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An Assessment of Broiler Litter Bacterial Diversity using Next Generation DNA Sequencing

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To the Graduate Council:

I am submitting herewith a thesis written by Jack Liu Davitt entitled "An Assessment of Broiler Litter Bacterial Diversity using Next Generation DNA Sequencing." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Environmental and Soil Sciences.

Shawn Hawkins, Major Professor

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An Assessment of Broiler Litter Bacterial Diversity using Next Generation DNA Sequencing

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Jack Liu Davitt
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DEDICATION

This page is dedicated to

My beloved parents,

Family,

Friends,

Shannon,

And

Lily.

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In the beginning, chickens were created. Now the broilers and layers were defined, broilers were fried and made to fillets, and layers were great origins for omelets. And there were problems associated with the deliciousness. There were wastes, and no one knew the complete bacteria within. With the help from my committee members Drs. Hawkins, Layton, Radosevich, Saxton, Smith, and Walker, there was the solution: sequencing! For that I'd like to thank all of you! I also like to thank the farms that allowed the sample collection, as well as the farming community; you are the backbone of America (Thank God for America)!

Additionally, I'd like to thank Stephen Techtmann, Dan Williams, and Benjamin Huangfu for helping with sample preparation and analysis. Special thanks to Dr. Crook for the support and encouragement along the way! My most sincere thank you to my beloved parents, I love you! Additionally, my friends and other family members, thank you all!

Finally, the words that kept me going: "I can do all things through Christ who strengthens me" Philippians 4:13.

ABSTRACT

Broiler chicken farms produce large amounts of litter, comprised mainly of used bedding and bird fecal waste, which is land applied. The regional concentration of broiler farms and land application of litter in the United States is a water quality concern. Geographically concentrated production also increases biosecurity concerns with potential pathogens and high nutrient contents as the litter is moved and land applied. Previous studies have attempted to characterize the bacterial population within litter because of these concerns. To date, this effort has not provided comprehensive information on the bacterial community structure within broiler litter. This lack of knowledge hinders the development of better water quality and biosecurity management efforts. This study characterized the bacterial community structure of broiler litter using Illumina MiSeq sequencing technology, specifically examining whether the litter bacterial community structure changed within in-house litter during a grow-out at one farm (fresh grouping), and whether the long-term stored (LTS) litter bacterial community structure varied in different geographic locations. Actinobacteria was the dominant in the fresh grouping (65.5% ± 10.2%) while only present at 22.2% ± 8% in the LTS. In contrast, the LTS samples were dominated by Firmicutes (77% ± 8%) which was only present at 31.4% ± 10% in the fresh grouping. Within the phylum Firmicutes, *Bacillaceae* (68% ± 11%) family was dominant in LTS across all of the geographic regions sampled, despite varying moisture content, integrators, and bedding/litter management practices. From ANOSIM, there were statistical differences among comparisons between integrators, and producers while no statistical difference was found among bedding materials. For the in-house comparisons, the bacterial community structure was uniform and dominated by families *Brevibacteriaceae* (19% ± 5%), *Dermabacteraceae* (15% ± 4%), *Staphylococcaceae* (14% ± 7%), and *Corynebacteriaceae* (13% ± 7%) despite the different

times of collection (days 1, 15, and 43). ANOSIM revealed no statistical differences among time of collections (p value > 0.05). *Staphylococcus* was the only family consistently present in both LTS and fresh samples that contains pathogens of biosecurity concerns. Two dominant bacterial families could be used for the development of broiler litter fecal source tracking: *Bacillaceae* and *Nocardiopsaceae* were identified.

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I. INTRODUCTION

Broiler chicken production is one of the most important components of agricultural production in the United States with the top ten states produced over 6 billion birds in 2012 [1]. The southern states are particularly active in broilers production; with Georgia, Arkansas, and Alabama among the top states in terms of bird numbers [1]. The large scale of broiler production leads to a great volume of waste called litter, which is a mixture of manure, wasted feed, feathers, and used bedding. Approximately 228 g of dry litter is produced per kg of live broiler weight (g/kg) [2]. This translates to a cumulative total of 3 billion kg of litter per grow-out from these states. Litter is almost exclusively land-applied as fertilizer due to its high nutrient content [3, 4].

The land application of litter can increase nutrient and fecal bacteria concentrations in rainfall runoff, thereby deteriorating surface water quality [5, 6]; this process also increases biosecurity concerns as the litter cleanout equipment is often used on multiple farms which can spread bird pathogens within the litter bacterial community [7, 8]. Polluted surface and ground waters result in over 175 million cases of infectious diseases in human each year [9]. In addition, pathogenic strains of *Salmonella enterica* can persist in litter amended soil for over 200 days [10]. One way to manage fecal pollution in surface waters is to identify the source. This is accomplished with microbial source tracking (MST), a group of methodologies that identifies and quantifies the sources of fecal contamination in surface waters [11]. These methodologies typically target a set of bacterial genetic sequences unique to a particular fecal waste origin. Different bacteria species have been proposed and shown to be effective in detecting general fecal wastes [12-14], as well as fecal waste from humans [12, 15-18], cattle [12, 15, 16, 19, 20], and swine [14, 21-25]. Previously *Bacteroidetes*, *Cl. Perfringens* and *D. hafniense* were

identified in poultry fecal waste but not in other fecal wastes [26]. However these species were only with detected in 40% of poultry wastes so they would not be completely effective in detecting surface waters polluted with poultry waste. More recently, *Brevibacterium avium* has been identified as a poultry specific source tracker with high specificity [27]. It was also suggested that the use of multiple species assays may improve poultry fecal waste MST [26, 28].

It was previously determined that the litter microbial count can be as high as 10^9 to 10^{11} cells per gram of litter [7, 29-31]. Diverse microorganisms such as bacteria and fungi have been found in the poultry litter [32, 33]. Moreover, culturing based studies targeting pathogenic organisms have detected *Listeria monocytogens, Salmonella* spp., *Campylobactor* spp., *Clostridia* spp., *Bordetella* spp., *Staphylococcus*, and *Escherichia coli* [7, 8] in litter samples. In a culturing study, *Staphylococcus* spp. was most prevalent in fresh broiler litter [7]. Additionally, litter from different states have significant variations in *Staphylococcus*, but not in *Escherichia coli* [7]. Interestingly, in another culture based study *Salmonella, E coli* 0157 and *Campylobacter* were all absent from broiler litter [34]. One of the biggest challenges associated with these studies is the dependency on culturing which identifies only a very small percent of the actual bacterial diversity present in environment samples [35]. Studies comparing the detection of *Campylobacter* in broiler litter using culturing versus quantitative PCR targeting of genetic markers clearly indicated that the genetic detection technique was more effective and accurate than culture based method [36, 37].

Culture independent sequencing studies targeting the 16S gene have demonstrated a complex and more comprehensive view of broiler litter bacterial community structure[29, 38, 39]. The 16S rRNA gene reveals more species within the environment than other methods (culturing), and low abundance species are best identified through 16S rRNA gene sequencing

[38]. Early sequencing studies have found *Actinomycetes* throughout a broiler house in Mississippi, with the presence of *Brachybacterium* sp., *Corynebacterium* sp., *Arthrobacter* sp., and *Brevibacterium* sp. [29]. In another study, DNA sequenced from 16S gene clone library revealed a total of 12 families that included *Lactobacillaceae, Aerococcaceae*, *Bacillus, Staphylococcus*, *Enterococcaceae*, and *Corynebacteriaceae* [39]. In addition, this study found uncharacterized strains, which suggests the high numbers of bacterial unknowns.

Next-generation (Next-Gen) DNA sequencing techniques targeting the 16S gene have been used in many different fields to provide fast and accurate results regarding environmental bacterial community structures. Studies that target the bacteria 16S gene within diverse environments are referred to as microbiomics. There are several different Next-Gen sequencing methods: 454 (pyrosequecing), Illumina (sequencing by synthesis), PacificBio (single-molecule real-time sequencing), Ion Torrent (Ion seminconductor), and SOLiD (sequencing by ligation) [40-46]. Even though the sequencing methodology varies across these methods, the general principals remain constant by targeting one or more of the nine hypervariable regions of the 16S gene with universal primers.

Through microbiomic related studies, the understanding of complex environments such as soil, the human gut, and cow digestive system has dramatically improved [47-51]. Despite the wide range of applications of this technique, there have only been a few poultry related studies using Next-Gen sequencing techniques. One study examined the broiler fecal bacteria community and found that *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* were the dominate phyla [52]. In another study, the soils under broiler houses and litter storage areas were examined and *Proteobacteria* were found to be the major phyla with no difference in bacterial species richness and diversity attributable to the poultry litter [53].

To date, there has only been one microbiomic study describing the bacterial community structure of broiler litter [54]. This study was conducted with a focus on gangrenous dermatitis related pathogens in poultry litter and used the 454 pyrosequencing technique targeting the V2 region of the 16S gene. The taxonomy of abundant 16S rRNA gene clusters found no major differences in the bacterial community structure between production houses with and without a history of gangrenous dermatitis; the top 5 operational taxonomic unit (OTU) clusters belonged to phyla Firmicutes and Actinobacteria [54].

None of the studies to date, either culturing or sequencing based, have studied the bacterial community structure of broiler litter throughout a production cycle or examined the changes to the community structure that may occur during litter storage. In order to better manage the movement and land application of broiler litter, a thorough understanding of the litter bacterial community structure appears to be necessary and helpful. The understanding of the in house litter samples could perhaps aid in understanding of bird intestinal bacterial structure [55]. The intestinal bacteria have a close association to bird health, bird performance, and well-being [56] [57-59]. Knowledge regarding stored litter bacterial community structure would be critical and important for better management practices, as well as developing potential fecal source trackers. The objectives for this study are: describe taxonomic diversity of broiler litter at family level, determine whether the broiler litter bacterial community structural changes over time during production, and assessing changes in broiler litter bacterial community from different producers. Because different stages of production and different producers have different management practices, and environmental factors we hypothesized that: 1. In-house litter bacterial community structure during a production cycle will change; 2. Stored litter bacterial community structures from different producers will differ. This study utilized an Illumina MiSeq

sequencer and targeted the V4 region of the bacterial 16S gene. The hypotheses were tested by examining the litter bacterial community structure within samples collected at days 1, 15, and 43 from a single grow-out and from litter stored across a wide geographic region spanning east Tennessee, north central Alabama, and west Tennessee.

II. MATERIALS AND METHODS

In-House Litter Samples

Litter samples were collected from a commercial farm containing 8 broiler production houses in Mohawk, TN (
Figure 1). Six of the houses were (≈ 25 years old) 40 x 400 feet (12 x 122 meters) curtained sided, cross ventilated houses with an open truss design. Two of the houses were (≈ 8 years old) 42 x 500 feet, tunnel ventilated houses with a drop ceiling design. All of the houses were heated with propane brooders. Chicks were placed and brooding was conducted in one end of the houses (the brood end) until approximately flock day 14, at which time the birds were allowed access to the full length of the house. Broilers in the houses were produced under contract with Koch Foods, Inc. located in Morristown and Chattanooga, TN. Summertime litter samples were collected between grow-outs from the center (CL) near the feed (FL) and water (WL) lines from all 8 houses during summer of 2012. All of these samples were pooled and described as lane series. Wintertime litter samples were collected from the brood end only of the new style houses between February and March, 2013 at days 1, 15 and 43 during a single wintertime grow-out (44 day growth period). These samples were denoted with the house-flock day as: H7D1, H7D15, H7D43, H8D1, H8D15, and H8D43. On day 1, the birds had just been delivered to the farm. On day 15 the curtains that divided the houses in half between the brood and non-brood ends were raised. Day 43 was the day before the birds left the farm for processing. The wintertime timed-series litter samples were collected from the brood end of the houses which was divided into 12 sections. Approximately thirty replicate litter sub-samples were collected within the 12 sections between the water and feed lines to a depth of 10 cm. Each of the section subsamples were placed into a bag and thoroughly mixed prior to collecting one composite section sample; the section composite samples were then pooled into final bag

from which one composite sample was taken for Next-Gen sequencing analysis. The remaining composite section samples were analyzed for moisture contents within 24 hours. The composite sample was stored at -20°C prior to DNA extraction.

Figure 1. Orthogonal image of the Mohawk, Tennessee farm from which in-house litter samples were collected.

Stored-Litter Samples

Stored litter samples were collected in December 2013 from 5 different farms in Tennessee and Alabama (Figure 2). At each farm, sub-samples were collected from 8 random locations within the stored litter piles using a shovel a depth at least 6" (15 cm) below the pile surface. The sub-samples were then mixed together and a single homogenous composite litter sample was collected. The composite stored litter samples were kept on ice during the 3 day travel period and frozen at -20°C prior to DNA extraction.

Litter was collected from a broad geographic region to evaluate whether the bacterial community structure of stored litter varied across different integrators, bedding materials, and litter management styles. All of the stored samples except one had been in storage between six weeks to three months. Collectively, these samples that were stored for an extended period of time were named long-term stored samples (LTS). One of the stored litter samples was collected from the same farm that we collected the in-house samples (JMF). This litter sampled provided a bridge for comparison between the in-house samples and stored litter samples. Samples collected from Wartrace and Shelbyville, TN were Tyson contracted producers. Wartrace samples consisted litter motility compost (MWT) and compost litter from houses using light saw dust as bedding material (CWT). Shelbyville samples consisted of old, stored litter (DOT) and recently cleaned (FCT). The FCT sample was collected right after litter was pulled out of the house, thus was classified with the in-house samples as fresh. The bedding material for Shelbyville samples was wood shaving. The sample (CLUC) collected from Union City, TN was a Tyson producer and the bedding material was rice hull. Snead, AL samples were collected from a Pilgrim Pride, Inc. producer who used wood shavings as the bedding material. One of the

samples was caked-litter samples (CASA), while the other one was a composed litter sample (COSA). Specific details regarding the samples can be found on Table 1.

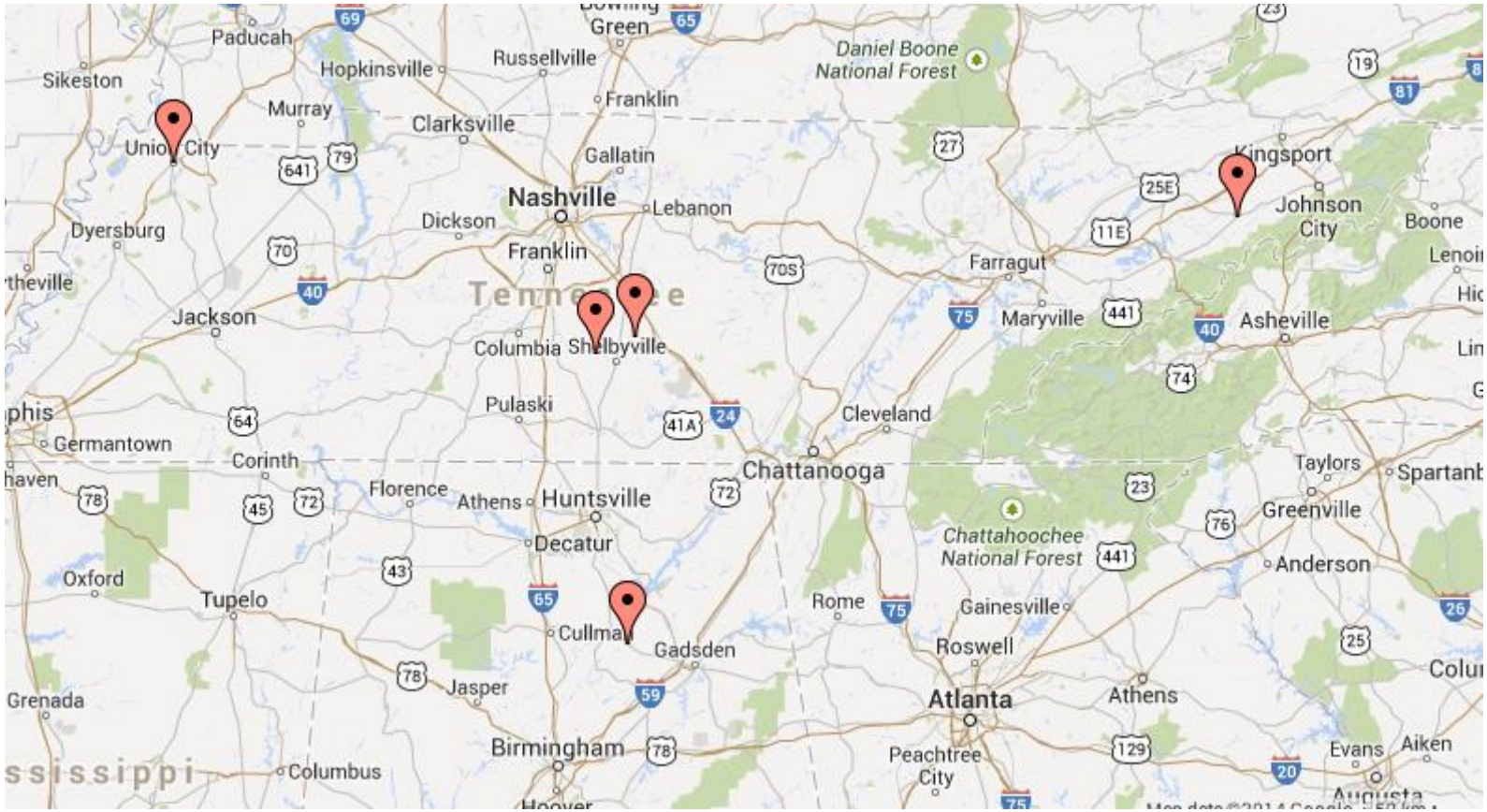

Figure 2. Geographic locations where stored litter samples were collected: Mohawk, TN, Wartrace, TN, Shelbyville, TN, Union City, TN, and Snead, AL.

Table 1. Broiler litter sample collection detail.

Sample_ID	Location	Description	Bedding Material	Integrator	Fresh/LTS
H7D1	Greenville, TN	Time-series	Wood Shaving	Koch Foods	Fresh
H7D15	Greenville, TN	Time-series	Wood Shaving	Koch Foods	Fresh
H7D43	Greenville, TN	Time-series	Wood Shaving	Koch Foods	Fresh
H8D1	Greenville, TN	Time-series	Wood Shaving	Koch Foods	Fresh
H8D15	Greenville, TN	Time-series	Wood Shaving	Koch Foods	Fresh
H8D43	Greenville, TN	Time-series	Wood Shaving	Koch Foods	Fresh
CL	Greenville, TN	Center Lane	Wood Shaving	Koch Foods	Fresh
FL	Greenville, TN	Feed Lane	Wood Shaving	Koch Foods	Fresh
WL	Greenville, TN	Water Lane	Wood Shaving	Koch Foods	Fresh
JMF	Greenville, TN	Stored	Wood Shaving	Koch Foods	LTS
CASA	Snead, AL	Stored	Sawdust	Pilgrim Pride	LTS
COSA	Snead, AL	Stored	Sawdust	Pilgrim Pride	LTS
CWT	Wartrace, TN	Stored	Wood Shaving	Tyson	LTS
MWT	Wartrace, TN	Stored w/ Mortality	Wood Shaving	Tyson	LTS
FCT	Shelbyville, TN	Recently Stored	Wood Shaving	Tyson	Fresh
DOT	Shelbyville, TN	Stored	Wood Shaving	Tyson	LTS
CLUC	Union City, TN	Stored	Rice Hull	Tyson	LTS

Moisture Content

Samples were weighed and dried in oven at approximately 107°C for 48 h. Dry weights of samples were determined and subsequently used for determining the moisture content using Equation 1.

$$\left[1-\left(\frac{net\_dry\_wt}{net\_wet\_wt}\right)\right]*100\%$$

Equation 1: Equation for computing moisture content

DNA Extraction

DNA was extracted in triplicate from the timed in-house and stored, while the in-house lane samples was extracted independently for houses 1~8 and then pooled to generate a composite of the lanes. DNA from all of litter samples were extracted using the Fast DNA Spin Kit for Soil (MP Biomedicals, LLC, Solon, OH) following manufacturer's instructions. Briefly, one gram of the litter sample was mixed with 5 ml of de-ionized water (5 PRIME, Gaitherburg, MD) and vortexed for 15 seconds; 0.25 ml of the mixed slurry was transferred into the kit Lysing Matrix E tube. Sodium Phosphate Buffer (978 µl) and MT Buffer (122 µl) was then added to the Matrix E tube and the mixture was homogenized in a Fast Prep® Instrument (model and manufacturer information) for 40 seconds at speed setting 6.0. The homogenized sample was then centrifuged at 14,000x*g* for 10 min and the supernatant was transferred to a clean 2ml microcentrifuge tube; Precipitation Solution (PPS-250 µl) was added to the tube which was then mixed by hand 10 times. The tubes were centrifuged again at14,000×*g* for 5 minutes and the supernatant was transferred to a clean 15 ml centrifuge tube and combined with 1 ml of re-suspended Binding Matrix Solution. The tubes were inverted by hand for 2 minutes and then allowed to settled for 3 minutes; 600 µl of the supernatant was transferred (without disturbing the

settled debris) and filtered through a SPIN™ Filter. SEWS-M (500-µl) was added to SPIN™ filter and the pellets in each tube were separated with the force from pippetting. The tubes were emptied, dried and replaced with new centrifuges tubes. The final DNA products were eluted using 100 µl of DES water (DNase-Pyrogen Free H_2O) and stored at -20°C prior to purification.

DNA Purification

DNA purification was conducted with the Genomic DNA Clean & Concentrator kit (Zymo Research, Irvine, CA) following manufacturer's protocol. The DNA extracts were combined 2:1 v/v with DNA binding Buffer and filtered through a Zymo-Spin™ Column. The columns were then washed twice with 200 µl DNA Wash Buffer. Finally, the DNA was eluted using 50 µl DNA elution buffer. The purified DNA concentration was measured with a NANO Drop™ 2000 (ThermoScientific, Wilmington, DE) for concentration and purity. If the NANO DROP™ 260/280 readings were less than 1.0 the purified DNA was processed with the OneStep™ PCR Inhibitor Removal Kit (Zymo Research) following manufacturer's instructions. The product was again measured with NANO Drop™ 2000 for quality assurance.

Barcode PCR

Purified litter sample DNA was PCR amplified by targeting the V4 region of the bacterial 16S gene as described by Caporaso et al. [60]. PCR was performed with the 515f forward primer with 5' Illumina adapter (AATGATACGGCGACCACCGAGATCTACAC), forward primer pad (TATGGTAATT), forward primer linker (GT) and forward primer (GTGCCAGCMGCCGCGGTAA), and reverse primer with the reverse complement of the 3' Illumina adapter (CAAGCAGAAGACGGCATACGAGAT), the barcode sequence (Table 2), reverse primer pad (AGTCAGTCAG), reverse primer linker (CC), and reverse primer (GGACTACHVGGGTWTCTAAT). The resulting PCR amplicons from each litter DNA sample

included different barcoded products. PCR reactions (50 µl) were performed using 2 µl of sample DNA, 1 µl of barcode primer and 47 µl of a mixture of 40 µl de-ionized water (5 PRIME, Gaitherburg, MD), 5 µl Invitrogen Pfx50™ buffer (Invitrogen, Carlstead, CA), 1 µl CAP 515 F, 1 µl dNTP (Invitrogen, Carlstead, CA), 1 µl Invitrogen Pfx50™ Polymerase (Invitrogen, Carlstead, CA) and 0.5 DMSO (Sigma, St. Louis, MO). A negative control sample was included that used 2 µl of deionized water (5 PRIME, Gaitherburg, MD) instead of the litter sample DNA. Thermocycling condition for the PCR included 94 °C for 3 min, followed 35 cycles of 94 °C for 45 s, 55 °C for 1 min, 72 °C for 90 seconds. The cycle was followed by 72°C for 10 min. The PCR reaction was performed on a GenePro thermocycler (Bulldog Bio, Portsmouth, NH).

Table 2. Sample IDs with barcode information.

#Sample ID	Barcode Sequence	#Sample ID	Barcode Sequence
H7D1_1	GAACACTTTGGA	**CASA1**	ATGTGCACGACT
H7D1_2	CATTCGTGGCGT	**CASA2**	CTGCTAACGCAA
H7D1_3	ATGATGAGCCTC	**CASA3**	ACCACATACATC
H7D15_1	TTGGGTACACGT	**COSA1**	TAGGATTGCTCG
H7D15_2	GGCCAGTTCCTA	**COSA3**	ATTGGGCTAGGC
H7D15_3	TGTCGCAAATAG	**COST2**	TCACGGGAGTTG
H7D43_1	TAATACGGATCG	**CWT1**	ATCCCGAATTTG
H7D43_2	CTATCTCCTGTC	**CWT2**	TCGAGGACTGCA
H7D43_3	TATACCGCTGCG	**CWT3**	GCTTCGGTAGAT
H8D1_1	GAGCCATCTGTA	**MWT1**	GTTGGTCAATCT
H8D1_2	TACTACGTGGCC	**MWT2**	CGGAGCTATGGT
H8D1_3	CGGTCAATTGAC	**MWT3**	TACAGATGGCTC
H8D15_1	AAGGCGCTCCTT	**FCT1**	GACTTTCCCTCG
H8D15_2	GATGTTCGCTAG	**FCT2**	TCTAGCGTAGTG
H8D15_3	GTGGAGTCTCAT	**FCT3**	TACTTCGCTCGC
H8D43_1	TCGGAATTAGAC	**DOT1**	ACGCGCAGATAC
H8D43_2	ACTCACAGGAAT	**DOT2**	TTAGGGCTCGTA
H8D43_3	AGTTGAGGCATT	**DOT3**	AATGTCCGTGAC
CL	GGAGACAAGGGA	**CLUC1**	TAGCTCGTAACT
FL	AATCAGTCTCGT	**CLUC2**	AAGAGATGTCGA
WL	AATCCGTACAGC	**CLUC3**	TGACCTCCAAGA
JMF1	ACGTGTACCCAA		
JMF2	AAGGAGCGCCTT		
JMF3	CGATCCGTATTA		

High Sensitivity CHIP Analysis

High sensitivity CHIP analysis was conducted after the barcode PCR reaction to ensure the quality was adequate for Next-Gen sequencing. CHIP analysis was conducted with the High Sensitive DNA kit following the manufactory's protocol on a model 2100 Bioanalyzer (Agilent, Santa Clara, CA). Amplicons from the litter sample DNA PCR reactions were confirmed to have the correct size (≈ 400 bp) and to be absent of primer dimmers. Furthermore, relative concentrations of the individual samples can be estimated based on the peak height at the appropriate size, and pooled to equal amounts.

Beads Clean up

The pooled products from the CHIP analysis were purified with either Agencour AMPure XP magnetic beads (Beckman Coulter, Inc., Indianapolis, IN) (in house timed litter samples) or SPRIselect (Beckman Coulter, Inc., Indianapolis, IN) (stored litter samples) following the manufacturer's protocol (Next-flex™ 16S V4 Amplicon Seq-kit manual). The products from clean up were analyzed again with High Sensitivity CHIP for quality assurance and verification of the removal of primer dimers.

Library Quantification

The Illumina Library Quantification kit (KAPA Biosystems, Boston, MA) was used to determine the concentration of pooled amplicons (e.g. library) before sequencing. Quantitative PCR was performed using the KAPA SYBR® FAST qPCR Master Mix (2X) and 6 DNA standards (20pM, 2pM, 0.2pM, 0.02pM, 0.002pM, and 0.0002pM). The concentration for each sample was determined based on amplicon adaptors. Accurate quantification of the number of amplifiable molecules in a library step is necessary to generate optimal sequencing reads using Illumina MiSeq sequencer. Each sample amplicon was diluted in TRIS (Invitrogen, Carlsbad,

CA) + TWEEN (Fisher Scientific, Hampton, NH) solution (10 mM TRIS with 0.05% TWEEN) to generate 1:1000, 1:5000, and 1: 10000 dilutions of the sample. Diluted sample amplicons, standards (4µl) and controls (4 µl de-ionized water instead of amplicons) were loaded onto a 96-well PCR plate contain 0.2 µl of master mix and ran in duplicate.

Illumina Sequencing

Sequencing was conducted on the Illumina MiSeq sequencer (Illumina, Inc., San Diego, CA). The amplicon library was diluted to a starting concentration of 10 nM sequenced by a trained professional technician following Illumina protocols.

Sequence Data Analysis

Sequences were assembled and analyzed using the QIIME Version 1.8.0-dev pipeline [60]. Prior to sequence assembly and OTU picking, individual reads were joined via "cat" in the command line. For the in-house timed samples, "FLASH" (Fast Length Adjustment of Short reads, version1.2.7) [61] was used to join the paired-ends from the sequencer. Barcode sequences were parsed and then joined into multiplexed reads for analyses in QIIME. Stored litter raw reads were joined together with provided barcode sequences. The sequences from in-house and stored litter samples were independently de-multiplexed and quality filtered using the default set up in QIIME. The post quality filter files were concatenated and analyzed.

Chimeras were detected and filtered via wrapped "usearch61" functions in QIIME [62] using the UCHIME algorithm, which performed chimera checking by searching for 3-way alignment of two segments of a query sequence that exhibited similarity to the two parent sequences. A cutoff was set (specific the cutoff); sequences than scored higher than the cutoff were considered chimeras and discarded from further analysis.

Operation Taxonomic Unit (OTU, 97% similarity) were picked using "UCLUST" [63] with *open_reference* as the picking process. This is the preferred method for OTU picking; all the reads are compared against a reference sequence collection and un-matched reads are then clustered as de novo. The resulting sequences were aligned using PyNAST [64] and taxonomy was assigned using RDP classifier [65] based on a Greengenes reference database (May 2013 Greengenes release). OTUs that were less than 0.005% were eliminated and remaining OTUs were rarified to 32,000 sequences per sample (the minimum reasonable number of remaining sequences in the samples). This provided an equal depth of sequence analysis to eliminate problems associated with increased richness associated with higher numbers of sequences. The Shannon's diversity index, Phylogenic Diversity (PD) whole tree and observed species were used to assess the sample diversity (richness measures absolute number of different species present and evenness measures number of different species making up the richness). The PD whole tree method evaluates the sample diversity based on phylogenic information. The observed species method provides the number of unique OTUs found in samples. Weighted Unifrac distance [66] was calculated, graphed with Principal Coordinate Analysis (PCoA) and visualized with EMPEROR [67]. Weighted Unifrac distance β diversity between samples was based on the branching of phylogenetic tree. This method considers species abundance of within the sample. The PCoA attempts to present the distances between samples and subsequently clusters similar samples. Analysis of similarities (ANOSIM) was conducted in QIIME with Weighted Unifrac distance matrices to determine the significance of groupings observed in the clustering. P-values were derived with 1,000 permutations. ANOSIM is a multivariate analysis using non-parametric method based on permutation test [68].

The assigned taxonomy was analyzed in STAMP v2.0.2 (Statistical Analysis of Metagenomic Profile) [69]. Coefficients of determination (R^2), which indicated how well data points fit a statistical model, was generated through a 1:1 fitted line. Welch's two-sided t-test was also used to compare different sample groupings and produced p-values for statistical analysis. To correct for the potential for false positives in our tests, the Benjamini-Hochberg FDR was used for multiple test correction [70]. JMP ® Pro 10 (SAS Institute Inc., Cary, NC) was also used in statistical analysis. The top families within each sample were examined. The top families in each sample were defined with ranking the families in a descending order and extracted the top families that cover at least 90% of total sequences.

III. RESULTS AND DISCUSSION

Sequences Recovered

This study employed the Illumina MiSeq high throughput DNA sequencing platform and targeted the V4 region of the 16S gene to assess the bacterial community structure of broiler litter. An average of 295,354 ± 247,435 sequences per sample were recovered from the litter DNA that met quality control standards. The sequences per sample were reduced to 271,620 ± 241,862 after chimera filtering and further reduced to 252,930 ± 231,806 after OTUs were removed that comprised less than 0.005% of the recovered sequences (Table 3). Two samples (H8D1, H8D15) had one extraction each with an insufficient number of sequences (<1,000) to analyze and were removed from downstream analysis. However, since these in-house by time point samples were analyzed in triplicate, duplicate values remained for these samples. One of the triplicates in CLUC was also removed due to contamination.

The remaining sequences were rarefied to 32,000 total sequences. To ensure the rarefied sequence dataset provided complete and thorough coverage of the litter samples, alpha rarefaction curves were produced using the Shannon index, PD whole tree, and observed species methods (Figure 3). All of the samples reached a plateau prior 32,000 sequences (at 5,000), which indicated the potential species within the sequences were adequately represented. Additionally, species richness and evenness was assessed with Shannon's index, PD whole tree, and observed species. These methods took an OTU and phylogenetic approach to assess population diversity. There was no statistical significance between any samples (corrected p values > 0.05).

Table 3. The number of sequences from the sequencer, post chimera/0.0005 filtering.

#Sample ID	# of Raw Sequences	Post Chimera	Post 0.0005 filtering**
H7D1	333,397	306,506	292,553
H7D1	211,778	189,378	176,260
H7D1	199,155	172,804	157,434
H7D15	418,561	394,075	374,177
H7D15	322,300	293,895	278,333
H7D15	138,237	120,918	112,051
H7D43	496,002	469,185	446,975
H7D43	248,426	218,014	203,375
H7D43	548,230	479,904	413,440
H8D1	250,555	227,931	220,224
H8D1	165,795	146,452	136,529
H8D1	28	27	25
H8D15	679,610	640,162	613,033
H8D15	245,102	217,248	203,579
H8D15	9	7	2
H8D43	513,948	477,973	457,030
H8D43	140,304	125,016	120,383
H8D43	278,716	237,875	215,041
CL	1,071,626	1,039,749	1,028,945
FL	1,104,287	1,070,566	1,055,964
WL	878,135	855,237	808,496
JMF1	35,544	34,753	33,652
JMF2	341,093	33,174	321,645
JMF3	354,030	344,093	332,790
CASA1	131,704	130,189	125,529
CASA2	430,222	426,335	412,204
CASA3	144,159	142,710	137,883
COSA1	82,859	81,191	77,602
COSA2	208,071	205,244	197,838
COSA3	119,244	117,093	112,503
CWT1	221,682	212,125	194,035
CWT2	140,782	136,041	129,667
CWT3	244,829	237,682	226,024
MWT1	249,275	237,793	215,511
MWT2	141,332	135,554	125,035
MWT3	204,468	196,956	179,944
FCT1	48,082	42,691	41,514
FCT2	192,906	179,190	170,962
FCT3	204,420	184,877	174,990
DOT1	102,849	99,517	94,799
DOT2	159,926	156,799	151,262
DOT3	140,931	137,388	131,994

Table 3 Continued.

#Sample ID	# of Raw Sequences	Post Chimera	Post 0.0005 filtering**
CLUC1	176,630	170,615	162,986
CLUC2	85,689	83,161	80,124
CLUC3	260,299	255,481	237,545
Average*	295,355	271,620	265,341
SD*	247,435	241,862	233,199
* The analysis does not include the grey colored outliers.H8D1, H8D15 did not amplify while CLUC3 was contaminated			
** Used for diversity analysis.			

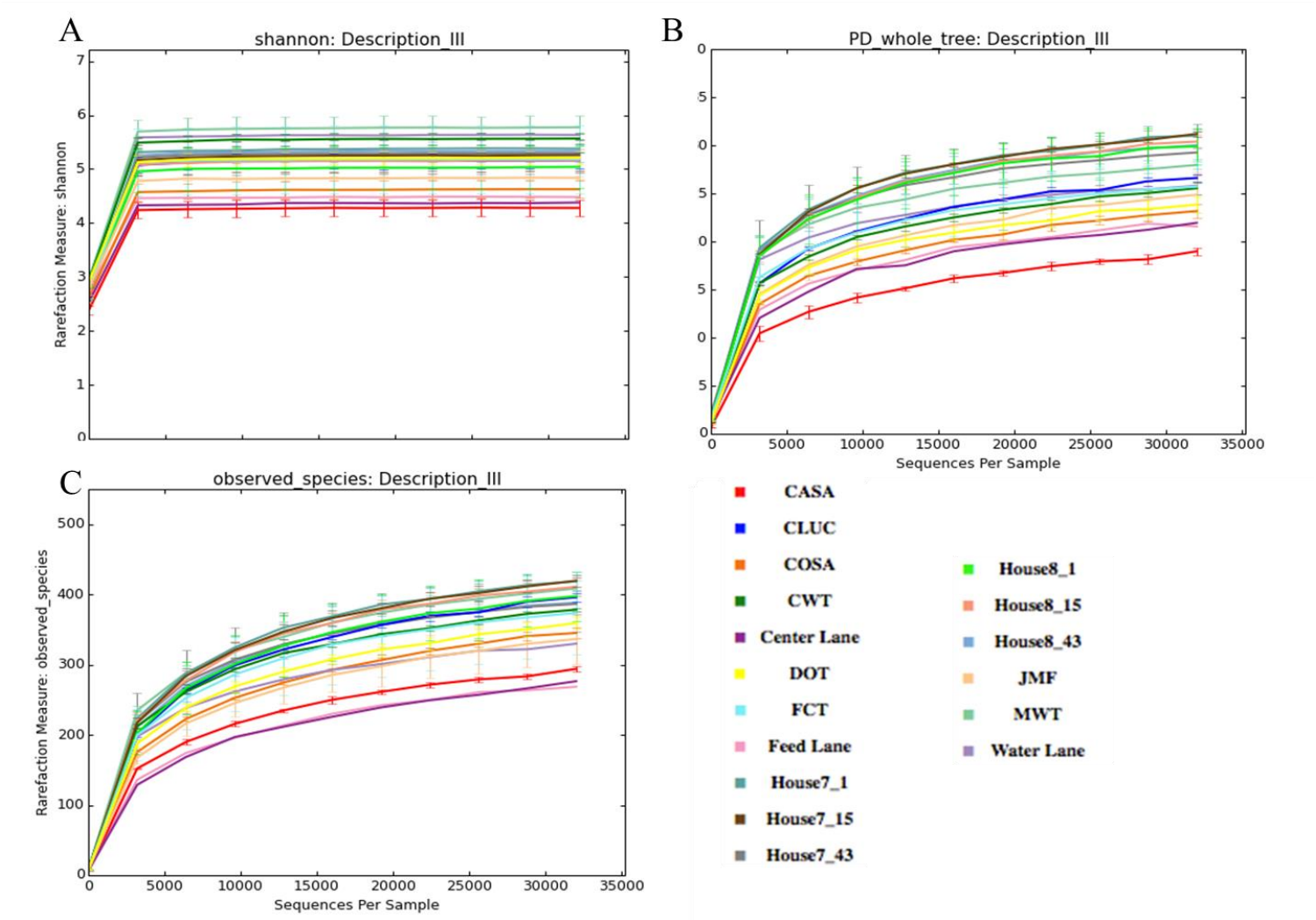

Figure 3. The litter α diversity indices for Shannon A) PD whole tree B) and observed species C) of each sample set

Weighted Unifrac-based (97% sequence identity) principal coordinate analysis (PCoA) revealed clustering within the samples (Figure 4). The weighted Unifrac method takes into consideration for the abundance of each species. While there was no significant difference observed among grouping based on bedding materials, locations or integrators (Figure 4, A, C, & D), there was clear clustering by grouping of fresh vs. stored (Figure 4). Most of the variations were explained by PC1 (66%), follow by PC2 (10%), and PC3 (6%) (Figure 5). The greatest amount of separation was observed at PC1 (66%), with a distinct gap between the fresh and the long-term stored (LTS) samples. Moreover, 2 out of 3 FCT (it was litter collected right after taken out of the broiler house) triplicates were grouped in between the stored and in-house samples; while the other sample extraction was grouped within the in-house samples. Additionally, while the MWT samples (sample collected from a motility litter pile in Wartrace, TN) were closer to the stored litter, they separated from the stored samples. This separation was observed with PC2, however only explains 10% of variation (Figure 5).

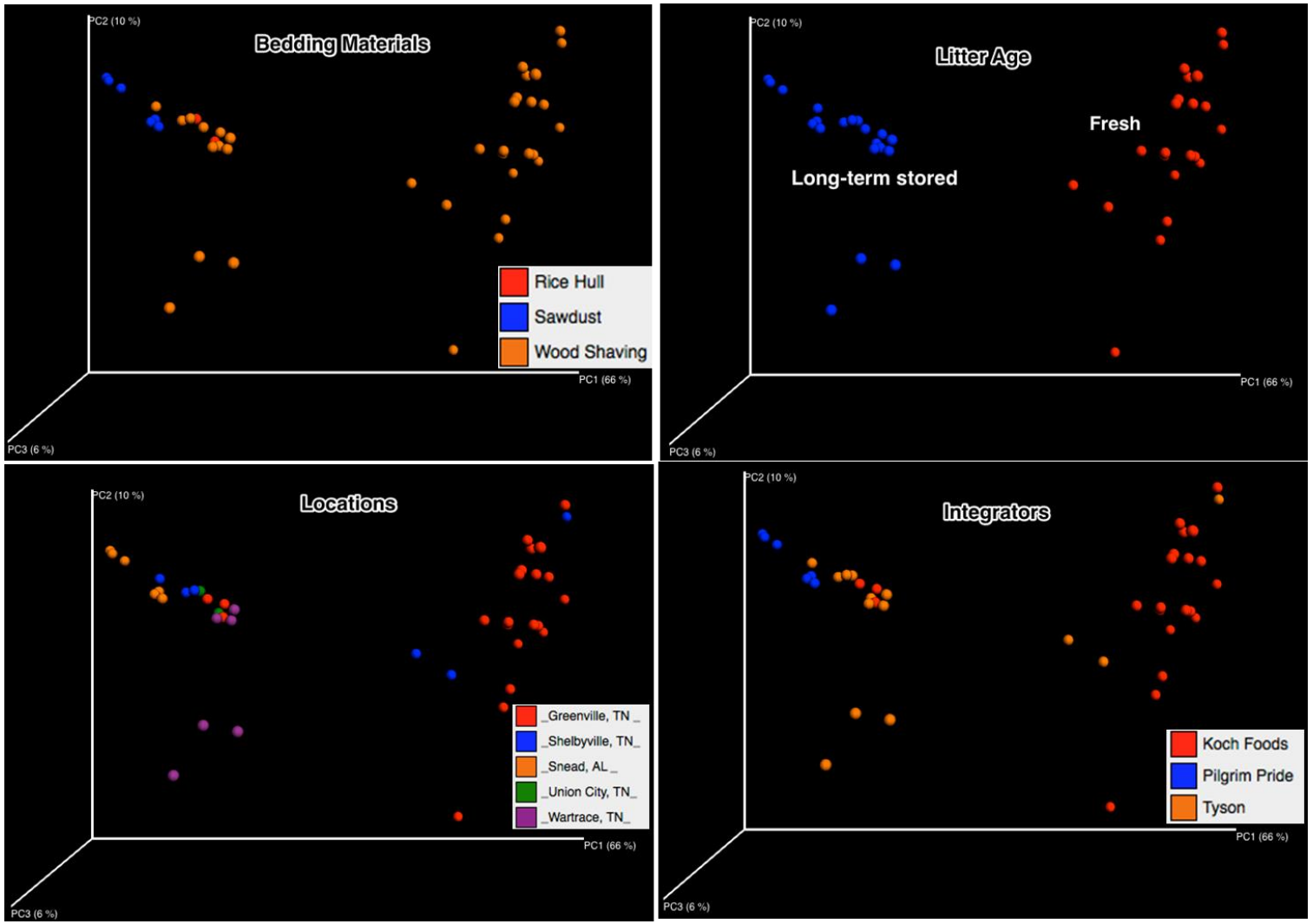

Figure 4. EMPEROR visualization of weighted Unifrac distance by A) Bedding materials, B) Litter activity, C) Locations, D) Integrators.

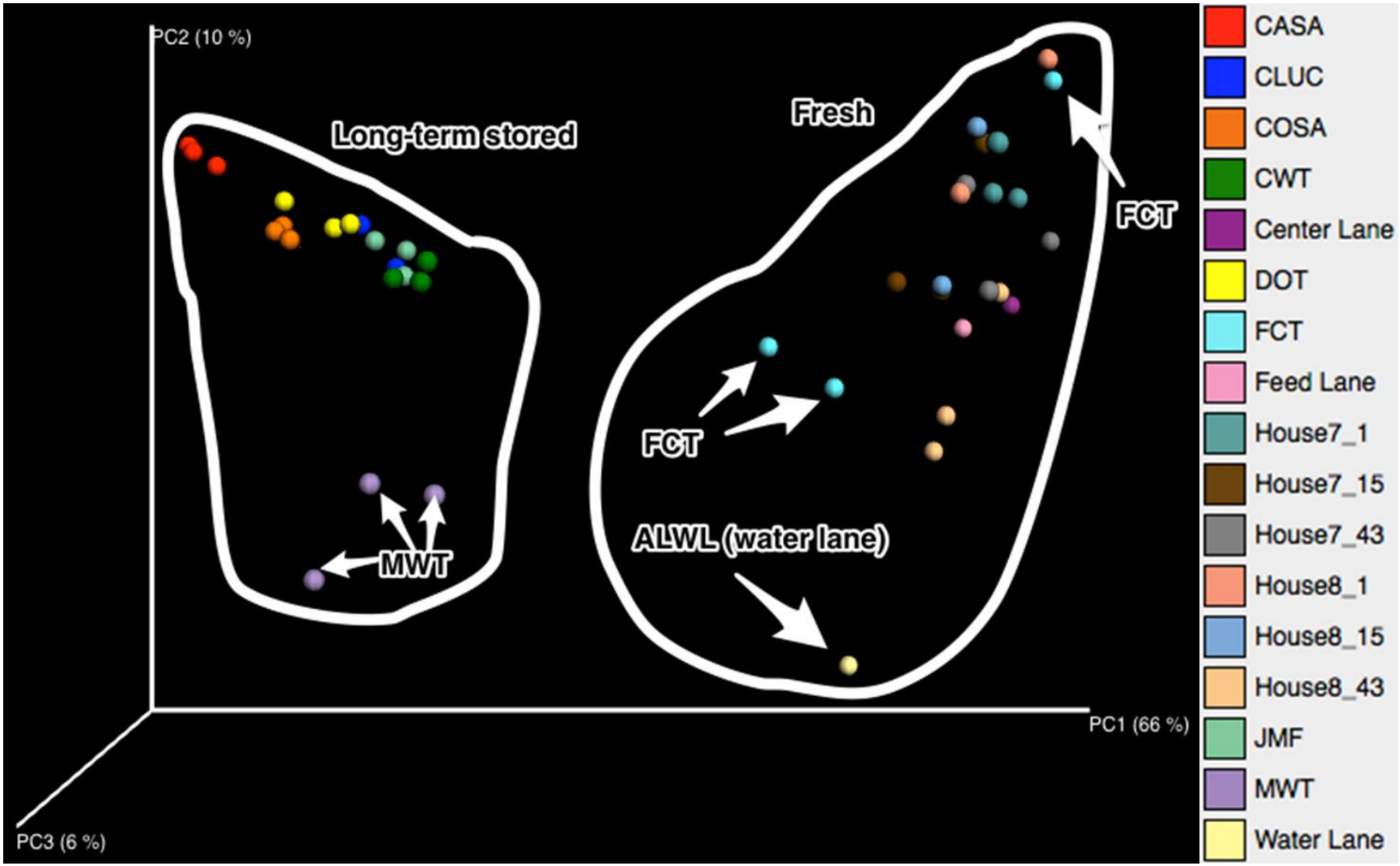

Figure 5. EMPEROR visualization of weighted Unifrac distance by samples.

Overall Bacteria Community Structure

The LTS samples included all of the stored samples except FCT (collected right after removal from the broiler house). While the fresh samples included all of the in-house (timed and lane samples) and FCT. Moreover, FCT and DOT were collected from the same producer, and JMF was also collected from the same producer as the in-house samples. In addition, MWT was grouped under the LTS samples; it was a motility waste pile with litter mixed.

About 0.03% ± 0.09% of sequences were unable to be classified below the level of kingdom. At the phylum level, all samples were dominated with phyla Actinobacteria and Firmicutes. For the LTS samples, Firmicutes was by far the largest phylum, accounting for 77% ± 8% of total sequences. Actinobacteria was the second largest phylum, accounting for 22.2% ± 8% of the total sequences. This distribution was reversed for the fresh sample grouping: Actinobacteria (65.5 % ± 10.2%) followed by Firmicutes (31.4% ± 10%). The differences in the distributions of Actinobacteria and Firmicutes between these two sample groupings was significant, both phylum had a corrected p-values less than $1x10^{-15}$ (Figure 6). These two phyla were also identified as the main phyla in Dumas et al. pyrosequencing study of broiler litter bacterial structure [54].

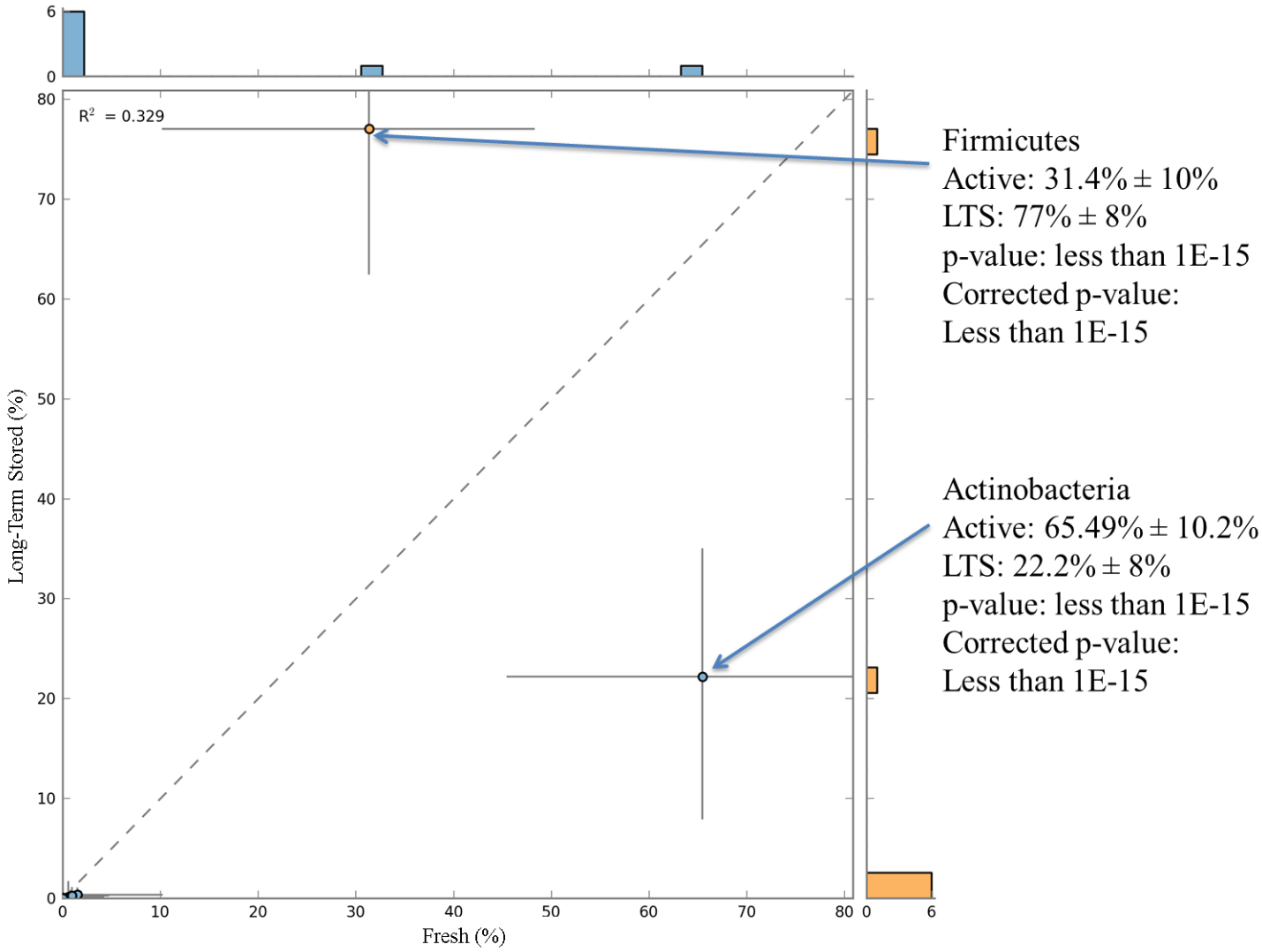

Figure 6. The comparison between the fresh and LTS litter samples at phylum level.

At the phylum level within the LTS grouping, sample CASA, one of the two samples collected from Snead, AL, had the highest percentage of Firmicutes (90.6% ± 0.88%), and the lowest percentage of Actinobacteria (9.27% ± 0.93). MWT, was the motility litter pile, had the lowest percentage of Firmicutes (69.82% ± 3.47%) but with the highest percentage of Firmicutes (90.6% ± 0.88%). The distributions of Firmicutes for other samples within LTS were JMF (74.32% ± 0.04), COSA (83.79% ± 1.75%), DOT (78.31% ±3.45%), CLUC (75.42% ± 0.91%), and CWT (66.41% ± 3.46%). The distributions of Actinobacteria for other samples within LTS were JMF (24.84% ± 0.31%), COSA (15.73% ± 1.76%), DOT (20.89% ± 3.31%), CLUC (24.01% ± 0.75%) and CWT (32.30% ± 2.79%).

For Firmicutes, CASA was found to differ (corrected p value <0.05) to COSA, CWT, JMF, and MWT; COSA was statistically different from CLUC, CWT, and JMF. For Actinobacteria, CASA was different from COSA, CWT, JMF, and MWT; COSA was different from CLUC, CWT, and JMF. Other sample comparisons within the phylum level were not statistically different. The corrected p-values from statistical comparisons of each sample within the phyla are summarized on Table 4.

Table 4. P-values for each sample comparison at phylum level (A). The LTS sample comparisons for Firmicutes. (B). The LTS sample comparisons for Actinobacteria

A.

Firmicutes	**CASA**	**CLUC**	**COSA**	**CWT**	**DOT**	**JMF**	**MWT**
CASA	--						
CLUC	0.016**	--					
COSA	0.067	0.049**	--				
CWT	0.029**	0.216	0.033**	--			
DOT	0.123	0.579	0.373	0.104	--		
JMF	0.006**	0.882	0.066	0.134	0.648	--	
MWT	0.010**	1.00	0.122	0.609	0.187	0.147	--

B.

Actinobacteria	**CASA**	**CLUC**	**COSA**	**CWT**	**DOT**	**JMF**	**MWT**
CASA	--						
CLUC	0.024**	--					
COSA	0.050**	0.011**	--				
CWT	0.031**	0.320	0.031**	--			
DOT	0.246	0.630	0.292	0.172	--		
JMF	0.005**	0.609	0.016	0.123	0.932	--	
MWT	0.049**	0.516	0.082	0.685	0.169	0.414	--

** The comparisons were statistically significant ($P<0.05$).

At the phylum level within the fresh sample grouping, over 95% of total sequences belong to two phyla: Actinobacteria (65.5 % ± 10.2%), and Firmicutes (31.4% ± 10%) (Figure 7). There were no statistically significant differences (corrected p-value > 0.05) between any of the sample for Firmicutes or Actinobacteria. It was important to note that while FCT sample was collected from a different producer at a different geographic location; it did exhibit very similar bacterial community structure at the phylum level to the in-house samples. Additionally, WL had the highest distributions of Bacteroidetes and Proteobacteria at 5.33% and 4.19% respectively. H7D1 also had a mean 4.15 % ± 4.23 % in Bacteroidetes, and could be contributed to one of the extractions contained soil from the broiler house. Both Bacteroidetes and Proteobacteria were observed in previous litter related sequencing studies [53, 54]. Moreover, in an Alabama broiler litter study, Proteobacteria was more prevalent (53% of total sequences) in soils with litter application, compared to 37% of total sequences directly under the production house. Also in this study, Bacteroidetes was found 22% of the sequences in soil under broiler houses [53].

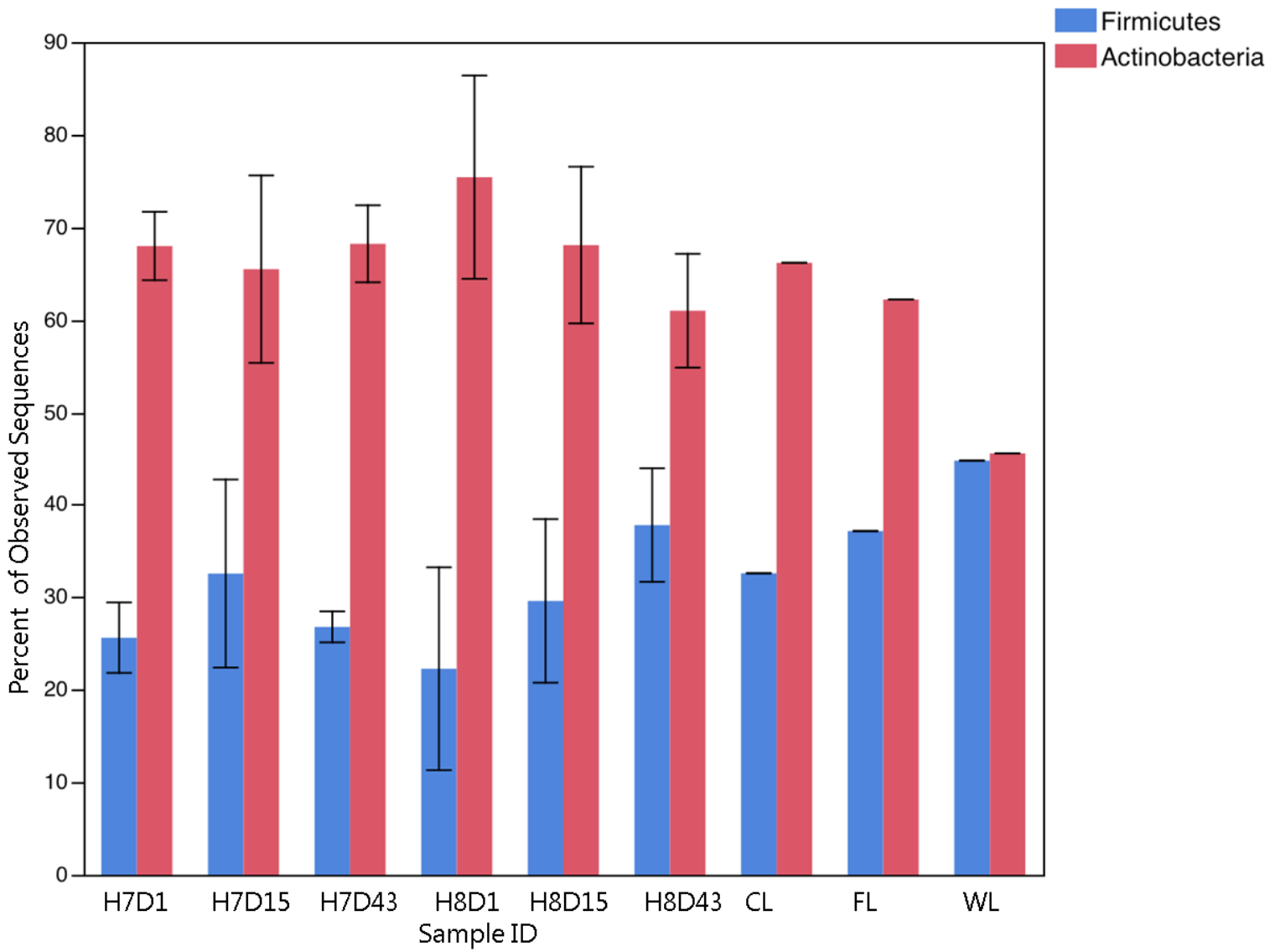

Figure 7. Major phyla in each sample set within the fresh sample grouping.

Overall Bacterial Community Structural Comparison at the Family level

The coefficients of determination (R^2) were calculated with STAMP v2.0.2 for comparisons of all of the litter samples collected in the study (Table 5). The mean R^2 was 0.98 ± 0.02 for each LTS sample compared to other LS samples. The lowest R^2 value within the LTS grouping was observed between CASA and MWT with an R^2 of 0.93. This suggested that while the samples were collected from different producers, the overall bacterial community structure at the family level remain similar. MWT, which was the sole mortality litter sample, was similar to other LTS samples (R^2 value greater than 0.9). For the entire fresh sample grouping (timed, lane series and FCT samples), the mean R^2 was 0.70 ± 0.2. Additionally, the mean R^2 for the in-house samples only (timed and lane series) comparisons yielded a mean of 0.73 ± 0.19. However, when the in-house samples were grouped separately into timed and lane series, the R^2s were 0.87 ± 0.072 and 0.92 ± 0.036, respectively. This indicated more similar bacterial community structures within the timed series and within the lane series than between the two groups. For the timed series, the lowest R^2 value was the comparison between H8D1and H8D43 at 0.716. For the lane samples, the lowest R^2 value was between CL and WL at 0.88. The FCT samples demonstrated some similarity to the in-house samples with a mean R^2 value of 0.586 ± 0.223. The R^2 mean increased to 0.70 ± 0.073 when compared solely within the timed series. In contrast, the FCT comparisons to LTS produced a lower R^2 value of 0.296 ± 0.046. These observations suggested a closer resemblance of FCT to the in-house samples, particularly to the in-house timed samples, than the LTS samples.

The comparison between the LTS and fresh sample groupings yielded an R^2 of 0.07. This indicated a great dissimilarity between the LTS and fresh samples. The comparison between LTS and fresh was further investigated for each family within samples.

Table 5 The family level R^2 values for comparisons of in-house versus in-house litter samples (light gray background), comparisons of stored litter versus stored litter samples (white background) and comparisons of in-house and stored litter samples (dark gray background

Family	H7D1	H7D15	H7D43	H8D1	H8D15	H8D43	ALCL	ALWL	ALFL	FCT	JMF	CASA	COSA	DOT	CWT	MWT	CLUC
H7D1	1																
H7D15	0.87	1															
H7D43	0.92	0.88	1														
H8D1	0.94	0.83	0.92	1													
H8D15	0.86	0.99	0.86	0.86	1												
H8D43	0.73	0.91	0.86	0.72	0.89	1											
ALCL	0.40	0.55	0.67	0.37	0.51	0.76	1										
ALWL	0.42	0.66	0.59	0.39	0.61	0.84	0.9	1									
ALFL	0.46	0.69	0.65	0.43	0.66	0.86	0.95	0.94	1								
FCT	0.72	0.64	0.81	0.74	0.6	0.64	0.52	0.05	0.52	1							
JMF	0.16	0.08	0.12	0.1	0.06	0.03	0.009	0.009	0.011	0.33	1						
CASA	0.09	0.03	0.06	0.04	0.02	0.004	0	0	0	0.23	0.98	1					
COSA	0.09	0.04	0.07	0.05	0.02	0.005	0	0	0.001	0.25	0.99	0.99	1				
DOT	0.21	0.05	0.09	0.07	0.03	0.01	0.002	0.001	0.002	0.28	0.995	0.99	0.997	1			
CWT	0.13	0.05	0.09	0.08	0.04	0.01	0.002	0.002	0.003	0.32	0.97	0.95	0.98	0.98	1		
MWT	0.17	0.07	0.12	0.11	0.05	0.02	0.009	0.008	0.008	0.36	0.959	0.93	0.96	0.96	0.99	1	
CLUC	0.15	0.07	0.17	0.09	0.05	0.02	0.006	0.006	0.007	0.305	0.995	0.99	0.99	0.996	0.97	0.96	1

In order to assess the significance in differences of specific families between the sample groupings, Welch's two-sided t-test was conducted. Benjamini-Hochberg FDR was used to correct the potential false positives due to multiple tests. There were a total of 32 families with statistical significance (corrected p value < 0.05). Table 6 listed the top 19 families (Table 6).

At the family level, LTS was dominated with *Bacillaceae* (68.33% ± 11.48%), while the fresh grouping was dominated with *Brevibacteriaceae* (19.1% ± 5.20%) and *Dermabacteraceae* (14.59% ± 4.29%) (Figure 8). These families were also statistically different with corrected p values of 5.35×10^{-22}, 1.02×10^{-14} and 1.34×10^{-12}, respectively. Statistically significant families with distribution of greater than 5% in either grouping were further investigated and assessed as families of interest. These families were *Yaniellaceae, Staphylococcaceae, Corynebacteriaceae,* and *Nocardiopsaceae* (Figure 9). While *Yaniellaceae* (7.58% ± 2.21%), *Staphylococcaceae* (14.05% ± 6.56%), *Corynebacteriaceae* (12.73% ± 7.01%) were dominant in the fresh sample grouping, they were only present at 3.07% ± 0.77%, 2.60% ± 1.26%, and 1.80% ± 1.22% respectively in the LTS sample grouping. *Nocardiopsaceae* was present at 7.28% ± 4.14% in the LTS grouping, but only present at 3.53% ± 3.43% in the fresh grouping. The large percentage of *Bacillaceae* found in LTS samples could be because this family contains both aerobic and facultative anaerobic chemo-organotrophic rods [71]. Additionally, members in this family can form endospores, in which can enable the bacteria to dormant for extended periods and reactivate itself when the environment condition become more favorable [72]. The unfavorable conditions found in LTS (colder outside environment) could contribute to large amount of spore forming in *Bacillaceae,* thus created an increase in its abundance.

Table 6. Statistically significant families between the long-term stored (LTS) and fresh sample groupings.

Phylum	Family	Fresh: mean (%)	Fresh: SD (%)	LTS: mean (%)	LTS: SD (%)	p-values (corrected); (LTS vs. fresh)
Actinobacteria	*Brevibacteriaceae*	19.09	5.20	2.28	1.89	1.02×10^{-14}
Actinobacteria	*Dermabacteraceae*	14.59	4.29	3.13	1.13	1.34×10^{-12}
Firmicutes	*Staphylococcaceae*	14.05	6.56	2.60	1.26	4.45×10^{-8}
Actinobacteria	*Corynebacteriaceae*	12.73	7.01	1.80	1.22	4.58×10^{-7}
Actinobacteria	*Yaniellaceae*	7.58	2.21	3.07	0.77	2.38×10^{-9}
Firmicutes	*Bacillaceae*	7.08	5.39	68.33	11.48	5.35×10^{-22}
Actinobacteria	*Other*	4.99	3.53	1.87	0.93	0.0024
Firmicutes	*Lactobacillaceae*	3.88	2.59	0.69	0.45	3.35×10^{-5}
Actinobacteria	*Nocardiopsaceae*	3.53	3.43	7.28	4.14	0.011
Firmicutes	*Aerococcaceae*	3.26	1.60	1.10	0.96	5.48×10^{-5}
Actinobacteria	*Dietziaceae*	1.00	0.38	0.12	0.09	8.45×10^{-11}
Actinobacteria	*Pseudonocardiaceae*	0.46	0.45	1.70	1.21	0.000375016
Firmicutes	*Other*	0.45	0.30	1.21	0.69	0.00021
Firmicutes	*Lachnospiraceae*	0.37	0.43	0.05	0.05	0.0083
Firmicutes	*Ruminococcaceae*	0.24	0.18	0.04	0.05	9.26×10^{-5}
Actinobacteria	*Intrasporangiaceae*	0.16	0.07	0.01	0.02	1.81×10^{-9}
Firmicutes	*Streptococcaceae*	0.15	0.10	0.04	0.03	0.00023
Firmicutes	*Other*	0.11	0.11	0.03	0.06	0.031
Firmicutes	*Leuconostocaceae*	0.09	0.10	0.03	0.05	0.0453

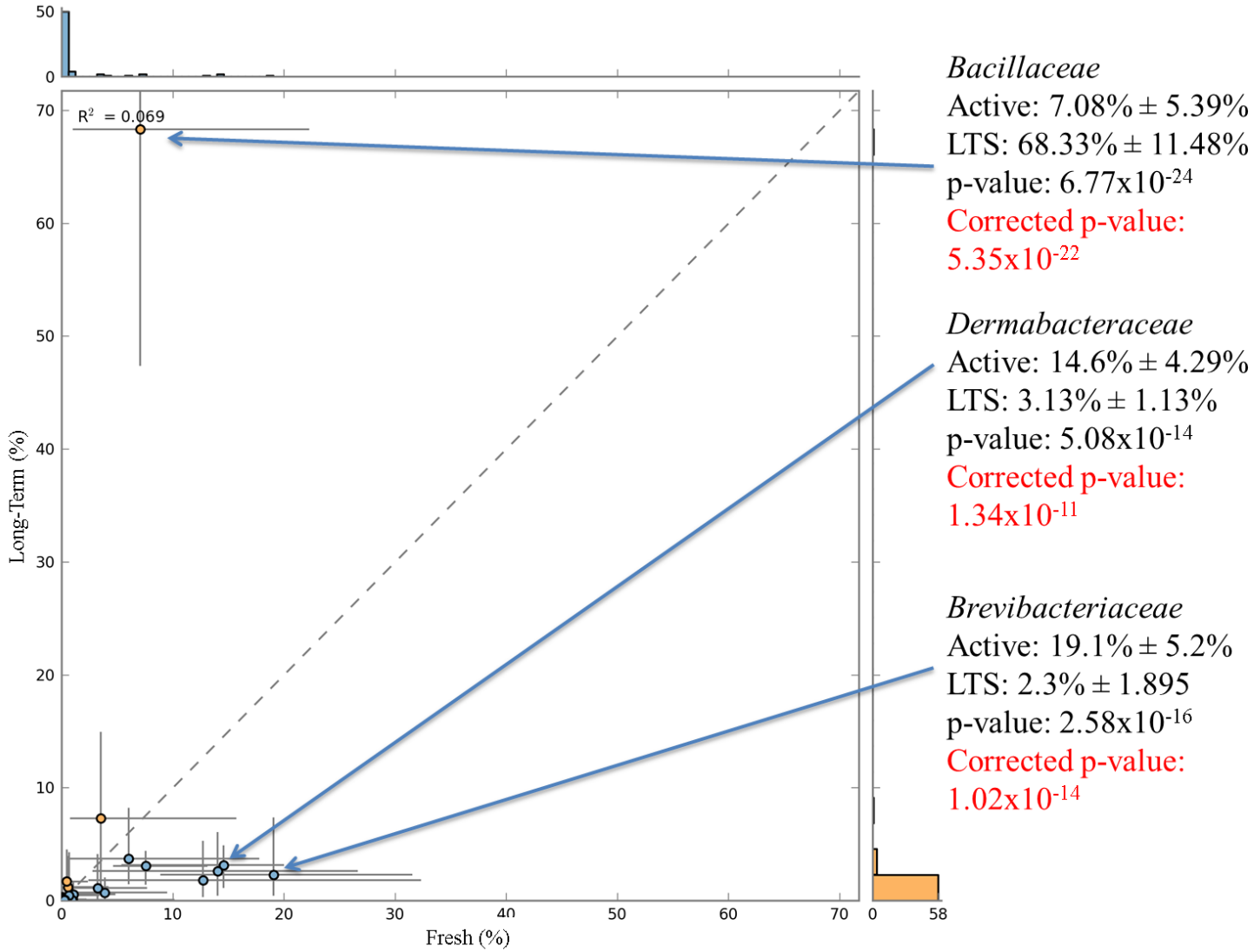

Figure 8. Family comparison between the LTS and fresh sample grouping

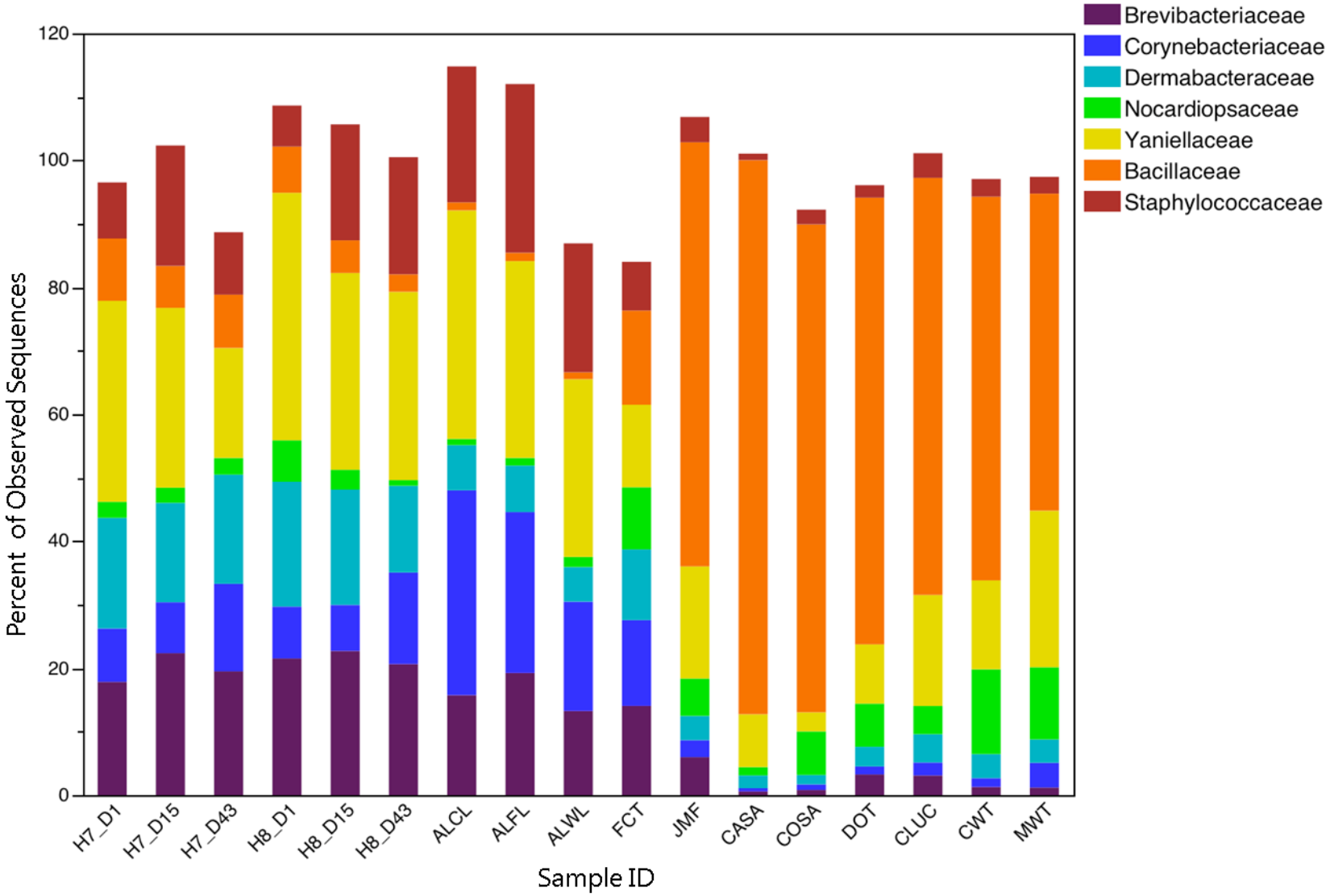

Figure 9. Most abundant bacterial families with statistical significance found in broiler litter.

The families *Bacillaceae*, *Brevibacteriaceae*, *Dermabacteraceae*, *Yaniellaceae*, *Staphylococcaceae*, and *Corynebacteriaceae* were all found in previous poultry litter studies [27, 39, 54]. All of these families belong to phyla Firmicutes and Actinobacteria. The family *Bacillaceae* is a heterogeneous collections of gram-positive rod shaped bacteria that contains both free-living and pathogenic species [73]. Whole genome sequencing of this family indicated the presence of five main lineages[73]. Members within this family had been found diverse habitats include soils, hot springs, human and animal bodies [73, 74], and have been associated with food poisoning, contamination of dairy products, and as an opportunistic pathogen in humans [75-79]. This family was found in Dumas et al. 16S DNA high throughput sequencing study [54], but was not clearly identified in Lu's 16S DNA library clone sequencing study [39].

The family *Brevibacteriaceae* may include poultry specific species *Brevibacterium avium* [27]. This family was found as the 4th largest OTU in Dumas' study (5.7%) [54] and at 7.06% in Lu's study [39]. The species sources as the only poultry broiler microbial source tracking (MST) organisms. The presence of *Staphylococcaceae* was not surprising, since it has been found on the skin of both healthy and diseased birds [80]. Additionally, it was previously found in all 44 litter samples using culturing method [81]. The litter microbiomic study in Delaware found that species within this family was the second most abundant OTUs [54].

The *Dermabacteraceae* family was one of the largest OTUs in Dumas' study (5.5%) [54]. In Lu et al. 16S DNA library clone sequencing study, it was also found in 5.3% clones [39]. *Dermabacteraceae* consists of Gram-positive, rod shape bacteria with high G+C content in DNA [82]. Strains within this family were isolated from human skin but were not considered as pathogenic [83].

The *Yaniellaceae* family, formally named *Yani* [84, 85], was found as one of the largest OTUs in Dumas et al. study (7.2%) [54]. However, members within this family were not found in Li et al. broiler litter study [39] but has been found in turkey fecal droppings [85].

Genera within the family of *Corynebacteriaceae* can be associated with humans, but less is known regarding this family of association with animals [86]. Species under this family were also found both in the Lu et al. study (9.71%) [39] and Dumas et al. study (2.33%) [54]. Members within this family were also found in the turkey gut [87].

The family *Nocardiopsaceae* was recently created to accommodate the genus *Nocardiopsis* [88] and members of this family have been found in soils [89]. Additionally, members in this family have been isolated from poultry feather wastes and have significant keratinolytic activity [90].

Bridge Comparisons

Some of the different geographic samples collected in this study were from farms with both LTS and fresh sample groupings. Specifically, DOT and FCT were collected from the same producer in Shelbyville, TN. This provided the same producer, bedding material and only differed in the age of the litter. DOT was the LTS sample, while FCT was fresh collected as the litter was removed from the house. Additionally, JMF was the stored version of the in-house samples, thus JMF vs. the day-43 of the house samples (H7D43 & H8D43) were specifically compared. These day-43 collection of house samples and JMF can provide more conclusive evidence regarding the changes in bacterial structural in broiler litter during storage.

For DOT vs. FCT comparison, there was no statistically significant at either phylum or family level, all of the corrected p-values were above 0.05. At the phylum level, the R^2 between FCT and DOT was 0.57, and was accompanied by the shift in phylum from Firmicutes to

Actinobacteria. The R^2 was reduced to 0.283 at the family level. DOT samples were dominated with the family of *Bacillaceae* (70.37% ± 2.91%) while this family was only present at 14.83% ±7.68% in FCT. The families of *Brevibacteriaceae* (DOT 3% ± 0.6%, FCT 14.06% ± 6.65%), *Dermabacteraceae* (DOT 11.14% ± 2.62%, FCT 3.10% ± 0.6%), and *Corynebacteriaceae* (DOT 1.26% ± 0.41%, FCT 13.51% ±3.38%) were also enriched in FCT samples. Although none of these comparisons were statistically significant.

The two sample sets had a low R^2 and the PCoA also demonstrated clustering based on Unifrac calculations. FCT samples were all clustering at the mid region between the in-house and LTS samples (Figure 5). Moreover, FCT seemed to be the connecting bridge between the stored and the in-house samples.

JMF vs. H7D43 and JMF vs. H8D43 were compared. At phylum level JMF vs. H7D43 comparison found statistical significance in both Actinobacteria and Firmicutes (corrected p-values 0.011 and 3.27×10^{-3} respectively). The phylum Actinobacteria decreased from 68.19% ± 3.40% of total sequences in H7D43 to 24.84% ± 0.31% of total sequences in JMF; the phylum Firmicutes was present at 26.79% ± 1.37% in H7D43 to 74.32% ± 0.04% in JMF. The comparison between JMF vs. H8D43 only had one statistical significant phylum: Actinobacteria (H8D43: 60.96% ± 5.02% to JMF 24.84% ± 0.31%).

At family level, the R^2 between H7D43 and JMF is 0.118; the family of *Bacillaceae* was clearly enriched in JMF (66.81% ± 2.27%) and only accounted at 8.39% ± 4.32% in H7D43 (corrected p-value of 0.027). *Dermabacteraceae* (p: Actinobacteria) decreased from 17.20% ± 1.27% in H7D43 to 3.82% ± 0.70% in JMF (corrected p-value 0.026) (Figure 10). These were the only statistical significant families.

The R^2 between H8D43 and JMF was very low (0.026). There were several statistically significant differences (p value < 0.05) in the families when comparing between JMF and H8D43 (Figure 11). The *Bacillaceae* family had a corrected p-value of 0.027 (H8D43: 2.76% ± 0.43%, JMF: 66.81% ± 2.27%). Additionally, the *Staphylococcaceae* family had a corrected p-value of 0.031 (H8D43: 18.44% ±1.33%, JMF 4.00% ± 1.43%). In addition, for H8D43 and JMF, families of *Brevibacteriaceae* (H8D43: 20.64% ±1.83%, JMF: 5.98% ± 0.92%) and *Dermabacteraceae* (H8D43: 13.66% ± 1.69%, JMF: 3.82% ± 0.70%) were also significantly different, with corrected p-values of 0.024 and 0.05 respectively.

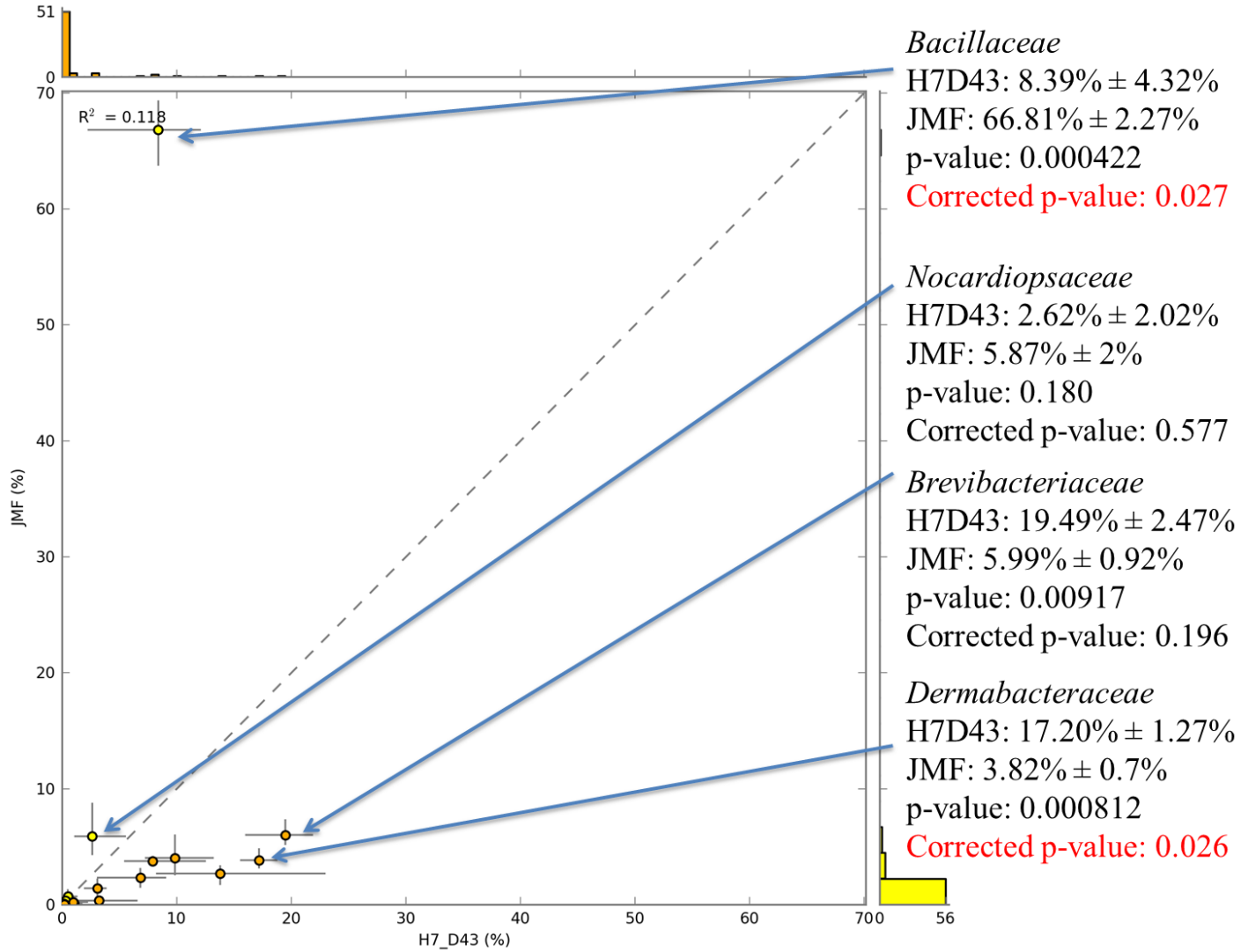

Figure 10. The comparison between the H7D43 and JMF samples at family level.

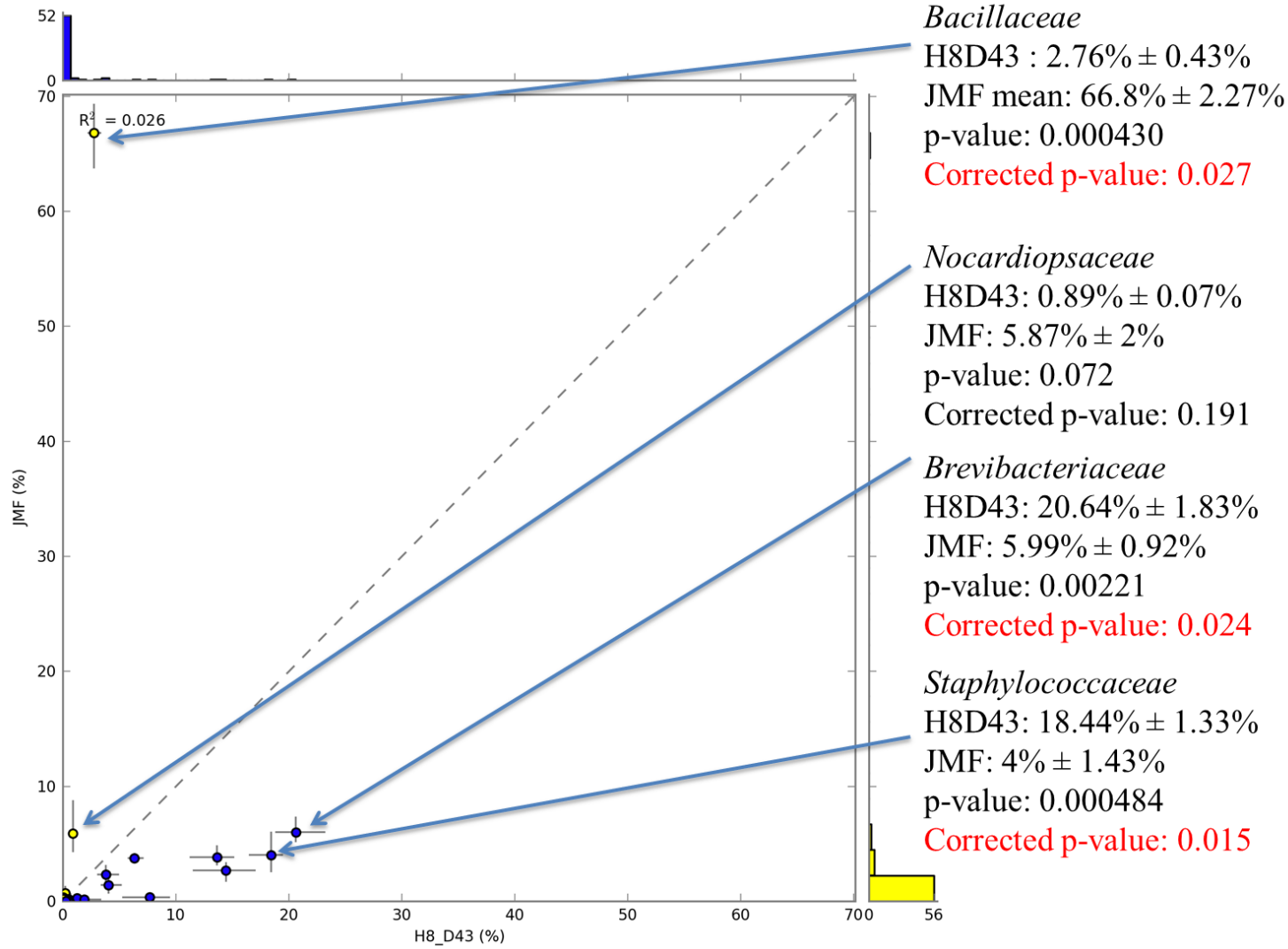

Figure 11. The comparison between the H8D43 and JMF samples at family level.

Comparisons Within LTS Grouping

All of the LTS samples had high R^2s when compared with each other at family level, which suggested a similar bacterial community structure. Samples under the LTS grouping consisted of long-term stored samples from a Koch foods contracted producer with wood shaving as bedding material from Mohawk, TN (JMF). Tyson foods producers used saw dust as bedding material from Wartrace (MWT, & CWT) and Shelbyville, TN (DOT), and rice hull as bedding material from Union City, TN (CLUC). Sample MWT was collected from mortality pile mixed with litter. Additionally, samples were also collected from a Pilgrims Pride contracted grower that used saw dust as bedding material from Snead, AL (COSA, & CASA). These samples were collected different geographical locations, integrators, and bedding materials. In addition, the moisture contents for these samples ranged from 24% to 49% pending on locations. Moisture contents for each sample are listed on Table 7. For statistical analysis, moisture contents between 25%~ 36% were grouped as medium, while 36%+ were classified as high.

Table 7. Moisture content of stored litter samples.

Sample ID	Moisture %
COSA	27
CASA	24
DOT	45
CWT	27
MWT	35
CLUC	26
JMF	42

At the family level, *Bacillaceae* (68.33% ± 11.48%) was by far the most dominant family. The largest families also included *Nocardiopsaceae* (7.28% ± 4.14%), *Dermabacteraceae* (3.13% ± 1.13%), *Yaniellaceae* (3.07% ± 0.77%), *Staphylococcaceae*, (2.60% ± 1.26%), and *Brevibacteriaceae* (2.28% ± 1.89%), and *Corynebacteriaceae* (1.80% ± 1.22%). In order to achieve the at-least 90% coverage, several other families were also necessary to include and listed on Table 8. Specifically, for MWT, families of *Aerococcaceae* (1.10% ± 0.96%), *Tissierellaceae* [name dispute] (0.45% ± 1.01%), an unknown family in the order of Thermoanaerobacterales (0.32% ± 00.86%) and an ambiguous family in the phylum of Firmicutes (0.35% ± 0.88%) that was unable to match with RDP classifier (Figure 12).

Table 8. The top largest families observed in LTS samples (%).

Taxon	**JMF**	**CASA:**	**COSA:**	**DOT:**	**CLUC:**	**CWT**	**MWT**
f_ *Bacillaceae*	66.81 ± 2.27	87.29 ± 0.67	76.87 ± 0.93	70.37 ± 2.91	65.70 ±3.77	60.45 ± 1.91	49.95 ± 2.50
f_ *Nocardiopsaceae*	5.87 ± 2.00	1.31 ± 0.23	6.87 ± 1.77	6.97 ± 1.73	4.44 ± 0.07	13.31 ± 1.07	11.37 ± 2.38
f_ *Corynebacteriaceae*	2.67 ± 0.67	0.52 ± 0.09	0.88 ± 0.21	1.26 ± 0.41	2.04 ± 0.20	1.39 ± 0.33	3.90 ± 0.96
f_ *Yaniellaceae*	3.74 ± 0.26	2.63 ± 0.14	1.86 ± 0.28	2.72 ± 0.38	3.70 ± 0.41	3.24 ± 0.28	3.82 ± 0.60
f_ *Dermabacteraceae*	3.82 ± 0.7	2.01 ± 0.56	1.48 ± 0.22	3.10 ± 0.60	4.47 ± 0.36	3.78 ± 0.41	3.68 ± 0.78
f_ *Staphylococcaceae*	4.00 ± 1.43	1.02 ± 0.39	2.32 ± 0.64	1.98 ± 0.5	3.89 ± 1.04	2.78 ± 0.93	2.65 ± 0.29
f_ *Brevibacteriaceae*	5.99 ± 0.92	0.58 ± 0.07	0.81 ± 0.15	3.26 ± 0.55	3.10 ± 0.36	1.28 ± 0.05	1.19 ± 0.20
o_ Bacillales; Other	0.33 ± 0.07	1.29 ± 0.13	0.98 ± 0.12	1.38 ± 0.2	1.00 ± 0.04	1.01 ± 0.09	2.42 ± 0.82
f_ *Pseudonocardiaceae*	0.01 ± 0.02	0.91 ± 0.23	1.62 ± 0.52	1.32 ± 0.20	2.22 ± 0.16	3.90 ± 0.64	2.00 ± 0.70
o_ Actinomycetales; Other	1.74 ± 0.54	0.97 ± 0.12	1.52 ± 0.21	1.58 ± 0.23	2.92 ± 0.41	3.21 ± 1.22	1.52 ± 0.32
p_* Firmicutes;* Other	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.00 ± 0	0 ± 0	2.35 ± 0.69
f_ Aerococcaceae	1.4 ± 0.60	0.18 ± 0.07	0.6 ± 0.07	0.7 ± 0.15	0.99 ± 0.5	0.81 ± 0.31	2.96 ± 0.75
f_[Tissierellaceae]	0.15 ± 0.08	0.016 ± 0.01	0.08 ± 0	0.05 ± 0.03	0.03 ± 0.02	0.05 ± 0.02	2.64 ± 1.09
*o_ Thermoanaerobacterales;f_*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.00 ± 0.00	0 ± 0	2.12 ± 1.08

**Samples with grey background were only necessary for MWT to reach 90% coverage.

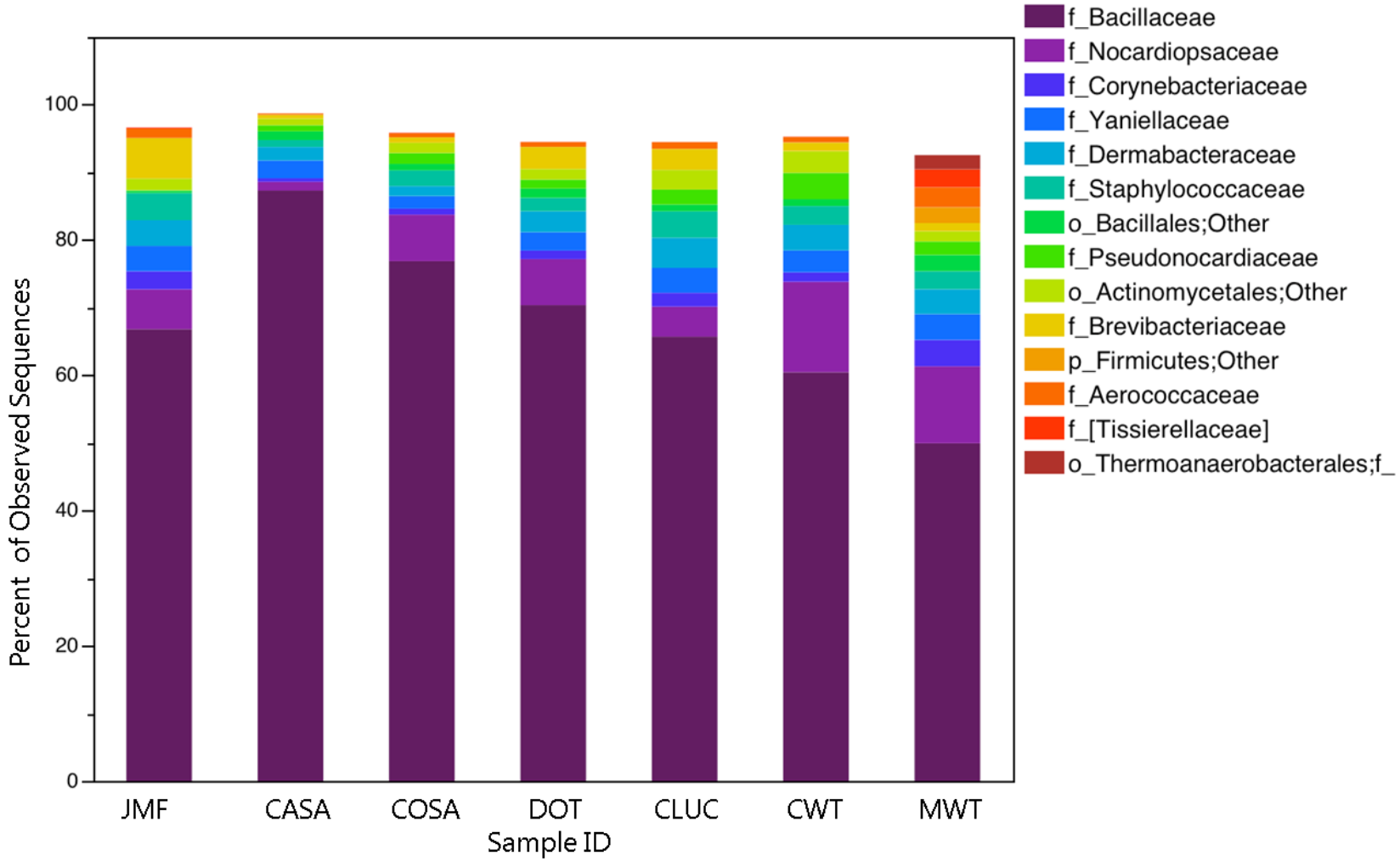

Figure 12. Top largest families within the LTS samples.

CWT had seven statistically different families when compared to CASA; *Brevibacteriaceae* (corrected p value < 0.00978), *Nocardiopsaceae*(corrected p value < 0.037), *Sphingobacteraceae* (corrected p value < 0.0005), *Bacillaceae* (corrected p value < 0.015), *Turicibacteraceae* (corrected p value < 0.0011), *Clostridiaceae* (corrected p value < 0.015), and *Alcalignenaceae* (corrected p value < 0.037). Similarly, CWT had five statistically different families when compared to COSA: *Alcalignenaceae* (corrected p value < 0.038), *Bacillaceae* (corrected p value < 0.038), *Pasteurellaceae* (corrected p value < 0.037), *Streptococcaceae* (corrected p value < 0.024), and, *Turicibacteraceae* (corrected p value < 0.02). CASA and COSA comparison has three statistically significantly different families: *Bacillaceae* (corrected p value < 0.029), *Nocardioidaceae* (corrected p value < 0.048), and *Streptococcaceae* (corrected p value < 0.033). CASA and CLUC comparison had two statistically different families: *Dietziaceae* (corrected p value < 0.00362), and *Nocardiopsaceae* (corrected p value < 0.036). COSA and JMF comparison had one statistically different family: *Flavobacteriaceae* (corrected p value < 0.00538). Among all these differences, only *Bacillaceae*, *Brevibacteriaceae* and *Nocardiopsaceae* were considered as major families (present at greater than 5% in any of the samples). Even though there were differences observed within LTS samples, *Bacillaceae* were consistently observed within all these samples. Additionally, despite difference in moisture content in various samples, the overall bacterial community structure remained largely unaltered. The overall bacterial community structural within the LTS sample grouping was similar. Even for MWT samples, all of the family level comparisons with other samples within the LTS had corrected p-values greater than 0.05.

Table 9. Families in LTS sample grouping that were statistically different when compared to the other groups.

Sample_ID	JMF	CASA	COSA	DOT	CLUC	CWT	MWT
JMF	-						
CASA	-	-					
COSA	*Flavobacteriaceae*	*Bacillaceae*; *Nocardioidaceae*; *Streptococcaceae*;	-				
DOT	-	-		-			
CLUC	-	*Dietziaceae*; *Nocardiopsaceae*;		-	-		
CWT	-	*Brevibacteriaceae*; *Nocardiopsaceae*; *Sphingobacteraceae*; *Bacillaceae*; *Turicibacteraceae*; *Clostridiaceae*; *Alcalignenaceae*;	*Alcalignenaceae*; *Bacillaceae*; *Pasteurellaceae*; *Streptococcaceae*; *Turicibacteraceae*;	-	-	-	
MWT	-	-	-	-	-	-	-

ANOSIM were used to test any potential statistical difference within the groupings of the bedding materials, producers, integrators, and moistures. The differences were observed with the comparisons of grouping with producers (p-value: 0.001, R-value:0.892) and integrators (p value: 0.042, R-value: 0.249). There were a total of 10 producers. The integrators consisted of Pilgrim Pride (n=6), Tyson (n=14), and Koch Foods (n=3). The closer the R values to 1, the stronger the grouping. The R-value of the grouping among producers was 0.892, indicated that the grouping of samples based on producers was strong. While the p-value was significant ($p < 0.05$) for the integrators grouping, the R-value was 0.249, which indicated a weaker grouping based on integrators. The comparison between different bedding materials yielded a p-value of 0.12, indicating the groupings by bedding materials was statistically insignificant. Additionally, the R-value obtained from this grouping was 0.14, which correlated with a low p value. This specific finding agrees with Fries et al. study that microflora in broiler litter was independent to bedding materials [91].

The moisture contents were observed as the main drive force for microbial community in soils [92]. In this study, while the moisture contents for different samples ranged from 24% to 46%, within the groupings of moisture contents, there was a lack of significance (p-value: 0.076, R value: 0.203) among the bacterial community structural.

Despite the statistical differences, all of the LTS samples were still similar in their overall bacterial community structural. In Torok et al. study on association between broiler gut microbial community and different bedding materials, found that factors such as bedding material and bird age in associations to broiler gut bacterial development, but this study also found that among all of the intestinal bacteria observed, OTUs over 4% had no statistically difference when compared within the grouping [93]. Similarly, in this study, while there were

changes in litter with less abundant families, overall the family structure was similar within the LTS grouping.

Fresh Sample Grouping Comparisons

The fresh sample grouping consisted of the FCT sample, which was collected from a Tyson contracted producer in Shelbyville, TN, three samples collected at three different times during one grow-out (Feb~ March, 2013) from a Koch food contracted producer in Mohawk, TN, and lane series samples collected from the same farm as the timed series samples during 2012. The lane series samples consisted of samples collected from center (CL), feed (FL), and water (WL) lanes in all 8 houses on one day in July of 2012. Moisture data was available for the FCT and in-house timed series samples. In FCT the moisture for litter was 48.8%. For the in-house timed series, at day 1, the moisture levels for houses 7 and 8 were 13.96% ± 5.06%, and 15.74 % ± 5.27%. Similarly, at day 15, the moisture content in houses 7 and 8 was 14.96% ± 4.5% and 16.51% ± 7.58% respectively. The moisture content increased at day 43, in houses 7 and 8 and was 29.44% ± 12.2% and 30.47% ± 15.48% respectively (Figure 13). For statistical analysis, within the in-house timed series, moisture contents between 10%~25% were grouped as low (day 1, & 15 samples), while 25%~ 36% were classified as medium (day 43 samples).

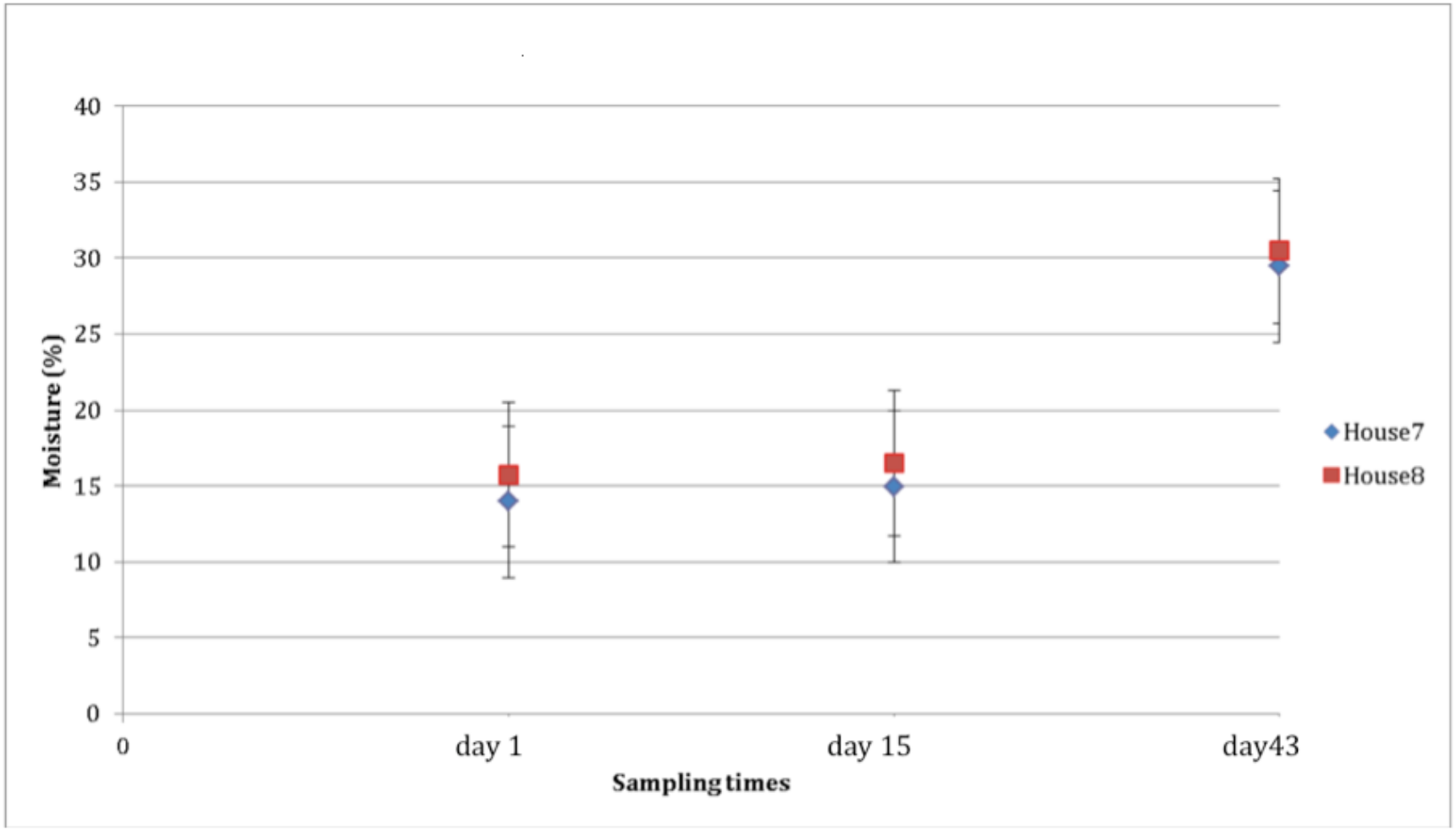

Figure 13 In-house timed litter sample moisture contents.

Within the fresh grouping, *Brevibacteriaceae* (19.08% ± 5.20%), *Dermabacteraceae* (14.59% ± 4.29%), *Staphylococcaceae* (14.05% ± 6.56%), *Corynebacteriaceae* (12.73% ± 7.01%), *Yaniellaceae* (7.58% ± 2.21%), and *Bacillaceae* (7.08% ± 5.39%) were the dominant families. The most abundant families needed to produce an at-least 90% coverage are listed in Table 10. The family of [*Balneolaceae*] (name dispute) was necessary for H7D1 to achieve the 90% coverage, while [*Tissierellaceae*], *Lachnospiraceae, Enterococcaceae, Enterobacteriaceae, Bacteroidaceae* and *Planococcaceae* were necessary for ALWL to achieve at least 90% coverage (Figure 14).

The family of *Corynebacteriaceae* was the highest in CL (32%); followed by FL (25.33%) and WL (17.22%). In addition to the fact that the lane samples were collected at the same farm from different houses and pooled together, these samples were collected during the summer, while the other fresh samples were collected during winter. In Lovanah's study on broiler microbial population distribution on Mississippi broiler farms suggested temperature and moisture affected the microbial diversity in houses [29]. Similarly, WL required six additional families to produce the at-least 90% coverage, which may due to the higher moisture content around the water lane areas.

Table 10. Top largest families observed in the fresh sample grouping.

Taxon	**H7D1(%)**	**H7D15(%)**	**H7D43(%)**	**H8D1(%)**	**H8D15(%)**	**H8D43(%)**	**ALCL (%)**	**ALFL (%)**	**ALWL (%)**	**FCT(%)**
Brevibacteriaceae	17.83 ± 1.36	22.36 ± 6.64	19.49 ± 2.47	21.53 ± 4.88	22.72 ± 4.70	20.64 ± 1.83	15.75	19.25	13.26	14.06 ± 6.65
Dermabacteraceae	17.43 ± 1.34	15.66 ± 1.55	17.20 ± 1.27	19.68 ± 0.27	18.18 ± 1.24	13.66 ± 1.69	7.11	7.34	5.45	11.14 ± 2.62
Staphylococcaceae	8.84 ± 2.35	18.97 ± 4.29	9.85 ± 2.45	6.47 ± 0.73	18.27 ± 3.74	18.44 ± 1.33	21.42	26.59	20.32	7.69 ± 3.44
Bacillaceae	9.81 ± 1.51	6.61 ± 2.14	8.39 ± 4.32	7.28 ± 2.24	5.15 ± 0.18	2.76 ± 0.43	1.25	1.32	1.08	14.83 ± 7.68
Corynebacteriaceae	8.41 ± 4.79	8.02 ± 1.88	13.80 ± 6.49	8.16 ± 3.49	7.23 ± 1.04	14.43 ± 2.22	32.27	25.33	17.22	13.51 ± 3.38
o_Actinomycetales; Other	10.08 ± 4.54	6.81 ± 1.50	5.28 ± 2.02	6.92 ± 1.64	5.67 ± 1.04	2.72 ± 0.18	0.57	0.74	0.63	2.69 ± 0.60
Yaniellaceae	6.96 ± 1.65	6.18 ± 0.73	7.91 ± 3.23	8.92 ± 0.46	7.04 ± 1.39	6.44 ± 0.54	8.27	6.67	6.01	10.62 ± 2.09
Nocardiopsaceae	2.52 ± 1.04	2.38 ± 0.43	2.62 ± 2.02	6.52 ± 1.51	3.11 ± 0.92	0.89 ± 0.07	0.98	1.20	1.60	9.79 ± 4.29
Lactobacillaceae	2.20 ± 0.76	2.91 ± 1.46	3.23 ± 2.58	2.23 ± 1.08	1.88 ± 0.60	7.69 ± 1.72	4.61	3.91	6.89	4.53 ± 3.01
Aerococcaceae	2.31 ± 1.18	1.82 ± 0.72	3.09 ± 0.78	2.67 ± 0.84	2.33 ± 1.14	4.04 ± 0.77	3.98	3.84	4.99	5.01 ± 2.37
Nocardioidaceae	2.69 ± 1.48	1.54 ± 0.44	0.50 ± 0.23	1.81 ± 0.28	1.55 ± 0.31	0.50 ± 0.04	0.09	0.13	0.10	0.28 ± 0.03
[*Balneolaceae*]	4.05 ± 4.26	0.98 ± 0.44	0.47 ± 0.47	0.71 ± 0.44	0.93 ± 0.31	0.29 ± 0.08	0.00	0.00	0.00	0.01 ± 0.01
[*Tissierellaceae*]	0.26 ± 0.23	0.16 ± 0.07	0.19 ± 0.07	0.50 ± 0.46	0.29 ± 0.27	1.90 ± 1.16	0.13	0.03	2.48	0.94 ± 0.73
Lachnospiraceae	0.34 ± 0.12	0.24 ± 0.03	0.26 ± 0.17	0.21 ± 0.15	0.23 ± 0.11	0.47± 0.10	0.31	0.44	2.19	0.12 ± 0.07
Enterococcaceae	0.15 ± 0.05	0.28 ± 0.12	0.13 ± 0.06	0.16 ± 0.11	0.16 ± 0.01	0.20 ± 0.02	0.27	0.24	2.18	0.13 ± 0.07
Enterobacteriaceae	0.05 ± 0.03	0.26 ± 0.13	0.01 ± 0.01	0.06 ± 0.04	0.38 ± 0.23	0.17 ± 0.10	1.14	0.40	2.09	0.08 ± 0.02
Bacteroidaceae	0.01 ± 0.0	0.08 ± 0.05	0.03 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.14	0.00	0.03	4.88	0.14 ± 0.19
Planococcaceae	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.0	0.03 ± 0.01	0.02 ± 0.00	0.03 ± 0.01	0.08	0.03	1.72	0.08 ± 0.05

**The light grey background was only necessary to include for H7D1 and the dark grey background was necessary to include for ALWL to achieve 90% coverage

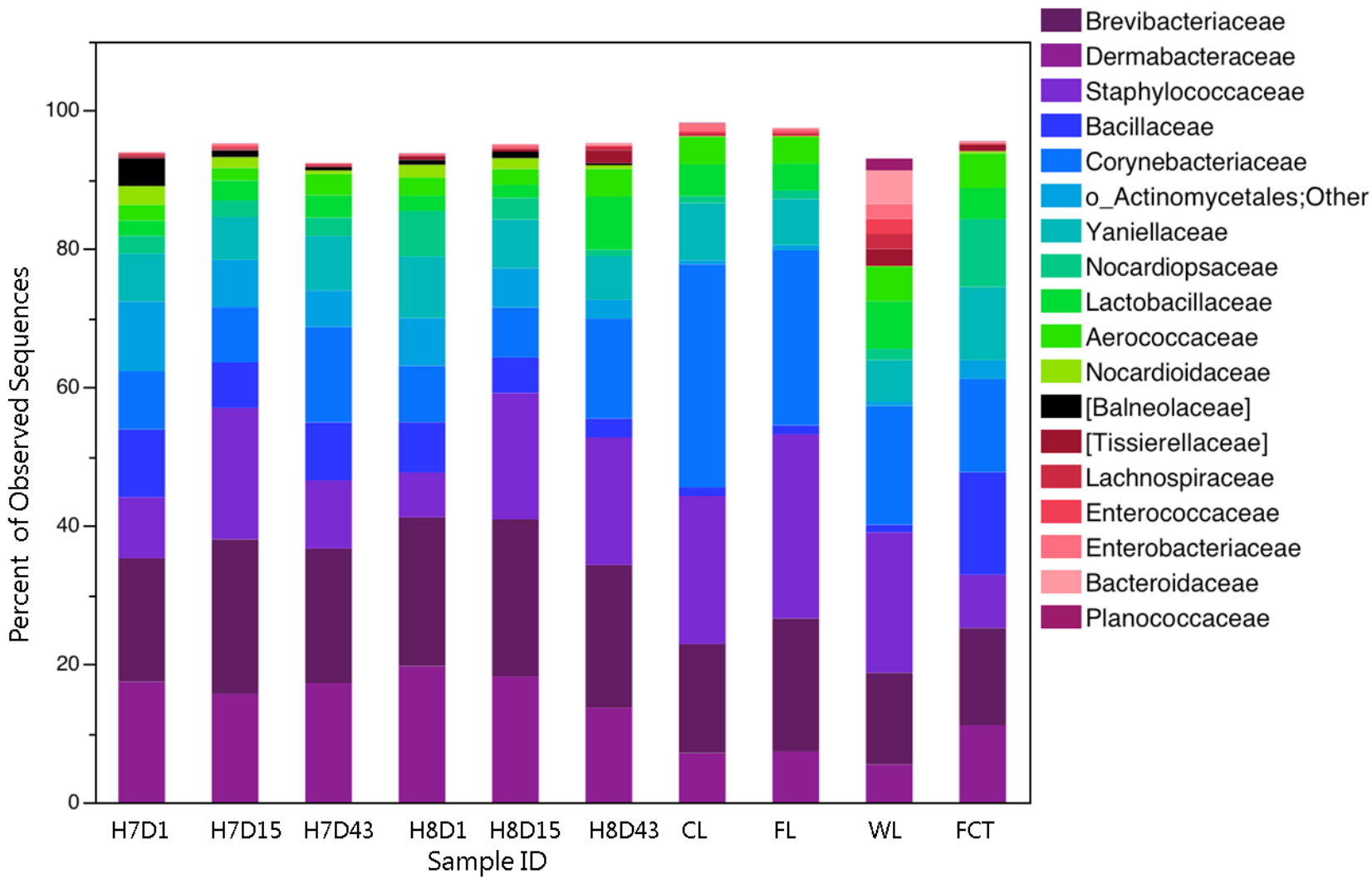

Figure 14. Top largest families within fresh sample grouping.

Among all these samples, the H7 and H8 samples were compared with each other. The families of *Brevibacteriaceae*, *Staphylococcaceae*, *Dermabacteraceae*, and *Corynebacteriaceae* were the largest families in all of the timed samples (Figure 15). *Brevibacteriaceae* was the highest in H8D15 (22.72% ± 4.70%) followed byH7D15 (22.36% ± 6.64%). Interestingly the family of *Staphylococcus* was less than 10% at day 1, and increased to over 15% by day 15, while at day 43 in house 7, this was dropped to less than 10%, it remained at over 15% in house 8 at day 43. Day 43 was the last day before birds were sent off for processing. The only statistically significant different comparison was generated between H7D43 and H8D43 (corrected p-value< 0.033). This difference was surprising, since both samples were collected from same style of houses at the same time. Both also had similar moisture content of the litter, and had the same management practices. Nonetheless this was the only statistically different results, and the R^2 values comparison between H7D43, and H8D43 yield 0.86, which suggest the strong similarity between the samples (Figure 16). The overall bacterial community structural at the end of production remained as *Brevibacteriaceae*, *Dermabacteraceae*, and *Corynebacteriaceae*. In addition, all of these families had p-values greater than 0.05, indicating that the major bacterial community structure was similar at day 43. Moreover, ANOSIM with Weighted Unifrac distances of the grouping based on time of collection within each houses showed no statistical difference (p-value: 0.066, R value: 0.186). Conversely, the moisture grouping found differences (p value < 0.05). These findings matched Wadud et al. study of microbial fingerprints generated from litter samples that showed a strong association to the moisture content while the microbial community appeared random to time [94].

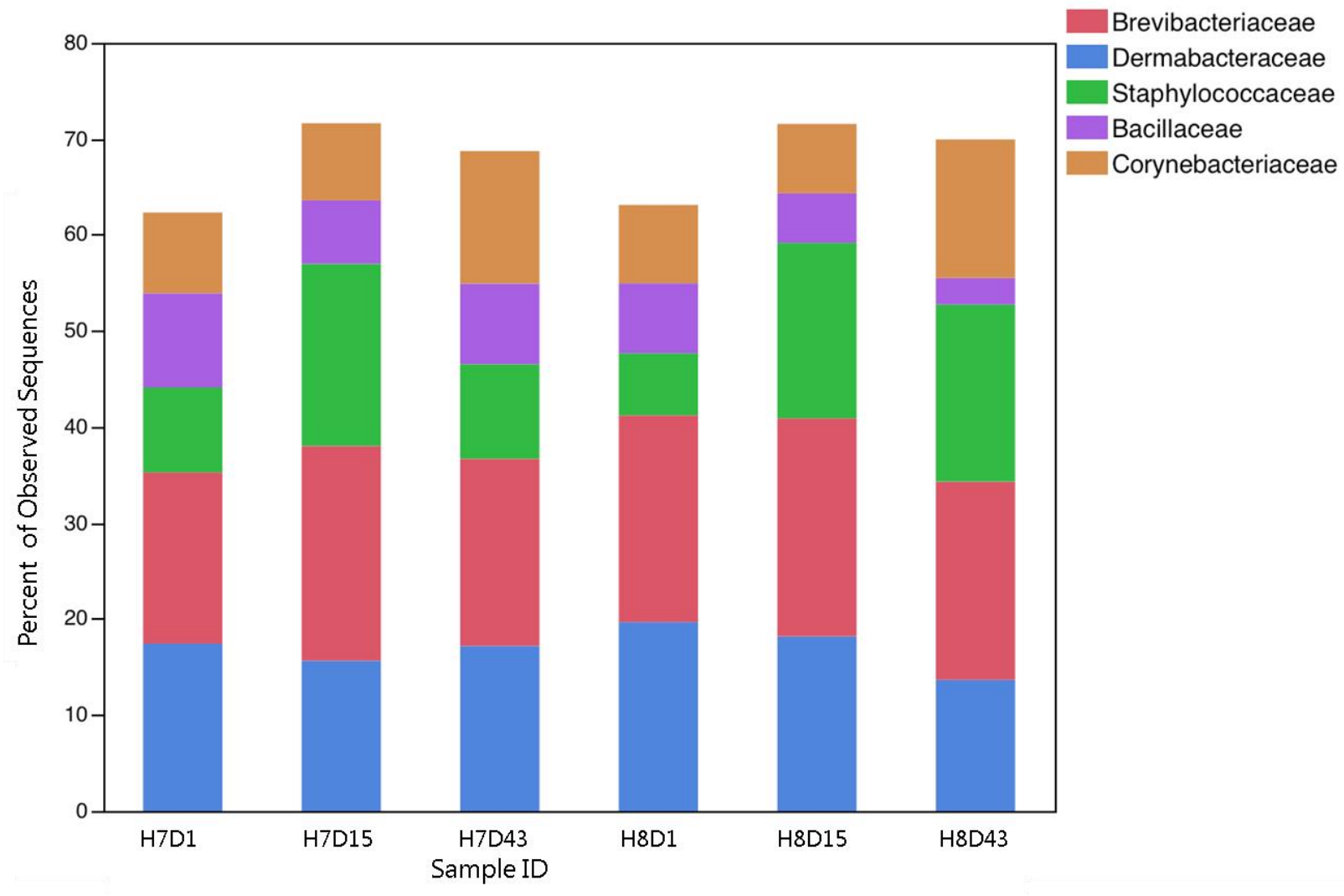

Figure 15. Dominant families in houses during the 44-day production

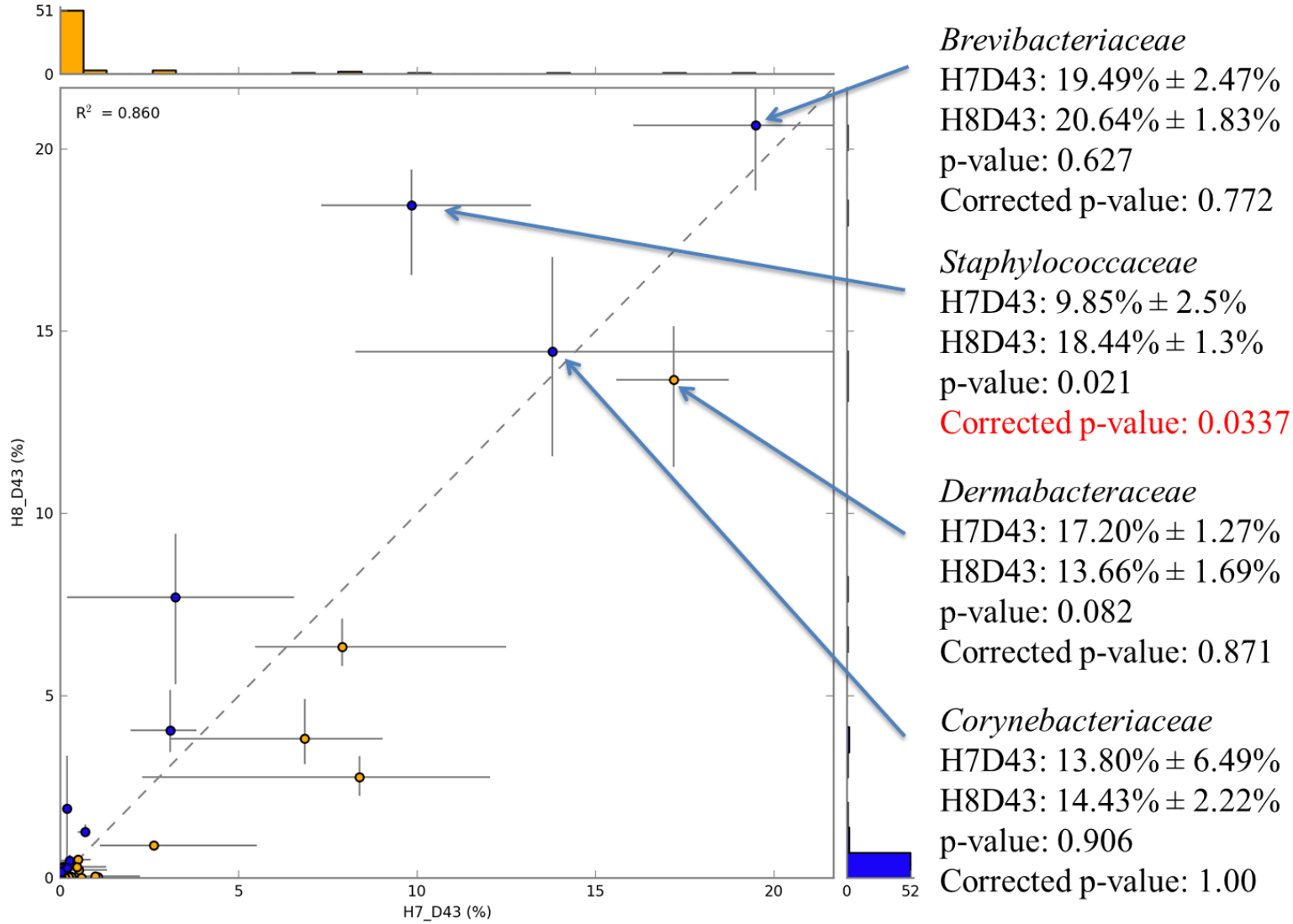

Figure 16. The scatter plot between Houses 7 & 8 at day 43.

FCT vs. H7D1, day 15 & 43 and H8D1, 15, &43 were compared to determine possible correlations for fresh sample between farms. This comparison can provide information regarding the litter bacterial community structure at a similar age from different locations, moisture, and producers. With Benjamini-Hochberg correction, there were no statistical differences among families for FCT vs. H7D1, 15, & 43 and H8D1, &15 comparisons. The lack of difference was indicative that the litter bacterial community structural was not greatly impacted by location, bedding material, or moisture content.

The comparison between FCT and H8D43 revealed statistically difference in families *Mycobacteriaceae, Micrococcaceae*, and *Intrasporangiaceae* (corrected p-values of 0.034, 0.036, and 0.034 respectively). All of these families belonged to the phylum of Actinobacteria. Nevertheless, these differences were observed for families with a low abundance (less than 1%). It was interesting to note that while FCT was collected from a different farm with different geographic area, moisture content and integrator; the overall bacteria structure still remained rather similar to the timed and lane series. However, FCT samples were collected right after the litter was removed from the house, which gave these samples a longer time absent of bird contact. Furthermore, the family of *Bacillaceae* was the highest in FCT (14.83% ± 7.68%) among all of fresh samples, and the large percentage of *Bacillaceae* seemed to be the most prominent trait among all of the LTS. This suggests that Bacillaceae systematically increases as broiler litter ages.

Potential Microbial Source Tracking Organisms and Pathogens

The Environmental Protection Agency uses methods that rely on the presence of indicator microorganisms including *E. coli*, Enterococci and Strepococci to detect fecal contamination of surface waters [95]. None of these traditional fecal pollution indicator microorganisms occurred at a high concentration the fresh or LTS samples. The genus that contained *E. coli* was most numerous in the water line sample, but represented only 1.3% of the total sequences recovered. All of sample sequences contained less than 0.5% of the family of Enterococcus, and at most 0.1% of the family of Streptococcus among the LTS samples.

The families of *Bacillaceae*, *Nocardiopsaceae*, and *Brevibacteriaceae* were used to generate the at least 90% coverage among all of our samples (Figure 17), both fresh and LTS. Species in *Brevibacteriaceae* was previously found to be specific to poultry and has been proposed as a poultry litter microbial source tracker [27]. Poultry litter is typically stored before being land application when crop nutrient demand is expected to be high. While the *Brevibacteriaceae* family was found in all samples, this family was present at a lower population level among the LTS samples. Population data from the current study indicates that other families, such as *Bacillaceae* and *Nocardiopsaceae*, could be examined for microbial source tracking genus/species specific to broiler litter (Figure 17); particularly, genus *Virgibacillus* in the family *Bacillaceae*, and the unknown genus in the family *Nocardiopsaceae*. Genus *Virgibacillus* has been reclassified from the genus Bacillus [96] and is present in both soil and water samples [97-100]. There is no known pathogenic or significant function associated with this genus. The family *Nocardiopsaceae* was only recently created to accommodate the genus *Nocardiopsis* [88], and members of this family have been often found in soils [89].

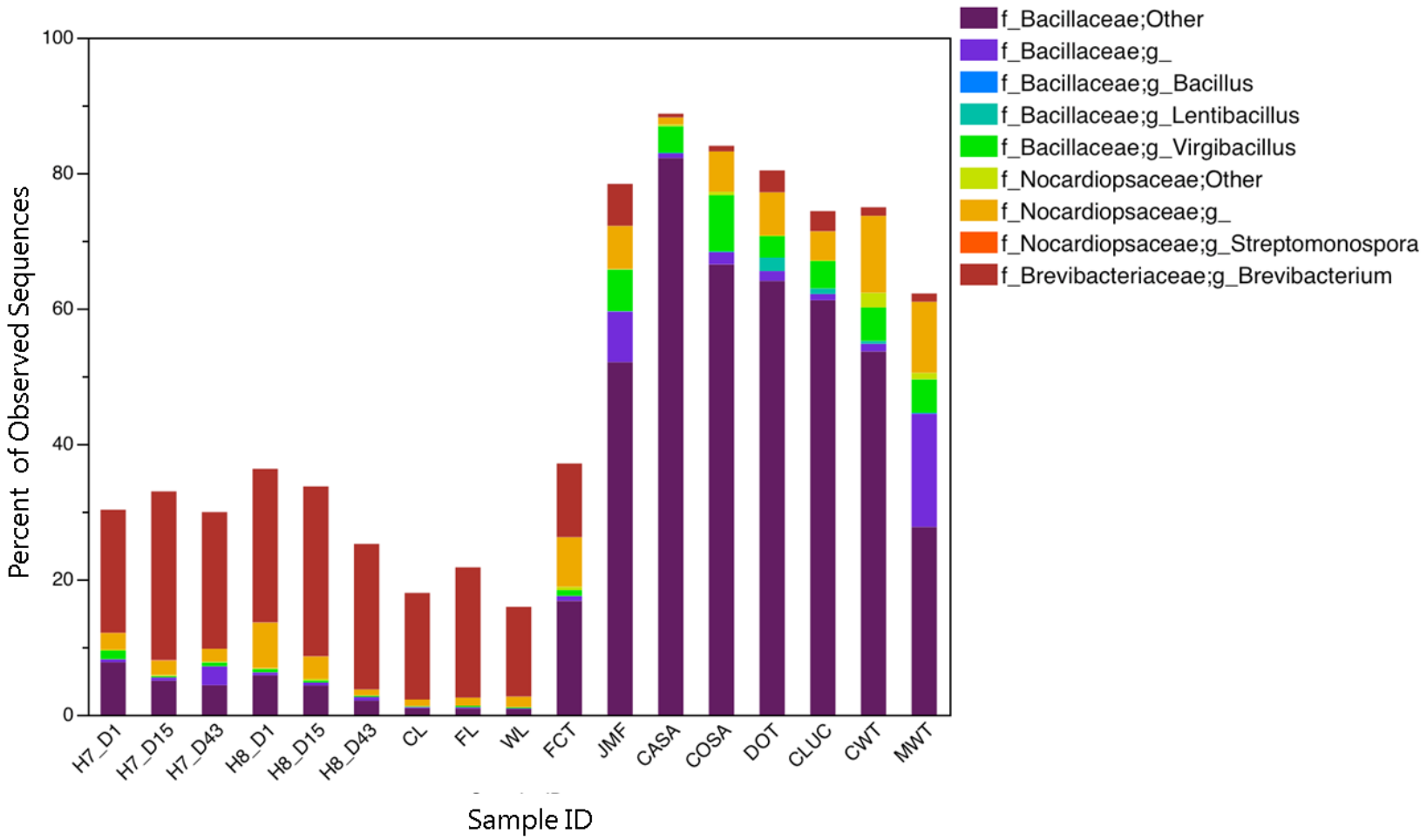

Figure 17. Potential Genus for microbial source tracking.

The litter microbiomic data was examined for the presence of common poultry pathogens, including the genera *Campylobacter*, *Clostridium*, *Escherichia*, *Salmonella*, and *Staphylococcus*. The species *Campylobacter Jejuni* is a gram-negative bacterial pathogen that is the leading cause of food borne illnesses in the U.S. and other developed countries [101]. Species in the genera *Clostridium* and *Staphylococcus* contain pathogenic strains associated with the deadly poultry disease known as gangrenous dermatitis (GD) [102, 103]. Additionally, certain *Clostridium* species can damage the poultry digestive system which in turns reduces weight gain [59]. *Escherichia coli* is naturally present in the intestinal tracts of warm-blooded animals [104]. In poultry, avian pathogenic *Escherichia coli* (APEC) causes colibacillosis, a complex syndrome characterized by multiple organ lesions [105]; it can also cause food poisoning [106]. There are only two species in the *Salmonella* genus, and both are responsible for human related diseases such as gastroenteritis and typhoid fever [107]. Staphylococcus is the most widely found pathogen in poultry litter [39, 81]; this pathogen is well known to be antibiotic resistant [108, 109]. Pathogenic *S. aureus* is associated with over 200,000 cases of food-borne illness annually [110].

The family *Staphylococcaceae* was found in all the litter samples tested. The highest relative proportion for the *Staphylococcus* genus occurred on day 15 in both houses (H7D15= 19% ± 5%, H8D15=18% ± 5%), as well as the lane series (ALCL=21%, ALFL=27%, and ALWL=20%). All of the lane samples had over 5% (in H7D15 and H8D15, this was over 9%). The feed lane had the highest distribution among all of our lane series at 9.4%, followed by center lane at 7.1%. This family was found at less than 4% in any of the LTS sample.

The genus *Escherichia* had the highest proportion of the recovered genetic sequences in the water lane sample (1.3%), followed by center lane (1%), and feed lane (0.4%). This genus

was only observed at a concentration less than 0.4% in the fresh and LTS samples. Similarly, Martin et al. could only detect *E. coli* in four out of 86 poultry litter [8].

Clostridium sequences constituted less than 0.3% of the sequences recovered in all of the poultry litter samples examined. Alexander et al. detected *Clostridia* spp. from 57% of litter samples [81]. In Lu et al's 16S gene targeted study, over 7% of all potential pathogenic strains were under *Clostridium* [39]. *Campylobacter* and *Salmonella* genetic sequences were absent in all of the poultry litter samples examined. *Campylobacter* has been inconsistently detected in poultry litter using PCR. In the Chinivasagam et al. study, *Campylobacter* was detected in all of litter samples (28 sheds) [111] but was absent in the studies conducted by Lu and Roberts [39, 112] . Similarly, in Australian broiler farms, *Salmonella* was detected in 83% of the farms that reused litter, and 68% of farms that performed total litter cleans outs [111]. This was different than Lu et al. study in which *Salmonella* was absent from all litter samples [39].

The lack of pathogenic sequences in this study could be due to the lack of previous disease outbreaks, but this fact did not necessarily mean the litter was totally absent of pathogenic bacteria. For example, in the Dumas et al. pyrosequencing study of broiler houses with a previous history of GD, there were no clear bacterial community structural differences compared with broiler houses that did not have a history of GD [54]. In their study, they found a low concentration of *Clostridium* spp. in all of the poultry houses and a high concentration of *Staphylococcus*. These findings were very similar to our findings that suggest the possible low prevalence of pathogens in litter.

IV. CONCLUSIONS AND RECOMMENDATIONS

To my knowledge, this is the only broiler litter bacterial community structure study that used Illumina MiSeq sequencing technology. This study successfully provided a much more detailed description of the poultry litter bacterial community within both fresh and stored litter samples. According to our data, Actinobacteria and Firmicutes were the main contributors to the overall community structural at phylum level. Actinobacteria was the dominant phylum in fresh samples, and Firmicutes was the dominant family in the long-term stored samples. While the family *Bacillaceae* was presented at greater than 50% in all of our LTS samples, it constituted less than 10% in fresh samples. The fresh samples had higher percentages of *Brevibacteriaceae, Corynebacteriaceae, Dermabacteraceae,* and *Yaniellaceae* than LTS. These families also accounted for the most changes between the fresh and LTS. To address the proposed hypotheses, the community structural at family level was evaluated for both LTS and fresh sample groupings. Additionally, ANOSIM was used to compare the sample groupings. The overall community structure at family level was similar within the LTS grouping, and was similar within the fresh sample grouping. ANOSIM revealed that within LTS samples, bedding materials across the farms were not statistically significant (p value > 0.05). There were differences observed among producers and integrators groupings (p-values < 0.05). Moreover, the groupings of producers were stronger than integrators (R values of 0.892, and 0.249 respectively). Within the fresh samples, there was no statistical differences observed between the 3 times during production (p- value > 0.05), but differences were observed within different moisture groupings (p value < 0.05). Thus, overall the bacterial community structural within houses during production was similar. The family *Nocardiopsaceae* was found to be more prevalent in LTS, and less prevalent among fresh samples. Due to the high distribution of

Bacillaceae and *Nocardiopsaceae* in both the fresh and LTS, I proposed that genus such as *Bacillus, Lentibacillus* and *Virgibacillus* in the family *Bacillaceae* and *Streptomonospora* in the family Nocardiopsaceae could be studied as potential organisms for broiler litter specific microbial source tracking.

In all of our samples, I did not observe a significant amount of pathogenic strains either. Despite the wealth of information presented regarding poultry bacteria community structure, I was not able to pin point any specific species with these experimental methods. Further experiments that target the specific organism would be ideal and necessary to detect the potential pathogens and species specificity to poultry. We did however, present here the overall bacteria structure for litter during and post production. This study also provides opportunity for further investigations in the specific bacterial species within litter.

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