HIGH-THROUGHPUT 16S AMPLICON ANALYSIS OF POULTRY LITTER MICROBIAL COMMUNITIES AND POSSIBLE LINKS WITH RECURRING OUTBREAKS OF GANGRENOUS DERMATITIS IN CHICKENS

by

Michael Dumas

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LIST OF ABBREVIATIONS

ATP - Adenosine Triphosphate

BLAST - Basic Local Alignment Search Tool

CCD – Charge-Coupled Device

CFU – Colony Forming Unit

DBI – Delaware Biotechnology Institute

DGGE - Denaturing Gradient Gel Electrophoresis

DNA - Deoxyribonucleic acid

dNTP - Deoxyribonucleotide triphosphate

DOTUR – Distance-Based OTU and Richness

RDP – Ribosomal Database Project

DPCoA – Double Principle Coordinate Analysis

EFM – Epifluorscent Microscopy

GD – Gangrenous Dermatitis

GIT – Gastrointestinal Tract

IBD –Infectious Bursal Disease

IGS – Institute for Genome Sciences

Infernal – INFERence of RNA ALignment

LSPN - List of Prokaryotic names with Standings in Nomenclature

LSU – Large subunit

MSA – Multiple Sequence Alignment

NAST – Nearest Alignment Space Termination

NCBI – National Center for Biotechnology Information

OTU - Operational taxonomic Unit

PBS – Phosphate Buffered Saline

PCA – Principle Component Analysis

PCR – Polymerase Chain Reaction

PFGE - Pulsed Field Gel Electrophoresis

RAPD - Random Amplification of Polymorphic DNA

RDP – Ribosomal Database Project

RNA - Ribonucleic acid

S – Svedberg unit

SBS –Sequencing By Synthesis

SOLiD – Sequencing by Oligonucleotide Ligation and Detection

SSU – Small subunit

WGS – Whole Genome Shotgun Sequencing

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ABSTRACT

The production of chicken for their meat (broilers) is a multi-billion dollar industry in which large firms contract the chicken rearing out to individual local farms. Poultry litter, composed of bedding material, chicken excrement and feathers, and spilled feed is often maintained on the floor of the house through many successive growing cycles. The litter generated by this process is used extensively as both feed for other domestic animals and as crop fertilizer. High-throughput sequencing was chosen as a means to assess the richness and diversity of the poultry litter environment.

Samples from under water lines (Wet) and from common areas away from water and feed lines (Dry) were collected from four commercial broiler houses in the Delmarva region. Over 22,000 bacterial 16S rRNA gene sequences were produced using 454 pyrosequencing. All 16S amplicon sequences were assigned to operational taxonomic units (OTUs) at the 95% identity level. 16S OTU clustering by house reveals that the dominant bacterial taxa are not distributed evenly across houses. Furthermore, the distribution of OTUs between Wet and Dry litter samples also reveal dramatic differences. Comparing the most abundant OTUs from each sample type reveals a greater amount of richness and diversity in the Wet litter, with 90% of the total abundance covered across the top 214 OTU clusters. In contrast, the top 50 clusters cover 90% of the total abundance in the Dry litter. This increased diversity is further supported by a number of high abundance OTUs found to be derived entirely from Wet litter sequence. One house, using a modern cooling system, contained the highest

number of unique OTU clusters. One of the unique clusters from this house was the sixth largest OTU cluster overall and classified as an *Arcobacter* sp., a potential human pathogen. Additionally, enumeration of both total virus and bacteria in litter by direct counts found a ten to one hundred-fold greater abundance compared to that found in soils.

Two of the broiler houses sampled had experienced recurring outbreaks of the poultry disease gangrenous dermatitis while the other two had no history of the disease. Although the primary pathogenic *Clostridial* and *Staphylococcal* species were not found in any house, there were 13 OTUs found primarily in houses affected by recurring GD. These results provide a starting point for future studies aimed at clarifying the relationship between the microbial community of litter and poultry disease.

RIBOSOME AND SEQUENCING REVIEW

Name Structure and function

Although electron microscope images in the 1940's and 50's first described the ribosome as an organelle (Palade 1954), the actual term "ribosome" was not coined until 1958 by R.B. Roberts at the inaugural symposium of the Biophysical Society. At that time, little was known about the ribosome besides its size, the fact that it was composed of both RNA and protein, and was involved in protein synthesis. Ever since these initial discoveries, researchers have been peeling away the mysteries of the ribosome and have come to understand its indispensable nature in all branches of life.

The ribosome is a complex molecular machine whose primary role is to facilitate the translation mRNA to protein (Alberts et al. 2002). Structurally, it can be broken down into two major subunits, appropriately named the large and small ribosomal subunits (LSU and SSU) (Fig 1.1). The LSU, called the 50S[1] subunit in bacteria is made of approximately 34 proteins and two RNA molecules, 5S and 23S. It is responsible for catalyzing the formation of a peptide bond in a growing chain of amino acids (Frank et al. 2000, Alberts et al. 2002).

[1] The abbreviation "S" stand for Svedberg unit. It is a measure of sedimentation coefficiency named after Nobel laureate Theodor Svedberg, one of the primary developers of analytical ultracentrifugation. Because early ribosomal study identified components based on their sedimentation rates, the various RNA components are still described in this manner. Since this is not a direct measure of size, the full bacterial ribosome is named "70S" while the two subunits are 50S and 30S.

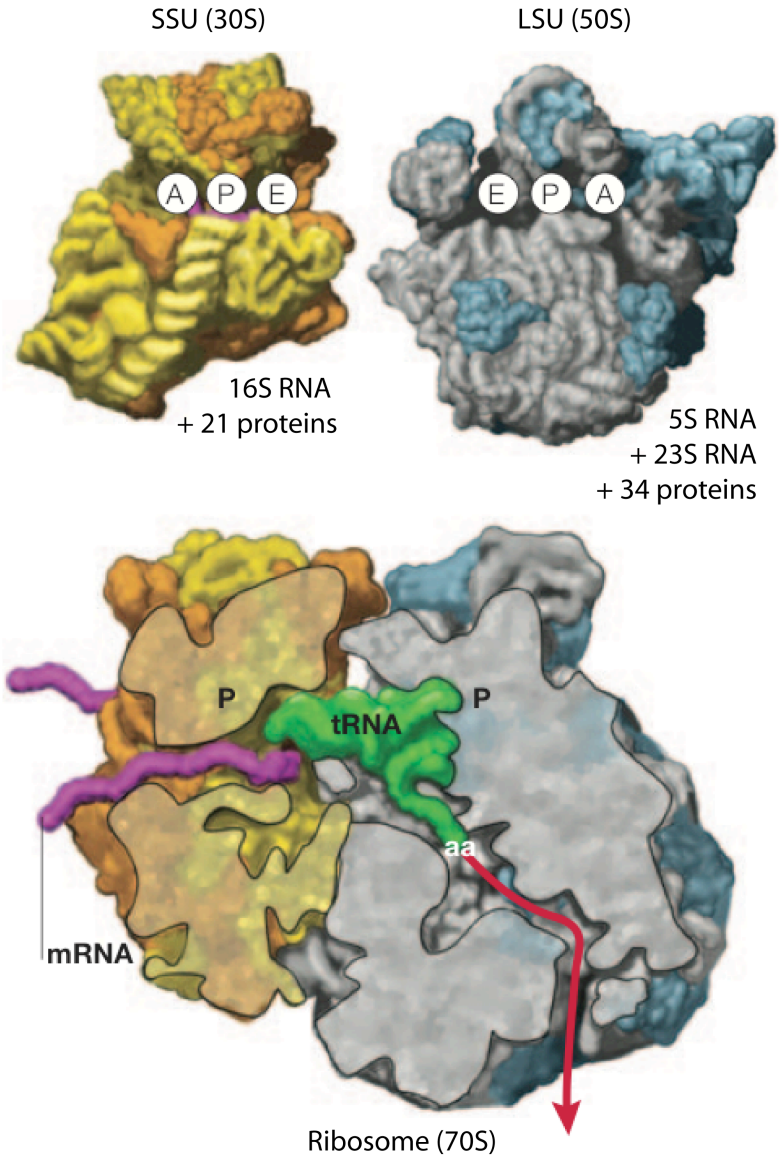

Figure 1.1 (Adapted from Poehlsgaard & Douthwaite 2005): Space filling model of the large and small ribosomal subunits (top). Yellow and gray represent RNA and orange and blue represent proteins. Below the subunits is a cutaway view of the full ribosome depicting where the mRNA (pink), tRNA (green) and growing amino acid chain (red arrow) fit.

The SSU, named the 30S subunit in bacteria is composed of a 16S RNA molecule and 21 proteins. It is responsible for ensuring accurate mRNA-tRNA interaction. Although around 50 proteins are integral parts of the ribosome, the RNA makes up nearly 2/3 of the volume (Frank 2000).

Based on our current understanding of cellular biology, all living organisms must have all of the genes coding for the RNA and protein components of the ribosome. The 16S RNA molecule in the SSU is ideally suited for inferring relationship between organisms. Because, as RNA, it serves its role with no need for translation, each base is directly tied to function rather than providing codon information for protein translation. Additionally, the 16S RNA was highly valued for its ease of purification and the fact that its sequence could be directly obtained using reverse transcriptase (Lane et al. 1985).

The SSU 16S rRNA gene is essential to protein synthesis and as a result contains numerous regions which are highly conserved across all taxa (Van de Peer et al. 1996, Woese 1983, Van de Peer et al. 1996). The conserved regions are under strong selective pressure as small changes in the 16S gene can inhibit protein synthesis thus slowing growth or causing complete lethality. The very slow rate of change in these regions can be used to infer deep branching phylogenetic relationships (Fox et al. 1980, Woese 1987). The regions of the 16S gene under weaker selection are equally important to phylogenetic classification. When the SSU RNA is correctly folded and complexed with its respective proteins, some regions are able to sustain mutations without loss of function. Researchers have identified nine such variable regions within the 1,540

bp 16S rRNA SSU gene (Van de Peer et al. (1996) (Fig 1.2). The variable regions, due to their higher mutability, can be used to infer phylogenetic difference at a much finer level than the conserved regions and can be used to differentiate bacteria at a species and even strain level (Schmidt et al. 1991, Chakravorty et al. 2007, Huse 2008).

The highly conserved regions of the gene have allowed researchers to develop “universal” primers which will bind to the ribosomal gene in all bacteria and allow amplification of the variable regions (Baker et al. 2003). Research groups must use caution if only selecting specific variable regions to analyze, as it has been shown that all regions are not equal in their ability to accurately classify the known bacterial genera (Mills et al. 2006, Chakravorty et al. 2008, Youssef et al. 2009). There is still considerable debate over which hypervariable regions should be used for the most accurate classification. Initial study found both the V2, V3, and V6 regions to be best suited for distinguishing between bacterial genera of pathogenic clinical isolates, with V4, V5, V7, and V8 being less helpful in accurate species classifications (Chakravorty et al. 2008). Other research using reference sequences from huma stool, microbial mats, and the murine distal gut found that the V1+V2, and V3 regions were best at accurately assigning correct taxonomy (Liu et al. 2008). A recent study concluded that the V1+V2 and V6 regions overestimated bacterial richness while the V3, V7, and V8

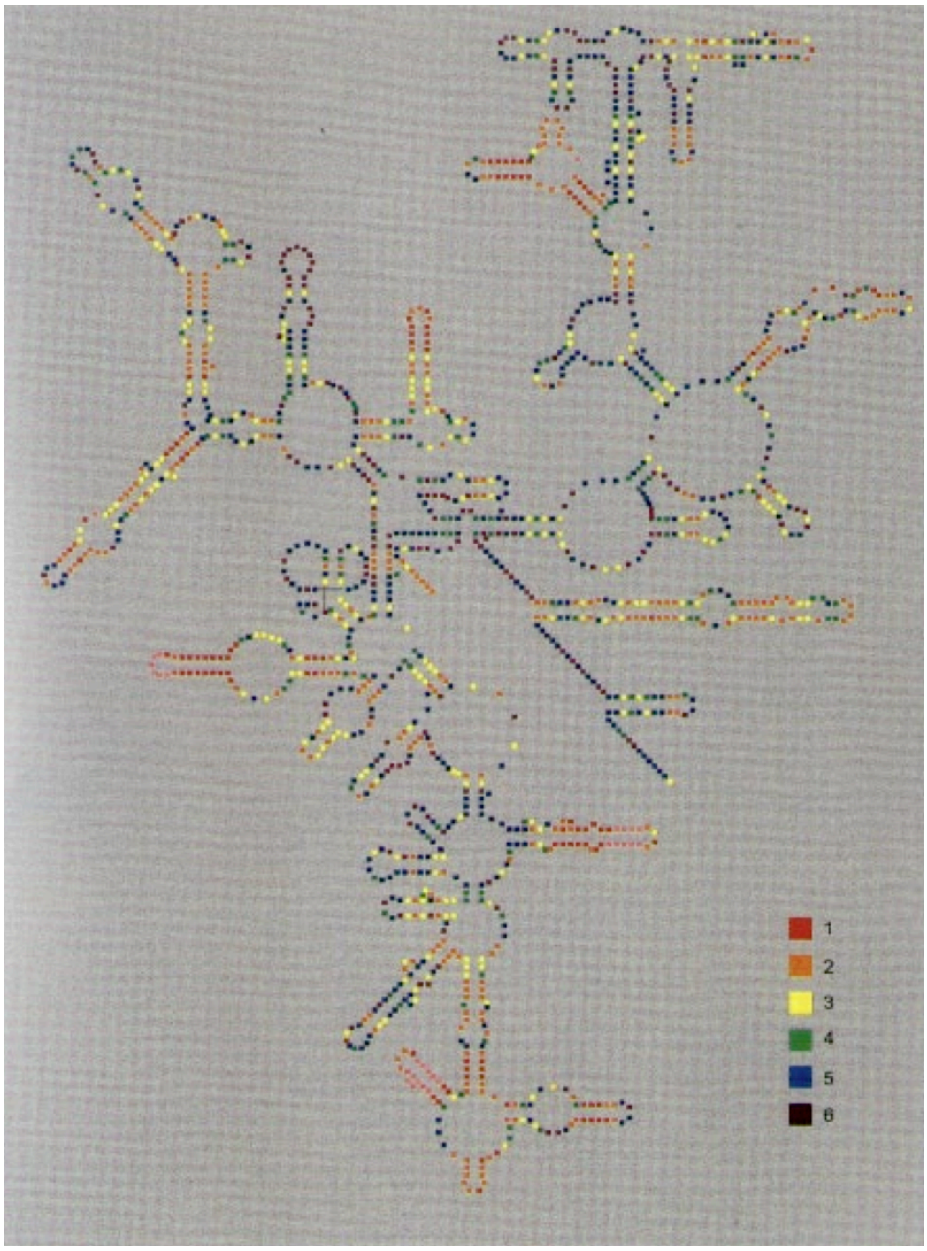

Figure 1.2 (From Van de Peer 1996): *Escherichia coli* Ribosomal 16S gene folded into its secondary structure. Colors red, orange, and yellow represent highly conserved residues. Colors blue, green, and purple represent less conserved residues.

regions underestimated richness (Youssef et al. 2009). Unlike the pathogen-based study, Youssef et al. (2009) recommended that V4, V5+V6, and V6+V7 were best for providing accurate richness estimates. This is clearly a significant problem facing many of the high throughput technologies in which the full-length 16S gene cannot yet be amplified. In this study, we chose the V1+V2 region based on the currently available information at the time and the fact that they had been used successfully to detect a variety of pathogens. As this study aimed to examine the litter community in relation to the incidence of recurring gangrenous dermatitis, this seemed to be the best choice.

Sequencing and Analysis

Sequencing Introduction

Over the past four decades, DNA sequencing has gone from a time consuming, arduous, and error prone process that yielded only a small amount of sequence information, to a high throughput, often highly automated pipeline producing millions of base pairs of sequence in a matter of hours. Multiple research group groups have developed tools and databases to deal with this onslaught of data. This review will focus on the relevant databases and methods used to produce and analyze 16S sequence data and highlight the ones used in this study.

In the Beginning: Maxam-Gilbert and Sanger sequencing

The era of "rapid" sequencing began in the 1970's and was based on research by Frederick Sanger, Allan Maxam, and Walter Gilbert. The Maxam-Gilbert or "chemical" sequencing method used a number of chemicals to cleave radiolabeled DNA between specific bases. The resulting fragmented DNA could then be run on a polyacrylamide gel and analyzed to determine the DNA sequences (Maxam & Gilbert 1977). This process was preferable to previous methods because it could be used for both single or double-stranded DNA, and did not require an initial cloning step to produce a single stranded DNA molecule. This approach became widely used in the late 1970's and early 1980's (Hutichson 2007).

The same year the Maxam-Gilbert method was published, what has become known as the Sanger, or chain-termination sequencing method was also developed and published (Sanger Et al. 1977). This method employs the use of dideoxynucleotides triphosphates[2] (ddNTPs) that inhibit the formation of the phosphodiester bond on a growing DNA strand. In vitro synthesis of a template strand of DNA using radiolabeled deoxynucleotides and dideoxynucleotides was carried out using a synthetic primer and DNA polymerase and synthesized products were run on a high resolution polyacrylamide gel. DNA amplicons which incorporate a ddNTP were terminated and appeared as different sized bands on the gel (Sanger et al. 1977). Initially the method employed

[2] In the initial experiment, araCTP instead of ddCTP was used as the chain terminator.

radiolabeling and four separate mixtures, each with a different ddNTP. Each of these four reactions were run separately.

Automation and Shotgun sequencing

Subsequent improvements to the Sanger method increased the throughput while simultaneously reducing the amount of reagents used per run. Instead of running a radiolabeled reaction in separate lanes for each ddNTP, a fluorescent dye covalently attached to each base was run in separate reactions, pooled, and then run on a single gel tube (Smith et al. 1986). The peaks in fluorescence were measured and stored on a computer, negating the need for autoradiographic gel imaging. Using single or multiple fluorophores instead of radiolabeled dNTPs was quickly adopted and in 1987, Ansorge et al. reported the development an automated sequencer using this method. There was some initial skepticism centered on the accuracy of base calling software. Many researchers had come to trust their workforce of dedicated graduate students to accurately assign sequence by studying gels. The “man vs machine” conflict was soon concluded as the efficiency and accuracy of the new automated sequencers became apparent (Hutichsom 2007).

Despite the switch to the use of fluorophores and automation, sequencing genomes larger than a small virus was limited by the laborious steps of cloning, selection of plasmids or cosmids, genome mapping by restriction digestion. Although obtaining sequence by random fragmentation had been employed by Sanger’s group, using this method for larger genomes was unfeasible due to the

genome size itself, the amount of sequencing need to obtain full coverage, and the lack of computer programs able to accurately assemble sequenced fragments (Fleischmann et al. 1995). Computing advances allowed these difficulties to be surmounted, and through use of a random DNA fragmentation and a cloning technique dubbed "shotgun sequencing", the first complete genome of an organism, *Heamophilus influenzae*, was produced (Adams et al. 1991, Fleischmann et al. 1995). Whole-genome shotgun sequencing (WGS) has been used to obtain sequences from many organisms including *Homo sapiens*[3] at a fraction of the time and cost of map-based sequencing approaches.

Metagenomics and Pangenomics

Because the process of shotgun sequencing does not require prior knowledge of the sequence for amplification, researchers can obtain previously unstudied DNA from one or multiple organisms. As throughput has increased, given adequate sequence coverage, entire genomes can be assembled from an environmental sample. Metagenomic studies have only further emphasized the idea that our current understanding of biological diversity for both bacteria and viruses is woefully shallow (Rusch et al. 2007, Frias-Lopez 2008).

It has also become clear that obtaining the "complete" sequence of an organism's genome does not encompass all genes present in that species. In

[3] The Privately funded Celera Genomics under the direction of Craig Venter was in direct competition with the government funded Human Genome Project for the first organization to determine the sequence of the "complete" human genome. The politics, policies, and controversies surrounding this competition are beyond the scope of this work, but it is generally agreed that the competition benefited both projects (Hutchison 2007).

bacteria especially, the total number of genes present in a population could be an order of magnitude greater than the average genome size (Medini et al. 2005). To fully understand a species and its role in the larger community it may be necessary to determine the pangenome that all the genes present within a species (Tettelin et al. 2005, Reno et al. 2009).

Determining the metagenome of a community and pangenome of a species is rapidly becoming feasible for modestly funded laboratories and institutions as opposed to large laboratories and sequencing centers. New generations of investigators will have unprecedented opportunities to obtain sequence data for their organism or microbial community of interest. Obtaining the complete sequence of a particular strain or subpopulation of organism under study may one day become a matter of gathering preliminary data rather than an endeavor warranting publication.

"Next generation" Sequencing

Although the term itself is rapidly becoming outdated, next generation sequencing generally refers to the first set of DNA sequencing instruments developed in the mid 2000's that are based on detecting nucleotide incorporation events as they happen, an approach called sequencing by synthesis (SBS). These technologies are more efficient in both cost and time per run. The first such platform released was the Genome Sequencer 20 (GS20) by 454 life sciences[4]. The next version of this platform, dubbed the GS FLX was used in

[4] Purchased by Roche diagnostics in 2007

this study. Other platforms that have been released in the last five years include the SOLiD system (Sequencing by oligonucleotide ligation and detection) from Life Technologies and the Genome analyzer by Illumina. The technologies vary in their sequencing method, throughput, read length, but all produce over 100Mb of sequence data per analytical run (Table 1.1) (Morozova and Marra 2008).

Table 1.1: Sequencing technologies: Sanger vs Next Generation sequencing

Technology	Approach	Read length (bp)	Bp per run	Company
Automated Sanger	dye terminators	Up to 900	96kb	ABI
454 Roche FLX	pyrosequencing	Up to 400	Up to 600Mb	Roche
Illumina	reversible terminators	Up to 100	Up to 50Gb	Illumina
SOLiD	ligation	Up to 75	Up to 300Gb	ABI

Adapted from Morozova & Marra 2008

454 pyrosequencing

The process of pyrosequencing was first reported by Ronaghi et al in 1996. Rather than sequencing by chain termination, as in the Sanger method, pyrosequencing is sequencing by synthesis. As its name implies, sequence identity is derived from the dNTPs being added sequentially by DNA polymerase. As a base is added the diphosphate cleaved by the polymerase is converted to ATP by ATP sulfurylase. The ATP is utilized by luciferase to produce light. Thus, each nucleotide incorporation event is recorded by light output. It was upon this process that a novel sequencing system was developed by Jonathan Rothberg at 454 Life Sciences Corp. The first machine was able to sequence 25 million bases with greater than or equal to 99% accuracy in four hours (Margulies et al. 2005). In the 454 sequencing process, DNA is first fragmented and

separated into single strands and ligated to DNA capture beads under conditions which promote only one bead per DNA fragment. Once bound to the bead, the fragment is amplified resulting in ten million copies bound to a single bead. The beads are deposited on a fiberoptic slide which contains wells designed to fit a single bead. A mixture of smaller beads bound to sulfurylase and luciferase are also added along with polymerase. The plate is then exposed to each dNTP in sequential order with washing in between. The result is that when a particular dNTP is exposed to the plate, any sequence with that complementary base will be added by polymerase and the resulting light will be captured by CCD image sensor. All wells are imaged simultaneously and an image is taken each time the plate is expose to a new base. In this manner, the sequence in each well is determined.

Benefits

The first iteration of the 454 sequencing platform was able to produce over 200,000 usable reads in a single four hour analytical run. The current platform produces over 1,200,000 (~550Mb of sequence) reads in a single 12h analytical run. In contrast, a 96-sample “high throughout” capillary array produces 96kb of Sanger sequence data in 3 hours (Morozova and Marra 2008). One of the largest benefits of the 454 technology is its *in vitro* amplification system. It is necessary in Sanger sequencing to amplify DNA fragments *in vivo* using bacterial hosts. This process is time consuming and labor intensive. It also introduces potential bias depending on the host and some elements of a genome

may be resistant to cloning. The emulsion PCR step used by 454 sequencing negates all of these issues.

Drawbacks

For small and even moderately sized universities, purchasing a pyrosequencing machine can be prohibitively expensive. Also, a full analytical run of 454 sequencing can cost over ten thousand dollars. However, many studies do not require a full plate to be run, so gaskets can be used to cover parts of the plate in order to cut down on reagent use. In lieu of having to wait on multiple 454 runs to produce sequence for studies requiring less than a full plate, small 6-8 bp unique sequences can be incorporated into the primers used for initial sample amplification. In this manner, each different sample will have a unique sequence tag or "barcode" when it is sequenced by the 454 run. After sequencing, software to detect the barcodes can separate all of the reads into their respective samples. Because of barcoded primers, hundreds of samples can be run in parallel on the same plate (Binladen et al. 2007, Meyer et al 2008). It is also important to note that the cost per base is around an order of magnitude lower than more traditional platforms (Wommack et al. 2008). Another drawback of the system is its inability to accurately determine the length of long single nucleotide homopolymers. Signal intensity increases in a roughly linear fashion as homopolymer length increases, but after eight or more bases, the signal is often misinterpreted (Marguiles et al. 2005). These errors can often be corrected when building a single genome through sequence coverage, but is more

problematic for study of unknown and environmental sequences. Short metagenomic sequence reads are often unable to be accurately matched to longer well-annotated, Sanger-derived sequences (Wommack et al. 2008). This issue is not fully overcome even with the increased sequencing depth that 454 sequencing provides.

Currently, accurate pyrosequencing read length is limited by two factors. One is residual nucleotides can be left over in a well and upon addition of the next base, part of the sequences on the bead will lose synchrony by incorporating the new base as well as the left over previous base, termed a "carry forward" effect. Loss of synchrony can also result from incomplete extension due to an insufficient amount of nucleotide in the well. Both of these effects are cumulative and thus limit accurate base calling after a certain point (Marguiles et al. 2005). Since its release, the system has seen improvements to both the platform and the chemistry involved in sequencing so that sequences have seen a 4-fold increase in accurate read length.

On the horizon: 3rd Generation Sequencing Technologies

The aforementioned next-generation sequencing systems are involved in upgrading their products to both increase sequence read length and throughput. At the same time, other companies have been developing new types of sequencing technology, sometimes referred to as "3rd generation" sequencing.

One such company is Helicos, a publicly traded company touting "single molecule sequencing". The process itself is similar to the pyrosequencing

strategy with two major differences. Instead of amplifying the target DNA on a bead prior to sequencing, the Helicos system sequences individual DNA strands and records the fluorescence base by base. Between bases, the florescent tag is removed and washed away, so unlike pyrosequecning, homopolymers are accurately described. This system also promises to be able to produce over a billion reads in a single analytical run. However, the average read length of this instrument is only 35bp leaving the question of how wide its potential applications are.

Another company employing a single molecule sequence strategy is Pacific Biosciences. In short, DNA polymerases and attached to the bottom of transparent wells and as each nucleotide is incorporated into a growing strand an image is captured. Again, fluorescence is measured, but unlike other systems the florescent tag is attached to the nucleotide instead of the base so when a base is added, the fluorescent tag is released and able to float out of the well. This negates the need for the "start-stop-wash" process used in the next generation platforms. This system has been shown to provide accurate reads averaging 1 kb in length. Refreshingly, since the initiation of this project in 2004, the developers have released numerous peer-reviewed publications as their work has progressed (Lundquist et al., 2008, Korlach et al. 2008, Eid et al., 2009).

Post-Sequencing Tools

The rapid advances in sequencing technology have encountered significant roadblocks not in the technology itself, but in the lack of computing

infrastructure to analyze increasingly large amounts of sequence data. Because of this, a number of independent research groups have shouldered the responsibility to develop these much needed tools. This can be both a boon and hindrance to a research group entering the high throughput arena. The multitude of analysis software available ensures that there are usually one or more programs capable of analyzing one's data. On the downside, different programs have different strengths and the tools themselves quickly change as their creators are publishing updates and improvements as often as possible. Finding a suite of sequence analysis tools that fits the researcher's needs typically has to be done on a case-by-case basis. Research groups must also be wary of becoming too attached to a particular suite of analysis approaches. Despite the sometimes steep learning curve for new software, newer programs may contain new more accurate methods for analysis or simply speed up the computing time significantly. The "race" to generate a highly accurate and flexible set of tools is still in full force and undoubtedly both new and existing software programs will become more efficient and better able to handle increasing amounts and different types of data.

Ribosomal Database Project (http://rdp.cme.msu.edu/)

The Ribosomal Database Project (RDP) provides a suite of tools for the curation, classification, and analysis of 16S rRNA gene sequence. Since its inception in 1993, the RDP project has matured from a service that used ftp and e-mail to transmit results, to a streamlined, semi-automated web application

which allows multiple download formats (Larsen et al. 1993, Cole et al. 2009). The first iteration of the RDP project offered a database to compare sequences from both the large and small ribosomal RNA subunits of bacterial, eukaryotic, and archaeal reference sequences (Larsen et al. 1993). It has since shifted its focus to only the 16S SSU rRNA of bacteria and archaea.

The current version, RDP 10, was released in May 2008 and consisted of 550,000 aligned 16S sequences. The most recent update to this release now consists of over 1.3 million aligned 16S sequences. This newest release includes aligned and annotated sequence data for archaeal sequences, which can be used in conjunction with the classifier and other tools. This release also introduced a new alignment strategy for both bacterial and archaeal sequences based on Infernal (INFERence of RNA ALignment) software (Nawrocki et al. 2009). This software generates alignments based on secondary structure and has been shown to be more sensitive than BLAST based methods and ~25X faster than the previously used alignment methods (Cole et al 2009, Nawrocki et al. 2009).

Following the boom in high throughput 16S analysis from bacterial communities, RDP has developed tools for dealing with large amounts of 16S rDNA sequence data obtained from 454 pyrosequening. In summary, raw sequence can be entered and samples can be sorted by their unique sequence tag. Sorted sequences can be filtered for quality based on length and ambiguous codons. Filtering can also identify and remove forward and reverse primer

sequence (Cole et al. 2009). Currently, two options exist for downstream analysis of 16S amplicon sequences

A taxonomy-based method can be employed using the RDP classifier to assign a phylum to genus level identification to each sequence. The RDP SeqMatch tool can provide the closest database match for each query sequence and RDP library compare tool works in conjunction with the classifier tool to identify taxa differently represented between samples (Cole et al. 2009).

In approaches for taxonomy independent analysis, sequences can be aligned using the aforementioned infernal software and clustered into operation taxonomic units (OTUs). Tools for calculating rarefaction, Shannon index, and chao1 are also available, although they have limited flexibility.

Classifier

The RDP classifier is a tool for classifying 16S rRNA gene sequences from the phylum down to genus level. This project, assaying the microbial communities of poultry litter, heavily utilized the RDP classifier for both pre and post OTU analysis. The classifier uses a naïve Bayesian classification in the context of Bergey's Manual of Systemic Bacteriology. The "naïve" stands for the fact that the different input parameters for calculating the genus probability are assumed to be independent. In general terms, Bayes theorem is used to estimate if a query sequence belongs to a particular taxa and assumes that all genera are equally probable (Wang et al. 2007). The tool is available for use online and also for download as a command line program. When run online,

thousands of sequences can be classified in only a few minutes (Wang et al 2007).

In addition to its rapid classification, two major factors make this tool highly desirable when classifying sequence data. One is the alignment-independent nature of the classifier. Multiple, non-aligned sequences can be entered at the same time with no effect on the classification accuracy. Alignment independence relates to the second major benefit of this tool, its assignment of confidence values based on bootstrapping. Rather than comparing each sequence to the entire database, the classification algorithm considers all possible 8-base subsequences and compares them to the query sequence. This is done 100 times, and each time 1/8 of the total possible 8-base "words" are chosen to compare to the query (Wang et al. 2007). This allows confidence values to be assigned for each taxonomic rank. Users can also assign a bootstrap cutoff value so that sequences that which below this threshold will be listed as unclassified. A bootstrap cutoff of 80% has often been used to ensure accuracy in classification. However, recent research in 2009 by Claesson et al. has shown that for partial 16S sequences smaller than 250bp, a 50% bootstrap cutoff can be used with relatively little loss in accuracy. Accurate classification at a lower bootstrap can be a boon to researchers that choose to use a taxonomic approach or those interested in diagnostic evaluation rather than bacterial community analysis of novel environments.

Greengenes (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi)

The Greengenes database is a relative newcomer to the realm of 16S sequence tools. Because of this, its primary goal was to generate a database that consolidates the taxonomies of the other databases as well as to check (in some cases recheck) for chimeras within these databases (DeSantis et al., 2006a) Greengenes offers tools for probe generation, sequence trimming, classification, and alignment. It also provides tools for analysis of phylochip data.

The Nearest Alignment Space Termination (NAST) tool can generate a multiple sequence alignment (MSA) for up to 500 sequences and this alignment can be merged with any other NAST aligned sequences. Alignment merging is possible because of the design of the NAST alignment tool. From the full Greengenes database, a high quality, full length "core set" of sequences that represent the consensus taxonomy can be used for sequence comparison. The query sequence is broken up into 7-base words (7mers) and run against the core set (DeSantis et al., 2006b). The returned alignment, even if derived from a partial sequence contains all the gaps pertaining to a full-length sequence. This way, a sequence can be aligned at any time and compared to any sequence aligned at a different time. NAST also offers the option to return not only the aligned sequence(s), but also the most closely related, non-chimeric sequences from the database. This project employed NAST alignment as a tool to prepare sequences for further analysis.

ARB and SILVA (http://www.arb-home.de/) (http://www.arb-silva.de/projects/living-tree/)

The ARB project is another set of free tools for use with ribosomal RNA and amino acid sequence data. It was initiated in the early 1990's by the Technical University of Munich with the two main goals of creating a well annotated database of rRNA sequences and a software tool for to interacting with the rRNA database. Since that time, the ARB team and collaborators at the Max Plank Institute for Marine Microbiology have created and maintained the SILVA database. As of February 2010, this database contains over 1.4 million aligned sequences of SSU and LSU rRNA from all domains of life. SILVA further separates its aligned sequences into a Ref database, which contains only high quality sequences with read lengths above 1,200bp. The Parc database contains partial 16S sequences along with lower quality full length sequences. A guide tree has been constructed based on Berges and LPSN taxonomy. Where applicable, sequences in the database are compared to RDP and Greengenes classification. The ARB program is run locally on a UNIX operating system and like RDP, offers a variety of tools for analyzing rRNA data. ARB has also been updated to handle both DNA and protein data. This project utilized ARB to generate a distance matrix of all 23,000 sequences as well as simple phylogenetic trees.

Distance-Based OTU and Richness (DOTUR)

DOTUR is a command line UNIX program that provides a highly flexible set of tools for statistically analyzing sequences based on a taxonomy independent approach. Three different methods are available for OTU generation, nearest neighbor, average neighbor and furthest neighbor. Each of these methods has its benefits and drawbacks (described in the next section) (Schloss and Handelsman 2005). Once a method is selected, DOTUR will generate OTUs at many user selected identity levels. Sequence rarefaction, bootstrapping, and jackknifing as well as diversity measures such as Shannon Index, Simpson index Chao-1, and ACE, will all be calculated at each identity level allowing for easy comparison between samples. Where applicable, a 95% confidence interval is also given at each identity. Furthermore, DOTUR will return a file listing the abundance of each OTU at each identity level. Before using DOTUR, sequences must first be aligned and a distance matrix must be generated for use as the input file. Recently, DOTUR has been replaced by MOTHUR, a free software package that includes more streamlined DOTUR software, as well as additional tools developed to analyze large amounts of sequence data (Schloss et al. 2009). This package also includes tools to edit pyrosequencing reads similar to the tools available online from RDP.

Operational Taxonomic Units

The term operational taxonomic unit (OTU) was coined in 1962 by Sneath and Sokal and used to describe a term in the newborn field of numerical taxonomy. Although principles of this field no longer rely on characterizing

physiological and metabolic data to determine phylogenetic relationships, the term itself still remains relevant. In molecular phylogeny, an OTU represents a group of sequences from an organism(s) in which they all are assumed to share a common ancestor. An OTU can encompass any number of sequences depending on the constraints in defining the OTU. The term itself is not constrained to being used solely for molecular phylogeny and can have a much broader definition depending on the context.

The primary benefit of analyzing sequence data by sorting into OTUs is that this approach is independent of existing taxonomic classification. Microbial ecology studies that discard the unclassifiable portion of their data risk losing information on the composition of the community. For example, the RDP classifier can classify sequences down to the genus level and give an accompanying confidence valve for each taxonomic level. Depending on the level of resolution desired, in most cases there will be some sequence that were classified with a low level of confidence. The researcher can then search multiple databases for the sequences that have the closest match to the unclassified query. In some cases this may help resolve the ambiguity, but other times the query sequence may not have a high degree of coverage or identity to any sequence in the current database.

Although knowing what is not known can be seen as a good thing[5], the more 16S data put to constructive use, means better understanding of the

[5] The quote often attributed to Socrates "True knowledge exists in knowing that you know nothing" is especially relevant for those researching bacteria and virus in the

microbial communities within a given environment. Analyzing OTUs first requires the generation of a distance matrix between all sequences. Subsequently, sequences can be clustered into OTUs using different algorithms. The program DOTUR offers three different algorithms for sequence clustering, although the math behind each algorithm is beyond the scope of this review, a summary of each method will be described[6]. The nearest neighbor, or single linkage algorithm, locates sequences that have a short distance (in terms of sequence identity) between them and will be at the most, X% different from the next closest sequence (neighbor). In this scenario, clustering tends to create fewer, larger OTUs because in many cases, within an OTU, there will always be subgroups of closely related sequence even if regions of homology occur at different parts of the molecule. In other words, the sequence only needs to be X% similar to one sequence already in the OTU cluster. In contrast, the furthest neighbor algorithm, or complete linkage algorithm clusters sequences into an OTU based on their X% distance from all other sequences in the OTU cluster. This method tends to make smaller clusters as the sequence being analyzed for potential incorporation has to be the same distance or less between the other sequences already in the OTU. The average neighbor method or Unweighted Pair Group Method with Arithmetic Mean (UPGMA) provides a compromise between the two previous methods, but can be prone to error if the sample size is small. It will

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environment. As in many sciences, new research is continually finding exceptions to the "rules" of what microorganisms are capable of.

[6] The remainder of this paragraph relies heavily on the 2005 Schloss & Handelsman paper describing DOTUR and on the accompanying user manual published on the DOTUR website.

measure the differences between two clusters as the average of the differences between all sequences in those clusters. As sequences are recruited to a new cluster, the average of difference between all clusters will change as will its relationship to the existing clusters. As a result of averaging, the total number of OTU clusters generated will usually fall between the nearest and furthest neighbor approaches.

As the existing taxonomy of bacteria continues to be expanded and revised, the need to cluster sequences by OTU will undoubtedly diminish. However, based on the current technology, for the foreseeable future, clustering sequence into OTUs will continue to be a useful approach to analyzing large datasets from different environments or cultures.

HIGH-THROUGHPUT ANALYSIS OF POULTRY LITTER

Introduction

High-throughput sequencing methods are allowing study of bacterial communities with an unprecedented amount of depth and clarity. Deep sequencing studies have consistently found bacterial taxa not covered by culture-based analyses in numerous environments, including ocean water (Sogin et al. 2006, Galand et al. 2009), soils (Roesch et al. 2007, Teixeria et al. 2010), and sediments (Hollister et al. 2010). The same findings are also replicated in clinical environments like those of chronic wounds (Price et al. 2009), the human intestine (Eckburg et al. 2004) and the human microbiome (Costello et al. 2009). These studies have redefined the true richness and diversity of microbial communities within these target environments and helped bring about the realization that less abundant members of a community can collectively make up a significant fraction of the total microbial population. Simultaneously, we have observed how environmental changes can alter the composition of microbial communities from addition of antibiotics (Dethlefsen et al. 2008) to changes in temperature (Miller et al. 2009) or pH (Bââth et al. 2010).

Previous culture and denaturing gradient gel electrophoresis (DGGE) based methods describing poultry litter microbes have only scratched the surface of understanding the microbial composition of this environment (Martin and McCann 1998, Terzich et al. 2000, Lu et al. 2003, Thaxton et al. 2003, Fries et al.

2005, Omeria et al. 2006). This study is the first to employ high-throughput 16S rRNA gene sequence analysis of bacterial communities in poultry litter, and aims to describe this community while searching for potential links to the nature of recurring gangrenous dermatitis (GD) that can occur in many flocks.

The broiler industry

Rapid advances in technology over the last century have greatly increased the scale of both crop and livestock agriculture. In the past 50 years, poultry production and consumption has increased by approximately 4% per year (Ollinger et al. 2005). The rearing of chickens is split into two groups, the layers, which provide eggs, and the broilers, which provide meat. In 2008, 50 billion pounds of broiler meat was produced in the United States valued at over 23 billion dollars (USDA 2009). In the same year, in the state of Delaware, 1.6 billion pounds of broiler meat was produced from 243 million chickens and was valued at 773 million dollars (USDA 2009).

The modern broiler industry is divided into two principle groups, which represent a clear division of labor. The integrators are large national or regional companies such as Perdue, Mountaire, and Tyson. These companies manage the hatching, slaughter and processing, feed and bedding distribution, and veterinary services for growing chickens. The actual chicken rearing, from just after birth until just before slaughter, is contracted out to poultry farmers, (termed growers). Because of this system, there is a high degree of farm heterogeneity as poultry houses owned by an individual grower can vary in size, layout, age,

and general state of repair (Boyd 2001). This lack of uniform rearing conditions can have an effect on the bird growth and health. Previous studies have found a high degree of variation in the overall microbial populations found in poultry litter of different farms (Martin & McCann 1998, Terzich et al. 2000, Thaxton et al. 2003) and within single houses (Lovnah et al. 2007). An unintended conclusion of this study is the apparent existence of a link between different house environments husbandry situations and the microbial communities of these houses.

Gangrenous dermatitis

In the 1960's a poultry disease termed gangrenous dermatitis (GD) became a considerable problem for farmers (Frazier et al. 1963). This ailment usually affects chicken 4-7 weeks in age. Onset may begin with redness and swollen patches on the skin and progress to large red and black patches of rotting tissue over the breast, wing tips, or thighs (Wilder et al. 2001). Disease progression is rapid and death will occur 12-72 hours after the first symptoms appear. As administration of antibiotics in the feed became common practice, the disease became less prevalent, but in recent years has again become a concern as cases affecting up to 4% of the flock per day have been reported (Schering-Plough Animal Health 2005).

The environment also plays a role in GD prevalence. Houses in the Delmarva area exhibit seasonality to GD outbreaks with the most occurring in the late spring and early summer (Don Ritter personal communication). GD outbreaks are more common in East coast poultry farms than West coast ones,

though there has been no in-depth comparison of litter composition and cleaning patterns between these areas.

Causes

Gangrenous dermatitis is believed to have a multifactoral pathogenesis, with numerous bacterial species believed to contribute to the disease state. The main species believed to cause the disease are *Clostridium septicum*, *Clostridium perfringens*, *Staphylococcus aureus*, and in some cases *Escherichia coli* (Willoughby et al 1996, Crevantes et al 1988). One or more of these species have been found to be present upon necropsy of affected chickens. One study that challenged chickens with *S. aureus* and *C. speticum* together, found that there was a much higher mortality rate using the combined inocculum than when administering each separately (Wilder et al. 2001). In fact, for chickens challenged with only *C. septicum*, there were no deaths reported (Wilder et al 2001). This contradicts early research that found *C.septicum* alone could produce the disease (Frazier et al. 1963) and more recent study in which isolated *C. septicum* from 108 turkeys and chickens that with gangrenous disease symptoms (Neumann & Rehberger 2009).

The molecular mechanisms that lead to the physiological manifestation of GD are not well characterized. It is hypothesized that the various infecting bacteria release exotoxins that lead to an immune response and subsequent tissue damage. One suspected toxin from *Staphylococcus* sp is dermonecrotoxin which produces a severe inflammatory immune response

(Cervantes et al. 1988). *C. septicum* produces a suite of toxins designated as alpha, beta, delta and gamma. The alpha toxin is a cytolysin and seen as the main disease causing agent (Neumann and Rehberger 2009). Once tissue becomes necrotic, the infecting bacteria will fill these areas causing further damage to surrounding live tissue. This can also obscure isolation of the bacteria responsible for the primary infection as secondary infection and environmental contamination of the gangrenous lesions are common.

Treatment

Once a GD outbreak has been recognized, the flock may be treated by the addition of iodine to the drinking water over three consecutive feeding periods (Clark et al. 2004). Adding aluminum sulfate or sodium sulfate to the litter has been used as a means of slowing an outbreak, but this treatment may damage equipment through corrosion as well as render the litter unfit for reuse as feed or manure. Initial work on creating a vaccine specific to GD pathogens has been performed with promising results. Birds immunized against *C. perf*ringens or *C. septicum*, *E.coli*, and *S. aureus* remained healthy as long as 10 days after being challenged with these same pathogens (Kaul et al. 2001). However, this type of vaccination would be unfeasible for dealing with a sudden outbreak. Preventative measures include a full clean out and disinfection of the house between flocks. This practice is rarely practiced in the United States, and multiple flocks are often raised consecutively on the same litter (Kim and Agblevor 2006).

Route of infection

Although the bacterial pathogens that cause GD have been tentatively identified, the factors leading to an outbreak and its severity have not been elucidated. Traditionally, an "outside-in" approach has been hypothesized as the main route of infection (Clark et al. 2004). Broken skin is often observed around the gangrenous lesions and it is thought that an initial skin injury provides an access point for infection by pathogens in the environment. More recently it has been suggested that the infection instead follows an "inside-out" progression. In this scenario, the disease causing agents, enter the bloodstream as the result of internal damage to the intestine or due to a high amount of immunosupression by a viral disease like Infectious Bursal disease (IBD).

Ultimately bacterial players responsible for GD come from the surrounding environment. The outside-in route of infection will come directly from the house and litter bacterial communities, while the inside-out route will also come from these communities as they establish bacterial succession in the gastrointestinal track (GIT). In this case the putative pathogens will potentially remain harmless until intestinal damage or disturbance occurs.

From either route, it is important to evaluate and understand the microbial community occurring within the house environment in order to provide a framework for disease pathogenesis. High throughput methods are poised to be able to reveal the true richness and diversity of these communities. It is likely that many of the conflicting results surrounding GD study are a reflection of not only the numerous bacterial species able to promote the disease state, but also a

suite of other players that facilitate or inhibit host-pathogen interactions. Physical factors such as weather, living conditions, and diet likely contribute to incidence of GD on a gross level, but potential differences in outbreak and mortality rates between houses of the same or neighboring farms may be due to subtle differences in the microbiological community.

Results and Discussion

To date, no study has analyzed the bacterial community of poultry litter using deep sequencing of bacterial 16S rDNA amplicons. Previous studies investigating both litter and intestinal microbial communities have employed denaturing gradient gel electrophoresis (DGGE), sequencing of 16S rDNA clone libraries, and cultivation-based assays (e.g. plate counting) (Martin and McCann 1998, Terzich et al. 2000, Lu et al. 2003, Thaxton et al. 2003, Fries et al. 2005, Omeria et al. 2006). With the depth of sampling that pyrosequencing allows; this research has resulted in the description of a litter microbial community with approximately 60-fold more sequence coverage than previous studies (Lu et al. 2003).

Quantitative litter properties

It is estimated that less than 1% of bacteria within most environmental samples are culturable using the current methods (Amann et al. 1995). Thus, cultivation methods severely constrain observations of both the full diversity and the true abundance of a given bacterial population. This study addressed both of

these issues by using a cultivation independent method to both enumerate and estimate the diversity of microbial communities in poultry litter.

Epifluorescence microscopy in conjunction with tower filtration was used to estimate bacterial abundance in litter. Contrary to cultivation-based methods, extracted litter bacterial cells were stained with the nucleic acid stain SYBR Gold, captured on a 0.02 µm filter, and directly counted (Shibata et al. 2006). Using this method, all litter samples contained around 10^{10} cells g dry wt^{-1} (Table 2.1). Mean bacterial abundance in Wet litter samples was approximately three times higher than abundance in Dry litter samples in when normalized to cells per gram dry weight. Previous studies employing culture-based methods to estimate abundance have been highly inconsistent, with estimates ranging from 10^3 to 10^{12} g^{-1} of litter, making comparison to direct counts difficult (Martin and McCann 1998, Terzich et al. 2000, Thaxton et al. 2003, Fries et al. 2005, Omeria et al. 2006). The only other litter study to use a culture independent method estimated total bacterial abundances of 10^8 to 10^{10} g^{-1} based on bacterial DNA from litter samples (Nandi et al. 2004). This is comparable to the bacterial abundance results obtained by this study.

Table 2.1 General properties of each sample

Sample name	Moisture content (%)	Bacterial abundance (g dry $wt^{-1}X10^{10}$) (SE)	Viral abundance (g dry $wt^{-1}X10^{10}$) (SE)	Virus to Bacteria ratio	Age of House in years	GD History	Ventilation system
Dry 1	25	2.4 (0.5)	5.0 (1.1)	2.1	30+	Yes	Suspended box fan
Wet 1	65	5.6 (0.5)	55.8 (14.6)	10.0			
Dry 2	22	2.5 (0.3)	25.5 (16.5)	10.2	30+	Yes	Suspended box fan
Wet 2	63	9.2 (1.4)	74.4 (17.4)	8.1			
Dry 3	19	1.3 (0.3)	ND[a]	ND	10+	No	Suspended box fan
Wet 3	67	4.6 (1.9)	199.4 (61.1)	43.4			
Dry 4	10	1.7 (0.4)	4.8 (1.5)	2.8	20b	No	Tunnel ventilation
Wet 4	43	4.6 (1.9)	94.2 (27.4)	20.5			

a No Data
b Ventilation system changed 10 years ago

To date, no study has examined viral abundance in poultry litter by direct counts. Although there are numerous research groups involved in identification of specific poultry and human viruses in litter and in/on chicken, their approaches give little indication of total viral abundance (Kabell et al. 2005, Khurana et al. 2009). In all samples extracted, viruses were stained and captured on a 0.02 µm filter and enumerated using epifluorescence microscopy. Across all samples viral abundance was 2 to 40-fold higher than corresponding bacterial abundance with values ranging from 10^{10} to 10^{12} g dry wt^{-1} (Table 2.1). Similar to the trend observed in bacterial counts, viral counts were higher in Wet litter samples as compared to Dry litter samples and the ratio of viral to bacterial abundance was highest in wet litter (Table 2.1). These viral abundance values are between two and three logs greater than those found in various Delaware Soils and Antarctic soil (Williamson et al. 2005, Williamson et al 2007) and five to six logs higher than lake and costal water (Chen et al. 2001). Viral extracts from Dry litter samples also contained a higher proportion of what was assumed to be humic

acids, which may have suppressed the true viral abundance through obscuring countable viruses. This issue was particularly acute for Dry litter samples from House 3. Issues with the interference of humic acids been have reported previously in a studies enumerating virus in both soils and sediments (Hewson & Fuhrman 2002, Williamson et al. 2003, Helton et al. 2006). Despite this difficulty, this study has shown that viruses within poultry litter can be extracted and enumerated in a reproducible manner.

Ideally, the moisture content in poultry litter should be fairly low and homogeneous throughout the house. The houses in this study employed a common setup in which two water lines with regularly spaced nipple drinkers spanned the length of the house. Litter beneath each nipple drinker was saturated, forming a distinct wet microenvironment in which different types of bacteria likely flourish.

Moisture content of Dry litter samples ranged from 10-25% and Wet litter from 43-67% (Table 2.1). Dry litter moisture content in this study was similar to previous studies where litter was collected randomly throughout a house (Martin et al. 1998, Terzich et al. 2000). One study that took samples specifically near a water dispenser measured ~50% moisture content, slightly lower, but similar to the measurements in this study (Lovanh 2007). Litter in House 4 had the lowest moisture content for both Dry and Wet samples, which was likely attributed to the high forced ventilation rate within this house.

16s amplification and pyrosequencing

This study employed pyrosequencing (Margulies et al. 2005) of 16S rRNA gene amplicon libraries to analyze the bacterial composition of poultry litter. Following bacterial genomic DNA extraction, the V1-V2 hypervariable region of the bacterial 16S small ribosomal subunit gene was PCR amplified from each sample. Amplicons were sequenced using GS-FLX 454 pyrosequencing. Resulting sequence reads were processed to remove reads lacking the appropriate barcode or linker sequence. This produced 22,673 sequences with an average read length of 236bp (Table 2.2). Library sizes ranged from 2,115 to 3,758 sequences (Table 2.2).

Table 2.2 Library clustering and unique clusters

Sample name	Number of reads	Average read length[a]	Number of OTU clusters[b]	Unique[c] OTU Clusters	Unique[d] OTU clusters in both GD affected houses (total sequences)	Unique[d] OTU clusters in both Non-GD affected Houses (total sequences)
Dry 1	2,616	240	156	122	13 (424)	
Wet 1	3,172	232	381			
Dry 2	2,115	238	197			
Wet 2	3,342	237	407	88		
Dry 3	2,499	238	202	96		12 (308)
Wet 3	2,849	233	325			
Dry 4	2,322	237	230			
Wet 4	3,758	236	529	172		

[a] Read length after trimming of primer and linker sequence.
[b] OTUs generated at 95% identity using UPGMA (average neighbor) clustering algorithm in DOTUR.
[c] Clusters found only in a single house after removal of all singleton clusters
[d] Clusters at a frequency less than 0.05% were discarded in the target library and clusters less than 0.02% in comparison libraries were included if applicable.

Classification with RDP

Amplicon libraries were classified from the phylum through genus levels using the classify tool available on the Ribosomal database project website (Larsen et al. 1993, Cole et al. 2009). Recent evidence suggests that for short reads covering only one or two variable regions, a 50% confidence cutoff maximizes the number of classifiable sequences in a library while maintaining high assignment accuracy (Claesson et al. 2009). Using these criteria, greater than 95% of the sequences were classified at the phylum through order levels, 85% at the family level, and 67% at the genus level (Table 2.3).

Table 2.3: Diversity at different taxonomic levels

Taxonomic level	Total number among samples	Range	Mean (SD)	% of library classified[a] (SD)	% of total phylogeny[b]
Phylum	9	4-7	5 (1)	99.2 (0.9)	23.7
Class	16	7-14	11 (3)	98.5 (1.6)	36.4
Order	38	8-29	18 (8)	97.0 (3.0)	39.2
Family	99	29-77	48 (17)	85.3 (5.3)	36.3
Genus	220	41-127	72 (32)	67.3 (10.4)	15.7

[a] Classified by RDP classifier at a bootstrap cutoff confidence interval of 50%
[b] Based on the RDP classification scheme (total sequences phylogeny/total possible).

Because this level of sequencing depth has not previously been applied to 16S rDNA amplicons from poultry litter, at all taxonomic levels new taxa were identified that have not been observed in previous studies of poultry litter or chicken intestine. When comparing the total number of phyla classified at each phenotypic level, the total number of RDP-classified taxa across all libraries was

greater than maximum that seen in any individual library. This immediately indicated that there were sample-specific unique reads as high as the phylum level.

Previous studies using both culture-derived classification and low throughput 16S amplicon clone library sequencing have reported the taxonomic composition of litter microbial communities within these broad classifications, high and low G-C gram positives and gram negatives (Lu et al. 2003, Fries et al. 2005, Enticknap et al. 2006, Lovanh et al. 2007). Although confounding factors such as flock size, litter age, and bedding material make comparisons difficult, the general trend in these low resolution studies reveals that poultry litter tends to have a high amount of gram positives with low G-C phyla dominating. The results of this study support the trend found in previous work (Lu et al. 2003, Enticknap et al. 2006, Lovanh et al. 2007). Seventy-seven percent of the RDP classified sequences were assigned to gram positive taxa split into 44% low G-C and 33% high G-C phyla. As compared to previous work, this study found a greater frequency of gram negative phyla, composed of 11% Proteobacteria and 9% Bacteroidetes. This is likely due to the sampling of wet litter which contained nearly all of the gram negative sequences. Although Lovnah et al. in 2007 noted a specific Dry-Wet split in their DGGE banding patterns of litter samples, the bands they selected and sequenced contained no gram negatives.

Operational Taxonomic Unit Analysis

At the genus level, a total of 7,401 16S sequences could not be classified by RDP with ≥50% confidence. Using an OTU based approach allowed the full dataset to be analyzed without having to discard reads unclassifiable by an existing taxonomy. At the 95% identity level, a total of 1,462 16S OTU clusters were generated from the V1-V2 16S amplicon sequences. Removal singleton OTUs dropped this total to 777 clusters. In this study, OTU data was analyzed by House and sample state (Dry or Wet).

House Breakdown

Rank abundance plots of bacterial species or OTUs are often used to describe the structure of bacterial communities (Schloss & Handelsman 2006, Frias-Lopez et al. 2008). OTU clusters were ranked according to the number of 16S sequences per OTU and plotted to examine OTU distribution across all houses (Fig 2.1A). Many of the top OTU clusters contained sequences from all houses, but the proportion of sequences that an individual house contributed to a given OTU cluster was dramatically different. For example, in OTU cluster 1, roughly 50% of the sequences were from House 2 while cluster 2 was dominated by sequences from House 1. Other top OTU clusters were made up of sequences from only single house. Most notably, OTU clusters 6 and 22 and 34, only occurred in House 4, and OTU cluster 21 in House 1. These house-to-house differences are further realized when applying double principle coordinate analysis (DPCoA) to the dataset (Pavoine et al. 2004). DPCoA shows houses diverge according to the identity and frequency of bacterial 16S OTUs within the

litter (Fig 2.2). The DPCoA found that 90% of the variation between houses was explained by the top two components. Principle component analysis (PCA) of DGGE fingerprints found that 83% of the in-house variation could be explained in the top two components with the best correlations related to moisture content and temperature or pH (Lovnah et al. 2007). Based on both RDP classification data and OTU analysis, moisture content is likely the strongest factor shaping the between house relationships seen in this study.

House-to-house estimates of bacterial richness and diversity according to 16S OTUs indicates that species richness was still increasing at ~6,000 sequences; however, the curves for diversity were essentially flat after 2,000-3,000 sequences (Fig 2.3A&C). Thus, increased sequencing would only reveal more rare 16S OTUs and would not improve estimates of total bacterial diversity in poultry litter.

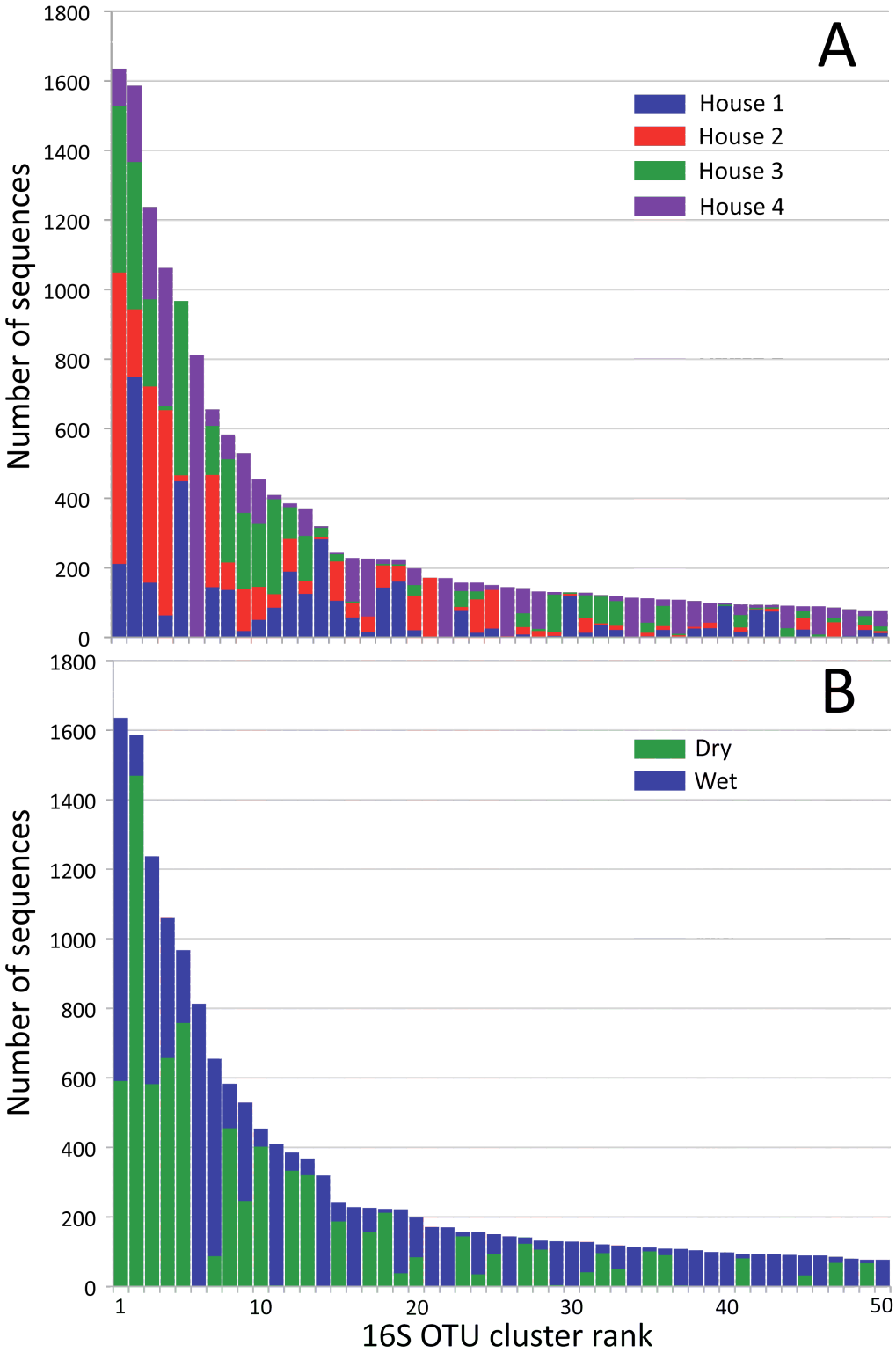

Figure 2.1 Rank abundance plots for the top 50 bacterial 16S OTUs split by house (A) and by sample type (B).

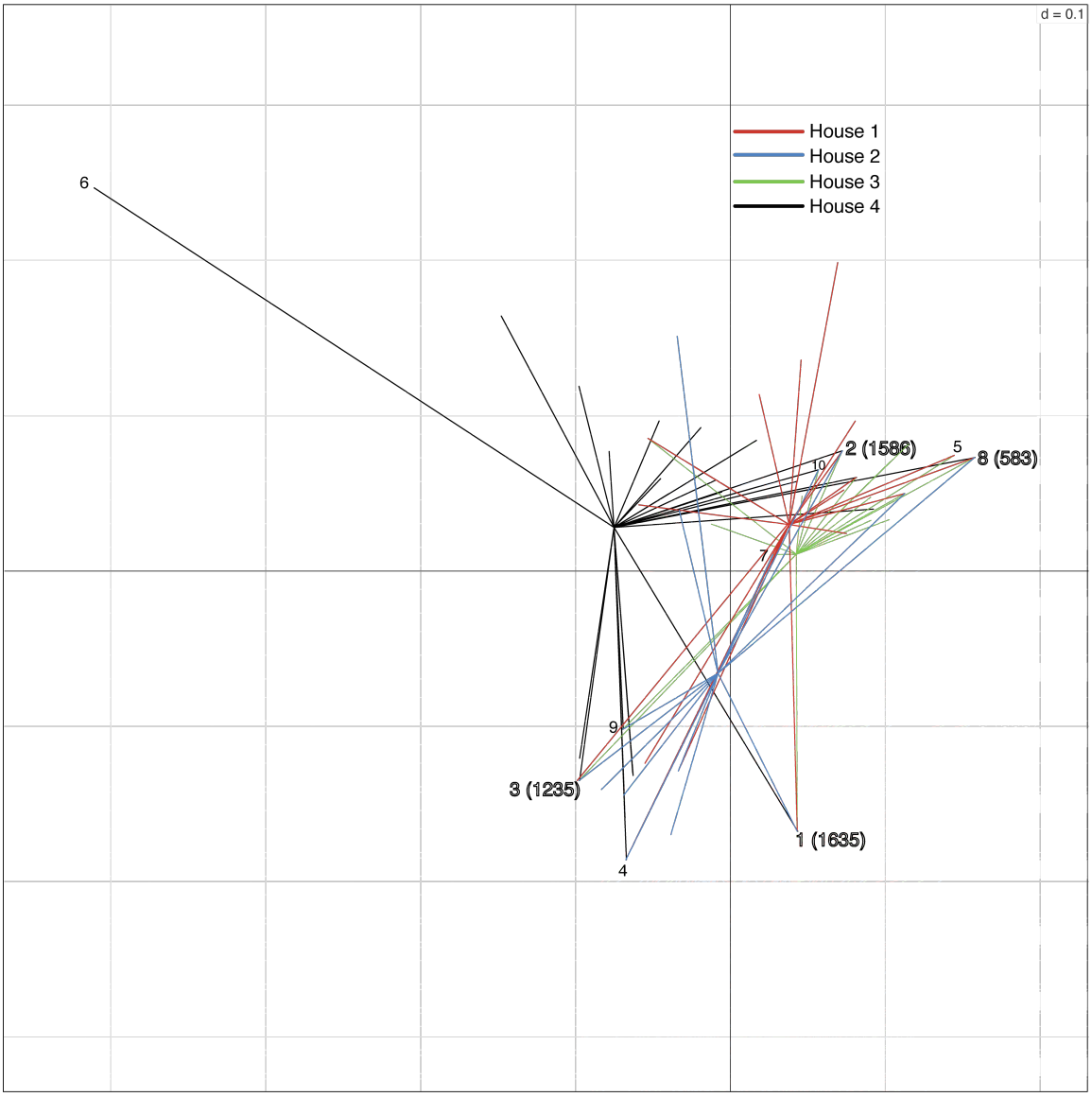

Figure 2.2 DPCoA displaying bacterial 16S OTUs with a frequency greater than 1% for each house. Positions of the ten most abundant OTUs are labeled and those shared by all houses are bold and accompanied by the total sequences in the OTU.

House 4 was of particular interest due to a number of distinct features revealed by both RDP classification data and OTU-based analysis. Most notably, the 6th largest 16S OTU was found only in the Wet litter of this house and classified to the genus *Arcobacter* in the relatively new family Campylobacteraceae (Fig 2.1) (Vandamme & DeLey 1991). This genus differs from *Campylobacter* in that these bacteria can tolerate oxygen and survive at lower temperatures. Both *Campylobacter* and *Arcobacter* are known to cause acute bacterial enteritis and improvements in medical diagnostics have revealed that A*rcobacter* infection can easily be misdiagnosed as *Campylobacter* infection (Vandenberg et al. 2004, Wybo et al. 2004). Unlike *Campylobacter*, the route of transmission of *Arcobacter* contamination is poorly characterized. Most studies have focused on the detection of arcobacterial contamination from the poultry farm to slaughterhouse to design strategies for preventing subsequent dispersion to consumers. Conflicting results on whether arcobacterial species are commonly found in the chicken gut have further confounded this issue (Gude et al. 2005, Driessche & Houf 2007, Ho et al. 2008). This is the first study to identify a large population of *Arcobacter* in poultry litter although some studies have identified it in broiler feces, a component of poultry litter (Ho et al. 2008). Although it is a common contaminant in poultry houses (Newell et al. 2003), in this study, 16S amplicon sequences classifiable as *Campylobacter* were only found in a single sequence from the dry litter of House 3.

The 22nd most abundant 16S OTU cluster was another group found only in the Wet litter sample of House 4. RDP classified the representative sequence

from this cluster to the genus *Azospira*. Belonging to the sub-phylum β-Proteobacteria, these gram-negative, non-spore-forming bacteria with a polar flagellum were first described in 1986, but only nominated as a separate genus in 2000 (Reinhold et al. 1986, Reinhold-Hurek & Hurek 2000). Currently, there are three described species of *Azospira* recognized along with a number of strains for, *Azospira oryzae*, the first species to be described (Tan & Reinhold-Hurek 2003, Coates & Achenbach 2004, Bea et al. 2007). This genus is of interest due to its potential for use in bioremediation. Strains of *Azospira* have been isolated that are able to reduce selenate and selenite to elemental selenium (Hunter 2007) and reduce the perchlorate to chloride (Van Trump & Coates 2009). Perchlorate reducing bacteria (putative *Azospira* sp and *Dechloromonas* sp) have been found in numerous soil and sediment environments (Coates et al, 1999), but this is the first study to report the presence of genus *Azospira* in a litter environment.

House 4 was also the sole only location for the 34th largest 16S OTU cluster. The representative sequence for this cluster was classified by RDP as the genus *Dysgonomonas* (100% confidence). In the phylum Bacteriodetes, the gram negative, non-motile rods described by this genus were formerly associated with CDC group DF-3, in which several isolates had been found in feces and soft tissue infections (Blum et al. 1992), but have since been assigned their own genus with four described species (Hofstad et al. 2000, Lawson et al. 2010, Shah et al. 2009). There have been very few positively identified isolates and these are derived almost exclusively from clinical samples including an abdominal

wound (Lawson et al. 2010) and a gall bladder infection (Hoftstad et al. 2000) and others (Hironaga et al, 2008). However, there are a number of published studies that have found similar 16S sequence (90-98% similarity) in biofilters (Friedrich et al. 2002) and more recently in bioreactors and microbial fuel cells (Borole et al. 2009, Zhang et al. 2009) and water adjacent to a hot spring sample (Kimura et al. 2003). These findings indicate that members of the genus have a more cosmopolitan distribution than previously suspected. Future study of a non-clinical environmental isolate would help resolve questions on how these organism fit into the existing taxonomy. Like *Azospria*, this is the first study to identify this genus in a litter environment.

House 4 also had the highest richness and diversity according to 16S OTU analysis (Fig 2.3A&C). This is supported by the fact that House 4 has the largest amount of unique OTU clusters (Table 2.2). However, the increased diversity is not simply the result of additional unique OTUs, the proportions of the most abundant OTUs differ as compared to the other three houses. In examining the DPCoA, for House 4, there are 14 OTUs that comprise greater than 1% of the total sequences for that house and occur at less than 1% abundance in any of the other houses (i.e. lines not converging with lines from another house) (Fig 2.2). By comparison, the other houses have fewer of these predominantly "house specific" OTUs (Houses 1&3 each have 7, House 2 has 5). All 14 of these House 4 dominated clusters are seen in the rank abundance plot (i.e. clusters 6, 16, 17, 22, 26-28, 34, 35, 37, 38, 44, 46, and 48) (Fig 2.1A).

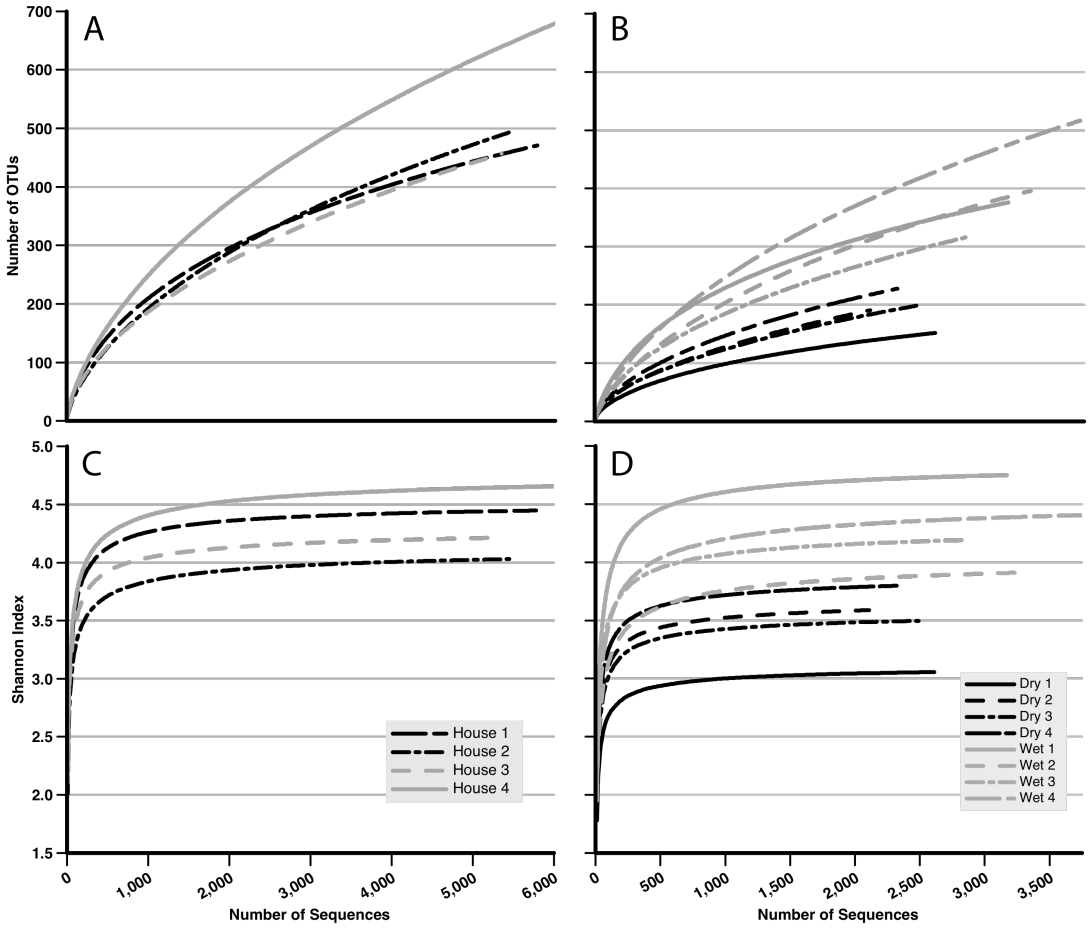

Figure 2.3 Rarefaction (A & B) and Shannon diversity index curves (C & D) for bacterial 16S OTUs at 95% similarity. A & C by poultry house; B & D by sample type and house number.

Because each poultry house sampled was managed by a single company, chickens were given the same initial bedding material, feed, and antibiotic regimen. Nevertheless, the conditions within each house were considerably different. The range of house conditions for broiler productions varies considerably between growers (Boyd 2001) and this study sought to sample a cross section of different houses (Table 2.1). The major difference between individual growers is the condition of the poultry house itself. Overall, our data indicate that house conditions have an impact on the composition of litter microbial communities. Houses 1 and 3 were dim with low lighting and some ambient light from vents/windows along the length of the house. The houses were kept cool using hanging fans and vents on the sides of the houses. These two houses shared roughly the same proportion of sequences in a number of the top 16S OTU clusters, including clusters 3, 5, 7, and 13 (Fig 2.1A). House 2 was of similar age to House 1, but was more open, allowing an abundance of light to penetrate. Interestingly, a number of the most abundant OTUs contained a disproportionately large number of sequences from House 2 (e.g. clusters 1, 3, 4, and 7). Perhaps greater exposure to environmental factors outside of this house had a beneficial influence on the already successful members of the microbial community. House 4 had the most modern construction and employed an evaporative cooling system and forced airflow along the length of the house. This house was tightly sealed and no natural light was allowed to enter.

Compared to all other houses, House 4 demonstrated a different overall composition and contained multiple, highly abundant OTUs comprised of only

House 4 sequences. These observations raise the question of whether the more modern husbandry practice encourages growth of genetically distinct microbial communities. These newer houses provide increased stability to the in-house environment and although this consistency is preferable for growing poultry, it may also promote a more virulent bacterial population. Further sampling of a greater cross section of houses could shed light on their potential to host unique pathogens.

Top 16S OTU Clusters

The most abundant 16S OTU clusters were further examined using the RPD to determine if these groups corresponded to the top groups classified by RDP prior to OTU processing. To this end, both BLAST from NCBI (Altschul et al. 1990) and RDP's SeqMatch tool were used to compare the reference sequence from each top OTU to 16S genes from other studies. The top five most abundant OTU clusters were divided into 2 phyla, clusters 1, 3, and 4 were classified as Actinobacteria, and clusters 2 and 5 were classified as Firmicutes. Together, the top five clusters composed 29% of all sequences across the study.

The representative sequence of the most abundant 16S OTU cluster was initially classified by the RDP classifier as belonging to the genus *Yaniella* (76% confidence). However, using the RPD seqmatch tool against the representative sequence of cluster one, there are numerous sequences from the genus *Arthrobacter* with a higher similarity score than the Yaniella sequences. This inconsistency highlights the potential classification problems associated with

sequencing a few 16S variable regions rather than the full 16S gene. While the resolution of OTUs clustered at 95% identity should ideally provide genus to species level of resolution, this is not always the case. Further investigation of the top OTU cluster using BLAST against sequences in the nr database found that unclassified sequences, from chicken litter (Enticknap et al 2006) and turkey feces (Lu & Domingo 2008) showed 94% identity to the representative sequence from cluster number one.

The representative sequence from the 2nd most abundant OTU cluster was classified by RDP as the genus *Staphylococcus*, which was not surprising as *Staphylococcus* spp. are often found on the skin and mucous membranes of both healthy and diseased chickens (Smyth & McNamee 2008). BLAST analysis of this sequence found 94% homology to an uncultured Firmicute isolate from a DGGE band produced from a chicken litter sample (Rothrock et al. 2008). There were also a number of BLAST hits to *Staphylococcus nepalensis* isolated from the GI tracts of monkey and pigs (Novakova et al. 2006). In the same study, Novakova et al. found that it was impossible to differentiate between *S. xylosus* and *S. nepalensis* by biological tests alone and additional 16S rRNA gene sequencing was required to differentiate these two species. This result raises the question of the true identity of the bacteria in a previous study which employed culture-based techniques and found *S. xylosus* to be the dominant *Staphylococcus* species in poultry litter (Martin & McCann 1998). *S. xylosus* has not been identified in some of the more recent 16S-based studies (Lu et al. 2003,

Lovnah et al. 2007), but both S. xylosus and *S. nepalensis* were described in litter isolates by Nandi et al. in 2004.

The 3rd largest OTU cluster was classified with confidence to the suborder Micrococcineae. Like cluster 1, unclassified sequences found in the Enticknap (2006) poultry litter study had the highest identity at 95%. Also among the top hits to this cluster were sequences classified as *Brachybacterium* from sewage sludge and the Bering Sea each with 91% identity (unpub GenBank acc #: AB210986.1, GU166125.1). Near perfect correlation was seen between RDP-classified *Brachybacterium* sequences and assignment of these sequences in 16S OTU clusters. Interestingly, the isolates used to describe the *Brachybacterium* genus were derived from "poultry deep litter" samples taken in the 1960's (Collins et al. 1988). Since that time, a number of new species have been described a variety of samples including coastal sand (Chou et al. 2007), medieval wall paintings (Heyrman et al. 2002), and the surface of ripened cheeses (Deetae et al. 2007). Deetae et al. found that Brachybacterium sp. play an important role in the distinctive odor of ripened cheddar and other cheeses through the production of hexanoic acid ethyl ester. As compared to previous poultry litter studies, *Brachybacterium* sp from the 16S rDNA clone isolated by Enticknap et al. (2006) only had 88% identity to the representative sequence of cluster three. In a litter study by Lu et al. 2003, 5% of the 16S poultry litter clones were classified as *Brachybacterium* sp.

The representative sequence for the 4th largest OTU cluster was classified by RDP to the Genus *Brevibacterium* with 88% confidence. Two separate

unpublished studies (one from untreated sludge in a waste waster treatment plant) reported sequences with 99% identity to the cluster four reference sequence and are both were classified as *Brevibacterium epidermis* (GenBank acc #': X76565, GU576981.1).

Sequences classified as uncultured *Brevibacterium* from poultry litter also matched with 99% identity (Lu et al. 2003). The same study by Lu et al. (2003) found 7% of their clones classified as *Brevibacterium sp*. In general, *Brevibacterium sp*. are not pathogenic including *B. epidermis*, however there are known pathogenic species like *B. avium* (Pascual & Collins 1999, Onraedt et al. 2005). Comparing the representative sequence from this study to the published *B. avium* found them to be 94% similar (in other words, not the same species). *Brevibacterium* sp. are economically important in their role of ripening cheeses (more so than *Brachybacterium*) and because of the high salt tolerance within this genus, *Brevibacterium* show potential for additional industrial applications (Onraedt et al. 2005).

The representative sequence from the 5th largest cluster was not confidently classified past the family level of Bacillaceae. Both RDP seqmatch and BLAST found no high similarity hits to any classified bacteria. Like cluster 1, the most similar sequences came from the 2006 Enticknap study (96% identity) and the 2008 Lu turkey study (92% identity). This cluster was also only found in Houses 1, 2, and 3 with over 98% of the sequences contributed evenly by Houses 1 and 3.

Examining the classification of 16S OTU representative sequences using RDP SeqMatch and BLAST confirmed that these approachs closely matched the genus-level classifications before clustering. Nevertheless, one large cluster (i.e. cluster five) was composed of non-classifiable sequences indicating thast unknown bacterial groups can be highly abundant in poultry litter. This result validates the utility of OTU-based approaches to analyze bacterial communities. It is also encouraging that 4 of the 5 top OTUs had highly similar matches to previous litter studies. Combining RDP, BLAST, and 16S OTU analyses may eventually lead to the defining the core microbial community of poultry houses.

Litter State

The distribution of 16S OTUs between Dry and Wet litter samples shows dramatic differences between these two microenvironments. A rank abundance plot reveals that only a few of the top 50 OTU clusters have a relatively even distribution of sequences between Dry and Wet litter samples (Fig 2.1B). OTU clusters 3, 4, 9, 20, 25, and 32 approach an even distribution while clusters 2, 5, 10, and 20 are examples of the uneven distribution found in the majority of clusters. Furthermore, a total of 20 of the top 50 OTU clusters (40%) contained sequences solely from Wet litter samples. In contrast, none of the top 50 OTU clusters were composed of sequences from only Dry litter. Although many bacteria are able to survive in both microenvironments, specific groups clearly thrive in one litter type or the other. The top five clusters all contained at least 100 sequences from both Dry and Wet libraries and the ability of these bacteria to survive in both conditions is perhaps part of the reason for their dominance.

Rank abundance distribution curves of 16S OTUs within dry and wet samples reveals that wet litter contains a higher richness and diversity of bacteria than dry litter (Fig 2.4). While both curves follow a trend seen in communities of higher organisms, ie, few, highly abundant organisms and many more rare organisms, (McGill 2007) when fit to a power law equation, it is apparent that the inflection of the curve for each sample type is distinctly different (Fig 2.4). This curve fit reveals that 90% of the bacterial abundance in Dry litter occurs within the top 50 OTUs. In contrast, 214 clusters comprise 90% of the bacterial abundance in Wet litter. Compared proportionally, 90% abundance is covered by only 19% of the total Dry clusters, while 90% abundance in Wet litter is covered by 36% of the total clusters. When examining Wet litter libraries individually this trend of greater, the richness and diversity occurs in all Wet litter samples (Fig 2.3B&D). Other studies employing deep sequencing in both soil and water have also sampled the “rare biosphere” which is comprised of a long tail of low abundance taxa that it is comprised of (Sogin et al. 2006, Elshahed et al. 2008, Galand et al. 2009). The presence and function of the long tail of bacterial 16S OTU groups as part of the overall microbial community is hotly debated. It has been proposed that the high rate of dispersion of microbes around the planet have lead to their ubiquitous presence in nearly all environments and thus the long tail is a reflection of the majority of bacteria which to not thrive in a given environment (Finlay & Clark 1999, Finlay 2002). Others have proposed that the long tail is maintained due to the low predation rate by selective protists and low infection rate by bacteriophage (Pedros-Alio 2006). A recent study which

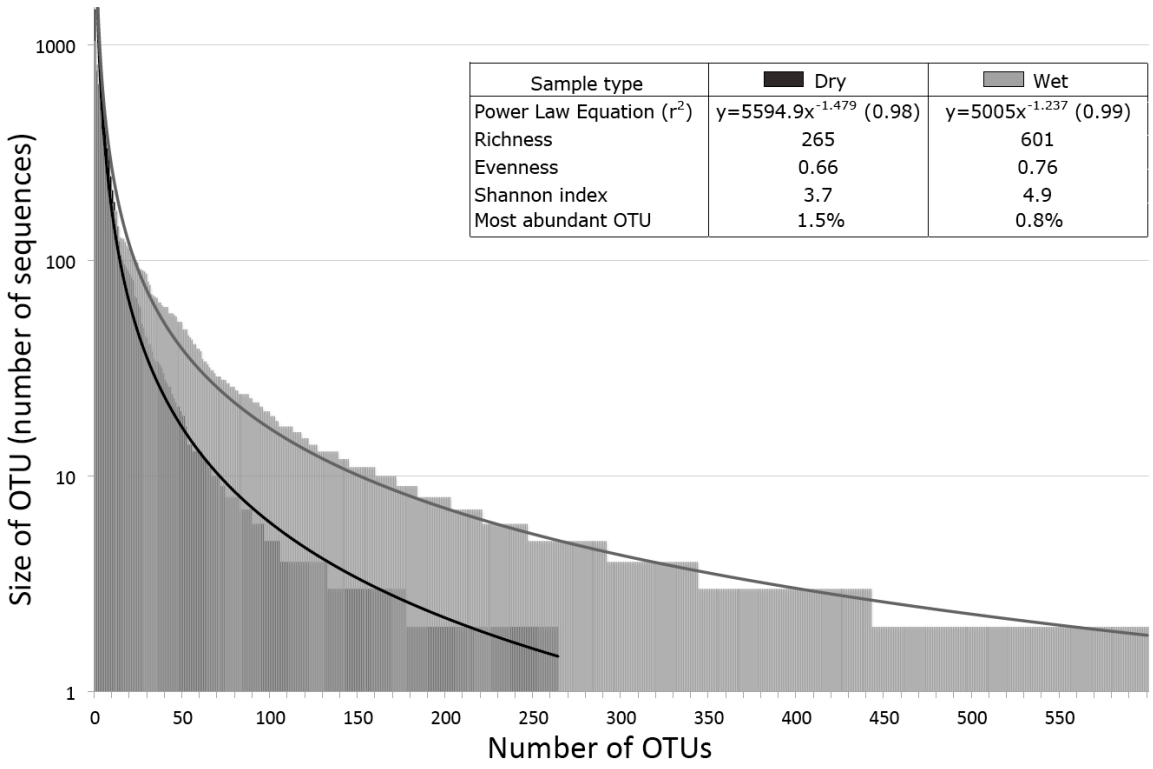

Figure 2.4 Bacterial 16S OTU rank abundance plot and power law curve fit for pooled Dry libraries (Black) and pooled Wet libraries (Gray). Singleton OTUs were removed prior to analysis. Richness (total OTUs observed), evenness (size distribution of OTUs), Shannon Diversity index, and most abundant OTU shown in table.

measured the distribution of both OTU rDNA and rRNA in a sample was able to assign the relative activity of a particular taxa along the rank abundance curve (Jones & Lennon 2010). They found that low abundance taxa were often more active than the highly abundant taxa, and theorized that the ability of bacterial species to enter dormancy allows them to remain highly abundant under oscillating environmental conditions. The ability of bacterial taxa to move along the abundance curve of through periods of dormancy and subsequent revival helps explain a number of phenomena, including seasonal succession in bacterial communities and the long tail itself (Jones & Lennon 2010). This concept could potentially explain the variability in poultry litter bacterial communities seen across houses and litter conditions. Although the in-house environment is kept as stable as possible, numerous factors such feeding regimen and growth of the birds themselves provide stimuli for continuous changes in the microbial composition of poultry litter.

Undoubtedly, the increased moisture in the Wet litter allows more types of bacteria to thrive. However, because moisture content also correlates with a suite of other physiochemical parameters known to play a role in microbial diversity including pH (Hogberg et al. 2007, Lauber et al. 2007), and availability of carbon (Wawrik et al. 2005, Monard et al. 2008) and nitrogen (Mendum et al. 1999) determining the predominant factor contributing to Wet litter bacterial diversity is not possible. Additionally, more types of bacterial metabolism become possible with the microenvironmental conditions provided by increased

moisture content. For example, saturate or near saturated conditions can create an anoxic environment only a few centimeters from the surface. Anoxic conditions occured in the Wet litter samples as evidenced by the occurrence of *Bacteroides* species in all the wet libraries. The Wet litter likely also exhibited a lower pH as was measured in other studies (Lovanh et al. 2007). Acidity can be a limiting factor for bacterial diversity and has been shown to be a predictor of soil diversity across a broad range of soil types (Lauber et al. 2009).

Wet litter areas provide an ideal location for disease spread as they are highly trafficked since the chicken must visit them regularly for feeding and hydration.

Gangrenous Dermatitis connection

One of the primary goals of this study was to examine the microbial communities of poultry houses affected by recurring outbreaks of gangrenous dermatitis (GD) and compare them to communities in houses with no history of GD. Previous research has determined the putative cause(s) of GD to be associated with *Clostridium septicum*, *Clostridium perfringens* and *Staphylococcus aureus* (Willoughby et al. 1996, Wilder et al. 2001). Examining the genus level classification from RPD, *Clostridium* was found at low levels in both GD and non-GD houses. *Staphylococcus* was found in high abundance in all houses, and in fact represented the second largest OTU cluster. However, overall, there was no clear trend in the microbial community structure when comparing the frequency of 16S OTU clusters between in GD and Non-GD

houses. Because the overall bacterial community structure appears to be heavily influenced by husbandry practice, we hypothesize that recurring GD may be attributable to the existence of one or multiple low abundance taxa rather than a single high abundance taxa. This idea is supported by the idea that lower abundance taxa can represent the more active fraction of bacterial communities (Jones & Lennon 2010). Within the dataset, thirteen, 16S OTU clusters were unique to GD houses and these encompassed 1.4% of all reads (Table 2.4). This is a first step in determining if there is a persistent low abundance population of bacteria that contribute to the disease prevalence and persistence within a given house. One or all of these unique clusters may foster microbiological the conditions favoring GD occurrence.

Classification of the representative sequence for each GD unique OTU indicates that nearly one third of clusters cannot be assigned to the family level and only 5 out of 13 can be classified to the genus level (Table 2.4). It is also important to consider that the majority of the unique clusters were derived from sequences in Wet libraries. This is not surprising considering the increased diversity of these libraries (Fig 2.3 B&D, Fig 2.4) and further highlights their potential for wet litter environments to harbor potential pathogens. Examination of the 16S OTU clusters with high confidence genus-level RDP classifications shows that *Anaerococcus* sp were the first and tenth largest unique GD clusters. These corresponded to the 30th and the 152nd most abundant clusters overall respectively. *Anaerococcu*s sp are encompassed in the larger loosely defined

group of gram-positive anerobic cocci (GPAC) which make up a large part of human microbial flora (Murdoch 1998, Song et al. 2003). There genus itself has

Table 2.4 GD Unique OTUs

Cluster number[a]	# of seqs	Majority Dry or Wet derived	Phylum (% confidence)[b]	Class (% confidence)	Order (% confidence)	Family (% confidence)	Genus (% confidence)
30	129	W	*Firmicutes* (100)	*Clostridia* (100)	*Clostridiales* (100)	*Incertae Sedis XI* (100)	*Anaerococcus* (100)
69	48	D	*Actinobacteria* (100)	*Actinobacteria* (100)	*Actinomycetales* (100)	Pseudonocardineae (85)	***Saccharomonospora* (35)**
69	48	W	*Firmicutes* (95)	*Bacilli* (83)	*Bacillales* (74)	***Bacillaceae* (46)**	***Halalkalibacillus* (13)**
91	31	W	*Bacteroidetes* (80)	***Sphingobacteria* (42)**	***Sphingobacteriales* (42)**	***Saprospiraceae* (36)**	***Haliscomenobacter* (22)**
104	26	W	***Deinococcus-Thermus* (26)**	***Deinococci* (26)**	***Thermales* (11)**	***Thermaceae* (11)**	***Vulcanithermus* (8)**
107	24	W	*Firmicutes* (97)	*Bacilli* (95)	*Bacillales* (92)	*Bacillaceae* (80)	***Salirhabdus* (8)**
114	23	W	*Bacteroidetes* (100)	*Bacteroidia* (78)	*Bacteroidales* (78)	*Bacteroidaceae* (75)	*Bacteroides* (75)
114	23	W	*Firmicutes* (100)	*Bacilli* (100)	*Lactobacillales* (100)	*Enterococcaceae* (100)	*Enterococcus* (100)
140	17	W	*Bacteroidetes* (93)	*Bacteroidia* (77)	*Bacteroidales* (77)	*Porphyromonadaceae* (77)	***Dysgonomonas* (45)**
152	15	W	*Firmicutes* (96)	*Clostridia* (96)	*Clostridiales* (96)	*Incertae Sedis XI* (96)	*Anaerococcus* (95)
161	14	W	*Firmicutes* (100)	*Clostridia* (100)	*Clostridiales* (100)	*Incertae Sedis XI* (91)	***Tepidimicrobium* (45)**
170	13	W	*Proteobacteria* (99)	*Alphaproteobacteria* (90)	*Rhodospirillales* (75)	*Rhodospirillaceae* (75)	*Fodinicurvata* (62)
170	13	D	*Actinobacteria* (93)	*Actinobacteria* (93)	*Actinomycetales* (90)	***Microbacteriaceae* (40)**	***Okibacterium* (33)**

a ranked by abundance
b based on RDP classifier

only recently been defined with future revisions probable (Ezaki et al. 2001, Song et al. 2007). Many Anaerococcus strains have clinical significance having been isolated from the penis and vagina microbiomes (Srinivasan & Fredrichs 2008, Price et al. 2010) and numerous diabetic ulcers and other infections (Song et al. 2007). Another GD unique OTU was classified as *Enterococcus*. Although the RDP classified sequences contained *Enterococcus* from all samples, this particular 16S OTU cluster was found predominantly in the GD houses. Only one sequence from each Non-GD house was recruited to this cluster so the cluster was considered unique, and likely represents a different species or strain than the ones found in the Non-GD houses. As a genus, *Enterococcus* has gained attention in recent years due to the isolation of increasingly antibiotic resistant stains from both clinical and industrial settings (Ghidan et al. 2004, Hayes 2004). *Enterococcus* species *faecium* and *feacalis* with resistance to numerous antibiotics have been isolated from both poultry litter and poultry transport containers (Hayes et al. 2004, Graham et al. 2009). SeqMatch and BLAST found matches with 96% identity to *E. asini* (GenBank acc. # GQ337016.1, de Vaux et al. 1998) as well as an unclassified sequence from an anaerobic sludge reactor from an unpublished study with 97% identity (DQ232856.1).

One GD unique OTU cluster had extremely low RDP classification confidence at the phylum level. The tentative classification was to the phylum Deinococcus –Thermus. Members of this phyla are share the ability to survive in extreme environments with the *Deinococcus* sp. able to survive high amounts of

ionizing radiation and the *Thermos* sp. able to survive high temperatures (Griffiths & Gupta 2007, Cox & Batista 2005, Heene et al. 2004). To date, there has been no report of any pathogenic species belong to this phyla. BLAST analyses of these sequences produced very few good identity hits. One sequence with 91% identity came from activate biomass from a high salinity common effluent treatment plant from an unpublished study identified the sequence as belonging to a *Thermus* sp. (GenBank acc#: DQ439630.1). Two other sequences also had 91% identity, one from a waste treatment plant (Khardenavis et al. 2009), and another from human skin (Dekio et al. 2005). Both were listed as unclassified.

Here we provide a starting point for further investigation into the influence of the litter microbial community in houses with recurring GD. There are numerous avenues for future studies to address questions concerning both GD and the full variability of the bacterial community in poultry litter allowed by heterogeneous husbandry practices. Temporal sampling over an extended time period will help clarify how stable the relative abundance of OTUs in a bacterial community really are. Sampling over time could also determine if the OTUs unique to GD are found consistently in GD affected house and continue to be not found in unaffected houses. Ideally, sampling litter from the onset of a GD outbreak and following the litter community through the course of treatment and subsequent return to “normal” in a flock would be invaluable in potentially isolating the key taxa that potentially facilitate the disease.

In addition to temporal sampling, increasing the sample size to include a wider variety of housing conditions will help further our understanding of how they can influence the microbial community. At the same time, taking samples from every house on a particular farm (assuming a similar construction and housing set-up) could reveal how much variation there is between litter samples collected from houses with close proximity. Widening the sampling size may also serve to narrow down the potential GD unique OTUs as some are subsequently found to be present in houses with no GD history.

High through-put sequencing of poultry feces and the chicken microbiome are another natural choice for research. Combining deep sequence studies from both the chicken environment and the chickens themselves is likely to help elucidate the pathogenesis of GD and other poultry diseases.

Conclusions and Future Work

Conclusions Summary

In this study, I have determined that poultry litter contains both bacterial cells and virus particles in abundances greater than both soil and aquatic environments.

The Wet litter beneath the water lines plays a large part in providing the conditions necessary for high bacterial diversity as shown by the shape of the rank abundance curve for the most abundant 16S OTUs (Fig 2.1) and in the high amount of Wet litter unique OTUs (Table 2.1). The wet litter environment also has the potential to harbor unique pathogens as seen by the *Arcobacter* sp. found in House 4. This knowledge could be useful for farmers and veterinary specialists in monitoring the potential for disease outbreak within a house. Alternatively, new water delivery strategies could be implemented to help limit the creation of Wet litter environments.

In addition to a Dry vs Wet dichotomy, this research has revealed the influence of poultry husbandry on litter bacterial communities. The house with the modern evaporative cooling and tunnel ventilation system contained the highest number of unique OTUs with numerous ones ranking in the top 50 most abundant overall. The distinct character of house 4 litter bacterial communities was best illustrated through DPCoA (Fig 2.2). While a house limiting exposure to the outside environment encourages the production of a bigger bird, the fairly static internal house environment could at the same time be allowing particular

populations to thrive in litter that otherwise would remain at low abundance. The house most exposed to the outside environment, House 2, had a distinctly different population compared to the other more closed houses also shown by the DPCoA.

Although there is no obvious trend between the large-scale community structure and incidence of recurring GD, I identified thirteen low abundance 16S OTUs representing a variety of taxa that were found primarily in houses affected by recurring GD. Further supporting the idea that pathogenic groups may come from wet litter, the majority of the GD unique OTUs were composed of sequences amplified from the wet litter samples. The GD unique OTUs found in this study provide a basis for future work investigating their involvement with GD etiology.

Examining the most abundant 16S OTU clusters revealed that representative sequences from four of the top five were highly similar to litter 16S sequences found in previous studies. Despite the differences between houses, four of the top 10 OTUs were found at an abundance greater than 1% and only one of the top ten was found in a single house. These trends for the largest clusters indicate the possible existence of a core group of bacterial species that characterize the litter environment.

Weaknesses of the current Study

One of the primary weaknesses of this study is the small sample size consisting of only eight samples from four houses. This study has provided the

most in-depth snapshot of the bacterial communities in poultry litter conducted thus far, but without data from more samples, rigorous statistical analyses connecting 16S OTU and taxonomy with GD cannot be used effectively. Although two samples were taken at each location, the duplicate samples were pooled prior to DNA extraction to maximize the capture of potential bacterial diversity in the house. As a result, there are no true replicates for the two sample types of each house.

There were also no additional chemical or physical measurements taken during sampling. This proved to be a hindrance when examining the results of DPCoA. Although samples were collected from two houses with recurring outbreaks of GD, at the time the samples were taken, the peak of GD outbreaks for the season had already passed and neither house had experienced mortality attributable to GD in a number of weeks. As a result, this study would have missed dynamic changes in the abundance of specific taxa occurring immediately before, during, and after a GD outbreak. This issue would have been difficult to address, as it is difficult to rapidly respond to an outbreak with uncertain timing and location. Moreover, the rapid mortality and relatively low morbidity experienced by a GD afflicted flock and also requires vigilance and quick action and communication by the farmer.

Future Work

As is often the case, this research raises more questions than answers concerning both the nature of the bacterial community in poultry litter and how it

affects the etiology of gangrenous dermatitis. Depending on the specific question, there are a number directions future research can pursue.

One of the main questions this study raises is the effect of poultry husbandry, e.g. housing conditions, on the exposure of the flock to population of bacterial pathogens. This work shows that the bacterial community structure from different houses supplied with the same feed, breed of chicken, and bedding material can be significantly different; and that this may be the result of how much exposure the flock has to the outside environment. Samples from a greater cross-section of houses needs to be analyzed to gain a greater sense of the underlying dynamics within the composition of the litter bacterial communities. Additionally, a wider collection of environmental metadata needs to be measured to tease apart the basis of these differences. Measurements as simple as pH, litter and air temperature could help to explain variability in litter bacterial communities. More intense measurements of the chemical properties of the litter such as carbon, phosphorus, and nitrogen abundance could also be useful. With the collection of these additional variables it could be possible to statistically determine the factors shaping the top components of a PCA or DPCoA distribution of bacterial communities (Fig 2.2) (Lovanh 2007).

Accounting for the influence the chickens themselves have on the litter microbial community is another potential obstacle to overcome. All growers working for a particular integrator will receive the same breed of chicken, but the growing cycle is not synchronized across all farms. The chickens inhabiting the houses sampled in this study ranged from 2 to 7 weeks in age. The intestinal

microflora of commercial poultry has been shown to change over the course of their short life spans (Lu et al. 2003), and assuming that this change also is reflected in fecal bacterial communities, the only way to normalize for this variation would be to sample houses in which chicken age was nearly synchronous (within one week difference). Since sampling fresh excrement is potentially both time and labor intensive, cloacal swaps could instead be used. Again, sample size would be an important consideration, since a typical poultry house can contain ca. 20,000 birds, sampling must be rigorous to account for potential variation between birds.

It is not known if bacterial succession and overall community structure is immutable after just one flock or if additional flocks can alter the litter community. However, it seems more likely that the litter bacterial community is dynamic and quickly responsive to even small changes in environmental conditions.

Sampling the same flock over a period of time could answer questions about the dynamism of the bacterial community. Sampling over time can also potentially address the question of how the bacterial community changes leading up to, during, and after an outbreak of GD or other flock-wide disease. As mentioned above, if the same GD-unique OTUs are found over temporal sampling, there is a stronger case for their potential role in fostering a the recurring incidence of GD within a house.

There are a number of additional samples that could be collected in addition to litter and feces/cloacal swap. Researchers could also take feather and/or skin samples, as these are also component of litter. Fresh bedding could

also be sampled along with fresh feed. Both of these samples types would be helpful if incidence of disease was seen systematically across many farms at the same time. Samples of the soil immediately outside of the poultry house (especially from an area that that is trafficked by the farmer before/after entering the house could provide a comparison of what bacteria could be easily transferred between the house and outside environment.

Another question that future studies may be able to answer is why some poultry houses are afflicted with GD outbreaks numerous times in a single season while houses very close by are never affected. This is a question that can only be approached by a combination of both spatial and temporal sampling. Bacterial communities could also be assayed for the presence of pathogenicity genes from isolates of GD regions. Such work could indicate whether litter communities serve as a reservoir for these genes.

Alternative paths of study: the viral assemblage

This study has shown that there is an abundance of viruses in poultry litter. This is not surprising given that work over the past three decades involving samples from a variety of environments (Bergh et al. 1989, Breitbart et al. 2004, Williamson 2005, Helton et al. 2006) indicates that viruses outnumber bacteria by more than tenfold. It has also been established that the viral assemblage in an environment can foster exchange of genetic information between otherwise unrelated organisms, and influence nutrient cycling on a global scale (Heenes & Simon 1995, Middelboe et al. 1996, Guixa-Boixereu et al. 1999). Understanding

more about the viral assemblage in poultry houses may not only provide insight into poultry health and husbandry practices, but also provide a proof of concept model for other investigations into the ecology and husbandry of other domesticated animals. Additionally, it could shed light on the various poultry diseases in which the route of infection is not readily apparent.

There are a number of ways to go about investigating the ecology of viral assemblages in poultry litter fitting various goals and budgets. In addition to measuring abundance, the diversity of a given viral assemblage can be assessed using viral concentrates made from extracted viruses which are run in a randomly amplified polymorphic DNA (RAPD) assay. In RAPD-PCR, the banding patterns of different viral concentrate samples can be compared and the similarity of viral assemblages can be compared through the presence or absence of bands RAPD-PCR bands (Winget & Wommack 2008). Noteworthy or unique bands from the gel can be isolated, amplified, and sequenced to provide further insight to what genes were highly amplified by the RAPD technique (Helton & Wommack 2009). Pulsed field gel electrophoresis (PFGE) (Serwer et al. 1993) can also be used to determine the relative sizes of the viral genomes found in poultry litter.

Researchers interested in the transfer of antibiotic resistance or pathogenicity genes among bacteria could assay not only the litter bacterial community, but the viral assemblage as well using gene-targeted PCR assays against litter viral extracts.

The methods mentioned above have been used in viral ecology studies of aquatic and soil environments and could provide excellent insight into key

questions about viral assemblages of poultry litter. An even better tool that is rapidly emerging is the use of deep sequencing to obtain a viral metagenome from an environmental sample. (Forest & Rohwer 2005). Using this method, viral genes can be detected and given adequate coverage; full viral genomes can be assembled. This approach could also be used to observe if there are antibiotic resistance or bacterial pathogenicity genes present in the viral metagenome and at what abundance.

The advances in high throughput sequencing technology will soon negate many of the limitations of the current study. Pyrosequencing reads are approaching a length that will cover the entire 16S rRNA gene and the cost-per-read continues to decrease. Third generation sequencing platforms are also likely to be able to provide exceptionally long and accurate reads. This will enhance both the resolution of the community under study and the accuracy of classification.

As was mentioned previously, packing many animals together provides an excellent environment for rapid dispersal of an easily communicable disease. Over reliance on antibiotics to both prevent poultry disease and boost poultry growth has generated new strains of bacteria highly resistant to many of the currently used antibiotics.

Understanding the microbial community of poultry litter will not be a panacea for all issues related to poultry health, but it will provide the basis for

developing new preventative practices and perhaps lead to improved husbandry methods.

METHODS

Sample collection

Samples were collected from four poultry houses in the Delmarva Peninsula between 10:30am and 1:05pm on August 22, 2008. A 15 cm spade rinsed with 70% ethanol was used to collect 4 scoops of litter from the top 3-6 inches of litter within in a 5-meter area and placed in 1 gal zip-loc bags. The process was repeated in another part of the house between 5 and 25 meters away and placed in a separate bag. Litter directly under the water lines was collected in the same manner as the dry samples and roughly parallel to where they were collected (Fig 3.1). All 16 samples were transported to the Delaware Biotechnology Institute and stored at 4° C until DNA extraction and enumeration.

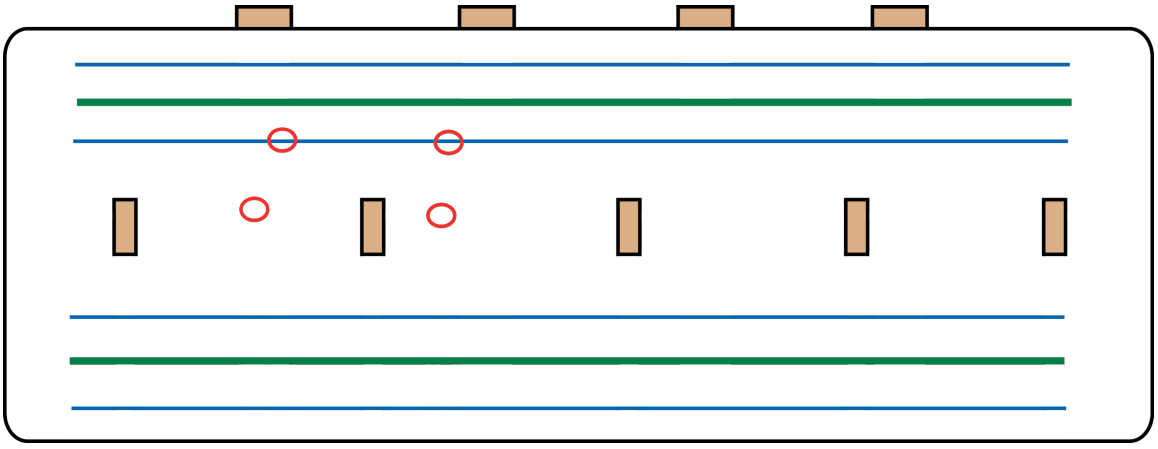

Figure 3.1 Simplified Diagram of a poultry house (not drawn to scale). Green lines represent feed lines, blue lines represent water lines. Brown boxes represent possible box fan configurations. Red circles represent examples of where sampling took place.

DNA Extraction

All samples were homogenized by hand and split into approximately equal amounts. One set samples was transported to the Institute for Genome Sciences at the University of Maryland School of Medicine where DNA was extracted in the laboratory of Dr. Jacques Ravel. The duplicate samples from each house and litter type were pooled to give a total of 4 Dry and 4 wet samples. Sterile PBS was used to bring the Dry litter to the same consistency as the wet. An enzyme cocktail optimized to lyse gram-positive bacteria was mixed with the litter samples (consisting of 0.15 g of litter, 5 μl lysozyme, 15 μl Mutanolysin, 33 μl lysostaphin 10 μL proteinase K, 50 μL 10% SDS in 1 mL of 0.05 potassium phosphate buffer). The mixture was shaken in a FastPrep FP120 (MP Bio) instrument for 40 s and allowed to sit for 5 min. DNA from the mixture was then purified using Zymo-Spin IV-HRC spin filters and accompanying kit reagents. DNA concentration of the elutant was measured by a nanodrop ND-1000 spectrophotometer (Thermo Scientific), aliquoted, and stored at -20º C.

Sequencing (as performed by members of the Ravel lab)

Each of the 8 samples was amplified using a barcoded universal bacterial 16S reverse primer with adaptors for 454 pyrosequencing (Roche). All samples used the same forward primer with accompanying 454 linkers (Table 3.1). The components for one, 25 μL PCR reaction are as follows: 0.1 μL Platinum Taq High Fidelity (Invitrogen), 2.5 μL 10X high fidelity PCR buffer, 1 μL 50 mM MgSO4, 0.5 μL 10mM dNTP Mix, 0.75 μL forward primer, 5 μL reverse primer,

50 ng amplified DNA sample (not to exceed 10uL), 5.15 μL nuclease-free water. PCR conditions are as follows: 94º C for 2 min, 94º C for 30 s, 52º C for 30 s, 68º C for 1 min (repeat temperature 2-4 30 times), 68º C for 5 min. Amplicons and negative controls were run on 2 separate 1% agarose gels (made with TAE and ethidium bromide) for 35 min at 105 V. A BioRad Geldoc XR system and accompanying software was used to determine the DNA concentration of each amplicon[7]. After the concentration was determined, 100 ng of DNA for each sample was pooled for use in the pyrosequencing pipeline.

Table 3.1 Primer Sequence for each sample

Sample name	Primer Sequence (5^1-3^1)[a]	
Dry 1	338R	GCCTCCCTCGCGCCATCAGACTCGTCTCATGCTGCCTCCCGTAGGAGT
Dry 2		GCCTCCCTCGCGCCATCAGACTGACTGCATGCTGCCTCCCGTAGGAGT
Dry 3		GCCTCCCTCGCGCCATCAGACTCGTCTCATGCTGCCTCCCGTAGGAGT
Dry 4		GCCTCCCTCGCGCCATCAGACTGCTCTCATGCTGCCTCCCGTAGGAGT
Wet 1		GCCTCCCTCGCGCCATCAGACTGACTGCATGCTGCCTCCCGTAGGAGT
Wet 2		GCCTCCCTCGCGCCATCAGAGAGACTGCATGCTGCCTCCCGTAGGAGT
Wet 3		GCCTCCCTCGCGCCATCAGAGACGTCTCATGCTGCCTCCCGTAGGAGT
Wet 4		GCCTCCCTCGCGCCATCAGAGAGCTCTCATGCTGCCTCCCGTAGGAGT
All samples	27F	GCCTTGCCAGCCCGCTCAGTCAGAGTTTGATCCTGGCTCAG

[a] blue region is the 454 linker, red region is the barcode, green region is the primer sequence

Bacterial Extraction

In triplicate for each sample, 4 g of litter was weighed and placed in a 50 mL centrifuge tube. Forty milliliters of autoclaved 1% potassium citrate buffer (containing 10 g potassium citrate, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, in 1 L H_20, pH: 7.0) was added to each tube, shaken for 5 s, and placed on ice for 10 min.

[7] The inaccuracy of this system is the primary reason for differences between library sizes for each sample.

The mixture was blended in a kitchen blender (Osterizer) for 3 min and transferred back to the centrifuge tube. Nine milliliters of the blended supernatant was transferred to an ultra centrifuge tube containing 2 mL nicodenz solution. The tubes were centrifuged at 9,000 rpm at 4° C using a SW 412 Ti rotor. Eight point five milliliters of supernatant in addition to the nycodenz was homogenized and transferred to 4.5 mL cryovials, adjusted to 1% gluteraldehyde and snap frozen in liquid nitrogen.

Viral Extraction

Virus was extracted in triplicate by placing 5 g of soil in a 50 mL centrifuge tube followed by the addition of 15 mL of 1% potassium citrate buffer (containing 10 g potassium citrate, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, in 1 L H_20, pH: 7.0). Tubes were vortexed for 5 s and placed on ice for 20 min. On ice, samples were sonicated (Branson S-450a) in three, 1 minute cycles, with 1 minute intervals in between each cycle. The mixture was then centrifuged at 3,000 g for 30 min. Supernatant from each sample was passed through a 0.22 μm sterivex filter (Millipore) into 4.5 mL cryovials and snap frozen in liquid nitrogen.

Bacterial/Viral Enumeration

One hundred microliter aliquots of virus or bacterial extract were suspended in 900 μL of sterile deionized water and further diluted between 100 and 1000-fold more before being vacuum filtered (~25 mm Hg) through a stack of 25-mm filters consisting of a 0.02-μm Anodisc (Whatman) for virus or 0.2 μm

membrane filters for bacteria (Millipore), a 0.22 μm Supor filter (Pall corporation), and a glass fiber filter (Pall Corporation). The anodisc filters containing trapped virus and membrane filters containing trapped bacteria were stained in the dark for 15 min with 400 μL of 1X SYBR Gold (Molecular probes). Filters were mounted on glass slides (Fisher Superfrost) along with 20 μL antifade solution (containing 20 mL PBS, 20 mL 100% glycerol, 400 μL p-phenyldiamine) to preserve fluorescent activity. Epifluorescent microscopy (EFM) was used to analyze the slides using an Olympus BX61 microscope (Olympus) with a flourescein isothiocynate excitation filter. Ten to fifteen fields per sample were imaged digitally at 1000x with a Retiga EXi camera (Q Imaging). Viruses were counted using iVision v4.0.8 software with a custom size-selection script. Bacteria were counted manually. Bacteria and virus counts were averaged based on counts from three replicate slides.

Post Sequence processing

Trimming by IGS

Raw sequences obtained by 454 pyrosequencing were processed at IGS by an automated pipeline which removed reads with a quality score less than 30 and trimmed off both linker and primer sequence. The resulting reads were sent to DBI for analysis.

Greengenes

Each library was aligned using the NAST alignment tool available online from the Greengenes website (greengenes.lbl.gov). The minimum length was set at 200bp and minimum identity at 75%.

ARB

ARB software v5.1 was used to generate a distance matrix for each library and for all libraries combined using the jukes-cantor substitution model.

DOTUR

Using the ARB-generated distance matrices, DOTUR was used to generate OTUs for all libraries and for each library with rarefaction. Output files containing OTU frequency, Shannon-index and rarefaction curves were parsed using custom PERL scripts and used to generate figures and tables.

Ribosomal Database Project

The RDP naïve Bayesian Classifier tool (Wang et al. 2007) was used to classify all sequences from the phylum through genus levels at the 80% (performed at IGS) and 50% (performed at DBI) confidence levels. The classifier was also used on the representative sequence of the most abundant and the unique OTUs generated from DOTUR. The RDP SeqMatch tool was used to compare representative sequence from individual OTUs to the RDP database.

BLAST

The BLASTn tool from NCBI was used to compare representative sequences from the top OTU clusters.

Programs used for Tables and Figures

Tables were generated using Microsoft Excel 2008 and Microsoft Word 2008 (Microsoft corporation). Figures were generated using Aabel 2.0 (Gigawiz), Microsoft Excel 2008 (Microsoft corporation), R ver2.6.2 with attached packages ade4, ape, ecodist, and gplots (The R project), and Adobe Illustrator CS2 (Adobe).

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