-tocopherol-acetate), 30 mg; vitamin B_1, 2 mg; vitamin B_2, 8 mg; vitamin B_6, 4 mg; vitamin B_{12} (cyanocobalamin), 0.025 mg; vitamin K_3 (bisulphatemenadione complex), 3mg; choline (choline chloride), 250 mg; nicotinic acid, 60 mg; pantothenic acid (D-calcium pantothenate), 15 mg; folic acid, 1.5 mg; betaíne anhydrous, 80 mg; D-biotin, 0.15 mg; zinc (ZnO), 80 mg; manganese (MnO), 70 mg iron ($FeCO_3$), 60 mg; copper ($CuSO_4 \cdot 5H_2O$), 8 mg; iodine (KI), 2 mg; selenium (Na_2SeO_3), 0.2 mg.

*All three-phase basal diet were top-dressed with the combination of of 3 levels of xylanase (0 BXU/kg, 8000 BXU/kg and 16000 BXU/kg) and 3 levels of xylooligosaccharides (0 g/ton, 50 g/ton and 100 g/ton) to yield 9 treatments.

3.2.3 Ileal mucosal histomorphometry

Ileal samples from d 42 (n=4/treatment) were transferred from 10% buffered formalin to 70% ethanol after fixation and send to histology core facility at John A. Burns School of Medicine for embedding and staining with Hematoxylin and Eosin (H&E). Each set of samples were cut into 6 sections of 5 μm on a microscopic slide for fixation, staining and mounting of the cover slip. The slides were then observed under 8X objective of an upright microscope (Olympus BX43, Olympus Co, Tokyo, Japan) for 3 sets of villus and crypts from each of 6 sections on every replicate slide. The height of villi and depth of crypts were recorded using image processing and analysis system of Infinity Analyze software (Lumenera Corporation, Ottawa,

ON, CA). The villus height and crypt depth were also used to calculate their ratio and average reading for each treatment was presented along with their standard error.

3.2.4 RNA extraction, reverse transcription, and real-time quantitative PCR

3.2.4.1 Ileal total RNA isolation

Ileal tissue from d 42 stored in cryovial at -80°C were transferred to dry ice for handling tissue for RNA extraction. Total RNA from ileal tissue of broilers were isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. The RNA isolation protocol included the following sequential steps: ileal tissue homogenization and lysis, RNA extraction using chloroform, triple-phase separation, RNA precipitation, RNA pellet washing, RNA pellet drying and resuspension in nuclease-free water. First, 9-11 Zirconia beads (BioSpec Products, Bartlesville, OK, USA) of 2.3 mm were taken in a safe lock nuclease free Eppendorf tube. A 300 µl of TRIzol® reagent was then added to the tube containing beads. A frozen ileal tissue (50-100 mg) was then dropped in the tube to be immersed in the TRIzol®. The tissue was then homogenized by bead beating on a vertical Bullet Blender® (Next Advance, Inc. Troy, NY) at speed 8 for 2-3 minutes. The microcentrifuge tubes containing homogenate and beads were then centrifuged at 10,000 rpm for 1 minute at 4°C. Approximately 250 µl of the supernatant was then transferred to a new microcentrifuge tube containing 750 µl of TRIzol®. The tube containing supernatant from homogenate and fresh TRIzol® was mixed gently and allowed to stand for 5 minutes at room temperature. Then, 0.2 ml chloroform per 1 ml of TRIzol® was added to the homogenate supernatant, vortexed briefly and allowed to incubate for 5 minutes at room temperature. The resultant mix was then centrifuged at 10,000 rpm for 15 minutes at 4°C. Following centrifugation, the solution distinctly separated into three layers: an upper aqueous containing total RNA, the bottom organic phase containing protein and the middle interface layer

containing DNA. The upper layer was pipetted in a new microcentrifuge tube without disturbing the interface layer and leaving some aqueous layer on top of the middle interface to ensure sufficiently pure RNA extraction. The extracted RNA was precipitated by adding 0.5 ml isopropanol to the aqueous phase followed by gentle mixing, incubation for 5 minutes and centrifugation 10,000 rpm for 10 minutes at 4°C. The supernatant solution was aspirated, and the pellet was washed with 1 ml 75% ethanol to remove any salts. Subsequently, the microcentrifuge tube containing ethanol was centrifuged for 5 minutes at 14,000 rpm at 4°C and the supernatant was discarded. The RNA pellet was dried for 10 minutes at room temperature by covering with sterile filter paper.

The RNA pellet was resuspended in nuclease-free water (Thermo Scientific, Waltham, MA) after the pellet was optimally dry and then the tubes were incubated on a block heating plate at 60°C for 10 minutes. The concentration of the isolated RNA was measured on a NanoDrop one microvolume UV spectrophotometer (Thermofisher Scientific, NY, US). The quality and integrity of RNA was assessed by running it through 2% agarose gel stained with ethidium bromide and observation of 28S and 18S bands ensured that the isolated RNA was intact and not degraded. The RNA samples were diluted to a concentration of 1000 ng/10 µl for cDNA synthesis and stored at -80°C until further processing.

3.2.4.2 Complementary DNA (cDNA)synthesis

The synthesis of first-strand cDNA was performed by reverse transcription of 1 µg total RNA (20 µl reaction of RT mixture) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The RNA samples used to synthesize cDNA was all normalized at a concentration of 100 ng/µl. A 10 µl of reverse transcriptase (RT) mix was prepared by combining 2 µl of 10X RT Buffer, 0.8 µl of 25X dNTP (100 nM), 2 µl of 10X RT

Random Primer and 1 µl of Multiscribe Reverse Transcriptase enzyme (Applied Biosystems, Foster City, CA).

The total reaction mix was prepared by mixing 10 µl of RNA sample and 10 µl of freshly prepared RT mix in PCR tubes. The tubes were loaded on a thermal cycler for the synthesis of single-stranded cDNA. The run program of thermal cycler was set at 25°C for 10 minutes followed by 37°C for 120 minutes then 85°C for 5 minutes and a final hold at 4°C. The newly synthesized cDNA (20 µl) in the PCR tube was then centrifuged briefly to remove the condensate on the inner side of tube lid and was diluted with 480 µl of nuclease-free water to yield 25X dilution. The diluted cDNA was then divided into two microcentrifuge tubes as stock and working solution and stored at - 20°C until preparation for qPCR assay.

3.2.4.3 Quantitative real-time PCR (qPCR) assay

The qPCR assay was conducted on a StepOne™ Plus real-time PCR system (Applied Biosystems, Foster City, CA) in a 10 µl reaction mixture containing 3 µl of sample cDNA and 7 µl of the qPCR mix. The master mix was prepared by mixing 5 µl of PowerUP SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and 1 µl each of forward and reverse primers specific to the target gene markers. The primers used in this study was designed on the NCBI primer blast tool and used from a previous study (Berrocoso et al., 2017). The final qPCR mixture consisting of 7 µl of master mix (500 nM of final primer concentration) and 3 µl of the individual sample was pipetted into a 96-well microtiter plate and sealed with clear optical adhesive films (Applied Biosystems, Foster City, CA). For running qPCR program, a run template file was designed on the StepOne™ Plus software v 2.3 (Applied Biosystems, Foster City, CA) with corresponding wells marked for the target gene and specific samples loaded on the PCR plate. The PCR plate was centrifuged for 20-25 seconds on a microplate centrifuge to

ensure proper mixing and to get rid of surface air bubbles in the well. The PCR plate was then inserted into the StepOne™ Plus machine for running PCR cycle. The amplification conditions were set as 50°C for 2 minutes, 95°C for 2 minutes followed by 40 cycles of 95°C for 15 seconds for denaturation, 60°C for 15 seconds for annealing and 72°C for 1 minute for the extension. A melting curve was generated to confirm the sequence-specific PCR products and primer efficiency. After each run, the data was downloaded for analysis and PCR plates were stored at 4°C for validation of PCR amplified products by running 2% agarose gel electrophoresis. The housekeeping gene Beta-actin (β-actin) was analyzed in triplicate and target genes were analyzed in duplicate and an average value was taken for each experimental replicate. The expression level of each gene was recorded for the cycle of threshold (Ct) values generated from the qPCR run analysis. The fold change for each gene was calculated using 2 exponential negative delta delta Ct ($2^{-\Delta\Delta Ct}$) method. The calculation was done from the Ct value of experimental tested (ET), experimental control (EC), experimental tested housekeeping (ETH) and experimental control housekeeping (ECH). The difference between ET and ETH (ΔCtT = ET-ETH), and EC and ECH (ΔCtC = EC-ECH) were calculated. The difference of ΔCtT and ΔCtC gave rise to ΔΔCt. The fold change value was presented as average with standard error on the bar diagram.

3.2.5 DNA extraction, 16S rRNA amplicon sequencing and data analysis

3.2.5.1 DNA extraction from cecal digesta

A total of 210 mg of frozen cecal contents of broilers from d 42 from each replicate samples were taken in a sterile Eppendorf tube and placed on ice. The reagents from QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) were used to extract DNA from the collected cecal contents according to the manufacturer's instructions. Briefly, 1 ml InhibitEX Buffer was added to each tube containing cecal contents and vortexed vigorously for 1-2 minutes

to homogenize the contents. The homogenized samples in the tubes were then centrifuged at 20,000 x g for 1 min to pellet the digesta particles. A 600 µl of the supernatant was taken in a new 2 ml tube containing 25 µl proteinase K and 600 µl of Buffer AL was added to it. The mixture was in the tube was vortexed for 15 seconds. The tubes were then incubated at 70°C for 10 min and centrifuged briefly to collect the condensed drops on the lid of tubes. A 600 µl of ethanol (96–100%) was added to the lysate and mixed by vortexing. The lysate was then loaded slowly (600 µl at a time) on the QIAamp® spin column fitted on a 2 ml collection tube and centrifuged at full speed for 1 min and repeated until all the lysate was loaded. The QIAamp® spin column was then washed with 500 µl Buffer AW1 by centrifuging for 1 min. The filtrate in the collection tube was discarded and the QIAamp® spin column was fitted on a new 2 ml collection tube. A 500 µl of Buffer AW2 was then added to the QIAamp® spin column and centrifuged for 3 min. The filtrate and the collection tube were discarded and the QIAamp® spin column was fitted on a new 2 ml collection tube and dried by centrifuging for 3 minutes. The QIAamp® spin column was then fitted on a new 1.5 ml Eppendorf tube and 200 µl of Buffer ATE was added directly on the QIAamp® spin column membrane. The column was incubated for 1 min at room temperature and then centrifuged at full speed for 1 minute to elute DNA. The quality of the extracted DNA was checked by running 2 µl of the aliquot on 1% agarose gel containing ethidium bromide and the bands were visualized under UV. The concentration of the DNA in ATE buffer was measured on NanoDrop one microvolume UV spectrophotometer (Thermofisher Scientific, NY, US). The final DNA concentration of all the samples was normalized to 10 ng/µl using nuclease-free water. The normalized samples were store at -20°C and transported on ice to a sequencing core facility at the University of Hawaii at Manoa for Illumina MiSeq sequencing.

3.2.5.2 Amplification of V3 and V4 region of 16S rRNA gene by Next Generation Illumina sequencing

In this study, we sequenced V3-V4 variable regions of the 16S rRNA gene. For 16S library preparation, amplicon PCR was performed using Platinum® Taq DNA Polymerase High-Fidelity (Invitrogen, Life Technologies Corporation, Grand Island, NY). The specific sequence of 16S rRNA gene was amplified from genomic DNA using forward primer (5′-CCTACGGGNGGCWGCAG-3′) and reverse primer (5′-GACTACHVGGGTATCTAATCC- 3′) according to Illumina MiSeq protocol (Klindworth et al., 2013). The overhang forward (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′) and reverse (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′) adapter sequences were added to 5′ end of the locus-specific primers. The amplicon PCR cycle consisted of initial denaturation step of heating PCR plate at 95°C on a thermal cycler for 3 minutes followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and an extension step at 72°C for 5 minutes, which was then followed by a hold at 4°C. The PCR amplicon was run on 1% agarose gel to ascertain the size of the amplified product to be around 550 bp. The PCR products were then cleaned according to Illumina 16S rRNA PCR Clean-Up protocol with some modifications, where Mag-Bind Total Pure NGS beads (Omega Bio-tek) were used instead of AMPure XP beads and 70% ethanol instead of 80% ethanol. After clean-up of the amplicon PCR product, an index PCR was performed for multiplexing by attaching Nextera XT dual indices and Illumina sequencing adapters to the target amplicon. The PCR conditions used for index PCR were 95°C for 3 minutes; 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds; followed by an extension at 72°C for 5 minutes and a final hold at 4°C. The cleaning of the index PCR product was carried similarly as done in the previous clean-up step. After PCR clean-up, the libraries were quantified using the Quant-iT PicoGreen dsDNA Assay

Kit, normalized, and pooled. The library pools (0.7 ng) were run on a Bioanalyzer High Sensitivity DNA chip to verify the size of the final library to be around 630 bp. The normalized and pooled amplicons were sequenced on the Illumina MiSeq desktop sequencer (2×300 bp paired-end run) at the University of Hawaii at Manoa Advanced Studies in Genomics, Proteomics and Bioinformatics core facility.

3.2.5.3 Metagenomic sequencing and data analysis

Demultiplexed paired-end R1 and R2 sequencing reads (approximately 300 bp in length) for each sample was generated on the MiSeq platform and downloaded via Illumina BaseSpace® Sequence Hub. The data processing was performed on Quantitative Insights Into Microbial Ecology (QIIME™ version 2.0 release 2019.4; Bolyen et al., 2019). The fastq.gz files for each sample were unzipped and imported using qiime tools import command in Casava 1.8 paired-end demultiplexed fastq format. Denoising, trimming and filtering of phiX reads along with chimeric sequences were done using DADA2 pipeline. The rooted and unrooted tree for phylogenetic diversity analysis was generated using align-to-tree-mafft-fasttree pipeline from qiime phylogeny plugin. Alpha and beta diversity analysis were conducted by applying core-metrics-phylogenetic method from qiime diversity plugin with a sampling depth of 10,000 frequency per sample. For alpha diversity, observed OTUs and Shannon Index were determined and for beta diversity, unweighted UniFrac and Bray Curtis metrics were evaluated.

3.2.6 Statistical analysis

All the variables were compared among treatments using the MIXED procedure of SAS V9.2 (SAS Institute Inc., Cary, NC) for growth performance, SCFA and ileal histomorphometry data. Differences among treatment means were considered significant at $P < 0.05$. Significant differences between treatment means were separated by Tukey test using pdmix macro of SAS.

For immune gene expression data, all the fold change was log-transformed and test variables were compared with control variables by TTest procedure of SAS. For microbial diversity analysis, Kruskal-Wallis pairwise test was used for alpha diversity and pairwise permanova was used for beta diversity in Qiime2.

3.3 Results

The proximate analysis of feeds from different phases was close to the estimated values from feed formulation (Table 3.1). Xylanase and XOS did not affect ($P > 0.05$) ADFI and FCR in any feeding phase from d 0-42 and did not have any interaction ($P > 0.05$) effect on growth parameters (Table 3.2). However, a trend ($P = 0.081$) was observed for the effect of xylanase on ADFI in the total period. Xylanase supplementation increased ADG in both finisher and total period ($P < 0.05$). The effect of 8000 BXU of xylanase supplementation on ADG was neither different from 16000 BXU of xylanase nor than that of 0 BXU xylanase control. Xylanase also increased the average total body weight of broilers by more than 170 g (ATW, 2940 & 2932 vs 2760 g) compared with control in the total period of 42 d ($P < 0.05$). In contrast, XOS did not affect ADG or ATW ($P > 0.05$).

The effect of xylanase and XOS was not observed on ileal mucosa as villus height and crypt depth was not different ($P > 0.05$) across treatments (Table 3.3). However, a trend ($P = 0.097$) was observed for the effect of xylanase on villus height to crypt depth ratio. Both xylanase and XOS increased ($P < 0.05$) cecal production of acetate on d 42 but no interaction effect between xylanase and XOS was observed (Table 3.4). Xylanase also increased the production of total SCFA ($P < 0.01$), while XOS exhibited a trend ($P = 0.052$). No significant ($P > 0.05$) effect of xylanase or XOS was observed on cecal propionate and butyrate production.

On d 42, none of the treatment had any significant effect ($P > 0.05$) on T-cell and B-cell immune markers in ileal tissue samples (Figure 3.1). The treatment containing 100 g/ton of XOS without xylanase expressed a higher level of IL-10 (a marker of anti-inflammatory cytokine) and IL-4 (a T-cell differentiation cytokine) genes compared with control ($P < 0.05$). No specific abundance of OTUs or bacterial communities was evident in response to xylanase and XOS supplementation, and no difference in the microbiota community was observed among treatments ($P > 0.05$; Figure 3.2 & 3.3).

Table 3.2 Effects of xylanase and xylooligosaccharides inclusion on growth performance of broilers from d 0-42 post-hatch.

Treatments	Variables												
	ADFI (g/day)				ADG (g/day)				FCR				ATW,g
	Day												
	0-14	15-28	29-42	0-42	0-14	15-28	29-42	0-42	0-14	15-28	29-42	0-42	0-42
0BXU xylanase													
Xylo-oligo 0g/t	36.0	109.0	171.2	105.4	27.0	75.6	91.9	64.9	1.34	1.44	1.87	1.63	2694
Xylo-oligo 50g/t	36.3	109.0	175.7	107.0	27.9	76.7	94.7	66.4	1.31	1.42	1.86	1.61	2800
Xylo-oligo 100g/t	36.4	109.9	173.7	106.7	28.2	77.5	92.9	66.2	1.29	1.42	1.88	1.61	2786
8000BXU xylanase													
Xylo-oligo 0g/t	36.3	110.5	177.5	108.1	27.7	77.5	97.9	67.7	1.31	1.43	1.81	1.60	2890
Xylo-oligo 50g/t	36.6	110.9	179.3	108.9	28.3	79.7	96.9	68.3	1.30	1.39	1.86	1.60	2959
Xylo-oligo 100g/t	36.7	111.6	179.2	109.2	28.7	80.2	98.6	69.2	1.28	1.39	1.82	1.58	2972
16000BXU xylanase													
Xylo-oligo 0g/t	36.7	111.4	179.4	109.2	28.5	78.3	98.8	68.5	1.29	1.43	1.82	1.60	2909
Xylo-oligo 50g/t	36.7	112.2	178.4	109.1	28.8	79.9	98.9	69.2	1.28	1.41	1.82	1.58	2946
Xylo-oligo 100g/t	36.8	111.2	179.2	109.0	29.1	80.5	98.5	69.4	1.26	1.38	1.82	1.57	2941
SEM (n=4)	0.50	1.70	3.47	1.56	0.87	1.76	2.44	1.33	0.04	0.02	0.04	0.02	65.02
Main effect factors													
Xylanase													
0BXU xylanase	36.2	109.3	173.5	106.4	27.7	76.6	93.2^{b}	65.8^{b}	1.31	1.43	1.87	1.62	2760^{b}
8000BXU xylanase	36.5	111.0	178.7	108.7	28.3	79.1	97.8^{ab}	68.4^{ab}	1.30	1.40	1.83	1.59	2940^{a}
16000BXU xylanase	36.7	111.6	179.0	109.1	28.8	79.6	98.7^{a}	69.0^{a}	1.28	1.40	1.82	1.58	2932^{a}
Xylooligosaccharides													
Xylo-oligo 0g/t	36.3	110.3	176.0	107.6	27.7	77.1	96.2	67.0	1.31	1.43	1.84	1.61	2831
Xylo-oligo 50g/t	36.5	110.7	177.8	108.3	28.3	78.8	96.8	68.0	1.29	1.41	1.84	1.59	2902
Xylo-oligo 100g/t	36.6	110.9	177.4	108.3	28.7	79.4	96.7	68.3	1.28	1.40	1.84	1.59	2900
SEM (n=12)	0.29	0.98	2.01	0.90	0.50	1.02	1.41	0.77	0.02	0.01	0.02	0.01	37.54

P-value													
Xylanase	0.490	0.247	0.114	0.081	0.317	0.108	0.020	0.015	0.480	0.395	0.305	0.197	0.003
Xylo-oligo	0.748	0.922	0.809	0.790	0.417	0.280	0.948	0.500	0.524	0.244	0.973	0.606	0.335
Xylanase×Xylo-oligo	0.993	0.978	0.959	0.985	0.998	0.999	0.935	0.992	0.990	0.980	0.954	0.995	0.983

Table 3.3 Effects of xylanase and xylooligosaccharides inclusion on ileum morphology of broiler chickens on d 42 post-hatch.

	Variables, µm		
Treatments	VH	CD	VH: CD
0BXU xylanase			
Xylo-oligo 0g/t	865.2	98.7	8.9
Xylo-oligo 50g/t	817.5	87.8	9.4
Xylo-oligo 100g/t	879.7	92.7	9.6
8000BXU xylanase			
Xylo-oligo 0g/t	883.9	96.4	9.3
Xylo-oligo 50g/t	995.4	102.4	9.8
Xylo-oligo 100g/t	886.0	91.6	9.8
16000BXU xylanase			
Xylo-oligo 0g/t	963.4	98.2	9.9
Xylo-oligo 50g/t	1101.6	111.3	10.0
Xylo-oligo 100g/t	928.8	93.0	10.1
SEM (n=4)	82.30	7.31	0.38
Main effect factors			
Xylanase			
0BXU xylanase	854.1	93.0	9.3
8000BXU xylanase	921.8	96.8	9.6
16000BXU xylanase	997.9	100.8	10.0
Xylooligosaccharides			
Xylo-oligo 0g/t	904.2	97.7	9.3
Xylo-oligo 50g/t	971.5	100.5	9.7
Xylo-oligo 100g/t	898.2	92.4	9.8
SEM (n=12)	47.51	4.22	0.22
P-value			
Xylanase	0.120	0.438	0.097
Xylo-oligo	0.489	0.399	0.248
Xylanase×Xylo-oligo	0.646	0.468	0.965

Table 3.4 Effects of xylanase and xylooligosaccharides inclusion on cecal SCFA (µM/g wet digesta) production in broilers on d 42 post-hatch.

	Variables, µM/g			
Treatments	Acetate	Propionate	Butyrate	Total SCFA
0BXU xylanase				
Xylo-oligo 0g/t	50.2	5.4	6.3	64.9
Xylo-oligo 50g/t	58.5	4.7	10.0	76.2
Xylo-oligo 100g/t	57.2	5.5	10.8	76.8
8000BXU xylanase				
Xylo-oligo 0g/t	68.0	6.5	9.7	87.5
Xylo-oligo 50g/t	66.6	5.9	8.0	84.1
Xylo-oligo 100g/t	71.0	6.0	10.1	90.7
16000BXU xylanase				
Xylo-oligo 0g/t	70.9	5.8	9.7	89.8
Xylo-oligo 50g/t	77.0	6.3	11.0	97.8
Xylo-oligo 100g/t	80.4	5.3	9.3	98.4
SEM (n=4)	2.74	0.92	1.18	3.82
Main effect factors				
Xylanase				
0BXU xylanase	55.3^{c}	5.2	9.0	72.6^{c}
8000BXU xylanase	68.5^{b}	6.1	9.3	87.4^{b}
16000BXU xylanase	76.1^{a}	5.8	10.0	95.3^{a}
Xylooligosaccharides				
Xylo-oligo 0g/t	63.0^{b}	5.9	8.5	80.7^{b}
Xylo-oligo 50g/t	67.4^{ab}	5.6	9.7	86.0^{ab}
Xylo-oligo 100g/t	69.6^{a}	5.6	10.1	88.6^{a}
SEM (n=12)	1.58	0.53	0.68	2.21
P-value				
Xylanase	<0.001	0.479	0.558	<0.001
Xylo-oligo	0.022	0.923	0.264	0.052
Xylanase×Xylo-oligo	0.409	0.907	0.102	0.404

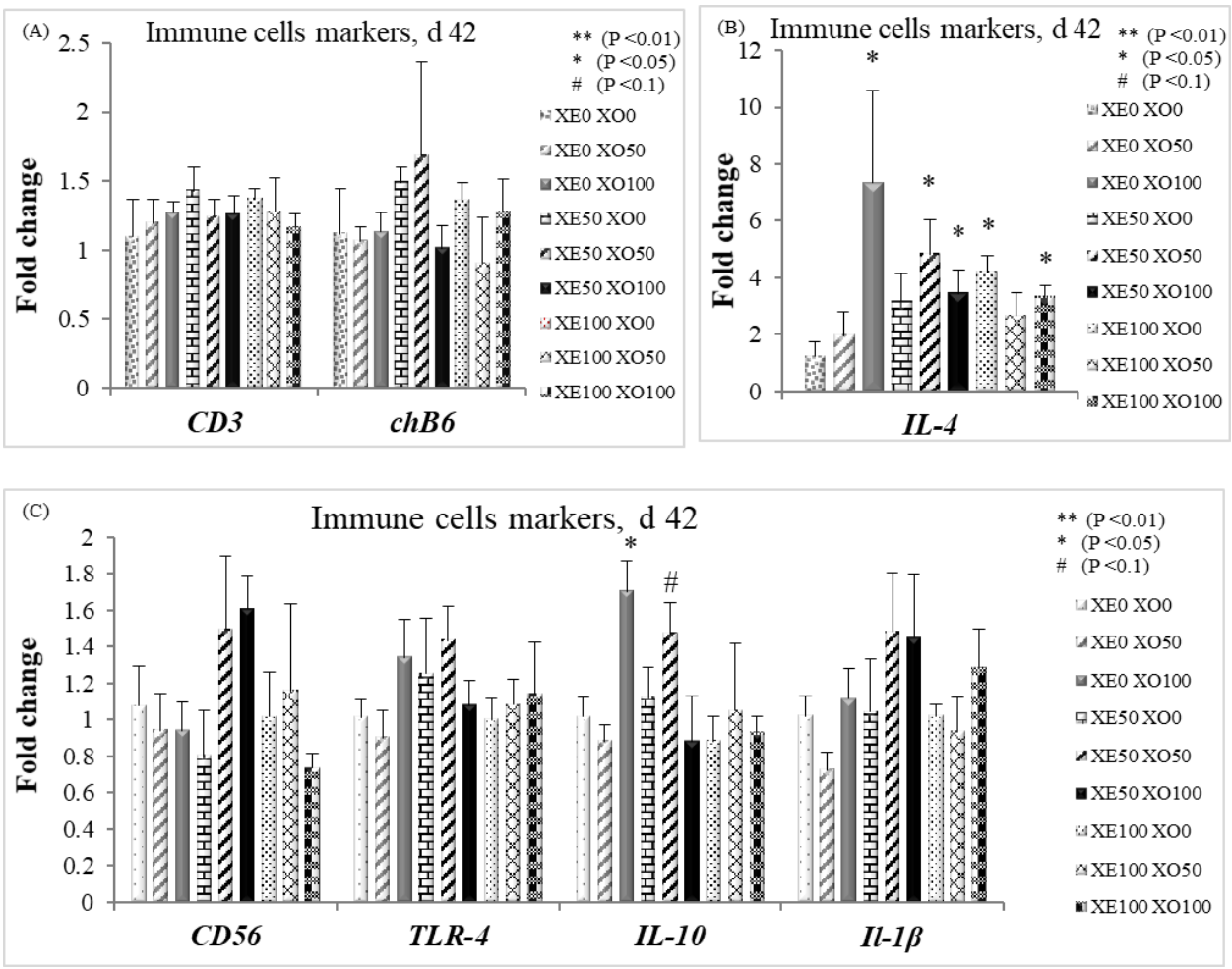

Figure 3.1 Effects of xylanase (XE) and xylooligosaccharides (XO) inclusion in feed on ileal gene markers of immune cells of broilers at 42 d of age. The expression of each gene was examined using RT-qPCR and expressed as fold change ratio to the β-actin housekeeping gene, with the level being set to 1 in broilers from xylanase 0 + xylooligosaccharides 0 group.

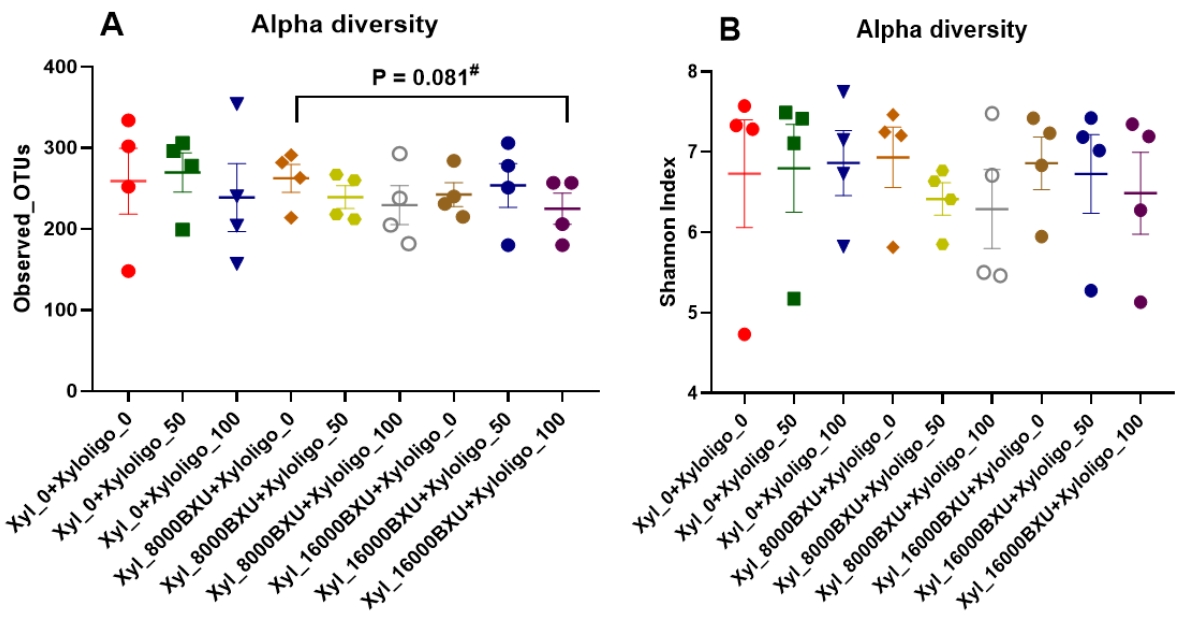

Figure 3.2 Alpha diversity analysis of different treatments at 10,000 reads depth per sample of cecal contents of broilers from d 42 post-hatch. A) Observed OTUs B) Shannon Index. Data represent mean ± SE. Statistical analyses were performed using Kruskal-Wallis test. Only the lowest p-value is shown. Xyl, xylanase; Xyloligo, xylooligosaccharides.

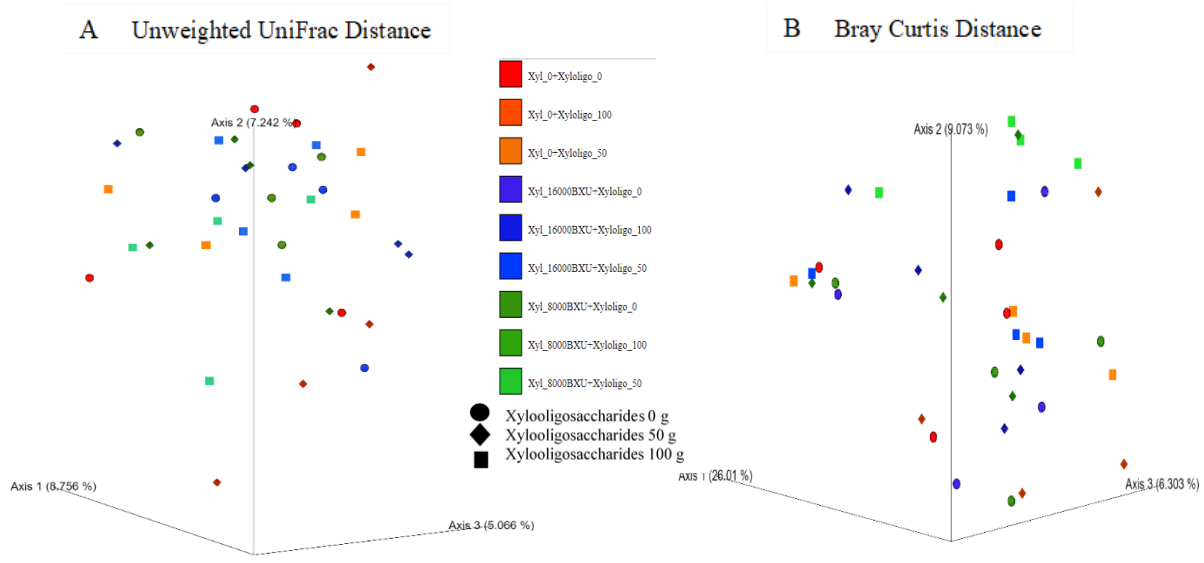

Figure 3.3 Principal coordinate analysis (PCoA) plot shows beta diversity analysis between different treatments at 10,000 reads depth per sample of cecal contents of broilers from d 42 post-hatch. A) Unweighted UniFrac distance B) Bray Curtis distance. The pairwise comparison did not show any significant differences. Xyl, xylanase; Xyloligo, xylooligosaccharides.

3.4 Discussion

The application of xylanase enzyme in wheat-based diet can improve AME and digestibility of amino acid (Hew et al., 1998) that can result in improved growth performance in broilers (Wu and Ravindran, 2004). Supplementation of xylanase enzyme in corn-based diets has also been reported to improve growth performance and nutrient digestibility in broilers (Cowieson et al., 2010; Kiarie et al., 2014). However, the level of improvement depends on the type of xylanase used and the amount of soluble and insoluble NSP present in the digesta (Choct et al., 2004).

In the present study, xylanase supplementation did not produce significant improvement in ADFI and FCR. The diets used in all the phases in this study were adequate in nutrients and metabolizable energy and there might have been less scope for improvement. Nevertheless, the improvement in ADG in the finisher and ADG and ATW total period substantiates the role of xylanase in enhancing nutrient utilization in broilers. It was also evident that addition half supplementation was able to produce similar benefit as full supplementation of xylanase, which further supports that there might have been a lack of availability of degradable substrates in the intestinal digesta. We did not observe any improvement in weight gain or FCR of broilers in response to XOS supplementation. This is in agreement with the findings of Samanta et al. (2017). In contrast, Zhenping et al. (2013) reported that XOS supplementation increased body weight gain in broilers compared with the control group. The XOS used in the study of Zhenping et al. (2013) was at a 10 times higher concentration than that was used in the present study and the variability in the extraction of different XOS might also have played some role.

It is interesting to note that both xylanase and XOS increased cecal acetate production that is indicative of their role in modulating microbial fermentation characteristics. XOS was

supplemented in a small amount which is not expected to yield enough SCFA on its own and thus is suggestive of a priming effect on cecal microbiota for selective utilization of fermentable substrates. The effect of higher concentration of XOS on IL-4 and IL-10 cytokines marker can validate claims that XOS can have an immunomodulating effect, but whether it is a direct role or via utilization by microbiota remains unexplained. In a study conducted on mice, XOS feeding did not produce any effect on intestinal immune-related genes, but it significantly reduced the level of IL-1β in blood, which suggests variation in immune response in different tissues and body sites (Hansen et al., 2013). We did not find the effect of xylanase or XOS on the diversity of cecal microbiota, whereas Samanta et al. (2017) had observed that XOS stimulated the population of *Bifidobacteria* and reduced the population of *Streptococci* and *E. coli* in the cecum of broilers. In the present study, XOS was supplemented in a low dose compared to other study and that can be one of the reasons for the absence of the influence of XOS on cecal microbiota. However, further studies on the effect of xylanase and XOS on cecal microbiota of broilers are warranted to elucidate variation in responses.

3.5 Conclusion

Thus, it is confirmed that xylanase can improve growth performance of broilers fed corn-SBM diets as well. Moreover, the results infer that both xylanase and XOS has potential to enhance cecal fermentation and the production of SCFA in broilers. However, these improvements may not necessarily depend on the enhancement of mucosal absorptive surface or cecal microbiota diversity in broilers.

CHAPTER 4: COMPARATIVE EFFECTS OF *IN OVO* INJECTION OF OLIGOSACCHARIDES (XYLOTRIOSE, XYLOTETRAOSE, MANNOTRIOSE, AND MANNOTETRAOSE) ON GROWTH PERFORMANCE AND GUT HEALTH OF BROILERS

Abstract

In ovo feeding of prebiotics is a novel strategy to enhance the gut health of broilers starting early post-hatch. To explore the beneficial role of prebiotics, four oligosaccharides (xylotriose, xylotetraose, mannotriose, and mannotetraose) were fed to the embryo in the egg of Cobb 500 broilers and the parameters of growth, cecal fermentation, histomorphometry, and immune-related genes were evaluated. A total of 192 fertilized eggs were divided into 6 treatment groups with 4 replicate trays containing 8 eggs per replicate. Group 1-4 was assigned to the respective oligosaccharides, group 5 to normal-saline (NS) control and group 6 to no-injection control. On 17.5 d of egg incubation, 3 mg of oligosaccharides (except for NS) dissolved in 0.5 ml of 0.85% NS was injected in the amniotic sac of eggs in respective treatments. The chicks were raised for 28 d post-hatch under standard husbandry practices and were fed a commercial broilers diet *ad libitum*. The feed intake and body weight were recorded weekly to determine feed conversion ratio and growth performance. Ileal samples were collected on hatch day, d 7 and 28 for histomorphometric and on hatch day and d 7 for gene expression analysis. Simultaneously, the cecal digesta were collected for DNA extraction and genomic sequencing, and short chain fatty acid (SCFA) determination. The hatchability, growth performance, and relative organ weights of breast, drumstick, liver and proventriculus were not different across the treatments ($P > 0.05$). The xylotriose injection increased the total SCFA production at 28 d compared with controls ($P < 0.05$). Villus height, crypt depth and their ratio were not different across treatments at 7 d and 28 d post-hatch ($P > 0.05$). However, the villus height to crypt depth ratio was significantly higher in the xylotetraose group compared with controls on hatch day ($P < 0.01$). The innate immunity-related proinflammatory and anti-inflammatory cytokines and adaptive immunity-related T-cell and B-cell marker genes were not

expressed differently across treatments at 7 d post-hatch. However, on the hatch day, the level of CD3 (a T-cell marker) was increased by xylotriose, while IL-10 (a marker of anti-inflammatory cytokine) was reduced by mannotetraose ($P < 0.05$) compared with NS and no injection controls. Xylotriose exhibited a trend of reduction for IL-10 ($P = 0.074$) compared with controls. Mannotetraose increased the counts of observed OTUs compared with normal saline ($P < 0.05$) and exhibited a trend ($P = 0.076$) of higher OTUs compared with no injection control for cecal microbiota on d 28 post-hatch. Mannotetraose also had more differentially abundant taxa including *Lactobacillales* and *Leuconostocaceae* ($P < 0.05$). The results of this study indicate that the effect of these oligosaccharides on ileal mucosa and immunity is transient, but the effect on fermentation and cecal microbiota is prolonged. Further research on the mechanism and potential of oligosaccharides prebiotics products is warranted before their extensive use alone or in combination with other additives as a gut health promoter in poultry.

Keywords: broilers, gut health, immunity, *in ovo*, oligosaccharides

4.1 Introduction

Dietary components are in intimate contact with the immune system in the intestine and the presence of nutrients in the intestine may be necessary for proper development and function of gut-associated lymphoid tissue (Ruthlein et al., 1992). Feeding of mannan oligosaccharide (MOS) is reported to increase *Lactobacillus* community diversity and decrease *Clostridium perfringens* and *E. coli* in the ileum of broilers (Kim et al., 2011) when fed in diet. Likewise, xylooligosaccharide (XOS), when added in broiler feed, can improve growth by stimulating butyrate-producing bacteria through cross-feeding of lactate (De Maesschalck et al., 2015). XOS, when fed in diet, also increases *Lactobacillus* and concentration of acetate and propionate in the cecum of broilers (Pourabedin et al., 2015). It is also reported that XOS improves body weight and feed conversion, *Clostridium* cluster XIVa in ceca and lactic acid bacteria in the colon of broilers when fed in feed and increases in vitro butyrate production in cecal inocula (De Maesschalck et al., 2015).

Besides therapeutic purposes, antibiotics are supplemented in poultry feeds as a growth promoter (AGP). However, the use of AGP is regulated or banned due to the public health concern of antibiotic resistance. Withdrawal of AGPs causes an increase in the incidence of diseases leading to reduced nutrient utilization, poor growth, and ultimately results in the economic loss in poultry production. Consequently, there is a growing demand for alternatives to AGPs and some products have shown potential in improving production while others require additional research. Several alternatives like organic acids, phytogenic compounds, direct-fed microbials, probiotics, enzymes, and prebiotics are being tried to generate similar benefits as AGPs. The objectives of this experiment were to evaluate the effect of *in ovo* injection of xylooligosaccharides and

mannooligosaccharides containing 3 and 4 monosaccharides units on growth performance, gut health, immune status, and microbiota diversity and quantitation.

4.2 Materials and methods

All animal care procedures were approved by the Institutional Animal Care and Use Committee of the University of Hawaii.

4.2.1 Experimental design and egg injection

192 fertile eggs (Cobb 500) from the 35-week breeding flock were obtained from a commercial hatchery (Asagi Hatchery Inc., Honolulu, HI) at 17th day of incubation. On arrival, the eggs were weighed and numbered and incubated at 37.5°C and relative humidity of 58% in an incubator (GQF incubator, Savannah, GA). After the eggs were acclimatized in the incubator for >8 hours, 32 eggs were randomly assigned from different weight groups to each treatment. The eggs were numbered for each treatment groups and were randomly allocated to 4 replicates of each of 6 treatments by location on the setter trays. For *in ovo* injection, each replicate group of eggs were taken out for injection in a biosafety cabinet and were placed out of the incubator for less than 15 minutes. On d 19 of incubation, the eggs were transferred to a hatcher set at 37°C temperature and relative humidity 75% following the instructions for pre-set hatcher (GQF incubator, Savannah, GA). Each replicate group of eggs from setter were again randomly assigned to one of 24 compartments in 6 hatcher trays separated by the dividers. After hatching, chicks from each treatment were weighed and tagged. Depending on the hatch, 25 chicks from each treatment were moved to the Small Animal Facility of the University of Hawaii at Manoa (Honolulu, HI) and randomly allocated to 5 replicate pens (5 chicks/pen).

4.2.2 *In ovo* injection

At d 17.5 broad end site of all eggs were disinfected with 10% povidone-iodine solution and then a tiny punch hole (shell perforation) was made using a stabbing awl with a fixed depth of 1mm made with a pipette sheath. After every punch, the tip of the awl was disinfected with 70% ethanol and wiped with sterile gauze. The injection was done in the amniotic sac of each egg using blunt tip 21-gauge sterile needle inserted to 2.80 cm length from the longest axis through the broad end and passing beyond the air sac. All the eggs were sealed using non-toxic glue. Each oligosaccharide treatment (xylotriose, xylotetraose, mannotriose and mannotetraose) was prepared at a concentration of 6 mg/ml in 0.85% Normal Saline and was injected at 0.5 ml per egg. All together there were six treatment groups: 1) 0.85% Normal Saline, 2) 0.5 ml 0.85% Normal Saline containing 3 mg xylotriose, 3) 0.5 ml 0.85% Normal Saline containing 3 mg xylotetraose, 4) 0.5 ml 0.85% Normal Saline containing 3 mg mannotriose, 5) 0.5 ml 0.85% Normal Saline containing 3 mg mannotetraose and 6) no injection control.

4.2.3 Post-hatch management, growth performance and organs relative weight

After hatch, the unhatched eggs were counted and opened to check the cause of death of embryo to rule out any infection or injury-related deaths and the hatchability was calculated for each treatment. The weighed and tagged chicks were placed randomly in 30 floor pens (5 birds per pen), making 5 replicates of each treatment. Birds in all floor pens were raised under standard commercial broiler rearing environment (temperature, humidity, and light). The temperature in the first week was maintained at 35°C and gradually decreased to 28°C by the end of the third week. All birds were fed commercial corn-soybean meal-based pellet diet during the 28-day post-hatch trial period (Table 4.1). The birds were fed *ad libitum* with unrestricted access to water. Bodyweight and feed consumption of the birds were measured by pen at 7, 14, and 21

and 28 d of age, and average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR, feed: gain) were calculated from these data by week and for total period. Feed wastage and bird's mortality were recorded daily and the feed consumption and FCR were adjusted for mortality and feed wastage. On d 28, five birds per treatment (1 bird per pen) were chosen randomly for the determination of organ weights and were dissected after euthanizing with CO_2 gas. The weight of breast muscle, drumsticks, gizzard, and proventriculus were recorded, and the relative weight (% of live body weight) was calculated.

Table 4.1 Determined nutrient composition of commercial starter diet fed to broilers from d 0-28 post-hatch.

Item	Inclusion level
Guaranteed analysis	
Crude Protein (Min)	22.00%
Lysine (Min)	1.00%
Methionine	0.45%
Crude Fat (Min)	3.50%
Crude Fiber (Min)	4.00%
Calcium (Ca) (Min)	0.90%
Calcium (Ca) (Max)	1.40%
Phosphorus (P) (Min)	0.60%
Salt (NaCl) (Min)	0.30%
Salt (NaCl) (Max)	0.80%
Total Selenium (Se) (Min)	0.60 ppm
Total Selenium (Se) (Max)	0.72 ppm
Phytase (A. Oryzae) (Min)	227 FYT/LB

One phytase unit (FYT) liberates one micromole of inorganic phosphorus per minute from sodium phytate at pH 5.5 and 98.6 F. Contains a source of phytase, Ronozyme HiPhos GT, which can hydrolyze phytate increasing the digestibility of phosphorus in diets containing phytate-bound phosphorus.

4.2.4 Sample collection for histology, gene expression, short chain fatty acid (SCFA), and microbiota analysis

On d 0, 7, 21 and 28, sections of 1 cm from ileum (defined as a section 1 cm distal to Meckel's diverticulum and 1 cm anterior to ileocecal junction) were collected from birds after

euthanasia and the tissues were fixed in 10% neutral buffered formalin (NBF) which were later preserved in 70% ethanol. On d 0 ileal tissue for histology was collected from 4 birds per treatment (1 bird per replicate cage) and on d 7, 21 and 28, the ileal tissue was collected from 5 birds, each from 5 replicate pens per treatment. On d 0 and 7, small sections (50-100 mg) of ileum were also collected in cryovials and snap-frozen in liquid nitrogen, and later stored at -80°C until further analysis. Moreover, on d 28, cecal contents were collected from broilers of each replicate pen and stored at -20°C from SCFA analysis and snap-frozen in liquid nitrogen and stored at -80°C for further analysis of microbiota.

4.2.5 Ileal mucosa histomorphometry

The ileal samples from all collection time point (d 0, n =4/treatment; d 7, 21and 28, n =5/treatment) fixed in NBF and preserved in 70% ethanol were outsourced to histology core facility at John A. Burns School of Medicine for embedding, sectioning and staining with Hematoxylin and Eosin (H&E). Six replicate sections of each sample were cut in 5 μm thickness and mounted on slides. After staining, the slides were observed under 8X objective of an upright microscope (Olympus BX43, Olympus Co, Tokyo, Japan), and villus height and crypt depth length were recorded using image processing and analysis system of Infinity Analyze software (Lumenera Corporation, Ottawa, ON, CA). Villus height and crypt depth were recorded from 3 sets of villus and crypt from each section of 6 sections from each replicate samples. The average measurement was calculated from all readings per sample replicate and villus height to crypt depth ratio was also determined. The data were presented as average for each treatment along with their standard error.

4.2.6 Determination of SCFA by gas chromatography

The calibration standards were prepared at different levels of concentration ranging from 0 to 8 mM for all individual SCFA from a standard stock of volatile free acid mix (Supelco®, Bellefonte, PA) containing 10 mM of each SCFA (formic, acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, hexanoic and n-caproic). All calibration standards contained 100 µl of 25% metaphosphoric acid (MPA) and 100 µl of 48.95 mM trimethylacetic acid (TMA, Pivalic acid from Sigma-Aldrich, St. Louis, MO) in a total volume of 1500 µl prepared in microcentrifuge tubes.

For sample preparation, at first 1 g of cecal content was mixed with 1 ml of distilled water in a centrifuge tube, vortexed and then centrifuged at 10, 000 ×g for 5 minutes at 4°C. Following centrifugation, 400 µl of supernatant was transferred into a new microcentrifuge tube containing 100 µl of 25% MPA and 100 ul of 48.95 mM TMA, and the final volume was made 1500 µl by adding distilled water. Next, the mixture was vortexed and centrifuged at 13,000 ×g for 10 minutes at 4°C. Following centrifugation, approximately 1000 µl of supernatant was transferred to a GC vial sealed using septa and screw cap and was then loaded on the carousel of autosampler for injection.

For determination of individual SCFA, the standards and samples were loaded on GC fitted carousel and the run method was set according to Zhao et al. (2006). Briefly, helium was supplied as a carrier gas at a flow rate of 14.5 ml/min and run time was set for 17.5 min. The initial oven temperature was 100°C held for 0.5 min, then ramped to 180°C at 8°C /min and held for 1 min, then again raised to 200°C at the rate of 20°C /min where it was finally maintained for 5 min. The temperature of the injector port was set at 200°C and that of detector port was set at 240°C. The flow rates of air, hydrogen and makeup gas (nitrogen) were 300, 30 and 20 ml/min

respectively. The samples injection volume was kept 0.5 µl in order to reduce the possibility of backflash and a large expansion of water used as a solvent. Isopropyl alcohol and distilled water were used as syringe wash solvent. Data handling and processing were performed on Chromeleon™ 7.2 software (Thermo Scientific, US). The processing of the sample was done after integrating the chromatogram for specific peaks and then comparing the peak area of each individual SCFA with the respective calibration curve of their standards.

4.2.7 RNA isolation, reverse transcription and real-time quantitative PCR

4.2.7.1 Total RNA isolation from the ileum

Ileal tissue from hatch day and from d 7 post-hatch was transferred from -80°C freezer to dry ice for convenient handling. Total RNA from ileal tissue of broilers were isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. The RNA isolation protocol included the following sequential steps: ileal tissue homogenization and lysis, RNA extraction using chloroform, triple-phase separation, RNA precipitation, RNA pellet washing, RNA pellet drying and resuspension in nuclease-free water. First, 9-11 Zirconia beads (BioSpec Products, Bartlesville, OK, USA) of 2.3 mm were taken in a safe lock nuclease-free Eppendorf tube. A 300 µl of TRIzol® reagent was then added to the tube containing beads. A frozen ileal tissue (50-100 mg) was then dropped in the tube to be immersed in the TRIzol®. The tissue was then homogenized by bead beating on a vertical Bullet Blender® (Next Advance, Inc. Troy, NY) at speed 8 for 2-3 minutes. The microcentrifuge tubes containing homogenate and beads were then centrifuged at 10,000 rpm for 1 minute at 4°C. Approximately 250 µl of the supernatant was then transferred to a new microcentrifuge tube containing 750 µl of TRIzol®. The tube containing supernatant from homogenate and fresh TRIzol® was mixed gently and allowed to stand for 5 minutes at room temperature. Then, 0.2 ml chloroform per 1 ml of

TRIzol® was added to the homogenate supernatant, vortexed briefly and allowed to incubate for 5 minutes at room temperature. The resultant mix was then centrifuged at 10,000 rpm for 15 minutes at 4°C. Following centrifugation, the solution distinctly separated into three layers: an upper aqueous containing total RNA, the bottom organic phase containing protein and the middle interface layer containing DNA. The upper layer was pipetted in a new microcentrifuge tube without disturbing the interface layer and leaving some aqueous layer on top of the middle interface to ensure sufficiently pure RNA extraction. The extracted RNA was precipitated by adding 0.5 ml isopropanol to the aqueous phase followed by gentle mixing, incubation for 5 minutes and centrifugation 10,000 rpm for 10 minutes at 4°C. The supernatant solution was aspirated, and the pellet was washed with 1 ml 75% ethanol to remove any salts. Subsequently, the microcentrifuge tube containing ethanol was centrifuged for 5 minutes at 14,000 rpm at 4°C and the supernatant was discarded. The RNA pellet was dried for 10 minutes at room temperature by covering with sterile filter paper.

The RNA pellet was resuspended in nuclease-free water (Thermo Scientific, Waltham, MA) after the pellet was optimally dry and then the tubes were incubated on a block heating plate at 60°C for 10 minutes. The concentration of the isolated RNA was measured on a NanoDrop one microvolume UV spectrophotometer (Thermofisher Scientific, NY, US). The quality and integrity of RNA was assessed by running it through 2% agarose gel stained with ethidium bromide and observation of 28S and 18S bands ensured that the isolated RNA was intact and not degraded. The RNA samples were diluted to a concentration of 1000 ng/10 µl for cDNA synthesis and stored at -80°C until further processing.

4.2.7.2 Complementary DNA (cDNA)synthesis

The synthesis of the first-strand cDNA was performed by reverse transcription of 1 µg total RNA (20 µl reaction of RT mixture) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The RNA samples used to synthesize cDNA was all normalized at a concentration of 100 ng/µl. A 10 µl of reverse transcriptase (RT) mix was prepared by combining 2 µl of 10X RT Buffer, 0.8 µl of 25X dNTP (100 nM), 2 µl of 10X RT Random Primer and 1 µl of Multiscribe Reverse Transcriptase enzyme (Applied Biosystems, Foster City, CA).

The total reaction mix was prepared by mixing 10 µl of RNA sample and 10 µl of freshly prepared RT mix in PCR tubes. The tubes were loaded on a thermal cycler for the synthesis of single-stranded cDNA. The run program of thermal cycler was set at 25°C for 10 minutes followed by 37°C for 120 minutes then 85°C for 5 minutes and a final hold at 4°C. The newly synthesized cDNA (20 µl) in the PCR tube was then centrifuged briefly to remove the condensate on the inner side of tube lid and was diluted with 480 µl of nuclease-free water to yield 25X dilution. The diluted cDNA was then divided into two microcentrifuge tubes as stock and working solution and stored at - 20°C until preparation for qPCR assay.

4.2.7.3 Quantitative real-time PCR (qPCR) assay

The qPCR assay was conducted on a StepOne^{TM} Plus real-time PCR system (Applied Biosystems, Foster City, CA) in a 10 µl reaction mixture containing 3 µl of sample cDNA and 7 µl of the qPCR mix. The master mix was prepared by mixing 5 µl of PowerUP SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and 1 µl each of forward and reverse primers specific to the target gene markers. The primers used in this study was designed on the NCBI primer blast tool and used from a previous study (Berrocoso et al., 2017). The final qPCR

mixture consisting of 7 µl of master mix (500 nM of final primer concentration) and 3 µl of the individual sample was pipetted into a 96-well microtiter plate and sealed with clear optical adhesive films (Applied Biosystems, Foster City, CA). For running qPCR program, a run template file was designed on the StepOne^{TM} Plus software v 2.3 (Applied Biosystems, Foster City, CA) with corresponding wells marked for the target gene and specific samples loaded on the PCR plate. The PCR plate was centrifuged for 20-25 seconds on a microplate centrifuge to ensure proper mixing and to get rid of surface air bubbles in the well. The PCR plate was then inserted into the StepOne^{TM} Plus machine for running PCR cycle. The amplification conditions were set as 50°C for 2 minutes, 95°C for 2 minutes followed by 40 cycles of 95°C for 15 seconds for denaturation, 60°C for 15 seconds for annealing and 72°C for 1 minute for the extension. A melting curve was generated to confirm the sequence-specific PCR products and primer efficiency. After each run, the data was downloaded for analysis and PCR plates were stored at 4°C for validation of PCR amplified products by running 2% agarose gel electrophoresis. The housekeeping gene Beta-actin (β-actin) was analyzed in triplicate and target genes were analyzed in duplicate and an average value was taken for each experimental replicate. The expression level of each gene was recorded for the cycle of threshold (Ct) values generated from the qPCR run analysis. The fold change for each gene was calculated using 2 exponential negative delta delta Ct ($2^{-\Delta\Delta Ct}$) method. The calculation was done from the Ct value of experimental tested (ET), experimental control (EC), experimental tested housekeeping (ETH) and experimental control housekeeping (ECH). The difference between ET and ETH (ΔCtT = ET-ETH), and EC and ECH (ΔCtC = EC-ECH) were calculated. The difference of ΔCtT and ΔCtC gave rise to ΔΔCt. The fold change value was presented as average with standard error on the bar diagram.

4.2.8 DNA extraction, 16S rRNA amplicon sequencing and data analysis

4.2.8.1 DNA extraction from cecal digesta

Frozen cecal contents from 28 d broilers were kept in microcentrifuge tubes and placed on ice. Genomic DNA was extracted from the cecal digesta of broilers by repeated bead beating and column method according to the protocol of Yu and Morrison (2004). Briefly, 0.25 g of cecal digesta was taken into a sterile 2 ml screw-cap tube containing 1 ml Qiagen Cell lysis buffer (Cat No./ID: 158908), and 0.3 g of 0.1 mm and 0.1 g of 0.5 mm pre-weighed zirconia beads. The tube containing a mixture of cecal digesta and beads in cell lysis buffer was homogenized for 3 min at maximum speed on a Mini-Beadbeater™ (BioSpec Products, Bartlesville, OK, USA). After homogenization, the tube was incubated at 70°C for 15 min and was gently shaken every 5 min. Later the tubes were centrifuged 4°C for 5 min at 16,000× g and the supernatant in each tube was transferred to a new 2 ml Eppendorf tube. A 300 µl of fresh cell lysis buffer was added to the lysis tube containing precipitate and the homogenization, centrifugation and collection of supernatant in each tube was repeated as above. Following homogenization and cell lysis, nucleic acid was precipitated using 10 M ammonium acetate and isopropanol. After centrifugation at 4°C for 15 min at 16,000× g, the supernatant was discarded by aspiration and the pellet was gently washed with 70% ethanol. The pellet was dried after removal of ethanol at room temperature for 10 min. The nucleic acid pellet was dissolved in 200 µl of TE buffer. After the dissolution of nucleic acid in TE buffer, removal of RNA and protein was done using the reagent from QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). The quality of the extracted DNA was ascertained by running 2 µl of the aliquot on 1% agarose gel electrophoresis. The staining was done using ethidium bromide and the bands were visualized under UV. The concentration of the DNA in AE buffer was measured on NanoDrop one microvolume UV spectrophotometer (Thermofisher Scientific, NY, US). The

final DNA concentration of all the samples was normalized to 10 ng/µl using nuclease-free water. The normalized samples were store at -20°C and transported on ice to a sequencing core facility at the University of Hawaii at Manoa for Illumina MiSeq sequencing.

4.2.8.2 Amplification of V3 and V4 region of 16S rRNA gene by Next Generation Illumina sequencing

We sequenced V3 and V4 hypervariable regions of the 16S rRNA gene. For 16S library preparation, amplicon PCR was carried out using Platinum® Taq DNA Polymerase High-Fidelity (Invitrogen, Life Technologies Corporation, Grand Island, NY). The specific sequence of 16S rRNA gene was amplified from genomic DNA using forward primer (5′-CCTACGGGNGGCWGCAG-3′) and reverse primer (5′-GACTACHVGGGTATCTAATCC- 3′) according to Illumina MiSeq protocol (Klindworth et al., 2013). The overhang forward (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′) and reverse (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′) adapter sequences were added to the 5′ end of the locus-specific primers. The amplicon PCR cycle consisted of initial denaturation step of heating PCR plate at 95°C on a thermal cycler for 3 minutes followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and an extension step at 72°C for 5 minutes, which was then followed by a hold at 4°C. The PCR amplicon was run on 1% agarose gel to ascertain the size of the amplified product to be around 550 bp. The PCR products were then cleaned according to Illumina 16S rRNA PCR Clean-Up protocol with some modifications, where Mag-Bind Total Pure NGS beads (Omega Bio-tek) were used instead of AMPure XP beads and 70% ethanol instead of 80% ethanol. After clean-up of the amplicon PCR product, an index PCR was performed for multiplexing by attaching Nextera XT dual indices and Illumina sequencing adapters to the target amplicon. The PCR conditions used for index PCR were 95°C for 3 minutes; 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C

for 30 seconds; followed by an extension at 72°C for 5 minutes and a final hold at 4°C. The cleaning of the index PCR product was carried similarly as done in the previous clean-up step. After PCR clean-up, the libraries were quantified using the Quant-iT PicoGreen dsDNA Assay Kit, normalized, and pooled. The library pools (0.7 ng) were run on a Bioanalyzer High Sensitivity DNA chip to verify the size of the final library to be around 630 bp. The normalized and pooled amplicons were sequenced on the Illumina MiSeq desktop sequencer (2×300 bp paired-end run) at the University of Hawaii at Manoa Advanced Studies in Genomics, Proteomics and Bioinformatics core facility.

4.2.8.3 Metagenomic sequencing and data analysis

Demultiplexed paired-end R1 and R2 sequencing reads (approximately 300 bp in length) for each sample was generated on the MiSeq platform and downloaded via Illumina BaseSpace® Sequence Hub. The data processing was performed on Quantitative Insights Into Microbial Ecology (QIIME™ version 2.0 release 2019.4; Bolyen et al., 2019). The fastq.gz files for each sample were unzipped and imported using qiime tools import command in Casava 1.8 paired-end demultiplexed fastq format. Denoising, trimming and filtering of phiX reads along with chimeric sequences were done using DADA2 pipeline. The rooted and unrooted tree for phylogenetic diversity analysis was generated using align-to-tree-mafft-fasttree pipeline from qiime phylogeny plugin. Alpha and beta diversity analysis were conducted by applying core-metrics-phylogenetic method from qiime diversity plugin with a sampling depth of 2000 frequency per sample. For alpha diversity, observed OTUs and Shannon Index were determined and for beta diversity, unweighted UniFrac and Bray Curtis metrics were evaluated. For assigning taxonomy, a Naive Bayes classifier pre-trained on the Greengenes 13_8 99% OTUs were applied to the sequences of the present study. We used classify-sklearn command of qiime feature-classifier plugin to

explore the taxonomic composition of the sample. To perform a differential abundance test at the specific taxonomic level, the taxonomy was added to the feature table by collapse method of qiime taxa pipeline. Once the feature table was collapsed at the specific species level, it was then exported to a biom file and later converted to a text file using biom convert. The text file was later used for linear discriminant analysis (LDA) using linear discriminant analysis effect size (LEfSe) tools of Galaxy web application.

4.2.9 Statistical analysis

All the variables were compared among treatments using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) for hatchability, growth performance, organ weight, histomorphometry and SCFA data. Hatchability was noted for all replicate and it was subjected to statistical analysis after arcsin square root transformation. Differences among treatment means were considered significant at $P < 0.05$. Significant differences between treatment means were separated by Tukey test using pdmix macro of SAS. For immune gene expression data, all the fold change was log-transformed and test variables were compared with control variables by TTest procedure of SAS. For microbial diversity analysis, Kruskal-Wallis pairwise test was used for alpha diversity in Qiime2. For presenting histogram and cladogram, statistical analyses were performed using linear discriminant analysis effect size (LEfSe) on Galaxy web application at a significance of $P < 0.05$.

4.3 Results

At d 21of incubation, most of the eggs in all the treatments group hatched within 12 hours and they were included in further growth performance study. The eggs that hatched late were included in hatchability calculation but were excluded from further experimental

procedures. The average percentage hatchability of xylotriose group was above 90% while it was lower in other groups but there was no significant difference (P >0.05) across treatments (Table 4.2). The growth performance parameters of ADFI, ADG and FCR during d 0-28 post-hatch were not different (P >0.05) across treatments (Table 4.3). The relative organ weights were calculated for liver, proventriculus, gizzard, drumstick and breast muscle with respect to the total live weight of birds on d 28 post-hatch (Table 4.4). The *in ovo* injection of oligosaccharides did not affect the relative weight of liver, proventriculus, drumstick and breast compared to NS and no injection control (P >0.05). However, the relative weight of gizzard was different across treatments and it was lowest in the xylotriose group and highest in mannotetraose group (P <0.05).

The villus height and crypt depth of ileum were not different (P >0.05) across treatments on hatch day, and on d 7, 21 and 28 post-hatch (Table 4.5). The villus height to crypt depth ratio was not different across treatments on d 7, 21 and 28 post-hatch (P >0.05). However, the villus height to crypt depth ratio was significantly different (P <0.01) on hatch day. The villus height to crypt depth ratio was highest for xylotetraose group and lowest for no injection control. The production of cecal acetate, butyrate and total SCFA on d 28 post-hatch were different (P <0.05) across treatments (Table 4.6). The *in ovo* injection of xylotriose enhanced the production of acetate, butyrate and total SCFA compared to both NS and no injection controls.

On hatch day, the level of gene expression of CD3 (a T-cell marker) was increased by xylotriose, while the level of IL-10 gene (a marker of anti-inflammatory cytokine) was decreased by mannotetraose (P <0.05) compared with NS and no injection controls (Figure 4.1). Xylotriose had a trend (P =0.074) on the reduction of IL-10 compared with controls. The immune markers of T-cell, B-cell, proinflammatory cytokines and anti-inflammatory cytokines were not expressed

differently (P >0.05) across treatments on d 7 post-hatch (Figure 4.2). *In ovo* injection of mannotetraose increased the frequency of observed OTUs compared with NS (P <0.05) and exhibited a trend (P =0.076) of increased OTUs compared with no injection control for cecal microbiota analyzed from cecal contents of d 28 post-hatch (Figure 4.3; Table 4.7). Mannotetraose also enhanced the frequency of differentially abundant (P <0.05) cecal microbial taxa including *Lactobacillales* and *Leuconostocaceae* compared with other treatments (Figure 4.4).

Table 4.2 Hatchability of eggs on d 21 of incubation in response to *in ovo* treatments.

Treatments	**%, Hatch**
Normal saline	79.3
Xylotriose	93.6
Xylotetraose	85.6
Mannotriose	82.9
Mannotetraose	82.0
No-injection	89.7
SEM (n=4)	8.67
P-value	0.86

Table 4.3 Effects of *in ovo* injection of oligosaccharides on growth performance of broilers from d 0-28 post-hatch.

	Treatments							
Variables	NS	X3	X4	M3	M4	NI	**SEM (n=5)**	**P-value**
ADFI (g/day)								
0-7 d	18	19	19	18	18	19	0.58	0.215
0-21 d	59	59	57	55	57	60	1.25	0.191
0-28 d	80	80	76	77	78	80	1.68	0.427
ADG (g/day)								
0-7 d	15	16	16	15	15	16	0.40	0.300
0-21 d	43	44	42	42	43	43	1.07	0.604
0-28 d	54	55	51	55	54	54	1.56	0.690
FCR								
0-7 d	1.20	1.20	1.17	1.17	1.18	1.18	0.04	0.996
0-21 d	1.37	1.34	1.37	1.32	1.32	1.40	0.03	0.137
0-28 d	1.49	1.48	1.48	1.41	1.45	1.48	0.04	0.747
ATW (g/bird)	1550	1569	1477	1572	1555	1556	43.26	0.666

ADFI- average daily feed intake, ADG- average daily gain, FCR-feed conversion ratio, ATW-average total weight, NS- normal saline, X3-xylotriose, X4-xylotetraose, M3-mannotriose, M4-mannotetraose, NI- no-injection.

Table 4.4 Effects of *in ovo* injection of oligosaccharides on relative organ weight (% of body weight) of digestive organ and meat portions at d 28 post-hatch.

	Treatments							
Relative weight (g, organ/body)	NS	X3	X4	M3	M4	NI	**SEM (n=5)**	**P-value**
Liver	3.12	2.95	3.06	3.04	3.15	2.89	0.11	0.557
Proventriculus	0.63	0.59	0.64	0.68	0.61	0.60	0.04	0.737
Gizzard	1.99^{ab}	1.90^{b}	2.21^{ab}	2.23^{ab}	2.28^{a}	2.20^{ab}	0.08	0.015
Drumstick	4.30	4.50	4.47	4.44	4.08	4.18	0.20	0.580
Breast	21.72	21.29	20.46	20.52	22.07	22.07	0.78	0.516

NS- normal saline, X3-xylotriose, X4-xylotetraose, M3-mannotriose, M4-mannotetraose, NI- no-injection.

Table 4.5 Effects of *in ovo* injection of oligosaccharides on ileum morphology of broiler chickens at hatch day, and d 7, 21 and 28 post-hatch.

	Treatments							
Parameters	NS	X3	X4	M3	M4	NI	**SEM (n=5)**	**P-value**
Hatch day								
VH,µm	371.0	410.8	405.6	412.9	421.8	330.7	27.5	0.232
CD, µm	69.5	58.7	53.4	54.9	71.0	65.8	5.1	0.128
VH: CD	5.5^{bc}	7.1^{abc}	7.8^{a}	7.6^{ab}	6.9^{abc}	5.1^{c}	0.5	0.006
7 d post-hatch								
VH,µm	533.7	475.8	517.0	492.2	486.0	502.3	29.2	0.752
CD, µm	81.4	78.5	84.5	78.8	79.4	85.0	2.7	0.362
VH:CD	6.6	6.1	6.2	6.3	6.2	6.0	0.3	0.854
21 d post-hatch								
VH,µm	744.5	722.4	767.9	739.4	761.1	720.1	30.2	0.831
CD, µm	101.2	99.5	109.8	103.8	99.9	102.1	3.1	0.229
VH: CD	7.4	7.4	7.1	7.3	7.7	7.1	0.3	0.701
28 d post-hatch								
VH,µm	793.2	936.0	861.0	919.4	804.8	875.7	52.3	0.323
CD, µm	99.4	104.8	99.7	103.8	103.0	101.5	2.7	0.659
VH: CD	8.1	9.0	8.8	9.0	8.0	8.7	0.5	0.475

VH, villus height; CD, crypt depth; VH: CD, villus height to crypt depth ratio.
NS- normal saline, X3-xylotriose, X4-xylotetraose, M3-mannotriose, M4-mannotetraose, NI- no-injection.

Table 4.6 Effects of *in ovo* injection of oligosaccharides on cecal short chain fatty acids (SCFA) in broilers at d 28 post-hatch.

	Treatments							
SCFA, µM/g	NS	X3	X4	M3	M4	NI	**SEM (n=5)**	**P-value**
Acetate	51.4^{b}	68.1^{a}	$61.^{ab}5$	58.9^{ab}	57.8^{ab}	51.0^{b}	3.50	0.019
Propionate	5.9	6.4	6.2	5.9	6.1	6.9	1.34	0.995
Butyrate	6.2^{b}	11.1^{a}	8.8^{ab}	6.1^{b}	7.0^{b}	7.1^{b}	0.92	0.006
Total SCFA	66.1^{b}	88.3^{a}	79.5^{ab}	73.3^{ab}	73.5^{ab}	67.8^{b}	4.31	0.015

NS- normal saline, X3-xylotriose, X4-xylotetraose, M3-mannotriose, M4-mannotetraose, NI- no-injection.

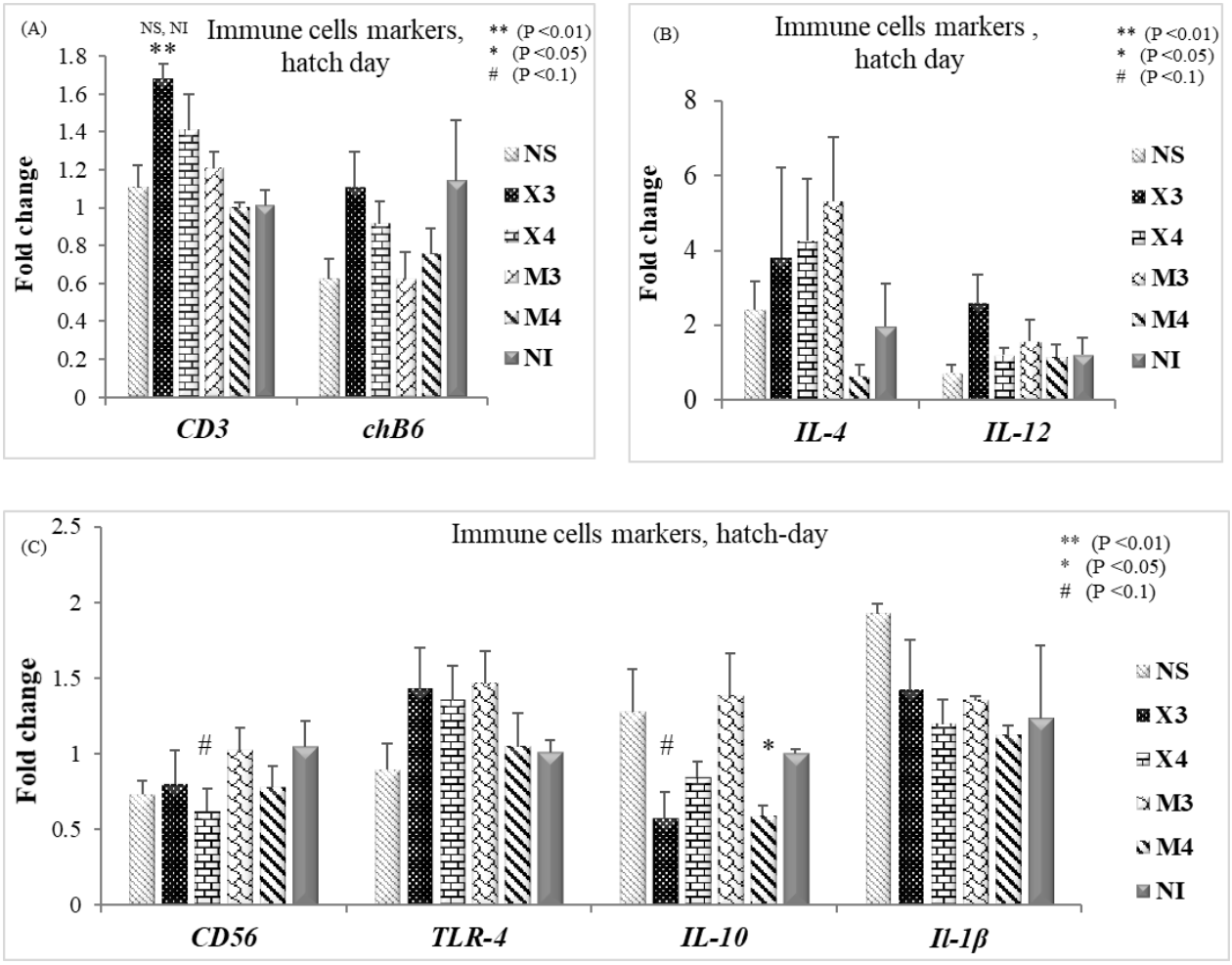

Figure 4.1 Effects of *in ovo* injection of oligosaccharides on gene markers of immune cells of broilers on the hatch day. The expression of each gene was examined using RT-qPCR and expressed as fold change ratio to the β-actin housekeeping gene, with the level being set to 1 in broilers from no injection group.

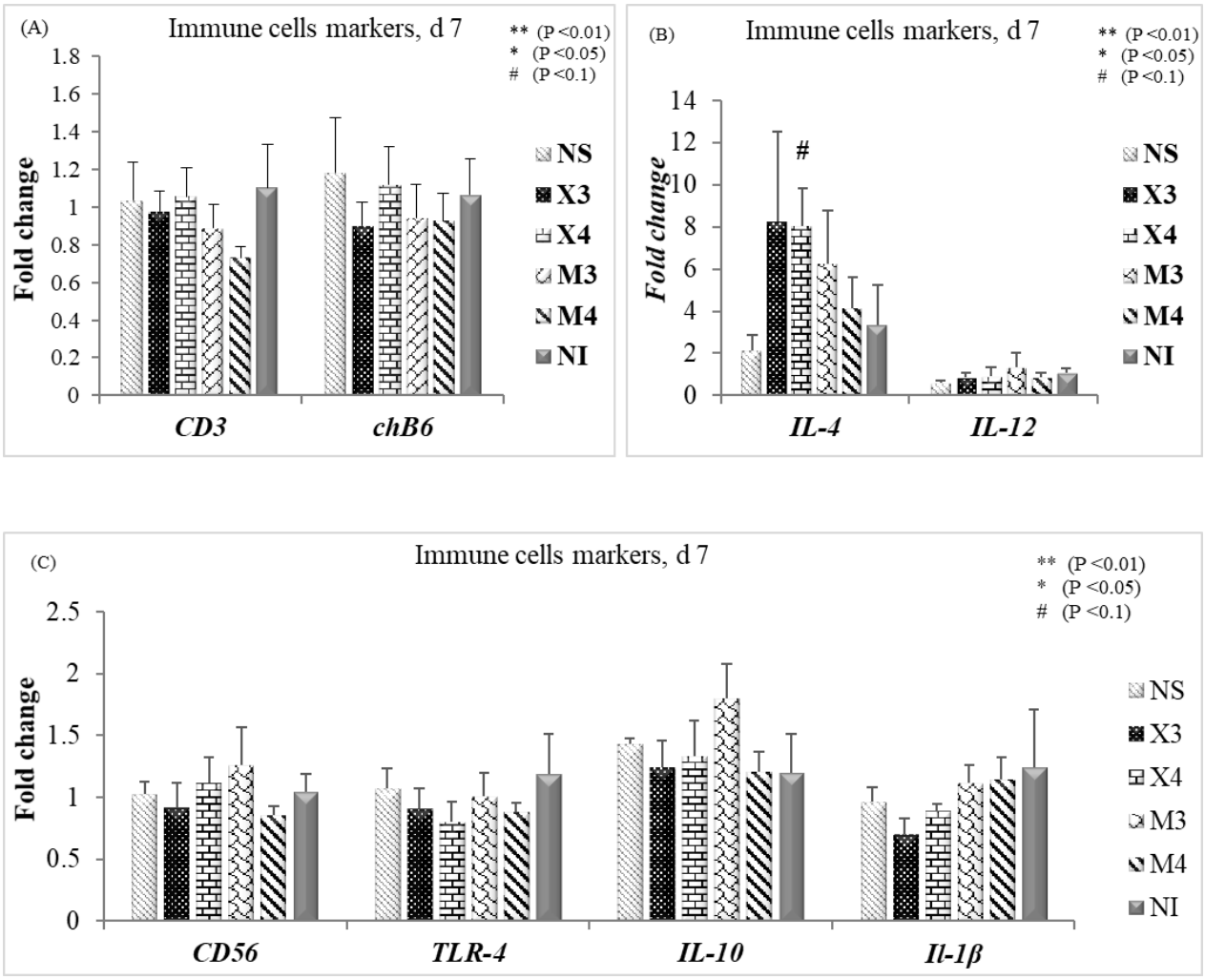

Figure 4.2 Effects of *in ovo* injection of oligosaccharides on gene markers of immune cells of broilers on d 7 post-hatch. The expression of each gene was examined using RT-qPCR and expressed as fold change ratio to the β-actin housekeeping gene, with the level being set to 1 in broilers from no injection group.

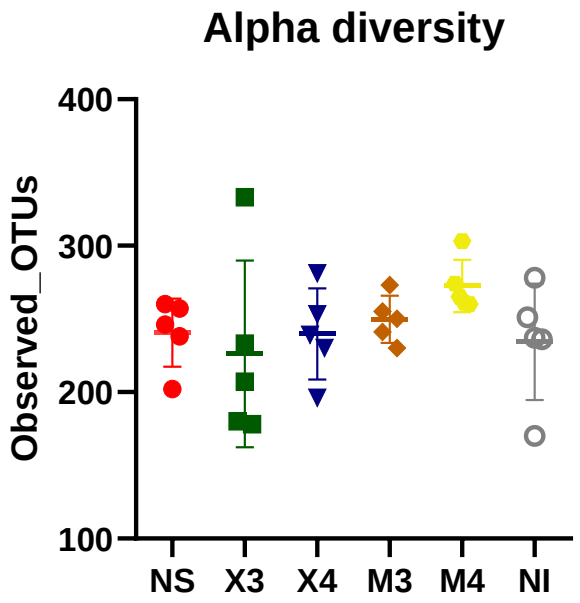

Figure 4.3 Alpha diversity analysis based on observed OTUs of different treatments at 2000 reads depth per sample of cecal contets of broilers at d 28 post-hatch in response to *in ovo* injection. Data represents mean ± SE. NS, normal saline; X3, xylotriose; X4, xylotetraose; M3, mannotriose; M4, mannotetraose; NI, no injection.

Table 4.7 Statistical analysis of alpha diversity based on observed OTUs of different treatments performed using Kruskal-Wallis test for cecal microbiota at d 28 post-hatch for *in ovo* injected broilers.

Group 1 (n=5)	Group 2 (n=5)	p-value
Control	Mannotetraose	0.076$^{\#}$
Mannotetraose	Mannotriose	0.047*
Mannotetraose	NS	0.012*
Mannotetraose	Xylotetraose	0.076$^{\#}$

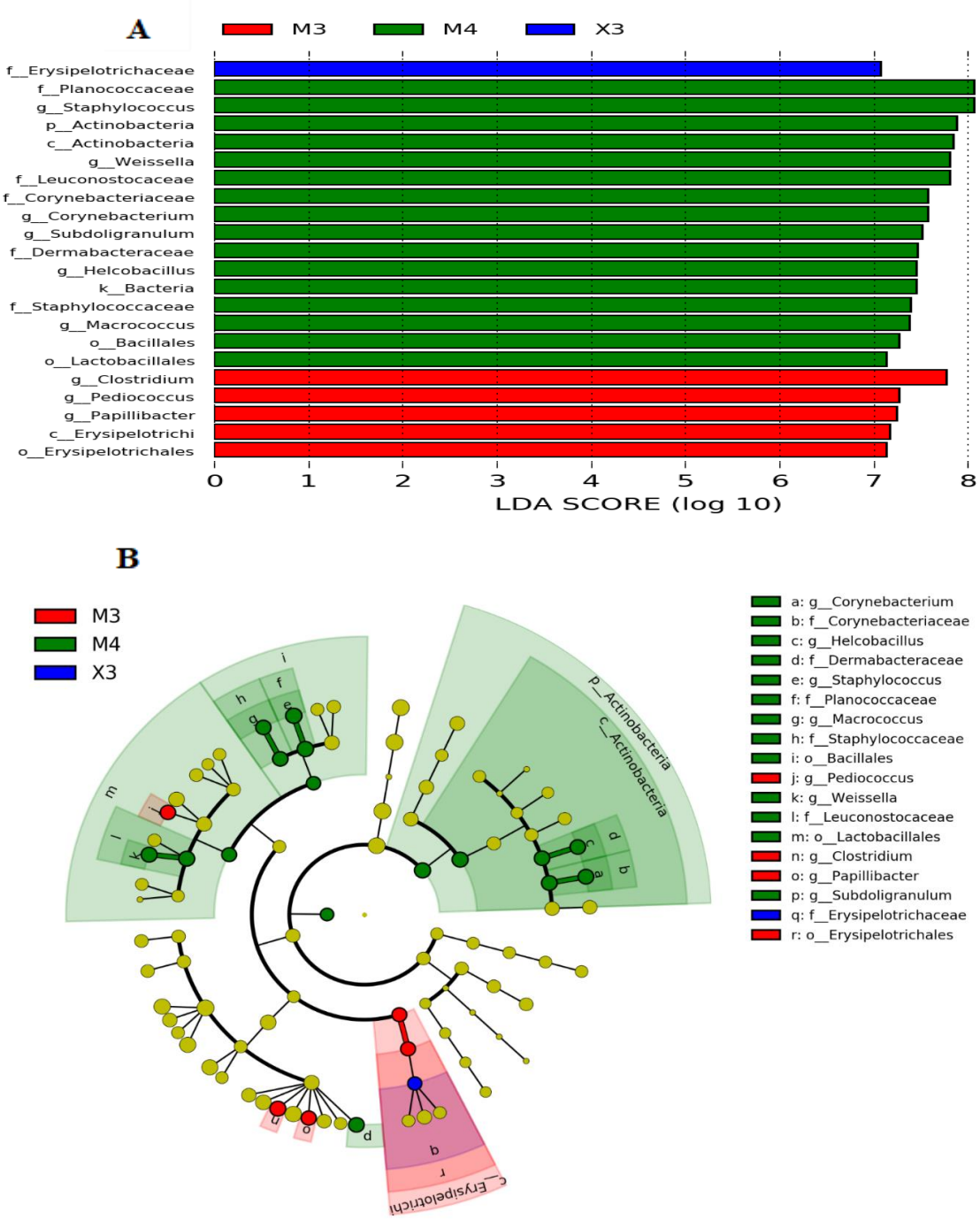

Figure 4.4 A) Histogram shows linear discriminant analysis (LDA) scores of taxa differentially abundant and B) Cladogram shows differentially abundant taxa (highlighted by small circles and by shading) at various taxonomic levels between different *in ovo* treatments in broilers at d 28 post-hatch. Statistical analyses were performed using linear discriminant analysis effect size (LEfSe). M3, mannotriose; M4, mannotetraose; X3, xylotriose.

4.4 Discussion

The hatchability was not different across treatments, which conveys the fact that no treatments had any negative impact on the livability of embryos. The growth performance and organ weights were not much influenced by the treatments. Similar to our finding, Maiorano et al. (2017) did not observe any significant improvement in the body weight and FCR of 42 d broilers in response to *in ovo* injection of trans-galactooligosaccharides or laminarin and fucoidan. The growth of broilers is more influenced by the density of nutrients in feed in a healthy flock. All the treatment groups were fed the same commercial diet throughout the growth period and the flocks remained unchallenged. The effect of improvement in other gut health parameters would therefore not have caused any major influence on the overall growth performance. The improvement in villus height to crypt depth ratio on hatch day by xylotriose was later leveled to other treatments with increasing age of the birds. However, the enhancement in the production of SCFA in response to xylotriose was evident at d 28 post-hatch. This suggests that the stimulant for ileal mucosa was removed with growth and passage of digesta, but it was adequate to cause a priming effect on gut microbiota to later utilize NSP from the diet for better fermentation.

Besides absorptive cells, broilers intestine also contains gut-associated lymphoid tissue (GALT) which consists of T and B-cells. It has been realized that such gut-associated immune cells can be modified by diet and intestinal microbiota (Honjo et al., 1993; Miyazaki et al., 2007), which could be a consequence of activation of dendritic cells in Peyer's patches that would then stimulate the circulating T-lymphocytes (Clancy, 2003). Xylotriose has also displayed its potential to stimulate cell-mediated immunity and support inflammatory response early on but the effect may not remain sustained if it is passed out of intestinal lumen along with

digesta. Likewise, Madej and Bednarczyk (2016) found that *in ovo* injection of trans-galactooligosaccharides prebiotics increased T-cells colonization in cecal tonsils at d 7, but the effect was lesser on d 21. They also reported that colonization of GALT by B-cells was more prominent in synbiotic groups compared with prebiotics. The early effect of prebiotics on immune cells is expected to be their direct effect on intestinal cells rather than being mediated through gut microbiota as the embryo's intestine is considered sterile and it takes some time for the establishment of stable gut microbiota (Cheled-Shoval et al., 2011; Yegani and Korver, 2008). Compared with XOS, mannotetraose did not exert stimulatory influence on immune cells, rather increased richness and differential abundance of bacterial OTUs in ceca of broilers. MOS supplementation in broiler feed in other studies has also been reported to have increased the counts of *Lactobacillus* and *Bifidobacteria*, while decreased that of *E. coli* (Baurhoo et al., 2007). It would be interesting to study the effect of mannotetraose on the gut microbiota of challenged flocks. Moreover, further investigations on the combined use of xylotriose and mannotetraose are necessary to define the aspects of the complementary effects of prebiotics. The results of this study indicate that the effect of xylotriose on immunity is transient, but its effect on fermentation is prolonged. To maintain an improved status of health and growth, a balance in the population of immune cells is required as immunodeficiency can render broilers vulnerable to many infectious diseases while an overactive immune response can cause autoimmune diseases.

4.5 Conclusion

This study provided some valuable insights into the functioning of prebiotics when introduced in the GIT of broiler through early feeding. It is intriguing to note that the enhancement of immunity during hatch must be the direct effect of oligosaccharides while the

increase in SCFA production requires the prebiotics to influence microbial fermentation. Further research on the mechanism and potential of prebiotic products is warranted before their extensive use alone or in combination with other additives as an alternative to AGPs.

CHAPTER 5: EFFECTS OF EARLY FEEDING WITH RESISTANT STARCH DURING POST-HATCH ON GROWTH PERFORMANCE AND GUT HEALTH PARAMETERS OF BROILERS

Abstract

Early feeding with special diets to chicks immediately after the hatch is becoming a popular and effective strategy to influence the overall growth and health of broilers. Resistant starch (RS) refers to the type of starch that resists digestion in upper GIT of host and can be fermented by the lower gut microbiota to produce short chain fatty acids (SCFA). This study investigated the effect of feeding RS type 2 (RS2) and type 4 (RS4) during the early post-hatch period on the parameters of growth, cecal fermentation, histomorphometry and immune parameters of Cobb 500 broilers. A total of 300 day-old chicks were randomly distributed to 30 cages, and 5 replicate cages were allocated to each of 6 treatments. The treatments included i) no feed (starved), ii) RS2, iii) RS4, iv) 50% commercial feed (CF) + 50% RS2, v) 50% CF + 50% RS4 (CF+RS), and vi) CF (control). The treatment diets were fed for two days post-hatch (48 hrs. ± 2 hrs.). Later, all birds were fed the same CF *ad libitum* for 21 d. The feed intake and body weight were recorded weekly to determine feed conversion ratio and growth performance. Ileal samples were collected on d 7 and 21 for histomorphometric and immune-related gene expression analysis. Simultaneously, the cecal digesta were collected for DNA extraction and genomic sequencing, and SCFA determination. At d 21, there was no difference in FCR among treatments ($P > 0.05$), but average daily feed intake (ADFI) and average daily gain (ADG) were higher in CF+RS2, CF+RS4 and CF groups compared with staved, RS2 and RS4 groups ($P < 0.01$). The treatments did not affect total cecal SCFA production at d 21 ($P > 0.05$). The ileal histomorphometric analysis at d 7 and 21 did not show a significant effect of any treatment on villus height, crypt depth, and their ratio ($P > 0.05$). It was found that RS4+CF group had higher expression of CD3 (a T-cell marker) genes while RS4 and RS4+CF groups exhibited a reduced level of IL-4 compared with control ($P < 0.05$) at d 7. At d 21, we did not find any differential

expression in the level of innate and adaptive immunity-related genes that were measured for T and B-lymphocytes, and pro- and anti-inflammatory cytokines ($P > 0.05$). A trend ($P = 0.083$) was observed for the Shannon Index of cecal microbiota diversity between RS2 and NF at d 21 post-hatch. RS2 also exhibited a significant ($P < 0.05$) Unweighted UniFrac metrics for beta diversity compared with NF, whereas a significant ($P < 0.05$) Bray Curtis metrics for beta diversity was observed between RS2+CF and NF group. The results indicate that the effect of early post-hatch feeding with RS on the immunity of chicks is transient, but the feeding of RS can have a prolonged effect on cecal microbiota diversity of broilers. Further research on the use of RS4 as a potential prebiotic that can act as an immunostimulant in broilers is necessary for its effective use in nutritional strategy to modulate the gut health of broilers.

Keywords: broilers, gut health, histology, immunity, resistant starch

5.1 Introduction

The perinatal period of early post-hatch is metabolically a very active period in broilers when chicks are shifting from lipid-based nutrition to carbohydrate diets. The digestive tract of embryo and chicks before the hatch is sterile and colonization by microbes starts immediately post-hatch. The microbes get entry into the GIT of chicks through air, feed and water intake. The colonization by beneficial microbes is critical for overall health and productivity of broilers (Jha et al., 2019). Early feed access and provision of a pre-starter diet containing essential nutrients is necessary for better growth and improved gut health, but the difference in productivity tends to disappear when birds are reared for longer duration in a non-challenged condition (Mateos et al., 2002). The use of antibiotic growth promoters (AGP) in broilers feed has been proven to improve the growth performance of broilers by suppressing the growth of pathogenic microbes in their gut. Compared to AGP, some phytogenic feed additives have also shown the promising result in increasing body weight, improving feed conversion ratio and boosting the establishment of beneficial microbial communities in broilers (Murugesan et al., 2015).

In response to increasing concerns of antibiotic growth promoters, alternatives to AGPs like prebiotics are being rapidly explored to support the growth of beneficial microbes (Chee et al., 2010). Prebiotics are non-digestible feed ingredients like peptides and carbohydrates which favors the growth of mainly non-pathogenic microbes in the lower gut of the host. Many forms of carbohydrates like oligosaccharides and resistant starch are potential candidates for use as prebiotics. Resistant starch (RS) that are being explored for prebiotic potential are either high amylose-based RS or retrograded starch or chemically modified starch. The contribution of RS to colonic fermentation is estimated to be greater than other non-starch polysaccharides (NSP) in human (Topping and Clifton, 2001). A diet high in resistant starch has also been reported to

modulate microbiota composition, enhance cecal and colonic SCFA concentration and influence gene expression in pigs (Haenen et al., 2013b).

The effect of inclusion of different prebiotics of NSP origin has been explored in broiler feeding and their responses in improving the gut health of broilers have also been encouraging in some studies. However, the use of RS in broiler feeding is limited and there is a lack of information on the influence of early feeding of RS on broiler performance and overall gut health parameters. The objective of this study was to investigate the effect of early feeding of RS type 2 and type 4 on growth performance, immunity, cecal fermentation and ileal histomorphometry and microbiota diversity of broilers.

5.2 Materials and methods

All animal care procedures were approved by the Institutional Animal Care and Use Committee of the University of Hawaii.

5.2.1 Experimental design and treatments

A total of 304 day-old chicks (Cobb 500) were obtained from a commercial hatchery (Asagi Hatchery Inc., Honolulu, HI). Four birds were randomly selected, and blood, liver, spleen, ileum and cecum samples were collected on d 0. The remaining birds were weighed, tagged and randomly allocated to one of 30 cages (10 birds/cage). Out of 30 cages, five replicate cages were assigned to one of six treatments. The treatments included i) no feed (NF), ii) RS type 2 (RS2), iii) RS type 4 (RS4), iv) 50% commercial feed (CF) + 50% RS2, v) 50% CF + 50% RS4, and vi) CF. The treatments were continued for 2 days of age. From d 3, all the groups were fed same commercial starter feed till d 21 post-hatch. Birds in all the cages were raised under standard commercial broiler rearing environment (temperature, humidity, and light). The

temperature in the first week was maintained at 35ºC and gradually decreased to 28ºC by the end of the third week. The birds were fed *ad libitum* and were provided unrestricted access to water.

5.2.2 Growth performance

Bodyweight and feed consumption of the birds were measured by cage on d 7, 14, and 21 post-hatch. The data of feed consumption and weekly weight of broilers were used to calculate average daily feed intake (ADFI), average daily gain (ADG) and feed conversion ratio (FCR) for each week and for the total period. Feed wastage and bird's mortality were recorded daily, and their feed consumption and FCR were adjusted for feed wastage and mortality.

5.2.3 Sample collection for histology, RNA isolation, short chain fatty acid (SCFA), and microbiota analysis

On d 7 and 21, sections of 1 cm from ileum (defined as a section 1 cm distal to Meckel's diverticulum and 1 cm anterior to ileocecal junction) were collected from 5 birds per treatment (1 bird per replicate cage) after euthanasia and the tissues were fixed in 10% neutral buffered formalin (NBF) which was later preserved in 70% ethanol. On d 7 and 21, small sections (50-100 mg) of ileum were also collected in cryovials and snap-frozen in liquid nitrogen, and later stored at -80°C until RNA isolation. On d 21, cecal contents were collected from birds from each replicate pen and stored at -20°C from SCFA analysis and snap-frozen in liquid nitrogen and stored at -80°C for further analysis of microbiota.

5.2.4 Ileal mucosa histomorphometry

The ileal samples (n= 5/treatment) fixed in NBF and preserved in 70% ethanol were outsourced to histology core facility at John A. Burns School of Medicine for embedding, sectioning and staining with Hematoxylin and Eosin (H&E). Six replicate sections of each sample were cut in 5 μm thickness and mounted on slides. After staining, the slides were

observed under 8X objectives of an upright microscope (Olympus BX43, Olympus Co, Tokyo, Japan) and villus height and crypt depth length were recorded using image processing and analysis system of Infinity Analyze software (Lumenera Corporation, Ottawa, ON, CA). Villus height and crypt depth were recorded from 3 sets of villus and crypt from each section of 6 sections from each replicate samples. The average measurement was calculated from all readings per sample replicate and villus height to crypt depth ratio was also determined. The data were presented as average for each treatment along with their standard error.

5.2.5 Determination of SCFA by gas chromatography

The calibration standards were prepared at different levels of concentration ranging from 0 to 8 mM for all individual SCFA from a standard stock of volatile free acid mix (Supelco®, Bellefonte, PA) containing 10 mM of each SCFA (formic, acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, hexanoic and n-caproic). All calibration standards contained 100 µl of 25% metaphosphoric acid (MPA) and 100 µl of 48.95 mM trimethylacetic acid (TMA, Pivalic acid from Sigma-Aldrich, St. Louis, MO) in a total volume of 1500 µl prepared in microcentrifuge tubes.

For sample preparation, at first 1 g of cecal content was mixed with 1 ml of distilled water in a centrifuge tube, vortexed and then centrifuged at 10, 000 ×g for 5 minutes at 4°C. Following centrifugation, 400 µl of supernatant was transferred into a new microcentrifuge tube containing 100 µl of 25% MPA and 100 ul of 48.95 mM TMA, and the final volume was made 1500 µl by adding distilled water. Next, the mixture was vortexed and centrifuged at 13,000 ×g for 10 minutes at 4°C. Following centrifugation, approximately 1000 µl of supernatant was transferred to a GC vial sealed using septa and screw cap and was then loaded on the carousel of autosampler for injection.

For determination of individual SCFA, the standards and samples were loaded on GC fitted carousel and the run method was set according to Zhao et al. (2006). Briefly, helium was supplied as a carrier gas at a flow rate of 14.5 ml/min and run time was set for 17.5 min. The initial oven temperature was 100°C held for 0.5 min, then ramped to 180°C at 8°C /min and held for 1 min, then again raised to 200°C at the rate of 20°C /min where it was finally maintained for 5 min. The temperature of the injector port was set at 200°C and that of detector port was set at 240°C. The flow rates of air, hydrogen and makeup gas (nitrogen) were 300, 30 and 20 ml/min respectively. The samples injection volume was kept 0.5 µl in order to reduce the possibility of backflash and a large expansion of water used as a solvent. Isopropyl alcohol and distilled water were used as syringe wash solvent. Data handling and processing were performed on Chromeleon™ 7.2 software (Thermo Scientific, US). The processing of the sample was done after integrating the chromatogram for specific peaks and then comparing the peak area of each individual SCFA with the respective calibration curve of their standards.

5.2.6 RNA isolation, reverse transcription and real-time quantitative PCR

5.2.6.1 Total RNA isolation from ileal tissue

Ileal tissue from d 7 and 21 stored in cryovial at -80°C were transferred to dry ice for easy handling of tissue for RNA extraction. Total RNA from ileal tissue of broilers were isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. The RNA isolation protocol included the following sequential steps: ileal tissue homogenization and lysis, RNA extraction using chloroform, triple-phase separation, RNA precipitation, RNA pellet washing, RNA pellet drying and resuspension in nuclease-free water. First, 9-11 Zirconia beads (BioSpec Products, Bartlesville, OK, USA) of 2.3 mm were taken in a safe lock nuclease-free Eppendorf tube. A 300 µl of TRIzol® reagent was then added to the tube containing beads. A

frozen ileal tissue (50-100 mg) was then dropped in the tube to be immersed in the TRIzol®. The tissue was then homogenized by bead beating on a vertical Bullet Blender® (Next Advance, Inc. Troy, NY) at speed 8 for 2-3 minutes. The microcentrifuge tubes containing homogenate and beads were then centrifuged at 10, 000 rpm for 1 minute at 4°C. Approximately 250 µl of the supernatant was then transferred to a new microcentrifuge tube containing 750 µl of TRIzol®. The tube containing supernatant from homogenate and fresh TRIzol® was mixed gently and allowed to stand for 5 minutes at room temperature. Then, 0.2 ml chloroform per 1 ml of TRIzol® was added to the homogenate supernatant, vortexed briefly and allowed to incubate for 5 minutes at room temperature. The resultant mix was then centrifuged at 10, 000 rpm for 15 minutes at 4°C. Following centrifugation, the solution distinctly separated into three layers: an upper aqueous containing total RNA, the bottom organic phase containing protein and the middle interface layer containing DNA. The upper layer was pipetted in a new microcentrifuge tube without disturbing the interface layer and leaving some aqueous layer on top of the middle interface to ensure sufficiently pure RNA extraction. The extracted RNA was precipitated by adding 0.5 ml isopropanol to the aqueous phase followed by gentle mixing, incubation for 5 minutes and centrifugation 10, 000 rpm for 10 minutes at 4°C. The supernatant solution was aspirated, and the pellet was washed with 1 ml 75% ethanol to remove any salts. Subsequently, the microcentrifuge tube containing ethanol was centrifuged for 5 minutes at 14, 000 rpm at 4°C and the supernatant was discarded. The RNA pellet was dried for 10 minutes at room temperature by covering with sterile filter paper.

The RNA pellet was resuspended in nuclease-free water (Thermo Scientific, Waltham, MA) after the pellet was optimally dry and then the tubes were incubated on a block heating plate at 60°C for 10 minutes. The concentration of the isolated RNA was measured on a

NanoDrop one microvolume UV spectrophotometer (Thermofisher Scientific, NY, US). The quality and integrity of RNA was assessed by running it through 2% agarose gel stained with ethidium bromide and observation of 28S and 18S bands ensured that the isolated RNA was intact and not degraded. The RNA samples were diluted to a concentration of 1000 ng/10 µl for cDNA synthesis and stored at -80°C until further processing.

5.2.6.2 Complementary DNA (cDNA)synthesis

The synthesis of the first-strand cDNA was performed by reverse transcription of 1 µg total RNA (20 µl reaction of RT mixture) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The RNA samples used to synthesize cDNA was all normalized at a concentration of 100 ng/µl. A 10 µl of reverse transcriptase (RT) mix was prepared by combining 2 µl of 10X RT Buffer, 0.8 µl of 25X dNTP (100 nM), 2 µl of 10X RT Random Primer and 1 µl of Multiscribe Reverse Transcriptase enzyme (Applied Biosystems, Foster City, CA).

The total reaction mix was prepared by mixing 10 µl of RNA sample and 10 µl of freshly prepared RT mix in PCR tubes. The tubes were loaded on a thermal cycler for the synthesis of single-stranded cDNA. The run program of thermal cycler was set at 25°C for 10 minutes followed by 37°C for 120 minutes then 85 °C for 5 minutes and a final hold at 4°C. The newly synthesized cDNA (20 µl) in the PCR tube was then centrifuged briefly to remove the condensate on the inner side of tube lid and was diluted with 480 µl of nuclease-free water to yield 25X dilution. The diluted cDNA was then divided into two microcentrifuge tubes as stock and working solution and stored at - 20°C until preparation for qPCR assay.

5.2.6.3 Quantitative real-time PCR (qPCR) assay

The qPCR assay was conducted on a StepOne™ Plus real-time PCR system (Applied Biosystems, Foster City, CA) in a 10 µl reaction mixture containing 3 µl of sample cDNA and 7 µl of the qPCR mix. The master mix was prepared by mixing 5 µl of PowerUP SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and 1 µl each of forward and reverse primers specific to the target gene markers. The primers used in this study was designed on the NCBI primer blast tool and used from a previous study (Berrocoso et al., 2017). The final qPCR mixture consisting of 7 µl of master mix (500 nM of final primer concentration) and 3 µl of the individual sample was pipetted into a 96-well microtiter plate and sealed with clear optical adhesive films (Applied Biosystems, Foster City, CA). For running qPCR program, a run template file was designed on the StepOne™ Plus software v 2.3 (Applied Biosystems, Foster City, CA) with corresponding wells marked for the target gene and specific samples loaded on the PCR plate. The PCR plate was centrifuged for 20-25 seconds on a microplate centrifuge to ensure proper mixing and to get rid of surface air bubbles in the well. The PCR plate was then inserted into the StepOne™ Plus machine for running PCR cycle. The amplification conditions were set as 50°C for 2 minutes, 95°C for 2 minutes followed by 40 cycles of 95°C for 15 seconds for denaturation, 60°C for 15 seconds for annealing and 72°C for 1 minute for the extension. A melting curve was generated to confirm the sequence-specific PCR products and primer efficiency. After each run, the data was downloaded for analysis and PCR plates were stored at 4°C for validation of PCR amplified products by running 2% agarose gel electrophoresis. The housekeeping gene Beta-actin (β-actin) was analyzed in triplicate and target genes were analyzed in duplicate and an average value was taken for each experimental replicate. The expression level of each gene was recorded for the cycle of threshold (Ct) values generated from the qPCR run analysis. The fold change for each gene was calculated using 2 exponential negative delta

delta Ct ($2^{-\Delta\Delta Ct}$) method. The calculation was done from the Ct value of experimental tested (ET), experimental control (EC), experimental tested housekeeping (ETH) and experimental control housekeeping (ECH). The difference between ET and ETH (ΔCtT = ET-ETH), and EC and ECH (ΔCtC = EC-ECH) were calculated. The difference of ΔCtT and ΔCtC gave rise to ΔΔCt. The fold change value was presented as average with standard error on the bar diagram.

5.2.7 DNA extraction, 16S rRNA amplicon sequencing and data analysis

5.2.7.1 DNA extraction from cecal digesta

A total of 210 mg of frozen cecal contents from each replicate samples were taken in a sterile Eppendorf tube and placed on ice. The reagents from QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) were used to extract DNA from the collected cecal contents according to the manufacturer's instructions. Briefly, 1 ml InhibitEX Buffer was added to each tube containing cecal contents and vortexed vigorously for 1-2 minutes to homogenize the contents. The homogenized samples in the tubes were then centrifuged at 20,000 x g for 1 min to pellet the digesta particles. A 600 µl of the supernatant was taken in a new 2 ml tube containing 25 µl proteinase K and 600 µl of Buffer AL was added to it. The mixture was in the tube was vortexed for 15 seconds. The tubes were then incubated at 70°C for 10 min and centrifuged briefly to collect the condensed drops on the lid of tubes. A 600 µl of ethanol (96–100%) was added to the lysate and mixed by vortexing. The lysate was then loaded slowly (600 µl at a time) on the QIAamp®spin column fitted on a 2 ml collection tube and centrifuged at full speed for 1 min and repeated until all the lysate was loaded. The QIAamp® spin column was then washed with 500 µl Buffer AW1 by centrifuging for 1 min. The filtrate in the collection tube was discarded and the QIAamp® spin column was fitted on a new 2 ml collection tube. A 500 µl of Buffer AW2 was then added to the QIAamp® spin column and centrifuged for 3 min. The filtrate

and the collection tube were discarded and the QIAamp® spin column was fitted on a new 2 ml collection tube and dried by centrifuging for 3 minutes. The QIAamp® spin column was then fitted on a new 1.5 ml Eppendorf tube and 200 µl of Buffer ATE was added directly on the QIAamp® spin column membrane. The column was incubated for 1 min at room temperature and then centrifuged at full speed for 1 minute to elute DNA. The quality of the extracted DNA was checked by running 2 µl of the aliquot on 1% agarose gel containing ethidium bromide and the bands were visualized under UV. The concentration of the DNA in ATE buffer was measured on NanoDrop one microvolume UV spectrophotometer (Thermofisher Scientific, NY, US). The final DNA concentration of all the samples was normalized to 10 ng/µl using nuclease-free water. The normalized samples were store at -20°C and transported on ice to a sequencing core facility at the University of Hawaii at Manoa for Illumina MiSeq sequencing.

5.2.7.2 Amplification of V3-V4 region of 16S rRNA gene by Next Generation Illumina sequencing

We sequenced V3 and V4 hypervariable regions of the 16S rRNA gene. For 16S library preparation, amplicon PCR was carried out using Platinum® Taq DNA Polymerase High-Fidelity (Invitrogen, Life Technologies Corporation, Grand Island, NY). The specific sequence of 16S rRNA gene was amplified from genomic DNA using forward primer (5′-CCTACGGGNGGCWGCAG-3′) and reverse primer (5′-GACTACHVGGGTATCTAATCC- 3′) according to Illumina MiSeq protocol (Klindworth et al., 2013). The overhang forward (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′) and reverse (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′) adapter sequences were added to 5′ end of the locus-specific primers. The amplicon PCR cycle consisted of initial denaturation step of heating PCR plate at 95°C on a thermal cycler for 3 minutes followed by 35 cycles of

95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and an extension step at 72°C for 5 minutes, which was then followed by a hold at 4°C. The PCR amplicon was run on 1% agarose gel to ascertain the size of the amplified product to be around 550 bp. The PCR products were then cleaned according to Illumina 16S rRNA PCR Clean-Up protocol with some modifications, where Mag-Bind Total Pure NGS beads (Omega Bio-tek) were used instead of AMPure XP beads and 70% ethanol instead of 80% ethanol. After clean-up of the amplicon PCR product, an index PCR was performed for multiplexing by attaching Nextera XT dual indices and Illumina sequencing adapters to the target amplicon. The PCR conditions used for index PCR were 95°C for 3 minutes; 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds; followed by an extension at 72°C for 5 minutes and a final hold at 4°C. The cleaning of the index PCR product was carried similarly as done in the previous clean-up step. After PCR clean-up, the libraries were quantified using the Quant-iT PicoGreen dsDNA Assay Kit, normalized, and pooled. The library pools (0.7 ng) were run on a Bioanalyzer High Sensitivity DNA chip to verify the size of the final library to be around 630 bp. The normalized and pooled amplicons were sequenced on the Illumina MiSeq desktop sequencer (2×300 bp paired-end run) at the University of Hawaii at Manoa Advanced Studies in Genomics, Proteomics and Bioinformatics core facility.

5.2.7.*3* Metagenomic sequencing and data analysis

Demultiplexed paired-end R1 and R2 sequencing reads (approximately 300 bp in length) for each sample was generated on the MiSeq platform and downloaded via Illumina BaseSpace® Sequence Hub. The data processing was performed on Quantitative Insights Into Microbial Ecology (QIIME™ version 2.0 release 2019.4; Bolyen et al., 2019). The fastq.gz files for each sample were unzipped and imported using qiime tools import command in Casava 1.8 paired-end

demultiplexed fastq format. Denoising, trimming and filtering of phiX reads along with chimeric sequences were done using DADA2 pipeline. The rooted and unrooted tree for phylogenetic diversity analysis was generated using align-to-tree-mafft-fasttree pipeline from qiime phylogeny plugin. Alpha and beta diversity analysis were conducted by applying core-metrics-phylogenetic method from qiime diversity plugin with a sampling depth of 10,000 frequency per sample. For alpha diversity, observed OTUs and Shannon Index were determined and for beta diversity, unweighted UniFrac and Bray Curtis metrics were evaluated.

5.2.8 Statistical analysis

All the variables were compared among treatments using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) for growth performance, SCFA and ileal histomorphometry. Differences among treatment means were considered significant at $P < 0.05$. Significant differences between treatment means were separated by Tukey test using pdmix macro of SAS. For immune gene expression data, all the fold change was log-transformed and test variables were compared with control variables by TTest procedure of SAS. For microbial diversity analysis, Kruskal-Wallis pairwise test was used for alpha diversity and pairwise permanova was used for beta diversity in Qiime2.

5.3 Results

In the first week, there was a difference ($P < 0.05$) in ADFI, ADG and FCR among treatments (Table 5.1). The group fed CF from d 0 had higher ADFI and ADG compared with NF and only RS fed groups from d 0-7, while FCR of CF group was only significantly ($P < 0.05$) lower than RS4 group. For the total period (d 0-21), ADFI and ADG were significantly ($P < 0.01$) affected by treatments but FCR was not different among treatments ($P > 0.05$). In total period, ADFI and ADG in CF group were different from NF and only RS fed groups but not from

RS+CF fed groups. Similarly, the average total weight (ATW) of the CF group was only different ($P < 0.05$) from NF and RS4 groups, but not from RS+CF fed groups. There was no effect ($P > 0.05$) of treatments on ileal histomorphometry of villus height, crypt depth and their ratio on d 7 and 21 post-hatch (Table 5.2). The cecal production of acetate, propionate, butyrate and total SCFA at d 21 was not different ($P > 0.05$) across treatments (Table 5.3).

At d 7 post-hatch, RS4+CF group had higher ($P < 0.05$) expression of CD3 (a T-cell marker) genes while RS4 and RS4+CF groups had a reduced ($P < 0.05$) level of IL-4 (a T-cell differentiation cytokine) compared with NF and CF controls (Figure 5.1). At d 21, RS4 group exhibited a trend ($P < 0.1$) for CD3 marker compared with CF control (Figure 5.2). For other immune-related genes measured at d 21, no RS or RS+CF groups had any differential expression in the level of innate and adaptive immunity genes or pro- and anti-inflammatory cytokines ($P > 0.05$).

A trend ($P = 0.083$) of low observed OTUs was found for RS2+CF compared with RS4+CF group cecal microbiota at d 21 post-hatch (Figure 5.3). RS2 also exhibited a trend ($P = 0.083$) for the lower value of the Shannon Index compared with the NF group. A significant ($P < 0.05$) Unweighted UniFrac distance was observed between RS2 and NF, and RS2+CF and NF, while a significant ($P < 0.05$) Bray Curtis metrics was also observed for beta diversity between RS2+CF and NF group (Figure 5.3; Table 5.4). There were trends observed for Unweighted Unifrac for RS4+CF vs NF ($P = 0.081$), and Bray Curtis distance for RS4 vs NF ($P = 0.076$) and RS2 vs RS4 ($P = 0.060$).

Table 5.1 Effects of early feeding of resistant starch (RS) on growth performance of broilers from d 0-21 post-hatch.

	Treatments							
Variables	NF	RS2	RS4	RS2+CF	RS4+CF	CF	SEM (n=5)	P-value
ADFI (g/day)								
0-7 d	12^{b}	13^{b}	12^{b}	18^{a}	17^{a}	19^{a}	0.54	<.0001
8-14 d	44^{c}	45^{bc}	45^{bc}	50^{a}	49^{ab}	49^{ab}	1.09	0.001
15-21 d	89	88	87	91	89	92	2.13	0.529
0-21 d	48^{b}	48^{b}	48^{b}	53^{a}	52^{ab}	53^{a}	0.94	0.0004
ADG (g/day)								
0-7 d	10^{b}	11^{b}	10^{b}	16^{a}	16^{a}	18^{a}	0.52	<.0001
8-14 d	35	35	35	38	39	37	1.26	0.100
15-21 d	63	64	64	65	64	67	2.12	0.892
0-21 d	36^{b}	37^{b}	36^{b}	40^{ab}	40^{ab}	41^{a}	0.90	0.003
FCR								
0-7 d	1.18^{ab}	1.16^{ab}	1.19^{a}	1.14^{ab}	1.11^{ab}	1.06^{b}	0.03	0.028
8-14 d	1.26	1.26	1.27	1.29	1.25	1.32	0.03	0.595
15-21 d	1.40	1.38	1.36	1.40	1.39	1.39	0.03	0.924
0-21 d	1.34	1.32	1.32	1.33	1.31	1.32	0.02	0.852
ATW (g/bird)	803^{b}	817^{ab}	811^{b}	880^{ab}	878^{ab}	898^{a}	18.85	0.003

ADFI- average daily feed intake, ADG- average daily gain, FCR-feed conversion ratio, ATW-average total weight, NF-no feed/starved, RS2-resistatnt starch type 2, RS4-resistant starch type 4, RS2+CF-50% resistant starch type 2 + 50% commercial feed, RS4+CF-50% resistant starch type 4 + 50% commercial feed, CF-commercial feed.

Table 5.2 Effects of early feeding of resistant starch (RS) on ileal mucosal morphology of broilers at d 7 and 21 post-hatch.

	Treatments							
Parameters	NF	RS2	RS4	RS2+CF	RS4+CF	CF	**SEM (n=5)**	**P-value**
7 d post-hatch								
VH,µm	492.8	499.0	504.5	525.3	510.1	495.0	19.98	0.870
CD, µm	78.9	81.5	79.7	84.1	81.7	82.3	4.46	0.969
VH:CD	6.3	6.2	6.4	6.4	6.4	6.1	0.30	0.953
21 d post-hatch								
VH,µm	790.2	788.2	788.5	690.5	782.9	785.3	31.22	0.198
CD, µm	91.9	90.2	90.9	84.0	87.1	90.9	3.18	0.497
VH:CD	8.7	8.8	8.8	8.3	9.1	8.8	0.32	0.719

VH, villus height; CD, crypt depth; VH:CD, villus height to crypt depth ratio. NF-no feed/starved, RS2-resistatnt starch type 2, RS4-resistant starch type 4, RS2+CF-50% resistant starch type 2 + 50% commercial feed, RS4+CF-50% resistant starch type 4 + 50% commercial feed, CF-commercial feed.

Table 5.3 Effects of early feeding of resistant starch on cecal short chain fatty acids (SCFA) in broilers at d 21 post-hatch.

	Treatments							
SCFA, µM/g	NF	RS2	RS4	RS2+CF	RS4+CF	CF	**SEM (n=5)**	**P-value**
Acetate	61.2	71.2	73.9	65.8	68.2	64.1	5.31	0.577
Propionate	4.0	4.0	4.2	2.9	4.1	4.1	0.60	0.621
Butyrate	9.2	10.3	11.5	7.9	9.6	9.6	1.63	0.751
Total SCFA	76.9	88.2	91.7	79.1	84.5	79.9	6.97	0.640

NF-no feed/starved, RS2-resistatnt starch type 2, RS4-resistant starch type 4, RS2+CF-50% resistant starch type 2 + 50% commercial feed, RS4+CF-50% resistant starch type 4 + 50% commercial feed, CF-commercial feed.

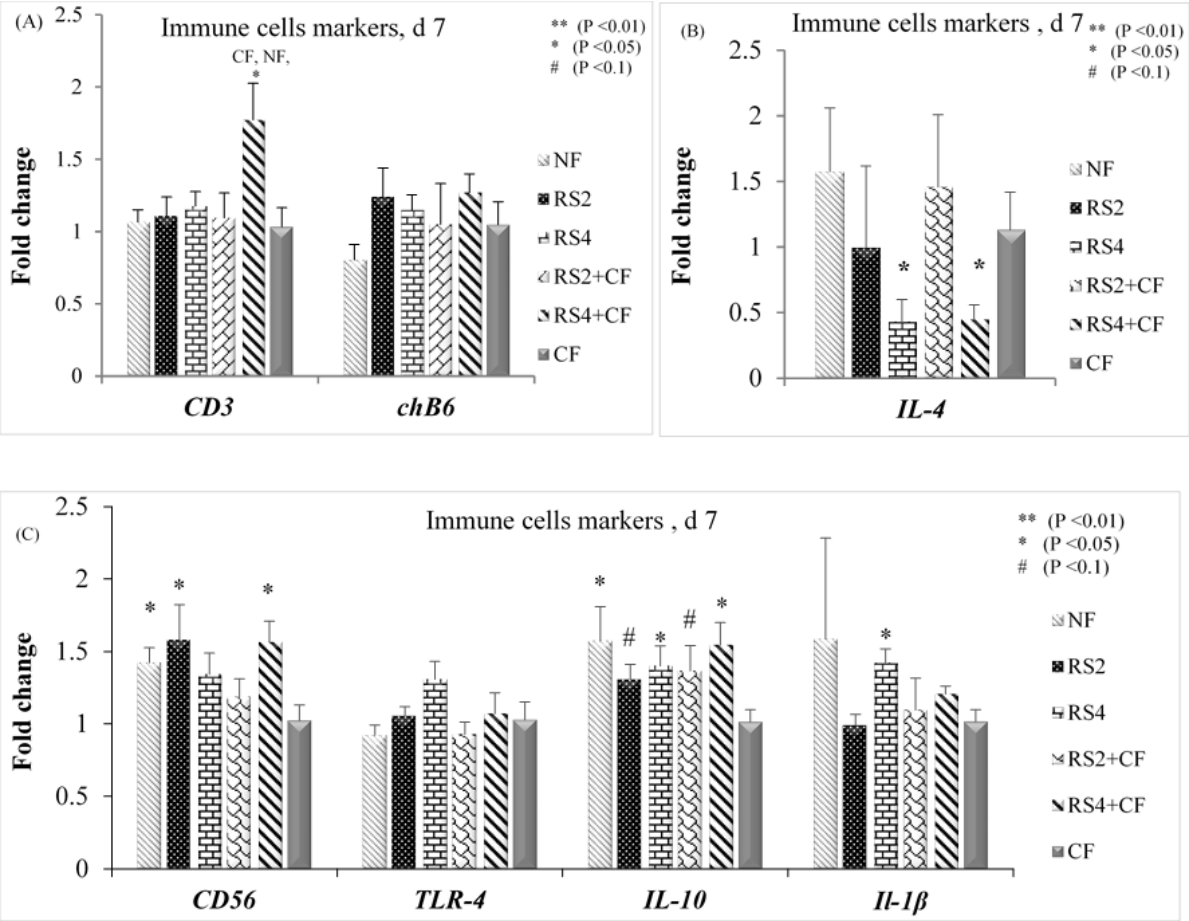

Figure 5.1 Effects of early feeding of resistant starch on gene markers of immune cells of broilers on d 7 post-hatch. The expression of each gene was examined using RT-qPCR and expressed as fold change ratio to the β-actin housekeeping gene, with the level being set to 1 in broilers from commercial feed (CF) groups. NF-no feed/starved, RS2-resistant starch type 2, RS4-resistant starch type 4, RS2+CF-50% resistant starch type 2 + 50% commercial feed, RS4+CF-50% resistant starch type 4 + 50% commercial feed, CF-commercial feed.

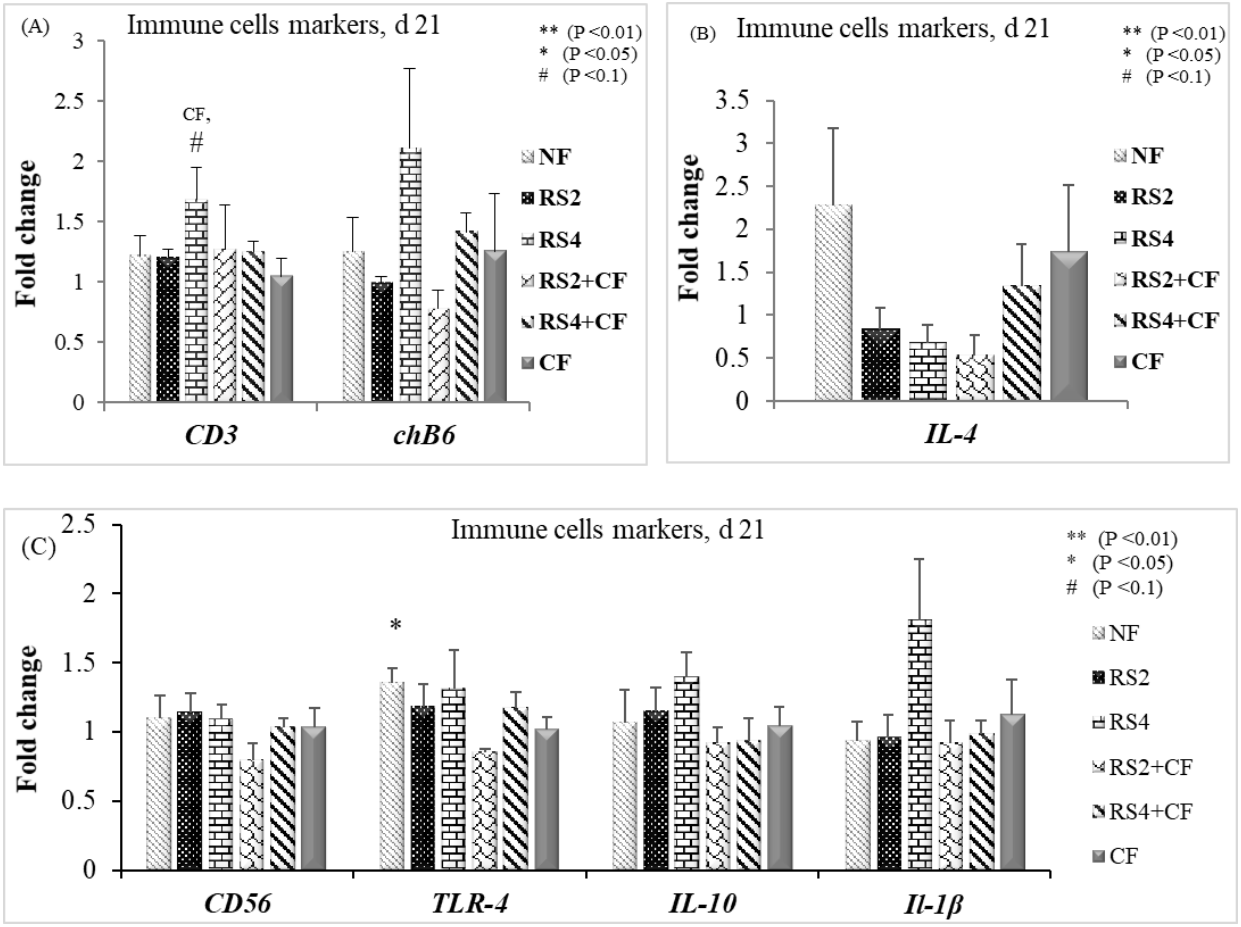

Figure 5.2 Effects of early feeding of resistant starch on gene markers of immune cells of broilers on d 21 post-hatch. The expression of each gene was examined using RT-qPCR and expressed as fold change ratio to the β-actin housekeeping gene, with the level being set to 1 in broilers from commercial feed (CF) groups. NF-no feed/starved, RS2-resistant starch type 2, RS4-resistant starch type 4, RS2+CF-50% resistant starch type 2 + 50% commercial feed, RS4+CF-50% resistant starch type 4 + 50% commercial feed, CF-commercial feed.

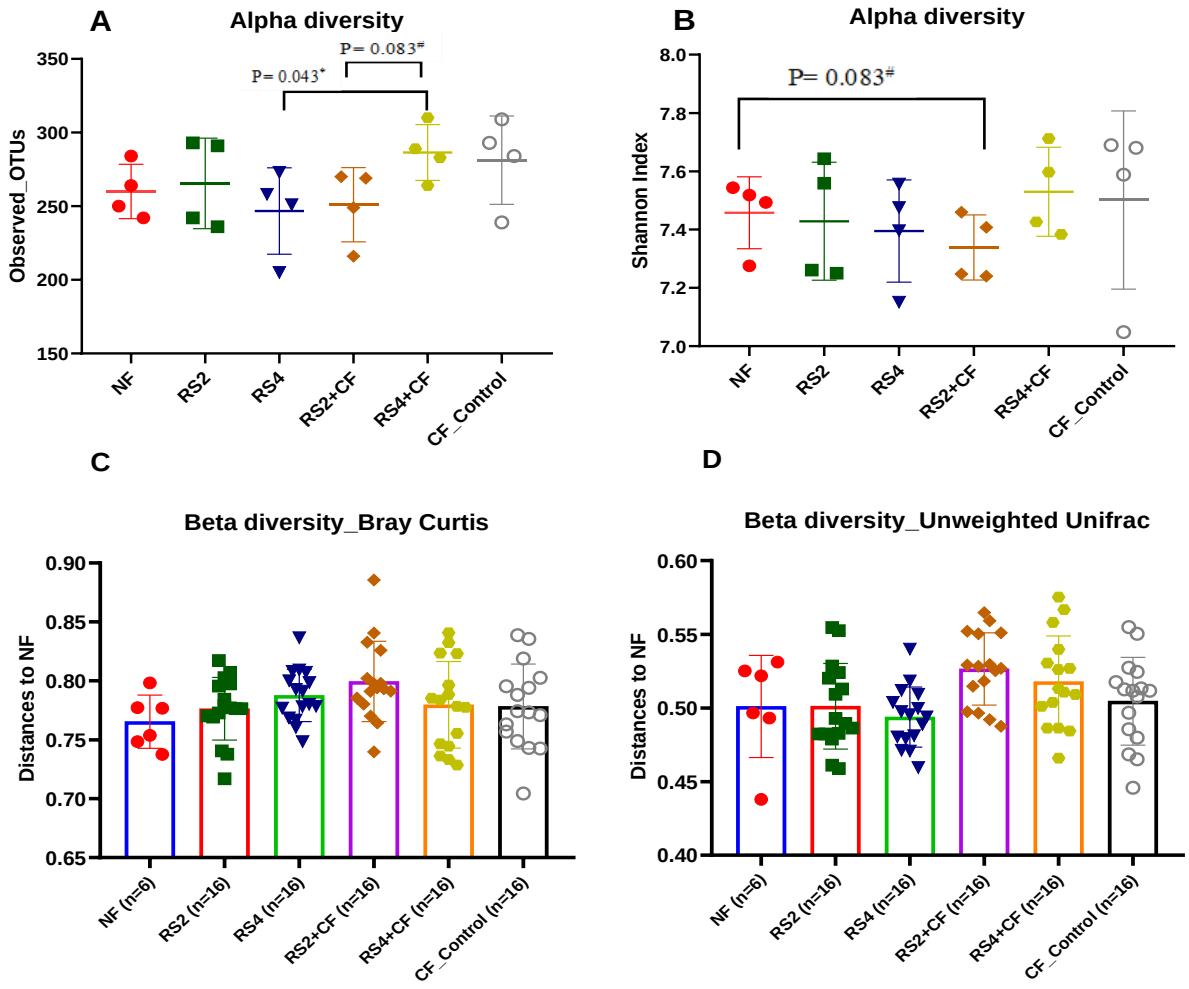

Figure 5.3 Effects of early feeding of resistant starch on cecal microbiota diversity of broilers at d 21 post-hatch at 10,000 reads per sample. A) alpha diversity, Observed OTUs (richness), B) alpha diversity, Shannon Index (evenness), C) beta diversity (Bray Curtis distance), D) beta diversity (Unweighted UniFrac distance). Data represent mean ± SE. Statistical analyses were performed using Kruskal-Wallis test for A and B and permanova for C and D. NF, no feed; RS2, resistant starch type 2, RS2+CF, 50% RS2 and 50% commercial feed; RS4, resistant starch type 4; RS4+CF, 50% RS4 and 50% commercial feed; CF, commercial feed.

Table 5.4 Statistical analysis for beta diversity analysis (n=8, refer to figure 5.3 C and D).

		Unweighted UniFrac	Bray Curtis
Group1	Group2	p-value	p-value
NF	RS2	0.029*	> 0.1
NF	RS2+CF	0.023*	0.030*
NF	RS4	> 0.1	0.076#
NF	RS4+CF	0.081#	> 0.1
RS2	RS4	> 0.1	0.060#

Statistical analyses were performed using pairwise permanova. #: $p < 0.1$ and *: $p < 0.05$. None of the other comparisons between treatment groups were statistically significant. NF-no feed/starved, RS2-resistatnt starch type 2, RS4-resistant starch type 4, RS2+CF-50% resistant starch type 2 + 50% commercial feed, RS4+CF-50% resistant starch type 4 + 50% commercial feed, CF-commercial feed.

5.4 Discussion

The importance of early feeding post-hatch is well understood in terms of supplying essential nutrients via feed to chicks immediately after hatch that could support the transition of yolk-based lipid to carbohydrate utilization for energy. Apart from proper organs development and growth in broilers, the modulation of the immune system and balance of gut microbiota are critical factors in managing the health and profitability of broiler production. The incorporation of prebiotic early in diets of chicks is sought to stimulate gut-associated lymphoid tissue (GALT) and encourage the establishment of beneficial gut microbiota.

RS has been extensively studied in human and pigs as prebiotics and different types of RS have been reported to contribute greater than NSPs in fermentation and colonic physiology (Topping and Clifton, 2001). There exists limited information about the role of RS in broilers and especially studies on their use in early feeding is very scarce. In the present study, we found that feeding RS at a very high inclusion level can cause a negative impact on growth as it does not get digested by the host enzymes. The difference in the performance of RS and RS+CF groups indicate that RS can be tolerated to a high inclusion level when feed can be balanced for nutrients. In an unchallenged study in broilers, Ariza-Nieto et al. (2012) also did not find the effect of retrograded RS on the growth performance of the birds. However, when birds challenged with *Eimeria* and *C. perfringens* were fed acetylated and butylated RS, an improvement in feed intake and weight gain was observed (M'Sadeq et al., 2015). In the present study, we did not find the effect of RS on gut mucosal growth and cecal SCFA production. It can be argued that early feeding of RS could get washed-out during the later growth period and it may not remain in the intestinal tract as a substrate for later fermentation.

The chemically modified RS (RS4) fed along with CF stimulated cell-mediated immunity at d 7 post-hatch, but only a trend was observed by d 21 post-hatch. It implies that RS has the potential to modulate the immune system even before the gut microbiota become diverse and established but the effect is transient. At d 21, RS2 fed group exhibited a trend for lower species evenness as well as a significant beta diversity compared with the early unfed group, which points towards the influence of RS2 on cecal bacterial colonization. RS can be expected to modify cecal bacteria and fermentation physiology in broilers as it has been reported to modulate microbiota composition and SCFA concentration in pig intestine (Haenen et al., 2013b; Sun et al. 2016).

5.5 Conclusion

The results indicate that RS4 has the potential to stimulate immunity, but its early feeding for a short period may not yield a long-standing impact on immunity or cecal fermentation in broilers despite its influence on microbial diversity. Moreover, RS2 exhibits a higher potential to modulate the diversity of gut microbiota and its longer feeding period should be investigated to determine its effect on the microbial colonization and cecal fermentation in broilers. Thus, RS can be one of the potential prebiotics that can be used in early feeding, but more research is warranted to utilize it in combination with probiotics for replacing AGPs.

CHAPTER 6: GENERAL DISCUSSION AND FUTURE DIRECTIONS

Because of fluctuating price and supply of conventional feed ingredients, alternative feedstuffs and agricultural co-products are being increasingly incorporated in broilers diet. To offset the effect of poor digestibility and reduction in AME due to the inherent presence of NSP in these alternative feedstuffs, exogenous carbohydrase enzymes like xylanase is used extensively. The use of xylanase in a wheat-based diet is expanding due to its effective role in degrading arabinoxylans and producing better weight gain and feed efficiency in broilers. The improvement in the corn-SMB diet in response to xylanase is comparatively moderate than that in the wheat-based diet. Besides the effect of xylanase on growth performance of broilers, its effect on the enhancement of cecal short chain fatty acids (SCFA) production in both corn and wheat diet has been immensely encouraging. The application of exogenous xylanase in the wheat-based diet (Chapter 2) and in the corn-based diet (Chapter 2 & 3) increased the production of cecal acetate and total SCFA, which is indicative of its effect on both soluble and insoluble arabinoxylans. Similar to xylanase, xylooligosaccharides (XOS) can also exert a modulating effect on cecal fermentation and result in increased production of cecal SCFA (Chapter 3). It is still important to understand whether xylanase and XOS could produce an additive effect when used in combination or if there would be any possibility of xylanase degrading XOS during the passage of digesta in the small intestine. It is also intriguing to note that xylanase modulated the diversity and abundance of certain taxa of cecal microbiota in 35 d study (Chapter 2), whereas it did not influence the cecal microbiota diversity during 42 d (Chapter 3). This variability in the profile of cecal microbiota in response to xylanase supplementation can partly be explained by the fact that the gut microbiota becomes diverse but more stable with an increase in age of broilers.

Along with the existing problem of antinutrient effect of NSP in broiler feed, the increasing concern against the use of antibiotic growth promoters (AGPs) is impelling researchers and nutritionist to find alternatives to AGPs in time. Out of several alternatives experimented, probiotics and prebiotics have been extensively used and studied and they have shown promising but inconsistent results. Because of the vulnerability of chicks to diseases as a consequence of withdrawal of AGP from feed, the early use of probiotics and prebiotics to modulate gut microbiota and improve gut health is gaining trend as a priority. Early feeding strategy in broilers comprises of i*n ovo* injection (Chapter 4) and early post-hatch feeding (Chapter 5). While comparing the prebiotic potential of different chain length XOS and mannanoligosaccharides (MOS), we found that there was no difference in growth performance in response to *in ovo* feeding of these oligosaccharides in broilers. The villus height to crypt depth ratio was increased by xylotetraose on hatch day and the production of cecal acetate, butyrate and total SCFA on d 28 was enhanced by xylotriose. The *in ovo* feeding of xylotriose also displayed the potential to stimulate the level of T-cell, which demonstrates its ability to modulate cell-mediated immunity. However, this effect on T-cell by xylotriose was not evident after hatch day and it indicates that the effect is rather transient. Compared with XOS, mannotetraose differentially enhanced the abundance of certain microbial taxa in the cecum. Thus, XOS and MOS show some prebiotic potential, but they produce different effects which might be the function of their physiochemical properties owing to different structure and chain length. *In ovo* nutrition has attracted focus in recent years because it provides an opportunity to address the constraint of limited nutrient reserves in eggs that cannot be otherwise changed once the eggs have been laid. *In ovo* technique has incredibly displayed a great potential to alter pre-hatch

nutritional programming in broilers, but it also faces limitation in supplying enough nutrients in a single dose and suffers from the inconvenience of injecting at multiple intervals.

To address this concern, required nutrients and additives can be supplemented during the early and immediately post-hatch period to take full advantage of the opportunity when chicks are still dependent on yolk-based nutrition and their gut microbiota has not been well established. In contrast to oligosaccharides prebiotics, resistant starch (RS) has not been adequately studied or utilized in broiler feeding. RS has proven to be a great prebiotic resource in human and pigs, owing to its preferred fermentation over other non-starch polysaccharides (NSP) and its butyrate shifting effect. An excessive inclusion of RS in early feeding can depress growth in broilers because of its low AME effect and matrix-associated imbalance in the density of nutrients in feed. Early short duration feeding of RS4 along with commercial feed in broilers showed that it can stimulate cell-mediated immunity, but the effect fades out with the passage of time. Likewise, RS2 fed broilers had a different level of cecal microbiota diversity compared with early unfed broilers. Early feeding of RS did not cause major changes in ileal histomorphometry and SCFA production when compared during 3rd week of post-hatch. Thus, the effect of RS on immunity and fermentation characteristics in broiler does not sustain for a longer period when it is discontinued from the diet.

To allow for the withdrawal of AGPs, it is essential that several alternate strategies are considered to improve growth performance, enhance feed efficiency and stimulate the immune response to reduce early mortality of chicks. The use of NSP enzymes, *in ovo* prebiotics, early feeding of prebiotics and long-term feeding of prebiotics post-hatch are plausible nutritional strategies to improve immune status, microbial colonization and gut health parameter in broilers. However, the level of improvement attained, and the inconsistencies encountered with the use of

these products do not make them an effective alternative to completely replace AGPs in the present scenario. The results obtained from these studies are still interesting and will be insightful in finding better combination of products and concerted nutritional strategies to minimize and gradually limit the use of AGPs in the broiler feeding program. It also opens the avenue for further research that can be conducted to discover if modification of these products could prove to be better alternatives or not than their use in present form. It will be further interesting to observe the effects of the application of prebiotics *in ovo* through a different route and at earlier incubation period. Alongside, the use of prebiotics, the concurrent application of probiotic bacteria that are promoted by prebiotics can also be one of the effective strategies to evaluate.

References

Abbas, A.K., Lichtman, A.H. and Pillai, S., 2018. Cellular and molecular immunology. 9th ed. Elsevier, Philadelphia, PA.

Adeola, O. and Cowieson, A.J., 2011. Board-invited review: opportunities and challenges in using exogenous enzymes to improve nonruminant animal production. Journal of animal science, *89*(10), pp.3189-3218.

Ahmed, S.T., Islam, M.M., Mun, H.S., Sim, H.J., Kim, Y.J. and Yang, C.J., 2014. Effects of *Bacillus amyloliquefaciens* as a probiotic strain on growth performance, cecal microflora, and fecal noxious gas emissions of broiler chickens. Poultry Science, 93(8), pp.1963-1971.

Akgül, A. and Kivanc, M., 1988. Inhibitory effects of selected Turkish spices and oregano components on some foodborne fungi. International Journal of Food Microbiology, 6(3), pp.263-268.

Ali, M.N., Abou, M.S. and El-kloub, M., 2008. Incorporation of wheat bran in broiler diets. International Journal of Poultry Science, 7(1), pp.6-13.

Alloui, M.N., Szczurek, W. and Świątkiewicz, S., 2013. The usefulness of prebiotics and probiotics in modern poultry nutrition: A review/Przydatność prebiotyków i probiotyków w nowoczesnym żywieniu drobiu–przegląd. Annals of Animal Science, 13(1), pp.17-32.

Almirall, M. and Esteve-Garcia, E., 1994. Rate of passage of barley diets with chromium oxide: influence of age and poultry strain and effect of β-glucanase supplementation. Poultry Science, 73(9), pp.1433-1440.

Amat, C., Planas, J.M. and Moreto, M., 1996. Kinetics of hexose uptake by the small and large intestine of the chicken. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 271(4), pp.R1085-R1089.

Amerah, A.M., Romero, L.F., Awati, A. and Ravindran, V., 2016. Effect of exogenous xylanase, amylase, and protease as single or combined activities on nutrient digestibility and growth performance of broilers fed corn/soy diets. Poultry Science, 96(4), pp.807-816.

Annison, G. and Choct, M., 1991. Anti-nutritive activities of cereal non-starch polysaccharides in broiler diets and strategies minimizing their effects. World's Poultry Science Journal, 47(3), pp.232-242.

Ao, Z., Kocher, A. and Choct, M., 2012. Effects of dietary additives and early feeding on performance, gut development and immune status of broiler chickens challenged with Clostridium perfringens. Asian-Australasian Journal of Animal Sciences, 25(4), p.541.

AOAC, 2006. Official Methods of Analysis, 18th ed. Association of Official Analytical Chemists, Arlington, VA, USA.

Apajalahti, J., Kettunen, A. and Graham, H., 2004. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. World's Poultry Science Journal, 60(2), pp.223-232.

Ariza-Nieto, C., Rodriguez, D., Ariza-Nieto, M. and Afanador, G., 2012. Effects of resistant starch of common and native potato on broiler performance. The FASEB Journal, 26(1) Supplement, 825.4.

Ashraf, S., Zaneb, H., Yousaf, M.S., Ijaz, A., Sohail, M.U., Muti, S., Usman, M.M., Ijaz, S. and Rehman, H., 2013. Effect of dietary supplementation of prebiotics and probiotics on

intestinal microarchitecture in broilers reared under cyclic heat stress. Journal of Animal Physiology and Animal Nutrition, 97, pp.68-73.

Audisio, M.C., Oliver, G. and Apella, M.C., 2000. Protective effect of *Enterococcus faecium* J96, a potential probiotic strain, on chicks infected with *Salmonella pullorum*. Journal of Food Protection, 63(10), pp.1333-1337.

Awad, W.A., Böhm, J., Razzazi-Fazeli, E., Ghareeb, K. and Zentek, J., 2006. Effect of addition of a probiotic microorganism to broiler diets contaminated with deoxynivalenol on performance and histological alterations of intestinal villi of broiler chickens. Poultry Science, 85(6), pp.974-979.

Awad, W.A., Ghareeb, K. and Böhm, J., 2011. Evaluation of the chicory inulin efficacy on ameliorating the intestinal morphology and modulating the intestinal electrophysiological properties in broiler chickens. Journal of Animal Physiology and Animal Nutrition, 95(1), pp.65-72.

Bach Knudsen, K.E., 2014. Fiber and nonstarch polysaccharide content and variation in common crops used in broiler diets. Poultry Science, 93(9), pp.2380-2393.

Barekatain, M.R., Choct, M. and Iji, P.A., 2013. Xylanase supplementation improves the nutritive value of diets containing high levels of sorghum distillers' dried grains with solubles for broiler chickens. Journal of the Science of Food and Agriculture, 93(7), pp.1552-1559.

Barnes, E.M., Mead, G.C., Barnuml, D.A. and Harry, E.G., 1972. The intestinal flora of the chicken in the period 2 to 6 weeks of age, with particular reference to the anaerobic bacteria. British Poultry Science, 13(3), pp.311-326.

Baurhoo, B., Ferket, P., Ashwell, C.M., de Oliviera, J. and Zhao, X., 2012. Cell walls of *Saccharomyces cerevisiae* differentially modulated innate immunity and glucose metabolism during late systemic inflammation. PloS ONE, 7(1), p.e30323.

Baurhoo, B., Letellier, A., Zhao, X. and Ruiz-Feria, C.A., 2007. Cecal populations of Lactobacilli and Bifidobacteria and *Escherichia coli* populations after in vivo *Escherichia coli* challenge in birds fed diets with purified lignin or mannanoligosaccharides. Poultry Science, 86(12), pp.2509-2516.

Bedford, M., 2000. Removal of antibiotic growth promoters from poultry diets: implications and strategies to minimise subsequent problems. World's Poultry Science Journal, 56(4), pp.347-365.

Bedford, M.R. and Classen, H.L., 1992. Reduction of intestinal viscosity through manipulation of dietary rye and pentosanase concentration is effected through changes in the carbohydrate composition of the intestinal aqueous phase and results in improved growth rate and food conversion efficiency of broiler chicks. The Journal of Nutrition, 122(3), pp.560-569.

Bedford, M.R., 1995. Mechanism of action and potential environmental benefits from the use of feed enzymes. Animal Feed Science and Technology, 53(2), pp.145-155.

Bedford, M.R., 2000. Exogenous enzymes in monogastric nutrition—their current value and future benefits. Animal Feed Science and Technology, 86(1-2), pp.1-13.

Bedford, M.R., Classen, H.L. and Campbell, G.L., 1991. The effect of pelleting, salt, and pentosanase on the viscosity of intestinal contents and the performance of broilers fed rye. Poultry Science, 70(7), pp.1571-1577.

Bermudez-Brito, M., Rösch, C., Schols, H.A., Faas, M.M. and de Vos, P., 2015. Resistant starches differentially stimulate Toll-like receptors and attenuate proinflammatory cytokines in dendritic cells by modulation of intestinal epithelial cells. Molecular Nutrition & Food Research, 59(9), pp.1814-1826.

Berrocoso, J.D., Kida, R., Singh, A.K., Kim, Y.S. and Jha, R., 2017. Effect of *in ovo* injection of raffinose on growth performance and gut health parameters of broiler chicken. Poultry Science, 96(6), pp.1573-1580.

Berry, C.S., 1986. Resistant starch: formation and measurement of starch that survives exhaustive digestion with amylolytic enzymes during the determination of dietary fibre. Journal of Cereal Science, 4(4), pp.301-314.

Bindels, L.B., Delzenne, N.M., Cani, P.D. and Walter, J., 2015. Towards a more comprehensive concept for prebiotics. Nature Reviews Gastroenterology & Hepatology, 12(5), p.303.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F. and Bai, Y. et al., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology, p.1.

Broekaert, W.F., Courtin, C.M., Verbeke, K., Van de Wiele, T., Verstraete, W. and Delcour, J.A., 2011. Prebiotic and other health-related effects of cereal-derived arabinoxylans, arabinoxylan-oligosaccharides, and xylooligosaccharides. Critical Reviews in Food Science and Nutrition, 51(2), pp.178-194.

Brown, I., Warhurst, M., Arcot, J., Playne, M., Illman, R.J. and Topping, D.L., 1997. Fecal numbers of Bifidobacteria are higher in pigs fed *Bifidobacterium longum* with a high

amylose cornstarch than with a low amylose cornstarch. The Journal of Nutrition, 127(9), pp.1822-1827.

Brown, I.L., Wang, X., Topping, D.L., Playne, M.J. and Conway, P.L., 1998. High amylose maize starch as a versatile prebiotic for use with probiotic bacteria. Food Australia, 50(12), pp.603-610.

Bruce, S.J., Guy, P.A., Rezzi, S. and Ross, A.B., 2010. Quantitative measurement of betaine and free choline in plasma, cereals and cereal products by isotope dilution LC-MS/MS. Journal of Agricultural and Food Chemistry, 58(4), pp.2055-2061.

Caballero-Franco, C., Keller, K., De Simone, C. and Chadee, K., 2007. The VSL# 3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. American Journal of Physiology-Gastrointestinal and Liver Physiology, 292(1), pp.G315-G322.

Campbell, J.M., Fahey Jr, G.C. and Wolf, B.W., 1997. Selected indigestible oligosaccharides affect large bowel mass, cecal and fecal short-chain fatty acids, pH and microflora in rats. The Journal of Nutrition, 127(1), pp.130-136.

Carvalho, A.F.A., de Oliva Neto, P., Da Silva, D.F. and Pastore, G.M., 2013. Xylo-oligosaccharides from lignocellulosic materials: chemical structure, health benefits and production by chemical and enzymatic hydrolysis. Food Research International, 51(1), pp.75-85.

Castanon, J.I.R., 2007. History of the use of antibiotics as growth promoters in European poultry feeds. Poultry Science, 86(11), pp.2466-2471.

Chee, S.H., Iji, P.A., Choct, M., Mikkelsen, L.L. and Kocher, A., 2010. Characterisation and response of intestinal microflora and mucins to manno-oligosaccharide and antibiotic supplementation in broiler chickens. British Poultry Science, 51(3), pp.368-380.

Cheled-Shoval, S.L., Amit-Romach, E., Barbakov, M. and Uni, Z., 2011. The effect of in ovo administration of mannan oligosaccharide on small intestine development during the pre- and posthatch periods in chickens. Poultry Science, 90(10), pp.2301-2310.

Cheng, M., Chen, Y., Xiao, W., Sun, R. and Tian, Z., 2013. NK cell-based immunotherapy for malignant diseases. Cellular & Molecular Immunology, 10(3), p.230.

Chiang, C.C., Yu, B. and Chiou, P.W.S., 2005. Effects of xylanase supplementation to wheat-based diet on the performance and nutrient availability of broiler chickens. Asian-Australasian Journal of Animal Sciences, 18(8), pp.1141-1146.

Chichlowski, M., Croom, W.J., Edens, F.W., McBride, B.W., Qiu, R., Chiang, C.C., Daniel, L.R., Havenstein, G.B. and Koci, M.D., 2007. Microarchitecture and spatial relationship between bacteria and ileal, cecal, and colonic epithelium in chicks fed a direct-fed microbial, PrimaLac, and salinomycin. Poultry Science, 86(6), pp.1121-1132.

Choct, M., 2006. Enzymes for the feed industry: past, present and future. World's Poultry Science Journal, 62(1), pp.5-16.

Choct, M., 2009. Managing gut health through nutrition. British Poultry Science, 50(1), pp.9-15.

Choct, M., Hughes, R.J. and Bedford, M.R., 1999. Effects of a xylanase on individual bird variation, starch digestion throughout the intestine, and ileal and caecal volatile fatty acid production in chickens fed wheat. British Poultry Science, 40(3), pp.419-422.

Choct, M., Hughes, R.J., Wang, J., Bedford, M.R., Morgan, A.J. and Annison, G., 1996. Increased small intestinal fermentation is partly responsible for the anti-nutritive activity of non-starch polysaccharides in chickens. British Poultry Science, 37(3), pp.609-621.

Choct, M., Kocher, A., Waters, D.L.E., Pettersson, D. and Ross, G., 2004. A comparison of three xylanases on the nutritive value of two wheats for broiler chickens. British Journal of Nutrition, 92(1), pp.53-61.

Chung, C.H. and Day, D.F., 2004. Efficacy of *Leuconostoc mesenteroides* (ATCC 13146) isomaltooligosaccharides as a poultry prebiotic. Poultry Science, 83(8), pp.1302-1306.

Clancy, R., 2003. Immunobiotics and the probiotic evolution. FEMS Immunology & Medical Microbiology, 38(1), pp.9-12.

Cleveland, J., Montville, T.J., Nes, I.F. and Chikindas, M.L., 2001. Bacteriocins: safe, natural antimicrobials for food preservation. International Journal of Food Microbiology, 71(1), pp.1-20.

Collado, M.C., Gueimonde, M., Hernandez, M., Sanz, Y. and Salminen, S., 2005. Adhesion of selected Bifidobacterium strains to human intestinal mucus and the role of adhesion in enteropathogen exclusion. Journal of Food Protection, 68(12), pp.2672-2678.

Collins, M.D., Lawson, P.A., Willems, A., Cordoba, J.J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. and Farrow, J.A.E., 1994. The phylogeny of the genus Clostridium: proposal of five new genera and eleven new species combinations. International Journal of Systematic and Evolutionary Microbiology, 44(4), pp.812-826.

Coloe, P.J., Bagust, T.J. and Ireland, L., 1984. Development of the normal gastrointestinal microflora of specific pathogen-free chickens. Epidemiology & Infection, 92(1), pp.79-87.

Corrigan, A., Horgan, K., Clipson, N. and Murphy, R.A., 2011. Effect of dietary supplementation with a *Saccharomyces cerevisiae* mannan oligosaccharide on the bacterial community structure of broiler cecal contents. Appl. Environ. Microbiol., 77(18), pp.6653-6662.

Courtin, C.M., Broekaert, W.F., Swennen, K., Lescroart, O., Onagbesan, O., Buyse, J., Decuypere, E., Van de Wiele, T., Marzorati, M., Verstraete, W. and Huyghebaert, G., 2008. Dietary inclusion of wheat bran arabinoxylooligosaccharides induces beneficial nutritional effects in chickens. Cereal Chemistry, 85(5), pp.607-613.

Cowieson, A.J. and Masey O'neill, H.V., 2013. Effects of exogenous xylanase on performance, nutrient digestibility and caecal thermal profiles of broilers given wheat-based diets. British Poultry Science, 54(3), pp.346-354.

Cowieson, A.J., Bedford, M.R. and Ravindran, V., 2010. Interactions between xylanase and glucanase in maize-soy-based diets for broilers. British Poultry Science, 51(2), pp.246-257.

Crotzer, V.L. and Blum, J.S., 2010. Autophagy and adaptive immunity. Immunology, 131(1), pp.9-17.

Crouch, A.N., Grimes, J.L., Ferket, P.R., Thomas, L.N. and Sefton, A.E., 1997. Enzyme supplementation to enhance wheat utilization in starter diets for broilers and turkeys. Journal of Applied Poultry Research, 6(2), pp.147-154.

Cuche, G., Cuber, J.C. and Malbert, C.H., 2000. Ileal short-chain fatty acids inhibit gastric motility by a humoral pathway. American Journal of Physiology-Gastrointestinal and Liver Physiology, 279(5), pp.G925-G930.

Cummings, J.H. and Stephen, A.M., 2007. Carbohydrate terminology and classification. European Journal of Clinical Nutrition, 61(S1), p.S5.

Dalloul, R.A., Lillehoj, H.S., Shellem, T.A. and Doerr, J.A., 2003. Enhanced mucosal immunity against *Eimeria acervulina* in broilers fed a *Lactobacillus*-based probiotic. Poultry Science, 82(1), pp.62-66.

Davidson, M.H. and McDonald, A., 1998. Fiber: forms and functions. Nutrition Research, 18(4), pp.617-624.

De Maesschalck, C., Eeckhaut, V., Maertens, L., De Lange, L., Marchal, L., Nezer, C., De Baere, S., Croubels, S., Daube, G., Dewulf, J. and Haesebrouck, F., 2015. Effects of xylo-oligosaccharides on broiler chicken performance and microbiota. Appl. Environ. Microbiol., 81(17), pp.5880-5888.

De Oliveira, J.E., Van der Hoeven-Hangoor, E., Van de Linde, I.B., Montijn, R.C. and Van der Vossen, J.M.B.M., 2014. *In ovo* inoculation of chicken embryos with probiotic bacteria and its effect on posthatch *Salmonella* susceptibility. Poultry Science, 93(4), pp.818-829.

Den Besten, G., van Eunen, K., Groen, A.K., Venema, K., Reijngoud, D.J. and Bakker, B.M., 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. Journal of Lipid Research, 54(9), pp.2325-2340.

Dethlefsen, L., McFall-Ngai, M. and Relman, D.A., 2007. An ecological and evolutionary perspective on human–microbe mutualism and disease. Nature, 449(7164), p.811.

Dibner, J.J. and Richards, J.D., 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poultry Science, 84(4), pp.634-643.

Ding, X.M., Li, D.D., Bai, S.P., Wang, J.P., Zeng, Q.F., Su, Z.W., Xuan, Y. and Zhang, K.Y., 2017. Effect of dietary xylooligosaccharides on intestinal characteristics, gut microbiota,

cecal short-chain fatty acids, and plasma immune parameters of laying hens. Poultry Science, 97(3), pp.874-881.

Donoghue, D.J., 2003. Antibiotic residues in poultry tissues and eggs: human health concerns?. Poultry Science, 82(4), pp.618-621.

Donohue, M. and Cunningham, D.L., 2009. Effects of grain and oilseed prices on the costs of US poultry production. Journal of Applied Poultry Research, 18(2), pp.325-337.

Dugas, B., Mercenier, A., Lenoir-Wijnkoop, I., Arnaud, C., Dugas, N. and Postaire, E., 1999. Immunity and probiotics. Immunology Today, 20(9), pp.387-390.

Dumonceaux, T.J., Hill, J.E., Hemmingsen, S.M. and Van Kessel, A.G., 2006. Characterization of intestinal microbiota and response to dietary virginiamycin supplementation in the broiler chicken. Appl. Environ. Microbiol., 72(4), pp.2815-2823.

Duncan, S.H., Louis, P. and Flint, H.J., 2004. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. Appl. Environ. Microbiol., 70(10), pp.5810-5817.

Eeckhaut, V., Van Immerseel, F., Dewulf, J., Pasmans, F., Haesebrouck, F., Ducatelle, R., Courtin, C.M., Delcour, J.A. and Broekaert, W.F., 2008. Arabinoxylooligosaccharides from wheat bran inhibit Salmonella colonization in broiler chickens. Poultry Science, 87(11), pp.2329-2334.

Engberg, R.M., Hedemann, M.S. and Jensen, B.B., 2002. The influence of grinding and pelleting of feed on the microbial composition and activity in the digestive tract of broiler chickens. British Poultry Science, 43(4), pp.569-579.

Engberg, R.M., Hedemann, M.S., Steenfeldt, S. and Jensen, B.B., 2004. Influence of whole wheat and xylanase on broiler performance and microbial composition and activity in the digestive tract. Poultry Science, 83(6), pp.925-938.

Englyst, H.N., Kingman, S.M. and Cummings, J.H., 1992. Classification and measurement of nutritionally important starch fractions. European Journal of Clinical Nutrition, 46, pp.S33-50.

Federation for Animal Science Societies, 2010. Guide for the Care and Use of Agricultural Animals in Research and Teaching. 3rd ed. FASS, Champaign, IL.

Fernandez, F., Hinton, M. and Gils, B.V., 2002. Dietary mannan-oligosaccharides and their effect on chicken caecal microflora in relation to *Salmonella* Enteritidis colonization. Avian Pathology, 31(1), pp.49-58.

Fuller, R., 1989. Probiotics in man and animals. Journal of Applied Bacteriology, 66(5), pp.365-378.

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

Ganguly, S., 2013. Supplementation of prebiotics, probiotics and acids on immunity in poultry feed: a brief review. World's Poultry Science Journal, 69(3), pp.639-648.

Gänzle, M.G., Hertel, C., van der Vossen, J.M. and Hammes, W.P., 1999. Effect of bacteriocin-producing lactobacilli on the survival of *Escherichia coli* and *Listeria* in a dynamic model of the stomach and the small intestine. International Journal of Food Microbiology, 48(1), pp.21-35.

Gao, F., Jiang, Y., Zhou, G.H. and Han, Z.K., 2007. The effects of xylanase supplementation on growth, digestion, circulating hormone and metabolite levels, immunity and gut

microflora in cockerels fed on wheat-based diets. British Poultry Science, 48(4), pp.480-488.

Gately, M.K., Renzetti, L.M., Magram, J., Stern, A.S., Adorini, L., Gubler, U. and Presky, D.H., 1998. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annual Review of Immunology, 16(1), pp.495-521.

Geier, M.S., Torok, V.A., Allison, G.E., Ophel-Keller, K. and Hughes, R.J., 2009. Indigestible carbohydrates alter the intestinal microbiota but do not influence the performance of broiler chickens. Journal of Applied Microbiology, 106(5), pp.1540-1548.

Gibson, G.R. and Roberfroid, M.B., 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. The Journal of Nutrition, 125(6), pp.1401-1412.

Gobinath, D., Madhu, A.N., Prashant, G., Srinivasan, K. and Prapulla, S.G., 2010. Beneficial effect of xylo-oligosaccharides and fructo-oligosaccharides in streptozotocin-induced diabetic rats. British Journal of Nutrition, 104(1), pp.40-47.

Gong, J., Forster, R.J., Yu, H., Chambers, J.R., Sabour, P.M., Wheatcroft, R. and Chen, S., 2002. Diversity and phylogenetic analysis of bacteria in the mucosa of chicken ceca and comparison with bacteria in the cecal lumen. FEMS Microbiology Letters, 208(1), pp.1-7.

Gopal, P.K., Sullivan, P.A. and Smart, J.B., 2001. Utilisation of galacto-oligosaccharides as selective substrates for growth by lactic acid bacteria including *Bifidobacterium lactis* DR10 and *Lactobacillus rhamnosus* DR20. International Dairy Journal, 11(1-2), pp.19-25.

Griffin, H.D. and Goddard, C., 1994. Rapidly growing broiler (meat-type) chickens. Their origin and use for comparative studies of the regulation of growth. International Journal of Biochemistry, 26(1), pp.19-28.

Haenen, D., Souza da Silva, C., Zhang, J., Koopmans, S.J., Bosch, G., Vervoort, J., Gerrits, W.J., Kemp, B., Smidt, H., Müller, M. and Hooiveld, G.J., 2013a. Resistant starch induces catabolic but suppresses immune and cell division pathways and changes the microbiome in the proximal colon of male pigs. The Journal of Nutrition, 143(12), pp.1889-1898.

Haenen, D., Zhang, J., Souza da Silva, C., Bosch, G., van der Meer, I.M., van Arkel, J., van den Borne, J.J., Pérez Gutiérrez, O., Smidt, H., Kemp, B. and Müller, M., 2013b. A diet high in resistant starch modulates microbiota composition, SCFA concentrations, and gene expression in pig intestine. The Journal of Nutrition, 143(3), pp.274-283.

Hansen, C.H., Frøkiær, H., Christensen, A.G., Bergström, A., Licht, T.R., Hansen, A.K. and Metzdorff, S.B., 2013. Dietary xylooligosaccharide downregulates IFN-γ and the low-grade inflammatory cytokine IL-1β systemically in mice. The Journal of Nutrition, 143(4), pp.533-540.

Havenaar R., Huis In't Veld J.H.J., 1992. Probiotics: a general view. in: Wood B.J.B. (eds) The Lactic Acid Bacteria Volume 1 (pp. 151-170). Springer, Boston, MA.

Havenstein, G.B., Ferket, P.R., Scheideler, S.E. and Larson, B.T., 1994. Growth, livability, and feed conversion of 1957 vs 1991 broilers when fed "typical" 1957 and 1991 broiler diets. Poultry Science, 73(12), pp.1785-1794.

Hew, L.I., Ravindran, V., Mollah, Y. and Bryden, W.L., 1998. Influence of exogenous xylanase supplementation on apparent metabolisable energy and amino acid digestibility in wheat for broiler chickens. Animal Feed Science and Technology, 75(2), pp.83-92.

Hmani, H., Daoud, L., Jlidi, M., Jalleli, K., Ali, M.B., Brahim, A.H., Bargui, M., Dammak, A. and Ali, M.B., 2017. A *Bacillus subtilis* strain as probiotic in poultry: selection based on in vitro functional properties and enzymatic potentialities. Journal of Industrial Microbiology & Biotechnology, 44(8), pp.1157-1166.

Hoffman, W., Lakkis, F.G. and Chalasani, G., 2016. B cells, antibodies, and more. Clinical Journal of the American Society of Nephrology, 11(1), pp.137-154.

Honjo, K., Hagiwara, T., Itoh, K., Takahashi, E. and Hirota, Y., 1993. Immunohistochemical analysis of tissue distribution of B and T cells in germfree and conventional chickens. Journal of Veterinary Medical Science, 55(6), pp.1031-1034.

Houssaint, E., Lassila, O. and Vainio, O., 1989. Bu-1 antigen expression as a marker for B cell precursors in chicken embryos. European Journal of Immunology, 19(2), pp.239-243.

Hsieh, C.S., Macatonia, S.E., Tripp, C.S., Wolf, S.F., O'garra, A. and Murphy, K.M., 1993. Development of TH1 CD4+ T cells through IL-12 produced by *Listeria*-induced macrophages. Science, 260(5107), pp.547-549.

Huang, M.K., Choi, Y.J., Houde, R., Lee, J.W., Lee, B. and Zhao, X., 2004. Effects of *Lactobacilli* and an acidophilic fungus on the production performance and immune responses in broiler chickens. Poultry Science, 83(5), pp.788-795.

Hunton, P., 2006. 100 years of poultry genetics. World's Poultry Science Journal, 62(3), pp.417-428.

Huyghebaert, G., Ducatelle, R. and Van Immerseel, F., 2011. An update on alternatives to antimicrobial growth promoters for broilers. The Veterinary Journal, 187(2), pp.182-188.

Igyártó, B.Z., Nagy, N., Magyar, A. and Oláh, I., 2008. Identification of the avian B-cell-specific Bu-1 alloantigen by a novel monoclonal antibody. Poultry Science, 87(2), pp.351-355.

Iji, P.A. and Tivey, D.R., 1998. Natural and synthetic oligosaccharides in broiler chicken diets. World's Poultry Science Journal, 54(2), pp.129-143.

Izydorczyk, M.S. and Biliaderis, C.G., 1995. Cereal arabinoxylans: advances in structure and physicochemical properties. Carbohydrate Polymers, 28(1), pp.33-48.

Jamroz, D., Jakobsen, K., Knudsen, K.E.B., Wiliczkiewicz, A. and Orda, J., 2002. Digestibility and energy value of non-starch polysaccharides in young chickens, ducks and geese, fed diets containing high amounts of barley. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 131(3), pp.657-668.

Jha, R. and Berrocoso, J.F., 2016. Dietary fiber and protein fermentation in the intestine of swine and their interactive effects on gut health and on the environment: A review. Animal Feed Science and Technology, 212, pp.18-26.

Jha, R., Singh, A.K., Yadav, S., Berrocoso, J.F.D. and Mishra, B., 2019. Early nutrition programming (*in ovo* and post-hatch feeding) as a strategy to modulate gut health of poultry. Frontiers in Veterinary Science, 6.

Joerger, R.D., 2003. Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. Poultry Science, 82(4), pp.640-647.

Jones, F.T. and Ricke, S.C., 2003. Observations on the history of the development of antimicrobials and their use in poultry feeds. Poultry Science, 82(4), pp.613-617.

Jørgensen, H., Zhao, X.Q., Knudsen, K.E.B. and Eggum, B.O., 1996. The influence of dietary fibre source and level on the development of the gastrointestinal tract, digestibility and energy metabolism in broiler chickens. British Journal of Nutrition, 75(3), pp.379-395.

Józefiak, D., Rutkowski, A. and Martin, S.A., 2004. Carbohydrate fermentation in the avian ceca: a review. Animal Feed Science and Technology, 113(1-4), pp.1-15.

Jozefiak, D., Rutkowski, A., Jensen, B.B. and Engberg, R.M., 2007. Effects of dietary inclusion of triticale, rye and wheat and xylanase supplementation on growth performance of broiler chickens and fermentation in the gastrointestinal tract. Animal Feed Science and Technology, 132(1-2), pp.79-93.

Jozefiak, D., Rutkowski, A., Kaczmarek, S., Jensen, B.B., Engberg, R.M. and Højberg, O., 2010. Effect of β-glucanase and xylanase supplementation of barley-and rye-based diets on caecal microbiota of broiler chickens. British Poultry Science, 51(4), pp.546-557.

Jung, S.J., Houde, R., Baurhoo, B., Zhao, X. and Lee, B.H., 2008. Effects of galacto-oligosaccharides and a Bifidobacteria lactis-based probiotic strain on the growth performance and fecal microflora of broiler chickens. Poultry Science, 87(9), pp.1694-1699.

Kabel, M.A., Kortenoeven, L., Schols, H.A. and Voragen, A.G., 2002. In vitro fermentability of differently substituted xylo-oligosaccharides. Journal of Agricultural and Food Chemistry, 50(21), pp.6205-6210.

Kaliński, P., Hilkens, C.M., Snijders, A., Snijdewint, F.G. and Kapsenberg, M.L., 1997. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. The Journal of Immunology, 159(1), pp.28-35.

Kaplan, H. and Hutkins, R.W., 2000. Fermentation of fructooligosaccharides by lactic acid bacteria and bifidobacteria. Appl. Environ. Microbiol., 66(6), pp.2682-2684.

Kato, S., Yuzawa, Y., Tsuboi, N., Maruyama, S., Morita, Y., Matsuguchi, T. and Matsuo, S., 2004. Endotoxin-induced chemokine expression in murine peritoneal mesothelial cells:

the role of toll-like receptor 4. Journal of the American Society of Nephrology, 15(5), pp.1289-1299.

Kermanshahi, H. and Rostami, H., 2006. Influence of supplemental dried whey on broiler performance and cecal flora. Int. J. Poult. Sci, 5(6), pp.538-543.

Khan, S.H. and Iqbal, J., 2016. Recent advances in the role of organic acids in poultry nutrition. Journal of Applied Animal Research, 44(1), pp.359-369.

Kiarie, E., Romero, L.F. and Ravindran, V., 2014. Growth performance, nutrient utilization, and digesta characteristics in broiler chickens fed corn or wheat diets without or with supplemental xylanase. Poultry Science, 93(5), pp.1186-1196.

Kidd, M.T., Ferket, P.R. and Garlich, J.D., 1997. Nutritional and osmoregulatory functions of betaine. World's Poultry Science Journal, 53(2), pp.125-139.

Kim, G.B., Seo, Y.M., Kim, C.H. and Paik, I.K., 2011. Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. Poultry Science, 90(1), pp.75-82.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Research, 41(1), pp.e1-e1.

Kobayashi, M., Fitz, L., Ryan, M., Hewick, R.M., Clark, S.C., Chan, S., Loudon, R., Sherman, F., Perussia, B. and Trinchieri, G., 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. Journal of Experimental Medicine, 170(3), pp.827-845.

Kocher, A., 2006. Interfacing gut health and nutrition: the use of dietary pre and probiotics to maximize growth performance in pigs and poultry. Antimicrobial Growth Promoters, p.289.

Konarzewski, M., Gavin, A., McDevitt, R. and Wallis, I.R., 2000. Metabolic and organ mass responses to selection for high growth rates in the domestic chicken (*Gallus domesticus*). Physiological and Biochemical Zoology, 73(2), pp.237-248.

Kühn, R., Löhler, J., Rennick, D., Rajewsky, K. and Müller, W., 1993. Interleukin-10-deficient mice develop chronic enterocolitis. Cell, 75(2), pp.263-274.

Kurts, C., Robinson, B.W. and Knolle, P.A., 2010. Cross-priming in health and disease. Nature Reviews Immunology, 10(6), p.403.

Kvietys, P.R. and Granger, D.N., 1981. Effect of volatile fatty acids on blood flow and oxygen uptake by the dog colon. Gastroenterology, 80(5), pp.962-969.

Lan, Y., Verstegen, M.W.A., Tamminga, S. and Williams, B.A., 2005. The role of the commensal gut microbial community in broiler chickens. World's Poultry Science Journal, 61(1), pp.95-104.

Leeson, S. and Summers, J. D, 2001. Digestion and nutrient availability. Scott's nutrition of the chicken. Univ. books, Guelph, Ontario, CA. pp. 5.

Leng, E., 1978. Absorption of inorganic ions and volatile fatty acids in the rabbit caecum. British Journal of Nutrition, 40(3), pp.509-519.

Li, L.L., Hou, Z.P., Li, T.J., Wu, G.Y., Huang, R.L., Tang, Z.R., Yang, C.B., Gong, J., Yu, H., Kong, X.F. and Pan, E., 2008. Effects of dietary probiotic supplementation on ileal digestibility of nutrients and growth performance in 1-to 42-day-old broilers. Journal of the Science of Food and Agriculture, 88(1), pp.35-42.

Liu, D., Guo, S. and Guo, Y., 2012. Xylanase supplementation to a wheat-based diet alleviated the intestinal mucosal barrier impairment of broiler chickens challenged by *Clostridium perfringens*. Avian Pathology, 41(3), pp.291-298.

Liu, N., Ru, Y.J., Tang, D.F., Xu, T.S. and Partridge, G.G., 2011. Effects of corn distillers dried grains with solubles and xylanase on growth performance and digestibility of diet components in broilers. Animal Feed Science and Technology, 163(2-4), pp.260-266.

Lowry, V.K., Farnell, M.B., Ferro, P.J., Swaggerty, C.L., Bahl, A. and Kogut, M.H., 2005. Purified β-glucan as an abiotic feed additive up-regulates the innate immune response in immature chickens against *Salmonella enterica* serovar Enteritidis. International Journal of Food Microbiology, 98(3), pp.309-318.

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J.J. and Lee, M.D., 2003. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Appl. Environ. Microbiol., 69(11), pp.6816-6824.

Lumpkins, B.S., Batal, A.B. and Dale, N.M., 2004. Evaluation of distillers dried grains with solubles as a feed ingredient for broilers. Poultry Science, 83(11), pp.1891-1896.

Luo, D., Yang, F., Yang, X., Yao, J., Shi, B. and Zhou, Z., 2009. Effects of xylanase on performance, blood parameters, intestinal morphology, microflora and digestive enzyme activities of broilers fed wheat-based diets. Asian-Australasian Journal of Animal Sciences, 22(9), pp.1288-1295.

Macfarlane, G.T., Gibson, G.R., Beatty, E. and Cummings, J.H., 1992. Estimation of short-chain fatty acid production from protein by human intestinal bacteria based on branched-chain fatty acid measurements. FEMS Microbiology Ecology, 10(2), pp.81-88.

Madej, J.P. and Bednarczyk, M., 2016. Effect of in ovo-delivered prebiotics and synbiotics on the morphology and specific immune cell composition in the gut-associated lymphoid tissue. Poultry Science, 95(1), pp.19-29.

Maiorano, G., Stadnicka, K., Tavaniello, S., Abiuso, C., Bogucka, J. and Bednarczyk, M., 2017. In ovo validation model to assess the efficacy of commercial prebiotics on broiler performance and oxidative stability of meat. Poultry Science, 96(2), pp.511-518.

Marteau, P., Pochart, P., Doré, J., Béra-Maillet, C., Bernalier, A. and Corthier, G., 2001. Comparative study of bacterial groups within the human cecal and fecal microbiota. Appl. Environ. Microbiol., 67(10), pp.4939-4942.

Masey O'Neil, H. V., Mathis, G., Lumpkins, B. S., and Bedford, M. R. 2012. The effect of reduced calorie diets, with and without fat, and the use of xylanase on performance characteristics of broilers between 0 and 42 days. Poultry Science, 91(6), pp.1356-1360.

Masey-O'Neill, H.V., Singh, M. and Cowieson, A.J., 2014. Effects of exogenous xylanase on performance, nutrient digestibility, volatile fatty acid production and digestive tract thermal profiles of broilers fed on wheat-or maize-based diet. British poultry Science, 55(3), pp.351-359.

Mateos, G.G., Lázaro, R. and Gracia, M.I., 2002. The feasibility of using nutritional modifications to replace drugs in poultry feeds. Journal of Applied Poultry Research, 11(4), pp.437-452.

Mccracken, V.J. and Gaskins, R.T. (1999) Probiotics and the immune system, in: Tannock, G.W.(Ed.) Probiotics: A Critical Review, pp. 85-111 (Horizon Scientific Presss, Wymondham, UK).

Medzhitov, R., 2001. Toll-like receptors and innate immunity. Nature Reviews Immunology, 1(2), p.135.

Meijer, K., de Vos, P. and Priebe, M.G., 2010. Butyrate and other short-chain fatty acids as modulators of immunity: what relevance for health?. Current Opinion in Clinical Nutrition & Metabolic Care, 13(6), pp.715-721.

Meimandipour, A., Shuhaimi, M., Soleimani, A.F., Azhar, K., Hair-Bejo, M., Kabeir, B.M., Javanmard, A., Muhammad Anas, O. and Yazid, A.M., 2010. Selected microbial groups and short-chain fatty acids profile in a simulated chicken cecum supplemented with two strains of *Lactobacillus*. Poultry Science, 89(3), pp.470-476.

Meng, Q.W., Yan, L., Ao, X., Zhou, T.X., Wang, J.P., Lee, J.H. and Kim, I.H., 2010. Influence of probiotics in different energy and nutrient density diets on growth performance, nutrient digestibility, meat quality, and blood characteristics in growing-finishing pigs. Journal of Animal Science, 88(10), pp.3320-3326.

Meng, X. and Slominski, B.A., 2005. Nutritive values of corn, soybean meal, canola meal, and peas for broiler chickens as affected by a multicarbohydrase preparation of cell wall degrading enzymes. Poultry Science, 84(8), pp.1242-1251.

Meng, X., Slominski, B.A., Nyachoti, C.M., Campbell, L.D. and Guenter, W., 2005. Degradation of cell wall polysaccharides by combinations of carbohydrase enzymes and their effect on nutrient utilization and broiler chicken performance. Poultry Science, 84(1), pp.37-47.

Miles, R.D., and Bootwalla, S.M., 1991. Direct-fed microbials in animal production. In Direct-Fed Microbials in Animal Production. A Review. National Food Ingredient Association: West Des Monies, USA, pp. 117-132.

Miyazaki, Y., Takahashi, K. and Akiba, Y., 2007. Developmental changes in mRNA expression in immune-associated cells of intestinal tract of broiler chickens after hatch and by dietary modification. Animal Science Journal, 78(5), pp.527-534.

Mookiah, S., Sieo, C.C., Ramasamy, K., Abdullah, N. and Ho, Y.W., 2014. Effects of dietary prebiotics, probiotic and synbiotics on performance, caecal bacterial populations and caecal fermentation concentrations of broiler chickens. Journal of the Science of Food and Agriculture, 94(2), pp.341-348.

Moran Jr, E.T., 2007. Nutrition of the developing embryo and hatchling. Poultry Science, 86(5), pp.1043-1049.

Mroz, Z., Koopmans, S.J., Bannink, A., Partanen, K., Krasucki, W., Øverland, M. and Radcliffe, S., 2006. Carboxylic acids as bioregulators and gut growth promoters in nonruminants. In Biology of Growing Animals (Vol. 4, pp. 81-133). Elsevier.

M'Sadeq, S.A., Wu, S.B., Swick, R.A. and Choct, M., 2015. Dietary acylated starch improves performance and gut health in necrotic enteritis challenged broilers. Poultry Science, 94(10), pp.2434-2444.

Murugesan, G.R., Syed, B., Haldar, S. and Pender, C., 2015. Phytogenic feed additives as an alternative to antibiotic growth promoters in broiler chickens. Frontiers in Veterinary Science, 2, p.21.

Mwangi, W.N., Beal, R.K., Powers, C., Wu, X., Humphrey, T., Watson, M., Bailey, M., Friedman, A. and Smith, A.L., 2010. Regional and global changes in TCRαβ T cell repertoires in the gut are dependent upon the complexity of the enteric microflora. Developmental & Comparative Immunology, 34(4), pp.406-417.

Nahm, K.H., 2003. Influences of fermentable carbohydrates on shifting nitrogen excretion and reducing ammonia emission of pigs. Critical Reviews in Environmental Science and Technology, 33(2), pp.165-186.

Nakae, S., Asano, M., Horai, R. and Iwakura, Y., 2001. Interleukin-1β, but not interleukin-1α, is required for T-cell-dependent antibody production. Immunology, 104(4), pp.402-409.

Neefjes, J., Jongsma, M.L., Paul, P. and Bakke, O., 2011. Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nature Reviews Immunology, 11(12), p.823.

Nian, F., Guo, Y.M., Ru, Y.J., Li, F.D. and Peron, A., 2011. Effect of exogenous xylanase supplementation on the performance, net energy and gut microflora of broiler chickens fed wheat-based diets. Asian-Australasian Journal of Animal Sciences, 24(3), pp.400-406.

Nugent, A.P., 2005. Health properties of resistant starch. Nutrition Bulletin, 30(1), pp.27-54.

Nutt, S.L., Hodgkin, P.D., Tarlinton, D.M. and Corcoran, L.M., 2015. The generation of antibody-secreting plasma cells. Nature Reviews Immunology, 15(3), p.160.

O'Neill, H.M., Mathis, G., Lumpkins, B.S. and Bedford, M.R., 2012. The effect of reduced calorie diets, with and without fat, and the use of xylanase on performance characteristics of broilers between 0 and 42 days. Poultry Science, 91(6), pp.1356-1360.

Olukosi, O.A. and Adeola, O., 2008. Whole body nutrient accretion, growth performance and total tract nutrient retention responses of broilers to supplementation of xylanase and phytase individually or in combination in wheat-soybean meal based diets. The Journal of Poultry Science, 45(3), pp.192-198.

Olukosi, O.A., Cowieson, A.J. and Adeola, O., 2007. Age-related influence of a cocktail of xylanase, amylase, and protease or phytase individually or in combination in broilers. Poultry Science, 86(1), p.77.

Patterson, J.A. and Burkholder, K.M., 2003. Application of prebiotics and probiotics in poultry production. Poultry Science, 82(4), pp.627-631.

Pauwels, J., Coopman, F., Cools, A., Michiels, J., Fremaut, D., De Smet, S. and Janssens, G.P., 2015. Selection for growth performance in broiler chickens associates with less diet flexibility. PloS ONE, 10(6), p.e0127819.

Peng, Y.L., Guo, Y.M. and Yuan, J.M., 2003. Effects of microbial phytase replacing partial inorganic phosphorus supplementation and xylanase on the growth performance and nutrient digestibility in broilers fed wheat-based diets. Asian-Australasian Journal of Animal Sciences, 16(2), pp.239-247.

Polizeli, M.L.T.M., Rizzatti, A.C.S., Monti, R., Terenzi, H.F., Jorge, J.A. and Amorim, D.S., 2005. Xylanases from fungi: properties and industrial applications. Applied Microbiology and Biotechnology, 67(5), pp.577-591.

Pomeranz, Y., 1988. Chemical composition of kernel structures. Wheat: Chemistry and Technology, 1, pp.97-158.

Porcheron, G., Kut, E., Canepa, S., Maurel, M.C. and Schouler, C., 2011. Regulation of fructooligosaccharide metabolism in an extra-intestinal pathogenic *Escherichia coli* strain. Molecular Microbiology, 81(3), pp.717-733.

Pourabedin, M. and Zhao, X., 2015. Prebiotics and gut microbiota in chickens. FEMS Microbiology Letters, 362(15), p.fnv122.

Pourabedin, M., Guan, L. and Zhao, X., 2015. Xylo-oligosaccharides and virginiamycin differentially modulate gut microbial composition in chickens. Microbiome, 3(1), p.15.

Psichas, A., Sleeth, M.L., Murphy, K.G., Brooks, L., Bewick, G.A., Hanyaloglu, A.C., Ghatei, M.A., Bloom, S.R. and Frost, G., 2015. The short chain fatty acid propionate stimulates GLP-1 and PYY secretion via free fatty acid receptor 2 in rodents. International Journal of Obesity, 39(3), p.424.

Pugh, D.M., 2002. The EU precautionary bans of animal feed additive antibiotics. Toxicology Letters, 128(1-3), pp.35-44.

Ratriyanto, A., Mosenthin, R., Bauer, E. and Eklund, M., 2009. Metabolic, osmoregulatory and nutritional functions of betaine in monogastric animals. Asian-Australasian Journal of Animal Sciences, 22(10), pp.1461-1476.

Ravindran, V., Morel, P.C., Partridge, G.G., Hruby, M. and Sands, J.S., 2006. Influence of an Escherichia coli-derived phytase on nutrient utilization in broiler starters fed diets containing varying concentrations of phytic acid. Poultry Science, 85(1), pp.82-89.

Regassa, A. and Nyachoti, C.M., 2018. Application of resistant starch in swine and poultry diets with particular reference to gut health and function. Animal Nutrition, 4(3), pp.305-310.

Rehberger, T.G. and Jordan-Parrott, D.S., 2009. AG Tech Products Inc. Method and composition for reducing *E. coli* disease and enhancing performance. U.S. Patent 7,618,640.

Rehman, H., Hellweg, P., Taras, D. and Zentek, J., 2008. Effects of dietary inulin on the intestinal short chain fatty acids and microbial ecology in broiler chickens as revealed by denaturing gradient gel electrophoresis. Poultry Science, 87(4), pp.783-789.

Ribeiro, T., Cardoso, V., Ferreira, L.M.A., Lordelo, M.M.S., Coelho, E., Moreira, A.S.P., Domingues, M.R.M., Coimbra, M.A., Bedford, M.R. and Fontes, C.M.G.A., 2018. Xylo-

oligosaccharides display a prebiotic activity when used to supplement wheat or corn-based diets for broilers. Poultry Science, 97(12), pp.4330-4341.

Ricke, S.C., 2003. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poultry Science, 82(4), pp.632-639.

Rinttilä, T. and Apajalahti, J., 2013. Intestinal microbiota and metabolites—Implications for broiler chicken health and performance1. Journal of Applied Poultry Research, 22(3), pp.647-658.

Romero, L.F., Sands, J.S., Indrakumar, S.E., Plumstead, P.W., Dalsgaard, S. and Ravindran, V., 2014. Contribution of protein, starch, and fat to the apparent ileal digestible energy of corn-and wheat-based broiler diets in response to exogenous xylanase and amylase without or with protease. Poultry Science, 93(10), pp.2501-2513.

Sakata, T., 1987. Stimulatory effect of short-chain fatty acids on epithelial cell proliferation in the rat intestine: a possible explanation for trophic effects of fermentable fibre, gut microbes and luminal trophic factors. British Journal of Nutrition, 58(1), pp.95-103.

Salminen, S., Isolauri, E. and Salminen, E., 1996. Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. Antonie Van Leeuwenhoek, 70(2-4), pp.347-358.

Samanta, A.K., Kotte, A.P., Elangovan, A.V., Dhali, A., Senani, S., Sridhar, M. and Jayapal, N., 2017. Effects of corn husks derived xylo-oligosaccharides on performance of broiler chicken. Indian J Anim Sci, 87, pp.640-643.

Samanya, M. and Yamauchi, K.E., 2002. Histological alterations of intestinal villi in chickens fed dried *Bacillus subtilis* var. natto. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 133(1), pp.95-104.

Saminathan, M., Sieo, C.C., Kalavathy, R., Abdullah, N. and Ho, Y.W., 2011. Effect of prebiotic oligosaccharides on growth of *Lactobacillus* strains used as a probiotic for chickens. African Journal of Microbiology Research, 5(1), pp.57-64.

Sato, T., Matsumoto, K., Okumura, T., Yokoi, W., Naito, E., Yoshida, Y., Nomoto, K., Ito, M. and Sawada, H., 2008. Isolation of lactate-utilizing butyrate-producing bacteria from human feces and in vivo administration of *Anaerostipes caccae* strain L2 and galacto-oligosaccharides in a rat model. FEMS Microbiology Ecology, 66(3), pp.528-536.

Schneeberger, E.E. and Lynch, R.D., 1992. Structure, function, and regulation of cellular tight junctions. American Journal of Physiology-Lung Cellular and Molecular Physiology, 262(6), pp.L647-L661.

Schouler, C., Taki, A., Chouikha, I., Moulin-Schouleur, M. and Gilot, P., 2009. A genomic island of an extraintestinal pathogenic *Escherichia coli* strain enables the metabolism of fructooligosaccharides, which improves intestinal colonization. Journal of Bacteriology, 191(1), pp.388-393.

Selle, P.H. and Ravindran, V., 2007. Microbial phytase in poultry nutrition. Animal Feed Science and Technology, 135(1-2), pp.1-41.

Selle, P.H., Huang, K.H. and Muir, W.I., 2003a. Effects of nutrient specifications and xylanase plus phytase supplementation of wheat-based diets on growth performance and carcass traits of broiler chicks. Asian-Australasian Journal of Animal Sciences, 16(10), pp.1501-1509.

Selle, P.H., Ravindran, V. and Partridge, G.G., 2009. Beneficial effects of xylanase and/or phytase inclusions on ileal amino acid digestibility, energy utilisation, mineral retention

and growth performance in wheat-based broiler diets. Animal Feed Science and Technology, 153(3-4), pp.303-313.

Selle, P.H., Ravindran, V., Ravindran, G., Pittolo, P.H. and Bryden, W.L., 2003b. Influence of phytase and xylanase supplementation on growth performance and nutrient utilisation of broilers offered wheat-based diets. Asian-Australasian Journal of Animal Sciences, 16(3), pp.394-402.

Servin, A.L. and Coconnier, M.H., 2003. Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. Best Practice & Research Clinical Gastroenterology, 17(5), pp.741-754.

Shanmugasundaram, R., Sifri, M. and Selvaraj, R.K., 2013. Effect of yeast cell product supplementation on broiler cecal microflora species and immune responses during an experimental coccidial infection. Poultry Science, 92(5), pp.1195-1201.

Silva, S.S.P. and Smithard, R.R., 2002. Effect of enzyme supplementation of a rye-based diet on xylanase activity in the small intestine of broilers, on intestinal crypt cell proliferation and on nutrient digestibility and growth performance of the birds. British Poultry Science, 43(2), pp.274-282.

Simon, O., Jadamus, A. and Vahjen, W., 2001. Probiotic feed additives-effectiveness and expected modes of action. Journal of Animal and Feed Sciences, 10, pp.51-68.

Singh, A., O'neill, H.M., Ghosh, T.K., Bedford, M.R. and Haldar, S., 2012. Effects of xylanase supplementation on performance, total volatile fatty acids and selected bacterial population in caeca, metabolic indices and peptide YY concentrations in serum of broiler chickens fed energy restricted maize–soybean based diets. Animal Feed Science and Technology, 177(3-4), pp.194-203.

Singh, A.K., Berrocoso, J.D., Dersjant-Li, Y., Awati, A. and Jha, R., 2017. Effect of a combination of xylanase, amylase and protease on growth performance of broilers fed low and high fiber diets. Animal Feed Science and Technology, 232, pp.16-20.

Singh, A.K., Tiwari, U.P., Berrocoso, J.D., Dersjant-Li, Y., Awati, A. and Jha, R., 2019. Effects of a combination of xylanase, amylase and protease, and probiotics on major nutrients including amino acids and non-starch polysaccharides utilization in broilers fed different level of fibers. Poultry Science. doi: 10.3382/ps/pez310.

Sinovec, Z. and Marković, R., 2005. Use of pre-biotics in poultry nutrition. Biotechnology in Animal Husbandry, 21(5-6), pp.235-239.

Sławinska, A., Siwek, M.Z. and Bednarczyk, M.F., 2014. Effects of synbiotics injected *in ovo* on regulation of immune-related gene expression in adult chickens. American Journal of Veterinary Research, 75(11), pp.997-1003.

Snel, J., Harmsen, H.J.M., Van der Wielen, P.W.J.J. and Williams, B.A., 2002. Dietary strategies to influence the gastrointestinal microflora of young animals, and its potential to improve intestinal health. Nutrition and Health on the Gastrointestinal tract, pp.37-69.

Sommer, F. and Bäckhed, F., 2013. The gut microbiota—masters of host development and physiology. Nature Reviews Microbiology, 11(4), p.227.

Stanley, D., Hughes, R.J. and Moore, R.J., 2014. Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. Applied Microbiology and Biotechnology, 98(10), pp.4301-4310.

Steed, E., Balda, M.S. and Matter, K., 2010. Dynamics and functions of tight junctions. Trends in Cell Biology, 20(3), pp.142-149.

Steenfeldt, S. and Pettersson, D., 2001. Improvements in nutrient digestibility and performance of broiler chickens fed a wheat-and-rye based diet supplemented with enzymes. Journal of Animal and Feed Sciences, 10(1), pp.143-158.

Sun, Y., Su, Y. and Zhu, W., 2016. Microbiome-metabolome responses in the cecum and colon of pig to a high resistant starch diet. Frontiers in Microbiology, 7, p.779.

Swain, S.L., Weinberg, A.D., English, M.I.C.H.E.L.E. and Huston, G.A.I.L., 1990. IL-4 directs the development of Th2-like helper effectors. The Journal of Immunology, 145(11), pp.3796-3806.

Swennen, K., Courtin, C.M. and Delcour, J.A., 2006. Non-digestible oligosaccharides with prebiotic properties. Critical Reviews in Food Science and Nutrition, 46(6), pp.459-471.

Takahashi, T., Oka, T., Iwana, H., Kuwata, T. and Yamamoto, Y., 1993. Immune response of mice to orally administered lactic acid bacteria. Bioscience, Biotechnology, and Biochemistry, 57(9), pp.1557-1560.

Taylor, I.L., 1993. Role of peptide YY in the endocrine control of digestion. Journal of Dairy Science, 76(7), pp.2094-2101.

Teng, P.Y. and Kim, W.K., 2018. Roles of prebiotics in intestinal ecosystem of broilers. Frontiers in Veterinary Science, 5.

Theander, O., Westerlund, E., Åman, P. and Graham, H., 1989. Plant cell walls and monogastric diets. Animal Feed Science and Technology, 23(1-3), pp.205-225.

Tiwari, U.P., Singh, A.K. and Jha, R., 2019. Fermentation characteristics of resistant starch, arabinoxylan, and β-glucan and their effects on the gut microbial ecology of pigs: A review. Animal Nutrition. doi: 10.1016/j.aninu.2019.04.003.

Topping, D.L. and Clifton, P.M., 2001. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiological Reviews, 81(3), pp.1031-1064.

Topping, D.L., Fukushima, M. and Bird, A.R., 2003. Resistant starch as a prebiotic and synbiotic: state of the art. Proceedings of the Nutrition Society, 62(1), pp.171-176.

Trowell, H., 1976. Definition of dietary fiber and hypotheses that it is a protective factor in certain diseases. The American Journal of Clinical Nutrition, 29(4), pp.417-427.

Uni, Z. and Ferket, P.R., Yissum Research Development Co of Hebrew University and North Carolina State University, 2003. Enhancement of development of oviparous species by *in ovo* feeding. U.S. Patent 6,592,878.

Uni, Z. and Ferket, R.P., 2004. Methods for early nutrition and their potential. World's Poultry Science Journal, 60(1), pp.101-111.

Uni, Z.., Ganot, S., and Sklan, D. 1998. Posthatch development of mucosal function in the broiler small intestine. Poultry Science, 77(1), pp.75-82.

Vahjen, W., Gläser, K., Schäfer, K. and Simon, O., 1998. Influence of xylanase-supplemented feed on the development of selected bacterial groups in the intestinal tract of broiler chicks. The Journal of Agricultural Science, 130(4), pp.489-500.

Van Acker, H.H., Capsomidis, A., Smits, E.L. and Van Tendeloo, V.F., 2017. CD56 in the immune system: more than a marker for cytotoxicity?. Frontiers in Immunology, 8, p.892.

Van der Wielen, P.W., Biesterveld, S., Notermans, S., Hofstra, H., Urlings, B.A. and van Knapen, F., 2000. Role of volatile fatty acids in development of the cecal microflora in broiler chickens during growth. Appl. Environ. Microbiol., 66(6), pp.2536-2540.

Vaziri, N.D., Liu, S.M., Lau, W.L., Khazaeli, M., Nazertehrani, S., Farzaneh, S.H., Kieffer, D.A., Adams, S.H. and Martin, R.J., 2014. High amylose resistant starch diet ameliorates oxidative stress, inflammation, and progression of chronic kidney disease. PloS ONE, 9(12), p.e114881.

Veldman, A. and Vahl, H.A., 1994. Xylanase in broiler diets with differences in characteristics and content of wheat. British Poultry Science, 35(4), pp.537-550.

Verma, D. and Satyanarayana, T., 2012. Molecular approaches for ameliorating microbial xylanases. Bioresource Technology, 117, pp.360-367.

Voragen, A.G.J., Gruppen, H., Verbruggen, M.A. and Vietor, R.J., 1992. Characterization of cereal arabinoxylans. In Proc. Progress in biotechnology 7: Xylans and xylanases, J. Visser et al.(eds.). Elsevier, Amsterdam (pp. 51-67).

Vulevic, J., Rastall, R.A. and Gibson, G.R., 2004. Developing a quantitative approach for determining the in vitro prebiotic potential of dietary oligosaccharides. FEMS Microbiology Letters, 236(1), pp.153-159.

Vyas, J.M., Van der Veen, A.G. and Ploegh, H.L., 2008. The known unknowns of antigen processing and presentation. Nature Reviews Immunology, 8(8), p.607.

Walugembe, M., Hsieh, J.C.F., Koszewski, N.J., Lamont, S.J., Persia, M.E. and Rothschild, M.F., 2015. Effects of dietary fiber on cecal short-chain fatty acid and cecal microbiota of broiler and laying-hen chicks. Poultry Science, 94(10), pp.2351-2359.

Wang, J., Cao, Y., Wang, C. and Sun, B., 2011. Wheat bran xylooligosaccharides improve blood lipid metabolism and antioxidant status in rats fed a high-fat diet. Carbohydrate Polymers, 86(3), pp.1192-1197.

Wang, Z.R., Qiao, S.Y., Lu, W.Q. and Li, D.F., 2005. Effects of enzyme supplementation on performance, nutrient digestibility, gastrointestinal morphology, and volatile fatty acid profiles in the hindgut of broilers fed wheat-based diets. Poultry Science, 84(6), pp.875-881.

Wegener, H.C., 2003. Antibiotics in animal feed and their role in resistance development. Current Opinion in Microbiology, 6(5), pp.439-445.

Wei, S., Morrison, M. and Yu, Z., 2013. Bacterial census of poultry intestinal microbiome. Poultry Science, 92(3), pp.671-683.

Widyaratne, G.P. and Zijlstra, R.T., 2007. Nutritional value of wheat and corn distiller's dried grain with solubles: Digestibility and digestible contents of energy, amino acids and phosphorus, nutrient excretion and growth performance of grower-finisher pigs. Canadian Journal of Animal Science, 87(1), pp.103-114.

Wilensky, A., Tzach-Nahman, R., Potempa, J., Shapira, L. and Nussbaum, G., 2015. Porphyromonas gingivalis gingipains selectively reduce CD14 expression, leading to macrophage hyporesponsiveness to bacterial infection. Journal of Innate Immunity, 7(2), pp.127-135.

Williams, M.P., Klein, J.T., Wyatt, C.L., York, T.W. and Lee, J.T., 2014. Evaluation of xylanase in low-energy broiler diets. Journal of Applied Poultry Research, 23(2), pp.188-195.

World Health Organization, 1997. The Medical impact of the use of antimicrobials in food animals: report of a WHO meeting, Berlin, Germany, 13-17 October 1997 (No. WHO/EMC/ZOO/97.4). Geneva: World Health Organization.

Wu, G., 1998. Intestinal mucosal amino acid catabolism. The Journal of Nutrition, 128(8), pp.1249-1252.

Wu, Y.B. and Ravindran, V., 2004. Influence of whole wheat inclusion and xylanase supplementation on the performance, digestive tract measurements and carcass characteristics of broiler chickens. Animal Feed Science and Technology, 116(1-2), pp.129-139.

Wu, Y.B., Ravindran, V., Thomas, D.G., Birtles, M.J. and Hendriks, W.H., 2004a. Influence of phytase and xylanase, individually or in combination, on performance, apparent metabolisable energy, digestive tract measurements and gut morphology in broilers fed wheat-based diets containing adequate level of phosphorus. British Poultry Science, 45(1), pp.76-84.

Wu, Y.B., Ravindran, V., Thomas, D.G., Birtles, M.J. and Hendriks, W.H., 2004b. Influence of method of whole wheat inclusion and xylanase supplementation on the performance, apparent metabolisable energy, digestive tract measurements and gut morphology of broilers. British Poultry Science, 45(3), pp.385-394.

Yang, Y., Iji, P.A. and Choct, M., 2009. Dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. World's Poultry Science Journal, 65(1), pp.97-114.

Yang, Y., Iji, P.A., Kocher, A., Mikkelsen, L.L. and Choct, M., 2008. Effects of xylanase on growth and gut development of broiler chickens given a wheat-based diet. Asian-Australasian Journal of Animal Sciences, 21(11), pp.1659-1664.

Yasar, S. and Forbes, J.M., 2000. Enzyme supplementation of dry and wet wheat-based feeds for broiler chickens: performance and gut responses. British Journal of Nutrition, 84(3), pp.297-307.

Yegani, M. and Korver, D.R., 2008. Factors affecting intestinal health in poultry. Poultry Science, 87(10), pp.2052-2063.

Yitbarek, A., Echeverry, H., Brady, J., Hernandez-Doria, J., Camelo-Jaimes, G., Sharif, S., Guenter, W., House, J.D. and Rodriguez-Lecompte, J.C., 2012. Innate immune response to yeast-derived carbohydrates in broiler chickens fed organic diets and challenged with Clostridium perfringens. Poultry Science, 91(5), pp.1105-1112.

Yu, Z. and Morrison, M., 2004. Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques, 36(5), pp.808-812.

Zeisel, S.H., Mar, M.H., Howe, J.C. and Holden, J.M., 2003. Concentrations of choline-containing compounds and betaine in common foods. The Journal of Nutrition, 133(5), pp.1302-1307.

Zhang, L., Xu, J., Lei, L., Jiang, Y., Gao, F. and Zhou, G.H., 2014. Effects of xylanase supplementation on growth performance, nutrient digestibility and non-starch polysaccharide degradation in different sections of the gastrointestinal tract of broilers fed wheat-based diets. Asian-Australasian Journal of Animal Sciences, 27(6), p.855.

Zhao, G., Nyman, M. and Åke Jönsson, J., 2006. Rapid determination of short-chain fatty acids in colonic contents and faeces of humans and rats by acidified water-extraction and direct-injection gas chromatography. Biomedical Chromatography, 20(8), pp.674-682.

Zhenping, S., Wenting, L., Ruikui, Y., Jia, L., Honghong, L., Wei, S., Zhongmie, W., Jingpan, L., Zhe, S. and Yuling, Q., 2013. Effect of a straw-derived xylooligosaccharide on broiler growth performance, endocrine metabolism, and immune response. Canadian Journal of Veterinary Research, 77(2), pp.105-109.

Zhou, Y., Zhang, T., Wang, X., Wei, X., Chen, Y., Guo, L., Zhang, J. and Wang, C., 2015. Curcumin modulates macrophage polarization through the inhibition of the toll-like receptor 4 expression and its signaling pathways. Cellular Physiology and Biochemistry, 36(2), pp.631-641.

Zou, X.T., Qiao, X.J. and Xu, Z.R., 2006. Effect of β-mannanase (Hemicell) on growth performance and immunity of broilers. Poultry Science, 85(12), pp.2176-2179.

Żyła, K., Gogol, D., Koreleski, J., Świątkiewicz, S. and Ledoux, D.R., 1999. Simultaneous application of phytase and xylanase to broiler feeds based on wheat: feeding experiment with growing broilers. Journal of the Science of Food and Agriculture, 79(13), pp.1841-1848.

APPENDIX-I

Table I Nucleotide sequences of primers used in real-time qPCR analyses in all studies.

Gene	Primer	Sequence	Genbank Accession number	Amplicon Size (bp)
Immune cell:				
CD3	Forward	5'-GGACGCTCCCACCATATCAG-3'	NM_205512	180
	Reverse	5'-TGTCCATCATTCCGCTCACC-3'		
CD56(NCAM1)	Forward	5'-GTTCATGAGCAGAGGGTGCT-3'	NM 001242604	196
	Reverse	5'-ACATGGCCTGGATGATGCAA-3'		
ChB6 (Bu-1)	Forward	5'-TACTTTGTCGGCCGAGTGTC-3'	NM 205182	197
	Reverse	5'-AGTCTGCAGTTCCATTGGGG-3'		
TLR4	Forward	5'-AGTCTGAAATTGCTGAGCTCAAAT-3'	NM_001030693	190
	Reverse	5'-GCGACGTTAAGCCATGGAAG-3'		
Cytokine:				
IL-1β	Forward	5′-CGCTTCATCTTCTACCGCCT-3'	NM_204524	144
	Reverse	5′-GATGTTGACCTGGTCGGGTT-3′		
IL-4	Forward	5′-TGTGCCCACGCTGTGCTTACA-3'	NM 001030693	155
	Reverse	5′-CTTGTGGCAGTGCTGGCTCTCC-3'		
IL-10	Forward	5′-TGTCACCGCTTCTTCACCTG-3′	NM_001004414	105
	Reverse	5′-CTCCCCCATGGCTTTGTAGA-3′		
IL-12	Forward	5′-AGACTCCAATGGGCAAATGA -3′	NM_213571	274
	Reverse	5′-CTCTTCGGCAAATGGACAGT -3′		
Reference:				
β-actin	Forward	5'- GAGAAATTGTGCGTGACATCA-3'	NM 205518	152
	Reverse	5'- CCTGAACCTCTCATTGCCA-3'		