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Authors: Shange, Raymon S., Ankumah, Ramble O., Zabawa, Robert, and Dowd, Scot E.

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ORIGINAL RESEARCH

Bacterial Community Structure and Composition in Soils Under Industrial Poultry Production Activities: An Observational Study

Raymon S. Shange[1,2], Ramble O. Ankumah[2], Robert Zabawa[2,3] and Scot E. Dowd[4]

[1]Carver Integrative Sustainability Center, Tuskegee University, Tuskegee, AL, USA. [2]Department of Agricultural and Environmental Science, Tuskegee University, Tuskegee, AL, USA. [3]George Washington Carver Agricultural Experiment Station, Tuskegee University, Tuskegee, AL, USA. [4]MR DNA (Molecular Research), Shallowater, TX, USA.
Corresponding author email: rshange2946@mytu.tuskegee.edu

Abstract: Confinement is the predominant method of producing poultry and eggs for consumption in the US. Because of its high-density approach, the potential health threats regarding pathogenesis in animals and humans have raised concerns. Although there best management practices exist to control the persistence and proliferation of pathogenic bacteria in poultry houses, very little is known about the bacterial communities, and poultry houses are potential pathogen sinks. We assessed the contribution of industrial poultry production to the structure and composition of bacterial communities in the soils at a poultry production site. Soil samples were collected from under poultry housing areas, litter storage areas, and an accompanying pasture adjacent to the production area; and environmental DNA was extracted from the samples. Following validation and amplification, DNA was sequenced using bacterial-tag encoded pyrosequencing. Bioinformatics analysis showed that the bacterial communities in the soils showed no significant differences in species richness according to observed and estimated operational taxonomic units (Chao1 and rarefaction). Proteobacteria were the major phyla present in all samples ranging from 37.1% in the soils under poultry houses to 53.4% of the sequences identified under pasture soils. Significant shifts in specific taxa were observed, including drops in the abundance of Acidobacteria observed from the poultry house to litter storage soils ($P < 0.05$) α-Proteobacteria increased from poultry house soil (10.9%) to pasture soils (32.8%, $P < 0.01$) and soils under litter storage (22.3%, $P < 0.05$). The phyla Bacteroidetes, which were observed between poultry house and pasture soils, dropped significantly from 21.8% to 7.2% ($P < 0.05$). Clustering exhibited a closer relationship between the soils under pasture and litter storage, while those under the poultry houses were unique. Pathogenic genera were also found in greater abundance under the poultry houses, which raises the question of persistence and re-colonization of bedding material even in the presence of mitigation attempts.

Keywords: poultry production, soil ecology, 16S rRNA, bacterial diversity

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Introduction

The importance of poultry production lies in its increasing significance to the economic and environmental well-being of agricultural systems in the United States. About 8.6 billion broilers are produced each year in the US, valuing $23.2 billion.[1] More than 65% of US broiler production is concentrated in the Southeastern States, with Alabama ranking third behind Georgia and Arkansas.[1] In 2011, Alabama produced roughly 1 billion birds with total cash receipts of $2.66 billion and representing an approximately 14% increase compared with 2002.[1] Poultry farmers reached this level of productivity by using confinement housing, in which high-density broilers are raised on litter in houses containing between 15,000 and 50,000 birds per batch.[2] As a result of the shift to fewer and larger confined animal operations, environmental and economic issues associated with utilization or disposal of animal manures and litters have become a focal point of conservation efforts.[3] A layer of wood shavings, sawdust, straw, peanut hulls, or other suitable bedding material is placed on the soil surface of poultry houses to mitigate the downward migration of excess nutrients, pathogens, and toxins. Dry/wet litter (cake) is removed after each flock with a complete clean-out performed once every 12 months or longer, depending on owner requirements. It is estimated that each of the 700,000 poultry houses for broiler production in the US generates approximately 180 tons of litter per year.[4]

Although land application is the most common and usually the most desirable method of utilizing manure because of nutrient and organic matter addition to soils, poultry litter has versatility in its uses as feed[5–7] and biomass for fuel production. However, concerns regarding pathogenic microorganisms commonly found in poultry litter (eg, Listeria monocytogenes, Salmonella spp., Escherichia coli, Clostridium spp., Campylobacter spp., Staphylococcus aureus and Bordetella spp.) raise issues related to management practices for reasons of food safety and public health. Researchers have previously identified these microorganisms among others as residential pathogens in poultry litter.[7–9] These and other bacterial species are pathogenic to humans and also may be pathogenic to poultry, causing serious infections that may lead to death and/or poor flock performance with no obvious symptoms. As stated above, recent trends in industrial broiler production have led to confined housing of thousands of birds, which has led to the practice of antibiotic management for purposes of treating anticipated and visible infections as well as improving flock growth. Recent concerns have been raised about the ability of pathogenic bacteria to develop resistance to antimicrobials used in food animal operations. Several reviews have addressed this issue;[10–13] specifically, zoonotic enteropathogens (ie, Salmonella, Campylobacter, Yersinia, and some strains of E. coli, such as serotype O157:H7) and commensals (ie, Enterococcus and other E. coli strains) have shown the capacity to develop antibiotic resistance in various animals.[14–21] The importance of these particular groups lies in their possible exposure to humans.[22]

Variations in on-site decisions, as well as the ability of some microbiota to survive adverse conditions, allow for the cultivation of potentially harmful microbial communities in litter as well as in underlying soils and surrounding water sources. Groundwater pollution by microbiota occurs through the percolation of microbes along with water (ie, rainwater or irrigation water) through the soil profile, reaching underground aquifers. The implications for such pollution have been shown, as between 1989 and 2002[23] 64% cases of waterborne diseases in the US were traceable to groundwater. Another survey showed that figure to be significantly higher (94%) for the years 2001–2002.[24]

There have been multiple studies directed at the microbial characterization of poultry litter,[7,8,25–27] as well as its effects on soil microbial communities through land application as a fertilizer.[28–31] Many microbial issues require improved management, and it is not clear to what extent poultry litter management influences soil microbial communities under confined poultry production operations.

The goal of this study was to characterize the microbial community structure of the soils in and around a confined broiler production system using pyrosequencing based on 16S rRNA gene sequences. Specifically, this study seeks to determine whether there are changes in bacterial community composition in structure between poultry houses, litter storage areas, and pastured areas. We also assessed the presence of pathogenic bacteria in each of these soils.

Material and Methods

Study sites

The study site was Wayne Farms broiler production unit located at 32° 4′ 2.2″ N and 85° 42′ 35.9″ W, on a 4 Hectare (Ha) land in Bullock County, AL, USA. The soil series of the study area were Alaga (loamy sand, thermic, coated Typic Quartzipsamments) and Conecuh (sandy loam, fine, smectic, thermic Vertic Hapludults). For the past 10 years, this land has been used as an industrial broiler production site. During each of those ten years, 5–6 batches (~80,000 broilers per batch) were produced with residual litter being removed annually and stored outside of the poultry houses at a designated site until a market could be established for the litter. In addition, there was approximately 1 Ha of pasture for a herd of 10 horses to graze. This area was only lightly grazed, as the horses were released onto this part of the land for only 2–3 days per week.

A preliminary geostatistical study of soil biochemical characteristics provided the initial evidence that soil biochemical and biological factors spatially vary with respect to land use type on this site (Table 1).[32] A stratified random sampling design was used in an effort to obtain a statistically useful dataset while being cost-efficient. The three sampling areas were constructed of different sizes, which were reflective of the amount of area covered by each on the farm and overlaid by sampling grids. Samples were randomly collected from vertices of the grid, such that the amount of samples collected was proportional to the size of the sampling areas.

Table 1. Selected soil properties amongst different land use strata.

Soil property	Broiler housing	Storage	Pastured
APA†	2.25a	2.81b	2.52ab
ACP†	1.75a	1.91b	1.90b
PD†	0.98ab	1.34b	0.71a
pH	6.39a	7.70b	6.55a
SOC	1.34	2.17	1.61
TN	0.22	0.26	0.18
Sand‡	0.72a	0.73a	0.79b
Silt & Clay‡	0.28	0.27	0.21

Notes: Different letters denote significant differences between measured variables at $P < 0.05$.
†Values for enzyme activity are in units of μmol p-nitrophenol g soil^{-1} hr^{-1}.
‡Values for particle size are expressed as a fraction of total soil particles (1.00).
Abbreviations: APA, acid phosphatase; ACP, alkaline phosphatase; PD, phosphodiesterase; SOC, soil organic carbon; TN, total nitrogen.

DNA extraction

Whole community DNA was extracted from approximately 0.25 g of soil (oven dried basis of field-moist soil) using the Power Soil Extraction Kit (MO BIO Laboratories, Soloana Beach, CA, USA) according to the protocol provided by manufacturer. Extracted DNA (2 μL) was checked for purity and concentration using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) as well as being run on an 0.8% agarose gel. Once quality and concentration were determined, 3 samples from each of the three land-use types were pooled at equal bacterial DNA ratios to create three pools of DNA, each representing a major category of land use across the agroecosystem. The samples were then submitted to Research and Testing Laboratories (Lubbock, TX, USA) for PCR optimization and pyrosequencing analysis. PCR, massively parallel pyrosequencing, and tag design were carried out according to a procedure described previously by Dowd et al.[33,34]

All DNA samples were diluted to 20 ng/μL from which a 20 ng (1 μL) aliquot of each sample DNA was used for a 25 μL PCR reaction: 5 min denaturing at 95°C, anneal for 30 cycles of 94°C for 30 sec, 52°C for 40 sec, 70°C for 40 sec, and final extension at 70°C for 5 min. Primers used were the 16S universal Eubacterial primers 28 F (5′-GGC GVA CGG GTG AGT AA) and 530 R (5′-CCG CNG CNG CTG GCA CS). The resulting amplicons were equally mixed and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). In preparation for pyrosequencing, the size and concentration of DNA fragments were measured using DNA chips under a Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad Laboratories, Hercules, CA, USA) and a TBS-380 Fluorometer (Promega Corporation, Madison, WI, USA). Samples of double-stranded DNA (9.6×10^6 molecules/μL with an average size of 625 bp) were combined with 9.6 million DNA capture beads for emulsion PCR. The resulting bead-attached DNAs were denatured with NaOH and sequencing primers were annealed. The 454 Titanium sequencing run was performed on a 70×75 GS PicoTiterPlate by using a Genome Sequencer FLX System (Roche, Nutley, NJ, USA).

Bioinformatics and statistical analysis

As a result of pyrosequencing services, quality trimmed sequences and hierarchal taxonomic data were provided following the bioinformatic pipeline described by Acosta-Martinez et al.[35] Each sequence was trimmed back to utilize only high-quality sequence information. Tags whose sequences designated individual samples were extracted from the FLX-generated multi-FASTA file, while parsing that file into individual sample-specific files. Tags that did not have 100% homology to the original sample tag designation as well as sequences that were less than 200 bp after quality trimming were not considered. Samples were then depleted of definite chimeras using B2C2 software that is described and freely available from Research and Testing Laboratory (Lubbock, TX, USA, USA). The resulting sequences were then evaluated using BLASTn[36] against a custom database derived from the RDP-II database[37] and GenBank (http://ncbi.nlm.nih.gov). The sequences contained within the curated 16S database were those considered to be high-quality based upon RDP-II[38] standards and which had complete taxonomic information within their annotations.

Identification at the species level for the purpose of this study was considered tentative, and these taxonomic groups are referred to as operational taxonomic units (OTUs) and not species. Following best-hit processing, a secondary post-processing algorithm was utilized to combine genus and other taxonomic designations generating compiled data with relative abundance of each taxonomic entity within the given sample. Phylogenetic assignments were based upon NCBI taxonomic designations. Further processing and out-based analyses were then carried out using the MOTHUR[39] suite of programs for sequence processing and diversity analysis [v.1.19.3]. Processing commands included those for identifying/consolidating unique sequences, removing low-quality sequences, filtering, chimera removal, multiple sequence alignment, distance matrix generation, and sequence clustering into OTUs. OTU-based analysis differentiates itself from other methods of phylogenetic analysis in that it quantifies richness, diversity, and similarity amongst and between samples. The resulting clusters were assessed at 3% dissimilarity to provide the data needed for downstream analysis given a previous explanation of the relationship percent dissimilarity and species estimation based upon rarefaction.[40]

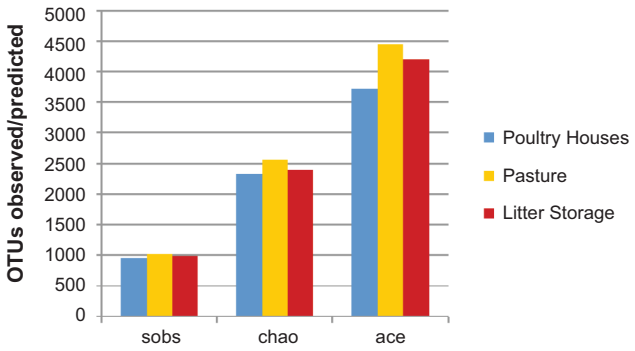

Figure 1. Richness/diversity estimators as calculated by mothur at levels of 3% dissimilarity.

Clusters at 3% were then utilized to generate rarefaction curves and the (diversity) indices ACE[41] and CHAO[42] as well as unweighted UniFrac for principle coordinate analysis (PCoA) plots.

Results and Discussion

Richness and diversity estimates

Figure 1 shows the observed and expected OTUs at 3% dissimilarity. The maximum OTUs detected across the soilscape at the site according to the observed clusters (sobs) at 3% dissimilarity was 1035 (Fig. 1), found in the pastured bacterial community. All Chao1 values reported in Figure 1 were comparable to the maximum OTUs predicted by rarefaction models (Fig. 2), while ACE estimators predicted significantly higher OTUs. No significant differences were observed between the bacterial communities under various areas for any of the estimators ($P < 0.05$). A distinct trend was detected in all estimators, suggesting that the highest richness was detected in the pastured area,

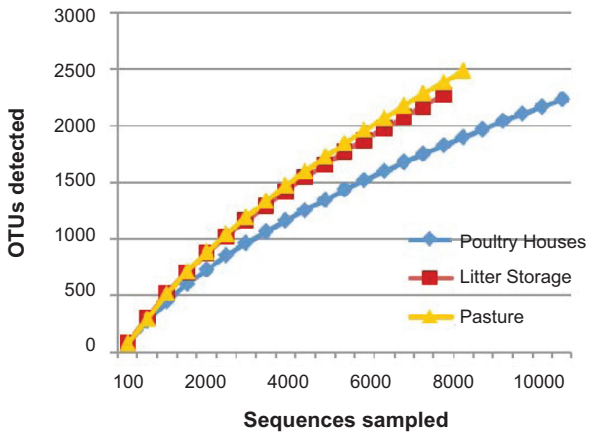

Figure 2. Richness as estimated by rarefaction in mothur at a level of 3% dissimilarity.

while the lowest richness was found in the bacterial community under the poultry houses.

The amount of species richness seemed to be similar among the different areas of the agricultural ecosystem, as significant differences in the soils were only observable using the ACE diversity index (Figs. 1 and 2). With the application of organic amendments (particularly poultry litter) to soils, researchers have reported changes in microbial communities found within these soils.[43,28] An important exception was that reported in a study by Acosta-Martinez and Harmel[28] who observed that that when poultry litter was applied to pasture surface soils and not incorporated into the soil, there was an obvious lack of response by microbial communities at the highest application rates, suggesting the need of some type of mechanical mixing of soil and litter to aid enhancement. This observation can be compared to that of the litter storage area, which tends to be higher in richness, but has no significant differences except for the ACE estimate. There is no frequent mechanical disruption to this area to allow for colonization of the soil by poultry litter microbes, thus resulting in a moderate level of diversity.

Bacterial taxa

The soils under the poultry litter houses showed significant shifts in the relative abundance of five of the eight top bacterial phyla (Fig. 3). Significant shifts ($P < 0.005$) occurred in the phylum Proteobacteria between the broiler house community (37.1%) and the pastured community (53.4%). Because of the observed dominance of Proteobacteria in soils, the major bacterial classes were assessed for significant shifts amongst the soil systems as well (Fig. 4). Among the five classes of Proteobacteria, α-Proteobacteria was the only class to show significant shifts. These shifts occurred between BRHS soil (10.9%) and soils under grazed pasture (32.8%, $P < 0.01$) and soils under litter storage (22.3%, $P < 0.05$). Another shift that occurred between the BRHS and grazed pasture soil systems was in the phyla Bacteroidetes, which dropped from 21.8% to 7.2% ($P < 0.05$). The classes Flavobacteria and Bacteroidetes showed similar trends (Fig. 4). Other major shifts observed between the broiler house area and the litter storage areas were a decrease in Acidobacteria (9.4% to 1.9% at $P < 0.005$) and an increase in Chloroflexi (3.0% to 10.8% at $P < 0.05$). Chloroflexi was the only phyla showing a significant decrease in relative abundance between the litter storage area and the grazed pasture soil system. Chloroflexi relative abundance actually dropped from 10.8% in the litter storage soil to 3.0% in the pasture soil ($P < 0.05$).

Proteobacteria remained the most dominant phyla under the different soil conditions, suggesting their central role in the soil ecosystem. Along with Actinobacteria and Bacteroidetes, Proteobacteria have been suggested to be a copiotrophic group of organisms;[44] as such, it would be expected that these organisms would be found in high abundance where there

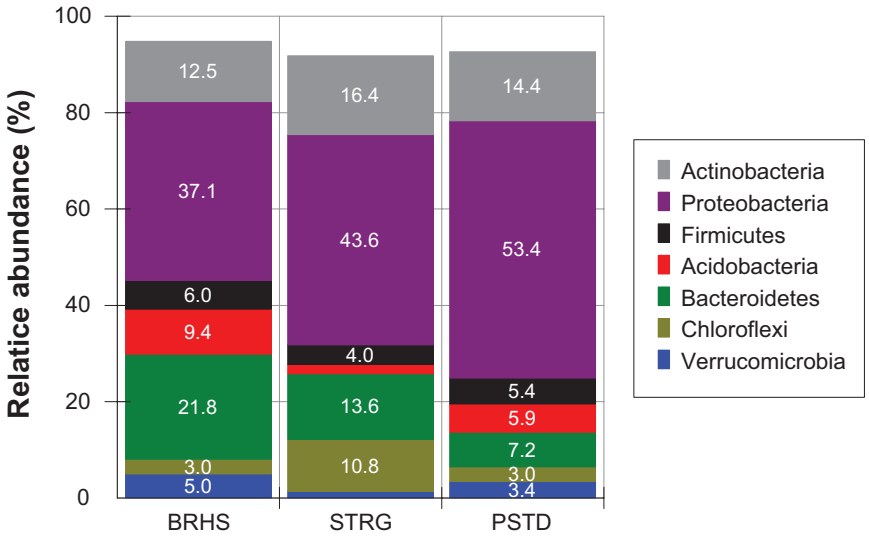

Figure 3. Relative abundance of major phyla across land use systems.

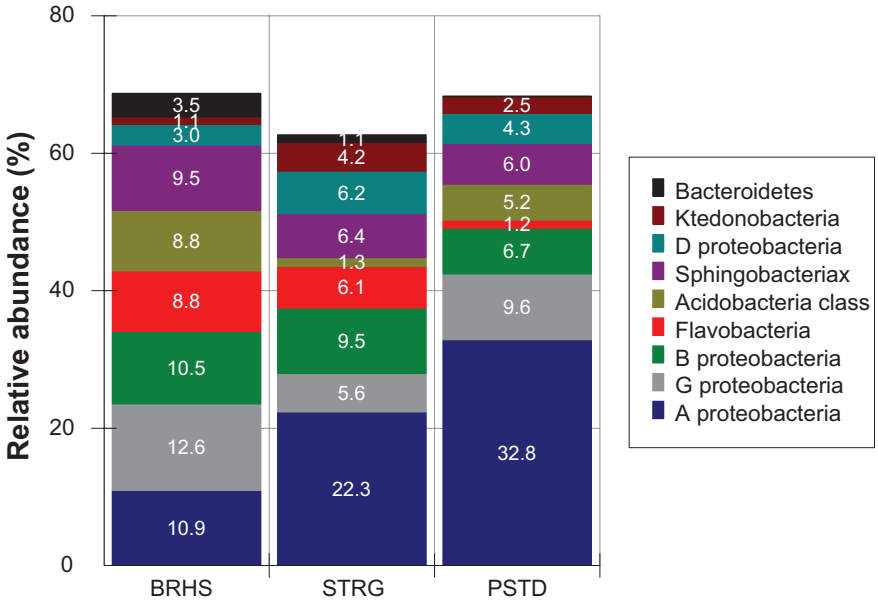

Figure 4. Relative abundance of classes from the most dominant phyla identified.

is access to plenty of organic carbon. It was observed that there was a significant decrease in the Proteobacterial phyla from the poultry house soil compared to the pastured soil. A further examination of Proteobacterial classes showed that the only significant shift occurred in one of the five classes, α-Proteobacteria (Fig. 4). Another important shift observed was in the phyla Bacteroidetes, which are found in soils, but are also largely associated with the internal and external flora of animals.[45] The classes that played major roles in this shift are Flavobacteria and Bacteroidetes. The class Flavobacteria has recently been described as predatory, since a growing number of its members are being characterized as such.[46–51] As there is no literature on the predator-prey relationship of Flavobacteria and other microbes, their increase could not be readily explained by shifts in other groups. More research in the area of Flavobacteria predation could shed more light on this ecological feature of the group. Bacteroidetes, one of the most widely studied classes, have been ecologically associated with animal intestines and feces, but they also contain genera that are known to be associated with soils.[52] Because both of these groups contain organisms that are considered opportunistic pathogens, and are associated with animal feces and soil colonies, the soils under the poultry house seem to be an optimal environment to find these copiotrophs, where there is a convergence of these two ecosystems. This convergence may provide the conditions necessary for cross colonization of the litter layer and soil layer under the poultry houses.

Clustering of soil samples

Bacterial classes were used in cluster analysis to generate the double dendrogram shown in Figure 5. The double dendrogram allowed visualization of the diversity found among microbial classes over the study site. The patterns appeared to show the community in the poultry house soils exhibiting a distinct pattern compared to in other systems. Even within the poultry house samples, sample BRHS_2 showed less similarity to the other poultry house samples. The relative abundance was more focused towards the classes at the top of the y-axis than in any of the other sampled areas. The individuality expressed by this sample was reflected in the clustering analysis, as this sample showed a closer relationship to the other 6 samples at a distance of 2.25. All other samples (litter storage and pasture) clustered together at ~1.75, while individual samples for each of these strata clustered at ~1.50, showing sample similarity according to their soil system. Further analysis using Unifrac metrics and PCoA supported this data.

The 3-dimensional plot visualized from the principle coordinates analysis based upon unweighted Unifrac metrics (Fig. 6) showed that the samples of the bacterial community contained under the poultry houses distinguished itself in response to the variation detected in the samples across three axes. The x, y, and

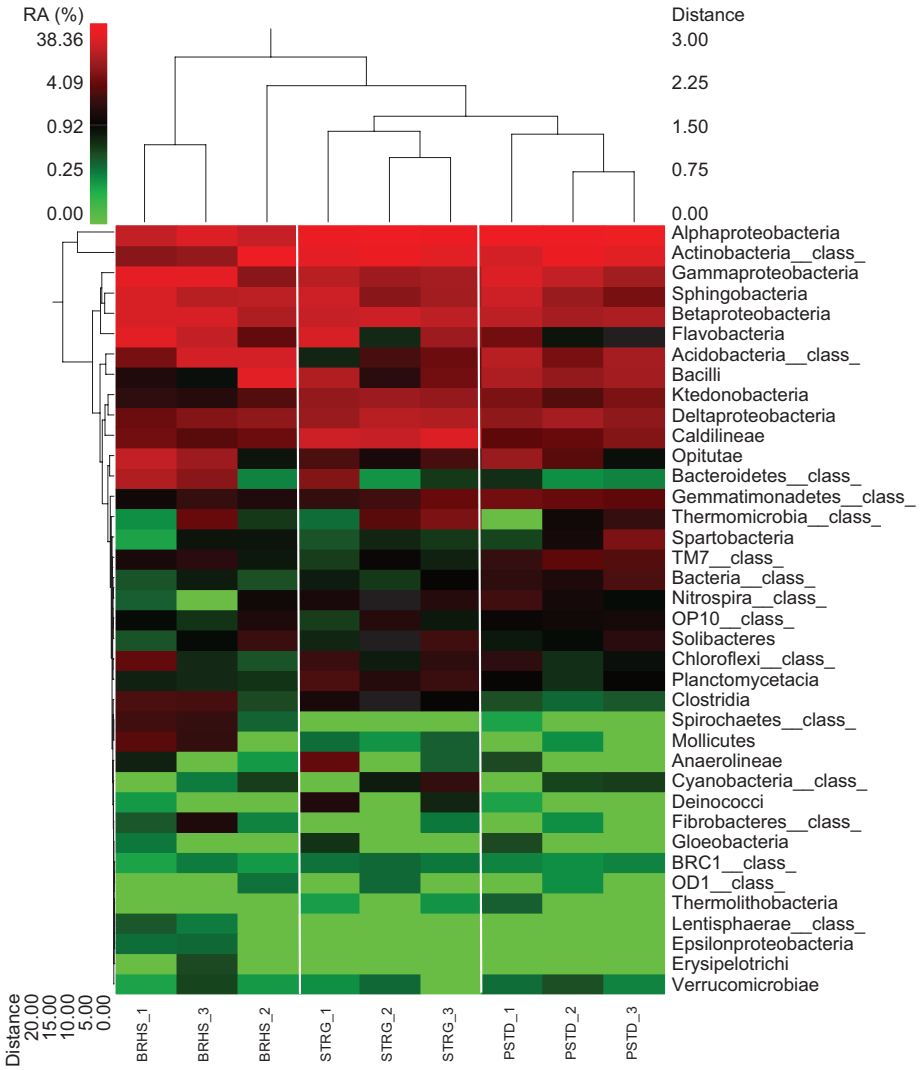

Figure 5. Hierarchal clustering based upon the relative abundance of orders across the 9 samples across the three land use systems. Clustering in the Y-direction is indicative of abundance, not phylogenetic similarity.
Abbreviations: RA, relative abundance; BRHS, poultry house samples; STRG, litter storage samples; PSTD, pasture samples.

z axes in the PCoA plots (Fig. 6) represented 16.8%, 16.1%, and 12.6% of variation, respectively. Similar clustering can be observed in Figure 4.5 in that the samples from the same type of land-use system clustered together, with the exception of the BRHS_2 sample, which differed in response to both axis 1 and primarily axis 3.

Genera of interest

Although the sequences represented in Table 2 did not have high relative abundances compared to other taxonomic classes, scale is important determining their potential environmental impact. In order to assess the significance of these specific genera for the environmental quality of the production system, average relative abundances were calculated using percentages. T-tests were conducted to determine if there were higher abundances in specified soil systems across the site. Major groups of interest were genera known for their contribution to pathogenesis in human and animal systems. With the exception of Mycobacterium, all pathogenic genera exhibited their highest abundance under poultry houses, with Brevibacterium and Staphylococcus being significant. Soils under the poultry houses showed the pattern of BRHS > PSTD > STRG representing 8.12%, 1.61%, and 0.85% of the total sequences in each system.

When considering the genera present in the soils, these data suggest that groups important to pathogenicity are present in all soils, though particularly

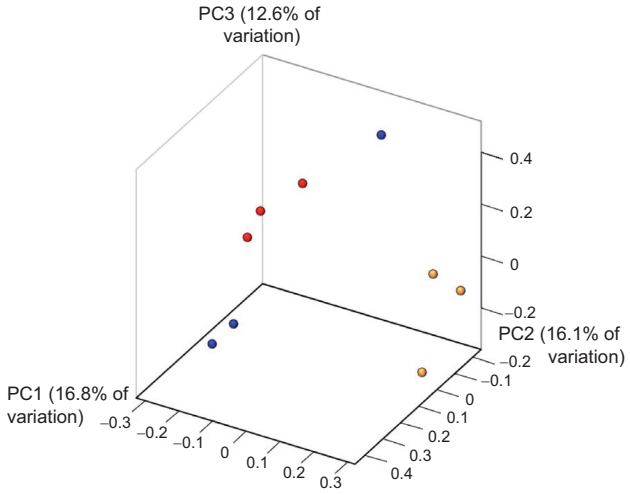

Figure 6. A 3-dimensional PCoA plot showing the clustering of samples around the first three axes of variation based on unweighted Unifrac scores.

high relative abundance was observed for specific genera in the soils under the poultry houses (Table 4.1). In the present study, relative abundances of genera did not surpass 8.97%, as only 23 of the genera identified had a relative abundance greater than 1%. When considering the pathogenic bacterial genus detected in the samples, the major contributor was Mycobacterium. Mycobacterium avium subsp. paratuberculosis (Map), an organism with excellent survivorship in the environment, as it has been detected for up to 600 days in water and soil. Although it can survive long periods without a host, the organism requires a host to grow and propagate.[53] There has been growing concern about the movement of pathogenic bacteria through the soil profile to contaminate groundwater.[54] Though this possibility exists for some microbes, the results of a recent study suggest that the potential for groundwater contamination by Map is low; however, the organism may remain bound to the soil near the surface where it can be ingested by grazing animals or be released during runoff events.[55]

Although the ability of the litter layer to prevent leaching and migration of nutrients and microorganisms, respectively, has been documented, it was observed that biochemical processes still take place under the layer at comparable levels to that in other areas. Pyrosequencing revealed that some of the same pathogenic genera present in studies characterizing microbial communities in the litter layer were present in the soil layer, but in differing amounts. The genera of note were Brevibacterium, Clostridium, Corynebacterium, Mycobacterium, Staphylococcus, and Streptococcus. Brevibacterium accounts for about 6% of the sequences that were found in the soils under the poultry houses, and two-thirds of those were Brevibacterium avium, which is thought to be a secondary invader of diseased animals.[56,57] We also detected Clostridium in samples from under the poultry house, but there were no hits for C. perfrigens or C. botulinum, which are infamous pathogens. Staphylococcus was found in abundance as it has been found in litter,[9,27,7] which included S. cohnii and S. endermititis, known pathogens to humans. Other Staphylococcus sp. included animal pathogens and non-pathogens, but species belonging to this genus and Enterococcus may serve as sinks for the transmission of antibiotic resistance to normal human commensalist flora.[9,58] Another pathogenic genus present was Streptococcus, which has been found in the ileum of chickens, along with Enterococcus and Clostridium.[26] S. constellatus was prominent and is part of the Streptococcus auginosus group (SAG) that has the propensity to cause disease in humans.[59] Bordetella, Enterococcus, and Escherichia were found, but they showed relative abundances of less than 0.1%. Although these populations were found in relatively small abundance, persistence of bacterial populations and the development of resistance is a complex ecological process, and perhaps easier to acquire and maintain for some species of bacteria than others.

Table 2. Salient genera of potentially pathogenic bacteria are expressed as mean relative abundance in each of the sampled areas. Significant differences are denoted by different letters in rows.

	BRHS	STRG	PSTD
Potentially pathogenic genera			
Bordetella	0.05	0.04	0.00
Brevibacterium	6.10a	0.00b	0.00b
Clostridium	0.36	0.20	0.02
Enterococcus	0.02	0.00	0.02
Escherichia	0.03	0.01	0.00
Mycobacterium	0.30a	0.41a	1.52b
Staphylococcus	1.11a	0.01b	0.00b
Streptococcus	0.15	0.18	0.05
Total	8.12	0.85	1.61

Conclusion

These data suggest that though there is a similarity in richness of the soil bacterial communities across the study site, the communities under the poultry houses are unique in their composition. Despite the attempt of the producer to adhere to best practices in litter management, bacterial populations appear to exist that could potentially contribute to pathogenicity. Soils on which poultry have been continuously raised may be considered long-term reservoirs of infectious pathogens and a potential risk to surface waters and public health. The complexity of antibiotic resistance, microbial persistence, and environmental migration of bacterial species pose a large risk to water quality (both groundwater and surface water sources), as well as to the human food chain. Further studies are required to assess a variety of poultry production sites, which differ according to management strategies to determine if there are similarities between the ecologies found at such sites as in this study. Further, the degree to which the results of 16S rRNA sequencing concerning pathogenic bacteria leads to pathogenesis in human and animal models should be investigated. These combined efforts could provide future mitigation techniques to dampen the impact of pathogens associated with food animal operations on public health.

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Author Contributions

Conceived and designed the experiments: RS, RA, and RZ. Analysed the data: RS, SD. Wrote the first draft of the manuscript: RS. Contributed to the writing of the manuscript: RS. Agree with manuscript results and conclusions: RS, RA, SD, RZ. Jointly developed the structure and arguments for the paper: RS, RA. Made critical revisions and approved final version: RA. All authors reviewed and approved of the final manuscript.

Competing Interests

Author(s) disclose no potential conflicts of interest.

Disclosures and Ethics

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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