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# Assessment of Microbial and Growth Response of Broilers Fed a Commercial Prebiotic

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Assessment of Microbial and Growth Response of Broilers Fed a Commercial Prebiotic

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science in Cell and Molecular Biology

by

Sang In Lee  
University of Arkansas  
Bachelors of Science in Animal Science, 2013

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This thesis is approved for recommendation to the Graduate Council

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## **Abstract**

Prebiotic refers to nondigestible food ingredients that enhance the health of the host by selectively promoting one or more number of beneficial bacteria. Gibson and Roberfroid (1995) initially described prebiotics as compounds that neither hydrolyzed nor absorbed in upper part of the gastrointestinal tract, selectively stimulates growth of beneficial bacteria, enhance health of the host and able to amend microorganism population to healthier states. Prebiotics are particularly attractive supplements in animal production due to their variety of effects including production of short chain fatty acids, pH amendments and suppression of pathogen colonization. In addition, prebiotics were emphasized as antibiotic alternatives due to raise of antibiotic resistant pathogens. This thesis consists of one review and two research parts: a comprehensive literature review that covers various prebiotics used in poultry industry and microbiome quantification techniques including multiplex polymerase chain reaction (PCR), quantitative PCR, denaturing gradient gel electrophoresis (DGGE), and next generation sequencing (NGS) (**chapter 1**), a research manuscript involves assessment of cecal microbiota, integron, fermentation response, and *Salmonella* frequency in broilers fed a commercial prebiotic (**chapter 2**), and a research manuscript involves growth performance and microbial population assessment by NGS approach (**chapter 3**).

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## **I. Introduction**

Antimicrobial growth promoters have been commonly used in agriculture production since 1950's to improve performance of livestock and to reduce pathogen colonization. However, over usage of antibiotics in the agriculture industry contributed to the rise of the antibiotic resistant pathogens thus, alternative supplements such as prebiotics were required and demanded by consumers and producers. Variety of compounds such as mannanoligosaccharides (MOS), galactooligosaccharides (GOS) and fructooligosaccharides (FOS) were evaluated and developed as prebiotics that potentially could replace in-feed antibiotics. This thesis focuses on the application of above prebiotics in poultry industry and molecular techniques that used to evaluate its effects on poultry. In addition, advantages and disadvantages of molecular techniques were described. Research manuscripts of this thesis are focused on MOS prebiotic in particular and emphasized on investigation of microbial population by polymerase chain reaction based denaturing gradient gel electrophoresis (PCR-DGGE) and next generation sequencing (NGS). Growth performance, processing responses including birds weights, feed conversion ratio (FCR), parts weight and white meat yield of the poultry are the major aspects that producers are concerned and these specific characteristics are affected by prebiotic through different mechanisms. Numerous studies were conducted to evaluate many aspects of poultry by prebiotics however, inconsistent results were observed. Understanding the mechanisms of variety of prebiotics, limitation and advantage of molecular technique will contribute to help choosing more adequate approaches and more consistent result in future study of prebiotics.



## **II. Chapter 1**

### **Application of prebiotics in poultry and molecular techniques for microbiome quantification**

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## **1. Abstract**

Antibiotics as a part of feed supplementation have been traditionally used in poultry production for a variety of reasons. Such feed supplements were considered an economical advantage because they appeared to directly benefit producers by decreasing mortality rates of farm animals, increasing animal growth rates and improving the feed conversion ratios. However, the concept of antibiotic alternatives such as prebiotics and probiotics emerged because it was believed that the use of antibiotics contributed to the increase of antibiotic resistant pathogens of public health concern. Prebiotics are known as nondigestible carbohydrates that selectively stimulate growth of beneficial bacteria, thus improving overall health of the host. Once prebiotics are introduced to the host, two major modes of action can potentially occur. First, the corresponding prebiotic reaches the intestine of the host without being digested in the upper part of the gastrointestinal tract by the host but are selectively utilized by certain bacteria considered beneficial to the host. Secondly, other gut activities occur in conjunction with prebiotic presence including generation of short-chain fatty acids and lactic acid as microbial fermentation products as well as increased height of intestinal villi leading to an increased absorption rate of nutrients, a decreased rate of pathogen colonization and inhibition of pathogen adherence.

A variety of molecular techniques have been utilized in food microbiology and safety because rapid detection and typing analysis are directly related to public health. Multiplex PCR is a modified version of conventional PCR that can amplify multiple DNA fragments simultaneously, thus have considerable utility for pathogen identification, SNP genotyping, and mutation analysis. One of the more recent methods to quantify genes and organisms is quantitative PCR. Quantitative PCR allows absolute or relative quantification of target sequence in a high-throughput format. Two types of signals are primarily utilized in qPCR. One of them is

SYBR green fluorescence which can bind to double stranded DNA. Another approach is to use the TaqMan probe which consists of a reporter and quencher group that are involved in emitting signals. Quantitative PCR can be used for diagnostic applications in clinical microbiology to detect infectious disease agents, to detect genetically modified organisms and to quantify genotype strains.

Characterizing the microbial community of the gut is important because gut health can directly impact the health of host. One of the many types of approaches to investigate the gastrointestinal microbiome is band pattern based analysis using denaturing gradient gel electrophoresis (DGGE) or DNA based quantification by next generation sequencing (NGS). Analysis by DGGE was first introduced to analyze the microbial diversity in mixed, complex microbial populations. However, modifications of DGGE and TGGE have greatly expanded usage of this technique for applications in genetic fingerprinting, monitoring microbial community status and subsequent enrichment of selected bacteria, gene detection, clone library screening and even determining PCR and cloning biases. Next generation sequencing, also referred to as parallel sequencing is a more recent sequencing technology developed after Sanger sequencing had served as the standard. Millions of DNA fragments from a mixed culture sample can be sequenced by NGS, thus application of this technology can be unlimited. Examples of applicable fields include therapeutic and clinical use to determine genetic disease.

## **2. Introduction**

Prebiotics were initially defined as nondigestible food ingredients that promote one or more number of beneficial bacteria such as *Bifidobacteria* and *Lactobacillus* and thus improving gut health which could in turn enhance the host's health (Gibson and Roberfroid, 1995; Kleessen, et al., 2001). Properties and requirements for substances to be classified as prebiotics were also described by Gibson and Roberfroid (1995) as those compounds which must be 1) neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract (GIT); 2) a selective substrate that stimulates growth or metabolic activity of one or more of what would be considered beneficial bacteria; 3) able to amend microbiota towards a healthier state; 4) able to induce luminal or systemic effects that are beneficial to the host. Suggested candidates that meet these criteria include nondigestible carbohydrates which are substrates that are not absorbed in the upper part of the GIT and utilized by endogenous colonic bacteria. Nearly 10 years later, Gibson et al. (2004) revised the definition of prebiotics by stating that they were "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" (Gibson, et al., 2004). By 2007, trans-galactooligosaccharide and inulin were considered the only substrates that fully met these requirements (Roberfroid, 2007). More recently, resistant starch, lactulose and mannanoligosaccharides (MOS) have also been considered prebiotics (Boler and Fahey Jr, 2012; Hutkins, et al., 2016; Ricke, 2015b).

## **3. Prebiotics**

### **a. Improving animal production - Historical perspectives**

Antibiotic growth promoters (AGP) have been used in animal production since the 1950's in order to improve their performance and control their GIT microbiota to make them less susceptible to pathogens (Groschke and Evans, 1950; Jones and Ricke, 2003; Moore, et al., 1946; Stokstad and Jukes, 1950; Thomke and Elwinger, 1998; Whitehill, et al., 1950). Limiting the occurrence of enteric disease associated pathogens is important not only because of public health concerns but productivity and mortality of livestock are believed to be directly related (Castanon, 2007; Gaggia, et al., 2010; Merrifield, et al., 2010; Patterson and Burkholder, 2003). However, it has been suggested that the frequency of antibiotic resistant bacteria may be increased by the continued use of antibiotic growth promoters in agricultural production (Barnes, 1958; Dibner and Richards, 2005; Gorbach, 2001; Lee and Lin, 2003; Marshall and Levy, 2011; Starr and Reynolds, 1951).

Therefore, interest in developing alternative approaches to retain progress in weight gain responses of farm animals continues to increase (Abbas, et al., 2014; Bachaya, et al., 2015; Biggs, et al., 2007; de los Santos, et al., 2005; Griggs and Jacob, 2005; Kasper, 1998; Nava, et al., 2005; Parnell and Reimer, 2010; Zaman, et al., 2012). In broiler production programs, it is essential to have broilers reach their market weight as soon as possible. Rapid growth of broilers not only saves labor and feed but also minimizes fixed cost of production (Austic and Nesheim, 1990). Bird growth rate is more likely to increase when broilers exhibit a high feed conversion efficiency thus feed additives such as prebiotics or probiotics if effective may reduce time prior to marketing (Timmerman, et al., 2006). By modifying the gut microbiota, producers tend to increase energy utilization of birds hence, increase performance of birds. According to De Maesschalck et al. (2015) xylo-oligosaccharides (XOS) which exhibit prebiotic like properties resulted in significant increases of villi length in the ileum, *Lactobacilli* numbers in the colon and

*Clostridium* in the ceca of broiler chicken. Since the GI tract is highly colonized (Apajalahti and Kettunen, 2006), microbial composition and corresponding microbial physiology is very important. Thus, the potential for enhancing short chain fatty acids (SCFAs) production by prebiotic selected microorganisms has been profiled in numerous studies (Blaut, 2002; Donalson, et al., 2008a; Gibson and Wang, 1994; Ricke, 2015b; Scholz-Ahrens and Schrezenmeir, 2002).

Prebiotics can also exhibit their beneficial effect on layer hen performance as well. According to Chen et al. (2005), a 1.0 % oligofructose-type commercial prebiotic supplementation and 1.0 % of inulin administered hens increased weekly egg production by 13.35 and 10.73% respectively. Also, both additives increased cumulative weekly egg weight by 12.50 and 10.96% as well as the feed conversion ratio of birds. Another study by Chen and Chen (2004) focusing on the ability to utilize oligofructose and inulin by layer hens also exhibited a beneficial effect from the prebiotics. Analyzing minerals in tibia and eggshell strength is important in layer production because it is directly related to mineral absorption rate of birds and retail sales. According to Chen and Chen (2004), oligofructose and inulin supplemented layer's serum calcium levels, eggshell weight, eggshell strength, total ash, calcium and phosphorus levels in the tibia were significantly increased.

#### **b. Impact of prebiotics on gut physiology and composition**

Prebiotics were first introduced in order to externally manipulate genetic composition of the microbiome via dietary feed additives (Gibson and Roberfroid, 1995). Manipulation of bacterial composition can also be initiated by introduction of bacteria which reside in the large intestine that goes through a series of fermentation reactions that in turn impacts gut physiology. Mechanisms of pathogen inhibition in the microbiome include production of SCFAs and lowering pH, competing for nutrients and binding sites on the epithelium of the intestine (Afr,

1989; Gibson and Fuller, 2000; Ricke, 2003; Rolfe, 2000). The SCFAs are produced by microorganisms during fermentation of complex sugar molecules (Cummings and Macfarlane, 1991; Cummings, 1981; Rerat, et al., 1987) can modulate physical properties of the villi in GIT, and thus increase absorption rate of minerals and molecules (Xu, et al., 2003). In addition, *Bifidobacterium* and *Lactobacillus* residing in the gut can restrict pathogen colonization by producing lactic acid (Orrhage and Nord, 1999; van der Wielen, et al., 2000).

Young birds are typically more susceptible to enteric pathogens due to their lack of gastrointestinal microbiota complexity which becomes more complex through maturity of birds (Milner and Shaffer, 1952; Palmer, et al., 2007; Stark and Lee, 1982; Stavric, 1987). Therefore, administration of feed amendments and biologicals such as probiotics or prebiotics to accelerate development of complexity of gastrointestinal microbiota in the newly hatched bird has been considered a means to reduce susceptibility to enteric pathogens (Corrier, et al., 1995; Nisbet, 2002; Nurmi and Rantala, 1973; Stavric, et al., 1985). Competitive exclusion treatment administered to newly hatched birds serves as the classic example of reduction in pathogen colonization by introducing microorganisms into the intestine (Nurmi and Rantala, 1973). Competitive exclusion (CE) are typically normal, intestinal adult fowl microorganisms that are directly introduced to the host (Corrier, et al., 1995; Fukata, et al., 1999; Nisbet, 2002; Nurmi and Rantala, 1973; Stavric, et al., 1985).

Several studies have proven that prebiotics possess modes of action that benefit gastrointestinal microbiota of the host but in a different manner than competitive exclusion methods (Patterson and Burkholder, 2003). While competitive exclusion approaches involves introduction of a external microbial population or even single microorganism to the host (Mead, 2000), a prebiotic indirectly influences the microbiota by increasing the concentration of

indigenous beneficial microorganisms (Gibson and Roberfroid, 1995). Prebiotics are known to increase mineral absorption rate (Scholz-Ahrens and Schrezenmeir, 2002), enhance immune function (Lomax and Calder, 2009) and possibly deter development of colon cancer (Geier, et al., 2006; Munjal, et al., 2009). In particular, selection for increased populations of *Bifidobacteria* and *Lactobacilli* represent the best known major beneficial effects of prebiotics because presence of these bacteria are considered an indicator of healthy microbiota due to their ability to suppress growth of putrefactive proteolytic bacteria (Afric, 1989; Rowland, 1992; Yusrizal and Chen, 2003).

According to Scholz-Ahrens et al. (2002), inulin type fructans led to increased SCFAs production, and villi height which ultimately increased the absorptive surface (Xu, et al., 2003). Production of SCFAs is significant because SCFAs introduce a variety of impacts on the gut microbiota of the host (Ricke, 2003; van der Wielen, et al., 2000). Some of the effects include reducing pH in the gut lumen to prevent pathogen colonization (Blaut, 2002; Gibson and Wang, 1994), increasing mucin production in order to enhance colon morphology (Barcelo, et al., 2000) and improving the immune response by sparing glutamine which serves lymphatic tissue as a preferred substrate (Jenkins, et al., 1999; Ouwehand, et al., 2005; Taylor, et al., 1990). Also, oligo-fructoses in particular can enhance solubility of minerals that consequently increase transportation rate of calcium and phosphorus (Scholz-Ahrens and Schrezenmeir, 2007).

Another beneficial role of prebiotics that has been proposed is that prebiotics may inhibit infectious bacteria colonization by competing for the receptor sites on intestinal epithelial cells. (Gibson, et al., 2005; Kunz, et al., 2000). Adherence mechanisms of pathogen are very important because they represent the initial step in the colonization process (Finlay and Falkow, 1989;



Karlsson, 1989). Several studies have demonstrated anti-adhesive properties associated with prebiotics in *Escherichia coli* (Aronson, et al., 1979; Buddington, et al., 2002; Firon, et al., 1987).

The most extensively characterized prebiotic groups are discussed in the current review, namely, fructooligosaccharides, galactooligosaccharides and mannanoligosaccharides (yeast based products) along with the less traditional nondigestible oligosaccharides.

Fructooligosaccharides are linear chains of fructose units which occur naturally in various plants for example, onion, chicory, galic, asparagus, banana, artichoke as well as other sources (Sabater-Molina, et al., 2009). Galactooligosaccharides are produced from lactose by  $\beta$ -galactosidase (Torres, et al., 2010; Wallenfels and Malhotra, 1960). Lastly, mannanoligosaccharides are derived from the cell walls of the yeast *Saccharomyces cerevisiae*. In the following subsections, individual groups of prebiotics will be discussed in more detail.

### **b-1. Fructooligosaccharides**

Fructooligosaccharides (FOS) qualify as prebiotics because they are not hydrolyzed in the upper part of GIT and can act as substrates for specific intestinal microorganisms (Hartemink, et al., 1997). Fructooligosaccharides can be fermented by *Bifidobacteria* and *Lactobacillus* thus, improving the gut health of the host as described previously (Bouhnik, et al., 1994; Gibson and Roberfroid, 1995; Hidaka, et al., 1986). Studies have shown that FOS may increase SCFAs and lactic acid concentration and also inhibit growth of pathogens such as *Clostridium perfringens* which can contribute to the high bird mortality rate in poultry production (Ricke, 2015b). Oyarzabal and Conner (1996) observed that *Salmonella* colonization was reduced by 19% when FOS was introduced into the drinking water of broilers. In addition, Bailey et al. (1991) demonstrated that enterobacteria such as *Salmonella* and *E. coli* will not utilize this particular prebiotic as a carbon source and they observed reduction of *Salmonella* colonization by 42%

when these compounds were combined with a competitive exclusion culture. In addition, reduction of two logarithms of *S. Typhimurium* populations were observed *in vitro* by addition of FOS to the feed substrate (Donalson, et al., 2007) which corresponds to the observations by Bailey et al (1991). The study by Ammerman et al. (1988) demonstrated a positive effect or improvement by FOS such as increased feed efficiency and weight gain of broilers. However, negative effects were observed in study of Waldroup et al. (1993) when cecal contents of broilers and *Salmonella* on broiler carcasses were observed.

Layers were also affected beneficially by prebiotic treatment resulting in increased production of cecal SCFAs and lactic acid (Donalson, et al., 2008a) when a mixture of 0.25g of either alfalfa meal or layer ration and 7.5% of FOS were added into cecal material. However, no significant effects were consistently observed in the study of Donalson et al. (2008b). Donalson et al. (2008b) concluded that there may be several possible reasons for limited laying hen responses to FOS including minimal utilization capability of the microbiome, more rapid fermentation of similar substrates or simply a low intake of the prebiotic. In terms of *Salmonella* colonization against FOS, Donalson et al. (2008b) reported that FOS containing diets to hens significantly reduced *Salmonella* concentration in ovaries and livers of hens subjected to feed withdrawal.

To counter this inconsistency, different dosages of prebiotics have been examined. Improvements in feed conversion ratio (FCR) have been positively related to increased enzymatic activity of leucine aminopeptidase (LAP), protease and amylase (Williams, et al., 2008; Xu, et al., 2003). However, negative effects of FOS were demonstrated by Ten Bruggencate et al. (2003), claiming that FOS may stimulate growth of not only some beneficial bacteria but also noxious bacteria. In addition, rapid fermentation of FOS can cause excessive

production of SCFAs that potentially induces colonic mucosal damage or inflammation due to their low pH, hence decreasing resistance to intestinal pathogens (Argenzio and Meuten, 1991; Remesy, et al., 1993). Ten Bruggencate et al. (2004) suggested that if an excessive number of beneficial bacteria already exist in the host, improvements may not occur after introduction of the prebiotic. Optimal levels of FOS were observed by Wu et al. (1999) when 2.5 to 5.0 g/kg was supplemented with beneficial effects such as increases in body weight gains and improved feed efficiencies occurring while over supplementation of FOS (10 g/kg) caused diarrhea, resulting in reduced production performance .

## **b-2. Galactooligosaccharides**

Prebiotic galactooligosaccharides (GOS) are produced from lactose by glycosyl transfer of the D-galactosyl unit to D-galactose moiety of lactose followed by catalysis of the hydrolysis of  $\beta$ -galactosides by  $\beta$ -galactosidase (Mahoney, 1998). Tzortzis et al. (2005) demonstrated that GOS not only elevated the growth rate of *Bifidobacteria* species by 0.9 log but also increased lactic (4 mM) and acetic acid (3 mM) production in the proximal colon of pigs 4 and 3 fold, respectively compared to the diet of negative control group which was completely free of antibiotic. This study was of particular interest because the effect of prebiotic was identified at the bacterial species level, versus to other conventional prebiotic studies where only quantity of specific bacterial genera were studied. Targeting and comparing bacteria at the species level is significant because even when genera and species of bacteria are the same, preferred substrates for fermentation may vary under different circumstances. For example, Ruiz-Moyano et al (2013) demonstrated a variance in the preferred substrates among subspecies of *Bifidobacterium*. *Bifidobacterium longum* subsp. *infantis* and where *Bifidobacterium bifidum* preferred human milk oligosaccharides while *B. longum* subsp. *longum* and *Bifidobacterium breve* did not.

Anti-adhesive properties of GOS have been described by Shoaf et al (2006) where they observed that the adherence of enteropathogenic *E. coli* (EPEC) to HEP-2 cells and Caco-2 cells were inhibited by 65% and 40 to 70% respectively. Among the many prebiotics compared by Shoaf et al. (2006), GOS exhibited the highest inhibition rate of adhesion activity of *E. coli* to tissue cells. Shoaf et al. (2006) suggested that differences in adherence inhibition were more likely due to the structural differences among the oligosaccharides. Enzymatic modification or chemical modification of GOS has been suggested as a means to obtain prebiotics which are similar to human breast milk oligosaccharides that possess high anti adherence activity against not only *E. coli* but also *Campylobacter jejuni* and *Helicobacter pylori* (de Araújo and Giugliano, 2000; Newburg, 1997; Palmeira, et al., 2001). Beneficial effects due to the introduction of GOS to broilers were described by Jung et al (2008) demonstrating that increased overall numbers of anaerobic bacteria including *Lactobacilli* and *Bifidobacteria*. According to Jung et al. (2008), GOS significantly promoted populations of *Lactobacilli* by 0.53 log units and *Bifidobacteria* by 1.32 log units.

### **b-3. Yeast as a prebiotic source**

Another alternative approach to feed additives resulting from the ban of in-feed antibiotics was the introduction of yeast cultures (YC) as feed amendments. The concept of YC as a feed additive was first introduced by Eckles and Williams (1925) utilizing *Saccharomyces cerevisiae* as a growth promoter for ruminants. Studies reported that introduction of YC can enhance ruminal fermentation (Alshaikh, et al., 2002; Lila, et al., 2004; Roto, et al., 2015; Sune, 1998). The YC can be differentiated from probiotics by including only cellular constituents, residual of viable cells, cell wall components, metabolites as well as the cultural media which the yeast were grown (Miles and Bootwalla, 1991). Introduction of YC to the digestive system can

greatly benefit the host by enhancing digestive function of the GIT as well as modifying microbiota (Beev, et al., 2007) therefore, commercial usage of YC became common practice in ruminant production. Subsequently, yeast products were used in poultry production and swine production (Bradley, et al., 1994; Hayat, et al., 1993; Shin, et al., 2005; Stanley, et al., 2004; Van Heugten, et al., 2003; Zhang, et al., 2005).

However the mechanisms of the yeast product when present in the GIT of animals remain unclear (Beev, et al., 2007). Stanley et al. (2004) reported that YC supplementation promotes beneficial effects by increasing commensal microorganisms or reducing pathogens however, inconsistent results were reported in other studies (Stanley, et al., 2004; White, et al., 2002). Gao et al (2008) hypothesized that the beneficial mechanism of YC is that mannan-oligosaccharide which is a component of the yeast cell wall enhances immunity of host, thus promoting growth of beneficial bacteria. In other studies it was reported that the amendment of YC improved phytate phosphorus utilization by poultry (Thayer, et al., 1978; Thayer and Jackson, 1975) and nutrient digestibility (Shin, et al., 2005). Also, intestinal mucosal development was improved by YC supplementation in broilers (Santin, et al., 2001; Zhang, et al., 2005). A more recent study by Gao et al. (2008) was focused on investigating effects of different concentrations of YC in broilers on performance and immunomodulatory functions. Dietary supplementation of YC resulted in increases of average daily gain and feed conversion ratio of broilers. In addition, digestibility of calcium and phosphorus were increased and villi height to crypt depth ratios in the duodenum and jejunum and ileum were also increased when broilers were fed 2.5g / kg of YC. Immunomodulatory functions were observed by vaccinating Newcastle disease vaccine to broilers along with YC and resulted in a beneficial effect. However, Gao et al. (2008)'s result demonstrated that the appropriate levels of YC is essential for broilers to exhibit a beneficial

effect. They noted that an either too high or low concentration of YC did not appear to have significant benefits.

#### **b-4. Mannanligosaccharides**

Mannanligosaccharides (MOS) are prebiotic compounds derived from yeast cell walls and they have been associated with enhanced growth performance of broilers (Hooge, 2004; Rosen, 2007). However, significant improvement of performance was only detected in young birds inhabited by less populated gut microbiota while older birds did not exhibit significant improvements because of their complex indigenous microbiota population. Studies have shown that the intestinal tracts of newly hatched birds stabilizes when birds are at least 2 weeks old (Amit-Romach, et al., 2004; Lee, et al., 2010; Ohimain and Ofongo, 2012). *Lactobacilli* for example, are not predominant in chickens less than 2 weeks old (Barnes, et al., 1972).

Once MOS is introduced to the host gut, its high affinity ligands compete with the receptors of pathogens (Ofek, et al., 1977). Also, MOS binds to mannose-specific type-1 fimbriae of pathogens and can prevent colonization of pathogens (Newman, 1994). Spring et al (2000) reported that MOS decreased intestinal *Salmonella* concentration by 25 fold (4.01 versus 5.40 log cfu/g) when compared to the control group of broiler chickens. Oyofe et al. (1989 a, b) specifically demonstrated reduction of *S. Typhimurium* colonization by adding mannose to the diets of young broilers.

Conversely, the effects of MOS were not as obvious in a study of laying hens provided with 0.11% of MOS. According to Zaghini et al. (2005), MOS treated birds produced lighter eggs in the second and third weeks of the trial compared to the negative control and 2.5 ppm of aflatoxin B1 (AFB1) treated birds which corresponded to the result reported previously by Rizzi et al. (2003). However, the reduced aflatoxin level found in the liver of birds demonstrated the

ability of MOS to degrade aflatoxins (Zaghini, et al., 2005). In addition, Zaghini et al. (2005) reported the shell thickness was negatively influenced when AFB1 and MOS were both administered, thus concluding that MOS influenced egg quality traits positively.

In addition, studies involving MOS supplementation have shown that they can aid livestock in retaining health by increasing immunoglobulin A production (Kudoh, et al., 1999; Swanson, et al., 2002). Similar to FOS, MOS administration has also exhibited some inconsistencies in animal performance responses. Stanczuk et al. (2005) and Pelicano et al. (2004) reported there were no significant effects of MOS found in terms of weight gain of turkeys and broiler chickens while Sim et al. (2004) reported improvements in live weights of turkeys. In a study on broilers, Hooge (2004) reported that compared to non-supplemented flocks, MOS treated birds exhibited significant increases in body weight and feed conversion ratio while mortality was reduced. However, when MOS fed birds were compared with antibiotic-supplemented birds, no significant increases in body weight and feed conversion ratio were observed (Hooge, 2004). According to Yang et al. (2007) and Hughes and Benedetto (2003), MOS as a feed amendment did not affect apparent metabolisable value. In addition Yang et al (2007) reported that MOS supplement increases body weight gain of broiler but did not affect the gut morphology and function compared to negative and positive control groups. Yang et al (2007) hypothesized that the inconsistent results may be due to differences in species, diets, and rearing conditions.

#### **b-5. Non-digestible carbohydrates and fiber dietary sources**

Besides the particular prebiotics discussed in the previous sections and the subsequently more chemically defined prebiotics such as fructans, oligofructose, inulin, lactulose and glutan, a few other substances have also proposed as possessing prebiotic-like properties. These include

resistant starch (RS), pectin and milk oligosaccharides (Hutkins, et al., 2016). The effects of RS were recognized and emphasized by Bird et al. (2010) along with its relationship with SCFAs, especially butyrate. According to Bird et al. (2010) dietary intake level of RS in a population at low risk of diet-related large bowel diseases was high compared to populations at high risk of disease. Also, reports indicated that consumption of RS increased concentration of SCFA, especially butyrate acid in large bowel and lowered colonocyte proliferation (Dronamraju, et al., 2009; van Munster, et al., 1994). Ricke et al. (1982) demonstrated the beneficial effect of various fibers including alfalfa cell wall, lignin, xylose, pectin and gum arabic to young chicken age of 1 and 8 days old. As result, alfalfa cell wall, and mixture of lignin, xylose and gum arabic significantly increased feed efficiencies of 1, 8 day old chicks.

Another fiber source being readily examined especially for layer hens is alfalfa. Because of the nature of commercial egg industry, layer hens go through a molting procedure prior to their next laying cycle to rejuvenate flocks and increase marketing profits (Donalson, et al., 2005). Historically, the most commonly used molting methods involved withdrawing feed to simulate molting behavior of wild birds for the generation of a second egg laying cycle. However, this feed withdrawal (FW) method compromised the health of layer hens making them susceptible to *S. Enteritidis* infection. Studies have shown that when alfalfa is mixed with feed of layer hens, it induced molt while inhibiting growth of *S. Enteritidis* due to its properties of high protein, high fiber, well balanced amino acids, vitamins and antioxidants.

Kim et al (2005), discovered that a molting treatment of 100% alfalfa significantly increased the egg production compared to 90% alfalfa treated birds. Also, shell weight of feed withdrawal group and 90% alfalfa diet groups exhibited heavier shell weight than 100% alfalfa fed birds at the end of the second laying cycle indicating heavier eggs are produced by molted



hens than younger nonmolted hens. In summary, alfalfa based diets could serve as an alternative method to induce molt in birds because inducing molt by feed withdrawal can alter the microbiome and the immune system of birds hence, making them more susceptible to *S. Enteritidis* colonization (Ricke, et al., 2013).

Since there are various types of prebiotic and their mechanisms are different, evaluation of the effects of prebiotics are essential. However, since many different microbiota species exhibit unique metabolism strategies, it is difficult to distinguish whether a prebiotic characteristic is having a beneficial impact on the microbiota of the host (Hutkins, et al., 2016). Several aspects can be evaluated such as pathogen identification and quantification of specific microorganisms and compositional analysis of microbiota. In the following sections, the more commonly used molecular methods to evaluate such aspects are discussed.

#### **4. Molecular methods for quantifying and identifying GIT pathogens**

##### **a. Introduction**

The importance of microbial ecology of variety of food animals has been highlighted and began with studies in order to understand ability of ruminants to subsist an cellulose (Ricke, 2015a). As awareness of food safety and interest of functional food increased by consumers, characterization of the GIT and an understanding of bacterial metabolism and the relationships with the host became essential and required consistent, rapid and accurate methodology to evaluate these aspects that were required for the food production industry. Therefore, a wide range of supplements such as prebiotics and probiotics have been examined and developed. However, characterization of highly populated regions of the GIT can be difficult.

In the past decade, the concept and the definition of prebiotics are being refined as methods for identifying and quantifying gut microbiota of the host that had been traditionally limited to culture-based methods in the past are now involving molecular based approaches (Hutkins, et al., 2016; Ricke and Pillai, 1999). However, while numerous approaches have been developed such as qPCR and next generation sequencing (NGS) methods, other microorganisms of interest, besides *Lactobacillus* and *Bifidobacterium* have been identified and subjected to evaluate the effect of prebiotic candidates.

### **b. Multiplex PCR**

Due to irreproducibility of various serotyping methods such as amplified fragment length polymorphism (AFLP) (Torpdahl and Ahrens, 2004), random-amplified polymorphic DNA (RAPD)-PCR (Hoorfar, et al., 2000) and PCR-single-strand conformation polymorphism (SSCP) (Nair, et al., 2002), multiplex polymerase chain reaction (PCR) approaches were developed. Identifying different strains by multiplex PCR is easier compared to conventional PCR because the procedure is essentially identical as conventional PCR but more rapid by hours due to its simultaneous amplification of multiple fragments. Multiplex PCR requires several primers that align with varying sizes of target genes and thus needs to be optimized so that all primer pairs can attach to the template strand at the same annealing temperature. This method was first introduced by Chamberlain et al. (1988) for detecting the majority of the deletions occurring in the Duchenne muscular dystrophy (DMD) gene. In addition, multiplex PCR has been utilized by Ballabio et al (1990), to screen for the steroid sulfatase (STS) gene in patients with STS deficiency. Multiplex PCR had also been modified for genotyping microsatellite (SSR) and single nucleotide polymorphism (SNP) by Hayden et al. (2008).

One of the advantages of multiplex PCR is that it provides internal controls (Edwards and Gibbs, 1994) which means by amplifying multiple fragments, amplicons can act as internal controls for each other and thus can reveal false negatives. For example, the failure of fragment amplification can be determined if the noncontiguous deletions show no detectable bands because major gene deletions are usually contiguous (Chamberlain, et al., 1992). Multiplex PCR can also be used as an indicator of template quality by determining several loci at the same time with higher sensitivity than Southern blot analysis. According to Chamberlain et al. (1992), multiplex PCR analysis revealed single exon deletion mutations which were not detected by Southern blot analysis and 82% of those deletions detected by Southern blot analysis were also detected by multiplex PCR. By targeting multiple sequences, evaluation of amplification efficiency can be more accurate.

#### **b-1. Microbial pathogen identification**

One of the major applications of multiplex PCR is the identification of bacterial pathogens. Because multiplex PCR amplifies various genes simultaneously, specific bacterial species or strains can be differentiated. Numerous research and review articles have been published on the application of multiplex PCR approaches to detect and identify bacterial pathogens in water, shellfish, animal feeds, respiratory tract, clinical and agricultural samples and other sources (Baker, et al., 2016a; Bej, et al., 1990; Brasher, et al., 1998; Jarquin, et al., 2009; Kim, et al., 2007; Kong, et al., 2002; Maciorowski, et al., 2005; Pacheco, et al., 2007; Panicker, et al., 2004; Park, et al., 2014). Commonly targeted pathogens include *E. coli* O157:H7, *Salmonella*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Vibrio parahaemolyticus* and fungi. Also, viral DNA can be amplified by multiplex PCR in order to screen tissue samples with infectious disease. Viruses including human papillomavirus (HPV), human immunodeficiency

virus type 1 (HIV-1), human T-cell leukemic viruses, human T lymphotropic virus types I and II, hepatitis B virus, parvovirus and hog viruses have been screened or detected by multiplex PCR assay in several studies (Repp, et al., 1993; Sevall, 1990; Sunzeri, et al., 1991; Vandenvelde, et al., 1990; Wattel, et al., 1992; Wirz, et al., 1993).

*Salmonella* and *E. coli* O157:H7 are typically classified as the foodborne pathogens of greatest concern and multiplex PCR technique enabled considerable reduction in detection time by days (Mahon, et al., 1994; Park, et al., 2014). Baker et al. (2016a) also suggested that multiplex PCR can be used to distinguish closely related microbial species by developing genus, species specific primers via BLAST search targeting. According to Oliveira and Lancaster (2002), multiplex PCR technique was proven to be rapid, robust and useful technique to differentiate *mec* elements among 18 different strains of *Staphylococcus aureus*.

## **b-2. Mutation detection by DNA typing**

The single nucleotide polymorphism (SNP) refers to single nucleotide variation in the genome between members of biological species or paired chromosomes (Brookes, 1999). Therefore, SNP genotyping is the measurement of genetic variation. Conventional methods to detect SNP consist of two steps, amplification of target sequence and detection of the SNP (Ye, et al., 1992, 2001). Since multiplex PCR can utilize several markers simultaneously, efficiency and speed of SNP detection is improved. Sanchez et al. (2003) reported a new SNP typing assay based on multiplex PCR and multiplex minisequencing. Similar to SNP detection, multiplex PCR can be utilized to analyze a mutation on the DNA strand as well. Cheng et al. (2004) employed multiplex PCR to rapidly detect mutations in *Mycobacterium tuberculosis*. Cheng et al. (2004) detected mutations in DNA fragments more rapidly within hours, compared to the parental single stranded conformation polymorphism approach which requires more than 10 h

(Cheng, et al., 2004). Duchenne/Becker muscular dystrophy (DMD/BMD) is an example of human disease caused by a gene deletion in a specific region (Chamberlain, et al., 1988). By targeting the steroid sulfatase gene with multiplex PCR, the respective deleted or altered gene could be identified (Ballabio, et al., 1990; Chamberlain, et al., 1988).

Another application of multiplex PCR is DNA typing to determine genetic linkage and mapping (Neff, et al., 1999; Towbin, et al., 1993). The DNA typing of an individual can be achieved more accurately by multiplex PCR targeting repetitive DNA polymorphisms because examining multiple loci decreases probability of identical alleles in two individuals. Amplifying short tandem repeats (STRs) in close proximity by multiplex PCR can be used to screen for disease linkages because STRs are highly polymorphic, numerous and may be co-amplified without overlapping size ranges (Beckmann and Weber, 1992; Edwards, et al., 1992).

### **c. Quantitative PCR**

Quantitative PCR (qPCR) is also known as real-time PCR and refers to a method to determine absolute or relative amount of target sequence in a high-throughput format. Real-time PCR has often been confused with RT-PCR which is the abbreviation for reverse transcriptase PCR (Mackay, 2007). Reverse transcriptase PCR is a commonly used technique to detect RNA expression through creation of complementary DNA (cDNA) (Freeman, et al., 1999). The qPCR approach is considered to be more sensitive, rapid and safer compared to conventional PCR because a gel for amplicon confirmation is not needed and a radioactive reagent or chemical such as ethidium bromide is not involved (Arya, et al., 2014). The qPCR commonly consists of three oligos, a pair of primers and a probe (Smith and Osborn, 2009). Probes are designed to hybridize 100% to the amplified sequence and typically exhibit higher melting temperatures than the corresponding primers to allow annealing during the extension phase (Smith and Osborn, 2009).

When hybridized probes are cleaved by nuclease activity, fluorescence signals are released which are proportional to the target copy numbers (Smith and Osborn, 2009). Two common methods of qPCR assay are utilization of SYBR green and TaqMan Probes (Holland, et al., 1991; Houghton and Cockerill, 2006; Livak, et al., 1995a; Wittwer, et al., 1997). The SYBR green molecules release high fluorescent signals when they bind to double strand DNA and weak signals will be detected if the molecules are not bound to the DNA strand (Wilhelm and Pingoud, 2003).

One of the pitfalls of the SYBR green assay is that the dye can also bind to non-specific double stranded DNA and thus generate false quantification values (Deprez, et al., 2002). To overcome this problem, melting curve analysis is essential. According to Wilhelm et al (2003) and Bustin et al (2002), primer dimers which are primer molecules that have hybridized to each other and non-specific double stranded DNA can be distinguished by melting curve analysis (Bustin, 2002; Smith and Osborn, 2009; Wilhelm and Pingoud, 2003). Taqman probes are considered as sequence specific DNA probes because they are designed to hybridize to an amplified sequence (VanGuilder, et al., 2008). The qPCR by Taqman probe is based on the use of the 5' nuclease assay described by Holland et al (1991) and dual-labeled fluorogenic hybridization probes (Bassler, et al., 1995; Holland, et al., 1991; Lee, et al., 1993; Livak, et al., 1995a, b). The Taqman probe consists of the reporter group at the 5' end and the quencher group at 3' end that hybridizes during the extension phase. Reporter groups do not emit signals when the quencher group is present (Heid, et al., 1996). However, once the quencher group is separated by nuclease activity the reporter group emits a fluorescence signal (Heid, et al., 1996). The qPCR can be applied for diagnostic and microbiological uses, gene quantification and genotyping (Espy, et al., 2006; Smith and Osborn, 2009). The disadvantages of qPCR includes

the requirement for sequence data to the target gene of interest, binding of the dye to non-specific amplicons, incompatibility of the system with some fluorogenic chemistries and inability to monitor amplicon size (Arya, et al., 2014; Smith and Osborn, 2009).

### **c-1. Clinical microbiology**

The introduction of qPCR methods in clinical microbiology has improved the detection of infectious disease agents and improved patient management and care (Sails, 2013). For example, a wide range of viral diseases have been diagnosed by quantitative PCR (Niesters, 2002). Also, qPCR has improved detection and quantification of numerous respiratory, gastrointestinal pathogens, enteric parasites, malaria parasites, leishmania organisms and even viruses (Polley, et al., 2011). Improved ability of qPCR is particularly useful when more slowly growing or poorly culturable bacteria need to be detected, for example, *Anaplasma phagocytophila*, *Bartonella henselae*, *Mycoplasma pneumoniae* or *Chlamydia* (Espy, et al., 2006). The conventional PCR approach to these bacteria is difficult because some organisms elicit substances that inhibit PCR chemistry, thus sensitivity is greatly compromised (Espy, et al., 2006). Increasing infection rate of methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* spp. (VRE) in U.S. hospitals (Diekema, et al., 2004) are considered tremendous threats to public health, thus the ability of qPCR to reduce detection time by several days compared to culture-based methods and simplified overall processes offers opportunities for more rapid appraisal (Niesters, 2002).

In addition, qPCR can be utilized in fields as diverse as food safety, food spoilage and fermentation and for the microbial risk assessment of water quality and in public health protection because molecular based qPCR approaches are faster, more sensitive and specific than previous standard approaches which are culture based methods (Postollec, et al., 2011). Due

to its advantages, the International Organization for Standardization (ISO) established the standard to detect foodborne pathogens utilizing PCR because of its ability to detect sub-dominant populations, dead or viable but non cultivable cells (Postollec, et al., 2011). In addition, dynamics and activities of genes can be studied by contrasting gene expression via combining qPCR with reverse transcript PCR (Postollec, et al., 2011). Lee et al (2007) were able to successfully demonstrate the power of reverse transcript PCR by analyzing gene associated with enterotoxin produced by *Staphylococcus aureus* suggesting reverse transcript PCR is very effective in evaluating gene expression.

### **c-2. Genotyping**

The utilization of qPCR in clinical facilities has allowed for the quantification and characterization of virus strains such as the Hepatitis B virus (Yeh, et al., 2004). Yeh et al (2004) reported an approach for one-step quantification of HBV genotypes A-G and genotyping of HBV genotypes B and C with one set of primers and probes that could overcome the difficulty of genotyping due to genetic diversity. Liu et al. (2003) successfully differentiated the number of gene copies in a mouse model using qPCR assay. Liu et al. (2003) also emphasized that the qPCR assay is less labor intensive than chromosome analysis (Davisson and Akeson, 1987; Liu, et al., 2003) while being more sensitive and reliable than other DNA based methods such as Southern blot analysis. In addition, quantitative PCR has allowed for the genotyping of mice at any age whereas the chromosomal method was limited to mice over 6 weeks of age.

### **c-3. Foodborne pathogen quantification and other applications**

Numerous studies have been conducted in order to quantify *Salmonella* from many food commodities including chicken carcass rinses, ground beef, ground pork and raw milk (Chen, et al., 1997). According to Hein et al. (2006), the limit of detection to quantify *Salmonella* from



food samples by qPCR was less than 5 CFU. In addition, Wang et al. (2007) demonstrated a rapid and simultaneous quantification method by combining qPCR and multiplex PCR and was able to successfully detect *E. coli* O157:H7, *Salmonella* and *Shigella* from ground beef samples. According to Wang et al. (2007), the detection range of multiplex qPCR was  $10^2$  to  $10^9$  CFU/mL,  $10^3$  to  $10^9$  CFU/mL and  $10^1$  to  $10^8$  for *E. coli*, *Salmonella* and *Shigella*, respectively. In addition, Elizacuível et al (2013) reported a modified qPCR approach in order to detect only viable *Listeria monocytogenes*, *Salmonella* and *E. coli* O157:H7 cells using propidium monoazide (PMA) (Elizacuível, et al., 2013).

The qPCR was also used to detect genetically modified organisms (GMO). As GMOs are introduced to the market, scientists and consumers were concerned about its safety and demand for analysis increased (Meyer, 1999). According to Van den Bulcke (2010), the requirement for GMO screening methods are 1) appropriate approaches with adequate performance, scope, and legal basis 2) suitable reference materials, and 3) decision support system which interprets the analytical results. The current approaches commonly used to identify specific single GMO is the PCR based technology (Hernández, et al., 2005; Holst-Jensen, et al., 2003). The GMOs are typically detected by species-specific PCR by reference targets for example, the lectin gene is targeted for detection of roundup ready soybean (Berdal and Holst-Jensen, 2001; Mafra, et al., 2008). According to Brodmann et al. (2002), qPCR detection methods were developed for the 4 approved genetically modified maize variants.

## **5. Molecular methodology for characterizing the microbiome**

### **a. Denaturing gradient gel electrophoresis (DGGE)**

Characterizing the microbiome is critical because such populations can be directly associated with gut health of the host and their performance (Gill, et al., 2006; Noverr and Huffnagle, 2004; Turnbaugh, et al., 2006). A 16S rRNA amplification-based approach is a widely used technique for identification and classification of microorganisms because it is present in most microorganisms (Kuczynski, et al., 2012). One of the initial techniques to fingerprint microbial ecology in environmental samples was electrophoretic separation via high resolution polyacrylamide gels of low molecular weight rRNA molecules. Denaturing gradient gel electrophoresis was first introduced by Muyzer et al. (1993) to differentiate mixed microorganisms in a consortia mixture consisting of *Escherichia coli*, *Desulfovibrio desulfuricans*, *Microcoleus chthonoplastes*, *Desulfovibrio sapovorans* and *Thiobacillus thioparus* from Leiden University, Wadden Sea sediment and Slufter sediment on the island of Texel respectively. The PCR-based DGGE could be used to separate PCR amplicons of uncharacterized microbial mixtures by their different melting points due to sequence variance of amplicons (Fischer and Lerman, 1979; Myers, et al., 1987), therefore allowing for a comparison of microbial communities (Hanning and Ricke, 2011; Hume, et al., 2003; Oviedo-Rondón, et al., 2006; Pedroso, et al., 2006). A key mechanism of this method was the attachment of a guanine and cytosine rich region, the so called the GC clamp in the amplified fragments. Subsequent studies demonstrated that the presence of GC clamp increased the detection rate of sequence variance substantially (Myers, et al., 1985; Sheffield, et al., 1989). Because of this GC clamp, melted amplicons were able to halt migration once they reached the melting point threshold of the respective amplicons in the acrylamide gel. Substitution of the GC clamp, the chemiclamp, attaches a photoactivatable compound to the 5' end of primer (Baker and Harayama, 2004). However, limits of the chemiclamp were clear. Initially the covalent bond PCR product could not

be amplified correctly and in addition, the possibility of UV light damaging PCR amplicons could not be resolved (Cariello, et al., 1988). Also, the chemiclamp may modify melting properties of PCR amplicons, making it hard to predict (Guldberg, et al., 1998).

Limitations of DGGE include restricted fragment size, time required to complete a run, requirement of a well-trained technician, co-migration and overestimation (Gafan and Spratt, 2005). Fragment separation by DGGE and TGGE limits the size of the fragments up to 500 bp (Myers, et al., 1985) which are also directly related to the amount of sequence information. In addition, Buchholz-Cleven et al (1997) and Vallaeys et al. (1997) reported that if an organism exhibits certain levels of sequence variation, separation of DNA fragments was not possible (Buchholz-Cleven, et al., 1997; Vallaeys, et al., 1997).

Co-migration is also one of the pitfalls of DGGE and TGGE. For example, Nubel et al. (1996) reported that DGGE and TGGE resulted in the overestimation of the number of bacteria when some bacteria possessed multiple *rrN* operon sequences. Usage of degenerate primers in the PCR prior to DGGE can also result in an overestimation (Kowalchuk, et al., 1997). Despite the development of NGS technologies which allow for a more directly quantitative statistical analysis of microbiome composition of the species-level, DGGE can still be a viable technique due to complicated procedures, costs and requirements of a well-trained technician to execute NGS. The advantage of DGGE is that it provides a simple, rapid visual profile of the microbial population of samples which can be very useful for pre-screening samples prior to NGS (Hanning and Ricke, 2011). The study by Yu et al. (2015) was conducted to study microeukaryotic community from sea sample by utilizing NGS and DGGE. The study revealed that the NGS method using Illumina MiSeq revealed higher densities of the microeukaryotic

community from samples compared to DGGE however, no significant differences were detected in diversity of species.

### **a-1. Genetic fingerprinting for environmental studies**

One of the major utilities of DGGE is characterizing microbial ecology. Microbial fingerprinting by DGGE was used by Muyzer et al. (1993) when they amplified the gene fragments from widely distributed in bacteria and archaea using conventional PCR (Saiki, et al., 1988). Even though the molecular size of the amplicons were the same, amplicons were successfully separated by acrylamide gel because of the variant melting point properties resulting from the number of hydrogen bonds associated in guanine and cytosine. Besides analyzing only the patterns produced by DGGE, more detailed information could be obtained by using radioactively-labelled oligonucleotide probes to sequence acrylamide gel fragments excised from DGGE (Amann, et al., 1992; Muyzer and de Waal, 1994). In addition, high-resolution melt (HRM) analysis profiling has been suggested as an additional approach post DGGE analysis for the identification of DGGE bands (Porcellato, et al., 2012). Teske et al. (1996) used DGGE to analyze distribution of sulfate reducing bacterial population at three different time points during growth. Also, Ferris et al (1997) utilized PCR-DGGE in order to characterize the seasonal distribution of bacteria in hot springs and re-establishment of a microbial mat after removal of the entire cyanobacterial layer. For example, Díez et al. (2001) successfully identified picoeukaryote diversity in natural marine assemblages. Also, Díez et al. (2001) compared the relative levels of specific microorganism rDNA using three different approaches including DGGE, T-RFLP and gene cloning. Considering the technical differences, relative level values were reasonably comparable among the three techniques. In addition, Díez et al. (2001)

mentioned that one of the pitfalls of DGGE is the variable quality of sequenced DGGE bands produced by Sanger sequencing.

## **a-2. Genetic fingerprinting in food production systems**

Understanding and monitoring of bacterial communities in food matrices has been suggested as a means to detect possible contamination by food safety relevant organisms. The PCR based DGGE technique has been utilized to study bacterial communities of various foods for example, applications based on DGGE was first utilized to examine bacterial communities in pozol, a Mexican fermented maize dough (Ampe, et al., 1999). Handschur et al. (2005) identified *Enterobacteriaceae*, *Pseudomonas libaniensis* from processed salad samples. Porcellato et al. (2012) and Donner et al (1996) reported the occurrence of species-level of lactic acid bacteria in dairy products and observed changes in the enzymatic activity of cellulases by DGGE analysis. As DGGE exhibit its effectiveness in identifying bacterial community, the technique was widely utilized to fingerprint a variety of microorganism populations in various samples.

Since many food processing plants regulate the safety of their product by temperature, it is essential to monitor and determine the safety of the product. In a study by Handley et al. (2010), the microbial populations of poultry carcasses were examined by assessing banding patterns generated from DGGE and they observed substantial transition over the time period of 44 to 50 h of post chill process. Band pattern analysis by Handley et al. (2010) reported high similarities within sampled groups. Also, the bands present from the beginning to the end of sampling time indicated which microbial groups were able to survive slaughter and evisceration process. Variation in detectable bands suggested that the environmental variation was occurring during processing, however, differences in bacterial levels and diversity may also be explained by other factors such as cross contamination from transport crates, equipments or environmental

conditions. In order to remove potential bias, Handley et al. (2010) noted that the processing procedures and equipment in the plants were identical.

Since food slicers are widely used in ready-to-eat products including cheese, vegetables, and bread, it is important to keep the equipment safe from cross contamination of foodborne pathogens. Koo et al. (2013) utilized DGGE to analyze cross-contamination possibilities by slicer equipment used in deli meat retail processes. The DGGE technique revealed the similarities of overall populations of bacteria from the samples collected from slicer. Koo et al. (2013) successfully identified *Lactococcus lactis* and *Streptococcus thermophilus* from swabbed samples taken from the surfaces of the slicer. Overall microbial diversity of slicers analyzed by DGGE were similar between slicers and the most densely populated part of the slicers was the blade guards.

When the DGGE banding patterns were compared in a study of microbiota of chickens by Wielen et al. (2002) they concluded that unique and complex banding patterns can be produced for different compartments of the intestine and different ages of chickens therefore, unknown host specific factors may play an important role in the development of the intestinal bacterial community. The PCR-based DGGE method has been utilized for a variety of animals for overall evaluation of prebiotic and probiotic impact of the gut bacterial community of hosts. Oviedo-Rondon et al. (2006) identified microbial communities of broilers vaccinated with mixed *Eimeria* species using DGGE. In their study by Oviedo-Rondón et al. (2006) constructed dendrograms to compare percentage similarity between differently treated groups . Oviedo-Rondón et al. (2006) concluded that the cocci vaccination in broilers caused changes in intestinal microbial community by 36.7, 55.4, and 36.2% similarity coefficients in duodenal, ileal, and cecal, respectively.

Park et al. (2013) and He et al. (2009), demonstrated an alteration of the gut bacterial community of mice and hybrid tilapia (*Oreochromis niloticus*) respectively. Digestive microbiota of molted and nonmolted hens were also examined as well as hens being fed different diets. (Hume, et al., 2003). By observing different compartments of digestive tract, jejunum, ileum, and colon of birds, Hume et al. (2003) demonstrated a shift of predominant microbial population occurring in birds of different ages. According to Hume et al. (2003) similar microbial compositions were observed from adjacent digestive compartment and the compartments that were further apart being composed of different microbial communities as expected. Dunkley et al. (2007) utilized a PCR-DGGE technique to monitor microbial profiles during the entire molting period of laying hens and observed considerable similarity of band patterns among full-fed and alfalfa crumbles fed birds.

### **a-3. Monitoring of enrichment and isolation of bacteria**

Originally, DGGE was developed to identify microbial communities and compare complexities among treatments however, it also proved to be suitable to assess mixed populations of microorganisms (Muyzer and Smalla, 1998). Santegoeds et al. (1996) and Ward et al. (1997) used DGGE to monitor enrichment cultures of bacteria in hot springs by 16S rRNA methods and successfully unveiled a diversity of bacterial populations in the enrichment cultures. By comparing bands produced by the respective 16S rRNA fragment, morphology and the presence of biochemical markers, Garcia-Pichel et al. (1996) demonstrated that *Microcoleus chthonoplastes* represented a single, well-defined taxon with a ubiquitous distribution. In addition, Teske et al. (1996) applied DGGE to analyze the constituents of a coculture by sequencing DGGE bands to design more selective conditions for isolation of *Desulfovibrio* and an *Arcobacter* isolate.

#### **a-4. Gene detection**

Since DGGE approaches can be used to incorporate practical uses of melting points to differentiate variations in sequences, DGGE is also suitable for detecting microheterogeneity of genes. According to Nubel et al. (1996), temperature gradient gel electrophoresis (TGGE) band patterns produced by 16S rRNA fragments from pure cultures of *Panibacillus polymyxa* exhibited distinct patterns. Different band patterns produced by pure cultures can serve as an indicator of sequence based biodiversity and could be used to construct the phylogenetic tree. According to van der Luijt et al. (1997), pathogenic mutations in the adenomatous polyposis cold (APC) gene which is responsible for familar adenomatous polyposis (FAP) could be identified by screening exons utilizing a DGGE technique combined with a protein truncation test and Southern blot analysis. van der Luijt et al. (1997) utilized DGGE for the small exon and used protein truncation test for the large exon and successfully identified 65 pathogenic mutations from 105 Dutch FAP kindreds. Consequently, frameshifts and single base substitution mutations could be identified.

#### **a-5. Clone library screening**

Colony hybridization and restriction fragment length polymorphism of cloned rRNA inserts (Moyer, et al., 1996) has been one of the more highly utilized strategies to screen clone libraries. The DGGE (Kowalchuk, et al., 1997) and TGGE (Felske, et al., 1998) analyses were also used to measure and estimate redundancy of cloned 16S rDNA inserts in the environment. Both DGGE and TGGE were employed to analyze PCR products which are amplified after 16S rRNA genes have been cloned in suitable vectors. By analyzing cloned inserts obtained from the environment, an indication of the representative members in the natural microbial community can be acquired. According to Muyzer and Smalla (1998), re-amplifying inserts with nested



PCR will cluster clones together in groups and each representative clone can be sequenced. Furthermore, DGGE analysis of PCR products from cloned inserts may give an indication of representative members in mixed culture samples. Kowalschuk et al. (1997) utilized DGGE to analyze PCR-amplified 16S rDNA fragments and successfully detected potential beta-subdivision ammonia oxidizers present in the dune samples

#### **a-6. Determining PCR and cloning biases**

Keohavong and Thilly (1989) applied DGGE to determine the error rate of different DNA polymerases during DNA synthesis. They reported that DGGE permitted direct enumeration and identification of a point mutation caused by T4, modified T7, a Klenow fragment of polymerase I, and *Thermus aquaticus* (*Taq*) during PCR. Fidelity comparison of DNA amplification in the study was suitable because the base pairs were small and mutations could be detected by different concentrations of denaturant required for each amplicon. According to Keohavong and Thilly (1989), the most predominant mutations were transitions of G and C to A and T or vice versa with error rates varying from  $3 \times 10^{-6}$  to  $2.1 \times 10^{-4}$ . Keohavong and Thilly (1989) emphasized that reaction conditions such as temperature, dNTP and concentration of salt may have an impact on mutations and error rate however, error rates and mutation that predominantly existed were highly similar in four different templates indicating that the fidelity of the enzyme essentially remains constant during DNA synthesis. In the study by Eckert and Kunkel (1991) fidelity of various polymerases were compared using three techniques, DGGE, cloning and M13mp2 an *in vitro* assay. The measured fidelities of PCR with *Taq* polymerases by DGGE were similar with fidelity measured by cloning PCR product and M13mp2 in an *in vitro* assay (Eckert and Kunkel, 1991).

#### **b. Next generation sequencing**

Nucleic acid sequencing refers to a method for determining order of nucleotides in DNA or RNA molecules (Sanger, et al., 1973). However, first generation sequencing or Sanger sequencing had obvious limits of time required and poor quality of beginning sequences. This resulted in demands for more economical and rapid methods in research and clinical labs which led to development of NGS approaches. The NGS provided a more high-throughput approach for the sequencing of millions of DNA fragments from a sample. Next generation sequencing also became known as parallel sequencing because NGS can perform millions of sequencing reactions in parallel by micro-reactors and/or solid surfaces or beads (Kwon and Ricke, 2011; Metzker, 2010; Reis-Filho, 2009). One of the notable differences between Sanger sequencing and NGS was the length of reads. While Sanger sequencing generated long reads (nearly a thousand bp), NGS generates millions of shorter reads (hundreds of bp) which can be quantified (Fullwood, et al., 2009; John and Grody, 2008; Morozova and Marra, 2008; Stratton, et al., 2009; Tucker, et al., 2009; Voelkerding, et al., 2009).

In the past decade, various NGS platforms have been developed including the Ion Torrent system of Life Technologies (Carlsbad, CA), MiSeq of Illumina (San Diego, CA), 454 pyrosequencing of Roche Diagnostics (Risch-Rotkreuz, Switzerland) and SOLiD of applied Biosystems (Foster City, CA). Ion torrent by Life Technology utilizes a hydrogen ion sensitive transistor and its throughput is approximately 320 Mb per one run which runs for approximately 8 hrs. The Illumina Miseq needs to be run for 24 h and produces read lengths of 150 bp and throughput is from 1.0 to 1.4 Gb. Conversely, Roche 454 pyrosequencing technology runs for 10 hours and is able to produce read lengths of 400 bp and a throughput of 400 Mb per run. SoLiD requires over a week of run time and produces 15G per day with a read length of 60 bp.

Next generation sequencing can be applied to clinical diagnostics and therapeutics to determine the genetic cause of a disease by sequencing the protein-coding region of a respective gene (Saunders, et al., 2012). Although whole genome sequencing is possible, sequencing hotspots for disease causing mutations can be also effective because it is more rapid and more cost effective. Targeting specific genomic regions can be accomplished by coupling NGS with DNA capturing methods (Ng, et al., 2009).

Besides sequencing DNA, RNA-seq also known as whole transcriptome shotgun sequencing (Morin, et al., 2008) has been developed and can be applied to the study of gene expression, RNA sequencing, paired-end RNA sequencing, and small and noncoding RNA sequencing (Reis-Filho, 2009; Wang, et al., 2009). In addition, RNA-seq has allowed for the determination of exon and intron boundaries and the capability of observing cellular pathway alteration during infection (Qian, et al., 2013). Eventually, RNA-seq has led to more in-depth understanding of RNA editing events when combined with massive DNA sequencing. In addition, several modifications of NGS allowed assessment of DNA methylation and acetylation (Lister and Ecker, 2009; Meissner, et al., 2008) along with immunochromatin microarray assays (Visel, et al., 2009).

### **b-1. Whole-exome sequencing**

The term exome refers to the sequences which are transcribed to RNA after introns are removed. Therefore, exome sequencing provides information on the protein-coding region of genome. Exon refers to the part of a gene that remains within the mature RNA after introns are removed. Human exons constitute approximately 1% of the total genome (Ng, et al., 2009) and sequencing exons are known to be an efficient strategy to determine the genetic basis for gene disorders (Bamshad, et al., 2011). Exome sequencing can also provide information of disease

causing mutations in pathogen. Lai-Cheong and McGrath (2011) successfully identified genes relevant to inherited skin disorders by exome sequencing. Disease-causing mutations in multiple genes have been identified by Cullinane et al (2011) in a patient with oculocutaneous albinism and congenital neutropenia. The study by Cullinane et al (2011) utilized whole exome sequencing to align DNA sequence fragment of patient to the corresponding reference genome to identify variation and detected 62,235 variations.

### **b-2. Targeted sequencing**

Sequencing of a specific region is preferred when the suspected disease is identified and the region of interest is already known. Compared to whole-exome sequencing, targeted sequencing is much more affordable, yields much higher coverage of genomic regions of interest, and reduces sequencing cost and time (Xuan, et al., 2013). Also, cancer-type specific treatments can be evaluated and aided by NGS targeting specific genomic region (Rehm, 2013). Rehm (2013) reported that utilization of NGS is gradually extending its ability into clinical laboratories especially in diagnostics for hereditary disorders and prognostics of somatic cancers.

### **b-3. Microbial community analysis**

High-throughput sequencing technology such as NGS, parallel sequencing has revolutionized the study of microbial community analysis because NGS approaches can produce extensive and detailed data on bacterial composition which could be considered significant to both the health of host and in fermented food. Prokaryotic 16S rRNA and fungal ITS genes are typically targeted in the molecular surveillance of microbial communities. Sequencing millions of reads in a single run by NGS provides an incomparable amount of data with high-resolution optics in molecular level. Hence, high computational power and storage are mandatory. Also, the gigabyte sized data produced by the NGS platform requires programs to process NGS raw data

to downstream analysis such as characterization of short-amplicons, filtration/demultiplexing, operational taxonomic units (OTUs) selection, taxonomic assignment and sequence alignment (Bokulich and Mills, 2012).

Three open source programs are available for processing data, QIIME (Caporaso, et al., 2010), Mothur (Schloss, et al., 2009) and MG-RAST (Meyer, et al., 2008). QIIME, Mothur and MG-RAST software are capable of analyzing data by trimming, screening and aligning sequences produced from community samples and furthermore able to calculate distance and OTUs between community sequence sample. According to Plummer et al (2015), there were no significant differences detected at the phylum level while genus levels exhibited differences when fecal sample of infants were analyzed targeting 16S rRNA gene. Across the three pipelines, a total of 90 distinct genera were identified and MG-RAST and Mothur shared the least genera at 39 while QIIME and Mothur exhibited higher similarity of 53 genera. Plummer et al (2015) highlighted that the QIIME and Mothur pipelines have more powerful statistical capabilities than MG-RAST however, Mothur excels greatest in terms of flexibility. However, Plummer et al (2015) concluded that QIIME was more user friendly, required less time than Mothur, and was preferred for analyzing large datasets. Approximate analysis time for specific dataset used by Plummer et al (2015) were 1 h, 10 h and 2 days for QIIME, mothur and MG-RAST respectively. The advantage of using MG-RAST pipeline is its accessibility to the public database and shotgun metagenomic datasets. Also, MG-RAST generates multi-fasta file for each sample, allowing the researcher to select particular reads for further analysis.

## **6. Conclusions**

In-feed antibiotics have become a worldwide concern due to the rise of antimicrobial resistant pathogens (Dibner and Richards, 2005; Jones and Ricke, 2003) thus alternatives such as prebiotics have been extensively studied in order to promote performance and to protect against numerous pathogens. Ever since the concept of prebiotic was first introduced by Roberfroid and Gibson (1995), a variety of prebiotics have been evaluated. However, the effect of prebiotics on growth performance have been, for the most part, inconsistent. Inconsistency may be due to differences including the housing systems of birds, concentration of prebiotic used and species of birds (Yang, et al., 2007). Also, studies have shown that each type of prebiotic excels in a distinctly different beneficial role. For example, FOS supplement appears to succeed in promoting beneficial gut bacteria therefore, establishing an acidic environment in the gut to inhibit pathogen colonization. A GOS supplement has also demonstrated its role for selectively promoting beneficial bacteria in gut of host. An MOS feed supplement was able to inhibit adherence activity of pathogens to epithelial cells by competing for a common receptor which the pathogen utilizes. Inconsistent results need to be overcome in future applications by evaluating effects of prebiotic more thoroughly via investigations at the molecular level of microorganism such as transcriptomics or NGS.

Because multiplex PCR requires several primer pairs with various properties such as specificity and optimal annealing temperature, optimization of the multiplex PCR can be difficult. However, once the assay has been optimized, the procedure of multiplexing becomes quite simple and provides more detailed information of template DNA because multiplex PCR can target species or strain specific fragments of DNA. Consequently, more rapid and accurate detection and characterization of pathogen in food safety or industry has become possible compared to previous enrichment culture based methods which historically were the gold

standard of detection. In addition, it can be applied to diagnose genetic and infectious disease accurately by screening for multiple loci simultaneously. The multiplex PCR assay can be also used to examine relationship of genetic linkage between two or more sequences, environmental association and host-parasite and disease-infection (Edwards and Gibbs, 1994).

Conventional PCR-agarose gel visualization is a useful technique however, specificity and sensitivity of conventional PCR can be compromised by the possibility of a false positive product (Sails, 2013). The development of enzyme and probe hybridization molecules for detecting amplified product has led to accurate quantitation of target genes and the generation of real-time instrumentation and chemistry for PCR. The quantitative PCR alone provides data equivalent to the combination of conventional PCR and Southern blot analysis which are highly sensitive and specific.

Next generation sequencing approaches developed following the wide scale application of Sanger sequencing. These are also referred to as parallel sequencing methods because of their mechanisms involving sequencing millions of fragments simultaneously. The NGS can be applied to unlimited fields of studies involving DNA or RNA based technology. In addition, NGS approaches can, not only be applied to sequence genome for genetic disorders, but can also applied to microbiome studies by targeting 16S rRNA while the flexibility of NGS is useful, it can be inefficient and the cost to implement NGS has historically been very expensive. Andersen et al (2014) developed a methodology of enhancing NGS by adopting barcodes to PCR products prior to NGS library construction. The barcoded library approach of NGS increased efficiency and decreased cost of library preparation by two thirds per sample. In addition to development of NGS, also called second generation sequencing, development of third generation sequencing and fourth generation sequencing platforms are emerging (Schadt, et al., 2010). The significance of

third generation sequencing is that amplification of template strands are no longer required which means availability of single molecule real time sequencing. Absence of amplification steps reduces the error that might occur during amplification (Ku and Roukos, 2013). Third generation sequencing technology includes the PcBio RS of Pacific BioSciences and the Heliscope sequencer of Helicos BioSciences. Fourth generation sequencing technology is known as nanopore-based technology and is highlighted by the cost effectiveness. Feng et al (2015) introduced this technology to improve the potential of sequencing entire human genome for less than \$1000 or even less than \$100.

Finally, molecular techniques such as DGGE, multiplex PCR, qPCR and NGS can be utilized for the investigation of microbial shift responses to supplementation such as prebiotics which require more detailed investigations due to some of the inconsistent results occurring among studies. Since the NGS method is becoming more accessible, microbiome analysis of poultry will reveal other key microorganisms other than *Bifidobacteria* and *Lactobacillus* and should be able to explain inconsistent result of prebiotic studies.

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## **8. Authorship statement for chapter 1**

**Sang In Lee** is the first author of the paper and completed at least 51% of the studies among coauthors, which the title is "Application of prebiotics in poultry and molecular techniques for microbiome quantification" in chapter 1.

Major Advisor: Dr. Steven C. Ricke

## **Published Paper**

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### III. Chapter 2

#### EFFECTS OF A COMMERCIAL PREBIOTIC IN BROILERS

**Assessment of cecal microbiota, integron occurrence, fermentation responses, and *Salmonella* frequency in conventionally raised broilers fed a commercial yeast-based prebiotic compound**

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## ABSTRACT

Prebiotics are defined as nondigestible food ingredients that can stimulate the growth of one or more beneficial bacteria in the gastrointestinal tract. The Biolex<sup>®</sup> MB40 is a commercial prebiotic that contains mannanoligosaccharides. The aims of this study were to evaluate the effects of prebiotic Biolex<sup>®</sup> MB40 on cecal microbiota of conventionally raised chickens using PCR-based denaturing gradient gel electrophoresis (DGGE) and assessing *Salmonella* prevalence. Chickens were randomly selected and distributed into three groups; a negative control (NC) and two treatment groups (T1 and T2). The NC group was fed a non-medicated feed, while the treatment groups were fed either T1 or T2, 0.05% antibiotic (BMD50) or 0.2% Biolex<sup>®</sup> MB40 respectively. During the study, cecal contents and bird feed were plated on selective media for *Salmonella*, yeast and mold prevalence analysis. Ten chickens from each group were randomly selected at 1, 2, 4 and 6 wk and ceca were extracted for DNA isolation for PCR-based DGGE. Also, short-chain fatty acids (SCFAs) were analyzed from collected cecal material by gas chromatography. Only 4.2 % of the samples were *Salmonella* positive. Presence of class 1 integron from cecal material were analyzed by PCR and 97.5% of the cecal samples were positive for integron presence, but no class I integrons were detected in the *Salmonella* isolates. According to the PCR-based DGGE analysis, the T2 group exhibited a cecal microbial population pattern that was similar to the T1 group prior to wk 4 and the T2 group appeared to be almost identical with the NC group after wk 4 but T2 exhibited less *Bacteroides rodentium* prior to wk 4. Overall results showed that the commercial prebiotic, MB40 did not lead to a detectable reduction of *Salmonella* but the general frequency of *Salmonella* was minimal in all treatments. However, feeding an MB40 supplement did result in similar DGGE band patterns as the T1 indicating that cecal microbiota were potentially similar in these 2 groups. Overall, it appears

that MB40 (T2) exhibited similar DGGE-cecal population patterns as BMD50 (T1) which suggests that these treatments may have influenced the populations in a comparable fashion.

**Key words:** *Salmonella*, integron, short-chain fatty acids, prebiotic, broiler.

## INTRODUCTION

Prebiotics are defined dietary supplements that impact the host gastrointestinal (**GI**) tracts by selecting for beneficial gastrointestinal bacteria such as *Bifidobacterium* and *Lactobacillus* which can inhibit establishment of pathogenic microorganisms through the production of lactic acid (Gibson and Roberfroid, 1995). In this study, brewers' yeast cell wall derived supplement, Biolex<sup>®</sup> MB40, was used as a prebiotic feed additive which contains a concentration of 1,3-1,6- $\beta$ -D-glucan and mannanoligosaccharides (**MOS**, Leiber GmbH, Bramsche, Germany). Previous studies have demonstrated that MOS can enhance the host's immune system by increasing immunoglobulin (Ig) A and increasing *Lactobacillus* in the gut (Swanson et al, 2002).

According to the report from FoodNet trend data of Center for Disease Control and Prevention (**CDC**) in 2013 (<http://www.cdc.gov>), *Salmonella* serovars have been considered one of the major pathogens causing infection in humans, followed by *Campylobacter*. Therefore, control of these pathogens is critical for the food industry. In addition, one of the concerns in food animal production is to prevent the rise of antimicrobial resistance in microorganisms. The presence of integrons allows bacteria to acquire a variety of antibiotic resistance genes, leading to a general increase in antimicrobial resistance of pathogens (Gillings et al, 2008). Class 1 integrons are not only widely spread in the bacteria isolated from clinical facilities and livestock (Martinez-Freijo et al, 1998; Goldstein et al, 2001) but also occur in many Gram-negative bacteria (Lee et al, 2002; Smith et al, 2007; Ahmed and Shimamoto, 2014). Integrons consist of recombinases which are a family of integrases that serve as promoters for direct transcription (Ochman et al., 2000). The presence of integrons has been suggested as a potential indicator of antibiotic resistance because of their ability to capture and excise genetic elements by their site-

specific recombination systems, thus playing a significant role in propagation of antimicrobial resistance genes among bacteria (Ochman et al, 2000; Roe and Pillai, 2003).

Amplification of the 16S ribosomal ribonucleic acid (**rRNA**) gene has been widely used for identification or classification of bacteria. A polymerase chain reaction based denaturing gradient gel electrophoresis (PCR-based **DGGE**) approach has been used to separate PCR amplicons of uncharacterized mixtures of bacteria based on DNA strands with different melting points due to their sequence variation, therefore allowing direct visual comparisons of microbial communities of the gut microbiota among different treatment groups (Hume et al, 2003, Hanning and Ricke, 2011).

The purpose of this study was to investigate potential microbiota compositional changes in the ceca of prebiotic or antibiotic treated broiler chickens by PCR-based DGGE. Along with the potential microbiota compositional changes, prevalence of *Salmonella* and class 1 integron genes were assessed by direct plating methods followed by PCR analysis.

## **MATERIALS AND METHODS**

### ***Chicken Housing***

Three houses (treatment per house) were utilized in this study. A total of 15,300 Hubbard X Cobb 500 straight –run broiler chicks were placed in each house. The breeder flocks used to supply the chicks were equally distributed among the three houses. The birds were identified with study number, house number, and assigned treatment. Each week, 10 birds from each house were randomly selected for sampling of cecal contents. Flock performance for each house (treatment) was monitored and recorded (data not shown) during the course of the trial. A negative control group was provided with feed without treatment additives while the other two

groups were administered feed supplemented with 0.05 % of BMD50 (**T1**) (BMD50, Zoetis, Floham Park, NJ, USA) or feed supplemented with 0.2 % of Biolex<sup>®</sup> MB40 (**T2**) (Leiber GmbH, Bramsche, Germany). Diets were typical commercial starter, grower, finisher 1 and finisher 2. Starter diets were fed 1.5 lb (0.68 kg) per bird, 3 lb (1.36 kg) per bird for grower, 4 lb (1.81 kg) per bird for finisher 1 and finisher 2 were fed as needed to market. The only differences in the diets were feed amendments T1 and T2. Randomly selected groups (10 birds in each group) were chosen for cecal removal at 1, 2, 4 and 6 wk of age. A review by the institutional animal care and use committee (**IACUC**) was exempted because the birds were raised in an off-campus commercial farm operation and the current study was restricted to microbiological evaluation of birds selected on site. The commercial cooperators used internal animal welfare protocols based on National Chicken Council (**NCC**) guidelines ([www.nationalchickencouncil.org](http://www.nationalchickencouncil.org)).

### ***Salmonella Isolation and Identification by Multiplex PCR***

During 1, 2, 4 and 6 wk of age, 10 chickens from each group were randomly selected and cecal contents were aseptically harvested for DNA extraction and pathogen isolation. Ceca containing several grams of cecal contents were stored at -20°C in sterile Whirl Pak bag for further analyses. One gram of cecal contents were added into 15 mL culture tubes and 9 mL of 0.1% peptone water was added to establish a 1:9 ratio of cecal contents and diluent. Cecal suspensions were subsequently inoculated into 9 mL of tetrathionate (TT, HIMEDIA, Mumbai, India) broth and incubated at 37°C for 24 h. After incubation, 10 µL of inoculated TT broth was streaked on xylose-lysine-tergitol 4 (**XLT-4**, BD Bioscience, Franklin Lakes, NJ, USA) and brilliant green (**BG**, BD Bioscience) selective media using a loop and incubated at 37°C for 24 h. To confirm positive colonies, one of the positive presumptive colonies on either XLT-4 or BG

media was suspended in 100  $\mu$ L of distilled water and colony PCR was performed for *Salmonella* confirmation.

For colony PCR, the 20  $\mu$ L reaction contained 2  $\mu$ L of cell suspension, 500 nM of each primers (F: TTT GGC GGC GCA GGC GAT TC; R: GCC TCC GCC TCA TCA ATC CG) (Kim et al, 2006) which amplifies the 423 bp fragment of the transcriptional regulator gene (STM3098) of *Salmonella*, 10  $\mu$ L Jump Start Ready Mix (Sigma-Aldrich, St. Louis, MO, USA) and 6  $\mu$ L of distilled water. After an initial denaturation of 94°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s followed, accordingly. Finally, 5 min of an elongation step at 72°C and a 5 min final hold step at 4°C was conducted. Aliquots (10  $\mu$ L) of each amplification product was electrophoretically separated on a 1% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer. The respective DNA aliquots were extracted from each of the 5 *Salmonella* positive samples and a multiplex PCR was applied for confirmation via colony PCR to identify specific serovars as described previously by Park and Ricke (2015).

### ***Yeast and Mold Prevalence***

Yeast and mold prevalence in chicken feeds used in this study was evaluated to confirm cross contamination of chicken feeds using a 3M<sup>TM</sup> Petrifilm (St. Paul, MN, USA). A 10 g sample of chicken feed from each wk's negative and two treatment groups were suspended with 90 mL of distilled water to establish a 1:9 dilution. Feed suspensions were mixed thoroughly and 1 mL of mixture was dispensed to the 3M<sup>TM</sup> Petrifilm plate. Petrifilm plates were duplicated and incubated at 25°C for 48 h. The total number of colonies appearing on plates was multiplied by the dilution factor and subsequently divided by total volume plated on petrifilm to obtain cfu/g.

### ***PCR prior to Denaturing Gradient Gel Electrophoresis (DGGE)***

Total DNA was extracted from 200 mg of ceca using a QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA), and concentration and purity of DNA were measured with a Nanodrop ND-1000 (Thermo Scientific, Marietta, OH, USA). For conducting PCR prior to DGGE, the 50  $\mu$ L PCR reaction volume contained 50 ng of template DNA, 400 nM of each of the primers (F: CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG; R: ATT ACC GCG GCT GCT GG) (Muyzer et al, 1993) which amplifies the 233 bp of V3 region of the 16S rRNA gene, 25  $\mu$ L of Jump Start Ready Mix (Sigma-Aldrich) and 19  $\mu$ L of distilled water. The PCR assay was first denatured at 94°C for 5 min and kept at 94°C for 1 min. The temperature was subsequently dropped to 67°C for 45 s. This temperature was decreased by 0.5°C for every additional cycle until the temperature reached 58.5 °C followed by an elongation step of 72°C for 1 min. After the first products were generated, 12 cycles of 94°C for 1 min and 58°C for 45 s followed. The final elongation step was at 72°C for 7 min and held at 4°C for 5 min. Aliquots of 10  $\mu$ L for each amplification product were electrophoretically separated on a 1.5% agarose gel in a 1X TAE buffer.

### ***DGGE***

The DGGE analyses were performed using a DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). The PCR products were applied on an 8% polyacrylamide gels in 1X TAE with gradients which were formed with 8% acrylamide stock solutions (acrylamide/bis, 37.5:1) and contained 35 to 60% denaturant. Electrophoresis was performed at 55 V and a temperature of 59°C. After 18 h of electrophoresis, the gel was stained with 25  $\mu$ L of SYBR green in 700 mL of 1X TAE on a shaker for 40 min and destained in



distilled water for 10 min. The gel was viewed with a transilluminator while minimizing the exposure time of UV light to prevent DNA damage. After running DGGE for each sample, different samples with identical banding patterns were pooled within each group to generate the number of samples so that all three groups could be run on one gel for comparison. The DGGE banding patterns were also used for phylogenetic tree analysis based on the unweighted pair group method with arithmetic mean (UPGMA) algorithm by Quantity One software (Bio-Rad).

### ***DGGE Gel Purification for Sequencing***

The DGGE gel bands were cut under UV light with a razor blade. Weak and closely adjacent bands were avoided to maximize DNA recovery rate for successful sequencing. Excised target bands were placed into 0.5 mL microcentrifuge tubes with pierced bottoms, placed in 2 mL microcentrifuge tubes and subsequently centrifuged at 16,000 x g for 5 min to disrupt the gel. A 300 µL aliquot of TE buffer was added to the disrupted gel and incubated at 65°C for 15 min. Contents were transferred to a Spin-X tube (VWR, Radnor, PA, USA) and centrifuged at 16,000 x g for 5 min. The contents were subsequently suspended with 133 µL of 7.5 M of NH<sub>4</sub>OAc, 60 ng of glycogen and 800 µL of 100% ethanol. Suspensions were placed at -70°C for 10 min followed by centrifugation at 16,000 x g for 15 min at room temperature. Pellets were washed twice with cold 70% ethanol and resuspended with a solution containing 5 µL of distilled water and 1.6 µM of reverse primer. Purified gel band DNA was submitted to the DNA Resource Center at the University of Arkansas, Fayetteville, AR (Park et al., 2014).

### ***Integron Detection in Cecal Contents***

Extracted DNA from cecal contents were used to identify integrons by PCR. A 25  $\mu$ L of PCR reaction volume containing 25 ng of DNA, 400 nM of each of the integron primers (F: GGC ATC CAA GCA GCA AG; R: AAG CAG ACT TGA CCT GA) targeting conserved sequence regions (Waturangi et al, 2003), 12.5  $\mu$ L of 2X premix ExTaq (Takara, Mountain View, CA, USA) was used. A 25  $\mu$ L portion of each aliquot was pre-denatured at 94°C for 5 min, followed by 30 cycles of 95°C for 1 min, 60°C for 30 s and 72°C for 2 min. After 30 cycles, the final elongation step was followed by 5 min at 72°C with a final hold step for 5 min at 4°C. Aliquots of 10  $\mu$ L of the respective PCR amplicons were separated on 1% agarose gel and appeared as visible bands while no amplicons were detected for the negative control. PCR products were purified by Diffinity Rapid Tip<sup>®</sup>2 (Sigma-Aldrich) and sequenced at the DNA Resource Center at the University of Arkansas, Fayetteville, AR. The class 1 integron sequence was confirmed by basic local alignment search tool (**BLAST**) analysis. *Salmonella* isolates were subjected to DNA extraction followed by class 1 integron PCR assessment under the same conditions as the cecal DNA analysis.

### ***Short Chain Fatty Acids (SCFAs) Analysis by Gas Chromatography (GC)***

A mixture of acetic acid, propionic acid and butyric acid was injected for analysis of the peak area generated by the GC in order to establish a standard curve. Due to limited cecal material available in samples from wk 1 and 2, only samples of wk 4 and 6 were analyzed for SCFA. The method of SCFA preparation and analysis by Kaur et al (2011) was used. A 1 g aliquot of ceca material were suspended in 9 mL distilled water and vortexed thoroughly. Two aliquots of 1.2 mL were centrifuged at 3,000 x g for 10 min. Supernatants of 900  $\mu$ L were transferred to new tubes and 100  $\mu$ L of reagents containing 50 mM of 4-methyl-valeric acid, 5%

of meta-phosphoric acid and 1.56 mg/mL of copper sulfate were added. Solutions were vortexed thoroughly for 1 min, incubated in room temperature for 10 min followed by centrifugation for 10 min at 11,000 x g. Finally, 100  $\mu$ L of supernatant was used for analysis. A 1  $\mu$ L of supernatant was used for GC (Shimadzu, Columbia, MD, US) analyses and the column was set to 100°C initially and increased by 4°C every 3 min until 120°C with a rate of 3°C per min up to 150°C afterwards. A splitter was set to 220°C with a split ratio of 30 and flame ionization detector was set to 230°C throughout the experiment.

### ***Statistical Analysis for Microbial Prevalence***

Microbial prevalence (yeast and mold) were analyzed by JMP<sup>®</sup> Genomics (SAS Institute, Cary, NC, USA) software. Means of each data were compared using **ANOVA** (one-way analysis of variance) test with an  $\alpha$  value (level of significance) of 0.05.

## **RESULTS**

### ***Salmonella Prevalence***

*Salmonella* prevalence among groups is shown in Table 1. All 5 samples amplified with the 423 bp PCR product from the colony PCR. Two out of five *Salmonella* positive samples were successfully amplified by the 216 bp product which would indicate that those isolates were *Salmonella* Heidelberg. The remaining 3 *Salmonella* isolates could not be amplified by any of the three *Salmonella* Typhimurium, Heidelberg and Enteritidis specific regions but were identified as *Salmonella* subspecies I.

### ***Yeast and Mold Prevalence***

Average cfu/g of feed for each of the groups were 34, 24 and 79, NC, T1 and T2 respectively. Based on the student's t test, there were no significant differences in yeast and mold prevalence among the NC and treatment groups.

### ***Analysis of Cecal Microbiota Using a PCR-based DGGE***

All cecal samples in this study amplified the 233 bp product via PCR prior to DGGE. Samples of wk 1, 2, 4 and 6 birds were separated by DGGE are shown in Figure 1. At wk 1, bright bands were concentrated around the center region of gel while some bands were spread more randomly among the treatment groups. Both treatment groups at wk 4 yielded a weak double banding pattern in the same region of the gel as double banding patterns in the wk 2 negative group. Samples of wk 6 were uniform among all groups except for a few specific bands appearing in the T1 groups. When phylogenetic trees were generated by Quantity One software based on banding patterns and intensity of the bands, most samples were clustered within the same group (Figure 2).

### ***DGGE Band Sequencing***

Sequence results of wk 1, 2, 4 and 6 are shown in Table 2. Based on BLAST analysis of the sequence, *Bacteroides dorei* and *Bacteroides rodentium* appeared in the negative control group for wk 2 and 4 samples but were less frequent in the wk 6 samples. Both treatment groups exhibited similar patterns for the wk 4 and 6 samples. In the T1 group, a partial 16s RNA sequence of *B. dorei* was detected in the wk 2 sample, but its band intensity decreased in wk 4 and 6. *Barnesiella viscericola* was predominantly detected in all groups at wk 4, while the

*Clostridiales* group of *Firmicutes* were detected in all treatment groups. *Proteobacterium* species was only observed in the T1 group at wk 6.

### ***Integron Prevalence***

Integrans were detected in 117 samples out of a total of 120 samples (97.5%). Most of them were the 1 kb amplicon in size while some samples also had an additional amplicon size of over 1.5 kb on a 1 % agarose gel. There were no class 1 integrans detected in DNA for any of the *Salmonella* isolates.

### ***SCFA Analysis***

The average and total concentrations of each SCFA are shown in Figure 3A to E. Overall, the average concentration of acetic acid found in cecal material was the highest among the fermentation acids, followed by butyric acid while the propionic acid concentration was the least (Figure 3E). However, there were no significant differences among treatment groups and NC in terms of acetic acid, propionic acid and butyric acid found in ceca material or between wk 4 and 6 when data was pooled among treatments. Among the three individual SCFAs, acetic acid constituted the highest average percentage at 64%, butyric acids at 22% followed by propionic acid at 14% (Table 3). Average concentration of each SCFA in wk 4 did not exhibit any significant differences when compared to wk 6 (data not shown).

## **DISCUSSION**

The purpose of this study was to investigate the microbiota compositional changes in the ceca of commercial prebiotic treated broiler chickens as well as prevalence of *Salmonella* and

occurrence of integron genes. The MB<sup>®</sup>40 prebiotic consists of MOS which is a complex sugar molecule containing mannose. Mannose based polymers have been examined and proven to block bacterial lectin, therefore inhibiting the colonization of pathogens such as *E. coli* and *Salmonella* (Ofek and Beachey, 1978; Spring et al, 2000). In addition, Onderdonk et al (1992) reported that Poly-[1-6]-D-glucoopyranosyl-[1-3]-D-glucoopyranose glucan (PGG), type  $\beta$ -Glucan reduced early mortality and increased leukocyte cell population in mice challenged with *E. coli* and *Staphylococcus*. Oligosaccharides are a fermentable carbohydrate source that leads to production of SCFAs which may play several beneficial roles in the host such as energy substrates for colonic epithelial cell (Roediger, 1982) and promoting colonic anastomosis (Scheppach, 1994). Consistency of SCFA concentration among all groups in this study indicates that there was no detectable impact on the fermentation upon introduction of feed amendments at least in the later stages of bird growth which corresponds to reports by Jozefiak et al (2008) where pH of ceca was measured when  $\beta$ -glucan supplemented 6 wk old broiler chickens were examined. However, Jozefiak et al (2008) reported that inulin and lactose supplemented birds exhibited lower pH than the control group which may, in turn, result from higher SCFA production. In the current study, the acetic acid concentration was significantly higher than other SCFAs which corresponds to previous studies by Ricke et al (1982) and Woodward et al (2005) when 8 day old chicks and 50 wk old hens were examined respectively. Also, Woodward et al (2005) reported that for 50 wk old hens, nonmolted bird groups for most trials exhibited the highest SCFA concentration in the ceca while no detectable differences in SCFA concentration were observed between feed withdrawal molt birds and the alfalfa molted birds. Results from previous studies propose that different feed amendments may favor production of certain SCFAs

at varied time points depending on the age and gut physiology of the birds (Ricke et al, 1982; Woodward et al, 2005; Jozefiak et al, 2008).

While *Salmonella* can be a common foodborne pathogen found in poultry operations (Corrier et al, 1999; Dunkley et al, 2009; Finstad et al, 2012; Foley et al, 2011, 2013), in the current study *Salmonella* species were relatively infrequent. Only 4.2% of the samples were *Salmonella* positive and no significant differences were detected among the treatment groups. However, because only 10 samples from each treatment and wk were selected for study, this may not be a sufficient number of samples to assess prevalence. To obtain sufficient numbers of *Salmonella* positive birds may require an infection study with known quantities of a recoverable *Salmonella* marker strain. There were no significant differences among the treatment groups in terms of yeast or mold prevalence which eliminates any bias or skewed results that may have been brought by distribution of unequal feed quality conditions. Feed quality control is important because if a batch of feed was contaminated, microbiota comparison among groups could be biased.

One of the major intentions of this study was to examine the potential impact of prebiotic supplementation on cecal microbial populations. The NC did not include feed amendments, while the two treatment bird groups were fed with either antibiotic or prebiotic consisting of MOS and  $\beta$ -D-Glucan. Antimicrobial feed additives have been used in poultry production to increase feed conversion ratio (**FCR**), animal growth and reduce morbidity and mortality due to clinical and subclinical diseases (Thomke et al, 1998). Control of pathogens in the food industry is important because they may be directly related to public health issues (Scallan et al, 2011). Also, antibiotic usage of in-feed supplements is one of the main concerns due to rise of antibiotic resistance (Dibner and Richards, 2005). Consequently, other feed supplements that provide

similar animal production responses have been examined (Kasper et al, 2001; Biggs et al, 2007). Following gut microbial population changes over time within conventional flocks treated with prebiotics is important because there are believed to be several host-microbiota interactions occurring as well as other factors such as greater bird stocking densities that can lead to extensive microbiota changes (Guardia et al., 2011; Stanley et al., 2014). According to the PCR-DGGE analysis in the current study, Firmicutes, a phylum of bacteria consisting of numerous Gram positive cells, such as *Clostridia*, *Bacteroides*, and *Alistipes* were found in all groups during the entire study. Also, more uniform banding patterns were observed as the birds matured.

The problem of drug resistant bacteria has been further highlighted with a report of two million illnesses and 23,000 deaths related to antibiotic resistant bacteria according to CDC. In response to this an executive order by the U.S. President was initiated in 2014 to encourage development of strategies to combat antimicrobial resistance occurring in the public health sector ([www.whitehouse.gov](http://www.whitehouse.gov)). Several studies have shown that class 1 integrons are fairly prevalent in pathogens and even in clinical facilities and livestock production (Martinez-Freijo et al, 1998; Goldstein et al, 2001). According to BLAST, identified class 1 integrons in this study are common in *Salmonella* Heidelberg (KJ756517.1), *Escherichia coli* (KJ594082.1) and *Staphylococcus aureus* (KF687971.1). Previous studies have shown that class 1 integron genes can be detected in anywhere from 20 to 100% in *E. coli*, 21% in *C. jejuni* isolates from broiler chickens (Lee et al, 2002; Smith et al, 2007), 39 and 68% in *Salmonella* from dairy and meat products (Ahmed and Shimamoto, 2014) and pasture raised broiler chickens (Melendez et al 2010), respectively. Prevalence of a class 1 integron from these previous studies was significantly lower than the 97.5% observed in this study but does correspond to a previous class 1 integron prevalence study of pasture raised flock receiving no antibiotics and fed the same



prebiotics (Park et al, 2014). This is somewhat expected because whole genomic DNA extracted from the ceca was used in the current study while only specific pathogen isolates were tested for class 1 integron in other studies. Integron sizes vary because of their ability to integrate into a wide variety of genes by site-specific recombination. Identification of antimicrobial resistance traits that may have been introduced by integrons was not conducted in this study. However, the significant finding of this study was that class 1 integron genes which potentially mobilize antimicrobial resistance do appear to commonly exist in the cecal microbiota of broiler chickens raised under commercial conditions whether fed an antibiotic or not. It remains to be determined what factors contribute to this prevalence.

Further assessment and quantification of *Bifidobacteria* or *Lactobacillus* are suggested because Baurhoo et al (2009) demonstrated an increase in *Bifidobacteria* from MOS fed chickens. In terms of the microbial population, MB40 (T2) exhibited similar patterns as BMD50 (T1) which suggests that both dietary treatments may have influenced the populations in a similar fashion. However, further investigation of the cecal microbial population by next-generation sequencing and metabolomics approaches (Park et al, 2013) are needed to detect more subtle but functionally distinguishable differences between AGP and prebiotic fed chickens.

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**Figure 1. Denaturing gradient gel electrophoresis (DGGE) gel (A: wk 1; B: wk 2; C: wk 4; D: wk 6) BLAST result for each numbered bands shown in Table 2.**

**Figure 2. Phylogenetic tree based on bands pattern of denaturing gradient gel electrophoresis (DGGE) gel (A: Wk 1; B: Wk 2; C: Wk 4; D: Wk 6). Each number represent the vertical lane from gel counting from left (Feed only: 1 to 7; BMD50: 8 to 14; MB40: 15 to 21)**

**Figure 3. A: Total short-chain fatty acid (SCFA) concentration; B, C, D: Average concentration of acetic, propionic, butyric acid respectively; E: Average SCFAs concentration comparison by Wk. Different letters indicates significant differences**

**Table 1. *Salmonella* prevalence in cecal contents of birds (n = 10)**

<i>Salmonella</i>					
	Wk 1	Wk 2	Wk 4	Wk 6	Total
NC <sup>1</sup>	0/10	0/10	1/10	0/10	1/40
T1 <sup>2</sup>	0/10	0/10	2/10	1/10	3/40
T2 <sup>3</sup>	0/10	0/10	1/10	0/10	1/40
Total	0/30	0/30	4/30	1/30	5/120 (4.2%)

<sup>1</sup>NC = Feed only

<sup>2</sup>T1 = Feed + 0.05% AGP (BMD50)

<sup>3</sup>T2 = Feed + 0.2% Biolex<sup>®</sup> MB40



**Table 2. DGGE sequence result for each wk**

Species	Band No. *			
	1 wk	2 wk	4 wk	6 wk
<i>Alistipes</i>		15	2, 8, 16, 18, 19	13, 14, 15
<i>Bacteroides</i>		2, 3, 15		
<i>Bacteroides dorei</i>	12	1, 7, 12	13	11
<i>Bacteroides ovatus</i>	1	9		
<i>Bacteroides rodentium</i>	14, 15	4, 8	4, 9	
<i>Bacteroides uniformis</i>			14	
<i>Barnesiella viscericola</i>			3, 10	1, 2
<i>Campylobacter jejuni</i>			5	
<i>Clostridiales</i>	2, 5, 6, 7, 9, 10 11, 13	11	1, 11, 12, 15	6, 8
<i>Crocinitomix</i>				10
<i>Escherichia coli</i>			7	
Firmicutes	2,8	6, 10, 14	6, 17	
<i>Flammevirga arenaria</i>				4
<i>Lachnospiraceae</i>	3, 4, 11			8
<i>Lactobacillus aviarius</i>				3
<i>Lactococcus bacillus</i>		5		
<i>Porphyromonadaceae</i>		13		12
<i>Proteobacterium</i>				5, 7, 9
<i>Rikenellaceae</i>			2	
Rumen Bacteria	16			
<i>Ruminococcus</i>	3, 5, 6, 7, 8, 10		1, 11, 12, 15	6

\* Band No. : Numbers are correspond to band numbers in Figure 1.

**Table 3. Molar percentage of each SCFA**

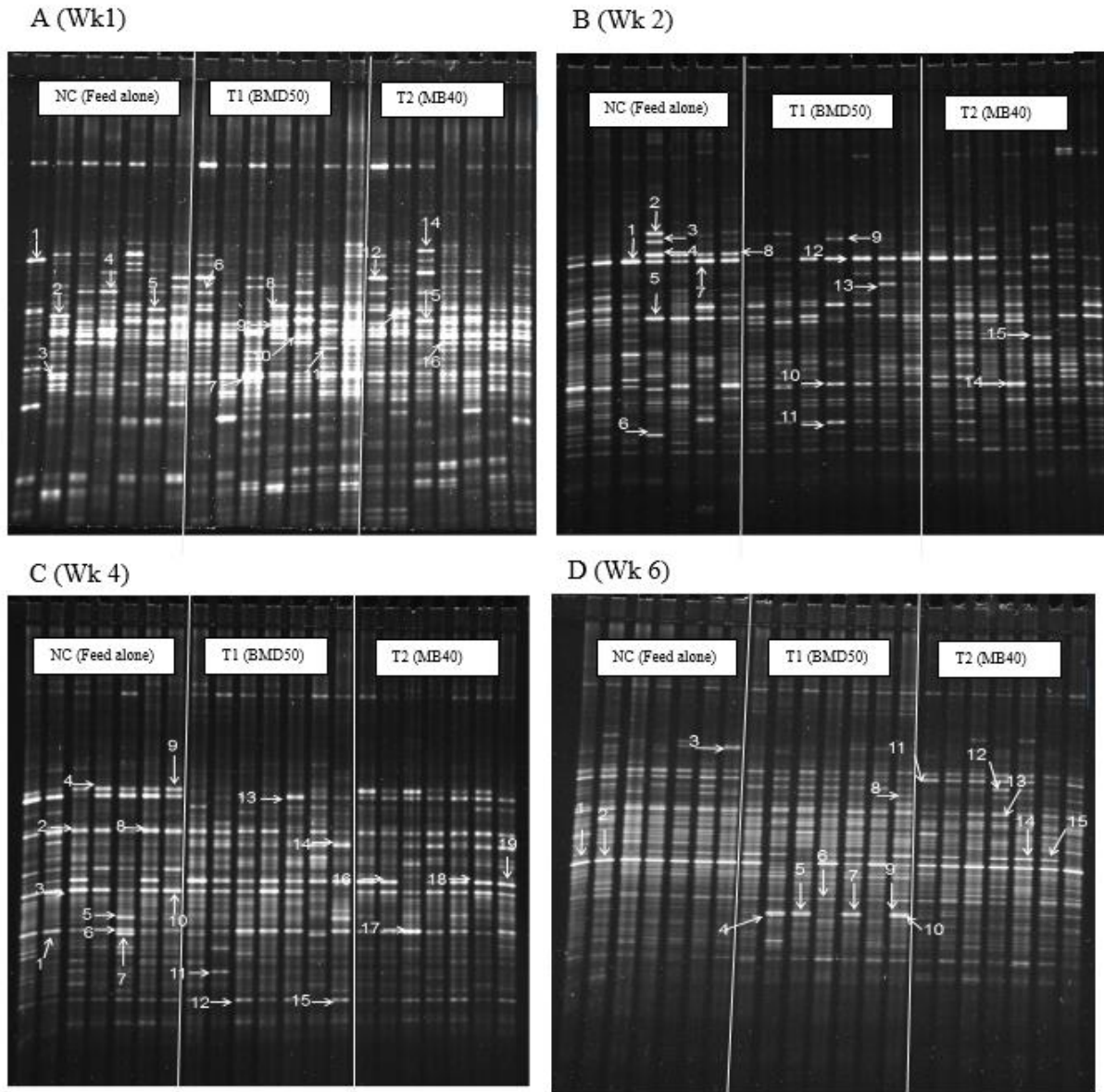
		Acetic acid (%)	Propionic acid (%)	Butyric acid (%)	Acetic acid/Propionic acid Ratio
Wk 4	NC <sup>1</sup>	61.2	13.2	25.6	4.63
	T1 <sup>2</sup>	66.2	13.6	20.2	4.11
	T2 <sup>3</sup>	60.9	14.8	24.3	4.56
Wk 6	NC	64.6	12.8	22.6	5.10
	T1	67.5	14.8	17.7	4.39
	T2	64.3	14.7	21.0	4.57
SE		± 0.48	± 0.16	± 0.52	± 0.06

<sup>1</sup>NC = Feed only

<sup>2</sup>T1 = Feed + 0.05% AGP (BMD50)

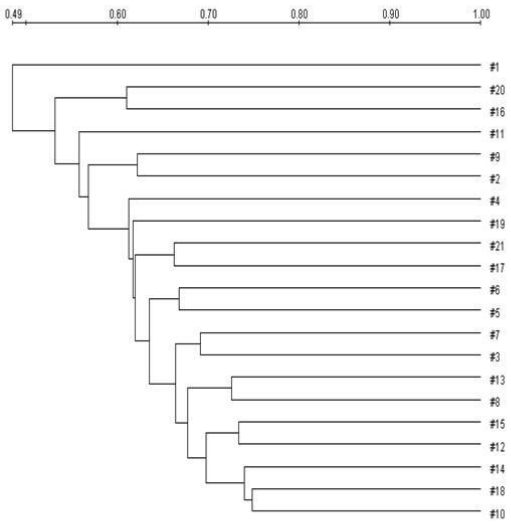
<sup>3</sup>T2 = Feed + 0.2% Biolex<sup>®</sup> MB40

Percentages were calculated by dividing each acid by sum of all three SCFAs.

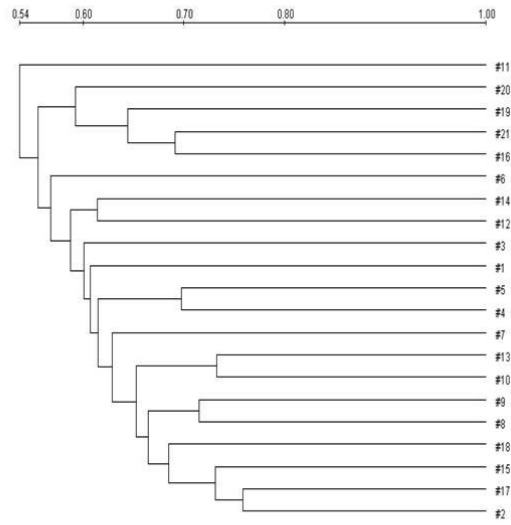


**Figure 1.**

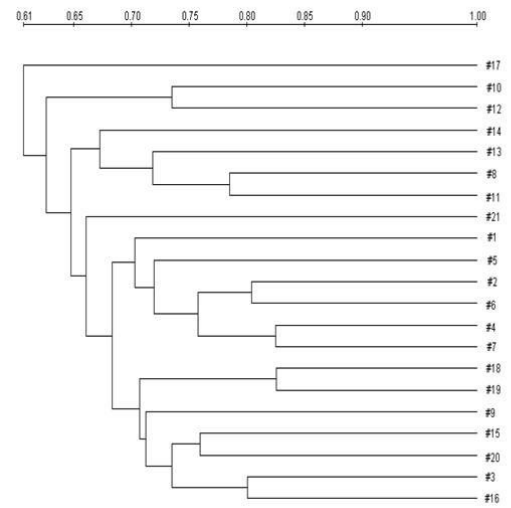
A (Wk 1)



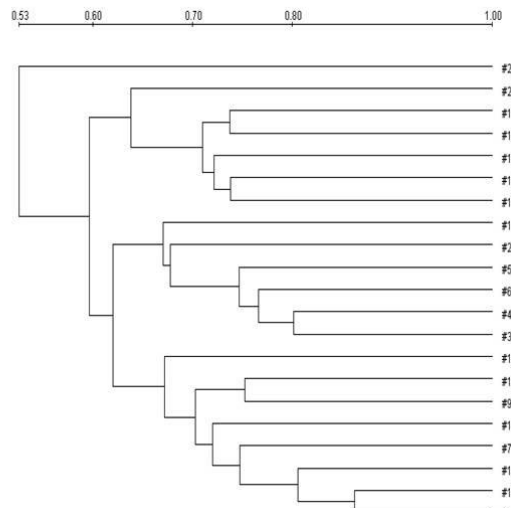
B (Wk 2)



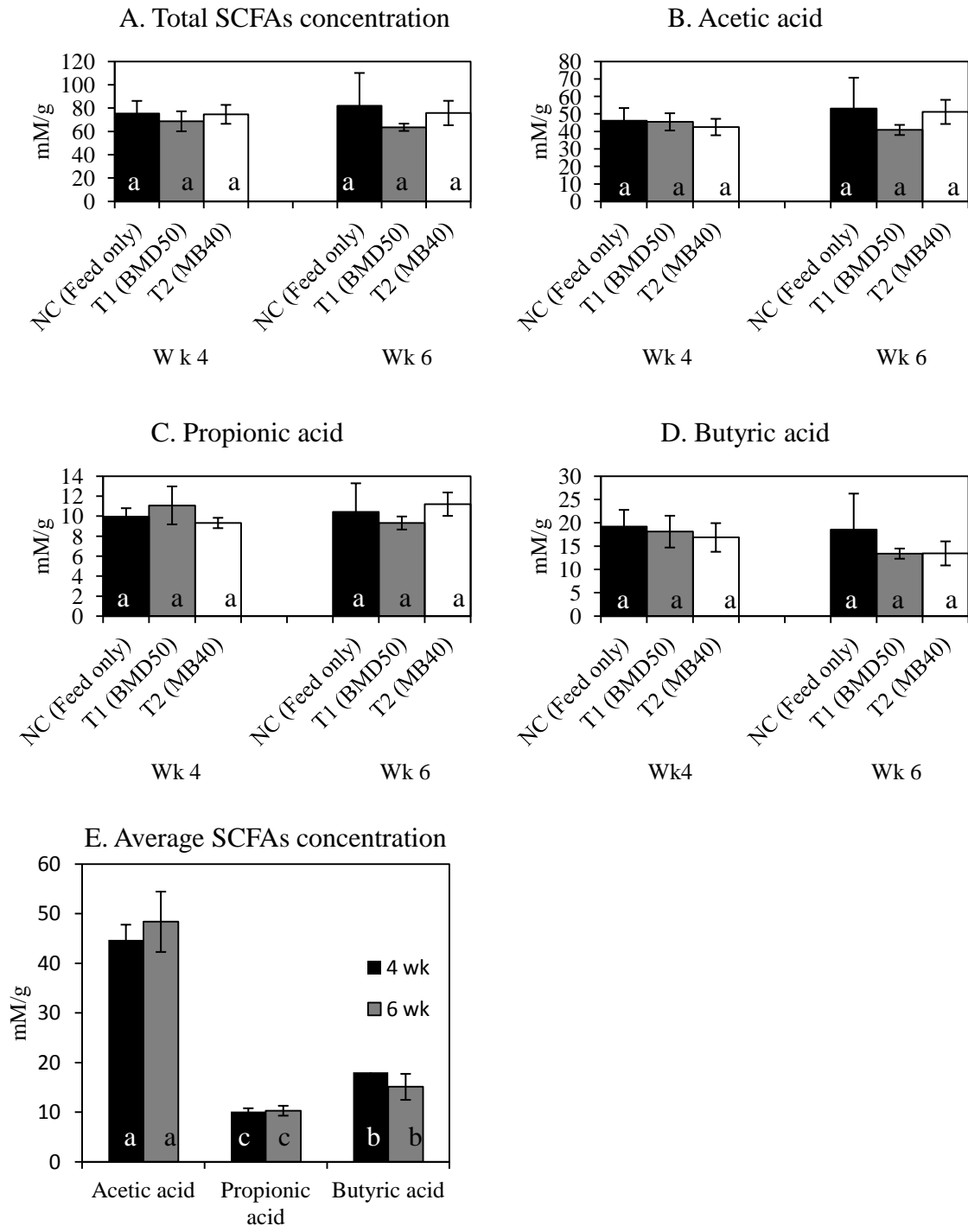
C (Wk 4)



D (Wk 6)



**Figure 2.**



**Figure 3.**

## **Authorship statement for chapter 2**

**Sang In Lee** is the first author of the paper and completed at least 51% of the studies among coauthors, which the title is "Assessment of cecal microbiota, integron occurrence, fermentation responses, and *Salmonella* frequency in conventionally raised broilers fed a commercial yeast-based prebiotic compound" in chapter 2.

Major Advisor: Dr. Steven C. Ricke

#### **IV. Chapter 3**

### **Effect of commercial prebiotic on gastrointestinal microbial population and growth performance of broilers**

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## 1. Abstract

Prebiotics are defined as fermentable food ingredients that selectively stimulate beneficial bacteria in the lower gastrointestinal tract of the host. The purpose of this study was to assess growth performance of broiler and the cecal microbial populations of an antibiotic, BMD50 (T1) supplemented birds compared to broiler chickens fed the prebiotic, Biolex<sup>®</sup> MB40 (T2). Data for weight responses of carcasses without giblets (WOG), wing, skin, white meats including breast and tenders, and feed conversion ratio (FCR) of the chickens were collected during processing. Extracted DNA from cecal contents of individual bird was utilized to construct a sequencing library and sequenced via an Illumina Miseq. Generated data were analyzed by a quantitative insights into microbial ecology (QIIME) pipeline to acquire operational taxonomic units (OTUs) tables. Each of the OTUs were assigned to specific microorganisms to determine taxonomic levels and subjected to alpha and beta diversity analysis. Subsequently, taxonomy data were imported to Microsoft Excel and JMP<sup>®</sup> Genomics for conducting a student t-test. In conclusion, there were no significant differences among groups in terms of parts weight and FCR but the white meat yield of Biolex<sup>®</sup> MB40 treated groups exhibited significant improvement compared to negative control and BMD50 group. In addition, supplementation of prebiotic MB40 significantly increased level of *Lactobacillus* in 2 wk compared to negative control and antibiotic BMD50 treated groups which may affect the mortality of birds. Retention of broiler performance and improvement of white meat yield suggest that the MB40 prebiotic appears to be a potential alternative to replace the antibiotic growth promoter.



## 2. Introduction

In 1995, Gibson and Roberfroid first defined prebiotics as nondigestible food ingredients that selectively promote beneficial bacteria such as *Bifidobacteria* and *Lactobacillus* (Kleessen, et al., 2001) that ultimately enhance health of the host by altering the microbial populations in the gut. Prebiotics are becoming more attractive as alternative feed supplements in animal production because it has been suggested that common usage of antibiotics in agricultural production could result in increases of antibiotic resistant bacteria (Barnes, 1958; Dibner and Richards, 2005; Elliott and Barnes, 1959; Gorbach, 2001; Lee and Lin, 2003; Marshall and Levy, 2011; Starr and Reynolds, 1951). A variety of prebiotics utilize various mechanisms to improve health of the host including short chain fatty acids (SCFAs) production, pH adjustment and competing for binding sites against pathogens (Afric, 1989; Gibson and Fuller, 2000; Rieke, 2003; Rolfe, 2000). For example, fructooligosaccharides (FOS) and galactooligosaccharides (GOS) are substrates for fermentation by *Bifidobacteria* and *Lactobacillus* thus, leading to increased SCFA production which in turn inhibits colonization and growth of pathogens (Bouhnik, et al., 1994; Gibson and Roberfroid, 1995; Hidaka, et al., 1986; Tzortzis, et al., 2005).

The commercial prebiotic evaluated in this study, Biolex<sup>®</sup> MB40, consists of 1,3-1,6-  $\beta$ -D-glucan and mannanoligosaccharides (MOS) which are derived from the cell walls of *Saccharomyces cerevisiae*, and several studies have shown their positive effects on growth performance of broilers (Hooge, 2004; Rosen, 2007). Growth performance of livestock is economically important to producers and poor performance is not acceptable by producers even if supplementation can reduce the level of foodborne pathogens (Jarquin, et al., 2007). According to Hooge (2004), supplementation of MOS exhibited statistically equivalent body weight with antibiotic amendment groups while significant improvements in final body weight were observed

compared to the negative control group. In addition, feed conversion ratio (FCR) significantly improved and mortalities were decreased in birds fed MOS diets by an average of 1.99% and 21.4% respectively compared to negative control groups but did not compared to the antibiotic supplemented group. Hooge (2004) noted that the greatest attribute of the MOS diet was the ability to decrease mortalities because it was the only attribute that was significantly different compared to the antibiotic diet fed group.

One of the distinct features to the MOS is the ability of bind to mannose-specific type-1 fimbriae of pathogen therefore, prevent colonization of pathogens (Newman, 1994). Receptor competition against pathogens is mediated by high affinity ligands derived from the yeast cell wall (Ofek, et al., 1977). One of the major foodborne pathogens, *Salmonella* utilizes this type-1 fimbriae thus, reduction of *Salmonella* concentration is expected upon introduction of MOS. Spring et al (2000) and Oyofe et al (1989a,b) observed *Salmonella* reduction in broilers by adding mannose to their diets. In a previous report Lee et al (2016) detected low levels of *Salmonella* from the same cecal samples used for the current study. In addition to improvement of growth performance and suppression of *Salmonella* colonization, supplementation of MOS also exhibited elevation of immunoglobulin A (IgA) and immunoglobulin G (IgG) level (Cetin, et al., 2005; Kudoh, et al., 1999; Swanson, et al., 2002).

The current study evaluates microbial population using NGS approaches on samples from the study by Lee et al (2016) where polymerase chain reaction based denaturing gradient gel electrophoresis (PCR-DGGE) had been utilized for comparing cecal microbial populations. According to the previous report using a PCR-DGGE approach, prebiotic supplemented group exhibited very similar patterns with antibiotic supplemented groups prior to wk 2 but were similar to negative control group as the birds became more mature. The objectives of the current

study includes performance evaluation and molecular analysis of the cecal microbiome of conventionally raised broilers fed with commercial prebiotic MB40 compared to negative control group and antibiotic BMD50 fed birds.

### **3. Materials and methods**

#### **Broiler housing**

Three houses were assigned for each treatment and each house contained 15,300 birds (Figure 1). The birds were identified with the name tag of the corresponding treatment and house number in order to avoid confusion. In addition, three pens in two locations were set up within in each house and twenty birds from each treatment group were randomly placed in one pen at each location to avoid a house effect (Figure 1). Diets for birds consisted of commercial starter, grower, finisher 1 and finisher 2. The only difference between diets was that T1 group received 0.05 % of BMD50 and T2 group consisted 0.2% of Biolex<sup>®</sup> MB40. Since the birds were raised in an off-campus commercial farm, the current study was exempted from review by the insitutional animal care and use committee (IACUC). In addition, the National chicken council (NCC) guidelines were ([www.nationalchickencouncil.org](http://www.nationalchickencouncil.org)) followed by the commercial cooperators to ensure internal animal welfare. Ten birds from each treatment were chosen for sampling of cecal contents as described previously (Lee, et al., 2016).

#### **Chicken performance**

Twenty birds that resided in the respective pens were weighed at 14, 28, 40 and 53 days of age and recorded. In addition, carcasses without giblets (WOG), wing, skin breast and tender weights were measured from randomly selected 100 birds (50 males and 50 females) that were located in the house but outside the pens at 53 days of age. Birds that lost tags or were

condemned by the USDA inspector were exempted from statistical analysis. Finally feed conversion ratio (FCR) was determined by feed intake and body weight at 28, 40 and 53 days of age. White meat yields were calculated by dividing sum of breast and tenders by live weight of randomly selected 100 birds within each house. Recorded data were imported by Microsoft Excel and JMP<sup>®</sup> Genomics for analysis of variance (ANOVA). *P*-values less than 0.05 were interpreted as significant differences among treatments.

### **Cecal microbial population assessment**

Cecal contents (200 mg) from each birds were collected for DNA isolation utilizing QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA). The concentration of extracted DNA was diluted to 10 ng/μL for the preparation of a sequencing library targeting the V4 region of 16S rRNA (Kozich, et al., 2013). Based on the recommendation of the manufacturer's protocol, isolated DNA samples were amplified and normalized using dual-index primers and SequelPrep<sup>™</sup> Normalization kit (Life Technology). The library was constructed following the method used by Park et al (2016) by combining 5 μL of each normalized aliquot samples for further assessment. Library concentration and product size were confirmed using a KAPA Library Quantification Kit (Kapa Biosystems, Woburn, MA, USA) and quantitative PCR (qPCR, Eppendorf, Westbury, NY, USA) and an Agilent 2100 Bioanalyzer system (Agilent, Santa Clara, CA, USA). The 20 nM of pooled library aliquot and the 20 nM of PhiX control v3 were combined with 0.2 N fresh NaOH and HT1 buffer and mixed a second time with 5% of the PhiX control v3. The 600 μL of the mixture containing pooled library aliquote, PhiX control v3, NaOH and HT1 buffer was subsequently loaded onto a MiSeq v2 Reagent cartridge to initiate sequencing.

### **NGS analysis by QIIME pipeline**

Sequencing read files were processed using quantitative insights with the microbial ecology (QIIME) pipeline (version 1.9.0) (Caporaso, et al., 2010; Park, et al., 2016). Operational Taxonomic Units (OTUs) tables were constructed by clustering sequences with 97% or higher identity based on Greengenes 16s rRNA gene database. In addition, OTUs that were not observed at least five times were excluded to eliminate possible erroneous reads from sequencing. Subsequently, OTUs tables were converted to taxonomic tables for further analysis. Weighted and unweighted version of UniFRac graphs and rarefaction plots were generated for beta and alpha diversity test respectively. Taxonomic level data acquired by QIIME was imported by Microsoft Excel and JMP<sup>®</sup> Genomics for analysis of variance (ANOVA) and a *P*-value of 0.05 to determine significant differences.

#### **4. Results and discussion**

The current study is the continued analysis of previously published research of Biolex<sup>®</sup> MB40 by Lee et al (2016) where fingerprinting of cecal microbiota were analyzed by PCR-DGGE. The current study focused on identification of the microbial population at the molecular taxonomic level along with the growth and processing performance of the birds.

##### **Broiler performance**

Growth performance including weight of the birds, FCR, weight of the parts and white meat yields of broiler is important since they are directly related to the market value of bird (Lubritz, 1997; Stevens, 1991). Average body weight and FCR are shown in Table 1. There were no significant differences among groups in terms of parts yield and FCR. These results agree with earlier studies by Waldroup et al (2003) and Midilli et al (2008) where no improvement of body weight and body weight gain were observed upon introduction of a MOS and yeast derived

prebiotic. No significant improvement of FCR by prebiotic was also observed by Ignacio (1995) however, FCR of broilers were significantly increased in the study by Midilli et al (2008) which agrees with observations by Sahane et al (2001) and Pelicia et al (2004) when broilers were supplemented with MOS or a mixture of probiotic and prebiotic. In addition, the study by Biggs et al (2007) reported that MOS did not exhibit any effect on metabolizable energy (ME) but reduced amino acid digestibility of broilers until day 7 of age when MOS, inulin, oligofructose, short-chain fructooligosaccharide (SCFOS), and transgalactooligosaccharide were compared. In addition, Pelicano et al (2004) observed better weight gain and increased FCR when MOS was supplemented however, positive effects in growth performance were only observed when amendments were introduced at 21 days of age. Pelicano et al (2004) speculated that absence of better weight gains at 35 and 42 days of age may be due to the level of stress and dilution effect by other compounds in the grains such as non-starch polysaccharides and non-digestible oligosaccharides. Midilli et al (2008) and Roshanfekar and Mamooee (2009) have hypothesized that the reason of inconsistent results among studies could be the differences in management, environmental conditions, stress and presence of unfavorable organisms.

Another study by Park et al (2014), on the same prebiotic used in this study, Biolex<sup>®</sup> MB40, observed similar results of pasture flock performance with no significant differences occurring in FCR and average bird body weights among treatment groups however, the prebiotic MB40 results did match the responses seen with antibiotic fed birds therefore, prebiotic MB40 potentially could replace the benefit seen with the antibiotic supplement. Significant weight gains of the birds were observed in the study by Roshanfekar and Mamooee (2009), when supplementation of MOS, Primalac (commercial probiotic) and a mixture of both increased FCR and average weight of the birds by 81.3, 73.5 and 148.8 g compared to the control group.

When comparing chicken processing responses including part weights and white meat yield, only white meat yield was significantly increased by supplementation of prebiotic MB40 compared to negative control group and antibiotic treated group (Table 2). According to Stevens (1991), white meats are the most economically valuable part of the broilers in Europe. The white meat or the breast meat is particularly important to producers because studies have shown high genetic correlation between white meat and the body weight of the birds (Le Bihan-Duval, et al., 1998). Roshanfekar and Mamooee (2009) observed significantly higher breast meat yield when the probiotic was supplemented however, the prebiotic supplemented group did not exhibit any improvement of breast meat yield.

### **Taxonomy summary**

Table 3 represent the total number of reads acquired by Illumina MiSeq and observation OTU count summary by the QIIME pipeline. The QIIME pipeline was able to identify and differentiate 16S rRNA fragment of bacteria from phylum level to species level and taxonomy bar graphs could be generated for each bacterial grouping from each sample. According to QIIME analysis, the most abundant bacteria at the phylum level was *Firmicutes* with average of 62.2 % and followed by *Bacteroidetes* with an average of 32.7 % (Figure 2A). *Proteobacteria* and *Tenericutes* were also detected with an average of 2.4 and 2.1% from the samples respectively (Figure 2A). *Clostridiales* were the most abundant microorganism and *Firmicutes*, and *Bacteroidales* were commonly identified along with *Bacteroidia*. (Figure 2B). In addition, 3.2% of the *Firmicutes* were identified as *Lactobacillales* (Figure 2B). *Proteobacteria* and *Tenericutes* were divided into more specific organisms at the order level consisting of *Enterobacteriales* and *Mollicutes* (Figure 2B). While the sum of *Bacteroidales* and *Clostridiales* comprised more than 90% of the total microbial population, *Bacteroidales* were found

predominantly in birds were more or equal to 2 wks of age despite the treatment. The level of *Bifidobacteriaceae* was low and was identified in very few samples, thus a statistical comparison was not possible.

When prebiotics were first introduced, only microorganisms were noted as beneficial bacteria were *Lactobacilli* and *Bifidobacteria* (Hutkins, et al., 2016). However, more detailed investigations using various molecular techniques such as qPCR or NGS have revealed more complex outcomes when specific prebiotics are introduced to the host. Numerous studies have reported that specific prebiotic supplementation not only promotes *Lactobacilli* and *Bifidobacteria* but also improves glucose homeostasis, leptin sensitivity and intestinal homeostasis (Everard, et al., 2011, 2014). In addition studies have suggested that prebiotic increases other beneficial microorganisms besides *Lactobacilli* and *Bifidobacteria* such as *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* which in turn benefits the host (Dewulf, et al., 2012; Heinken, et al., 2014; Miquel, et al., 2014; Ramirez-Farias, et al., 2009).

### **Microbial Population Shifts**

According to Lee et al (2016), antibiotic, BMD 50 and prebiotic, MB40 treated groups exhibited greater similarities in early stages of bird growth based on the phylogenetic trees generated from analysis of band patterns produced by DGGE. Base on QIIME analysis, most of the bacteria did not exhibit significant differences in yield among different treatment groups except *Barnesiellaceae* which is microorganism commonly existed throughout the samples according to PCR-DGGE approach (Lee et al, 2016). However, bacterial composition varied significantly by age of the birds. *Firmicutes* at the phylum level and *Clostridiales* at the order level decreased as birds became older with no significant differences between treatments (Table 4). Out of a total population of microorganisms, *Firmicutes* and *Bacteroidetes* constituted more



than 90 percent of the population and this is consistent with the fact that *Firmicutes* and *Bacteroidetes* are the most abundantly found microorganisms and associated with energy resorption rate in the gut (Ley, et al., 2006a, b). *Bacteroidales* however, increased significantly each week until 4 wk and kept a consistent level until 6 wk with no significant improvements among treatments. *Mollicutes* yield was low in 2 and 4 wk but significantly higher levels were detected in 1 and 6 wk. However, the level of *Bacteroidaceae* increased significantly in 2 wk and decreased afterwards.

According to QIIME, 67 of 120 samples identified *Campylobacter* within them however, 90% of *Campylobacter* were found in birds with 4 and 6 wk of age which indicates *Campylobacter* colonizes within birds in their later phases of growth. Yield of *Campylobacter* among all microorganisms were minimal as 0.3% and there were no significant differences in *Campylobacter* yield within distinct treatment groups (Data not shown). Studies have shown that colonization of *Campylobacter* can be suppressed by introduction of *Lactobacillus* and *Bifidobacteria* and a previous study by Kaakoush et al (2014) demonstrated the negative correlation between *Campylobacter* and *Lactobacillus* however, no statistical relationship was found in the current study (Baffoni, et al., 2012; Ganan, et al., 2013; Tareb, et al., 2013; Cean, et al., 2015).

One of the major bacteria considered to be beneficial, *Lactobacillus* was identified among all samples with an average of 3%. Levels of *Lactobacillus* observed in negative control and prebiotic treated groups were significantly greater compared to antibiotic treated groups in 2 wk (Figure 3). In addition, prebiotic MB40 was the only group that exhibited significantly higher levels of *Lactobacilli* in 2 wk however, statistical improvements disappeared after 2 wk. According to previous probiotic studies involving *Lactobacillus* culture, introduction of

*Lactobacillus* appears to exhibit significant effect on mortality reduction in broilers (Jin, et al., 1998; Vicente, et al., 2007)

### **Alpha and beta diversity analysis**

Alpha diversity analysis were carried out by the QIIME pipeline to measure microbial diversity within each sample. Figure 4A and B represent rare classes detected for each of the sequences obtained from the samples based on 'chao1' and 'observed OTUs' metric system respectively, which are commonly used for assessment of organism diversity. Both graphs exhibited prebiotic and antibiotic treated groups closer together compared to NC group and higher rarefaction were observed which indicates prebiotic or AGP amendments supported a more complex microbial diversity compared to the feed amendment group and the prebiotic MB40 and antibiotic BMD50 resulted in similar richness of species in cecal material of broiler. High similarity of microbial diversity between antibiotic, T1 and prebiotic, T2 groups agreed with the previous research result by Lee et al (2016) where the diversity of microbial populations were assessed by PCR-DGGE.

Figure 5A and B represent weighted and unweighted PCoA plots. Weighted PCoA plots (Figure 5A) were created using relative abundance of OTUs and each of the treatment groups were widely dispersed. Dispersion or no cluster of samples by treatment in weighted PCoA plots indicates similarity of OTU diversity within samples are more heavily affected by other factor besides treatments. Unweighted PCoA plot (Figure 5B) exhibits considerable clusters by age of the birds which implies maturity of the bird have greater impact to create a more uniform diversity of microorganisms compared to treatments used in the current study. In addition, cluster by samples from bird of younger age was more widely scattered than older birds, indicating a potential stabilization of the cecal microbiome as the bird gets ages. Observation of

complex but highly similar diversity in older birds agrees from previous studies where DGGE exhibited similar patterns as birds reach their marketing age.

## **5. Conclusions**

No statistical differences were detected in FCR and parts yield which leads to the conclusion that there were no variation in terms of chicken performance when BMD50 (T1) and MB40 (T2) were compared. Consistency of body weight, FCR and parts yields among prebiotic, antibiotic and control groups were also observed in previous studies (Baurhoo, et al., 2009; Morales-López, et al., 2009). The growth performance between treatment and negative control groups were not statistically different except for the white meat yield.

Overall, commercial prebiotic, MB40 significantly improved white meat yield in conventionally raised broilers but did not exhibit significant enhancement in other aspects including body weight, FCR and parts weight. According to the NGS approach to microbiome analysis significant increases in *Lactobacillus* level occurred in birds receiving prebiotic MB40 supplementation at 2 wk which may positively affect the mortality of birds. No significant reduction of chicken performance was observed thus, MB40 could be a viable alternative of in-feed antibiotic supplementation in concern of antibiotic resistant bacteria.

## **6. Acknowledgement**

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**Table 1. Broiler body weight and FCR responses in broilers**

	Age (Day)	Treatment		
		NC (Feed only)	T1 (BMD50)	T2 (MB40)
Bird weight (lb)	14	1.50 ± 0.02	1.49 ± 0.00	1.49 ± 0.01
	28	3.53 ± 0.02	3.44 ± 0.04	3.47 ± 0.03
	40	5.85 ± 0.02	5.76 ± 0.04	5.81 ± 0.04
	53	8.17 ± 0.07	8.15 ± 0.16	8.48 ± 0.15
FCR	28	1.51 ± 0.01	1.53 ± 0.02	1.56 ± 0.03
	40	1.80 ± 0.01	1.81 ± 0.01	1.90 ± 0.07
	53	1.74 ± 0.03	1.66 ± 0.02	1.74 ± 0.04
	53(Mort Adj <sup>1</sup> )	1.69 ± 0.01	1.66 ± 0.02	1.68 ± 0.02

**Table 2. Chicken processing responses**

		Treatments		
		NC (Feed only) n = 91	T1 (BMD50) n = 96	T2 (MB40) n = 97
Parts (lb)	WOG	7.14 ± 0.86	7.36 ± 0.75	7.37 ± 0.90
	Wing	0.70 ± 0.10	0.70 ± 0.10	0.70 ± 0.10
	Skin	0.32 ± 0.06	0.33 ± 0.05	0.33 ± 0.06
	Breast	1.62 ± 0.23	1.65 ± 0.19	1.71 ± 0.23
	Tender	0.33 ± 0.04	0.34 ± 0.03	0.35 ± 0.04
		NC (Feed only)	T1 (BMD50)	T2 (MB40)
White meat				
yield (%)		24.36 ± 0.18 <sup>b</sup>	24.35 ± 0.17 <sup>b</sup>	25.15 ± 0.17 <sup>a</sup>

Different superscript letters indicates significant difference

<sup>1</sup>Mort Adj includes total mortality weight in FCR calculation

**Table 3. Sequence reads and BIOM table summary**

Illumina MiSeq	BIOM table (observation counts)				
Total reads	Total observation	Min *	Max *	Median *	Mean *
26,665,292	10,030,751	42,204	134,689	82,611	83,589

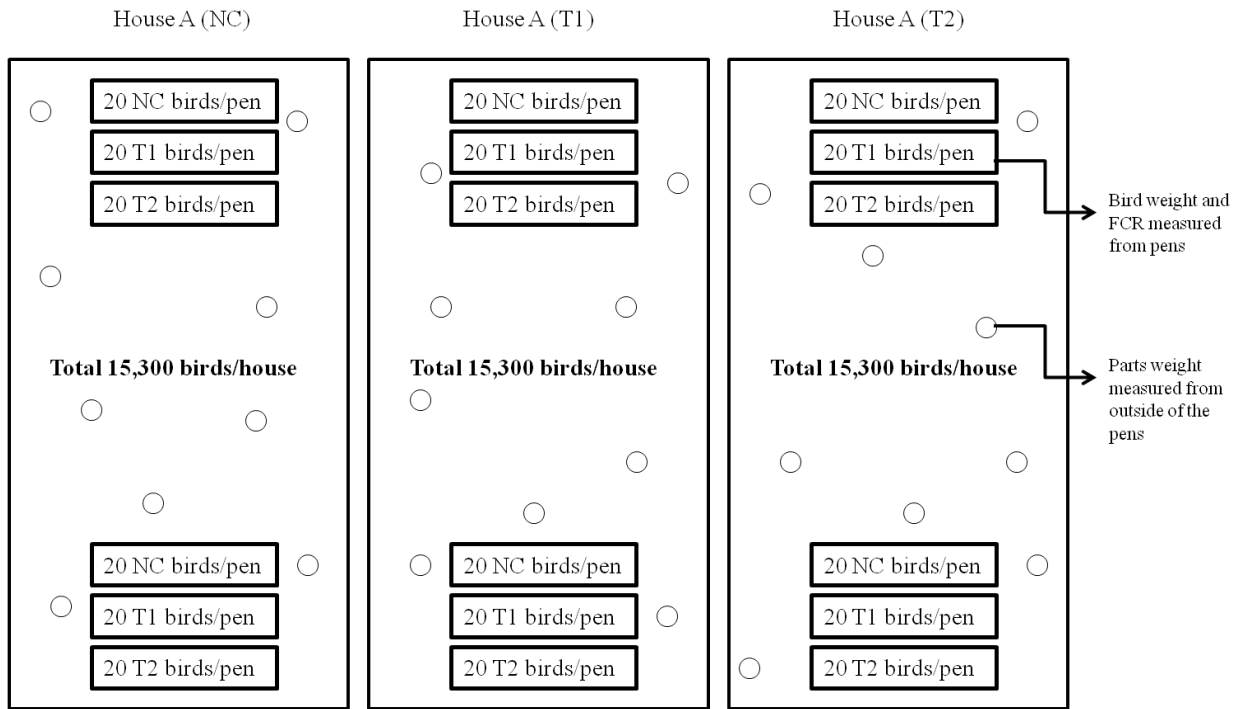
\*Counts per Samples

**Table 4. Phylum populations of microorganisms (%)**

Organisms	Average percentage			
	1 wk	2 wk	4 wk	6 wk
<i>Firmicutes</i>	83.15 ± 1.69 <sup>a</sup>	64.82 ± 1.66 <sup>b</sup>	50.29 ± 1.66 <sup>c</sup>	51.68 ± 1.66 <sup>c</sup>
<i>Clostridiales</i>	77.06 ± 1.94 <sup>a</sup>	58.29 ± 1.94 <sup>b</sup>	48.88 ± 1.94 <sup>c</sup>	49.37 ± 1.94 <sup>c</sup>
<i>Bacteroidales</i>	11.47 ± 1.72 <sup>c</sup>	31.91 ± 1.72 <sup>b</sup>	43.98 ± 1.72 <sup>a</sup>	43.47 ± 1.72 <sup>a</sup>
<i>Mollicutes</i>	3.16 ± 0.23 <sup>a</sup>	1.35 ± 0.23 <sup>b</sup>	01.41 ± 0.23 <sup>b</sup>	2.51 ± 0.23 <sup>a</sup>
<i>Bacteroidaceae</i>	11.44 ± 1.46 <sup>c</sup>	26.77 ± 1.46 <sup>a</sup>	17.84 ± 1.46 <sup>b</sup>	9.60 ± 1.46 <sup>c</sup>

\*Different superscript letters indicates significant difference

**Figure 1. Housing diagram**



NC: Feed only

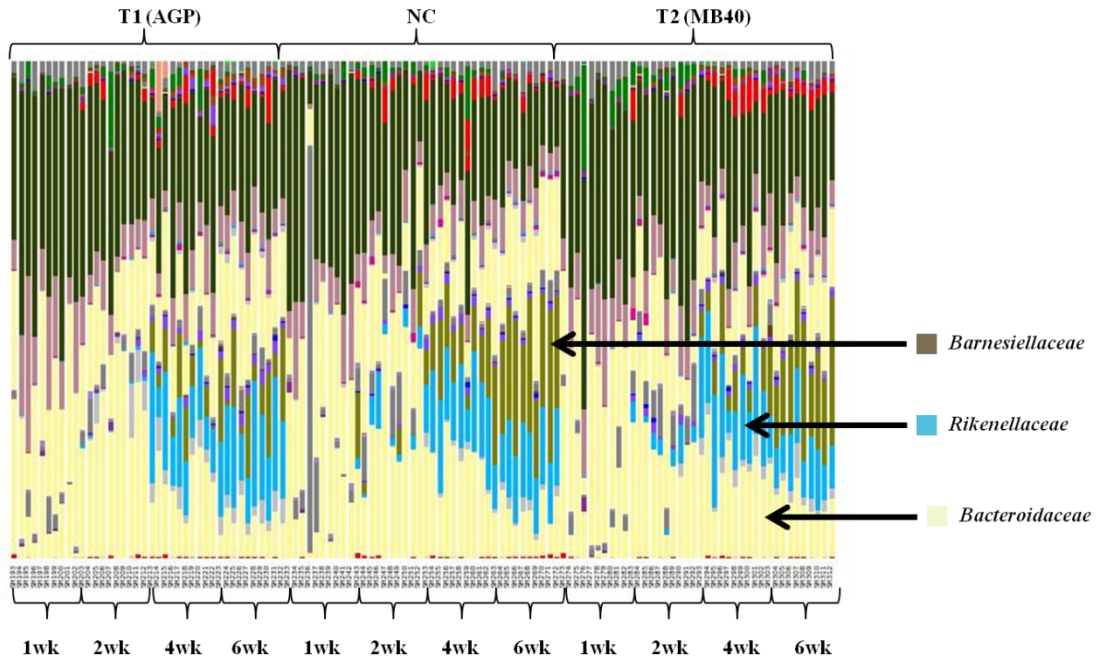
T1: 0.05% BMD 50

T2: 0.2% Biolex<sup>®</sup> MB40

Figure 2. Taxonomy bar graphs

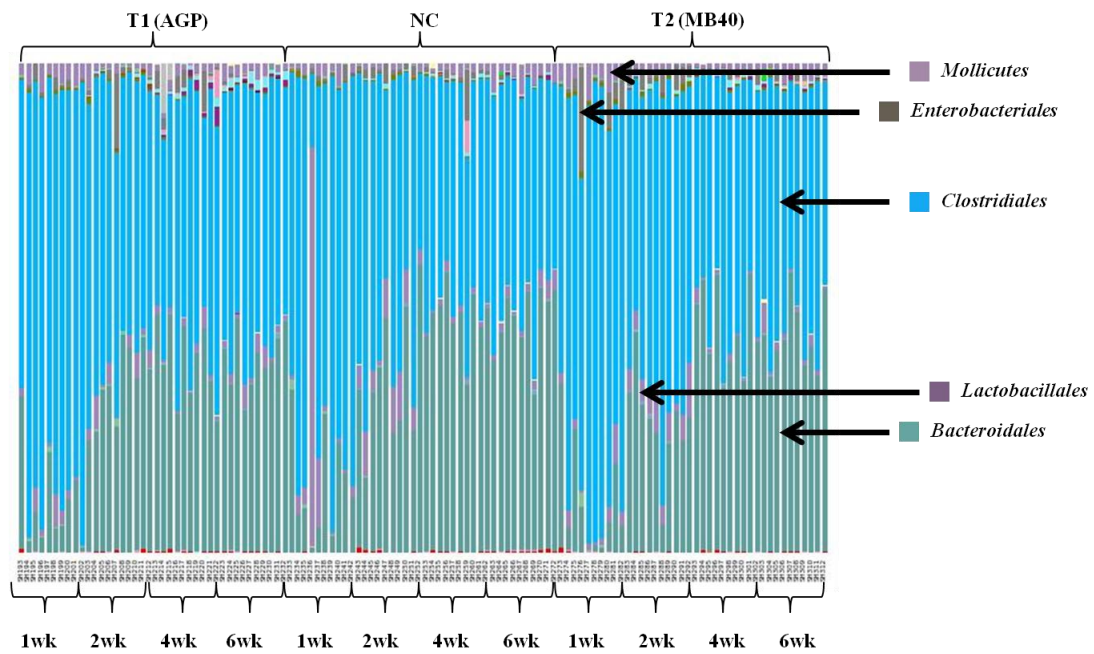
A

Family



B

Order





C

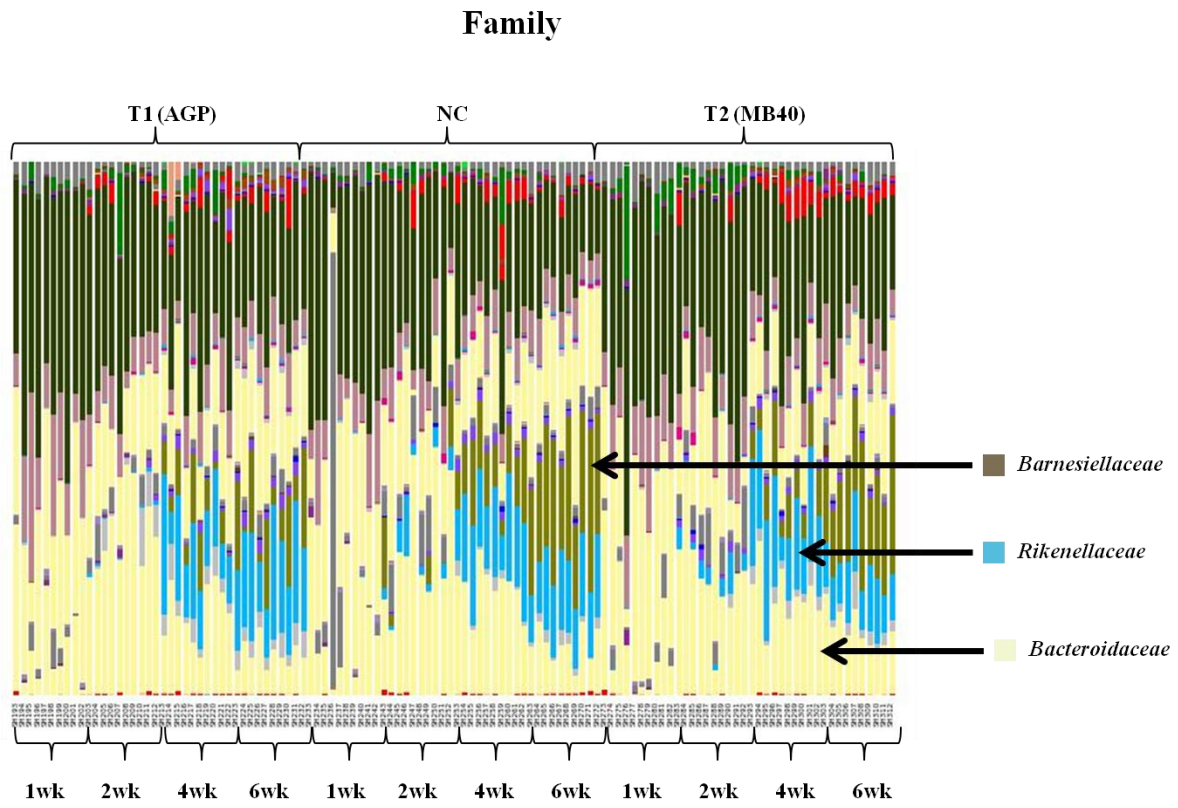
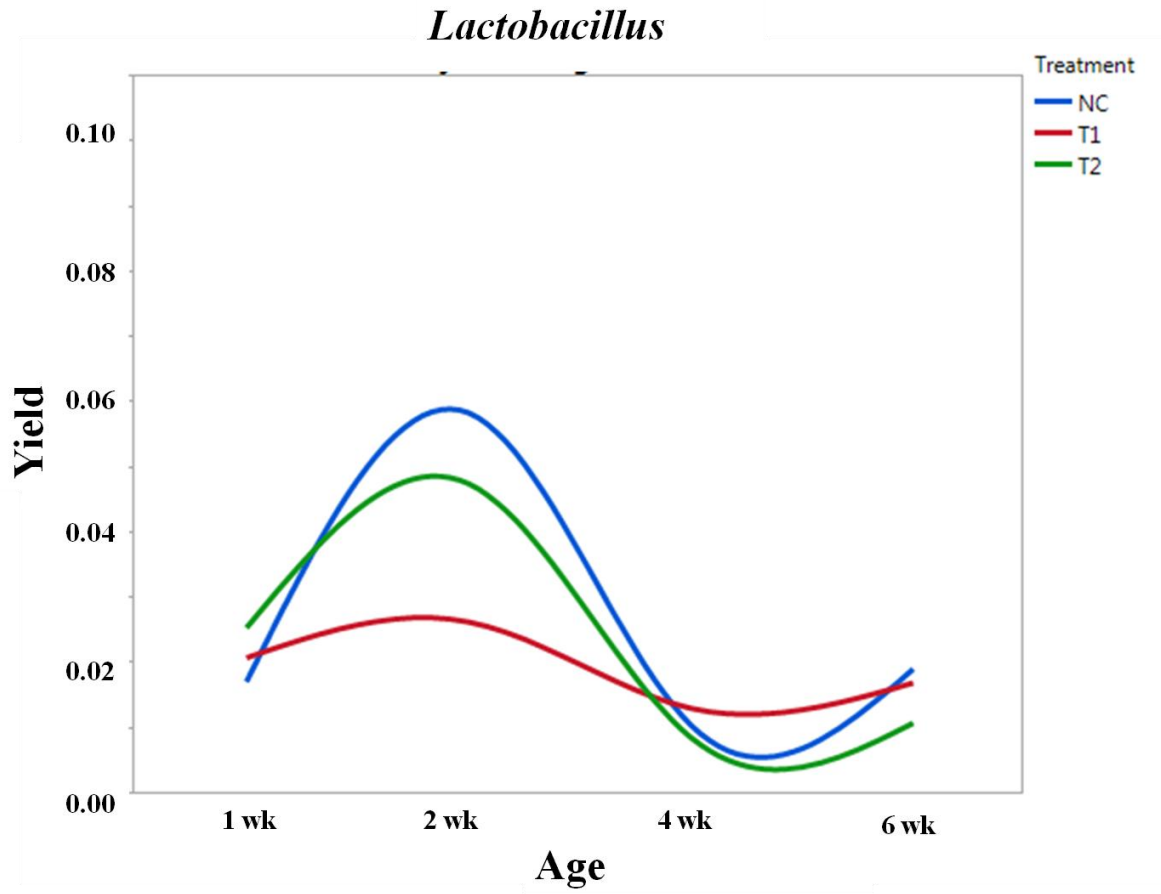


Figure 3. *Lactobacillus* yield



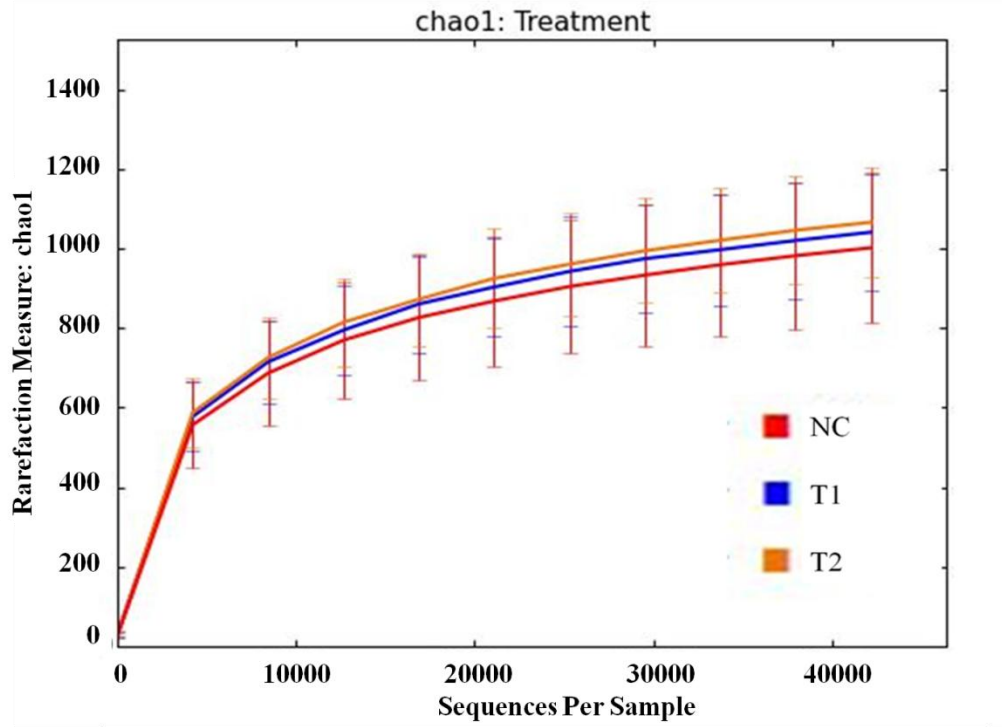
NC: Feed only

T1: 0.05% BMD 50

T2: 0.2% Biolex<sup>®</sup> MB40

Figure 4. Rarefaction plots

A



B

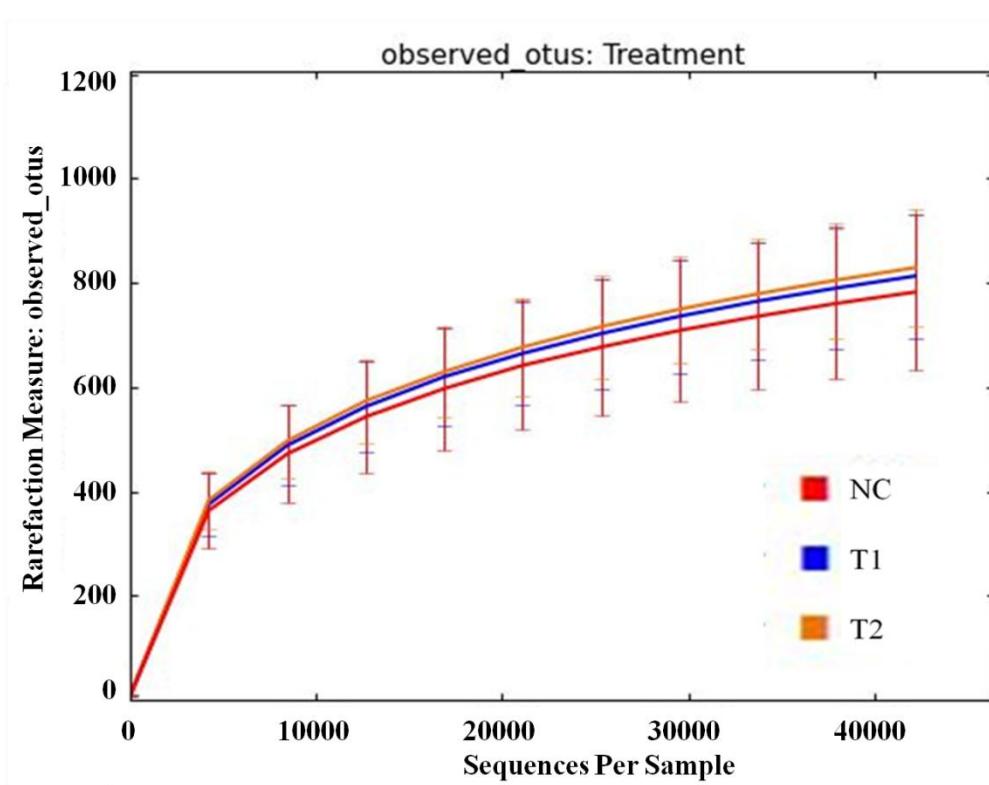
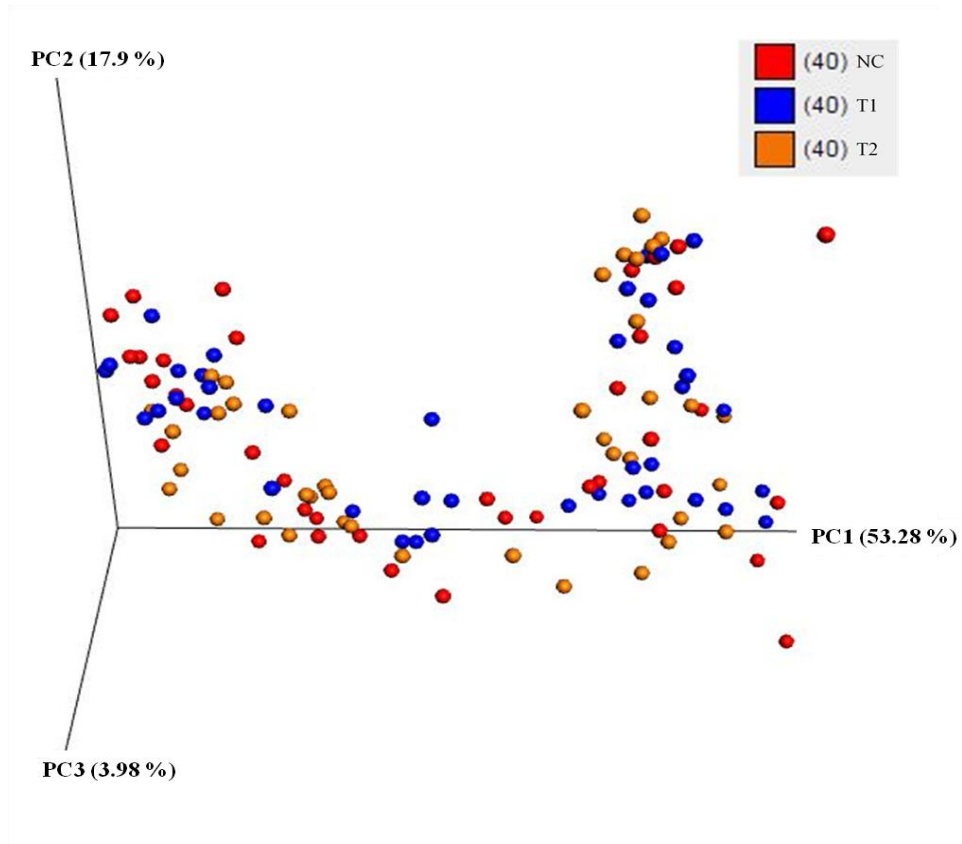
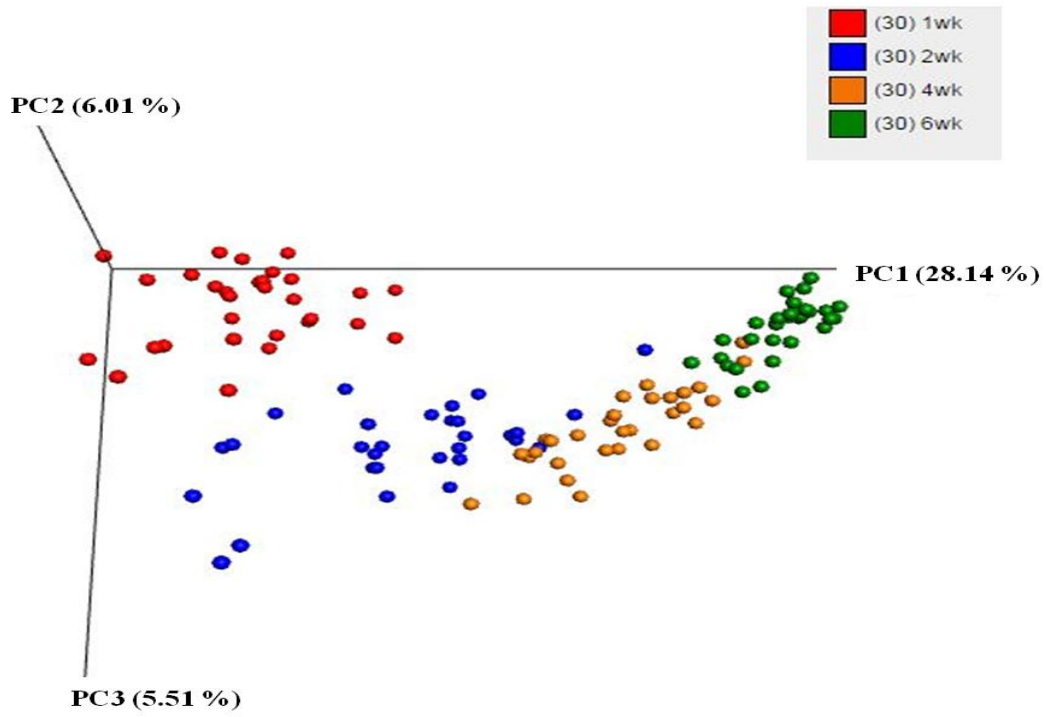


Figure 5. PCoA plots

A



B



### **8. Authorship statement for chapter 3**

**Sang In Lee** is the first author of the paper and completed at least 51% of the studies among coauthors, which the title is "Effect of commercial prebiotic on gastrointestinal microbial population and growth performance of broilers" in chapter 3.

Major Advisor: Dr. Steven C. Ricke

## V. Conclusion

As new prebiotics are developed and discovered, definition of prebiotics has been widened. In addition, different dosages of prebiotics and housing environments make the precise evaluation of prebiotics difficult. However, new technologies are also being developed that allow scientists to approach the investigation in variety of ways. For instance, NGS approach produce incomparably large amount of detailed data compare to DGGE which only produces a brief snapshot of microbiome and a very limited sequence result. As new approaches are conducted, there are possibilities to discover new aspects or factors affecting gut microbiome of the host either directly or indirectly thus, inconsistency between studies may overcome.

Since the core attribute of prebiotic is to alter the microbiome of the host towards healthier state thus, fingerprinting of the microbiome is essential. The objectives of the research in this thesis was to compare effects of prebiotics to commonly used in-feed antibiotics. The results did not exhibit any significant difference in integrons, SCFAs and *Salmonella* frequency among treatment groups however, white meat yields were significantly increased by the prebiotic and microbial populations were highly similar between prebiotic and antibiotic treatment groups according to PCR-DGGE and NGS methods. Similar microbial diversity as birds fed antibiotics and no significant reduction growth performance in broilers suggest that the particular prebiotic used in the studies, MB40, appears to be a potential alternative to an antibiotic growth promoter.

## VI. Appendix

### a. IACUC exemption Letter

Here is the email on the 2 projects not needing IACUC approval.

Corliss

To All: After much discussion at the meeting and input from both Billy Hargis, who was familiar with the nature of your projects and Jason Apple, who was familiar with the Jeff Chewning's operation (he had high praise for it) ; it was decided to return the two protocols with a decision of "Of No Action Required of the IACUC" with the request, citing the following portion of the UAF Policy on Use of Animals in Research and Teaching "*There is one exception to this policy, which is that specific Animal Use Protocols shall not be required for agricultural teaching applications involving the non-stressful observation of farm animals, demonstration of judging techniques, demonstration of accepted farm management practices, or normal use of farm animals in production. **Instead, standard operating procedures detailing such practices and procedures shall be kept on file in the office of Research Support and Sponsored Programs [Office of Research Compliance] and of the Associate Vice President for Agriculture-Research, and shall be incorporated into the Policies and Procedures of the Dale Bumpers College of Agricultural, Food and Life Sciences and the Agricultural Experiment Station***", that you send a memo to the IACUC (which I will distribute to the Committee) that includes a Standard Operating Procedure for the transport and euthanasia of birds that will be taken as samples from the production birds set for these kinds of studies. The IACUC would like for you to consult with Dr. Hargis as to the preparation of this memo and the SOP and how best to proceed with these kind of projects in the future.

This has certainly been a learning experience for all of us!

b. IBC approval Letter



UNIVERSITY OF  
ARKANSAS

Office of Research Compliance

May 19, 2014

MEMORANDUM

TO: Dr. Steven Ricke

FROM: W. Roy Penney  
Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 08030

Protocol Title: "Numbers and variations in species of Campylobacter on raw chicken carcasses"

Approved Project Period: Start Date: June 30, 2008  
Expiration Date: June 29, 2017

The Institutional Biosafety Committee (IBC) has approved the renewal of Protocol 08030, "Numbers and variations in species of Campylobacter on raw chicken carcasses". You may continue your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.






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