Towards a Better Understanding of Poultry Intestinal Microbiome through Metagenomic and Microarray Studies

DISSERTATION

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By
Shan Wei
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Dissertation Committee:
Dr. Zhongtang Yu, Advisor
Dr. Hua Wang
Dr. Michael Lilburn
Dr. Macdonald Wick
Abstract

The intestinal microbiome in poultry tracts functions as an interface between the host and the feed ingested and has important roles in nutrition and health of the host. This intestinal microbiome consisting of bacteria, archaea, fungi and few protozoa, with bacteria being the most abundant domain and greatly affecting the performance of the host. Modulation of the intestinal microbiome of poultry through dietary and managerial interventions has been used to improve poultry growth and health. However, the diversity and complexity of the intestinal microbiome hinders understanding of the mechanism of these interventions and achieving the intended modulations. Cultivation-based methods have been applied to intestinal microbiome studies, providing useful information. However, the fact that the majority of poultry intestinal microbiome are uncultivable limits the knowledge that can be learned about this microbiome. PCR-Denaturing Gradient Gel Electrophoresis (DGGE) and cloning and sequencing are primary cultivation-independent 16S rRNA gene-targeted molecular techniques commonly used in characterizing diversity and composition of poultry intestinal microbiome. However, they can only provide limited information because neither can provide detailed information on most of the bacteria present in the microbiome in a cost-effective manner. Recently, microarray and next-generation sequencing (NGS) technologies have been widely applied to investigation of microbiomes in various habitats, including guts of
humans and animals, greatly advancing our knowledge on microbiomes. However, few metagenomics or microarray studies have been conducted to investigate the poultry intestinal microbiome. The objectives of my study were to i) achieve a comprehensive understanding of the intestinal microbiomes of broiler chickens and turkeys, ii) establish a global phylogenetic framework of bacterial diversity of this microbiome, iii) examine how litter conditions and bacitracin (as a model antimicrobial growth promoter) affect colonization and abundance of common enteric pathogens in broiler chickens, and iv) develop a habitat-specific phylochip that can support comprehensive studies on poultry intestinal microbiome.

In the first study (chapter 3), a naïve-analysis of all the available 16S rRNA gene sequences of poultry gut origin archived in the public databases was performed. By analyzing high quality sequences of chicken and turkey gastrointestinal origin (3,184 and 1,345, respectively) collected from the GenBank, Ribosomal Database Project (RDP), and Silva database, I identified 915 and 464 species-equivalent operational taxonomic units (OTUs, defined at 0.03 phylogenetic distance) in the chicken and the turkey intestinal microbiomes, respectively. *Firmicutes, Bacteroidetes*, and *Proteobacteria* were the largest phyla in both chicken and turkey, accounting for > 90% of all the sequences. The estimated coverage of bacterial diversity of chicken and turkey reached 89% and 68% at species-equivalent level, respectively. More than 7,000 bacterial sequences from each bird species would be needed to reach 99% diversity coverage for either bird species.
In the second study (chapter 4), we used 454 pyrosequencing in investigating the bacterial diversity of cecal content and ileal mucosa samples of chickens and turkeys with two technical replicates. The objectives of this study were to uncover the major bacterial composition of poultry intestinal microbiome and to investigate the variations of bacteria diversity as affected by different sequencing runs and data processing pipelines. The 338,177 sequences analyzed represented on average 3,401 and 125 OTUs in chicken cecal content and ileal mucosa as well as 1,687 and 16 OTUs in turkey cecal content and ileal mucosa, respectively. The sequences from each bird species reached > 95% bacterial diversity in the intestinal microbiomes, except for the turkey mucosa microbiome. When compared to the public databases, this study identified 39 and 50 new genera in the chicken and turkey cecal microbiome, respectively. Noticeable variations were observed from the number of OTUs revealed by different sequencing runs and data processing pipelines. This study provided comprehensive perspectives on the chicken and turkey intestinal microbiota.

In the third study (chapter 5), a habitat-specific phylochip microarray (referred to as PITChip) was developed based on the sequences collected in the first and the second studies. The PITChip enables simultaneous detection and semi-quantification of 1,848 different bacterial OTUs and 105 genera of bacteria. After careful validation, the PITChip was used to analyze microbiomes of ileal mucosa and cecal content of broiler chicken reared on fresh and reused litters to assess how litter conditions affected the intestinal microbiome. More than 85 groups of bacteria were found to be influenced by litter status
in each sampling location. This study indicated that litter management can modulate the intestinal microbiome of broiler chickens and may have a profound effect on bird health and performance. This is the first phylochip developed for comprehensive studies of the intestinal microbiome of poultry. The PITChip may be used in future integrated studies to investigate interrelations between intestinal microbiomes, diets, feed additives, and managerial factors to promote flock health and performance.

In the last study (chapter 6), we further investigated the effects of supplemental bacitracin and litter management (fresh vs. reused) on the abundance of *Campylobacter*, *Clostridium perfringens*, and *Salmonella* in broiler chickens using specific quantitative PCR (qPCR) assays. This is the first study that has examined the effect of dietary bacitracin and litter conditions on the prevalence of these three common enteric pathogens. Bacitracin was shown to be effective to reduce abundance of *C. perfringens*, but it had no effect on *Campylobacter* or *Salmonella*. A somewhat negative correlation was noted between the abundance of *C. perfringens* and *Salmonella*. Unless contaminated by pathogens from a previous flock, litter reuse did not appear to increase the risk of necrotic enteritis caused by *C. perfringens* or *Salmonella* infection in subsequent flocks.

Taken together, the findings of the series of studies on poultry intestinal microbiome advanced our knowledge on the bacterial communities of poultry, provided new insight into the effects of litter management and bacitracin on the intestinal microbiome of
broiler chickens, and established a new microarray that can be used to support comprehensive analysis of the intestinal microbiome of chickens and turkeys that are often needed in studies of poultry nutrition and host health.
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Vita

Feb 1985 .......................................................... Qingyuan, China

2003 ............................................................. B.S. Biology Sciences, Nankai University

Graduate Research Associate, Department of Animal Sciences, The Ohio State University

Publications


Fields of Study

Major Field: Animal Sciences

Focus: Poultry Intestinal Microbial Ecology
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Microbiome in poultry intestinal tract plays important roles in nutrition and health of the host. The diversity and variable abundance of the individual microorganisms in this microbiome create a challenge for any effort to understand its members comprehensively. Determining the true diversity and actual composition of the intestinal microbiome in poultry has been a topic of research for microbiologists for decades. Microscopy was of limited utility because the morphologies of bacteria are too simple to be informative in their identification and characterization. Cultivation-based methods combined with physiological and biochemical tests were then used to identify bacteria, yielding reliable identification, classification, and characterization (Amann et al., 1995). However, cultivation-based methods have inherent limitations when applied to studies of intestinal microbiota because most of the intestinal bacteria are not readily cultivable in laboratory. Cultivation-independent methods, such as DNA reassociation and 16S rRNA gene sequencing, overcome the limitation of cultivation-based methods (Schloss and Handelsman, 2006). The most commonly used molecular marker of cultivation-independent method is 16S rRNA gene. As 16S rRNA gene consists of conserved and hypervariable regions, it can be amplified with universal primers or specific primers by PCR. Moreover, large public databases of 16S rRNA genes, such as the RDP,
Greengenes, GenBank, and Silva databases, are valuable resources for 16S rRNA gene-based studies.

PCR-Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer et al., 1993; Gong et al., 2007; Zhou et al., 2007; Cressman et al., 2010) and cloning and sequencing library (Bjerrum et al., 2006; Scupham et al., 2008a; Cressman et al., 2010) have been the most common 16S rRNA gene-based molecular techniques used in studying poultry intestinal microbiome. Although they can overcome the limitation of cultivation and have advanced knowledge on poultry intestinal microbiomes, several limitations restricted their application to exploring microbiome. Firstly, limited number of data, either bands or clones, can be resolved in an individual study. Thus, only predominant microorganisms or those of interest, such as pathogens or probiotics, can be identified (Kim, 2012). Second, DGGE does not allow identification of the bacteria represented by individual bands unless followed by another time-consuming cloning and sequence library analysis is performed.

Additionally, some of the recovered 16S rRNA gene sequences have been deposited in public databases but have not been reported in literatures, contributing little to characterizing and understanding the intestinal bacterial diversity of poultry (Wei et al., 2013a). Furthermore, as shown for the ruminal microbiome (Edwards, et al., 2004; Kim, et al., 2011), individual studies can bias towards or against certain bacterial phyla due to the methodology used. To assess the status of species richness that has been revealed and
synthesize the fragmented and biased knowledge on the intestinal microbiome of chickens and turkeys, a naïve analysis was performed on the publically available 16S rRNA gene sequences that were generated with the Sanger DNA sequencing technology from intestinal samples of both chicken and turkey (Wei et al., 2013a). We also estimated the current coverage of the bacterial diversity already identified in these two domesticated bird species and identified particular gaps in knowledge and understanding of the bacterial populations in these birds. Finally, the bacterial composition was compared between chickens and turkeys. The high quality sequences retrieved from this study served as a major source on developing a poultry-specific phylotype microarray (PITChip).

The 16S rRNA gene sequences of poultry gut origin collected from public databases were estimated to represent 89% and 68% bacterial diversity at species-equivalent level present in chicken and turkey, respectively. To gain a better understanding of the bacterial diversity of poultry intestinal microbiome, the next-generation sequencing (NGS) technology enabled by the 454 pyrosequencing FLX system was used to detail the species richness and composition of the cecal microbiomes of both broiler chickens and turkeys. Greater than 95% coverage of bacterial diversity was achieved for both bird species. Factors that can potentially affect the results were evaluated by comparing two independent pyrosequencing runs, different downstream analyses including sequence alignment and OTU clustering algorithms. The comprehensive information obtained in this study greatly expanded our knowledge of poultry intestinal microbiome. The
pyrosequencing reads and the 16S rRNA gene sequences collected from public databases have been archived in an in-house ARB database, which was then used to develop a microarray dedicated to analysis of poultry gut microbiome. Our results also showed that microbiome profiling can be screwed by variations among different pyrosequencing runs and downstream analyses procedures and programs.

Litter management practiced in commercial poultry production can affect the development of the intestinal microbiome of young chicks, including colonization by pathogenic bacteria. Bacitracin is among the most commonly used antimicrobial growth promoters (AGPs) used in the poultry industry. In a previous study, chickens reared on reused litter has been found to have more intestinal bacteria of intestinal origin while those reared on fresh litter have increased concentrations of intestinal bacteria of environmental origin (Cressman et al., 2010). Reused litter has also been reported to increase coliform levels and coccidial outbreaks in poultry flocks (Stanley et al., 2004) and increase the intestinal inflammatory response (Shanmugasundaram et al., 2012). To examine the effect of litter conditions and bacitracin on the loads of common enteric pathogens in broiler chickens, quantitative real-time PCR (qPCR) assays were used to quantify the abundance of Campylobacter, Clostridium perfringens, and Salmonella in samples collected from ileal mucosa, cecal content, and litter at two different ages of broiler chickens. Our results suggest that reused poultry litter does not necessarily increase the load of these pathogens.
A poultry-specific phylochip microarray can facilitate comprehensive investigation of poultry intestinal microbiome as affected by different factors, including diet, feed additives, and litter management. The major challenge of developing a host-specific phylochip microarray for the analysis of the intestinal microbiomes of poultry is the vast microbial diversity of microbiomes and lack of comprehensive information on the poultry intestinal microbiomes. Hence, the 16S rRNA gene sequences collected from public database were combined with the 454 pyrosequencing reads we generated into a database to represent the best diversity knowledge on poultry intestinal microbiome. Using this in-house database, we developed an oligonucleotide phylochip microarray, termed PITChip, dedicated to comprehensive studies of poultry intestinal microbiome. The PITChip contained more than 1,800 probes targeting all the known phylotypes, 105 bacterial genera, and common enteric pathogens that have been identified in poultry. It was validated for specificity, detection limits, and linear dynamic range. The utility of this PITChip was tested by analyzing the chicken intestinal microbiome as affected by litter management. The PITChip analysis of the ileal and cecal microbiomes of broiler chickens reared on fresh versus reused litter management indicated that litter management can be an effective approach to modulate the chicken intestinal microbiome. To our knowledge, this is the first phylochip dedicated to analysis of poultry gut microbiome, and it may be used in comprehensive analysis of poultry intestinal microbiome to support studies on poultry performance, health and nutrition.
Collectively, the series of studies reported in this dissertation have advanced our understanding on the diversity of the intestinal microbiome in chickens and turkeys, provided new light on the effects of litter management on the intestinal microbiome of broiler chickens, determined to what extent litter conditions and bacitracin affect colonization and abundance of common enteric pathogens of poultry, and developed a powerful analytical tool which can facilitate studies of poultry intestinal microbiome.
Chapter 2: Literature Review

2.1 Poultry intestinal microbiome: who is out there and what do they do?

The unique microbial community (or microbiome) in poultry intestinal tract plays an important role in poultry nutrition and health. It has been referred to as a forgotten organ (O'Hara and Shanahan, 2006), which functions as an interface between the host and the feed ingested by the host. The intestinal microbiome of poultry contributes to the wellbeing of host in a range of aspects including nutrition, immune system development, competitive exclusion, and detoxification (Kohl, 2012).

The intestinal microorganisms is dominated by bacteria which can be ranked pathogenic or commensal bacteria according to their effects on the host. Certain pathogens cause intestinal bacterial diseases that are costly to the poultry industry or of public health concern. For example, *Campylobacter jejuni* is the leading cause of bacterial food-borne diarrheal disease in humans (Mead et al., 1999). *Clostridium perfringens* is a commensal bacterium in healthy poultry digestive tract. However, under certain conditions (young age and diets), *C. perfringens* growth increases and secretes toxins, destroying the lining of digestive tract (Van Immerseel et al., 2009) This typically leads to necrotic enteritis and increased mortality. Besides single pathogens, severe alternation of intestinal microbiota can also cause Crohn’s disease (Roediger, 2004. Sartor, 2008). In these cases, commensal intestinal microbiota is critical in maintaining intestinal homeostasis and
eliminating proliferation of pathogens. A comprehensive understanding of the poultry intestinal microbiota can guide researchers and the poultry industry to improve poultry nutrition and health.

The working mechanisms of intestinal microbiome have been well established in mammals; the mechanisms working in poultry, although assumed to be similar to those of mammals, have not been fully established and require further research (Brisbin et al., 2008). Here a brief introduction to the known microbial diversity and functions of poultry intestinal microbiome will be provided.

2. 1.1 Diversity of poultry intestinal microorganisms

Microbiologists have spent decades to investigate the microbial diversity of poultry gut. Cultivation-based studies on poultry intestinal microbiome have primarily been used in investigating intestinal microorganisms, especially species of special interest or pathogens (Kohl, 2012). However, it is estimated that 60-90% of the chicken intestinal bacterial community cannot be cultivated under current laboratory conditions (Lu et al., 2003). Culture-independent methods overcome the limitation of cultivation-based methods, providing new insight into the bacterial diversity of poultry intestinal microbiome. 16S rRNA gene based molecular methods are the major culture-independent methods identifying bacterial diversity. By analyzing all the publicly available 16S rRNA gene sequences of chicken and turkey gut origin, the poultry intestinal microbiomes were found highly similar at phylum level. *Firmicutes, Bacteroidetes* and *Proteobacteria* were
the three most predominant phyla in both chicken and turkey (Wei et al., 2013a). It agreed with a previous finding that the common ancestor of amniotes maintained a microbial community mostly consisting of *Firmicutes* and *Bacteroidetes* (Costello et al., 2010; Kohl, 2012). As for archaea in poultry gut, only phylum *Euryarchaeota* was represented by a very small number of sequences, corroborating low abundance of methanogens in the gut of chickens (Saengkerdsub et al., 2007). Several next-generation sequencing studies have also been reported that investigated the intestinal microbiomes of chicken or turkey (Qu et al., 2008; Callaway et al., 2009; Lee, et al., 2011; Stanley et al., 2012a, b). While *Firmicutes*, *Proteobacteria* and *Bacteroidetes* were still identified as the predominant intestinal bacteria phyla, the percentage of the recovered 16S rRNA gene sequences assigned to each phyla varied among studies. At this point, comparing the abundance of bacteria across studies is not feasible due to several reasons: 1.) a limited number of species recovered from conventional 16S rRNA gene sequencing methods; 2.) different methods, such as PCR primers and experimental conditions, used to recovered the sequences; 3.) non-quantitative characteristic of both conventional cloning libraries or pyrosequencing profiles of bacterial diversity. It would be useful to adopt standardized approaches or a comprehensive quantitative analytical tool for future inventories of poultry intestinal microbiomes to investigate the effects of different factors on intestinal microbial communities across different studies (Kohl, 2012).
2.1.2 Poultry Intestinal microbiome affects host nutrition

Bacteria play the most important role in converting ingested feed to nutrients. Bacteria also contribute to the nitrogen supply to the host birds. Although cellulolytic bacteria are the focus of many studies on the rumen-like microbiome because they degrade cellulose, but they are not the major microbes in poultry feed digestion (Kim, 2012; Kohl, 2012).

In many mammals, microorganisms in intestinal microbiome carry out complex metabolic activities recovering energy and absorbable substrates for the host, and provide energy and nutrients for bacterial growth and proliferation (O'Hara and Shanahan, 2006). The situation in birds is different to that in mammal. Fibrolytic microbes, such as *Propionibacterium acnes*, *Ruminococcus flavefaciens*, and *Fibrobacter*, are mainly presenting in birds maintaining large, fibrolytic fermentation chambers, such as hoatzin (Godoy-Vitorino et al., 2010) and ostrich (Matsui et al., 2010a; Matsui et al., 2010b) and can contribute to ≥ 75% of the energy sources in these birds (Jozefiak, 2004; Kohl, 2012). On the other hand, energy recovered from this group of bacteria only contributes about 8% of the energy in chicken (Jozefiak, 2004). Cellulolytic bacteria has been occasionally reported in the ceca of chicken and turkey, but other study suggested little cellulolytic activity in chicken (Vispo and Karasov, 1997).

The major bacteria residing in the intestines of chicken and turkey are saccharolytic bacteria instead of cellulolytic bacteria (Kohl, 2012). Saccharolytic bacteria facilitate the host in utilization of sugar substrates. Microbial amylase activity has been detected in the crops of the chicken and turkey (Pinchasov, 1994; Kohl, 2012). These microbes may
conduct microbial fermentation of starches and simple sugars, which provides relatively less energy to the birds than hydrolysis by enzymes (Stevens and Hume, 2004), but may still increase net energy extraction for the host (Kohl, 2012).

In the ceca of birds, a large population of bacteria can utilize uric acid, converting it to volatile fatty acids (VFA) and ammonia. Birds cannot convert urea to ammonia and thereafter amino acids by themselves, and uricolytic bacteria help the bird to utilize urea in feed as a nitrogen source (Braun and Campbell, 1989). The refluxing of uric acid into the ceca and its subsequent degradation by bacteria provides an effective mechanism for the reclamation of carbon and nitrogen from the urine (Braun and Campbell, 1989) and water reabsorption from urinary water (Vispo and Karasov, 1997). The process of uric acid metabolism mediated by uricolytic bacteria is thought to be especially important for conserving nitrogen, especially in species with low protein diets (Kohl, 2012).

Microbes are known to increase nutrient absorption in mammals (O'Hara and Shanahan, 2006). However, the epithelial fortification may actually inhibit nutrient absorption in poultry (Kohl, 2012). Poultry has relatively smaller intestinal surface area compared to mammals, and relies more on the paracellular pathway of transportation of water-soluble nutrients between intestinal epithelial cells (Lavin and Karasov, 2008; Kohl, 2012). Studies using germ-free chickens have indicated that that colonization by microorganisms decreases total absorption of glucose and vitamins (Ford and Coates, 1971; Kohl, 2012). The mechanisms of balancing between microbial colonization and nutrient absorption in birds remain to be further investigated.
2.1.3 Poultry Intestinal microbiome affects host Immune function

Based on comparative studies of germ-free animals and colonized animals, intestinal microorganisms have been reported to greatly affect the intestinal physiology and development of mucosal immune system (O'Hara and Shanahan, 2006). Interactions between intestinal microbes and the immune systems of avian hosts have been assumed to be similar to mammals, yet they have been largely undescribed (Brisbin et al. 2008). Given that birds have a unique immune system compared to mammals, the limited information of microbe-host immunity interactions hinders better understanding of influence of poultry intestinal microbiome on immune system. The available studies of effects of poultry intestinal microbiome on the host immune functions were summarized in a recent review (Kohl, 2012).

The primary site of B lymphocyte development in birds is in the bursa of Fabricius. The bursa of Fabricius is a diverticulum on the proctadael region of the cloaca, and it’s also colonized by microorganisms after hatching (Kimura et al., 1986). These microorganisms may either serve as antigens or induce production of cytokines, leading to the proliferation of maturation of bursal B cells (Ratcliffe, 2006; Kohl, 2012). Ligation of the bursal tract prior to hatch lead to lower antibody production, suggesting that intestinal microorganisms can influence host immunity systemically through the interaction with the bursal tract (Ekino et al., 1985). However, further investigation is necessary to confirm whether intestinal microbiome up-regulates B cell development through this mechanism (Kohl, 2012).
Birds also possess unique characteristics of cell-mediated immunity. Fewer families of the T-cell receptor genes have been identified in birds (Lahti et al., 1991), which may indicate a lower diversity of peptides recognized by avian T cells (Mwangi et al., 2010; Kohl, 2012). The shifting of T-cell receptor (TCR) from polyclonal to oligoclonal occurs through the deletion of T cells that recognize food antigens or commensal microorganisms to avoid costly or over-reactive immune responses (Probert et al., 2007; Kohl, 2012). While the shifting TCR can occur throughout the life of mammals, it can only occur in birds during a restricted period, usually about one week after hatching (Friedman et al., 2008; Mwangi et al., 2010; Kohl, 2012). Germ-free chickens tend to maintain a polyclonal TCR repertoire. Therefore, delayed colonization of intestinal microbiome may alter the TCR repertoire, leading to costly and over-reactive immune responses to food and commensal microbial antigens (Probert et al., 2007; Mwangi et al., 2010; Kohl, 2012). Moreover, as expected, the complexity of the intestinal microbiome also affects TCR repertoires by influencing the deletion of T cells at the early age (Mwangi et al., 2010).

2.1.4 Poultry intestinal microbiome and detoxification

Some secondary metabolites from plant or invertebrate can be toxic to birds when being absorbed. Many of these compounds can be metabolized by bacteria to be less toxic to the host (Vispo and Karasov, 1997). For example, chicken intestinal microbiome has been found to be able to metabolize several mycotoxins, reducing the toxicity to the host (Young et al., 2007). On the other hand, intestinal microbes can also make some toxic
compounds in feed more toxic to the host. For example, glucosinolates in mustard family plants can be absorbed more readily in the gut after metabolized by cecal bacteria, and hence more toxic to the bird (Vispo and Karasov, 1997). However, limited studies have been executed to investigate roles of poultry intestinal microbiome in detoxification. Further investigation are in need to achieve a comprehensive understanding of roles of poultry intestinal microbiome in detoxification.

2.1.5 The role of microbiome in reducing pathogens

Poultry has been considered as an important source of zoonotic infection for over 100 years. A stable and healthy intestinal microbiome can limit the colonization of poultry gut by exogenous pathogens. Studies on Enterococcus faecium and some Lactobacillus isolates indicated that commensal bacteria can suppress the proliferation of pathogens not only by direct competition for available nutrients, but also competitive exclusion (Yeoman et al., 2012), by which the colonization of commensal bacteria prior to pathogen infection occupied the adhesion sites available on intestinal epithelium, leading to inability of pathogen colonization (Yeoman et al., 2012). Therefore, maintaining a healthy poultry intestinal microbiome is important for limiting pathogen proliferation. Some bacteria are known to produce bactericides or creating adverse environment for other bacteria, such as Bacillus subtilis and Lactobacillus spp., and they can be employed as potential approaches to limit pathogens.
2.2 Accessing the inaccessible: high throughput molecular tools for investigating poultry intestinal microbiome.

Poultry intestinal microbiome is rich in microbial abundance and complex in microbial composition. The fact that the majority of microorganisms are not cultivable under current experimental conditions limit the application of cultivation-based methods to microbiome studies. Cultivation-independent methods, especially 16S rRNA gene based methods have been used for three decades to investigate bacteria within various microbial communities. Ribosomal RNA gene was introduced as a phylogenetic marker because it is phylogenetically conserved and not horizontally transferred (Woese et al., 1983). 16S rRNA gene consists of hypervariable and conserved regions (Yu and Morrison, 2004). The hypervariable regions can be used for species identification, while the conserved regions flanking the hypervariable regions can be used to design universal primers, supporting studies of microbiomes at different phylogenetic resolutions (Yu and Morrison, 2004; Kim, 2012).

Cloning libraries of 16S rRNA gene sequences is one conventional cultivation-independent molecular method for investigating poultry intestinal microbiomes (Scupham et al., 2008a). Thousands of 16S rRNA gene sequences retrieved from cloning libraries have been archived in public databases, such as Ribosomal Database Project (RDP), GenBank, and Silva, providing a potential source of extensive knowledge of poultry intestinal microbiome (Kohl, 2012). However, a naive-analysis of all the
available 16S rRNA gene sequences of poultry origin suggested that around 7,000 sequences of 16S rRNA gene would be necessary to cover the majority (99% of bacteria diversity) of poultry intestinal microbiome (Wei et al., 2013a). The use of 16S rRNA and other highly conserved genes as phylogenetic markers has greatly expanded our view of intestinal microbiota. Because of the complexity of the intestinal microbiota and extremely variable population sizes, a large number of clones need to be sequenced to achieve an adequate coverage. However, the conventional one-clone-one-sequence approach enabled by the Sanger sequencing technology does not allow for cost-effective or efficient sequencing of hundreds of thousands of clones. The most ambitious cloning library effort reported to date sequenced 10,932 clones from libraries constructed from 10 poultry intestinal microbiomes, generating 370 distinct sequences in total (Dumonceaux et al., 2006). Substantially more sequences are required to reveal the full diversity.

2.2.1 Next generation sequencing

454 Pyrosequencing is one of the popular next generation sequencing (NGS) techniques. Next generation sequencing technique features extra high throughput sequencing capacity which can do parallel sequencing for millions of sequences. 454 pyrosequencing, while retaining the high throughput capacity, generates the longest next generation sequence reads by far. A GS FLX system can generate 400,000 250bp sequence reads in 7.5 hours; a GS FLX Titanium system generates over 1 million 400-500bp sequence reads (700bp with an updated version) in 10 hours. 454 pyrosequencing has proven to be a powerful
tool in studying genomics and metagenomics (Margulies et al., 2005). The NGS has changed our perspective of microbiome and made comprehensive investigations of microbiome possible.

The recent application of 454 pyrosequencing to simultaneously sequence thousands of 16S rDNA sequences (pyrotags) per sample has revolutionized the characterization of complex microbial communities. To date, studies based on 454 pyrotags have dominated the field of microbiome studies, but other NGS sequencing platforms, such as the Illumina platform, that generates many more sequence reads at much lower costs, have been developed. Dengan and Ochman (2012) reported a study using paired-end library of hyper-variable 16S rDNA fragments amplified from samples that varied in contents from a single bacterium, an artificial community, and a highly complex natural community. Beside the shorter sequence reads, there are three major obstacles in the application of Illumina platform to microbiota studies: (1) contamination, (2) the utility and classification of short read lengths, and (3) sequencing error rates (Degnan and Ochman, 2012). However, the great amount of sequence reads promises investigation to a deeper level and broader application in the future.

2.2.2 DNA microarray

DNA microarray is another high throughput tool that is popular in genomic and metagenomic studies. By assembling specific probes on the same chip, it allows simultaneous detection of thousands of targets of interest. Several host-specific
phylogenetic microarrays have been developed and they have greatly facilitated the investigation of microbiome (Harrington et al., 2008; Rajilić-Stojanović et al., 2009). Although pyrosequencing showed high throughput capacity in identifying the diversity in a microbiota, its current applications to typical ecological studies are limited for a few reasons. First, a single run of 454 pyrosequencing costs over $10,000 currently, which will be cost-prohibitive in most studies where multiple samples need to be analyzed comparatively. Pooling multiple samples using barcoded technology is an option to reduce the cost, but such pooling will also reduce coverage, diminishing the advantage of pyrosequencing. Second, extensive phylogenetic analyses are needed to interrogate the huge sequence datasets generated. These time-consuming analyses requires powerful computing capacities that often exceed the capacities of personal computers. Programmers and online servers are still struggling to develop improved software to handle the huge datasets generated by pyrosequencing, and there is no standardized data processing pipeline that a researcher can follow. On the other hand, microarrays can eliminate the above limitations. Additionally, microarrays eliminate the redundant information generated in pyrosequencing, making the data analysis relatively straightforward and user-friendly. Further, microarray-based analysis is especially suitable for comparative studies. Besides providing relative quantitative information, a microarray can be directly applied to nucleic acid samples, so potential PCR bias will not compromise the qualitative aspect of analyses of microbiomes.
2.3 In the -omics era: what did metagenomics analysis uncover?

16S rRNA gene sequence analysis is primarily used for microbial inventory and generates bacterial diversity information about microbial community. As the second and third generation high throughput sequencing platforms become more affordable and capable, it’s possible to explore microbiomes at unprecedented phylogenetic depth (Yeoman et al., 2012). A comparison of the bacterial profiles as revealed by the naïve-analysis of 16S rRNA gene and 454 pyrosequencing studies can identify new insights obtained in the NGS-facilitated metagenomic analysis of poultry intestinal microbiome.

The bacterial diversity in the chicken cecum microbiome has been investigated recently using 454 pyrosequencing (Qu et al., 2008; Callaway et al., 2009; Lee et al., 2011; Stanley et al., 2012a, b). The high throughput sequencing capacity of the NGS sequencing technology allows deeper coverage of bacterial diversity than the conventional Sanger sequencing technology in time- and cost- manners. The bacterial profiles in chicken cecum varied considerably among these studies (Wei et al., 2013a). Even so, all the genera that have been reported in 454 pyrosequencing of 16S rRNA gene amplicons studies (Callaway et al., 2009; Lee et al., 2011; Stanley et al., 2012a, b) were included in the sequence dataset from public databases. Therefore, even if the coverage of the individual studies was low, the global sequence collection represents the majority of bacterial diversity present in the chicken cecum and can serve as a phylogenetic framework of the bacterial diversity of chicken cecum (Wei et al., 2013a). The 16S rRNA gene sequences archived in the database of the chicken cecum was also compared to the
16S sequences recovered from chicken cecum by shotgun pyrosequencing (Qu et al., 2008) deposited on the MG-RAST server (Wei et al., 2013a). When the global sequence database of chicken cecal bacteria identified 59 bacterial genera, the shotgun pyrosequencing dataset only detected 21 bacterial genera, and 7 (Corynebacterium, Paracoccus, Helicobacter, Trabulsiella, Candidatus phytoplasma, and Akkermansia) of them were not represented in the 16S rRNA gene sequences archived in the database (Wei et al., 2013a). This might reflect the bias of individual studies that hindered a comprehensive knowledge of the composition of intestinal microbiome (Wei et al., 2013a).

The predominant genera represented in the 16S rRNA gene sequences archived in databases (of cecum origin) also differed from those identified by 454 pyrosequencing studies. Ruminococcus, Lactobacillus and Bacteroides were the most predominant genera in the 16S rRNA gene sequences archived in databases and in two 454 pyrosequencing studies (Qu et al., 2008; Stanley et al., 2012a). However, Bacteroides and Prevotella were found to be the most predominant genera in the cecum by Callaway et al. (2009), while Butyricimonas and Faecalibacterium were more predominant than other genera in the study by Nordentoft et al. (2011). The relative abundance of Lactobacillus, Clostridium and Ruminococcus varied in the 16S rRNA gene sequences archived in databases and in 454 pyrosequencing profiles of chicken intestinal microbiomes (Qu et al., 2008; Callaway et al., 2009; Nordentoft et al., 2011; Stanley et al., 2012a, b). However, comparison of the relative abundance of individual genera among different
studies should be interpreted with caution and accurate comparison among studies would not be feasible until a standardized quantitative method has been adopted by every studies (Wei et al., 2013a). Differences in host, feed, and biases associated with the analysis techniques used might all contribute to discrepancy among studies. All the major enteric pathogenic bacteria were represented in the 16S rRNA gene sequences archived in databases, but *Campylobacter* and *Shigella* were not detected in any of the 454 pyrosequencing datasets.

Metagenomic studies based on 16S rRNA gene provides comprehensive taxonomic information of poultry intestinal microbiome. Functional metagenomic analysis based on the functional genes present in the microbial community provided further information of the physiological roles of the microbiome. For example, genes encoding glycoside hydrolase (GH), polysaccharide lyase (PL), and carbohydrate esterase (CE) enzymes and other proteins participated in carbohydrate metabolism have been identified by a shotgun pyrosequencing study, representing by about 20% of the total genes identified (Qu et al., 2008; Yeoman et al., 2012). Bacterial Genes involved in lipid metabolism were also detected in chicken cecum, suggesting microbial participation in metabolism of lipid (Qu et al., 2008; Yeoman et al., 2012). Thus, functional metagenomic analysis is informative for illustrating the functions of poultry intestinal microbiome.

Microarray is a qualitative and quantitative high throughput method in studying intestinal microbiome. Host-specific phylogenetic microarrays have been developed for several
hosts, including human, mouse and porcine (Rajilić-Stojanović et al., 2009). The development and application of the human intestinal tract chip (HITChip) demonstrated its powerful qualitative and relative quantitative ability in examining human intestinal microbiomes. It has greatly facilitated the assessment of intestinal microbiomes in hypothesis-driven comparative studies and more than 800 samples have been examined by HITChip to date (Rajilić-Stojanović et al., 2009; Rajilić-Stojanović et al., 2010). A poultry-specific intestine tract chip (PITChip) has not been reported and the development and application of PITChip will be described in Chapter 5.

There should be no doubt that the high throughput techniques make it possible to access the comprehensive dynamics of a microbiome in the –omics era. The microarray techniques makes it possible to provide qualitative and quantitative information of poultry intestinal microbiome. The metagenomic data, in combination with genomic and transcriptomic information, can provide further elucidate of the working mechanism of poultry intestinal microbiome, leading to a better understanding of the composition and functions of microbiome.
Chapter 3: Bacterial census of poultry intestinal microbiome

3.1 Abstract

The objective of this study was to generate a phylogenetic diversity census of bacteria identified in the intestinal tract of chickens and turkeys using a naïve analysis of all the curated 16S rRNA gene sequences archived in public databases. High quality sequences of chicken and turkey gastrointestinal origin (3,184 and 1,345, respectively) were collected from the GenBank, Ribosomal Database Project (RDP), and Silva comprehensive ribosomal RNA database. Through phylogenetic and statistical analysis, 915 and 464 species-equivalent operational taxonomic units (OTUs, defined at 0.03 phylogenetic distance) were found in the chicken and the turkey sequence collections, respectively. Of the 13 bacterial phyla identified in both bird species, Firmicutes, Bacteroidetes, and Proteobacteria were the largest phyla, accounting for > 90% of all the sequences. The chicken sequences represent 117 established bacterial genera, while the turkey sequences represent 69 genera. The most predominant genera found in both the chicken and the turkey sequence datasets were Clostridium, Ruminococcus, Lactobacillus, and Bacteroides, but with different distribution between the two bird species. The estimated coverage of bacterial diversity of chicken and turkey reached 89%

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1 Poultry Science, in press.
and 68% at species-equivalent and 93% and 73% at genus-equivalent levels, respectively. Fewer than 7,000 bacterial sequences from each bird species from various locations would be needed to reach 99% coverage for either bird species. Based on annotation in the sequences, the cecum was the most sampled gut segment, from which 972 and 958 high quality sequences were recovered for chickens and turkeys, respectively. Chickens and turkeys were shown to have distinct intestinal microbiomes, sharing only 16% similarity at species-equivalent level. The bacterial census generated in this study may serve as a framework for future studies and development of analytic tools.

3.2 Introduction
The intestinal track of poultry harbors a complex and dynamic microbial community (or microbiome) consisting primarily of bacteria (Zhu et al., 2002). This microbiome has been recognized to have an important role in host growth performance and health (Brisbin et al., 2008; Jankowski et al., 2009; Yegani and Korver, 2008). The bacteria present in this microbiome can be categorized as commensal or pathogenic bacteria, both of which can be affected by a range of factors, such as host, litter management, diet, and feed additives. Numerous efforts, especially dietary intervention and litter management, have been attempted to modulate this intestinal microbiome to enhance feed conversion and gut health (Owens et al., 2008; Ruiz et al., 2008; Yegani and Korver, 2008). Although limited success has been achieved, few of these interventions have achieved consistent and sustainable improvement. It is now recognized that a better understanding
of the interactions of intestinal microbiome with host and with ingested feed is required to further enhance poultry nutrition and gut health. However, the lack of sufficient knowledge on the bacterial diversity (both phylogenetic and functional) in poultry intestines is considered one of the major knowledge gaps that hinder understanding of such interactions.

The composition and diversity of the poultry intestinal microbiome, like other microbiomes, were primarily investigated using cultivation-based methodologies (Barnes et al., 1972; Salanitro et al., 1974) until 16S rRNA gene-targeted analyses were applied in early 2000’s (Gong et al., 2002; Zhu et al., 2002). In addition to pathogenic bacteria, these cultivation-based studies helped identify some culturable commensal bacteria, especially facultative anaerobic and aerotolerant anaerobic bacteria. However, it soon became evident that only some of the intestinal bacteria can be cultured in laboratory media (Barnes et al., 1972; Salanitro et al., 1974). The use of DNA-based molecular biology techniques, primarily closing and sequencing of 16S rRNA genes, have provided opportunities to comprehensively characterize the intestinal microbiomes of poultry, primarily chickens and turkeys because of practical consideration (Bjerrum et al., 2006; Gong et al., 2002; Lu and Domingo, 2008; Scupham 2007a, b; Scupham et al., 2008; Zhu et al., 2002). These studies revealed a complex and diverse intestinal microbiome than previously thought and greatly expanded the perspective on poultry intestinal microbiome in terms of species composition, diversity, and community structure.
Until recently, all the 16S rRNA gene sequence datasets reported were generated using the Sanger DNA sequencing technology. Due to cost constraints, most studies produced relatively small numbers of sequences (a few hundred or less per sample) each, thus revealing only a small portion of the full diversity present in the intestinal microbiome. Besides the limited depth of coverage of diversity, the scope of these studies was also narrow with respect to numbers of birds sampled, types of diets and dietary additives fed, housing system and litter management used, and geographic regions surveyed. Additionally, some of the recovered 16S rRNA gene sequences have been deposited in public databases, but have not been reported in the literature, contributing little to characterizing and understanding the intestinal bacterial diversity of poultry. Furthermore, as shown for the ruminal microbiome (Edwards et al., 2004; Kim et al., 2011), individual studies can bias towards certain bacterial phyla due to the methodology used. As such, the knowledge on intestinal microbiome of chickens and turkeys remains to be fragmented and biased. We hypothesize that the general bacterial diversity of the intestinal microbiome of poultry can be better defined by analyzing all the 16S rRNA gene sequences (both published and unpublished) collected from all the intestinal microbiomes ever analyzed worldwide. In this study, we performed a naïve analysis of all the publically available 16S rRNA gene sequences that were generated with the Sanger DNA sequencing technology from intestinal samples of both chicken and turkey. We also estimated the current coverage of the bacterial diversity already identified in these two domesticated bird species and identified particular gaps in knowledge and understanding.
of the bacterial populations in these birds. Finally, the bacterial composition was compared between chickens and turkeys.

3.3 Materials and Methods

3.3.1 Sequence Data Collection

The 16S rRNA gene sequences of chicken and turkey origin were retrieved from the three public databases of nucleic acids including GenBank (http://www.ncbi.nlm.nih.gov/), Silva comprehensive ribosomal RNA database (Silva, http://www.arb-silva.de/), and Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/) in Jan and Feb, 2012 using the search terms ‘chicken’, ‘chickens’, ‘chick’, ‘chicks’, ‘poultry’, ‘broiler’, ‘hen’, ‘hens’, ‘turkey’, and ‘turkeys’. The sequences for chickens and turkeys were downloaded separately. Sequences shorter than 250bp were removed from the dataset to avoid uncertainties in comparing and classifying short sequences that have little or no sequence overlap. Possible chimeric sequences were identified using Chimera Slayer and UCHIME in the Mothur package (Schloss et al., 2009b; Edgar et al., 2011; Haas et al., 2011) and removed. The database record information associated with each of the sequences was examined, and the sequences of not poultry gut origin were removed manually. The final sequence datasets were deposited at the MG-RAST server (http://metagenomics.anl.gov/) and accessible through the collection Poultry_GIT_DB (4491721.3 through 4491724.3).
3.3.2 Phylogenetic Diversity Analysis

All the sequences that satisfied the above criteria were aligned using the sequence aligner in Mothur (V1.22) with the Silva SSU Ref NR 108 dataset (Ludwig et al., 2004) as reference sequences (Schloss et al., 2009a). Sequences that could not be aligned due to short overlap with the Silva reference dataset were removed. To generate a detailed phylogenetic tree using the Neighbor-Joining method (Saitou and Nei, 1987), the resultant aligned sequences were inserted into the Silva ARB tree constructed from the Silva reference dataset, with each sequence being inserted into a branch with which the sequence had the greatest sequence similarity. The sequences used in this study are maintained in an in-house ARB database dedicated to intestinal microbiome of chickens and turkeys and is available from the corresponding author. Krona charts (Ondov et al., 2011) were generated from the sequences for chicken and turkey (and their cecum) using the MG-RAST server (Meyer et al., 2008) to illustrate the composition of intestinal microbiomes of chickens and turkeys. A genus-level taxonomy tree each was also constructed for the sequences from the ceca of chicken and turkey using the MG-RAST server (Meyer et al., 2008) to compare the two cecal microbiomes.

3.3.3 Diversity Estimates

To minimize fragment effect of sequences corresponding to different regions of 16S rRNA gene, the aligned sequences were initially clustered based on the Silva bacterial sequence templates using the Cluster.fragment function of Mothur (Schloss et al., 2009b). Based on the classifications determined by the Classifier program in Mothur (Wang et al.,
distance matrices were computed within ARB with the Jukes-Cantor correction applied for the following bacterial groups: total bacteria, the phylum *Bacteroidetes*, the phylum *Firmicutes*, the phylum *Proteobacteria*, and sequences of cecum origin. Separate distance matrices were computed for chickens and turkeys. One distance matrix each was constructed at 0.03 (equivalent to species, OTU<sub>0.03</sub>), 0.05 (genus, OTU<sub>0.05</sub>), 0.10 (family), and 0.20 (phylum) phylogenetic distances (Schloss and Handelsman, 2004). The Mothur program (Schloss et al., 2009b) was also used to cluster sequences into OTUs, generate rarefaction curves, and determine the nonparametric ACE and Chao1 estimates of maximum richness from each of the distance matrices. The distance matrices were computed 3 times and the median was chosen in calculating these indices to avoid under- or over-estimation.

The maximum number of OTUs present in the intestinal and cecal microbiomes of each bird species was estimated using the non-linear models procedure (PROC NLIN) of SAS (V9.2, SAS Inst. Inc., Cary, NC). This method fits the monomolecular function to the rarefaction output generated by Mothur to determine the asymptote that serves as the upper bound of the curves as previously described (Larue et al., 2005). The value defined by the asymptote is an estimate of the expected maximum species richness complementary to the ACE and Chao1 richness estimates and has been used previously to estimate maximum species richness in different types of microbiomes (Larue et al., 2025; Kim et al., 2011; Nelson et al., 2010; Youssef and Elshahed, 2008). The percent coverage was calculated by dividing the observed number of OTUs by the maximum
number of OTUs (Kim, et al., 2011). The number of sequences that would be required to provide 99% coverage at 0.03 and 0.05 phylogenetic distances was estimated using the same non-linear model (Larue et al., 2005; Kim et al., 2011).

3.3.4 Comparison of Intestinal Microbiomes between Samples

The intestinal microbiomes of chickens and turkeys were compared using 3 methods: weighted UniFrac distance, which measures the phylogenetic distance between sets of taxa as phylogenetic trees (Lozupone and Knight, 2005; Lemos et al., 2012); the SONS function in the Mothur package, which compares two microbiomes by taking into consideration of OTU richness, membership, and structure (Schloss and Handelsman, 2006; Schloss, et al., 2009b); and Krona charts that allows comparison between microbiomes based on detailed phylogenetic composition. The cecal microbiomes between chicken and turkey was compared on a RDP annotated taxonomy tree at genus level using the MG-RAST server.

3.4 Results and Discussions

In total, 33,598 16S rRNA gene sequences of chicken and turkey gut origin were retrieved from GenBank, RDP, and Silva databases using the search terms. Of these sequences, 3,184 from chickens and 1,345 from turkeys passed the selection criteria and were analyzed in this study (Table 3.1), reflecting a fact that more than 85% of the 16S rRNA gene sequences archived in public databases are of short length or poor quality, or without a clear record of poultry gut as the sampling location. These sequences represent
13 existing bacterial phyla, besides 5.3% and 6.8% of the chicken and the turkey sequences, respectively, that could not be classified to any of the phyla within the Bergey’s taxonomy implemented in the RDP database (Fig. 3.1, Fig. 3.2). The sequences of chicken origin were assigned to 915 species-equivalent OTUs0.03 within 655 genus-equivalent OTUs0.05, whereas the sequences recovered from turkeys were grouped into 464 OTUs0.03 within 364 OTUs0.05. The sequences from chicken gut represented 12 existing phyla of bacteria (Fig. 3.1), while the sequences from turkey gut represented 8 recognized bacterial phyla (Fig. 3.2). Compared to the gut microbiome of other animals, the numbers of sequences recovered from both chickens and turkeys, and the diversity represented by these sequences are relatively small. The fast transit and thus short retention time in the poultry gut (approximately 4 hours for chickens) might be a major reason accountable for such relatively low diversity.

3.4.1 The Global Diversity of Intestinal Microbiome Sampled from Chickens

Of the 12 phyla of bacteria represented by the 3,184 high-quality 16S rRNA gene sequences of chicken origin, Firmicutes was the most predominant phylum and accounted for nearly 70% of all the bacterial sequences of chicken origin (Fig. 3.1). The Firmicutes sequences were grouped into 713 OTUs0.03 within 495 OTUs0.05 (Table 3.1). Bacteroidetes (12.3% % of the bacterial sequences) and Proteobacteria (9.3% of the bacterial sequences) were the second and third most predominant phyla, represented by 172 and 157 OTUs0.03 within 139 and 124 OTUs0.05, respectively. Other ‘minor’ phyla were only represented by a small number of OTUs, each of which was represented by
small numbers of sequences. The predominance of *Firmicutes* documented in chicken gut was much greater, whereas that of *Bacteroidetes* was smaller, than in the gut of other domesticated food animals sampled.

In total, 117 established genera of bacteria were represented by the sequence collection, with most genera belonging to the phyla *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* (Fig. 3.1). However, most of these genera were represented by a small number of sequences. Within phylum *Firmicutes*, genera *Clostridium*, *Ruminococcus*, *Lactobacillus*, *Eubacterium*, *Faecalibacterium*, *Butyrivibrio*, *Ethanologenens*, *Alkaliphillus*, *Butyricicoccus*, *Blautia*, *Hespellia*, *Roseburia*, and *Megamonas* were represented by more than 1% of the total bacterial sequences (in descending order). Most of these predominant genera are common intestinal residents, but the relatively high prevalence of *Ethanologenens*, a genus of ethanol-producing bacteria, is intriguing. Within phylum *Proteobacteria*, genus *Desulfohalobium* was represented by the most sequences (0.7% of the bacterial sequences), while within phylum *Bacteroidetes*, most of the sequences were classified into order *Bacteroidales*, and only genera *Bacteroides*, *Prevotella*, *Parabacteroides*, and *Alistipes* were each represented by > 1% of the bacterial sequences. Of the minor phyla, genus *Actinobacteria* was the most predominant, but only the genus *Bifidobacterium* was represented by > 1% of sequences within this phylum (Fig. 3.1). Other minor phyla, including *Cyanobacteria*, *Spirochaetes*, *Synergistetes Fusobacteria*, *Tenericutes*, and *Verrucomicrobia*, were only represented by no more than several sequences, suggesting their low abundance or prevalence in the gut of chickens. As for
archaea, only phylum *Euryarchaeota* was represented by a very small number of sequences, corroborating low abundance or prevalence of methanogens in the gut of chickens (Saengkerdsub et al., 2007).

The numbers of OTUs clustered at different phylogenetic distances were examined using rarefaction analysis (Fig. A1 A). At 0.03 phylogenetic distance or above, the rarefaction curves approached plateau, but continued to project upwards at ‘unique’ level. These results suggest that the diversity at sub-species level has not been completely sampled. The parametric (rarefaction) and non-parametric (Chao1 and ACE) estimates of richness were similar (Table 3.1). Based on these estimates, 904 - 1,028 bacterial OTU$_{0.03}$ and 703 - 834 OTU$_{0.05}$ likely exist in the gut of chickens collectively. As in the case of the observed numbers of OTUs, most of the predicted OTUs were within phylum *Firmicutes*. Based on the parametric estimate, the dataset has documented at least 89% of the OTU$_{0.03}$ and 93% of the OTU$_{0.05}$, and approximately 6,800 additional sequences from multiple chicken flocks in different geographic regions would probably allow for identification of 99% of the OTU$_{0.03}$ (Table 3.1), with most of the sequences to be recovered from members of *Firmicutes*. The acquisition of these new sequences will probably reveal all the OTU$_{0.05}$.

### 3.4.2 The Global Diversity of Intestinal Microbiome Sampled from Turkeys

The 1,345 bacterial 16S rRNA gene sequences of turkey gut origin represented eight phyla of bacteria, and 93.2% of these sequences were classified to existing phyla (Fig.
The most predominant phyla included *Firmicutes* and *Bacteroidetes*, accounting for approximately 60.4% and 28.8% of the total sequences from turkeys, respectively. Except *Proteobacteria* and *Actinobacteria*, each of the other ‘minor’ phyla was represented by only a small number of bacterial sequences. The turkey sequences were grouped into 464 OTU<sub>0.03</sub> within 364 OTU<sub>0.05</sub> (Table 3.1), as in the case of chicken sequences, most of which were found within *Firmicutes* and *Bacteroidetes*. However, phylum *Bacteroidetes* in turkeys was represented by a higher proportion of total bacterial sequences than in chickens. The increased proportion of *Bacteroidetes* was at the expense of the proportion of *Firmicutes*. Because the diets between domesticated chickens and turkeys are quite similar, the above differences in gut bacterial diversity might be mainly attributed to host differences.

The taxonomic composition of the turkey bacteria was detailed at the genus level in the Krona chart (Fig. 3.2). The turkey sequence dataset identified 69 genera of bacteria; however, 20 of them were represented by only a single sequence (Fig. 3.2). *Firmicutes* alone was represented by 37 genera, but only *Ruminococcus*, *Clostridium*, and *Lactobacillus* each represented more than 5% of all the sequences of this phylum. Other genera which were represented by more than 1% of the total bacterial sequences included (in descending order): *Megamonas*, *Bacillus*, *Faecalibacterium*, *Virgibacillus*, *Blautia*, *Eubacterium*, *Butyrivibrio*, *Ethanoligenes*, *Butyricoccus*, and *Clostridiales Family XI Incertae Sedis*. *Bacteroides* was the most predominant genus, accounting for 79% of the sequences, in the phylum *Bacteroidetes*. Other relatively predominant genera in this
phylum included *Prevotella* and *Paraprevotella*. Within phylum *Proteobacteria*, *Desulfohalobium*, and *Aeromonas* were the most predominant genera.

The numbers of OTUs observed at phylogenetic distances $\geq 0.03$ tended to approach plateau, but not at unique level (Fig. 3.1B). Unlike in the case of the chicken sequences, the Chao1 and ACE estimates of richness were greater than the parametric rarefaction estimate for most of the bacterial groups (Table 3.1). Based on the rarefaction estimate, at least $681 \text{ OTU}_{0.03}$ within $497 \text{ OTU}_{0.05}$ might be found in the gut of turkeys collectively, with most of them being within phyla *Firmicutes* and *Bacteroidetes*. The sequence dataset of turkey provided lower coverage than that of chicken because of the smaller number of sequences that have been recovered from turkeys. To achieve 99% coverage of diversity at phylogenetic distance 0.03, at least 5,652 sequences might need to be collected from multiple turkey flocks based on the non-linear rarefaction curve model (Laura et al., 2005).

### 3.4.3 The Global Diversity of Intestinal Microbiome Sampled from Chicken Cecum

The sampling locations of chicken gastrointestinal tract were not all clearly documented in the databases. Among the 16S rRNA gene sequences annotated with sampling locations, 972 were sampled from chicken cecum. These sequences represented 10 known bacterial phyla and accounted for 92.8% of the chicken cecal sequences (Fig. 3.3). The most predominant phyla included *Firmicutes* and *Bacteroidetes*, accounting for approximately 78% and 11% of the total cecal sequences, respectively. Except for
Proteobacteria and Actinobacteria, the other ‘minor’ phyla each were represented by only a small number of bacterial sequences. The sequences from chicken cecum were grouped into 532 OTU\textsubscript{0.03} within 400 OTU\textsubscript{0.05} (Table 3.1).

The cecal sequences from chicken identified 59 bacterial genera; however, 26 of them were represented by only a single sequence (Fig. 3.3). Firmicutes alone contained 31 genera, but only Ruminococcus, Clostridium, and Eubacterium each represented ≥ 5%, of all the sequences of this phylum. Other genera that contained more than 1% of the total cecal bacterial sequences included (in descending order): Faecalibacterium, Blautia, Butyrivibrio, Lactobacillus, Megamonas Roseburia, Ethanoligenes, Hespellia, Veillonella, and Anaerostipes. Bacteroides was the most predominant genus in the phylum Bacteroidetes, accounting for 40% of the cecal sequences in this phylum. Other relatively predominant genera in this phylum included Prevotella and Paraprevotella, Tanneralla, and Riemeralla. Within phylum Proteobacteria, Desulfohalobium, Escherichia/Shigella, and Neissenia were the most predominant genera.

The numbers of species-equivalent OTU\textsubscript{0.03} tended to approach plateau, but not at unique level (Fig. A1 C). Based on the rarefaction, Chao1 and ACE estimates, 785 – 903 OTU\textsubscript{0.03} within 530-901 OTU\textsubscript{0.05} might be found in the cecal microbiome of chicken collectively, with most of them being within phyla Firmicutes and Bacteroidetes. The diversity coverage for chicken cecum was lower than that for the entire chicken gut, because of the smaller number of sequences that have been recovered from the cecum. To
achieve 99% coverage of the diversity at species-equivalent level, at least 4,597 sequences might need to be collected from multiple flocks.

The bacterial diversity present in the chicken cecum has been investigated recently using 454 pyrosequencing (Qu et al., 2008; Callaway et al., 2009; Lee, et al., 2011; Stanley et al., 2012a, b). The massive parallel sequencing capacity of this technology allows for deeper coverage of diversity than the Sanger sequencing technology. The bacterial profiles revealed in the chicken cecum varied considerably among these studies with respect to number of OTUs and genera detected and their relative proportion. Even so, all the genera that have been identified from the 454 pyrosequencing of 16S rRNA gene amplicons (Callaway et al., 2009; Lee, et al., 2011; Stanley et al., 2012a, b) were represented in the global sequence dataset. Therefore, even though the coverage of the individual studies was low, the global sequence dataset represents much of the diversity present in the chicken cecum and can serve as a phylogenetic framework of the bacterial diversity of chicken cecum. The global sequence dataset of the chicken cecum was also compared to the 16S sequences recovered from chicken cecum by shotgun pyrosequencing (Qu et al., 2008) on the MG-RAST server. When the global sequence database of chicken cecal bacteria identified 59 bacterial genera, the shotgun pyrosequencing dataset only detected 21 bacterial genera, and 7 (Corynebacterium, Paracoccus, Helicobacter, Trabulsiella, Candidatus phytoplasma, and Akkermansia) of them were not represented in the global sequence dataset. This might reflect the bias of
individual studies that hindered a comprehensive knowledge of composition of intestinal microbiome.

The predominant genera represented in the global sequence dataset (of chicken cecum origin) also differed from those identified by 454 pyrosequencing studies. *Ruminococcus, Lactobacillus, and Bacteroides* were the most predominant genera in the global sequence dataset and in two 454 pyrosequencing studies (Qu et al., 2008; Stanley, et al., 2012a). However, *Bacteroides* and *Prevotella* were found to be the most predominant genera in the cecum by Callaway et al. (2009), while *Butyricimonas* and *Faecalibacterium* were more predominant than other genera in the study by Nordentoft et al. (2011). The relative abundance of *Lactobacillus, Clostridium, and Ruminococcus* in the global sequence dataset of chicken cecum was 3%, 14%, and 18%, respectively, whereas their relative abundance ranged from < 2% to > 20% among the 454 pyrosequencing studies (Qu et al., 2008; Callaway et al., 2009; Nordentoft et al., 2011; Stanley et al., 2012a, b). Differences in host, feed, and biases associated with the analysis techniques used might all contribute to the discrepancy. Thus, comparison of the relative abundance of individual genera or OTUs among different studies should be interpreted with caution. All the major enteric pathogenic bacteria were represented in the global sequence dataset, but *Campylobacter* and *Shigella* were not detected in any of the 454 pyrosequencing datasets. It might indicate a lower risk of *Campylobacter* and *Shigella* proliferation in healthy chicken.
3.4.4 The Global Diversity of Intestinal Microbiome Sampled from Turkey Cecum

Among the sequences annotated with sampling locations, 958 bacterial 16S rRNA gene sequences were sampled from turkey cecum. Most of these sequences (99.8%) were assigned to seven bacterial phyla (Fig. 3.4). The most predominant phyla included Firmicutes and Bacteroidetes, accounting for approximately 55% and 37% of the total turkey cecal sequences, respectively. As in the case of chicken cecal microbiome, except for Proteobacteria and Actinobacteria, the other ‘minor’ phyla each was represented by a small number of bacterial sequences. The sequences from turkey cecum were grouped into 350 OTU$_{0.03}$ within 275 OTU$_{0.05}$ (Table 3.1).

The sequences from turkey cecum identified 50 bacterial genera, 15 of which were represented by only a single sequence (Fig. 3.4). In the phylum Firmicutes, genera Ruminococcus, Clostridium, Faecalibacterium, and Megamonas each represented ≥ 5% of all the turkey cecal sequences, while genera Blautia, Butyrivibrio, Butyricoccus, Alkaliphillus, Eubacterium, and Pectinatus were each represented by ≥ 1% of the Firmicutes sequences (in descending order). Bacteroides was the most predominant genus, accounting for 80% of the Bacteroidetes sequences of turkey cecum. Other relatively predominant genera in this phylum included Prevotella and Paraprevotella. The remaining phyla were only represented by several sequences.

The numbers of species-equivalent OTU$_{s0.03}$ tended to approach plateau, but not at a unique level (Fig. A1 D). Based on the rarefaction estimation, at least 596 OTU$_{s0.03}$
within 400 OTUs might be found in the cecum of turkeys collectively. As in the case of chicken cecum, the bacterial diversity coverage for turkey cecum was lower than that for the entire turkey gut because of the small number of sequences that have been recovered. To achieve 99% coverage of bacterial diversity at species-equivalent level, at least 5,137 sequences need to be collected from multiple flocks.

3.4.5 Comparisons of Global Diversity of Intestinal Microbiome between Chickens and Turkeys.

The intestinal microbiomes of chickens and turkeys represented by the composite sequence datasets analyzed in this study appeared to be significantly different based on UniFrac significance analysis and P test (p < 0.01). When compared with respect to OTU richness, membership, and structure using the SONS function within the Mothur program, the Yue-Clayton similarity index (θyc, ranging from 0 for two completely different communities to 1 for two identical communities. Yue and Claton, 2005) was only 0.16 at species-equivalent and 0.59 at phylum level, indicating two distinct intestinal microbiomes (Table 3.2). The two intestinal microbiomes were more similar with respect to phylum *Proteobacteria*, followed by *Firmicutes*. On the other hand, the two microbiomes shared little similarity with respect to phylum *Bacteroidetes*. As expected, greater community similarities were shared at higher phylogenetic distances. Noticeably, the cecal microbiomes of the two bird species shared a lower θyc similarity index at each difference level when compared to the total gut intestinal microbiomes, suggesting that the cecal microbiomes of the two bird species were also distinct and the microbiomes in
other intestinal segments might share a relatively higher similarity between the two birds (Table 3.2). However, an in-depth analysis of the microbiomes from other gut sections was not feasible because only small numbers of sequences have been recovered from other intestinal segments of these two species.

Recently, Lemos et al. (2012) noted that OTU-based approaches, when applied to datasets with low sequence coverage, may lack the resolution to detect overlapping species between microbiomes. On the other hand, weighted UniFrac distances was suggested to be a reliable index when comparing both the diversity and structure of bacterial communities for all sequencing datasets, even the ones with a relatively small number of sequences. Comparison using weighted UniFrac distances revealed that the intestinal microbiomes of chickens and turkeys shared less than 50% overall similarity in diversity and phylogenetic structure (Table 3.2), and *Proteobacteria* was the phylum that was shared the most between the two bird species, which agreed with the results of the SONS analysis.

Besides differences in sequence predominance between the intestinal microbiomes of the two bird species, the distribution and relative abundance of the bacterial genera in the cecum also differed between chicken and turkey (Fig. 3.5). Although the cecal microbiomes of both bird species shared the major phyla, *Actinobacteria*, *Bacteriodes*, *Firmicutes*, and *Proteobacteria*, they showed distinct distribution and relative abundance at genus level. One extreme example is in *Actinobacteria* in which no genus was shared
between the two bird species. More genera of *Bacteroidetes* were found in the turkey cecal microbiome and six of them (*Porphyromonas, Paraprevotella, Capnocytophaga, Elizabethkingia, Flavobacterium, and Ornithobacterium*) were not found in the cecal microbiome of chicken. On the other hand, chicken cecal microbiome contained more genera of *Proteobacteria*, and nine of them were not found in the cecum of turkey. Being the most predominant phyla in both bird species, *Firmicutes* consisted of 42 genera but only 24 genera were shared by both bird species, leaving 13 genera exclusively found in the chicken cecum and 5 genera only identified in the turkey cecum.

Differences in host (genetics, breeds, anatomical features of gut, physiology, etc.) and feeds may be attributable to the observed differences. For examples, the intestines are larger in diameter in turkeys than in chickens. It is also known that turkeys have a more viscous digesta and a slower digesta passage rate (i.e. longer retention time) than chickens (Palander, et al., 2010). These factors may result in lower partial O$_2$ pressure and redox potential in the gut of turkeys than in the gut of chickens. These factors may explain, at least partially, the greater predominance of *Bacteroides* and *Faecalibacterium*, which are two strictly anaerobic genera predominant in the gut of mammalian animals, but smaller predominance in facultatively anaerobic genera, such as *Enterococcus, Streptococcus, Blautia, Subdoligranulum*, and several unclassified bacteria in the gut of turkeys than in the gut of chickens (Fig3, 4, and 5). Further, domesticated turkeys are grown primarily in the USA and Canada, and most of the turkey sequences in the sequence dataset were generated in several comprehensive studies conducted in the USA.
(Lu and Domingo, 2008; Scupham, 2007b; Scupham, et al. 2008) (Scupham, 2007a, b; Lu and Domingo, 2008; Scupham et al., 2008b) (Lu and Domingo, 2008; Scupham, 2007a; Scupham, 2007b; Scupham, et al., 2008). The narrower geographic regions of turkeys than chickens that have been sampled might be another reason contributing to the observed differences in the two microbiomes between chickens and turkeys. The differences in intestinal microbiome between these two bird species have important implications. Approaches to manipulate the intestinal microbiome may not be equally applicable to both species of birds. Indeed, chicken-derived competitive exclusion cultures effectively protected chicks, but not young turkeys, from infection with *Salmonella kedougou* or *S. typhymurium* (Hollister et al., 1994). It should also be noted that nearly all the turkey sequences were represented by uncultured bacteria, reflecting the lack of cultivation-based studies on the intestinal bacteria in turkeys.

3.4.6 Towards a Comprehensive Perspective of Poultry Intestinal Microbiome

Including sequences recovered from different chickens and turkeys fed different diets in different countries using a range of methodologies, this sequence dataset can serve as a global phylogenetic framework of bacterial diversity identified in chickens and turkeys. According to the estimates from the sequence datasets, less than 7,000 new sequences each from chickens and turkeys will probably allow nearly complete (99%) coverage at both species-equivalent and genus-equivalent levels. It should be noted, however, these new sequences need to be recovered from different flocks of chickens and turkeys fed different diets across a broad geographic regions. Otherwise, the data will be biased
towards one or a few groups of birds fed a few diets in limited narrow regions, and thus the sequence data will not represent true global diversity, even with increased depth of coverage afforded by the next generation DNA sequencing technologies. Furthermore, the current coverage might be an underestimate because with increasing number of sequences the predicted species richness tends to increase (Roesch et al., 2007; Yuet al., 2006). Thus, more than 7,000 new sequences from each bird species might be needed to achieve relatively high coverage of the bacterial diversity. Nevertheless, with the advancement of next-generation DNA sequencing technologies, it is feasible, both technically and fiscally, to generate sufficient new sequences. Given the bias noted in 454 pyrosequencing profiles, a coordinated effort from researchers is needed to sample chickens and turkeys fed diverse diets from different countries. The knowledge of the full diversity of gut microbiome can provide a diversity framework to assess the significance of individual populations in poultry gut and development of new analytic tools.

3.5 Acknowledgement

The study was partially supported by a grant awarded to Z.Y. (2006-06146) from the USDA/CSREES Midwest Poultry Research Program.
Table 3.1. The number of OTUs for predominant bacterial phyla and groups, their percentage coverage, diversity index, and number of sequences needed to reach 99% coverage.

<table>
<thead>
<tr>
<th>Bacterial phyla or groups</th>
<th># of seqs</th>
<th>Observed # of OTUs (% coverage)</th>
<th>Maximum # of OTUs</th>
<th># of sequences needed to reach 99% asymptote</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rarefaction asymptote</td>
<td>Chao1</td>
</tr>
<tr>
<td><strong>Chicken</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total bacteria</td>
<td>3184</td>
<td>915 (89) 655 (93)</td>
<td>1028</td>
<td>703</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>391</td>
<td>172 (58) 139 (66)</td>
<td>296</td>
<td>212</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>2192</td>
<td>713 (84) 495 (90)</td>
<td>856</td>
<td>551</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>295</td>
<td>157 (84) 124 (54)</td>
<td>415</td>
<td>230</td>
</tr>
<tr>
<td>Cecal bacteria</td>
<td>972</td>
<td>532 (63) 400 (76)</td>
<td>846</td>
<td>530</td>
</tr>
<tr>
<td><strong>Turkey</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total bacteria</td>
<td>1345</td>
<td>464 (68) 364 (73)</td>
<td>681</td>
<td>497</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>387</td>
<td>99 (60) 90 (65)</td>
<td>167</td>
<td>138</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>812</td>
<td>294 (70) 213 (76)</td>
<td>423</td>
<td>280</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>80</td>
<td>29 (77) 24 (79)</td>
<td>38</td>
<td>31</td>
</tr>
<tr>
<td>Cecal bacteria</td>
<td>958</td>
<td>350 (59) 275 (68)</td>
<td>596</td>
<td>405</td>
</tr>
</tbody>
</table>
Table 3.2. Comparisons of intestinal bacterial diversity between chickens and turkeys

<table>
<thead>
<tr>
<th>Source</th>
<th>Distance level</th>
<th># of OTUs Shared</th>
<th>$\Theta_{yc}$ (lci, hci)</th>
<th>UniFrac Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria</td>
<td>0.03</td>
<td>191</td>
<td>0.161 (0.130, 0.191)</td>
<td>0.598</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>180</td>
<td>0.187 (0.154, 0.220)</td>
<td>0.596</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>25</td>
<td>0.587 (0.544, 0.630)</td>
<td>0.721</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>0.03</td>
<td>14</td>
<td>0.085 (0.041, 0.129)</td>
<td>0.736</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>19</td>
<td>0.115 (0.064, 0.165)</td>
<td>0.754</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>4</td>
<td>0.631 (0.548, 0.714)</td>
<td>0.946</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>0.03</td>
<td>144</td>
<td>0.239 (0.188, 0.291)</td>
<td>0.567</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>131</td>
<td>0.307 (0.251, 0.364)</td>
<td>0.570</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>14</td>
<td>0.756 (0.711, 0.802)</td>
<td>0.630</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>0.03</td>
<td>13</td>
<td>0.416 (0.267, 0.564)</td>
<td>0.524</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>12</td>
<td>0.473 (0.309, 0.637)</td>
<td>0.611</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>6</td>
<td>0.762 (0.642, 0.881)</td>
<td>0.814</td>
</tr>
<tr>
<td>Cecal</td>
<td>0.03</td>
<td>91</td>
<td>0.081 (0.052, 0.111)</td>
<td>0.656</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>88</td>
<td>0.117 (0.082, 0.152)</td>
<td>0.690</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>20</td>
<td>0.374 (0.324, 0.424)</td>
<td>0.738</td>
</tr>
</tbody>
</table>

\[ \theta_{yc} = \frac{\sum_{i=1}^{S_{Tr}} a_i b_i}{\sum_{i=1}^{S_{Tr}} (a_i - b_i)^2 + \sum_{i=1}^{S_{Tr}} a_i b_i} \]  
(Yue and Clayon, 2005)

where, $S_{Tr}$ = the total number of OTUs in communities A and B;  
$a_i$ = the relative abundance of OTU i in community A;  
$b_i$ = the relative abundance of OTU i in community B.
Figure 3.1. Krona chart of the bacteria represented by 16S rRNA sequences recovered from chicken gut (from 3,030 of the 3,184 total chicken sequences).
Figure 3.2. Krona chart of the bacteria represented by 16S rRNA sequences recovered from turkey gut (from 1,254 of the 1,345 total turkey sequences).
Figure 3.3. Krona chart of bacteria from chicken cecum (from 919 of the 972 chicken cecal sequences).
Figure 3.4. Krona chart of bacteria from turkey cecum (from 956 of the 958 turkey cecal sequences).
Figure 3.5. Distribution bacterial genera identified in the cecal microbiomes of chicken and turkey. Red bars: chicken cecum; green bars: turkey cecum.
4.1 Abstract

We investigated the phylogenetic diversity of bacteria present in cecal content and ileal mucosa of chickens and turkeys. Amplicons of the V3 hypervariable region of the 16S rRNA gene was amplified from samples of cecal content and ileal mucosa and sequenced by the 454 pyrosequencing GS FLX system. The objectives of this study were to uncover the major bacteria present in the intestinal microbiome of each bird species and to investigate the variations between two independent pyrosequencing runs and two data processing pipelines. The 338,177 sequences analysed represented on average 3,401 and 125 operational taxonomy units (OTUs, defined at a 0.03 phylogenetic distance) in chicken cecal content and ileal mucosa, respectively, as well as 1,687 and 16 OTUs in turkey cecal content and ileal mucosa, respectively. Good’s coverage analysis indicated that the sequences from each intestinal location reached a high coverage (> 95%) of bacterial diversity, except for turkey ileal mucosa. Besides bacteria that have been identified or reported in the cecum of chickens and turkeys, 39 additional genera of bacteria were identified in the chicken cecal microbiome, including *Butyricimonas*, *Odoribacter*, *Hydrogenoanaerobacterium*, *Moryella*, *Parasporobacterium*, and *Ruminococcus*, and 50 genera of bacteria were detected in turkey cecal microbiome,
including *Gemmiger, Olsenella, Moryella, Bilophila, Hydrogenoanaerobacterium, Akkermansia, Collinsella, Staphylococcus, Ruminococcus, Slackia, and Sporacetigenium*. Noticeable variations were observed in the number of OTUs between different pyrosequencing runs and data processing pipelines. Comparison between the two bird species revealed distinct intestinal microbiome associated with each bird species. However, the microbiome of the same gut origin of the two bird species shared greater community similarity than the microbiomes of different gut origins within each bird species. Identifying most of the bacterial diversity of broiler chickens and turkeys, this study provided a detailed perspective of the intestinal microbiome of these two importance poultry species. The data may also be useful to the development of other analytical tools, such as phylochips.

4.2 Introduction

Poultry gastrointestinal track harbors a dynamic microbial community consisting of a huge number species, primarily bacteria. This community is believed to be pivotal to host health and growth performance. Microorganisms in gastrointestinal tracts can be roughly grouped into pathogenic or commensal groups (Yegani and Korver, 2008). Pathogenic bacteria damage the host by causing localized or systemic infections, intestinal putrefaction, and toxin production (Jeurissen et al., 2002). Commensal bacteria can benefit the host by providing nutrients, metabolism facilitation, and competitive exclusion (Wagner 2006; Rehman et al., 2007). Knowledge of bacterial composition and activity as well as understanding the working mechanism of indigenous bacteria in
modulating the intestinal environment are required to improve host health and feed utilization.

Started many decades ago, studies on poultry intestinal microbiome relied on cultivation and characterization of intestinal bacteria. During the past two decades, however, 16S rRNA gene has been used as the primary biomarker for bacterial identification in various environments, including poultry gut, to overcome the limitation of culture-dependent methodologies, allowing identification of unculturable bacteria. Studies using individual 16S rRNA gene clone libraries provided insights into the diverse gut microbiome of poultry gut, but these studies were restricted by the relatively small numbers of sequences that can be afforded by the researchers. Consequently, a comprehensive perspective on the diversity and community composition of poultry intestinal bacterial community is beyond the reach of studies using traditional 16S rRNA gene clone libraries.

High-throughput next-generation sequencing technologies have been proven to be a powerful tool in comprehensive analysis of complex microbiomes (Krause et al., 2008; Petrosino et al., 2009). Pyrosequencing can generate large amounts of sequencing data at a low cost. It also allows sequencing of environmental DNA without a prior cloning step, eliminating cloning bias (Edwards et al., 2006; Turnbaugh et al., 2006). The unprecedented sequencing capacity support identification of bacteria that are present in low abundance in a microbiome. The 454 pyrosequencing technology has been widely applied to analysing intestinal microbiomes of human and animals and as well as
environmental microbiomes (Zhang et al., 2009; Kirchman et al., 2010; Larsen et al., 2010; Fierer et al., 2011). There are only a few studies, however, that focused on comprehensive studies on the intestinal microbiomes of chickens and turkeys to date (Qu et al., 2008; Callaway et al., 2009; Lee et al., 2010; Stanley et al., 2012b; Stanley et al., 2012a). In addition, these studies only reported a relatively small number of sequences per samples (less than 10,000 reads), resulting in low coverage and incomplete knowledge on the diversity of poultry intestinal microbiomes.

Despite the fact that 454 pyrosequencing can produce large datasets, it has been recognized that this technology also has its inherent limitations, including sequencing errors, artifactual sequences, chimeric sequences (add two refs. Probably move a few from the next sentence). In addition, different pipelines used in analysing 454 sequences use different algorithms in sequence alignment, distance computation, and OTU clustering, which can lead to different results (Huse et al., 2007; Kunin et al., 2010; Haas et al., 2011a; Quince et al., 2011; Schloss et al., 2011), including alien estimation of OTUs (Schloss, 2010). Moreover, little attention has been given to the repeatability of the technology in different pyrosequencing runs. Thus, the objective of this study was to reveal the major composition of the intestinal bacterial communities in chicken and turkey using 454 pyrosequencing technique, with a secondary objective to evaluate the repeatability of the technique and the effects of different data processing pipeline.
4.3 Methods and materials

4.3.1 Samples collection

Five broiler chickens were randomly chosen from each of three flocks (n=15) at six weeks of age and eight turkeys were chosen from one flock (n=8) at 14 weeks of age. The samples were collected at the Poultry Research Farm located at the Ohio Agricultural Research and Development Center (OARDC), Wooster, OH. The chicks and turkeys were fed standard corn-soybean-meal-based diets that contained NRC (1994) suggested nutrient levels. Cecal content was collected from each bird and pooled by species. Ileal mucosa samples were collected from the region between Meckel’s diverticulum and the ileocecal junction from each bird and pooled based on species as described by Cressman et al., (2010). Each composite sample was mixed to represent each species and location.

4.3.2 DNA extraction and PCR reaction

Community DNA was extracted from each of the four composite samples (2 bird species (chicken vs. turkey) x 2 locations (ileal mucosa vs. cecal content)) using the repeated bead beating plus column purification method (Yu and Morrison, 2004a). The V3 region of 16S rRNA gene in the metagenomic DNA was amplified with barcoded universal primer sets as listed in Table 4.1. Every forward primer consists of three parts: a 19-nt degenerated primer (357F), a 10-nt barcode, and the pyrosequencing adapter A. The reverse primer consisted of an 18-nt degenerated primer (519R) and a 19-nt
pyrosequencing adapter B. The degenerated primers were modified so that they were more inclusive towards the sequences in the RDP database.

For each PCR reaction, 400 ng of metagenomic DNA template was added to a 49 µl master mix that contained 1x PCR buffer, 1.75 mM MgCl₂, 670 ng/µl bovine serum albumin, 200 µM dNTP, 500 nM of each primer, and 0.625 U Platinum Taq high-fidelity polymerase (Invitrogen Corporation, Carlsbad, CA). The PCR thermal program consisted of an initial denaturation at 95°C for 10 min; 20 to 25 cycles (20 cycles for cecal content samples and 25 cycles for ileal mucosa samples) of a 30s denaturation step at 95°C, a 35s annealing step at 55°C, and a 35s elongation step at 72°C; and a final extension step at 72°C for 7 min, before a 4°C hold.

The quality of the PCR product was examined with agarose gel (1.2%) electrophoresis, and the expected PCR products approximately 200bp were gel purified using a Qiagen Gel Purification Kit (Qiagen, Valencia, CA, USA). The concentration of the purified products was quantified using a Quant-it Kit (Invitrogen Corporation, Carlsbad, CA, USA) and confirmed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

4.3.3 Pyrosequencing and data analysis

Given the expected higher diversity in the cecal samples than in the ileal mucosa samples, the amplicons from the former and the latter were mixed in 9:1 ratio for each
bird species. Then the amplicons from the chickens and turkeys were pooled in a 2:1 ratio. The pooled amplicon samples were divided and sequenced in two independent pyrosequencing runs on one half of a picotiter plate each using a 454 Life Sciences Genome Sequencer FLX machine (Roche, Basel, Switzerland) at University of Illinois at Urbana-Champaign facility. The raw data were provided to us as sff files.

The quality of the 454 pyrosequencing data was evaluated using the raw sff data files following the standardized operating procedure (SOP) proposed by Schloss et al. (2011). Briefly, the flow file was generated from the sff file of each sample using the sffinfo program of the GS Analysis Software (Version 2.5, 454 Life Sciences Corporation, Branford, CT, USA). The pyrosequencing noise of each flow file was removed by the AmpliconNoise function implemented in mothur (Quince et al., 2009; Schloss et al., 2009b; Quince et al., 2011; Schloss et al., 2011). The denoised sequences were trimmed off the primer 357F and 519R, which results in sequences of the V3 hypervariable region of 16S rRNA genes (minimum length, 100bp). The trimmed sequences were aligned using the mothur aligner (Schloss et al., 2009a; Schloss et al., 2009b) with the Silva_SSU_Ref_NR_108 dataset (Ludwig et al., 2004) as reference sequences, with a -4 score penalty for gapopen and -3 score penalty for mismatch. Sequences that could not be aligned due to short overlap with the Silva reference dataset were removed. The common gaps in the sequence alignment were filtered out, and the sequences were preclustered to remove sequences that contain possible pyrosequencing errors (Huse et al., 2010; Schloss et al., 2011). Possible chimeric sequences were identified using
UCHIME implemented in the mothur package (Schloss et al., 2009b; Edgar et al., 2011; Schloss et al., 2011).

A distance matrix of each dataset was computed using the ARB database environment with the Jukes-Cantor correction (Ludwig et al., 2004) applied. The mothur and USEARCH were used to cluster sequences into OTUs at 0.03, 0.05, and 0.20 phylogenetic distances, generate rarefaction curves, and determine the nonparametric ACE and Chao1 estimates of maximum richness from each of the distance matrices. The maximum number of OTUs likely present in each of the samples was also estimated using the non-linear models procedure (PROC NLIN) of SAS (V9.2, SAS Inst. Inc., Cary, NC), which fits the monomolecular function to the rarefaction output to determine the asymptote that serves as the upper bound of the curves as previously described (Larue, et al., 2005). Each distance matrix was computed 3 times and the median was chosen in calculating these indices to avoid under- or over-estimation.

A representative sequence of each OTU0.03 defined at 0.03 genetic distances was obtained using the “get.oturep” command in the mothur package V1.22 (Schloss et al., 2009b). These OTU0.03 representative sequences were imported into ARB, and a phylogenetic tree was constructed for each sample by inserting each sequence into the reference tree of the 286,858 Silva reference sequences (SSU_111_Ref_NR, http://www.arb-silva.de/) as described previously (Wei et al., 2013a). The phylogenetic tree was then used for weighted UniFrac analysis as described in library comparison below. The sequences used
in this study are maintained in an in-house ARB database dedicated to the intestinal microbiome of chickens and turkeys and is available upon request. The representative sequences of each sample were also archived in the MG-RAST server under the project of Poultry_MID_DB (4508915.3 to 4508920.3). The OTU0.03s were classified using the RDP classifier (Wang et al., 2007) and the composition of each microbiome was visualized as a taxonomy tree constructed using MEGAN (Huson et al., 2011).

The intestinal microbiomes of chickens and turkeys were compared using 4 different methods: weighted UniFrac distance, which measures the phylogenetic distance between sets of taxa as phylogenetic trees (Lozupone and Knight, 2005; Lemos, et al., 2012); the SONS function in the mothur package, which compares two microbiomes by taking into consideration of OTU richness, membership, and structure (Schloss and Handelsman, 2006; Schloss, et al., 2009); RDP library comparison, which compares 2 libraries side by side based on the represented taxa and computes the likelihood that the frequency of membership in a given taxon is the same (Cole et al., 2009); and MEGAN phylogenetic tree, which allows comparison between microbiomes based on detailed phylogenetic composition (Huson et al., 2011).

The numbers of raw sequence reads and numbers of quality-checked sequences were compared between the two independent pyrosequencing runs to assess the variations between them. The quality-checked sequences were aligned using the Silva_bacteria provided on mothur’s webpage (http://www.mothur.org/) as references by the RDP Pyro
aligner (http://pyro.cme.msu.edu/) and the mothur aligner to assess the effect of different aligners on OTUs clustering. One distance matrix was computed for each alignment with the Jukes-Cantor correction applied, and OTUs_{0.03} were clustered using mothur.

4.4 Results and Discussion

4.4.1 Overview of the 454 pyrosequencing results and the variation

In total, 402,247 DNA sequence reads were obtained, of which 338,177 were successfully assigned to the corresponding samples based on the barcode information (Table 4.2). The data from the two pyrosequencing runs did not differ significantly in terms of numbers of raw sequences or sequences resulted from the denoising step. However, after the preclustering step, which was designed to reduce the effect of pyrosequencing errors (Huse et al., 2010), the second run produced much fewer sequence reads than the first run, about 55% less raw sequence reads and 82% less denoised sequences. These results suggest that significant variations in sequencing quality and numbers of useable sequences can result from the same amplicon library and the same pyrosequencing system.

Both the RDP Pyro aligner and the mothur aligner align 16S rRNA sequences based on their secondary structure and can align a massive amount of sequences in a short time (Cole et al., 2009; Nawrocki et al., 2009; Schloss et al., 2009a; Schloss, 2010). The RDP Pyro aligner does not align the hypervariable regions while the mothur aligner aligns the 16S rRNA sequences using the Silva alignment as reference sequences. Both aligners
have been commonly used in analysis of pyrosequencing data. So the two aligners were evaluated using the same dataset. The mothur aligner resulted in more (about twice) OTUs when the default alignment setting was used than when penalties were given to gapopen and mismatch. The RDP aligner yielded more than 3 folds more OTUs than the mothur aligner when the penalty options applied. The RDP aligner has been reported to result in more OTUs from all but the hypervariable regions except the V3 and V4 of 16S rRNA genes when compared to the Silva aligner (Schloss et al., 2011). However, in this study, the V3 region was used and more OTUs also resulted from the RDP Pyro aligner. In addition, even though the SOP of mothur includes several quality screening procedures, the default setting may not be the best setting for every dataset. As demonstrated in this study, the introduction of penalties for gapopen and mismatch can significantly reduce the risk to overestimate diversity. It is recommended that researchers test more than one setting in each step to avoid inflation of diversity. Hence, the mothur aligned sequences with penalties for gapopen and mismatch were used in assessing the effect of different clustering algorithms on OTUs clustering.

Species richness of metagenomic data is typically expressed as numbers of OTUs at different phylogenetic distances. In this study, mothur and USEARCH, both of which are commonly used in clustering OTUs (Schloss et al., 2009b; Caporaso et al., 2010; Edgar, 2010), were compared using the same datasets of all the for microbiome samples. The USEARCH method generated twice as many OTUs than mothur (Table 4.2). These results suggest that different clustering methods can produce different species richness.
Comparisons between different studies on the same types of microbiomes, especially those that used different clustering and alignment methods, should be done with caution. Additionally, different phylogenetic distances might be needed when different clustering methods are used to produce comparable species richness. In this study, the mothur was used to cluster OTUs from all the four datasets.

4.4.2 Bacterial diversity of chicken cecal content microbiome

The cecum is the main reservoir of bacteria in poultry. The first and the second pyrosequencing runs produced 3,973 and 2,829 OTUs0.03, respectively (Table 4.2). The estimated asymptotes of OTU0.03 reached 1.5-fold of the number of observed OTU0.03s. Both the pyrosequencing runs achieved a high level of coverage (Good’s coverage > 95%) of the bacteria diversity in the chicken cecal microbiome. The RDP classification of OTU0.03s from both pyrosequencing runs were combined and imported into MEGAN to generate a taxonomy tree of the major bacteria (Fig. 4.1). In total, we identified 9 identified bacterial phyla (Firmicutes, Proteobacteria, Bacteroides, Synergistetes, Fusobacteria, Actinobacteria, Deferrribacteres, Tenericutes, and Lentisphaerae) and 84 known genera (data not shown). The Firmicutes was the most predominant phylum, accounting for 57.8% of the total bacterial sequences. Within this phylum, 30.9% of the OTU0.03s could not be classified to any known taxa. The Bacteroidetes and Proteobacteria were less predominant, accounting for 5.4% and 4.3% of the total bacteria sequences, respectively. No significant difference was observed at phylum or class level between the two pyrosequencing runs except for the class
Gammaproteobacteria (P<0.01). However, the major difference surfaced in unclassified Enterobacteriaceae. In the first pyrosequencing run, Bifidobacteriaceae, Bacillaceae, Staphylococaceae, Carnobacteriaceae, Enterococaceae, Hydrogenoanaerobacterium, and Synergistaceae were found to be represented by more than 5 OTUs, while Selenomonadales was the only taxon or group that was represented by >5 OTUs. These results indicate that significant variations in diversity results could arise from different runs, and quantitative interpretation of pyrosequencing data should be exercised with caution.

A recent study established a global diversity framework of the poultry intestinal microbiome by using a naive analysis of 16S rRNA gene sequences of poultry gut bacteria that have been recovered worldwide (Wei et al., 2013a). When compared to the global cecal bacterial diversity, 29 genera were missing from this pyrosequencing study. These include Salmonella, Megamonas, Subdoligranulum, and Paraprevotella, each of which was presented by more than 10 sequences in the public databases. On the other hand, this pyrosequencing study identified 39 bacteria genera that were not represented in the global cecal bacterial diversity, including Butyrivibrio, Odoribacter, Hydrogenoanaerobacterium, Moryella, Parasporobacterium, Ruminococcus, and many minor genera represented by less than 5 OTUs (data not shown). Overall, this study expanded the number of bacterial genera identified in the cecum of chickens by 80%. This is likely attributed to the increased sequencing depth we intentionally achieved.
Most of these “new” genera found in the cecum might be present at low abundance. Future studies can further elucidate their functions and importance to the host.

4.4.3 Bacterial diversity of chicken ileal mucosa microbiome

Few pyrosequencing studies on the chicken ileal mucosa microbiome have been reported. In this study, more than 5,000 sequences were obtained from each of the two pyrosequencing runs, resulting in 135 and 114 OTUs from the first and second runs, respectively (Table 4.2). Although the Good’s coverage also reached > 95%, the estimated asymptotes of OTUs were up to 1.9 folds of the observed numbers of OTUs. Thus, the diversity in the ileal mucosa has not been completely sampled. It should be noted that about half of the original sequencing reads from the ileal mucosa appeared to be 18S rRNA genes of the host. The presence of these host sequences was probably due to the broad specificity of primers 357f and 519r, both of which can anneal to 18S rRNA genes (Weisburg et al., 1991).

The OTUs from chicken ileal mucosa were classified into 7 bacterial phyla: Actinobacteria, Bacteroidetes, Cyanobacteria/Chloroplast, Firmicutes, Proteobacteria, Synergistetes, and TM7). Firmicutes and Proteobacteria were the major phyla, accounting for 72.6% and 11.1% of total sequences, respectively (Fig. 4.2). Significant differences between the two pyrosequencing runs were not observed at taxonomic ranks from phylum to genus. However, some minor groups were only identified in one of the two pyrosequencing runs. For example, Bacteroides, Enterococcaceae, Lachnospiraceae,
and *Gammaproteobacteria* were only identified in the first run, and each of them was represented by at least 5 OTU0.03s, whereas *Prevotella, Salinicoccus*, and another 10 genera, each of which was represented by one or two OTU0.03s, were only identified in the second run. *Lactobacillus* was the largest genus in the chicken ileal mucosa, accounting for 11% of the total sequences. In a previous study using 16S rRNA gene clone libraries, *Lactobacillus* was found to account for 75% (63/84) of the ileal mucosa bacterial sequences among 7-day-old chicks (Cressman et al., 2010). The differences in bird age and methodologies used might explain the discrepancy in *Lactobacillus* predominance witnessed in the ileal mucosa. About 99% of the bacteria identified in jejunal microbiome by pyrosequencing were classified as *Lactobacilli* in Australia (Stanley et al., 2012b). The greater predominance of *Lactobacillus* was expected for the jejunum than for the ileum. The PH of different gut section may play an important role in shaping the bacterial communities. The pH of the chicken intestinal tract contents increases from 4.5 in the crop, to 5.7–6.0 in the duodenum/jejunum, to 6.3–6.4 in the ileum/rectum, and up to pH 7.0 or higher in the cecum (Denbow, 1999; Siragusa et al., 2008). In the upper part of the chicken gastrointestinal tract, only the *Lactobacilli* can tolerate the acidic environment; the PH in ileum is in favor of a broader range of bacteria.

4.4.4 Bacterial diversity of turkey cecal content microbiome

In total, 1891 and 1481 OTU0.03s were obtained from the first and second pyrosequencing runs, respectively (Table 4.2). Similar to the chicken cecal microbiome,
the estimated asymptotes of OTU0.03s were about 1.5-fold of the number of observed OTU0.03s, while the Good’s coverage reached > 95%. The OTU0.03s were classified into 8 bacterial phyla (Firmicutes, Bacteroides, Actinobacteria, Proteobacteria, Verrucomicrobia, Synergistetes, Elusimicrobia, and Lentisphaerae) and 85 known genera (data not shown). Phyla represented by more than 5 OTU0.03s were shown on the taxonomy tree (Fig. 4.3). Firmicutes, Proteobacteria, and Actinobacteria were the most predominant phyla in the turkey intestinal microbiome, accounting for 66.3%, 7.4%, and 3.2% of the total bacterial sequences. A significant difference was observed between the two pyrosequencing trials in phyla Firmicutes and Proteobacteria according to RDP Library comparison. In total, there were 17 genera or groups that were recovered only in the first sequencing run, including Olsenella, Clostridiaceae, Roseburia, Hydrogenoanaerobacterium, Selenomonadales, Bilophila, Enterobacteriaceae, and other groups, each of which was represented by less than 5 OTU0.03s (Fig. 4.3). On the other hand, 14 genera or groups were identified only in the second sequencing run, including Porphyromonadaceae and other minor genera. Interestingly, only one OTU0.03 was classified as Escherichia/Shigella, a common genus of enteric bacteria. This might be caused by its low abundance in the turkey cecal microbiome. Quantitative PCR analysis on this OTU0.03 may help confirm its abundance in the turkey cecal microbiome.

The bacterial profile of the turkey cecal microbiome was also compared to the global bacterial diversity framework of poultry (Wei et al., 2013). Twenty one genera, including Megamonas, Prevotella Paraprevotella, Subdoligranulum, Hallella,
Phascolarctobacterium and minor genera representing less than 10 sequences, were not detected by the current pyrosequencing study. This lack of these genera or groups might be explained by the sampling of only one flock of turkey in the present study. On the other hand, the current pyrosequencing study uncovered 50 bacterial genera that were not represented in the global dataset. The major genera include Gemmiger, Olsenella, Moryella, Bilophila, Hydrogenoanaerobacterium, Akkermansia, Collinsella, Staphylococcus, Ruminococcus, Slackia, Sporacetigenium, and a number of genera represented by less than 5 OTU0.03s. Future studies are needed to further understand the importance and contribution of these new genera to host health and nutrient utilization. It should be noted, however, some of the identified bacterial genera contain food-borne pathogens, such as Bilophila, which is associated with several infections such as perforated and gangrenous appendicitis (Baron, 1997). Thus, deep pyrosequencing analysis not only support better understanding of the turkey cecal microbiome and developing novel inventions to modulate microbiome, but also provides opportunities to identify potential risk factors which have been overlooked. Future studies are needed to understand the factors that govern the populations of these pathogens.

4.4.5 Bacterial diversity of turkey ileal mucosa microbiome

This study is the first reported investigation of the turkey ileal mucosa microbiome using the pyrosequencing technique. Most of the sequencing reads turned to be host sequences rather than bacterial 16S rRNA genes (Table 4.2). This is likely attributed to the low proportion of bacterial DNA in the DNA extract and possibly low PCR cycles. (25
cycles). The bacteria population of ileal mucosa was reported to be about 1% of that of the cecal content (\(10^8\text{ - }10^9\) copies/g of sample vs. \(10^{10}\text{ - }10^{11}\) copies/g of sample) (Ahmed et al., 2007; Yeoman et al., 2012). It was more difficult to recover the bacterial diversity using low-cycle-number PCR, which was designed to eliminate the sequence error introduced by PCR amplification. The experimental condition need to be optimized to reach a delicate balance between the sequence error and the PCR product yield of mucosal samples in the future. In total, only about 30 bacterial 16S rRNA gene sequences were obtained from each of the two pyrosequencing runs, resulting in less than 20 OTUs0.03 (Table 4.2). The recovered OTU0.03s was classified as the phyla Firmicutes, Proteobacteria, and Bacteroidetes, representing 59.3%, 25.0% and 6.3% of the total bacterial sequences (Fig. 4.4). In total 12 genera of bacteria were found in the ileal mucosa microbiome of the turkeys. No significant difference between the two pyrosequencing runs was observed due to the small datasets, but 7 genera were identified by the first pyrosequencing run while only 3 genera were identified by the second sequencing run.

Except Lactobacillus, all the identified genera were represented by only one OTU0.03. Lactobacillus and Alistipes were found in both the pyro sequencing runs. The coverage of bacteria diversity of turkey ileal mucosa microbiome was far from complete, and the bacterial diversity of turkey ileal mucosa microbiome was not discussed further in this study. Future studies need to maximize amplification of bacterial 16S rRNA genes while reducing amplification of host DNA.
Comparisons between chicken and turkey microbiome

The OTU0.03s from both the pyrosequencing runs were combined for each of the four microbiomes and compared. Overall, the intestinal microbiomes of chickens and turkeys were significantly different when compared using UniFrac significance analysis (p < 0.01, data not shown). When compared with respect to OTU richness, membership, and structure using the SONS within the mothur program, these two intestinal microbiomes were also distinct, only sharing 18.6% Yue and Clayton index (θyc similarity) (Yue and Clayton, 2005) at 0.03 distance level and 60.0% at 0.20 distance level (Table 4.3). As expected, greater community similarities were shared at higher phylogenetic distances.

The weighted UniFrac distances computed from the OTU representatives were used to access the structure of poultry intestinal microbiomes (Table 4.3). Weighted UniFrac distance was chosen over OTU-based approaches because the latter lacks the resolution to detect overlapping species between microbiomes when datasets with low sequence coverage were used, while the former is a reliable index when comparing sequencing datasets with varied sample sizes (Lemos et al., 2012). Consistent with the SONS analysis, the weighted UniFrac distances indicated that microbiomes from the same gut section of the two bird species (i.e., chicken cecal digesta, CD vs. turkey cecal digesta, TD) shared a greater similarity in phylogenetic structure than the microbiomes from the different locations of the same host (i.e., CD vs. chicken ileal mucosa, CM).

Differences in major genera were evident between the intestinal microbiomes of the two bird species. The distribution and relative abundance of the major bacterial genera in the
cecum differed between the chickens and turkeys (Fig. A2). Although the cecal microbiomes of both bird species shared the major phyla (Actinobacteria, Bacteriodetes, Firmicutes, and Proteobacteria), significant difference was revealed in Firmicutes, Actinobacteria, and Bacteriodetes by RDP library comparison (data not shown). Fusobacteria and Deferrribacteres were minor phyla only identified in the chicken cecal microbiome, while Verrucomicrobia and Elusimicrobia were minor phyla only identified in the turkey cecal microbiome. At genus level, Barnesiella and Odoribacter were significantly more diverse in the chicken cecal microbiome while the opposite was observed for Olsenella and Rikenella. In total, there were 22 genera which were only identified in chicken cecal microbiome including Mucispirillum and Phascolarctobacterium, each of which represented more than 5 OTU0.03s. Another 23 genera were only identified in the turkey cecal microbiome, including Olsenella, Akkermansia, Sporacetigenium, and several other genera, each of which was represented by > 5 OTU0.03s.

As discussed in our earlier study (Wei et al., 2013a), differences in host (genetics, breeds, anatomical features of gut, physiology, etc.) and diets might be attributable to the difference the distinct cecal microbiome of chicken and turkey. For example, turkeys have a larger intestinal diameter, a more viscous digesta, and a slower digesta passage rate (i.e. longer retention time) than chickens (Palander et al., 2010). These factors may lead to lower partial O₂ pressure and redox potential in the gut of turkeys than in the gut of chickens. From this point of view, it is interesting to see the distinct distribution of
Actinobacteria in turkey and chicken cecum. The major genera of Actinobacteria in the turkeys included Micrococcineae and Coriobacteriaceae (Yassin et al., 2011), which help to decompose food residues, while Bifidobacteriaceae was the predominant genus of Actinobacteria in the chicken cecal microbiome. It remains to be determined if the distinct microbiomes observed in this study are common among other broiler and turkey flocks, and to what extent these distinct intestinal microbiomes differ in contributions to host health and nutrient utilization.

4.5 Conclusions

Microbiome profiling by pyrosequencing analysis can be biased due to variations among runs and different pipeline programs used to process the sequencing data. By deep sequencing, a large number of genera and OTUs were found in the cecum and ileal mucosa of broiler chickens and turkeys, expanding our knowledge on the intestinal microbiome of these two bird species, especially when they are reared under the dietary and managerial conditions common in the North America. Some of the bacterial groups unique to each bird species might be important to host health and performance. A comprehensive knowledge of poultry intestinal microbiome and the differences between bird species may be useful in modulating gut microbiome to improve host health and growth performance.
4.6 Acknowledgement

This study was partially supported by an AFRI grant (award number: 2008-35204-18845).
### Table 4.1. Barcoded degeneracy primers for V3 amplicons

<table>
<thead>
<tr>
<th>Forward</th>
<th>B-A-D16-357F:</th>
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<tr>
<td></td>
<td>GCCTCCCTCGCGCCATCAGACGCTCGACACWYCTACGGRDGGC</td>
</tr>
<tr>
<td></td>
<td>WGCAG</td>
</tr>
<tr>
<td></td>
<td>C-A-D16-357F:</td>
</tr>
<tr>
<td></td>
<td>GCCTCCCTCGCCATCAGAGACGCACTCCWYCTACGGRDGGC</td>
</tr>
<tr>
<td></td>
<td>WGCAG</td>
</tr>
<tr>
<td></td>
<td>D-A-D16-357F:</td>
</tr>
<tr>
<td></td>
<td>GCCTCCCTCGCGCCATCAGAGCACTGTAGCWYCTACGGRDGGC</td>
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<td>WGCAG</td>
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<tr>
<td></td>
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<tr>
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<td>WGCAG</td>
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<tr>
<td>Reverse</td>
<td>B-D4-519R:</td>
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<td></td>
<td>GCCTTGCCAGCCCGCTCAGGTNTTACCGCGGCTGCTG</td>
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Table 4.2. Summary of the 454 pyrosequencing data.

<table>
<thead>
<tr>
<th>Sample</th>
<th># of raw seqs assigned</th>
<th># of sequences after screening</th>
<th># of preclustered sequences after screening</th>
<th>Observed OTU0.03s</th>
<th>Maximum # of OTUs</th>
<th>Good's coverage**</th>
</tr>
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<tbody>
<tr>
<td>CD-1</td>
<td>98021</td>
<td>91456</td>
<td>21080</td>
<td>3973</td>
<td>8899</td>
<td>6001</td>
</tr>
<tr>
<td>CD-2</td>
<td>98238</td>
<td>91227</td>
<td>12437</td>
<td>2829</td>
<td>5162</td>
<td>4278</td>
</tr>
<tr>
<td>CM-1</td>
<td>5457</td>
<td>2714</td>
<td>598</td>
<td>135</td>
<td>324</td>
<td>259</td>
</tr>
<tr>
<td>CM-2</td>
<td>5273</td>
<td>2506</td>
<td>330</td>
<td>114</td>
<td>199</td>
<td>205</td>
</tr>
<tr>
<td>TD-1</td>
<td>56959</td>
<td>53527</td>
<td>10304</td>
<td>1891</td>
<td>4252</td>
<td>2779</td>
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<tr>
<td>TD-2</td>
<td>57442</td>
<td>49836</td>
<td>6685</td>
<td>1484</td>
<td>2706</td>
<td>2188</td>
</tr>
<tr>
<td>TM-1</td>
<td>8104</td>
<td>30</td>
<td>23</td>
<td>17</td>
<td>19</td>
<td>63</td>
</tr>
<tr>
<td>TM-2</td>
<td>8683</td>
<td>33</td>
<td>19</td>
<td>15</td>
<td>16</td>
<td>19</td>
</tr>
</tbody>
</table>

CD, chicken cecal digesta; CM, chicken ileal mucosa; TD, turkey cecal digesta; TM, turkey ileal mucosa.

* estimated by \( \text{Number of phyotypes} = a \left(1 - \beta \times e^{-\kappa \times n} \right) \)

** estimated by \( \text{Coverage of diversity} = (n - N) / n \times 100\% \).
Table 4.3. Comparisons of intestinal bacterial diversity between chickens and turkeys.

<table>
<thead>
<tr>
<th>Source</th>
<th>Distance level</th>
<th># of OTUs Shared</th>
<th>øyc(^b) (lci,hci)</th>
<th>UniFrac Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD vs CM</td>
<td>0.03</td>
<td>74</td>
<td>0.030 (0.023,0.038)</td>
<td>0.627</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>76</td>
<td>0.052 (0.034, 0.068)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>31</td>
<td>0.280 (0.211, 0.350)</td>
<td></td>
</tr>
<tr>
<td>CD vs TD</td>
<td>0.03</td>
<td>743</td>
<td>0.186 (0.170, 0.202)</td>
<td>0.364</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>758</td>
<td>0.332 (0.303, 0.358)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>69</td>
<td>0.600 (0.564, 0.630)</td>
<td></td>
</tr>
<tr>
<td>CM vs TM</td>
<td>0.03</td>
<td>11</td>
<td>0.076 (0.025, 0.127)</td>
<td>0.517</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>13</td>
<td>0.149 (0.091, 0.207)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>13</td>
<td>0.568 (0.267, 0.870)</td>
<td></td>
</tr>
</tbody>
</table>

\[ øy_{yc}^{b} = \frac{\sum_{i=1}^{S_T} a_i b_i}{\sum_{i=1}^{S_T} (a_i - b_i)^2 + \sum_{i=1}^{S_T} a_i b_i} \]  
(Yue and Clayon, 2005)

where,

- \( S_T \) = the total number of OTUs in communities A and B;
- \( a_i \) = the relative abundance of OTU \( i \) in community A;
- \( b_i \) = the relative abundance of OTU \( i \) in community B.
Figure 4.1. Bacteria diversity of chicken cecal content microbiome. Genera represented by > 5 OTU0.03s were displayed and the size of each node reflects the total number of OTUs. The relative proportion of the OTU0.03s from the two pyrosequencing runs was shown by different colors: red, OTU0.03s from the first run; blue, OTU0.03s from the second run.
Figure 4.2. Bacterial diversity of chicken ileal mucosa microbiome. Genera represented by > 5 OTU0.03s were displayed and the size of each node reflects the total number of OTUs. The relative proportion of the OTU0.03s from the two pyrosequencing runs were distinguished by color: red, OTU0.03s from the first run; blue, OTU0.03s from the second run.
Figure 4.3. Bacteria diversity of turkey cecal content microbiome. Genera represented by > 5 OTU0.03s were displayed and the size of each node reflects the total number of OTUs. The relative proportion of the OTU0.03s from the two pyrosequencing runs were distinguished by color: red, OTU0.03s from the first run; blue, OTU0.03s from the second run.
Figure 4.4. Bacteria diversity of turkey ileal mucosa microbiome. The size of each node reflects the total number of OTUs. The relative proportion of the OTU0.03s from the two pyrosequencing runs were distinguished by color: red, OTU0.03s from the first run; blue, OTU0.03s from the second run.
Chapter 5: Development and application of a poultry intestinal tract chip (PITChip), a phylogenetic microarray, for analysis of poultry intestinal microbiomes as affected by litter management

5.1 Abstract

In this study we developed a phylogenetic microarray and tested its utility in comprehensive analysis of the intestinal microbiomes of broiler chickens. This microarray, referred as the poultry intestinal tract chip (PITChip), was designed based on the global diversity of the intestinal microbiome of poultry (both turkeys and chickens), which was depicted by about 3000 16S ribosomal RNA gene sequences (rrs) of poultry origin archived in the RDP database and > 400,000 pyrosequencing reads we generated from both broiler chickens and turkeys. The PITChip allows simultaneous detection and semi-quantification of 1848 operational taxonomic units (OTUs) of bacteria and 105 bacterial genera. These PITChip was evaluated for probe specificity, detection limits and linear dynamic range using 2 sets of rrs clones of chicken intestinal microbiome. On average, the PITChip had a detection limit of $1.8 \times 10^6$ copies and a linear dynamic range from $1.8 \times 10^6$ to $1.8 \times 10^9$ copies of a target per hybridization reaction. The PITChip was used to analyze six chicken intestinal microbiomes that had been analyzed previously using rrs clone libraries to examine the relationship between the litter conditions (fresh vs. reused) and the microbiomes of ileal mucosa and cecal content in broiler chickens at
ages of 7, 14, 21, and 42 days. Collectively, *Anaerofilum*, *Porphyromonadaceae*, *Roseburia*, *Bifidobacteriaceae*, *Clostridales*, *Lachnospiraceae*, *Shigella*, *Coprococcus*, and *Salmonella* appeared to be the common and major groups of bacteria detected in the samples. Differences in microbiome structure and diversity were detected with respect to age and litter conditions. As expected, more bacteria were found in the cecal content with increasing age. Surprisingly, however, more bacteria were detected in the ileal mucosa of younger chicks compared with older birds. Compared to the fresh litter, the reused litter led to significant decreases in *Pseudomonas*, *Salmonella*, *Roseburia*, *Lachnospiraceae*, *Oribacterium*, *Ruminococcaceae*, *Shigella*, *Mollicutes*, and *Bacteroidetes*, but increases in *Clostridiales*, *Lactobacillaceae*, and *Barnesiella*. In summary, litter management can modulate the intestinal microbiome of broiler chickens and may have a profound effect on bird health and performance. The PITChip may be a useful tool in future studies to investigate the relationships between the intestinal microbiome and diet, feed additives, litter management, flock health, and performance.

5.2 Introduction

Chickens and turkeys ingest considerable amount of litter material, and consequently the conditions (both a biotic and biotic) can impact the health and growth of poultry birds primarily through effects on intestinal microbiome. Thus, litter management can be used to modulate intestinal microbiota of chickens to improve growth and health. However, the vast diversity of the intestinal microbiome hinders adequate understanding of how
litter conditions affect the intestinal microbiome. Moreover, the as-yet uncultivated status of most intestinal bacteria hinders comprehensive characterization and quantification of even the major bacteria in the intestinal microbiome (He et al., 2007). Our knowledge on the changes of the microbiome as affected by dietary and managerial interventions was limited to the quantities information of certain enteric pathogens of interest, such as *Salmonella* spp. and *Clostridium perfringens* (Wei et al., 2013b). A comprehensive understanding of the structure, functions, stability and adaptations of the intestinal microbiome is critical to assess how individual bacteria responses to interventions. Our primary objective in this study was to develop a habitat-specific phylogenetic chip dedicated to comprehensive analysis of poultry intestinal microbiome.

Microarray is a comprehensive analytical tool that can detect and semi-quantify thousands or more genes simultaneously (Bilitewski, 2009). Microarray was first developed to examine gene expression (Schena et al., 1995; Schena et al., 1996; Talla et al., 2003), but it has been rapidly adapted to other fields, such as pathogen detection and genotyping (Wang et al., 2002; Cai et al., 2005) and exploring the function and composition of microbial communities (Franke-Whittle et al., 2005; He et al., 2007). The throughput capability of microarray makes it a powerful analytical tool to identify bacteria of intestinal microbiome. Several host-specific phylotype microarrays, including human gut-specific microarrays, have been developed to monitor the global changes within the microbiome (Brodie et al., 2007; Palmer et al., 2007; Kang et al., 2010). When compared to the Next-Generation sequencing (NGS) technique, another popular high
throughput technique for microbiome studies (Margulies et al., 2005; Voelkerding et al., 2009; Barriuso et al., 2011), phylotype microarray is more advantageous by providing high-throughput semi-quantitation information on the microbiome (Paliy and Agans, 2012). Microarray-based genomic technologies have revolutionized our understanding towards biological systems (He et al., 2007).

In this study, we developed a poultry-specific phylotype microarray (referred to as PITChip) for comprehensive examination of the effects of litter management on the intestinal microbiomes of broiler chickens. After validation of probe specificity, detection limits and linear detection range, the PITChip was used in analysis of chicken intestinal microbiome as affected by litter management approaches. To our knowledge, PITChip is the first phylochip dedicated to analysis of the intestinal bacterial communities.

5.3 Results and discussion

5.3.1 PITChip probe design

From the RDP database, only 2,954 high-quality sequences of poultry gut origin were found. Of these sequences only 888 had the V3 region. These sequences were classified using the RDP Classifier (Wang et al., 2007) into 145 genera/groups, 105 of which are named genera. A consensus sequence was generated for each genus using ARB software (Ludwig et al., 2004). To develop a microarray that enables comprehensive analysis of the bacteria present in the intestinal microbiomes of both chickens and turkeys, a
pyrosequencing study (targeting the V3 hypervariable region in 16S rRNA gene only) was done on the cecal microbiomes of both broiler chickens and turkeys (Chapter 4). The above two datasets were combined, and 1,904 OTUs were clustered at 0.10 phylogenetic distance. Specific probes were designed for these OTUs. 29 specific genes of poultry pathogens and 31 genes of the virulence factors were introduced in PITChip. 19 of the pathogen-specific genes used for pathogen identification were not 16S rRNA genes and these probes for pathogen identification were only applicable when analysing metagenomics DNA or RNA directly without amplification. To allow the application of 16S rRNA gene based investigation of poultry intestinal microbiome, the corresponding 16S rRNA gene of 19 pathogens were retrieved from RDP database, despite that the documented strains were not recovered from poultry origins.

Probes designed from human mitochondrial sequence were used as negative controls on PITChip. Probe was designed based on Human mitochondrion cytochrome oxidase subunit II (COII), Asp-tRNA and Lys-tRNA genes and Homo sapiens mitochondrial mRNA for AD 1 specifically. On the other hand, probes designed from chicken mitochondrial sequence were used as positive controls on PITChip, based on the sequences of Gallus varius NADH dehydrogenase subunit 2 (ND2) gene and Gallus gallus 12S ribosomal RNA gene.

Finally, the 18S rRNA gene of *Eimeria tenella*, *Eimeria acervulina*, and *Eimeria maxima* were included for PITChip probe design. *Eimeria* spp. are coccidiosis-causing protozoa
residing in poultry gut that can cause reduction of body weight gain and intestinal lesions (Ryley, 1980; Martynova-Vankley et al., 2008). Similar to the case of genes for pathogen identification, the probes for *Eimeria* won’t be able to detect targets in 16S-rRNA-gene-based investigations.

Picky software (Chou et al., 2004) was primarily used for probe design for the 1904 phylotypes in this study. Picky allows rapid and efficient determination of gene-specific oligos based on the sequence and thermodynamic characteristics of given gene sets, and can be used for large, complex dataset (Chou et al., 2004; Chou, 2010). It was reported that Picky was one of the optimal software for probe design which performed best specificity estimation (Adebiyi, 2007; Lemoine et al., 2009). The thermodynamic calculation for target and non-target hybridization alongside the probe design also facilitate the optimization of the hybridization condition. Some factors are known to be important for the quality of a probe: specificity, sensitivity, G+C% of a probe, melting temperature, secondary structure, sequence repetition, composition of the first and last base (Reymond, 2004). Parameters of the Picky software was set to ensure specificity and sensitivity and the maximum Tm for a non-target hybridization was controlled below 45°C. Antisense short oligonucleotide (20-30bp) and medium oligonucleotide probes (30-50bp) were designed for the phylotypes to ensure of higher coverage.

Probes were designed for both consensus sequences and representative sequences of the 1904 phylotypes in poultry intestinal microbiome. In total 1932 PITChip probe were
designed for poultry intestinal microbiomes. Subsequent to the probe design, the specificity and the potential targets of each PITChip probe was examined by matching the probes with the phylotypes of poultry intestinal microbiome using Picky ‘examine oligos’ function (Chou et al., 2004). To better determine the potential targets of each probes, it was also matched with the total 16S rRNA gene of poultry intestinal microbiome using and the information of potential targets of each probe was included in the list of probe information.

Based on the in silico test result, three types of probes were included on PITChip: 1) unique probes that hybridize only specifically to their targets, 2) shared probes that can hybridize to more than one targets under the same stringency at which these probes will not hybridize to non-targets (Chou, 2010). A shared probe will be informative when several targets share a greater phylogenetic similarity at a particular position. 3) multi-target probes that hybridize to more than one target with different thermodynamic properties which could still be detected under experimental condition according to in silico calculation. A multi-target probe was only included when no specific or shared probe could be designed for a target. Eventually, Picky generated 517 unique probes, 53 shared probes, and 216 multi-target probes from the consensus sequences, which provided ≥ 57% coverage of the consensus sequences. Meanwhile, 545 unique probes, 95 shared probes, and 398 multi-target probes were designed from the representative sequences, providing ≥ 83% coverage of the dataset. Ninety nine genus-specific probes were also designed based on the full-length RDP consensus sequences that target 105
genera out of the 145 genera/groups. In addition, 86 probes targeting 34 pathogen, 3 probes targeting eimeria, and 4 controls probes were also included on the PITChip. Each PITChip had 6 microarrays in a 6X7K format with each probe being represented in three replicates. Six samples can be analyzed simultaneously using one PITChip.

5.3.2 Validation and optimization of the PITChip

The specificity, detection limits of PITChip were evaluated using clones of 16S rRNA gene from a former study (Cressman et al., 2010). 112 representative sequences at 0.03 genetic distance level were randomly selected from the 453 reported 16S rRNA gene clones and divided into two pools. *In silico* test of PITChip probes against the clones indicated that 71 clones had unique PITChip probes, 23 clones had PITChip probes with mismatches, and 18 of the clones could not be detected under the experimental condition. In pool 1, 50 ng of 50 out of 56 clones, 5 ng of 4 clones, and 0.5ng of 2 clones were as the template of *in vitro* transcription. In pool 2, 50ng of 52 out of 56 clones, 5 ng of 2 clones, and 0.5ng of 2 clones were as the template for *in vitro* transcription. As the reproducibility of *in vitro* transcription had been reported to be 99.6% (Rajilić-Stojanović et al., 2009), the in vitro transcription efficiency for the same type of gene was assumed to be highly similar. Two pools of cRNA were subjected to PITChip examination with a technical replicate. No significant differences were observed between technical replicates (data not shown). And the microarray profile of two pools of 16S rRNA clones showed distinct profile (Fig. 5.1), which indicated good specificity of PITChip probes in distinguishing species level 16S rRNA genes.
To better determine the specificity of PITChip probes and the influence introduced by mismatches between probes and targets, 5 probes with perfect match to the clones, 5 probes with one mismatch to the targets and 4 probes with more than 1 mismatches were randomly selected for the investigation (table 5.1). First, the signal intensity was examined under the hybridization and washing condition in the manufacturer’s protocol (MYcroarray, Ann Arbor, MI, USA) using constant amount of targets, and probes with one mismatch to the target could not be distinguished from perfect match. Thus the experimental condition was modified to a more stringent level with higher hybridization temperature, higher temperature of washing buffer and one more washing using the washing buffer at different stringency levels. Under the reported optimized experimental condition, a > 30% reduction of signal intensity from one mismatch hybridization was noticed when compared to that of a perfect match and minimal signal intensity was still detectable from a two-mismatch hybridization. It’s important to note that as the PITChip probes were designed for phylotypes with ≥ 90% similarity at the V3 region of 16S rRNA gene, mismatches should be expected when being applied to 16S rRNA genes belonging to the same phylotypes in the real world.

A strong variation of the hybridization signal intensity from probe-target complexus with similar predicted hybridization behavior has been addressed by several studies (Loy et al., 2002; Pozhitkov et al., 2006; Rajilić-Stojanović et al., 2009). The variation of signalling intensity has also been noticed during the validation of PITChip. 12 positive clones at the
same concentration were randomly selected from pool 1 and the hybridization signal intensity varied $\leq 2 \log$ (Fig. 5.2), which agreed with previous finding, but with a smaller range of signal intensity variation (Palmer et al., 2006). However, the relative change of the hybridization signal of PITChip probes was shown to be proportional to the log10 value of concentrations of corresponding phylotypes in pool, similar to the human intestinal tract chip (HITChip) study (Rajilić-Stojanović et al., 2009). This investigation was performed by analysing phylotypes at 100%, 10%, and 1% concentrations (data not shown). These result showed that the hybrid

The detection limit of PITChip was determined using 1:10 series dilution of the clone pool 1 from $10^{-1}$ to $10^{-6}$ of the original concentration, i. e., about $1.81 \times 10^9$ to $1.81 \times 10^4$ copies per clone in 54 µl hybridization reaction system (Fig. 5.2). The signal down to $1.81 \times 10^4$ copies per clone was detectable on PITChip, but a linear range of the log10 value of signal intensity of each probe was observed from $1.81 \times 10^9$ to $1.81 \times 10^6$ copies per clone on each array. Moreover, as mentioned before, $\leq 2 \log$ of signal variation were observed for all the tested probes at each concentration, but good linearity was observed within each probe, which qualified the PITChip hybridization signal intensity (log transferred) as an indicator of the changes in the relative abundance of a phylotype within the linear detection range. But the direct interpretation of signal intensity as abundance of phylotypes would not be feasible without validation of FISH, qPCR or other quantitative methods due to the variation of signal intensity (Rajilić-Stojanović et al., 2009; Kim, 2012)
5.3.3 Comparison of PITChip and cloning library

To test the detection power of the PITChip as a phylogenetic fingerprinting tool, the bacterial profile of 6 chicken microbiomes were examined by PITChip. Two flocks of chicks were subjected to fresh or reused litter treatment and the ileal mucosa, cecal content and litter samples were collected at 7 days of age. Overall, 217 probes based on representatives, 169 probes based on consensus sequences 11 pathogen-specific probes shown positive signal (Fig. 5.3). The hierarchical clustering heatmap analysis suggested that the microbiome of the same sampling location shared more similarity of microbial composition regardless the treatment chicks received. The core bacteria of intestinal microbiome at the younger age, the bacteria significantly affected by litter management as well as sample collecting locations can be displayed by the PITChip profile. As the DGGE and cloning library profiles of these samples had been reported (Cressman et al., 2010) and were compared with the PITChip profiles to test the applicability of PITChip. DGGE is a commonly used technique for microbial profiling by distinguishing sequences based on the GC content. Overall DGGE profiling resolved a significantly lower number of bands when comparing the results of PITChip profiling. This may suggest improved resolution when adopting PITChip for microbiome analysis instead of DGGE. Moreover, DGGE profile could not provide annotation information for each band resolved, which weaken it’s power on microbiome study. Cloning library has been used for microbiome study for the past decades as the major culture-independent technique. For the Cloning library study, due to the restriction of time, labor, and cost, about 70 clones were
recovered from each microbiomes. A network comparison among the profiles as revealed by PITChip as well as cloning library illustrated the massive information newly revealed by PITChip which may have been missed by the conventional cloning library method (Fig. 5.4). However, a few clones were not detected by PITChip, ranging from 3 to 11 clones for each microbiome. The cloning sequencing library information was not available in a public database by the time the of PITChip design. It’s an undeniable fact that a microarray cannot detect a target without a corresponding probe. It is therefore critical to ensure of the coverage of the original sequence collection. The high coverage of the PITChip to poultry intestinal bacterial diversity lead to at least 86% (11/79) coverage to a brand new poultry microbiome as indicated by this study. Last but not least, PITChip provided relative abundance information of the bacteria in 7-day-old chicks’ microbiomes, which could not be resolved by the cloning library. Collectively, the PITChip analysis is superior as a user-friendly high throughput analytical tool for investigating poultry intestinal microbiome by providing both qualitative and quantitative information.

5.3.4 Microbiomes as affected by litter managements

The potential of the PITChip to generation relative quantitative information on the poultry intestinal microbiomes qualified it for comprehensive comparative analysis on poultry intestinal microbiomes which were expected to harbor difference in relative abundance (Rajilić-Stojanović et al., 2009; Rajilić-Stojanović et al., 2010). As mentioned above, PITChip has been used to access the microbiomes from chicks reared on fresh or
reused litter to provide extensive quantitative and qualitative information of microbiomes. Studies on human intestinal microbiomes at different ages indicated that the age of the host can lead to principle difference in the microbial profiles (Mueller et al., 2006; Rajilić-Stojanović et al., 2009). Due to the low throughput of cloning library method in resolving microbial profiles, Cressman et al., (2010) only reported the cloning library of microbiomes of 7-day-old chick. To monitor the changes to the microbial profiles initiated by litter managements at different ages, ileal mucosa, cecal content, and litter samples were collected at day 7, day 14, day 21 and day 42 and subjected the PITChip investigation. The hierarchical clustering heatmap analysis suggested that the sampling location, i. e., ileal mucosa, cecal content, or litter, was the primary factor shaping the chicken intestinal microbiomes, following by the age of the host and eventually litter management (Fig. 5.5).

In ileal mucosa samples, mature microbiomes shared more similarity in microbial profiles (Fig. 5.6A). The stepping down of community distance may have mirrored the development towards a stable intestinal mucosal microbiome. Fig. 5.6B listed the 98 genes which were significantly reduced by fresh litter management as revealed by t-test (p < 0.05). Fresh litter had a noticeable major negative effect on the diversity as well as abundance on mucosal microbiomes, as expected. On the other hand, reused litter increased Akkermansia, Faecalibacterium, Sutterlla, Staphylococcaceae, Ruminococaceae, Clostridiales, Erysipelotrichaceae, and Bacteroidales.
The chicken cecal content samples collected from the same age shared more similarity (Fig. 5.7A). It is interesting to notice that cecal content microbiomes collected at week 1 and 6 shared more similarity, which might be indicating more complex mucosal microbiota at day 7 than we thought (Cressman et al., 2010). Fig. 5.7B outlines the 85 genes significantly affected by litter status as indicated by ANOVA analysis (p<0.05). Reused litter reduced *Clostridiales, Lachnospiraceae, Dorea, Pseudomonas, Roseburia, Bifidobacteriaceae, Coprococcus* as indicated by PITChip signal.

The chicken litter samples were also investigated using PITChip to provide background information of the environment. Similar to the situation in chicken ileal mucosa microbiomes, mature microbiomes tend to share more community structural similarity (Fig. 5.8A). Litter management altered the composition of litter microbiota by changing the most numbers of phylotypes (Fig. 5.8B). Reused litter led to significant decrease in *Pseudomonas, Salmonella, Roseburia, Lachnospiraceae, Oribacterium, Ruminococcaceae, Shigella, Mollicutes*, and *Bacteroidetes*. On the other hand, reused litter increased *Staphylococcus, Clostridiales, Lactobacillaceae, and Barnesiella*.

Although each composite sample carried a unique microbiome as indicated by the HCL heatmap analysis (Fig. 5.5), it was feasible to identify a range of phylotypes that persist within chicken microbiomes, regardless of the sampling location, age of host or treatment the chickens received. This group of phylotypes, so-called universal core of the microbiomes, was considered as functionally important to maintain the basic function of
microbiome (Zoetendal et al., 2008; Rajilić-Stojanović et al., 2009). To further adapt this concept, the PITChip phylotype profiles of the 24 poultry composite samples were clustered into groups using CAST, the cluster affinity search technique0x000A5006 MeV (Saeed et al., 2006). 48 phylotypes were identified across all chicken microbiomes, including phylotypes in Salmonella or Escherichia/Shigella, *Lawsonia, Osillibacter, Clostridiales, Bacteroidales, Bifidobacteriaceae, Porphyromonadaceae, Propionibacterium, Roseburia, Coprococcus, Ruminococcaceae, and Erysipelotrichaceae*. Moreover, phylotypes in the Salmonella/Escherichia/Shigella group, *Porphyromonadaceae, Bacilli, and Lawsonia* were detected to be at high relative abundance across all samples, which might suggest a fundamental rule of these phylotypes in chicken gut.

### 5.4 Conclusion

In this chapter, the development and validation of PITChip, a poultry specific phylotype microarray for intestinal microbiome studies was presented. By covering the known major phylotypes recovered from poultry intestinal microbiomes, this technique is highly valuable for comprehensive analysis of poultry intestinal microbiomes based on 16S rRNA genes. By providing relative quantitative information of phylotypes, comparative analysis can be easily carried out for multiple samples in a time, labor and cost efficient way. PITChip is user-friendly and the results of PITChip are easy to be interpreted without requirement of extensive computational skills. By simultaneously targeting more than 1900 phylotypes and pathogen specific gene, the PITChip is the first microarray specifically designed to support both qualitative and semi-quantitative analysis of
intestinal microbiota in chickens and turkeys. The first application of the comparative studies on the intestinal microbiotas as affected by litter management shed light on how these managerial and dietary treatments affect poultry intestinal microbiota. PITChip can serve as powerful tool to facilitate fundamental understanding of the microbiota important to both nutrition and health.

5.5 Experimental procedures

5.5.1 Sequence sources

The sequences used for the microarray design consisted were collected from four sources. Firstly, the 16S rRNA genes sequences of poultry origin were retrieved from RDP database (Release 24) using the search terms ‘chicken(s)’, ‘chick(s)’, ‘poultry’, ‘broiler(s)’, ‘hen(s)’, ‘turkey(s)’. The sequences of poor quality were discarded as described in our previous study (Wei et al., 2013a). In total, 888 16S rRNA sequences that contain the V3 hypervariable region were retained, and the V3 region were delineated and sliced out for probe design. Secondly, over 400,000 sequences of the V3 region representing > 90% of the bacterial diversity of chicken and turkey microbiomes were obtained, which represent 8795 operational taxonomic units (OTUs) of bacteria (97% sequence similarity). The sequences from the above two sources were combined and archived into an in-house ARB database. Representatives and consensus sequences were generated for each OTU defined at 90% similarity (He et al., 2007). Thirdly, the 16S rRNA gene of 34 known poultry pathogens and genes encoding their relative virulence factors were obtained from GenBank. Lastly, genes on human
mitochondria was used as a negative control, while genes of chicken mitochondrial gene was used as positive control.

5.5.2 *Probe design and fabrication of PITChip*

Antisense oligonucleotide probes were designed based on the reverse complement of the representative and consensus sequences of the V3 hypervariable region using the probe design program Picky (version 2.0) (Chou et al., 2004; Chou, 2010). Oligonucleotide probes (20-30 bps) were designed for the representative and consensus sequences, with the highest melting temperature (Tm) with non-targets being lower than 45°C. For the representative and consensus sequences from which not probe was found, longer oligonucleotide probes (30-50 bp) were designed with the same criteria. All the 16S rRNA gene sequences were classified using the Classifier program at the RDP database. One consensus sequence was generated using ARB program, and the genus-specific oligonucleotide probe was also using Picky as described above with the same criteria. Oligonucleotide probes also were designed from the 16S rRNA gene sequences or virulent gene sequences using Picky. Positive and negative control probes were designed from the 16S rRNA gene sequences of human and chicken mitochondria. The information about the probes and their targets is available upon request. The specificity of the probes was evaluated by in silico analysis using Blast search against the in-house ARB database. Slides of PITCip 1 were custom-fabricated by MYcroarray (Ann Arbor, MI, USA) as a 6X7K format with each probe having three 3 replicate probes.
5.5.3 Preparation of pools of 16S rRNA gene clones

Two pools of clones (56 clones each) were prepared from the 16S rRNA gene clone libraries of chicken intestinal microbiomes that had been analyzed in a previous study (Cressman et al., 2010). The 16S rRNA gene of each clone was using T7-Bac-27F (5’-TGAATTGTAATACGACTCTATAGGGGTTTGATCCTGGCTCAG-3’, the underlined region is the T7 promoter region) and Bac-1525R (5’-AAGGAGGTGWTCARCC-3’) (Weisburg et al., 1991). The PCR reaction mix and the thermal profile of the PCR reactions were the same as previously reported by Cressman et al., (2010).

5.5.4 Metagenomic DNA extraction

Metagenomic DNA was extracted from samples of ileal mucosa, cecal content, and litter using the RBB+C method as described in previous study (Yu and Morrison, 2004). The DNA quality was accessed using agarose gel (1.0%) electrophoresis, and DNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The concentration of an aliquot each DNA extract was adjusted to 100 ng/µl using Tris-EDTA (TE) buffer (pH 8.0). The DNA was stored at −20 °C before use.

5.5.5 Complementary RNA (cRNA) preparation

The 16S rRNA gene was amplified from the metagenomics DNA using the primers T7-Bac-27F and Bac-1502R as described above. The PCR products were purified using a
QIAquick PCR Purification kit (QIAGEN, Inc., Valencia, CA) and quantified using a NanoDrop ND-1000 spectrophotometer as described above.

*in vitro* transcription of the amplified 16S rRNA gene was performed using an Ambion T7 MEGAscript kit (Life Technologies Co. Grand Island, NY, USA) following the manufacturer’s instructions. For each reaction, 100 ng of the purified PCR product was used as the template in a 10μL reaction. The *in vitro* transcription was incubated at 37°C for 4 h. Then, the DNA template was degraded by 0.5μL Turbo DNase following an incubation for 15-20 min at 37°C. The in vitro transcribed cRNA was purified using an Ambion MEGAclean kit (Life Technologies Co. Grand Island, NY, USA), and the cRNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

The cRNA was labelled with Cy™5, which covalently attaches to any reactive heteroatoms (the non-carbon or non-hydrogen atoms on the backbone of the molecule) within rRNA molecules, using a Mirus Label IT µArray® Labelling Kits as described by Kim (2011). For each labelling reaction, 500ng cRNA was used in a 25μL reaction containing 1X buffer M in the kit and 1μL Cy5 dye. The Cy5-labeled cRNA was purified using an Ambion MEGAclean kit (Life Technologies Co. Grand Island, NY, USA), eluted with 30μL elution solution twice, and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). All the above procedures were done in dark to avoid light bleaching of the Cy5 dye.
5.5.6 Microarray hybridization

The hybridization was performed as described by Kim (2011). Briefly, the hybridization solution was prepared following the protocol provided by MYcroarray. Each hybridization solution (60μL) consisted of 20X SSPE (18μL), formamide (6.0μL), 1% bovine serum albumin (BSA, 0.6μL), 1% tween-20 (0.6μL), Control-oligos (0.6μL), positive controls (1.0μL), and Cy5-labelled cRNA (33.2μL). Microarray hybridization was performed using Agilent Technologies’ Hybridization gasket slides that are compatible with the MYcroarray slides. The hybridization solution was incubated at 65°C for 5 min, followed by immediate cooling on ice for 5 min. The Agilent Hybridization Cassette, the Agilent gasket slide, and the MYcroarray slide were assembled and preheated at 65°C. Then, 54 μL hybridization solution was applied on each array following MYcroarray’s protocol. The hybridization was carried out in an HB-1000 hybridization oven (UVP, LLC) at 45°C for 20h with rotation set at 24 rpm.

The microarray assembly was opened in 1X SSPE at room temperature, washed in 1X SSPE twice at 45°C for 5 min, following by washing twice at 45°C in 0.25X SSPE for 30s. The microarray slide was rotated upon changing the washing buffer to avoid uneven washing at one side of the array. The microarray slide was finally dried for 10 min using a microarray spinner. All the microarray experiment procedures were performed in dark to avoid light bleaching of the light-sensitive Cy5 dye.
5.5.7 Microarray data extraction and analysis

Microarrays were scanned using a GenePix 4000B microarray scanner (Molecular Devices, LLC, Sunnyvale, CA, USA) with 0.01% tolerance to oversaturation and photomultiplier sensitivity set at auto-PMT gain. The information of each probe spot on the scanned images was extracted by fitting the Gal file, which carries the annotation information of each spot on a microarray, onto the images using GenePix pro 6.0. Probe spots of negative detection were flagged out by the auto-alignment function of the GenePix software. The probe spots with oversaturation, bad shape, or contamination were flagged correspondingly manually.

The GenePix extracted results were stored as a gpr format and then converted to a mev format using ExpressConvertor to allow for downstream analysis using the TM4 microarray suite of tools (Saeed et al., 2003). The median signal intensity of each spot subtract background were kept for downstream analysis using ExpressConvertor and replicates were consolidated using Ginkgo software in TM4 (Saeed et al., 2003). The mean value of replicates was used to represent the median signal intensity after consolidation and was then normalized to the MYcroarray manufacturer’s positive control.

The normalized data were imported into MeV (V4.7) within the TM4 suite (Saeed et al., 2003) for statistical analysis. Microarray results were then normalized by the total signal intensity and log10 transferred to improve normality. ANOVA test was used to examine
the overall difference of datasets, and t-test was performed to identify genes significantly affected by the treatments in each sample (p<=0.05). Hierarchical clustering of the PITChip profiles was computed to estimate the distance between samples based on Manhattan metric using average linkage clustering. Clustering Affinity Search Technique (CAST) in MeV were used to cluster genes according to the pattern similarity.
Table 5.1. Signal intensity as effected by mismatch.

<table>
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<th>Ave F635 (Protocol)</th>
<th>Ave F635 (Optimized)</th>
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<tr>
<td>0</td>
<td>5</td>
<td>11400</td>
<td>102123</td>
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<tr>
<td>1</td>
<td>5</td>
<td>11909</td>
<td>69300</td>
</tr>
<tr>
<td>more</td>
<td>4</td>
<td>5765</td>
<td>9433 (1831)</td>
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Figure 5.1. Flowchart of the development of PITChip.
Figure 5.2. Partial PITChip signal counts for minimum detection limitation assessment. 12 clones were randomly picked up to illustrate the detection limits. Each clone were diluted from 10E-06 to 10E-01 folds from left to right. The microarray has a detection limit of $1.8 \times 10^6$ copies of targets (in a 54ul system), which is 10E-04 fold of the original samples, and a linear range of 3 to 4 logs.
Figure 5.3. Hierarchical clustering analysis of Poultry GutArray profiles of 6 chicken microbiota samples. LR, reused litter; LF, fresh litter; IR, ileal mucosa of reused-litter chicks; IF, ileal mucosa of fresh-litter chicks; CR, cecal content of reused-litter chicks; CF, cecal content of fresh-litter chicks.
Figure 5.4. Network comparison of the microarray profile and the published clone sequencing profile. LR, reused-litter clones; LF, fresh-litter clones; IR, ileal clones of reused-litter birds; IF, ileal clones of fresh-litter birds; CR, cecal clones of reused-litter birds; CF, cecal clones of fresh-litter birds. A round core stands for result from microarray analysis; a diamond core stands for sequencing results from Cressman’s study (Cressman et al., 2010).
Figure 5.5. Hierarchical clustering analysis of PITChip profiles of 24 chicken microbiota samples collected at wk 1, wk 2, wk 3 and wk 6. LR, reused litter; LF, fresh litter; IR, ileal mucosa of reused-litter chicks; IF, ileal mucosa of fresh-litter chicks; CR, cecal content of reused-litter chicks; CF, cecal content of fresh-litter chicks.
Figure 5.6. The microbiomes of chicken ileum mucosa as affected by litter status.

A. Sample HCL tree

B. 98 phylotypes significantly reduced by fresh litter.

Figure 5.6. The microbiomes of chicken ileum mucosa as affected by litter status.
Figure 5.7. The microbiomes of chicken cecal content as affected by litter status.

A. Sample HCL tree

B. 85 phylotypes significantly affected by litter status
Figure 5.8. The microbiomes of chicken litter as affected by litter status.
Chapter 6: Abundance of pathogens in the gut and litter of broiler chickens as affected by bacitracin and litter management

6.1 Abstract

*Clostridium perfringens*, *Salmonella* spp. and *Campylobacter* spp. are food-borne enteric pathogens that are commonly associated with poultry. The objective of this study was to investigate the effects of supplemental bacitracin and litter management (fresh vs. reused) on the abundance of these pathogens in commercial broiler chickens. Specific quantitative PCR (qPCR) assays were used to quantify *C. perfringens*, virulent *C. perfringens* that carried the genes encoding α-toxin (*cpa*) and NetB-toxin (*netB*), *Salmonella*, and *Campylobacter* in samples of ileal mucosa, cecal content, and litter. *Campylobacter* was not detected in any of the samples collected. The abundance of *Salmonella* was not affected by either bacitracin or litter condition. Generic *C. perfringens* was detected in the ileal mucosa at very low level at 10 days of age but was much higher at 35 days. Chickens reared on reused litter tended to have a lower abundance of generic *C. perfringens* compared with those reared on fresh litter. In the ileal mucosa, no *cpa* or *netB* was detected at day 10 but was detected at day 35 in the chickens that were not fed supplemental bacitracin. Chicks fed supplemental bacitracin

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2 Submitted to Veterinary Microbiology.
had reduced abundance of generic *C. perfringens* as well as the *cpa* and *netB* genes in the ileal mucosa, cecal content, and litters. A strong positive correlation was found between the abundance of all three measurements of *C. perfringens*. The abundance of *Salmonella* spp. and *C. perfringens* was also shown to be correlated. This is the first study that has examined the effect of dietary bacitracin and litter conditions on the prevalence of these three common enteric pathogens. Unless contaminated by pathogens from a previous flock, litter reuse does not appear to increase the risk of necrotic enteritis or *Salmonella* infection in subsequent flocks.

6.2 Introduction

*Campylobacter* species (particularly *C. jejuni* and *C. coli*), *Salmonella* species, and *Clostridium perfringens* are important bacterial pathogens associated with chickens. These pathogens are typically carried in the intestine of chickens but are also found in poultry meat and eggs (Golden et al., 2009; Melero et al., 2012; Temelli et al., 2012). When consumed with poultry meat or other poultry products, these pathogens can cause serious infection (Adzitey et al., 2012). Indeed, campylobacteriosis caused by *C. jejuni* has been reported to be the most common cause of bacterial gastrointestinal disease (Altekruse et al., 1999). Likewise, salmonellosis is another major public health concern. *C. perfringens* ranks as the third most common cause of food-borne diseases in the United Kingdom and the United States (Warrell, 2003; Adzitey et al., 2012). In addition to their risk to human health, both *C. perfringens* and *Salmonella* can infect chickens and
cause considerable economic losses via increased mortality and reduced growth. For example, *C. perfringens* can contribute to necrotic enteritis, an enteric disease among young chicks that is the most common and costly bacterial disease in commercial broiler and turkey flocks, largely due to increased mortality and reduced productivity (Van Immerseel et al., 2004). *Salmonella* can also cause mortality and decreased growth in young chicks.

In modern broiler and turkey production, dietary subtherapeutic antibiotics are often used for prophylactic reasons and as antimicrobial growth promoters (AGP). The mode of action of AGPs are not fully understood even though they have been used for over 50 years in commercial poultry production (Dibner and Richards, 2005; Niewold, 2007). The use of AGPs has been shown to be effective in preventing enteric diseases and enhancing performance, particularly feed efficiency (Coates et al., 1963; Miles et al., 2006). However, there is increasing concern and often contentious debate over the potential risk posed by the increased development and subsequent dissemination of antimicrobial resistance (Witte, 2000; Hammerum and Heuer, 2009; Marshall and Levy, 2011). Such concern led the ban of dietary AGP in Europe in 2006 while market/consumer concerns also result in decrease in the use of AGP in the US. Non-antibiotic alternatives (Huyghebaert et al., 2011) are being evaluated to replace, at least partially, the AGPs that have been used for many years.
Litter management is one aspect of commercial poultry production that can potentially affect the intestinal colonization of young chicks by bacteria, including pathogenic bacteria. In a previous study, we reported that chickens reared on reused litter had more intestinal bacteria of intestinal origin while those reared on fresh litter had increased concentration of intestinal bacteria of environmental origin (Cressman et al., 2010). Reused litter has also been reported to increase coliform levels and coccidial outbreaks in poultry flocks (Stanley et al., 2004) and increase the intestinal inflammatory response (Shanmugasundaram et al., 2012). In a preliminary study, we showed that reused litter effectively delayed intestinal *C. perfringens* colonization in chicks at 7 days of age. The objective of the current study was to investigate the effects of litter management (reused; fresh) and supplemental bacitracin on the abundance of *C. perfringens* (both generic and toxin-producing) in broiler chickens as well as their effect on *Campylobacter* and *Salmonella*.

6.3 Materials and methods

6.3.1. Animals and in vivo study design

The feeding experiment used a 2 x 2 factorial arrangement of treatments: litter management (fresh vs. reused litter) and dietary bacitracin (with vs. without) (Shanmugasundaram et al., 2012). Newly hatched chicks (1 day old) were randomly assigned to one of four treatment combinations: fresh pine shavings (referred to as fresh litter) and no dietary bacitracin (NF); fresh litter and bacitracin at 0.04% of feed (BF);
reused litter and no dietary bacitracin (NR); reused litter and 0.04% dietary bacitracin (BR). Each treatment combination was consisted of six replicate pens with 36 chicks per pen at the start of the study. The eggs from which the chicks were hatched were not disinfected. In the hatchery, all chicks were subjected to the anti-coccidial spray vaccine (Coccivac-B, Schering-Plough Animal Health) at 87% of the recommended spray dose (21 ml per 1,000 chicks). Chickens were fed standard corn-soybean-meal-based diets which met the NRC (1994) suggested nutrient levels. Ileal mucosa and cecal content samples were collected from five birds per pen at 10 and 35 days of age. The ileal mucosa samples were collected from the region between Meckel’s diverticulum and the ileocecal junction, and cecal content samples were collected from each bird and pooled by pen as described by Cressman et al. (2010). Subsamples of litter were collected on day 35 from each of four locations within a pen: the brood area, beneath the water and feed lines, and along the walls and pooled to represent the average litter condition of each treatment. The study was repeated over three flocks with the litter being completely replaced in the fresh litter treatments.

6.3.2. DNA extraction

Microbial community DNA was extracted from the mucosal, cecal, and litter samples using the RBB+C method (Yu and Morrison, 2004). The DNA quality was evaluated using agarose gel (0.8%) electrophoresis, and DNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).
concentration of an aliquot of the extracts was adjusted to 100 ng/µl using Tris-EDTA (TE, pH 8.0) buffer prior to PCR amplification. The DNA was stored at −20 °C before use.

6.3.3 Screening for enteric pathogens

Primer sets specific for the *invA* gene of *Salmonella* spp., the 16S rRNA gene of the genus *Campylobacter*, the 16S rRNA gene of species *C. perfringens*, and the *cpa* gene and *netB* gene of virulent strains of *C. perfringens* were used to screen all the samples for the presence of the above pathogens using endpoint PCR amplification (Table 6.1). For each PCR reaction, 0.5 µl DNA (100 ng/µl) was added to a 24.5 µl master mix that contained 1x PCR buffer, 1.75 mM MgCl₂, 670 ng/µl bovine serum albumin, 200 µM dNTP, 500 nM of each primer, and 0.625 U Platinum Taq high-fidelity polymerase (Invitrogen Corporation, Carlsbad, CA). The PCR thermal program consisted of an initial denaturation step at 94°C for 5 min; 40 cycles of a 30s denaturation step at 95°C, a 30s annealing step at respective temperature (Table 6.1), and a 40s elongation step at 72°C; and a final extension step at 72°C for 7 min before a 4°C hold. The expected PCR amplification products were confirmed by agarose gel (1.0%) electrophoresis. No-template control and positive control samples were included in all the PCR screening.
6.3.4 Quantitative real-time PCR (qPCR)

The genomic DNA of *Salmonella enterica* and *Campylobacter jejuni* was used to amplify the gene fragment of *invA* and 16S rRNA of *Salmonella* and *Campylobacter*, respectively, using their specific primer set (Table 6.1). Fragments of the 16S rRNA gene of species *C. perfringens*, *cpa* and *netB* of virulent strains of *C. perfringens* were amplified using each respective specific primer pair from the metagenomic DNA samples that were positive for each target (Table 6.1). Each PCR product was purified using a QIAquick PCR Purification kit (QIAGEN, Inc., Valencia, CA) and then cloned using a TOPO TA cloning kit for sequencing (Invitrogen Inc., Carlsbad, CA). For each pair of primers, one clone was selected and linearized by *Nco*I restriction enzyme (New England Biolabs, Inc., Ipswich, MA) that only cuts the vector region. Each of the linearized recombinant plasmids was purified using ethanol precipitation and dissolved in TE buffer. The concentration of each qPCR standard was determined using the Quant-it Kit (Invitrogen Corporation, Carlsbad, CA, USA). Copy-number concentration was calculated from the mass concentration and the length of each standard. Serial dilutions (1:10) were made in TE buffer prior to each qPCR assay. Each of the qPCR standards was also used as the positive control in the screening experiment (section 2.4).

Each qPCR assay was performed using respective specific primers (Table 6.1) as described previously (Chen et al., 2007). Briefly, 0.5 µl of 100 ng/µl metagenomic DNA each was used as the template in each qPCR reaction in 25 µl volume. The reaction
mixture was the same as the end PCR used in the screening experiment except a reduce primer concentration (250 nM each primer), the addition of SYBR (0.013x final concentration) and ROX (0.003% final concentration). Serial dilutions (10^1 - 10^7 copies) of each of the qPCR standards were included on the same qPCR plate. All the qPCR assays were performed in three replicates for both the DNA samples and the corresponding qPCR standard. The thermal program for the qPCR assays was the same as described previously (Chen et al., 2007). The gene copy number of the target in each reaction was calculated from the standard curves (the R^2 \geq 0.993), while the abundance of each target (gene copies/g sample) was calculated from the copy number per reaction and the number of reactions that can be performed from the DNA sample derived from one g of each sample. The detection limits of the pathogens were above 10^3 copies/g of sample.

6.3.5 Statistical Analysis

The qPCR data were log_{10} transformed to improve normality. Data were analyzed using the MIXED procedure of SAS (V9.2, SAS Inst. Inc., Cary, NC) based on a Split Plot with Double Repeated Measures Model. Uni-variance analysis was carried out for each sampling location. The abundance of each qPCR measurement from different sampling locations (ileal mucosa, cecal content, litter) was calculated for the four treatments (NF, NR, BF, and BR) and the two ages (days 10 and 35). The NF treatment was considered the control, and the abundance of the other three treatments were compared against that of the control using one-way ANOVA with Tukey’s multiple comparison test on
GrahPad Prism 6 (GraphPad Software, San Diego, CA, USA). The CORR procedure in SAS (V9.2, SAS Inst. Inc., Cary, NC) was used to compute the correlation of the abundance of the pathogens analyzed by sampling location, dietary bacitracin, and litter management. Statistical significant was declared at $P \leq 0.05$, while tendency was declared at $P \leq 0.10$.

6.4 Results

6.4.1 Effects of bacitracin and litter management on poultry performance.

The average body weight determined at day 35 ranged from 2.58 to 2.64 kg and did not differ among the different bacitracin-litter treatment combinations (Fig. 6.1). The birds that were fed supplemental bacitracin, however, had significantly lower mortality than those that did not received the medicated feed ($P < 0.0001$; Fig. 6.2). On the other hand, litter management alone did not significantly affect the mortality rate though the reused litter trended to increase mortality. There was greater variability in mortality among pens of chickens that were not fed supplemental bacitracin (data not shown).

6.4.2 Abundance of enteric pathogens in chicken ileal mucosa.

*Campylobacter* spp. were not detectable in the chicken ileal mucosa samples, but both *Salmonella* and generic *C. perfringens* were detected in nearly all the samples analyzed (Fig. 6.3). The abundance of *Salmonella* was not affected by either the dietary bacitracin or litter management at both sample ages. The abundance of generic *C. perfringens*,

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however, tended to be lower among the chickens reared on reused litter when compared with those reared on fresh litter at both ages. Bacitracin also decreased the abundance of this species numerically at day 10 and significantly at day 35. As the birds aged, the abundance of generic *C. perfringens* increased considerably. *C. perfringens* that carried the *cpa* or *netB* genes were found in the chickens that were fed bacitracin and reared on fresh litter but was not detected in other treatments with the exception of *netB* being detected in the chickens that were reared on reused litter without bacitracin. Uni-variance analysis showed a significant bacitracin x reused litter interaction with respect to abundance of *cpa* and *netB* genes (p<0.0001) but not the abundance of generic *C. perfringens*.

6.4.3 Abundance of enteric pathogens in chicken cecal content.

Genus *Campylobacter* was not detected in any of the cecal content samples but generic *C. perfringens* and *Salmonella* spp. were detected. As in the case of the ileal mucosa, reused litter tended to reduce the abundance of generic *C. perfringens* in the cecal content at day 10 except in the pens that received bacitracin (Fig. 6.4). Bacitracin also tended to reduce generic *C. perfringens* at day 10 and reduced this bacterium significantly (p<0.0001) at day 35, and generic *C. perfringens* increased in abundance in the cecal content as the bird aged. In the cecal content both *cpa* and *netB* genes were detected in the pens that received no bacitracin, with a greater abundance being found in pens that used fresh litter. No *cpa* or *netB* genes were detected in the chickens that were fed
bacitracin with the exception of a low \textit{cpa} gene level in the bacitracin plus fresh litter treatment at day 35. There was no bacitracin x litter treatment interaction observed with respect to the abundance of generic \textit{C. perfringens} (P < 0.0947) or \textit{cpa} gene (P < 0.135). However, a significant bacitracin x litter treatment interaction was noted with respect to the abundance of the \textit{netB} gene of \textit{C. perfringens} and \textit{invA} gene of \textit{Salmonella} (P <0.0001).

6.4.4 \textit{Abundance of enteric pathogens in chicken litter.}

The litter samples of the four treatment groups were collected only at day 35. The litter samples were \textit{Campylobacter} negative based on end-point PCR analysis. Bacitracin significantly reduced the abundance of generic \textit{C. perfringens} (P <0.0001), and both the \textit{cpa} gene and \textit{netB} gene were not detectable in the litter samples from chickens fed bacitracin. Interestingly, litter conditions did not significantly affect the abundance of any of the measured microbial groups. A significant bacitracin x reused litter interaction was noted with respect to the abundance of generic \textit{C. perfringens}, \textit{netB} gene and \textit{Salmonella} spp. (P <0.0001). The abundance of \textit{Salmonella} did not appear to be affected by either litter treatment or dietary bacitracin.

6.4.5 \textit{Correlations of the behavior of enteric pathogens.}

Potential correlations between the individual qPCR measurements were examined using Pearson correlation coefficient (r) based on sampling location, bacitracin treatment, and
litter management. A strong positive correlation was found in abundance among generic *C. perfringens*, *cpa* and *netB* genes (*r*=0.648 to 0.940, *P*<0.0001) in the ileal, cecal content, and litter samples, irrespective of the litter treatment and supplemental bacitracin. The abundance of *Salmonella* spp. was slightly negatively correlated with that of generic *C. perfringens*, *cpa* gene, and *netB* gene (*r*= -0.260 to -0.316, *p*=0.03 – 0.010) in the cecal content samples from chickens reared on reused litter. In the ileal mucosa, however, the abundance of *Salmonella* and generic *C. perfringens* was positively correlated (*r*=0.309, *p*=0.008), irrespective of litter treatment or dietary bacitracin.

6.5 Discussion

Litter status (Cressman et al. 2010) and dietary antibiotics (Smirnov et al., 2005) are important factors that can influence the chicken intestinal microbiome, including enteric pathogens. In this study, we systematically examined and compared the effects of litter condition and dietary bacitracin on the most common enteric pathogens found in broiler chickens. As expected, mortality was significantly reduced by dietary bacitracin, but not the litter conditions used in this study. The reduced mortality in the chickens fed bacitracin mirrors the reduced abundance of generic *C. perfringens*, *cpa* gene, and *netB* genes in all the three sampling locations: ileal mucosa, cecal content, and litter (both fresh and reused). As a Gram-positive bacterium, *C. perfringens* is sensitive to bacitracin. The MIC(90) of bacitracin-resistant *C. perfringens* strains (Charlebois et al., 2012) was much lower (256 µg/ml) than the bacitracin level used in this study. Therefore, the
supplemented bacitracin might have directly inhibited *C. perfringens*. However, the abundance of *C. perfringens* might have also been affected by a bacitracin-induced alteration in the intestinal microbiome. This hypothesis has been reported in several studies (Engberg et al., 2000; Geier et al., 2010). In a separate study, dietary bacitracin was found to lower the inflammatory response in cecal tonsils compared to birds fed no bacitracin (Shanmugasundaram et al., 2012). It remains to be determined, however, if the reduced inflammatory responses were related to the reduced abundance of *C. perfringens*.

The abundance of generic *C. perfringens*, *cpa* gene, and *netB* gene were positively correlated in all three sampling locations irrespective to the dietary bacitracin or litter condition. These results suggest that strains that carry the *cpa* and (or) *netB* genes have similar ecology to the generic *C. perfringens* population and a reduction in total *C. perfringens* will reduce the risk of necrotic enteritis. It was also noted that the abundance of *cpa* gene and *netB* genes were not detected in the same abundance in most of the samples. Such a discrepancy may be attributed to the fact that *cpa* is carried on a chromosome (Canard and Cole, 1989) whereas *netB* is carried on a 85kb plasmid that might be conjugative (Lepp et al., 2010). The differences in the abundance of *cpa* and *netB* also suggest that the *cpa* and the *netB* genes may not always be carried by the same strains of *C. perfringens*.

Broiler chickens may consume up to 4% of their daily intake as litter (Malone, 1992). Thus, the litter microbiome can serve as an important entry point for bacteria that gain
access to the gut and colonize therein. Indeed, fresh litter and reused litter were found to affect the composition of the gut microbiome in broiler chickens, especially at an early age (Cressman et al., 2010). In this study, the reused litter tended to delay colonization of the ileal mucosa, especially at day 10 when compared with fresh litter. It remains to be elucidated if the microbiome present in the reused litter served as competitive exclusion cultures that delayed the colonization of the gut in young chicks by C. perfringens. On the other hand, if the previous flock sheds C. perfringens or other pathogens, then reused litter may become source of pathogens for the subsequent flocks, increasing the risk of developing enteric diseases.

There was a relatively high level of Salmonella spp. in the ileal mucosa and litter microbiomes. Neither the litter conditions nor the dietary bacitracin reduced the abundance of Salmonella spp. However, the reused litter tended to reduce the abundance of Salmonella in the cecal content in the absence of bacitracin but not in the presence of the antibiotic. By inhibiting peptidoglycan synthesis, bacitracin is more inhibitory against Gram-positive bacteria than against Gram-negative bacteria. In the chickens that were fed supplementary bacitracin, Gram-positive intestinal bacteria were probably inhibited, and consequently, Gram-negative bacteria, including Salmonella, may have proliferated due to the reduced competition for nutrients available in the gut. Indeed, Gram-positive bacteria were reduced while Gram-negative bacteria were increased in the rumen of cattle.
fed monensin (unpublished data). A study is underway to comprehensively examine the effect of bacitracin on intestinal microbiome in chickens.

6.6 Conclusion

Bacitracin can effectively reduce the abundance of generic *C. perfringens* as well as the virulent strains that produce α-toxin and NetB-toxin, resulting in reduced mortality among broiler chickens. The gut of young chicks may not be colonized by *C. perfringens* until an age of older than 10 days, and reused litter may delay its colonization. In general, the abundance of generic *C. perfringens* is positively correlated with that of the genes that encode α-toxin and NetB-toxin, but the two toxin genes may differ in abundance due to different residence (chromosome vs. plasmid). Bacitracin may result in a negative correlation between the abundance of *C. perfringens* and that of *Salmonella* due to the differential effect of bacitracin on these two bacteria. Unless contaminated by enteric pathogens shed from a previous unhealthy flock, litter reuse will not increase the risk of enteric disease in subsequent flocks.

6.7 Acknowledgement

We thank Dr. Norman St-Pierre for his assistance with the statistical analysis. This study was partially supported by an AFRI grant (award number: 2008-35204-18845).
Table 6.1. Specific primers used for different assays.

<table>
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<th>Primer</th>
<th>Target gene</th>
<th>Sequence (5’ → 3’)</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon length (bp)</th>
<th>References</th>
</tr>
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<tr>
<td>InvA-F</td>
<td>invA of genus</td>
<td>GAAATTATATGACACRTTCGGGCAA TCATCGCACCCTCAAARGARCC</td>
<td>52</td>
<td>282</td>
<td>Hein et al., 2006</td>
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<tr>
<td>invA-R</td>
<td>Salmonella</td>
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<td></td>
</tr>
<tr>
<td>Campy-F</td>
<td>rrs of genus</td>
<td>GGATGACACTTTTCGGGAG AATTCCATCTGCTCTCC</td>
<td>48</td>
<td>246</td>
<td>Rinttila, et al., 2004</td>
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<tr>
<td>Campy-R</td>
<td>Campylobacter</td>
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<td></td>
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<tr>
<td>Cper-164F</td>
<td>rrs of C.</td>
<td>TGAAGATGCGCATCATTCAAC GGTACCGTCATTATCTTCCCCAAA</td>
<td>50</td>
<td>282</td>
<td>This study</td>
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<tr>
<td>Cper-446R</td>
<td>perfringens</td>
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</tr>
<tr>
<td>Cpa-F</td>
<td>cpa of C.</td>
<td>TCATCTGCTAATGTTACTG CCACTAGTTGATATGTAAGC</td>
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<td>392</td>
<td>Sheedy, et al., 2004 (modified)</td>
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<td>perfringens</td>
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<td>AKP78</td>
<td>netB of C.</td>
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Figure 6.1. The average body weight (± SEM) of chickens from 3 growth trial.
Figure 6.2. Average mortality rate (± SEM) of chicken from 3 growth trials. (**) $p < 0.01$
Figure 6.3. The abundance of enteric pathogens and toxin genes in chicken ileal mucosa. Results are the average (± SEM) of growth trial 2 and 3. (*p < 0.05; **p < 0.01)
Figure 6.4. The abundance of enteric pathogens and toxin genes in chicken cecal content. Results are the average (± SEM) of growth trial 2 and 3. (*p < 0.05; **p < 0.01)
Figure 6.5. The abundance of enteric pathogens and toxin genes in litter samples. Results are the average (± SEM) of growth trial 2 and 3. (*p < 0.05; **p < 0.01)
Chapter 7: General Discussion

Cultivation-independent 16S rRNA gene-based techniques have greatly advanced our knowledge of microbial diversity of poultry intestinal microbiome. Both metagenomic and habitat-specific phylochip microarray technologies were used to investigate the poultry intestinal microbiome in the series of studies we reported here. The goal of these studies was to achieve a better understanding of the composition of the poultry intestinal microbiome and the effects of dietary and managerial interventions on poultry intestinal microbiomes. The collective knowledge provided a new insight into the composition of poultry intestinal microbiome and may help improve host health and growth in the future.

PCR-DGGE and 16S rRNA gene clone libraries have been used to investigated poultry intestinal microbiome in for the past two decades. The inability to identify the bacteria by DGGE and the limited diversity coverage afforded by clone libraries were the major limitations of these techniques. In addition, the scope of individual explorations on the poultry intestinal microbiome was narrow with respect to number of birds sampled, dietary conditions investigated, managerial systems employed, and geographic locations. The PCR that both technologies rely on also has inherent bias that can screw the diversity and composition profile of the microbiomes. The naïve analysis of all the 16S rRNA gene sequences of poultry gut origin available in public databases enabled us to establish a
global framework of bacterial diversity in poultry (Chapter 3). By identifying 915 and 464 OTUs of bacteria in the gut of chickens and turkeys, respectively, this global diversity frame greatly advanced our knowledge on the bacterial diversity present in both chickens and turkey in general. We also managed to estimate the bacterial diversity of poultry intestinal microbiomes currently witnessed (89% for chickens and 68% for turkeys) and the future sequencing effort (about 7,000 high quality sequences from various flocks) needed to determine the complete diversity. The global diversity framework also served as the foundation of the in-house ARB database dedicated to the intestinal microbiome of poultry. An unexpected finding of this study was that more than 85% of the 16S rRNA gene sequences recovered from public databases are of poor quality, very short sequences, or incomplete record of sampling locations, making them of little value in any naïve analysis. Therefore, efforts are needed to improve not only sequence quality but also proper annotation information of sequences in the future. MG-RAST, an online server for metagenomic data deposition and analysis, has made an effort in enforcing authors to provide the Minimum Information about any (X) Sequence (MIxS) developed by the Genomic Standards Consortium (GSC) when submitting metagenomic sequences (Meyer et al., 2008). Similar convention should also be adopted by other public databases in the future.

Next-generation sequencing (NGS) technologies have become increasingly powerful and cost-effective. Using the NGS empowered by the 454 FLX system, we performed a deep pyrosequencing study on the intestinal microbiomes of both chickens and turkeys reared
under typical dietary and managerial conditions with a goal to identify most of the bacteria present in the gut of these two important bird species (Chapter 4). As we intended, we were able to identify most of the bacteria (> 95%) present therein. This study greatly expanded our view on the intestinal microbiome in both chickens and turkey, at least chickens and turkeys reared in North America. Besides, the sequencing data also expanded the ARB database of the poultry intestinal microbiome. Future analytical tools or techniques may be developed from this comprehensive database. We also demonstrated the importance of using multiple pyrosequencing runs and evaluation of multiple analysis pipelines, and parameters in reducing analysis errors. We further pointed out that because the pyrosequencing studies reported to date employed amplicons prepared using different primers, different quality screening, and data processing pipelines, different sorts of errors and the magnitude of these errors are likely associated with different datasets. Thus, as pointed out by Meyer et al. (2008), it could be risky to compare the results from different deep sequencing studies directly. Therefore, it was recommended to introduce a set of standard quality screening and control procedures and data processing pipelines, such as the pipeline we described in Chapter 4, in future studies to improve data consistence and facilitate comparative analysis of different studies.

Pyrosequencing profiling was not designed for quantification purpose because it has inherent bias that makes it impossible to translate the frequency of a sequence into relative abundance of the bacterium that sequence represents. FISH and qPCR (Rajilić-
Stojanović et al., 2009) are quantitative tools, but they do not support comprehensive analysis of multiple target bacteria simultaneously. The PITChip we developed is the first phylochip dedicated to the analysis of poultry intestinal microbiomes. By providing 57% coverage to the consensus sequences and 83% coverage of the representative sequences of the global diversity of poultry intestinal microbiomes, the PITChip can support comprehensive analysis of multiple target bacteria at both OTU and genus levels (Chapter 5). As more sequences become available and longer in length (particularly pyrosequencing reads), new and improved probes can be added to the PITChip in the future.

Litter management is believed to be an effective strategy to modulate chicken intestinal microbiome. The application of the PITChip to the analysis of the samples of ileal mucosa and cecal content identified more bacteria that were impacted by fresh litter and reused litter than the study that used clone libraries. The core chicken microbiome was also identified that may play essential roles in the gut of poultry. On the other hand, the variable microbiome responding to litter conditions may be further investigated as to their effect on host health and growth. By the same token, the PITChip can be used to screen for potential targets of interest under different dietary conditions, at different growth performance, and the identified target bacteria can be further investigated using quantitative techniques (e.g., qPCR) with respect to their importance and correlation to host nutrition and health. Therefore, the PITChip may find broad applications in future
studies that require comprehensive and quantitative analysis of intestinal microbiome of poultry.

Enteric pathogens in chicken and turkeys not only affect host performance, but also pose serious health risk to humans. Antimicrobial growth promoters (AGPs) have been widely used by the poultry industry to control the proliferation of enteric pathogens and enhance chicken performance. Non-antimicrobial alternative approaches are sought after to control enteric pathogens due to increased restriction on AGPs. Our quantitative study on the effect of litter management and dietary bacitracin (Chapter 6) showed that bacitracin is effective in reducing abundance of Gram-positive *C. perfringens*, but it has no effect on *Salmonella*. A surprising finding was increased abundance of *Salmonella* correlated to decreased abundance of *C. perfringens*. Although further studies are needed to confirm this negative correlation, this finding has important implication because salmonellosis has been a major health risk. The finding that reused litter tended to delay colonization of ileal mucosa by *C. perfringens* and to reduce the abundance of *Salmonella* in the cecal content in the absence of bacitracin also has important implication. Reused litter may serve as a ‘natural’ source of competitive exclusion cultures that can reduce colonization of the gut in young chicks by *C. perfringens*. A better understanding of the effects of litter management and bacitracin on chicken intestinal microbiome can be achieved by using metagenomic and PITChip analysis in future studies.
A better understanding of the intestinal microbiome of chickens and turkeys is needed to further improve nutrient utilization and host health, especially when use of AGPs is under increasing scrutiny and tight regulation. The series of investigations reported in this dissertation advanced our knowledge and understanding of poultry intestinal microbiome, while the PITChip developed may be very useful in future mechanistic studies that investigate the interactions of the intestinal microbiome with host, diet, and environmental conditions.


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Appendix: Supplemental data
A1. Rarefaction profiles of poultry gut origin 16S rRNA gene sequences
A2. Comparison of chicken and turkey intestinal microbiomes. Number of OTUs of each microbiome was normalized. Genera represented by > 5 OTU0.03s were displayed and the size of each node reflects the total number of OTUs. The relative proportion of the OTU0.03s from each microbiome was shown by different colors: red, chicken cecal content; blue, turkey cecal content; green, chicken ileal mucosa; yellow, turkey ileal mucosa.