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Investigation of Microbiota in Health and Disease of Poultry

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Investigation of Microbiota in Health and Disease of Poultry

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ABSTRACT

The microbiotas play vital roles in health and diseases of both humans and animals. 16S rRNA genes sequence analysis is one of the most popular and commonly used methods in the analysis of microbiotas associated with hosts. In this dissertation, the microbiotas of chickens (broilers, breeders, and layers) and turkeys were evaluated by 16S rRNA gene sequencing. Characterization of the culturable subpopulations of *Lactobacillus* in the chicken gut can serve as a valuable resource for probiotic development. In Chapter 2, *Lactobacillus* subpopulations recovered on MRS from chicken gut were defined comprehensively for the first time using 16S rRNA gene profiling, where they varied with different regions (cecum vs. ileum) and locations (lumen vs. mucosa) with in the same region. In Chapter 3, we investigated the effect of cell densities as determined by varying levels of sample dilution on the culture-enriched microbiota profiles using MRS agar medium as a model system. The dilution levels of original samples was found to alter the resulting culture-enriched microbiota profiles via unknown density-dependent mechanisms. In chapter 4, *Bacillus* isolates (*B. subtilis* and *B. amyloliquefaciens*) were used to evaluate their therapeutic and prophylactic effects against *Salmonella* Enteritidis, and found their potentialities to reduce *S.* Enteritidis colonization and improve the intestinal health in broiler chickens possibly through altering the composition and functions of gut microbiota. In chapter 5, we investigated the cecal microbiota and egg production in two strains of Hy-Line (Brown and W-36) housed in conventional cages (CC) and enriched colony cages (EC), and noticed differences in egg production and cecal microbiota between strains and housing types. In chapter 6, we performed a comprehensive survey of the litter microbiotas using booty swab samples in the 5 commercial turkey farms of the Northwest Arkansas. The litter microbiotas were found to differ between farms, and flocks which were further affected by the ages of turkeys. In Chapter 7, we

developed and evaluated the nested TaqMan probe based qPCR assay for the quantitative detection of *Clostridium septicum* that targets the alpha toxin gene (*csa*).

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DEDICATION

I would like to dedicate this dissertation to my beloved parents; Mrs. Laxmi Devi Adhikari and late Mr. Basanta Adhikari, whose continuous selfless love, support, and encouragement helped me to achieve this degree.

TABLE OF CONTENTS

PUBLISHED, SUBMITTED, AND IN-PREPARATION PAPERS

Chapter two is from:

Adhikari, B., and Kwon, Y. M. (2017). Characterization of the Culturable Subpopulations of *Lactobacillus* in the Chicken Intestinal Tract as a Resource for Probiotic Development. *Frontiers in Microbiology* 8, 1389. doi: 10.3389/fmicb.2017.01389

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Chapter seven is from:

Adhikari, B., Tellez-Isaias, G., and Kwon, Y. M. Development of real time PCR assay for quantitative detection of *Clostridium septicum*. In preparation.

CHAPTER ONE

1.1 Review of Literature

1.1.1 Common Terminologies Used in the Microbial Community Analysis

After the initiation of the Human Microbiome Project (HMP) in 2007 (Turnbaugh *et al*., 2007), intensive researches were focused on gut microbiome, and it is now widely accepted that the gut microbiome affects health and physiology of mammalian hosts through their various roles in nutrition, immunology, gut development, and regulation of host physiology. Microbiome studies have significantly increased nowadays because of the decrease in the cost of sequencing and advancement in computational methods.

There are different terminologies used in microbiome studies and sometimes people used those terms interchangeably, although there are differences. In 2015, Marchesi and Ravel described the terms such as microbiome, microbiota, metagenome, and metagenomics, and emphasized the need of uniform use of those vocabulary in the microbiome research (Marchesi and Ravel, 2015).

The term microbiota, which was first defined by Lederberg and McCray (Lederberg and McCray, 2001), is the collection of microorganisms existing in a defined environment. When microbial community is analyzed through amplifications and sequencing of certain marker genes such as 16S ribosomal RNA (rRNA) genes, 18S rRNA genes, or other marker genes and genomic regions, this should be termed as microbiota. In contrary, the term microbiome denotes to both biotic and abiotic factors and includes the microorganisms (bacteria, viruses, archaea, and eukaryotes), their genomes, and the environmental conditions. On the other hand, metagenome

refers to the assemblage of genes and genomes of microbiota. Thus, the term metagenome highlights the genetic capacity and potentials of the microorganisms, while the term microbiome emphasizes both genes and genomes of the microbiota, their products and the host environment which is characterized through one or combinations of approaches such as metagenomics, metabolomics, metatranscriptomics, and metaproteomics in combination with clinical or environmental metadata (Marchesi and Ravel, 2015).

Alpha diversity is defined as a diversity within a sample or community. Richness and evenness are two main factors that need to be taken account for calculating alpha diversity of samples (Kim *et al*., 2017). Richness measures number of different species present in a sample, whereas evenness measures the relative abundance of different species present in a sample. Thus, evenness compares how uniformly different species are distributed within a sample. Beta diversity refers to the diversity among different samples or communities. It is used to compare the diversity among samples based on the distance or dis (similarity) between each sample pair (Kim *et al*., 2017).

1.1.2 Status and Limitations of the Current Research on Animal Gut Microbiota

Almost any metazoan, either invertebrates or vertebrates harbor gut microbiota (Lee and Hase, 2014). Previously, around 10^{14} bacteria was estimated to be present in the alimentary tract of the human (Luckey, 1972), and the ratio of total bacteria to the total number of somatic and germ cells present in human was estimated to be 10:1. However, recent study shows the variations in gut bacterial number from 10^{7} (Stomach, Duodenum, and Jejunum) to 10^{14} (Colon), and estimates the ratio of total bacteria to total number of human cells as ~1:1 (Sender *et al*., 2016). The human genome contains around 20,000 genes (Turnbaugh *et al*., 2007) whereas around 3.3

million non-redundant genes are found to be present in metagenome of the gastrointestinal tract. More than 99% of these genes belongs to 1,000 to 1,150 different bacterial species (Qin *et al*., 2010) representing diverse and complex human gut microbiota. Like human, different animals have also abundant and diverse gut microbiotas. Based on sequencing of 16S rRNA gene, around 375 unique OTUs were reported in pig gastrointestinal tract (Leser *et al*., 2002), and around 613 OTUs were reported in the rumen of cows (Kong *et al*., 2010). In addition, 915 and 464 OTUs have been described in chicken and turkey, respectively (Wei *et al*., 2013).

Traditionally, gut microbiota composition was studied using culture-dependent methods. Since most of the bacterial species in gut (around 80%) are unculturable (Eckburg *et al*., 2005), culture dependent methods cannot provide comprehensive information on gut microbiota composition. Recently, microbiome studies (16S rRNA gene profiling) have been increased along with the development and application of speedy and cost-effective sequencing platforms like Roche 454 pyrosequencing and Illumina MiSeq/HiSeq (Guinane and Cotter, 2013). For taxonomic classification of gut microbiota, 16S rRNA gene has been most frequently targeted because of its universal presence in all prokaryotes, and variable regions. However, due to the limited resolution of 16S rRNA gene based microbiome profiling method, need of genome-wide approaches to characterize intraspecies strains diversity in human have been recently described (Ellegaard and Engel, 2016).

In recent years, studies focusing on gut microbiome have been increased in livestock like chicken, pig, and cattle. However, they are very less as compared to human, and mainly based on 16S rRNA based profiling method (Kim *et al*., 2011; Isaacson and Kim, 2012; Yeoman *et al*., 2012; Waite and Taylor, 2015). Beside livestock, microbiome of various wildlife species like black howler monkey, red and giant panda, koala, and Tasmania devil have been recently studied (Xue

et al., 2015; Amato *et al*., 2013; Kong *et al*., 2014; Alfano *et al*., 2015; Cheng *et al*; 2015). In addition to terrestrial animals, gut microbiome studies have also been extended to both marine and fresh water aquatic species (King *et al*., 2012; Lyons *et al*., 2016). Regarding animal model, the laboratory mice are most commonly used in order to study the impacts of gut microbiota on physiology, and the development of diseases on host (Clavel *et al*., 2016). However, invertebrates like drosophila, and honeybee have also been gaining popularity as a model for gut microbiota since their gut microbiota are less complex, and greater coverage of all microbiota can be assessed through sequencing of metagenome samples (Ellegaard and Engel, 2016).

Based on the above information, we can say that several studies related to gut microbiota have been conducted in a wide range of animals with the objectives of either identifying their own gut microbiome and their various roles in host, or as a model animal to get valuable information for human gut microbiota. Initially, more researches were focused on characterization of microbiota throughout various regions and locations of the gastrointestinal tract of the animals (Yeoman and White, 2014). Nowadays, researchers are more concerned to investigate different factors that can affect microbiome of animals in order to address their differences between ecosystems, species, and/or populations (Bahrndorff *et al*., 2016). Host genetics, diet, environmental exposure, and health have already been identified as some of the contributing factors for microbiome evolution (Yeoman *et al*., 2011). However, limited number of studies have been conducted to investigate the role of gut microbiota on animal's health as compared to human. Similarly, most of the researches on animal's microbiota are based on 16S rRNA gene sequencing, and there are very few based on metagenomics approaches (Yeoman *et al*., 2012) as compared to human. Because of the limited resolution of 16S rRNA sequencing method, mostly information of animal's gut microbiota is limited to genus level.

1.1.3 Characterization of Microbial Community by 16S rRNA Genes Analysis

The 16S rRNA gene sequence was used by Carl R. Woese and George E. Fox in 1977 for the first time in phylogenetic studies, which proposed the eubacteria, archaebacterial, and ukaryotes as three important aboriginal lines of descent (Woese and Fox, 1977). Sequence analysis of 16S rRNA genes is the most commonly used method for the study of microbial community residing in the host. All the procedures that involve in the 16S rRNA genes sequence analysis can be broadly categorized into two steps; 1. Activities that are carried out in the lab for library preparation and sequencing and 2. Computational work for sequence data analysis.

1.1.3.1 Library Preparation and Sequencing

After the proper experimental design and completion of the experiment, the samples need to be collected aseptically, brought to the lab maintaining cold temperature or using preservatives and processed immediately or stored at -20 °C/-80 °C depending upon the time of analysis. It is very important to follow proper preservation methods as they can impact stability (Song *et al*., 2016) and eventually gut microbiota profiling (Zhao *et al*., 2011; Chen *et al*., 2019), although the effect is small as compared to the DNA extraction methods (Costea *et al*., 2017). On contrary, the composition and diversity of stool microbiota were not affected significantly after preservation for 3 or 7 days at four different temperatures (-80, 7, 22, and 37°C) either in dry or RNAlater® (Al *et al*., 2018). A wide range of commercial kits are available for the extraction of microbial genomic DNA. Depending upon the sample types, judicial selection of DNA extraction kits is strongly recommended because the DNA extraction methods have significant effects on microbiota composition and diversity (Costea *et al*., 2017). Moreover, inclusion of mechanical disruption step such as bead beating is also desirable for more comprehensive profiling of gut microbiota (Lim *et*

al., 2018). Likewise, extraction of DNA from samples should be carried out with a negative control at each time, since the reagents and laboratory contamination impact both 16S rRNA gene sequence and shotgun metagenomics analysis to a greater extent (Salter *et al*., 2014).

Since 16S rRNA gene sequence analysis is polymerase chain reaction (PCR) based method, it is necessary to either design the new primer sets based on the 16S rRNA gene sequences or use the previously designed primers available elsewhere. Nine hypervariable regions (V1-V9) are found in bacterial 16S rRNA genes, which contain substantial sequence variations among different bacterial species and can be used for their identification (Van de Peer *et al*., 1996). The 16S rRNA gene is around 1,550 base pairs and also contain well-conserved regions between variable regions and thus allows designing primers that target the hypervariable regions (Clarridge, 2004). Various primers that amplify the different variable regions of 16S rRNA gene were already developed and used in the study of composition and diversity of microbial community (Baker *et al*., 2003; Clarridge, 2004; Chakravorty *et al*., 2007; Klindworth *et al*., 2013; Barb *et al*., 2016). Six different primers sets that target V2, V3, V4, V6-7, V8, and V9 regions of 16S rRNA gene were compared using mock samples and reported variations on the performance of primers for the proper identification of bacterial family and genus of mock communities (Barb *et al*., 2016). Among these, primers sets that target V2, V4, and V6-7 gave the lowest divergence, while primer set that target V9 produced the highest divergence as compared to the mock samples.

The primers should be designed in such a way that they can amplify most bacterial 16S rRNA gene sequences ("universal primers") and allow maximum phylogenetic resolution (Fuks *et al*., 2018). However, none of the primer pairs were perfect and universal, and thus right primer pairs should be selected to avoid accumulative bias (Klindaworth *et al*., 2013). For sequencing of large number of samples in a single run, PCR is performed using primer sets that contain unique

barcodes on both forward and reverse primers or only in one primer followed by pooling samples together at equimolar concentration (Multiplexing). Several library preparation protocols for high throughput next generation sequencing such as "MiSeq Wet Lab SOP" (Kozich *et al*., 2013) and "Earth Microbiome Project 16S Illumina Amplicon Protocol" (Thompson *et al*., 2017) are readily available online. In our projects, previously described dual index primers (27F and 533R) that target the V1-V3 regions of 16S rRNA gene were used (Mandal *et al*., 2016; Adhikari and Kwon, 2017), in addition, single index primer sets 515F (Parada *et al*., 2016) and 806R (Apprill *et al*., 2015) that target V4 region of 16S rRNA gene were also used. Chapters two, three, and four are based on the dual index primers, while chapters 5 and 6 are based on single index primers.

Minor changes during library preparation, sequencing procedures and platforms, and sequence analysis can significantly alter the results which demands the proper use of quality controls and standard operating procedures throughout laboratories (Hiergeist *et al*., 2016; Sinha *et al*., 2017; Bender *et al*., 2018). In each run of PCR, negative control should be included like in DNA extraction steps and all negative controls should be sequenced along with samples for the purpose of quality control. In addition, mock sample that contain the known microbial communities should be included in each sequencing run and its analysis. Illumina is the most commonly used sequencing platform for 16S rRNA genes analysis, however, other DNA sequencing platforms such as 454 pyrosequencing, Ion Torrent and Pacific Biosciences were also widely used.

1.1.3.2 Computational Analysis of 16S rRNA Gene Sequences

1.1.3.2.1 Quality Filtering and Preprocessing of Reads

Approximately 20-25 million paired end reads are obtained from a single Illumina MiSeq run using MiSeq reagent kits v3. The sequence reads are in FASTQ format whose quality needs to be checked using algorithms such as FastQC (Andrews, 2010) before further processing of reads. The adaptor and primer sequences should be removed using NGS read preprocessing tools such as Cutadapt (Martin, 2011) followed by trimming and filtering of low quality reads using tools such as Trimmomatic (Bolger *et al*., 2014). Further processing and analysis of amplicon reads can be done either independently or using established software. Among different options, QIIME1 (Caporaso *et al*., 2010), which has been now succeeded by QIIME2 (Bolyen *et al*., 2018), and Mothur (Schloss *et al*., 2009) are the two most popular software that contain comprehensive packages of tools and algorithms necessary for the thorough analysis of amplicon reads. Another important step is the removal of chimeric sequences since chimeric sequences are formed during PCR which can contribute to false identification of taxa and inflated estimation of sample alpha diversity (Haas *et al*., 2011). Some of the commonly used tools for chimeric detection of 16S rRNA sequences include DECIPHER (Erik *et al*., 2011), USEARCH (Edgar, 2010), and VSERACH (Rognes *et al*., 2016).

1.1.3.2.2 Taxonomic Composition Analysis

Further analysis of amplicon data starts with construction of operation taxonomic units (OTUs) by clustering of reads that differ by less than certain percentage of dissimilarity, which is most commonly 3% (Westcott and Schloss, 2015; Kopylova *et al*., 2016). Although the OTUs based methods have still been used, new methods including DADA2 (Callahan *et al*., 2016),

UNOISE2 (Edgar, 2016), and Deblur (Amir *et al*., 2017) have recently been developed that can distinguish amplicon sequence variants (ASVs) differing by a single nucleotide. The ASV based methods remove Illumina amplicon sequencing errors, and moreover, ASVs are reusable through studies, reproducible and are not restricted by incomplete reference database as compared to the OTUs. Therefore, it is argued that these methods should replace OTUs based methods (Callahan *et al*., 2017). Furthermore, these methods can perform quality filtering and chimera detection while clustering the unique sequences in the reads. Figure 1 shows simple illustration of OTUs and ASVs based clustering.

After the generation of OTUs table or ASV feature table (containing ASVs and their counts), those OTUs or ASVs should be assigned into different levels of taxonomy using different pairs of the classifiers and databases. For taxonomic assignment of the OTUs or ASVs, QIIME1 use UCLUST clustering method by default (Edgar, 2010), while QIIME2 use a naïve Bayes classifier (Bokulich *et al*., 2018). In addition, Mothur uses the naïve Bayesian RDP classifier (Wang *et al*., 2007) for the taxonomic assignments of OTUs. These classifier use different reference database such as Greengenes (McDonald *et al*., 2012), NCBI (Federhen, 2012), RDP (Cole *et al*., 2013), or SILVA (Yilmaz *et al*., 2013) for taxonomic classification of query sequences. Following taxonomic assignments, microbial taxa at different levels of taxonomy can be summarized and statistical analysis is performed using various methods such as Univariate statistics (t-test/ANOVA or Mannn-Whitney/Kruskal-Wallis test) metagenomoeSeq (Paulson *et al*., 2013), edgeR (Robinson *et al*., 2009), DESeq2 (Love *et al*., 2014), ANCOM (Mandal *et al*., 2015), LEfSe (Segata *et al*., 2011), and Random Forest (Breiman *et al*., 2001) to identify important features or taxa differentially present among different groups. Since the sequencing data contain high level of systematic variability which can reduce the statistical power and introduce false

positive, it is highly desirable to rarefy and/or normalize the OUT/feature table before summarization of the taxa, calculation of diversity, and any statistical comparisons. Alternatively, several normalization methods have been developed and compared (Pereira *et al*., 2018) including cumulative sum scaling (Paulson *et al*., 2013) and rarefaction (McMurdie *et al*., 2013).

1.1.3.2.3 Microbial Diversity Analysis

1.1.3.2.3.1 Alpha Diversity Analysis

The microbial diversity within a sample or community is called alpha diversity. Richness and evenness are two important factors that need to be considered for calculating alpha diversity of samples (Kim *et al*., 2017). Richness measures number of different species present in a sample, whereas evenness measures the relative abundance of different species present in a sample. Thus, evenness compares how uniformly different species are distributed with in a sample. Normally, alpha diversity is calculated as a certain numerical value for each sample. In 16S rRNA genes analysis, alpha diversity is usually calculated at OTUs or ASVs level. Commonly used metrics for calculating alpha diversity are described below.

Chao1

Chao1 is a nonparametric estimator of total species richness in a sample (Chao, 1984). It is also called a qualitative metric because it only consider presence or absence of species rather than the frequencies of each species in a sample (Lozupone and Knight, 2008). However, it considers the frequency of singletons (species having only one count) and doubletons (species having only two count) to incorporate information of rare species in a sample. Thus, it is simply calculated by adding frequency of rare species on the number of observed species by the equation shown below:

$$S_{Chao1} = S_{obs} + F_1 (F_1 - 1)/ 2(F_2 + 1)$$

where S_{obs} refers to the observed species and F_1 and F_2 refers to the frequencies of singletons and doubletons, respectively.

Shannon Index (H′)

Shannon Index is a quantitative diversity metric which measures both species richness and evenness. Thus, it accounts for both the number of species and their frequencies present in a given sample or community. Although, it estimates both species richness and evenness, it provides more emphasis on species richness (Kim *et al*., 2017). The value increases along with the increase in number of species and their evenness distribution in a sample, and higher value of Shannon index indicates higher diversity (Lemos *et al*., 2011). It is calculated by the following formula:

$$H' = -\sum_{i=1}^{s} (p_i \ln p_i)$$

Where *s* refers to the number of OTUs and p_i refers to the proportion of the community associated with OTUi.

Simpson Index (D)

Like Shannon index, Simpson index also measures the richness and evenness present in a sample or community. However, it gives more weightage to the evenness than richness (Kim *et al*., 2017). The sample or community having equal abundance of most species are considered to be more even. In contrary to evenness, dominance refers to those highly abundant species present in a sample or community. Simpson index refers the probability of choosing two individuals from same species randomly, and thus indicates species dominance (Lemos *et al*., 2011). Its value

ranges from 0 to 1, where higher value indicates lower diversity and vice versa. It is calculated by the following formula:

$$D = 1/\sum_{i=1}^{s} p_i^2$$

Where *s* refers to the number of OTUs and p_i refers to the proportion of the community associated with OTUi (Simpson, 1949; Kim *et al*., 2017). The data needs to be normalized before calculating both Shannon and Simpson's index to avoid biasness due to variation of sequences among samples.

Phylogenetic Diversity (PD)

Phylogenetic diversity (PD) has been defined as "the minimum total length of all the phylogenetic branches required to span a given set of taxa on the phylogenetic tree" (Faith, 1992; Faith and Baker, 2007). It is based on phylogenetic differences among different taxa and thus, it accounts for an evolutionary history of taxa. Higher value of PD indicates higher diversity.

In sum, different metrics based on species richness, evenness, or phylogenetic relationship have been used to calculate certain value as a measure of alpha diversity which is summarized in table 1.

1.1.3.2.3.2 Beta Diversity Analysis

Beta diversity refers to the diversity among different samples or communities. It is used to compare the diversity among samples based on the distance or dis (similarity) between each sample pair. While comparing more than two samples, it is calculated for each pair and create a distance/dissimilarity matrix. Simply, it can be calculated based on the overlapping taxa/OTUs by the equation $\beta = (n_1 - c) + n_2 - c)$, where n_1 and n_2 represents the number of taxa in samples 1 and 2 respectively, and c represent the shared taxa between them (Morgan and Huttenhower,

2012). However, there are different beta diversity metrics available which are broadly divided based on two main categories: a) either phylogenetic (eg. UniFrac metrics) or non-phylogenetic/species based (eg. Bray-Curtis and Jaccard index), b) either quantitative (using sequences abundance, eg. weighted UniFrac and Bray-Curtis) or qualitative (based on presence or absence of sequences, eg. unweighted UniFrac and Jaccard index), as described earlier (Goodrich *et al*., 2014). Some of the commonly used beta diversity metrics in microbiome study are described below.

Bray-Curtis Index

It is a non-phylogenetic statistical method that measures compositional dissimilarity of different samples or communities, based on their sequences counts. This method is an abundance based method which was developed by J. Roger Bray and John T. Curtis in 1957 (Bay and Curtis, 1957). It is commonly used as either similarity or dissimilarity index (1-similarity index). It is a modified version of Sørensen index by including additional abundance information (Chao *et al*., 2006). The Bray-Curtis dissimilarity value ranges from 0 to 1 where 0 stands for no difference in species composition and 1 stands for complete difference in species composition between two communities. The Bray-Curtis dissimilarity index is calculated by the following formula.

$$\mathrm{BC}_{ij} = 1 - \frac{2C_{ij}}{S_i + S_j}$$

Where S_i and S_j are the number of species present in populations *i* and *j*, Cij is the sum of lesser values for only those common species of both sites.

Although, this method is widely used to identify compositional dissimilarity between communities, it bears large bias when sampling fractions are unequal, and thus cannot be recommended to use unless sampling fractions are equal (Chao *et al*., 2006).

Unique Fraction Metric (UniFrac)

Nowadays, Unifrac distances between communities is the most common and widely used statistical method to measure beta diversity (Lozupone *et al*., 2011). UniFrac measures a distance between microbial communities based on phylogenetic information of OTUs/taxa present in a phylogenetic tree (Lozupone and Knight, 2005). Unifrac is either weighted or unweighted.

Unweighted UniFrac

Unweighted UniFrac only considers presence or absence of an OUT/taxa in a sample or community rather than its abundance, and thus, it is a qualitative measure of beta diversity. The UniFrac distance between two communities is measured as the fraction of branch length in a phylogenetic tree that leads to members of either community but not both (Lozupone *et al*., 2011). The unweighted UniFrac has always values between 0 and 1 for identical and nonoverlapping communities, respectively. Unweighted UniFrac is more useful while comparing communities that differ primarily by what present inside them and thus, it can be better suited to detect the effects of various founding populations such as, the effect of temperature on microbial growth, sources of newborn mice gut colonization etc. (Lozupone *et al*., 2007).

Weighted Unifrac

Weighted Unifrac is a quantitative measure of beta diversity which measures weights of the branches of a phylogenetic tree based on the abundance information (Lozupone *et al*., 2007).

Thus, it considers not only which taxa or OTUs are present but also their abundance, which can be an important factor for describing community changes. While calculating weighted UniFrac, the raw value is calculated by the following equation:

$$u = \sum_{i}^{n} bi \times \left| \frac{A_i}{A_T} - \frac{B_i}{B_T} \right|$$

Where *n* represents the total number of branches in a tree and b_i represents the length of branch *i*. Likewise, A_i and B_i, represents the number of sequences from branch *i*, and A_T and B_T represents the total number of sequences, in communities A and B, respectively. In some situations such as, for correcting unequal sampling effort or difference in evolutionary rates between taxa, normalization of weighted UniFrac can be done by the average distance of members of the two communities to the root. This gives the value of normalized weighted UniFrac between 0 and 1 as unweighted UniFrac (Lozupone *et al*., 2007). Weighted UniFrac is more useful while comparing communities that differ primarily by the relative taxon abundance and thus, it can better detect the effects of transient factors such as nutrient availability where certain taxa can flourish because of the availability of limiting nutrient (Lozupone *et al*., 2007).

1.1.3.2.3.3 Principal Coordinate Analysis (PCoA)

In order to compare beta diversity in more than two samples, a distance/dis (similarity) matrix is created by comparing every pair of samples. In general, to visualize distances between *N* samples, we need *N-1* dimensions which will be hard to visualize. Thus, for better visualization of data present in beta diversity distance matrix, two or three dimensional scatter plots are created by assigning each sample a location known as PCoA plots. PCoA converts distance matrix into a new sets of orthogonal axes known as principal coordinate axes which preserve the distances of each individuals (Gower, 1966). The main difference between PCoA and principal component analysis

(PCA) is in the type of dataset they used as input. PCA uses a table containing the frequency of each phylotype observed in each sample or environment, whereas PCoA uses a dis (similarity) matrix as input (Lozupone *et al*., 2007).

Each axis has an eigenvalue whose magnitude reflects the fraction of variation in the data set explained by that axis. Each axis eigenvalue is used to calculate the proportion of variation captured by them in comparison to the total eigenvalues. Thus, the percentage in each axis of PCoA plots is defined as the percentage of variations in the data set explained by that axis. The first axis (PC1/axis 1) explains maximum amount of variation in the data set followed by PC2/axis2 and so on.

Further comparing of diversity between two groups, there are different statistical tests like Adonis (Oksanen *et al*., 2007), PERMANOVA (Anderson, 2001) and Analysis of similarities (ANOSIM) (Clarke, 1993). ANOSIM is a nonparametric tests that is used to compare the statistical significances among groups through permutations. It gives *R* and *P* values as shown in Figure 5. *R* is a test statistic whose values varies from 0 to 1, where 0 indicates no difference between groups analyzed, whereas 1 indicates complete different between groups. $P < 0.05$ indicates statistical significance.

1.1.3.2.4 Functional Prediction of 16S rRNA Gene Sequences

Metagenomic content and putative biological functions of microbial community can be predicted through linking 16S rRNA gene sequences with the available microbial genomes. For this purpose reference based OUT table is needed. There are already different tools and packages such as PanFP (Jun *et al*., 2015), PICRUSt (Langille *et al*., 2013), PAPRICA (Bowman *et al*., 2015), Piphillin (Iwai *et al*., 2016), SINAPS (Edgar, 2017), Tax4Fun (Aßhauer *et al*., 2015),

Vikodak (Nagpal *et al*., 2016) that can predict functional potentialities of marker gene data. There are different functional databases available, among them KEGG (Kanehisa *et al*., 2008) and MetaCyc (Caspi *et al*., 2006) are very popular and widely used. For statistical analysis of predicted functional profiles, STAMP (Parks *et al*., 2014) is a graphical software package that is widely use among others.

1.1.4 Research Approaches to Improve 16S rRNA Based Sequencing Resolution

In an approach to improve taxonomic resolution of 16S rRNA based amplicon sequencing, a new supervised computational method called "Oligotyping" was developed which was initially used to investigate the diversity of *Gardnerella vaginalis* (Eren *et al*., 2011), and later validated in environmental samples too (Eren *et al*., 2013). This method uses Shannon entropy as a default method to identify small but reproducible nucleotide variation within 16S rRNA gene sequences of same operational taxonomic units (OTUs), which is then used to generate oligotypes for distinguishing closely related organism beyond species level. Similarly, based on the same core principle of oligotyping, minimum entropy decomposition (MED) algorithm was developed in 2015 which is unsupervised, and don't require prior clustering, and pairwise alignment of the sequences in comparison to oligotyping (Eren *et al*., 2015). Another method, commonly known as low-error amplicon sequencing (LEASeq), was reported in 2013 (Faith *et al*., 2013) in order to demonstrate the stability of bacterial strains in human feces over time through sequencing of 16S rRNA gene. This method was based on initial tagging of template DNA at one end using diluted primer in a linear PCR extension, followed by exponential PCR. By doing so, they claimed reduction in PCR errors, and assigned taxonomy up to strain level with high precision and depth. Recently, an attempt was made to sequence nearly full length of 16S rRNA genes from human skin samples using Illumina MiSeq platform (Burke and Darling, 2016). This approach was the

modification of the previous method (Faith *et al*., 2013), and used dual tagging of template DNA at both ends instead of previous single end tagging followed by tagmentation, and amplification of both ends before sequencing and assembly of reads. This method can be robust in removing chimeras and PCR errors, however sequencing error from this method is unclear.

In addition to the above methods, single-molecule analysis technologies that can provide longer read lengths have been adopted during these days. Pacific Biosciences (PacBio) platform based on single molecule sequencing technology has been used successfully for sequencing 16S rRNA gene for few years (Fichot and Norman, 2013; Mosher *et al*., 2014; Schloss *et al*., 2016; Singer *et al*., 2016). Initially, PacBio sequencing platform possessed higher sequencing error rates, and low throughput (Fichot and Norman, 2013). Besides, increased in read length that can be sequenced by PacBio, the sequencing error rates had claimed to be low and comparable with those of other most widely used sequencing platforms, like Roche 454 and illumina's MiSeq platform (Schloss *et al*., 2016; Wagner *et al*., 2016). However, high quality reads obtained from PacBio platform was less as compared to those obtained from MiSeq platform. Recently, PacBio circular consensus sequencing was used in combination of DADA2 sequence analysis pipelines to identify full-length 16S sequence variants with near-zero error rate (Callahan *et al*., 2018). Besides PacBio, a portable MinION™ sequencing platform was developed by Oxford Nanopore Technologies (ONT) in 2014 which was also based on single-molecule analysis technology, and was used previously for sequencing complete bacterial genome (Quick *et al*., 2014). Recently, species level identification in mock community was reported using the same platform (Benítez-Páez *et al*., 2016). Although they were able to construct almost full length of 16S rRNA sequences, there is still need to improve per base accuracy and nucleotide bias. Thus, continuous efforts have been

made to improve 16S rRNA based sequencing methods to achieve longer quality read lengths for higher taxonomic resolution and functional profiling of microbiomes.

1.1.5 An introduction to Clostridial Dermatitis (Cellulitis) in Turkey

The frequency and severity of clostridial dermatitis, often called as cellulitis, has increased within the last two decades and has become a serious problem of the commercial turkey industry (Lighty *et al*., 2016).

Clostridium septicum (CS) is considered as a primary causative agent of cellulitis in commercial turkeys (Tellez *et al*., 2009). Although *C. septicum* has been reported as a primary causative agent of cellulitis in turkey, *C. perfringens*, *C. sordellii*, and *S. aureus* have also been described as potential etiological agents (Tellez *et al*., 2009; Clark *et al*., 2010; Thachil *et al*., 2010; Lighty *et al*., 2016). Unlike other diseases, cellulitis in turkey do not fulfill Koch's postulates because not all isolates of CS recovered from cellulitis lesions caused cellulitis after intravenous injection of those isolates in health turkeys. In addition, the authors weren't able to isolate CS in every filed cases of turkey cellulitis (Tellez *et al*., 2009). Various factors such as, flock type, breed, weight, litter condition, stress, and stocking density can affect the incidence of cellulitis in turkey (Clark *et al*., 2010; Huff *et al*., 2013; Lighty *et al*., 2016).

Because of limited availability of experimental data, the pathogenesis of cellulitis in turkey is still poorly understood. There is still debate among researchers regarding the involvement of "inside-out" or "outside-in" theory associated with turkey cellulitis. Through damaged intestinal wall, pathogenic Clostridia, toxin, or both can enter into blood stream, localize under skin, and produce enterotoxins causing cellulitis. In addition, Clostridia from contaminated environment can cause infection through oral route. This is called as "inside-out" theory. Alternatively, Clostridia

can enter directly through skin abrasions which is known as "outside in" theory. (Clark *et al*., 2010).

Any factors described above, can serve as stressor which can affect on intestinal permeability (Caso *et al*., 2008; Gareau *et al*., 2008) resulting localization of pathogenic *Clostridia* under skin via hematogenous route. *C. septicum* isolate was isolated from blood of asymptomatic turkey, which may suggests the possibility of hematogenous route of infection during turkey cellulitis (Neumann and Rehberger, 2009). However, "outside-in" theory also cannot be neglected and more studies should be conducted to understand the detail mechanism of pathogenesis in turkey cellulitis in future.

1.1.6 An Introduction to Food Borne Pathogens with Emphasis on *Salmonella*

There are several foodborne pathogens that are typically asymptomatic to animals, however, can cause severe illness in humans. Once these pathogens are shed in the feces, they are transmitted to animals, humans, and food products through different vectors. Centers for disease control and prevention (CDC) estimates around 48 million people become sick, out of which 128,000 gets hospitalized and 3,000 die annually from foodborne illness in the United States (CDC, 2017). A study that was published in 2011 reported 31 pathogens that are known to cause food borne illness in the United States (Scallan *et al*., 2011). Among those pathogens, Norovirus was reported to contribute highest foodborne illness while nontyphoidal *Salmonella* spp. was reported as a major causative agent for hospitalization and deaths of patients. In addition, they reported Norovirus, nontyphoidal *Salmonella* spp., *Clostridium perfringens*, *Campylobacter* spp., *Toxoplasma gondii*, and *Listeria monocytogenes* as major pathogens responsible for either illness, hospitalization or deaths. Overall health-related cost associated with food borne illness from those

pathogens was estimated to be around $51.0 and $77.7 billion based on basic and enhanced model respectively, as described earlier (Scharff, 2012).

Among different food borne pathogens, *Salmonella* is a genus of gram-negative rod-shaped bacilli associated with Enterobacteriaceae family that are facultative anaerobes, motile and non-spore formers. It was previously broadly divided into three different species: *S. typhi*, *S. cholera-suis*, and *S. enterica* (Hanes, 2003). However, recent nomenclature has divided Salmonella genus into two species: *S. bongori* and *S. enterica*, where the latter is further divided into six subspecies (Su and Chiu, 2007) which contain more than 2,500 serotypes based on O (somatic) and H (flagellar) antigens. *S. enterica* subspecies are associated with warm blooded animals whereas *S. bongori* with cold blooded animals (Tortora, 2008). *S. enterica* subsp. *enterica* contains both nontyphoidal serovars (*S*. Typhimurium and *S*. Enteritidis) and typhoidal serovars (*S*. Typhi and *S*. Paratyphi), and are mostly associated with food borne illness.

Salmonella contamination has been reported in meat and meat products of chicken, turkey, and other animal species. *Salmonella* was detected at a higher percentage in ground turkey (49.9%) and chicken (44.6%) meat. In addition, it was also detected at ready to eat meats (3.1%), ground beef (7.5%), market hogs (8.7%), steers and heifers (1%), and pasteurized eggs (14.6%) (Naugle *et al*., 2006). *Salmonella* contamination can occur at any stages of food chain from farm to table as reviewed earlier (Rajan *et al*., 2017). For instances, possible routes of contamination start from agricultural practices, primary breeder farms, broiler farms, feed production, transportation, slaughter house operation, processing plants, distribution channels etc. (Rajan *et al*., 2017).

Salmonella species are prevalent as a normal inhabitant of gastrointestinal tract (GIT) in most of the livestock species including poultry, cattle, swine and sheep (Doyle and Erickson, 2006). The typhoidal strains of *Salmonella* cause enteric fever whereas non typhoidal strains cause

food poisoning manifested by typical symptoms of gastrointestinal illness including diarrhea, fever and abdominal pain, and thus serve as most common pathogen of gastroenteritis worldwide (Chen *et al*., 2013; Gal-Mor *et al*., 2014). A study that was conducted in US based on data available from 2000-2008 and population of 2006 estimates 11% of foodborne illness contributed by non typhoidal *Salmonella* spp. Similarly, these species were found to contribute 35% and 28% respectively, among those are hospitalized and deaths, which is equivalent to approximately one million of illness, 19,000 hospitalized, and 380 deaths every year (Scallan *et al*., 2011) with an estimated cost of $4,312 and $11,086 per case based on basic and enhanced model respectively, as described earlier (Scharff, 2012). Similarly, another study conducted by Majowicz *et al*. estimated 93.8 million cases of gastroenteritis and 155,000 deaths caused by *Salmonella* species annually (Majowicz *et al*., 2010). Interestingly, 80.3 million cases were estimated to be foodborne suggesting *Salmonella* as a notorious food borne pathogen and burden to both developed and developing countries. A total of 69,663 cases of human Salmonellosis was reported in EU/EEA, 2015 by 20 serovars of *Salmonella*, where Enteritidis, Typhimurium and Monophasic Typhimurium (1,4,[5],12:i:-) were three most contributing serovars (EFSA and ECDC, 2016).

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1.3 Tables and Figures

Table 1. Summary of different metrics used for calculation of alpha diversity.

Metrics	Qualitative/ Quantitative	Other features	Limitations
Chao 1	Qualitative	Species richness	Only species richness
Shannon's index	Quantitative	Species richness and evenness	More weightage on species richness
Simpson's index	Quantitative	Species richness and eveness	More weightage on species eveness
Phylogenetic Diversity (PD)	Qualitative	Phylogeny based	Challenges to address phylogenetic tipping points, and some cases PD losses.
Other metrics like ACE, Rarefaction	Qualitative	Species richness	Only species richness

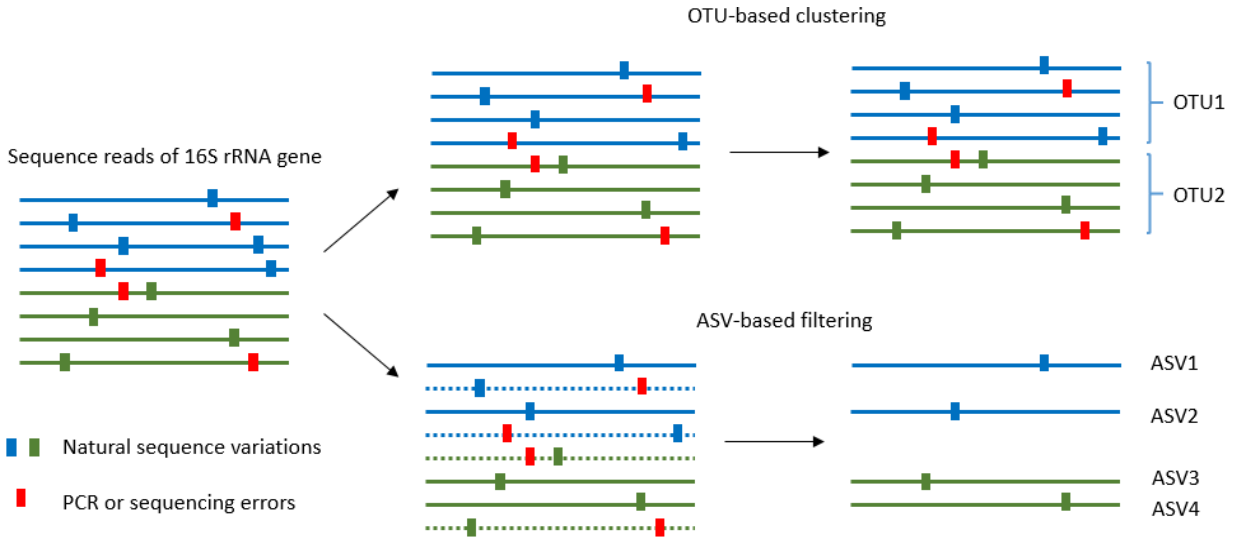

Figure 1. Illustration of the simple concept behind the generation of Operational taxonomic units (OTUs) and amplicon sequence variants (ASVs).

CHAPTER TWO

Characterization of the Culturable Subpopulations of Lactobacillus in the Chicken Intestinal Tract as a Resource for Probiotic Development

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2.1 Abstract

To gain better understanding of the distributions of the culturable *Lactobacillus* species in the chicken intestinal tract, we collected ceca, and distal ileum from 10 3-weeks-old broiler chickens. *Lactobacillus* strains from cecal lumen contents (M-CL), and those associated with mucosa of ceca (M-CM) and ileum (M-IM) were recovered on de Man, Rogosa and Sharpe (MRS) agar plates, and used for microbiota analysis. The total cecal content (T-CL) was also used directly for microbiota analysis. We purposefully focused on MRS-recovered populations to gain understanding of the culturable subpopulations of *Lactobacillus*, since the culturability is an important phenotype in order to exploit the chicken gut microbiota as a resource for development of probiotics. The V1–V3 regions of 16S rRNA gene was amplified from genomic DNA samples, and the pooled amplicons were analyzed by MiSeq sequencing with paired-end read 300 cycle option. Among MRS groups, *Firmicutes* were significantly higher in M-IM and M-CL as compared to M-CM, whereas *Proteobacteria*were significantly higher in M-CM as compared to M-IM and M-CL at $p < 0.05$. Among *Lactobacillus*, *L. salivarius* (36%) and *L. johnsonii* (21%) were higher in M-IM as compared to M-CL (*L. salivarius*, 28%; *L. johnsonii*, 15%), and M-CM (*L. salivarius*, 20%; *L. johnsonii*, 11%). *L. crispatus* was found significantly higher in M-CL as compared to M-IM ($p < 0.01$) whereas *L. gasseri* was found significantly higher in M-IM as compared to M-CM ($p < 0.05$). *L. aviarius*, and *L. fornicalis* were only observed in T-CL. In summary, *Lactobacillus* populations recovered on MRS vary with different regions and locations in chicken GIT, which might indicate their distinct functional roles in different gastrointestinal tract (GIT) niches, and some species of *Lactobacillus* are not culturable on MRS agar media. This study is the first attempt to define culturable *Lactobacillus* subpopulations in the chicken intestinal tract comprehensively using 16S rRNA gene profiling, and the findings of this study will be used

as a platform to develop a new strategy for isolation of effective *Lactobacillus* probiotic candidates based on comparative analyses of chicken gut microbiota.

Keywords: broiler, gastrointestinal tract, *Lactobacillus*, microbiota, probiotics

2.2 Introduction

Due to the increased risk associated with the development of antibiotic resistance in bacteria, the use of antibiotic growth promoters (AGPs) in animal industry has been completely banned in Europe since January 1, 2006 and has been in the process of reduction or complete elimination in several countries, including the United States (Dibner and Richards, 2005; Huyghebaert et al., 2011). The use of probiotics as an alternative to AGP has been rapidly increasing in recent years (Ahasan et al., 2015). Microbes that are commonly used as probiotics include various species of the genera *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* (Moreira et al., 2005). Although the microbial communities are distributed throughout the GIT, their composition was found heterogeneous along the different regions of GIT in chicken (Yeoman et al., 2012; Choi et al., 2014; Ranjitkar et al., 2016), pigs (Looft et al., 2014), and cattle (Mao et al., 2015). The variations in microbial composition can occur not only in different segments along GIT, but can also at different locations (lumen vs. mucosa) in the same region (Gong et al., 2002; Looft et al., 2014). Diverse groups of microbes reside in various regions and locations of the GIT and this might indicate differential functional roles they play in maintaining host health. Thus, in this study we characterized the bacterial communities across the different regions and locations of the GIT of chickens with a focus on the genus *Lactobacillus*, which have been most commonly considered for probiotics, through microbiota analysis of the bacterial cells recovered on MRS agar plates. By characterizing bacterial cells recovered on MRS agar plates, we eliminate unculturable *Lactobacillus* strains from the downstream analysis, retaining only culturable strains. If necessary, this step can be followed by identification and isolation of the species that demonstrate promising utility as probiotics based on comparative metagenomic analysis (16S rRNA gene profiling, and/or shot-gun metagenomics). For example, when a comparative

microbiota/microbiome analysis indicates particular species (or strains) as effector species (or strains), the culturability of the corresponding species can be first confirmed by the presence of corresponding DNA signatures in culture-recovered bacterial populations before any attempt can be made to isolate the target species (strains) for further evaluation as promising probiotics. It is important to note that current method for 16S rRNA gene profiling using Illumina sequencing has a limited resolution and often cannot differentiate even at a species-level, while a strain-level analysis is impossible. It is mainly due to short lengths of the target regions in 16S rRNA gene that are sequenced, and inevitable sequencing errors from PCR and sequencing step. However, with the increasing interest in exploring intra-species variations, novel methods have been developed to overcome the current limitations enabling microbiota analysis at a strain-level (Ellegaard and Engel, 2016).

Lactobacillus strains were found to enhance tight junctions, and thereby reducing intestinal permeability in both *in vitro* studies with Caco-2 cells (Anderson et al., 2010; Miyauchi et al., 2012) and *in vivo* study with mice (Xu et al., 2016). However their distribution at species level, and functional activity may differ in different regions and locations of the GIT. *Lactobacillus* strains that are tightly associated with mucosa might possess better properties as probiotics than those found in lumen, and detailed characterization of *Lactobacillus* populations in both lumen and mucosa of different regions may be very helpful in the quest for isolating good probiotic candidates. Although MRS agar is the most commonly used medium for isolation of *Lactobacillus* strains, the scope of the culturability on MRS agar for diverse *Lactobacillus* species has not been systematically evaluated. In addition, since the use of candidate *Lactobacillus* strains for probiotic applications would require the culturability of the

strains, in this study we adopted the approach of characterizing *Lactobacillus* strains recovered on MRS agar plates.

The precise identification of *Lactobacillus* isolates by phenotypic method is difficult, because phenotypic properties beyond the common fermentation tests are often required, and around 17 phenotypic tests are required to identify *Lactobacillus* at species level (Moreira et al., 2005). Only around 30% of the total vaginal and intestinal lactobacilli from humans were identified correctly at the species level by the most commonly used commercially available biochemical kit (Song et al., 1999). Alternatively, taxonomic identification of the strains belonging to genus *Lactobacillus* can be performed at species level with high accuracy based on DNA sequencing of the variable regions in 16S ribosomal RNA (16S rRNA) gene (Woo et al., 2002; Piotrowska et al., 2016).

Hence, the main aim of this study is to analyze bacterial populations recovered on MRS agar media via deep sequencing of the V1–V3 region of 16S rRNA gene in order to better understand the structure and distribution of the culturable subpopulations of *Lactobacillus*in different regions and locations of the GIT of broiler chickens.

2.3 Materials and Methods

2.3.1 Sample Collection and Processing

Cobb 500 broiler chickens were provided *ad libitum* access to water and an antibiotic-free corn-soybean meal diet. At the age of 3 weeks, 10 birds were humanely sacrificed, and ceca and distal end of ileum (5 cm) were aseptically collected according to the animal use protocol approved by the IACUC committee at the University of Arkansas. The age of 3 weeks was chosen because

the gut microbiota are established stably around this age (Ranjitkar et al., 2016). Cecal lumen contents were serially diluted and plated on MRS agar plates. To isolate bacteria associated with cecal mucosa or ileal mucosa, each mucosa sample was washed in sterile PBS buffer (pH 7.4) after removing luminal contents for four times, and homogenized in 20 ml PBS using Bullet Blender® (Next Advance). The supernatant was collected, serially diluted, and plated on MRS agar plates. The MRS agar plates were incubated overnight at 37°C under microaerophilic condition. Bacterial pellets were recovered from MRS plates with lowest dilutions (1 plate per sample) by resuspending all colonies in 5 ml PBS followed by centrifugation. The lowest dilutions were used to maximize the number of colonies collected for each sample: 10-fold dilution was used for M-CL and the supernatant without dilution was for M-CM and M-IM. The average $\log_{10}$CFUs per sample (mean ± standard error) was 6.02 ± 0.18, 3.71 ± 0.18, and 3.23 ± 0.21 for M-CL, M-CM, and M-IM samples, respectively.

2.3.2 DNA Extraction and PCR

Genomic DNA was extracted from each pellet (equal amount) by using DNeasy Blood and Tissue Kit (Qiagen). Genomic DNA of total bacteria in cecal lumen was also extracted directly without culturing on MRS plates using QIAamp Fast DNA Stool Minikit (Qiagen). Thus, we had altogether 40 genomic DNA samples: 10 MRS-recovered cells from each of cecal lumen (M-CL), cecal mucosa (M-CM), and ileal mucosa (M-IM), and 10 total bacterial cells from cecal lumen (T-CL). The V1–V3 region of the 16S rRNA gene was amplified from the genomic DNA samples using barcode-tagged universal primers; 27F (5′-AGRGTTYGATYMTGGCTCAG-3′) and 533R (5′-TTACCGCGGCTGCTGGCAC-3′) with attached Illumina adapters. Details regarding primers, enzymes, and PCR conditions were previously described (Mandal et al., 2016). The amplicons were purified from 0.7% agarose gel electrophoresis after verifying the length of

amplicons. After the concentration of each amplicon sample was measured using Qubit dsDNA broad range assay kit (Life Technologies, United States), the amplicons were pooled at an equal amount. The pooled sample was gel-purified from 6% TBE gel (Invitrogen, United States), and sent for Illumina sequencing at the University of California (Riverside, CA, United States) using MiSeq paired-end reads with 300 cycles.

2.3.3 Data Analysis

All MiSeq paired-end sequence reads were analyzed by Quantitative Insights into Microbial Ecology, QIIME version 1.9.1 (available at http://qiime.sourceforge.net/; Caporaso et al., 2010). General pipelines for data analysis was previously described in details (Mandal et al., 2016). Forward and reverse ends sequences were joined together by using join_paired_ends.py command followed by formatting barcodes using customized Perl script, before extracting barcodes using extract_barcodes.py option. Demultiplexing and quality filtering were performed by split_libraries_fastq.py with default options. OTU picking was performed by using reference sequences from NCBI RefSeq 16S RNA database (O'Leary et al., 2016) and Swarm algorithm (Mahé et al., 2014). Taxonomic classification was performed by using reference taxonomy file from NCBI RefSeq 16S RNA sequences and SortMeRNA algorithm (Kopylova et al., 2012). NCBI RefSeq 16S RNA sequences are curated, non-redundant and quality controlled (Pruitt et al., 2007; O'Leary et al., 2016). We used this database instead of greengenes database for better taxonomic assignment at species level. Cumulative sum scaling (CSS) method with QIIME was used to normalize the OTU BIOM (biological observation matrix) before taxonomic assignment and alpha diversity calculation. Beta diversity estimates were calculated by using beta_diversity_through_plots.py options of QIIME with even sampling depth of 8000. Analysis of similarities (ANOSIM) between groups were performed using unweighted UniFrac distance

metric (compare_categories.py, QIIME). Statistical significance in alpha diversity indices and different taxa among various groups were measured using one-way analysis of variance (ANOVA) followed by *post hoc* Student's *t*-test using JMP Genomics 7 software.

2.4 Results

After demultiplexing and quality filtering, there was 1,350,414 assembled sequence reads ranging from 444 to 574 bp with median sequence length 546 bp. Summarizing raw vs. CSS normalized otu biom table resulted in mean sample depth of 33,760.35 ± 3,311.22 and 1,488 ± 11.72 reads per sample, respectively. CSS normalized otu biom table was used further for taxonomy assignment and alpha diversity analysis.

2.4.1 Taxonomy Assignment

2.4.1.1 Phylum Level

Taxonomic analysis among MRS groups revealed *Firmicutes* (83.83%) as the predominant phylum followed by *Proteobacteria* (13.83%). *Firmicutes* were found significantly higher in cecal lumen (M-CL) and ileal mucosa (M-IM) as compared to cecal mucosa (M-CM) at $p < 0.05$ (Figure 1), but there was no significant difference between M-CL and M-IM. On the contrary, *Proteobacteria* were found significantly higher in M-CM as compared to M-IM and M-CL at $p < 0.05$ (Figure 1).

2.4.1.2 Genus Level

Relative abundance of different genera recovered from MRS groups (≥1% of all MRS groups) is shown in Figure 2. *Lactobacillus*, *Enterococcus*, and *Citrobacter* were the major

predominant genera recovered from MRS groups. *Lactobacillus* was observed significantly higher in M-IM and M-CL as compared to M-CM ($p < 0.01$), whereas *Citrobacter* was significantly higher in M-CM as compared to M-IM ($p < 0.05$). Although *Lactobacillus* was predominant genus in each MRS group, recovery of other genera demonstrated that MRS agar medium also supports the growth of the strains belonging to *Enterococcus* and *Citrobacter*.

2.4.1.3 Species Level

Among the major *Lactobacillus* species identified, relative abundance of *L. salivarius* was highest in all three groups followed by *L. johnsonii*. Both *L. salivarius* (36%) and *L. johnsonii* (21%) were higher in M-IM as compared to M-CL (*L. salivarius*, 28%; *L. johnsonii*, 15%) and M-CM (*L. salivarius*, 20%; *L. johnsonii*, 11%) as shown in Figure 3. *L. crispatus* was found higher in M-CL as compared to M-CM and M-IM, but significant difference was found only between M-CL and M-IM ($p < 0.01$). Similarly, *L. gasseri* was found significantly higher in M-IM as compared to M-CM ($p < 0.05$).

2.4.1.4 OTU Heatmap at Species Level

The OTU heatmap that consists of only *Lactobacillus* species, constructed with QIIME, revealed that *L. aviarius* and *L. fornicalis* were detected only from the total bacterial group (T-CL) as shown in Figure 4. Although these species were found only in a subset of T-CL samples, their relative abundance was significantly high as indicated by the green colors. Some other *Lactobacillus* species such as *L. aviarius*, *L. equigenerosi*, *L. agilis*, *L. gallinarum*, *L. satsumensis*, and *L. capillatus* were also detected in negligible amounts, in only one or two samples of the total bacterial group or MRS groups, which may be due to the errors during PCR or Illumina sequencing step.

2.4.2 Alpha Diversity

The observed OTUs ranged from 20 to 71 for all samples together. The alpha diversity measured with observed OTU metric was not significantly different among M-CL, M-CM, and M-IM. But as expected, the alpha diversity was significantly higher in the samples for which genomic DNA was directly isolated from total bacteria (T-CL) as compared to the samples recovered from MRS medium at $p < 0.01$ as shown in Figure 5.

2.4.3 Beta Diversity

Unweighted unifrac distance metric was used to calculate ANOSIM. ANOSIM results showed that there were significant differences in bacterial community structure among different groups (M-CL, M-CM, M-IM, and T-CL; $R = 0.67$, $p = 0.001$) as illustrated in PCoA plot in Figure 6A. Similarly, the difference in bacterial community structure was observed among the groups of samples isolated from MRS medium (M-CL, M-CM, and M-IM; $R = 0.13$, $p = 0.01$) as shown in Figure 6B, and also between cecal and ileum mucosal samples ($R = 0.18$, $p = 0.02$) as shown in Figure 6C.

2.5 Discussion

Although the use of different species of *Lactobacillus* as probiotics in chickens has shown beneficial effects (Zhang et al., 2007; Mappley et al., 2013; Saint-Cyr et al., 2017), there is still a lack of solid scientific basis for probiotic actions, and thus effective strategies to isolate promising probiotic strains. Comprehensive investigation of *Lactobacillus* populations in chicken GIT might provide important insights for better understanding of their roles in host function, and therefore for development of better screening strategies to identify more effective probiotic strains.

Comprehensive characterization of chicken gut microbiota through the use of high throughput next generation sequencing (HT-NGS) has been limited as compared to human gut microbiota (Shaufi et al., 2015). It has already been reported that the relative abundance of *Lactobacillus* varies among different segments of the GIT in chickens (Gong et al., 2007; Ranjitkar et al., 2016) using culture independent method. Only one study reported the analysis of mucosa associated microbiota in chicken GIT via high-throughput sequencing of 16S rRNA gene sequences (Gong et al., 2007). Thus, there is very limited information available regarding topological differences of *Lactobacillus* population found in chicken GIT.

Gong et al. (2002) reported differences in bacterial populations between lumen and mucosa of chicken caeca through terminal restriction fragment length polymorphism (T-RFLP). The 16S rRNA gene-based analysis of mucosa-associated bacterial populations in chicken GIT revealed *Lactobacillus* as a predominant genera in upper GIT where *L. salivarius* and *L. aviarius* were predominant species in genus *Lactobacillus* (Gong et al., 2007). Similarly previous studies reported *Lactobacillus* species higher in ileum than cecum (Ranjitkar et al., 2016; Wang et al., 2016). We also noticed higher percentage of *L. salivarius* and *L. johnsonii* in ileal mucosa as compared to cecal lumen and cecal mucosa, albeit there was no significant differences among them. This is in agreement with our findings at phylum level where *Firmicutes* were higher in ileal mucosa as compared to cecal lumen and cecal mucosa, but significant difference was observed only between ileal mucosa and cecal mucosa. Observation of other genera that do not belong to lactic acid bacteria (LAB), such as *Citrobacter* and *Bacillus*, among the MRS groups suggests the limited selectivity of MRS agar for LAB strains as demonstrated earlier (Hartemink and Rombouts, 1999; Quartieri et al., 2016). Our report on the limited selectivity of MRS agar should be considered carefully when MRS agar is used as a means to estimate CFUs of LAB strains

present in animal GIT samples. We reported *L. salivarius* to be a predominant species in all regions and locations of the GIT, which is in agreement with recent studies in chickens that reported higher percentage of *L. salivarius* in both cecum and ileum at the age of 36 (Ranjitkar et al., 2016), and at ileal mucosa at the age of 35 (Wang et al., 2016). These recent findings are in agreement with the previous reports that *L. salivarius* are consistently detected in older birds (Knarreborg et al., 2002; Guan et al., 2003). In this study, *L. crispatus* was found significantly higher in cecal lumen than ileal mucosa whereas *L. gasseri* was found significantly higher in ileal mucosa as compared to cecal mucosa. *L. crispatus* can be found in vertebrate GIT and is a *Lactobacillus* species frequently isolated from human vaginal tract (Witkin et al., 2007; El Aila et al., 2009). However, we should consider different factors including age, diet, litter type, horizontal gene transfer, chicken type, geography, climate, environment, feed additive, etc. before direct comparison of the present study with other findings, since these factors can affect chicken GIT microbiota (Qu et al., 2008; Danzeisen et al., 2011; Wang et al., 2016).

We observed *L. aviarius* and *L. fornicalis* only in total bacterial group. Failure to recover these species from MRS agar may be due to the followings reasons; these species either (i) require strictly anaerobic condition (*L. aviarius*), or (ii) grow well under anaerobic condition although being facultative anaerobic (*L. fornicalis*) as compared to microaerophilic condition at 37°C, which was used in this study (Fujisawa et al., 1984; Dicks et al., 2000; Baele et al., 2003). Alternatively, some of these species may have unique metabolic requirements that are not provided in MRS media. Observation of significantly higher alpha and beta diversity in total bacterial group (T-CL) as compared to MRS groups is obvious. Among the MRS groups (M-CL, M-CM, and M-IM), alpha diversity was observed higher in cecal lumen followed by cecal mucosa and ileal mucosa, although there was no significant difference. This was in agreement with ANOSIM results

which showed differences in bacterial community structure among different MRS groups. Thus results from both alpha and beta diversity revealed difference in bacterial diversity between cecum and ileum, which is similar with the previous findings (Shaufi et al., 2015; Ranjitkar et al., 2016).

In summary, *L. salivarius* was found as a dominant species in all three regions of the GIT. Relative abundance of *Lactobacillus* not only varied with different regions of the GIT but also varied between lumen and mucosa of the same region. All the *Lactobacillus* species present in chicken GIT samples may not be cultured on MRS agar media. Analysis of alpha diversity and beta diversity revealed differences in the structure of MRS-recovered bacterial communities among different regions and locations of the GIT.

To our knowledge, in most studies to isolate effective probiotics in poultry as well as in other food-producing animals, the first step is isolation of strains that belong to the target taxonomic group (e.g., *Lactobacillus* genus), followed by a screening of the strains for various desirable phenotypes, including resistance to acidic pH or bile acid, ability to inhibit the growth of pathogenic bacteria *in vitro*, and particular enzymatic properties among others (Asghar et al., 2016; Kizerwetter-Świda and Binek, 2016). However, this approach has the following inherent limitations: (1) the screening is conducted with randomly picked strains from a large pool of bacterial strains, (2) the number of strains screened is critically limited due to the labor and time required for the process, and (3) the suitability of the screening criteria for *in vivo* efficacy remains questionable (Morelli, 2000). For these reasons, this current approach remains ineffective, limiting our ability to exploit the gut microbiota as a rich resource for development of more effective probiotics.

On the other hand, the use of culture-independent approaches (16S rRNA gene profiling, and shot-gun metagenome analysis) have provided new insights on the function of gut microbiota in overall body functions (Singh et al., 2014; Choi et al., 2015; Yan et al., 2017), and are expected to reveal some core members of gut microbiota that play crucial roles in promoting gut health and thus growth performance in poultry. For example, Stanley et al. (2016) attempted to identify probiotic candidates for broilers based on their association with desirable productivity outcomes using microbiota analyses. Although the lack of consistency in the microbial shifts across the three animal trials was shown as a major challenge for this effort, this new approach demonstrated in Stanley et al. (2016) has a great potential for identification of effective probiotics. On the other hand, Buffie et al. (2015) identified *Clostridium scindens* as a species associated with resistance to *C. difficile*gut colonization in both mice and humans using comparative microbiota analysis and mathematical modeling, and experimentally demonstrated that oral administration of *C. scindens* significantly enhanced resistance to *C. difficile* colonization in mice.

In the study by Buffie et al. (2015) the use of the *C. scindens* strain originated from different source was successful in demonstrating the probiotic efficacy, suggesting that the genetic capacity conferring resistance is probably well-conserved within the *C. scindens*species. However, an increasing body of studies are pointing to the fact that intra-species variations on genetic capacity is quite common (Greenblum et al., 2015). In some cases, different strains from the same species can act in an opposite manner as previously reported by Fåk and Bäckhed (2012) that *L. reuteri* ATCC PTA 4659 was linked to weight loss while *L. reuteri* L6798 was linked to weight gain in mice. These findings suggests that the probiotic candidates identified by comparative microbiota analysis should be strain-specific in some cases and thus need to be isolated from appropriate samples used for the microbiota analysis.

However, when the target species or strains are identified, the next step to isolate the strains represented by the identified signature DNA sequences (e.g., specific 16S rRNA gene sequences) would encounter multiple challenges to overcome, primarily due to the complex microbiota background from which the target strains are to be isolated. One major challenge can be the culturability of the target strains, because DNA sequence data do not provide information regarding culturability of each member of a microbiota. However, a comparative microbiota analysis between culture-recovered bacteria such as shown in our study (e.g., M-CL) and direct microbiota (e.g., T-CL) can identify the culturable members in the microbiota as illustrated in Figure 4. This information would ensure that the efforts to retrieve target strains is an achievable goal, although the practical strategies to isolate the strains based on DNA signatures still remains to be developed.

We reason that the conventional approach to isolate probiotics should move toward this new direction to fully exploit gut microbiota in poultry as a valuable resource to develop probiotics that would be more effective in positively modulating gut microbiota, thereby preventing diseases, and promoting health and growth performance in poultry. Our study is conducted on a small scale, but it is the first attempt to define MRS-recovered *Lactobacillus* subpopulations in GIT of chickens with the long-term goal of developing more effective *Lactobacillus* probiotic candidates based on system-wide comparative microbiota analyses.

2.6 Author Contributions

BA and YK designed the experiment. BA conducted the study, analyzed the data and wrote the manuscript. BA and YK revised the manuscript.

2.7 Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.8 Acknowledgements

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Figures

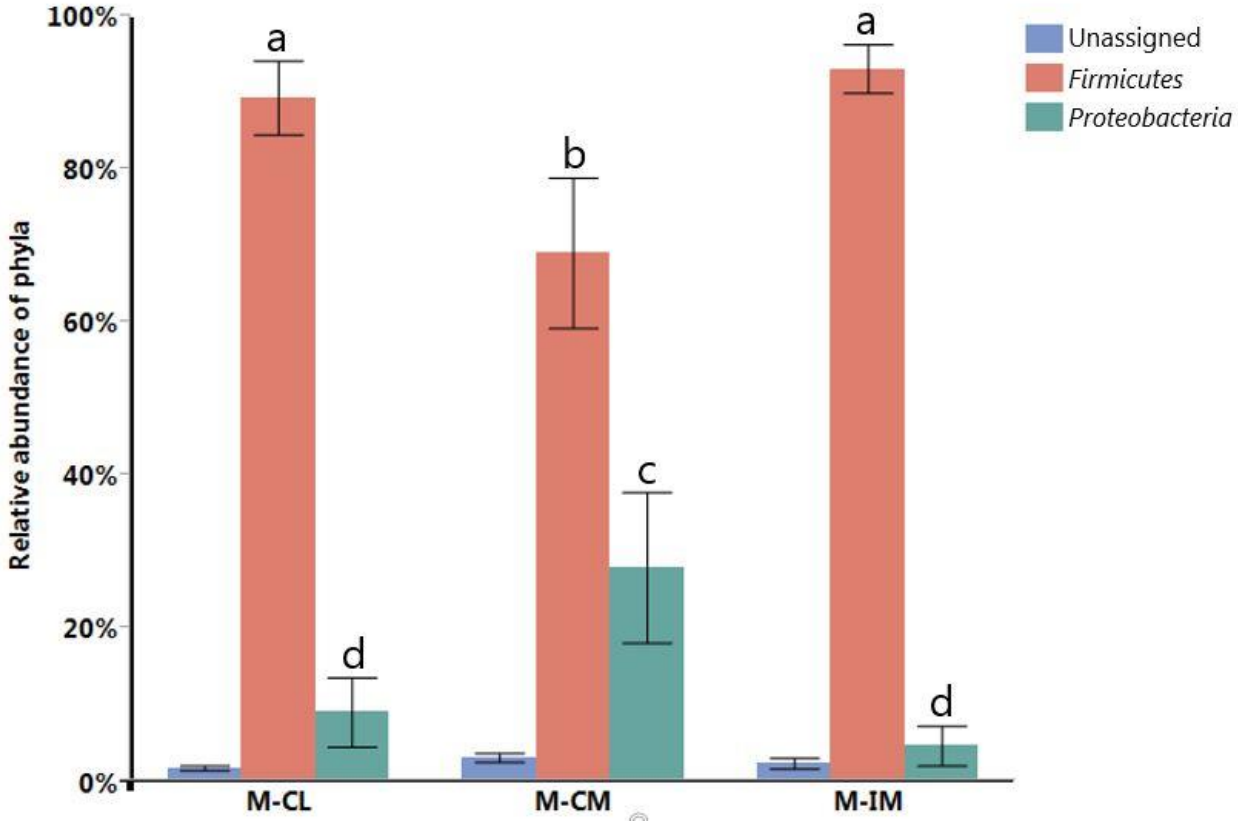

FIGURE 1. Relative abundance of different phyla. Different letters indicate significance at $p < 0.05$. Total bacterial cells from cecal lumen (T-CL). MRS-recovered cells from cecal lumen (M-CL), cecal mucosa (M-CM), and ileal mucosa (M-IM).

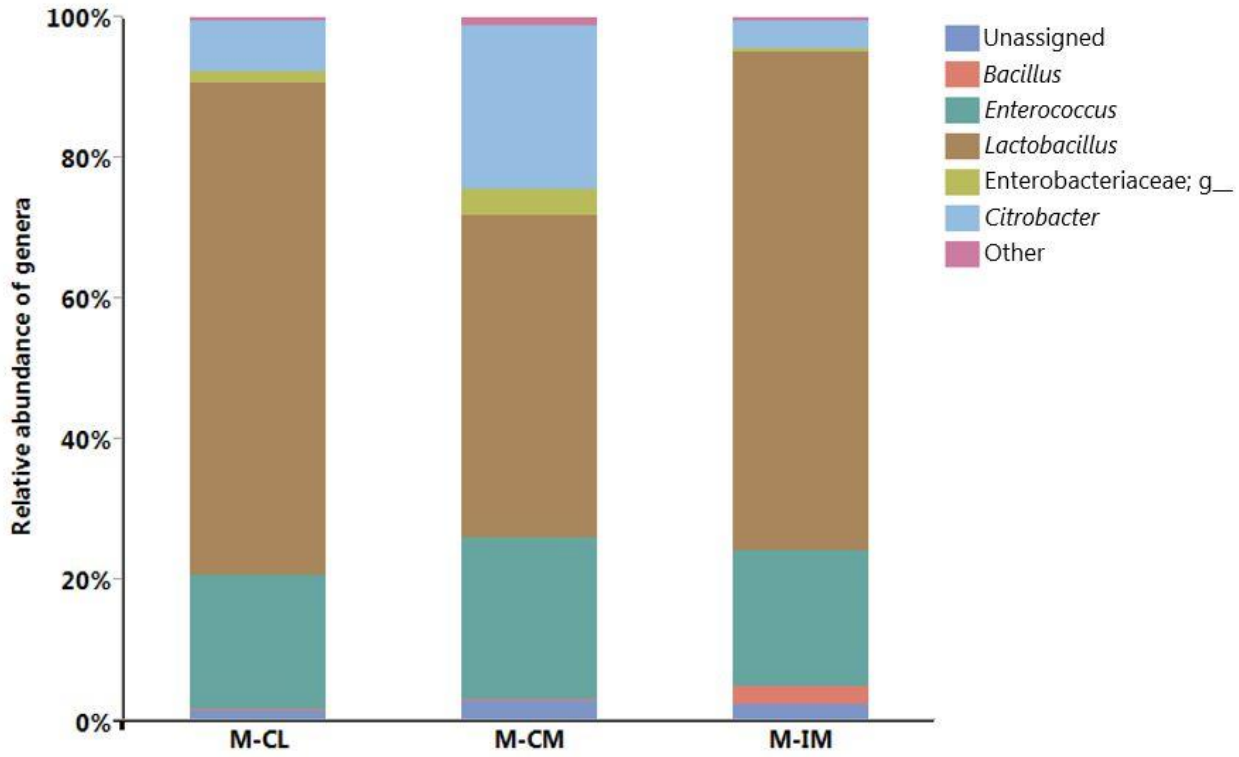

FIGURE 2. Relative abundance of different genera. MRS-recovered cells from cecal lumen (M-CL), cecal mucosa (M-CM), and ileal mucosa (M-IM).

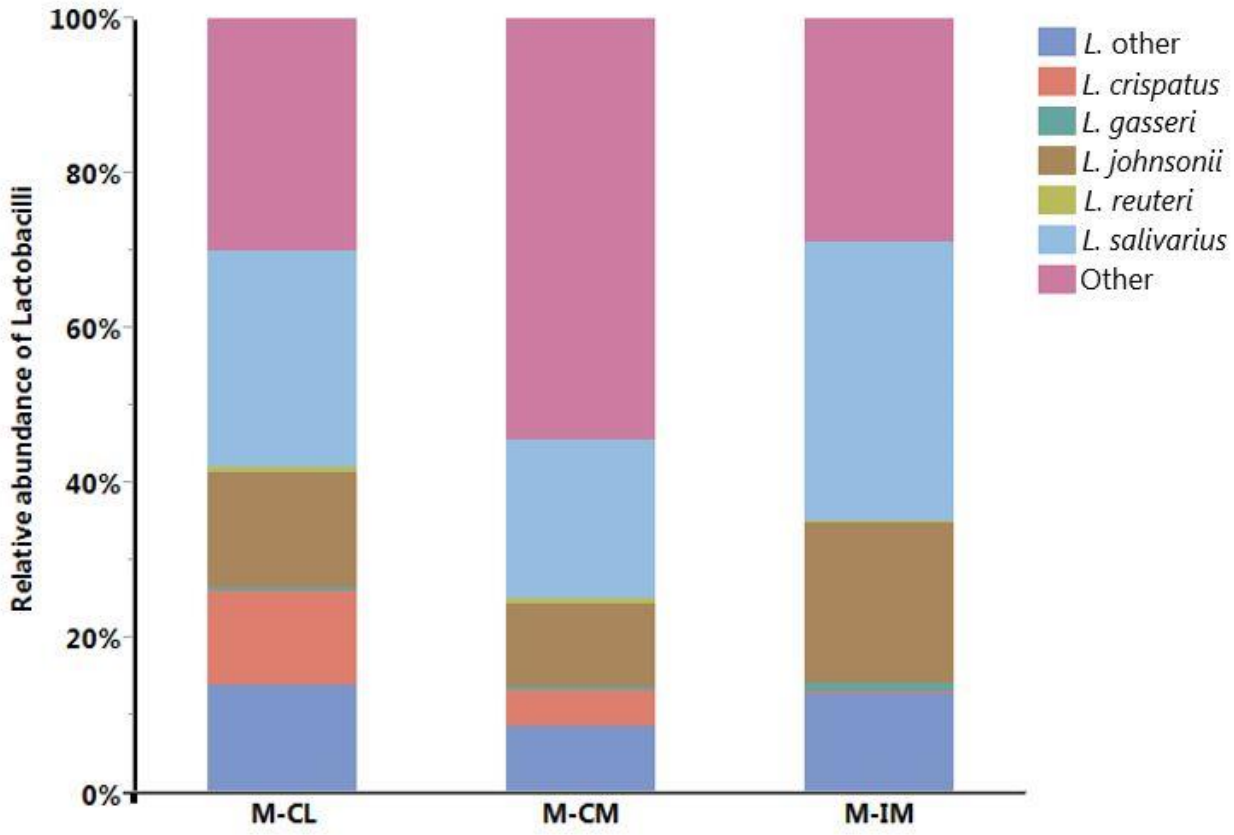

FIGURE 3. Relative abundance of different *Lactobacillus* species. MRS-recovered cells from cecal lumen (M-CL), cecal mucosa (M-CM), and ileal mucosa (M-IM).

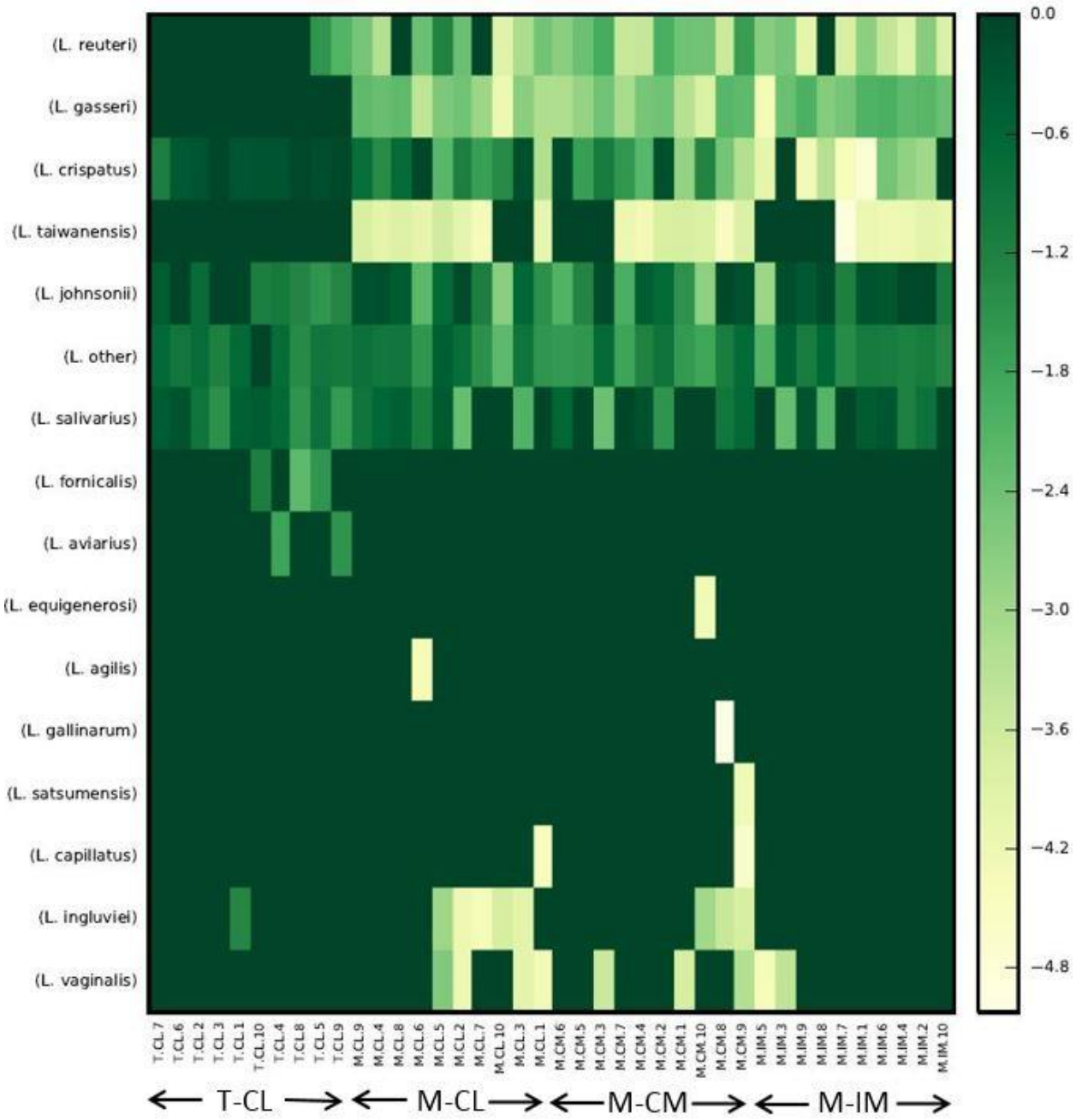

FIGURE 4. Heatmap of normalized OTU table consisting of *Lactobacillus* species only. Heatmap was constructed with make_otu_heatmap.py option of QIIME with log transformation where all zeros were set to a small value (1/2 the smallest non-zero entry), and data was translated to non-negative after log transformation, and num_otu_hits was set to 0. The abundance of *Lactobacillus* species decreases as the intensity of color decreases from green to yellow. Total bacterial cells from cecal lumen (T-CL). MRS-recovered cells from cecal lumen (M-CL), cecal mucosa (M-CM), and ileal mucosa (M-IM).

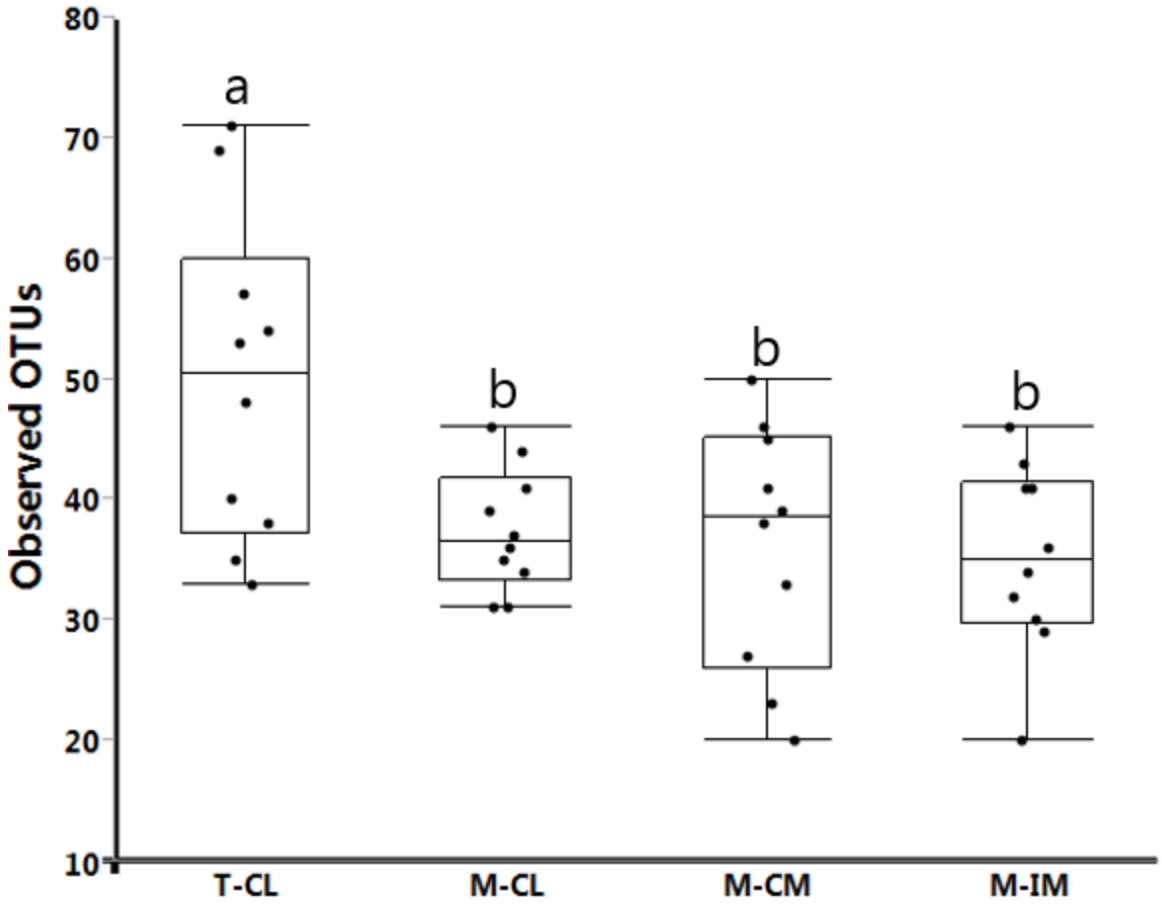

FIGURE 5. Alpha diversity in different groups measured with Observed_otus metric. Bars with different letters represent statistical significance at $p < 0.01$. Total bacterial cells from cecal lumen (T-CL). MRS-recovered cells from cecal lumen (M-CL), cecal mucosa (M-CM), and ileal mucosa (M-IM).

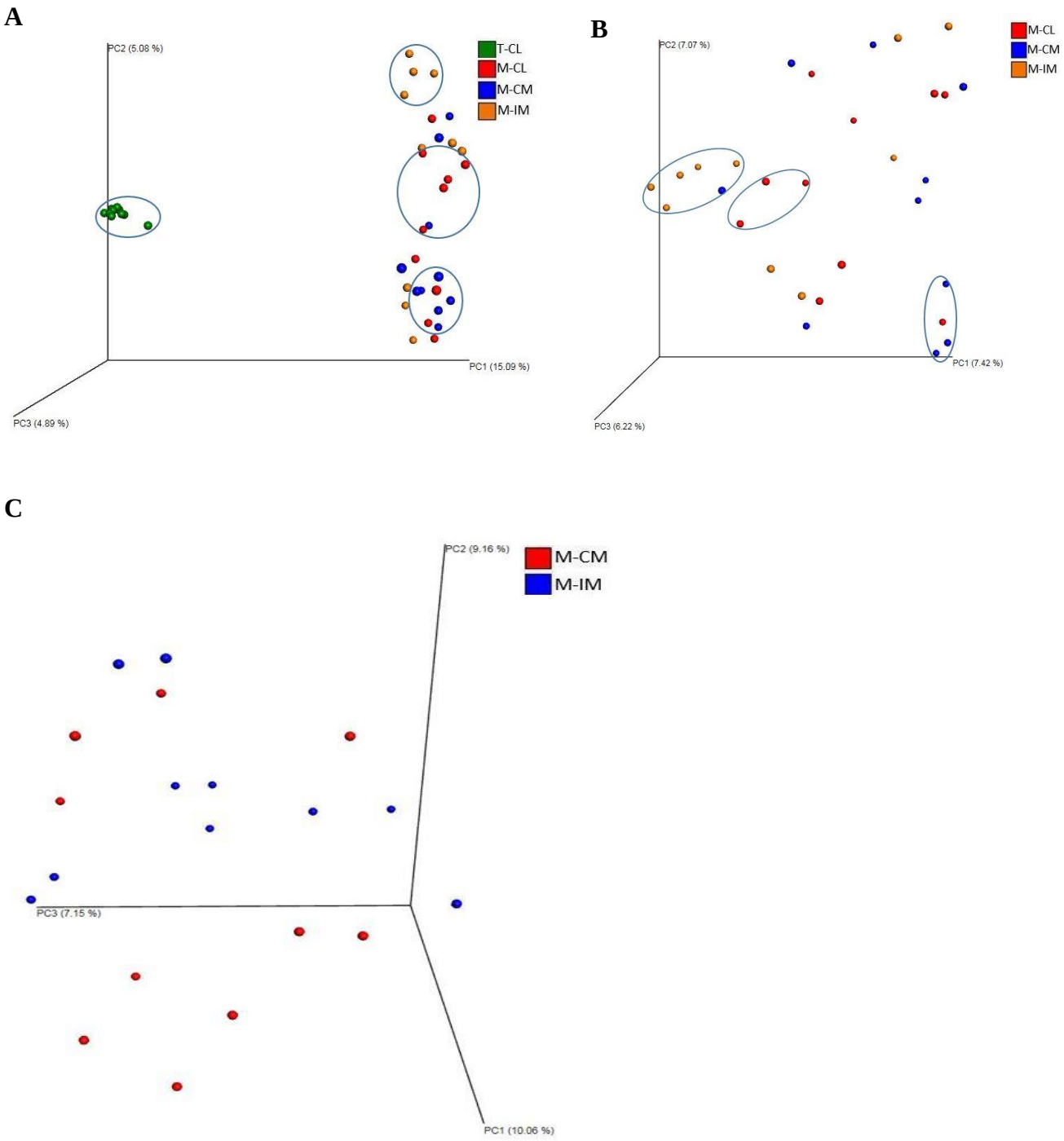

FIGURE 6. PCoA plots showing significant difference in bacterial community structure. **(A)** Among all groups analyzed; MRS-recovered cells from cecal lumen (M-CL), cecal mucosa (M-CM) and ileal mucosa (M-IM), and total bacterial cells from cecal lumen (T-CL) (R = 0.67, p = 0.001). **(B)** Among MRS groups; M-CL, M-CM, and M-IM (R = 0.13, p = 0.01). **(C)** Between two different regions of gut; M-CM and M-IM (R = 0.18, p = 0.02).

CHAPTER THREE

Cell density alters bacterial community structure in culture-enriched 16S rRNA gene microbiota profiling

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3.1 Abstract

Microbial community profiling using 16S rRNA gene has provided invaluable insights into diverse microbial communities. Recently a few studies have attempted to use 16S rRNA gene microbiota profiling in combination with the conventional culture methods to explore bacterial communities. In this "culture-enriched microbiota profiling" approach, microbes in a sample are cultured on solid media, and the resulting colonies are combined and subjected to 16S rRNA gene microbiota profiling. In this study, we investigated the effect of cell densities as determined by varying levels of sample dilution on the culture-enriched microbiota profiles using De Man, Rogosa and Sharpe (MRS) agar medium as a model system. Cecal samples collected from 10 healthy chickens were serially diluted to 10^2 fold (M-LOW), 10^4 fold (M-MEDIUM), and 10^6 fold (M-HIGH), and the dilutions were plated on MRS agar. 16S rRNA gene profiling showed that the relative abundance of certain genera showed gradual increase (*Pediococcus* and *Enterococcus*) or decrease (*Lactobacillus* and *Turicibacter*) with higher dilutions, though it was significant only for *Pediococcus* ($p<0.05$). The result indicates that the dilution levels of original samples can alter the resulting culture-enriched microbiota profiles via unknown density-dependent mechanisms, and thus should be considered for designing experiments using culture-enriched microbiota profiling.

Key words: microbiota, culture-enriched, cell density, 16S rRNA gene sequencing, MRS agar

3.2 Introduction

Studies on gut microbiota have been expanded greatly during recent years due to the increasingly common use of high-throughput sequencing for 16S rRNA gene-based microbiota profiling of gut microbiotas. Studies focused on chicken gut microbiota have also increased remarkably during these years, though they are fewer in comparison to humans and other vertebrates[1]. Similar to other species, chickens also harbor complex and diverse gut microbiota dominated by bacteria[2,3]. These diverse and complex communities of gut microbes were shown to play an important role in maintaining health, development, immune systems, and productivity of animals[4,5].

One of the goals of exploring gut microbiota in food-producing animals is to exploit the abundant bio-resources in gut microbiota and environment to promote gut health, control of enteric diseases and thus overall growth performance of the animals[6–8]. Microbiota profiling using MiSeq sequencing of 16S rRNA gene will continue to be an indispensable tool to accomplish the goal. However, some inevitable limitations in 16S rRNA gene microbiota profiling approach and the need for retrieval of cultured live bacteria for subsequent use for research purpose and as probiotics have created the need to combine culture-independent microbiota profiling approach with conventional culture methods[9,10]. This new branch in microbiomics, called "culture-enriched molecular profiling" or "culture-enriched microbiota profiling", attempts to use the culture methods to grow live microbes, which are then further analyzed by culture-independent 16S gene microbiota profiling method[11].

In the study by Sibley et al. (2011), the authors directly evaluated the cultivability of the airway microbiota by analyzing samples from 6 cystic fibrosis patients in depth using culture-enriched molecular profiling, which combines culture-based methods with the molecular profiling

methods using terminal restriction fragment length polymorphisms (T-RFLP) and 16S rRNA gene sequencing. The results of the study demonstrated that combining culture-dependent and culture-independent approaches enhances the sensitivity of either approach alone. In a more recent study by Lau et al. (2016), the similar approach was used to investigate the portions of the fecal microbiotas that were readily recovered on culture media[12]. By applying 16S rRNA gene sequencing method to culture-enriched bacteria using 66 culture conditions as well as directly to the fecal metagenomic DNA samples, they demonstrated that the majority of OTUs detected from metagenomics DNA could be detected through culture-enriched molecular profiling, and culture-enriched profiling detected greater diversity than culture-independent method[12]. The utility of the culture-enriched molecular profiling was further demonstrated by successful target culturing of the family *Lachnospiraceae* based on the microbiota profiles indicating specific growth conditions where the relative abundance of this family was significantly enriched among 66 conditions evaluated[12].

In another study employing this approach, Browned et al. (2016) studied human fecal microbiota by culturing bacteria on a broad-range agar medium, and analyzing the recovered colony populations by MiSeq sequencing of 16S rRNA gene[13]. When these culture-enriched molecular profiles were compared to those obtained directly from metagenomic DNA, there was a statistically significant correlation between the two types of profiles at the species level [13]. In another study, similar approach was used to investigate bacterial populations recovered on aerobic plate count (APC) Petrifilm and Campy-Cefex selective media[14]. Our group also previously analyzed the bacterial populations recovered on MRS agar by MiSeq sequencing of 16S rRNA gene to compare the lactic acid bacterial populations in different regions of chicken GIT[15].

On the other hand, Lagier et al. (2016) combined a culture method representing diverse growth conditions with a rapid method for taxonomic identification such as MALDI-TOF to enable high-throughput taxonomic identification of hundreds of thousands of recovered colonies. The study showed that the use of "culturomics" allowed the culture of microbes corresponding to sequences previously unidentified by comparatively analyzing the results of the metagenomic and culturomic analyses[16].

We expect this new trend in the study of microbial communities of employing conventional culture methods will continue to grow in its applications to understand and exploit gut microbiotas in humans as well as food-producing animals. From this perspective, we wanted to explore the experimental variables that might have influence on the microbiota profiles obtained from culture-enriched bacterial populations. Specifically, we were interested in cell density as determined by dilution levels of the microbiota samples as a potentially important variable in assessing the structure of culture-recovered bacterial populations. In this study, we used MRS agar medium as a simple model system to study the role of the dilution factor in the composition and structure of MRS-recovered bacterial populations originated from chicken cecal contents.

3.3 Materials and Methods

3.3.1 Cecal Sample Collection and Processing

Ten breeder hens of 32 weeks old were slaughtered humanely, and one whole cecum from each hen was collected aseptically according to the animal use protocol approved by the IACUC committee at the University of Arkansas. The cecal contents were removed, serially diluted with 1X PBS to 10^2 fold (M-LOW), 10^4 fold (M-MEDIUM), and 10^6 fold (M-HIGH) dilutions. These dilutions were plated on MRS agar plates and incubated for 24 hours under a microaerophilic

condition at 37°C. The average $\log_{10}$ colonies forming units (CFUs) per ceca recovered on MRS plates was 9.84±0.157 (mean ± standard error). There were on average 125± 27.76 CFUs/plate on M-HIGH group for 10 cecal samples.

3.3.2 DNA Extraction and PCR

Pellets recovered from MRS agar plates were used to extract genomic DNA using QIAamp DNA Mini Kit, Qiagen. In addition, DNA was also extracted from cecal contents directly without culturing using QIAamp Fast DNA Stool Minikit, Qiagen which represent total bacterial group (T-ZERO). Thus, altogether 40 DNA samples were used to amplify V1-V3 region of 16S rRNA gene using barcode-tagged universal primers: 27F (5′-AGRGTTYGATYMTGGCTCAG-3′) and 533R (5′-TTACCGCGGCTGCTGGCAC-3′) with attached Illumina adapters as described previously[15,17]. The amplicons of desired length were purified from 0.7% agarose gel and the concentration of each amplicon was measured by Qubit^{R} DNA broad range assay kit (Invitrogen^{TM}, USA). The amplicons were pooled together by mixing in an equal amount, purified from 6% TBE gel, and sent for MiSeq sequencing at the University of California (Riverside, CA, USA) with paired-end read 300 cycle option.

3.3.3 Data Analysis

Quantitative Insights into Microbial Ecology, QIIME version 1.9.1 was used to analyze the MiSeq Illumina paired- end reads[18]. After joining two ends by join_paired_ends.py script, barcodes were formatted using customized Perl script and extracted using extract_barcodes.py script of QIIME. Quality filtering and demultiplexing were performed by split_libraries_fastq.py option of QIIME with default option. Reference sequences and taxonomy file from NCBI RefSeq 16S RNA database were used for picking operational taxonomic unit (OTU)[19] and taxonomic

classification using closed OTU picking options of QIIME (pick_closed_reference_otus.py). Since closed OTU picking method was used which keeps only those sequences that are present in reference database (curated and chimera checked), we skipped the chimeric checking step. OTU BIOM (biological observation matrix) table was normalized with cumulative sum scaling (CSS) method with QIIME[20]. Beta diversity estimate was calculated by using beta_diversity_through_plots.py options of QIIME. Analysis of similarities (ANOSIM) between groups was performed using both Weighted and Unweighted UniFrac distance metrics (compare_categories.py, Qiime)[21]. Statistical significance of alpha diversity indices and different taxa among various groups were measured by using one-way analysis of variance (ANOVA) and post-hoc Tukey-Kramer HSD.

3.4 Results

3.4.1 Summary of Sequencing Analysis and Composition of Microbiotas

There were total 1,707,295 reads after demultiplexing and quality filtering whose sizes ranged from 410 to 580 bp with median sequence length of 546 bp. Summarizing OTU biom table after removing low coverage samples (<100) and CSS normalization resulted mean sample depth of 115.71±6.93 reads per sample. Taxonomic analysis among MRS selected groups revealed mainly two major phyla: *Firmicutes* (93.31%) and *Proteobacteria* (6.41%), where *Firmicutes* was significantly higher ($p<0.0001$) as compared to *Proteobacteria* in all dilution groups. However, no significant difference was observed in regards to each of both phyla among the dilution groups as shown in **Figure 1**. At genus level, there were mainly five major genera (>1%) recovered on MRS agar plates from three different dilutions as shown in **Figure 2**. Among them, *Lactobacillus* (76.16%) was dominant genera followed by *Enterococcus* (11.59%), *Citrobacter* (4.97%), *Turicibacter* (2.03%), and *Pediococcus* (1.67%). Occurrence of different genera that do not belong

to Lactic acid bacteria (LAB) suggested non-stringent selectivity of MRS agar plates, which confirms our previous observation[15]. When compared at species level, among the major *Lactobacillus* species recovered on MRS agar from different dilutions, *L. salivarius* (21.44%) was the predominant one followed by *L. agilis* (12.62%), *L. crispatus* (11.21%), *L. gasseri* (10.07%), *L. ingluviei* (6.77%), *L. johnsonii* (4.09%), and *L. saerimneri* (3.17%). Additionally, *L. helveticus* (2.75%), *L. amylovorus* (1.90%), *L. ultunensis* (0.98%), and *L. reuteri* (0.87%) were also recovered as minor members from MRS agar plates as shown in **Figure 3**. *L. salivarius* and *L. agilis* were consistently predominant across all dilutions. The detailed information of all OTUs detected in MRS dilution groups is shown in **Table S1** with their taxonomic assignment and relative abundance levels in each group.

3.4.2 Comparison of Alpha Diversity

The result of alpha diversity analysis as measured by observed OTUs metric showed that the alpha diversity was similar among the 3 MRS groups, while T-ZERO group had significantly higher alpha diversity as compared to the 3 MRS dilution groups ($p<0.05$)(**Figure S1**). The result agrees with the expectation, because only subset of bacterial species in the cecal samples can grow on MRS agar medium while T-ZERO should capture all species that are represented in the extracted metagnomic DNA.

3.4.3 Impact of the Dilution Levels on the Structure of MRS-Recovered Bacterial Communities

To investigate the effect of cell density in cecal samples as determined by dilution levels on the relative abundance of different taxonomic groups, we performed statistical analysis as summarized in **Table 1**. The relative abundance of all OTUs found in MRS groups were also determined from the directly isolated DNA samples (T-ZERO) and included in the statistical

analysis as a reference for comparison. At the phylum level, there was no significant difference in the relative abundance of either Firmicutes or Proteobacteria across directly isolated DNA (T-ZERO) and different dilution groups (M-LOW, M-MEDIUM, and M-HIGH) (**Table 1**). Although there was no statistical significance, the relative abundance of Firmicutes was consistently higher in MRS groups as compared to T-ZERO, which is largely due to the enrichment of the dominant genus *Lactobacillus* on MRS agar plates as expected. At genus level, *Turicibacter* showed the clear trend of decreasing relative abundance levels as the dilution level increased (11.8%, 3.1%, 1.9%, and 1.2% in T-ZERO, M-LOW, M-MEDIUM, and M-HIGH, respectively). In case of *Lactobacillus*, similar decreasing trend was observed with increasing dilutions among MRS groups (81.2%, 77.8%, and 70.0% in M-LOW, M-MEDIUM, and M-HIGH, respectively). On the contrary, two genera *Enterococcus* and *Pediococcus* showed increasing levels of relative abundance as the dilution increased. However, statistical difference was observed only with *Pediococcus* across the different groups ($p<0.05$). Interestingly, no *Pediococcus* was found in both T-ZERO and M-LOW, while it increased to 1.3% (M-MEDIUM) and 3.5% (M-HIGH) with higher dilutions. In addition to *Pediococcus*, other genera such as *Streptococcus* and *Bacillus* were not also recovered from the T-ZERO, while recovered on MRS groups. When the relative abundance of all LAB (*Enterococcus*, *Pediococcus*, and *Streptococcus*) excluding genus *Lactobacillus* was compared, it showed consistently increasing trends as the dilution increased ($p<0.05$). At species level focused only on the genus *Lactobacillus*, *L. johnsonii* and *L. ultunensis*, which were not detected in T-ZERO, were found at variable levels in MRS groups with no clear correlation with the dilution levels. On the contrary, *L. reuteri* present at 6.8% in T-ZERO was significantly lower or not detected among MRS groups (<0.05).

3.4.4 Comparison of Beta Diversity

To understand the difference in microbial community structure due to the sample dilution levels, we conducted beta diversity analysis (Unweighted UniFrac distance metric) using all 4 groups (**Figure S2**). As expected, T-ZERO group was clustered separately away from other MRS dilution groups. When the same analysis was conducted only for the 3 MRS groups, we observed that the different dilution samples originated from the same cecal samples were tightly clustered together for 3 samples, indicating the community structure was not altered by sample dilutions in those samples (**Figure S3**).

We reasoned that the separation of T-ZERO from MRS dilution groups (shown in **Figure S2**) could be due to the OTUs that were exclusively present in T-ZERO because they could not be cultured on MRS agar plates. Therefore, we filtered the reads in T-ZERO to retain only the OTUs that were also present in MRS dilution groups, which was then used for beta diversity analysis along with MRS dilution groups. The PCoA plot based on Weighted UniFrac distance metric showed that the separate clustering of T-ZERO disappeared and T-ZERO group shared the similar space with MRS groups (**Figure 4**). The similar PCoA plot based on Unweighted UniFrac distance metric is also shown in **Figure S4**.

3.5 Discussion

Since the 16S rRNA gene profiling by high-throughput sequencing was developed and became easily accessible to the researchers, this culture-independent method to study the bacterial communities has dominated the field of microbiota analysis[22]. This advance has greatly increased our understanding on the microbial communities from diverse environmental niches. Due to the straightforward and comprehensive nature of the approach, researchers have assumed that culture-dependent approach using deep sequencing of 16S rRNA gene can provide a comprehensive

nonbiased analysis of the complex microbial communities. However, further investigations of the microbiota profiles have revealed that 16S rRNA gene sequencing approach suffers many biases that are originated during multiple steps of the sample and data processing[23]. Other studies have shown that 16S rRNA gene profiling method failed to capture certain members of bacterial communities for various reasons, low efficiency in DNA extraction and limited coverage of the PCR primer pairs being the major ones[9,13,23].

On the other hand, recent approaches attempting to characterize microbial community in a high-throughput manner using bacterial colonies recovered on various agar media have successfully isolated novel bacterial species and spore-formers that have escaped detection by culture-independent method alone[9,12]. The culture-enriched microbiota profiling using various media was used successfully to enrich rare target bacterial species, which was on the list for the most wanted from the Human Microbiome Project (HMP)[10]. In addition, multiple studies using culturomics approach has successfully isolated numerous novel species, which remained previously uncultured members [16,24–26]. On the contrary, studies in which the microbiota profiles were compared between culture-dependent and culture-independent approaches have reported that each approach captured unique subsets of micoorganims[10,27]. Although the presence of microoganisms that are difficult to culture was predicted, detection of microorganisms only by culture in the studies was rather surprising. One plausible explanation was that a large majority of the culture only strains belong either spore formers or species with cell membranes that are difficult to lyse[9]. These studies strongly suggest that the limitation of 16S rRNA gene profiling approach can be overcome at least partially by the use of culture-enriched microbiota profiling or culturomics approaches. These studies also suggest that the research communities on microbiota analysis will increasingly use these approaches in the coming years.

In the present study, we sought to evaluate the hypothesis that the relative abundance levels of bacterial taxa in microbial communities as determined by 16S rRNA gene profiling of culture-enriched bacteria change with different levels of sample dilution. The hypothesis was built on the followings: (1) there are a number of antagonistic mechanisms operating among the bacterial cells in microbial community, including colicins, bacteriocins, contact-dependent growth inhibition systems, or type VI secretion systems among others [28], and (2) the assumption that the cell density of the samples, which in turn changes the physical distance between the cells on solid medium when plated, would influence those antagonistic interactions during formation of colonies on solid media.

In the recent studies using culture-enriched microbiota profiling, the researchers used slightly different procedures to recover the bacterial colonies to represent taxa that are recovered on a solid media in terms of the dilution levels of the original samples. For example, Browne et al. (2016) plated serial dilutions of the samples, and the lowest dilutions that allowed the growth of distinct colonies on agar plates were used to collect the colonies for microbiota profiling[13]. Rettedal et al. (2014) combined multiple dilutions (2-3 consecutive dilutions) of the human fecal samples from each media in equal proportions to better represent the bacteria capable of growing on each media, and cells were typically recovered from samples diluted 100,000 to 1,000,000-fold[10]. In our previous study, bacterial colonies recovered on MRS agar plates plated with 10-fold dilution of intestinal samples were used to perform 16S rRNA microbiota profiling. Similarly, the chicken carcass rinsates, which are similar equivalent to 10-fold dilution, were used for plating on APC Petrifilim or Campy-Cefex selective media, and the recovered colonies were used for 16S rRNA microbiota profiling[14].

The results in this study demonstrated that the levels of dilution of the chicken cecal samples plated on MRS plates changed the resulting microbiota profiles in a dilution level-dependent manner. The changes in many taxa at phylum, genus and species levels were not random, but they followed the patterns closely associated with the level of dilution, suggesting that the observed changes in relative abundance are based on cell concentration-dependent mechanisms. There are number of antagonistic mechanisms among the members in microbial communities, including colicins, bacteriocins, contact-dependent growth inhibition systems, or type VI secretion systems among others[28]. One of the clear trend observed was that the relative abundance of the genus *Lactobacillus* decreased consistently as the dilution increased, indicating the presence of concentration-dependent inhibition mechanism by *Lactobacillus* against non-Lactobacillus (**Table 1**). However, closer examination at species level revealed that the responses are dependent on specific species of *Lactobacillus*. The result in **Table 1** shows that the different *Lactobacillu*s species displayed different patterns of relative abundance in relation to varying levels of sample dilution. For example, unlike other *Lactobacillus* species, *L. reuteri* was 6.7% in T-ZERO, but was reduced significantly in all MRS-dilution groups ($p<0.05$). On the contrary, *L. johnsonii* and *L. ultunensis*, which were not detected in T-ZERO, became detectable in MRS-groups at various levels. Although the relevant explanation is lacking for these observations, future studies based on these observations will lead to the discovery of the underlying inhibitory mechanisms. It was interesting to observe that some genera such as *Pediococcus*, *Streptococcus*, and *Bacillus* were detected only in MRS groups, while undetected in T-ZERO. More interestingly, the similar observation was made for particular species of *Lactobacillus*, such as *L johnsonii* and *L. ultunensis*. The reasons for these observations are currently unknown, but they challenge some

of the assumptions we currently have regarding culture-dependent and culture-independent microbiota profiling approaches.

Since the antagonistic action would be more effective in a close physical distance, the colony growth on the plates with the samples of high cell density would be altered by the inhibition mechanisms. On the contrary, when the samples are diluted to an appropriate level the inhibitory effects would be reduced significantly or completely disappeared, leading to unhindered growth of all colonies. This line of reasoning suggests that the microbiota profiles from the samples highly diluted would resemble the profiles of the direct profiling more closely. However, the result shown **Figure 4** does not support this hypothesis in a clear way. It might be possible that the samples in M-LOW (10^2-fold diluted) were already diluted sufficiently to allow unhindered growth of the colonies.

This study was conducted in a small scale using only MRS media as a model system. Therefore, it remains to be tested if similar concentration-dependent changes of culture-enriched microbiota profiles would happen when different microbiota samples and culture conditions (e.g. media and gas atmosphere) are used. However, considering the common presence of various mechanisms of cell-to-cell interactions suggests that similar result would be expected in general when other microbiota samples are analyzed using various culture conditions. Therefore, it would be important to consider dilution factors for future studies using culture-enriched microbiota profiling approach.

3.6 Acknowledgements

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3.7 Author Contributions

BA and YK – designed the experiments; BA – performed experiments; BA – analyzed data; BA and YK – wrote the manuscript.

3.8 Competing Interests

The authors declare no competing interests.

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3.10 Tables and Figures

Table 1. Summary of the relative abundance levels of different taxonomic groups.

Level	Taxa	T-ZERO (%)	M-LOW (%)	M-MEDIUM (%)	M-HIGH (%)
Phylum	Firmicutes	(82.03±7.52)^{a}	(93.35±3.82)^{a}	(94.91±3.97)^{a}	(91.68±5.84)^{a}
	Proteobacteria	(17.97±7.52)^{a}	(6.12±3.33)^{a}	(5.09±3.97)^{a}	(7.98±5.85)^{a}
Genus	Lactic acid bacteria (LAB)				
	Lactobacillus	(69.01±6.15)^{a}	(81.21±4.48)^{a}	(77.82±5.27)^{a}	(70.01±5.07)^{a}
	Enterococcus	(1.24±1.24)^{b}	(5.62±2.31)^{ab}	(12.11±3.66)^{a}	16.37±5.64)^{a}
	Pediococcus	(0.00±0.00)^{b}	(0.00±0.00)^{b}	(1.31±0.67)^{ab}	(3.50±1.47)^{a}
	Streptococcus	(0.00±0.00)^{a}	(1.78±1.22)^{a}	(0.49±0.49)^{a}	(0.66±0.66)^{a}
	Other than LAB				
	Bacillus	(0.00±0.00)^{a}	(0.59±0.59)^{a}	(0.00±0.00)^{a}	(0.00±0.00)^{a}
	Turicibacter	(11.76±1.72)^{a}	(3.11±1.85)^{b}	(1.93±0.99)^{b}	(1.16±0.77)^{b}
	Citrobacter	(1.69±1.69)^{a}	(4.03±3.40)^{a}	(4.03±4.03)^{a}	(6.74±5.96)^{a}
	Other grouping				
	Non *Lactobacillus*	(30.99±6.15)^{a}	(18.79±4.48)^{a}	(22.18±5.27)^{a}	(29.99±5.07)^{a}
	LAB other than *Lactobacillus**	(1.24±1.24)^{c}	(7.99±2.98)^{bc}	(13.91±4.08)^{ab}	(20.52±5.24)^{a}
	Other than LAB	(29.74±6.00)^{a}	(10.80±4.80)^{b}	(8.27±3.90)^{b}	(9.47±5.76)^{b}
Species	*L. johnsonii*	(0.00±0.00)^{b}	(4.66±1.44)^{a}	(4.56±1.23)^{a}	(3.12±1.39)^{ab}
	L. reuteri	(6.76±1.77)^{a}	(1.70±0.84)^{b}	(0.00±0.00)^{b}	(1.00±1.00)^{b}
	L. salivarius	(16.38±1.56)^{b}	(18.85±1.74)^{ab}	21.66±2.13)^{ab}	(23.53±3.19)^{a}
	L. ultunensis	(0.00±0.00)^{b}	(0.64±0.64)^{ab}	(1.72±0.87)^{a}	(0.54±0.54)^{ab}

Values are presented in means ± SEM (Standard Errors of Means). Different letters across each row show statistically significance at $P<0.05$ (ANOVA, Student t-test). *L. acidophilus* only present on M-HIGH, absent in all other groups (0.33±0.33) %. Other species didn't show any significant differences among different groups.

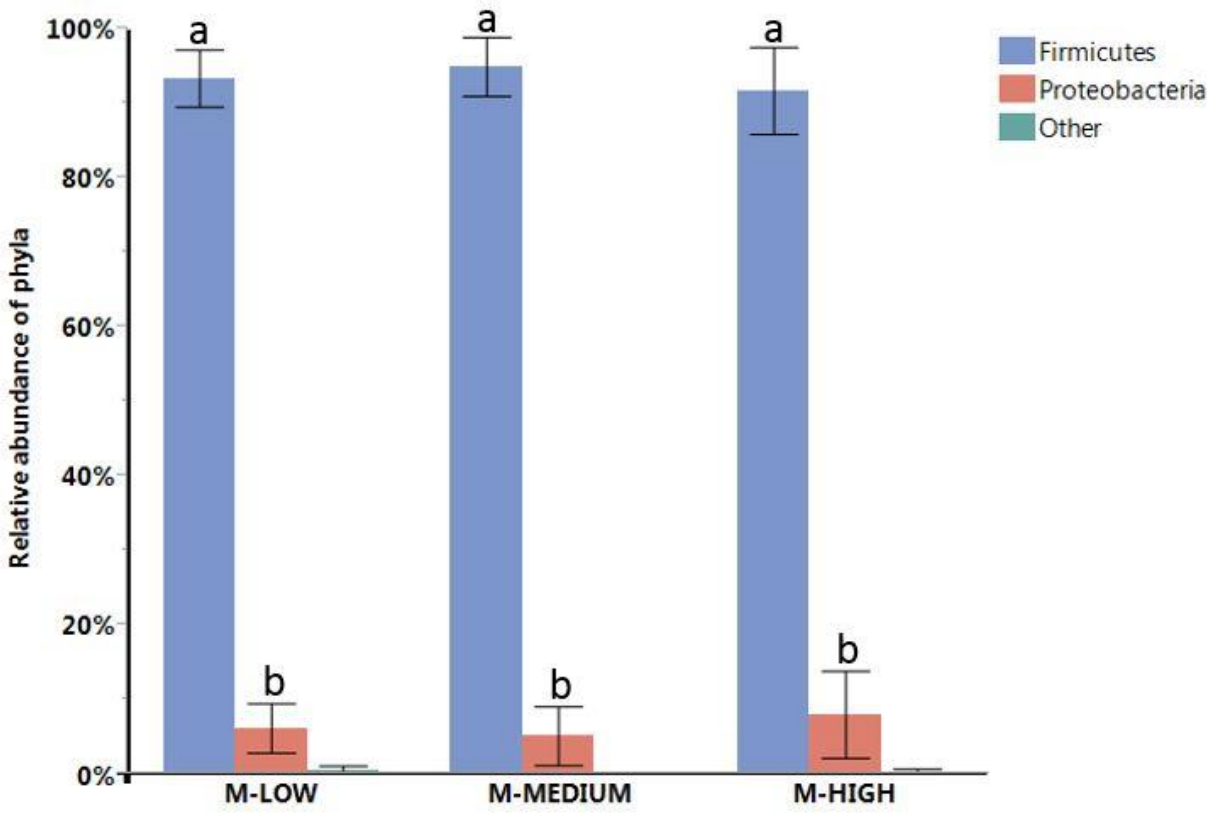

Figure 1. Relative abundance of different phyla. Different letters indicate significance at $p < 0.0001$. M-LOW, M-MEDIUM, and M-HIGH represent bacterial population recovered on MRS from 10^2, 10^4, and 10^6 fold dilutions respectively.

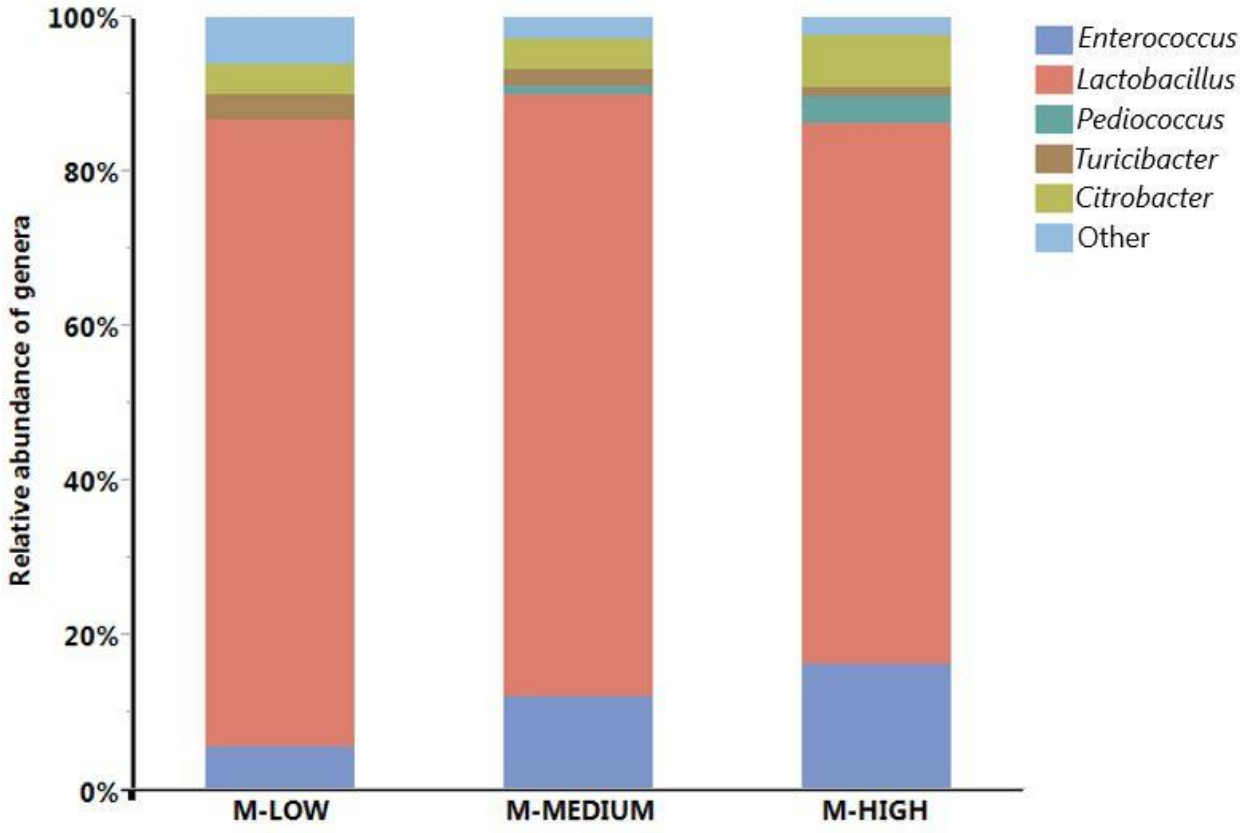

Figure 2. Relative abundance of major bacterial genera recovered on MRS plates from different dilutions. M-LOW, M-MEDIUM, and M-HIGH represent bacterial population recovered on MRS from 10^2, 10^4, and 10^6 fold dilutions respectively.

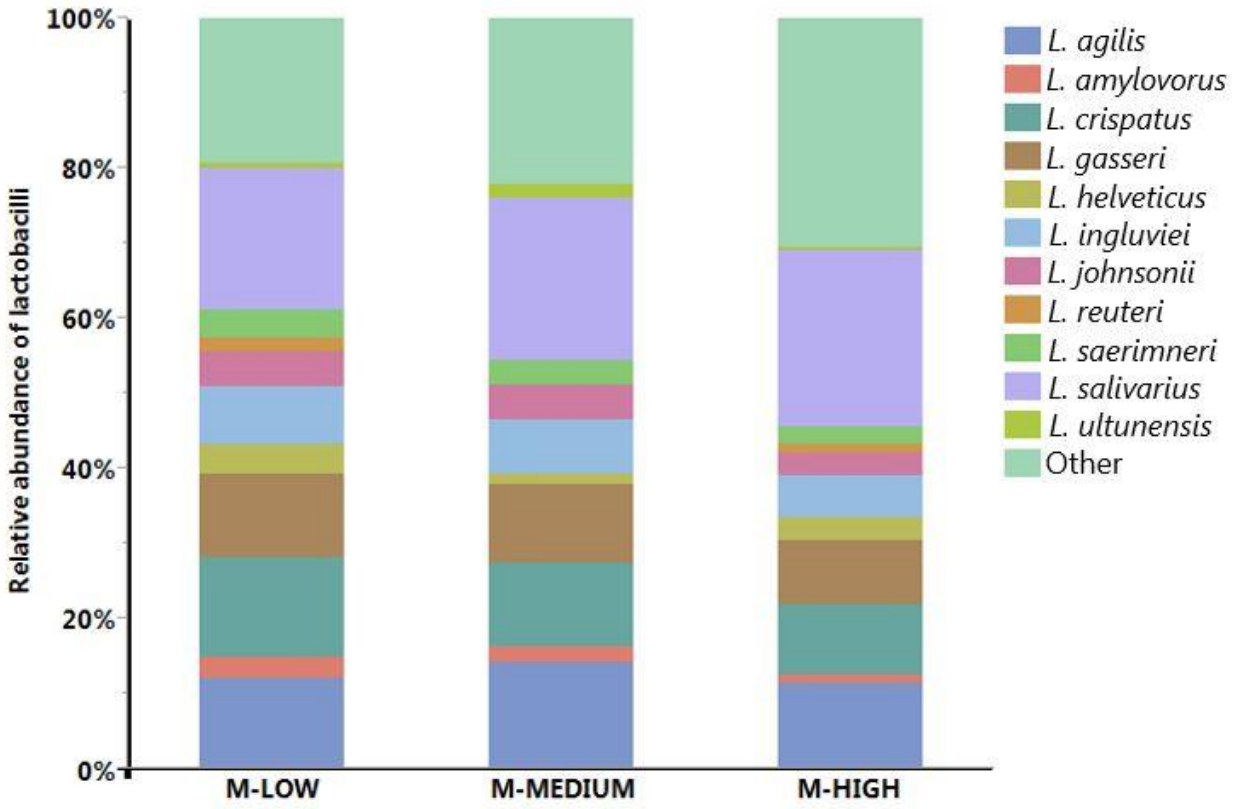

Figure 3. Relative abundance of major *Lactobacillus* species recovered on MRS plates from different dilutions. M-LOW, M-MEDIUM, and M-HIGH represent bacterial population recovered on MRS from 10^2, 10^4, and 10^6 fold dilutions respectively.

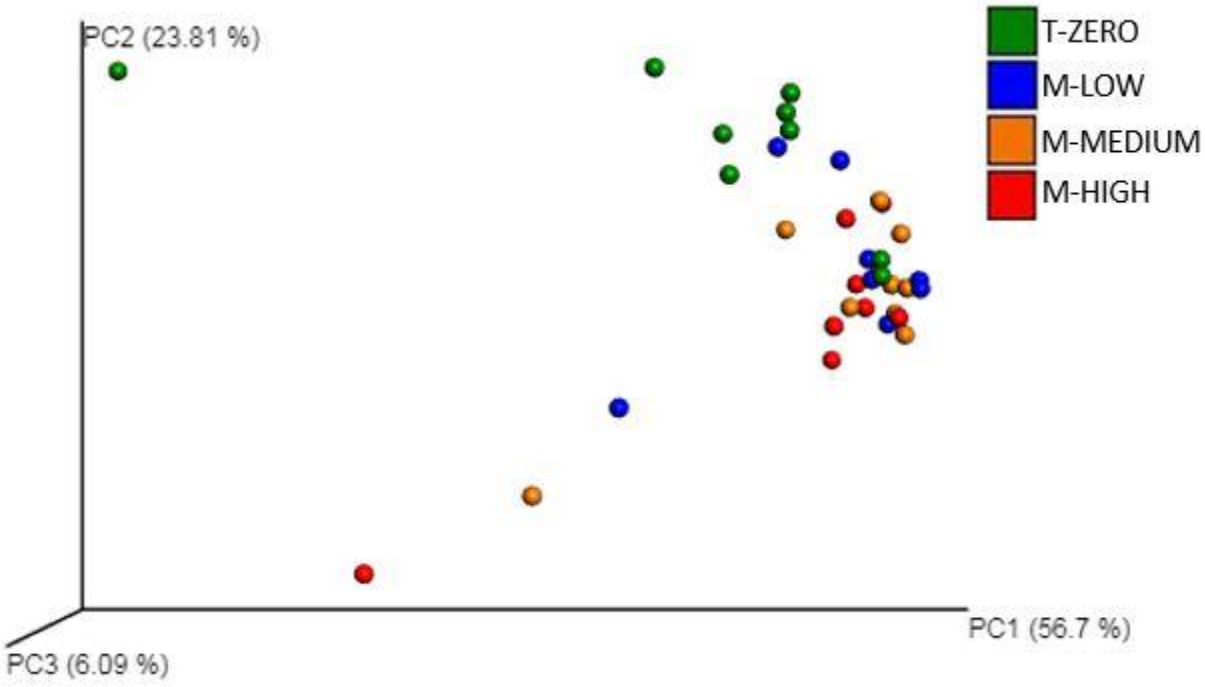

Figure 4. PCoA plot showing the distances among total bacteria (T-ZERO) and MRS-selected dilution groups (M-LOW, M-MEDIUM, and M-HIGH) based on Weighted UniFrac distance metric. For T-ZERO in this analysis, only the OTUs in T-ZERO that were also found in MRS-dilution groups were used.

CHAPTER FOUR

Evaluation of the antimicrobial and anti-inflammatory properties of *Bacillus*-DFM (Norum^{TM}) in broiler chickens infected with *Salmonella* Enteritidis

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4.1 Abstract

Restrictions of in-feed antibiotics use in poultry has pushed researches towards finding their appropriate alternatives such as Direct-Fed Microbials (DFM). In this study, previously tested *Bacillus* isolates (*B. subtilis* and *B. amyloliquefaciens*) were used to evaluate their therapeutic and prophylactic effects against *Salmonella* Enteritidis in broiler chickens. For this purpose, initial antibacterial activity of *Bacillus*-DFM (10^4 spores/g or 10^6 spores/g) against *S.* Enteritidis colonization in crop, proventriculus and intestine was investigated using *in vitro* digestive model. Furthermore, to evaluate therapeutic and prophylactic effects of *Bacillus*-DFM (10^4 spores/g) against *S.* Enteritidis colonization, 60 and 30 1-d old broiler chickens were randomly allocated to either DFM or Control group (without *Bacillus*-DFM), respectively. Chickens were orally gavaged with 10^4 cfu of *S.* Enteritidis per chicken at 1-d old, and cecal tonsils (CT) and crop were collected at 3 and 10 days later during therapeutic study, whereas they were orally gavaged with 10^7 cfu of *S.* Enteritidis per chicken at 6-d old and CT and crop were collected 24 h later from two independent trials during prophylactic study. Serum superoxide dismutase (SOD), FITC-d and intestinal IgA levels were reported for both chicken studies, in addition of cecal microbiota analysis from therapeutic study. DFM significantly reduced *S.* Enteritidis concentration in intestine compartment, and in both proventriculus and intestine compartments as compared to the Control when used at 10^4 spores/g and 10^6 spores/g, respectively ($p<0.05$). DFM significantly reduced FITC-d and IgA, and SOD and IgA levels ($p<0.05$) as compared to the Control in therapeutic and prophylactic studies, respectively. Interestingly, in the therapeutic study, there was significant difference in bacterial community structure between DFM and Control. Likewise, phylum Actinobacteria and the genera *Bifidobacterium*, *Roseburia*, *Proteus*, and cc_115 were decreased, while the genus *Streptococcus* was enriched significantly in DFM group as compared to the

Control (MetagenomeSeq, $p<0.05$). Thus, the overall results suggest that the *Bacillus*-DFM can reduce *S.* Enteritidis colonization and improve the intestinal health in chickens through mechanism(s) that might involve the modulation of gut microbiota and their metabolic pathways. The prophylactic and therapeutic effects of *Bacillus*-DFM at higher dose (10^6 spores/g) in broiler chickens are currently being evaluated.

4.2 Introduction

Antibiotics have been widely used in animal production for decades not only for therapeutic purposes, but also as antimicrobial growth promoters (AGPs) to enhance growth rate and feed conversion efficiency (Dibner and Richards, 2005; Huyghebaert et al., 2011). Although the use of AGPs has a significant positive economic impact in commercial animal production systems, there is a greater concern regarding possibilities of their use in developing antimicrobial resistance (AMR) in bacterial populations. Because of this reason, the use of in-feed antibiotics has been completely banned in Europe since January 1st, 2006 (EC Regulation No. 1831/2003) and has also been restricted to several non-European countries including Taiwan and South Korea (Maron et al., 2013). Since January 2017, medically important antibiotics to human health are no longer allowed in animal production for growth promotion or feed efficiency in the United States, and require licensed Veterinarian prescription to use them for prevention, control, and treatment of animal diseases (FDA's Guidance #213).

Poultry industry is the fastest growing animal industry and is expected to grow continuously as demand for meat and eggs is accelerating due to growing populations, increasing incomes and urbanization (Mottet and Tempio, 2017). However, due to ban or restrictions on AGPs, there are growing challenges for poultry industry to cope up with enteric pathogens such as *Salmonella*. This has created huge demands for finding alternatives to AGPs and thus, several possible alternatives such as enzymes, (in) organic acids, probiotics, prebiotics, etheric oils, and immunostimulants have already been widely studied (Huyghebaert et. al., 2011; Hernandez-Patlan et al., 2019a).

Among those alternatives, probiotics or Direct-Fed Microbials (DFM) which were defined as "a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989) have generated significant interest during the last two decades to all sectors of animal production. The majority of microbes used as DFM are bacteria that belong to around 40 different species in 7 bacterial genera including *Lactobacillus*, *Bifidobacterium*, *Propionbacterium*, *Enterococcus*, *Pediococcus*, *Bacillus*, and *Bacteroides*. In addition to these bacteria, yeast (*Saccharomyces cerevisiae*) and molds (*Aspergillus niger* and *Aspergillus oryzae*) were also reported as DFM (Buntyn et al., 2016). Moreover, certain strains of *Clostridium* such as *Clostridium butyricum* MIYAIRI 588 was also used as potential probiotic (Hagihara et al., 2018). Unlike other bacteria whose vegetative cells are used as DFM, spores from *Bacillus* sps. can be used as DFM because they are more stable and heat tolerant (Nicholson, 2002; Setlow et al., 2006; Moeller et al., 2009), and thus well suited for its application in pelleted feeds (Wolfenden et al., 2011). Previous studies reported the ability of *Bacillus* spores to germinate and enumerate within the gastrointestinal tract of the poultry (Lu et al., 2003; Barbosa et al., 2005; Latorre et al., 2014). In poultry, several studies have reported beneficial effects of *Bacillus* isolates when used as DFM on production parameters and pathogens inhibition (Fritts et al., 2000; Vilà et al., 2009; Dersjant-Li, 2014) which might be achieved through increasing nutrient digestibility, improving intestinal morphology, balancing intestinal microbiota, and modulating immunity (Lee et al., 2013; Lei et al., 2015; Latorre et al., 2017). Moreover, our previous studies based on the selected candidates of *Bacillus* sps. reported the reduction in the recovery of *Salmonella* Typhimurium in both chicks and poults after experimental infection in preliminary laboratory trials (Shivaramaiah et al., 2011), as well as in poults during the brooding phase of commercial turkey production (Wolfenden et al., 2011). However, the modes of action for improved performance by

Bacillus species were not well defined, and performance parameters were varied within species or strains, demanding appropriate screening and characterization of *Bacillus* isolates prior to commercialization (Grant et al., 2018).

Norum^{TM} (Eco-Bio/Euxxis Bioscience LLC, Fayetteville, AR) is a *Bacillus* spore direct DFM culture consisting of two isolates of *B*ac*illus amyloliquefaciens* and one isolate of *Bacillu subtilis* which were isolated in our laboratory and screened based on *in vitro* enzyme production profiles and *Clostridium perfringens* reduction (Latorre et al., 2015a). In addition, these isolates were shown to reduce digesta viscoscity, bacterial translocation, improve performance, bone quality parameters, and balance intestinal microbiota in chickens raised with rye based diets or corn distillers dried grains with solubles (Latorre et al., 2015b, 2017). However, the effect of dietary supplementation of Norum^{TM} has not been evaluated *in vivo* in an established *Salmonella* challenge model until now. Thus, the objectives of this study were to evaluate the antimicrobial effects of Norum^{TM} DFM against *S*. Enteritidis in an *in vitro* digestion model that simulates the pH and enzymatic conditions present in the crop, proventriculus and intestine of broiler chickens, as well as the therapeutic and prophylactic effects against *S*. Enteritidis colonization in crop and cecal tonsil (CT), aside from its effects on intestinal health parameters, and cecal microbiota composition in broiler chickens.

4.3 Materials and Methods

4.3.1 Preparation of Treatments and Diets

Norum^{TM} (Eco-Bio/Euxxis Bioscience LLC, Fayetteville, AR) is a *Bacillus* spore DFM culture, consisting of three isolates: two *Bacillus amyloliquefaciens* and one *Bacillu subtilis.* The product contains a concentration of stable *Bacillus* spores (~3 X 10^{11} spores/g). DFM was added

into the feed to obtain the experimental diet with a final concentration of 10^4 or 10^6 spores/g feed. Samples of feed containing the DFM were subjected to 100 °C for 10 min to eliminate vegetative cells and validate the number of spores per gram of feed after inclusion and mixing steps. Following heat-treatment, 10-fold dilutions of the feed samples were plated on TSA, letting spores in the feed sample germinate to vegetative cells after incubation at 37 °C for 24 h, hence representing the number of spores present per gram of feed. The experimental diet used in this study was formulated to approximate the nutritional requirements of broiler chickens as recommended by the National Research Council (1994), and adjusted to breeder's recommendations (Cobb-Vantress Inc., 2015). No antibiotics were added to the diet (**Table 1**). All animal handling procedures complied with the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas, Fayetteville.

4.3.2 Bacterial Strain and Culture Conditions

The organism used in all experiments was a poultry isolate of *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis), bacteriophage type 13A, obtained from the USDA National Veterinary Services Laboratory, (Ames, IA, United State). This strain was resistant to 25 µg/mL of novobiocin (NO, catalog no.N-1628, Sigma) and was selected for resistance to 20 µg/mL of nalidixic acid (NA, catalog no.N-4382, Sigma) in our laboratory. For the present studies, 100 µL of *S.* Enteritidis from a frozen aliquot was added to 10 mL of tryptic soy broth (Catalog no. 22092, Sigma) and incubated at 37°C for 8 h, and passed three times every 8 h to ensure that all bacteria were in log phase as previously described (Lin et al., 1995). Post-incubation, bacterial cells were washed 3 times with sterile 0.9% saline by centrifugation at 1,864 × *g* for 10 min, reconstituted in saline, quantified by densitometry with a spectrophotometer (Spectronic 20D+, Spectronic Instruments Thermo Scientific, Rochester, NY, United States), and finally diluted to an approximate

concentration of 1×10^8, 4×10^4 and 4×10^7 cfu/mL. Concentrations of *S.* Enteritidis were further verified by serial dilution and plating on brilliant green agar (BGA**,** Catalog no. 70134, Sigma) with NO and NA for enumeration of actual cfu used to in the experiments.

4.3.3 Experiment 1. *In vitro* Digestion Model

In this experiment, the antimicrobial activity of two different concentrations of DFM (10^4 or 10^6 spores/g) against *S.* Enteritidis was determined using an *in vitro* digestion model previously described (Annett et al., 2002; Latorre et al., 2015a) that simulates the pH and enzymatic conditions present in the crop, proventriculus, and intestine of broilers. Experiments were run in quintuplicate. Briefly, 5 g of feed with or without DFM were placed inside 50 mL polypropylene centrifuge tubes, followed by the addition of 1 ml of 1×10^8 cfu/ml *S.* Enteritidis suspension in each tube. Subsequently, the media and corresponding enzymes to simulate each compartment of the *in vitro* digestion model were added to the tubes, respecting the stirring conditions and incubation times established. Finally, in each compartment 1 mL of sample was collected to enumerate *S.* Enteritidis.

4.3.4 Experiment 2. Effect of Therapeutic Administration of DFM on *Salmonella* Enteritidis

This experiment was performed to evaluate the therapeutic effect of 10^4 spores/g DFM in broiler chickens infected with *S.* Enteritidis. Sixty one-day-old male Cobb-Vantress broiler chickens (Fayetteville, AR, USA) were challenged with 1×10^4 *S.* Enteritidis cfu per bird and randomly allocated to one of two groups (n=30 chickens/group): 1) Control group challenged only with *S.* Enteritidis and 2) DFM group challenged with *S.* Enteritidis and also with 10^4 spores/g Norum^{TM}. On days 3 and 10 post-*S.* Enteritidis challenge, 15 chickens were euthanized by CO_2 inhalation, and the crop and CT from 12 birds per group were aseptically collected to evaluate *S.*

Enteritidis recovery. Blood samples were collected from the femoral vein and centrifuged (1000×g for 15 min) to separate the serum for the determination of fluorescein isothiocyanate-dextran (FITC-d) concentration and superoxide dismutase (SOD) activity at day 10. The concentration of FITC-d administered was calculated based on group body weight at day 9 post-*S.* Enteritidis challenge. Furthermore, intestinal samples for total intestinal IgA levels were also collected.

4.3.5 Experiment 3. Effect of Prophylactic Administration of DFM on *Salmonella* Enteritidis

In this experiment, two independent trials were conducted to evaluate the prophylactic administration of 10^4 spores/g DFM in reducing the incidence of *S.* Enteritidis in broiler chickens. In each trial, 30 day-of-hatch male Cobb-Vantress broiler chickens (Fayetteville, AR, USA) were randomly allocated to one of two groups (n = 15 chickens): 1) Control group challenged only with *S.* Enteritidis and 2) DFM group challenged with *S.* enteritidis and also with 10^4 spores/g Norum^{TM}. Chicks were placed in heated brooder batteries with a controlled age-appropriate environment and provided with their respective diet and water *ad libitum*. At day 6, all chickens were orally gavaged with 1×10^7 cfu of *S.* Enteritidis per bird. Chicks were euthanized by CO_2 inhalation 24 h post-*S.* Enteritidis challenge, and the crop and CT from12 birds per group were aseptically collected to evaluate *S.* Enteritidis recovery. Blood samples were collected from the femoral vein and centrifuged (1000×g for 15 min) to separate the serum for the determination of FITC-d and SOD. The concentration of FITC-d administered was calculated based on group body weight at 6-d old. Furthermore, intestinal samples for total intestinal IgA levels were also collected.

4.3.6 *Salmonella* Recovery

The crop and ceca-cecal tonsils collected in experiments 2 and 3 were homogenized and diluted with saline (1:4 w/v), and ten-fold dilutions were plated on BGA with NO and NA,

incubated at 37°C for 24 h to enumerate total *S.* Enteritidis colony forming units. Following plating to enumerate total *S.* Enteritidis, the crop and CT samples were enriched in double strength tetrathionate enrichment broth and further incubated at 37°C for 24. Enrichment samples were streaked onto Xylose Lysine Tergitol-4 (XLT-4, Catalog No. 223410, BD DifcoTM) selective media for confirmation of *Salmonella* presence.

4.3.7 Serum Determination of FITC-d Leakage

FITC-d (MW 3-5 KDa; Sigma-Aldrich Co., St. Louis, MO) was used as a marker of paracellular transport and mucosal barrier dysfunction (Yan et al., 2009; Baxter et al., 2017). In both *in vivo* experiments, 1 h before the chicks were euthanized by CO_2 inhalation, 12 broiler chickens from each group were given an oral gavage dose of FITC-d (8.32 mg/kg of body weight), and the rest were used as controls. The concentrations of FITC-d from diluted sera (1:5 PBS) were measured fluorometrically at an excitation wavelength of 485 nm and an emission wavelength of 528 nm (Synergy HT, Multi-mode microplate reader, BioTek Instruments, Inc., VT, USA). FITC-d concentrations were reported as ng of FITC-d/mL of serum (Baxter et al., 2017).

4.3.8 Enzyme-Linked Immunosorbent Assay for Total IgA Levels

Total IgA levels in both *in vivo* experiments were determined in 12 gut rinse samples each as previously described (Merino-Guzmán et al., 2017). A commercial indirect ELISA set was used to quantify IgA according to the manufacturer's instructions (Catalog No. E30-103, Bethyl Laboratories Inc., Montgomery, TX 77356). 96-well plates (Catalog No. 439454, Nunc MaxiSorp, Thermo Fisher Scientific, Rochester, NY) were used, and samples diluted 1:100 were measured at 450 nm using an ELISA plate reader (Synergy HT, multi-mode microplate reader, BioTek

Instruments, Inc., Winooski, VT, USA). Total intestinal IgA levels obtained were multiplied by the dilution factor (100) to determine the amount of chicken IgA in the undiluted samples.

4.3.9 Serum Superoxide Dismutase Determination

Serum superoxide dismutase activity was measured in 12 serum samples per group using a commercial assay kit (item No. 706002, Cayman chemical company, Ann Arbor, Michigan, United States) following the manufacturer's instructions. The three types of SOD (Cu/Zn, Mn, and FeSOD) were determined in samples diluted 1:5. Samples were measured at 450 nm using an ELISA plate reader (Synergy HT, multi-mode microplate reader, BioTek Instruments, Inc., Winooski, VT, USA).

4.3.10 Data and Statistical Analysis

Log cfu/g of *S.* Enteritidis, total intestinal IgA, SOD activity and serum FITC-d concentrations were subjected to analysis of variance (ANOVA) as a completely randomized design, using the General Linear Models procedure of SAS (SAS Institute Inc., 2002). Significant differences among the means were determined by Duncan's multiple-range test at $P<0.05$. Enrichment data were expressed as positive/total chickens (%), and the percent recovery of *S.* Enteritidis was compared using the Chi-Squared test of independence (Zar, 1984), testing all possible combinations to determine the significance ($P<0.05$).

4.3.11 Cecal Microbiota Analysis

4.3.11.1 DNA Extraction and PCR

Six cecal samples from each group (Control and DFM groups) from the therapeutic study at day 10 post-*S.* Enteritidis challenge were used for the cecal microbiota study. About 200 mg of ileal content from each sample was used for genomic DNA extraction using QIAamp® fast DNA stool mini kit (Qiagen, Catalog # 51604) following manufacturer's instructions with addition incorporation of bead beating step. For bead beating, pellet from each sample was resuspended in 1 ml inhibit Ex buffer provided with kit and transferred to 2 ml microcentrifuge tubes with screw cap (Thermofisher Scientific, Catalog # 3468) containing 0.25 ml of sterile 0.1mm glass leads (BioSpec, Mfr # 11079101). Bead beating was performed using Bead mill 24 (Fisher Scientific) for 6 cycles where each cycle contained run time 0.30 sec and stopping time 0.11 sec between each cycle. V1-V3 region of 16S rRNA gene from each 10 ng genomic DNA samples was amplified by using unique barcoded universal primers as described previously (Adhikari and Kwon, 2017). PCR was performed using Q5® High-Fidelity DNA Polymerase (NEB; New England Biolabs) in a final volume of 50 µl following manufacturer's instructions. The PCR condition included initial denaturation at 98 °C for 30 sec followed by 30 cycles of exponential amplifications using denaturation at 98 °C for 10 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 30 sec, and final extension at 72 °C for 2 min. Amplicons were purified from 0.7% agarose gel, measured concentration using Qubit dsDNA broad range assay kit (Life Technologies, United States), and equal concentration (20 ng/µl) of amplicons were pooled together. The purified pooled amplicons were sequenced using MiSeq illumina 300 cycle paired end options at University of California (Riverside, CA, United States).

4.3.11.2 16S rRNA Gene Sequence Analysis

Raw sequence reads were analyzed using Quantitative Insights into Microbial Ecology, QIIME version 1.9.1 (Caporaso et al., 2010) at Jetstream cloud computing platform (Towns et al. 2014; Stewart et al. 2015). Paired end reads were joined together using join_paired_ends.py command of QIIME with fastq-join option (Aronesty, 2011). After joining, barcodes positions were formatted using customized Perl script and barcodes were removed using extract_barcodes.py command of QIIME. Split_libraries_fastq.py command of QIIME was used for demultiplexing and quality filtering of joined reads. Reads having Phred quality score less than 20 were discarded. The chimeric sequences were identified using USEARCH version 6.1.544 (Edgar, 2010) and chimeric sequences along with shorter sequences (<100 bp) were excluded for downstream analysis. The OTU picking was performed using pick_open_reference_otus.py command of QIIME with uclust method (Edgar, 2010). Taxonomy was assigned based on green genes taxonomy and reference database version 13_8 (DeSantis et al., 2006) with RDP classifier (Wang et al., 2007).

For further statistical analysis and visual exploration, OTU table with taxa in plain format and metadata file were uploaded to the MicrobiomeAnalyst tool (Dhariwal et al., 2017). Data were filtered using options: minimum count 4 and low count filter based on 20% prevalence in samples. Alpha diversity analysis was calculated based on Shannon Index. Data were normalized using cumulative sum scaling before any statistical comparisons (Paulson et al., 2013). Significant differences in alpha diversity among different groups were calculated based on ANOVA/T-test where significant difference level was set at $p<0.05$. Beta diversity was calculated based on Weighted UniFrac distance metric (Lozupone et al., 2011) and statistical comparisons among groups were performed with Analysis of Similarities method (ANOSIM). To determine

differentially abundant phyla and genera among different groups, MetagenomeSeq (Paulson et al., 2013) that uses zero-inflated Gaussian fit model was used, where the level of significance was set at $p<0.05$. PICRUSt ver. 1.1.3 (Langille et al., 2013) was further utilized to predict the functional pathways from 16S rRNA gene sequencing data using closed OTU table created with the Greengenes database 13.8. The statistical analysis and visualization in the third level KEGG pathways predicted by PICRUSt between two groups were performed using the Statistical Analysis of Metagenomic Profiles (STAMP ver. 2.1.3) (Parks et al., 2014).

4.4 Results

4.4.1 *In vitro* Digestion Model

The antibacterial effect of DFM at two different concentrations **(**10^4 spores/g and 10^6 spores/g) against *S*. Enteritidis colonization in crop, proventriculus, and intestine using the *in vitro* digestive model is shown in **Table 2**. When DFM was used at 10^4 spores/g of feed significantly reduced *S*. Enteritidis colonization in intestinal compartment ($p<0.05$), while at higher concentration (10^6 spores/g) significantly reduced *S*. Enteritidis colonization in both proventriculus and intestine ($p<0.05$) as compared to the control group (**Table 2**). However, the antibacterial effect of DFM was more pronounced at higher dose and especially in intestine, where it reduced the *S*. Enteritidis colonization by more than 7 $\log_{10}$ and brought to the undetectable level.

4.4.2 Prophylactic Effects of DFM

4.4.2.1 Effect on *Salmonella* Enteritidis Cecal Tonsil (CT) and Crop Colonization

The prophylactic effect of DFM (10^4 cfu/g) on *Salmonella* Enteritidis cecal tonsil (CT) and crop colonization in broiler chickens is shown in **Table 3**. Although there were no significant

differences, there were clear tendencies in reducing *S.* Enteritidis count and its incidence in both trials and tissues of chickens in DFM group as compared to the control group (**Table 2**). In trial 1, the *S.* Enteritidis incidence was reduced by 17% in both CT and Crop in DFM group as compared to the Control. Similarly, in trial 2, the *S.* Enteritidis recovery was decreased by 17 and 23% respectively in CT and Crop in DFM group in comparison with the Control group. In addition, *S.* Enteritidis count was reduced by less than half $\log_{10}$ and more than 1 $\log_{10}$ in CT and Crop, respectively in both trials when comparing the DFM group with control group (**Table 3**).

4.4.2.2 Superoxide Dismutase (SOD) Activity, Serum FITC-d Concentration and Total Intestinal IgA Levels

The SOD activity, serum FITC-d concentration and total intestinal IgA levels in broiler chickens with or without receiving DFM into the diet are shown in **Table 4**. DFM significantly reduced SOD activity and total intestinal IgA levels as compared to the control group ($p<0.05$). However, no significant difference was observed with FITC-d between two groups as shown in **Table 4.**

4.4.3 Therapeutic Effects of DFM

4.4.3.1 Effect on *Salmonella* Enteritidis Cecal Tonsil (CT) and Crop Colonization

The therapeutic effect of DFM (10^4 cfu/g) on *S.* Enteritidis cecal tonsil (CT) and crop colonization in broiler chickens is shown in **Table 5**. Although there were no significant differences, there were tendencies in reducing *S.* Enteritidis count and its incidence in both ages and tissues of chickens in DFM group as compared to the control group (**Table 5**). At 3-d old, the *S.* Enteritidis count and its incidence in CT were reduced by ~2 $\log_{10}$ and 25%, respectively by

DFM group as compared to the control group. In addition, at 10-d old, DFM reduced the *S.* Enteritidis count in CT and crop by more than 1 $\log_{10}$ as compared to the control group, while the incidence of *S.* Enteritidis was decreased by 17 and 16%, respectively (**Table 5**).

4.4.3.2 SOD Activity, Serum FITC-d Concentration and Total Intestinal IgA Levels

The SOD activity, serum FITC-d concentration and total intestinal IgA levels in broiler chickens with or without receiving DFM into the diet at day 10 post-*S.* Enteritidis challenge are shown in **Table 6**. DFM significantly reduced FITC-d and intestinal IgA levels as compared to the control ($p<0.05$). In case of SOD activity, there was numerical reduction in DFM group compared to the control group, however, no significant difference was observed.

4.4.4 Cecal Microbiota

Summarization of the OTU table resulted a total of 441,934 reads that ranges from 27,654 to 43,856 reads per sample. The total number of OTUs after data filtering was 1,108.

4.4.4.1 Cecal Microbiota Composition at Phylum Level

Firmicutes was found as a predominant phylum in both groups (Control group, 88.71%; DFM group, 86.68%) followed by Proteobacteria and Actinobacteria as shown in **Figure 1**. Actinobacteria was significantly reduced in DFM group as compared to the Control group ($p<0.05$).

4.4.4.2 Cecal Microbiota Composition at Genus Level

The relative abundance of different genera present in Control and DFM groups is shown in **Figure 2**. *Ruminococcus* was found as a predominant genus in both groups (Control group,

14.48%; DFM group, 19.14%) followed by *Lactobacillus* (Control group, 8.91%; DFM group, 3.40%) and *Streptococcus* (Control group, 0.15%; DFM group, 3.68) in Control and DFM, respectively.

The genera *Bifidobacterium*, *Roseburia*, *Proteus*, and cc_115 were significantly decreased, while the genus *Streptococcus* was significantly enriched in DFM group as compared to the Control group (MetagenomeSeq, $p<0.05$). In addition, some of the notable genera such as *Enterococcus*, *Dorea*, *Coprobacillus*, *Coprococcus*, *Eubacterium*, and *Blautia* were numerically reduced in DFM group as compared to the Control group.

4.4.4.3 Alpha Diversity

Alpha diversity of Control and DFM groups as measured by Shannon index is shown in **Figure 3**. The average Shannon index in the Control group was 4.61±0.09 (Mean±SE), while 4.27±0.22 in case of the DFM group. However, there was no significant difference observed between both groups.

4.4.4.4 Beta Diversity

Beta diversity between Control and DFM groups as measured by Unweighted UniFrac metric is illustrated in PCoA plot (**Figure 4**). Analysis of similarities (ANOSIM) result showed significant difference in microbial community structure between the two groups ($R=0.35$, $p<0.01$).

4.4.4.5 Functional Potentialities of Cecal Bacterial Community

The predicted functions of cecal microbiota in the Control and DFM groups by PICRUSt and their analysis by STAMP are shown in the **Figures 5** and **6**. The PCA plot shows that the third

level KEGG pathways of DFM group are relatively distinct in comparison to the Control group (**Figure 5**). More specifically, many bacterial genes that are involved in various metabolic pathways such as bile acid synthesis (primary and secondary), carbohydrate metabolism (pentose phosphate pathway and other glycan degradation,), and nucleotide metabolism (purine) were predicted to be enriched in the Control group. On the other hand, bacterial genes that could involve in amino acid metabolism (Glycine, Serine, and Threonine) and alkaloids biosynthesis (isoquinoline, tropane, piperidine, and pyridine alkaloids) were predicted to be enriched in the DFM group (**Figure 6**).

4.5 Discussion

Previous study reported nontyphoidal *Salmonella* sps., *Clostridium perfringens*, *Campylobacter* sps., and *Escherichia coli* as some of the most important foodborne bacterial pathogens in the United States. (Scallan et al., 2011). Overall health-related cost associated with the food borne illness from those pathogens was estimated to be around $51.0 and $77.7 billion based on basic and enhanced model respectively, as described earlier (Scharff, 2012). Nontyphoidal *Salmonella* sp. was reported as a major causative agent for hospitalization and deaths of patients in the United States (Scallan et al., 2011). *S. enterica* serotype Enteritidis (*S.* Enteritidis) that emerged as an important human illness during 1980s is currently one of the most common non typhoidal *Salmonella* serotypes worldwide, especially in developed countries (Patrick et al., 2004). Poultry and their products (eggs and meat) are considered as one of the most important source of *S.* Enteritidis infection in humans, however, *S.* Enteritidis was also isolated from non-poultry sources such as market hog carcass, steer and heifer carcass, cow and bull carcass, and ground beef (White et al., 2007; Gantois et al., 2009; Antunes et al., 2016).

Several studies have been conducted with the objective to reduce *S.* Enteritidis load in poultry and their products using various approaches such as antibodies, bacteriophages, probiotics, prebiotics, vaccines, and integrated farm management (Fulton et al., 2002; Fiorentin et al., 2005; Donalson et al., 2008; Trampel et al., 2014; Kilroy et al., 2016). Although several approaches have already been studied, there is still need to find better products that can work effectively with reproducible results. In the present study, we evaluated the effects of Norum^{TM} (DFM) to reduce *S.* Enteritidis colonization using both *in vitro* and *in vivo* trials in broiler chickens. Our previous study using *in vitro* digestion model showed reduction of *C. perfringens* by the isolates used in Norum^{TM} in different non-corn based diets demonstrating their antibacterial property against this Gram-positive bacteria (Latorre et al., 2015a). The antimicrobial activity of various species of *Bacillus* including *B. subtilis* and *B. amyloliquefaciens* were studied elsewhere and found to be effective mainly against Gram-positive bacteria (Cladera-Olivera et al., 2004; Yilmaz et al., 2006; Baindara et al., 2013; Kadaikunnan et al., 2015). In the current study, we also observed the reduction of *S.* Enteritidis by DFM in the intestinal compartment simulated in the model and in both proventiculus and intestinal compartments, when using 10^4 spores/g and 10^6 spores/g DFM, respectively. These findings further suggest that DFM exhibit a wide range of antibacterial activities which can be effective for both Gram-positive and negative bacteria. Although the detailed mechanism is not well understood, these antibacterial properties of DFM might be achieved not only through competitive exclusion and production of antimicrobial peptides (AMPs), but also might be indirectly through one or several beneficial effects exhibited by them including secretion of exogenous enzymes, alternation of immunity, gut microbiota and morphology (Latorre et al., 2015a; Latorre et al., 2016; Nawawi et al., 2017; Grant et al., 2018). The AMPs secreted by *Bacillus* sps. are diverse in nature with different chemical structure

(Cladera-Olivera et al., 2004) and include bacteriocins, glycopeptides, lipopeptides, and cyclic peptides (Baindara et al., 2013).

The antibacterial activity of *Bacillus* isolates in Norum^{TM} against *Clostridium perfringens* (Latorre et al., 2015a), *S*. Enteritidis, *Escherichia coli*, and *Clostridium difficile* (Latorre et al., 2016) was evaluated earlier using *in vitro* model and reported as promising DFM candidates. In addition, this was found to mitigate the negative impacts of necrotic enteritis in broiler chickens using a laboratory challenge model (Hernandez-Patlan et al., 2019b). In this study, we evaluated the therapeutic and prophylactic effectes of those isolates in Norum^{TM} against *S*. Enteritidis CT and crop colonization in broiler chickens. Although there were no significant differences, there were tendencies in reducing *S*. Enteritidis count and its incidence in both ages (3d and 10 d) and tissues (CT and Crop) of chickens by DFM as compared to the control during therapeutic study. Similar tendencies were also reported in both trials during the prophylactic study. This may be due to the lower dose of *Bacillus* spores (10^4 spores/g of feed) used during the *in vivo* trials, because the antibacterial effect was more pronounced with higher dose compared to the lower dose as demonstrated by *in vitro* digestion model (**Table 2**). A similar dose dependent antimicrobial response of *Bacillus*-DFM against Necrotic enteritis was observed earlier where higher dose (10^6 cfu/g of feed) mitigated negative impacts of NE more than the lower dose (10^4 cfu/g of feed) (Tactacan et al., 2013). The antibacterial effect of Norum^{TM} against *S*. Enteritidis with higher dose is currently under evaluation.

Several enteric pathogens including *Salmonella* sps. disrupt the intestinal tight junctions leading to the increase in gut permeability; commonly known as “leaky gut” (Berkes et al., 2003; Awad et al., 2017). Serum FITC-d increases with inflammation and is considered as a good indicator to measure enteric inflammation induced gut permeability in broiler chickens (Vicuña et

al., 2015). The significant reduction of serum FITC-d level by DFM as compared to the control group in the therapeutic study might be due to the alleviation of negative impacts of *S.* Enteritidis by increasing the regulation of tight junction proteins (Chichlowski et al., 2007; Grant et al., 2018) . Antioxidant enzymes such as SOD play a vital role to degrade superoxide anions and hydrogen peroxide produced during an inflammatory process. There was significant and numerical increase of SOD activity in Control group of the prophylactic and therapeutic study, respectively. The increased SOD activity in Control group could be related to the response of increase in oxidative stress due to severe intestinal damage caused by *S.* Enteritidis, since SOD play a key role in lowering oxidative stress (Carillon et al., 2013). Similarly, the significant increase in IgA level in both *in vivo* trials might be associated with disruption of intestinal epithelium, since secretion of intestinal IgA serves as the first line of defense to protect the intestinal epithelium from enteric toxins and pathogenic microorganism, as well as to antagonizes the inflammatory processes and enhance the nonspecific defense mechanisms (Mantis et al., 2011; Merino-Guzmán et al., 2017). In contrary, the decrease of SOD activity and IgA level by DFM could be related to its anti-inflammatory and immune modulating properties to mitigate the negative impacts of *S.* Enteritidis, reducing the gut morphological and immunological alterations through expression of the cytoprotective proteins and modulation of various cytokines (Lee et al., 2010; Lee et al., 2013; Dersjant-Li et al., 2016; Wang et al., 2017; Grant et al., 2018; Wu et al., 2018).

Along with the advancement in sequencing technologies, the cost of sequencing has significantly reduced during these days making the microbiota studies more affordable. It is now well accepted fact that the gut microbiota plays a key role in health and diseases of both humans and animals which have been reviewed elsewhere (Sekirov et al., 2010; Liang et al., 2018; Adhikari et al., 2018; Brugman et al., 2018). Although detailed mechanisms are unknown, the

supplementation of various alternatives to antibiotics including *Bacillus*-DFM can improve overall intestinal health and growth in chickens. Modulation of gut microbiota is one of the important mechanism of action exhibited by alternatives to antibiotics in order to exert beneficial effects on the host (Huyghebaert et al., 2010; Allen et al., 2014; Tellez and Latorre, 2017; Grant et al., 2018; Kim et al., 2018). Moreover, inclusion of *Bacillus*-DFM have shown to alter the cecal (Lei et al., 2015) and ileal (Latorre et al., 2017) microbiota in broiler chickens.

The cecum of chicken harbors the greatest bacterial diversity and is an important organ for water regulation and production of short chain fatty acids (SCFA) through carbohydrate fermentation (Oakley et al., 2014; Grant et al., 2018). The ceca of young chickens are mainly dominated by the phylum Firmicutes, Proteobacteria, and Actinobacteria, whereas the relative abundance of Bacteriodetes increase with age and was detected only after 15 days in broiler chickens (Ranjitkar et al., 2016). We also reported Firmicutes as dominated phyla in both groups followed by Proteobacteria and Actinobacteria. Actinobacteria was significantly lowered by the DFM which could be due to the antibacterial activity of DFM against *S*. Enteritidis since Actinobacteria was increased in chickens infected with *S*. Enteritidis (Mon et al., 2015; Hernandez-Patlan et al., 2019). The genus *Proteus* that was previously reported on intestinal dysbiosis (Janssens et al., 2018) was significantly higher in the Control group. Similarly, the genus cc_115 that belong to the family Erysipelotrichaceae was also significantly higher in the Control group. The bacterial family Erysipelotrichaceae was found to be associated with several diseases including ulcerative colitis, irritable bowel syndrome, and colorectal cancer (Janssens et al., 2018). Thus, increase of *Proteus* and cc_115 in the Control might be associated with gut dysbiosis and inflammation caused by *S*. Enteritidis (Videnska et al., 2013), whereas their decrease in DFM group might be due to the antibacterial property of DFM. This is further supported by the numerical

increase of intestinal dysbiosis associated genera such as *Enterococcus*, *Dorea*, *Coprobacillus*, *Coprococcus*, and *Blautia* in the Control group (Janssens et al., 2018). Furthermore, increase of *Bifidobacterium* and *Roseburia* in the Control group might be due to the inflammatory response, since these genera were found to have anti-inflammatory properties (Scott et al., 2015; O'Callaghan et al., 2016). Although some of the species of *Streptococcus* cause infection in poultry (Chadfield et al., 2005; Sekizaki et al., 2008) they are commensal organism present in the GI tract of chickens and have been used as potential probiotics (Owings et al., 1990; Herrera et al., 2012) because of their ability to reduce pathogen colonization through competitive exclusion and reduction of the pH through lactic acid production (Roto et al., 2015). Thus, increase in *Streptococcus* by DFM in the present study may be playing a vital role in reducing the colonization and incidence of *S.* Enteritidis, however, higher resolution to the strain level is needed to understand the actual effects as two strains of same species can do complete opposite roles (Fåk and Bäckhed, 2012).

DFM not only affected the bacterial composition in the ceca of broiler chickens, but also the community structure as indicated by the beta diversity analysis. However, in case of alpha diversity, although there was numerically higher diversity in the Control group, but no significant difference was observed between the two groups. This may be related to one of the theories that the DFM promotes growth of the host by reducing the number and diversity of the commensal microbiota which will allow increase nutrient utilization by intestinal epithelial cells and lower detrimental effects of microbial metabolites (Gadde et al., 2017). These regulations by DFM might be achieved through changes in bacterial genes involved in various metabolic pathways (**Figures 5 and 6**). One of the important metabolic pathway predicted to be enriched in the Control group was bile acid synthesis. Bile acids are considered as important regulators of the gut microbiota and reduced levels of bile acids in the gut are associated with bacterial overgrowth and intestinal

inflammation (Ridlon et al., 2014; Jia et al., 2018). Enrichment of bile acid synthesis pathway in the Control group might be a response to lower level of bile acids and inflammation caused by *S.* Enteritidis and other dysbiosis associated bacteria colonization in the gut. Similarly, other glycan degradation pathway was enriched in the Control group, this might be related to the response of mucinogeneis as a result of *S.* Enteritidis inflammation and the overgrowth of *Bifidobacterium* in the Control group which can degrade the host derived glycans (Zúñiga et al., 2018). Amino acids serve as precursors for microbial derived SCFA such as acetate, proprionate, and butyrate which has been reviewed elsewhere (Lin et al., 2017). Increased in the metabolic pathways associated with aminoacid metabolism (glycine, serine, and threonine) in the DFM group could be related to the amino acid fermenting ability of the *Bacillus*-DFM (Neis et al., 2015) to produce SCFA. SCFA serves as nutritents for colonocytes and other gut epithelial cells, and plays a key role in shaping the gut microbiota of the host (Koropatkin et al., 2012). Future investigation of the effects of DFM in the *Salmonella* challenged model by metagenomics and metabolomics analysis will reveal more functional potentialities of DFM.

In summary, the overall results of the present study suggest that the *Bacillus*-DFM (Norum^{TM}) can be used for the prevention and treatment of *S.* Enteritidis infection, since it has potential to reduce *S.* Enteritidis colonization and mitigate its negative effects in broiler chickens. These effects of Norum^{TM} could be achieved through mechanism(s) that might involve the modulation of gut microbiota and their metabolic pathways. Effects of Norum^{TM} against *S.* Enteritidis at a higher dose (10^6 spores/g) may disclose more promising results, and is currently under evaluation.

4.6 Conflict of Interests

Author Margarita A. Arreguin-Nava was employed by Eco-Bio LLC. All other authors declare no competing interests.

4.7 Author Contributions

B.A. and G.T. designed the experiments and wrote the first version of the manuscript, B.A., D.H. B.S. performed the experiment. Y.K., M.A., J.L. and B.H. aided in the analysis and interpretation of data and supervised the project, G.T., B.A., J.L. and X.H. contributed to editing the final version of the manuscript. All the authors reviewed and finally approved the manuscript.

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4.9 Tables and Figures

Table 1. Ingredient composition and nutrient content of a basal starter diet used in the experiments on as-is basis.

Item	Corn soybean-based diet
Ingredients (g/kg)	
Corn	574.5
Soybean meal	346.6
Poultry oil	34.5
Dicalcium phosphate	18.6
Calcium carbonate	9.9
Salt	3.8
DL-Methionine	3.3
L-Lysine HCL	3.1
Threonine	1.2
Choline chloride 60 %	2.0
Vitamin premix[1]	1.0
Mineral premix[2]	1.0
Antioxidant[3]	0.5
Calculated analysis	
Metabolizable energy (MJ/kg)	12.7
Crude protein (g/kg)	221.5

[1]Vitamin premix supplied per kg of diet: Retinol, 6 mg; cholecalciferol, 150 µg; dl-α-tocopherol, 67.5 mg; menadione, 9 mg; thiamine, 3 mg; riboflavin, 12 mg; pantothenic acid, 18 mg; niacin, 60 mg; pyridoxine, 5 mg; folic acid, 2 mg; biotin, 0.3 mg; cyanocobalamin, 0.4 mg. [2]Mineral premix supplied per kg of diet: Mn, 120 mg; Zn, 100 mg; Fe, 120 mg; copper, 10 to 15 mg; iodine, 0.7 mg; selenium, 0.2 mg; and cobalt, 0.2 mg. [3]Ethoxyquin.

Table 2. Evaluation of the antibacterial activity of different DFM ratios on *Salmonella* Enteritidis[†] in an *in vitro* digestive model using the plating method.[‡]

Treatment	**Crop**	**Proventriculus**	**Intestine**
Control	7.78 ± 0.00 [a]	5.03 ± 0.12 [a]	7.23 ± 0.00 [a]
DFM (10^4 spores/g)	7.78 ± 0.00 [a]	5.11 ± 0.03 [a]	5.31 ± 0.10 [b]
DFM (10^6 spores/g)	7.66 ± 0.01 [ab]	4.22 ± 0.04 [b]	0.00 ± 0.00 [c]

[a,b] Values within treatment columns for each treatment with different superscripts differ significantly ($P < 0.05$).
[*]Each mean is represented by five observations (n=5) ± *S.* Enteritidis.
[†]Inoculum used 10^8 cfu/ml of *S.* Enteritidis.
[‡]Data expressed in $\log_{10}$ cfu/ml.

Table 3. Effect of prophylactic administration of DFM (10^4 cfu/g) on *Salmonella* Enteritidis cecal tonsil (CT) and crop colonization in broiler chickens.

Treatments	CT Log_{10} cfu/g	CT + / - (%)	Crop Log_{10} cfu/g	Crop + / - (%)
		Trial 1		
Control	4.01 ± 0.29 [a]	12/12 (100%)	2.68 ± 0.47 [a]	9/12 (75%)
DFM	3.72 ± 0.55 [a]	10/12 (83%)	2.11 ± 0.66 [a]	6/12 (58%)
		Trial 2		
Control	3.94 ± 0.22 [a]	12/12 (100%)	2.69 ± 0.48 [a]	9/12 (75%)
DFM	3.75 ± 0.56 [a]	10/12 (83%)	2.08 ± 0.64 [a]	5/12 (42%)

[1]Data expressed in Log_{10} cfu /g (Mean ± SE) of tissue from 12 chickens, where different letters indicate statistical significant difference at $P < 0.05$.
[2]Chickens were orally gavaged with 10^7 cfu of *S.* Enteritidis per chicken at 6-d old, samples were collected 24 h later.
[3]Data expressed as positive/total chickens (%).

Table 4. Evaluation of Superoxide dismutase (SOD) activity, serum fluorescein isothiocyanate-dextran (FITC-d) concentration and total intestinal IgA in broilers chickens with or without consuming DFM into the diet[1].

Treatments	**SOD (U/mL)**	**FITC-d (μg/mL)**	**IgA (μg/mL)**
Control	4.50 ± 0.31 [a]	0.591 ± 0.055 [b]	14.21 ± 0.83 [a]
DFM	1.97 ± 1.85 [b]	0.664 ± 0.063 [b]	10.57 ± 0.82 [b]

[1]Data expressed Mean ± SE from 12 chickens, where different letters indicate statistical significant difference at $P < 0.05$.

Table 5. Effect of therapeutic administration of DFM (10^4 cfu/g) on *Salmonella* Enteritidis cecal tonsil (CT) and crop colonization in broiler chickens.

Treatments	CT Log_{10} cfu/g	CT + / - (%)	Crop Log_{10} cfu/g	Crop + / - (%)
		Trial 3-d		
Control	6.44 ± 0.15 [a]	12/12 (100%)	3.18 ± 0.46 [a]	10/12 (83%)
DFM	4.66 ± 0.82 [a]	9/12 (75%)	3.05 ± 0.45 [a]	10/12 (83%)
		Trial 10-d		
Control	6.61 ± 0.21 [a]	12/12 (100%)	2.93 ± 0.65 [a]	7/12 (58%)
DFM	5.49 ± 0.76 [a]	10/12 (83%)	1.78 ± 0.65 [a]	5/12 (42%)

[1]Data expressed in Log_{10} cfu /g (Mean ± SE) of tissue from 12 chickens, where different letters indicate statistical difference at $P < 0.05$.
[2]Chickens were orally gavaged with 10^4 cfu of *S.* Enteritidis per chicken at 1-d old, samples were collected 3 and 10 days later.
[3]Data expressed as positive/total chickens (%).

Table 6. Evaluation of Superoxide dismutase (SOD) activity, serum fluorescein isothiocyanate-dextran (FITC-d) concentration and total intestinal IgA in broilers chickens with or without receiving DFM into the diet at day 10 post-*S.* Enteritidis challenge[1].

Treatments	**SOD (U/mL)**	**FITC-d (μg/mL)**	**IgA (μg/mL)**
Control	10.34 ± 0.67 [a]	0.700 ± 0.020 [a]	14.34 ± 2.81 [a]
DFM	9.29 ± 0.88 [a]	0.531 ± 0.013 [b]	6.21 ± 2.31 [b]

[1]Data expressed as Mean ± SE from 12 chickens, where different letters indicate statistical significant difference at $P < 0.05$.

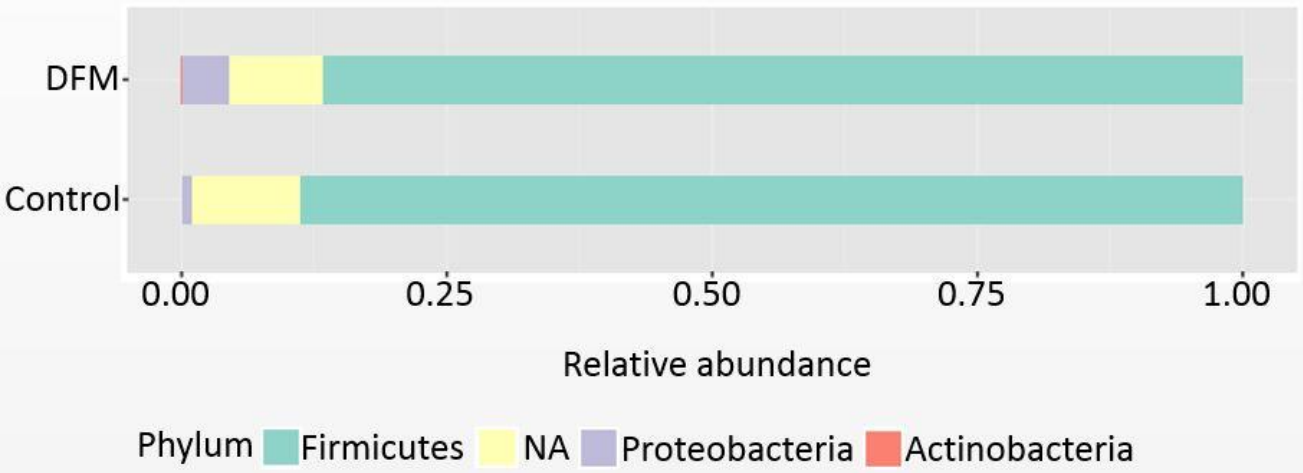

Figure 1. Relative abundance of major phyla in two different treatment groups (Control and DFM). NA refers to those reads that aren't assigned to any phyla.

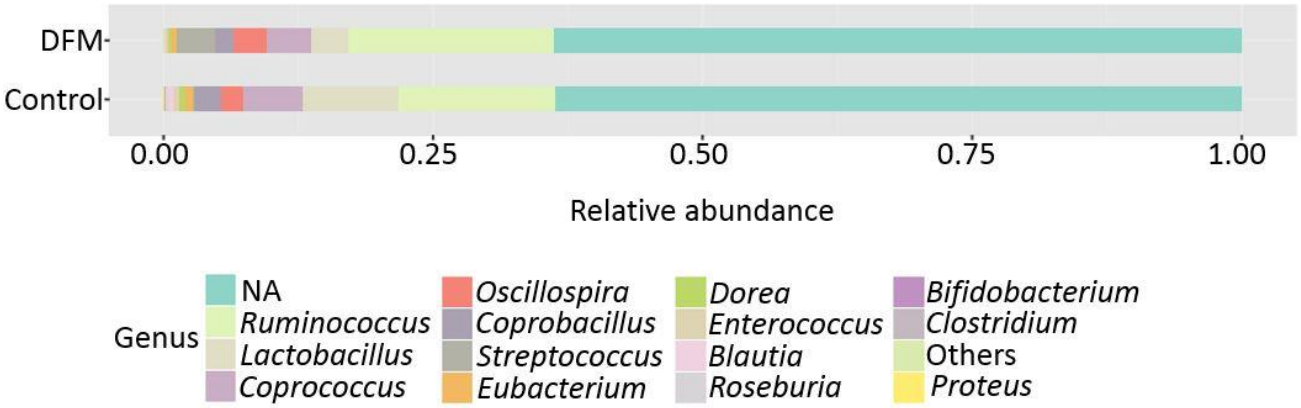

Figure 2. Relative abundance of major genera in two different treatment groups (Control and DFM). NA refers to those reads that aren't assigned to any genera. Genera having counts less than 100 are merged together in "Others".

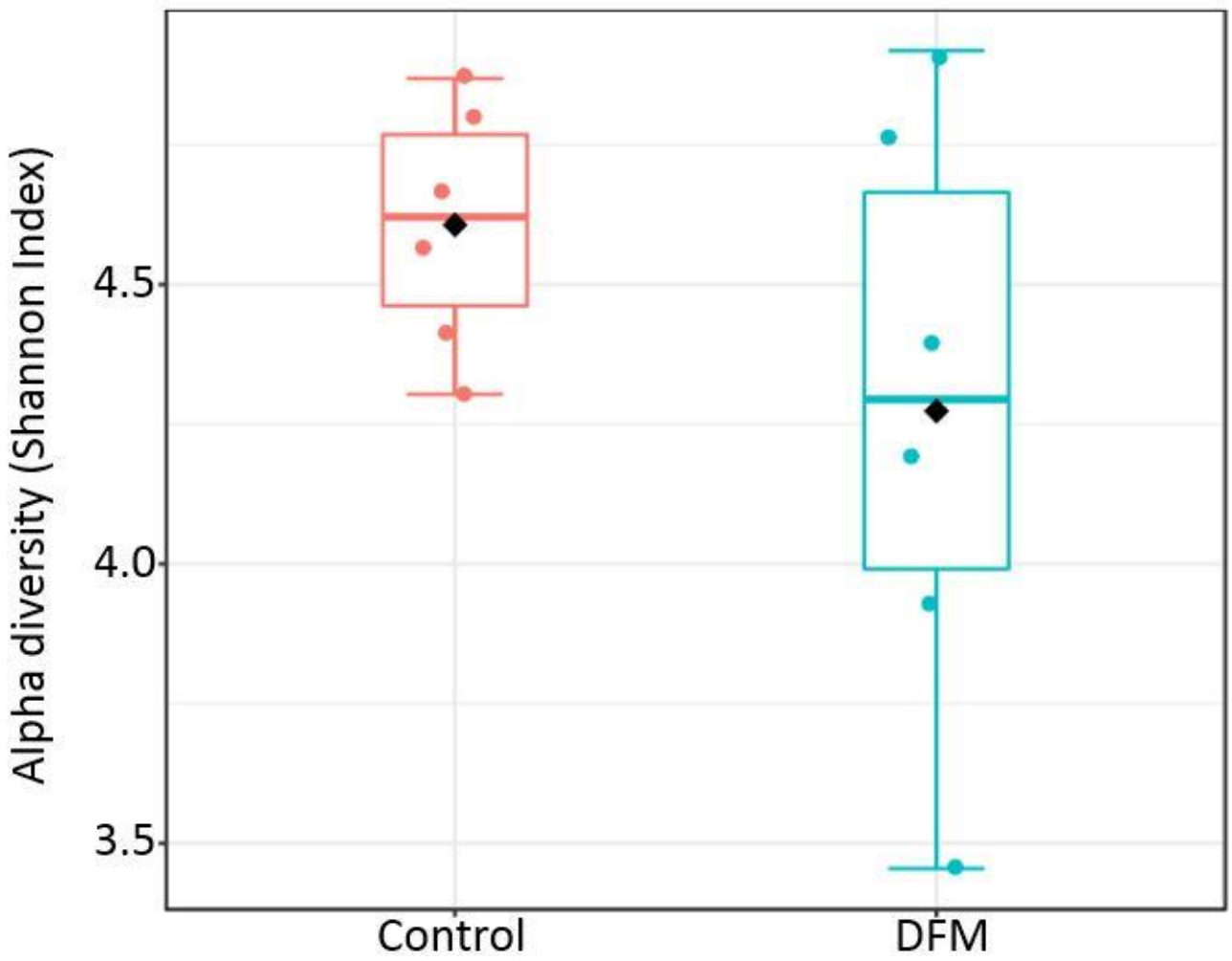

Figure 3. Alpha diversity of two different groups (Control and DFM) as measured by Shannon Index. No significant difference was observed between them (T-test, $p>0.05$).

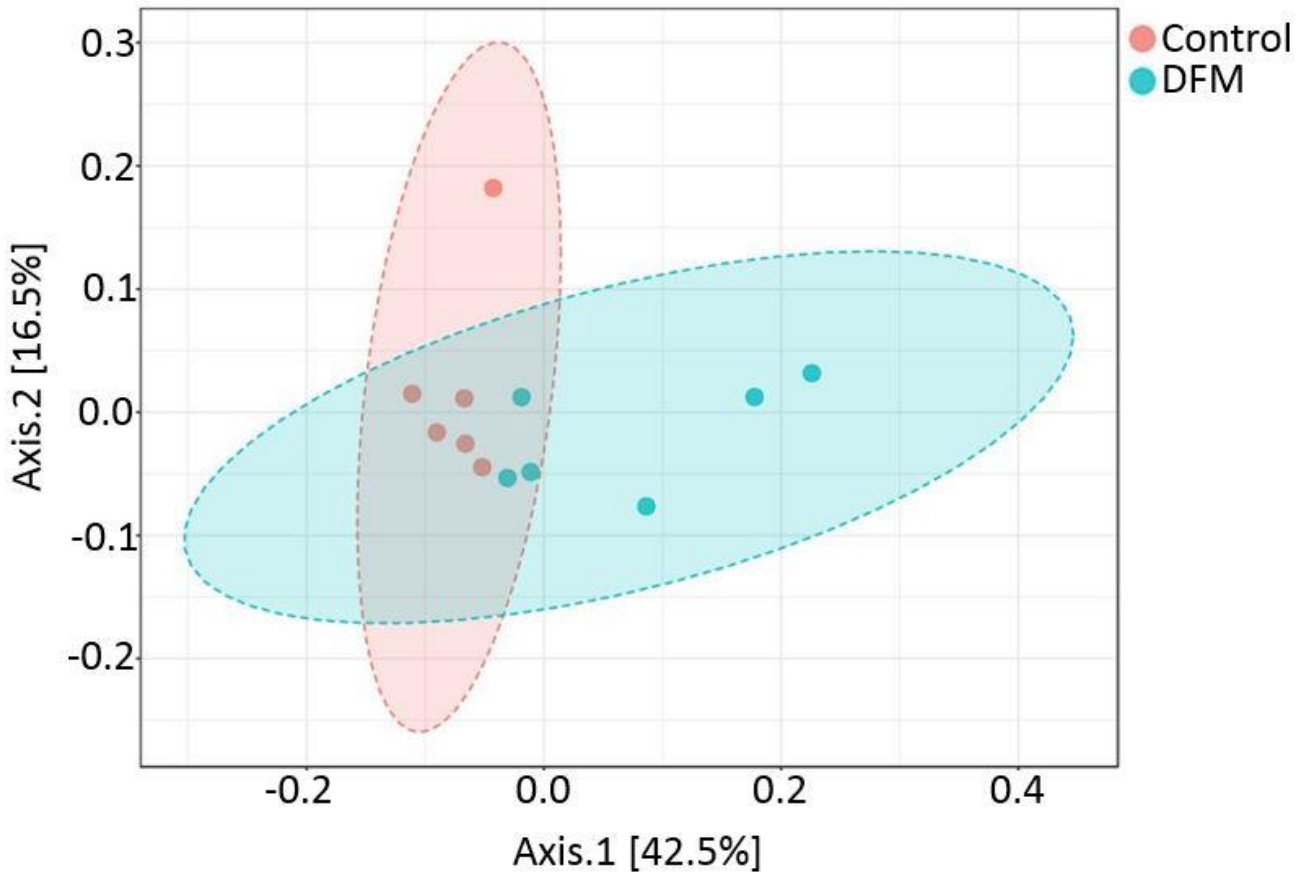

Figure 4. PCoA plot showing difference in microbial community structure between Control and DFM groups (ANOSIM; R = 0.35 and $p<0.01$).

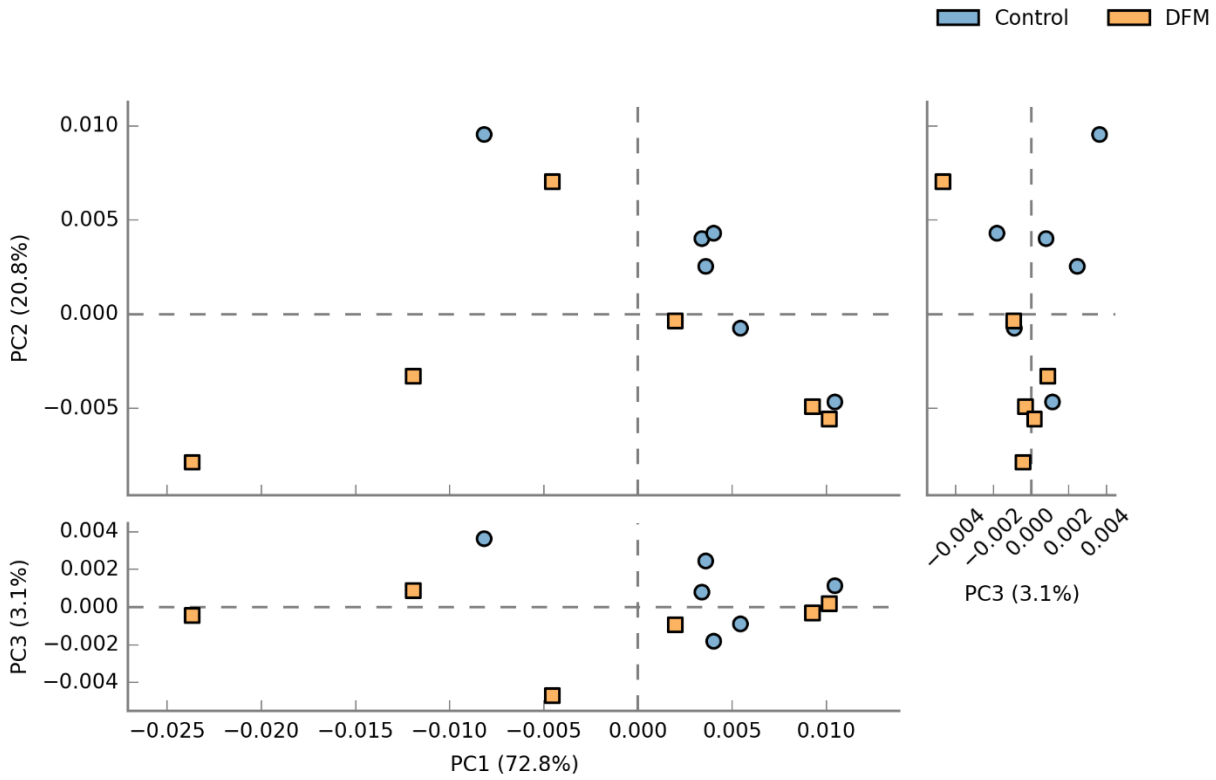

Figure 5. PCA plot comparing third level KEGG pathways between Control and DFM groups. The third level KEGG pathways were predicted using PICRUSt followed by the generation of PCA plot using STAMP.

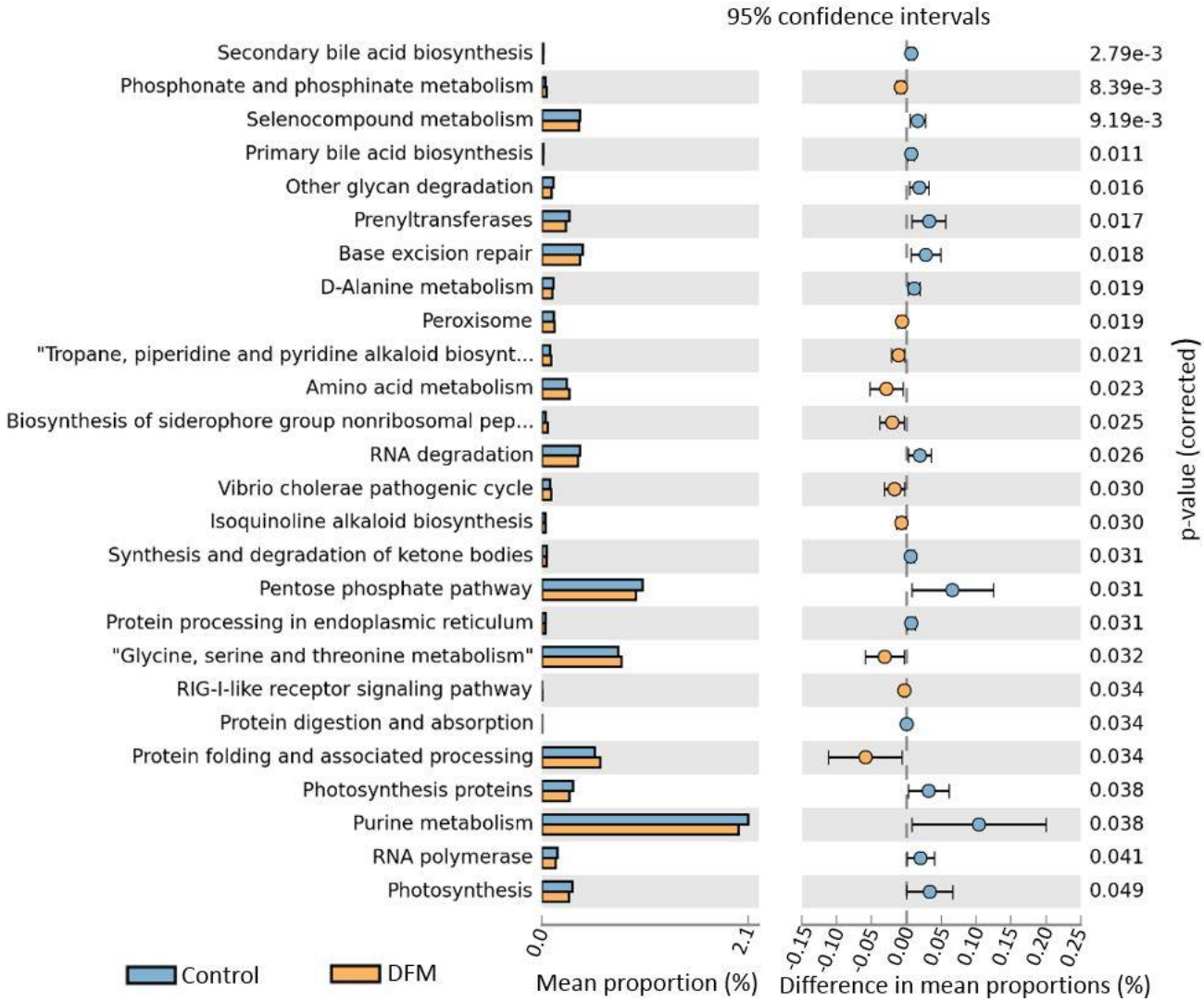

Figure 6. Extended error bar plot generated by STAMP showing differential abundant third level KEGG pathways between Control and DFM group. Only significant features with $p<0.05$ (Welch's t-test) were included in the plot.

CHAPTER FIVE

Effects of housing types on egg production and cecal microbiota of two different strains of laying hens during the late production stage

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5.1 Abstract

Due to animal welfare issues, EU banned the use of conventional cages (CC) for laying hens, and non-EU countries including the US are also under constant public pressure to restrict their use in egg production. Enriched colony cages (EC) were developed to provide hens more comfort movement and allow natural behaviors. Although previous studies have investigated the performance parameters and welfare of laying hens housed in CC and EC, there is very limited information regarding the changes in gut microbiota and their possible roles in egg production. Thus, this study was conducted to explore the effects of CC and EC on egg production and cecal microbiota of two commercial laying hen strains, Hy-Line W36 White Leghorn (WL) and Hy-Line Brown (HB). Hens were assigned in a 2x2 factorial arrangement in a completely randomized design: HB in CC (120) and EC (311), and WL in CC (120) and EC (355). Hen-day egg production (HDEP) was recorded weekly, and cecal samples (n=6/group) were collected at 53, 58, 67, and 72 weeks of age for microbiota analysis by MiSeq sequencing of 16S rRNA gene. Statistical analysis of HDEP data was carried out in a 2x2 factorial design for each week with significance level set at $P<0.05$, whereas sequence reads were analyzed using QIIME2 ver. 2018.8. Differentially abundant taxa were identified by LEfSe ($P<0.05$, LDA score>2.0) analysis. Although, hens housed in CC had higher HDEP compared to EC throughout all time points, no significant differences were observed. On contrary, significant interaction effect of house and strains was observed at 53 weeks, where HDEP of WL hens was significantly higher as compared to the HB in CC housing ($P<0.05$). Likewise, the main effect of strains was observed at 72 weeks, where WL had significantly higher HDEP as compared to HB (65% vs. 56%). Moreover, the composition and diversities of cecal microbiota were affected by breed, housing, and age in descending order. At phylum level, Actinobacteria was significantly enriched in WL at all time points as compared to HB, while Synergistetes, Spirochaetes, and both were significantly higher in HB as compared to

WL at 53, 58, and 67 weeks, respectively. However, Firmicutes was significantly higher in CC as compared to EC at 67 weeks. In contrast, Spirochaetes at 53 and 58 weeks, and Bacteroidetes and Proteobacteria at 67 and 72 weeks, respectively were higher in EC as compared to CC. At genus level, 51, 48, 58, and 15 differentially abundant taxa were revealed between HB and WL at 53, 58, 67, and 72 weeks, respectively. Interestingly, *Bifidobacterium* was significantly enriched in WL at all time points, and in addition, butyrate producing genera such as *Butyricicoccus* and *Subdoligranulum* were significantly higher in WL as compared to HB at 58 and 72 weeks. Moreover, 13, 8, 23, and 8 differentially abundant taxa between CC and EC housing were observed at 53, 58, 67, and 72 weeks, respectively. At 72 weeks, the phylum Proteobaceria and its associated genera such as *Campylobacter* and the unknown genus of family Campylobacteriaceae and Helicobacteriaceae were significantly enriched in EC which might be associated with reduced egg production in EC. Likewise, there were significant differences in both alpha and beta diversity between HB and WL at all time points, while a significant difference was observed between CC and EC only at 67 week ($P<0.05$). Moreover, functional metabolic pathways associated with energy and nucleotide metabolism, and amino acids and vitamin B biosynthesis were differentially presented between CC and EC in a strain dependent manner. The overall results suggest that the difference in egg production between HB and WL, and CC and EC might be achieved at least partially through alterations of cecal microbiota.

Keywords: laying hens, egg production, enriched colony cage, convention cage, cecal microbiota

5.2 Introduction

Poultry industry is the fastest growing animal industry which is expected to grow continuously since demand for meat and eggs is increasing as a result of growing human populations (Mottet and Tempio, 2017). In order to feed the growing human population which is expected to reach 9.8 billion by 2050 (UNDESA, 2017), there is a huge demand to accelerate animal production including poultry. Traditionally, people focused mainly on the strategies to maximize the profit and productivity of poultry, and conventional cage (CC) system is one of those strategies developed during 1930s and has been used in the traditional egg production since 1950s (Yilmaz Dikmen et al., 2016). Although the CC system has been considered as one of the most efficient housing method of laying hens for a long time, it is now widely accepted to have negative impacts on the welfare of hens (Craig and Swanson, 1994; Tactacan et al., 2009; Lay et al., 2011; Yilmaz Dikmen et al., 2016; Hartcher and Jones, 2017). The negative impacts of CC are mainly due to the limited space for movement that can cause musculoskeletal weakness, and low complexities of the environment which can abolish many of their natural behaviors such as nesting, roosting, dust bathing, perching, and foraging (Baxter, 1994; Lay et al., 2011; Hartcher and Jones, 2017).

Because of the increased public concerns about animal welfare, conventional cage systems have been banned in EU since 2012 (Council Directive 1999/74/EC). In addition, non-EU countries including USA, Canada, and Australia are also under constant public pressure to restrict the use of convention cage systems for egg production (Van Staaveren et al., 2018). As an alternative, enriched cages were developed that provide more space for movement and comfort behaviors, and allow for some dust bathing, nesting, foraging, and perching (Appleby et al., 2002). Although previous studies have conducted to investigate the performance parameters and welfare

of laying hens in conventional and enriched cages (Tactacan et al., 2009; Karcher et al., 2015), there is very limited information regarding the changes in intestinal microbiota with those housing systems. Furthermore, several host factors such as breeds or strains within the same breed can affect the intestinal microbiota in chicken (Kers et al., 2018), but those variations were less studied in laying hens in comparison to broilers. Thus, the aim of this study was to investigate the effects of conventional cage (CC) and enriched colony cage (EC) systems on egg production and cecal microbiota of two commercial laying hen strains, Hy-Line Brown (HB) and Hy-Line W36 White Leghorn (WL).

5.3 Materials and Methods

5.3.1 Hens and Husbandry

The animal care experimental protocol was approved by the Institutional Animal Care and Use Committee at Mississippi State University. Both strains (HB and WB) of hens were purchased from Mansfield Pullet Co., at MO. Hens were reared in top two tiers of both A- frame type conventional cage (CC; dimension: 1.6’ x 2’) and enriched colony cage (EC; dimension: 4’ x 12’) at Mississippi State University Poultry Research Farm located in Starkville, MS. Enriched colony had scratch pads, perches and nesting area for hens. Hens were housed with four hens per cage in CC and 50 per cage in the EC system. Four groups of hens were assigned as WL in CC (120), HB in CC (120), WL in EC (355) and HB in EC (311). The lighting schedule was 16 h light and 8 hour darkness and were provided *ad libitum* commercial laying hen ration according to the Hy-Line management guide recommendation containing 2,760 Kcal ME/kg and 16% CP (Table 1). Hen-day egg production (HDEP) data was calculated from eggs collected from every week continuously from 53 to 72 weeks of age. The number of samples in each group used for egg production analysis is summarized in Table 2.

5.3.2 Cecal Microbiota Analysis

5.3.2.1 Sample Collection and Processing

At 53, 58, 67 and 72 weeks of age, six hens per group were humanely euthanized with Co_2. One cecum from each hen was collected aseptically and stored at -20°C until microbiota analysis. The number of samples from each group used for microbiota analysis is summarized in Table 2.

5.3.2.2 DNA Extraction, PCR, and Library Preparation for Sequencing

Quick-DNA™ Fecal/Soil Microbe Kits (Catlog No. D6012, ZymoResearch, USA) was used to extract genomic DNA from approximately 150 mg of ileal content per sample following the manufacturer's instructions. V4 region of 16S rRNA gene from genomic DNA of each sample was amplified using the primers 515F (Parada et al., 2016) and 806R (Apprill et al., 2015). The library of amplicons for sequencing was prepared according to the 16S Illumina PCR protocol described in the Earth Microbiome project (http://www.earthmicrobiome.org; Thompson et al., 2017) with slight modifications. In brief, Platinum™ II Hot-Start Green PCR Master Mix (2X) (Thermofisher Scientific, Catalog No. 14000013) was used to conduct PCR in a 25 µl final reaction volume through 30 cycles. The thermocycling condition of PCR included an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of 0.5 min at 94 °C, 0.5 min at 60 °C, and 0.5 min at 68 °C, and a final extension of 5 min at 68 °C.

The length of amplified products was confirmed with 1% agarose gel electrophoresis and equal amounts (~300 ng) of amplicons from all sample as measured by Qubit dsDNA BR Assay Kit (ThermoFisher Scientific, Catalog No. Q32850) were pooled together. The pooled amplicons were finally ran on 1% agarose gel electrophoresis, purified using Zymoclean Gel DNA Recovery

Kit (Zymo Research, Catalog No. D4007), and sequenced with Illumina MiSeq paired end 300 cycle options at University of California at Davis.

5.3.3 Data Analysis

5.3.3.1 Egg Production

Data were analyzed in a 2×2 factorial arrangement (house type × strain) using JMP Genomics 9, where the significance level was set at $P < 0.05$. The values are presented as LS means ± Standard Error (SE), where the mean difference was separated using Tukey HSD.

5.3.3.2 Amplicons Sequence Analysis

Nebula cloud computing platform of the University of Arkansas was used to process raw sequencing reads in QIIME 2 version 2018.8 (Bolyen et al., 2018) utilizing the pipelines developed for paired-end data types. In sum, "demux emp-paired" method of q2-demux plugin was used to demultiplex sequencing reads followed by quality filtering and denoising with "dada2 denoise-paired" method of q2-dada2 (Callahan et al., 2016) plugin available at QIIME 2. The truncation length of forward and reverse reads was set at 240 and 200 bp, respectively, which is based on the quality score criteria (≥30). Taxonomic assignments was performed using a Naive Bayes classifier (Pedregosa et al., 2011) pre-trained with Greengenes (Version 13.8) 99% OTUs (DeSantis et al., 2006) and q2-feature-classifier plugin, where the sequences have been trimmed to include only the V4 region of the 16S rRNA gene bound by the 515F/806R primer pair. The core-metrics-phylogenetic method at a sampling depth of 31,060 was used to analyze Alpha and Beta diversity. Shannon's diversity index (Shannon, 1948) and UnWeighted UniFrac distance metric (Lozupone et al., 2011) were used to calculate alpha and beta diversity, respectively. All figures were created using ggplot2 packages of R (Wickham, 2016). Statistical differences among treatment groups at

different taxonomic assignments were calculated using LEfSe. The significant differences in alpha diversity were calculated using the alpha-group-significance command of QIIME2 which uses Kruskal-Wallis test. In the contrary, statistical differences in beta diversity among groups were calculated by PERMANOVA (Anderson, 2001) test using the beta-group-significance command of QIIME2 with pairwise option. For both diversities analysis, the corrected *P* values for multiple comparisons (*q*) were used to report a significant difference between two groups, where the level of significance was set at $q < 0.05$. PICRUSt2 (Langille et al., 2013) was used to predict the metabolic pathways of cecal microbiota and MetaCyc database (Caspi et al., 2016) was used to describe the predicted pathways. Differentially abundant features were identified using Welch's t-test inbuilt in STAMP software (Parks et al., 2014), where features were filtered using $P>0.05$ and difference in mean proportions (%) <0.03 criteria.

5.4 Results

5.4.1 Egg Production

There was a significant interaction effect of house and strain type at 53 weeks, where WL had significantly higher HDEP as compared to the HB raised in CC housing (89% vs 72%, $P<0.05$). At 58 and 67 weeks, although the HDEP of WL was numerically higher than HB, no significant differences were observed. At 72 weeks, the main effect of strain was observed, where HDEP of WL was significantly higher as compared to HB (65% vs. 56%). On the contrary, although the hens reared in CC had numerically higher HDEP compared to those housed in EC throughout all four time points, no significant differences were observed between the two groups. The results of egg production are summarized in Table 3.

5.4.2 Cecal Microbiota

Summarization of the feature table resulted 5,568,578 number of sequence reads from 90 samples that range from 31,060 to 88,097 reads per sample. The median and mean±SE reads per sample were 63,893.50 and 61,873.09±1,270.94, respectively. In addition, there were altogether 1,759 unique features (amplicon sequence variants) from these samples. The summary of average reads per sample in different groups is summarized in Table 2.

5.4.2.1 Cecal Microbiota Composition at the Phylum Level

Taking consideration of all samples, 99.36% of total sequence reads were assigned to 15 different bacterial phyla, while 0.63% of total sequence reads were assigned to domain Archaea. In addition, 0.01% of total sequence reads which were only assigned to Kingdom Bacteria but not assigned to the lower level of taxonomy. Among those phyla, Bacteroidetes (49.05%) was the predominant phylum followed by Firmicutes (45.05%). Other important phyla whose relative abundance was greater than 0.2% were Actinobacteria (2.70%), Proteobacteria (0.77%), Spirochaetes (0.52%), Synergistetes (0.41%), and WPS-2 (0.34%). The relative abundance levels of major phyla that were presented in two different housing at four different time points were shown in Figure 1. The relative abundance of Bacteroidetes was the highest followed by Firmicutes in both HB and WL irrespective of housing types and ages, except in WL hens housed in CC housing at 67 weeks where the Firmicutes (51.96%) was found as a predominant phylum (Figure 1). Likewise, the relative abundance of Actinobacteria was found higher especially in WL irrespective of housing as shown in Figure 1.

5.4.2.2 Differentially Abundant Phyla

The differentially abundant phyla in two different hen strains and housing types as identified by LEfSe (P<0.05 and LDA score > 2.0) are summarized in Table 4. The phylum Actinobacteria was significantly enriched in WL group throughout all four different ages as compared to the HB group. However, the phyla Synergistetes and Spirochaetes were significantly abundant in HB group at 53 and 58 weeks, respectively, and both Synergistetes and Spirochaetes at 67 weeks as compared to WL. At 72 weeks, no significant difference was observed at any phyla between HB and WL groups.

Regarding housing effects, the phylum Spirochaetes was significantly higher in EC group in both 53 and 58 weeks as compared to the CC. On the contrary, Bacteroidetes and Firmicutes were significantly enriched in the EC and CC group, respectively, at 67 weeks. At 72 weeks, Proteobacteria was significantly higher in EC as compared to the CC group.

5.4.2.3 Cecal Microbiota Composition at the Genus Level

Out of 99.36% of total sequence reads that were assigned to one of the bacterial phyla, 68.45% were properly assigned to one of the 89 bacterial genera while taking account of all samples. The remaining reads were assigned to low level of bacterial taxa such as family, order, class, and phylum. Among those genera, *Bacteroides* (17.60%) was the predominant genus followed by *Prevotella* (10.20%), *Ruminococcus* (7.91%), *Lactobacillus* (4.83%), *Fecalibacterium* (3.60%), *Phascolarctobacterium* (3.41%), and *Megamonas* (3.37%). Other notable genera included *Coprococcus*, *Blautia*, *Peptococcus*, genus S24-7, and *Turicibacter* whose relative abundance ranges from 1.21 to 1.91%. The relative abundance of major genera that were presented in two different housing and breed types at four different time points were shown in

Figure 2. *Bacteroides* that ranged from 13.57% (EC-WL at 53 weeks) to 21.69% (CC-HB at 58 weeks) was the predominant genus in both hen strains housed in either CC or EC except in WL housed at EC at 53 weeks and 58 weeks, where *Prevotella* (16.13%) and *Lactobacillus* (15.65%) were the predominant genera in respective ages (Figure 2). The relative abundance of *Prevotella* ranged from 5.49% to 9.78% in HB (Figure 2; left half), whereas it ranged from 8.62% to 16.13% in WL (Figure 2; right half). Similarly, the relative abundance of *Ruminococcus* ranged from 4.83% to 9.75% in HB, while it ranged from 5.93% to 9.84% in WL. In addition, *Lactobacillus* ranged from 2.59% to 4.72% in HB, but it ranged from 2.35% to 15.65% in WL. Another important observation was the genus *Megamonas* which was found the highest (13.75%) in WL housed at 67 weeks in CC housing.

5.4.2.4 Differentially Abundant Genera in Two Different Hen Strains

The strains effect was more pronounced than housing effect and the bacteria taxa that were differentially abundant in WL and HB strains at 53, 58, 67, and 72 weeks are shown in Figures 3-6, respectively. The number of bacterial taxa at the genus level that were significantly higher in WL were 15, 27, 4, and 8 at 53, 58, 67, and 72 weeks, respectively. The genus *Bifidobacterium* was significantly enriched in WL as compared to the HB throughout all time points. In addition, *Butyricicoccus* (except, 67 weeks), unidentified genera of phylum Actinobacteria (except, 67 weeks), *Bulleidia* and *Pseudoramibacter-Eubacterium* (except 72 weeks) were significantly higher in WL at all time points. Other notable genera that were significantly abundant in WL were Candidatus Arthromitus (except 58 and 67 weeks) and *Subdoligranulum* (except 53 and 67 weeks.) as shown in Figures 5-8. Moreover, *Prevotella*, *Collinsella*, *Flexispira*, and *Slackia* were presented significantly higher in WL only at 58 weeks, whereas *Succinatimonas* was presented significantly higher only at 72 weeks.

On the contrary, the number of bacterial taxa at the genus level that was significantly higher in HB were 36, 21, 54, and 7 at 53, 58, 67, and 72 weeks, respectively. *Turicibacter*, genus 02d06 of Clostridiaceae family, the unidentified genus that belongs to family Barnesiellaceae, and that belong to phylum Verrucomicrobia were significantly enriched in HB throughout all time points as shown in Figures 3-6. In addition, the genus *Akkermansia*, and unidentified genera that belong to phylum Synergistetes, and that belong to family Christensenellaceae were also significantly higher in HB at all time points except at 72 weeks. Similarly, *Paraprevotella*, *Clostridium*, *Dehalobacterium*, and the unidentified genera that belong to family Ruminococcaceae, Preptostreptococcaceae, and that belong to order Bacteroidales were significantly higher in HB as compared to WL only at 53 and 72 weeks. Moreover, *Megamonas*, *Oscillospira*, *Desulfovirbrio*, *Megasphaera*, *Treponema*, *Alistipes*, cc_115, *Butryricicoccus*, *Collinsella*, and *Coprobacillus* were presented significantly higher in HB, but only at 67 weeks of age.

Interestingly, some of the archaeal taxa were also found to be differentially presented between two strains of laying hens throughout all time points except at 72 weeks. Methanobrevibacter and 3 unknown genera that were assigned as Methanobacteria, Methanobacteriales, and Methanobacteriaceae respectively were significantly higher in WL (at 53 and 58 weeks), while unknown genera that were assigned as Methanomicrobia, Methanomicrobiales, and Methanocorpusculaceae were significantly higher in HB (except 72 weeks).

5.4.2.5 Differentially Abundant Genera in Two Different Housing Types

The significantly abundant bacterial taxa at genus level which are identified by LEfSe between two housing at 53, 58, 67, and 72 weeks are shown in Figures 7-10, respectively. At 53 and 72 weeks of age, the significantly abundant bacterial taxa were found only with EC housing,

while at 58 and 67 weeks, both housing had differentially abundant bacterial taxa. The bacterial genus *Treponema* and the unknown genera of order Spirochaetales, Spirochaetes, and Spirochaetes were significantly enriched in EC as compared to CC at both 53 and 58 weeks. On the contrary, *Campylobacter* and other unknown genera of family Campylobacteraceae were significantly higher in EC at both 67 and 72 weeks. In addition, bacterial genera such as *Ruminococcus*, *Corynebacterium*, *Sutterella*, and unknown genera that were assigned at order Burkholderiales and Actinomycetales, and family Corynebacteriaceae and Alcaligenaceae were significantly abundant in EC at 53 weeks. Similarly, the genus *Flexispira*, *Anaerobiospirillum*, and unknown genera that were assigned at family Helicobacteraceae were significantly enriched in EC at 72 weeks.

However, the differentially enriched bacterial taxa in CC were observed only at 58 and 67 weeks with more number at 67 weeks. At both 58 and 67 weeks, the unknown genera that were assigned at class 4c0d_2 and order YS2 of phylum Cyanobacteria were significantly higher in CC as compared to the EC. In addition, Megamonas was significantly higher in CC at 58 weeks, while genera such as Mucispirillum, Succinatimonas, and Sutterella were significantly higher at 67 weeks.

5.4.2.6 Alpha Diversity

The bacterial diversity within a group (alpha diversity) was calculated by Shannon index. The word significant refers to the statistically significant differences between the two groups at adjusted *P* value (*q*) <0.05. The alpha diversities for two different breed and housing types across four different ages of birds are shown in Figure 11 and Figure 12, respectively. The alpha diversity was highly affected by breed in comparison to housing. The alpha diversities in HB breed was significantly higher as compared to WL throughout all four ages as shown in Figure 11. The alpha diversities increased with increase in age of both breeds with more noticeable in HB, where the

alpha diversity of HB breed at 72 weeks was significantly higher in comparison to HB at 53 weeks of age as shown in Figure 11.

Like in breed, as age of birds increased, the alpha diversity also increased in both housing types with more pronounced increase in EC housing, where the alpha diversity of birds at 67 weeks was significantly higher as compared to those at 53 weeks as shown in Figure 12. Although, the alpha diversities in birds housed in EC were numerically higher in comparison to those housed in CC across all four ages, however, the significant difference between EC and CC was found only at 67 weeks of age.

5.4.2.7 Beta Diversity

The beta diversity of two different breeds and housing types across four-time points is shown in the PCoA plot (Figure 13). The PERMANOVA results showed that the microbial community structure in laying hens was significantly affected by all three variable analyzed; age (P=0.028), housing (P=0.001), and breed (P=0.001). Pairwise PERMANOVA results showed that although there was a tendency of microbial community structure difference between EC and CC throughout four ages, the housing effect was more pronounced at 67 weeks of age where there was significant difference observed between EC and CC. This is in accordance with egg production and alpha diversity results. Furthermore, in concord with taxonomic composition and alpha diversity, the breed effect was more prominent on beta diversity too, where there was significant difference in beta diversity between HB and WL throughout all four ages (adjusted P<0.05). In contrary to housing, increased in age resulted in significant difference in beta diversity even within the same breed, which was more noticeable in HB (53 vs 67, 58 vs 67 and 72, and 67 vs 72) than WL (53 vs 67). Moreover, the cecal microbiota community structure was affected by housing types in both Brown (Figure 14) and White laying hens (Figure 15) at P<0.00.

5.4.2.8 Functional Predictions of Cecal Microbiota

The PCoA plot illustrating the microbial functional diversity between two different housing and breed types across four different time intervals is shown in Figure 16. The factors age, housing and breed not only affected community diversity but also affected functional diversity of cecal microbiota ($P<0.001$). However, functional diversity of cecal microbiota was less affected by the breed than their community structure as visualized in Figure 16, where the breed effect was significant at all ages except at 72 weeks (PERMANOVA pairwise, $P<0.05$). On contrary, housing affected functional diversity more than the community structure, where there was significant difference in functional diversity between CC and EC at both 67 and 72 weeks (PERMANOVA pairwise, $P<0.05$).

Differentially abundant predicted metabolic pathways of cecal microbiota between HB and WL hen strains are shown in Figure 17. Among 17 differentially abundant pathways between HB and WL, 13 pathways were significantly enriched in WL while 4 pathways were significantly enriched in HB. In WL, metabolic pathways related to TCA cycle, sucrose degradation, hexitol fermentation (lactate, formate, and ethanol), amino acids biosynthesis (arginine, L-phenylalanine, and L-tyrosine), the *Bifidobacterium* shunt, and peptidoglycan biosynthesis were significantly enriched in WL. However, pathways related to pyruvate fermentation to acetone, and biotin synthesis, palmitate biosynthesis were highly abundant in HB (Figure 17).

Moreover, differentially abundant microbial metabolic pathways between CC and EC housing systems in HB and WL laying hens are shown in Figure 18 and Figure 19, respectively. In HB group, altogether 22 metabolic pathways (8 in CC and 14 in EC) were differentially presented between CC and EC housing systems after filtering pathways with $P>0.05$ (Welch's t-test) and effect size (% difference in mean proportions) <0.03 using STAMP (Figure 18).

Specifically, pathways of TCA cycle, amino acid biosynthesis (L-serine and L-glycine), starch degradation, adenosylcobalamin (also known as vitamin B12 or coenzyme B12) biosynthesis, and 6-hydroxymethyl-dihydropterin diphosphate biosynthesis (precursor of vitamin B9 synthesis) were significantly enriched in CC group, whereas pathways of glycerol degradation, methanogenesis, amino acid biosynthesis (L-lysine, L-threonine, L-methionine, and L-aspartate), and purine and pyrimidine biosynthesis were significantly enriched in EC group.

In WL group, altogether 37 metabolic pathways (22 in CC and 15 in EC) were differentially presented between CC and EC housing systems as shown in Figure 19. Like in HB group, pathways of TCA cycle and 6-hydroxymethyl-dihydropterin diphosphate biosynthesis (precursor of vitamin B9 synthesis) were significantly higher in CC group, while pathways of purine nucleotide and amino acids (L-lysine and L-aspartate) biosynthesis in EC group. In contrary, biosynthesis pathways of amino acids such as L-ornithine, L-tryptophan, L-arginine, L-tyrosine, L-histidine, and L-phenylalanine were significantly enriched in CC group. The other important observations were significantly enrichment of glycolysis, acid fermentation and *Bifidobacterium* shunt pathway in EC group, while significant enrichment of pathways associated with various vitamins biosynthesis such as K2 (menaquinol-8 biosynthesis) and B12 (tetrapyrrole biosynthesis I) was observed in CC group (Figure 19).

5.5 Discussion

The intestinal microbiotas of chickens are affected by various factors such as age, breed, gut region, sex, feed, housing, hygiene, medication, temperature, litter, location, and maternal factors as reviewed earlier (Kers et al., 2018). Among these factors, the effect of feed on intestinal microbiota composition of chickens is widely studied. In laying hens, different dietary supplementations such as threonine (Dong et al., 2017), rapeseed meal (Long et al., 2017),

probiotics (Guo et al., 2016; Guo et al., 2018; Yan et al., 2019), calcium (Dastar et al., 2016), and flaxseed oil (Lee et al., 2016) have been found to modulate the intestinal microbiota. However, there is very limited information regarding the changes in intestinal microbiota composition of laying hens due to the housing systems.

To our knowledge, this is the first study that reported the effects of CC and EC on alterations of cecal microbiota in two important commercial strains of laying hens, WL and HB. In the present study, we found changes in cecal microbiota composition, their diversities and predicted functional pathways in both laying hen strains housed in CC and EC housing systems during the late production stage. Previous study reported higher number of *Clostridium perfringens* in ileum and cecum of broiler chickens raised in organic farms as compared to the conventional farms (Bjerrum et al., 2006). However, they suggested that the lower count of *C. perfringens* in conventional farms might be achieved due to the application of Salinomycin in the conventional feed that has antibiotic properties. In addition, they found an increase in Lactobacilli, while a decrease in Enterobacteriaceae counts in the ileal contents of the chickens from organic farms (Bjerrum et al., 2006). Another study reported enrichment of *Bifidobacterium* in both ileum and ceca of broiler chickens which were provided free daytime access as compared to those chickens which were kept at indoors range (Gong et al., 2008). Furthermore, both the composition and functions of cecal microbiota were different in Dagu chickens raised in free-range as compared to those raised in cages (Xu et al., 2016). Firmicutes/Bacteroidetes ratio was higher in cecum of cage-raised chickens, while the abundance of Bacteroidetes was higher in free range chickens (Xu et al., 2016). Although no direct comparisons can be made between the studies, we also reported the higher abundance of Bacteroidetes in EC where hens have more access to movement, while the higher abundance of Firmicutes in CC where they have restricted movement, especially at 67

weeks of age. In addition, we also reported significantly higher abundance of Proteobacteria in EC at 72 weeks of age which might be correlated with the tendency of decrease in egg production between CC and EC (P=0.06). Many gram-negative pathogenic bacteria such as *Escherichia*, *Salmonella*, *Campylobacter*, *Helicobacter*, and *Vibrio* are included under the phylum Proteobacteria whose increase can be considered as a potential indicator of gut dysbiosis (Shin et al., 2015). This was also reflected at genus level where *Campylobacter* and unknown genera of family Campylobacteraceae and Helicobacteraceae were significantly higher in EC at 72 weeks. Xu *et al.* also reported a higher abundance of cecal microbiota functions associated with amino acids and glycan metabolic pathways in Dagu chickens from free-range (Xu et al., 2016).

Recently, a study compared the cecal microbiota of You chickens (a Chinese native breed) reared in cages and free-range system at 45 weeks of age and reported the difference in their composition, diversity, and metabolic functions between the two systems (Chen et al., 2019). More specifically, the alpha diversity was decreased in chickens housed in cages as compared to those from free range. In addition, most of the KEGG pathways of cecal microbiota associated with various functions such as metabolism, alkaloid biosynthesis, and amino acids degradation were down-regulated in cages-reared chickens. In this study, the alpha diversity was significantly higher in EC compared to CC at 67 weeks of age and was numerically higher throughout all ages. Likewise, several metabolic pathways were differentially enriched between CC and EC in the current study which further depended on laying hen strains. For instance, 22 metabolic pathways (8 in CC and 14 in EC) were differentially abundant in HB strain, while 37 metabolic pathways (22 in CC and 15 in EC) in WL strain suggesting more pronounced effects of housing in WL. Specifically, pathways related to energy and nucleotide metabolism, and amino acids and vitamin

B biosynthesis were differentially presented between two housing systems in strains dependent manner.

The phylum Actinobacteria and its genus *Bifidobacterium* were significantly enriched in WL as compared to the HB throughout all four-time points. Bifidobacteria are common probiotic bacteria whose effects on hosts' health and diseases are studied elsewhere (Jung et al., 2008; O'Callaghan and van Sinderen, 2016), and are widely considered to confer beneficial effects on hosts through their metabolic activities. Specifically, bifidobacteria are well known for their ability to ferment complex carbohydrates in the lower part of the intestine that bypasses the degradation in the upper parts through various carbohydrate-degrading enzymes (Pokusaeva and Fitzgerald, 2011). They can ferment diverse complex carbon sources including gastric mucin, (trans)-galactooligosaccharides, xylo-oligosaccharides, malto-oligosaccharides, fructo-oligosaccharides, pectin, soybean oligosaccharides, and other plant derived-oligosaccharides. However, their ability to degrade particular carbon source is species/strain dependent (De Vrese and Schrezenmeir, 2008). Through fermentation, bifidobacteria can degrade complex carbohydrates to monosaccharides which are further degraded to intermediates of the hexose fermentation pathway (also known as Bifidobacterium shunt or fructose-6-phosphate shunt) (De Vries and Stouthamer, 1967), and finally converted to short-chain fatty acids, especially acetate and lactate (O'Callaghan and van Sinderen, 2016). In the current study, carbohydrate degradation was significantly enriched in WL as compared to the HB. In addition, the Bifidobacterium shunt pathway was significantly enriched in WL as compared to the HB.

Similarly, butyrate producing genera such as *Butyricicoccus* and *Subdoligranulum* were significantly higher in WL as compared to HB at 58 and 72 weeks. Butyrate, a metabolite of intestinal microbiota is considered as an important feed additive in animal production due to its

several beneficial effects such as improvement of performance parameters and maintenance of gut health by controlling the proliferation of bacterial pathogens and enhancement of intestinal development (Guilloteau et al., 2010; Bedford and Gong, 2018). Other important observations were time-dependent enrichment of phyla Synergistetes and Spirochaetes and genera such as *Clostridium* and *Paraprevotella* in HB compared to WL. In details, there were 36, 21, 54, and 7 differentially abundant genera in HB at 53, 58, 67, and 72 weeks, respectively as compared to WL. Interestingly, the differences in cecal microbiota between WL and HB not only observed in their composition but also in both community and functional diversities, which might explain the variations in egg production between the two strains. Moreover, both egg production and cecal microbiota variations in hen strains depended on housing types. Significant interaction effect of housing and laying hen strains on egg production were also reported earlier (Singh et al., 2009).

In sum, egg production, cecal microbiota composition, diversities, and their functional pathways were affected by housing type which further varied between two commercial laying hen strains, HB and WL. This suggests that both housing and strains should be considered while selecting alternative housing systems.

5.6 References

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5.7 Tables and Figures

Table 1. Diet formulation and calculated composition of diet fed to Hy-Line hens.

Ingredient	**Amount (%)**
Corn	57.00
Soybean Meal	21.79
Limestone	11.06
Corn DDGS	5.00
Poultry Fat	2.84
Dicalcium Phosphate	1.44
Common Salt	0.30
DL-Methionine	0.23
Vitamin Premix	0.13
Mineral Premix	0.13
L-Lysine HCL	0.09
Total	100.00
Calculated composition	
ME (Kcal/kg)	2.760
CP (%)	16
Ca (%)	4.6
Available P (%)	0.40

Table 2. Summary of samples and reads distribution across different groups. The first and second number in brackets represent number of samples used for microbiota and egg production analysis, respectively. The values in each cell represent an average number of reads/sample (Mean±SE) for that particular group.

Variables	**53 weeks**	**58 weeks**	**67 weeks**	**72 weeks**
House				
CC	63,697.3±4,212.0 (12, 10)	61,876.4±5,055.5 (9, 10)	56,769.6±3,649.8 (12, 10)	59,954.4±3,174.7 (12, 10)
EC	66,289.4±3707.3 (12, 8)	61,392.5±2,759.5 (11, 9)	58,962.2±2,287.3 (12, 10)	66,829.9±3,865.4 (10, 10)
Strain				
HB	67,706.5±3,773.8 (12, 10)	56,247.0±3,870.6 (11, 10)	56,402.4±2,576.2 (12, 10)	59,455.7±3,996.4 [b] (11, 10)
WL	62,280.2±4,024.3 (12, 8)	68,165.3±2,134.0 (9, 9)	59,329.3±3427.1 (12, 10)	66,703.5± 2,840.0 [a] (11, 10)
House-Strain				
CC-HB	62,531.5±6,031.7 (6, 5)	54,635.2±7409.1 (5, 5)	54,942.5±3,271.5 (6, 5)	58,006.8±5,730.9 (6, 5)
CC-WL	64,863.0±6,413.6 (6, 5)	70,928.0±3,563.5 (4, 5)	58,596.7±6,824.6 (6, 5)	61,902.0±3,160.0 (6, 5)
EC-HB	72,881.5±3,954.2 (6, 5)	57,590.2±4,188.2 (6, 5)	57,862.3±4,200.8 (6, 5)	61,194.4±6,098.6 (5, 5)
EC-WL	59,697.3±5,239.9 (6, 3)	65,955.2±2,455.0 (5, 4)	60,062.0±2,211.1 (6, 5)	72,465.4±3,763.7 (5, 5)

Table 3. Hen-day egg production (HDEP %) of two laying hen strains kept in conventional (CC) and enriched colony cages (EC) from 53 to 72 weeks of age. Data was analyzed in a 2*2 factorial design using JMP Genomics 9, where the pairwise comparison of means was performed by Tukey HSD test. The values are presented as LS means ± standard error. Different letters with in a column represent a significant difference between two groups at $P<0.05$.

Group	**53 weeks**	**58 weeks**	**67 weeks**	**72 weeks**
House				
CC	80.57±2.49	87.69±2.26	70.56±3.09	63.46±2.07
EC	77.09±2.87	85.07±2.40	66.09±3.09	57.73±2.07
Strain				
HB	74.89±2.49	83.69±2.26	66.68±3.09	56.34±2.07 [b]
WL	82.76±2.87	89.07±2.40	69.97±3.09	64.85± 2.07 [a]
House*Strain				
CC x HB	72.14±3.52 [b]	88.11±3.20	66.13±4.38	61.77±2.93
CC x WL	89.00±3.52 [a]	87.26±3.20	75.00±4.38	65.15±2.93
EC x HB	77.65±3.52 [ab]	79.28±3.20	67.23±4.38	50.91±2.93
EC x WL	76.52±4.54 [ab]	90.87±3.58	64.95±4.38	64.55±2.93
P - value				
House	0.3758	0.4423	0.3231	0.0690
Strain	0.0578	0.1252	0.4634	0.0106
House × Strain	0.0331	0.0795	0.2216	0.1000

Table 4. Summary of differentially abundant phyla identified by LEfSe ($P<0.05$, LDA score >2.0).

Group	**53 weeks**	**58 weeks**	**67 weeks**	**72 weeks**
House				
CC	-	-	Firmicutes	-
EC	Spirochaetes	Spirochaetes	Bacteroidetes	Proteobacteria
Strain				
HB	Synergistetes	Spirochaetes	Synergistetes, Spirochaetes	-
WL	Actionobacteria	Actionobacteria	Actionobacteria	Actionobacteria

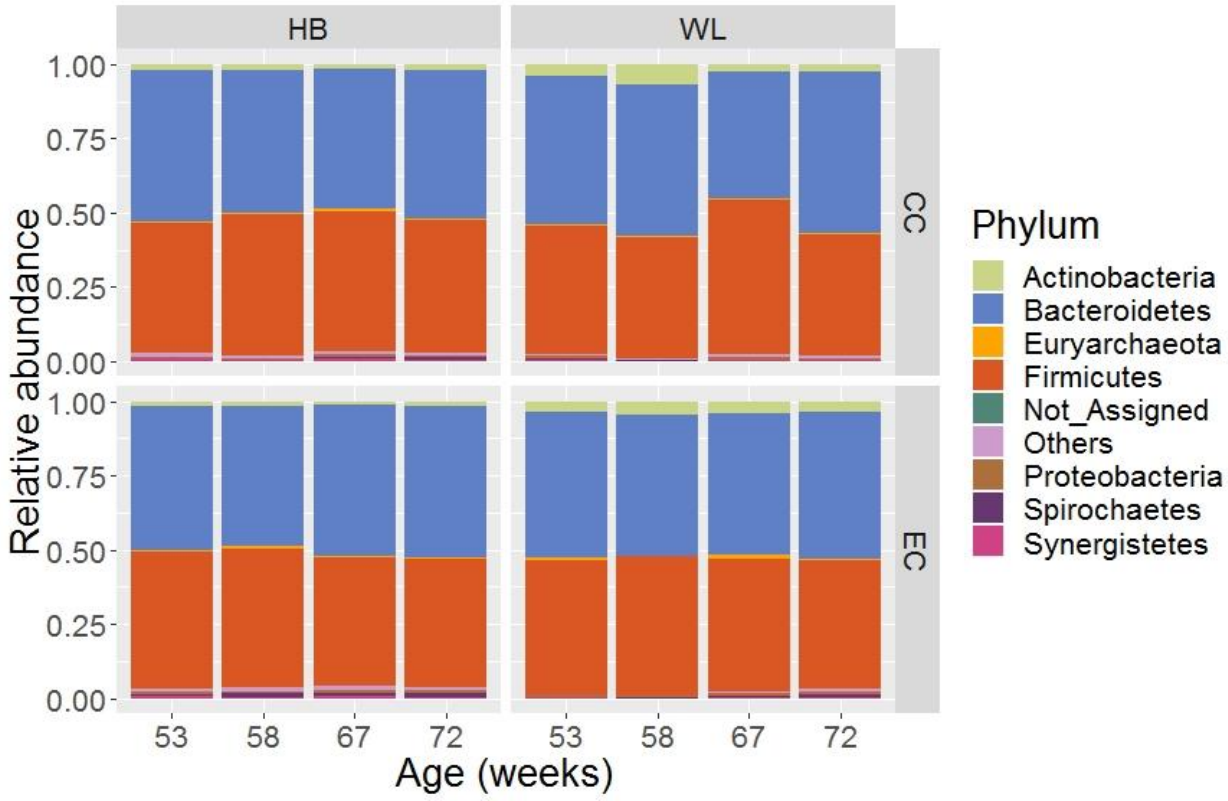

Figure 1. The relative abundance of cecal microbiota at phylum level. HB and WL represent Hyline Brown and White Leghorn, while CC and EC represent Conventional Cage and Enriched Colony Cage, respectively. Not_Assigned represent the reads that weren't assigned at any phyla, where "Others" represent the phyla which were present less than <0.4% on average of all samples.

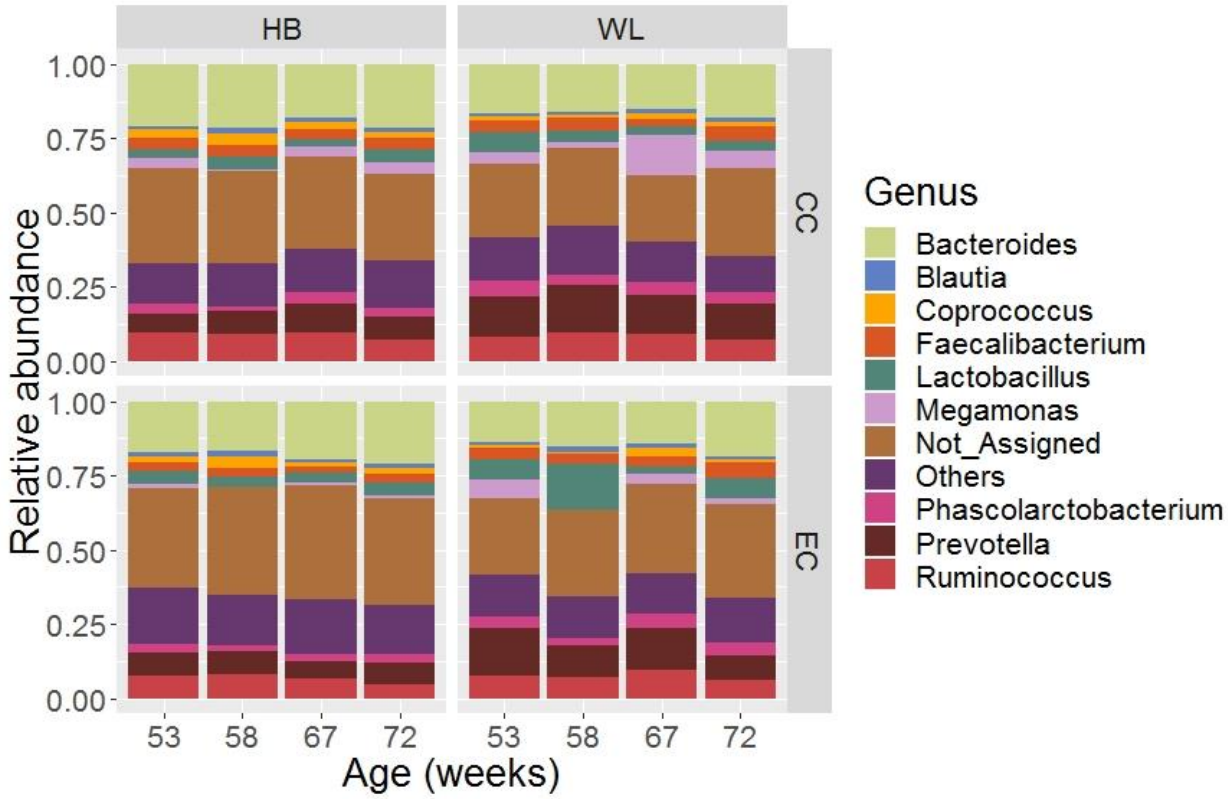

Figure 2. The relative abundance of cecal microbiota at genus level. HB and WL represent Hyline Brown and White Leghorn, while CC and EC represent Conventional Cage and Enriched Colony Cage, respectively. Not_Assigned represent the reads that weren't assigned at genus but assigned at higher taxonomic level. Others represent the genera which were present less than <1.0% on average of all samples.

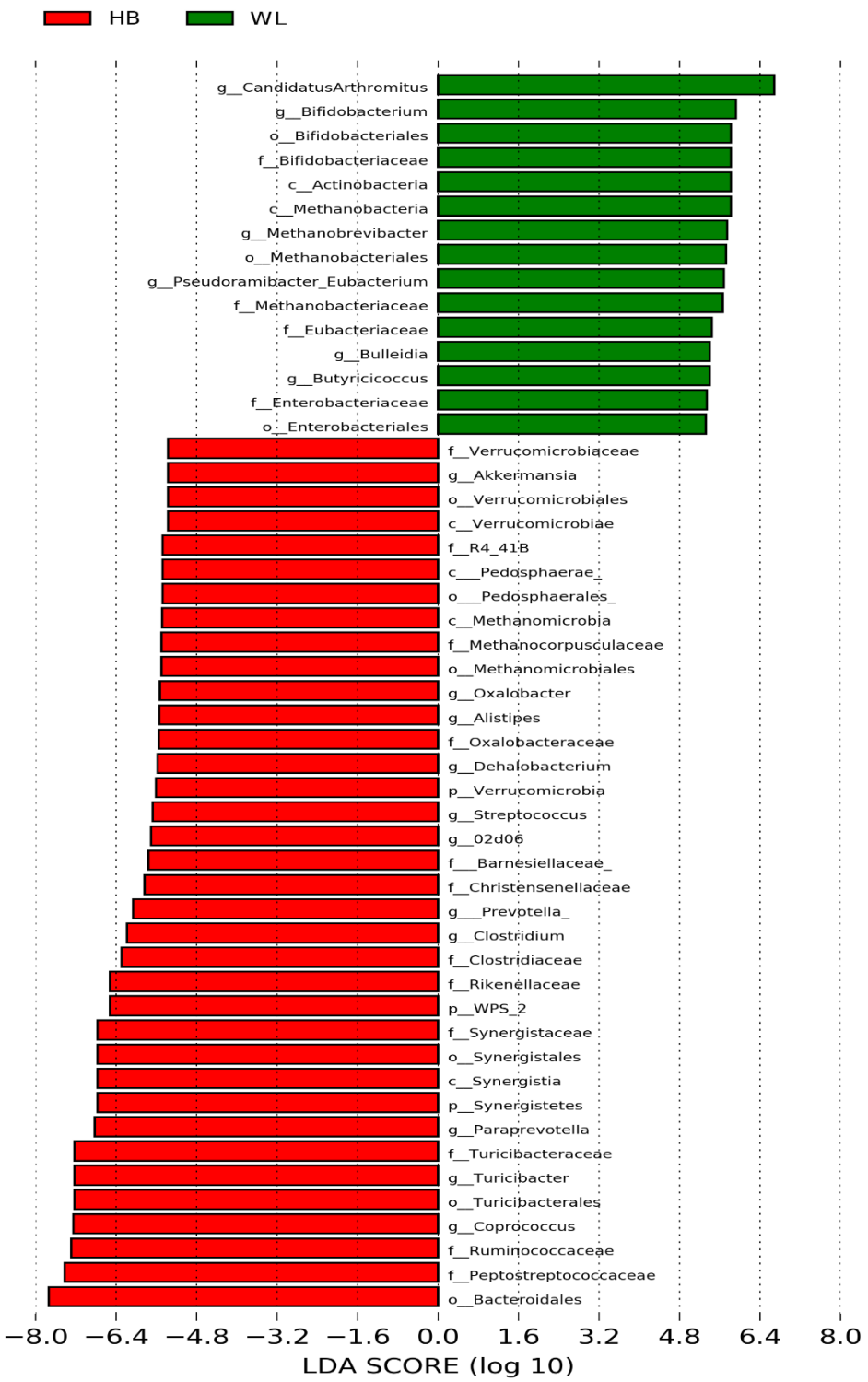

Figure 3. Differentially abundant taxa that were assigned at the genus level and identified by LEfSe (P<0.05, LDA score>2.0) between Hyline Brown (HB) and White Leghorn (WL) at 53 weeks.

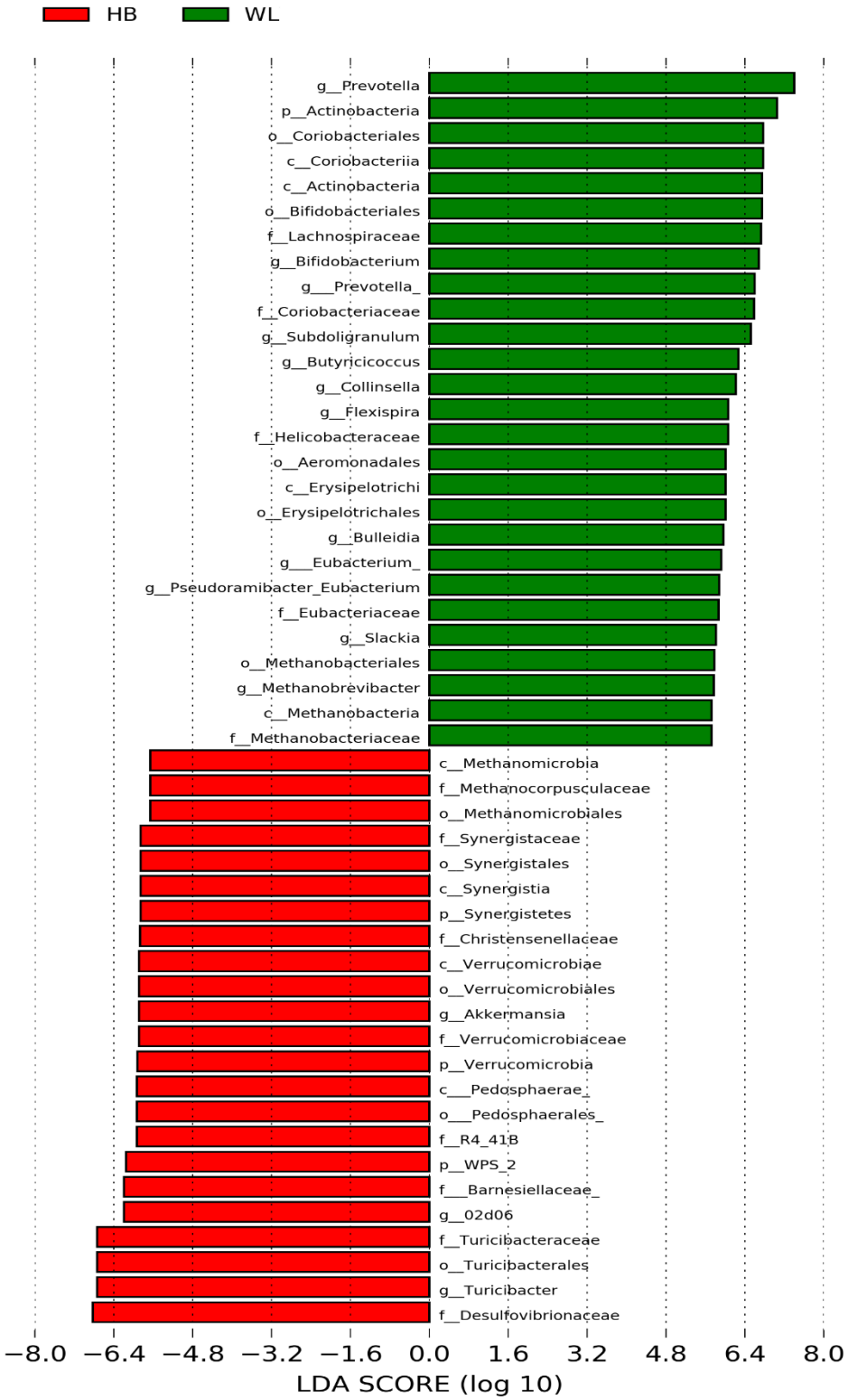

Figure 4. Differentially abundant taxa that were assigned at the genus level and identified by LEfSe (P<0.05, LDA score>2.0) between Hyline Brown (HB) and White Leghorn (WL) at 58 weeks.

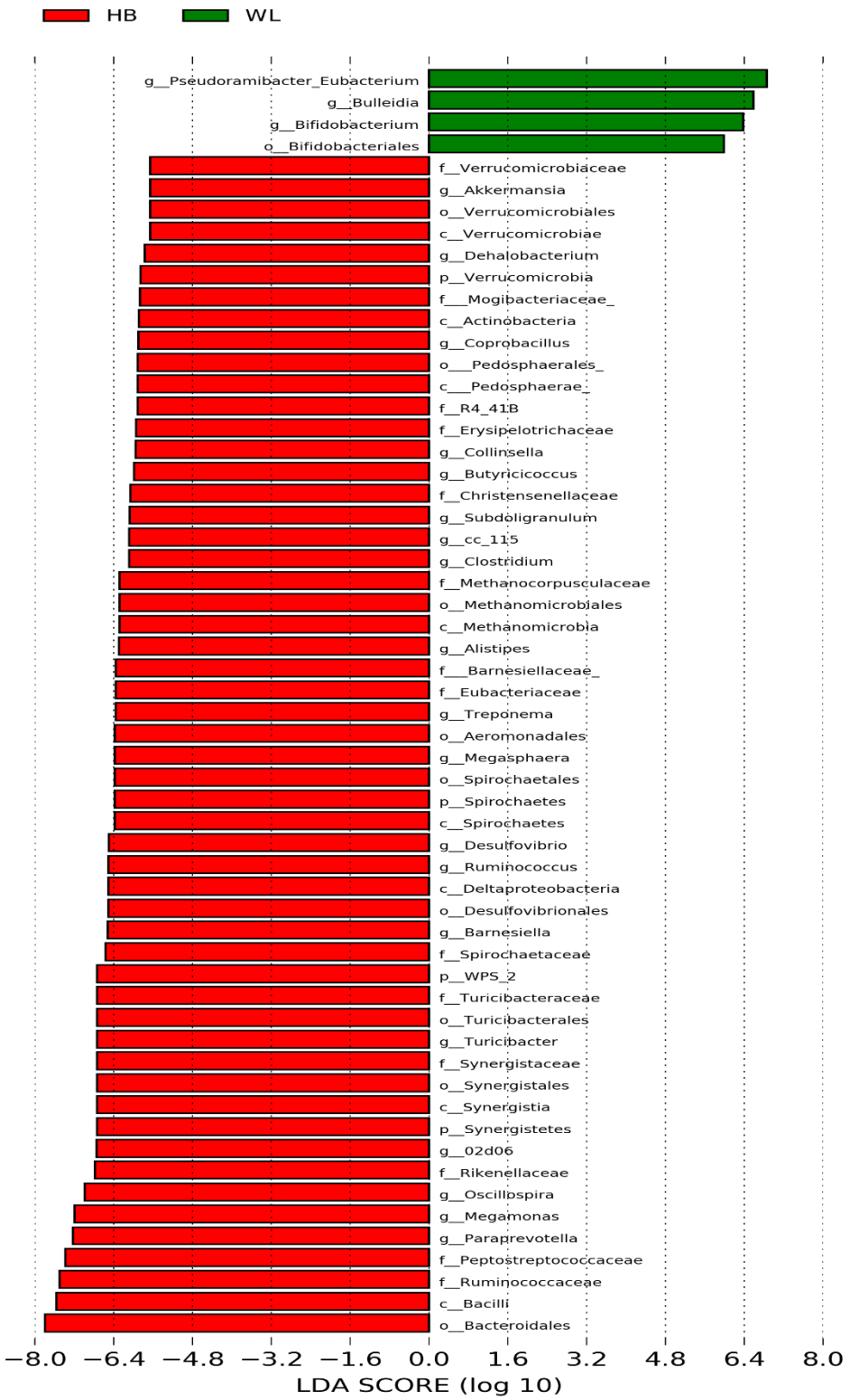

Figure 5. Differentially abundant taxa that were assigned at the genus level and identified by LEfSe (P<0.05, LDA score>2.0) between Hyline Brown (HB) and White Leghorn (WL) at 67 weeks.

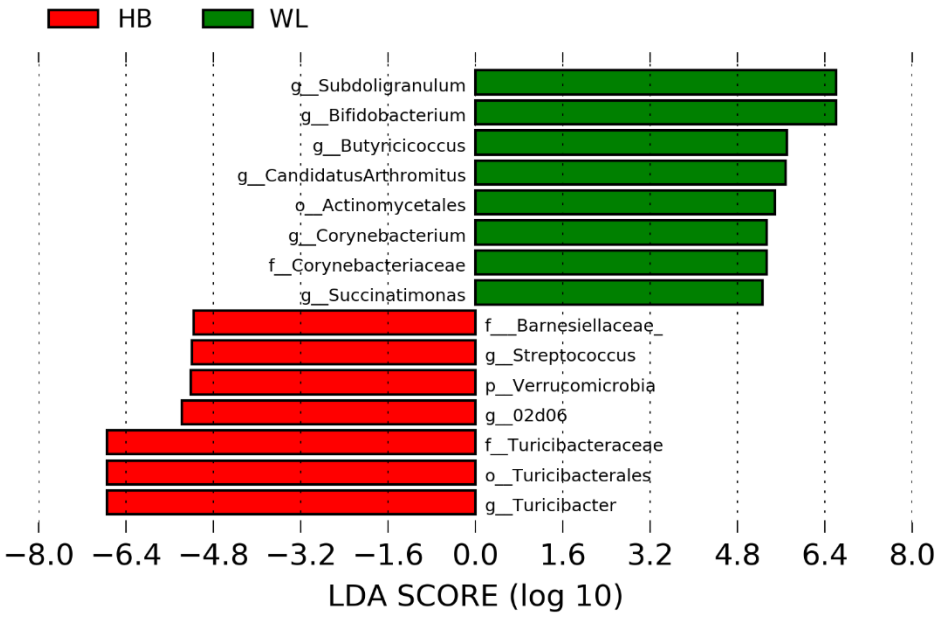

Figure 6. Differentially abundant taxa that were assigned at the genus level and identified by LEfSe (*P*<0.05, LDA score>2.0) between Hyline Brown (HB) and White Leghorn (WL) at 72 weeks.

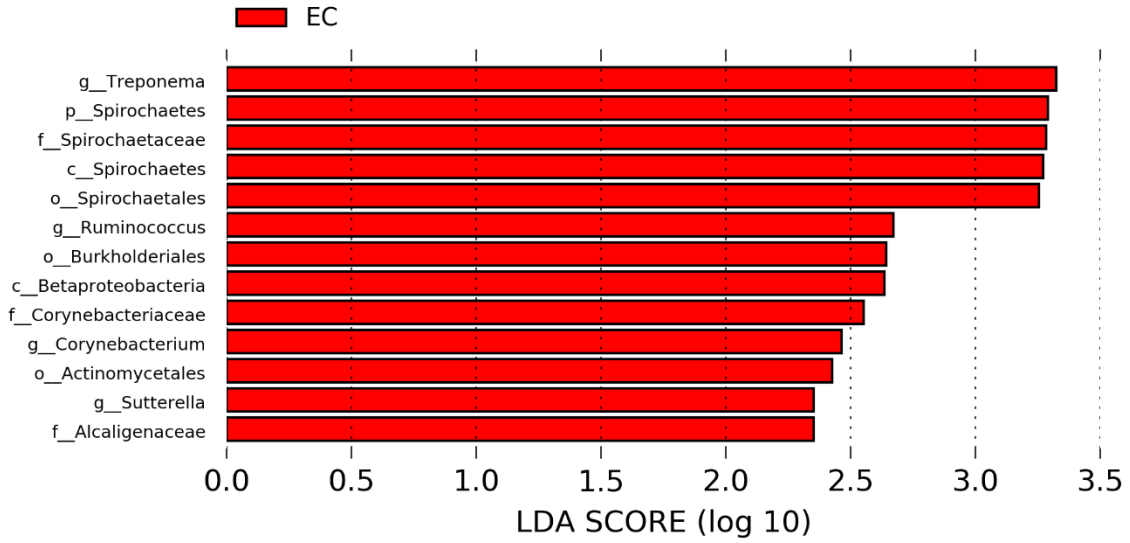

Figure 7. Differentially abundant taxa that were assigned at the genus level and identified by LEfSe (*P*<0.05, LDA score>2.0) between Conventional Cage (CC) and Enriched Colony Cage (EC) housing systems at 53 weeks.

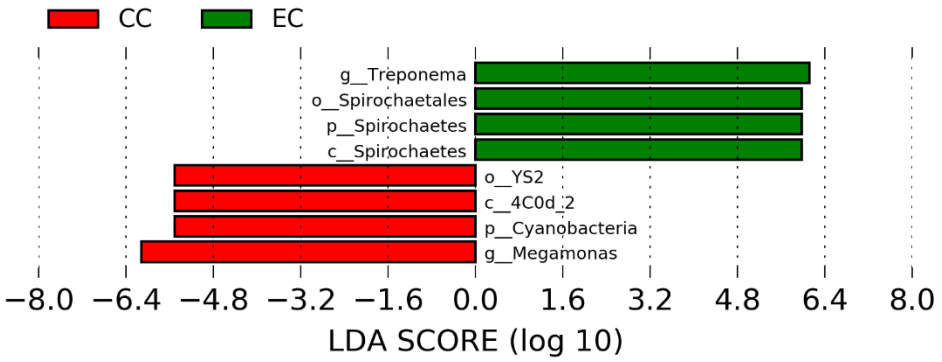

Figure 8. Differentially abundant taxa that were assigned at the genus level and identified by LEfSe (P<0.05, LDA score>2.0) between Conventional Cage (CC) and Enriched Colony Cage (EC) housing systems at 58 weeks.

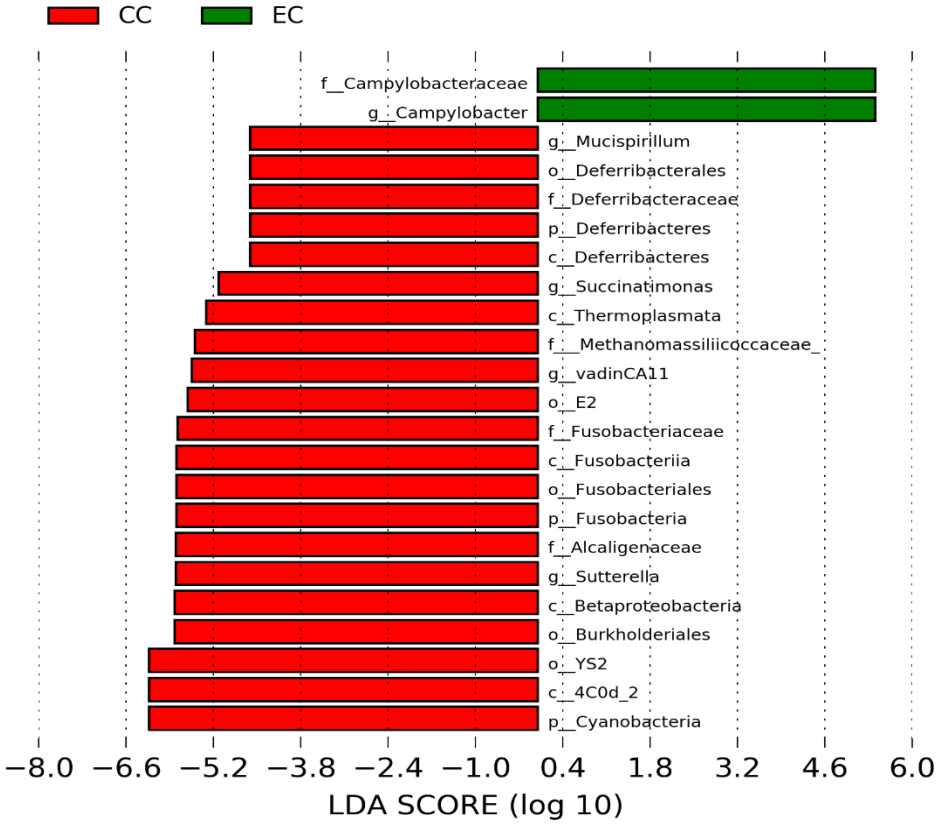

Figure 9. Differentially abundant taxa that were assigned at the genus level and identified by LEfSe (P<0.05, LDA score>2.0) between Conventional Cage (CC) and Enriched Colony Cage (EC) housing systems at 67 weeks.

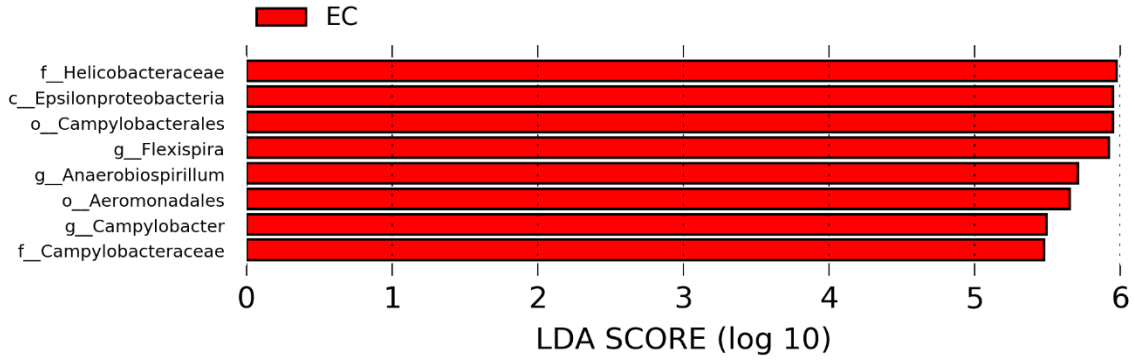

Figure 10. Differentially abundant taxa that were assigned at the genus level and identified by LEfSe (P<0.05, LDA score>2.0) between Conventional Cage (CC) and Enriched Colony Cage (EC) housing systems at 72 weeks.

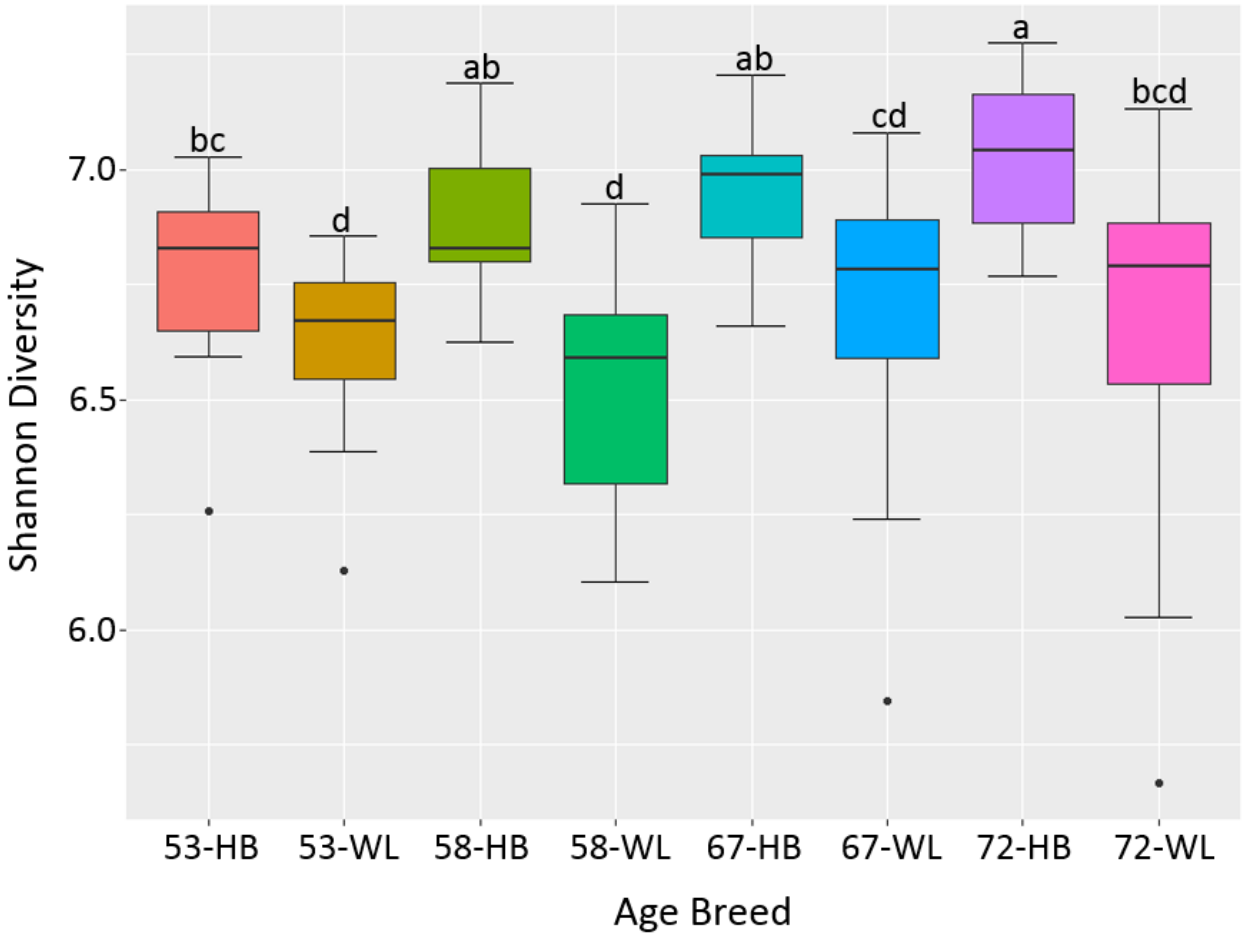

Figure 11. The difference in alpha diversity as measured by Shannon's diversity between Hyline Brown (HB) and White Leghorn (WL) at 53, 58, 67, and 72 weeks of hens' ages.

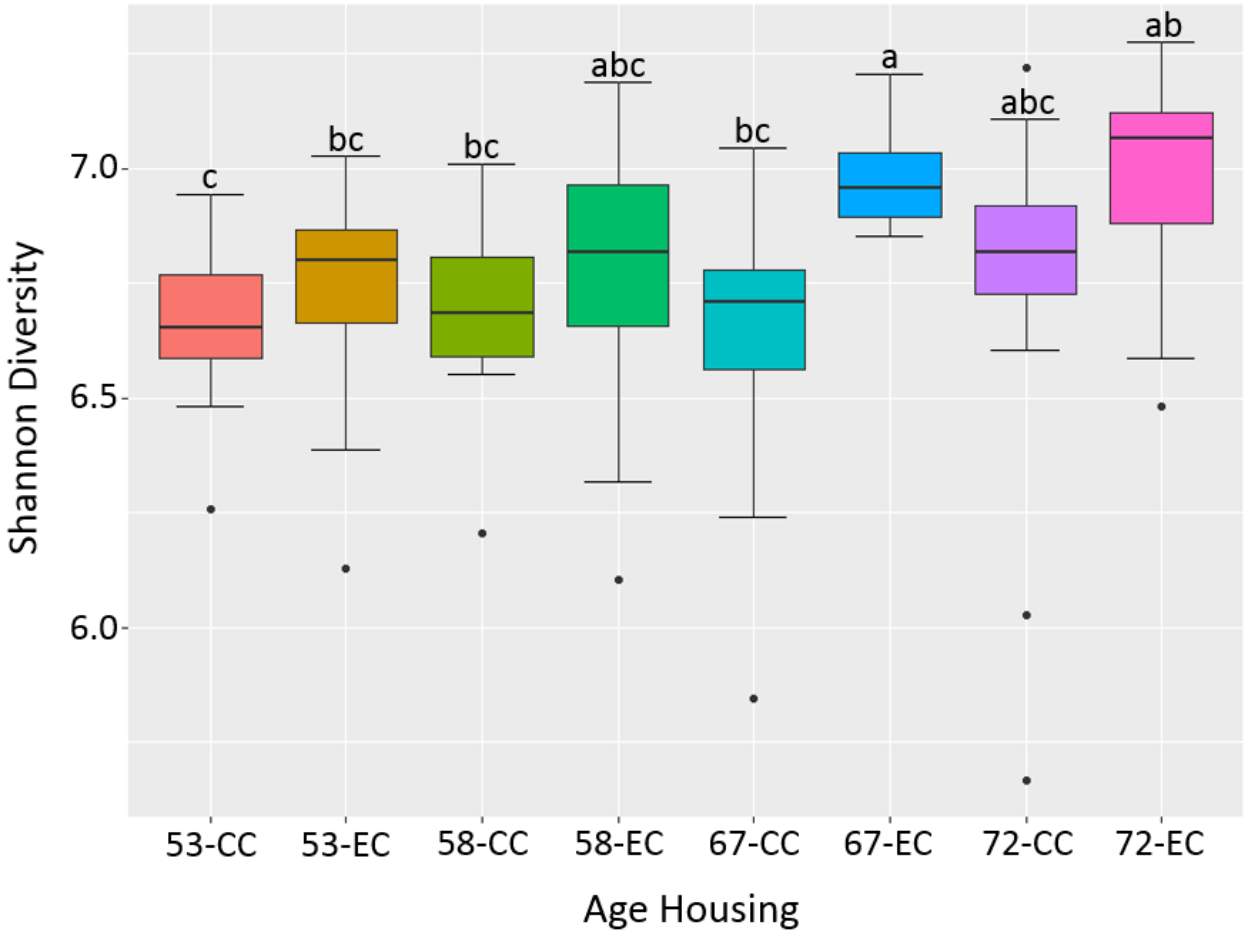

Figure 12. The difference in alpha diversity as measured by Shannon's diversity between hens housed in Conventional Cage (CC) and Enriched Colony Cage (EC) systems at 53, 58, 67, and 72 weeks of hens' ages.

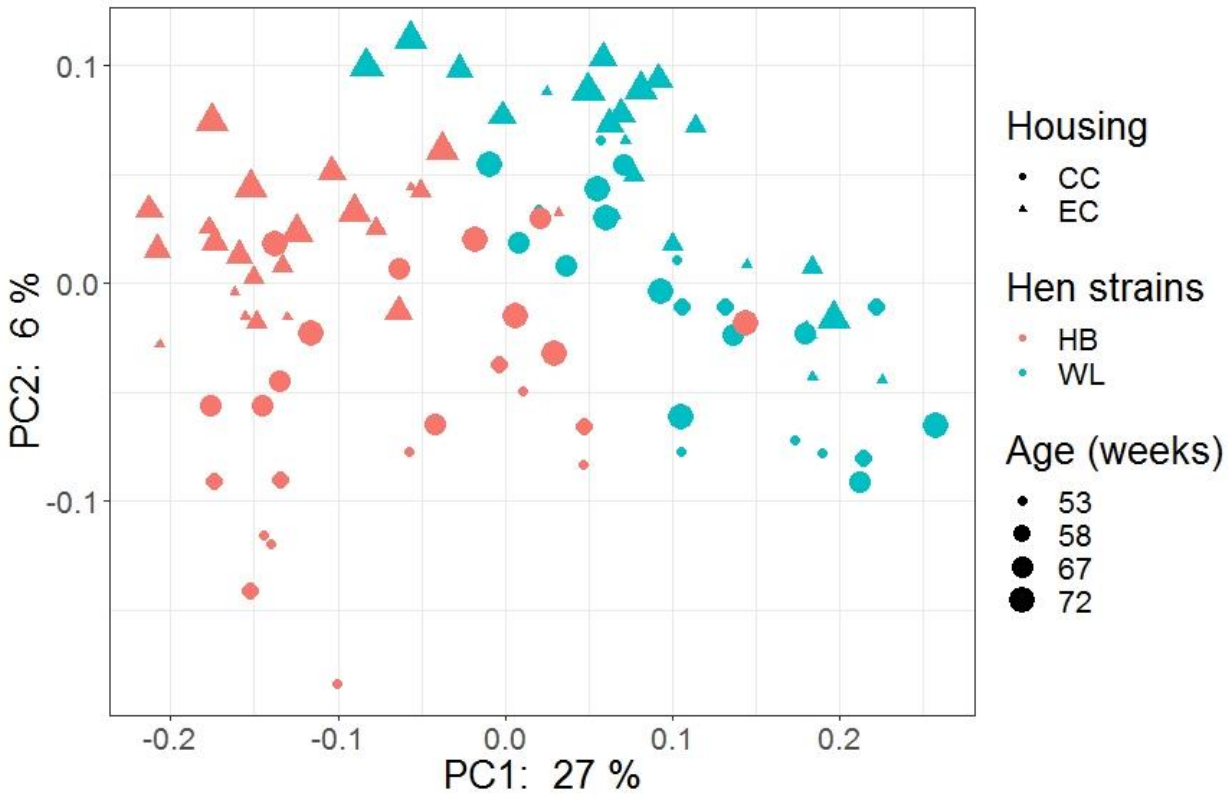

Figure 13. PCoA plot showing cecal microbiota community structure between two different housing (CC; Conventional Cage and EC; Enriched Colony Cage) and breed types (HB; Hyline Brown and WL; White Leghorn) at 53, 58, 67, and 72 weeks of hens' ages. The plot was generated using unweighted distance metric.

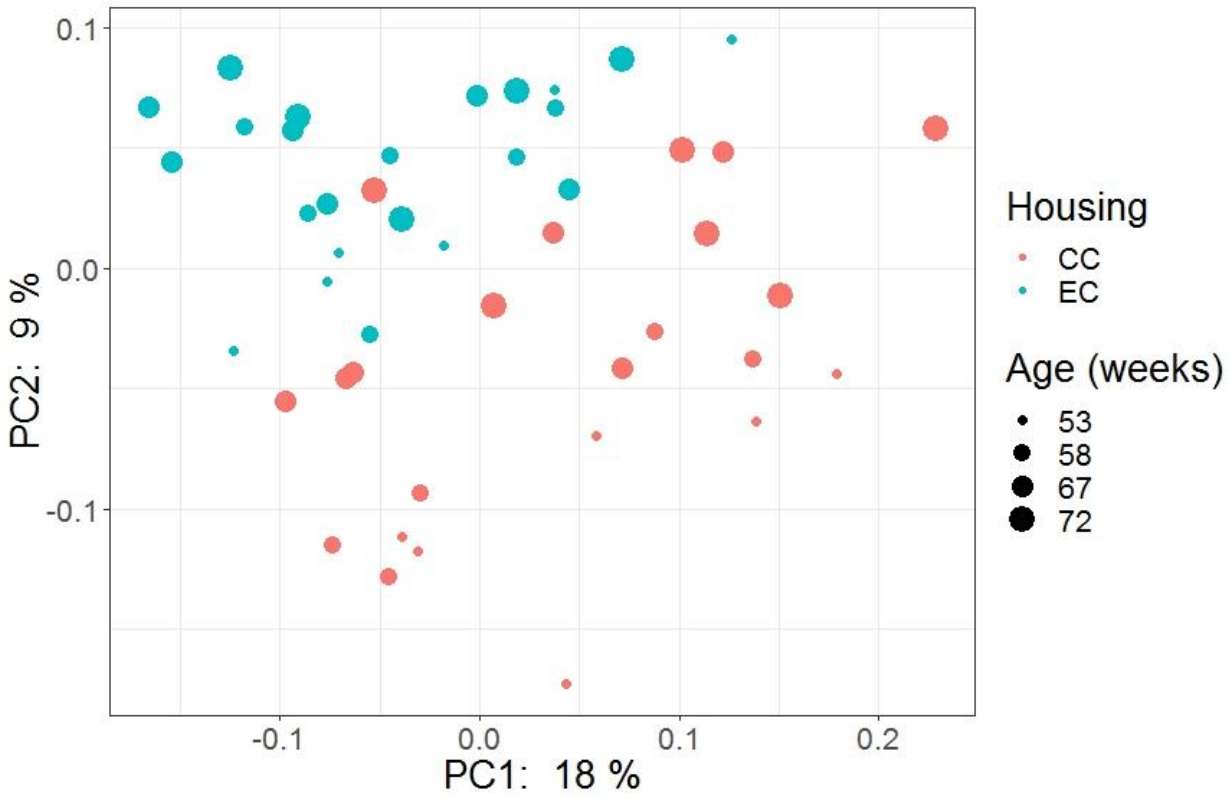

Figure 14. PCoA plot showing cecal microbiota community structure in Hyline Brown (HB) housed in Conventional Cage (CC) and Enriched Colony Cage (EC) systems.

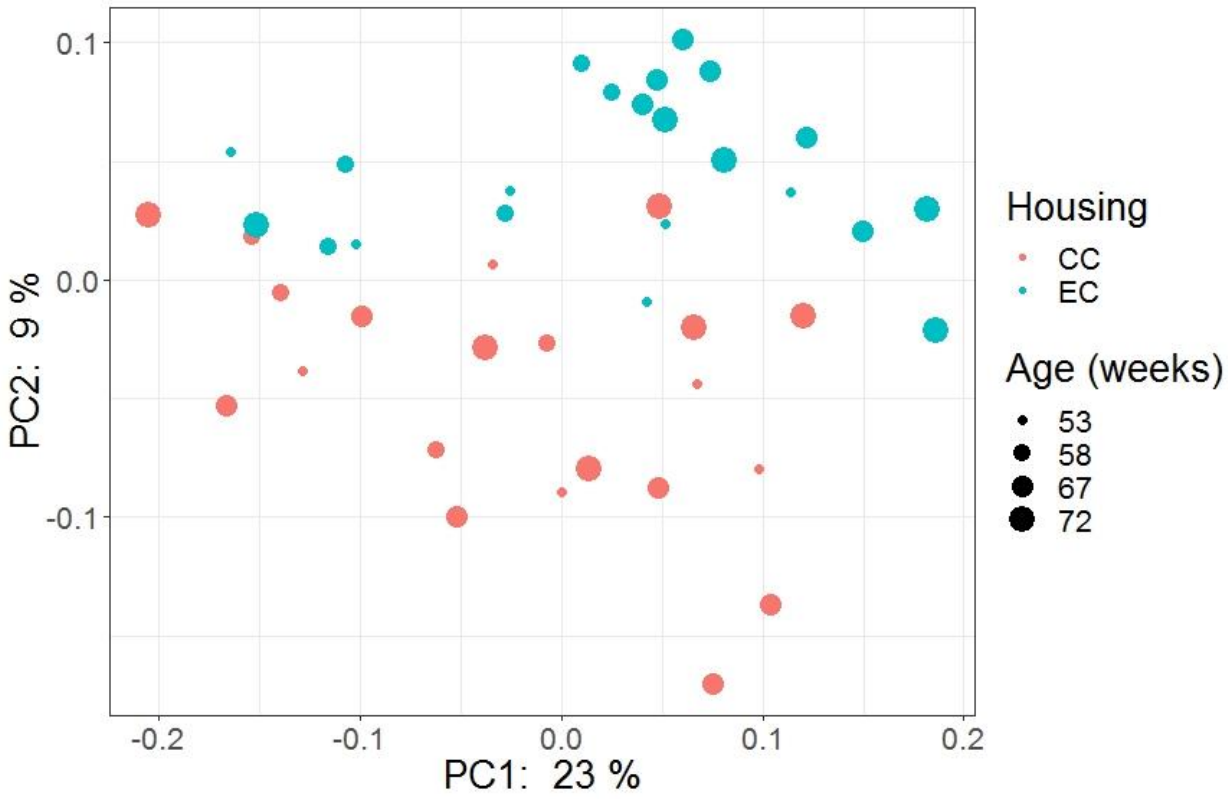

Figure 15. PCoA plot showing cecal microbiota community structure in White Leghorn (WL) housed in Conventional Cage (CC) and Enriched Colony Cage (EC) systems.

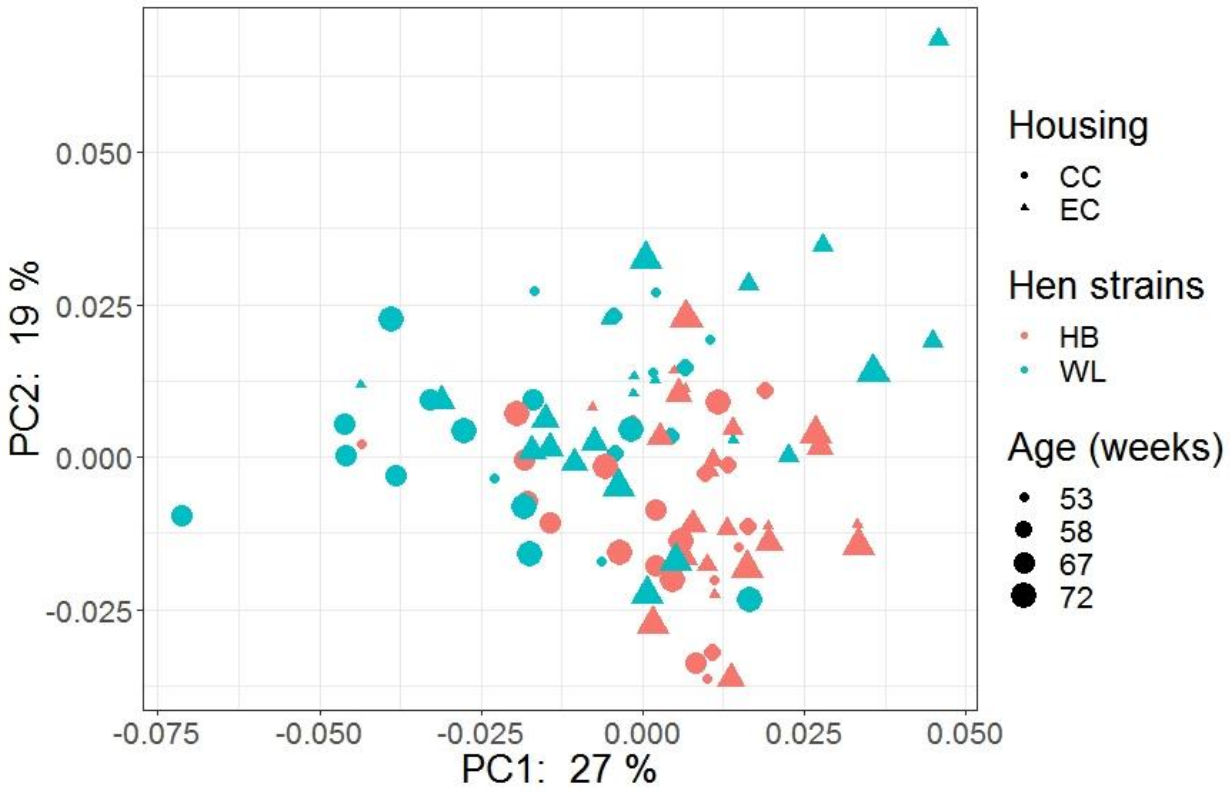

Figure 16. PCoA plot showing cecal microbiota functional diversity between two different housing (CC; Conventional Cage and EC; Enriched Colony Cage) and breed types (HB; Hyline Brown and WL; White Leghorn) at 53, 58, 67, and 72 weeks of hens' ages. The plot was created using Bray Curtis distance metric generated from metabolic pathways predicted by PICRUSt2.

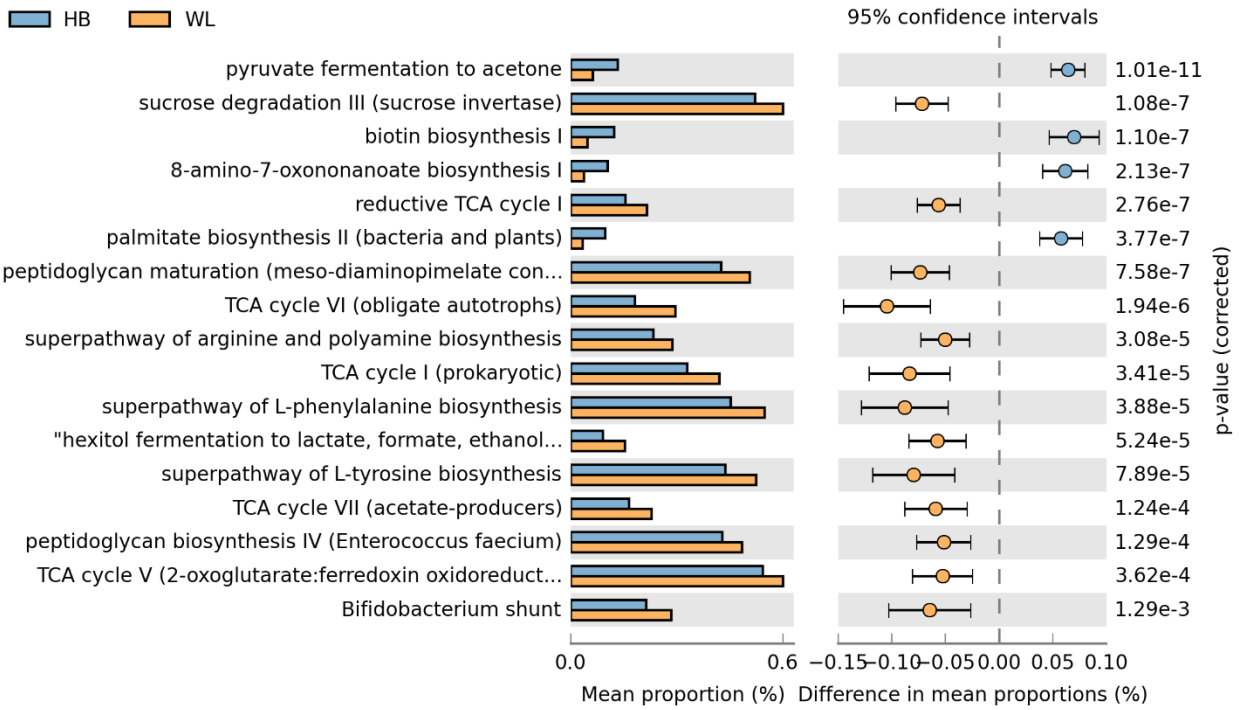

Figure 17. Differentially abundant metabolic pathways of cecal microbiota between Hyline Brown (HB) and White Leghorn (WL). STAMP software was used to identify differentially abundant features using Welch's t-test, where features were filtered using $P>0.05$ and difference in mean proportions (%) <0.05 criteria.

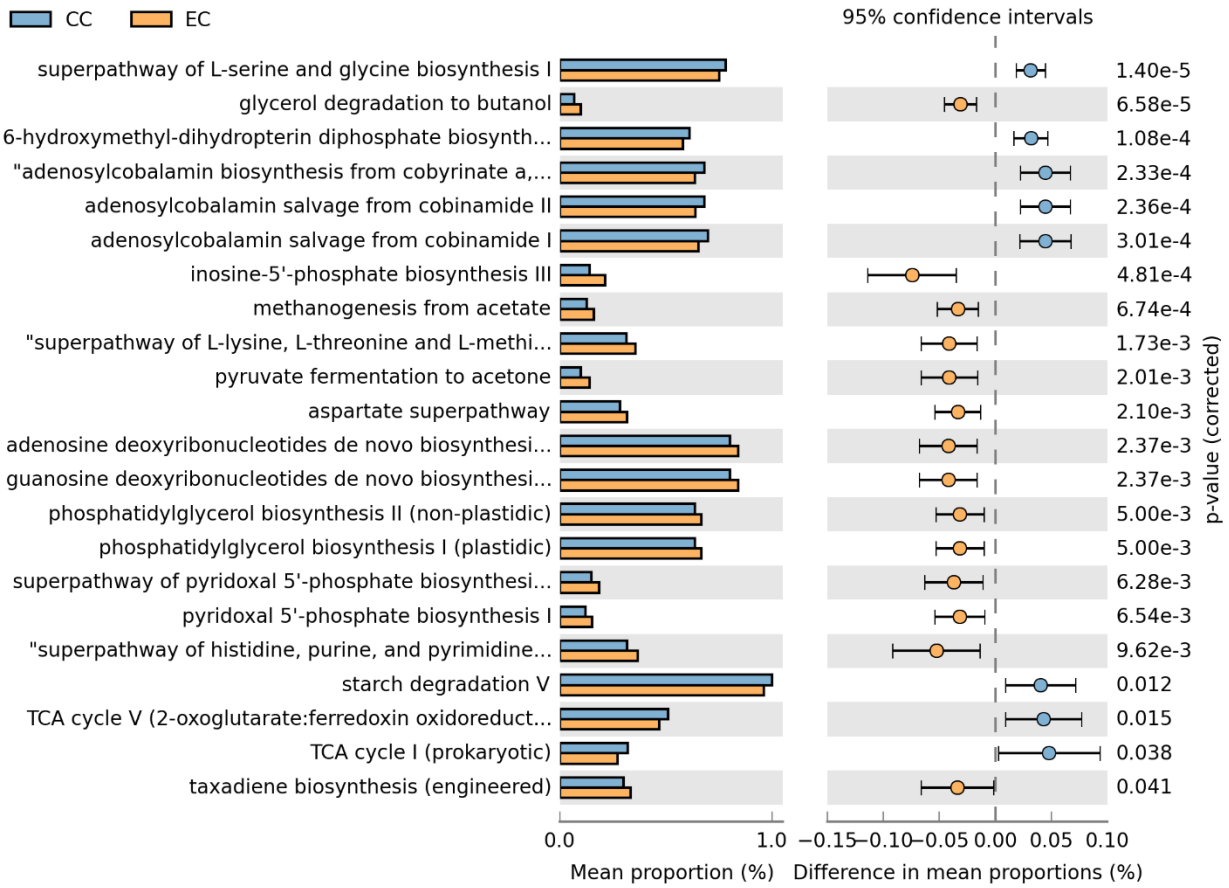

Figure 18. Differentially abundant metabolic pathways of cecal microbiota in Hyline Brown (HB) housed in Conventional Cage (CC) and Enriched Colony Cage (EC) systems. STAMP software was used to identify differentially abundant features using Welch's t-test, where features were filtered using $P>0.05$ and difference in mean proportions (%) <0.03 criteria.

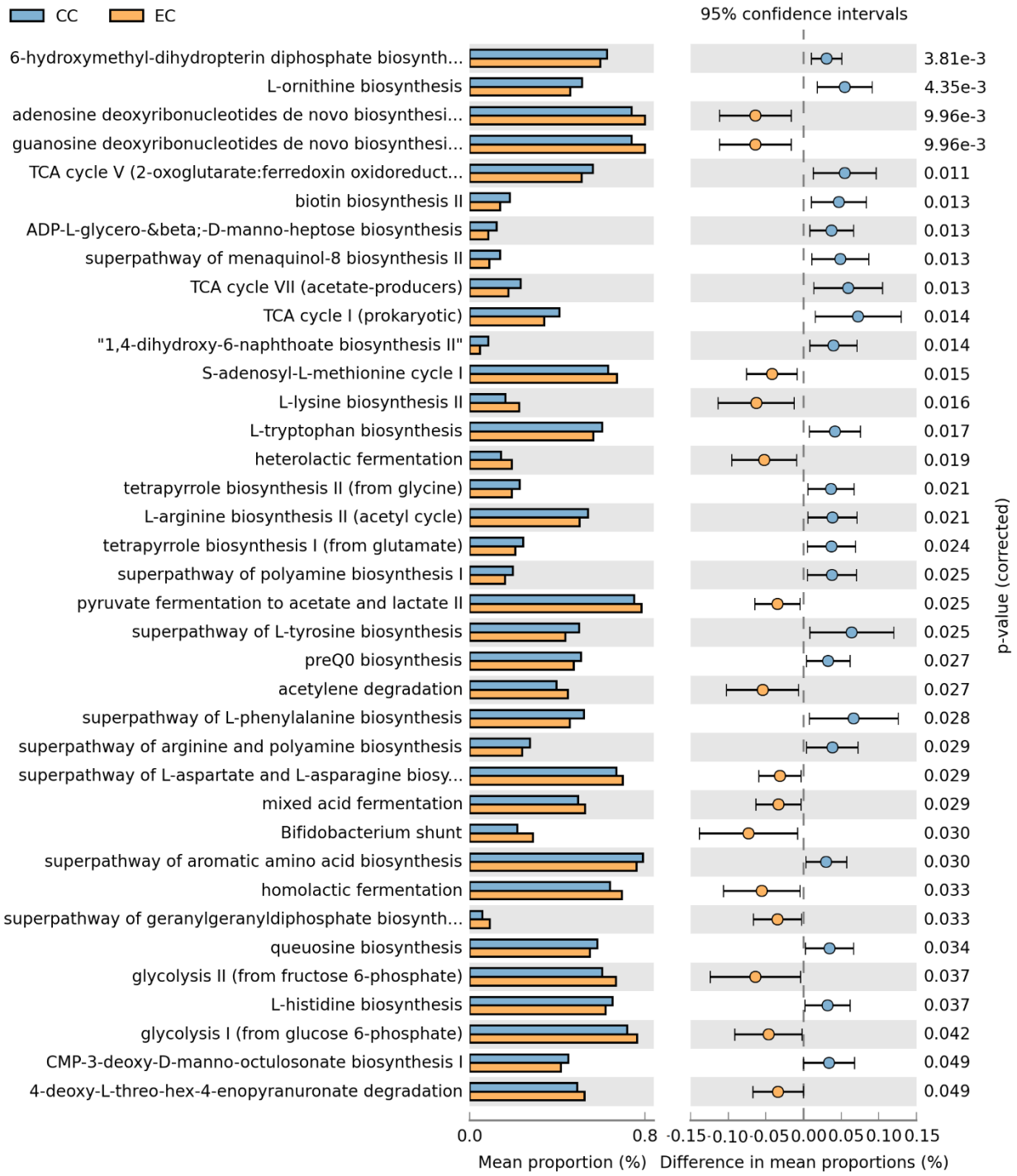

Figure 19. Differentially abundant metabolic pathways of cecal microbiota in White Leghorn (WL) housed in Conventional Cage (CC) and Enriched Colony Cage (EC) systems. STAMP software was used to identify differentially abundant features using Welch's t-test, where features were filtered using P>0.05 and difference in mean proportions (%) <0.03 criteria.

CHAPTER SIX

Comprehensive survey of the litter bacterial communities in commercial turkey farms

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6.1 Abstract

The importance of microbiota in the health and diseases of farm animals has been well documented for diverse animal species. However, studies on microbiotas in turkey and turkey farms are limited. In this study, we performed a comprehensive survey of the microbiotas in the 5 commercial turkey farms of the Northwest Arkansas (H, M, V, K, and R) including one farm with positive incidence of cellulitis (R farm). Altogether 246 boot swabs were used for 16S rRNA gene profiling of the microbial communities in the litter of the turkey farms. Altogether 3,057 unique features (amplicon sequence variants; ASVs) were identified from 10,863,650 sequences. At phylum level, 11 major bacterial phyla (≥0.01%) were recovered along with one phylum (Euryarchaeota; 0.08%) of division archaea. At genus level, 13 major bacterial genera were found whose relative abundance were > 2%. The microbial composition at both phylum and genus level as well as their diversities varied across different farms and among different flocks within the same farms, which were further affected by the ages of turkeys. Generally, the Firmicutes were found higher in the flocks of younger birds, while the Actinobacteria and Bacteroidetes were found higher in the flocks of the older birds. The Proteobacteria were highly enriched (47.97%) especially in K farm housing 56 days old turkeys (K-56), but Bacteroidetes, were found the highest in the flock C of M farm housing 63 days old turkeys (M-C-63; 22.38%), followed by K-84 group (17.26%). Such variations were also reflected at the genus level where the genus *Escherichia-Shigella* that belong to the phylum Proteobacteria was highly abundant in K-84 (42.83%). Similarly, the genus *Bacteroides* was reported the highest in M-C-63 group (13.70%). On the contrary, *Corynebacterium* (0.97%) and *Staphylococcus* (1.07%) were found the lowest in M-C-63 group. 20 core bacterial genera were identified based on the 95% samples prevalence, while only 4 core genera (*Staphylococcus*, *Brevibacterium*, *Brachybacterium*, and *Lactobacillus*) were

identified based on 100% samples prevalence. In contrast, 24 core bacterial genera were found based on 100% samples prevalence in cellulitis associated samples including *Corynebacterium*, an unknown genus of family Bacillaceae, *Clostridium* sensu stricto 1 (>97% similarity with *C. septicum*), and *Ignatzschineria* beside others, suggesting their possible roles in etiopathogenesis of cellulitis in turkeys. To our knowledge, this is the first study that investigated the turkey litter microbial communities using boot swabs and the overall results of this study may provide valuable insights for future studies targeting the health and diseases of turkeys.

6.2 Introduction

During the last decade, the decrease in sequencing costs coupled with innovations in computational technologies and approaches (Muir et al., 2016) has advanced our analysis and understanding of the composition and function of microbial communities residing in diverse environments (Jovel et al., 2016). Although the roles of microbiota in health and diseases have been well documented in wide range of animals, very limited microbiome studies have been conducted so far in turkeys.

A study that was published in 2007 that investigated the succession of intestinal microbiota in the ceca of male turkeys, where they reported decrease in clostridia species and increase in *Bacteroides uniformis* over time (Scupham, 2007). A period of microbial community transition was detected at 12 weeks of age with significant increase in the abundance of *Campylobacter coli*. In addition, increased in age of birds resulted increase in the species richness in trial 1, but it was not noticed in trial 2. Likewise, the cecal bacterial succession in relation to the *Campylobacter jejuni* and *Campylobacter coli* loads has also previously been studied (Scupham, 2009). Similar with the previous findings, the cecal bacterial communities were changed in a time-dependent manner and *Campylobacter* loads were correlated with the acute microbial community transition. In another study, considerably divergence of the cecal bacterial genera was found in the domestic turkeys as compared to the wild ones, though higher level bacterial compositions were similar (Scupham and Patton, 2008). Although, these studies provide valuable insights regarding intestinal microbiota in turkeys, they are based on low-resolution molecular fingerprinting methods, such as terminal restriction fragment length polymorphism (T-RFLP) or automated ribosomal intergenic spacer analysis (ARISA) (Scupham, 2007: Scupham and Patton, 2008; Scupham, 2009). These

methods possess some limitations in terms of accurately depicting microbial diversity in samples, especially for those samples with higher taxon richness (Jami et al., 2014).

Along with the advancement in sequencing technologies, the intestinal microbiota of turkey has been investigated using high throughput next generation sequencing of 16S rRNA genes (Danzeisen et al., 2013; Danzeisen et al., 2015; Andreano et al., 2017; Wilkinson et al., 2017). These studies were conducted in turkeys to characterize the microbiota along the gastrointestinal tract (Wilkinson et al., 2017), litter microbiotas (Danzeisen et al., 2015), and their relation in terms of body weight gain (Danzeisen et al., 2013), antibiotics treatment (Danzeisen et al., 2015), and hemorrhagic enteritis virus (Andreano et al., 2017). Mostly, these studies were conducted in experimental animal settings which might not properly reflects the turkey microbiotas in commercial farms, demanding the need of more comprehensive survey of turkey microbiota in commercial farms. Furthermore, the microbiotas of turkey's litter were more closely related to the ileal microbiotas (Danzeisen et al., 2015) which suggests that the litter microbiotas can reflects the changes in intestinal microbial communities of turkeys.

In this study, we characterized the litter microbiota from different flocks of five different commercial farms at different time points of turkey production. Moreover, we used the boot swab samples for better representation of microbiota from individual birds.

6.3 Materials and Methods

6.3.1 Collection of Samples

The samples were collected from each side of the barn's quadrant by walking with a boot with sponge attached at the bottom. For instance, each barn has four quadrants and thus from each barn 8 (4x2=8) samples were collected. Samples were collected from five commercial turkey farms

(H, M, V, K, and R) of Northwest Arkansas at different time points including one farm (R) that has incidence of cellulitis. From R farm, four sponge samples directly from the birds (RB) and four boot sponge samples from the surrounding area (RL) were collected. The summary of the samples including farms, flocks, age of birds, and number of samples is shown in Table 1.

6.3.2 DNA Extraction

We developed the protocol for the extraction of metagenomics DNA in boot swab samples. For this purpose, each sponge swab sample was transferred to the sterile stomacher bag with filter (Seward), poured 20 ml sterile PBS buffer, and stomached for 2 min in a stomacher (Lab Blender 400 series). In order to obtain uniformity in sponge samples, litter debris attached to each samples were removed aseptically before transferring to stomacher bags. The filtered contents from each samples after stomaching were transferred to 15 ml sterile tube and centrifuged @8000 rpm for 10 min to make pellets. The supernatant from each samples after centrifugation was removed, whereas pellets containing bacterial cells were used for DNA extraction using QIAamp. Fast DNA Stool Minikit (Qiagen, Catlog # 51604). All the procedures for DNA extraction were followed according to the manufacturer's instructions except incorporation of additional bead beating step. Bead beating step was incorporated in the protocol because bead beating was reported to affect DNA yield and taxon abundances (Knudsen et al., 2016). For bead beating, pellets from each samples were resuspended in 1 ml inhibit Ex buffer provided with kit and transferred to 2 ml microcentrifuge tubes with screw cap (Thermofisher Scientific, Catlog # 3468) containing 0.25 ml of sterile 0.1mm glass leads (BioSpec, Mfr # 11079101). Bead beating was performed using Bead mill 24 (Fisher Scientific) for 6 cycles where each cycle contained run time 0.30 sec. and stopping time 0.11 sec between each cycle. After bead beating, samples were incubated at 70 °C for 10 min

and followed manufacturer's protocol for downstream steps and DNA was eluted in 30 µl of elution buffer.

6.3.3 PCR and Library Preparation for Sequencing

V4 region of 16S rRNA gene from genomic DNA of each sample was amplified using the primers 515F (Parada et al., 2016) and 806R (Apprill et al., 2015). The library of amplicons for sequencing was prepared according to the 16S Illumina PCR protocol described in the Earth Microbiome project (http://www.earthmicrobiome.org; Thompson et al., 2017) with slight modifications. In brief, Platinum™ II Hot-Start Green PCR Master Mix (2X) user guide protocol (Thermofisher Scientific, Catalog No. 14000013) was used to conduct PCR in a 25 µl final reaction volume and 35 amplification cycles. The thermocycling condition of PCR included an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of 0.5 min at 94 °C, 0.5 min at 60 °C, and 0.5 min at 68 °C, and a final extension of 5 min at 68 °C. The length of amplified product was confirmed with 1% agarose gel electrophoresis and equal concentration (~300 ng) of amplicons from each sample as measured by Qubit dsDNA BR Assay Kit (ThermoFisher Scientific, Catalog No. Q32850) were pooled together. The pooled amplicons were finally ran on 1% agarose gel electrophoresis, purified using Zymoclean Gel DNA Recovery Kit (Zymo Research, Catalog No. D4007), and sequenced with Illumina MiSeq paired end 300 cycle options at University of California (Davis, CA).

6.3.4 Amplicons Sequence Analysis

Nebula cloud computing platform of the University of Arkansas was used to process raw sequencing reads in QIIME 2 version 2018.8 (Bolyen et al., 2018) utilizing the pipelines developed for paired-end data types. In sum, "demux emp-paired" method of q2-demux plugin was used to

demultiplex sequencing reads followed by quality filtering and denoising with "dada2 denoise-paired" method of q2-dada2 (Callahan et al., 2016) plugin available at QIIME 2. The truncation length of forward and reverse reads was set at 240 and 200 bp, respectively, which is based on the quality score criteria (≥30). Taxonomic assignments was performed using a Naive Bayes classifier (Pedregosa et al., 2011) pre trained with SILVA (Version 132) 99% OTUs (Quast et al., 2013; Yilmaz et al., 2014) and q2-feature-classifier plugin, where the sequences have been trimmed to include only the V4 region of the 16S rRNA gene bound by the 515F/806R primer pair. The core-metrics-phylogenetic method at a sampling depth of 17,000 was used to analyze Alpha and Beta diversity. Alpha diversity calculated by Shannon's diversity index (Shannon, 1984) and Observed OTUs metric, while beta diversity calculated by unweighted UniFrac distance metric (Lozupone et al., 2011) and Bray Curtis (Bay and Curtis, 1957) were presented. All figures were created using ggplot2 packages of R (Wickham, 2016). The significant differences in alpha diversity were calculated using alpha-group-significance command of QIIME2 which uses Kruskal-Wallis test. In contrary, statistical differences in beta diversity among groups were calculated by PERMANOVA (Anderson, 2001) test using beta-group-significance command of QIIME2 with pairwise option. For both diversities analysis, the corrected P values for multiple comparisons (q) were used to report significant difference between two groups, where the level of significance was set at corrected $P< 0.05$.

6.4 Results

6.4.1 Overview of the Samples

We collected litter samples from commercial turkey farms in Northwest Arkansas using boots swab method. We used the subset of 246 farm samples for analysis of bacterial communities

using 16S rRNA gene profiling targeting V4 region. The summary of the samples included in the microbiota analysis is shown in Table 1.

6.4.2 Summary of DNA Sequencing Analysis

Summarization of the feature table resulted in total 10,863,650 sequence reads from the 246 farm samples that ranges from 17,134 to 82,383 reads per sample. The median and mean ± SE reads per sample were 42,949.5 and 44,161.2 ± 787.9, respectively. In addition, there were altogether 3,057 unique features (amplicon sequence variants) from all samples.

6.4.3 Phylum Level Composition of Litter Microbial Communities

At phylum level, eleven major bacterial phyla and one phylum (Euryarchaeota; 0.08%) that belongs to the domain archaea were detected from four farm samples (excluding positive farm samples) which constituted around 99.96% of the total sequences. Among the major bacterial phyla, Firmicutes was the predominant phylum (51.10%) followed by Actinobacteria (31.69%), Proteobacteria (8.30%), and Bacteroidetes (8.18%). Other minor phyla included Cyanobacteria, Synergistetes, Epsilonobacteraeota, Kiritimatiellaeota, Tenericutes, Fusobacteria, Verrucomicrobia whose relative abundance ranges from 0.01 to 0.24% and constituted <1% in total. The relative abundance of the major phyla across four different farms is shown in Figure 1. Irrespective of farms, the Firmicutes was the predominant phylum which was found the highest in the H farm (55.47%), while it was found the lowest in the K farm (34.49%) as shown in Figure 1. On the contrary, Proteobacteria was found the highest in K farm (26.92%), whereas the Actinobacteria was found the most in V farm (41.51%). The phylum Bacteroidetes was found the highest in M farm (12.04%) as shown in Figure 1.

In addition, the composition of microbial phyla were also differentially present between flocks with in the same farm as illustrated in Figure 2. The variations in the relative abundance of major phyla between flocks of same farm was further achieved due to differences in the ages of birds as illustrated in Figure 3. Generally, Firmicutes was found higher in each flock of the farms rearing younger birds, while the Actinobacteria and Bacteroidetes were found higher in the flocks rearing the older birds (Figure 3). However, their relative abundance varied depending upon the farms and flocks within the same farm and are not linear at all the time points. Similarly, the Proteobacteria was highly enriched (47.97%) especially in K farm housing 56 days old turkeys as shown in Figure 3. In case of Bacteroidetes, this phylum was found the highest in the flock C of M farm housing 63 days old turkeys (22.38%) followed by K farm having turkeys at 84 days old (17.26%).

From the positive farm samples (R farm), Firmicutes was detected as the predominant phylum (66.06%) followed by Proteobacteria (17.77%), Actinobacteria (14.44%), and Bacteroidetes (1.47%) which constituted around 99.97% of the total sequences. Although no direct comparisons can be made, the relative abundance of phyla Firmicutes and Proteobacteria were increased, while the relative abundance of phyla Actinobacteria and Bacteroidetes were decreased in positive farm samples in comparison to the rest of the farm samples. The distribution of the relative abundance of major four phyla across different samples from R farm is shown in Figure 4. The phylum Bacteroidetes was significantly reduced in birds swab samples (RB; 0.19%) as compared to the litter swab samples (RL; 2.75%) at $P<0.05$ (Kruskal-Wallis test). In addition, Proteobacteria was numerically enriched in RB (26.22% vs. 9.31%), whereas Firmicutes (72.15% vs. 59.98%) and Actinobacteria (15.60% vs. 13.28%) were numerically abundant in RL.

6.4.4 Genus Level Composition of Litter Microbial Communities

At genus level, thirteen major bacterial genera were identified whose average relative abundance were greater than 2% when summed across all four farm samples excluding R farm. Among these genera, the relative abundance of the genus *Corynebacterium* (16.66%) was found the highest followed by *Staphylococcus* (11.03%), *Brevibacterium* (6.01%), *Megamonas* (5.13%), *Brachybacterium* (4.83%), *Jeotgalicoccus* (4.76%), *Lactobacillus* (3.72%), *Bacteroides* (3.66%), *Escherichia-Shigella* (3.33%), *Aerococcus* (2.62%), Prevotellaceae UCG-001 (2.27%), *Pseudogracibacilibacillus* (2.24%), and *Oceanisphaera* (2.04%). The relative abundance of the major genera across four different farms is shown in Figure 5. The genus *Corynebacterium* was the predominant genus in H (21.78%) and V (17.30%) farm, however, the genera *Megamonas* (12.39%) and *Escherichia-Shigella* (17.79%) were significantly higher in the M and K farm, respectively. Moreover, the composition of bacterial genera vary not only between the flocks of the same farm (Figure 6), but also affected by ages of birds with in the same flock and same (Figure 7). For instance, the genus *Megamonas* was highly enriched in flock C of the M Farm rearing turkeys of 28 (19.02%) and 63 days old (27.60%), but very lower amount of *Megamonas* was detected at the same flock rearing 98 days old (1.95%) turkeys. Similarly, the genus *Escherichia-Shigella* was highly abundant in K farm having the turkeys of 56 days old (42.83%) (Figure 7). Similarly, the genus Bacteroides was reported the highest from the flock C of M Farm having turkeys of 63 days old (13.70%). Regarding *Corynebacterium* and *Staphylococcus*, they were present at significant amount throughout all ages and flocks of the farms (Figure 7) except at the flock C of M Farm having turkeys of 63 days old where they were found 0.97% and 1.07%, respectively.

The top 14 major genera whose relative abundances were on average >2% when summed across all samples recovered from samples of R farm are shown in Figure 8. On the contrary to the other farm samples, the positive farm samples constituted unknown genera of the family Bacillaceae (15.05%) followed by the *Ignatzschineria* (14.58%) which were presented only 1.67% and 0.035% in rest of the farm samples, respectively. Other important genera included *Staphylococcus* (10.60%), *Corynebacterium* (9.65%), *Clostridium* sensu stricto 1 (6.34%), *Pseudogracilibacillus* (5.95%), *Nosocomiicoccus* (4.28%), *Jeotgalicoccus* (3.88%), Atopostipes (3.69%), *Lactobacillus* (2.55%), *Enteractinococcus* (2.54%), *Virgibacillus* (2.20%), *Sporosarcina* (2.09%), and *Aerococcus* (2.06%). Although direct comparisons cannot be made, it seems that different genera were differentially abundant between positive farm samples with the rest of the farm samples (Figure 5 and Figure 8). Moreover, as seen in Figure 8, there exists differences in the relative abundance of major bacterial genera between RL and RB groups. For instance, the genera *Enteractinococcus*, *Pseudogracilibacillus*, *Virgibacillus*, *Nosocomiicoccus*, and *Lactobacillus* were significantly higher in RL group, while the *Clostridium* sensu stricto 1 was significantly higher in RB group (Kruskal-Wallis test, $P<0.05$).

When all ASVs that belong to the *Clostridium* sensu stricto 1 were compared with *Clostridium septicum* 16S rRNA gene sequence, they showed >97% similarity. Thus, we believed that the sequences of *Clostridium* sensu stricto 1 belong to *C. septicum* as C. septicum is considered as the primary etiological agent of cellulitis in turkeys (Tellez et al., 2009). It is further confirmed by the qPCR results in Chapter 7.

6.4.5 Core Bacterial Genera in Litter of Farm Samples Excluding Positive Farm samples (R Farm)

The number of core bacterial genera that were presented in the 50-100% of the farm samples is shown in Figure 9. There were 90 core bacterial genera found in 50% of the samples, while only 4 genera (*Staphylococcus*, *Brevibacterium*, *Brachybacterium*, and *Lactobacillus*) were found in all samples (Figure 9). In addition, 20 core bacterial genera were identified in 95% of the samples which include *Corynebacterium*, *Staphylococcus*, *Jeotgalicoccus*, *Brevibacterium*, *Brachybacterium*, *Lactobacillus*, *Bacteroides*, *Pseudogracilibacillus*, *Aerococcus*, *Atopostipes*, *Virgibacillus*, an unknown genus of Lachnospiraceae, *Facklamia*, *Weissella*, *Escherichia-Shigella*, *Bifidobacterium*, *Enterococcus*, *Phascolarctobacterium*, *Sellimonas*, and *Subdoligranulum*.

6.4.6 Core Bacterial Genera in Litter of Positive Farm Samples (R Farm)

The number of core bacterial genera that were presented in the 50-100% of the farm samples with positive incidence of cellulitis is shown in Figure 10. As shown in Figure 10, 73 core bacterial genera were detected in 50% of samples, whereas 24 genera were present in all 100% samples. These genera include unknown genus of Bacillaceae, *Staphylococcus*, *Corynebacterium*, *Pseudogracilibacillus*, *Nosocomiicoccus*, *Ignatzschineria*, *Jeotgalicoccus*, *Atopostipes*, *Enteractinococcus*, *Lactobacillus*, *Virgibacillus*, *Sporosarcina*, *Aerococcus*, *Weissella*, *Brevibacterium*, an uncultured genus of Bacillaceae, *Bifidobacterium*, *Brachybacterium*, an unknown genus of Lachnospiraceae, *Salinicoccus*, *Subdoligranulum*, *Blautia*, *Sellimonas*, and *Romboutsia*.

6.4.7 Alpha Diversity

Alpha diversity of the microbial communities was measured using Shannon and observed OTUs indices. When the Shannon index was compared among the 4 different farms, no significant difference was observed in alpha diversity (Figure 11A). However, when the Shannon index was compared across different flocks within the same farms, all pairwise comparisons among the 3 flocks (A, B, and C) in M Farm showed significant differences. Similarly, the two flocks (A and B) in V Farm showed significant difference in the Shannon index (Figure 11B).

Similar, yet slightly different results were observed with observed OTU index. There was significant difference in alpha diversity between H and M Farms (Figure 12A). When the flocks within the same farms were compared, significant difference was observed between the flock A and B in H Farm, between the flock B and C in M Farm, and between the flock A and B in V Farm (Figure 12B).

6.4.8 Beta Diversity

Beta diversity of the microbial communities was measured by Bray-Curtis and unweighted distance metrics. All pairwise combinations of various flocks from four turkey farms showed significant difference in microbial communities among the groups as indicated by both unweighted distance metric (Figure 13A; adjusted $P<0.001$) and Bray-Curtis distance metric (Figure 13B; adjusted $P<0.01$).

In addition, within H farm, all possible pairwise comparisons of flocks and ages combinations showed significantly different microbial community structure in terms of both unweighted distance metrics (Figure 14A) and Bray-Curtis (Figure 14B) at adjusted P (q)<0.001.

6.5 Discussion

In the current study, we characterized the microbiota associated with the litter from five different commercial farms of the Northwest Arkansas including a farm with positive incidence of cellulitis. To our knowledge, this is the first study that used boot swab samples for comprehensive survey of litter microbiota in commercial turkey farms. Previously, boot swab was used for the detection of *Mycobacterium avium* subsp. paratuberculosis (MAP) in cattle herds (Eisenberg et al., 2013). By the culture of boot swab samples, they were able to isolate MAP from 90.6% of MAP confirmed cattle herds. We also noticed significant enrichment of *Clostridium* sensu stricto 1 in farm samples with positive incidence of cellulitis. When sequences of all ASVs identified as *Clostridium* sensu stricto 1 were compared with *C. septicum* 16S rRNA gene sequence, they shared >97% sequence identity. Furthermore, the nested qPCR results from the assay that target the alpha toxin gene (*csa*) of *C. septicum* gave strong amplification signals from the same farm samples with incidence of cellulitis (Chapter 7). Thus, we believe that the sequences that were classified as *Clostridium* sensu stricto 1 belong to *C. septicum*, since cellulitis in turkey is considered to be primarily caused by *C. septicum* (Tellez et al., 2009). This further suggests that the boot swab samples can serve as an easy and cost effective technique for the collection of environmental samples for the detection of various pathogens as well as the study of litter microbiota. Moreover, studies on litter microbiota can reflect the changes in the microbial communities of the poultry as the litter microbial communities correlate with the communities residing in the hosts (Danzeisen et al., 2015), which are further affected by the litter types (Cressman et al., 2010).

It was found that the different flocks with in the same farm attributed differences in the composition and structures of litter microbial communities, which are further affected by the ages of turkeys. In addition, those variations are further depended upon the environmental conditions

(Farms). Age as a major driving factor of turkey microbiota was also reported previously (Danzeisen et al., 2013; Danzeisen et al., 2015). Differences in environmental conditions can play a vital role in the initial maturation of turkey microbiome, in addition with the flocks types (Danzeisen et al., 2013). Although the trend is not linear, we noticed the higher abundance of Firmicutes from the flocks rearing younger age of birds, while Actinobacteria and Bacteroidetes were reported higher from the flocks rearing older birds.

Interestingly, the phyla Proteobacteria and Bacteroidetes were highly enriched in the flock C of the M farm with 63 days old turkeys (MN-C-63) and K farm housing 56 days old turkeys (K-56), respectively. This was reflected at the genus level by increasing the abundance of *Escherichia-Shigella* and *Bacteroides* in the respective farms. The Proteobacteria is the phylum that contains several pathogenic Gram negative genera such as *Escherichia* and *Shigella* whose increase is generally considered as the signature of gut dysbiosis (Shin et al., 2015). So, increase in the relative abundance of the phylum Proteobacteria and the subsequent increase of genera *Escherichia-Shigella* in the K farm (K-56) might be correlated with the health and diseases of turkeys, though we are lacking those data for confirming our hypothesis. Another important observation was that the genera *Bacteroides* and *Megamonas* were present the most in the M-C-63 group. The increase in the relative abundance of *Bacteroides* in the particular farm was explained by the highest abundance of the phylum Bacteroidetes in that farm. In addition, the genus *Staphylococcus* was highly reduced in M-C-63 as compared to the other groups. The *Bacteroides* is a genus of Gram negative bacteria that are well known for its ability to degrade complex plant carbohydrates and host derived glycan. This group of bacteria can play beneficial effects on the hosts' health and maintain gut homeostasis, however, the effects were found to vary between the studies and strains of *Bacteroides* (Wexler et al., 2017; Janssens et al., 2018). The increase in the abundance of the

genera Bacteroides and Megamonas might be associated with the reduction of *Staphylococcus* in M-C-63 group. Although *C. septicum* is considered as primary etiological agent, *Staphylococcus aureus* was also reported to be associated with cellulitis in turkeys (Gornatti-Churria et al., 2018). This was further supported by our results from the farm with positive incidence of cellulitis, where the *Staphylococcus* was detected in all samples suggesting the possible association of *Staphylococcus* in cellulitis of turkeys.

Moreover, only 4 core genera (*Staphylococcus*, *Brevibacterium*, *Brachybacterium*, and *Lactobacillus*) were found in all samples of 4 farms excluding R farm, whereas 24 core genera were present in all samples from R farm that had cellulitis. The important core genera in positive samples were *Corynebacterium*, an unknown genus of family Bacillaceae, *Clostridium* sensu stricto 1 (>97% similarity with *C. septicum*), and *Ignatzschineria* beside others. These genera should be considered while describing the etiopathogenesis of cellulitis in turkeys. The genus *Ignatzschineria* was noticeably enriched in some of the positive samples especially in RB3 (51.97%), RB4 (29.91%), and RL3 (21.70%) as shown in Figure 8. *Ignatzschineria* is a genus of Gram-negative bacteria that has been associated with necrotizing wounds colonized by maggots (Barker et al., 2014; Le Brun et al., 2015; Muse et al., 2017). This group of bacteria are common isolates from the larvae of the parasitic flesh fly (*Wohlfahrtia magnifica*) and two species, *I. indica* (Barker et al., 2014; Muse et al., 2017) and *I. ureiclastica* (Le Brun et al., 2015) were isolated from the bacteremia following maggots infestation of the wounds in humans. This suggests that if the cellulitis is not properly treated in a timely manner, it can create further complications including septicemia.

In sum, boot swab samples were successfully used to investigate the litter microbial communities of the commercial turkey farms of the Northwest Arkansas. Majority of the microbial

taxa identified using boot swabs belong to the microbiota residing in the gut of the poultry which suggests that the litter microbiota can be used to reflect the microbial changes in the hosts. The composition and diversities of litter microbial communities varied even between the flocks of the same farm which are further affected by the age of birds. The core bacterial genera from samples with cellulitis differed as compared to the rest of the farm samples. In addition, several bacterial genera such as *Corynebacterium*, *Staphylococcus*, *Ignatzschineria*, unknown genus of family Bacillaceae and other that were identified as core members in the positive samples might be correlated with incidence of cellulitis beside *C. septicum*.

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6.8 Tables and Figures

Table 1. Summary of the farm samples used for microbiota analysis

Farm	Flock	Age (days)	No. of Samples
H	A	33	8
		84	16
		105	16
	B	49	8
		70	16
		103	16
M	A	84	16
	B	98	16
	C	28	8
		63	16
		98	16
V	A	58	8
		112	14
	B	59	8
		80	16
		115	16
K	-	28	8
		56	8
		84	8
R*	-	60	8

* represents farm with positive incidence of cellulitis, where 4 sponge samples from birds and 4 booty sponge samples from the surrounding areas were collected.

Figures

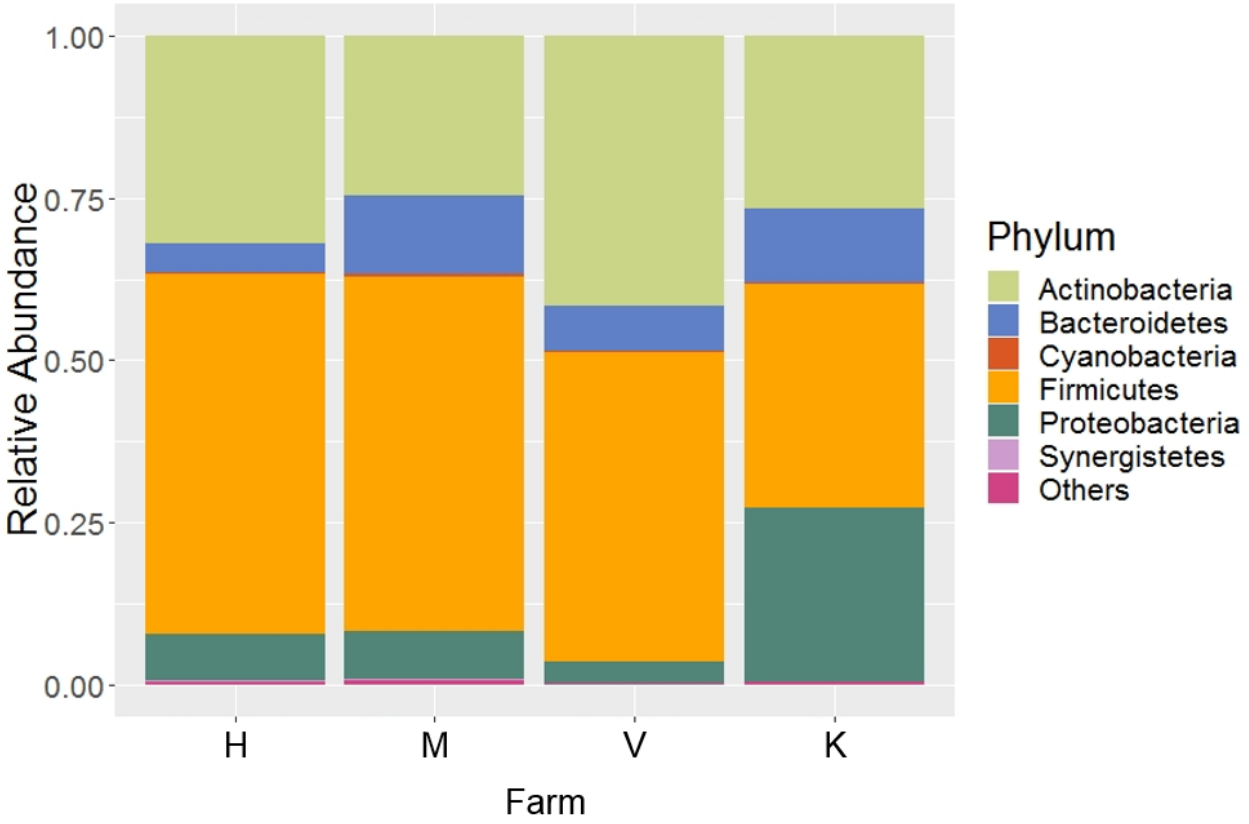

Figure 1. Composition of the litter microbiotas at phylum level in four different commercial turkey farms of Northwest Arkansas. “Others” represent the minor phyla whose relative abundance were less than 0.1%.

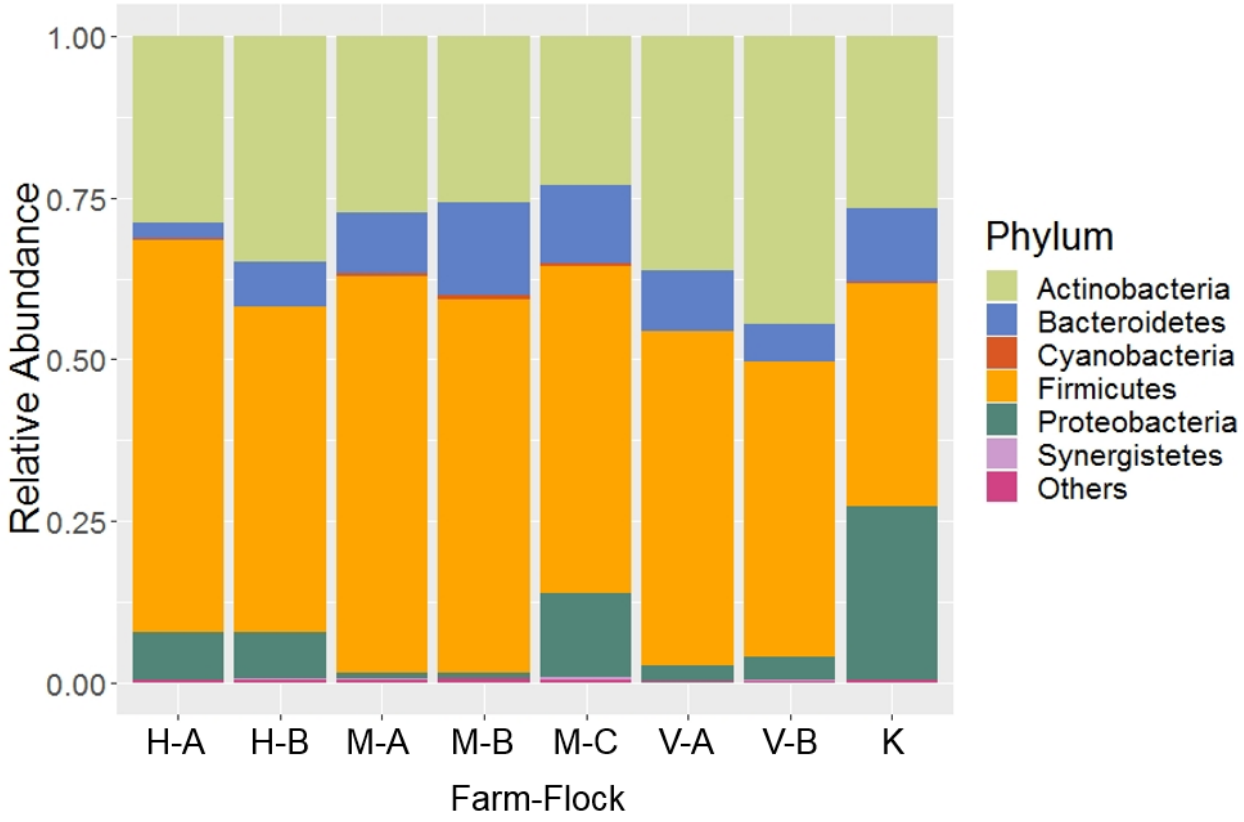

Figure 2. Composition of the litter microbiotas at phylum level in different flocks of four different commercial turkey farms of Northwest Arkansas. A, B, and C represent different flocks. “Others” represent the minor phyla whose relative abundance were less than 0.1%.

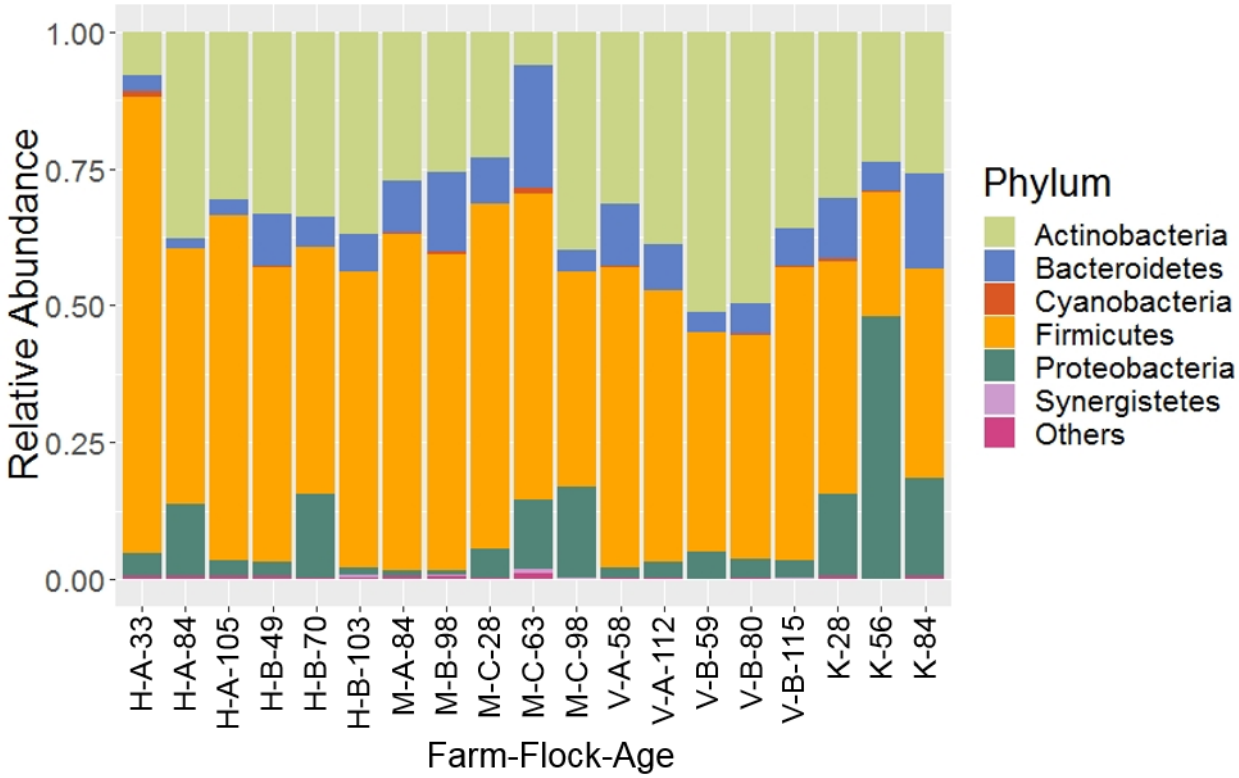

Figure 3. Composition of the litter microbiotas at phylum level in different ages of turkeys rearing in various flocks of four different commercial farms of Northwest Arkansas. A, B, and C represent different flocks. The numbers represent ages of turkeys when samples were collected. "Others" represent the minor phyla whose relative abundance were less than 0.1%.

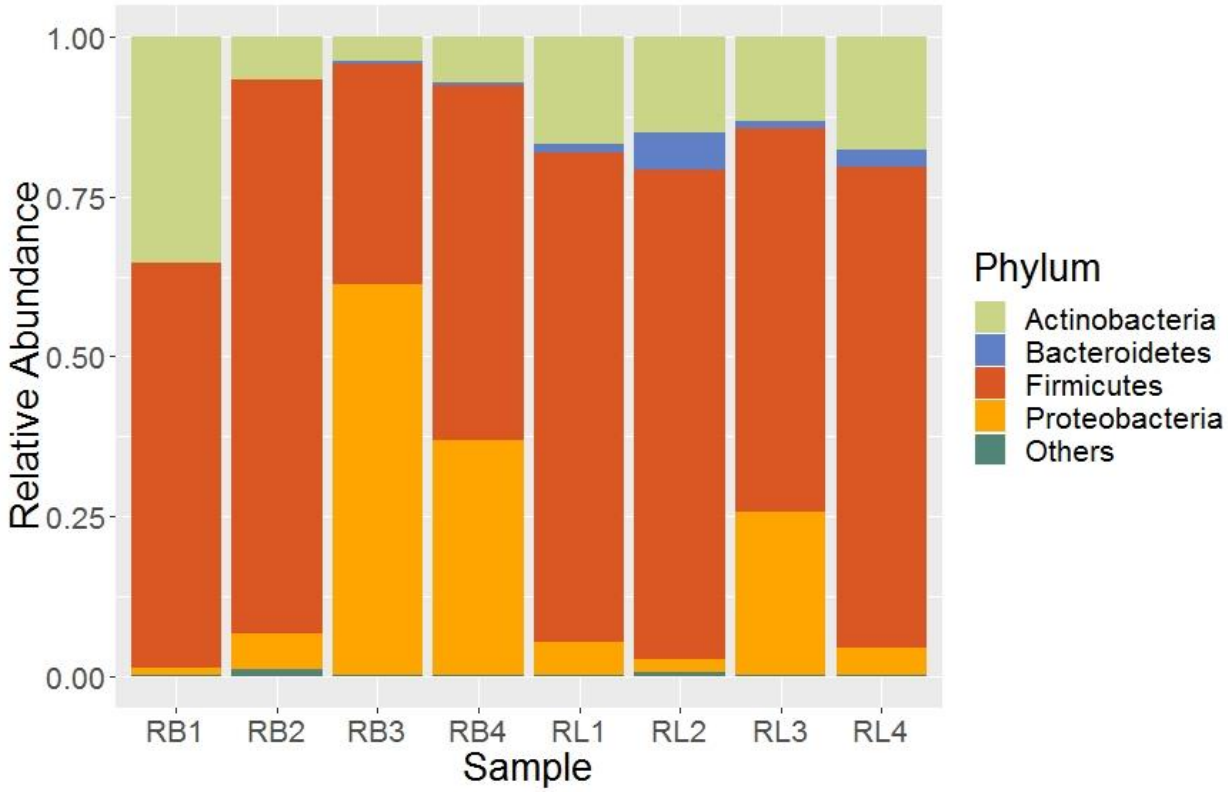

Figure 4. Composition of the litter microbiotas at phylum level in different samples of R farm with incidence of cellulitis. RB and RL represent sponge swab samples collected directly from the birds and boot sponge swab samples collected from the surrounding areas. “Others” represent the minor phyla whose relative abundance were less than 0.1%.

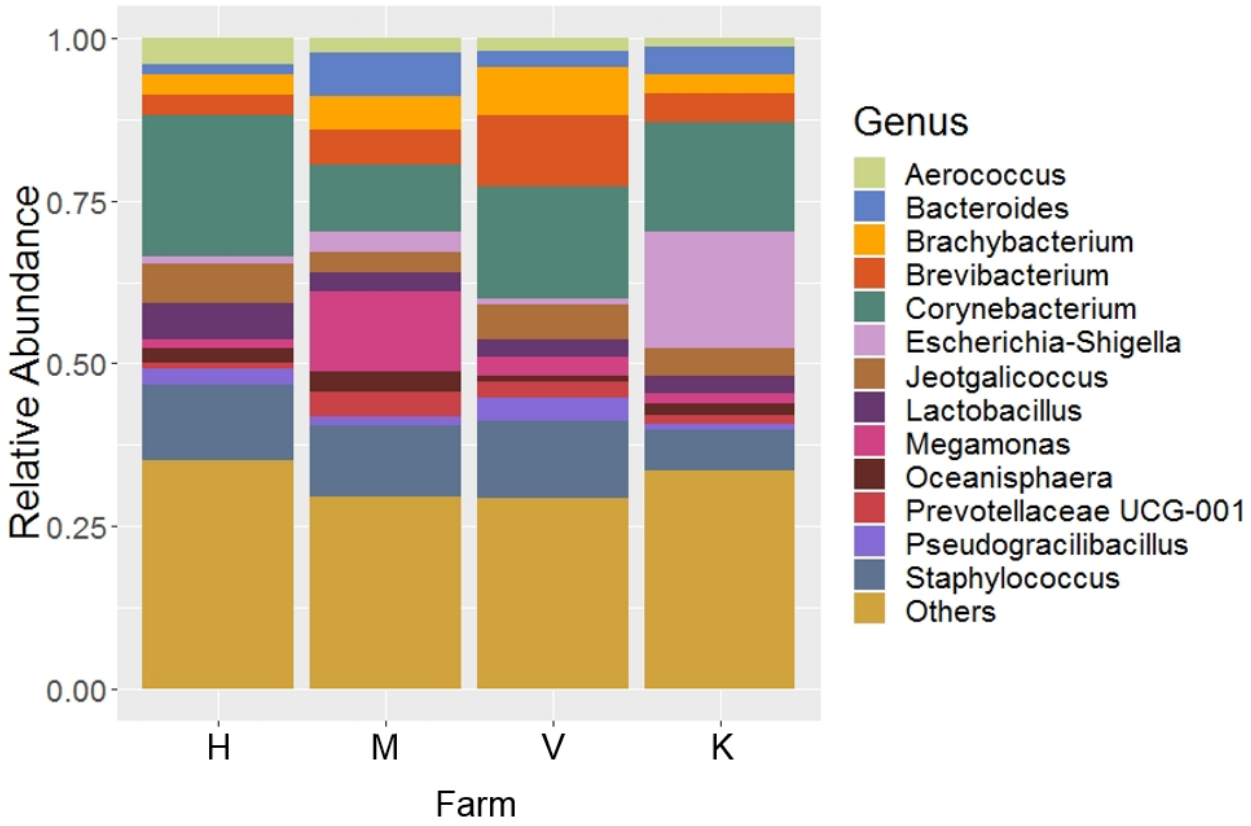

Figure 5. Composition of the litter microbiotas at genus level in commercial turkey farms of Northwest Arkansas. "Others" represent the minor genera whose relative abundance were less than 2.0 %.

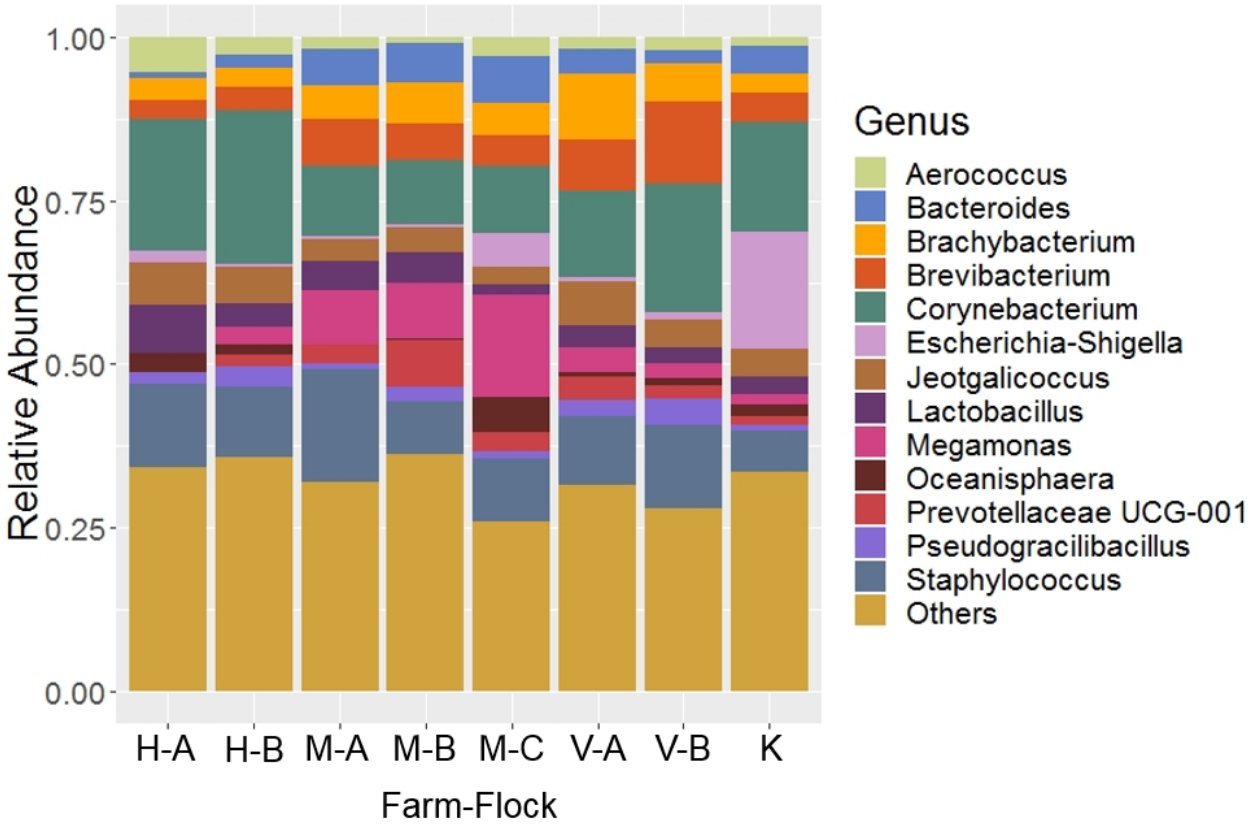

Figure 6. Composition of the litter microbiotas at genus level in different flocks of four different commercial turkey farms of Northwest Arkansas. A, B, and C represent different flocks. “Others” represent the minor genera whose relative abundance were less than 2.0 %.

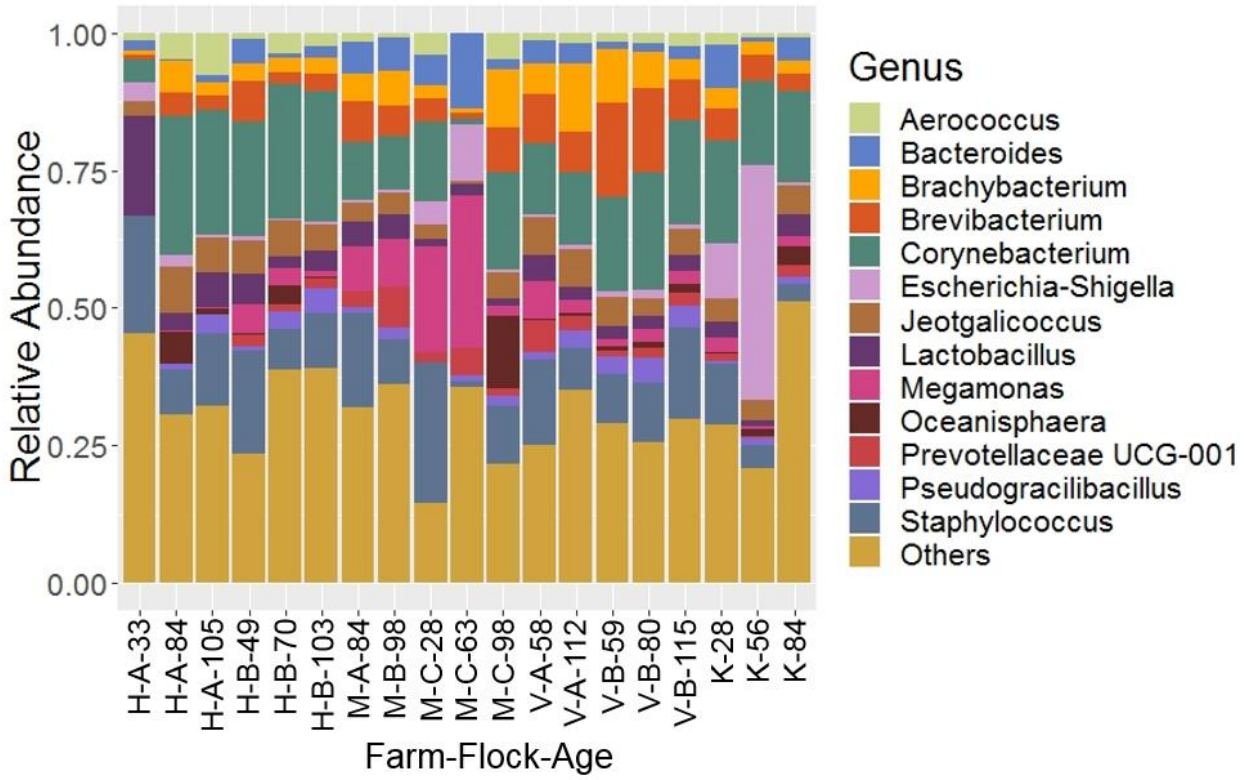

Figure 7. Composition of the litter microbiotas at genus level in different ages of turkeys rearing in various flocks of four different commercial farms of Northwest Arkansas. A, B, and C represent different flocks. The numbers represent ages of turkeys when samples were collected. "Others" represent the minor genera whose relative abundance were less than 2.0 %.

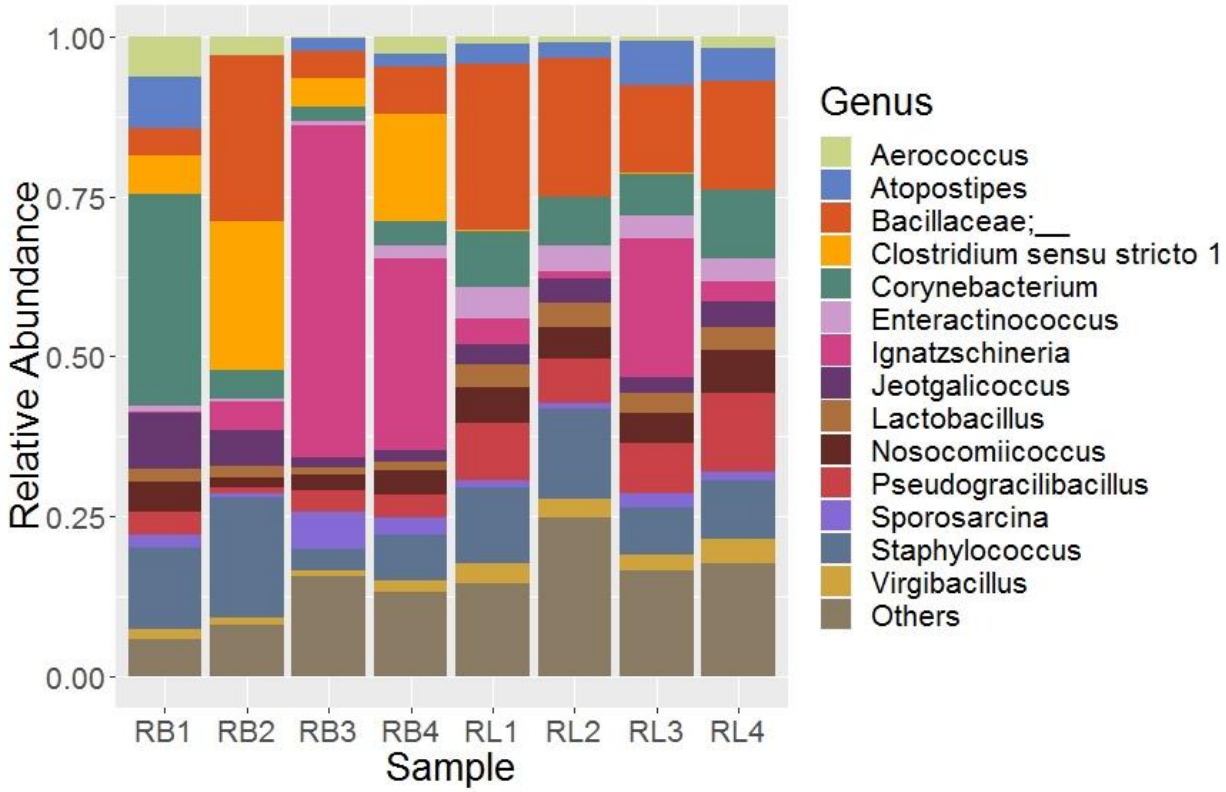

Figure 8. Composition of the litter microbiotas at genus level in different samples of R farm with incidence of cellulitis. RB and RL represent sponge swab samples collected directly from the birds and boot sponge swab samples collected from the surrounding areas. “Others” represent the minor genera whose relative abundance were less than 2.0 %.

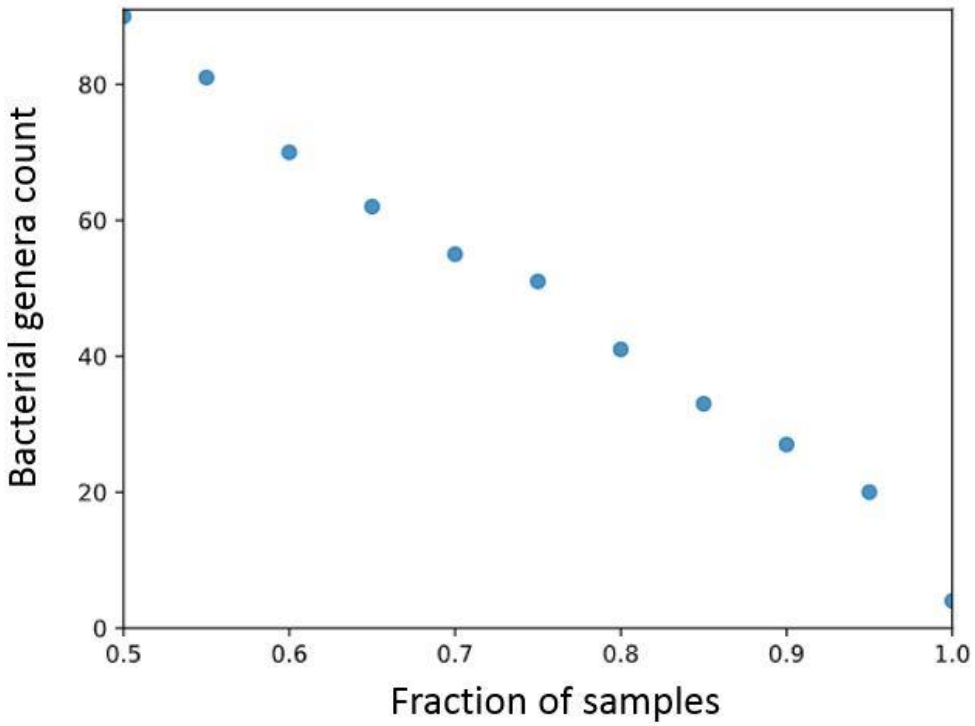

Figure 9. The number of core bacterial genera identified from four different farms of turkeys (H, M, V, and K) and the fraction of samples from which they are recovered.

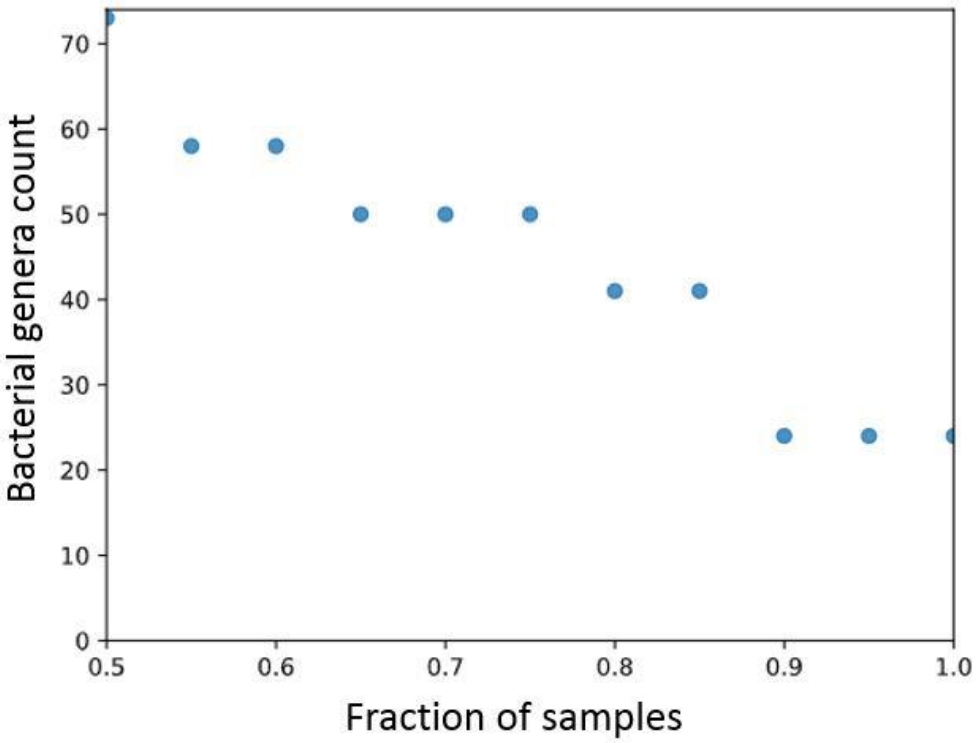

Figure 10. The number of core bacterial genera identified from R farm that had an incidence of cellulitis and the fraction of samples from which they are recovered.

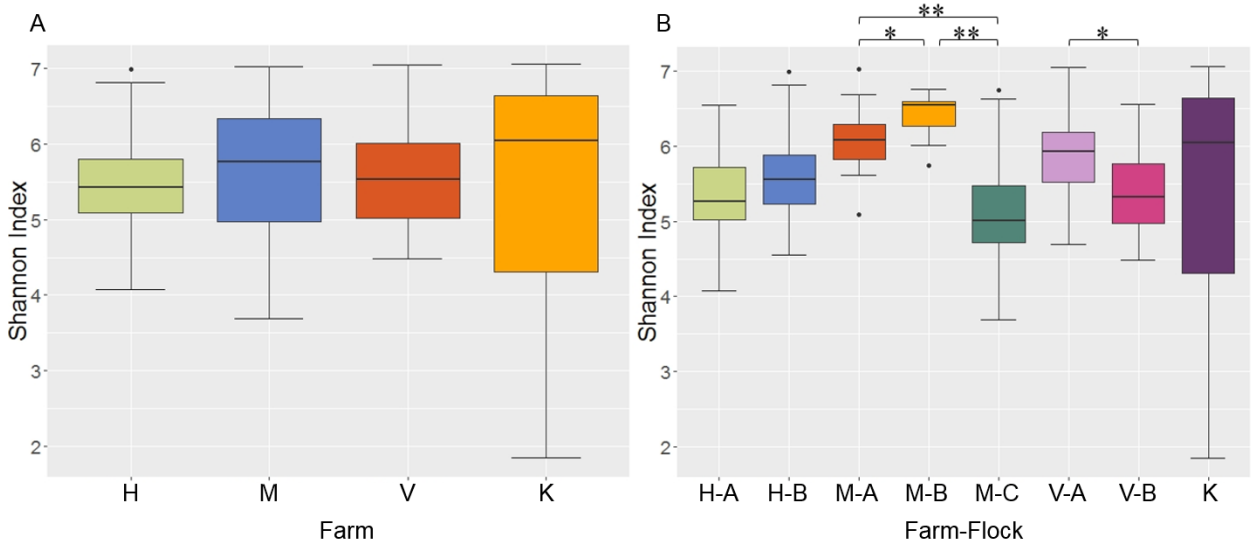

Figure 11. Alpha diversity in different farms (A) and flocks (B) as measured by Shannon Index. Significant difference is indicated at adjusted *P* (*q*) < 0.05 (*) or < 0.01(**).

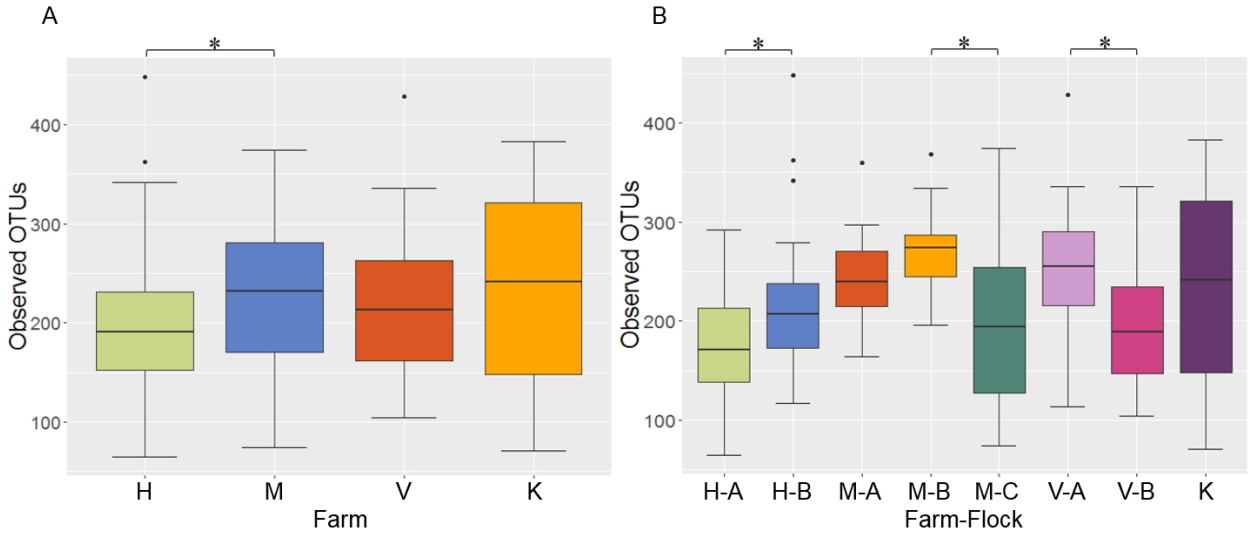

Figure 12. Alpha diversity in different farms (A) and flocks (B) as measured by Observed OTUs index. Significant difference is indicated at adjusted *P* (*q*) < 0.05 (*).

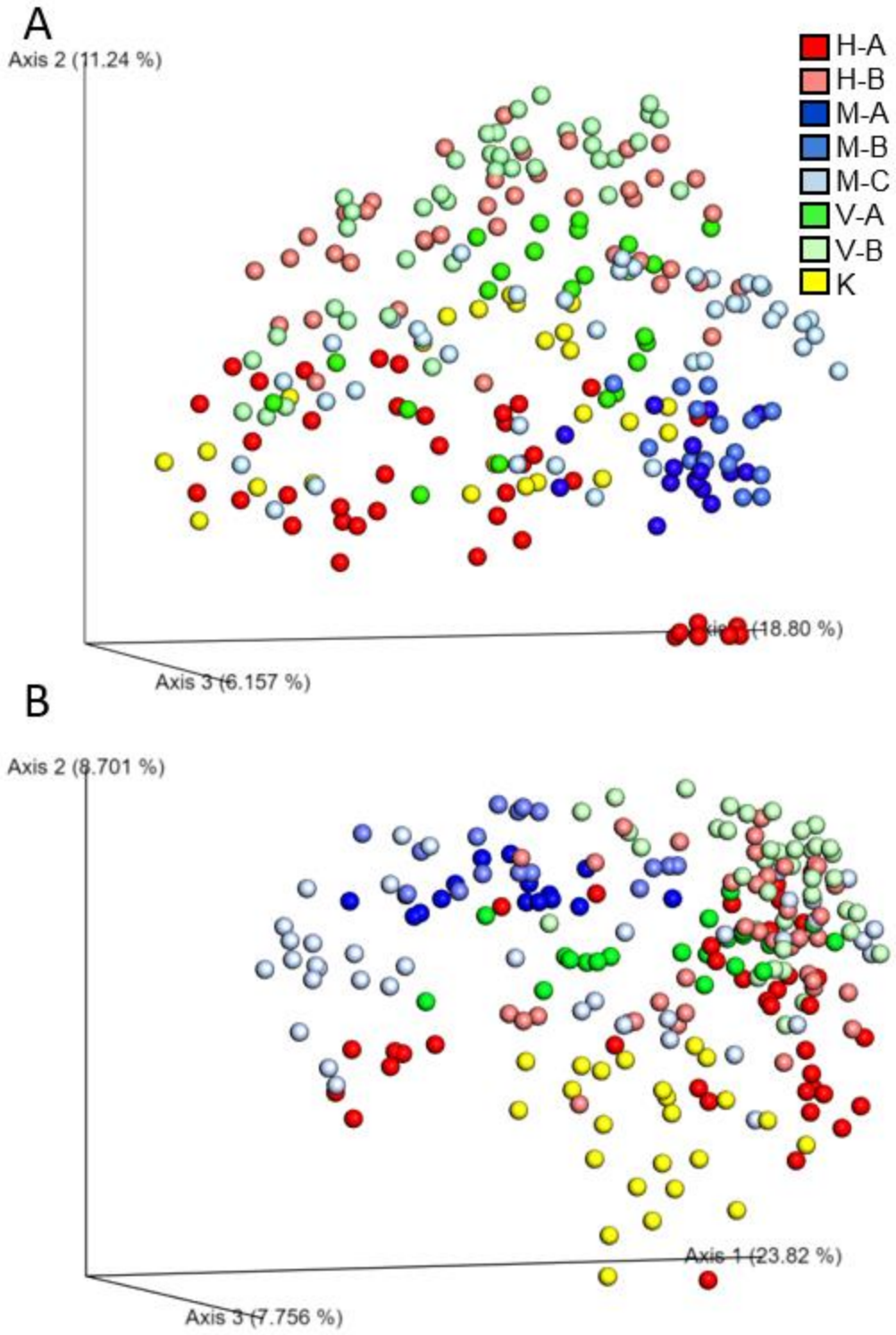

Figure 13. Emperor plot showing beta diversity distances among the different samples from different flocks of four farms and as measure by (A) unweighted UniFrac distance and (B) Bray-Curtis distance indices.

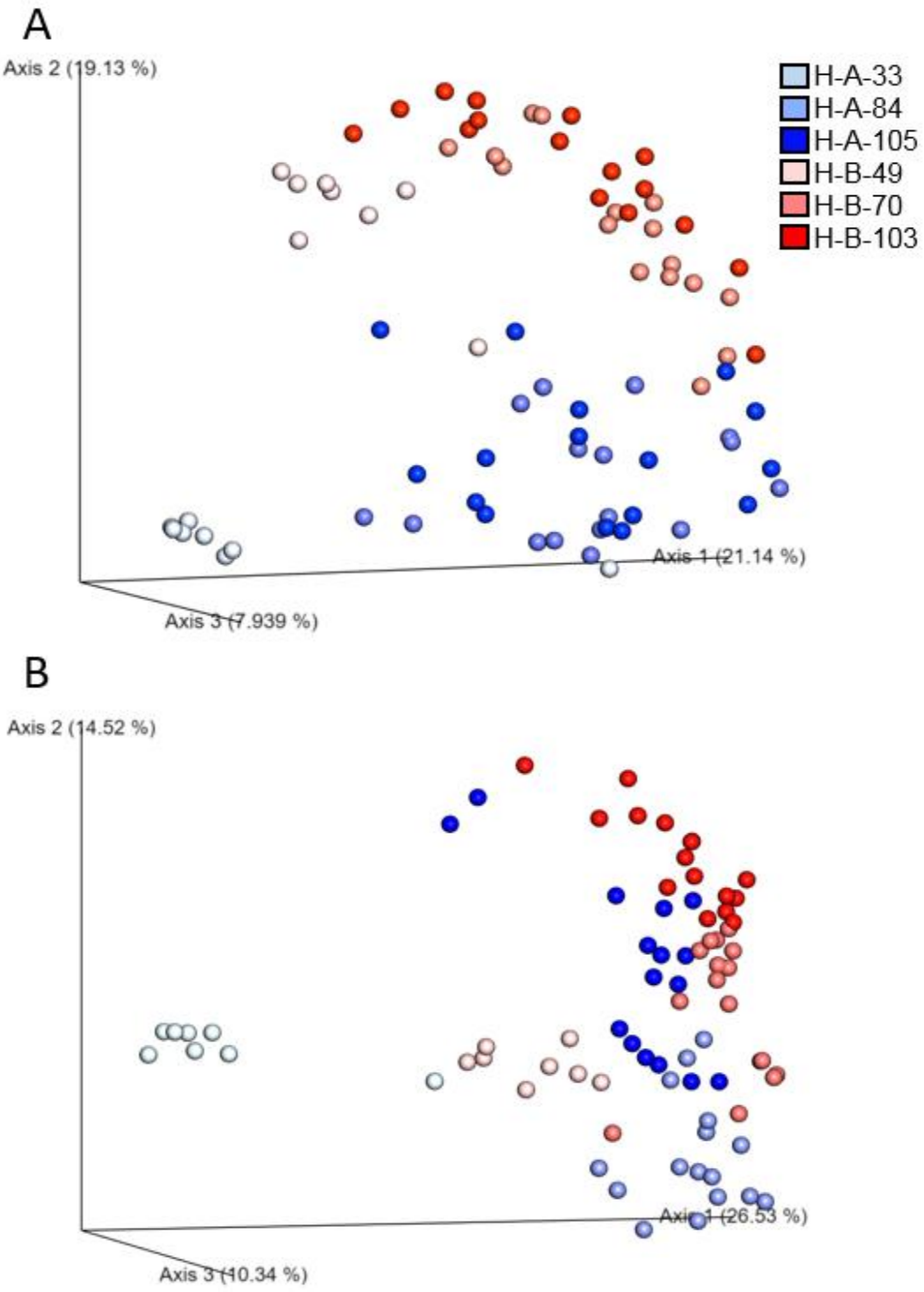

Figure 14. Emperor plot showing beta diversity distances among the different samples in H farm as measured by (A) unweighted UniFrac distance and (B) Bray-Curtis distance indices. A and B represent two different flocks of H farm, whereas the number represents the ages of turkeys when samples were collected.

CHAPTER SEVEN

Development of real time PCR assay for quantitative detection of *Clostridium septicum*

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7.1 Abstract

Cellulitis is an important disease in commercial turkey farms associated with significant economic loss. Although etiology of cellulitis is not fully elucidated, *Clostridium septicum* (*C. septicum*) is one of the main causes of this infectious disease. In this study, we report the development of a quantitative PCR assay targeting the alpha toxin gene (*csa*), which involves a prior 15-cyle PCR using nested primers to increase the detection sensitivity. Additionally, TaqMan probe was used to increase the specificity of the assay. The performance of our nested qPCR assay was evaluated by using *Clostridium* isolates from turkey farms, representing both *septicum* and non-*septicum* species as well as sponge swab samples from turkey farms. Our step-by-step development of the assay showed that the *csa* gene is a suitable target for specific-detection of *C. septicum* strains and that the inclusion of nested PCR step significantly increased the detection sensitivity of the final qPCR assay. The performance of the assay was also validated by high correlation between the quantification cycles of the qPCR assay with the relative abundance of *C. septicum* read counts in 16S rRNA gene microbiota profiling of the samples from turkey farms.

7.2 Introduction

The frequency and severity of clostridial dermatitis has increased during the last two decades and has become a serious problem of the commercial turkey industry (Lighty et al., 2016). The clostridial dermatitis, which is often called as cellulitis in turkeys, is considered to be caused primarily by *Clostridium septicum* (Tellez et al., 2009). However, *C. perfringens*, *C. sordellii*, and *Staphylococcus aureus* have also been described as potential etiological agents (Tellez et al., 2009; Clark et al., 2010; Thachil et al., 2010; Lighty et al., 2016). In contrast to the other diseases, cellulitis in turkey does not fulfill Koch's postulates because not all isolates of *C. septicum* recovered from cellulitis lesions caused cellulitis after intravenous injection of those isolates in healthy turkeys. Moreover, the authors were not able to isolate *C. septicum* in all field cases of turkey cellulitis (Tellez et al., 2009). Additionally, various factors such as flock type, breed, weight, litter condition, stress, and stocking density can affect the incidence of cellulitis in turkey (Clark et al., 2010; Huff et al., 2013; Lighty et al., 2016).

The pathogenesis of cellulitis in turkey is still poorly understood because of the limited availability of experimental data. Thus, there is still debate among scientists regarding the validity of the pathogenesis model between "inside-out" and "outside-in" theory associated with turkey cellulitis. Pathogenic clostridia, toxin, or both can enter into blood stream through damaged intestinal wall, localize under skin, and produce enterotoxins causing cellulitis. Furthermore, clostridia from contaminated environment can cause infection through oral route. This is called as "inside-out" theory. On the other hand, clostridia can enter directly through skin abrasions causing cellulitis, which is known as "outside-in" theory (Clark et al., 2010).

Any factors described above can serve as stressors and affect the intestinal permeability (Caso et al., 2008; Gareau et al., 2008). This results in the localization of pathogenic *Clostridium*

under skin via hematogenous route. *C. septicum* isolates were isolated from blood of asymptomatic turkey, which may suggest the possibility of hematogenous route of infection during turkey cellulitis (Neumann and Rehberger, 2009). However, "outside-in" theory also cannot be ignored and more studies should be conducted in future to understand the detail mechanisms of pathogenesis in turkey cellulitis.

For the prevention and control of cellulitis, rapid and sensitive detection of *C. septicum* is important. Several studies have been conducted to develop PCR primers and quantitative PCR assays for detection of *C. septicum* (Halm et al., 2010; Lange et al., 2010; Neumann et al., 2010). In these studies, various target genes were used for development of the assay, including *csa* (alpha toxin) gene (Neumann et al., 2010), 16S rRNA gene (Halm et al., 2010) and *spo0A* gene (Lange et al., 2010).

In this study, we developed the real time PCR assay for specific detection of *C. septicum* species based on the *csa* gene with an additional step of prior nested PCR step as an effective means to increase the sensitivity of the detection. In addition, we used TaqMan probe for improved specificity of the assay.

7.3 Materials and Methods

7.3.1 Isolation and Identification of Bacterial Strains

We have obtained *Clostridium* strains that belong to either the species *septicum* or non-*septicum* species isolated from commercial turkey farms in Northwest Arkansas. Samples consisting of litter samples or tissues from clinically ill birds were submitted to Northwest Arkansas Veterinary Services for anaerobic culture.

Litter samples were weighed and suspended in Buffered Peptone Water to give a 1:10 dilution. A 50 ml aliquot of the suspension was pasteurized at 70°C for 10 minutes to kill non-spore formers. 20 ul of the heat-treated sample was plated onto Tryptic Soy Agar (TSA) w 5% sheep red blood cells (Hardy Diagnostics) and Columbia Agar with Colistin and Naladixic (CNA) Acid w 5% sheep red blood cells (Hardy Diagnostics). Plates were incubated in anaerobic jars with Mitsubishi Anaero-pack sachets for 48 hrs at 37°C. Colonies suspected to be anaerobic were sub-cultured on TSA and incubated under both anaerobic and aerobic conditions at 37°C to confirm isolates were anaerobic. Obligate anaerobic isolates were identified to species using RAPid anaerobic panels (Remel).

Tissue samples were surface seared with a propane torch. A sterile cotton tipped swab was used to collect a sample from the subcutis and a second swab was used to collect a sample from deep muscle tissues. Swabs were plated on TSA w5% sheep blood and Columbia CAN Agar with 5% sheep red blood cells. After plating the swabs were placed into Chopped Meat Glucose Broth (CMG Difco). Plates and CMG tubes were incubated anaerobically at 37 °C as described for litter samples. Isolates were selected and confirmed as obligate anaerobes as described for litter samples. RAPid panels were used to identify each isolate. Isolates were maintained under anaerobic conditions on TSA blood agar plates.

7.3.2 Collection of Farm Samples

The farm samples used in this study were described in detail in the previous chapter (Chapter 6).

7.3.3 DNA Extraction of Litter Swab Samples and *Clostridium* strains

We developed the protocol for the extraction of metagenomics DNA in boot swab samples. For this purpose, each sponge swab sample was transferred to the sterile stomacher bag with filter (Seward), followed by adding 20 ml sterile PBS buffer, and stomaching for 2 min in a stomacher (Lab Blender 400 series). In order to obtain uniformity in sponge samples, litter debris attached to each samples were removed aseptically before transferring to stomacher bags. The filtered contents from each samples after stomaching were transferred to 15 ml sterile tube and centrifuged @8000 rpm for 10 min at 4°C to make pellets. The supernatant from each samples after centrifugation was removed, whereas pellets containing bacterial cells were used for DNA extraction using QIAamp Fast DNA Stool Minikit (Qiagen, Catlog # 51604). All the procedures for DNA extraction were followed according to the manufacturer's instructions except incorporation of additional bead beating step. Bead beating step was incorporated in the protocol because bead beating was reported to improve DNA yield and taxon abundances (Knudsen et al., 2016). For bead beating, pellets from each samples were resuspended in 1 ml inhibit Ex buffer provided with the kit and transferred to 2 ml microcentrifuge tubes with screw cap (Thermofisher Scientific, Catlog # 3468) containing 0.25 ml of sterile 0.1mm glass leads (BioSpec, Mfr # 11079101). Bead beating was performed using Bead mill 24 (Fisher Scientific) for 6 cycles where each cycle contained run time 0.30 sec. and stopping time 0.11 sec between each cycle. After bead beating, samples were incubated at 70°C for 10 min and processed following the manufacturer's protocol for downstream steps and finally DNA was eluted in 30 µl of elution buffer.

For DNA extraction of clostridial isolates, the colonies grown on agar plates were resuspended in 1.5 ml sterile PBS buffer, and the cell suspension was transferred to 2 ml sterile Eppendorf tubes. The suspensions were centrifuged at 13,000 rpm for 1 min at 4°C and the

supernatant was removed. The pellets were resuspended in a 1.5 ml sterile PBS buffer, centrifuged, and removed the supernatant. This washing process was repeated for additional two times. After washing the colonies for three times, the pellets were used for the DNA extraction following the same procedures as described above.

7.3.4 Design of the Primers and Probes for Quantitative Real-Time PCR (qPCR) Assay

We wanted to develop a quantitative real time PCR assay (qPCR) to detect and quantify *Clostridium septicum* strains using TaqMan probe targeting the alpha toxin gene (*csa*). For the design of the primers and probe, we obtained DNA sequences of the *csa* gene from 5 different strains of *C. septicum* that are publicly available in the NCBI database (AB083434.1, EU482188.1:315-1646, HM051335.1, FJ212777.1, KU726861.1:1078-1677). Multiple sequence alignment was performed using CLUSTAL Omega (1.2.4), where primers (csa-F1 and csa-R1) and probe (csa-Probe) were selected from the conserved region among the 5 *csa* gene sequences (Figure 1) using PrimerQuest tool of integrated DNA technologies (IDT). ZEN Double-Quenched Probe from IDT that contain a 5′ fluorophore (FAM), 3′ Iowa Black FQ (IBFQ) quencher, and proprietary, internal ZEN quencher from ID was synthesized through IDT and used in this study. For nested qPCR, the primers that anneal outside of the csa-F1 and csa-R1 as shown in Figure 1 were designed. Primers and probe sequences are listed in Table 1.

7.3.5 Normal PCR, qPCR, and Nested qPCR

For normal PCR, each 2.5 µl DNA sample from clostridial isolates or farm samples was amplified using *Taq* DNA Polymerase (0.25 µl) with standard *Taq* buffer (NEB) in a 50 µl final reaction volume. The primers (csa-F1 and csa-R1) and dNTPs were used at the final concentration of 0.2 µM and 200 µM, respectively. The thermocycling condition of PCR included an initial

denaturation step at 94 °C for 2 min, followed by 35 cycles of 0.5 min at 94 °C, 0.5 min at 55 °C, and 1 min at 68 °C, and a final extension of 5 min at 68 °C. The qPCR assay included 2X PrimeTime Gene Expression Master Mix from IDT (12.5 µl), 1.5 µl each of 10 µM csa-F1 and csa-R1 primers, 5 µM csa-Probe (1.25), and 2.5 µl DNA sample in a 25 µl reaction volume. The qPCR was performed using the 7500 real-time PCR system (Applied Biosystems). The thermos cycling conditions were: one cycle at 95 °C for 10 min, 40 cycles of amplification at 95 °C for 15 s, 60 °C for 1 min. For nested qPCR, PCR amplification of the samples using nested primers (csa-F1-Nested and csa-R1-Nested) was performed for 15 cycles, and 2 µl PCR reaction from nested PCR was used for subsequent qPCR using TaqMan probe as described above.

7.4 Results

7.4.1 Evaluation of the Primers and Probes for Quantitative PCR Assay

To check the specificity of the designed primers (csa-F1 and csa-R1), PCR was performed using DNA templates from pure culture of both *C. septicum* (n=13) and non-*C. septicum Clostridium* (n=12) isolates from various turkey farms. The length of amplicons (148 bp) was confirmed using 1% agarose gel electrophoresis (Figure 2). As summarized in the Table 2, the primer pair resulted in 100% amplification from all *C. septicum* strains and 0% amplification from non-septicum *Clostridium* strains, supporting high specificity of the PCR primers in detecting *C. septicum* species.

In addition, the swab samples from turkeys with severe cellulitis (n=4) and the surrounding litters (n=4) were tested as positive controls using the PCR assay. All 8 samples showed strong positive results, suggesting the high efficiency of the PCR for the farm samples as well.

7.4.2 Result of the First qPCR Assay

We performed an initial evaluation of the qPCR assay using the primers and probe described above with some of the representative litter swab samples from farms as well as both *C. septicum* (positive control) and non-*C. septicum* (negative control) isolates (Table 3). All samples were ran in duplicates. V4 and V2221 were litter swab samples from V farm with the ages of birds 58 days and 16 weeks, respectively. Similarly, H8 and H2212 were litter swab samples from H farm with the ages 33 days and 12 weeks, respectively. RL1 was the sample from R farm where there was reported positive incidence of cellulitis, which was chosen to serve as the positive control sample. CS2B and CS3B were pure culture samples of *C. septicum* serving as the positive controls, whereas pure culture samples of *C. novyi* and *C. butyricum* served as the negative controls. In this run, our aim was to investigate the general performance (specificity and sensitivity) of the qPCR assay rather than quantifying the signals. Positive controls from both farm and pure culture showed lower Cq value than negative controls as expected, where it was much lower for the two culture positive samples as compared to the litter positive sample from the farm (TABLE 3). On the contrary, negative controls (no template, *C. novyi*, and *C. butyricum*) showed either very higher Cq values or could not be determined at all.

7.4.3 Evaluation of the Primers for Nested PCR

Since Cq value especially from the positive farm sample was quite high (27.80±0.04 for RL1), there was need to improve the sensitivity of the qPCR assay. Thus, we designed the other set of primer that anneal just outside of the csa-F1 and csa-R1 as shown below (Figure 1) to be used for nested PCR reaction.

The specificity of the nested primers was also tested using PCR followed by 1% agarose gel electrophoresis with proper positive (farm and pure *C. septicum* isolates) and negative (no template and non *C. septicum* isolates) controls. As expected, all the positive farm controls and *C. septicum* isolates showed amplifications with desired band length (~235 bp) and negative controls showed no amplification in 1% agarose gel.

7.4.4 Evaluation of the Improved qPCR Assay using Nested Primers

For initial evaluation of the nested qPCR assay, the nested qPCR was performed using relevant samples, including *C. septicum* and non *C. septicum* isolates. For nested qPCR, PCR amplification of the samples using nested primers (csa-F1-Nested and csa-R1-Nested) was performed for 15 cycles, and 2 µl PCR reaction from nested PCR was used for subsequent qPCR using TaqMan probe. To evaluate the improvement of including nested PCR step, the same assay was conducted with and without nested PCR step prior to qPCR assay. The summary of Cq values from same samples with or without nested PCR is summarized in Table 4. As we can see in Table 4, the sensitivity of qPCR assay increased to a greater extent by using nested qPCR as indicated by consistently lower Cq values in comparison to normal qPCR.

7.4.5 Evaluation of the Nested qPCR Assay Using Turkey Farm Samples

The sequences of *Clostridium* sensu stricto 1 as identified by microbiota analysis (Chapter six) were ≥97.6% identical to *C. septicum* sequence, and thus we considered these as *C. septicum* sequences. The results of nested qPCR from farm samples that contained *Clostridium* sensu stricto 1 are summarized in the Table 5. Spearman correlation test was performed using JMP Genomics9 to test the relationship between sequences of *C. septicum* and resulting Cq values. For this purpose, rarefied sequence counts were used and Cq value 40 was given to those samples which

quantification cycles were not determined. The quantification cycles of the qPCR assay were negatively correlated (Spearman's ρ/r_s=-0.54, P<0.0001) with the abundance of *C. septicum* read counts in 16S rRNA gene microbiota profiling of the samples from turkey farms (Table 5, Figure 3). Increased in *C. septicum* sequence counts resulted decrease in Cq values and vice versa.

7.5 Discussion

In the present study, we developed nested qPCR assay that was able to detect the *C. septicum* isolates in pure culture as well as from farm samples. Cellulitis in turkeys is considered as one of the emerging diseases of commercial turkey industry with the top most concerns of poultry Veterinarian (Clark et al., 2010; Lighty et al., 2016). Cellulitis in turkeys is primarily considered to be caused by *C. septicum* (Tellez et al., 2009), which normally starts at 13-16 weeks of age and continued until the market age (Carr et al., 1996). The typical mortality due to cellulitis varies from few birds to 3% daily (Gornatti-Churria et al., 2018) and 1-2% per week (Carr et al., 1996). But in some flocks, mortality up to 60% was also reported (Gornatti-Churria et al., 2018). Likewise, increase in down-grading and condemnation rates of turkey carcasses at slaughter have been associated with cellulitis (Gornatti-Churria et al., 2018). Moreover, cellulitis resulted increase in cost of production by 0.031 to 5.5 cents per kilogram of meat produced (Lighty et al., 2016). Thus, early detection of *C. septicum* from farm samples may help to reduce economic losses associated with cellulitis.

Although, previous studies developed qPCR assay for the detection of *C. septicum* based on *csa* (alpha toxin) gene (Neumann et al., 2010), 16S rRNA gene (Halm et al., 2010) and *spo0A* gene (Lange et al., 2010), there is still room to improve the sensitivity and selectivity of the assay. Specifically, the qPCR assay based on *spo0A* gene was developed for the simultaneous detection of both *C. septicum* and *C. chauvoei* species (Lange et al., 2010). Another qPCR assay based on

16S rRNA gene was developed for the purpose of differentiating *C. septicum* and *C. chauvoei* by the use *C. septicum* and *C. chauvoei* specific probes (Halm et al., 2010). However, this assay was not able to 100% differentiate the *C. septicum* from other clostridial species. Clostridial species such as *C. quinii*, *C. celatum*, *C. difficile* (DSM 5566), *C. haemolyticum* (DSM 5565), *and C. histolyticum* gave amplification signals when *C. septicum* specific probe assay was used (Halm et al., 2010).

On the contrary, the qPCR assay that targeted the alpha toxin gene (*csa*) which is believed to be presented in all strains of *C. septicum*, was able to differentially detect *C. septicum* from all tested non *C. septicum* and other closely related isolates (Neumann et al., 2010). This qPCR assay utilized the SYBR Green I, a nonspecific fluorescent dye that can bind to any double stranded DNA and can generate false positive signals. In addition, the length of amplicons also affect the intensity of the amplification in SYBR Green based qPCR assay (Cao and Shockey, 2012). However, TaqMan probe can only bind to the DNA sequence between the two PCR primers which enables to generate a fluorescent signal only from the specific PCR product, and thus increases the specificity (Cao and Shockey, 2012).

In the present study, we used the double quenched probe from IDT which was claimed to increase the sensitivity, specificity, and precision of qPCR experiment (IDT). Using normal PCR, we got 100% amplifications from all of the *C. septicum* (n=13) strains, while 0% amplifications from non-septicum *Clostridium* strains (n=12) which supports the high specificity of PCR primers in detecting *C. septicum*. Furthermore, all 8 swab samples (4 litters and 4 birds) collected from the farm having the higher incidence of cellulitis got amplified. This supports that the primers are not limited for the detection of pure cultures of *C. septicum* but can also be extended to the detection of *C. septicum* from farm samples. However, the nature of our farm samples is unique in the sense

that they were collected using swabs attached to the boots which contain lots of litter debris and the biomass of microbes may be very less as compared to other types of samples such as intestinal digesta or fecal samples. This is reflected in the first qPCR study where the Cq values from positive farm sample and pure culture of *C. septicum* strains were higher as expected (Table 3) demanding the need of improving the sensitivity of the assay. Thus, we designed the nested qPCR since nested qPCR was found to significantly increase the sensitivity of qPCR assay as reported earlier (Neuberger et al., 2016; Tran et al., 2014). In agreement with these studies, we also reported significant increase in the sensitivity of the nested qPCR assay (Table 4). Moreover, the nested qPCR assay was successfully applied to the farm samples where the Cq values correlated with the sequence counts of *C. septicum* as identified by the microbiota analysis (Table 5). Hence, the nested qPCR assay presented here can be successfully applied to detect and quantify the *C. septicum* strains in wide range of samples, which can help to prevent and treat the diseases associated with them by enabling their early detection.

7.6 Acknowledgements

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7.9 Tables and Figures

Table 1. Sequences of nested primers.

Primers	**Sequences**
csa-F1	GGGCAAATGTAGCTCATTCATTA
csa-R1	GGATCATTTGGATTGTATCTAGCAG
csa-Probe	CTGTTCCACCGCACCATCCAAATC
csa-F1-Nested	AAAATATTTGGATATGAAGACAATGA
csa-R1-Nested	CATAGAAAGTCTATCTTTTGCACGA

Table 2. Summary of PCR results.

***Clostridium* isolates**	**PCR results**
C. septicum-2	Positive
C. septicum-3	Positive
C. septicum-4	Positive
C. septicum-1B	Positive
C. septicum-2B	Positive
C. septicum-3B	Positive
C. septicum-4B	Positive
C. septicum-5B	Positive
C. septicum-6B	Positive
C. septicum-10B	Positive
C. septicum-B.D	Positive
C. septicum-C1	Positive
C. septicum-C2	Positive
C. bifermenticus	Negative
C. subterminale	Negative
C. perfringens	Negative
C. butyricum	Negative
C. novyi-1	Negative
C. novyi-2	Negative
C. limosum-1	Negative
C. limosum-2	Negative
C. limosum-3	Negative
C. limosum-4	Negative
C. cochlearium-1	Negative
C. cochlearium-2	Negative
Farm Samples	
RL (1-4)	Positive
RB (1-4)	Positive

*The strains that belong to *C. novyi*, *C. limosum*, *C. cochlearium*, and *C. butyricum* were identified through Sanger sequencing of 16S rRNA genes. RB and RL represent swab samples from turkey with severe cellulitis and the surrounding litters, respectively.

Table 3. Summary of qPCR results from first run using primers (csa-F1 and csa-R1) and probe (csa-Probe).

Sample	**Cq (Mean±SD)**
V4	Undetermined
V2221	Undetermined
H8	Undetermined
H2212	Undetermined
RL1	27.80±0.04
C. septicum CS2B	14.37±0.07
C. septicum CS3B	14.29±0.01
C. novyi	Undetermined
C. butyricum	34.22±1.10
NC	Undetermined

Note: SD; represent standard deviation, NC; represent negative control (no template).

Table 4. Summary of qPCR results obtained with and without nested PCR.

Sample	**Cq (Mean±SD) Normal**	**Cq (Mean±SD) Nested**
C. septicum CS2B	13.69±0.01	4.71±0.03
C. septicum CS3B	13.43±0.01	4.86±0.04
RL2	29.78±0.01	20.94±0.05
RB1	28.03±0.15	20.50±0.06
H8	Undetermined	Undetermined
H2212	Undetermined	Undetermined
V4	Undetermined	Undetermined
V2221	Undetermined	Undetermined
C. novyi	35.90±1.69	27.57±0.01
C. bifermenticus	34.16±0.04	28.26±0.11
NC	Undetermined	Undetermined

NC represents negative control (no template DNA), other samples are same as described above.

Table 5. Summary of qPCR results from farm samples with *Clostridium* sensu stricto 1 sequences count as determined by microbiota analysis.

Sample	**Cq (Mean±SD)**	**Sequence Count**
CS2B	8.38±0.22	NA
RL1	19.94±0.05	5
RL3	23.55±0.18	4
RB1	19.47±0.06	1035
RB2	24.32±0.16	3987
RB3	17.39±0.14	737
RB4	17.55±0.07	2857
M3321	25.66±0.20	53
M3322	26.53±0.08	31
M3341	26.93±0.05	17
M3312	27.54±0.17	22
M3331	28.14±0.13	18
M3241	28.35±0.20	26
M2321	28.36±0.10	12
M3311	29.20±0.11	19
M3212	29.67±0.22	21
M3211	30.72±0.18	33
K335	31.63±0.16	8
H3341	Undetermined	9
M3231	Undetermined	30
M3342	Undetermined	14
M3221	Undetermined	19
M2231	Undetermined	9
M3222	Undetermined	15
M2311	Undetermined	9
M2322	Undetermined	10
M3232	Undetermined	15
M3242	Undetermined	12
M2341	Undetermined	11
M2212	Undetermined	8
M2211	Undetermined	4
M2242	Undetermined	14
M2331	Undetermined	6
M2241	Undetermined	5
M2342	Undetermined	16
M2312	Undetermined	9
M2222	Undetermined	5
M6231	Undetermined	2

Table 5 Cont. Summary of qPCR results from farm samples with *Clostridium* sensu stricto 1 sequences count as determined by microbiota analysis.

Sample	**Cq (Mean±SD)**	**Sequence Count**
H2342	Undetermined	2
H2332	Undetermined	2
H3331	Undetermined	5
H3322	Undetermined	5
H3342	Undetermined	6
H3311	Undetermined	8
K336	Undetermined	29
K332	Undetermined	16
K318	Undetermined	16
H2312	Undetermined	3
H3332	Undetermined	2
H2341	Undetermined	28
K311	Undetermined	5
K334	Undetermined	13
K338	Undetermined	8
K337	Undetermined	3
K333	Undetermined	2
K314	Undetermined	3
K331	Undetermined	2
V635	Undetermined	1
NC1	Undetermined	NA
NC2	Undetermined	NA

NC represents negative control. Other samples are same as described above and Chapter six. NA represent not applicable. The counts are from rarefied table.

Figures

GGGGATATGCAAATYATAATAATGCTTCTTCAATTAAAATATTTGGATATGAAGA
CAATGARGATTTAAAAGCTAAAATTATTCAAGATCCAGAGTTTATAASAAAYTGG
GCAAATGTAGCTCATTCATTAGGATTTGGATGGTGCGGTGGAACRGCTWWWCCWR
RHRTTGGASAAGRYTTTGAATTTAAAAGAGAAGTTGGGGCAGGTGGAAAAGTATC
TTATTTATTATCTGCTAGATACAATCCAAATGATCCTTATGCAAGTGGATATCGT
GCAAAAGATAGACTTTCTATGAAAATATCAAATGTTAGATTTGTTATTGATAATG

Figure 1. DNA sequence of the *csa* gene region showing the design and locations of the primers and probe used for the nested qPCR assay described in this study. The regions corresponding to the primers and probe are shown in different colors: nested-F1, csa-F1, Taqman probe, csa-R1, and nested-R1. C was common to both csa-R1 and Nested-R1. The oligonucleotides were designed based on the csa gene sequences of the following *C. septicum* strains: AB083434.1, EU482188.1:315-1646, HM051335.1, FJ212777.1, KU726861.1:1078-1677.

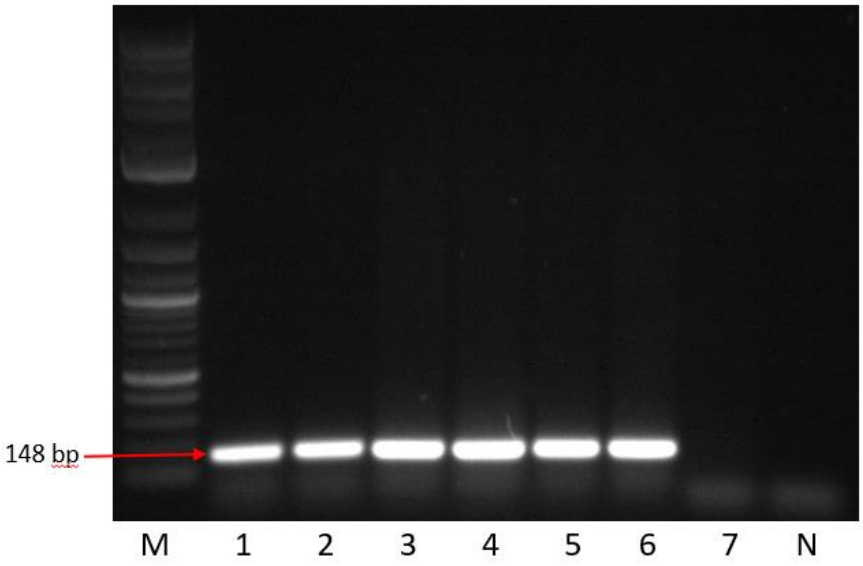

Figure 2. PCR products of the *csa* gene separated on 1.0% agarose gel. M: 2-log ladder, 1: *C. septicum*-2B, 2: *C. septicum*-3B, 3: *C. septicum*-4B, 4: *C. septicum*-5B, 5: *C. septicum*-6B, 6: *C. septicum*-10B, 7: *C. novyi*, and N: no template (Negative Control).

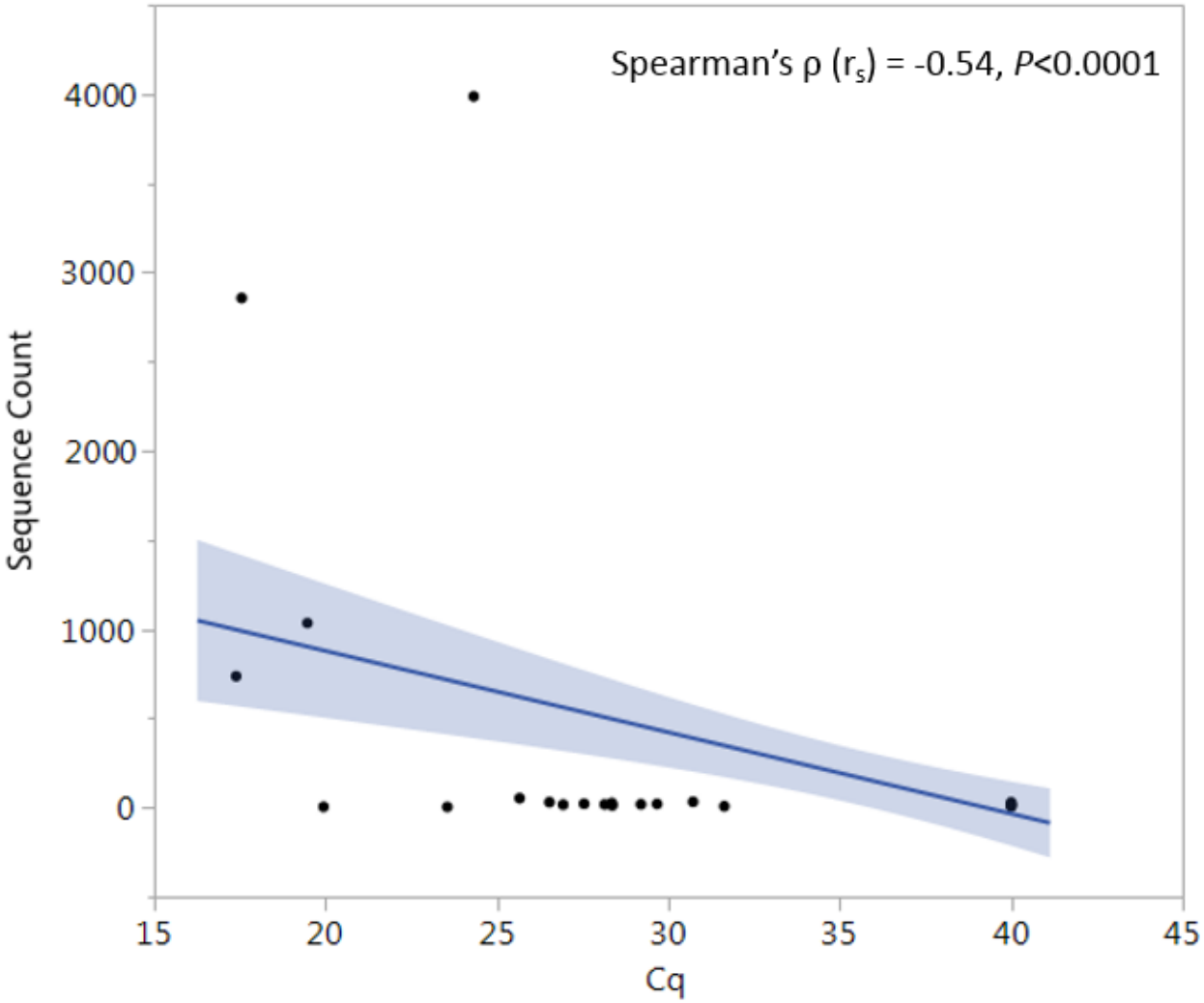

Figure 3. The scatter plot showing the correlation between the sequence counts (rarefied) of *C. septicum* and the quantification cycles (Cq). Spearman correlation test showed increase in sequence counts of *C. septicum* resulted decrease in Cq values and vice versa (r_s=-0.54, *P*<0.0001).

CONCLUSION

In this dissertation, the microbiotas in diverse samples collected from chickens (broilers, breeders, and layers) and turkeys were investigated by 16S rRNA genes sequences analysis. The culturable *Lactobacillus* subpopulations recovered on MRS agar from the chicken gastrointestinal tract (GIT) showed variations with in the different regions (cecum vs. ileum) and locations (lumen vs. mucosa) of the GIT, indicating their distinct functional roles in different GIT niches. Some species of *Lactobacillus* were not culturable, while other non-lactic acid bacteria grew on MRS agar media which suggest that the MRS agar are not strictly selective to lactic acid bacteria only. While investigating the effect of cell densities as determined by varying levels of sample dilution on the culture-enriched microbiota profiles, the dilution levels of original samples were found to alter the resulting microbiota via unknown density-dependent mechanisms. Thus, cell densities of samples should be considered for designing experiments using culture-enriched microbiota profiling. Direct-Med Microbials (DFM) based on *Bacillus* isolates (*B. subtilis* and *B. amyloliquefaciens*) were found to reduce *S.* Enteritidis concentrations in the intestinal compartments as compared to the control using *in vitro* digestive model. In addition, DFM improve intestinal health by reducing the permeability as measured by serum FITC-d levels and other markers of intestinal health such as IgA and superoxide dismutase (SOD) using *in vivo* trials. When egg production performance and cecal microbiota were compared between two strains of Hy-Line (Brown and W-36) housed in conventional or enriched colony cages, there was significant interaction effect of strains and housing types on egg production in addition with significant changes in composition, diversities, and functional potentialities of cecal microbiota between strains and housing types during the late production stage. The overall results of this study suggest that the differences in egg production between hens' strains and housing types might be achieved

at least partially through alterations of cecal microbiota. Moreover, comprehensive microbiota analysis of 246 boot swabs collected from five commercial turkey farms of Northwest Arkansas revealed variations in the litter microbiota compositions and their diversities among farms and flocks which were further affected by the ages of turkeys. Interestingly, 24 core bacterial genera were found to present in all farm samples with positive incidence of cellulitis including *Corynebacterium*, an unknown genus of family Bacillaceae, *Clostridium* sensu stricto 1 (>97% similarity with *C. septicum*), and *Ignatzschineria* beside others, while only 4 core bacterial genera were reported from all rest of the farm samples (*Staphylococcus*, *Brevibacterium*, *Brachybacterium*, and *Lactobacillus*). The differences in bacterial genera recovered in positive samples and rest of the farm samples suggest the possible roles of other bacteria beside *C. septicum* in etiopathogenesis of cellulitis in turkeys. We also developed and evaluated nested qPCR assay for the quantitative detection of *C. septicum* that targets the alpha toxin gene (csa). The assay was sensitive to detect *C. septicum* from the pure culture as well as from the farm samples.

APPENDIX

Table S1. The relative abundance of all OTUs found in MRS groups were also determined from the directly isolated DNA samples (T-ZERO) and included in the statistical analysis as a reference for comparison.

#OTU ID	CL.1a	CL.1b	CL.1c	CL.2a	CL.2b	CL.2c	CL.3a	CL.3b	CL.3c	CL.4a	CL.4b	CL.4c		CL.6b	CL.6c	CL.7a	CL.7b	CL.7c	CL.8a	CL.8b	CL.8c	CL.9a	CL.9b	CL.9c	CL.10a	CL.10b	CL.10c	Top-hit Species
Lactic Acid Bacteria: *Lactobacillus*																												
265678423	19.87	16.48	16.34	20.84	20.03	22.07	19.50	19.27	18.72	19.47	21.59	21.95		19.08	17.99	19.00	19.58	19.80	12.44	19.76	17.69	22.44	21.50	19.60	22.84	20.58	21.51	*L. salivarius*
343206111	12.48	11.79	0.00	16.03	14.51	12.00	11.37	9.97	10.27	10.27	11.33	6.81		8.54	13.11	12.27	10.74	14.30	9.09	17.19	14.13	17.37	19.05	13.75	13.55	12.38	14.36	*L. agilis*
444439671	9.05	2.25	0.00	12.66	10.82	11.63	14.83	10.90	9.55	11.19	11.42	9.12		15.86	17.31	14.13	16.24	14.00	8.83	8.16	0.00	12.15	10.14	10.97	10.14	9.81	11.28	*L. crispatus*
343198491	8.54	4.96	1.94	14.77	9.86	9.35	18.21	13.51	12.94	9.85	10.47	0.00		9.19	9.92	13.93	10.55	8.73	8.51	8.97	7.04	11.99	10.43	10.83	10.67	10.23	12.54	*L. gasseri*
265678507	6.45	0.00	0.00	10.20	0.00	0.00	14.07	9.30	5.09	9.80	11.21	12.41		10.27	7.30	8.43	8.33	10.13	0.00	9.97	0.00	9.97	6.98	5.51	8.97	11.87	11.34	*L. ingluviei*
444439749	0.00	0.00	0.00	9.47	4.67	5.48	14.23	8.15	7.64	4.99	6.73	0.00		6.15	0.00	10.60	8.16	0.00	0.00	0.00	0.00	7.27	0.00	4.54	0.00	6.00	7.56	*L. johnsonii*
265678780	0.00	0.00	0.00	5.90	5.64	0.00	8.34	5.48	0.00	0.00	6.73	0.00		6.87	7.30	8.70	5.20	13.58	0.00	0.00	0.00	7.86	0.00	0.00	6.98	0.00	8.19	*L. saerimneri*
343201713	0.00	0.00	0.00	5.90	0.00	5.48	7.83	0.00	0.00	4.99	0.00	0.00		8.35	10.67	8.11	10.16	7.74	6.52	0.00	0.00	0.00	0.00	5.51	0.00	0.00	0.00	*L. helveticus*
343198690	0.00	0.00	0.00	5.90	0.00	0.00	7.60	0.00	0.00	4.99	5.75	0.00		8.45	10.46	6.13	9.25	4.97	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*L. amylovorus*
343202487	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.51	0.00	0.00	0.00	0.00		6.15	8.29	7.12	8.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*L. ultunensis*
343201103	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.03	0.00	0.00		4.60	7.30	5.15	0.00	0.00	8.51	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*L. crispatus*
444439721	0.00	0.00	0.00	5.90	0.00	0.00	5.06	0.00	0.00	0.00	0.00	6.81		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.28	0.00	0.00	0.00	0.00	0.00	*L. reuteri*
Lactic Acid Bacteria: non-*Lactobacillus*																												
310975058	3.74	0.00	0.00	0.00	0.00	0.00	6.61	0.00	0.00	0.00	0.00	0.00		9.54	5.01	7.12	6.76	5.95	0.00	12.41	12.59	11.12	8.78	15.60	6.98	5.03	0.00	*Enterococcus durans*
343201328	0.00	0.00	0.00	0.00	0.00	0.00	5.06	0.00	0.00	0.00	0.00	0.00		6.55	6.57	0.00	0.00	4.97	0.00	7.75	9.53	8.59	7.97	11.81	0.00	0.00	5.99	*Enterococcus faecium*
310975218	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.73	7.80		8.71	8.55	0.00	6.76	0.00	0.00	9.16	0.00	0.00	0.00	6.49	0.00	0.00	7.56	*Enterococcus hirae*
343201331	0.00	0.00	0.00	0.00	0.00	8.03	0.00	0.00	0.00	0.00	0.00	0.00		4.60	8.43	0.00	5.20	10.01	0.00	6.18	0.00	0.00	0.00	0.00	0.00	0.00	10.11	*Pediococcus acidilactici*
507147983	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.51	0.00	0.00	0.00	0.00		5.57	5.99	0.00	0.00	0.00	0.00	0.00	0.00	6.28	0.00	8.18	0.00	0.00	4.45	*Enterococcus faecium*
343200102	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	9.31	10.60		0.00	0.00	0.00	0.00	0.00	0.00	6.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*Enterococcus fecalis*
343201094	0.00	0.00	0.00	5.90	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.97	3.53	7.56	*Streptococcus alactolyticus*
444439707	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	7.17	7.04	0.00	0.00	9.00	0.00	0.00	0.00	*Enterococcus hirae*
Non-Lactic Acid Bacteria																												
265678513	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		7.87	0.00	7.70	6.76	7.25	11.55	0.00	0.00	6.28	6.98	0.00	0.00	0.00	5.99	*Turicibacter sanguinis*
265678383	11.89	9.92	9.96	0.00	0.00	0.00	6.61	0.00	4.52	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*Citrobacter rodentium*
444439588	12.35	10.33	11.60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*Citrobacter rodentium*
444304126	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00	5.01	5.15	0.00	0.00	6.52	7.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*C. jejuni subsp doylei*
219846899	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	6.52	6.18	0.00	0.00	5.99	0.00	0.00	0.00	0.00	*Cl. disporicum*
253680771	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.00	6.71	5.20	6.52	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*Helicobacter pametensis*

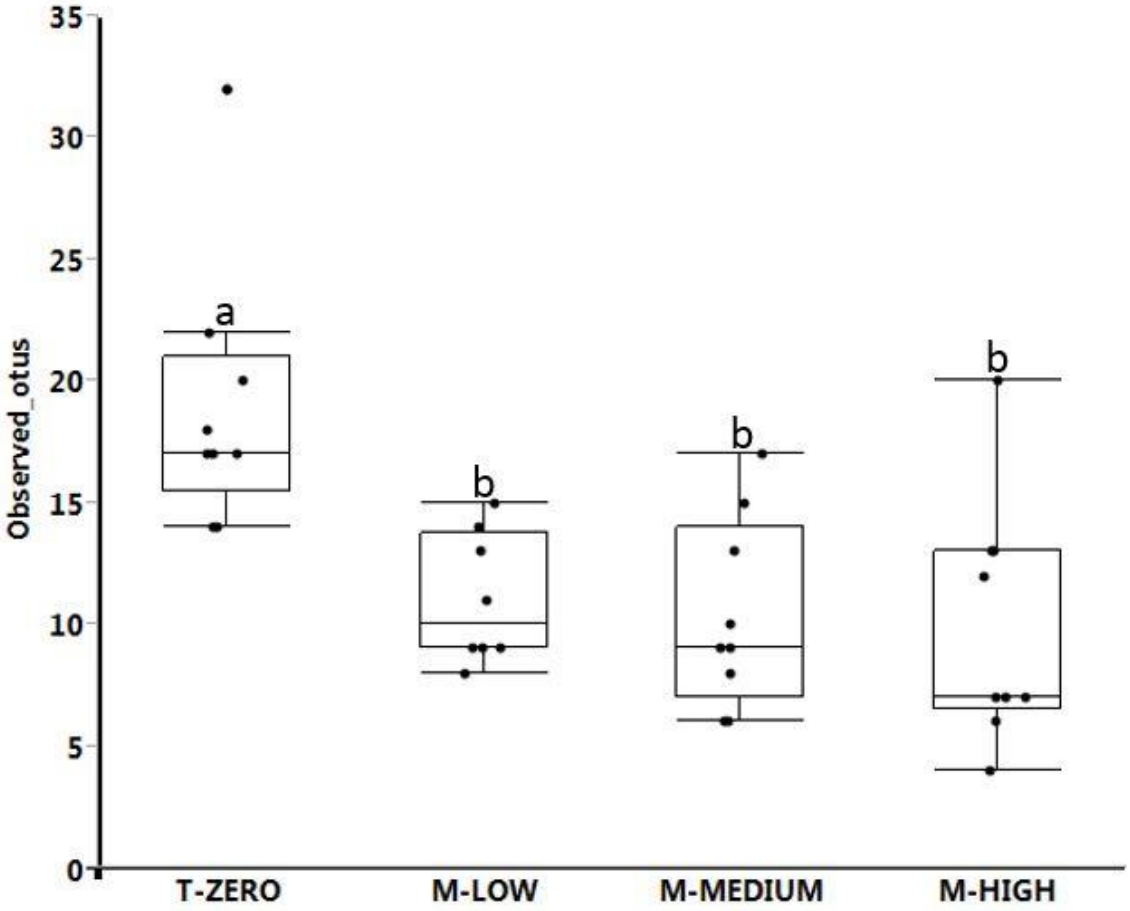

Figure S1. Alpha diversity of the different groups as measured by observed OTUs. Bars with different letters represent statistically significant at $p<0.05$. T-ZERO represent total bacterial populations recovered directly from cecal contents whereas M-LOW, M-MEDIUM, and M-HIGH represent bacterial population recovered on MRS from 10^2, 10^4, and 10^6 fold dilutions respectively.

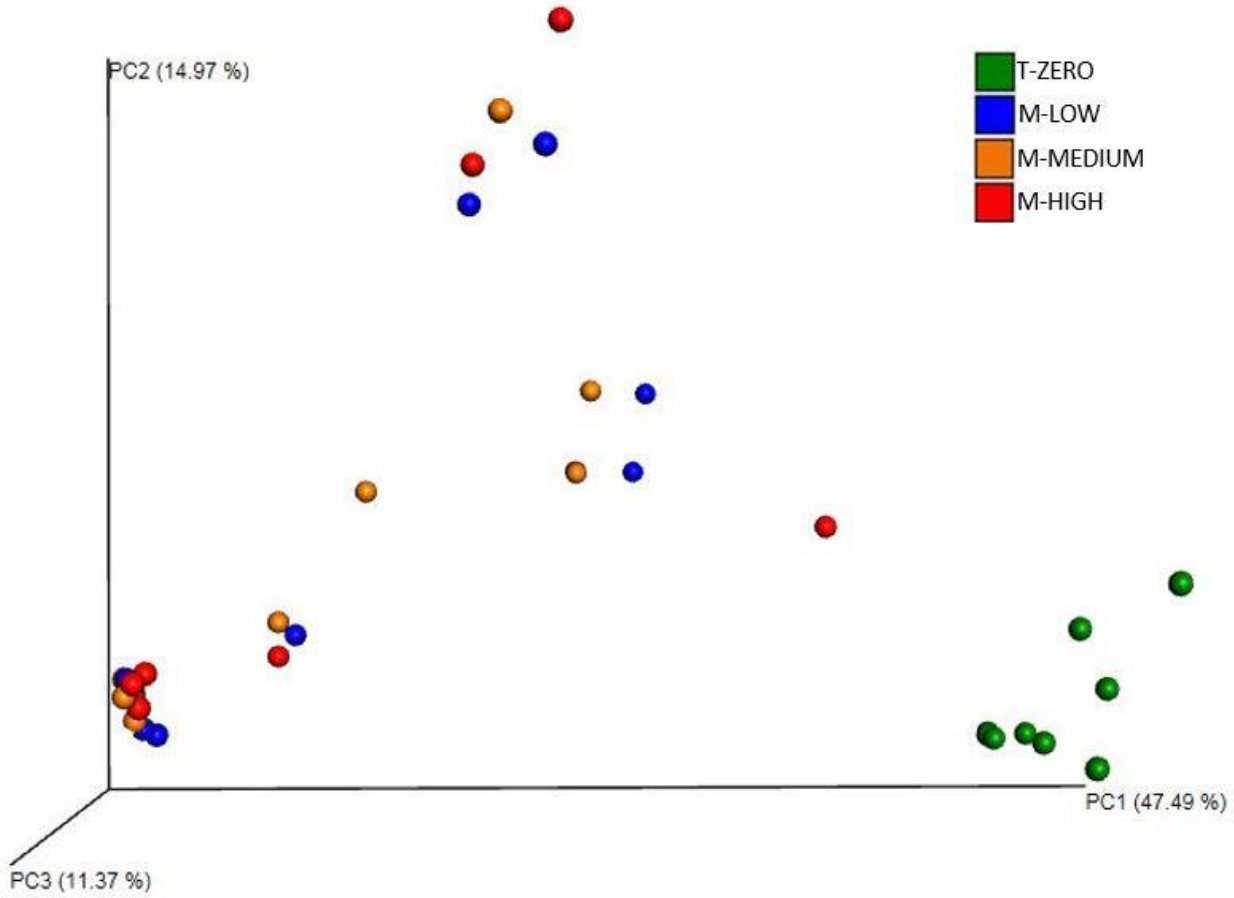

Figure S2. PCoA plot showing the distances among total bacteria (T-ZERO) and MRS-selected dilution groups (M-LOW, M-MEDIUM, and M-HIGH) based on Unweighted UniFrac distance metric (ANOSIM: R = 0.48, p = 0.001).

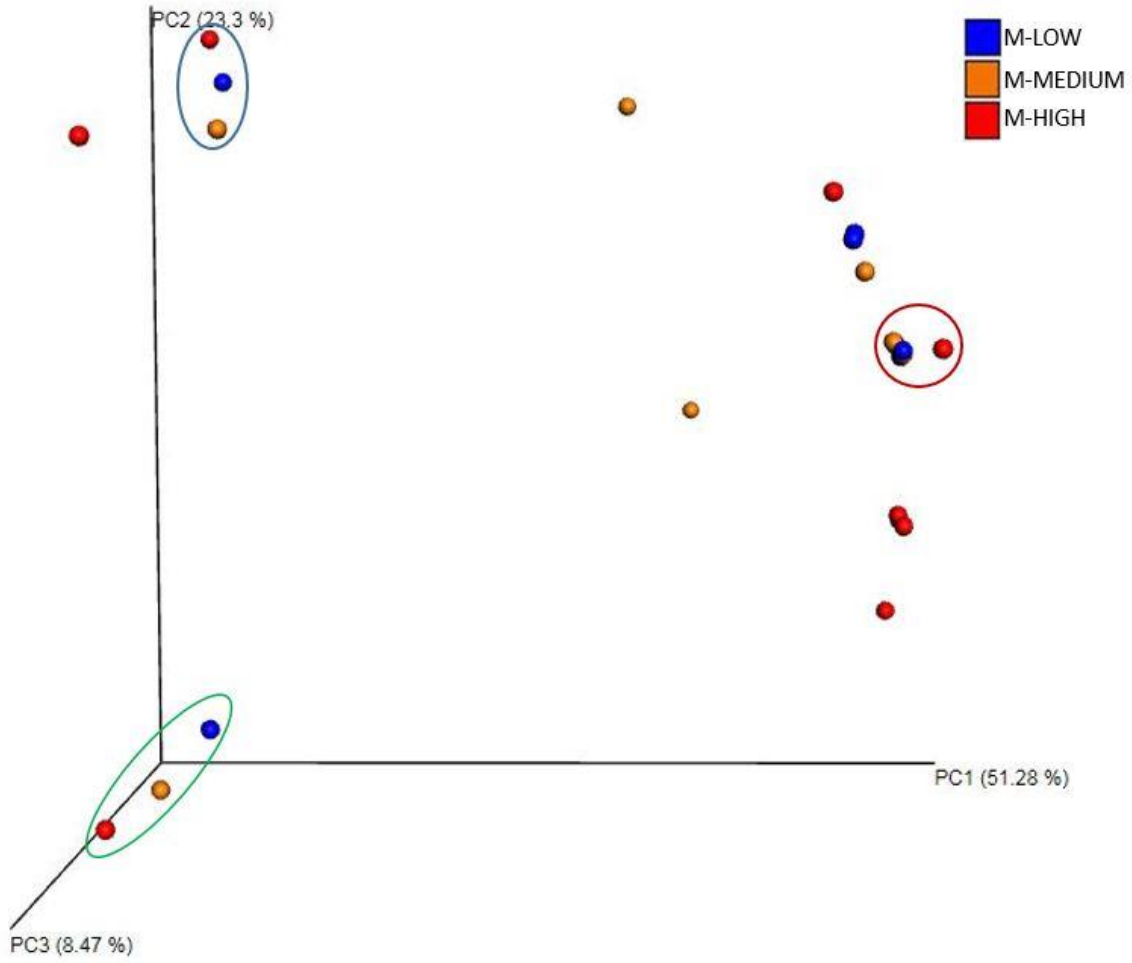

Figure S3. PCoA plot showing the distances among the MRS-enriched dilution groups based on Unweighted UniFrac distance metric. M-LOW, M-MEDIUM, and M-HIGH (ANOSIM: R = -0.05, p = 0.85). The circles indicate the different dilution samples originated from the same cecal samples.

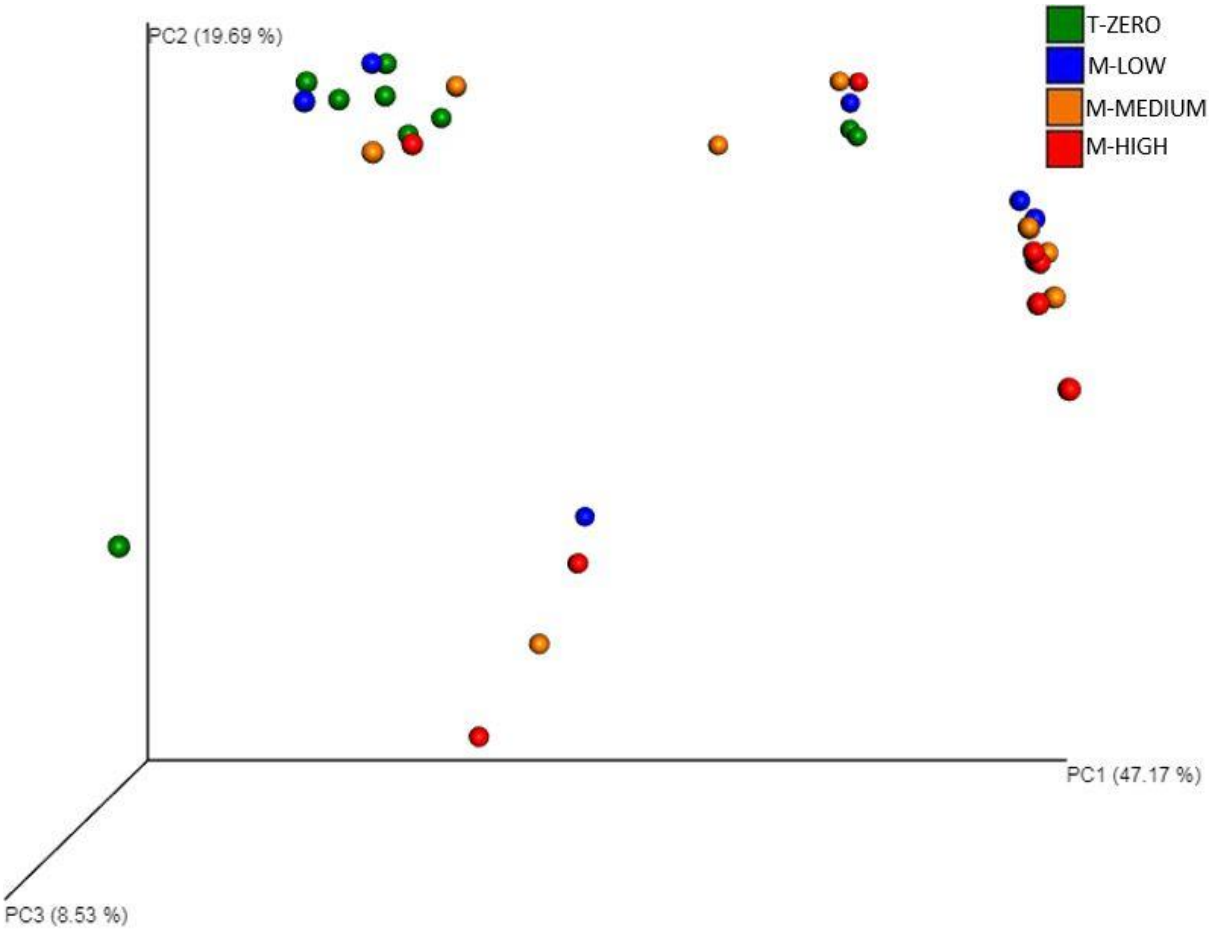

Figure S4. PCoA plot showing the distances among total bacteria (T-ZERO) and MRS-selected dilution groups (M-LOW, M-MEDIUM, and M-HIGH) based on Unweighted UniFrac distance metric. For T-ZERO in this analysis, only the OTUs in T-Zero that were also found in MRS-dilution groups were used.

IACUC Approval (protocol # 16049)

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To:	Young Kwon
Fr:	Craig Coon
Date:	April 2, 2018
Subject:	IACUC Approval
Expiration Date:	April 6th, 2019

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **16049:** *Rationale design for stain-specific prebiotics for promotion of gut health and growth performance in chickens*.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond April 6th, 2019 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Deepti Samarth, Young Kwon, Yichao Yang, and Bishnu Adhikari. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp

16049

IACUC Approval (protocol # 18030)

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To:	Billy Hargis
Fr:	Craig Coon
Date:	October 19th, 2017
Subject:	IACUC Approval
Expiration Date:	October 5th, 2020

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18030:** *Enteric inflammation models for investigation of antibiotic alternatives in poultry*.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond October 5th, 2020 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Billy Hargis, Guillermo Tellez, Cheryl Lester, Brittany Mahaffey, Amanda Wolfenden, Lucas Graham, and Kyle Teague. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp

18030

http://vpredweb.uark.edu/iacuc-webapp/mods/letter.php?ID=1170&PROTOCOL=18030

IBC approval (protocol # 16020)

UNIVERSITY OF ARKANSAS | Research & Economic Development
Research Compliance

February 23, 2016

MEMORANDUM

TO:	Billy Hargis
FROM:	Ines Pinto, Biosafety Committee Chair
RE:	New Protocol
PROTOCOL #:	16020
PROTOCOL TITLE:	Experimental studies to evaluate efficacy of probiotic culture alternatives
APPROVED PROJECT PERIOD:	Start Date 02/11/2016 Expiration Date 02/10/2019

The Institutional Biosafety Committee (IBC) has approved Protocol 16020, "Experimental studies to evaluate efficacy of probiotic culture alternative". You may begin your study.

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

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

CURRICULUM VITAE

BISHNU ADHIKARI

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Email: bxa015@uark.edu, bishnuadhikari323@gmail.com
www.linkedin.com/in/bishnuadhikari

EDUCATION

PhD in Poultry Science **2019**
University of Arkansas, Fayetteville, AR, USA
Committee: Billy M. Hargis, Guillermo Tellez-Isaias, Jiangchao Zhao, & Young Min Kwon (chair)
Dissertation title: "Investigation of Microbiota in Health and Disease of Poultry"

MSc in Sustainable Animal Nutrition and Feeding **2015**
Aarhus University, Aarhus, Denmark

MSc in Sustainable Animal Nutrition Agricultural Engineer **2015**
University of Debrecen, Debrecen, Hungary
Thesis title: "Impact of Pre and Postnatal Malnutrition on Glucagon Regulation and Signalling in Sheep"
Committee: Prabhat Khanal, Mette Olaf Nielsen, László Babinszky, & Mogens Vestergaard
Program details: http://sites.wageningenur.nl/en/emsanf.htm

BSc in Veterinary Science and Animal Husbandry/Doctor of Veterinary Medicine (DVM) **2010**
Institute of Agriculture and Animal Science (IAAS), Tribhuwan University, Nepal
Thesis title: "Prevalence of *Salmonella* Isolated from Water Used in Retail Goat Meat Shop of Kathmandu Valley and Rupandehi District of Nepal"
Advisor: Hom Bahadur Basnet

RESEARCH INTEREST

- Analysis of NGS data, functional genomics, host-pathogen interactions, microbial ecology, immunology, microbial physiology and genetics
- Interested in investigating the interactions of Nutrition, Microbiome, and Hosts for the control and prevention of animal diseases, development of alternatives to antibiotics, and overall improvement of animal health.

PROFESSIONAL EXPERIENCE

Research Assistant
Department of Poultry Science, University of Arkansas **2015-Present**

- Worked with "Microbiome" on a large scale to investigate the roles of microbiota in health and diseases of animals (broilers, layers, turkeys, and pigs) such as necrotic enteritis in broilers and cellulitis in turkeys
- Utilized different bioinformatics tools to analyse Next Generation Sequencing (NGS) data
- Characterized *Lactobacillus* subpopulations in chicken intestinal tract as a resource for probiotic development
- Investigated the antimicrobial and anti-inflammatory properties of boric acid and *Bacillus*-DFM for the control and treatment of *Salmonella* Enteritidis infections and necrotic enteritis in chickens, as well as evaluated the effects of different alternatives to antibiotics products from different company such as Kemin, Anpario, Chr. Hansen etc. in the modulation of gut microbiota and their association with performance parameters
- Investigated microbiome and quantify *C. septicum* from litter of different commercial turkey farms with/without supplementation of antibiotics alternatives and varying degree of cellulitis at different stages of growth
- Perform genome-wide screening for *C. perfringens* HN13 strain using functional genomic approach
- Published **9** manuscripts in peer reviewed journals and expected to publish more including those under review (**6**) and in preparation (**8**)

Teaching Assistant
Department of Poultry Science, University of Arkansas **2017-2018**

- Taught lab session of the graduate level course entitled "Molecular Analysis of Foodborne Pathogens"
- Provided students hands-on experiences regarding different tools and techniques used in the molecular analysis of foodborne pathogens such as, sample processing, DNA extraction, PCR, and library preparation for Sanger/Illumina sequencing
- In addition, trained students to use bioinformatics tools for Sanger/Illumina sequence analysis

Research Assistant
Department of Veterinary, Clinical and Animal Science, University of Copenhagen **2014-2015**
- Investigated the impacts of early life nutritional manipulations on glucagon regulation and signalling in sheep where the study found that the pancreatic α-cell compared to β-cells may be less sensitive towards late gestation malnutrition, whereas hepatic glucagon signalling appears to be a target of prenatal programming.

Internship
Department of Veterinary, Clinical and Animal Science, University of Copenhagen **2014**
- Validated Mercodia glucagon ELISA assay (Mercodia AB, Uppsala, Sweden) for the first time to determine glucagon in sheep plasma.

Veterinary Officer/TechnoMarketing Executive **2011-2013**
Triveni Feed Industries Pvt. Ltd. Kathmandu, Nepal
- Visited different poultry farms and provided technical support in both aspects of management and diseases as well as reported performance parameters to the company in a routine way
- Conducted various technical trainings to farmers and related stake holders
- Used to frequently visit distributors across the different parts of the country to promote the company products

Internship
Veterinary Teaching Hospital, IAAS, Tribhuwan University **2010**
- Studied different microbiological techniques such as sample collection, plating, culturing of bacteria, Gram's staining, and some basic biochemical tests
- Learnt different diagnostic techniques of diseases related to large animals, pets, and poultry and their appropriate treatment methods
- Collected water samples used for cleaning meats from different retail goat meat shops in Nepal and investigated the prevalence of *Salmonell*a in those water samples

VOLUNTEERING
- Judged for the biology undergraduate student posters in Arkansas IDeA Network of Biomedical Research Excellence, INBRE, 2017 at University of Arkansas
- Tiger population monitoring in Terai Arc landscape of Nepal conducted by World Wildlife Fund (WWF) from November 2008 to April 2009

AD HOC REVIEWER
- *Frontiers in Veterinary Science* since November, 2017 to present

RESEARCH SKILLS

Laboratory skills
- Isolation of various infectious and food borne pathogens such as *Salmonella*, *Escherichia*, *Clostridium*, *Campylobacter* etc. from wide variety of sources and their identification utilizing both culture dependent and independent methods
- Experience on handling of BSL2 pathogens and have knowledge on BSL3/4 pathogens
- Expertise on PCR, qPCR, RT-qPCR, cloning, DNA and RNA extraction, electrophoresis, ELISA, transposon mutagenesis, library preparation for Illumina sequencing, anaerobic bacterial culture, and other general microbiological tools and techniques etc.
- Familiar with various animal diseases challenge models: *in vivo* model of Necrotic Enteritis and *Salmonella* Enteritidis as well as *in ovo* model of *E. coli* transmission

Bioinformatics skills

- Proficient in Linux commands, and cloud computing
- Familiar with version control (Git)
- Programming languages: Intermediate level experiences on Perl, Python, R, and Bash scripting
- Bioinformatics tools: QIIME, MOTHUR, BOWTIE, BWA, Samtools, Prokka, Sourmash, Trimmomatic, FastQC, Plink, VCF tools, DADA2, Deblur, VSEARCH etc.
- Next generation sequence analysis: Proficient on analysis of 16S rRNA microbiome data and have hands on trainings on analysis of shotgun metagenomics, transcriptomics, genome and transcriptome assembly, GWAS, Variant calling, and transposon sequencing (TnSeq)

Veterinarian skills

- Necropsy, general examination and handling of animals, vein puncture and infusion, familiar with animal management and diseases etc.

Nutrition-related skills

- Proximate analysis of feed, general feed formulation, in vitro testing of feed additives etc.

Statistical software

- SAS, JMP, R (intermediate), and Sigma Plot

LEADERSHIP SKILLS

President

Nepali Association of Northwest Arkansas (NANA) **2016-2017**

- Served as a president of a registered student association at University of Arkansas for the term 2016/2017
- Played a lead role to get NANA actively involved in different University events such as International Bazaar to show our cultural diversity
- Conducted different social and cultural events such as "Nepali New Year"
- Provided guidance, support, and suggestions for Nepalese students living in Northwest Arkansas

President and Exchange Officer **2009-2010**

Nepal Veterinary Student Association (NVSA)

- Played a lead role of organization and coordinated with University to identify any sorts of problems faced by students and their solutions
- Played a vital role in exchange of information between NVSA and International Veterinary Students Association (IVSA).
- Coordinated with Research and Extension Committee (RECOM) to conduct various outreach activities such as rabies vaccination program, deworming program and Blue Cross Editorial committee to publish 11th edition of "The Blue Cross" which is an annual publication of NVSA
- Conducted various technical seminars (eg. Avian Influenza) with in the University
- Continuously coordinated with Nepal Veterinary Association (NVA) and actively participated in their programs such as celebration of World Veterinary Day 2009 with a theme "One World, One Health" which got "World Veterinary Day Award" by World Veterinary Association (WVA) in partnered with World Organization for Animal Heath (OIE)
- Conducted various social activities such as "Welcome" and "Farewell" programs for newcomers and graduates, respectively

ACHIEVEMENTS AND AWARDS

Department of Poultry Science's Outstanding Ph.D. Graduate Student

- Recipient of "Outstanding Ph.D. Graduate Student Award" by Department of Poultry Science, Dale Bumpers College of Agricultural, Food & Life Sciences, University of Arkansas for the year 2019

Travel Awards

- Poultry Science Association (PSA) annual meeting: July 15-18, 2019, Montreal, Quebec, Canada
- Metagenomics Workshop: September 29-30, 2016, Noble Foundation, Ardmore, OK
- Midwest Big Data Summer School: June 20-24, 2016, Ames, IOWA

First place in poster competition

- The poster entitled "Analysis of *Lactobacillus* species in the ceca of breeder hens" was recognized with People's choice award in Bumpers College Honors Student Board Research Poster Competition, held on April 10-12, 2017, University of Arkansas, Fayetteville

Erasmus Mundus Scholarship Award

- Selected for highly competitive and prestigious Erasmus Mundus Scholarship Award (48000 Euros) of European Union to pursue double MSc degree. Only 16 students from all over the developing countries were selected for this scholarship; only Nepalese representative

Rotary Shrijana Veterinary Award

- Awarded by Nepal Veterinary Association (NVA) of Nepal for being first in the third year of B.V.Sc. & A.H/DVM program

Kiran Memorial Award

- Awarded by "Kiran Memorial Trust" for securing highest percentage in whole Chitwan district at higher secondary school level final exam of 2003 taken by Higher Secondary Education Board, Nepal

Full Scholarship in BSc

- Selected for full scholarship to study B.V.Sc. & A.H/DVM program for the term 2005-2010 through a competitive exam and received meritorious student award throughout the period

District Topper Award

- Awarded by District Development Committee, District Education Committee, and Amarapuri Village Development Committee for securing highest percentage in whole Nawalparasi district in School leaving Certificate exam of 2002, Nepal

Mr Genius Award

- For securing first position in Intra College Chess tournament conducted by NVSA, Nepal in 2005

CONFERENCES/WORKSHOPS ATTENDED

Bioinformatics workshops

Arkansas Bioinformatics Consortium (AR-BIC): February 25-26, 2019, Little Rock, Arkansas

- Attended scientific program related to "Bioinformatics in Food and Agriculture"

A Gentle Introduction to Bayesian Statistics: December 1-2, 2018, Chicago, Illinois

- A workshop at CRWAD, 2018 which provided hands experiences in the basics of the Bayesian approach, including Bayes theorem and its practical applications, linear and logistic regression, and mixed models, taught using practical examples and real data in R

Cloud Computing Workshop: September 4, 2018, University of Arkansas, Fayetteville, Arkansas

- Hands-on training on "Nebula", a cloud computing facility in University of Arkansas

Arkansas Bioinformatics Consortium (AR-BIC): April 23-24, 2018, Little Rock, Arkansas

- Workshop by TriNetX, attended talks on different aspects of bioinformatics that ranges from shotgun metagenomics (MG-RAST) and RNA-seq to text mining and natural language processing

Jetstream/Transcriptomics/Metagenomics Workshop: Sept. 11 - 12, 2017, University of Arkansas, Fayetteville, Arkansas

- Hands-on training on how to apply bioinformatics tools in the analysis of genomic data (transcriptomics and shotgun metagenomics) within the Jetstream environment

Data Intensive Biology Summer Institute (DIBSI), Next-Generation Sequence Analysis Workshop: June 26 - July 8, 2017, University of California, Davis, USA
- Intensive two weeks hands on trainings on next generation sequence analysis (NGS)
- Training included but not limited to use of cloud computer, download and transfer files, running command-line BLAST, running RStudio using command line and its use to analyse data, RANseq expression analysis, Genome assembly, Bacterial genome annotation, automation, K-mers analysis, RMarkdown, Variant calling, Genome wide association analysis (GWAS), Jupyter Notebook and Python, Public databases, assessing and assembling Nanopore data, denovo transcriptome assembly and annotation etc.

Python Workshop: May 18 - 19, 2017, University of Arkansas, Fayetteville, Arkansas
- Hands-on training on Shell scripts, python programming, and version control with Git

Metagenomics Workshop: September 29 - 30, 2016, Noble Foundation, Ardmore, OK
- Amplicon sequence analysis with QIIME and shotgun metagenomics with MG-RAST

Midwest Big Data Summer School: June 20 - 24, 2016, Ames, IOWA
- Hands-on training on Python and R programming, talks on text mining, management and access of big data, machine learning etc.

Scientific Conference Presentations
- Poultry Science Association (PSA) Annual Meeting: July 15-18, Montreal, Quebec, Canada
- Conference of Research Workers in Animal Diseases (CRWAD): December 2-4, 2018, Chicago, Illinois.
- Arkansas Nutrition Conference: September 11-13, 2018, Rogers, Arkansas
- International Poultry Scientific Forum (IPSF) at International Production and Processing Expo (IPPE): January 29 - 30, 2018, Atlanta, Georgia
- Symposium on Gut Health in Production of Food Animals: November 14 - 16, 2016, St. Louis, Missouri
- Poultry Science Association (PSA) annual meeting: July 11 - 14, 2016, New Orleans, Louisiana
- Asian conference of Veterinary students: August 22-27, 2011, Tokyo, Japan. This event was organized by International Veterinary Students Association, Japan chapter; gave a talk on "Current Status of Veterinary Education in Nepal"

OTHER TRAININGS

- Broiler Farmers' Training- Training of Trainers (TOT): September 25-27, 2011, conducted by Practical Action Consulting, Nepal
- TOT on Commercial Broiler Production and Management: May 24-27, 2011, conducted by International Finance Corporation (IFC), Nepal
- Commercial Poultry Production: July 25-29, 2010, conducted by Nepal Veterinary Association Chitwan Chapter, Nepal
- Statistical Analysis: August 11-13, 2009, conducted by Agriculture Students' Liaison Forum, Nepal
- Participatory Research Methods (RRA/PRA): July 24-31, 2009, conducted by Farmers' Institute for Participatory Research and Development, Nepal
- Project Concept Notes and Proposal Writing: July 10-11, 2009, conducted by Research and Extension Committee, NVSA, Nepal

PUBLICATIONS

Published Papers (10)
- **Adhikari**, **B**., Kim, S.W., & Kwon, Y.M. (2019). Characterization of microbiota associated with digesta and mucosa in different regions of gastrointestinal tract of nursery Pigs. *International Journal of Molecular Sciences* 20:1630. doi:10.3390/ijms20071630
- Hernandez-Patlan, D., Solis-Cruz, B., Pontin, K. P., Hernandez, X., Merino-Guzman, R., **Adhikari**, **B**., et al. (2019). Impact of a *Bacillus* direct-fed microbial on growth performance, intestinal barrier integrity, necrotic

enteritis lesions and ileal microbiota in broiler chickens using a laboratory challenge model. *Frontiers in Veterinary Science* 6:108. doi: 10.3389/fvets.2019.00108

- Hernandez-Patlán, D., Solis-Cruz, B., **Adhikari, B**., Pontin, K.P., Latorre, J.D., Baxter, M.F.A., et al. (2018). Evaluation of the antimicrobial and intestinal integrity properties of boric acid in broiler chickens infected with *Salmonella* Enteritidis: Proof of concept. *Research in Veterinary Science*. https://doi.org/10.1016/j.rvsc.2018.12.004
- **Adhikari**, **B**., Kwon, Y.M., Hargis, B.M., & Tellez G. (2018). Prokaryotes rule the world. In A. Evrensel, *Gut Microbiota - Brain Axis*. London, United Kingdom: IntechOpen. doi: 10.5772/intechopen.77953
- Latorre, J.D., **Adhikari**, **B**., Park, S.H., Teague, K.D., Graham, L.E., Mahaffey, B.D., et al. (2018). Evaluation of the epithelial barrier function and ileal microbiome in an established necrotic enteritis challenge model in broiler chickens. *Frontiers in Veterinary Science* 5:199. doi: 10.3389/fvets.2018.00199
- **Adhikari**, **B**., Kwon, Y.M., Hargis, B.M., & Tellez G. (2018). How trillions of microbes residing on gastrointestinal tract maintain homeostasis with host cells? *Food & Nutrition Journal* FDNJ-170. doi: 10.29011/2575-7091. 100070
- Kim, J.Y., Kwon, Y.M., Kim, I.S., Kim, J.A., Yu, D.Y., **Adhikari**, **B**., et al. (2018). Effects of the Brown Seaweed *Laminaria japonica* Supplementation on Serum Concentrations of IgG, Triglycerides, and Cholesterol, and Intestinal Microbiota Composition in Rats. *Frontiers in Nutrition* 5:23. doi: 10.3389/fnut.2018.00023
- **Adhikari**, **B**., Khanal, P., & Nielsen, M.O. (2018). Impacts of pre- and postnatal nutrition on glucagon regulation and hepatic signalling in sheep. *Journal of Endocrinology* 238(1), 1-12. doi: 10.1530/JOE-17-0705
- **Adhikari**, **B**., & Kwon, Y.M. (2017). Characterization of the culturable subpopulations of Lactobacillus in the chicken intestinal tract as a resource for probiotic development. *Frontiers in Microbiology* 8:1389. doi: 10.3389/fmicb.2017.01389
- **Adhikari**, **B**., (2012). Prevalence of *Salmonella* Isolated from Retail goat meatshop. *LAMBERT Academic Publishing*, Germany. ISBN: 365921731X

Under Review (6)

- **Adhikari**, **B**., & Kwon, Y.M. Cell density alters microbial community structure in culture-enriched microbiome profiling. *Scientific Reports*.
- **Adhikari**, **B**., Hernandez-Patlán, D., Solis-Cruz, B., Kwon, Y.M., Arreguin, M.A., Latorre, J.D., et al. Evaluation of the antimicrobial and anti-inflammatory properties of *Bacillus*-DFM (Norum^{TM}) in broiler chickens infected with *Salmonella* Enteritidis. *Frontiers in Veterinary Science*.
- Kim, I.S., Lee, S.H., Kwon, Y.M., **Adhikari, B**., Kim, J.A., Yu, D.Y., et al. Oral administration of β-glucans and *Lactobacillus plantarum* LM1004 alleviates the atopic dermatitis-like symptoms. *Journal of Medicinal Food*.
- An, S.J., Kim, J.Y., **Adhikari**, **B**., Yu, D.Y., Kim, I.S., Hong, Y.H., Kwon, Y.M., et al. Modulation of Intestinal Microbiota by Supplementation of Fermented Kimchi in Rats. *Journal of Functional Foods*.
- Arreguin, M.A., Graham, B.D., **Adhikari**, **B**., Agnello, M., Selby, C.M., Hernandez-Velasco, X., et al. (2019). *In ovo* administration of a lactic acid base probiotic drives a change to a protective microbiota composition against a virulent *E. coli* horizontal infection in the hatching cabinet in broiler chickens. *Scientific Reports*.
- Arreguin, M.A., Graham, B.D., **Adhikari**, **B**., Agnello, M., Selby, C.M., Hernandez-Velasco, X., et al. (2019). Evaluation of *in ovo Bacillus spp.* based probiotic administration on horizontal transmission of virulent *E. coli* in neonatal broiler chickens. *Poultry Science*.

In Preparation (8)

- **Adhikari**, **B**., Jun, S.R., Kwon, Y.M., Kiess, A.S., & Adhikari, P. Effects of housing types on egg production and cecal microbiota of two different strains of laying hens during the late production stage. In Preparation to submit to *Microbiome*.
- **Adhikari**, **B**., Samarth, D., Chai, J., & Kwon, Y.M. Exploring spore-former subpopulation in chicken gut microbiota. In Preparation to submit to *Applied and Environmental Microbiology*.
- **Adhikari**, **B**., Liu, S.Y., Rochell, S.J., Kidd, M.T., & Kwon, Y.M. Changes in the ileal microbiota of broiler chickens in response to different levels of dietary lysine. In Preparation to submit to *Poultry Science*.

- **Adhikari**, **B**., Jourdan, A., Rochell, S., & Kwon, Y.M. Effects of alternatives to in-feed antibiotics on intestinal microbiome in broiler chickens. In Preparation to submit *Poultry Science*.
- **Adhikari**, **B**., Jourdan, A., Rochell, S., & Kwon, Y.M. Evaluation of *Leifsonia xyli* as a live spike-in control and its use for quantitative profiling of jejunal microbiotas in broiler chickens. In Preparation to submit *Poultry Science*.
- **Adhikari**, **B**., & Kwon, Y.M. Characterization of microbiome and quantification of *Clostridium septicum* from litter of different commercial turkey farms with/without supplementation of antibiotics alternatives and varying degree of cellulitis. In Preparation.
- **Adhikari**, **B**., Tellez-Isaias, G., Teague, K.D., & Kwon, Y.M. Are chicken embryos sterile? An investigation through both culture dependent and independent methods. In Preparation.
- **Adhikari, B**., & Kwon, Y.M. Growing needs, challenges and opportunities for strain-level microbiome analysis in understanding gut microbiomes of food production animals – a review. In Preparation.

Abstracts (10)

- **Adhikari**, **B**., Jun, S.R., Kwon, Y.M., Kiess, A., & Adhikari, P. (2019, July). Effects of housing types on egg production and cecal microbiota of two different strains of laying hens during the late production stage. Oral presentation at *Poultry Science Association* (PSA) Annual Meeting, Montreal, Quebec, Canada.
- **Adhikari**, **B**., Samarth, D., Chai, J., & Kwon, Y.M. (2019, July). Exploring spore-former subpopulation in chicken gut microbiota. Poster session presented at *Poultry Science Association* (PSA) Annual Meeting, Montreal, Quebec, Canada.
- **Adhikari**, **B**., Hernandez-Patlán, D., Solis-Cruz, B., Kwon, Y.M., Arreguin-Nava, M.A., Latorre, J.D., et al. (2019, July). Evaluation of the antimicrobial and anti-inflammatory properties of Bacillus-DFM (NorumTM) in broiler chickens infected with Salmonella Enteritidis. Poster session presented at *Poultry Science Association* (PSA) Annual Meeting, Montreal, Quebec, Canada.
- Hernandez-Patlán, D., Arreguin-Nava, M.A*., Solis-Cruz, B., **Adhikari**, **B**., Latorre, J., Hernández-Velasco, X., et al. (2019, July). Therapeutic effect of boric acid against Salmonella Enteritidis infection, intestinal permeability, total IgA concentration, and cecal microbiome composition in broilers chickens. Poster session presented at *Poultry Science Association* (PSA) Annual Meeting, Montreal, Quebec, Canada.
- **Adhikari, B**., Hernandez-Patlán, D., Solis-Cruz, B., Latorre, J.D., Arreguin-Nava, M.A., Hargis, B.M., et al. (2018, December). Evaluation of *Bacillus* Direct-fed microbial for control of necrotic enteritis in chickens. Oral Presentation at *Conference of Research Workers in Animal Diseases* (CRWAD), Chicago, Illinois.
- **Adhikari, B**., Hernandez-Patlán, D., Solis-Cruz, B., Latorre, J.D., Arreguin-Nava, M.A., Hargis, B.M., et al. (2018, September). Evaluation of in-feed inclusion of a *Bacillus* Direct-fed microbial on growth performance, lesion score, gut permeability, and ileal microbiome in chicken model of necrotic enteritis. Poster session presented at *Arkansas Nutrition Conference*, Rogers, Arkansas.
- Kwon, Y.M*., & **Adhikari, B**. (2018, July). Future directions for exploring poultry gut microbiomes: challenges and opportunities. Oral Presentation at *Poultry Science Association* (PSA) Annual Meeting, San Antonio, Texas.
- **Adhikari**, **B**., Tellez-Isaias, G., Teague, K.D., & Kwon, Y.M. (2018, January). Are Chicken embryos sterile? An investigation through both culture dependent and independent methods. Poster session presented at International Poultry Scientific Forum (IPSF) at *International Production and Processing Expo* (IPPE), Atlanta, Georgia.
- **Adhikari**, **B**., & Kwon, Y.M. (2016, November). Analysis of Lactobacillus species in the ceca of breeder hens. Poster session presented at *Symposium on Gut Health in Production of Food Animals*, St. Louis, Missouri.
- **Adhikari**, **B**., Mandal, R.K., & Kwon, Y.M. (2016, July). Characterization of lactic acid bacteria population associated with different regions in gastrointestinal tract of chicken. Poster session presented at *Poultry Science Association* (PSA) Annual Meeting, New Orleans, Louisiana.