# THE ANIMAL MICROBIOME IN HEALTH AND DISEASE

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# THE ANIMAL MICROBIOME IN HEALTH AND DISEASE

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REVIEW
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# A Review of Prebiotics Against *Salmonella* in Poultry: Current and Future Potential for Microbiome Research Applications

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Prebiotics are typically fermentable feed additives that can directly or indirectly support a healthy intestinal microbiota. Prebiotics have gained increasing attention in the poultry industry as wariness toward antibiotic use has grown in the face of foodborne pathogen drug resistance. Their potential as feed additives to improve growth, promote beneficial gastrointestinal microbiota, and reduce human-associated pathogens, has been well documented. However, their mechanisms remain relatively unknown. Prebiotics increasing short chain fatty acid (SCFA) production in the cecum have long since been considered a potential source for pathogen reduction. It has been previously concluded that prebiotics can improve the safety of poultry products by promoting the overall health and well-being of the bird as well as provide for an intestinal environment that is unfavorable for foodborne pathogens such as *Salmonella*. To better understand the precise benefit conferred by several prebiotics, "omic" technologies have been suggested and utilized. The data acquired from emerging technologies of microbiomics and metabolomics may be able to generate a more comprehensive detailed understanding of the microbiota and metabolome in the poultry gastrointestinal tract. This understanding, in turn, may allow for improved administration and optimization of prebiotics to prevent foodborne illness as well as elucidate unknown mechanisms of prebiotic actions. This review explores the use of prebiotics in poultry, their impact on gut *Salmonella* populations, and how utilization of next-generation technologies can elucidate the underlying mechanisms of prebiotics as feed additives.

**Keywords: prebiotics, *Salmonella*, poultry, microbiomics, metabolomics, fructooligosaccharides, mannanoligosaccharides, galactooligosaccharides**

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## INTRODUCTION

*Salmonella* can be spread through the fecal-oral route (1, 2), and is a concern for pathogenic contamination of poultry meats and eggs used for human consumption. Previously this concern had been mitigated through the use of antibiotics, which also promoted animal growth (3). However, with the rise of multidrug-resistant bacteria (4–6), the food industry has been pursuing alternative control measures for pathogenic *Salmonella* contamination. These approaches include

but are not limited to chemical-based interventions, such as organic acids and essential oils, or biological-based treatments, such as bacteriophage, probiotic, and prebiotic therapies.

The recent use of prebiotics has been well documented. The term "prebiotic" was first coined by Gibson and Roberfroid in 1995 and defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (7). Gibson and Roberfroid (8) demonstrated that the intake of prebiotics could regulate specific gastrointestinal tract (GIT) microorganisms to alter the microbiome. Over the years, further findings have led to several suggested modifications of the definition such as the addition of the term "selectively fermentable" (9) or the term "nonviable" (10, 11). More recently, an expert consensus from the International Scientific Association for Probiotics and Prebiotics (ISAPP) defined prebiotics as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (12).

Prebiotics have been used to influence the growth of reported beneficial bacteria in the GIT, such as *Bacteroides* and *Bifidobacterium* (13–16). Van Loo et al. (17) detailed several natural sources of prebiotics including garlic, onions, and asparagus. Typically including fiber and oligosaccharides (18), prebiotics in chickens increase amylase production in the GIT and therefore improve the overall growth rate of broilers (16). They reduce colonization of *Salmonella* during hen molting (19). Some prebiotics have also influenced protection against *Salmonella* by providing binding sites for bacteria to be flushed out of the digestive tract (18). Numerous studies have also seen the reduction of *Salmonella* populations by increasing short chain fatty acids (SCFAs) concentrations (20–22) which can be accomplished through prebiotic administration (23, 24).

Furthermore, several studies (25–29) investigated prebiotic effects on the GIT microbiota through 16S microbiome sequencing. By also noting changes in metabolite concentrations or metabolomics, this approach may be able to correlate changes in the microbiome to changes in the metabolite concentration such as SCFAs and other, possibly unknown, metabolites that can stymie *Salmonella* growth. The scope of this paper to provide an overview of the literature linking the use of prebiotics to the overall reduction in the number of foodborne *Salmonella* and the repression of virulence factors. The scope of this paper will not detail the other benefits of prebiotics in poultry such as impact on growth performance or antioxidant capacity, as they are covered extensively in Dhama et al. (30, 31), Yadav et al. (32), and other literature reviews. By investigating SCFA production, microbiomic, and metabolomic technologies, and currently utilized prebiotics, notably oligosaccharides, this review attempts to elucidate novel avenues of research into the reduction of virulent pathogens via prebiotics, which may improve the safety of the poultry industry and improve the overall public health by reducing the incidence and or severity of poultry-acquired salmonellosis.

## THE POULTRY GASTROINTESTINAL TRACT

The gastrointestinal tract of chickens is complex due to the bird's large energy requirements (33). The chicken GIT includes the crop, gizzard, duodenum, ileum, and cecum, which are microbiologically abundant with over 900 documented bacterial species (34). Included in the upper segment of the GIT, is the crop, which is used for fermentation, hydrolysis of starch to sugar, food storage, and as an acid barrier with a pH of ∼4.5. The gizzard grinds food particles in a highly acidic environment (pH 2.6) (35–38). While the mean retention time throughout the GIT is ∼6 h, feed can remain in the crop and gizzard for as little as 8 and 50 min, respectively (39). The crop contains numerous anaerobic bacteria attached to the epithelium, including *Lactobacillus*, and they produce SCFA's and lactic acid (40, 41). The continuous layer of *Lactobacillus*, enterococci, coliforms, and yeast promote digestion of most carbohydrates, with the remainder digested in the ceca after passage through the lower GIT (37, 42).

Lower in the GIT is the duodenum, ileum, and cecum. Digestive enzymes and bile from the pancreas and gallbladder are added to the duodenum to break down food further, allowing for better absorption into the bloodstream through the villi (43). This process is continued through the ileum in the lower small intestine (43). The small intestine is dominated by anaerobic bacteria (44), and contains *Lactobacillus* and *Bifidobacterium* species in high concentrations as well as *Enterococcus faecium* and *Pediococcus* spp. (35, 45, 46). However, despite the presence of these bacteria in the small intestine, the concentrations of bacteria in the ceca are reported to be the highest in the chicken GIT, at $\sim 10^{11}$ bacteria/g (35, 47, 48).

The ceca are located where the small and large intestines meet, and while they serve no identifiable purpose for digestion in mammals, it is important in chickens for fermentation and overall animal health (33, 35, 43). Due to culturing poultry cecal microbiota on arabinoxylan, it has been suggested the cecum may be involved in the breakdown of grains (42). The cecum plays additional roles in water adsorption and urea recycling, although the full nutritional significance remains unclear (49, 50). However, despite its importance, in an experiment involving ligation of the cecum, it was shown that while nitrogen availability was disturbed by a cecectomy, it was not necessary for survival (51, 52). The ceca, from a food safety standpoint, is also of major significance because it is one of the leading sites for *Salmonella* colonization along with the crop (53–55).

*Salmonella* can be found in varying concentrations in all regions of the poultry GIT of challenged chickens (56, 57). In Fanelli et al. (56), 1 day after the birds were challenged with *Salmonella*, the duodenum and the small intestines were examined, and 5–45% of the samples tested positive depending on the region viewed. However, cecal samples in this study were nearly 100% positive for *Salmonella* colonization (56). This trend continued throughout the 13-day trial. Additional studies found that, when challenged with a lower concentration, *Salmonella* was not recoverable from the duodenum and small

intestine despite being isolated from the crop, because bacteria were often destroyed in passaging through the acid lumen of the proventriculus and gizzard (58). While other studies have focused on the crop and even the gizzard as colonization sites of *Salmonella,* the ceca remain the most commonly investigated section of GIT for *Salmonella* (39, 55, 58, 59). This is likely because of the relatively high bacterial counts of up to $10^{11}$ cells/g of digesta by the day three post-hatch (35, 60). Other reasons may include the ceca being the environment in the GIT most advantageous for *Salmonella* to colonize (56), and because the ceca can be ruptured during processing. However, it should be noted, Hargis et al (55) found that crops was 86 fold more likely to rupture than ceca during processing. Despite this focus on the ceca, with the potential for each organ's microbial composition to influence the next downstream, it is vital to understand the microbiota of each region of the avian GIT.

Stanley et al. (35) compiled data from several papers detailing the most prevalent microbial groups in each of the GIT regions. They found that while *Lactobacillus* was prominent, if not dominant in all systems, a myriad of differences was reported, including *Clostridiaceae* and *Enterococcus* in the crop and gizzard, and that a majority of cecal bacteria were not culturable or described. However, these profiles can vary greatly, as it has been suggested that host genotype, sex, and age play an important role in determining microbial composition (61). Furthermore, a majority of the collected papers reported information using community-fingerprinting techniques such as temporal temperature gradient electrophoresis (TTGE) and terminal-restriction fragment length polymorphism (T-RFLP), as well as culture-based methods. These techniques provide useful information, such as the application of T-RFLP in Torok et al. (25), which helped identify the presence of over 600 bacteria species and 100 distinct genera in the GIT of chickens. However, each of these techniques exhibits significant issues. Community fingerprinting techniques in general, are considered only semi-quantitative and are only capable of detecting taxa in abundance of >1% (61, 62). Additionally, culture-dependent methods are particularly limited. For example, in the cecum, only 10–60% of bacterial strains have been cultured (63, 64). Therefore, while these techniques have generated valuable information, to accurately detail the complex and minute changes to the microbiota under the effect of prebiotics, further investigation with more sensitive methodologies is needed. The changes, however, often depend on the type of prebiotic utilized.

## COMMONLY USED PREBIOTICS

Prebiotic studies have focused largely on oligosaccharides such as mannanoligosaccharides (MOS), galactooligosaccharides (GOS), and fructooligosaccharides (FOS) including inulin (12, 24, 65–67). Oligosaccharides are polymer chains with 3 to 10 of simple sugars (**Figure 1**) (68). Oligosaccharides and fiber have been combined and amended with feed products to create commercially viable sources of prebiotics in the poultry industry with a range of results. Illustrations of the modes of action of prebiotics within poultry can be found in Yadav et al. (32) and Pourabedin and Zhao (67).

Several commercial prebiotics have been studied and utilized, such as Biolex® MB40 and Leiber® ExCel (Leiber, Hafenstraße 24, Germany), which are brewer's yeast cell walls composed of MOS (27–29, 69). These products were found to reduce *Campylobacter* concentrations and alter the microbiome, and there is an expectation of MOS-based products to reduce pathogens that utilize mannose-specific type 1 fimbriae such as *Salmonella* (28, 70). Furthermore, Lee et al. (71) did evaluate the effect of these products against *Salmonella* in commercially raised broilers, and while a lower prevalence was noted, only 10 samples were utilized, and a challenge study was not performed. As another example, the commercialized yeast-fermentate product XPC (Diamond V, Cedar Rapids, IA), has reduced *Salmonella* in chickens and increase butyrate in the GIT (27, 29, 72–74). Furthermore, during a *Salmonella* challenge experiment, the addition of XPC, which is comprised of 25% fiber, to chicken feed decreased the expression of virulence factor *hilA*, which is a regulator and promoter within a pathogenicity island (SPI-1) (72, 74). These findings imply that XPC may reduce *Salmonella* virulence and invasion.

While these effects are detectable, synergistic effects can also be created by combining probiotics and prebiotics to create synbiotics. Probiotic products such as All-Lac® have been used in conjunction with Bio-MOS® to alter the microbiome, whereas Fasttrack® (Fasttrack, Conklin, Kansas City MO) and PoultryStar® (PoultryStar, BIOMIN GmbH, Herzogenburg, Austria), contain FOS and have been shown to reduce *Salmonella* and improve feed conversion efficiency (65, 75–77). These products, along with numerous others, have been found to improve poultry GIT health, increase animal weight, and inhibit *Salmonella* and *Campylobacter*. As a consequence, because of the range of available prebiotic products, methodologies of application, and the yield of numerous and sometimes inconsistent results (24, 78, 79), it is vital to understand these prebiotics better. Moreover, it is essential to detail their currently elucidated or suggested mechanisms to refine further ways to improve poultry health and production practices. To capture the effects of the breadth of prebiotics available, several types of prebiotics and their impact on *Salmonella* in poultry will be discussed in this section.

Mannanoligosaccharides (**Figure 1A**) are found in the cell wall of numerous fungal species including brewer's yeast (*Saccharomyces cerevisiae*) and *Saccharomyces boulardii*, as well as certain plants (66, 67). Comprised of mannose oligomers linked via β-1,4 glycosidic bonds, MOS have been demonstrated to suppress enteric pathogens and enhance the poultry immune system (80). Broiler chickens do not possess enzymes to break down MOS, as such it is suggested that bacteria in the lower GIT, such as the ceca, are responsible for their digestion (67). One particular advantage of MOS as a prebiotic is its stability as a pellet during steaming, which allows it to be easily added to feed (66). Studies have shown that *Salmonella* possessing type 1 fimbriae can be sensitive to the presence of MOS, which can disrupt attachment and adhesion from the intestinal lining by encouraging attachment to the mannose in the lumen (69, 81).

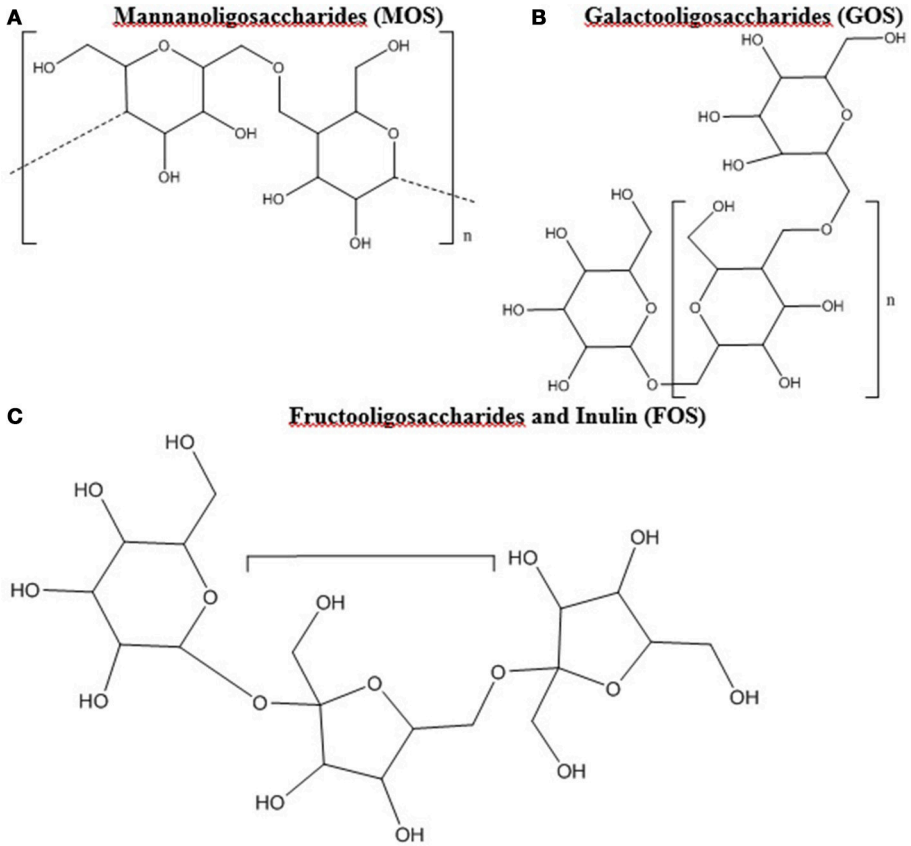


**FIGURE 1 | (A–C)** Chemical Structure of oligosaccharides. All chemical structures were drawn in ChemBioDraw Ultra (PerkenElmer, Waltham, MA).

The disruption of attachment and adhesion was reported for 53% of tested *Salmonella* strains (81, 82). However not every *S.* Typhimurium strain possesses type 1 fimbriae, as out of 13 tested strains by Mirelmann et al. (83), only 4 expressed type 1 fimbriae.

Mannanoligosaccharides have also been reported to improve overall gut health through increasing villi length and providing an adjuvant-like effect by acting as a microbial antigen (66, 84, 85). One study in particular exhibited a reduction in *Salmonella* ceca population by day 10 in challenged chicks fed a diet consisting of (0.40%) MOS (86). Stanley et al. (87) also demonstrated a one to three log reduction of cecal *Salmonella* counts in 21-day old chicks when supplemented with 0.05% MOS and $MgSO_4$. A meta-analysis, which was designed to increase power by combining results from multiple studies, was performed by Hooge (66), which indicated MOS addition to feed generated improved body weight, feed conversion ratios, and survivability. This meta-analysis listed seven selection criteria including date of publication and age of bird and consisted of 29 pen trials from separate studies that were analyzed using a paired *T*-test. However, some discrepancies were noted in MOS ability to improve beneficial microorganisms (80), and there was no set standardization among studies involving the administration of the amount of the prebiotic.

Fructooligosaccharides (**Figure 1C**) are naturally occurring, typically of plant origin, contain β-(2,1) linkages, and can be food ingredients, functional foods, and prebiotics (8, 88). Due to the β-(2,1)-linkages, enzymatic degradation is difficult in the upper GIT, leading to primary breakdown occurring in the ceca (8, 24, 89). Fructooligosaccharides support the growth of *Lactobacillus* and *Bifidobacterium,* resulting in an increase in SCFAs and lactate, an enhancement of the immune system, and the reduction of *Salmonella* colonization (23, 24, 90, 91). The elucidated mechanism of action for many of these benefits is that FOS is fermented by *Lactobacillus* and *Bifidobacterium* which increases SCFAs and lactate in the cecum resulting in lower *Salmonella* colonization (23, 24). The ability to ferment FOS is present in most strains of *Lactobacillus* and *Bifidobacterium* (24, 92, 93). However, only 8 of 55 strains tested by Rossi et al. (94) were capable of using inulin, which is a long chain FOS derivative, as the sole carbon source.

Furthermore, it was suggested that adverse consequences might exist with the implementation of FOS in poultry feed. Ten Bruggencate et al. (95) demonstrated, in rats, a decrease in *Salmonella* resistance occurred due to an increase in intestinal permeability. Additionally, SCFAs may lead to an enhanced expression of *Salmonella* virulence genes despite reductions in colonization (20, 96). However, inulin amended diets have

yielded middling results with Rehman et al. (93) demonstrating that inulin supplementation did not significantly impact the microbial community of the chicken cecum and Ramnani et al. (97) showed no impact on SCFA production in human diets supplemented with inulin. The effectiveness of FOS and inulin is dependent on a number of factors including the composition of the basal diet, degree of FOS polymerization, the presence of *Bifidobacteria* strains, host animal characteristics, and even host stress factors (91, 98). The FOS amended diets in poultry studies have appeared to yield inconclusive results; however, it has been demonstrated that FOS, when supplemented with probiotics, can produce consistently significant reductions in *Salmonella* (24, 79). This potential synergism has led to its implementation in products such as PoultryStar™ that directly impact aspects of the GIT (76, 99).

Galactooligosaccharides (**Figure 1B**) can be naturally found in human and cow milk, and consist of β-(1,6) and β-(1,4) linkages that avoid digestion in the upper GIT (100–103). Commercially, GOS can be prepared through hydrolyzing lactose from cow's milk and often commercial products contain lactose and a myriad of GOS oligomers (104–106). For instance, Bimuno (Clasado Ltd) is composed of varying concentrations of lactose and di-, tri-, tetra-, and pentose oligomers of GOS (104, 106, 107). Bimuno, *in vitro* and in mice ileal gut loops, caused reduction of *S.* Typhimurium adhesion and invasion, and but not when GOS was removed from the Bimuno mixture (107). Despite these positive effects, no significant differences in *Salmonella* concentrations was found when poultry was provided feed amended with 1% GOS, although significant alterations to the cecal microbiome were observed (108).

Despite this contrast, while GOS has not been as well studied in poultry compared to FOS and MOS (67), several publications have suggested some potential for GOS as a prebiotic in poultry. A bifidogenic effect has been observed by showing increased counts of *Bifidobacterium* in feces of birds fed 3 g of GOS per 25 kg of feed for 40 days (100). The addition of GOS to feed has also been shown to increase the *Lactobacillus* population in cecal contents (109), and when compared to xylooligosaccharides (XOS), FOS, and MOS, GOS significantly improved *L. reuteri* growth on minimal media (110). Besides promoting the growth of *Bifidobacterium* and *Lactobacillus*, GOS has demonstrated other potentially beneficial effects such as reducing heat stress in the jejunum, but not the ileum (111). GOS has been demonstrated to significantly alter the poultry transcriptome when injected *in ovo* compared to the addition of inulin and *Lactococcus lactis* (112), and also improve cell-mediated immunity when in low concentrations (0.1%) (109).

Additionally, GOS has been utilized as part of a synbiotic in some studies. Synbiotics are defined as a combination of probiotics and prebiotics (113). When *Bifidobacterium* was added to poultry feed along with GOS, this synbiotic affected total anaerobic microbial populations in feces, increasing them from 9.71 to 10.26 log colony forming units per gram (CFU/g) (100). This addition also increased *Lactobacillus* and *Bifidobacterium* fecal counts by 0.53 log and 1.32 log units, respectively (100). When injected *in ovo*, commercialized GOS and *Lactococcus lactis* elevated the body weight of broilers at the end of the rearing period (102, 113). This data differed from Biggs et al. (114) which used only the prebiotic, and by Jung et al. (100) and Abiuso et al. (115), which found no change in body weight when GOS was administered in feed. A cursory examination suggests this variation may be due to the differences in the basal diet and genetic variation of the chickens but more in-depth studies must be performed to ascertain the reason.

Other prebiotics have also been investigated to varying degrees. The implementation of 2 g/kg of XOS increased *Lactobacillus* and acetate in the cecum and after a 5-week treatment, significantly reduced cecal colonization and spleen translocation of *S.* Enteritidis (92, 116). Approximately a one log reduction of *S.* Enteritidis in the cecum was found by Pourabedin et al. (117) when XOS was implemented, but this was lower than the reduction observed by MOS (1.6 log reduction). Additionally, it was found that isomaltooligosaccharides (IMO) improved growth of *Lactobacillus in vitro*, exhibited a bifidogenic effect, and inhibited *Salmonella in vitro* (110, 118, 119). Thitaram et al. (120) found that diets supplemented with 1% IMO could reduce *Salmonella* by a two-log reduction and enhance growth during the first 3 weeks of growth, as well as increasing butyrate concentrations in the jejunum (121).

The effects of dietary fiber has also been investigated and suggested to possess prebiotic properties in poultry (10, 122). Fiber, depending on the derivative, source, and concentration, can accelerate feed passage and can alter the weight of the organs of the poultry GIT in a way that is indicative of improved functioning of the GIT (122–125). Organic acids, such as SCFAs, are a by-product of anaerobic fermentation of dietary fiber, and this suggests the possibility of inhibiting *Salmonella* growth in the GIT (126). As a consequence, there is some discussion if fiber should be considered a prebiotic (10). In Japan, while the term prebiotic is not defined, fiber, along with oligosaccharides are considered "foods to modify the gastrointestinal conditions" and can be considered "foods with specific health uses" (10, 127). Dietary fiber does meet the definition of a prebiotic purported in Gibson et al. (12). However, Roberfroid 128 suggests the need for several additional criteria such as resistance to gastrointestinal absorption, fermentation by intestinal microbiota, and selective stimulation of growth or activity of beneficial bacteria. Under this definition fiber, as well as inulin does not match the criteria for being a prebiotic, despite having some prebiotic effects (46, 128). As such, regulatory agencies such as the FDA and the European Food Safety Authority (EFSA) do not currently consider fiber to be a prebiotic (10, 129).

Regardless of their defined role from a regulatory consideration, there is an apparent variance in the effects these molecules have on the chicken GIT. Due to the complexity of some of these molecules such as fiber, and their effects, to elucidate their mechanisms on *Salmonella* reduction, the changes in the gut microbiota must be observed. To capture these alterations, microbiomic technologies can be employed.

## MICROBIOMICS

With the advent of whole genome and 16S rRNA genomic sequencing, researchers have been able to more accurately quantify microbial population shifts and host responses to the addition of prebiotics (25). By sequencing portions of the highly conserved 16S rRNA gene, such as the V1-V3 or the V4 region, and comparing it to databases, such as the Greengenes database, accurate identification of the microbiome can be determined efficiently and at a relatively lower cost (130, 131).

It should be noted that the rapid advancement in DNA sequencing technologies is continuously allowing for higher throughput at a lower cost (132, 133), and this section will attempt to provide as recent information as possible. Currently, Illumina-based microbiome sequencing can provide Operational Taxonomic Unit (OTU) detection at a very low abundance due to sequencing short DNA strands up to 300 bp. With the Illumina MiSeq Benchtop sequencer (Illumina, San Diego, CA, USA), a three-day sequencing run can return 7.5 Gb from 15 million 300-base paired-end reads to yield bulk data for small-scale projects (132). This efficiency is only increasing as technology allows for faster returns of more substantial data. Large-scale projects to study numerous samples can also use the Illumina HiSeq which allows for parallel sequencing at a comparably lower cost (132). The Illumina HiSeq returns 1,500 Gb from 5 billion 150 base paired-end reads but is typically only considered for production scale laboratory studies (132). Additionally, the Ion Torrent PGM system operates by detecting hydrogen ions that are released during DNA synthesis to sequence the genome israpid and easily scalable (Thermo Fisher Scientific, Waltham, MA, USA) (134–136). To analyze this ever-expanding capacity for bulk genomic data, bioinformatics programs are be employed such as Quantitative Insights Into Microbial Ecology (QIIME) and mothur (131, 137). Despite several differences, such as the programing language utilized, both programs have been shown to compile genomic data and evaluate species richness and equality with little statistical variation (131, 138–141). Using these bioinformatic programs, data can be efficently processed and changes in the GIT microbiome can be elucidated.

Investigative research into prebiotics greatly benefits from the sensitive high throughput technology that can quantitatively measure the differences between testing conditions. Park et al. (26) utilized Illumina based technology and the QIIME pipeline program to assess the changes in the cecal microbiota when subjected to the yeast-based prebiotics, Biolex® MB40, and Leiber® ExCel. They found significant changes in concentrations of *Campylobacteraceae*, *Faecalibacterium*, and, on the whole, in the phyla *Firmicutes* and *Proteobacteria* (26). This data was supported by Rastall and Gibson (142), and Park et al. (28), which also found an increase in *Faecalibacterium* OTU's during prebiotic treatment and suggested this increase helped facilitate a healthy microbiome, as an increase in *Faecalibacterium* has been linked to health benefits in poultry. Additional investigations into prebiotics found that MOS implementation can significantly alter the bacterial community phylogenetically (143, 144). Park et al. (28) also reported that FOS increased species diversity in pasture flock chickens demonstrated the prominence of *Firmicutes* across all trials, and showed that *Bacteroidetes* decreased in birds fed with diets amended with FOS and GOS. This study also investigated the use of fiber and found it increased the presence of the butyrate-producing *Fusobacterium* (28).

However, these changes only represent broad stroke differences in previously identified major taxa of importance. The aforementioned studies, as well as studies such as Pan (145), have generated not only general information about major taxa shifts but also seemingly negligible differences in the abundance and presence or absence of previously undetailed bacterial strains. While it is important to report changes in previously identified taxa of importance, Illumina sequencing allows for investigation into more nuanced changes or differences found in previously undescribed taxa. For instance, in Park et al. (26), several bacteria that could only be classified to the order *Bacteroidales* were present in chickens fed Biolex® MB40, but were not noted in the control group or birds fed with Leiber® ExCel. These unspecified species may play a potential role in the overall health of the GIT and may have previously gone undetected by culture and community fingerprinting techniques. Some of these nuanced differences can be attributed to variation in individual chicken microbiomes, but, when taken in composite, these data may yield vast and potentially vital information for understanding changes in the avian GIT incurred by prebiotics.

Currently, through analysis of clustered data, it appears the predominant driver of the poultry microbiota composition is host age (28). This deterministic variable was independent of treatments with feeds amended with 1 kg of FOS or plum fibers per ton and 2 kg of GOS per ton (28). While Original XPC™ was able to reduce *Salmonella* cecal populations in Park et al. (27), the microbiota was impacted more by the age of the bird even when in the presence of a coccidiosis vaccine (27, 29). These findings agree with previous assertions regarding the age of the poultry GIT, as it is reported that at birth the GIT is colonized by aerobic organisms followed by anaerobic microbial domination (146). Despite the strong influence of age and other uncontrollable variables such as gender (61), data still indicate that the microbiome can be shifted due to feed amendments. Therefore, because prebiotics can still be utilized to shift the microbial composition of poultry GIT, it is possible to generate environments that are unfavorable for *Salmonella* colonization. This can be accomplished by increasing populations of "healthy" bacteria, preventing space for *Salmonella* colonization as well as increasing SCFA production (67). To understand how these environments can be chemically altered, microbiome technologies can be employed in conjunction with investigative metabolomics technologies.

## METABOLOMICS

Metabolomics is the qualitative and quantitative identification of all metabolites in a biological system such as the GIT. Metabolites are the final products of cellular processes and can be quantified through a number of instruments such as nuclear magnetic resonance (NMR) and mass spectrometry (MS) (147,

148). Due to its high selectivity, NMR is widely accepted as the primary choice for metabolite elucidation. However, MS is more sensitive comparatively, allowing for detection down to femtomolar ($10^{-15}$) concentrations. Because of this sensitivity, for mixed samples, such as cecal and fecal contents, MS analysis is more readily utilized (147, 149, 150). Mass Spectrometry can also be coupled with chromatography to elucidate the macro-contents of complex mixtures (151). Gas Chromatography (GC) coupled with MS has allowed for the analyses of both volatile and nonvolatile compounds (152). Using GC-MS, Rubinelli et al. (153) investigated the effects of rice bran on *Salmonella* in cecal cultures *in vitro* and detected 578 metabolites. Of these, 367 were unknown, and the change in metabolite concentration was causally linked to the reduction of *Salmonella*. Liquid chromatography has also been used to identify thermolabile molecules in the form of high-pressure liquid chromatography (HPLC) which demonstrated FOS when fed to layers, could reduce cholesterol in eggs (154).

Metagenomic outputs in Sergeant et al. (155) indicated over 200 enzymes that can degrade non-starch polysaccharides in cecal contents, some of which are involved in pathways that produce SCFAs and are vital to the mechanistic understanding of modifying the environment. Unfortunately, one significant drawback to this methodology is the current inability to incorporate genomic information by providing definitive linkages between genotypes and the metabolome (147). Furthermore, the dynamic range of current MS technologies resolving power is $\sim 10^6$, which is far below the estimated concentration of cellular metabolites (147). However, with advances in both high throughput microbiome sequencing and mass spectrometry, it may be possible to derive causal relationships between the presence of phylogenetically related species and concentrations of metabolites.

## CONCLUSIONS

The potential for prebiotics to alter the GIT of broiler chickens has been demonstrated with previous generation technologies such as DDGE, T-RFLP, and conventional plating techniques (35). However, despite the success of altering the microbiome, the precise mechanisms, and changes, such as the exact impact of SCFAs on the cecal microbiota, were historically undetermined due to the incomplete analysis offered by the technologies available at the time (156). Furthermore, with a range of variables such as age, type of bird, and genotype, the underlying mechanisms affecting the GIT seemed unlikely to be elucidated. However, with the rising use and affordability of "omic" technologies such as metagenomics and metabolomics, new investigative strategies can be employed. Through the use of bioinformatics pipeline applications on the bulk deep-sequencing data produced by these technologies, there is potential to produce a complete image of the GIT affected by prebiotics. This image may provide predictive power and allow for the understanding and creation, through prebiotics, of an environment that controls for and inhibits *Salmonella* colonization and growth. Moreover, while *Salmonella* is not the only pathogen of concern in the poultry industry, with the potential for virulence gene repression, it is likely prebiotics will continue to play a role in the control of this pathogen. With the ability to utilize next-generation technologies and more fully understand the complexity of the microbiome of poultry GIT, impacts of prebiotics on pathogen control will continue to be elucidated, investigated, and utilized in food safety.

## AUTHOR CONTRIBUTIONS

AM, SF, and SR have made substantial, direct and intellectual contribution to the work, and approved it for publication. HP and DM have been involved in the editing process and approved it for publication.

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# Evaluation of the Epithelial Barrier Function and Ileal Microbiome in an Established Necrotic Enteritis Challenge Model in Broiler Chickens

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Necrotic enteritis (NE) is a recognized multifactorial disease that cost annually to the poultry industry around $2 billion. However, diverse aspects related to its presentation are not completely understood, requiring further studies using known induction experimental models. Therefore, the purpose of this study was to measure the changes occurring in performance, intestinal integrity and ileal microbiome using a previously established NE-challenge model. Chickens were assigned to a negative control group (NC) or a positive control group (PC). In the PC, broilers were orally gavaged with *Salmonella* Typhimurium (ST) ($1 \times 10^7$ cfu/chick) at day 1, *Eimeria maxima* (EM) ($2.5 \times 10^4$ oocyst/chick) at day 18 and *Clostridium perfringens* (CP) ($1 \times 10^8$ cfu/chick/day) at 23–24 days of age. Weekly, body weight (BW), body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) were evaluated. Morbidity and mortality were determined throughout the study, and NE lesion scores were recorded at day 25. Additionally, blood and liver samples were collected to measure gut permeability as determined by levels of serum fluorescein isothiocyanate-dextran (FITC-d) and bacterial translocation (BT). Ileal contents were processed for 16S rRNA gene-based microbiome analysis. Performance parameters and intestinal permeability measurements were negatively impacted in the PC resulting in elevated serum FITC-d and BT with a −6.4% difference in BWG. The NE lesion score in PC (1.97 vs. 0.00) was significantly higher in comparison to NC, although there was no difference in mortality. The microbiome analysis showed a dramatic shift of ileal microbiomes in PC groups as compared to NC (ANOSIM: $R = 0.76$, $P = 0.001$). The shift was characterized by reduced abundance of the phylum Actinobacteria ($P < 0.01$), and increased abundance of the genera *Butyrivibrio*, *Lactobacillus*, *Prevotella* and *Ruminococcus* in PC compared to NC ($P < 0.05$). Expectedly, *Clostridium* was found higher in PC ($2.98 \pm 0.71\%$) as compared to NC ($1.84 \pm 0.36\%$), yet the

difference was not significant. In conclusion, results of the present study showed the different intestinal epithelial and microbiological alterations occurring in an established NE-challenge model that considers paratyphoid *Salmonella* infections in young chicks as an important predisposing factor for presentation of NE.

**Keywords: broiler chickens, *Clostridium perfringens*, intestinal permeability, microbiome, necrotic enteritis**

## INTRODUCTION

*Clostridium perfringens* (CP) is a Gram-positive anaerobe, spore-forming pathogen with a short replication rate in thioglycolate medium, and the capacity to produce more than 16 different toxins/enzymes with diverse modes of action (1–3). In mammals, it has been demonstrated that CP alpha-toxin is a key virulence factor in the pathogenesis of gas gangrene, since the injection of the alpha-toxin or injection of a beneficial *Bacillus subtilis* expressing the alpha-toxin can induce gangrene and tissue necrosis (4, 5). Alpha-toxin targets the liposomes of the cell membrane, as it contains phospholipase C and sphingomyelinase, disrupting the most important defensive organelle of the cell (6). Alpha-toxin is also responsible for hemolysis, tissue necrosis, epithelial barrier dysfunction, and severe inflammation as it activates the arachidonic acid pathway, the nuclear factor kappa beta pathway (NF-κβ), and the release of proinflammatory cytokines such as interferon gamma (IFN)-γ and tumor necrosis factor alpha (TNF)-α (7, 8). These physiological responses to alpha-toxin can also lead to edema due to increased vascular permeability (9, 10).

In chickens, CP type A and C are recognized as the primary causative agent of necrotic enteritis (NE), a multi-factorial disease that has a significant economic impact on the poultry industry with annual losses of ∼2 billion dollars (11). However, even though alpha-toxin has been extensively studied in the pathogenesis of the disease (12–14), its role as the main virulent factor is no longer an accepted dogma. Few other toxins (NetB, Tpel) are now considered to be more important than alpha toxin in NE pathogenesis (3, 15). In fact, CP NetB-toxin has been also reported to induce NE without the presence of alpha-toxin (16, 17). In chickens, NE is characterized by high mortality, rapid loss in performance, depression, and a severe necrosis of the intestinal mucosa (18, 19). CP is ubiquitous and is harbored in the intestinal tract of metazoans. Hence, any condition that changes the normal microbiota (dysbacteriosis), could favor CP overgrowth and cause their toxins to rise leading to severe epithelial damage and necrosis of the intestinal absorptive surface (20–22).

Recent investigations have shown significant changes in the microbiome of chickens affected by NE when compared with healthy control chickens (23, 24). In this multi-factorial disease, coccidial infections, in particular with *Eimeria maxima* (EM) are recognized as pre-requisites in the pathogenesis of NE (11, 12). Likewise, diets with a high content of non-starch polysaccharides (NSP), immunosuppression, and withdrawal of antibiotic growth promoters or anticoccidials have been reported as factors related to the increased incidence of NE in chickens (25). The use of probiotics have been shown to reduce the incidence and severity of NE (24, 26), and these studies suggest that probiotics improve intestinal gut barrier function. However, to this date, there are no consistent results to prove this hypothesis. In an attempt to develop a reliable NE chicken model, our laboratory integrated different predisposing factors for presentation of NE that included a neonatal *Salmonella* Typhimurium (ST) challenge, followed by EM and CP challenges (27). Hence, the objectives of the present study were to evaluate the effect of NE on intestinal permeability and intestinal microbiome changes in broiler chickens in a laboratory challenge model that could be used to evaluate future alternative feed additive candidates to control the presentation of this important enteric disease.

## MATERIALS AND METHODS

### Animal Source and Diets

For the NE challenge study, a total of 80 day-of-hatch Cobb 500 male broiler chicks were obtained from a commercial hatchery (Siloam Springs, AR, USA). Chickens were neck-tagged and randomly located to one of eight floor pens (206 × 104 cm) with new pine shavings as litter in an environmentally controlled room. The temperature was maintained at 34°C for the first 5 days and was then gradually reduced until a temperature of 23°C was achieved at day 21 of age. Lighting was provided for 18 h/day. Broilers chicks were fed with mash corn-soybean based diets. Starter (0–7 days) and grower (8–25 days) diets were formulated to approximate the nutritional requirements for broiler chickens as recommended by the National Research Council (28), and adjusted to breeder's recommendations (29). Water and feed were provided *ad libitum*. No antibiotics or anticoccidials were added to the feed. In the present study, all animal handling procedures were in compliance with the University of Arkansas, Institutional Animal Care and Use Committee (IACUC, approved protocol: 15006).

### *Salmonella* Typhimurium

A poultry isolate of ST selected for resistance to 25 μg/mL nalidixic acid (NA, Sigma, St. Louis, MO) and 20 μg/mL novobiocin (NO, Sigma, St. Louis, MO) was used during this study. An aliquot of ST was thawed and 100 μL of culture was inoculated into 10 mL of tryptic soy broth (TSB, Sigma, St. Louis, MO) and incubated at 37°C for 18 h. This was followed by three more passages at intervals of 8 h into fresh TSB to ensure that all bacteria were in log phase. Post-incubation, bacterial cells were washed three times in sterile saline (0.9%) by centrifugation at 1,864 × *g*, 4°C for 15 min. The approximate concentration of ST was quantified spectrophotometrically (Spectronic 20D+, Spectronic Instruments Thermo Scientific, Madison, WI) at 625 nm and diluted with sterile saline to reach a challenged

concentration of $\sim 10^7$ cfu/mL. Additionally, this concentration was also determined retrospectively, by serial dilution and plating on brilliant green agar (BGA, Sigma, St. Louis, MO) with NA (25 μg/mL) and NO (20 μg/mL) for determination of actual ST colony forming units.

### *Eimeria maxima*

Oocysts of the previously described EM Guelph strain (EM-GS) were donated by Dr. John. R. Barta, University of Guelph, Canada. The EM-GS strain is a single oocyst-derived isolate that has been maintained at the Ontario Veterinary College since 1973 (30). EM-GS oocysts were propagated *in vivo* in chickens experimentally inoculated with $10–30 \times 10^4$ sporulated oocysts (31). The methods for detecting and recovering oocysts from infected chickens, oocyst sporulation as well as preparation of infective doses, have been previously described (32). A preliminary dose titration study was performed, offset by 1 week, to determine the EM-GS challenge dose before starting the NE study. At 13 days of age, all broilers were weighed, divided into four groups ($n = 15$/group) and challenged with three different doses (25,000, 40,000, and 50,000) of sporulated oocysts per mL by oral gavage. A group of chicks was sham challenged with saline as a negative control. Five days post-challenge, body weight (BW) and body weight gain (BWG) were recorded. Based on the criterion that the challenge dose caused sub-clinical coccidiosis, consisting of a reduction on performance parameters without the presentation of clinical signs, the lowest dose providing 25,000 oocyst per mL that caused a 24 % reduction in BWG was chosen for the present NE challenge model study. Doses corresponding to 40,000 and 50,000 oocysts per mL reduced BWG in a 27 and 28% respectively, but results were not significantly different from the lowest EM-GS challenge dose (data not shown).

### *Clostridium perfringens*

For CP challenge, a previously described strain used in a NE model was kindly donated and confirmed alpha-toxin positive using a multiplex PCR assay (27, 33). The primer pair used for detection of CP toxin gene *cpa* was: Forward sequence: 5′ TGCATGAGCTTCAATTAGGT 3′; Reverse sequence: 5′ TTAGTTTTGCAACCTGCTGT 3′. A frozen aliquot was amplified in TSB with sodium thioglycolate (Becton Dickinson, Sparks, MD). The broth culture was plated on phenyl ethyl alcohol agar (PEA) plates (Becton Dickinson, Sparks, MD) with 5% sheep blood (Remel, Lenexa, KS) to confirm purity. Aliquots were made with 25% sterile glycerol and stored at −80°C until further use. A single aliquot was individually amplified in TSB with sodium thioglycolate overnight for the NE challenge study and the challenge dose was confirmed by plating 10-fold dilutions on tryptic soy agar (TSA, Becton Dickinson, Sparks, MD) with sodium thioglycolate.

## Experimental Design

In the NE challenge trial, 80 neonatal broiler chicks were randomly assigned to either a negative control non-challenged group (NC) or a positive control group (PC). Each experimental group had four replicates of 10 broilers. In the PC group, chickens were orally challenged with ST ($1 \times 10^7$ cfu/chick) at day 1, followed by EM-GS ($2.5 \times 10^4$ oocyst/chick) at day 18 and CP ($1 \times 10^8$ cfu/chick/day) at 23–24 days of age, according to a previously published experimental model (27) and the results of the *E. maxima* dose titration study described above. The NC group was sham challenged twice with saline and once with TSB with sodium thioglycolate to simulate handling and challenge conditions of the PC. Weekly, all broilers were individually weighed and BW, BWG and pen feed intake (FI) were noted at the end of each phase to calculate the feed conversion ratio (FCR) for starter (0–7 days), grower (8–25 days), and overall (0–25 days) experimental phases. Additionally, chickens were also weighed before EM-GS and CP challenge to evaluate the possible impact of each pathogen on performance. At 25 days of age, all the animals were euthanized by cervical dislocation. Chickens with similar BW where randomly selected to collect the different samples. Liver tissue was obtained for determination of aerobic and anaerobic bacterial translocation (BT) from 3 birds per pen ($n = 12$/group). In the case of the evaluation of gut permeability, blood samples were collected to measure serum fluorescein isothiocyanate-dextran levels (FITC-d) from 5 chickens per replicate ($n = 20$/group). Determination of ileal microbiome was performed based on 16S rRNA gene sequence analysis from 3 birds per pen ($n = 12$/group). CP lesion scores ($n = 40$/group) were evaluated according to Prescott et al. (34): 0 = no lesions; 1 = thin-walled and friable intestines; 2 = focal necrosis, gas production and ulceration; 3 = extensive necrosis, hemorrhagic and gas-filled intestines; and 4 = generalized necrosis typical of field cases, marked hemorrhage. Morbidity was evaluated as negative (bright and alert) or positive (reduce spontaneous activity, isolation or lethargy) according to Shojadoost et al. (35). Details about measurement techniques are described below.

## Bacterial Translocation

Briefly, the right half of the liver was removed from each chicken, collected into sterile bags, weighed, homogenized, and 1:4 w/v dilutions were made with sterile 0.9% saline. Ten-fold dilutions of each sample were subsequently made in a sterile 96 well Bacti flat bottom plate, and the diluted samples were plated on TSA with and without sodium thioglycolate for evaluation of anaerobic and aerobic BT. Anaerobic samples were incubated at 37°C for 24 h using an anaerobic chamber (GasPak™, Becton Dickinson, Sparks, MD). Aerobic samples were incubated under aerobic condition using the same temperature and time parameters (37°C for 24 h). Bacterial translocation was expressed in colony forming units ($Log_{10}$ cfu/gram of tissue).

## Determination of Serum FITC-d Levels

Serum levels of fluorescein isothiocyanate dextran (FITC-d), a measurement of enteric inflammation and mucosal permeability were determined after all chickens received a single oral gavage dose of FITC-d (8.32 mg/kg). Blood samples were collected from the femoral artery 1 h post FITC-d administration and allowed to clot under room temperature for 3 h. Samples were subsequently centrifuged ($1,000 \times g$ for 15 min) to separate serum from red blood cells. The serum samples were then diluted in phosphate buffer saline (1:5), and fluorescence was measured at 485 nm excitation and 528 nm emission (Synergy HT, multimode micro

plate reader, Bio Tek Instruments, Inc., VT, USA). Levels of fluorescence in the samples were converted to respective FITC-d ng per mL of serum based on a standard curve (36).

## Preparation of the 16S rRNA Gene Amplicon Library for MiSeq Sequencing

Ileal contents (200 mg) from each bird were collected for DNA isolation utilizing QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA). The concentration of extracted DNA was diluted to 10 ng $\mu L^{-1}$ for the preparation of a sequencing library targeting the V4 region of the 16S rRNA gene (37). Isolated DNA samples were amplified via a PCR using dual-index primers and normalized the amplicons with a SequalPrep™ Normalization kit (Life Technology, Carlsbad, CA) according to the manufacturers' recommendation. The library was constructed by combining 5 μL of each normalized aliquot sample for further assessment. Library concentration and product size were confirmed using a KAPA Library Quantification Kit (Kapa Biosystems, Woburn, MA) via quantitative PCR (qPCR, Eppendorf, Westbury, NY) and an Agilent 2100 Bioanalyzer system (Agilent, Santa Clara, CA), respectively. The 20 nM of pooled library aliquot and the 20 nM of PhiX control v3 were combined with 0.2 N fresh NaOH and HT1 buffer and mixed a second time with 5% of the PhiX control v3. The 600 μL of the mixture containing pooled library, PhiX control v3, NaOH and HT1 buffer was subsequently loaded onto a MiSeq v2 reagent cartridge to run sequencing.

## Analysis of Microbiome Sequencing Data by QIIME Pipeline

Raw sequencing read files were processed using quantitative insights into microbial ecology (QIIME) pipeline version 1.9.1[available at http://qiime.sourceforge.net/; (38)] at Jetstream cloud computing platform (39). Demultiplexed reads were joined together using fastq-join (40) option of QIIME. Reads that were quality filtered using multiple_split_libraries_fastq.py option of QIIME were used for chimeric sequences identification using USEARCH version 6.1.544 (41). After removing chimeric sequences, the operational taxonomic unit (OTU) picking and taxonomy assignment were performed using pick_open_reference_otus.py command of QIIME with uclust method (41). Taxonomy was assigned based on green genes taxonomy and reference database version 13_8 (42). Sequences that belong to Chloroplast and mitochondria were removed from OTU table as they are not the part of microbial communities and possible contamination of Chloroplast was previously described (43). The OTU table was normalized using cumulative sum scaling (44) before summarization and statistical comparisons of any taxa and diversity analyses. Beta diversity was calculated using weighted UniFrac metric with even sampling depth of 7,000 reads and statistical comparisons were made using analysis of similarities (ANOSIM) method.

## Statistical Analysis

All data were subjected to one-way ANOVA as a completely randomized design using the GLM procedure of SAS (45). For evaluation of growth performance parameters (BW, BWG, FI, and FCR), each of the replicate pens was considered as the experimental unit ($n = 4$/group), whereas data on BT ($n = 12$/group), serum FITC-d level ($n = 20$/group) and ileal microbiome population assessment ($n = 12$/group) were based on randomly selected broilers from all replicates of each group. Treatment means were partitioned using Duncan's multiple range test at $P < 0.05$ indicating statistical significance. Mortality and morbidity were compared using the chi-square test of independence to determine significance ($P < 0.05$). Taxonomic and alpha diversity data analyzed by QIIME was imported to Microsoft Excel and JMP® Genomics 9 to determine significant differences using Wilcoxon test where level of significance was set at $P < 0.05$.

# RESULTS

## Overall Performance

The results of the evaluation of BW, BWG, FI and FCR in broiler chickens in a NE challenge model are summarized in **Table 1**. After 7 days of the neonatal ST challenge, there was a significant difference in BWG of 19 g between experimental groups. During the overall experimental period a significant reduction in BW (1149 vs. 854 g) and BWG (1107 vs. 811 g) were observed in the PC compared with NC group. FI was significantly reduced in the challenged group at 7 days (142 vs. 123 g) and 25 days (1754 vs.

**TABLE 1 |** Evaluation of body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) in broiler chickens in a Necrotic enteritis challenge model[c].

| Item | Negative control non-challenged | Positive control challenged |
|---|---|---|
| **BW, g/broiler** | | |
| day 0 | 42.4 ± 0.40[a] | 43.1 ± 0.83[a] |
| day 7 | 148.3 ± 2.30[a] | 130.1 ± 0.40[b] |
| day 14 | 390.5 ± 6.20[a] | 357.5 ± 0.75[b] |
| day 25 | 1149.0 ± 11.75[a] | 854.2 ± 19.60[b] |
| **BWG, g/broiler** | | |
| days 0–7 | 106.0 ± 3.00[a] | 87.0 ± 0.40[b] |
| days 7–14 | 242.3 ± 3.85[a] | 227.4 ± 1.15[b] |
| days 14–25 | 758.5 ± 5.55[a] | 496.8 ± 20.35[b] |
| days 0–25 | 1106.7 ± 12.15[a] | 811.1 ± 18.75[b] |
| **FI, g/broiler** | | |
| Days 0–7 | 141.6 ± 1.64[a] | 123.4 ± 1.68[b] |
| Days 7–14 | 364.6 ± 2.61[a] | 344.5 ± 37.50[a] |
| Days 14–25 | 1248.2 ± 2.04[a] | 1114.0 ± 52.96[b] |
| Days 0–25 | 1754.3 ± 6.30[a] | 1576.4 ± 86.66[b] |
| **FCR** | | |
| Days 0–7 | 1.337 ± 0.019[a] | 1.419 ± 0.026[a] |
| Days 7–14 | 1.510 ± 0.014[a] | 1.517 ± 0.173[a] |
| Days 14–25 | 1.650 ± 0.010[b] | 2.240 ± 0.015[a] |
| Days 0–25 | 1.586 ± 0.012[b] | 1.949 ± 0.069[a] |

[a,b] *Means in each row with different superscripts are significantly different ($P < 0.05$).*
[c] *Data are expressed as mean ± SE; n = 40/group.*

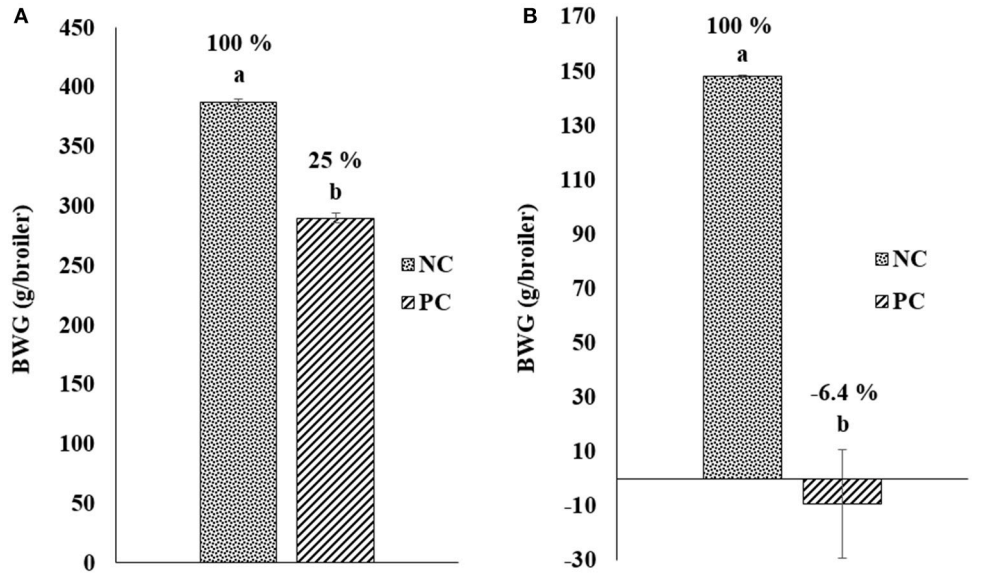


**FIGURE 1 |** Change in body weight gain (BWG; g/broiler) after challenge with **(A)** *Eimeria maxima* and **(B)** *Clostridium perfringens* in a Necrotic enteritis model.

1576 g) of age, resulting in a significant increase in the FCR from day 14–25 (1.650 vs. 2.240) and the overall experimental period in the PC compared to the unchallenged NC group (1.586 vs. 1.949).

## Body Weight Gain After *E. maxima* and *C. perfringens* Challenge

**Figure 1** shows the results of the change in BWG after challenge with *E. maxima* and *C. perfringens* in a NE model. Chickens in PC group were weighed and challenged at day 18 of age with EM-GS ($2.5 \times 10^4$ oocysts/mL), and 5 days post-inoculation were weighed again to calculate the BWG difference. A reduction of 25.0% was observed in BWG in the PC with respect to non-challenged chickens in NC group (**Figure 1A**). Therefore, showing similar results as those of the preliminary dose titration study with the lowest 25,000 oocysts dose, resulting in a BWG difference of 24% (data not shown). Additionally, when broilers were orally administered for two consecutive days with CP, the PC showed a lost in BWG of −6.4% in comparison to the NC (**Figure 1B**). Therefore, the impact of CP administration in BWG was evidently higher compared to the EM-GS challenge alone, confirming the presentation of a synergistic detrimental effect on intestinal health.

## Intestinal Permeability and Lesion Scores

**Table 2** shows the results of the evaluation of gut permeability in a NE model measuring BT to the liver and serum FITC-d levels. A significant increase in both, aerobic (2.25 vs. 3.76 $Log_{10}$ cfu/g) and anaerobic (0.61 vs. 2.28 $Log_{10}$ cfu/g) BT to the liver tissue were observed in the PC challenge chickens when compared with the NC non-challenged chickens. Similarly, a significant increase in FITC-d leakage from the intestinal lumen to the serum was detected in challenged chickens at a magnitude 13.5-fold higher compared with unchallenged chickens (15.05 vs. 203.23 ng/mL).

**TABLE 2 |** Evaluation of gut permeability and ileal lesion scores in a necrotic enteritis challenge model.

| Item | Negative control | Positive control |
|---|---|---|
| Aerobic bacterial translocation[c] ($Log_{10}$ cfu/g) | 2.25 ± 0.38[b] | 3.76 ± 0.49[a] |
| Anaerobic bacterial translocation[c] ($Log_{10}$ cfu/g) | 0.61 ± 0.49[b] | 2.28 ± 0.42[a] |
| FITC-d[d] (ng/mL) | 15.05 ± 6.90[b] | 203.23 ± 27.86[a] |
| Lesion Score[e] (0–4) | 0.0 ± 0.0[b] | 1.97 ± 0.12[a] |

[a,b] *Means in each row with different superscripts are significantly different ($P < 0.05$).*
[c] *Total recovered bacteria translocated to the liver; data are expressed as mean ± SE; n = 12/group.*
[d] *Fluorescein isothiocyanate dextran (FITC-d); data are expressed as mean ± SE; n = 20/group.*
[e] *Ileal lesion score data are expressed as mean ± SE; n = 40/group.*

In the case of NE lesion scores, the NC showed no lesions (0.0), while the average lesion score for PC was 1.97, presenting focal necrosis, gas production and ulcerations in the intestinal mucosa.

## Morbidity and Mortality

The results of morbidity and mortality of broiler chickens in the NE model are summarized in **Table 3**. As expected, no mortality or clinical signs were observed in the non-challenge group. Nevertheless, at day 23 of age in the PC group, 12.5% of the chickens started to show reduced spontaneous activity ($P < 0.05$). At the second day of CP challenge, 100% of the chickens exhibited clinical signs that included either reduce activity, isolation or pronounced lethargy ($P < 0.01$). Interestingly, a significant increase in mortality was expected by 25 days of age, however, no mortality was recorded in any of the evaluated groups.

**TABLE 3 |** Morbidity and mortality progression of broiler chickens in Necrotic enteritis model.

| Item | Negative control (%) | Positive control (%) |
|---|---|---|
| Morbidity (day 22) | 0 | 0 |
| Morbidity (day 23) | 0 | 12.5* |
| Morbidity (day 24) | 0 | 100** |
| Mortality (day 25) | 0 | 0 |

*Means in each row with different superscripts are significantly different ($P < 0.05$); Morbidity and mortality are expressed as total percentage; n = 40/group.
**Means in each row with different superscripts are significantly different ($P < 0.01$); Morbidity and mortality are expressed as total percentage; n = 40/group.

## Summary and Comparision of Significant Taxa at Different Levels

The results showing relative abundance of major phyla, families, and genera are summarized in **Figure 2**. In addition, differentially abundant phyla, families, and genera at $P < 0.05$ are listed in **Table 4**. Firmicutes were found as a predominant phylum in both groups (NC; 68.46%, PC; 66.58%) followed by Proteobacteria (NC; 16.60%, PC; 18.69%), Bacteroidetes (NC; 5.72%, PC; 6.38%), and Actinobacteria (NC; 5.18%, PC; 2.91%) as shown in **Figure 2A**. Although, Firmicutes and Actinobacteria were found higher in NC group while Proteobacteria and Bacteroidetes were higher in PC group, significant difference was observed only with Actinobacteria ($P < 0.01$). The relative abundance of major families (≥1% in total) found on both groups is summarized in **Figure 2B**. In total, Lactobacillaceae (10.70%), Enterobacteriaceae (10%), Lachnospiraceae (9.20%), Clostridiaceae (7.40%), Ruminococcaceae (6%), Bacillaceae (4.7%), Turicibacteraceae (3.30%), and Peptostreptococcaceae (3.2%) were some of the major predominant families. As shown in **Table 4**, Brevibacteriaceae, Clostridiaceae, Flavobacteriaceae, Hyphomicrobiaceae, Microbacteriaceae, Moraxellaceae, Peptostreptococcaceae, Phyllobacteriaceae, Sphingobacteriaceae, Staphylococcaeae, and Turicibacteriaceae were significantly higher in NC group as compared to PC group ($P < 0.05$). On the contrary, Christensenellaceae, Enterobacteriaceae, Eryipelotrichaceae, Lactobacillaceae, Leuconostocaceae, Prevotellaceae, and Ruminococcaceae were significantly higher in PC group as compared to NC group ($P < 0.05$). Similarly, the relative abundance of top 18 major genera found on both groups is summarized in **Figure 2C**. In total, *Lactobacillus*, (10.58%), *Turicibacter* (3.33%), *Enterococcus* (2.65%), *Ruminococcus* (2.78%), *Clostridium* (2.41%), *Bacillus* (2.25%), *Coprococcus* (1.37%), and *Oscillospira* (1.19%) were predominant genera. As shown in **Table 4**, *Brevibacterium*, *Devosia*, *Epulopiscium*, *Ochrobactrum*, SMB53, and *Turicibacter* were found significantly higher in NC group as compared to PC group ($P < 0.05$). On the contrary, *Butyrivibrio*, *Dorea*, *Lactobacillus*, *Mogibacterium*, *Oscillospira*, *Prevotella*, *Proteus*, PSB-M-3, cc_115 of family Erysipelotrichaceae, and *Ruminococcus* were found significantly higher in PC group as compared to NC group ($P < 0.05$). Although not significant, *Clostridium* was found higher in PC group (2.98 ± 0.71%) as compared to NC group (1.84 ± 0.36%). Likewise, *Gallibacterium* was also found numerically higher in PC (0.79 ± 0.37%) as compared to NC (0.59 ± 0.35%).

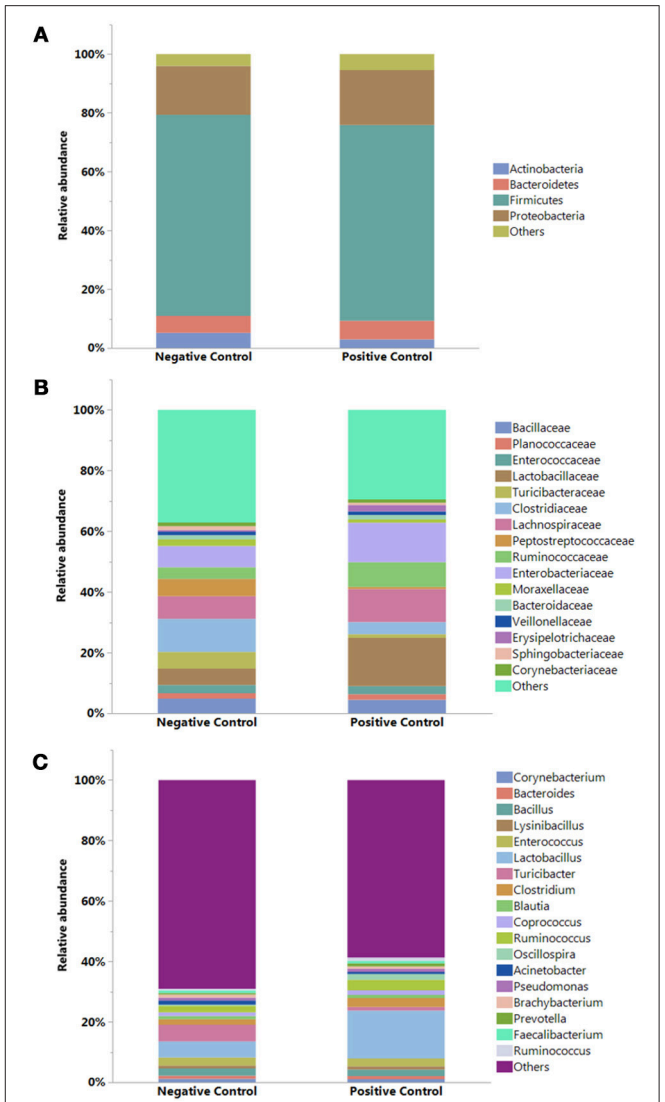


**FIGURE 2 |** Relative abundance (%) of bacteria at **(A)** Phylum, **(B)** Family, and **(C)** Genus levels in the ileum of negative and positive control (with induced necrotic enteritis) groups. Minor bacteria genera including unassigned values were included as "others".

## Diversity Analyses

There was no significant difference in alpha diversity calculated by all three metrics (chao1, PD_whole_tree, and Observed_otus available in QIIME) between NC and PC groups (data not shown). However, ANOSIM result ($R = 0.76$, $P = 0.001$) showed significant difference in beta diversity measured by weighted UniFrac metric between NC and PC groups as demonstrated in PCoA plot (**Figure 3**). In addition, samples in NC group were more clustered together as compared to PC group in PCoA plot, suggesting less sample wise variations existed in the microbial communities in NC group than PC group.

**TABLE 4 |** Relative abundance of phylum, family, and genera differentially present in the ileal microbiomes between negative control vs. positive control with induced necrotic enteritis[a].

| | Negative control (%) | Positive control (%) | *P*-value |
|---|---|---|---|
| **BACTERIAL PHYLUM** | | | |
| Actinobacteria | 5.18 ± 0.57 | 2.91 ± 0.39 | 0.0055 |
| **BACTERIAL FAMILY** | | | |
| Brevibacteriaceae | 0.87 ± 0.12 | 0.23 ± 0.09 | 0.0008 |
| Christensenellaceae | 0.02 ± 0.20 | 0.20 ± 0.06 | 0.0258 |
| Clostridiaceae | 10.86 ± 0.86 | 4.01 ± 0.80 | 0.0002 |
| Enterobacteriaceae | 6.98 ± 0.76 | 12.98 ± 1.30 | 0.0018 |
| Erysipelotrichaceae | 0.58 ± 0.21 | 2.08 ± 0.30 | 0.0013 |
| Flavobacteriaceae | 0.42 ± 0.10 | 0.08 ± 0.05 | 0.0118 |
| Hyphomicrobiaceae | 0.75 ± 0.13 | 0.01 ± 0.01 | 0.0002 |
| Lactobacillaceae | 5.37 ± 1.23 | 15.95 ± 2.34 | 0.0009 |
| Leuconostocaceae | 0.11 ± 0.06 | 0.83 ± 0.12 | 0.0002 |
| Microbacteriaceae | 0.34 ± 0.13 | 0.05 ± 0.05 | 0.0339 |
| Moraxellaceae | 2.15 ± 0.35 | 1.08 ± 0.15 | 0.0370 |
| Peptostreptococcaceae | 5.69 ± 0.93 | 0.75 ± 0.14 | <0.0001 |
| Phyllobacteriaceae | 0.28 ± 0.13 | 0.00 ± 0.00 | 0.0164 |
| Prevotellaceae | 0.52 ± 0.13 | 1.02 ± 0.58 | 0.0344 |
| Ruminococcaceae | 3.83 ± 0.65 | 8.12 ± 1.21 | 0.0042 |
| Sphingobacteriaceae | 1.22 ± 0.22 | 0.76 ± 0.38 | 0.0183 |
| Staphylococcaceae | 0.97 ± 0.22 | 0.55 ± 0.28 | 0.0415 |
| Turicibacteraceae | 5.55 ± 0.71 | 1.11 ± 0.20 | <0.0001 |
| **BACTERIAL GENUS** | | | |
| *Brevibacterium* | 0.87 ± 0.12 | 0.23 ± 0.09 | 0.0008 |
| *Butyrivibrio* | 0.08 ± 0.05 | 0.29 ± 0.08 | 0.0305 |
| cc_115 | 0.08 ± 0.03 | 0.44 ± 0.11 | 0.0174 |
| *Devosia* | 0.75 ± 0.13 | 0.01 ± 0.01 | 0.0002 |
| *Dorea* | 0.50 ± 0.11 | 0.95 ± 0.16 | 0.0453 |
| *Epulopiscium* | 0.54 ± 0.15 | 0.03 ± 0.03 | 0.0039 |
| *Lactobacillus* | 5.30 ± 1.21 | 15.85 ± 2.28 | <0.0001 |
| *Mogibacterium* | 0.00 ± 0.00 | 0.27 ± 0.06 | 0.0004 |
| *Ochrobactrum* | 0.63 ± 0.17 | 0.05 ± 0.04 | 0.0061 |
| *Oscillospira* | 0.44 ± 0.14 | 1.94 ± 0.63 | 0.0178 |
| *Prevotella* | 0.52 ± 0.13 | 1.02 ± 0.16 | 0.0344 |
| *Proteus* | 0.15 ± 0.07 | 0.70 ± 0.14 | 0.0040 |
| PSB-M-3 | 0.03 ± 0.03 | 0.48 ± 0.08 | 0.0005 |
| *Ruminococcus* | 2.10 ± 0.32 | 3.45 ± 0.31 | 0.0085 |
| SMB53 | 0.90 ± 0.09 | 0.10 ± 0.04 | <0.0001 |
| *Turicibacter* | 5.50 ± 0.71 | 1.16 ± 0.20 | 0.001 |

[a]*Relative abundance data are expressed as mean ± SE; n = 12/group. Wilcoxon test was conducted to identify differentially abundant taxa where significant level was set at P < 0.05.*

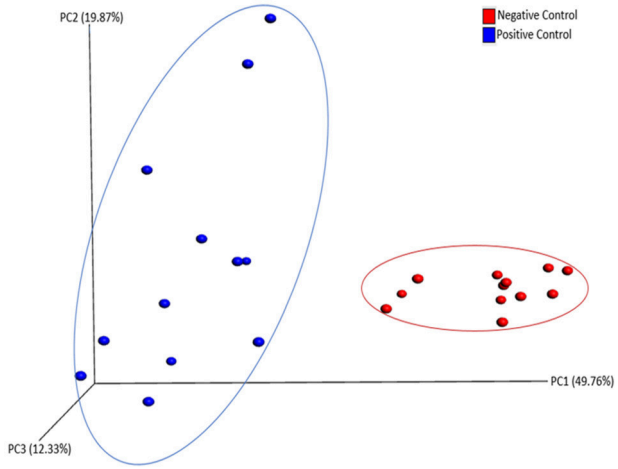


**FIGURE 3 |** PCoA plot showing beta diversity between Negative and Positive control (with induced necrotic enteritis) as measured by weighted UniFrac metric. ANOSIM results ($R = 0.76$, $P = 0.001$) showed significant difference in community structure between groups.

## DISCUSSION

The decrease in the use of antibiotics in the poultry industry has created an opportunity for the presentation of multi-factorial diseases such as the devastating NE (46, 47). Factors that increase the presentation of this enteric disease include inappropriate management and nutritional practices or presentation of coccidiosis, leading to chronic stress and breakdown of the fragile gut microbiome. Therefore, triggering an unfavorable state of dysbacteriosis, characterized by alterations in pH, increased mucus secretion, reduced transit time and most importantly, shifting of the bacterial community (48, 49). This series of changes create ideal conditions for the rapid growth of CP, which synthesizes a collection of over 16 toxins and enzymes, most of them targeting the cell membrane of the enterocytes (1, 50). The principal CP toxins will disrupt the cell membrane by altering the cellular permeability and osmotic pressure (alpha, beta, and epsilon toxins), or by destroying the actin cytoskeleton (iota toxin) (3, 15). Interestingly, CP enterotoxin (CPE) has a different target and mode of action, as it binds the claudin family of the tight junction (TJ) proteins, causing obliteration of TJ thus increasing paracellular permeability across the enterocytes (2, 51, 52). Nevertheless, it is also crucial to mention that not all bacteria from the genus *Clostridium* are considered pathogenic. Interestingly, most of commensal *Clostridia* play decisive roles in the physiology, immunology, and even cognitive activities as some of the most important butyric acid producing bacteria of the GIT (53–57).

In the case of the current study, three different pathogens were used to successfully induce NE by disrupting the intestinal homeostasis state. The NE model included a ST challenge in neonatal broiler chickens followed by an EM oral-gavage at day 18 of age and 2 consecutive days of CP administration (27). In contrast to the high mortality and severe macroscopic lesions reported previously, the macroscopic lesions observed in PC chickens were mild, and no mortality was observed in any of the two experimental groups. However, positive control chickens were challenged with a low virulent strain of EM (Guelph strain) in contrast to the highly virulent EM (M6) used by Shivaramaiah et al. (27). Nevertheless, in the

present experiment, PC challenge chickens showed a significant reduction in performance parameters; increased morbidity; and enhanced gut permeability, evidenced by liver aerobic and anaerobic BT as well as higher serum leakage of FITC-d. Alterations in gut permeability are linked with translocation of bacterial from the intestinal lumen to the portal circulation in several pathological conditions that induce leaky gut and systemic inflammation (58, 59). In the current study, PC chickens contained 13.5-fold more FITC-d in the serum when compared with NC non-challenged chickens. The relevance of this finding is that due to its large size (3–5 kDa), FITC-d does not leak into circulation under normal conditions. However, any impairment of TJ increases the permeability of FITC-d into the blood after oral administration (60–62).

The immunosuppressive effect of ST in neonatal chickens has been previously reported (63). Hence, early infection with ST in our current model has been a more reliable way to induce NE rather than using immunosuppressive viruses (18, 25). Furthermore, ST induces activation of the NF-κβ and alteration in TJ (64–66). Additionally, because the life cycle of *Eimeria* spp. involves intracellular (asexual), and extracellular (sexual) phases, a severe immune response mediated by NF-κβ activation results in the release of IFN-γ and TNF-α that contribute to the pathophysiology of coccidiosis in chickens (67, 68). Under these conditions, TNF-α increases gut permeability by downregulating TJ proteins (69–71). Furthermore, *Eimeria* infections have been also reported to induce dysbiosis. In the present study, EM challenge had a significant impact in BWG that was even more profound after the CP challenge. The increased gut permeability observed in PC group may be the result of a synergistic effect of all three pathogens involved in the NE model used in this study (ST, EM, and CP). Compromising the intestinal permeability, the largest and most important barrier against external environmental agents, the absorption of nutrients was also negatively affected, which impacted the BWG observed in the PC group as has been previously reported (72–74). Another predisposing factor to induce the presentation of NE is the utilization of cereal grains with a high content of NSP (75–77). However, in this NE study the diets provided to the animals were based on the most common feed ingredients used in the poultry industry around the world to simulate commercial conditions.

Additionally, the bacterial taxonomy results obtained in the present study are fascinating. At the phylum level, the NC group showed a higher abundance of Firmicutes and Actinobacteria as compared to PC group, however, a significant difference was observed only with Actinobacteria. Similar changes of the Actinobacteria population have been previously observed in chickens challenged with CP and coccidia in comparison to non-challenged birds (23). The most relevant observation in NC chickens regarding the Actinobacteria phylum was the significantly higher abundance of the family Brevibacteriaceae and the genus *Brevibacterium* as compared to PC chickens. Likewise, *Turicibacter,* a genus of Gram-positive bacteria that has been recognized as an important butyric acid producer was also found significantly higher in NC chickens (78, 79). The NC treatment also showed a significantly higher abundance of Peptostreptococcaceae, a family of Gram-positive bacteria in the class Clostridia that represents another important group of butyric acid-forming bacteria (80, 81).

In contrast, the PC group showed numerically higher relative abundance of Proteobacteria and Bacteroidetes compared to NC group. A similar increment in the population of Proteobacteria and Bacteroidetes has been reported before in severe cases of NE (24). The phylum Proteobacteria contain many opportunistic pathogens including bacteria from the genera *Escherichia*, *Salmonella*, *Campylobacter*, *and Proteus.* Therefore, an increase in Proteobacteria could be related to a probable presentation of gut dysbiosis (82). Among Proteobacteria, Enterobacteriaceae at the family level and *Proteus* at the genus level were significantly higher in PC group as compared to NC group. *Proteus* is a genus that contains opportunistic pathogens including *P. mirabilis* which has been associated with urinary tract, wound, and nosocomial infections in humans and has been considered an important zoonotic pathogen with a wider host range (83, 84). In addition, *P. mirabilis* has been isolated from chicken carcasses (85) and droppings (86), and has shown multiple drug resistance to antibiotics including tetracycline, nalidixic acid, gentamycin, and ampicillin trimethoprim-sulfamethoxazole (86).

The most notable genera among the phylum Bacteroidetes was *Prevotella*, a genus of Gram-negative bacteria which was found significantly higher in PC group compared to NC group. On the other hand, in agreement with previous findings, *Dorea* that belongs to a genus of Gram-positive bacteria of the phylum Firmicutes was significantly higher in PC group as compared to NC group (23). Similarly, *Ruminococcus*, a genus of Gram-positive bacteria associated with phylum Firmicutes and family Ruminococcaceae was significantly higher in PC group. This is in agreement with a previous NE challenged study which reported significantly higher abundance of *Ruminococcus* in the ileum of PC chickens (24). In addition, *Ruminococcus* was also found to be associated with enteritis in humans (87, 88). The association of *Ruminococcus* to enteritis in both humans and chickens may be due to its ability to utilize mucin glycans as an energy source for proliferation (89). Furthermore, a numerically higher proportion of the genus *Gallibacterium* was also observed in PC chickens as compared to NC chickens. This treatment also showed higher proportions of *Clostridium* ($P > 0.05$) and *Lactobacillus* ($P < 0.0001$). It is unclear why PC group had a higher relative abundance of *Lactobacillus*, however, it could be related to the astonishingly rapid recovery of morbid birds in the PC at day 25. Similar to our findings, *Lactobacillus* was also reported significantly higher in ceca of chickens in a different NE model that were challenged with *C. perfringens*, coccidia, and supplemented with fishmeal in the diet as compared to the non-challenged group (23). On the contrary, *Lactobacillus* was significantly higher in NC group compared to PC group in another NE challenged model study (24). On the other hand, the beta diversity analysis shows clear differences between NC and PC groups, suggesting a major shift in the gut microbiome in PC groups due to the challenges used to induce NE.

In summary, the results of the present study suggest that NE impairs the gut epithelial barrier function and induces

microbiome alterations in broiler chickens in a laboratory challenge model. This additional information related to the pathogenesis and development of NE could be helpful to understand in a commercial scenario the mechanism of action of some alternative feed additives used as a replacement of antibiotic treatments to control or prevent the presentation of this important enteric disease. Studies to evaluate the dietary inclusion of a *Bacillus subtilis* direct-fed microbial (*Bacillus*-DFM) selected for production of multiple exogenous enzymes are currently in progress using the previously described NE disease model. This *Bacillus*-DFM has shown to reduce both viscosity and CP proliferation under an *in vitro* digestive system and could be one of many alternatives available to mitigate or prevent NE development without the inclusion of in-feed antibiotics (90, 91).

## ETHICS STATEMENT

All animals handling procedures were in compliance with the Institutional Animal Care and Use Committee at the University of Arkansas (IACUC, approved protocol: 15006).

## AUTHOR CONTRIBUTIONS

JL, GT conceived and planned the study. JL, GT supervised all research. LB provide the *Clostridium perfringens* strain. KT, LG, BM, and MB collected data. JL, SP, GT, BA, YK, and BH analyzed and interpreted data. JL, BA, and XH-V prepared the tables and prepared the figures. JL, BA, YK, XH-V, SR, and GT wrote and/or revised the manuscript. All the authors approved the final version of the manuscript.

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# Fecal Hyodeoxycholic Acid Is Correlated With Tylosin-Induced Microbiome Changes in Growing Pigs

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The changes in the gut microbiome play an important role in the promoting effects of antibiotics, such as tylosin, to the health, and productivity of farm animals. Microbial metabolites are expected to be key mediators between antibiotics-induced microbiome changes and growth-promoting effects. The objective of this study was to extend the identification of tylosin-responsive microbes to the identification of tylosin-responsive metabolites in growing pigs. The feeding trial was conducted on a commercial farm using two pens of pigs fed diets with and without tylosin (40 mg/kg of diet). Fecal samples were collected from 10 pigs per pen at weeks 10, 13, 16, 19, and 22 of age, and subsequently analyzed using liquid chromatography-mass spectrometry (LC-MS) analysis. The multivariate model of LC-MS data showed that time-dependent changes occurred in the fecal metabolome of both control and tylosin-treated pigs. More importantly, the metabolomic profiles were similar between the tylosin treatment and control groups in weeks 10 and 22, but diverged during weeks 13–19. Subsequent analyses of the fecal metabolites contributing to the separation of two groups of pigs showed that hyodeoxycholic acid (HDCA), together with tylosin and its metabolites in feces, was greatly increased during weeks 13–19 ($P < 0.05$) in the group of pigs fed tylosin. The integration of current metabolomics data and the microbiome data from a previous study revealed the consistency between HDCA and a specific genus of microbes in the *Clostridia* family. Further studies are required to determine the causative relations between tylosin-elicited changes in HDCA and the microbiome as well as the role of HDCA in the growth promoting effects of tylosin.

**Keywords: antibiotics, bile acids, metabolomics, microbiome, pigs**

## INTRODUCTION

Current food animal production systems have been able to supply animal products (e.g., milk, eggs, meat) at lower cost than ever before. Likewise, efficiency of food animal production is greater in modern production systems than in the past, while also decreasing environmental impact (1). Modern farms attained such efficiencies in productivity in part because of the implementation of technologies such as utilization of antibiotics as growth promoters. Sub-therapeutic levels of

antibiotics in feeds have been used in swine and poultry diets since the 1940s to improve growth performance of animals while also reducing sub-clinical disease (2, 3). However, the use of antibiotics also increases selective pressures responsible for the evolution of antibiotic resistant bacteria (4, 5). The One Health framework suggests that animal health is closely linked to human health and consequently, the use of antibiotic growth promoters increases the risk of antibiotic resistant bacteria in humans (6). Therefore, it is necessary to develop strategies that maintain and improve animal productivity while reducing the usage of antibiotics in the production of livestock.

The mechanism(s) whereby antibiotics improve growth and efficiency of pigs is still not completely understood, making it difficult for nutritionists, veterinarians, and food animal producers to identify antibiotic alternatives that can produce similar improvements in growth performance without using sub-therapeutic levels of antibiotics. Early experiments in poultry showed that germ-free chicks fed sub-therapeutic levels of antibiotics did not have improved growth compared to the ones fed control diets, indicating that the microbiome plays a significant role in the growth promotion process (7). The microbiome affects numerous physiological processes of animals including protection against some pathogens, development of the immune system and stimulation of immune responses, development of the epithelium, nutrient digestion, and nutrient metabolism (8). Because of the multiple roles ascribed to the microbiome in animals and the complexity of the composition of the microbiome, it has been difficult to define specific mechanisms of antibiotic growth promotion. To fully understand the impact of sub-therapeutic levels of antibiotics on animal growth, research is needed that integrate growth with the metabolome.

Previous studies identified that pigs fed the antibiotic tylosin, had prominent shifts in their fecal microbiome in both abundant and less abundant species compared with the pigs fed an antibiotic-free control diet (9). These results also showed that the composition of the microbiome converged over time, and tylosin appeared to increase the rate at which the microbiome matured. We hypothesized that this shift in microbiome maturation and ultimate convergence would also be represented in the functionality of the microbiome, especially the production of specific bacterial metabolites (10). The objective of this study was to determine tylosin-induced changes in the fecal metabolome of growing pigs and also to correlate these metabolic changes with tylosin-induced changes in the microbiome for a better understanding of the mechanisms mediating antibiotic growth promotion.

# MATERIALS AND METHODS

## Animal Experiment

The animals were housed in conventional confinement facilities on a commercial farm located in southwestern Minnesota for the duration of the experiment [farm 2 in (9)]. Only samples collected from farm two of the experiment previously reported by Kim et al. were used for further analysis (2012). Two pens containing 50 pigs each were used in the experiment. Ten pigs in each pen were randomly chosen, ear tagged for identification and were sampled throughout the sampling period. Pigs in one pen received tylosin in their feed at a concentration of 40 mg/kg beginning at 10 weeks of age and continuing for 12 weeks. Tylosin was chosen as the antibiotic because of its frequent use for growth promotion in the swine industry. The second pen of pigs served as a control and pigs were fed the same feed except that tylosin was not included in it. None of the pigs were given any additional antimicrobials through the duration of the experiment, and all pigs were fed the same standard commercial corn-soybean meal diet. Fresh feces were collected directly from the rectum of the 20 pigs at 10, 13, 16, 19, and 22 weeks of age. Samples were stored at −80°C until analysis. The stability of bile acids in fecal samples after long-term storage has been demonstrated in a previous study (11). This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota (Protocol 0705A09361).

## Metabolomics Analysis

### Chemicals and Reagents

LC–MS-grade water and acetonitrile (ACN) were purchased from Fisher Scientific (Houston, TX); triphenylphosphine (TPP) and 2- hydrazinoquinoline (HQ) from Alfa Aesar (Ward Hill, MA); 2,2′-dipyridyl disulfide (DPDS) from MP Biomedicals (Santa Ana, CA); tylosin tartrate from Ark Pharm (Arlington Heights, IL); acetic acid-$d_4$ from Sigma-Aldrich (St. Louis, MO); glycocholic acid-$^{13}C_1$ from C/D/N Isotopes (Quebec, Canada). The metabolite standards used for structural confirmation were from Sigma-Aldrich, Fisher Scientific, AlfaAesar, Ark Pharm (Libertyville, IL), respectively.

### Fecal Sample Preparation

Fifty mg of pig fecal samples were mixed with 50% aqueous ACN containing 5 μM glycocholic acid-$^{13}C_1$ in 1:10 (w/v) ratio and sonicated for 10 min. The samples were then subjected to further mixing using a vortex mixer and then were centrifuged at 18,000 ×g at 4°C for 10 min to obtain fecal sample extracts. The extracts were stored at −80°C prior to further analysis.

### Derivatization of Short-Chain Fatty Acids (SCFAs) in Fecal Samples

Short-chain fatty acids (SCFAs) in the pig fecal samples were derivatized with HQ prior to LC-MS analysis using a modification of (12). Two microliters of the extract supernatant were mixed with 70 μL of acetonitrile containing 7.5 μM acetic acid-$d_4$, 10 μL DPDS, 10 μL TPP, and 10 μL HQ. The mixture was incubated at 60°C for 30 min, chilled on ice, and mixed with 100 μL $H_2O$. The mixture was then centrifuged at 18,000 ×$g$ for 10 min. Five microliters of the supernatant were injected into the UPLC system.

### LC–MS Analysis

Fecal extracts were analyzed in both non-derivatized form and derivatized form. Non-derivitized fecal extracted were separated a BEH C18 column (Waters, Milford, MA) using a mobile phase gradient containing 0.1% formic acid (A) and ACN containing

0.1% formic acid (B). For SCFAs analysis, HQ-derivitized fecal samples were separated a BEH C18 column (Waters, Milford, MA) using a mobile phase gradient containing 2 mM ammonium acetate and 0.05% acetic acid, v/v (A), and $H_2O$/ ACN = 5:95, v/v, containing 2 mM ammonium acetate and 0.05% acetic acid, v/v (B). The LC eluant was introduced into a Xevo-G2-S quadrupole time-of-flight mass spectrometer (Waters) for accurate mass measurement and ion counting. Capillary voltage and cone voltage for electrospray ionization was maintained at 0.1 kV and 5 V for negative-mode detection, and at 3 kV and 30 V for positive-mode detection. Source temperature and desolvation temperature were set at 120 and 350°C, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (800 L/h), and argon was used as collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution with a mass-to-charge ratio (m/z) of 50–1,000 and monitored by the intermittent injection of the lock mass leucine enkephalin ($[M + H]^+ = 556.2771$ *m/z* and $[M - H]^- = 554.2615$ *m/z*) in real time. Mass chromatograms and mass spectral data were acquired and processed by MassLynx software (Waters) in centroided format. The concentration of individual compounds was determined by calculating the ratio between the peak area of compound and the peak area of internal standard and fitting with a standard curve using QuanLynx software (Waters).

### Chemometric Analysis and Biomarker Identification

The chromatographic and spectral data of fecal extracts were deconvoluted by MarkerLynx software (Waters). A multivariate data matrix containing information on sample identity, ion identity (retention time and *m/z*), and ion abundance was generated through centroiding, deisotoping, filtering, peak recognition, and integration. The intensity of each ion was calculated by normalizing the single-ion counts (SIC) vs. the total-ion counts (TIC) in the whole chromatogram. The data matrix was further exported into SIMCA-P+ software (Umetrics, Kinnelon, NJ) and transformed by *Pareto* scaling, and then analyzed by unsupervised principal component analysis (PCA), supervised partial least squares-discriminant analysis (PLS-DA), and supervised orthogonal partial least squares-discriminant analysis (OPLS-DA). Major latent variables in the data matrix were described in a scores scatter plot of the established multivariate model. Metabolites affected by tylosin were identified by analysis of ions contributing to the separation of tylosin and control samples in the loadings plot the models. The chemical identities of compounds of interest were determined by accurate mass measurement, elemental composition analysis, MSMS fragmentation, and comparisons with authentic standards if available.

## Microbiome Correlation Analysis

Isolation of DNA**,** PCR amplicon production, sequencing, and analysis were all completed and analyzed previously (9). Since the microbiome data analysis only used pooled data, the average relative abundance of metabolites for each time point and treatment were used in this calculation. This allowed the data sets to be equally compared for the correlations analysis. The average values for the metabolomics samples were only used for the correlation analysis. Weighted Bray-Curtis beta diversity metrics were calculated using the vegan package in the statistical software R (13). The dissimilarity distance matrix for both the microbiome and metabolome data was calculated after relative abundance transformations to account for non-normal distributions. Correlations between microbiome and metabolome data were calculated using the mantel test and procrustes analyses, also within the vegan R package (13). A multiple correlation analysis approach, based on Spearman correlation coefficients and adjusted using false discovery rate (fdr) methods for multiple testing using the microbiome R package (14), was also conducted to assess how the abundance of identified bacterial taxonomic units covaried with the abundance of identified bile acid metabolites.

# RESULTS

## Metabolomic Comparisons

The distribution of fecal samples in the score plot of a PLS-DA model showed that time-dependent changes in the fecal metabolome occurred in both control and tylosin-treated pigs (**Figure 1A** and **Figure S1**). Between the two treatment groups, the metabolome profiles were comparable at 10 and 22 weeks of age, but different during weeks 13–19 (**Figure 1A** and **Figure S1**). The metabolites contributing to the separation between control and tylosin groups in 13, 16, and 19 weeks of pigs were defined in the S-plot of a OPLS-DA model (**Figure 1B**). As expected, tylosin and its metabolites contributed to the separation of two groups of pigs in the models (**Figure 1C**). More importantly, HDCA, a bile acid, was identified as another prominent marker associated with tylosin feeding (**Figure 1D**).

Following the observation of HDCA as a tylosin-responsive metabolite, the levels of bile acids in feces were quantified. Based on their concentrations, HDCA and lithocholic acid (LCA) are major bile acids while deoxycholic acid (DCA) and cholic acid (CA) are minor ones in pig feces (**Figures 2A–D**). More importantly, the results showed that the concentrations of fecal bile acids were relatively stable in the control group between week 10 and 22, but significantly and differently affected by tylosin in the treatment group (**Figures 2A–D**). HDCA and CA shared a comparable time-course profile, since the concentrations of both bile acids were elevated by tylosin during weeks 13 and 19, but became comparable to the controls on week 22 (**Figures 2A,D**). In contrast, DCA, and LCA were only increased by tylosin treatment during weeks 19 and 22 (**Figures 2B, C**). Besides bile acids, short chain fatty acids (SCFA) in these fecal samples, including acetic acid, propionic acid, butyric acid, and valeric acid, were also quantified. The acetic acid concentrations were different ($P < 0.05$) between the control and treatment group at the 10 weeks of age (**Figure 3A**). This pre-existing difference between groups cannot be simply explained by tylosin treatment because the antibiotic was only added a few hours before the fecal samples were collected. Aside from this difference at a single time point, there were no differences in the concentration of any SCFA between the treatment and control group (**Figures 3A–D**).

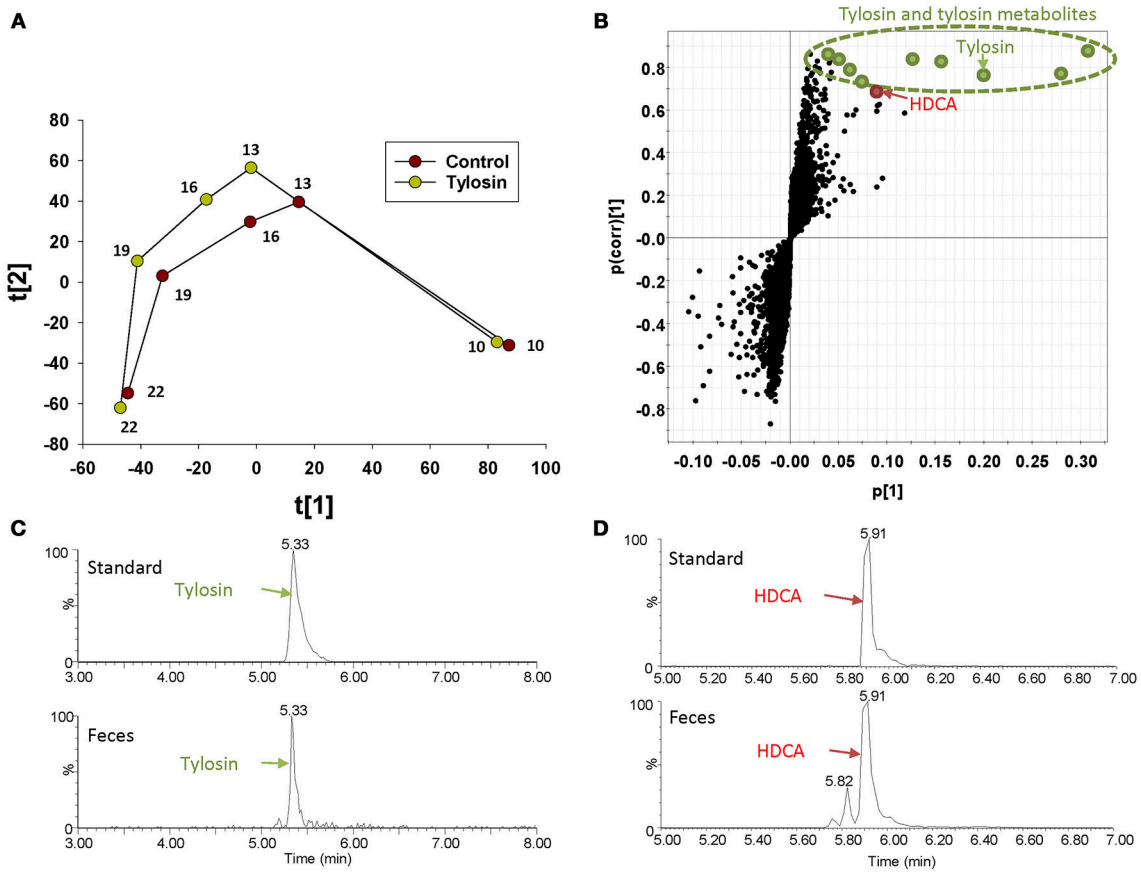


**FIGURE 1 |** Identification of fecal metabolites induced by tylosin treatment through LC-MS-based metabolomics. **(A)** Scores plot of a PLS-DA model on fecal samples from the tylosin-treated and control pigs. The t(1) and t(2) values represent the scores of each data point in the principal component 1 and 2 of the model, respectively. These values are the averages of 10 pigs under the same treatment at weeks 10, 13, 16, 19, and 22. **(B)** S-plot of an OPLS model on week 13–19 control and tylosine treatment samples. The fecal metabolites contributing to the separation of two groups of pigs are labeled. The p(1) axis represents the magnitude of the fecal ions. The p(corr)(1) axis represents the correlation of the ions toward the predictive variation induced by tylosin treatment. **(C)** Extracted chromatograms of tylosin standard and a fecal sample. **(D)** Extracted chromatograms of HDCA standard and a fecal sample.

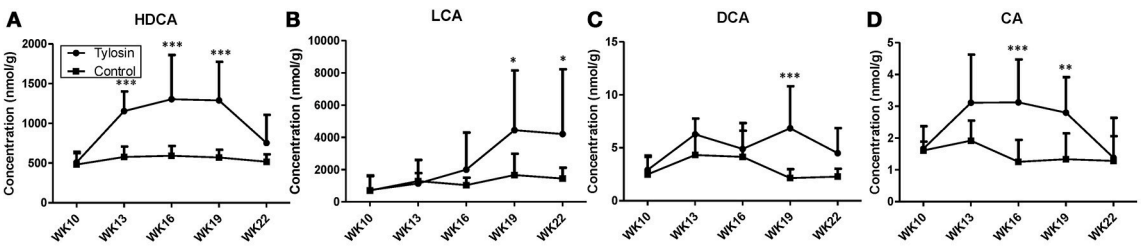


**FIGURE 2 |** Concentrations of bile acids in fecal samples from control and tylosin-treated pigs from week 10 to week 22. **(A)** HDCA. **(B)** LCA. **(C)** deoxycholic acid (DCA). **(D)** CA. Values are mean ± S.D. (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

## Comparison of Metabolome and Microbiome

There was a positive correlation ($r = 0.78$, $P = 0.001$) between the microbiome composition and metabolomic patterns at all-time points for both treatments, revealed by a Mantel test. Procrustes analysis based on the Spearman method further confirmed this correlation (correlation in a symmetric Procrustes rotation = 0.88, $P = 0.001$, m12 squared= 0.22). Multiple correlation analysis was also used to detect associations between identified metabolites and OTUs in the microbiome. Significant correlations ($Q < 0.05$) were observed between bile acid metabolites and limited OTUs (**Table S1**). HDCA is

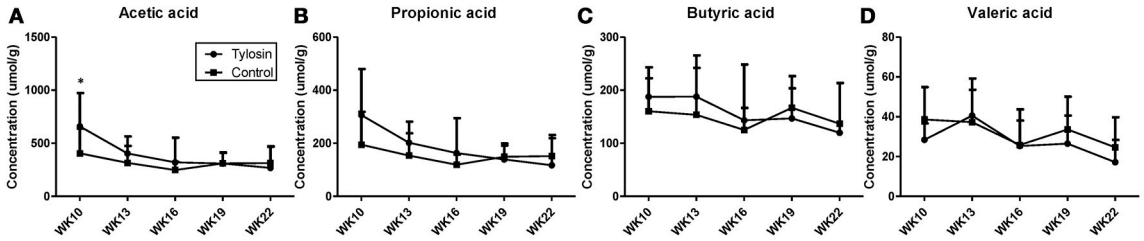


**FIGURE 3 |** Concentrations of SCFAs in fecal samples from control and tylosin-treated pigs from week 10 to week 22. **(A)** Acetic acid. **(B)** Propionic acid. **(C)** Butyric acid. **(D)** Valeric acid. Values are mean ± S.D. (*$P \leq 0.05$).

**TABLE 1 |** Significant correlations (Q < 0.05) between tylosin-responsive bile acids and tylosin-responsive families in swine feces.

| Taxa | Metabolite | r-value | Q-value[a] |
|---|---|---|---|
| k__Bacteria..p__Firmicutes.. c__Clostridia..o__Clostridiales.. f__Lachnospiraceae..g__*Coprococcus*.. s__.60 | LCA | 0.939394 | <0.0001 |
| k__Bacteria..p__Firmicutes.. c__Clostridia..o__Clostridiales.. f__Ruminococcaceae.. g__*Ruminococcus*..s__.102 | LCA | 0.951515 | <0.0001 |
| k__Bacteria..p__Firmicutes..c__Clostridia ..o__Clostridiales..f__Lachnospiraceae.79 | HDCA | 0.963263 | 0.0313 |

[a]*Adjusted for multiple comparisons in the model through false discovery rate.*

the most common bile acid in pigs, and was associated with the abundance of the family *Lachnospiraceae,* which belong to order of Clostridiales. LCA was associated with *Coprococcus* and *Ruminococcus* in both treatment and control groups (**Table 1**). To further evaluate these relationships over time, the concentrations of bile acid metabolites were plotted against the abundances of their correlated bacterial species (**Figure 3**). For LCA, even though metabolite concentration between treatment and control deviated in later weeks, the abundance of *Coprococcus* remained similar between groups but increased over time. The association between LCA and *Ruminococcus* appeared to be more direct, meaning the concentration of LCA increased over time in the tylosin treatment group, as did the abundance of *Ruminococcus* (**Figure 4**). There was an initial increase in the levels of *Lachnospiraceae* and HDCA in pigs fed tylosin, but at subsequent time points, the levels of both decreased to match the control group at 22 weeks (**Figure 4**).

## DISCUSSION

Because metabolites can function as energy carriers and signaling initiators of the growth and wellbeing of host and gut microbes, metabolomic analysis could provide useful insights on the connections between growth promoting effects and microbiome modulating effects of antibiotics. In this study, the composition of the fecal metabolome was similar between tylosin-treated pigs and control pigs at weeks 10 and 22, but different at weeks of 13, 16, and 19 of age (**Figure 1**). This observation resembles our previous observation on the fecal microbiome of these pigs, because the differences in the distribution and quantity of microbes between the control and tylosin treated group were also observed at weeks 13, 16, and 19, but not weeks 10 and 22 (9). This phenomenon suggested that tylosin might cause the microbiome to mature at a faster rate and then stabilize by week 22 (9). Interestingly, this "maturation" of the microbiome was observed in a different set of animals and samples (farm 1) compared to the samples used for the metabolomics analysis presented in this paper (farm 2) (9). The authors explained this variation in microbiome between farms as a technical issue from more in-depth sequencing on farm two, variation in the microbiome between farms that could respond differently to antibiotics, or inaccuracy of the maturation hypothesis. Though this pattern was not as clear in the microbiome on farm two, our results from metabolomic analysis still support this hypothesis, showing similar metabolite compositional patterns between groups at 10 and 22 weeks, with convergence of the metabolome profiles at 22 weeks. When evaluating this phenomenon from an ecological perspective, it has been proposed that the microbiome is always driven to return to a stable state, even after the impact of a stressor, such as antibiotic exposure (15, 16). Our results suggest that the functionality of the microbiome may also follow this pattern, as reflected by convergence of metabolome profiles between the tylosin treated pigs and control pigs.

Because of the role that the microbiome plays in converting primary bile acids to secondary bile acids, we hypothesized that the concentration of secondary bile acids would be altered after exposure to tylosin (17). Previous studies have shown that antibiotics can impact secondary bile acid secretion in humans and rats (18, 19). Furthermore, previous research has also identified that variation in the gut microbiome between germ-free and conventional mice impacts primary bile acid synthesis in the liver through interactions between gut microbes and the nuclear receptor Farnesoid X receptor (20). For this reason, it was also hypothesized that we would observe variations in primary bile acid secretion between the tylosin treatment and the control group. Although the concentration of CA (primary bile acid) was different between treatment and control group, this difference was only present for weeks 16 and 19. It is still unknown which species of bacteria are most involved in the regulation of the Farnesoid X receptor pathway, and we were unable to confirm

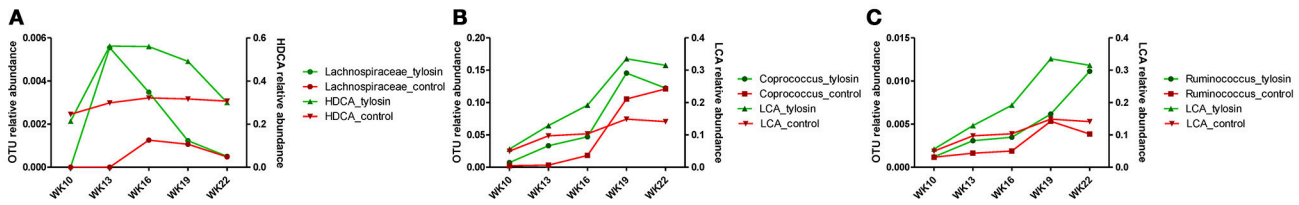


**FIGURE 4 |** Comparison of relative abundance for significantly correlated bacterial species over time for HDCA **(A)** and LCA **(B,C)**.

if antibiotic induced changes in the microbiome impacted this pathway.

Our data also showed differences in HDCA concentration between the tylosin treatment and control group. Because HDCA is produced in germ-free pigs, it has been considered to also be a primary bile acid (21). However, previous research has also demonstrated that a healthy microbiome is capable of producing significant amounts of HDCA, indicating it could also be considered to be a secondary bile acid (22). Based on these conflicting results, it is unclear if HDCA should be considered a primary or secondary bile acid. For this reason, it is difficult to determine which mechanism may be impacting the increased concentration of HDCA in our experiment (i.e., action from the microbiome or interaction with the liver and primary bile acid production). We also found differences in the concentration of LCAs between treatments, which is another secondary bile acids in pigs. The abundance of bacterial class *Clostridia* have been shown to be correlated with intestinal secondary bile acids (23). It has also been reported that bacteria from the family *Clostridia* plays a critical role in bile acid deconjugation (24). In our experiment, we identified a significant, positive correlation with the secondary bile acids LCA and HDCA that were associated with three bacterial species in the class *Clostridia* (Class *Coprococcus, Ruminococcus,* and *Lachnospiraceae*). Thus, we suggest that feeding sub-therapeutic levels of Tylosin may lead to increases in the abundance of *Clostridia*, and ultimately increasing the production of secondary bile acids. It is also worth noting that the majority of previous studies that have reported changes in bile acid metabolism, fed greater antibiotic doses compared to sub-therapeutic levels of tylosin fed in the current study (18, 23, 25). Our experiment found similar alterations in the microbiome, leading to consequential changes in the animals' metabolome, even with a relatively low dose of tylosin.

SCFA are a major group of microbial metabolites in the large intestine (26). Influences of antibiotic exposure on SCFA production have been observed in both human and animal studies. For example, the concentrations of SCFA in feces were reduced by the 6-day treatment of a variety of antibiotics in healthy human subjects (27), while feeding sub-therapeutic levels of antibiotics increased concentrations of SCFA in cecum of treated mice (28). In contrast to these observations, no difference in fecal SCFA concentrations was observed between control and tylosin-treated pigs in this study. It is possible that bacteria responsible for SCFA production might not be sensitive to the dose of tylosin in this study. Tylosin is macrolide-class broad spectrum antibiotic commonly used for its activity against gram-negative bacteria, but is also effective against a select number of gram-positive bacteria (29) Specific gram-positive bacteria from the families *Propionibacteriaceae, Bifidobacteriaceae,* and *Veillonellaceae* have been shown to play a role in SCFA production (30). It is possible that tylosin did not have a bacteriostatic effect on some of these bacteria, allowing them to continue SCFA production without major changes.

One of the main limitations of our experiment was our inability to correlate the change in bile acid synthesis to a change in growth performance or health of pigs, because body weights, mortality, and morbidity data were not collected during the experiment. There is currently limited research available that has reported a direct change in growth performance of swine as a result of increased bile acid synthesis, but some previous research suggests that the mechanism for growth promotion when feeding antibiotics are due to changes in bile biotransformation (31, 32). However, this proposed mechanism suggests that increased bile acid secretion decreases average daily gain in the animal, which has been demonstrated in swine with LCA (25). Our results showed that the concentrations of LCA and other bile acids increased in pigs fed tylosin, which suggests that these differences may be specific to tylosin. Various antibiotics target different types of bacteria, which suggests that the mechanisms of growth promotion through modulation of the gut microbiome will vary between antibiotics used (3, 20, 23). Without growth performance data being available from this experiment, we are unable to determine the impact of the altered bile acid concentrations on growth of these pigs.

In conclusion, inclusion of sub-therapeutic levels of tylosin in the diet of growing pigs impacted bile acid concentration in the feces, but this change tended to diminish in subsequent time periods. These observations warrant further investigation to better understand the role of bile acids in growth and development of pigs, and whether these observations may be correlated with the mechanisms of growth promotion when supplementing diets with sub-therapeutic levels of antibiotics for pigs.

## AUTHOR CONTRIBUTIONS

MT, YZ, FL, CC, and RI all contributed equally to this work. MT, FL, RI, GS, and PU conceived and designed the experiments. YZ and CC performed the experiments. MT, YZ, and AG analyzed the data. CC contributed reagents, materials, analysis tools. MT, YZ, FL, AG, CC, PU, GS, CC, and RI wrote the paper.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2018.00196/full#supplementary-material

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

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# Organic Acids and Potential for Modifying the Avian Gastrointestinal Tract and Reducing Pathogens and Disease

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Recently, antibiotics have been withdrawn from some poultry diets; leaving the birds at risk for increased incidence of dysbacteriosis and disease. Furthermore, mortalities occurring from disease contribute between 10 to 20% of production cost in developed countries. Currently, numerous feed supplements are being proposed as effective antibiotic alternatives in poultry diets, such as prebiotics, probiotics, acidic compounds, competitive exclusion products, herbs, essential oils, and bacteriophages. However, acidic compounds consisting of organic acids show promise as antibiotic alternatives. Organic acids have demonstrated the capability to enhance poultry performance by altering the pH of the gastrointestinal tract (GIT) and consequently changing the composition of the microbiome. In addition, organic acids, by altering the composition of the microbiome, protect poultry from pH-sensitive pathogens. Protection is further provided to poultry by the ability of organic acids to potentially enhance the morphology and physiology of the GIT and the immune system. Thus, the objective of the current review is to provide an understanding of the effects organic acids have on the microbiome of poultry and the effect those changes have on the prevalence of pathogens and diseases in poultry. From data reviewed, it can be concluded that the efficacy of organic acids on shifting microbiome composition is limited to the time of administration, the composition of the organic acid product, and the current health conditions of poultry.

**Keywords: poultry, organic acids, lactic acid producing bacteria, prebiotics, microbiome**

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## INTRODUCTION

With the removal of antibiotics from some poultry integrators and the implementation of antibiotic-free birds (ABF), the industry is challenged with identifying a valid alternative to antibiotics with similar capabilities to that of antibiotics (1, 2). As antibiotics have been noted to improve weight gain, through the reduction of subclinical and clinical infection by mitigating the presence of bacteria in the gastrointestinal tract (GIT) and consequently reducing nutrient competition, immune stimulation, thinning the intestinal wall, and enhancing nutrient digestibility (3, 4), these are considered the qualities expected of an effective alternative. Many antibiotic alternative products improve growth performance characteristics of poultry by directly impacting the environment of the GIT, such as altering the bacterial populations, physiology, and the pH of the GIT (5). Although there are numerous alternatives currently on the market, organic acids are a

valid alternative with the capability to reduce pathogenic bacteria and increase nutrient digestibility through their effect on the pH in the GIT (5–7). Because the digestive process extensively includes microbial fermentation, organic acids are commonly produced by beneficial bacteria (probiotics) present in the crop, intestines, and ceca (3). Furthermore, the supplementation of prebiotics has the potential to increase the production of organic acids by probiotic bacteria. Thus, there are several application methods to alter the GIT: the dietary introduction of acidic compounds either directly via feed supplements containing organic acids, or indirectly as a shift in fermentation originating from the presence of probiotics, prebiotics, or combined as synbiotics in the GIT. The current review aims to elaborate on the use of organic acids and organic acid stimulating dietary supplements, probiotics and prebiotics, and their subsequent effects on pathogen prevalence and the developing avian GIT microbiome.

## Antibiotics in the Livestock Industry

After the rapid expansion of the poultry industry in the 1940s, there was a need for basic feed components. Due to this accelerated growth in the commercial poultry industry, there was a shortage of fishmeal and other animal protein sources (8–10). With the necessity for more animal protein sources, the industry sought to determine what the Animal Protein Factor (APF), the factor in animal protein sources that promoted increased poultry performance, consisted of and to find a suitable alternative (8). APF was later discovered to be Vitamin $B_{12}$ in 1948 (8–10). Ultimately, the search to find an effective alternative to APF helped fuel the discovery of antibiotic growth promoters (AGPs).

Alexander Fleming, an English scientist, discovered penicillin in 1928 when he was testing the ability of mold to reduce staphylococci on agar plates (8). However, it took until the early 1940s for scientists, Ernst Chain and Howard Florey, to isolate a sufficient quantity of penicillin to be tested and validated as an effective treatment for illnesses (8). Shortly after the discovery of antibiotics, a growth promoting component of fungal mycelia, an antibiotic, was observed outperforming APF, vitamin $B_{12}$ (8, 11–14). Moore et al. included antibiotics in chicken feed and was the first research group to show an increase in weight gain due to the inclusion of antibiotics (15). Later, the use of antibiotics in feed would be coined as the term "AGPs" and be utilized for prophylactic purposes that prevented or reduced the risk for infection, as well as promoted growth in broilers.

AGPs in the poultry industry are administered in the diet when there is no clinical sign of infection, however the risk still exists. Prophylactic application of AGPs have resulted in improved weight gain, reduced bacterial presence in the GIT, reduced nutrient competition, and reduced immune stimulation (4). After the introduction of AGPs to the industry, there were concerns for the residues in meat and fungal overgrowth in animals. However, since the poultry industry does not employ antibiotics that are absorbed by the digestive tract, the concern for antibiotic residues in meat and meat products was not considered a direct concern (16, 17). As time progressed, the concerns have evolved due to consumer perception and scientific reports (8).

## Removal of Antibiotics From the Poultry Industry

The poultry industry began to turn away from the use of antibiotics due to growing public concern over antibiotic resistant pathogens. As early as the late 1960s, the Swann Committee in the European Union (EU) researched the possibility of bacterial resistance due to the use of antibiotics in livestock diets (18). It was found in the years between 1963 and 1965 that the resistance to antibiotics could be transferable to other bacteria, as was seen in the epidemic of antibiotic resistant *Salmonella* Typhimurium (18). The epidemic of *S.* Typhimurium led the United Kingdom (UK) government to appoint the Swann Committee to monitor and identify possible resistance of pathogenic bacteria to antibiotics from animal origins (18). The Swann Committee later recommended in 1969 that the antibiotics used as growth promoters in feed diets be those that "have little or no application as therapeutic agents in man or animals and will not impair the efficacy of a prescribed therapeutic drug or drugs through the development of resistant strains of organisms" (18). The Swann Committee in that same statement deemed the use of chlortetracycline, oxytetracycline, penicillin, tylosin, and the sulphonamides as unsuitable for growth promotion (18). The statement was later adopted by the UK in 1998 (19). As the continued concerns grew in the UK and across the world, the poultry industry experienced extreme pressure to terminate the use of AGPs in the diet of poultry and other livestock.

The first country in the EU to officially ban the use of AGPs was Sweden in 1985 (18). Sweden, after joining the EU in 1995, heavily campaigned for the termination of the use of antibiotics as growth promoters in animal feed in the EU (18). In 1996, the United States (US) implemented the National Antimicrobial Resistance Monitoring System (NARMS), which monitored the antimicrobial resistance in bacteria (8). Within that same time period (1997 and 1998), the World Health Organization (WHO) and Economic and Social Community of the European Union deemed the use of antimicrobials in food animals as a public health concern, citing risks to the long-term use of antibiotics, such as resistance to antibiotics (17).

The EU finalized the ban on AGPs with the creation of Regulation 1831/2003 which eliminated the use of all AGPs as of January 1, 2006 (17). Although the overall use of antibiotics has decreased by 55% from 1986 to 1999 in conjunction with a low prevalence of antimicrobial resistance (20), there is still concern for the increase in use of therapeutic antibiotics due to the increase in infections (21).

Current concerns over antibiotic resistance have been backed by the prevalence of antibiotic resistance stemming from livestock origin. Poultry have been linked to the resistance of *Campylobacter* and *Salmonella* to multiple antibiotics. For example, a few years after the introduction of fluoroquinolones in The Netherlands, there was an increase in fluoroquinolone-resistant *Campylobacter* of poultry origin (22). The EU also experienced gentamicin resistance in *Campylobacter* from broiler meat origins that ranged from 0 to 6.3% (23). The US has seen *Campylobacter coli* resistance to gentamicin increase from 1% in 2007 to 18% in 2011 from chicken meat isolates and

an increase from 1 to 6% between 2007 and 2011 from chicken isolates at slaughter (24). In addition, *Salmonella* spp. have been noted to develop a multi-drug resistance to antibiotics such as tetracyclines, sulfonamides, streptomycin, kanamycin, chloramphenicol, and some β-lactam antibiotics (25–27). However, there has been a relatively stable reporting of resistance among these antibiotics since 1996 (4). The resistance to other antibiotics has increased relatively, as seen in amoxicillin/clavulanic acid and ceftiofur, which have been associated with increases from <2 to 15% from 1998 to 2005, respectively (28).

Currently, the US poultry industry has initiated phasing out AGPs partly due to the increase in consumer concern over the usage of AGPs and the increase in AGP free exportation requirements. However, numerous growers have observed an increase in "dysbacteriosis," a condition in which the small intestines' experience bacterial overgrowth (3). The solution is to find alternatives with similar effects as AGPs such as: (1) reducing the number of incidences and the amplitude of subclinical infections; (2) reducing the use of nutrients by bacteria; (3) improving absorption through the thinning of the intestinal wall; and (4) by reducing the amount of "growth-depressing metabolites" produced by Gram-positive bacteria (3).

## ALTERNATIVES TO ANTIBIOTICS: ORGANIC ACIDS

Several alternatives have been proposed to replace AGPs in the poultry industry including exogenous enzymes, competitive exclusion products, prebiotics, probiotics, herbs, essential oils, acidic compounds, and bacteriophages (3, 29). Currently, the more common alternatives applied in broiler diets are prebiotics, probiotics, and organic acids. All are utilized with the ultimate goal of ameliorating the condition of the poultry GIT by mitigating the presence of enteric bacteria present in the GIT and improving the performance of the bird (29). It is of interest to determine how each alternative product specifically achieves improvement in bird gut health. Both organic acids and probiotics appear to have similar mechanistic impacts on bird health as many probiotics improve the physiology and anatomical structure of the intestinal cell wall, enhancement of immunological functions in the GIT, and the increased resistance to enteropathogenic bacteria activity (3). This occurs either by direct introduction of organic acids including short chain fatty acids (SCFA) in the feed or in the case of probiotic bacteria generating SCFA, hydrogen peroxide, and intermediary metabolites with antimicrobial activity once they become established in the GIT (3). Organic acids include not only SCFAs but also lactic and formic acids as well as longer carbon chain acids. Prebiotics are also of interest, as they stimulate the proliferation and maintenance of beneficial bacteria such as *Lactobacillus*, which in return increases the production of SCFA (30). Thus, organic acid, probiotic, and prebiotic supplements are interlinked because of their role in the production of SCFA and other fatty acids (31–33).

Organic acids are organic compounds that retain acidic properties (5). Most organic acids consist of carboxylic acids (-COOH). Organic acids are primarily composed of SCFAs (≤C6), also commonly referred to as volatile short-chain fatty acids (VSCFA), such as fumaric, propionic, acetic, lactic, butyric, and others. Other organic acids consist of medium-chain fatty acids (MCFA; C7 to C10), and long-chain fatty acids (LCFA; ≥C11) (**Table 1**).

**TABLE 1 |** A list[1] and description of straight-chain monocarboxylic acids[2,3,4] and their derivatives[5].

| Acid | Chemical name | Formula | MW | pKa |
|---|---|---|---|---|
| Formic[2] | Formic Acid | HCOOH | 46.03 | 3.75 |
| Acetic[2] | Acetic Acid | $CH_3COOH$ | 60.05 | 4.76 |
| Propionic[2] | 2-Propanoic Acid | $CH_3CH_2COOH$ | 74.08 | 4.88 |
| Butyric[2] | Butanoic Acid | $CH_3CH_2CH_2COOH$ | 88.11 | 4.82 |
| Lactic | 2-Hydroxypropanoic Acid | $CH_3CH(OH)COOH$ | 90.08 | 3.83 |
| Sorbic | 2,4-Hexandienoic Acid | $CH_3CH:CHCH:CHCOOH$ | 112.14 | 4.76 |
| Fumaric | 2-Butenedioic Acid | $COOHCH:CHCOOH$ | 116.07 | 3.02 |
| Caproic[2] | 1-Hexanoic Acid | $CH_3CH_2CH_2CH_2CH_2COOH$ | 116.16 | 4.88 |
| Malic | 2-Hydroxybutanedioic Acid | $COOHCH_2CH(OH)COOH$ | 134.09 | 3.40 |
| Caprylic[3] | 1-Octanoic Acid | $CH_3CH_2CH_2CH_2CH_2CH_2CH_2COOH$ | 144.21 | 4.89 |
| Tartaric | 2,3-Dihydroxy-Butanedioic Acid | $COOHCH(OH)CH(OH)COOH$ | 150.09 | 2.93 |
| Capric[3] | Decanoic Acid | $CH_3(CH_2)_8COOH$ | 177.26 | 4.90 |
| Citric | 2-Hydroxy-1,2,3-Propanetricarboxylic Acid | $COOHCH_2C(OH)(COOH)CH_2COOH$ | 192.14 | 3.13 |
| Lauric[4] | Dodecanoic Acid | $CH_3(CH_2)_{10}COOH$ | 200.32 | 5.30 |

[1] *Adapted from Dibner and Buttin and Cherrington et al. (6, 34).*
[2] *Classified as a short-chain fatty acid (SCFA; ≤C6).*
[3] *Classified as a medium-chain fatty acid (MCFA; C7-C10).*
[4] *Classified as a long-chain fatty acid (LCFA; ≥C11).*
[5] *Derivatives of saturated straight chain fatty acids: unsaturated (sorbic), hydroxylic (citric, lactic), multicarboxylic (fumaric, malic, tartaric, and citric).*

Due to the lipophilic nature of LCFA, their antimicrobial properties may be a constituent of their potential to incorporate themselves into target cell membranes and promote leakage of cellular protons or ions, such as in Gram-positive bacteria (35–37). However, it has been demonstrated by Shue and Freese that the resistance possessed by Gram-negative species to MCFA and LCFA is in part due to the presence of the lipopolysaccharide (LPS) layer in the cell wall (38). Thus, LPS prevents MCFA and LCFA from crossing the cell membrane and into the cell (34). Further, Gram-negative bacteria, such as *E. coli*, possess the ability to assimilate MCFA and LCFA into the cell and subsequently metabolize them per the β-oxidation cycle (39).

Alternatively, specific bacterial groups such as *Salmonella* and *Escherichia coli* are capable of utilizing SCFA as energy sources (39–43), whereas fermentative bacteria produce organic acids when oxygen is not available (29). More specifically, acetic acid is a source of carbon and energy for bacteria by activating enzymes of the glyoxylate pathway, isocitrate lyase, and malate synthase (39). Furthermore, lactobacilli, streptococci, lactococci, and enterococci are all capable of fermenting sugars to produce lactate; however, if sugar is scarce these bacteria are capable of generating acetate, formate, and ethanol from fermentation to enhance ATP production (44).

Organic acids were initially added to feed for sanitization purposes such as to reduce fungal contamination in feed and as a preventative against salmonellosis in poultry (45–47). However, in the past 30 years, formic and propionic acid have been examined for bactericidal activity, *in vivo*, of poultry (48). Organic acids utilized in feed are not only capable of decontaminating feed but have the potential to reduce enteric bacteria internally in poultry.

Weak organic acids (C1-C7) with a pKa between 3 and 5 are explicitly used for their antimicrobial activity (5). There are two major types of organic acids (**Table 2**). The first group (lactic, fumaric, citric) are capable of generally lowering the pH of the stomach, thus reducing the acid sensitive bacteria present indirectly. The second group (butyric, formic, acetic, propionic, and sorbic) lower the pH in the GIT by directly acting upon the cell wall of Gram-negative bacteria (5, 49). Organic acids ameliorate the conditions of the GIT through the reduction of GIT pH, promoting proteolytic enzyme activity and nutrient digestibility, intensifying pancreatic secretions, encouraging digestive enzyme activity, creating stability of the microbial population and stimulating the growth of beneficial bacteria, and by being bacteriostatic and bactericidal to pathogenic bacteria (5). With the need to find a suitable alternative to AGPs, a wide range organic acids have been utilized in poultry diets for the potential to mitigate pathogen prevalence in the GIT of poultry.

**TABLE 2 |** Two different mechanisms of organic acids on altering the pH of the gastrointestinal tract (GIT) and its subsequent effect on pathogens.

| Acids | Effect |
|---|---|
| Lactic, fumaric, citric | Indirectly mitigating or eliminating pathogens by decreasing the environmental pH in the GIT[1]. |
| Butyric, formic, acetic, propionic, and sorbic | Directly mitigating or eliminating pathogens by acting upon the cell wall of Gram-negative bacteria and subsequently lowering the pH in the GIT[1]. |

[1]*Adapted from Papatisiros et al. and Diener et al. (5, 49).*

## ORGANIC ACIDS AS FEED ADDITIVES

As previously mentioned, organic acids can benefit poultry internally by their ability to lower the pH of the gastrointestinal tract. It has been found that organic acids such as fumaric, propionic, lactic, and sorbic acid have the ability to reduce the colonization of pathogenic bacteria and the production of toxic metabolites through acidification of the diet (50). Although the crop and gizzard are the locations in which propionic and formic acid are confined to, the crop is one of the initial locations for *Salmonella* establishment that can lead to subsequent infection of the bird (51). It has also been demonstrated that most *Salmonella* spp. are killed when the pH value is the equivalent to that of the crop and proventriculus, *in vitro* (52). In addition, the vertical transmission and initial colonization of chicks with *Salmonella* can be reduced through the dietary inclusion of organic acids (53). Although organic acids can indirectly have an impact on pathogenic bacteria by lowering the pH of the GIT, they can also elicit non-pH direct toxic effects on bacterial metabolism.

Although the most noted benefit of organic acids is its ability to lower the pH of the GIT, organic acids can also prevent pathogen livability on the cellular level. Organic acids possess the ability to target the cell wall, cytoplasmic membrane, and particular functions of metabolism in the cytoplasm associated with replication, protein synthesis, and function (48, 54). VSCFA, consisting of weak organic acids that are bacteriostatic without affecting intestinal microbiota, are not regarded as acidifiers as their mode of action is to directly diffuse across the cell membrane of bacteria in the undissociated form without lowering the bowel pH (55). VSCFA, once diffused across the bacterial cytoplasm, lower the internal pH of the bacteria (55).

The specific effectiveness of a particular organic acid relies heavily on several factors such as: type and acidity of the SCFA, inclusion rate of acids, diet composition and buffering within the diet, level of "intraluminal production of acids" by lactic acid producing bacteria (LAB) in GIT, feed palpability, receptor on the epithelial villi for bacterial colonization, vaccinate immunity, welfare, and age (5).

Some concerns for the use of organic acids include their inability to affect the lower part of the GIT, bacteria's ability to create a resistance against organic acids, and their hindering effect on host beneficial bacteria such as LAB. Much of their bacterial impact is related to their effective concentration present in different compartments of the GIT. For example, Thompson and Hinton noted that as SCFAs move along the digestive tract, their concentration decreases due to digestion and metabolism (51). It has also been reported by Hume et al. that most of the propionic acid that was in the treated feed did not get past the crop, proventriculus, and gizzard and thus never reached the

small intestines (48, 56). Most organic acids will dissociate before reaching the lower GIT and thus having little to no effect on the GIT (56). Although it was stated earlier the initial site of *Salmonella* colonization is the crop, it is important for organic acids to enter the lower GIT, as the ileum and ceca are also considered primary sites of infection. Furthermore, it is unlikely for organic acids to prevent a large infectious dose of *Salmonella* from getting past the crop (51).

To combat the potential decrease in effective concentrations of organic acids as they traverse the GIT, encapsulation of organic acids offers the potential to not only protect them but control their subsequent release as they pass through the poultry GIT. It has been demonstrated that the dietary inclusion of encapsulated butyric acid has the capability to improve digestion and absorption (57), reduce the infection of *S.* Enteritidis throughout the GIT (55) and reduce stress-induced catabolism and oxidative injury of tissues (58) of broilers.

## ORGANIC ACIDS PRODUCED BY INTRODUCTION OF PROBIOTICS AND PREBIOTICS TO THE GIT

Although SCFA can be experimentally provided as feed additives in poultry diets, they are also naturally produced by the GIT, and their relative concentrations and types can be altered. In chickens, ruminants, and humans the production of SCFA in the GIT has been reported as high as 190 mM (59–62). Research has demonstrated that both probiotic and prebiotics stimulate the production of SCFAs in the GIT of poultry (3, 63–66) either through the direct production of SCFA by lactic acid producing bacteria (LAB), a type of probiotic, or through the administration of prebiotic substances which increase the presence of LAB and their production of SCFA (67, 68). Thus, probiotic and prebiotic supplementation can enhance SCFA production and, in turn, their impact on the avian microbiome. The following subsection provides discussion of specific studies that illustrate this impact.

### Probiotics and Their Influence on SCFA Production

Lilley and Stillwell originally conceived the term probiotics as "a substance produced by one microorganism which stimulated the growth of another" in 1965, well after the discovery of antibiotics (69). Although the term was not coined until after the discovery of antibiotics, probiotics had been around since the early Twentieth century (70). Over time as more knowledge was obtained on the nature of what represented a true probiotic culture, the definition started to change to define their usefulness and application better. In 1989, the definition was modified to a "live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" by Fuller (70). Three years later in 1992, Havenaar and Huis in't Veld extended the definition to "a mono or mixed culture of live microorganisms which, applied to animal or man, affect the host beneficially by improving the properties of the indigenous microflora" (70, 71). The definition of probiotics has now been established by Fuller as "a preparation consisting of live microorganisms or microbial stimulants which affects the indigenous microflora of the recipient animal, plant or food in a beneficial way" (70).

Microorganisms that have been considered as probiotics include: lactic acid producing bacteria, avirulent mutants of *E. coli*, *Clostridium difficile*, *S.* Typhimurium, yeasts, fungi, viruses, and bacteriophages (70). Current research using probiotic bacteria include: *Bifidobacterium*, *Lactobacillus*, *Bacillus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, and *Saccharomyces* (5). Probiotics serve to protect the GIT microbiota through bacterial antagonism, bacterial interference, barrier effect, competitive exclusion, and colonization resistance (70).

The most common probiotic spp. utilized in poultry diets are from the genera *Lactobacillus*, *Enterococcus*, *Pediococcus*, and *Bacillus;* however, extensive research has been conducted on *Lactobacillus* species (63, 72, 73). Further, various probiotic spp, differ in their ability to colonize the GIT of animals. Those that are considered colonizing species are *Lactobacillus* and *Enterococcus* spp., while *Bacillus* spp. and *Saccharomyces cerevisiae* are free-flowing and do not colonize the GIT (3). As stated earlier, the benefits of probiotic supplementation include: onset of changes of the physiology and anatomical structure of the intestinal cell wall; enhancement of immunological functions in the GIT; and the enhanced resistance to enteropathogenic bacteria (3). These actions are typically accomplished via coupling with the production of SCFA, hydrogen peroxide, and intermediary metabolites with antimicrobial activity (3).

Probably the best-characterized group of probiotics are lactic acid producing bacteria (LAB), such as *Lactobacillus*. LAB generate lactic acid *in vitro* and the lactic acid produced is utilized for the production of butyric acid by Clostridial clusters, which supports the concept of cross-feeding (3). *Lactobacillus* spp. have been found to reduce pathogenic attachment to the ileal epithelial cells through exclusion and competition (72). *Lactobacillus* also elicits antibacterial effects by producing lactic acid (63). Lactic acid, an organic acid, can lower the GIT pH, thus creating a hostile environment for resident pathogenic bacteria. *Lactobacillus acidophilus* is found to be the most sufficient candidate as a dietary appurtenance (71). *L. acidophilus* has the potential to decrease the external pH to lower values than other lactic acid producing bacteria and can reach a medium pH of 3.5 (64). Thus, *Lactobacillus* spp. are considered excellent candidates as AGP alternatives.

### Prebiotics Influence on SCFA Production

Prebiotics have also been considered as valid AGP alternatives (74). Prebiotics are described as beneficial non-digestible feed ingredients that when fed selectively enhance populations of bacteria in the GIT (59). More recently, prebiotics have been described as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (75). Thus, prebiotics influence the GIT by acting as substrates for beneficial bacteria. Prebiotics include non-digestible carbohydrates such as oligosaccharides and polysaccharides, particular peptides, proteins, and specific lipids (76). Poultry research investigating the application of prebiotics as antibiotic alternatives typically revolves around the administration of oligosaccharides which

include mannanoligosaccharides (MOS) galactooligosaccharides (GOS), and fructooligosaccharides (FOS) (32, 33, 75, 77–79).

The dietary inclusion of prebiotics has been demonstrated to influence the microbial profiles of the avian GIT. Kim et al., reported the increased concentration of lactobacilli at the the ileal cecal junction of 4 week old broiler chickens fed diets containing 0.25% FOS and 0.25% MOS and a decrease in the populations of *Clostridium perfringens* in birds fed diets containing 0.25% FOS, 0.05% MOS, and avilamycin (80). The dietary inclusion of MOS (5 g/kg) and FOS (5 g/kg) have also been shown to change the jejunal, ileal, and cecal *Lactobacillus* community profiles of 25 d Cobb 500 broilers, with differences in *Lactobacillus* communities being noted between MOS and FOS treated broilers (81). Although research has demonstrated the effect prebiotics have on bacteria, performance has been shown to not be improved by the dietary inclusion of MOS or FOS (80, 81). In contrast, the addition of Bio-Plus 2B® into diets of Ross 308 broilers improved the feed conversion ratio (FCR) throughout the entirety of the study (42 d) compared to those fed control diets (82).

In addition, the dietary supplementation of prebiotics has demonstrated the enhanced production of organic acids in the GIT. The dietary inclusion of FOS has demonstrated the ability to increase populations of *Bifidobacterium* and *Lactobacillus* in the intestinal and cecal digesta of 49 d old male Avian Farms broilers (83). Thus, the increase of LAB species such as *Lactobacillus* and *Bifidobacterium* may enhance the production of SCFA in the GIT. Furthermore, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Bifidobacterium longum*, and *Bifidobacterium bifidus* in the presence of millet dietary fibers have exhibited the ability to produce SCFAs such as acetate, propionate, and butyrate, *in vitro*, with the production of acetate being most significant (84). A study investigating the inclusion of inulin (1%)in the diets of 42 d old Cobb 500 broilers reported the increased concentration of acetate in the jejunum and an increase in the proportions of n-butyrate and n-valerate in the cecal digesta in broilers fed diets containing inulin compared to those fed control diets (85). In short, the dietary supplementation of prebiotics appears to contribute to the increased production of SCFA *in situ*.

## MECHANISMS OF ORGANIC ACIDS VS. LACTIC ACID PRODUCING BACTERIA

Research has demonstrated that both organic acids and LAB have the capability to improve broiler performance and reduce pathogenic bacteria (86–90). Since the modes of action for both organic acid supplements and LAB involve the lowering of the pH of the GIT, many of their benefits appear to be similar. However, LAB and organic acids should also still be considered in some respects considerably different in their effectiveness, mechanisms, and interaction with one another.

Although LAB do not directly destroy enteric bacteria, LAB are able to inhibit colonization and further growth and establishment of pathogenic bacteria. Furthermore, LAB byproducts beyond SCFAs, such as hydrogen peroxides, and intermediary metabolites also contribute to the reduction of pathogens present in the GIT. In fact, research has demonstrated when *S.* Enteritidis at $10^6$ CFU and *L. salvarius* at $10^8$ CFU were gavaged orally and simultaneously into the proventriculus of 1-day old broiler chicks, at 21 days of age all birds were negative for *Salmonella* (91). It has been noted that SCFAs when interacting with Gram-negative bacteria are not only bacteriostatic but can also be bactericidal (51). Furthermore, organic acids such as SCFAs are produced in millimolar concentrations in the GIT of animals due to the prevalence of anaerobic bacteria. Organic acids, being SCFA, also possess the ability to lower the pH of the GIT and improve broiler performance similar to LAB. Thus, previous research has seen both methods to be beneficial in the reduction of pathogenic bacteria (86–90).

Another challenge of using organic acids as an alternative to AGP is the resistance bacteria can develop to stressful environments. It has been reported that *E. coli* and *Salmonella* can elicit a tolerance to environments that induce stress such as an acidic environment created by the use of organic acids (48). In addition, Diez-Gonzalez and Russell have reported the increased resistance to extreme acidic conditions of *E. coli* O157:H7 after exposure to SCFAs (92). Likewise, Conner and Kotrola previously observed that *E. coli* exhibited the ability to live in acidic condition (pH $\geq$ 4.0) below 4.0°C and for up to 56 d, however, the temperature and type of acidifier affect their survival (93). In addition, pH-independent tolerance is also possible. For example, Kwon and Ricke reported the increased acid resistance displayed by *S.* Typhimurium occurred after exposure to a single SCFA at high concentration but neutral pH (94). Furthermore, it has been reported that the proportions of SCFAs within the large intestines can influence the cross-resistance of *S.* Typhimurium 14028s to other stressors such as an extreme pH (pH 3.0), 2.5 M NaCl, and 20 mM $H_2O_2$ (95).

Not only can bacteria build a resistance to organic acids, but pathogenic bacteria can also lower their internal pH to protect themselves from the acidic properties of organic acids, thus rendering them ineffective in being bactericidal against pathogenic bacteria (48). Furthermore, fermentative bacteria have the ability to lower their intracellular pH in the event that the extracellular pH becomes highly acidic. If the intracellular pH is lowered, the bacterium has a much smaller pH gradient across the cell membrane and will be protected from anion accumulation (53).

The most significant challenge to organic acid feed additive use is their potentially detrimental effect on LAB. In previous research, the use of organic acids in the diet reduced not only the amount of lactic acid but the LAB present in the GIT. As early as 1989, Impey and Mead reported that adding 1.0% formic acid into a food slurry containing *Salmonella* and *Lactobacilli*, not only killed *Salmonella* but *Lactobacilli* as well (pH $<$ 4.0; 37°C) (96). It was also observed by Hume et al. that organic acids reduced LAB (56). The finding by Hume et al. is consistent with the conclusion by Thompson and Hinton that LAB were reduced by the inclusion of organic acids (51, 56). In one of the studies conducted by Thompson and Hinton, 68% formic acid and 20% propionic acid product, was added to a poultry diet and resulted in an increase of propionic and formic acid, as well a decrease in lactic acid in the crop (51). This interaction suggests

that propionic and formic acid inhibit LAB and thus reduced lactic acid. Consequently, SCFA may be counterproductive to the overall development of microbiota in the GIT of broiler chickens.

Poultry are born without an established microbiota in their GIT (97) and are removed from maternal care to be incubated in a controlled environment. Thus, poultry housed in modern production facilities have difficulty in establishing beneficial microbiota associated with a mature GIT microbiota (70). This corresponds with research where intestinal infections affect germ-free animals more than those with an established microbiota (98, 99). Probiotics have been especially important in improving the microbiota composition of poultry, as well as protecting poultry from intestinal infections and are recognized as an alternative to AGPs. If the use of organic acids in poultry reduces the concentration of LAB present in the GIT, it could increase the chance of *Salmonella* colonizing the GIT. This especially could occur as organic acids are limited to the crop and may not be able to handle a high inclusion of *Salmonella* (51). Probiotics (LAB) serve to protect the GIT microbiota through bacterial antagonism, bacterial interference, barrier effect, competitive exclusion, and colonization resistance (70). LAB are not only beneficial in protecting the bird from pathogens but also provides the bird with physical enhancements to the GIT. These enhancements include strengthening the gut wall integrity, enhance anti-inflammatory response, and correct dysbacteriosis (29). With all of the benefits that LAB provide to poultry, it is vital to ensure their survival and utilization in poultry.

As both organic acids and LAB are potential alternatives to AGPs, it is imperative to understand the specific effect attributable to each method that can be associated with bird performance and welfare, as well as the interactions they have on one another within the bird GIT.

**TABLE 3 |** Predominant phyla and bacterial population in the chicken gastrointestinal tract[1].

| Gastrointestinal segment | Phylum | Genus |
|---|---|---|
| Crop | Firmicutes | *Lactobacillus* |
| | Actinobacteria | *Bifidobacterium* |
| | Proteobacteria | *Enterobacter* |
| Proventriculus | Firmicutes | *Acetanaerobacterium, Clostridium, Faecalibacterium, Lactobacillus, Megamonas, Peptococcus, Pseudobutyrivibrio, Ruminococcus, Sporobacter, Subdoligranulum* |
| | Fungi | *Candida* |
| Ventriculus | Firmicutes | *Lactobacillus, Enterococcus* |
| Small Intestine | Firmicutes | *Candidatus Arthromitus, Clostridium, Lactobacillus, Ruminococcus* |
| | Proteobacteria | *Enterococcus, Escherichia* |
| Ceca[2] | Bacteroidetes | *Bacteroides* |
| | Proteobacteria | *Bilophila, Escherichia* |
| | Archea | *Methanobrevibacter, Methanobacterium, Methanococcus, Methanopyrus, Methanosphaera,Methanothermobacter, Methanothermus,* |
| Large Intestines | Fimicutes | *Lactobacillus* |
| | Proteobacteria | *Escherichia, others* |

[1] *Data was adapted from Qu et al. (107), Saengkerdsub et al. (108, 109), Gong et al. (110), and Yeoman et al. (111).*

[2] *Taxa of the ceca was constricted to the most pertinent phyla and genera although many more have been described.*

## SCFA AND POTENTIAL APPLICATION OF AVIAN MICROBIOME RESEARCH TECHNOLOGIES

Due to current poultry industry practices, prior to hatch, the GIT of chicks are presumed relatively sterile (100). However, immediately after hatch, the chick's microbiome begins to develop as the colonization of the GIT occurs until a diverse and dynamic microbiome is established (101). Previously, research has indicated that on day 0 (post-hatch) of age the cecal microbiome of broilers consists of 50 genera, whereas, by 42 days of age the cecal diversity is increased to over 200 genera (102). Additionally, shortly after post-hatch, the chick's nutrient source is shifted from the yolk to the carbohydrate- and protein-based diet (103, 104). The shift in the nutrient source is accompanied by the rapid development on the GIT and associated organs which can be directly affected by the gut microbiome (105, 106) Thus, it is imperative to alter the GIT microbiome at an earlier age, before what would be considered the adult diverse microbiome becomes stabilized.

Currently, the application of next-generation sequencing technologies to delineate the gut microbiome of poultry is becoming more routine and this, in turn, has resulted in an enhanced understanding of how bacteria of the GIT may influence the development and performance of poultry. The prominent phylum in the crop, gizzard, small intestines, and ceca is the bacterial phylum Firmicutes [**Table 3**; (107, 112, 113)]. The proportion of Firmicutes, primarily Lactobacilli, has been reported to be >90% in the GIT (112, 114). Thus, the microbiome of the small intestines consists mainly of *Lactobacillus*, *Enterococcus*, and *Clostridiaceae* species (97, 112, 115–118). However, the greatest diversity and quantity of bacteria is located within the ceca, where microbial fermentation is also the most active (112). The ceca are characterized by possessing a high proportion of Firmicutes, 50–90% of all taxa (107, 113). The predominant phyla in the ceca have been reported as Bacteroidetes (23–46 %), Proteobacteria (1–16 %), and Archaea (0.81 %) (107–110).

The GIT microbiome has a fundamental role in the production of SCFA (119). The ceca, especially, generate SCFA through various fermentation pathways and may recover up to 10% of energy available in the diet (120, 121). In the ceca, a vast majority of the Families within the phylum Firmicutes belongs are members of the Clostridiales, a significant component of SCFA metabolism (107, 122). In the ceca, SCFA production is derived from the hydrolysis and fermentation of non-starch polysaccharides [NSP; (123)].

The bacterial fermentation of NSPs in the ceca has been reported to consist primarily of acetic acid, propionic acid,

and butyric acid (124). Sergeant et al. identified fermentation pathways encoded in the cecal metagenome that are responsible for the production of acetate, propionate, and butyrate (125). The authors identified gene clusters, encoding enzymes methylmalonyl-CoA epimerase and methylmalonyl-CoA decarboxylase, from Bacteroidetes and Firmicutes to be involved in the production of propionate in the chicken ceca (125). Further, Sergeant et al. speculated the involvement of *Megamonas* and *Dialister* in a novel propionate fermentation pathway (125). The butyrate fermentation pathway is encoded in the BCD/ETF complex and phosphotransbutylase/butyrate kinase genes of butyrate-producing bacteria and sequences are reported to be from Bacteroidetes (125).

There is limited research investigating the effect organic acids have on the GIT microbiome of poultry. Early research demonstrated the negative correlation between Enterobacteriaceae and organic acids such as acetate, butyrate, and propionate in the ceca of broilers (126). More recently, Oakley et al. supplied organic acids as a dietary feed additive (propionic acid and MCFA), in the water supply (formic acid, propionic acid, ammonium formate, MCFAs, an emulsifier, and, propylene glycol), or a combination of the two and examined the subsequent change in the cecal microbiome of Ross × Cobb male broilers over a 42-d period (102). Oakley et al. reported that treatment had little to no effect on the cecal microbiome (102). Instead, the authors demonstrated that the drastic changes in the cecal microbiome occurred as a function of bird age (102) which agrees with the increase in cecal microbiome diversity with age observed by others (127–130). Furthermore, Oakley et al. identified the cecal microbiota to primarily consist of *Flavonifractor*, *Pseudoflavonifractor*, and *Lachnospiracea* on d 7, *Faecalibacterium* (23–55 % of sequences) on d 21, and *Faecalibacterium* and *Roseburia* on d 42 (102). Also, on d 42, *Lachnospiracea incertae sedis* and *Oscillibacter* were recorded as being abundant (102). Some members of *Lachnospiracea incertae sedis* and *Oscillibacter* have been identified as SCFA producers (131, 132). In another study, the dietary supplementation of a microencapsulated feed additive consisting of a phenolic essential oil, thymol, and an organic acid, sorbic acid, resulted in the decrease of *Campylobacter jejuni* and a reduction in the abundance of *Streptococcus* in Ross 308 broilers inoculated with $10^4$ CFU of *C. jejuni* (A2008a and G2008b) (133).

Although the microbial diversity in the ceca increases with age (102, 127–130), it has been demonstrated that organic acids reach their highest concentrations in the GIT of broilers on d 15 (126). As mentioned previously, SCFA production can be enhanced with the dietary supplementation of prebiotics (83–85). The increase in SCFA production is a consequence of the increased colonization of LAB species within the GIT. Birds fed diets containing FOS have been reported to alter the microbiome by enhancing the production of *Bifidobacterium* and *Lactobacillus* which in return enhance digestive enzyme activity and suppress pathogens such as *E. coli* (83). Therefore, by increasing the population of LAB or by supplementing diets with SCFA, the concentration of SCFA in the GIT may be enhanced beyond the peak experienced on day 15.

Future studies should be aimed at evaluating the potential of novel feed additives, organic acids, probiotics, prebiotics, on their potential to change the concentration of specific microbiome populations that may enhance or hinder the performance of poultry. More specifically, studies should focus on the change of *Enterobacteriaceae*. A negative correlation between the concentration of SCFAs such as acetate, propionate, and butyrate and the concentration of *Enterobacteriaceae* has been observed in the ceca of broilers (133). Furthermore, the ratio of Firmicutes to Bacteroides has been identified as a potentially significant index due to its possible correlation with performance. In mice, it has been reported that an increase in Bacteroidetes has been linked to a decrease in nutrient absorption, while the increase in Firmicutes has resulted in an increase in nutrient absorption (134). Another population for monitoring is *Lactobacillus* spp. as their presence in the lower small intestines has been associated with reduced performance (135).

## CONCLUSIONS

As the poultry industry is faced with increased demand for ABF, an alternative to antibiotics needs to be identified that enhances the GIT microbiome of poultry. It is also crucial that this alternative is easily integrated into nutrition, genetics, housing, and veterinarian care for future application. Thus, it is imperative for research to be conducted to determine the most effective method in reducing pathogenic bacteria in the gut, improving broiler performance, and improving gut morphology. To accomplish this will require the application of methodologies that increase the understanding of the avian GIT microbiota. Indeed, the availability of microbiome sequencing offers opportunities to characterize the poultry GIT microbial community in response to organic acids. However, it will be essential to profile GIT populations along the entire GIT from crop to ceca to get a better understanding of where organic acids are eliciting their effects and how this influences bird performance and control of pathogen colonization in the GIT. Once more becomes understood, it should be possible to develop more precisely targeted strategies for employing organic acids as feed additives and eventually optimizing multiple hurdle combinations of probiotics and organic acid combinations.

## AUTHOR CONTRIBUTIONS

DD contributed thoughts and wrote the review. AK contributed financial support of the graduate stipend of the first author, DD, and supervised the development and revision of this review with the assistance of SR.

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# Development of the Tonsil Microbiome in Pigs and Effects of Stress on the Microbiome

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Tonsils, lympho-epithelial tissues located at the junction of the oropharynx and nasopharynx, play a key role in surveillance, colonization, and persistence of inhaled and ingested pathogens. In pigs, the tonsils are a reservoir for numerous bacteria and viruses, including host-specific pathogens and potential zoonotic pathogens as well as commensal organisms. However, there are no in depth studies of the development of the tonsillar microbiome in pigs, or any mammal, over time. The goal of this study was to follow the development of the tonsil microbiome in healthy pigs from birth to market weight. Samples were collected using tonsil brushes from 16 piglets (4 each from 4 sows) at newborn, 1, 2, 3, and 4 weeks of age, and from 8 of those piglets at 6, 8, 10, 12, 16, and 19 weeks of age. Bacterial DNA was isolated from each sample and 16S rDNA genes were amplified and sequenced. Sequence analysis showed that members of the *Streptococcaceae*, *Pasteurellaceae,* and *Moraxellaceae* were present at all time points and represent the three most abundant families identified. Other community members appeared transiently or increased or decreased significantly with disruption events or stress. We observed four significant shifts in the tonsil community that coincided with well-defined disruption events: weaning plus addition of Carbadox plus movement to the nursery at week 3, removal of Carbadox and addition of Tylan at week 5, removal of Tylan and habitat change at week 9, and habitat change at week 16. Weaning triggered a bloom of *Streptococcaeae* and decrease of *Moraxellaceae*. The shift from Carbadox to Tylan led to reduction in *Proteobacteri*a and *Streptococcaceae* but an increase in other *Firmicutes*, accompanied by a dramatic increase in community richness. Cessation of Tylan coincided with a return to a less rich community, and a bloom in *Clostridiales*. The final shift in habitat was accompanied by a decrease in *Clostridiales* and increase in *Proteobacteria.* The tonsillar microbiome of older pigs resembled the previously described mature core tonsillar microbiome. This study demonstrates a temporal succession in the development of the pig tonsillar microbiome, and significant community shifts that correlate with disruption events.

**Keywords: microbiome, tonsil, pig, development, stress**

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## INTRODUCTION

Numerous bacteria and viruses can access the host using the oropharynx and nasopharynx as portals of entrance. One of the first lympho-epithelial tissues these organisms encounter is the tonsils, located at the junction of these two major portals of entry. Tonsils play a key role in immunologic surveillance of pathogens that access the host via these routes and in the initial process of pathogen-host colonization (1). Tonsils frequently serve as a reservoir of host-specific pathogens as well as zoonotic pathogens highly transmissible to humans, such as *Streptococcus suis, Salmonella enterica*, and swine influenza virus (2). Multiple pathogenic microorganisms, including bacteria and viruses, are regularly isolated from tonsils of asymptomatic animals. Pathogens residing in the tonsils can spread systemically or be transmitted to other animals including humans, with such transmission often triggered by stressful conditions such as transport (3). The resident tonsillar microbiome likely interacts with incoming pathogens, inhibiting colonization via competitive exclusion (4–7) as well as functioning to regulate immune homeostasis that is critical in providing resistance to infection (8, 9).

There are only limited numbers of studies addressing the tonsillar microbiome in humans or pig (10–17), in contrast to the growing number of studies on the intestinal microbiome in different species. Studies have suggested a gradual and sequential process in the development of the intestinal microbiome in humans and animals (18–21), where certain taxa persisted and became stable while others were acquired over time or only transiently. Further, microbiome development has been suggested to be based on specific bacterial interactions and not on random assembly of microorganisms (18). Intestinal microbial communities tended to achieve an adult-like profile as time progressed (18, 20). This trend was seen despite the fact that during development there were significant shifts in the structure of the population (20) as well as in the diversity (21), and many of these shifts were associated with life events or stresses such as diet changes and antibiotic treatment (18).

It has been demonstrated that common management practices such as the use of antibiotic treatments can significantly affect microbial communities and predispose the host to infections (22). However, the microbiota also can be shifted toward a microbial community that would protect the host from potential infections, as in the case of altering the intestinal microbiota through fecal microbiota transplantation (6). Despite the relevant role that the microbiota can play in maintaining good health status in the host and the key role played by the tonsils in both the respiratory and gastrointestinal tracts, there is a lack of knowledge about the development of tonsillar microbial communities of pigs or of the effects of stresses such as diet changes or antibiotic usage on the development, composition, and diversity of these communities.

Two studies have described the normal tonsillar microbiome in finishing (13, 14) and a third has described the metabolically active microbiome of slaughter pigs (15). The core tonsil microbiome in 18–20 week old grower-finisher pigs was comprised of members of the families *Pasteurellaceae, Moraxellaceae, Streptococcaceae, Fusobacteriaceae*, *Veillonellaceae, Enterobacteriaceae, Neisseriaceae,* and *Peptostreptococcaceae,* as well as the order *Clostridiales* (14). Whether a successional process is involved in how this core microbial community in the adult tonsils is established and develops over time and what role it plays in the acquisition and carriage of pathogens and thus in host health and disease is not known at this time.

We recently completed a study of the composition and development of the tonsil microbiome in piglets from birth up to 4 weeks of age (16). The tonsil microbial communities initially clustered by litter, but then converged by 3 weeks of age, regardless of litter or housing. These communities were comprised mainly of microorganisms acquired from the sow vaginal tract and teat skin, with a sequential succession observed over time. The combined stress of weaning, shift in food and housing, and addition of the growth promoter Carbadox® at 3 weeks of age led to a major shift in the microbiome at 4 weeks of age.

The goal of the current study was to extend this study, utilizing high-throughput sequencing of 16S rRNA genes to follow and describe the development of the tonsillar microbial communities in pigs through market age. This characterization of the development of the swine tonsillar microbiome lays a base for future studies that judiciously manipulates this microbiome to reduce pathogen load and improve overall animal health.

## MATERIALS AND METHODS

### Animals

This study and all animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee. The pigs used in this study were from a high health status farrow-to-finish herd with ∼200 sows, housed at the Michigan State University Swine Teaching and Research Center. Relevant medical history for this herd included no recent respiratory disease; freedom from *Actinobacillus pleuropneumoniae, Mycoplasma hyopneumoniae*, and porcine respiratory and reproductive syndrome virus (PRRSV); a recent outbreak of porcine epidemic diarrhea virus (PEDV), under control prior to this study; and routine vaccination against erysipelas (*Erysipelas rhusiopathiae* bacterin ER Bac Plus, Zoetis Inc, Kalamazoo MI, administered intramuscularly) and porcine circovirus type 2 (Porcine Circovirus Vaccine, type 2, killed baculovirus vector, Ingelvac Circoflex, Boehringer Ingelheim Vetmedica, Inc, St. Joseph MO, administered intramuscularly). Four crossbred sows (Yorkshire X Hampshire) of different parity (number of pregnancies) were selected for this study and included sow 1700 (first parity), sow 1631 (second parity), sow 1445 (fifth parity) and sow 1711 (tenth parity). Four randomly selected piglets from each of the four sows were sampled within a period no longer than 8 h after birth (newborn) and the same piglets were sampled subsequently at 1, 2, 3, 4, 6, 8, 10, 12, 16, and 19 weeks of age.

Newborn piglets received a single intramuscular injection of Iron-Dextran (100 mg ANEM-X 100, Aspen Veterinary Resources, Ltd, Liberty MO) during their first week of life.

Between the third and fourth weeks of age (21–24 days—average weight 18 pounds) piglets were weaned, vaccinated, and moved from the farrowing room where they were housed with the sow and littermates to a nursery room, with litters maintained as pen mates. At this time the piglets were weaned from milk to a solid pellet ration diet (Pig 1300®, Akey Nutrition, Brookville OH) supplemented with Carbadox® at a dose of 50 g/ton. Two weeks after being moved to the nursery facility (at 5 weeks of age), Carbadox® supplementation was removed from the feed, and food was changed to a ground ration supplemented with Tylan® at a dose of 100 g/ton. At ∼9 weeks of age (63–67 days—average weight 60 pounds), piglets were moved to a finishing room and were assigned to different pens based on criteria such as gender and weight; separation by litter was no longer maintained. At this time, Tylan® supplementation was discontinued and a ground ration without supplementation was provided. Finally, at ∼eighteen weeks of age, piglets were moved again to another finishing room (with another mixing of prior penmates) where they remained until being moved to the slaughterhouse (average weight 240 pounds). These management practices are summarized in **Figure 1**.

A total of 128 pig tonsil microbiome samples from pigs at birth through market age were sequenced and analyzed. Of these, 64 samples were collected from piglets before weaning (newborn—third week), which included samples from 16 piglets (4 per sow) at each time point. In addition, 64 samples were collected from pigs after weaning, which included samples from 16 piglets at week 4 and 8 piglets (2 per sow) at all subsequent time points (**Table 1**). In a previous study (16), we found a strong litter effect on the tonsil microbiome that disappeared by 3 weeks of age; therefore, we analyzed 4 pigs per litter initially but reduced this number to 2 pigs per litter (8 in total) for subsequent sampling times.

## Collection of Microbiome Samples

Tonsil brushes developed by our group and validated in previous studies (14) were used to collect tonsil microbiome samples from sows and larger piglets, while Cytosoft™ cytology brushes (Medical Packaging Corporation, Camarillo, CA) were used for smaller piglets (16). Collection and storage of samples was as previously described (14).

### Isolation of Community DNA

Extraction of community DNA from samples was performed using a PowerSoil DNA Isolation Kit and PowerBead tubes (MoBio Laboratories, Carlsbad, CA) as previously described (13, 14, 16).

### Illumina Sequencing and Sequence Analysis

Sequencing was performed at the MSU Research Technology Support Facility (RTSF) as previously described (16). Negative controls consisting of DNA-free water or MoBio C6 reagent were used as "blank library controls" (16) and included in each sequencing run. Briefly, uniquely indexed primers were used to amplify the V4 region of the 16S rRNA gene from the community DNA, as described by Caporaso (23). A SequalPrep normalization plate (Invitrogen) was used to normalize the amplification products, which were then pooled and the reaction cleaned using AMPure XP beads. The pooled sample was sequenced on an Illumina MiSeq v2 flow cell using a 500 cycle v2 reagent kit (PE250 reads). Base calling was performed using Illumina Real Time Analysis Software (RTA) v1.18.54 and output of RTA demultiplexed and converted to FastQ files using Illumina Bcl2fastq v1.8.4.

The open-source, platform-independent, community-supported software program mothur v.1.35.0 (http://www.mothur.org) (24) was used for amplicon analysis. Raw sequencing data was processed according to the mothur standard operating procedure (http://www.mothur.org/wiki/MiSeq_SOP) (25) and aligned using the mothur-formatted version 123 of Silva 16S ribosomal gene database (26). After sequences were classified, all sequences classified as Chloroplast, Mitochondria, unknown, Archaea, or Eukaryota were removed from the data set. Subsampling at 7000 sequences per sample was done, followed by a preclustering of the sequences and removal of chimeric sequences using a mothur formatted version of the Ribosomal Database Project (RDP) training set version 14 and uchime, based on mothur protocol. A cutoff of ≥97% sequence identity was used to classify sequences into Operational Taxonomic Units (OTUs). Singleton and doubleton reads were removed before the final analysis. For the final analysis of the data, samples were subsampled to 5179 reads per sample. The full data set analyzed and the mother code used are available as a supplemental file at https://figshare.com/s/7147a352573045d7cf5c.

The samples for piglets from birth through 4 weeks of age were a subset of a larger set of samples used in a prior study (16).

## Diversity and Statistical Analysis

A clustering cutoff of 3% for the processed sequences was used in the statistical analysis. Mothur output files were used to estimate alpha diversity (sobs) and beta diversity indexes, as well as representative sequences, all of which were calculated in mothur v.1.35.0 (http://www.mothur.org) (24). PAST3 (Version 3.14; http://folk.uio.no/ohammer/past/) was used for statistical analysis of the samples. FigTree (Version 1.4.3; http://tree.bio.ed.ac.uk/software/figtree/) was used for construction of dendrogram figures. The area of the ellipses for the two dimensional scatter plot was measured using ImageJ (27). RStudio (Version 0.99.446; https://www.rstudio.com/) and libraries: gplots (https://CRAN.R-project.org/package=gplots) were used to generate heatmaps. Inkscape 0.91 (https://inkscape.org/en/download/mac-os/), was used to process images and edit labels. Taxonomy tables and OTU plots were generated in Microsoft® Excel® 2011, where the analysis of samples was done with data that represented higher than 0.1% of the total reads for the samples analyzed.

## Availability of Supporting Data

Raw sequence data and metadata is available at NCBI database (SRA accession number: SRP144702). Reviewer / collaborator link to metadata, valid through 8-8-18: ftp://ftp-trace.ncbi.nlm.nih.gov/sra/review/SRP144702_20180508_081000_cb5ae17636e975f9bf71ddf5bc542075.

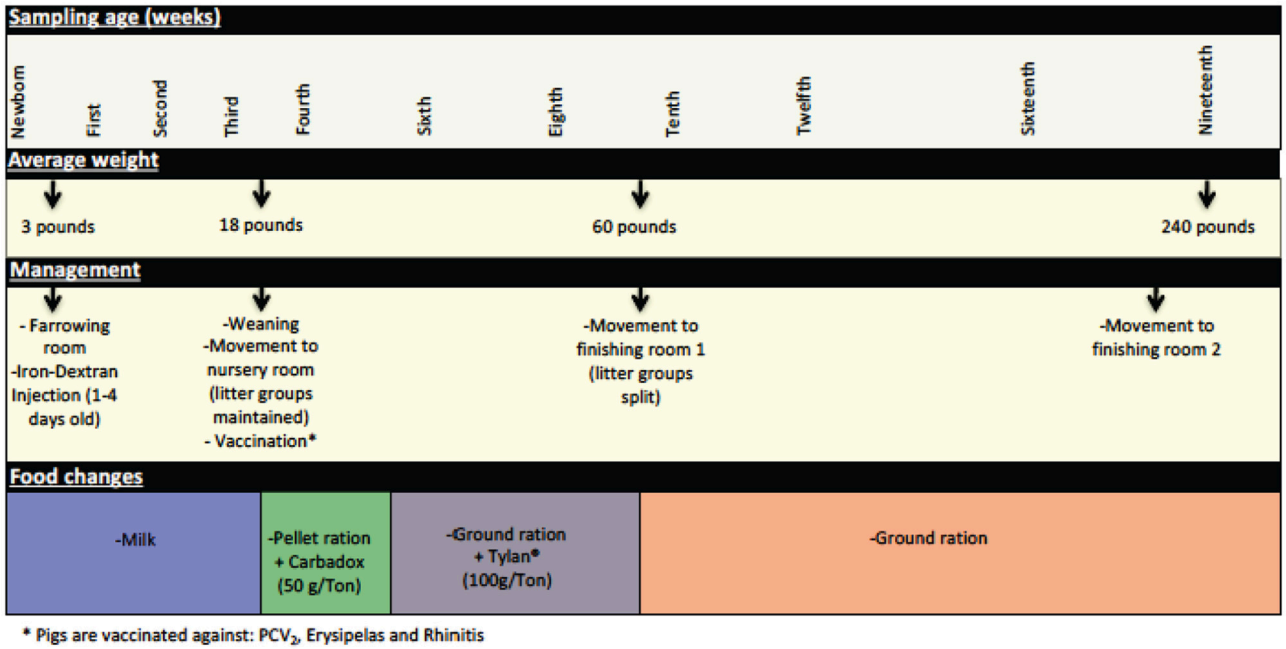


**FIGURE 1 |** Significant management practices at the swine farm during the life of the pigs in this study. General management features experienced by the pigs during their life at the farm are depicted here, including changes in feed, use of in feed antibiotics, and movement to new housing.

**TABLE 1 |** Samples processed by sampling time and litter.

| | Sow ID | 1700 | 1631 | 1445 | 1711 |
|---|---|---|---|---|---|
| | No. sow parity | 1 | 2 | 5 | 10 |
| | Litter members | 10,11, 12,13 | 15,16, 17,18 | 36,39, 40,42 | 22,23, 24,26 |
| ID | Sampling time | Number of samples analyzed per sampling time | | | |
| A | Newborns | 4 | 4 | 4 | 4 |
| B | First week | 4 | 4 | 4 | 4 |
| C | Second week | 4 | 4 | 4 | 4 |
| D | Third week | 4 | 4 | 4 | 4 |
| Total samples before weaning | | 16 | 16 | 16 | 16 |
| E | Fourth week | 4 | 4 | 4 | 4 |
| F | Sixth week | 2 | 2 | 2 | 2 |
| G | Eighth week | 2 | 2 | 2 | 2 |
| H | Tenth week | 2 | 2 | 2 | 2 |
| I | Twelfth week | 2 | 2 | 2 | 2 |
| J | Sixteenth week | 2 | 2 | 2 | 2 |
| K | Nineteenth week | 2 | 2 | 2 | 2 |
| Total samples after weaning | | 16 | 16 | 16 | 16 |

# RESULTS

## Management Practices Are Related With Changes in Population Diversity

A total of 128 tonsil samples for microbiome analysis were collected at 11 time points during the life of the pigs in this study. Some of the sampling times were chosen specifically to represent times associated with management practices significant in the life of the pigs, including immediately prior to and after weaning, alteration in feed and in-feed growth promoters, and movement to new rooms (**Figure 1**).

Analysis of the alpha diversity (species richness at a specific site) of the tonsil microbiome, as measured by the total number of species observed (sobs), and the relation with the different changes experienced by the pigs during their life showed that the alpha diversity varied widely (**Table 2**). For newborn piglets, the average value of sobs was 110. The average sobs value decreased steadily in the following weeks (first to third week), dropping to a value of 83. This was accompanied by a marked decrease in the standard deviation to 24, indicating that the microbiome became very similar in all pigs by 3 weeks of age. In contrast, from week 4 to 10 there was a substantial increase in diversity that coincided with specific challenging events experienced by the piglets. Between the third and fourth week, the piglets were weaned, and at the same time they were moved to a nursery room, vaccinated and their diet was changed. These changes were reflected in a slight increase in the average and maximum sobs as well as the standard deviation. However, the biggest change in diversity occurred during the period where Carbadox® was removed from the diet and Tylan® was supplemented. Diversity increased to over three times the previous registered values for average sobs. Conversely, the removal of Tylan®, accompanied by the transfer of pigs to a finishing room where they were no longer segregated by litter, led to a trend of decreasing diversity. By week 19, this progressive decrease in the diversity led to a value of average sobs of 108. Overall, there was a pattern demonstrating

**TABLE 2 |** Number of observed OTUs[a] (sobs[b]) during the different sampling times.

| Sampling time | No. Samples analyzed | Average sobs | Min sobs[c] | Max sobs[d] | Standard deviation sobs |
|---|---|---|---|---|---|
| Newborn | 16 | 110 | 26 | 376 | 100.6 |
| First week | 16 | 104 | 37 | 223 | 58.1 |
| Second week | 16 | 107 | 49 | 242 | 59.0 |
| Third week | 16 | 83 | 57 | 132 | 23.8 |
| Weaned, Moved to nursery, Vaccinated, Carbadox supplementation. | | | | | |
| Fourth week | 16 | 111 | 53 | 175 | 38.7 |
| Carbodox removed, Tylan supplementation added | | | | | |
| Sixth week | 8 | 368 | 215 | 434 | 69.8 |
| Eighth week | 8 | 355 | 276 | 408 | 46.5 |
| Tylan removed, Moved to finishing room 1, litters split | | | | | |
| Tenth week | 8 | 257 | 123 | 377 | 96.6 |
| Twelfth week | 8 | 139 | 53 | 234 | 64.1 |
| Sixteenth week | 8 | 132 | 83 | 177 | 35.7 |
| Moved to finishing room 2 | | | | | |
| Nineteenth week | 8 | 108 | 73 | 178 | 35.2 |

[a] *OTU,Operational Taxonomic Unit.*
[b] *sobs,total number of species observed, measured as the number of observed OTUs.*
[c] *Min sobs, Minimum number of observed sobs.*
[d] *Max sobs, Maximum number of observed sobs.*

that extended time under constant conditions led to fewer sobs and reduced standard deviation.

## Challenging Management Conditions During Development of the Pigs Generated Disruption in the Microbiome

We wondered if the development of the tonsillar microbiome followed a temporally dependent successional pathway and to what degree, if any, dietary antibiotics, and management pratices influenced the microbiome. An unrooted dendrogram based on a Bray-Curtis analysis (**Figure 2**) shows the clustering of the pig tonsillar microbiome samples from newborn through the nineteenth week. Samples from newborn piglets were mainly distributed in two groups, one corresponding to pigs from a first parity sow (4/16; 25%) and the other from pigs of multiparous sows (10/16; 62.5%), the remaining two samples were clustered with microbiome samples of older pigs. At 1 week of age, the microbiome samples were clustered by litter in four different groups.

The following weeks showed that as pigs aged, their tonsil microbiomes tended to become more similar. During the second week, the samples clustered together in three related groups. In the third week, all sixteen samples clustered together in one group, which also included ten of the week 2 samples. The fourth week, which marked a transitional time after a challenge, i.e., weaning plus movement to new housing plus addition of Carbadox® to the new solid feed, showed a split of the previously tightly clustered samples into four separate groups, which were not clustered by litter. The sixth week, again marked by a transition after a challenge, i.e., removal of Carbadox® and addition of Tylan® to the feed, again showed samples clustered in four separate groups, which were neither clustered by litter nor the same, with one exception, as the groups for the fourth week samples. However, for the 8 week, samples clustered in only two groups. Once again, in the tenth week, which marked the transition after a challenging condition, i.e., removal of Tylan® from feed and reassignment to new finishing rooms with litter groups broken up, showed a major disruption in the clustering pattern with samples falling into six different groups. The sampling times corresponding to weeks 12 and 16, a time of stability for the piglets, once again showed coalescing of the microbiota phylogenetic compostion; the twelfth week samples clustered into three groups, while the sixteenth week samples all clustered into a single group. Finally, for the last sampling period corresponding to the nineteenth week, also a transitional time after a challenge, i.e., movement to new finishing rooms with another re-assortment of the piglets, the samples once again showed a split into three different groups. The clustering pattern also showed that as the pigs aged, most samples clustered with tonsillar samples from sows, despite no longer having contact with the sows. Based on the above analysis we identified three sampling times (third, eighth and sixteenth weeks), which were immediately before a challenging condition, where the microbiome tended to be more similar between pigs. Statistical support for this clustering pattern is shown in an unrooted dendrogram based on Bray-Curtis analysis (**Figure 3**), where samples derived from the third, eighth and sixteenth weeks formed three distinct groups which were supported by bootstrap values higher than 70.

We also analyzed the clustering shown in **Figure 2** to determine whether there were effects of litter or of pen on the clustering. Samples from newborn and 1 week old pigs clustered by litter, but older animals did not. We saw no correlation of the clustering with groups of piglets in the same pens except as related to the litter effect seen in newborn and 1 week old animals.

## Tonsil Microbiome Membership Throughout the Life of the Pigs

To visualize how the membership of the tonsillar microbiome changes through the life of the pigs, we plotted the proportion of the 20 most commonly identified bacterial families in piglets at each sampling time, as well as in sows (**Figure 4**). Members of the phyla *Actinobacteria* (Family *Micrococcaceae*), *Bacteroidetes* (Families *Bacteroidaceae*, *Porphyromonadaceae*, *Prevotellaceae*, *Flavobacteriaceae*), *Firmicutes* (Families *Bacillaceae 1*, *Staphylococcaceae*, *Streptococcaceae*, *Clostridiaceae 1*, *Clostridiales Incertae Sedis XI*, *Lachnospiraceae*, *Peptostreptococcaceae*, *Ruminococcaceae*, *Erysipelotrichaceae*, *Veillonellaceae*), *Fusobacteria* (Family *Fusobacteriaceae*), and *Proteobacteria (*Families *Burkholderiaceae*, *Neisseriaceae*, *Pasteurellaceae,* and *Moraxellaceae*) were identified as the most abundant bacterial phyla and families in pig tonsils. The distribution and proportions of these bacterial families

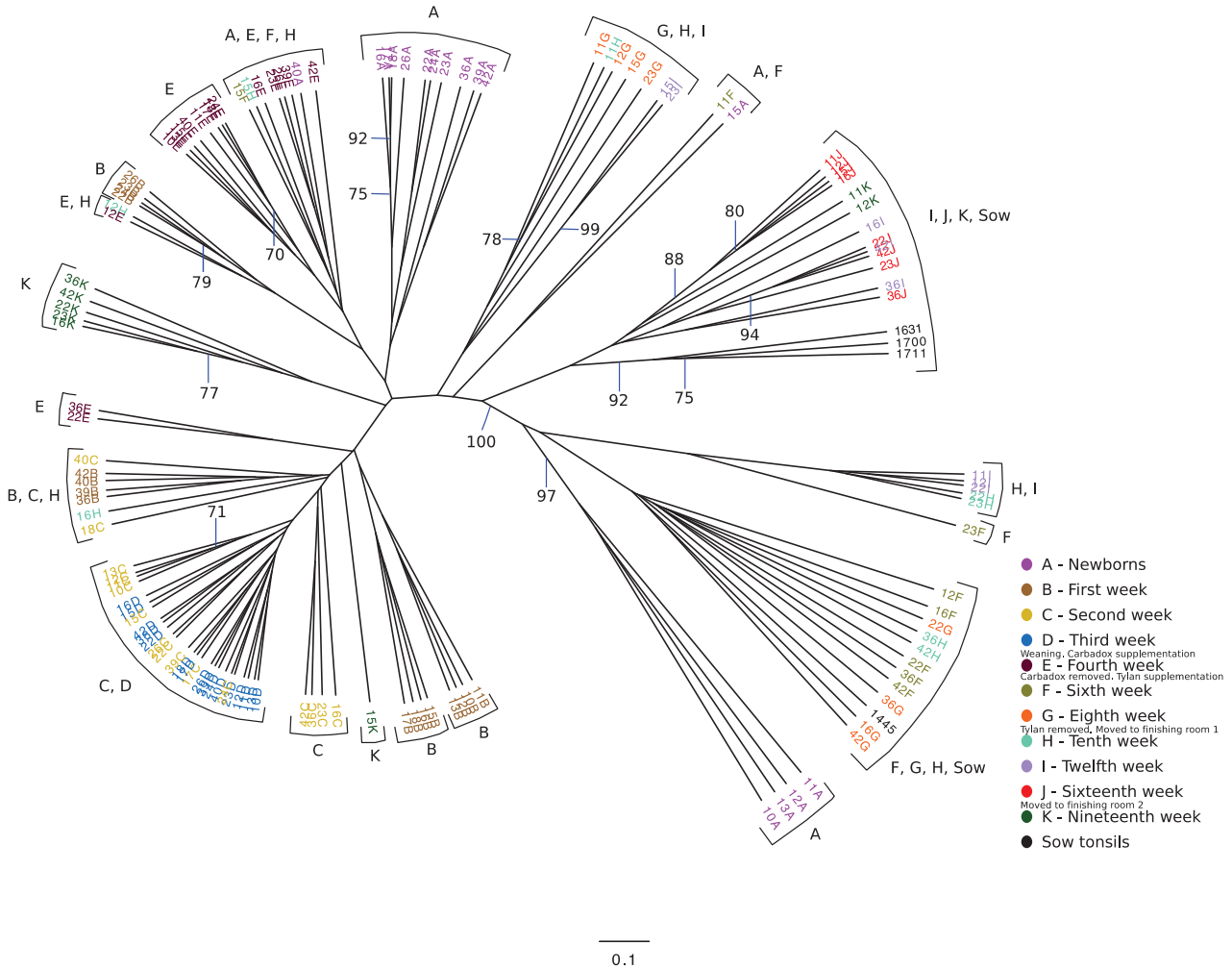


**FIGURE 2 |** Unrooted Bray-Curtis dendrogram for all sampled weeks. The dendrogram shows the clustering of the samples collected from pigs from newborn through 19 weeks, as well as sow tonsillar samples. Samples are color coded by week of sampling. Small legends indicate some of the challenges that took place in specific times.

fluctuated through the sampling period (**Table 3**), with the largest shifts related with challenging conditions experienced by the pigs. Three families that consistently represented a major portion of the tonsil microbiome across all time points were the *Streptococcaceae, Pasteurellaceae,* and *Moraxellaceae*.

The microbiome of newborns was characterized by the abundant presence of the families *Streptococcaceae*, *Moraxellaceae*, *Staphylococcaceae*, and *Micrococcaceae*, each representing 10 to 23% of the total; members of families *Pasteurellaceae*, *Burkholderiaceae,* and *Bacillaceae* as well as members of the order *Clostridiales* were identified in smaller proportions. In the first week, *Pasteurellaceae* and *Porphyromonadaceae* increased dramatically, to 25 and 8.1%, respectively. *Moraxellaceae* also increased slightly, while there was a slight decrease in *Streptococacceae.* A more dramatic decrease was evident for *Staphylococcaceae,* which almost disappeared, and *Micrococcaceae*. Over the next 2 weeks, members of the *Streptococcaceae* continued to decrease, and *Micrococcaceae* virtually disappeared. In contrast, members of *Moraxellaceae* continued to increase. Members of *Pasteurellaceae* remained constant. *Fusobacteriaceae* appeared in week 2 and remained present in week 3. Multiple members of the order *Clostridiales* (*Clostridiaceae 1*, *Clostridiales Incertae Sedis XI*, *Lachnospiraceae*, *Peptostreptococcaceae,* and *Ruminococcaceae*) were present in proportions lower than 1%, each, throughout the first 3 weeks of life in these piglets.

The transition between the third and fourth weeks, when the piglets were weaned, moved to new housing, and shifted to solid food containing Carbadox®, was marked by drastic shifts in the tonsil microbiome. *Moraxellaceae* decreased dramatically from 31.2% in week 3 to 7.9% in week 4, *Streptococcaceae* bloomed from 7.4 to 41.6%, while *Pasteurellaceae* and *Clostridiales* remained steady. Members of *Fusobacteriaceae* and *Porphyromonadaceae* almost disappeared.

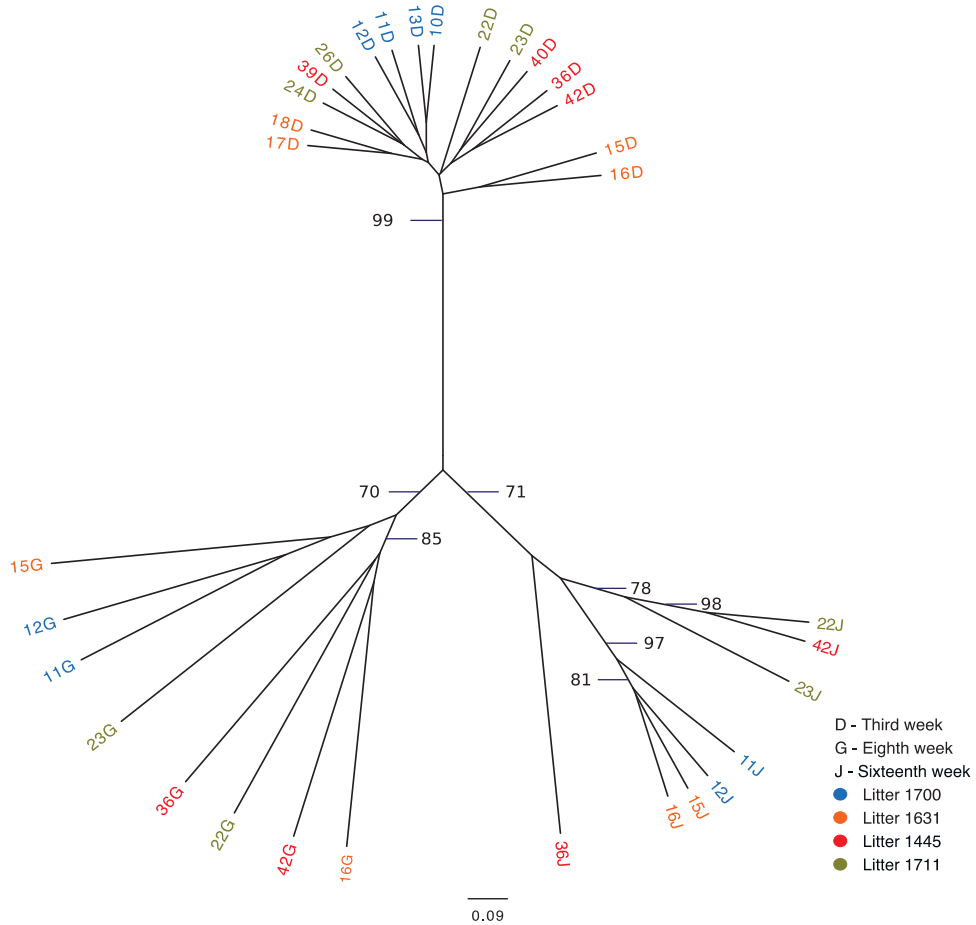


**FIGURE 3 |** Unrooted Bray-Curtis dendrogram for three pre-transition times. The dendrogram shows the clustering of tonsil microbiome samples from pigs at three times immediately before challenging events: week 3, week 8, and week 16. Samples are color coded by week of sampling. Bootstrap values higher than 70 are shown.

Week 6, after another major transition when Carbadox® was removed from feed and Tylan® added, was again marked by drastic shifts in the tonsil microbiome. Overall sobs, as described above, increased from 111 to 368 (**Table 2**), indicating a massive increase in diversity. Members of the *Streptococcaceae* and *Pasteurellaceae* both decreased dramatically, from 41.6 to 11.6% and 23.2 to 10%, respectively, and *Moraxellaceae* decreased and almost disappeared. However, members of *Bacillaceae 1* and some members of the order *Clostridiales* (*Clostridiales Incertae Sedis XI*, *Lachnospiraceae,* and *Ruminococcaceae*) began to flourish and increased substantially, particularly *Bacillaceae* 1 which increased from 0.9 to 13.1%. Interestingly, almost 44% of the members of the tonsillar microbiome did not fit into these twenty most abundant bacterial families for this time point, again indicative of an overall increase in diversity.

In the eighth week, the decreasing trend for *Streptococcaceae* and *Pasteurellaceae* continued and each family dropped to a relative abundance of 7%. *Moraxellaceae* remained in very low abundance. However, anaerobic organisms including *Clostridiales,* particularly *Clostridiaceae 1*, and *Bacteroidales* increased. The proportion of identified bacterial families that were not included in the twenty most abundant was still close to 40%.

The tenth week, which corresponded to another significant transition period for the pigs, i.e., removal of Tylan® from feed as well as movement to finishing rooms and reassortment of litter members, was again marked by a major shift in the microbiome. The three predominant families, *Pasteurellaceae, Streptococcaceae,* and *Moraxellaceae,* all increased, particularly the *Pasteurellaceae* that increased from 7% to 30.7%. In contrast, members of the *Clostridiales* (*Clostridiaceae 1*, *Clostridiales Incertae Sedis XI*, *Lachnospiraceae,* and *Ruminococcaceae*) and *Prevotellaceae* decreased, as did the proportion of the microbiome classified as "Others."

Over the next 6 weeks, represented by sampling times at 12 and 16 weeks, the tonsil phylogenetic structure of the microbiome in all of the pigs coalesced to a common core (**Figure 2**). Overall, there was a massive increase

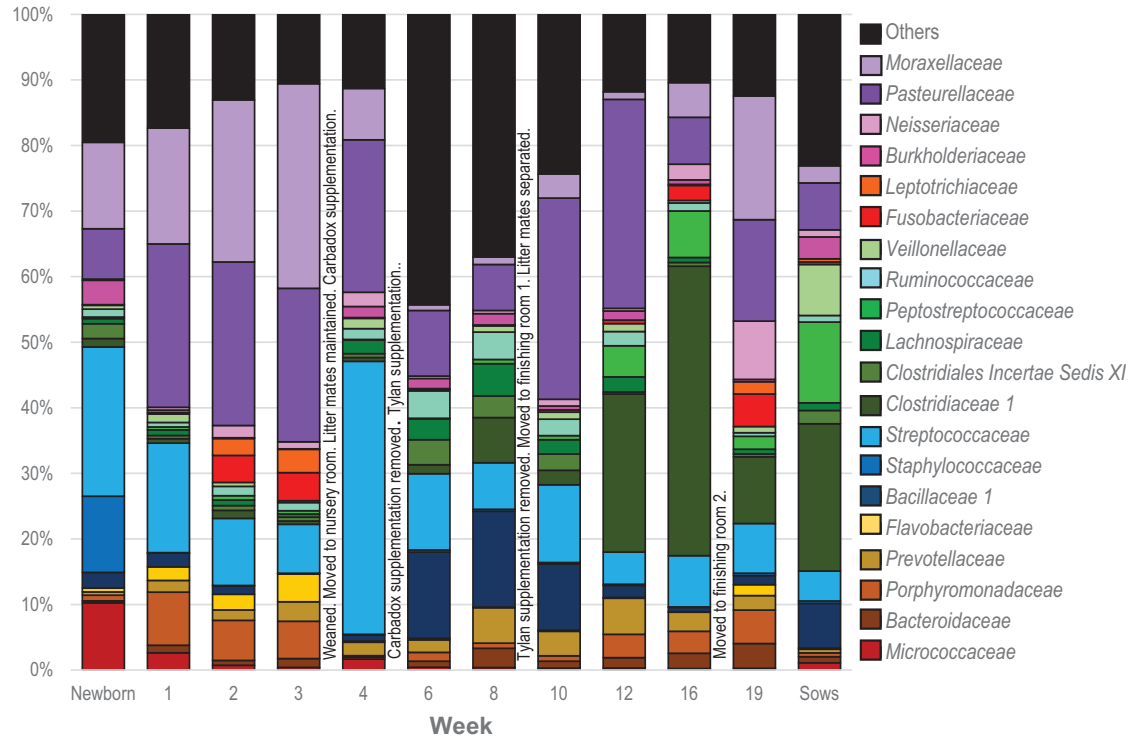


**FIGURE 4 |** Twenty most abundant families identified in the tonsillar microbiome of pigs from newborn to market age and in the sows. Colored bars illustrate the percentage of the total reads classified into specific families at each sampling time and are the average of all animals at each time point. Text boxes in the plot indicate times when a challenging event occurred. "Others" represents members of bacterial families different from the 20 most abundant families identified.

in the *Clostridiales,* particularly *Clostridiaceae 1,* and *Peptostreptococcaceae*, from 2.2 and 0.6% in week 10 to 44.1% and 7%, respectively in week 16. Over the same period, *Pasteurellaceae* decreased from 30.7 to 7.1%, and *Bacillaceae 1* decreased from 10.1% to 0.6%. *Streptococcaceae, Moraxellaceae,* and *Bacteroidales* remained relatively stable. The proportion of identified bacterial families that were not included into the twenty most abundant families decreased to ∼10%. By week 16, *Fusobacteriaceae* and *Neisseriaceae* reappeared in low proportions.

Finally, the nineteenth week, which coincided with a transitional period in which penmates were reassorted into new rooms, was marked by another significant disruption of the microbiome. Overall, an increase in *Pasteurellaceae, Moraxellaceae, Neisseriaceae,* and *Fusobacteriaceae* was paired with a dramatic decrease in *Clostridiales*, particularly *Clostridiaceae 1* and *Peptostreptococacceae*.

It should be noted that, after the first 3 weeks and weaning, these shifts in the microbiome were not synchronous in all piglets. **Figure 2** shows several clusters that contain samples from sequential weeks, e.g., weeks 6, 8 and 10; weeks 8, 10, and 12; and weeks 10, 12, and 16, indicating that common microbiomes, represented by the clusters, were reached at different times in different pigs. This is further illustrated in **Figure 5**, which shows the most abundant microbial families over time in 4 different pigs. As examples, a microbiome with a preponderance of *Pasteurellaceae* is seen in week 10 in pig 23, week 12 in pig 11, both weeks 10 and 12 in pig 22, and not at all in pig 36. A microbiome with a preponderance of *Clostridiaceae*, mainly *Clostridiaceae 1* and *Peptostreptococcaceae*, is seen in weeks 12 and 16 in pig 36, week 16 in pigs 22 and 23, and weeks 16 and 19 in pig 11.

The tonsillar microbiome of sows was dominated by members of *Clostridiaceae 1* (∼23%) and *Peptostreptococcaceae* (∼12%). Other families present in proportions between 1 and 8% included *Erysipelotrichaceae*, *Pasteurellaceae*, *Bacillaceae 1*, *Streptococcaceae*, *Burkholderiaceae*, *Moraxellaceae*, *Neisseriaceae*. *Micrococcaceae,* and other members of the *Clostridiales* (*Clostridiales Incertae Sedis XI*, *Lachnospiraceae,* and *Ruminococcaceae*).

## Distribution of Specific OTUs Throughout the Life of the Pigs

While presentation of the microbiome data at the taxonomic level of family gives the best overview of the data over time, we also examined the presence and abundance of specific OTUs over time (**Figure 6**). At the family level, *Pasteurellaceae*, *Streptococcaceae,* and *Moraxellaceae* predominate throughout the life of the pigs. However, within the top 40 OTUs there were three OTUs of *Pasteurellaceae* seen, including OTU0001, which was present in high concentration during weeks 1–4 but never lost, OTU0016 which appeared in weeks 6 and

**TABLE 3 |** Top 20 most abundant families at each sampling time (% of total).

| | Week | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Family** | **Newborn** | **1** | **2** | **3** | **4** | **6** | **8** | **10** | **12** | **16** | **19** | **Sow** |
| *Corynebacteriaceae* | 2.02 | 0.14 | 0.07 | 0.07 | 0.22 | 0.68 | 0.68 | 0.28 | 0.10 | 0.25 | 0.30 | 1.50 |
| *Micrococcaceae* | 10.25 | 2.63 | 0.74 | 0.42 | 1.68 | 0.43 | 0.38 | 0.29 | 0.30 | 0.23 | 0.26 | 1.11 |
| *Bacteroidaceae* | 0.30 | 1.14 | 0.74 | 1.33 | 0.29 | 0.93 | 2.94 | 1.07 | 1.60 | 2.33 | 3.78 | 0.93 |
| *Porphyromonadaceae* | 0.85 | 8.11 | 6.11 | 5.69 | 0.21 | 1.34 | 0.78 | 0.81 | 3.54 | 3.34 | 5.11 | 0.59 |
| *Prevotellaceae* | 0.51 | 1.79 | 1.58 | 2.97 | 2.05 | 1.90 | 5.40 | 3.75 | 5.52 | 2.92 | 2.21 | 0.55 |
| *Rikenellaceae* | 0.24 | 0.07 | 0.02 | 0.00 | 0.04 | 1.16 | 0.54 | 0.70 | 0.04 | 0.02 | 0.11 | 0.00 |
| *Flavobacteriaceae* | 0.59 | 2.06 | 2.38 | 4.22 | 0.18 | 0.25 | 0.11 | 0.17 | 0.10 | 0.08 | 1.67 | 0.18 |
| *Chitinophagaceae* | 0.17 | 0.01 | 0.00 | 0.01 | 0.04 | 0.15 | 0.10 | 0.14 | 0.56 | 0.28 | 0.04 | 0.11 |
| *Bacillaceae 1* | 2.40 | 2.10 | 1.29 | 0.13 | 0.94 | 13.13 | 14.60 | 10.07 | 1.83 | 0.65 | 1.37 | 6.83 |
| *Bacillales Incertae Sedis XI* | 0.19 | 1.32 | 1.93 | 1.22 | 0.51 | 0.06 | 0.14 | 0.09 | 0.04 | 0.44 | 0.82 | 0.76 |
| *Planococcaceae* | 0.15 | 0.16 | 0.10 | 0.03 | 0.10 | 0.82 | 0.88 | 0.65 | 0.08 | 0.24 | 0.11 | 1.25 |
| *Staphylococcaceae* | 11.60 | 0.07 | 0.06 | 0.03 | 0.08 | 0.34 | 0.30 | 0.25 | 0.19 | 0.08 | 0.36 | 0.35 |
| *Aerococcaceae* | 0.36 | 0.57 | 0.18 | 0.07 | 0.09 | 0.00 | 0.00 | 0.04 | 0.17 | 0.33 | 1.23 | 0.15 |
| *Lactobacillaceae* | 0.16 | 0.60 | 0.31 | 0.03 | 2.25 | 0.55 | 0.21 | 0.19 | 0.23 | 0.22 | 0.06 | 0.08 |
| *Streptococcaceae* | 22.77 | 16.71 | 10.23 | 7.45 | 41.64 | 11.63 | 7.09 | 11.86 | 4.90 | 7.83 | 7.58 | 4.56 |
| *Clostridiaceae 1* | 1.30 | 0.67 | 1.21 | 0.49 | 0.58 | 1.35 | 6.90 | 2.21 | 24.10 | 44.14 | 10.19 | 22.48 |
| *Clostridiales Incertae Sedis XI* | 2.25 | 0.49 | 0.73 | 0.59 | 0.59 | 3.82 | 3.29 | 2.48 | 0.32 | 0.56 | 0.43 | 2.03 |
| *Lachnospiraceae* | 0.78 | 0.87 | 0.85 | 0.49 | 2.06 | 3.21 | 4.92 | 2.18 | 2.30 | 0.77 | 0.72 | 1.17 |
| *Peptostreptococcaceae* | 0.24 | 0.45 | 0.67 | 0.50 | 0.11 | 0.08 | 0.65 | 0.63 | 4.75 | 7.09 | 1.97 | 12.31 |
| *Ruminococcaceae* | 1.21 | 0.67 | 1.41 | 1.24 | 1.64 | 4.18 | 4.19 | 2.52 | 2.17 | 1.21 | 0.53 | 1.00 |
| *Erysipelotrichaceae* | 0.69 | 0.47 | 0.41 | 0.32 | 0.54 | 0.06 | 0.29 | 0.25 | 0.53 | 1.33 | 0.39 | 7.77 |
| *Veillonellaceae* | 0.59 | 1.28 | 0.61 | 0.28 | 1.57 | 0.27 | 0.93 | 1.05 | 1.20 | 0.39 | 0.95 | 0.39 |
| *Fusobacteriaceae* | 0.09 | 0.23 | 4.11 | 4.29 | 0.12 | 0.01 | 0.13 | 0.34 | 0.54 | 2.28 | 4.96 | 0.49 |
| *Leptotrichiaceae* | 0.00 | 0.00 | 2.58 | 3.55 | 0.00 | 0.06 | 0.01 | 0.00 | 0.01 | 0.16 | 1.88 | 0.49 |
| *Caulobacteraceae* | 2.20 | 0.05 | 0.02 | 0.07 | 0.26 | 0.06 | 0.07 | 0.14 | 0.04 | 0.00 | 0.00 | 0.48 |
| *Sphingomonadaceae* | 0.31 | 0.10 | 0.01 | 0.03 | 0.31 | 0.52 | 0.51 | 0.33 | 0.19 | 0.03 | 0.20 | 0.98 |
| *Burkholderiaceae* | 3.73 | 0.38 | 0.13 | 0.08 | 1.68 | 1.54 | 1.73 | 0.62 | 1.39 | 0.69 | 0.35 | 3.32 |
| *Comamonadaceae* | 0.75 | 0.17 | 0.11 | 0.02 | 0.27 | 0.97 | 1.11 | 0.73 | 0.40 | 0.18 | 0.04 | 0.98 |
| *Neisseriaceae* | 0.17 | 0.43 | 1.87 | 1.07 | 2.17 | 0.36 | 0.48 | 1.01 | 0.40 | 2.39 | 8.90 | 1.08 |
| *Succinivibrionaceae* | 0.04 | 0.01 | 0.02 | 0.00 | 0.05 | 0.05 | 1.45 | 0.03 | 0.06 | 0.02 | 0.00 | 0.14 |
| *Enterobacteriaceae* | 0.93 | 0.49 | 0.25 | 0.04 | 0.49 | 2.32 | 1.10 | 0.86 | 0.37 | 0.43 | 0.27 | 0.78 |
| *Pasteurellaceae* | 7.68 | 24.90 | 24.93 | 23.40 | 23.25 | 10.00 | 7.02 | 30.70 | 31.87 | 7.16 | 15.47 | 7.17 |
| *Moraxellaceae* | 13.19 | 17.67 | 24.72 | 31.22 | 7.85 | 0.87 | 1.15 | 3.68 | 1.16 | 5.28 | 18.87 | 2.61 |
| *Pseudomonadaceae* | 0.12 | 0.06 | 0.01 | 0.01 | 0.09 | 0.33 | 0.71 | 0.45 | 0.91 | 0.03 | 0.00 | 0.38 |
| *Xanthomonadaceae* | 1.15 | 0.16 | 0.05 | 0.01 | 0.23 | 1.47 | 0.95 | 0.67 | 0.34 | 0.11 | 0.04 | 0.36 |
| *Spirochaetaceae* | 0.23 | 0.22 | 0.47 | 0.11 | 0.08 | 0.56 | 1.39 | 0.57 | 0.33 | 0.35 | 1.50 | 0.81 |
| Others | 9.80 | 12.76 | 9.07 | 8.51 | 5.71 | 34.54 | 26.88 | 18.22 | 7.43 | 6.15 | 7.33 | 13.84 |

*Red, families that represent the top twenty most abundant families for a specific period or sample.*
*Blue, families that were not part of the top 20 families for that sampling period.*
*Black, other members of the microbiome not listed in the table.*

10–12; and OTU0031, which was mainly seen in 1 week old piglets. Similarly, there were three OTUs of *Streptococcaceae,* including OTU002 which was seen throughout the lives of the pigs but was particularly dominant in week 4, OTU009 which was seen in newborns and weeks 1–4, and OTU0024, which was seen mainly in older piglets. Finally, there were three OTUs of *Moraxellaceae*, including OTU003, which was a major component of the microbiome in newborns through week 4 and then decreased to return in the week 10 and 19 samples; OTU0006, which was present in lower amounts than OTU0003 in weeks 1–4 but in much higher amounts in weeks 16 and 19; and OTU0046, which was a minor component of the microbiome.

## Aerobic, Anaerobic, and Facultatively Anaerobic Organisms in the Tonsils

An analysis of the distribution of the bacterial families identified in the tonsils based on their classification by use

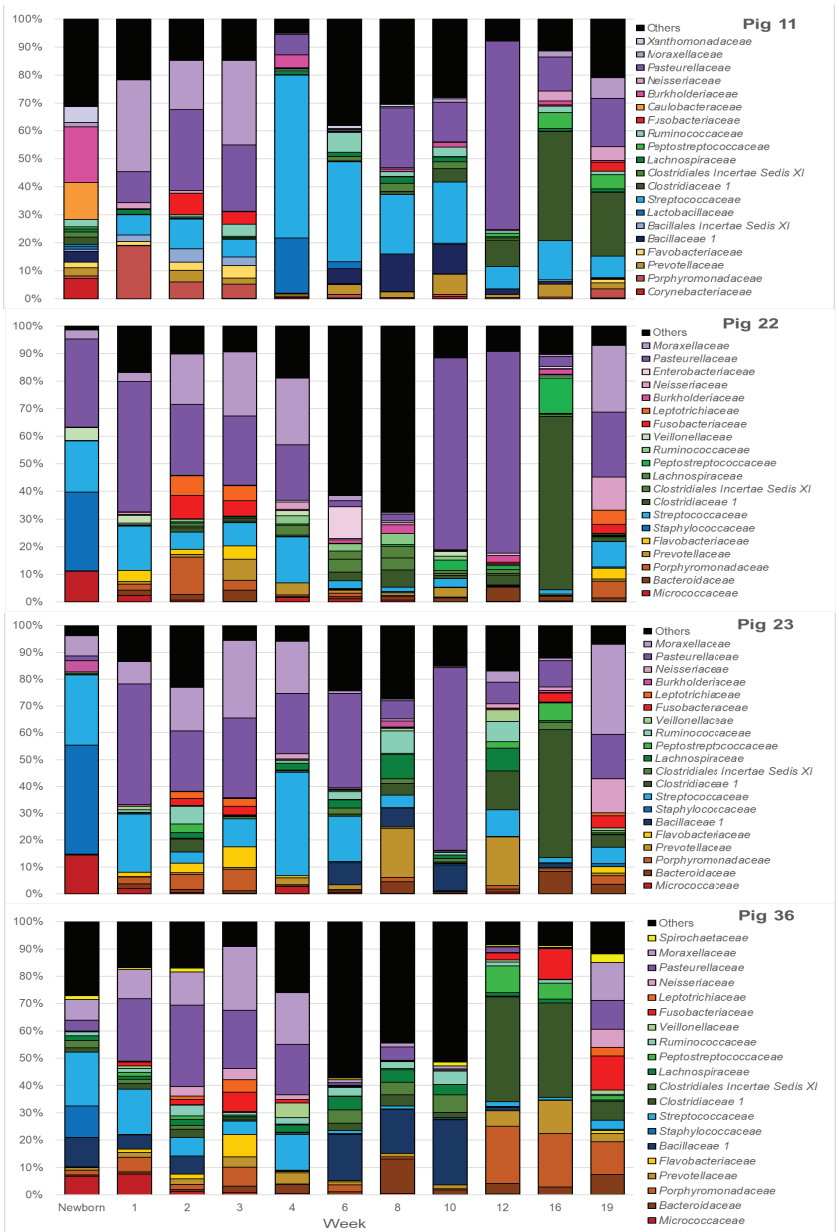


**FIGURE 5 |** Twenty most abundant families in the tonsilar microbiome per sampling time for 4 selected pigs. Colored bars illustrate the percentage of the total reads classified into specific families at each sampling time from newborn through market age for pig 11, pig 22, pig 23, and pig 36.

of oxygen as aerobes, anaerobes or facultative anaerobes (**Figure 7**) showed that in piglets aged newborn to 4 weeks the microbial population was comprised of ∼70% aerobes and facultative anaerobes. The abundance of facultative anaerobes decreased from birth through week 3, but increased after weaning, most likely due to the bloom in *Streptococcaceae*. The proportion of anaerobes increased after the weaning period, with a concomitant decrease in facultative anaerobes and aerobes, and reached ∼65% of the total microbiome in week 16.

## DISCUSSION

We have previously characterized the tonsil bacterial microbiome in healthy 18–20 week old grower-finisher pigs (14) and have recently described the development of the tonsillar microbiome in pigs from birth to weaning (16). In this study, we sought to extend this research to characterize how that tonsil microbial community develops and matures during the life of pigs from newborn to market age. In particular, we wished to determine when specific members of the tonsil microbial community

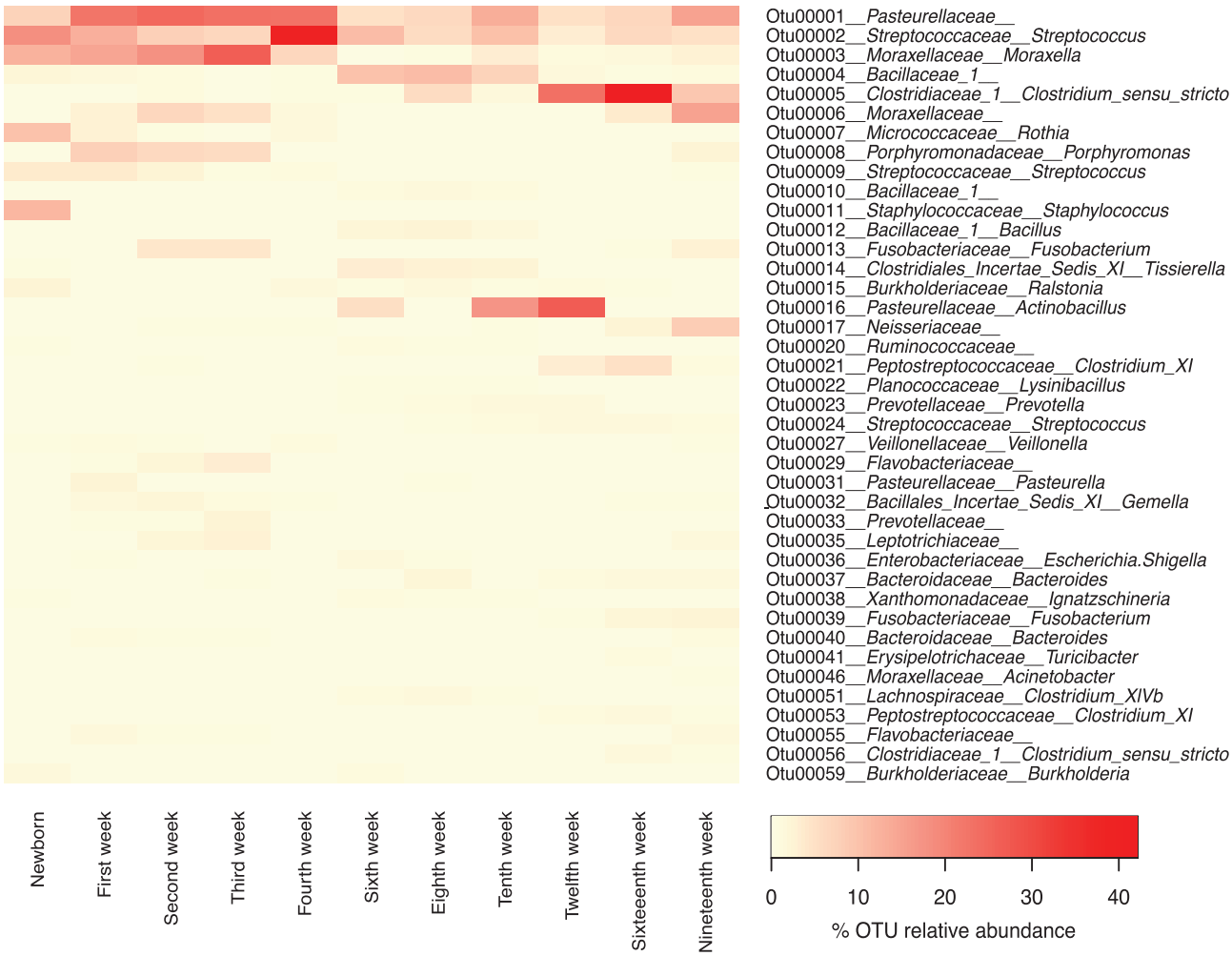


**FIGURE 6 |** Forty most abundant Operational Taxonomic Units (OTUs) for pigs through different sampling times. Heat-map showing the relative abundance of the top 40 OTUs identified per sampling time through the life of these pigs. Note that any OTUs identified as "unclassified" at the family level were not included in this list.

appeared and disappeared, whether there was a temporal succession in the development of the community, and whether stressful events such as alteration of feed or housing, addition or removal of in feed antibiotics, or mixing of pigs into new social groups affected the structure and composition of the tonsillar microbiome. Although clearly there are other microbes such as fungi and viruses present in porcine tonsils, we focused on the bacterial communities in this study.

There are strong parallels between our current data and that from the prior study on 18–20 week old pigs (14). In both studies, members of the tonsil microbiome were found to predominantly belong to 5 phyla: *Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria,* and *Actinobacteria*, with *Proteobacteria* and *Firmicutes* together representing 85-90% of the tonsil microbiome. In both studies, *Pasteurellaceae, Moraxellaceae, Neisseriaceae, Streptococcaceae, Peptostreptococcaceae, Veillonallaceae,* and *Fusobacteriaceae* were identified as among the most abundant bacterial families found. In the current study, many families in the orders *Clostridiales* and *Bacteroidetes* were also found to be among the most abundant taxa seen. Improvements in both the sequencing technology and the databases that facilitate identification of bacteria via 16s rRNA gene sequencing likely account for these differences. In the earlier study, it was not possible to identify most *Clostridiales* below the order level, which is now possible. Further, in that study it was recognized that *Bacteroidetes* were underrepresented in the final data, possibly due to amplification bias with the primers used in that study (14).

We collected samples from eleven different sampling periods from newborn to 19 weeks of age (**Figure 1**) as well as the tonsillar microbiome of the sows. In our analysis of the taxa (at the family level and the OTU level) in these samples, we observed that the development of tonsillar communities in pigs followed a successional process. Some members of the community were acquired during the birth process, from the sow vaginal tract, or within the first few hours of life from the

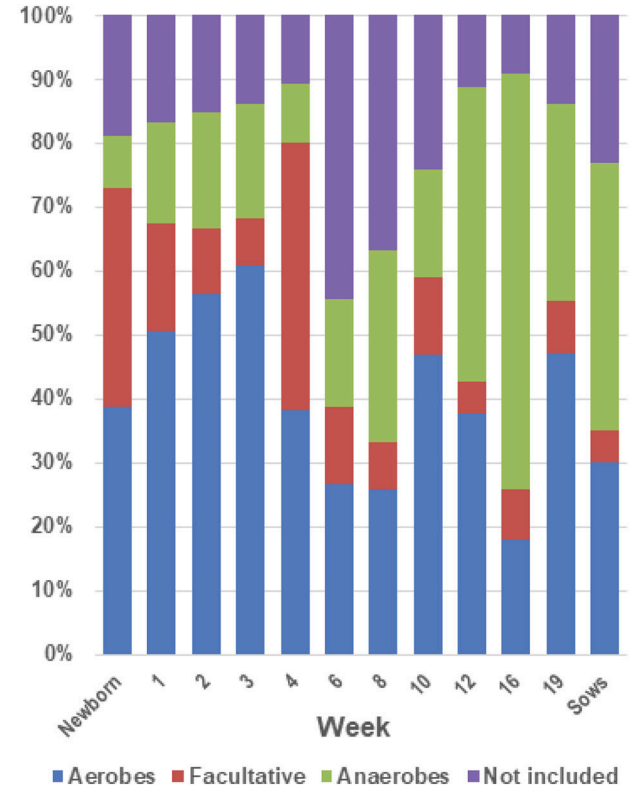


**FIGURE 7 |** Proportions of aerobes, anaerobes and facultative bacteria in the tonsil microbiome. The twenty most abundant families identified in the tonsillar microbiome of the sampled pigs from newborn through market age, as well as sows, were classified as aerobes, anaerobes or facultative organisms based on Bergey's Manual of Systematic Bacteriology (28). The chart illustrates the proportion of the microbiome classified as aerobes, anaerobes, and facultative anaerobes at each sampling time.

sow teat skin or milk (16), while others were acquired later. Some members of the community, particularly *Streptococcaceae, Pasteurellaceae*, and *Moraxellaceae*, were present throughout the life of the pigs, while others such as *Staphylococcaceae, Micrococcaceae,* and *Fusobacteriaceae* seemed to be transient (**Figure 4**). Further, specific OTUs of some of these major taxa also appeared to be either permanent or transient (**Figure 6**). The relative proportions of the major members of the microbiome did change through time, however, as pigs aged, their microbiome seemed to become more similar to the microbiome of older pigs (**Figure 4** and **Table 3**). This process was not always synchronous (**Figure 5**), but the overall progression was very similar in most pigs.

As we examined the changes in the microbiome over time, it became clear that at certain time points, e.g., at 3, 8, and 16 weeks, the phylogenetic structure of the microbiomes of all of the animals became very similar (**Figures 2**, **3** and **Table 2**). When we analyzed this in comparison to management of the pigs (**Figure 1**), we concluded that stretches of time with constant conditions, such as newborn through week 3, led to the convergence of microbiome structure across samples. This convergence occurred regardless of litter source for the pigs or room in which they were housed. In contrast, times where there were changes in management conditions, such as addition or removal of in feed growth promoting antibiotics or movement of pigs to new housing, and especially weaning, led to perturbations in the microbiome (e.g., weeks 4, 6,10, and 19). The taxonomic data was supported by an analysis of the alpha diversity (**Table 2**). Whether these perturbations occurred in response to specific stresses, such as presence of antibiotics, or were adaptations of the microbiome to new conditions, such as new feed, or resulted from exposure to new microorganisms when groups of piglets were reasserted to new rooms, or a simultaneous combination of such stresses, remains unclear. However, absence of challenges or disruptions led to stabilization of the microbiome, with most pigs developing similar microbiomes over times with constant conditions.

Our recent study that followed the development of the tonsillar microbiome of piglets from newborn to weaning, focusing on the source of members of the microbiome and the litter effect as well as the overall development and the effect of weaning (16), and the current study that extends that work, are the only studies available that describe the development of the tonsillar microbiome of pigs or other mammals. Most of the available data following the development of microbial communities in mammals has been focused on the gastrointestinal tract. Pajarillo et al (29) assessed the fecal bacterial diversity of healthy piglets during the weaning transition, and suggested that this period was related to a trend of increasing bacterial diversity, which may be related with the changes in diet. However, they did not discard a possible additional influence of stress or disruption associated with the weaning period. Another study describing the bacterial diversity of pig feces over time followed the development of the fecal microbiome of pigs 10–22 weeks old and identified that calculated diversity indices suggested similar diversity profiles for all the samples (30). Although these prior studies examined the fecal microbiome, they support our results of increased bacterial diversity when the piglets were weaned, which decreased after 10–12 weeks, as well as following challenges or disruptions, such as addition or removal of in feed antibiotics.

There is extensive research data showing that the balance of microbial communities is altered by the use of antibiotic treatments (22, 31). Rettedal et al. (32) studied the effect of the growth promoter chlortetracycline on the ileal microbiota of pigs and found an association with a significant shift in the gut microbiota. However, Poole et al. (33) did not find changes in diversity in feces associated with a similar dose of chlortetracycline. In-feed supplementation of pigs with a mixture of antibiotics known as ASP250, containing chlortetracycline, sulfamethazine, and penicillin, was correlated with a shift in the bacterial phylotypes present in the intestine, where microbial community membership changed over time, mainly showing a decrease in *Bacteroidetes* abundance and an increase in *Proteobacteria* (34). Carbadox® supplementation in-feed was associated with significant changes in community structure and bacterial membership in the intestinal microbiota of pigs (35). An

immediate effect was noticed, although the microbiome structure recovered later despite the continued use of the medication. The authors reported a relative increase in *Prevotella* associated with Carbadox® administration, while Carbadox® withdrawal was associated with an increase in the *E. coli* population (35). The use of the growth promoter Tylosin (also known as Tylan®) was associated with a pronounced shift in the intestinal microbiome distribution and quantity, altering the abundance of specific genera such as *Lactobacillus* among others. These changes occurred at specific times in the growing pig as the pigs aged (36). These prior studies have tried to identify the effects of medicated food on the gut/feces microbiome, but there are no studies that characterize the effect of ingested medications on the tonsillar microbiome. However, it can be concluded that regardless of the medication, the administration of antibiotics or growth promoters in food exerts an effect on the bacterial communities. In this study, we observed large shifts in the tonsil microbiome related to specific periods where medicated food was added, changed or removed. However, because in feed medication was not an isolated factor but supplementary to other changes at the same time, we cannot make a definitive conclusion about the specific effect of the administration of this medication. We do consider the microbiome shifts seen to be relevant and the potential subject of further research.

We identified the first major shift associated with supplementation of Carbadox® coinciding with a huge bloom in members of *Streptococcaceae* and a decrease in *Moraxellaceae, Fusobacteriaceae,* and *Porphyromonadaceae*, reported previously by our group (16). Another shift was associated with the removal of Carbadox® and supplementation with Tylan®, with a major decrease in members of *Streptococcaceae*, *Moraxellaceae,* and *Pasteurellaceae* with a concurrent increase in members of *Clostridiales* and *Bacillaceae 1*. The removal of Tylan® from the diet was associated with a slight increase in members of *Streptococcaceae* and *Moraxellaceae*, parallel to a higher increase in members of *Pasteurellaceae*. It is important to highlight that the presence of Tylan® in the diet is associated with an increase in the bacterial diversity (**Table 2** and **Figure 4**). We emphasize that our goal was not directed toward the identification of specific effects of antibiotics or growth promoters in the development of the tonsil microbiome, but instead toward characterization of the development of tonsillar microbiome of pigs from a healthy farm under normal management. Our results open an avenue for future research on the specific effect of these medications on the tonsillar microbiome and how they can potentially influence the acquisition of pathogenic flora.

Another big change experienced by the pigs was dietary, particularly at weaning. In human infants, the introduction to a new diet associated with cessation of breast feeding has been shown to be associated with profound changes in the composition of the intestinal microbiome (37). It has been suggested that the diet to which an individual has been exposed rapidly alters the structure of the intestinal microbial communities (38). Similarly, in pigs it has been shown that the diet can have an effect in the intestinal microbiome (39), and in particular that the diet supplemented after weaning in piglets can alter the fecal microbiota considerably. A diet supplemented with fermentable carbohydrates was related with greater bacterial diversity when compared to control diets (39). Diet changes during weaning transition can exert an effect on the composition of the intestinal microbiota (40, 41) where bacterial community structure can change as the diet changes (42).

It is not clear whether the bloom in *Streptococcaceae* seen at 4 weeks was related to weaning and removal from the sows, supplementation with Carbadox®, or both. The most common OTU of the *Streptococcaceae* in this bloom was identified as *Streptococcus suis*, an organism that is both normal microbiota of swine tonsils and a cause of severe infections including meningitis and polyarthritis in recently weaned piglets (43, 44). *S. suis* is also an emerging pathogen of humans (43, 45). Attempts to eradicate *S. suis* from pig herds by segregated early weaning were not successful, likely due to transmission of this organism during birth from the sow vagina tract to the oropharynx of piglets (16, 46). Many swine farms employ in feed antibiotics to reduce problems with *S. suis*. Our data suggest that Carbadox® is not effective and indeed may exacerbate the problem.

Finally, the environmental changes experienced by the pigs could play a role in the development of the tonsillar microbiome. These can include both changes in the physical environment and exposure to new penmates after reassortment of pigs into new housing. The immediate environment in which pigs grow has been suggested to have a profound influence on the initial acquisition and development of fecal and colonic microbiota (47). A recent study following the development of gut microbiota and the effect of early changes in the environment demonstrated that microbial diversity was disturbed by changes in environmental hygiene, and that the effect of the generated changes remained for a long time in the affected animals (8). In our study, we saw increased fecal anaerobes, such as *Clostridiales,* in the tonsils (**Figures 4**, **7**) after weaning and especially after Tylan® was removed from feed. Pigs are coprophagic, and it is likely that these anaerobes were acquired from ingestion of feces from the pen floors. In the older pigs, crypt abscesses in the deeper areas of elongating crypts might provide a niche for colonization by the acquired anaerobes, or conversely these anaerobes may cause the formation of the crypt abscesses. We have previously observed that pigs housed in a very clean high biosecurity environment had almost no *Clostridiales* in the tonsils (unpublished data). Conversely, the absence of deep crypts in very young pigs as seen by scanning electron microscopy suggests an absence of appropriately anaerobic sites within young tonsils for colonization by anaerobes (48).

In the sixteenth week, members of *Clostridiales, especially Clostridiaceae 1,* and *Peptostreptococcaceae,* comprised ∼51% of the identified members of the microbiome for this period. Our results compare with those of Bokulich et al. (31), who studied the development of fecal microbiota in children during early life and associated the administration of antibiotics in children during first months of life with a deficit in members of the *Clostridiales*. Further, the authors associated a gradual increase in members of this order with the introduction to solid food. Our findings become especially relevant when compared with recent findings reported by Kim et al. (7), which found that the presence of members of *Clostridiales* in the enteric microbiota of

mice is critical to prevent the growth of enteric pathogens in the intestine. We do not know how this finding can be translated to the tonsillar microbiome of pigs, but it is interesting to see that one of the most vulnerable periods for pigs to acquire diseases (weaning through eighth week) was marked by a low abundance of members of the *Clostridiales*.

In this study, we found that some bacterial families dominated the tonsillar microbiome throughout the life of pigs; however, their relative abundance often changed significantly after the challenging events. Similarly, other bacterial families appeared and/or disappeared at specific ages. A longitudinal study of bacterial diversity in feces of commercial pigs found that some phyla dominated the microbiome regardless of the age of the animals, supporting our findings in the tonsils. Further, it was observed that a small group of organisms were the most prevalent microbes as pigs aged, and their microbiome converged with the time when they were maintained under similar conditions (30). Although this study was focused on the fecal microbiome, it supports our results in the development of tonsillar microbiome, where we identified some bacterial families that dominated and were present throughout the study period, as well as other bacterial families that were transient and appeared at different times, and further saw a convergence of the tonsil microbiome in all the pigs when they were maintained under constant conditions.

Jensen et al. (11) characterized the microbiome of tonsillar crypts of human patients either with chronic tonsillitis or tonsils from healthy patients which were removed because of hyperplasia. The authors could identify a core microbiome population at the species level in the crypts of humans independent of their health status and age, which involved the genus *Streptococcus*, *Prevotella*, *Fusobacterium*, *Porphyromonas*, *Neisseria*, *Parvimonas*, *Haemophilus*, *Actinomyces*, *Rothia*, *Granulicatella,* and *Gemella*. The above identified genera are members of the families *Streptococcaceae*, *Prevotellaceae*, *Fusobacteriaceae*, *Porphyromonadaceae*, *Neisseriaceae*, *Clostridiales Incertae Sedis XI*, *Pasteurellaceae*, *Actinomycetaceae*, *Micrococcaceae*, *Carnobacteriaceae,* and *Bacillales Incertae Sedis XI*, respectively. Similarly, other studies identifying the human microbiome have recognized members of families *Streptococcaceae*, *Prevotellaceae,* and *Fusobacteriaceae* as abundant in the tonsillar microbiome of healthy humans (10, 17, 49). Although we did not characterize specifically the microbiome of tonsillar crypts and we were not able to characterize the members of the community further than family or genus level for some taxa, our results also show that members of the above mentioned families, except *Actinomycetaceae* and *Carnobacteriaceae,* comprised some of the most abundant families identified in pig tonsils. It was found that members of the genus *Staphylococcus* were present only in low proportions in human tonsils (11). Similarly, we identified that members of the family *Staphylococcaceae* were abundant only in the newborns and decreased noticeably and almost disappeared in the following weeks.

A Bray-Curtis analysis of the development of the pig tonsil microbiome from birth to market age (**Figure 2**) showed us that as the pigs were getting older, the acquired microbial population tended to be more similar to the microbiome present in adult pigs, i.e., the tonsillar microbiome of sows (**Figure 4**). We identified that between the sixth to tenth week, some samples clustered with a sample from the tonsillar microbiome of sows. However, a higher percentage of samples from older pigs, especially between twelfth to nineteenth weeks, were clustered together with samples from tonsillar microbiome of sows. These findings demonstrate both that there is a succession in the development of tonsillar microbiome in pigs and that the final status of the microbiome in grower/finisher pigs develops to resemble that of adult animals. Similar findings were reported by other authors studying the development of the human intestinal microbiota (20, 31), which found that as infants aged, their gut microbiome began to look like the adult microbiome, although it did not reach a mature stage found in adults. Our results show that although the microbiome of older pigs was more similar to the microbiome of the sows, there are still observable differences in the abundance of certain families, as the case of members of families *Peptostreptococacceae*, *Erysipelotrichaceae* and *Burkholderiales* which were more prominent in sow microbiome. Many other studies have also shown that there is a succession/sequentiality in the development of microbial communities in mammalian tissue (18, 21, 30, 50, 51).

In conclusion, this study provides baseline information on the development of the tonsillar microbiome of piglets from newborn to market age, as well as the tonsillar microbiome of sows. We demonstrate that there was a succession in the development of the tonsillar microbiome of piglets as they age, which was not synchronous on all pigs but was highly similar. The tonsil microbiome tended to stabilize and become very similar in all animals over times where management conditions were constant. However, the challenges associated with management procedures typical in a swine farm generated prominent changes in the microbiome composition and the abundance of diverse bacterial families. Nonetheless, over time the microbiome of these young pigs tended to be more similar to the microbiome of older animals. We do not know if the observed patterns would be similar for all pigs from this farm, or if the same pattern would be observed independent of the breed or the specific farm. This study lays the baseline for future research to examine the effect of specific conditions, such as use of antibiotics, on the development of the tonsil microbiome and of acquisition of specific pathogens on the tonsil microbiome and conversely of the effect of the composition and structure of the tonsil microbiome on acquisition of pathogens. Manipulation of the tonsil microbiome to provide enhanced resistance to acquisition and carriage of pathogens is a potential outcome of these studies.

## AUTHOR CONTRIBUTIONS

LP, MM, and JF conceived and designed the study. LP, RL, JF, and MM collected the samples. LP and RL processed the samples. LP, TM, RL, and MM performed the data analysis and interpretation. LP, TM, and MM drafted the manuscript. All authors revised and

edited the manuscript, gave final approval for the version to be published, and agreed to be accountable for all aspects of the work.

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# Microbiome and Blood Analyte Differences Point to Community and Metabolic Signatures in Lean and Obese Horses

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Due to modern management practices and the availability of energy dense feeds, obesity is a serious and increasingly common health problem for horses. Equine obesity is linked to insulin resistance and exacerbation of inflammatory issues such as osteoarthritis and laminitis. While the gut microbiome is thought to play a part in metabolic status in horses, bacterial communities associated with obesity have yet to be described. Here we report differences in metabolic factors in the blood of obese, normal and lean horses correlated with differences in gut microbiome composition. We report that obese horses had higher levels of leptin, triglycerides, glucose, and cortisol in their blood, and more diverse gut microbiome communities with higher relative abundance of Firmicutes, and lower numbers of Bacteroidetes and Actinobacteria. Network analyses of correlations between body condition, blood analytes, and microbial composition at the genus level revealed a more nuanced picture of microbe-host interactions, pointing to specific bacterial species and assemblages that may be signatures of obesity and leanness in the horse gut. In particular, bacteria groups positively associated with two blood analytes and obesity included *Butyrivibrio* spp., Prevotellaceae, *Blautia* spp., two members of Erysipelotrichaceae, and a Lachnospiraceae taxa. These results are an important first step in unraveling the metabolic differences between obese and lean horse gut communities, and designing targeted strategies for microbial intervention.

**Keywords: equine gut microbiome, obesity, 16S rRNA, network analysis, insulin, leptin, triglycerides, glucose**

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## INTRODUCTION

As hindgut fermenting, obligate herbivores, horses rely on the gut microbiome to access nutrients and energy from dietary complex carbohydrates. Short chain fatty acids produced by microbial metabolism have been estimated to provide as much as 42% of equine energy needs (1, 2). Surveys of the equine gut microbiome using 16S rDNA sequencing have revealed communities dominated by Firmicutes, and Bacteroidetes (comprising 75% or greater relative abundance), with less abundant Proteobacteria, Verrucomicrobia, Spirochaetes, Actinobacteria, and Fibrobacteres (3–7). As with other animal and human studies, the horse gut microbiome is sensitive to diet, specifically consumption of starch (8–11), fiber (9, 12–14), and high fat (9, 15), or following a rapid change in diet (14, 16). Both age (17) and exercise (18, 19) have also been shown to impact the composition of the equine gut microbiome.

Paralleling human health trends, equine obesity is a growing problem for horse owners, managers, and veterinarians despite greater awareness of body condition assessment, and the availability of specialized feeds for weight management (20–23). A recent study of 300 horses in Virginia using a standardized 1–9 scale to estimate body condition score (BCS) (24), found as many as 51% to be over-conditioned or obese (22). Indications suggest that this estimate of obesity is not an isolated trend (20, 23, 25).

A primary component of Equine Metabolic Syndrome (EMS), obesity contributes to insulin resistance (26–30), predisposes horses to laminitis (30–32), exacerbates heat intolerance(33), reduces performance (34, 35), and increases joint stress (36, 37). A breed effect in the incidence of EMS indicators has been demonstrated, with higher prevalence in ponies, Standardbreds, Andalusians (38), and Rocky Mountain Horses (39), and lower rates in Thoroughbreds, Quarter Horses, and mixed breeds (39).

While human and mouse studies comparing the gut microbiomes of lean and obese individuals have shown a higher Firmicutes: Bacteroidetes ratio correlated with obesity (40–42), comparisons of fewer than 10 obese and lean horses have observed no difference in the ratio of these groups (12, 43). One comparative EMS study of 20 horses found specific genera associated with obesity, including: *Clostridium* cluster XI, *Lactobacillus*, *Cellulosilyticum*, *Elusimicrobium*, and members of the phyla Verrucomicrobia, while *Fibrobacter*, *Ruminococcus*, *Saccharofermentans*, *Anarovorax,* and members of Lachnospiraceae and Rhodospirillaceae families were correlated with normal controls (12).

Several metabolic markers in blood have been shown to be correlated with high BCS in horses, namely higher levels of resting insulin, glucose, leptin, adiponectins, and triglycerides (39, 44–46). Higher leptin levels have been shown to be especially pronounced in horses fed diets rich in cereals or fat (38), but no response was seen in obese horses fed varying levels of non-structural carbohydrates in hay (47). Additionally, horses with higher levels of leptin showed elevated insulin (44, 46) and cortisol (especially mares) (44).

While levels of obesity associated blood analytes have been described in horses, studies to identify differences in the gut microbiomes of obese and lean horses have been few and limited to a small number of horses. The purpose of the present study is to correlate blood metabolites related to EMS (insulin, glucose, triglycerides, leptin, ACTH, and cortisol) with gut microbiome differences in a set of 78 horses: lean ($n = 24$), normal ($n = 17$), and obese ($n = 37$).

# MATERIALS AND METHODS

## Fecal Sample Collection

Fecal samples were collected manually midrectum from horses before breakfast, and stored in ice for no more than 2 h prior to storage at −80°C. Sampling was done in the January–April of 2015 or 2016, before horses had access to fresh, spring grass. Pasture-fed horses were of various breeds, aged 2–20 years, from three university herds: (University of Massachusetts, Amherst, MA, University of Illinois, Champaign-Urbana, IL, or Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA) or private horse owners from five different farms. To minimize variation due to location or owner, no facility contained fewer than three horses. Horses that had received antibiotic or anthelmintic medication within 60 days of collection were removed from the study. Metadata collected for each horse included: breed, gender, diet, housing type, and age. Diet was divided into three categories depending on primary feed with no distinction made for quantity or quality. Diet categories included: Pasture (P), Hay (H), Hay, and concentrate (HC). Age was divided into two categories: 10 years or less (Age_Y, $n = 29$), and over 10 years (Age_M, $n = 49$). BCS (1-9) was determined by the average of at least three observers using the Hennecke scale (24). Horses with divergent BCS across body regions were not sampled due to the possibility of metabolic issues. Classifications of obese, normal, or lean were assigned to each horse based on score: 7 or higher, between 6 and 7, and 5.5 and less, respectively. The demographics of horses participating in this study are summarized in **Table 1**.

## Blood Sample Collection

Whole blood was collected via venipuncture into untreated Vacutainer tubes (serum) and EDTA tubes (plasma) (BD, Franklin Lakes, NJ). Tubes were chilled for no more than 2 h before processing. Serum tubes were allowed to return to room temperature and clot before spinning. Plasma tubes were spun for 20 min at 850 g at 4°C. Serum tubes were spun for 20 min at 850 g at room temp. Plasma or serum layers were removed and stored at −80°C prior to analysis. All analysis was done at the Cornell Animal Health Diagnostic Center, Ithaca, NY. ACTH, cortisol, insulin, and leptin were measured from plasma samples, and glucose and triglycerides were measured from serum samples.

## DNA Extraction and Sequencing

Fresh fecal samples were collected midrectum from each horse, kept on ice for no more than 2 h prior to storage at −80°C. DNA was extracted utilizing either a modified CTAB-bead beating method (48–50), or Mobio Power Fecal DAN extraction kit (MoBio Laboratories, Carlsbad, CA), and stored at −80°C prior to sequencing.

Amplification of the V4-V5 region of the 16S rRNA gene and attachment of indexes for multiplexing samples were done using region specific primers (515F/926R) as described elsewhere (51). PCR products were pooled and sequenced using the MiSeq platform at either the University of Illinois Biotechnology Center, Urbana, IL, or RTL Genomics, Lubbock, TX. Paired ends were joined using FLASh (v. 1.2.11) (52). Quality and chimera filtering, taxonomic assignment, diversity analysis, and identification of shared and unique taxa were done using the QIIME (53) pipeline as applied previously (54).

## Statistical Analysis

Relative abundance of bacterial groups and alpha diversity measures by body condition group were compared using pair-wise, two-tailed *t*-tests (assuming unequal variances), and Kruskal-Wallis rank sum test in R (55). Differential abundance between lean, normal, and obese horses at the taxa level was modeled using a negative binomial distribution in the DESeq

**TABLE 1 |** Demographics of horses included in this study (for complete horse list, see **Table S1**).

| Farm | Horses | Gender | | | Age | | BCS category | | | Feed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total | Stallion | Mare | Gelding | Y | M | Lean | Normal | Obese | P | H | HC |
| IU | 6 | 3 | 3 | 0 | 4 | 2 | 1 | 0 | 5 | 6 | 0 | 0 |
| UM | 14 | 0 | 4 | 10 | 5 | 9 | 0 | 0 | 14 | 0 | 14 | 0 |
| VM | 25 | 0 | 17 | 8 | 4 | 21 | 11 | 0 | 14 | 25 | 0 | 0 |
| PO-DE | 18 | 0 | 14 | 4 | 15 | 3 | 10 | 7 | 1 | 0 | 0 | 18 |
| PO-NH | 15 | 0 | 5 | 10 | 2 | 13 | 2 | 10 | 3 | 0 | 0 | 15 |
| Total | 78 | 3 | 43 | 32 | 30 | 48 | 24 | 17 | 37 | 31 | 14 | 33 |

*Farm: University of Illinois (UI), University of Massachusetts (UM), Private Owner (PO state), VA-MD Regional College of Veterinary Medicine (VM). BCS: (1-9) according to the Hennecke scale. BCS cat: 1-5.5 (Lean), 6-6.5 (Normal), 7+ (Obese). Feed: Pasture (P), Hay (H), Hay/Concentrate (HC). Age: 10 years or less (Y), and over 10 years (M).*

**TABLE 2 |** 16S rRNA sequence counts after removal of low quality and short reads.

| Counts/Sample summary | |
|---|---|
| Number of samples | 78 |
| Minimum count | 4165 |
| Maximum count | 102594 |
| Median count | 44498.5 |
| Mean count | 40361.795 |
| Std. dev. | 21151.638 |

package (56) in R (55). Spearman correlations of all pairs of taxa, blood analytes, metadata, and relative abundance of bacterial taxa were calculated in JMP (Pro 13.0.0).

## Network Construction

Networks of significant Spearman correlations were visualized in Cytoscape (version 3.6.0). Taxa nodes were mapped to their phylogeny, colored by phyla, and assigned a two-letter code (**Table 3**). Border thickness of taxa nodes was proportional to Relative Abundance (RA). Significant positive and negative Spearman correlations were represented by red and blue edges, respectively. Edge thickness was proportional to correlation coefficient values ranging from +1 to +0.3 and from −1 to −0.3. Networks of nodes of differentially abundant taxa, were constructed by selecting first neighbors for all the specified nodes. In complex networks, edges representing pairwise correlations with values <0.5 were de-emphasized (faded).

# RESULTS

## 16S rRNA Sequencing

Summary statistics for 16S rRNA sequencing following filtering for low quality and length can be found in **Table 2**. The average read length was 412 bp, and the total number of reads was 3,148,220. Sequence data has been deposited in Genbank BioSample SAMN09917936.

## Bacterial Abundance Profiles

16S rRNA sequences were clustered at 97% similarity against the latest Greengenes database (13_5). The resulting operational taxonomic units (OTUs) were filtered for singletons and doubletons. **Table 3** lists the 51 bacterial OTUs with abundance >0.10% with their corresponding 2 letter codes. All taxa included in the subsequent analysis are found in **Table S2**.

At the phyla level, comparison of communities of lean, obese, and normal horses showed no significant differences in variance (Kruskal-Wallis test, $p$-value $> 0.05$) (**Figure S1**), however pairwise differences were detected between obese and lean and obese and normal horses in relative abundance of Bacteroidetes and Firmicutes (two-tailed $t$-test assuming unequal variances, $p$-value $< 0.05$) (**Figure 1**). Specifically, the relative abundance of Bacteroidetes was less in obese horses, while the relative abundance of Firmicutes was higher. Consequently, the Firmicutes/Bacteroidetes ratio was higher for obese horses. Comparison of Bacteroidetes families in the gut microbiome of obese, lean, and normal horses show differences in unspecified Bacteroidales family and Porphyromonadaceae, while difference were seen in six Firmicutes families: Christensenellaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Mogibacteriaceae, and Ruminococcaceae (**Figure S2**).

Differentially abundant taxa were identified (padj $< 0.05$) using a negative binomial distribution in DESeq for pairwise BCS groups and All BCS groups together (**Table 4**). All but four differentially abundant taxa were members of Actinobacteria, Firmicutes, or Bacteroidetes. There were 5, 6, and 24 differentially abundant taxa between Obese/Lean, Normal/Lean, and Obese/Normal groups respectively. Nine taxa were found to be differentially abundant in two or more pair-wise comparisons, and three taxa were identified as differentially abundant in comparisons of all BCS categories. Differentially abundant taxa with relative abundance >0.01% were compared by BCS group (**Figure 2**), and found to collectively constitute between 20 and 30% of total bacterial abundance.

## Bacterial Diversity

Obese horse samples were higher than both normal and lean for all measures of alpha diversity, including richness (Chao1 and Observed OTUs), richness and evenness (Shannon Index), and phylogenetic diversity (PD-whole-Tree) (**Figure 3**).

**TABLE 3 |** Taxa identified and the total relative abundance in the obese, normal, and lean horse samples.

| Taxon lineage | Code | Relative abundance (%) |
|---|---|---|
| Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales;f__;g__ | QE | 16.244410 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Ruminococcaceae;g__ | LK | 15.893878 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Lachnospiraceae;g__ | OJ | 14.452446 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__;g__ | QI | 11.473584 |
| Unassigned;Other;Other;Other;Other;Other | AA | 3.780830 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Ruminococcaceae;g__Ruminococcus | OK | 3.094827 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Mogibacteriaceae;g__ | BL | 2.678472 |
| Bacteria; Spirochaetes; Spirochaetes; Spirochaetales;f__Spirochaetaceae;g__Treponema | FR | 2.670410 |
| Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales;f__Prevotellaceae;g__Prevotella | DF | 2.435187 |
| Bacteria; Fibrobacteres; Fibrobacteria; Fibrobacterales;f__Fibrobacteraceae;g__Fibrobacter | EH | 2.356017 |
| Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales;f__Paraprevotellaceae;g__CF231 | KF | 1.715601 |
| Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales;f__Paraprevotellaceae;g__YRC22 | LF | 1.563672 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Lachnospiraceae;Other | NJ | 1.352235 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Veillonellaceae;g__Phascolarctobacterium | UK | 1.251141 |
| Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales;f__Paraprevotellaceae;g__ | JF | 1.212778 |
| Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales;f__RF16;g__ | EF | 1.168953 |
| Bacteria; Actinobacteria; Coriobacteriia; Coriobacteriales;f__Coriobacteriaceae;g__ | YD | 1.071085 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Lachnospiraceae;g__Coprococcus | SJ | 1.030847 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Clostridiaceae;g__Clostridium | ZI | 1.009161 |
| Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales;f__Bacteroidaceae;g__BF311 | UE | 0.882039 |
| Bacteria; Firmicutes; Bacilli; Lactobacillales;f__Streptococcaceae;g__Streptococcus | MI | 0.845326 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Lachnospiraceae;g__Blautia | QJ | 0.732944 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Lachnospiraceae;g__Pseudobutyrivibrio | YJ | 0.666919 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Clostridiaceae;g__ | VI | 0.659299 |
| Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales;f__BS11;g__ | RE | 0.642893 |
| Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales;f__Paraprevotellaceae;g__Prevotella | MF | 0.582616 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Ruminococcaceae;g__Oscillospira | NK | 0.550106 |
| Bacteria; Actinobacteria; Coriobacteriia; Coriobacteriales;f__Coriobacteriaceae;g__Adlercreutzia | ZD | 0.497567 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Christensenellaceae;g__ | TI | 0.466590 |
| Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales;f__Porphyromonadaceae;g__Paludibacter | XE | 0.443382 |
| Bacteria; Proteobacteria; Alphaproteobacteria; f__;g__ | MM | 0.410118 |
| Bacteria; Cyanobacteria; 4C0d-2; YS2;f__;g__ | NG | 0.404619 |
| Bacteria; Tenericutes; Mollicutes; RF39;f__;g__ | CS | 0.361142 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Mogibacteriaceae;g__Mogibacterium | DL | 0.351654 |
| Bacteria; Firmicutes; Erysipelotrichi; Erysipelotrichales;f__Erysipelotrichaceae;g__RFN20 | WL | 0.324342 |
| Bacteria; Firmicutes; Clostridia;Clostridiales;f__Eubacteriaceae;g__Pseudoramibacter_Eubacterium | JJ | 0.306375 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;Other;Other | PI | 0.260408 |
| Bacteria; Firmicutes; Erysipelotrichi; Erysipelotrichales;f__Erysipelotrichaceae;g__ | PL | 0.255276 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Veillonellaceae;g__ | RK | 0.223319 |
| Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales;f__S24-7;g__ | HF | 0.217790 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Lachnospiraceae;g__Dorea | TJ | 0.214578 |
| Bacteria; Firmicutes; Bacilli; Lactobacillales;f__Lactobacillaceae;g__Lactobacillus | KI | 0.210029 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Lachnospiraceae;g__Epulopiscium | UJ | 0.202495 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Clostridiaceae;Other | UI | 0.190916 |
| Bacteria; Firmicutes; Erysipelotrichi; Erysipelotrichales;f__Erysipelotrichaceae;g__p-75-a5 | BM | 0.185225 |
| Bacteria; Spirochaetes; Spirochaetes; Sphaerochaetales;f__Sphaerochaetaceae;g__Sphaerochaeta | DR | 0.170744 |
| Bacteria; Firmicutes; Erysipelotrichi; Erysipelotrichales;f__Erysipelotrichaceae;g__Eubacterium | YL | 0.160846 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Lachnospiraceae;g__Ruminococcus | BK | 0.157950 |
| Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales;f__Bacteroidaceae;g__Bacteroides | VE | 0.122171 |
| Bacteria; Firmicutes; Clostridia; Clostridiales;f__Lachnospiraceae;g__Roseburia | ZJ | 0.119148 |
| Bacteria; Tenericutes; Mollicutes; Anaeroplasmatales;f__Anaeroplasmataceae;g__Anaeroplasma | AS | 0.106584 |

*Two-letter codes designations used in the network analysis and total relative abundance over 0.10% are shown. For all taxa, see* ***Table S2****.*

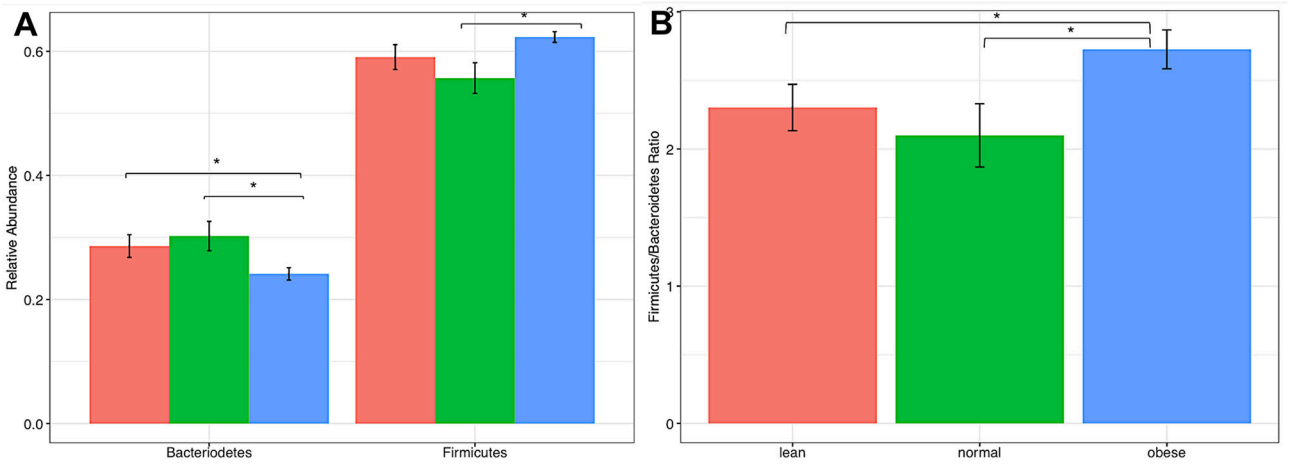


**FIGURE 1 |** Comparison of firmicutes and bacteriodetes from lean, normal, and obese horses. **(A)** Relative abundance of each Phla. **(B)** Firmicutes /Bacteriodetes ratio. Error bars represent standard error. Significantly different groups (two tailed *t*-test assuming unequal variances) art indicated*.

**TABLE 4 |** Differentially abundant taxa.

| Taxa lineage | Code | Number of connections | Relabund% | Obese/Lean | Obese/Normal | Lean/Normal | All BSC |
|---|---|---|---|---|---|---|---|
| Firmicutes;Clostridiaceae;SMB53 | AJ | 29 | 0.01 | | X | | |
| Anaeroplasmataceae;Anaeroplasma | AS | 10 | 0.11 | | X | X | |
| Actinobacteria;Coriobacteriaceae;Collinsella | BE | 74 | 0.01 | | X | | |
| Actinobacteria; Microbacteriaceae;Microbacterium | CC | 109 | 0.01 | | X | X | |
| Firmicutes;Peptococcaceae;g__ | CK | 56 | 0.03 | | X | | |
| Firmicutes; Mogibacteriaceae;Mogibacterium | DL | 76 | 0.35 | | X | | |
| Bacteroidetes;Bacteroidales;RF16 | EF | 23 | 1.17 | | X | | |
| Fibrobacteraceae;Fibrobacter | EH | 9 | 2.36 | | X | | |
| Firmicutes;Lactobacillales;Other | EI | 19 | 0.00 | | X | | |
| Firmicutes;Clostridiales;f__EtOH8 | EJ | 5 | 0.01 | | X | | |
| Spirochaetaceae;Treponema | FR | 15 | 2.67 | | X | | |
| Firmicutes;Peptostreptococcaceae;g__ | HK | 38 | 0.01 | | X | | |
| Verrucomicrobia;RFP12;g__ | HS | 23 | 0.03 | X | X | | X |
| Actinobacteria;Micrococcaceae;g__ | IC | 102 | 0.03 | | | X | |
| Bacteroidetes;Paraprevotellaceae;Other | IF | 17 | 0.01 | | X | | |
| Bacteroidetes;Paraprevotellaceae;g__ | JF | 19 | 1.21 | X | X | | X |
| Firmicutes;Bacillaceae;Bacillus | JH | 88 | 0.02 | | X | X | |
| Firmicutes;Streptococcaceae;Streptococcus | MI | 27 | 0.85 | | X | | |
| Cyanobacteria;YS2;f__;g__ | NG | 25 | 0.40 | | X | | |
| Firmicutes;Ruminococcaceae;Oscillospira | NK | 65 | 0.55 | | X | X | |
| Firmicutes;Clostridiales;Other;Other | PI | 3 | 0.26 | | X | | |
| Bacteroidetes;Bacteroidales;f__;g__ | QE | 69 | 16.24 | X | X | | |
| Firmicutes;Veillonellaceae;g__ | RK | 74 | 0.22 | X | X | | X |
| Actinobacteria;Nocardiaceae;Rhodococcus | TC | 119 | 0.01 | | X | X | |
| Firmicutes;Lachnospiraceae;Epulopiscium | UJ | 28 | 0.20 | | X | | |
| Firmicutes;Erysipelotrichaceae;Eubacterium | YL | 48 | 0.16 | X | | | X |

*Taxa identified in whole group and pairwise comparisons (padj < 0.05) using linear binomial distribution in DESeq. Network connectivity is estimated by numbers of connections in correlation network.*

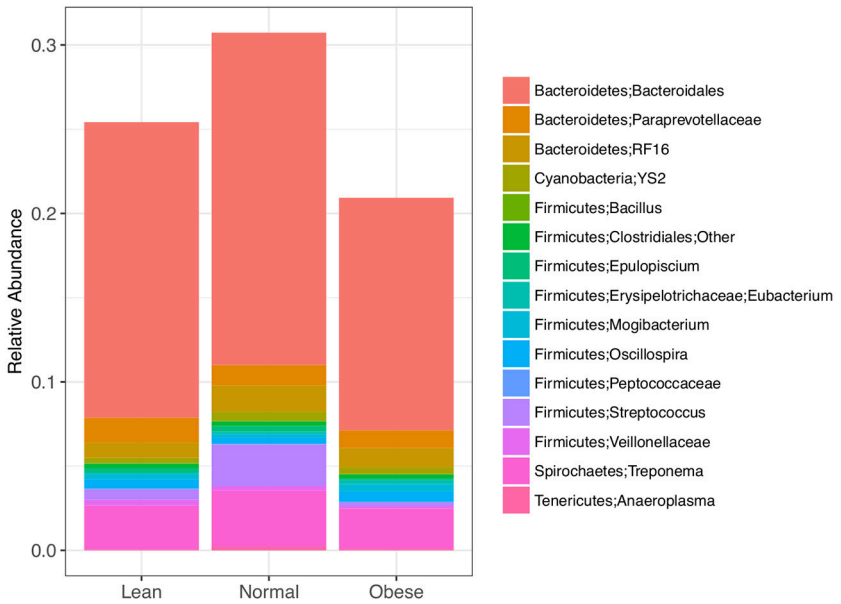


**FIGURE 2 |** Differently abundant bacterial taxa between lean, normal, and obese horses as determined using a negative binomial distribution, $p$-value $< 0.05$. Taxa with relative abundance $\geq 0.01$ % are shown.

## Blood Analytes

Measurements of insulin, glucose, ACTH, cortisol, leptin, and triglycerides were measured from either serum or plasma, and summarized by BCS group in **Table 5**. All blood analytes by horse are reported on **Table S3**. Blood levels of cortisol were higher for obese horses than normal or lean horses. Levels of leptin increased with increasing BCS. Triglyceride and glucose levels were similar between normal and obese horses, and lower for lean horses (**Figure 4**). Statistical difference was not seen between horse groups for resting insulin or ACTH (not shown).

The relationships between pairs of blood factors was plotted with 95% confidence intervals to identify patterns based on BCS (**Figure 5**). At the ranges measured, clear differences were seen in the trend for insulin and glucose in obese, normal, and lean horses. A positive slope for obese and normal samples showed that glucose and insulin levels increased proportionally. An opposite trend was shown for lean horses, as glucose dropped with increasing insulin levels. There was no overlap between confidence intervals for lean and either normal or obese horses. Between normal and obese horses, overlap occurred for only the upper confidence interval. Linear modeling of triglycerides and leptin showed a more positive relationship and leptin response in the obese horses, and nearly constant leptin levels in normal and lean horses. Confidence intervals did not overlap between the obese group and either the lean or normal horses, which were more consistent with each other.

## Correlation Analysis

Spearman rank correlation coefficients analysis performed in JMP (Pro 13.0.0) or R (55) included all 446 taxa, six blood analytes and four metadata variables: Feed, Age, BCS, and Owner. The default alpha value for the initial pairwise analysis was 0.05. 105,570 correlations were found. Correlations with $p$-values $\leq 0.01$ and coefficient values in the range of $-0.3$ to $-1.0$ and $+0.3$ to $+1.0$ resulted in 9,353 significant pairwise interactions for network analysis.

## Network Analysis

Networks of significant Spearman correlations were visualized in Cytoscape (version 3.6.0). This step resulted in a network composed of 458 nodes and 9,353 edges. The first neighbor network, showing significant correlations between all blood analytes, metadata, and taxa (**Figure 6**), showed positive correlations between BCS_O (obese) and blood analytes leptin, cortisol, triglycerides, and glucose, but no correlation with ACTH or insulin. BCS_O was positively correlated with Feed_H (hay), negatively correlated with Feed_HC (hay-concentrate), and not connected with Feed_P (pasture). Focusing on the differentially abundant taxa, the microbial network positively associated with BCS_O included 32 taxa, primarily from Actinobacteria, Firmicutes, and Bacteroidetes. BCS_O had only a few negatively associated bacteria, including highly connected members of the Veillonellaceae (RK), and Lachnospiraceae (UJ).

BCS_L (lean) showed negative correlations with leptin, glucose, and triglycerides, and no correlation with any feed group. The microbial network negatively associated with BCS_L included taxa positively associated with BCS_O or BCS_N, specifically Anaeroplasma (AS), Eubacterium (JF), and Paraprevotellaceae (YL).

BCS_N (normal) was not connected to any blood analyte, but showed positive correlation to Feed_HC and negative correlation to Feed_H. Negative correlations were shown for

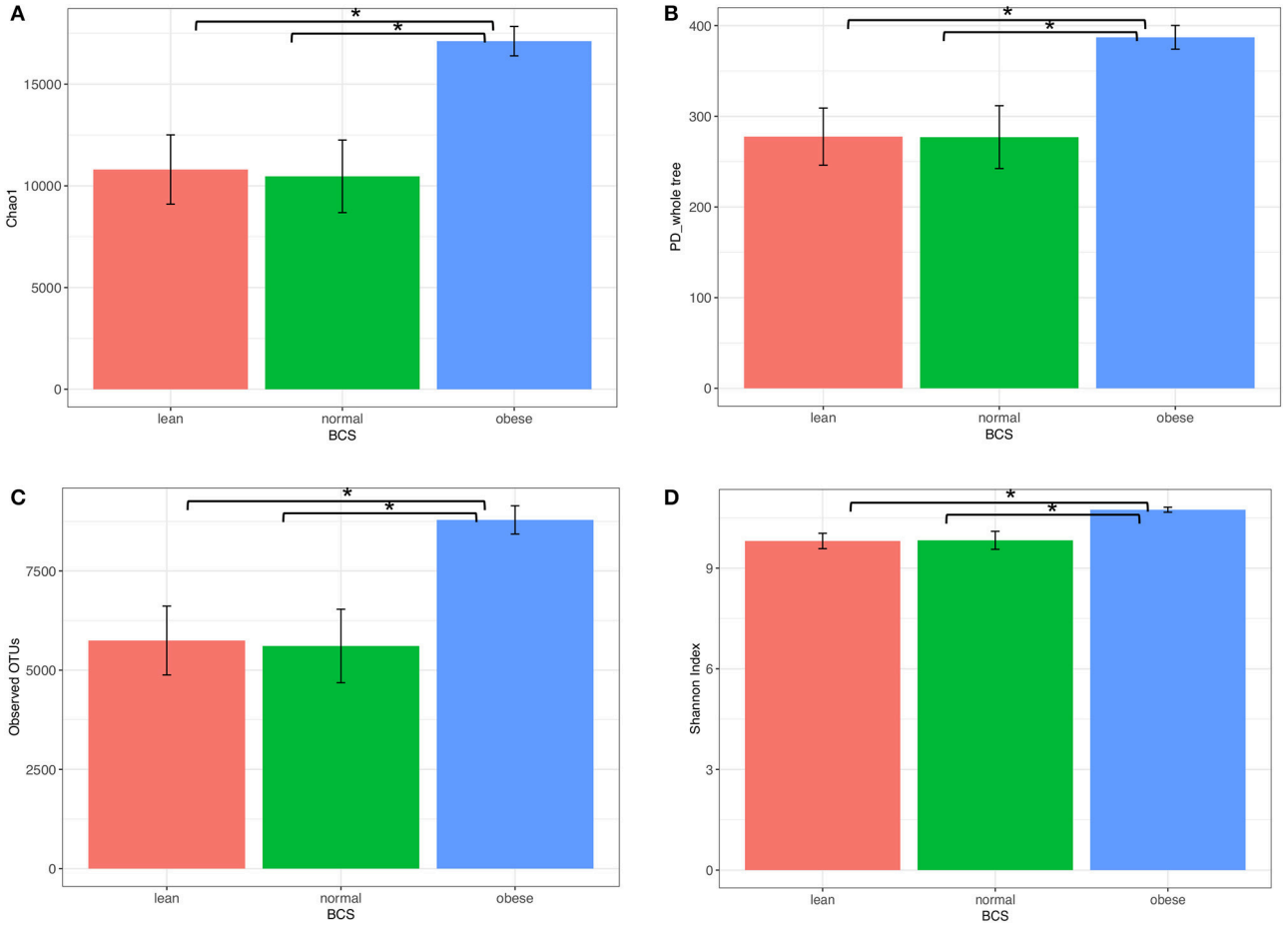


**FIGURE 3 |** Alpha diversity of bacterial communities from obese, normal, and lean horses. **(A)** chaol, **(B)** PD_whole _tree, **(C)** observed OTUs, **(D)** Shannon Index. Error bars represent standard error. Significantly different groups ( two tailed *t*-test assuming unequal variances) art indicated*.

**TABLE 5 |** Summary of blood analyte measurements based on BCS category.

| | Lean *n = 24* | | Normal *n = 17* | | Obese *n = 37* | |
|---|---|---|---|---|---|---|
| **Measurement** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| BCS | 4.56 | 0.60 | 6.16 | 0.24 | 7.46 | 0.48 |
| Insulin (uIU/ml) | 10.78 | 8.64 | 15.74 | 9.76 | 15.49 | 16.33 |
| ACTH (pg/ml) | 21.53 | 8.84 | 20.21 | 7.12 | 24.21 | 10.63 |
| Cortisol (ug/dL) | 3.73 | 1.55 | 3.49 | 1.44 | 4.67 | 1.33 |
| Leptin (ng/ml) | 4.32 | 1.94 | 6.56 | 4.64 | 12.63 | 7.59 |
| Glucose (mg/dL) | 86.00 | 7.39 | 90.71 | 8.14 | 93.92 | 8.80 |
| Triglycerides (mg/dL) | 23.26 | 7.93 | 30.06 | 12.36 | 36.24 | 12.84 |

twenty taxa, including differentially abundant Oscillospira (NK), Microbacterium (CC), Bacillus (JH), and Rhodococcus (TC).

Each of the blood analytes had a small sub-network of associations, except insulin, which showed negative correlations with over 50 bacterial taxa, and no connection to BCS. Insulin did show a positive correlation to Feed_HC, and a negative correlation with Age_Y (young).

The first neighbor network of the differentially abundant taxa for all BCS groups (**Figure 7**) showed the connectivity of these four taxa. Veillonellaceae (RK) was positively correlated with a Bacteroidetes (QE), a highly abundant (16.24%) taxa in the dataset, but negatively associated with 24 taxa that were all positively associated with an Erysipelotrichaceae (YL), suggesting a strong relationship between these two taxa. RK was also

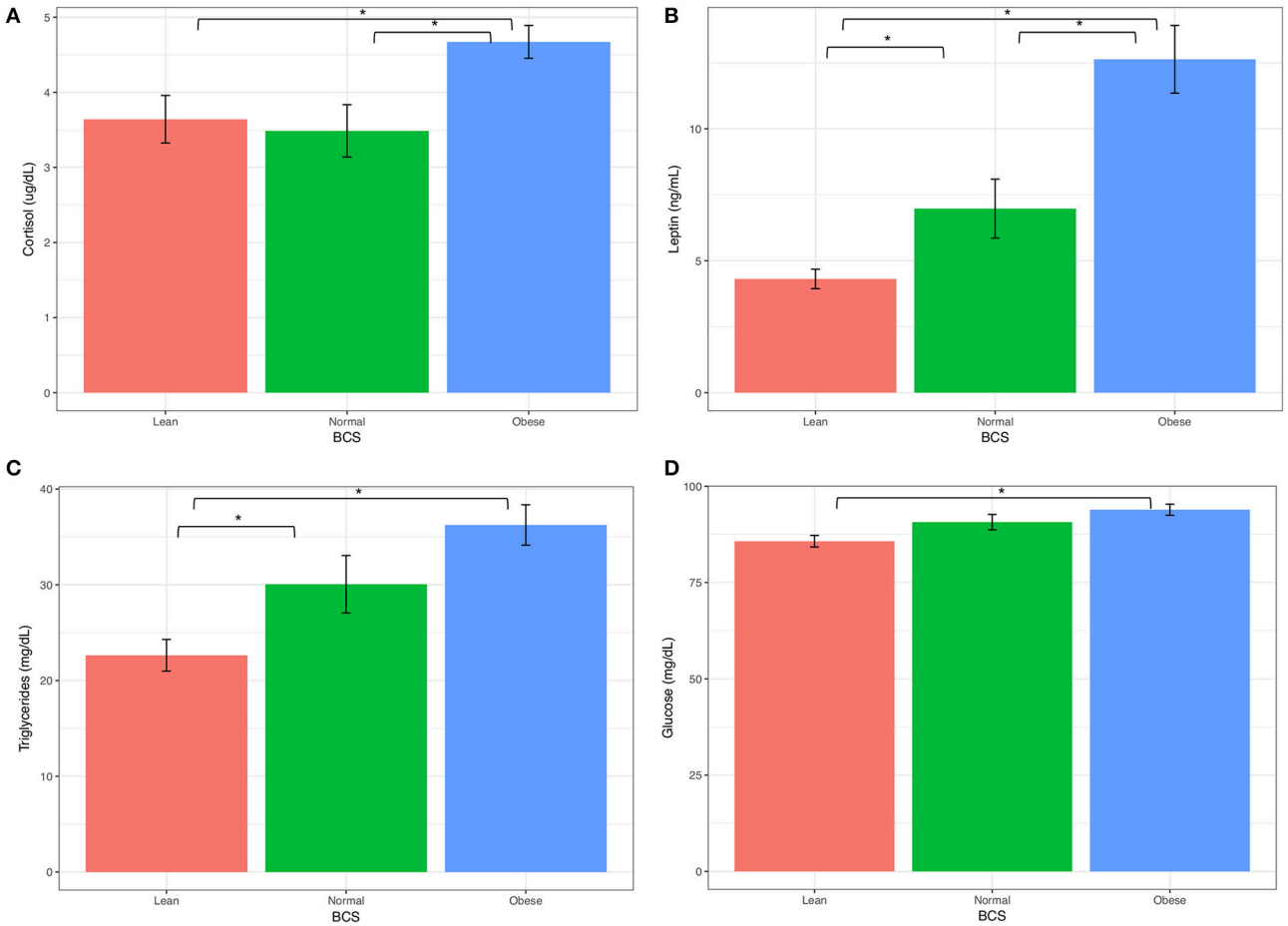


**FIGURE 4 |** Keyboard analytes from obese, normal, and lean horses **(A)** Corsitol, **(B)** Leptin, **(C)** Triglycerides, **(D)** Glucose. Error bars represent standard error. Significantly different groups (two tailed *t*-test assuming unequal variances) art indicated*.

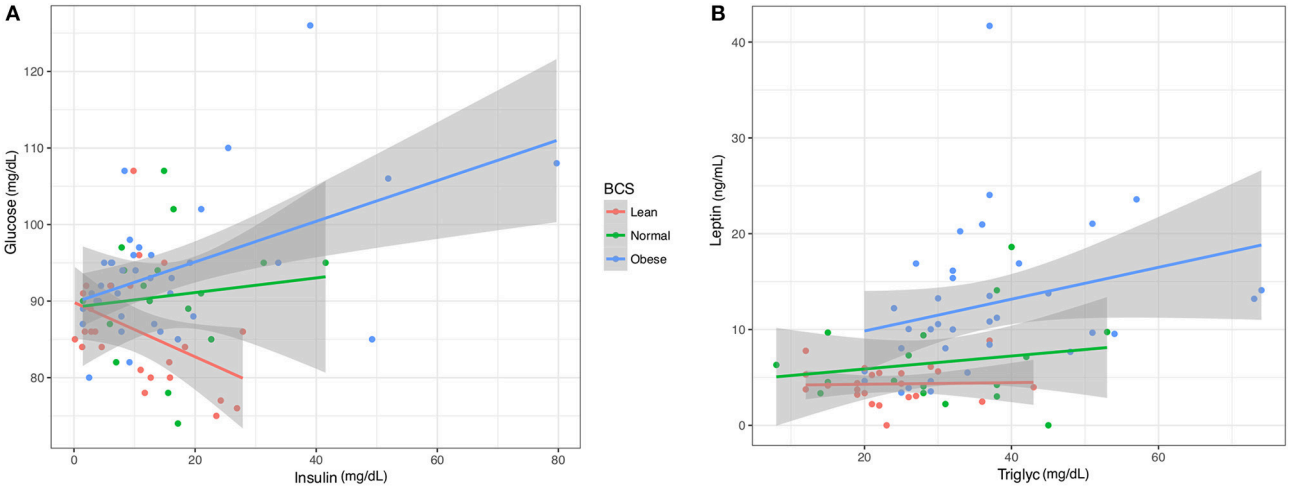


**FIGURE 5 |** Linear models of key blood analytes from obese, normal, and lean horses. **(A)** Insulin vs. Glucose. **(B)**. Triglycerides vs. Leptin. Gray region represents 95% confidence intervals.

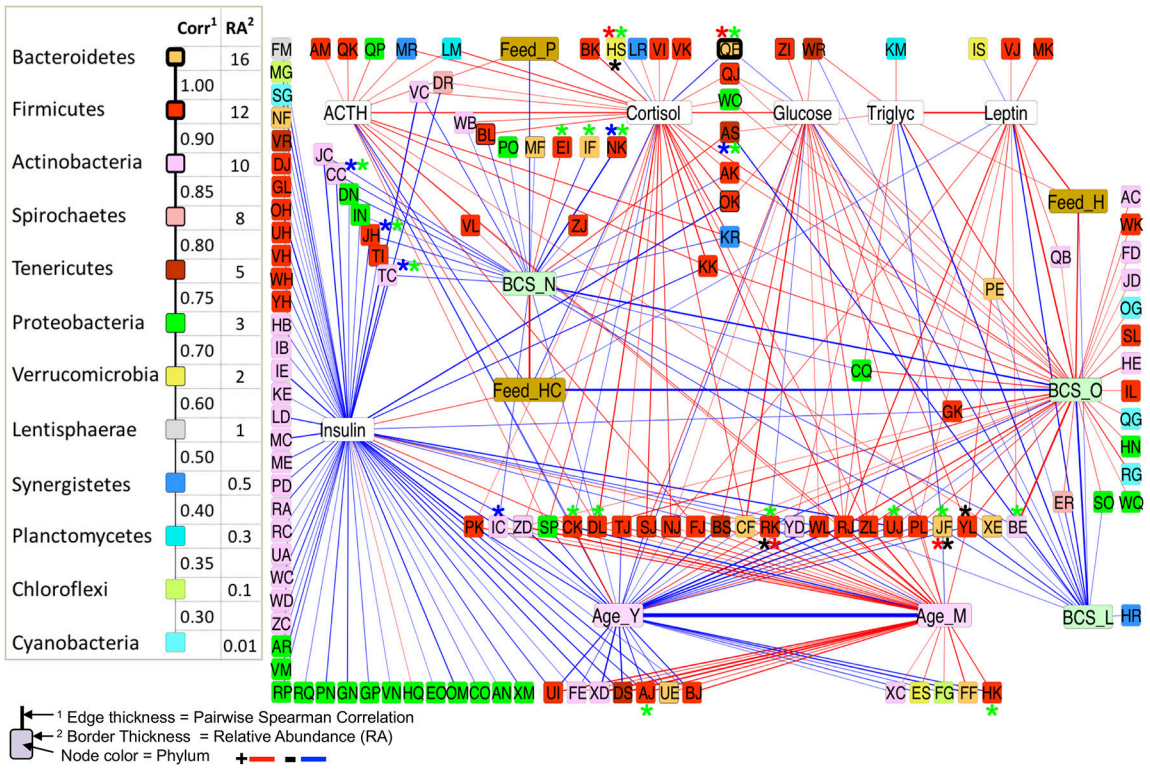


**FIGURE 6 |** First-neighbors networks of significant pairwise correlations for body condition score, blood analytes and age with differentially abundant (DA) taxa highlighted. Among All BCS*, between L/O horses*, L/N horses*, and O/N horses*.

positively associated with insulin and Feed_HC, and negatively correlated with glucose and Feed_P, while YL was positively correlated with glucose, leptin, Feed-H, BCS_O, and Age_M (middle aged), and negatively associated with Age_Y and BCS_L.

A network of bacteria containing only positive correlations with two or more blood analytes points to key taxa which are also associated with BCS_O and the older age group (**Figure 8**). This group contained nine Firmicutes, two Synergistetes, and one each of Bacteroidetes, Planctomycetes, and Proteobacteria. Of special interest were two taxa: *Firmicutes, Lachnospiraceae, Butyrivibrio,* and *Firmicutes, Lachnospiraceae, Other* which were positively correlated to two and four pairs of associations respectively.

## Analysis of Additional Metadata Factors

Significant correlations were found between owner and feed type, but not owner and BCS or any other blood analyte presumably due to consistence in management methods between farms. Three taxa were found to be uniquely correlated with owner: two Bacteroidetes (Rikenellaceae and Paraprevotellaceae, *YRC22*), and a Firmicutes (*Streptococcus* spp.) (**Table 6**). These were found in the dataset at 1.56, 0.012, and 0.845% respectively.

# DISCUSSION

This research compares the diversity and structure of gut microbiome communities of obese, lean, and normal horses, and correlates bacterial community assembly with blood analytes associated with obesity and metabolic issues in horses. The blood marker results (higher leptin, triglycerides, glucose, and cortisol levels, and trends toward higher insulin in obese horses) mirror what has been shown in other studies (45, 47, 57), but this is the first report correlating BCS, blood analytes, and microbial community composition in horses.

Similar to surveys of obese individuals in other systems, we report higher phylogenetic diversity and greater richness of bacteria in the gut microbiomes of the BCS_O horses (40, 41, 58). Specific Firmicutes groups (members of the Ruminococcaceae and Lachnospiraceae families) were positively correlated with two or more key blood analytes, increasing age, and obesity (**Figure 8**). While collectively this highly connected network of bacteria comprises <5% of the relative abundance of sequences in the data set, they could be providing beneficial metabolic products and ecosystem services.

We report obese BCS in horses to be positively correlated to four blood analytes: glucose, cortisol, triglycerides, and leptin, and lean BCS to be negatively correlated to glucose, tryglycerides, and leptin. These values were similar to prior studies in horses (27, 59, 60), and have been used in diagnostic panels for EMS. In humans, it has been estimated that the gut microbiome could explain 4.5–6% of the variation in BMI and triglyceride levels (61), specifically 114 taxa, 95 of which were members of Firmicutes (Lachnospiraceae, Ruminococcaceae,

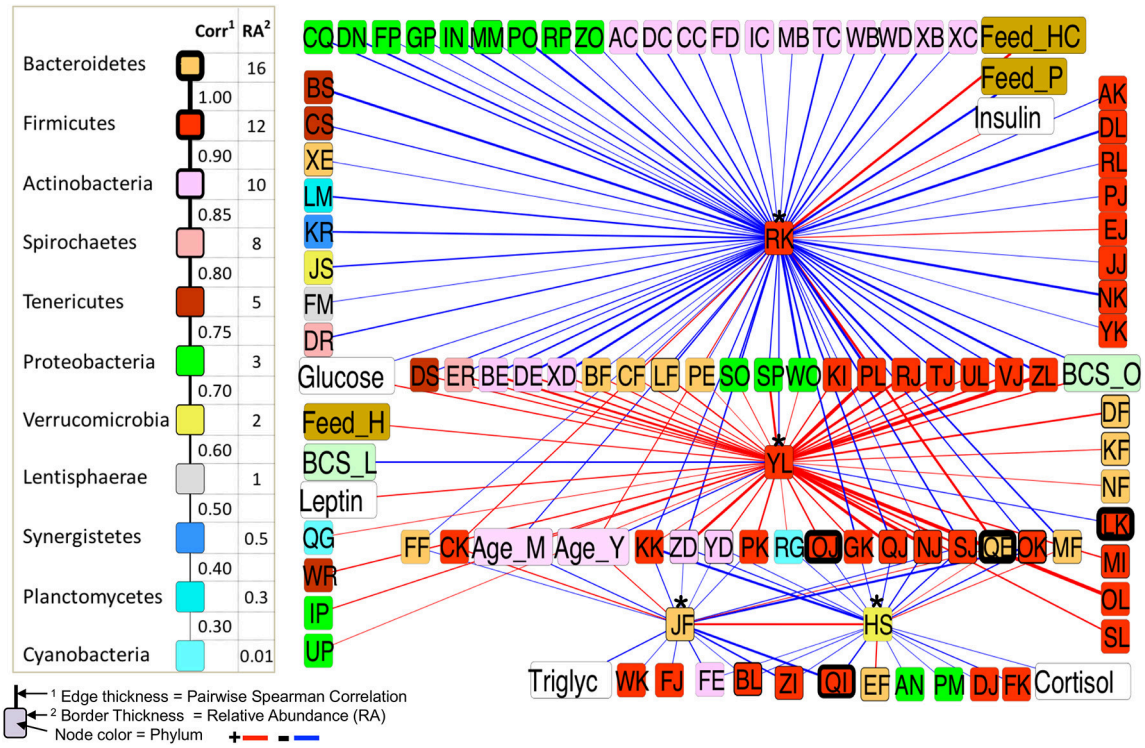


**FIGURE 7 |** First-neighbor network for Spearman significant pairwise correlations of all differentially abundant (DA) taxa among all BCS categories.

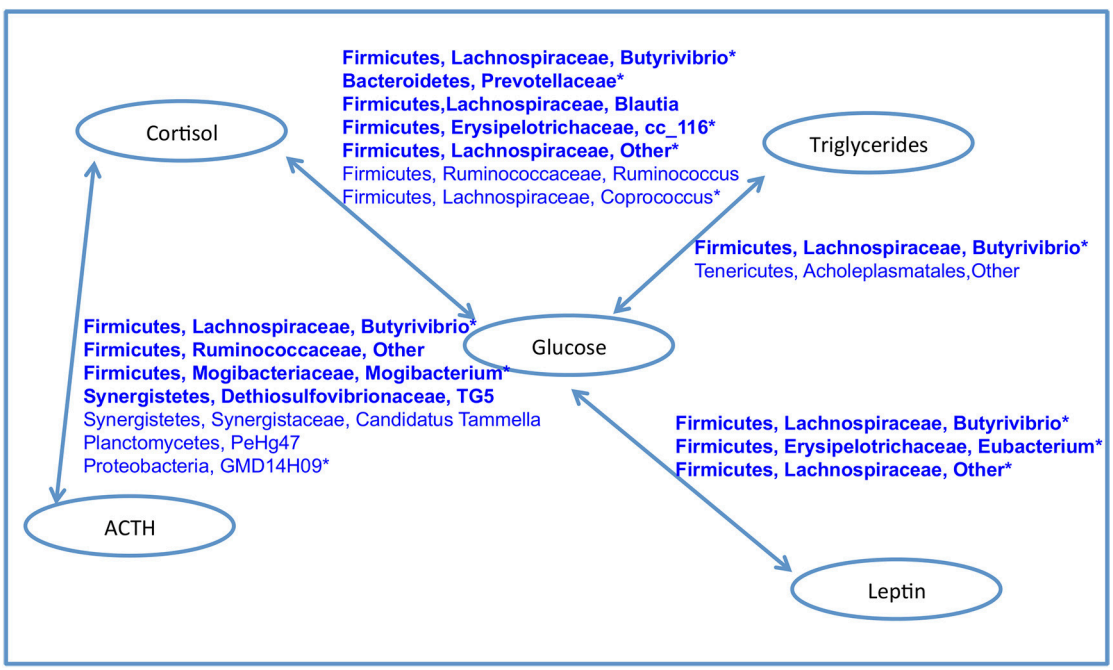


**FIGURE 8 |** Network of bacteria with positive associations with both blood analytes in each connected pair. Taxa in bold were positively correlated with BCS_O. Starred taxa were positively associated with the older age group. No bacterial taxa was positively associated with BCS_O, BCS_N and any pair of analytes. No bacterial taxa was positively with insulin and any other analyte.

**TABLE 6 |** Spearman correlations between owner, metadata factors and bacteria.

| Factor | Correlation |
|---|---|
| Age | 0.164 |
| Feed | 0.508* |
| BCS | 0.294 |
| Insulin | 0.113 |
| ACTH | −0.135 |
| Cortisol | −0.110 |
| Leptin | −0.223 |
| Glucose | 0.176 |
| Triglycerides | −0.007 |
| Bacteroidetes,Paraprevotellaceae,YRC22 (LF) | 0.332* |
| Bacteroidetes,Rikenellaceae (FF) | 0.332* |
| Firmicutes,Lactobacillales,Streptococcus (MI) | −0.367* |

*Only bacteria uniquely correlated with owner are shown. Significant correlations (coefficients >0.3 or <-0.3) are indicated *.*

Christensenellaceae, and others). While horses typically consume a relatively low fat diet, obese BCS gut microbiomes were found to be enriched in six triglyceride associated bacterial taxa, while the lean BCS group was not positively correlated with any of these taxa. Specific obesity related taxa from human studies were positively associated with obese BCS in this study, in particular *Campylobacter* spp., *Collinsella* spp., Prevotellaceae, *Selenomonas* spp., *Blautia* spp., and *Mogibacterium* spp. (62, 63), three taxa of Cyanobacteria, and *Adlercreutzi* spp. (64), four Erysipelotrichaceae taxa associated with obesity (65) and aromatic amino acid metabolism in high fat diet (66), and Dethiosulfovibrionaceae, a family of sulfate reducing bacteria (64, 66–68). That the normal and lean BCS groups were either negatively or not correlated with all of these taxa suggests distinguishing community differences in horses based on BCS, and points to similarities in host-microbial dynamics underlying metabolic disease between horses and humans.

At the same time, four taxa associated with healthy gut status were significantly correlated with obese BCS, specifically Propionibacteriaceae (propionate producer), *Butyrivibrio* spp. (butyrate producer), Ruminococcaceae (fiber degrader), and *Sutterella* spp. (function unclear) (62). *Butyrivibrio* spp. was of special interest because it was significantly correlated with all four pairs of blood analytes (**Figure 8**). While its abundance is <1% in the dataset, the high connectivity of this bacteria suggests that it could play an important role in host interactions, regulation, or immune status related to obesity.

The lack of correlation between resting insulin and bacterial taxa abundance found in this study reflected the difficulty in estimating blood insulin values using a resting measurement (27, 46), or suggested a more complex picture. Horses with high blood insulin and glucose levels are often, but not always obese (27, 39, 47). A more complete model of the gut microbiome and insulin dynamics would be possible by comparing the microbiomes of both lean and obese horses with a wider range of insulin levels.

While gut microbiome differences were seen in horses based on diet, it was not possible to associate feed with BCS as it is a driver for management decisions, especially given the relatively small numbers of owners and the consistency of their feeding patterns. The obese BCS horses were largely being fed hay or pasture only, and the lean and normal BCS horses were consuming hay/concentrate, resulting in a significant association between owner and feed (**Table 6**). Significant correlations were also noted based on age, but were inconclusive since the categories were broadly divided and included no horse above 20 years. Managing older horses will continue to be a challenge in the future as the numbers of aged horses increases, therefore future work to identify bacteria correlated with obesity and blood markers associated with age-related metabolic issues is warranted.

This research points to differences in the gut microbiomes of lean, normal, and obese horses that are significantly correlated to key blood analytes associated with BCS. Network analysis points to signature species for each body condition category, laying the foundation for experiments leading to a mechanistic understanding, and more targeted microbial solutions to the issue of obesity and metabolic syndrome in horses.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the USDA Animal Welfare Act and the NIH Public Health Service Policy on the Humane Care and Use of Animals, University of Illinois Institutional Animal Care and Use Committee (IACUC). The protocol was approved by the University of Illinois Institutional Animal Care and Use Committee (IACUC).

## AUTHOR CONTRIBUTIONS

AB obtained and prepared blood and fecal samples, gained funding, analyzed 16S rRNA sequence data, and wrote the manuscript. J-FT and ZF performed the pairwise correlation analysis in JMP and prepared the Cytoscape networks.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2018.00225/full#supplementary-material

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# A Cohort Study of the Milk Microbiota of Healthy and Inflamed Bovine Mammary Glands From Dryoff Through 150 Days in Milk

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The objective of this longitudinal cohort study was to describe the milk microbiota of dairy cow mammary glands based on inflammation status before and after the dry period. Individual mammary quarters were assigned to cohorts based on culture results and somatic cell count (**SCC**) at dryoff and twice in the first 2 weeks post-calving. Mammary glands that were microbiologically negative and had low SCC (<100,000 cells/mL) at all 3 sampling periods were classified as Healthy ($n = 80$). Microbiologically negative mammary glands that had SCC ≥150,000 cells/mL at dryoff and the first post-calving sample were classified as Chronic Culture-Negative Inflammation (CHRON; $n = 17$). Quarters that did not have both culture-negative milk and SCC ≥ 150,000 cells/mL at dryoff but were culture-negative with SCC ≥ 150,000 at both post-calving sampling periods were classified as Culture-Negative New Inflammation (NEWINF; $n = 6$). Mammary glands with bacterial growth and SCC ≥ 150,000 cells/mL at all 3 periods were classified as Positive (POS; $n = 3$). Milk samples were collected from all enrolled quarters until 150 days in milk and subjected to microbiota analysis. Milk samples underwent total DNA extraction, a 40-cycle PCR to amplify the V4 region of the bacterial 16S rRNA gene, and next-generation sequencing. Healthy quarters had the lowest rate of PCR and sequencing success (53, 67, 83, and 67% for Healthy, CHRON, NEWINF, and POS, respectively). Chao richness was greatest in milk collected from Healthy quarters and Shannon diversity was greater in milk from Healthy and CHRON quarters than in milk collected from glands in the NEWINF or POS cohorts. Regardless of cohort, season was associated with both richness and diversity, but stage of lactation was not. The most prevalent OTUs included typical gut- and skin-associated bacteria such as those in the phylum Bacteroidetes and the genera *Enhydrobacter* and *Corynebacterium*. The increased sequencing success in quarters with worse health outcomes, combined with the lack of bacterial growth in most samples and the high PCR cycle number required for amplification of bacterial DNA, suggests that the milk microbiota of culture-negative, healthy mammary glands is less abundant than that of culture-negative glands with a history of inflammation.

**Keywords: milk microbiome, milk microbiota, mastitis, somatic cell count, 16S sequencing**

## INTRODUCTION

Mastitis, or inflammation of the mammary gland, is a common disease of dairy cattle that causes decreased milk production (1, 2). Mastitis is characterized based on the magnitude of the inflammatory response and is classified as clinical mastitis (**CM**), when milk or the udder has visible abnormalities; or subclinical mastitis (**SM**), when milk appears visually normal but the somatic cell count (**SCC**) exceeds normal levels. Producers often collect milk samples from affected mammary glands for culture-based microbiological analysis to determine the etiology (3), but many samples result in no growth in aerobic culture (4–6). As such, culture-negative, visually normal milk with a low SCC (<100,000 cells/mL) is often considered healthy and was previously considered sterile due to the lack of growth in culture (7).

In recent years, as researchers have identified the importance of the microbiota in other dairy cow organ systems (8, 9), culture-independent sequencing techniques have been applied to culture-negative milk samples with the objective of determining if milk has a microbiota similar to that of other systems and if the milk contains pathogens undetectable by traditional means. These reports indicate that healthy milk contains DNA from bacteria not previously associated with milk, such as members of the family Lachnospiraceae and genera *Faecalibacterium* (10, 11) and *Enhydrobacter* (12). Early studies also suggest that milk samples obtained from presumably healthy mammary glands have greater bacterial richness and diversity, as compared to the microbiota of milk collected from glands experiencing CM (10, 13, 14). However, the concept of a healthy milk microbiota has been questioned due to the physiology of the mammary gland and the low concentration of viable bacteria or bacterial DNA in milk collected from apparently healthy glands (15).

In intensive dairy systems of the Northern Hemisphere, the risk of mastitis is associated with cow characteristics such as parity (older cows are at greater risk), stage of lactation (earlier lactation has greater risk), and season (cows are at greater risk in summer) (16–18) and the associated microbiota composition may also be correlated to these factors. However, prior reports of the milk microbiota have often not included descriptions of the cow population or environment (10, 13, 14). For example, bedding is a major source of bacterial exposure for the mammary gland, and a cross-sectional study of the milk microbiota in relation to bedding type found that, although diversity did not differ by bedding type, there were differences with respect to overall community composition (12). Age is also another likely factor, as older cows are more susceptible to mastitis (16, 18), and milk samples collected from glands with CM often have lower bacterial richness and diversity than milk samples collected from apparently healthy glands; however, associations between parity and microbiota status are yet unknown.

A significant challenge in understanding the milk microbiota, as it relates to mastitis, is that previous work has not tracked this microbiota longitudinally, even though mastitis is a temporal condition. In other mammals such as humans, the milk microbiota has been reported to change across the first 6 months of lactation (19), but the longest study reported to date of the milk microbiota of cows is 2 weeks (14). In that study, the richness and diversity of apparently healthy milk did not change across those 2 weeks, but the SCC and culture results of the milk were not reported (14), rendering the actual health status of the mammary quarters indeterminate. To address this apparent gap in knowledge, we undertook a prospective longitudinal cohort study to describe the milk microbiota from bovine mammary quarters from dryoff through the first 150 days of the next lactation. Somatic cell count and microbiological status were assessed for each of these quarters prior to dryoff and milk samples were subjected to 16S rDNA microbiota sequencing. Our study represents the first extensive longitudinal study of the milk microbiota.

## MATERIALS AND METHODS

### Quarter Selection and Enrollment

This study was approved and performed based on University of Wisconsin-Madison IACUC protocol A01548-08-13. All mammary gland quarters of all cows completing a lactation at the University of Wisconsin-Madison dairy research herd were screened for potential enrollment at the final milking prior to dryoff (**DR**), at 4–7 days in milk (**DIM**) of the next lactation (**C1**), and again at 11-14 DIM (**C2**). Milk samples were collected by researchers for microbiological analysis and SCC determination (CombiFOSS 6000, Foss Food Technology Corp., Hillerød, Denmark). Milk samples from quarters enrolled into predefined cohorts were collected weekly for SCC and every 4 weeks for microbiota analysis until the cows reached 150 DIM. Individual quarters within a cow were assigned to cohorts based on SCC and culture status at the DR, C1, and C2 samples (**Table S1**). Quarters that had a low SCC (< 100,000 cells/mL) and no growth at all 3 sampling periods were assigned to the Healthy cohort. Quarters with SCC ≥ 150,000 cells/mL but no bacterial growth from milk samples collected at DR and C1 were classified as chronically inflamed (CHRON). Quarters with a variable DR sample and SCC ≥ 150,000 cells/mL without bacterial growth in milk samples collected at both C1 and C2 were classified as having new culture-negative inflammation (NEWINF). Quarters with bacterial growth and SCC ≥ 150,000 cells/mL at DR, C1, and C2 were classified as positive (POS). After quarters were enrolled in a cohort, weekly milk samples were collected for determination of SCC and aseptic milk samples were collected for microbiological analysis monthly until quarters were 150 DIM (**M2**, **M3**, **M4**, and **M5**). The herd used computerized health records for all animals (Dairy Comp 305, Valley Agricultural Systems, Tulare, CA) and lactating cows were housed in sand-bedded freestall barns. Some cows ($n = 12$) were transferred to a different sawdust-bedded tiestall facility within the same herd from calving through early lactation. Sample collection began in February 2014 and ended in July 2015.

### Milk Sample Collection and Culturing

Milk samples were aseptically collected by researchers in the milking parlor immediately prior to the morning milking. Research personnel wiped udders with a clean, dry cloth to remove bedding and visible contaminants. Milking personnel

then performed standard pre-milking sanitation: 2–3 streams of foremilk per teat were discarded, then a 0.5% iodine predip solution (Theratec Plus, GEA, Columbia, MD) was applied, 30–60 s contact time was allowed, and teats were dried with a dry cloth towel. The researcher then put on clean nitrile gloves, discarded 2–3 streams of milk, scrubbed the teat with 70% isopropanol, allowed the isopropanol to dry, discarded another 2–3 streams of milk, and collected approximately 40 mL milk into a sterile sample vial. Next, 40 mL milk was collected into a nonsterile plastic vial containing a bronopol tablet for later SCC analysis. New gloves were used for aseptic collection of milk from each mammary gland. In accordance with normal herd health protocols, after the dryoff milking, every quarter was infused with an intramammary antimicrobial containing 1,000,000 IU penicillin and 1.0 g dihydrostreptomycin (Quartermaster, West Agro, Inc., Hamilton, NY) and administered a teat sealant containing 4 g bismuth subnitrate (Orbeseal, Zoetis, Parsippany, NJ).

All milk samples were placed on ice and transported to the laboratory to be cultured within 12 h of collection following National Mastitis Council procedures (20). One hundred microliter of milk were inoculated on half of a plate containing trypticase soy agar with 5% sheep blood (BD, Franklin Lakes, NJ) and half of a MacConkey agar plate (BD, Franklin Lakes, NJ). Inoculated agars were incubated aerobically at 37°C for 48 h. Samples with 0 or 1 colony at 48 h were considered negative. Samples with >2 colonies of a single type were considered positive and subjected to further biochemical testing. Samples with more than 2 colony types, regardless of the number of colonies, were considered contaminated (20). For positive samples, a single colony was selected for biochemical testing. Colonies were Gram-stained and examined with brightfield microscopy for cellular morphology. Gram-positive colonies were tested for catalase production; catalase-positive colonies were then tested for coagulase production and mannitol salt agar reaction while catalase-negative colonies were tested for triple sugar iron agar reaction, citrate utilization, motility, indole production, and ornithine production.

## DNA Extraction, PCR, and Sequencing

DNA was extracted from milk samples using buffers from a QIAamp DNA Stool Mini Kit (Qiagen, Frederick, MD) as previously described (12). Four milliliters whole milk was centrifuged at 13,000 × *g* for 20 min at 4°C. The milk fat layer and supernatant were discarded. Buffer ASL was added and samples were frozen and thawed 5× with liquid $N_2$ and a 37°C water bath. Samples were then incubated with lysozyme for 30 min in a 37°C water bath. Next, Proteinase K and Buffer ASL were added for 10 min and then absolute ethanol was added. Samples were transferred to spin columns, washed with Buffer PE, and eluted with Buffer AE. Eluted DNA was lyophilized, suspended in 20 μL nuclease-free water, and quantified using a Qubit (ThermoFisher, Waltham, MA).

DNA was diluted to 0.625 ng/μL and 8 μL DNA was added to each PCR reaction along with 6.6 μL Phusion Master Mix (New England BioLabs, Ipswich, MA), 1.0 μL of 10 μM forward universal bacterial barcoded primer (5′-GTGCCAGCMGCCGCGGTAA-3′), and 1.0 μL of 10 μM reverse universal bacterial barcoded primer (5′-GGACTACHVGGGTWTCTAAT-3′) for the 16S V4 region. Nuclease-free water was added to bring the reaction volume to 20 μL. The initial PCR denaturing step was 30 s at 98°C, and was followed by 8 s of denaturing at 98°C, 20 s of annealing at 58°C, and a 20-s extension step at 72°C. Forty PCR cycles were performed, and the PCR was completed with 5 min of extension at 72°C. Negative controls of nuclease-free water were subjected to PCR and sequencing along with experimental samples.

All PCR products were visualized on an agarose gel. Visible bands were excised from the gel and extracted with a Zymoclean gel DNA recovery kit (Zymo Research, Irvine, CA). After gel extraction, DNA was quantified with high-sensitivity Qubit reagents and pooled at equimolar concentrations prior to sequencing with 10% PhiX control DNA on an Illumina MiSeq (San Diego, CA) (9). Raw sequences were obtained in fastq format and cleaned using mothur v 1.38.1 (21) as described previously (12). Sequences were aligned to the SILVA 16S rRNA gene reference database (Release 128) (22). All sequences have been deposited in the National Center for Biotechnology Information's Sequence Read Archive under BioProject ID PRJNA478482.

## Statistical Analysis

Individual mammary quarters were the experimental units analyzed using SAS 9.4 (Cary, NC). Sample collection dates were used to classify data by season (fall: Sept.-Nov.; winter: Dec.-Feb.; spring: Mar.-May; summer: Jun.-Aug.). Parity was based on lactation at the subsequent calving (not DR). Sequencing success was defined as having a visible PCR amplicon band that produced sequence reads on an Illumina MiSeq. To test the hypothesis that sequencing success did not differ among cohorts, a logistic model was constructed in Proc LOGISTIC with sequencing success as the outcome variable. The explanatory variables parity (2, 3, 4–7), facility at time of sampling (sand-bedded, sawdust-bedded), sampling period (DR, C1, C2, M2, M3, M4, M5), and season (fall, winter, spring, summer) were tested for significance and variables with greatest *P*-values were eliminated in a backwards stepwise manner until only variables with *P*-values $\leq 0.05$ remained. To test the hypotheses that Chao richness or Shannon diversity did not differ among cohorts, regression models were constructed in Proc MIXED with the same explanatory variables and backwards stepwise elimination as in the logistic model. Canonical discriminant analysis was performed in Proc CANDISC using the relative abundances of operational taxonomic units (**OTUs**).

# RESULTS

## Herd Characteristics and Enrolled Quarters

The herd consisted of approximately 665 lactating cows with a rolling herd average production of 12,100 kg milk and a bulk tank SCC of approximately 180,000 cells/mL. A total of 1,078 quarters from 270 cows were screened for enrollment at dryoff and twice in the first 2 weeks of the next lactation. Because the first sample period was at the final milking of a

lactation, every quarter was parity 2 or greater at the subsequent calving. No cows were sampled during the first 150 DIM of the first lactation. Twelve cows (16 enrolled quarters) were transferred to the sawdust-bedded facility within the university herd for part of the subsequent lactation. The Healthy cohort contained 80 quarters from 55 cows, the CHRON cohort had 17 quarters from 14 cows, the NEWINF cohort had 6 quarters from 6 cows, and the POS cohort had 3 quarters from 3 cows (**Table 1**). Quarters enrolled to the Healthy cohort were from cows that were younger than cows enrolled to the CHRON, NEWINF, or POS cohorts (**Table 1**). Milk collected from cows in the Healthy cohort had the lowest SCC throughout lactation, while milk from cows enrolled in the CHRON cohort had low SCC after the C1 sample. Milk from cows enrolled in both the NEWINF and POS cohorts had increased SCC throughout the enrollment and follow-up periods ($P < 0.0001$) (**Figure S1**) with more CM ($P < 0.0001$) (**Figure S2**). Three cows, each with one quarter in the Healthy cohort, one cow with one quarter in the CHRON cohort, and a cow with one quarter from the NEWINF cohort died during the study period.

## DNA Extraction and PCR Success

In total, 723 milk samples were collected for microbiota analysis. Seventeen samples had insufficient sample volume for DNA extraction and 14 samples collected on a single day were erroneously discarded prior to extraction. Of the 692 samples subjected to PCR and sequencing, 397 (57.4%) were successful (**Table 2**). Sequencing success ranged from 53.2% in Healthy samples to 83.3% in NEWINF samples ($P < 0.001$) (**Figure 1**). Milk samples from NEWINF and CHRON quarters were 4.56 times more likely (95% CI: 1.85–11.2) and 1.79 times more likely (95% CI: 1.16–2.77) to have sequencing success, as compared to milk samples collected from Healthy quarters ($P < 0.001$) (**Table S2**). The odds of successful sequencing were greater for milk samples collected at M5 as compared to milk samples collected at any other time point ($P = 0.044$) (**Table S2**).

## Microbiota

### Sequences and Diversity

A total of 26,442,947 raw reads were generated for an average of 66,606 reads per sample. 10,256,631 reads (25,835 reads/sample) were retained after cleanup. Due to the large number of PCR cycles required to amplify DNA, reads matching those found in our negative controls (5 total OTUs) were removed from all sample results (**Figure S3**). Data were then normalized to 3,000 sequences per sample, which represents the lowest sequence amount for all samples, and a sequence clustering analysis of the normalized samples produced 11,304 OTUs. Chao richness was determined for these samples with cohort, season, and parity group retained in the final regression model due to significance, while sampling period was forced into the model. Chao richness was greater in Healthy quarters than other quarters ($P = 0.016$) and did not differ based on sampling period ($P = 0.38$) (**Figure 2A**) but increased from winter to summer ($P = < 0.0001$) (**Figure 2B**). Shannon diversity was greater in milk samples collected from Healthy and CHRON quarters as compared to milk samples collected from NEWINF and POS quarters ($P = 0.0019$). Stage of lactation was not associated

**TABLE 2 |** Sequencing success by cohort and sampling period.

| | DR | C1 | C2 | M2 | M3 | M4 | M5 |
|---|---|---|---|---|---|---|---|
| Healthy | 39/79 | 44/73 | 46/77 | 30/71 | 46/76 | 29/75 | 44/72 |
| CHRON | 13/17 | 9/15 | 9/17 | 10/15 | 13/17 | 9/15 | 12/16 |
| NEWINF | 3/4 | 6/6 | 3/5 | 5/5 | 5/6 | 4/6 | 4/4 |
| POS | 2/3 | 2/3 | 1/3 | 3/3 | 1/3 | 3/3 | 2/3 |

*Healthy, Quarters (n = 80) that had culture-negative milk with SCC < 100,000 cells/mL at DR, C1, and C2; CHRON, Quarters (n = 17) that had culture-negative milk samples with SCC ≥ 150,000 cells/mL at DR and C1; NEWINF, Quarters (n = 6) that had new culture-negative inflammation with variable DR milk sample results and culture-negative milk samples with SCC ≥ 150,000 cells/mL at C1 and C2; POS, Quarters (n = 3) that had bacterial growth in culture and SCC ≥ 150,000 cells/mL at DR, C1, and C2; Sampling periods, DR, dryoff, C1, first week of lactation, C2, second week of lactation, M2, second month of lactation, M3, third month of lactation, M4, fourth month of lactation, M5, fifth month of lactation.*

**TABLE 1 |** Descriptive statistics of enrolled bovine mammary glands.

| Cohort | *n* | Parity | Previous clinical mastitis[a] (*n*) | DR[b] SCC[c] | C1 SCC | C2 SCC | Incident clinical mastitis cases (*n*)[d] | Incident positive monthly culture cases (*n*)[e] |
|---|---|---|---|---|---|---|---|---|
| Healthy | 80 | 3 | 5 | 4.45 | 4.46 | 4.17 | 2 | 8 |
| CHRON | 17 | 4 | 5 | 5.72 | 5.45 | 4.68 | 3 | 6 |
| NEWINF | 6 | 4 | 2 | 5.42 | 5.80 | 5.69 | 3 | 6 |
| POS | 3 | 5 | 0 | 5.69 | 5.98 | 5.96 | 1 | 3 |
| Total | 106 | 4 | 12 | 5.66 | 5.59 | 5.06 | 9 | 23 |

[a] *Quarters that were treated for clinical mastitis with an intramammary antimicrobial during the previous lactation according to herd records.*
[b] *DR, dryoff, C1, first week of lactation, C2, second week of lactation.*
[c] *All values are the $log_{10}$SCC.*
[d] *Number of quarters that developed clinical mastitis during the follow-up period according to herd records.*
[e] *Number of quarters that had an aseptic milk sample with positive bacterial growth at one or more monthly milk samples.*
*Healthy, Quarters that had culture-negative milk with SCC < 100,000 cells/mL at DR, C1, and C2; CHRON, Chronically inflamed quarters that had culture-negative milk samples with SCC ≥ 150,000 cells/mL at DR and C1; NEWINF, Quarters that had new culture-negative inflammation with variable DR milk sample results and culture-negative milk samples with SCC ≥ 150,000 cells/mL at C1 and C2; POS, Quarters that had bacterial growth in culture and SCC ≥ 1,50,000 cells/mL at DR, C1, and C2.*

with Shannon diversity ($P = 0.77$) (**Figure 2C**) and Shannon diversity tended to be greater in spring than in winter ($P = 0.089$) (**Figure 2D**).

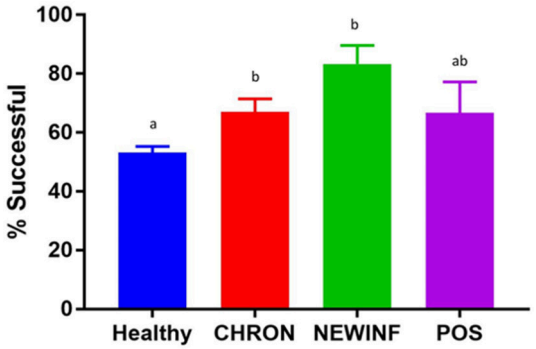


**FIGURE 1 |** Proportion of successful PCR and sequencing by cohort. Healthy, Quarters ($n = 80$) that had culture-negative milk with SCC < 100,000 cells/mL at DR, C1, and C2. CHRON, Quarters ($n = 17$) that had culture-negative milk samples with SCC ≥ 150,000 cells/mL at DR and C1; NEWINF, Quarters ($n = 6$) that had new culture-negative inflammation with variable DR milk sample results and culture-negative milk samples with SCC ≥ 150,000 cells/mL at C1 and C2; POS, Quarters ($n = 3$) that had bacterial growth in culture and SCC ≥ 150,000 cells/mL at DR, C1, and C2; Sampling periods, DR, dryoff, C1; first week of lactation; C2, second week of lactation. a,b Columns with different superscripts differ ($P < 0.05$).

## Prevalent OTUs

The 142 most common OTUs comprised 99.9% of the total sequences across all samples (**Figure 3**). Among the top OTUs, g_*Staphylococcus* ($P = < 0.0001$), g_*Knoellia* ($P = 0.041$), f_*Aerococcaceae* ($P = 0.0083$), and g_*Coxiella* ($P < 0.0001$) were associated with cohort (**Table 3**; **Figure 4**). The Positive cohort had the greatest prevalence of *Staphylococcus* sequences, with 16% of the sequences belonging to this genus. In contrast, only 0.75% of the sequences in the Healthy samples were classified to the genus *Staphylococcus* (**Figure 4**). The prevalence of *Coxiella* sequences was greater in New Inflammation quarters (6.8%) than in any other cohort (0.13–1.2%) (**Table 3**; **Figure 4**). In sum, 11 of the top 20 OTUs comprising 1% or more of the total sequences varied seasonally (**Table 3**). These OTUs included unclassified Bacteroidetes ($P = 0.0002$) and *Enhydrobacter* ($P = 0.0035$) (**Figure 4**). Overall community composition varied seasonally within Healthy quarters and also within CHRON quarters (**Figure 5**). The prevalence of some OTUs varied by stage of lactation, but this variation was less significant than the variation associated with season (**Table 3**). Among the 3 inflamed cohorts (CHRON, NEWINF, and POS), 105 of the top 142 OTUs were found in all cohorts and only the CHRON cohort had unique OTUs (**Figure 6**).

## Culture and Sequencing

All samples from the Healthy cohort were culture-negative at DR, C1, and C2. All CHRON samples were culture-negative at

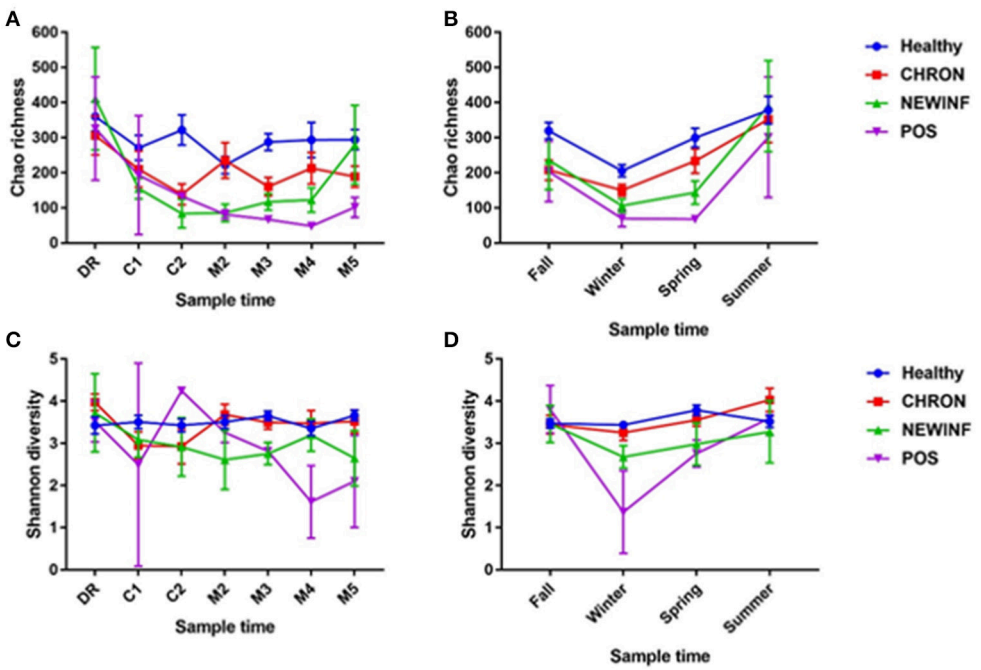


**FIGURE 2 |** Richness across lactation **(A)** and by season **(B)** and diversity across lactation **(C)** and by season **(D)** for each cohort. Healthy, Quarters that had culture-negative milk with SCC < 100,000 cells/mL at DR, C1, and C2. CHRON, Chronically inflamed quarters that had culture-negative milk samples with SCC ≥ 150,000 cells/mL at DR and C1. NEWINF, Quarters that had new culture-negative inflammation with variable DR milk sample results and culture-negative milk samples with SCC ≥ 150,000 cells/mL at C1 and C2. POS, Quarters that had bacterial growth in culture and SCC ≥ 150,000 cells/mL at DR, C1, and C2. DR, dryoff; C1, first week of lactation; C2, second week of lactation; M2, second month of lactation; M3, third month of lactation; M4, fourth month of lactation; M5, fifth month of lactation.

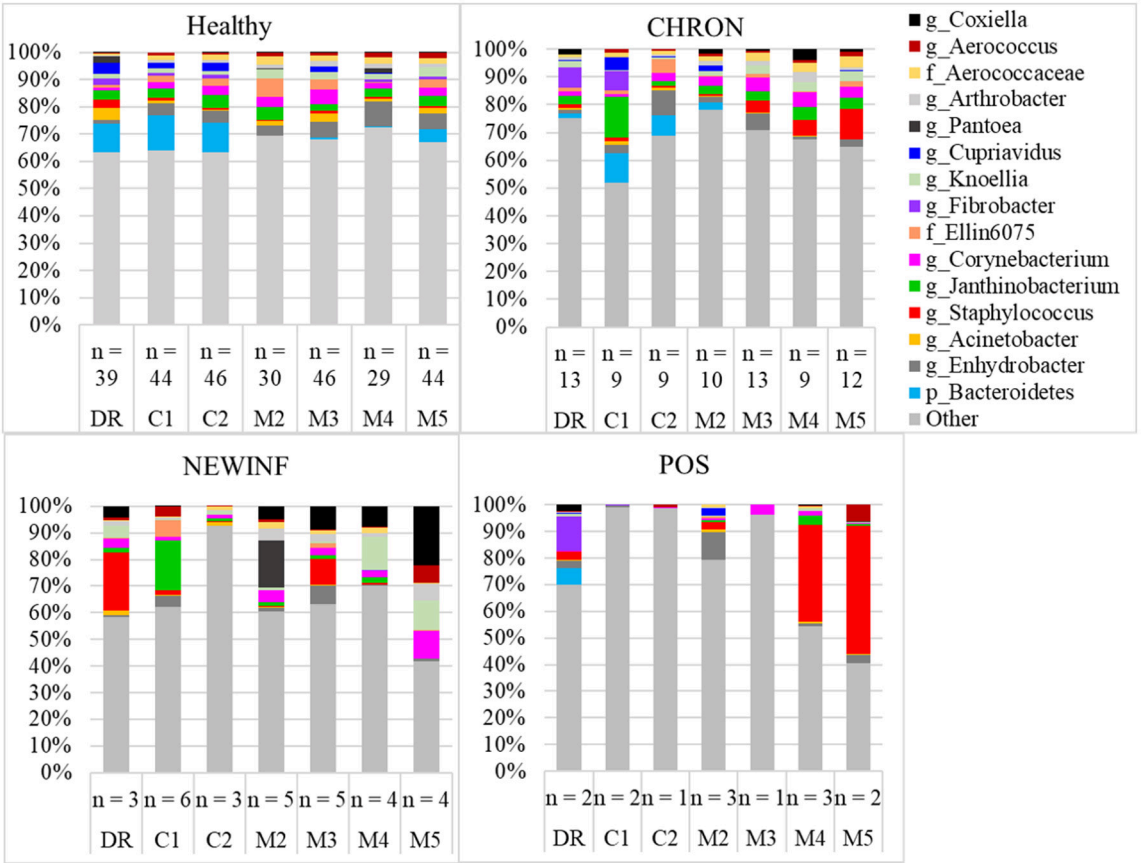


**FIGURE 3 |** Relative abundance of OTUs in milk samples from each cohort. Healthy, Quarters that had culture-negative milk with SCC < 100,000 cells/mL at DR, C1, and C2; CHRON, Chronically inflamed quarters that had culture-negative milk samples with SCC ≥ 150,000 cells/mL at DR and C1; NEWINF, Quarters that had new culture-negative inflammation with variable DR milk sample results and culture-negative milk samples with SCC ≥ 150,000 cells/mL at C1 and C2; POS, Quarters that had bacterial growth in culture and SCC ≥ 150,000 cells/mL at DR, C1, and C2; DR, dryoff, C1, first week of lactation, C2, second week of lactation, M2, second month of lactation, M3, third month of lactation, M4, fourth month of lactation, M5, fifth month of lactation.

DR and C1, and all NEWINF samples were culture-negative at C1 and C2. The samples from the POS cohort were culture-positive at all 3 enrollment samples. Of the 56 culture-positive milk samples, 40 were successfully sequenced (71.4%). Of these 40, 3 had growth of yeast in culture and were not subjected to a comparison of sequencing results and culture results. For milk samples with positive bacterial growth in culture, culture and sequencing results were generally in agreement, with one of the top 5 OTUs matching culture results for 24 of 37 (64.9%) culture-positive milk samples. Only 3 (8.1%) milk samples did not have the cultured bacteria represented in the top 20 OTUs detected from sequencing.

## DISCUSSION

To our knowledge, this is the most extensive longitudinal study of the dairy cow milk microbiota to date, as other studies have only monitored animals from dryoff to 7 DIM (23) or for 2 weeks during lactation (14). Here, we sampled cows from a sand-bedded university research herd that is similar in size and production to an average Wisconsin, USA dairy farm (24). Dairy cows spend a significant portion of their time lying down with their udders in contact with bedding (25), which is known to be densely populated with bacteria (26, 27). Sand is a commonly used bedding material that generally has lower bacterial counts in culture than other bedding materials, such as manure solids, due to the lower amount of available organic matter (12, 26). As expected, the Healthy cohort quarters had very low SCC throughout lactation and a low incidence of CM (16), providing us with an opportunity to study the microbiota of healthy milk through the first half of lactation. This observational study was conducted in a low-SCC herd, which resulted in the uneven distribution of quarters into cohorts.

Importantly, we cultured a large volume of milk (100 μL) because we wanted to maximize specificity when determining which samples were culture-negative (28), rather than diagnose intramammary infection. Previous reports did not culture all milk samples used for microbiota analysis (14, 29). Culturing is an important way to verify milk samples are not contaminated, as sampling within a milking parlor or barn is difficult with contamination rates in mastitis studies reaching nearly 20%

**TABLE 3 |** *P*-values for top OTU associations with Cohort, Season, or Sample Period.

| OTU | *P*-value | | |
|---|---|---|---|
| | Cohort | Season | Sample period |
| p_Bacteroidetes | * | **** | *** |
| g_Enhydrobacter | * | ** | * |
| g_Acinetobacter | * | * | * |
| g_Staphylococcus | **** | * | * |
| g_Janthinobacterium | * | * | * |
| g_Corynebacterium | * | **** | ** |
| f_Ellin6075 | * | **** | ** |
| g_Fibrobacter | * | **** | * |
| g_Knoellia | ** | ** | ** |
| g_Cupriavidus | * | ** | *** |
| g_Pantoea | * | * | * |
| g_Arthrobacter | * | **** | * |
| f_Aerococcaceae | *** | *** | * |
| g_Aerococcus | * | * | ** |
| g_Coxiella | **** | * | * |
| f_Rhodocyclaceae | * | **** | **** |
| o_Solibacterales | * | *** | * |
| g_Brevundimonas | * | ** | * |
| g_Psychrobacter | * | * | * |
| g_Burkholderia | * | * | * |

*$P > 0.05$.
**$0.05 \geq P > 0.01$.
***$0.01 \geq P > 0.001$.
****$P \leq 0.001$.
*Cohort, Healthy, Quarters (n = 80) that had culture-negative milk with SCC < 100,000 cells/mL at DR, C1, and C2; CHRON, Quarters (n = 17) that had culture-negative milk samples with SCC ≥ 150,000 cells/mL at DR and C1; NEWINF, Quarters (n = 6) that had new culture-negative inflammation with variable DR milk sample results and culture-negative milk samples with SCC ≥ 150,000 cells/mL at C1 and C2; POS, Quarters (n = 3) that had bacterial growth in culture and SCC ≥ 150,000 cells/mL at DR, C1, and C2; Sampling periods, DR, dryoff, C1, first week of lactation, C2, second week of lactation, M2, second month of lactation, M3, third month of lactation, M4, fourth month of lactation, M5, fifth month of lactation.*

(4, 6). The culturing methods recommended by the National Mastitis Council are limited to aerobic cultures near bovine physiological temperature for up to 48 h (3, 20). We could not determine whether samples were culture-negative because the bacteria are unculturable in our culture conditions, or whether the samples were culture-negative because the bacteria we detected with sequencing were non-viable. Many of the most prevalent OTUs found in this and other milk microbiota studies will not grow in commonly used culture conditions and may require colder temperatures (30) or additional nutrients not found in milk (31). Future milk microbiota research could incorporate additional culturing methods to determine if some bacteria detected using sequencing are viable when cultured using appropriate conditions. For example, many of the OTUs we detected likely have a slow doubling time at the normal temperature found within the bovine mammary gland (12, 32).

The lack of growth in culture, combined with our low sequencing success rate indicates low concentrations of bacterial DNA in our milk, which supports the theory that the milk microbiota is not a highly prolific bacterial community (15). The high cycle number required to achieve amplification is similar to the 35 (14, 29) and identical to the 40 (12, 33) cycles used in previous studies. The 53% sequencing success rate for our conventionally collected healthy milk samples is similar to the 40% sequencing success rate in our previous study (12). A previous study also reported difficulty in amplifying DNA from milk but did not report the rate of sequencing success (29), which would have been a valuable metric for comparison. We suggest that this metric should be reported in future studies. The greater sequencing success in milk samples collected from quarters with a history of inflammation and high cycle number required for PCR suggests that the healthy milk microbiota is minimal and does not have the high bacterial abundances found in other bovine sites. Bacteria in the rumen are found at approximately $2.7 \times 10^9$ cfu/g in rumen liquids and up to $5.6 \times 10^{11}$ cfu/g in rumen solids (8). This density far outnumbers that of individual quarter-milk samples, although one study reported $10^2$ to $10^5$ copies of the 16S rRNA gene in milk but did not specify the concentration of the gene copies (14). With such low concentrations of bacterial DNA, we may be detecting DNA that has been imported to the mammary gland within leukocytes (33), DNA from bacteria that were phagocytosed by leukocytes upon infiltrating the mammary gland via the teat canal, or DNA from bacteria that are trapped within the keratin of teat canal (15).

Seasonal changes in richness, tendencies toward seasonal changes in diversity, and overall community composition changes across seasons implicate bedding as a potential source of exposure for bacterial DNA found in milk. Bacteria counts in cultured bedding samples are often greater in summer than in winter (27, 34). Previous studies have also reported that bacterial richness increases in summer and that greater richness is often associated with improved health status (10, 14, 35), but mastitis incidence often increases in summer, likely due to increased exposure to pathogens (18). Similar seasonal trends observed in our cohorts support an external source of bacterial DNA found in the mammary gland that is universal among cows, regardless of inflammation status, as all cows were housed on the same farm and exposed to similar environmental conditions. Exposure to bacteria in bedding has been shown to be associated with milk microbiota composition, as overall bacterial community composition differs among bedding types in milk samples collected directly from the cistern of the mammary gland (12). As such, bedding should be investigated longitudinally with culture-independent methods to record seasonal changes in bacterial community composition and compare these changes in the milk microbiota.

We also found differences in richness, diversity, and community composition among quarters with different inflammation status, suggesting possible cow-related sources of bacterial DNA in milk. In humans, orally administered probiotic strains of *Lactobacillus salivarius* or *Lactobacillus fermentum* can later be cultured from milk (36). Dendritic cells have been suggested as a vehicle for transporting bacteria from the gut to the mammary gland in humans (37) but

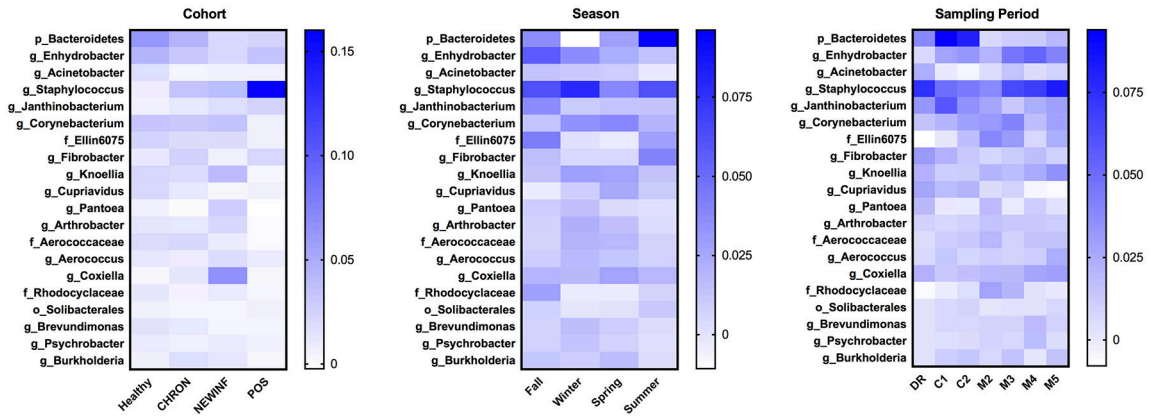


**FIGURE 4 |** Heat maps of the relative abundances of the 20 most prevalent OTUs in milk samples by cohort, season, or sampling period. Values included in the heat maps are LSM estimates generated from the models containing cohort, season, and sampling period as predictor variables for the prediction of the proportion of sequences represented by each OTU. The predicted values are included in the heat maps, even when the predicted proportion of sequences is < 0. Healthy, Quarters that had culture-negative milk with SCC < 100,000 cells/mL at DR, C1, and C2; CHRON, Chronically inflamed quarters that had culture-negative milk samples with SCC ≥ 150,000 cells/mL at DR and C1; NEWINF, Quarters that had new culture-negative inflammation with variable DR milk sample results and culture-negative milk samples with SCC ≥ 150,000 cells/mL at C1 and C2; POS, Quarters that had bacterial growth in culture and SCC ≥ 150,000 cells/mL at DR, C1, and C2; DR, dryoff, C1, first week of lactation, C2, second week of lactation.

this pathway has not been demonstrated in cattle. The major OTUs we found, such as unclassified Bacteroidetes, *Enhydrobacter*, and *Acinetobacter*, have all been reported in the bovine gut (9, 38). These gut-associated OTUs did not differ in relative abundance among cohorts, but did change seasonally (unclassified Bacteroidetes, *Enhydrobacter*) or across lactation (unclassified Bacteroidetes) (**Figure 4**). Unclassified Bacteroidetes was a major OTU that varied in prevalence across lactation, with an increase from DR to C1 and C2, followed by a dramatic reduction in prevalence after calving. The change in unclassified Bacteroidetes prevalence may be related to the compositional changes between colostrum and mature milk (39) or these changes in prevalence may be related to dry cow therapy or physiological factors during the transition period.

All quarters of all cows in our study were treated with an intramammary antimicrobial and a teat sealant at dryoff. Dry cow therapy is standard on more than 75% of US dairy farms as a method for curing existing intramammary infections and preventing new infections that may occur during the dry period (40, 41). The dry cow antimicrobial used in our herd contains penicillin and dihydrostreptomycin and is labeled for treatment and prevention of infections caused by *Staph. aureus*, but is expected to be effective against many Gram-positive pathogens (42). The most prevalent OTUs we detected are not included in the labeling for this drug, but we cannot determine whether antimicrobial therapy has an effect on the prevalence of these OTUs because every quarter was treated.

The greater richness and diversity we found in milk collected from quarters with lower SCC and better health outcomes across lactation is consistent with previous reports comparing milk collected from glands with CM to milk collected from apparently healthy glands (10, 13, 14), and with a report comparing the microbiota of milk collected from quarters that had previously experienced CM to milk collected from quarters that had not (35). As with better-characterized systems, a small number of OTUs did not dominate the healthier systems (43, 44). Our richness and diversity values were lower than those reported in other studies with different sequencing and analysis methods (13, 14), but the richness and diversity of our Healthy quarters were similar to healthy first-lactation cows that were subjected to the same sequence and analysis pipeline as the milk samples used in this study (12).

The OTUs reported in healthy milk from other studies include *Faecalibacterium*, *Lachnospiraceae*, *Propionibacterium*, and *Aeribacillus* (10, 11). *Lachnospiraceae*, *Propionibacterium*, and *Faecalibacterium* comprised well under 1% of the total sequences in our milk samples, while *Aeribacillus* was not detected at all. We did, however, detect DNA of known contaminant *Pseudomonas* in our negative controls and removed this from our sample results (12, 45). *Pseudomonas* has been reported as a prevalent OTU in healthy milk samples that were subjected to whole-genome amplification prior to 16S rDNA-targeted PCR (13) and may have been a contaminant rather than a genuine contributor to the milk microbiota. The differences in diversity and OTUs that we detected in milk illustrate that a "core" microbiota cannot be assumed for dairy cow milk, especially when other studies use different methods and report different bacterial composition. Studies of the human microbiota have used standardized methodologies (46) and we suggest that the development of such methods for dairy milk microbiota analysis would help resolve the vast differences observed across different studies.

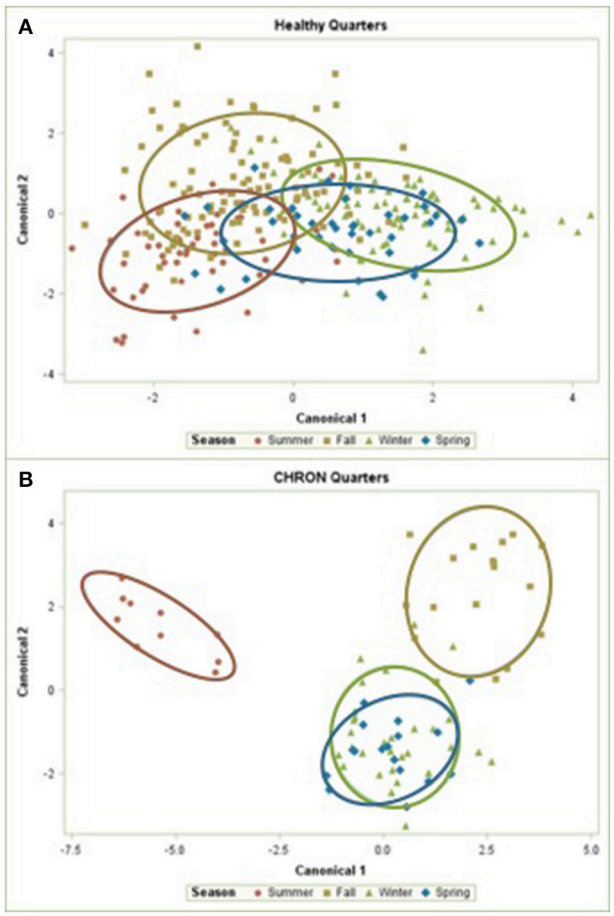


**FIGURE 5 |** Canonical discriminant analysis of the relative abundances of the top 50 OTUs in Healthy or CHRON bovine milk samples by season. Each symbol represents one milk sample. Healthy, Quarters that had culture-negative milk with SCC < 100,000 cells/mL at DR, C1, and C2; CHRON, Chronically inflamed quarters that had culture-negative milk samples with SCC ≥ 150,000 cells/mL at DR and C1; Sampling periods, DR, dryoff, C1, first week of lactation, C2, second week of lactation.

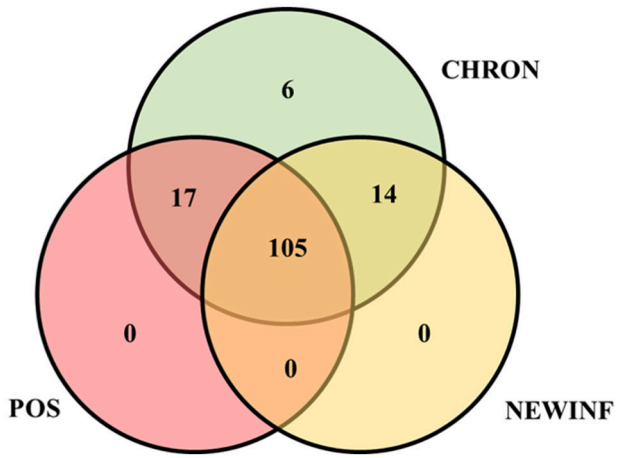


**FIGURE 6 |** Venn diagram of the 142 most prevalent OTUs found in milk samples collected from inflamed mammary quarters from dryoff to 150 DIM. CHRON, Quarters ($n = 17$) that had culture-negative milk samples with SCC ≥ 150,000 cells/mL at DR and C1; NEWINF, Quarters ($n = 6$) that had new culture-negative inflammation with variable DR milk sample results and culture-negative milk samples with SCC ≥ 150,000 cells/mL at C1 and C2; POS, Quarters ($n = 3$) that had bacterial growth in culture and SCC ≥ 150,000 cells/mL at DR, C1, and C2; Sampling periods, DR, dryoff, C1, first week of lactation, C2, second week of lactation.

An unexpected finding in our study was the presence of *Coxiella* in the milk microbiota, as this genus contains a single species, *Coxiella burnetii*, which causes the zoonotic disease Q fever (47). *Coxiella* sequences were found in the NEWINF quarters, with 6.7% of NEWINF sequences belonging to *Coxiella*. A 2011 human outbreak of Q fever, which can cause endocarditis or atypical pneumonia (48), was linked to consumption of raw cow milk (49). The majority of infected cattle show no signs of disease, even when shedding bacteria (48). Those animals in our study with a high prevalence of *Coxiella* sequences did not exhibit signs of clinical mastitis or abortion, according to herd records, but we cannot exclude the occurrence of this disease in our herd, as Q fever is difficult to diagnose in dairy cows (48).

In addition to having decreased SCC compared to the other inflamed cohorts (NEWINF and POS), milk samples collected from quarters in the CHRON group were the only inflamed cohort to contain unique OTUs. These unique OTUs included the rumen-associated genera *Oscillospira* (50), *Clostridium* (51), and *Selenomonas* (52), and each were present at <1% of the total sequence abundance in CHRON milk samples. In this study we were unable to determine if the microbiota caused changes in SCC or vice versa; we could only determine that the two are associated. *Clostridium* spp., an unexpected member of the milk microbiota, can be found in the rumen (51), but are more often associated with bulk tank milk and dairy farm soil rather than aseptically collected quarter-milk samples. *Clostridium* spp. can survive pasteurization and should be kept to a minimum in milk, especially milk intended for processing into infant formula (53). Like many other OTUs we identified, these ruminal species are difficult to grow in culture (51, 52) relative to the common mastitis pathogens for which our culture techniques were developed (20).

With the NMC culturing techniques we used, we were able to culture bacteria such as non-*aureus Staphylococcus* spp. and *Streptococcus*-like organisms. The 16S rRNA gene sequencing we used provided a greater resolution of bacterial identification for some of our cultured organisms, including *Streptococcus*-like spp. which were identified with sequencing as g_*Lactococcus*, g_*Enterococcus*, or g_*Streptococcus*. This genus-level resolution of *Staphylococcus* spp. is less beneficial than the biochemical identification we used. Although biochemical identification will not distinguish among non-*aureus Staphylococcus* spp., but can distinguish between *Staphylococcus aureus* and other *Staphylococcus* spp. The distinction between *S. aureus* and non-*aureus Staphylococcus* spp. is critical on a dairy farm due to the contagious nature of *S. aureus*.

Other researchers have generally found concordance between culture results and sequencing results in culture-positive milk samples (11, 14, 54). Though the milk microbiota is sparse, associations exist between the microbiota detected with next-generation sequencing and health outcomes in the mammary gland.

## CONCLUSIONS

The bovine milk microbiota is associated with inflammation status from dryoff through the first 150 DIM, but the milk microbiota is sparse in the healthiest bovine mammary quarters. The microbiota is more abundant but less diverse in mammary quarters that have inflammation or a history of inflammation. The cow population and housing of cows should be described in studies of the milk microbiota so that researchers can gain a better understanding of how the milk microbiota is related to cow and environmental factors. The differences in prevalent OTUs of the milk microbiota across studies indicates that methodologies for examining the milk microbiota should be compared to examine whether differences in results are related to laboratory methods or to the cow populations. Many types of bacteria that have not been previously associated with milk or mastitis are now potential targets for future research into the milk microbiota.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Protocol Number A01548-08-13, University of Wisconsin-Madison Institutional Animal Care and Use Committee. The protocol was approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee.

## AUTHOR CONTRIBUTIONS

LH, GS, and PR designed the study. LH, GS, and PR provided experimental and laboratory resources. SM conducted the study, sample collection, and DNA extraction. All authors developed PCR and sequencing methods. JS performed library construction and sequencing. SM performed data analysis. SM, LH, GS, and PR interpreted results. SM wrote the manuscript. LH, GS, and PR reviewed and edited the manuscript. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2018.00247/full#supplementary-material

**Figure S1 |** Weekly log10SCC for enrolled quarters. Values with different superscripts (a–d) differ.

**Figure S2 |** Survival to clinical mastitis for enrolled dairy cow mammary quarters.

**Figure S3 |** Negative control sequences prior to contamination removal.

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# Intestinal Bacteria as Powerful Trapping Lifeforms for the Elimination of Radioactive Cesium

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In March 2011, an accident at the Fukushima Daiichi Nuclear Power Plant led to major problems, including the release of radionuclides such as Cesium (Cs)-137 into the environment. Ever since this accident, Cs-137 in foods has become a serious problem. In this study, we determined the concentration of Cs-137 in the feces, urine, and ruminal contents of cattle and demonstrated the possibility of its elimination from the body by intestinal bacteria. The results revealed a high Cs-137 concentration in the feces; in fact, this concentration was higher than that in skeletal muscles and other samples from several animals. Furthermore, intestinal bacteria were able to trap Cs-137, showing an uptake ratio within the range of 38–81% *in vitro*. This uptake appeared to be mediated through the sodium–potassium ($Na^+$-$K^+$) ion pump in the bacterial cell membrane. This inference was drawn based on the fact that the uptake ratio of Cs-137 was decreased in media with high potassium concentration. In addition, it was demonstrated that intestinal bacteria hindered the trapping of Cs-137 by the animal. Cattle feces showed high concentration of Cs-137 and intestinal bacteria trapped Cs-137. This study is the first report showing that intestinal bacteria contribute to the elimination of Cs-137 from the body.

**Keywords: cesium (Cs)-137, feces, gut microbiome, cattle, ruminants, elimination, Fukushima Daiichi Nuclear Power Plant**

## INTRODUCTION

On March 11, 2011, the Pacific coast of Tohoku was hit by a gigantic earthquake, often referred to as the Great East-Japan Earthquake. This triggered a tsunami that seriously damaged the Tohoku region of northeastern Japan (1). In particular, the Fukushima Daiichi Nuclear Power Plant (FNPP), located on the coastal area, was struck by the tsunami, resulting in one of the worst nuclear accidents at a power plant, followed by widespread fall-out by various radionuclides (2–5).

After the FNPP accident, an evacuation zone was set up within a 20-km radius from the power plant. Many of the local population were forced to take refuge in unaffected areas and had to live in unfamiliar places. However, ∼3,400 head of cattle, 31,500 pigs, and 630,000 chickens were left behind in the area (6). On April 22, 2011, the Government of Japan ordered the Fukushima prefectural government to euthanize livestock within the evacuation zone, preventing people from eating meat with radionuclides. Meanwhile, outside this zone, radioactive Cesium (Cs) was detected in foods at concentrations exceeding the reference limit. Therefore, food shipments from parts of Fukushima Prefecture were restricted to allay concerns about foods with radionuclide (7, 8).

Many research papers have mentioned the impact of the FNPP accident and the internal exposure (9–13). Our group reported the distribution of radioactive substances in abandoned cattle, revealing that the highest distribution of Cs-137 was in the skeletal muscle (6). Furthermore, we found that some radionuclides showed organ-specific distribution, such as in the liver, blood, and kidneys (6).

Understanding the Cs-137 distribution is important for evaluation of food safety and to study the biological effects due to the exposure to radioactive substances. The dynamics of Cs-137 in the body are being revealed only gradually. It was previously thought that the major routes of Cs-137 excretion in humans are through urine and feces (14). In addition, livestock excrete Cs-137 via their milk (7). In the intestinal tract, the uptake of inorganic substances takes place against an electrochemical potential difference (15–17). Moreover, the amount of inorganic substances in the intestinal tract differs based on the dietary habits (16). In this study, we postulated that the fecal route is as important as the urinary system for excreting Cs-137. Therefore, we decided to examine the contribution of intestinal bacteria to Cs-137 excretion.

In the intestinal tract, Cs-137 encounters up to $10^{14}$ bacteria in the mammalian intestine (18). We postulated that the process of Cs-137 uptake was mediated through the metabolic system of the intestinal bacteria. Bacteria transport ions and metabolic products, through channel and membrane transport proteins that exist on their cell surface. These proteins maintain the intracellular conditions of the bacterial cell. However, potassium (K) ion channel does not transport sodium (Na) ions, despite having ion radius larger than that of Na and both ions belong to the same family of elements. The K channel acts as an ion selectivity filter, transporting only $K^+$ (19). However, it has been reported, that Cs ions can enter cells through the Na–$K^+$ pump (14). The rate of Cs transport is no more than ∼0.25 times that of K. Furthermore, estimates of the relative selectivity of K and Cs by the K channels and Na pump have been described (14), with most studies reporting that the Cs:K selectivity ratio varies from <0.02 to ∼0.2. Moreover, the typical Cs:K selectivity ratio for the Na pump is 0.25 (14). It is thus clear, that cells transport Cs ion. There have been some publications discussing uptake of Cs by microorganisms as well as proposed mechanisms (20–23). Therefore, we hypothesized that intestinal bacteria can also take up Cs-137 like other bacteria.

In this study, we used *Bifidobacterium*, *Bacteroides*, and *Clostridium* species for the Cs-137 uptake assays. These species were selected as they are dominant in the bovine intestine (24). We investigated whether feces were associated with Cs-137 elimination from the bodies, and whether intestinal bacteria indeed take up the radionuclide. We also checked for any competitive uptake between Cs-137 and K. These examinations should help in clarifying the contribution of feces in Cs-137 elimination.

**Abbreviations:** FNPP, Fukushima Daiichi Nuclear Power Plant.

## MATERIALS AND METHODS

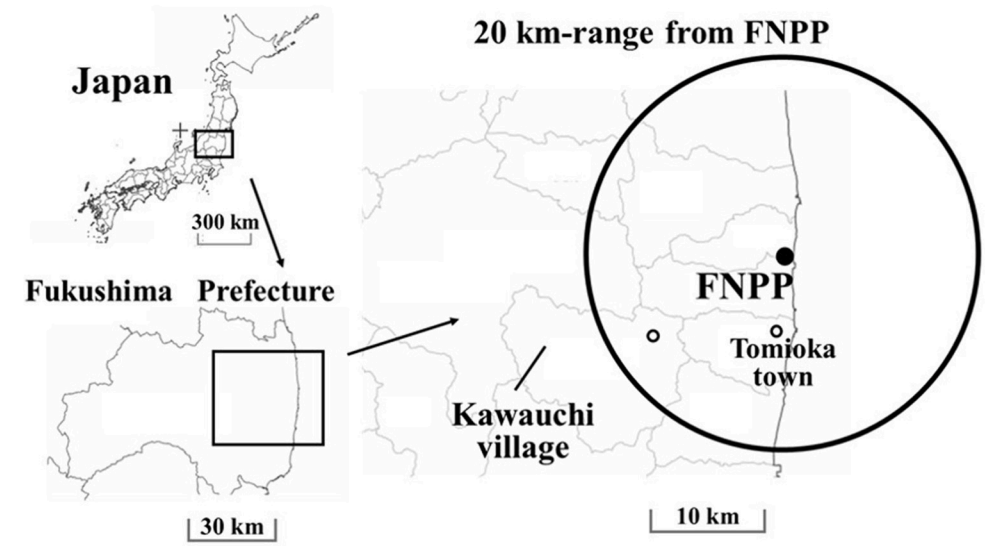
### Samples

During the period between October 20, 2011 and March 6, 2012, we sampled the skeletal muscle (longissimus muscle) from a total of 23 cattle (15 female and 8 male) in Kawauchi village and Tomioka town. We also obtained the fecal samples from 6 in Kawauchi village and 1 in Tomioka town. Urine was obtained from 3 cattle. In Tomioka town, feces, stomach content, and muscles were obtained from Inobuta (mixed kind; pig and wild boar) samples. Boar–pig hybrids are the hybridized offspring of a cross between the wild boar (*Sus scrofa*) and any domestic pig (*Sus scrofa domesticus*). Inobuta meat is known as a healthy alternative to other main meat products because it is tasty and low in fat. The number of wild Inobuta was increased in Fukushima after the Great East Japan Earthquake.

It has been reported that the highest distribution of Cs-137 is in the skeletal muscle (6). Therefore, by comparing the Cs-137 concentrations in the skeletal muscle and feces, the distribution of the radionuclide in the feces can be determined. In this experiment, we examined if Cs-137 was discharged via the feces and urine in the Fukushima cattle. In order to consider the discharge route, we compared the Cs-137 concentrations in the feces and urine. Furthermore, we assumed that the Cs-137 concentration in ruminal digests would fluctuate due to the digestive processes. Therefore, examination of the Cs-137 concentration at an intermediate point between food intake and excretion of feces was essential. For this purpose, the Cs-137 concentration in the ruminal content was also determined. In addition, we suspected that through the Cs-137 uptake activity, the intestinal flora might play an important role in inhibiting the body's absorption of the radionuclide from the intestine.

Soil and grass samples were collected at the place where the cattle were caught. Soil samples were taken in a square 30 × 30 cm from the surface to the depth of 10 cm. Radioactivity concentration was calculated into kBq/$m^2$ by the method previously described (6). Only the leafy portions of grasses were sampled and analyzed.

Kawauchi village and Tomioka town represented two different contamination levels of radionuclides (**Figure 1**). Air dose rate in Kawauchi village was more than 1.0 μSv/h, and 9.5 μSv/h and fewer at sampling time, while that of Tomioka town was more than 3.8 μSv/h, and 19.0 μSv/h and fewer at sampling time, respectively (URL: https://ramap.jmc.or.jp/map/#lat=37.457027049337896&lon=140.83407992880714&z=11&b=




| Sampling site | Cattle No. |
|---|---|
| Tomioka town | A405, A408, A440, A442, B16, B51 |
| Kawauchi village | A1012 |

**FIGURE 1 |** Study sites. Sites of cattle sampling in Fukushima Prefecture, within a range of 20-km from the Fukushima Daiichi Nuclear Power Plant (FNPP). ○: The locations of sampled cattle, Tomioka town, and Kawauchi village. These locations had different air dose rates. The maps were prepared from open-access base maps freely available for public and academic use (source: http://maps.gsi.go.jp, from the Geographic Information Authority of Japan).

**TABLE 1 |** Demographics of the sampled cattle.

| Cattle No. | Sampling sites | Sampled date | Birth | Gender | Age (months) | Ear tag numbers |
|---|---|---|---|---|---|---|
| A405 | K | Nov. 29, 2011 | Jul. 5, 2010 | F | 16 | 1253570241 |
| A408 | K | Dec. 9, 2011 | Sep. 1, 2007 | F | 50 | 463501274 |
| A440 | K | Nov. 29, 2011 | Jan. 29, 2010 | F | 10 | ※ |
| A442 | K | Nov. 29, 2011 | Aug. 1, 2007 | F | 51 | 0240672852 |
| A1012 | T | Mar. 6, 2012 | Aug. 8, 2010 | M | 19 | 1335275224 |
| B16 | K | Oct, 20. 2011 | Oct. 19, 2010 | M | 12 | 1335275323 |
| B51 | K | Oct. 20, 2011 | Aug. 8, 2010 | F | 14 | 1235270289 |

*Cattle No: Cattle were numbered to adjust the data. Sampling sites: K and T represent Kawauchi village and Tomioka city, respectively. Sampled date and Birth: All cattle had identifying ear tags with unique 10-digit numbers indicating their date of birth. Age: The age of the animal at euthanization. ※ indicates that A440 was not provided with ear tag numbers because it was born from A442.*

std&t=undefined&s=25,0,0,0&c$=$20110429_dr). The cattle demographics are presented in **Table 1**. We have tried to examine the excretion route of Cs-137 and measure the radioactivity in samples.

## Cs-137 Determination in Cattle's Organ

The radioactivity in the bovine skeletal muscle, ruminal contents, urine, and feces were measured with a gamma-ray spectrometer, using a high-purity Germanium detector (Ortec Co., Oak Ridge, TN, USA), as described in our previous study (6, 12). Feces and urine were sampled directly from rectum and bladder of euthanized cattle.

## Bacterial Strains, Media, and Cultures

*Bifidobacterium longum* subsp. *longum* JCM 1217, *Clostridium perfringens* JCM 1290, *Clostridium ramosum* JCM 1298, *Bacteroides fragilis* RIMD0230001, and *Bacteroides vulgatus* JCM 5826 were used as the major intestinal bacteria (25–31). These bacterial strains were propagated in 10 mL of Brain-Heart Infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA) or

**TABLE 2 |** Distribution of Cs-137 in skeletal muscle, feces, urine, and 1st stomach contents.

| Cattle No. | Cs-137 (Bq/kg) | | | |
|---|---|---|---|---|
| | Skeletal muscle | Feces | Urine | Ruminal contents |
| A405 | 604.2 ± 10.3 | 226.1 ± 6.2 | 98.8 ± 3.4 | 101.7 ± 1.9 |
| A408 | 669.5 ± 13.9 | 419.0 ± 6.0 | N. D. | N. D. |
| A440 | 748.4 ± 16.5 | 494.6 ± 9.9 | N. D. | N. D. |
| A442 | 663.7 ± 14.9 | 167.4 ± 4.0 | N. D. | N. D. |
| A1012 | 2447.9 ± 48.1 | 23213.5 ± 172.5 | N. D. | N. D. |
| B16 | 635.0 ± 23.9 | 213.0 ± 13.0 | 169.5 ± 3.5 | 85.1 ± 1.4 |
| B51 | 414.6±18.0 | 1887.1±17.1 | 35.3 ± 1.3 | 35.3 ± 3.6 |

*Cattle No.: The individual identification number for each animal. Data are presented as the mean ± SD. N. D., Non data.*

Gifu Anaerobic Medium (GAM) broth (Nissui Pharmaceutical Co., Tokyo, Japan) using 1% (v/v) inoculums. BHI medium was used for incubation of *B. longum*, *C. perfringens* and *C. ramosum*, and GAM medium was used for *B. fragilis* and *B. vulgatus* incubation. All bacteria were incubated anaerobically in Anaero-Pack systems (Mitsubishi Gas Chemical, Tokyo, Japan) at 37°C for 24 h. The BHI and GAM broths were sterilized at 121°C for 15 min and 115°C for 15 min, respectively.

## Cs-137 Determination in Bacteria

Muscles were sampled from the cattle living within 20-km range from the FNPP and muscle extract was prepared by boiling. BHI agar and GAM agar containing 10% (v/v) of the Cs-137-containing extract were used as the incubation media for the bacterial Cs-137 uptake assay. The number of viable bacteria was adjusted to $10^8$ colony forming units (CFUs)/ml, and 100 μl of each bacterial strain was inoculated into the respective incubation medium and incubated at 37°C for 48 h under anaerobic conditions. After the incubation, the media were washed three times using 1 ml of sterile Dulbecco's phosphate-buffered saline (PBS) (Nissui Pharmaceutical Co., Tokyo, Japan). The bacterial suspension was recovered and poured into U8 (100 mL) polypropylene containers (Yamayu, Osaka, Japan). The agar medium was also melted and poured into a separate U8 polypropylene container. The agar medium from the three Petri dishes was poured into U8 containers. The concentration of radioactive Cs in the bacterial cells and media was detected using Germanium gamma-ray spectrometry. Results of radioactivity in several organs were expressed as Bq/fresh weight. Furthermore, blank test was conducted to examine how Cs-137 was extracted by washing. Blank test was done using the same protocol without inoculating it with bacteria. Risk assessment was performed for the handling of radioactive substances among the people involved. In addition, when we measured the sample's radiation using survey meter, results were within the background levels.

## Inhibition of Cs-137 Uptake

To examine the Cs-137 uptake inhibition caused by $K^+$ in the medium, $K_2HPO_4$ was added to BHI and GAM agar at a final concentration of 1,500 ppm. After cultivation under anaerobic conditions, the concentration of Cs-137 was detected in both bacterial cells and the media as described above. To confirm the $K^+$ concentration in the BHI and GAM medium, a LAQUA Twin Compact Water Quality Meter (HORIBA Ltd., Kyoto, Japan) was used according to the provided protocols.

## Statistical Analysis

Differences of Cs-137 concentration between female and male were examined by Student *t*-test. Differences of Cs-137 concentration among feces, skeletal muscle and stomach contents were calculated by Turkey-Kramer test. Differences in the uptake ratio of Cs-137 in media with or without added K were analyzed with the two-way analysis of variance (ANOVA). Furthermore, significant differences among the strains were calculated with the Turkey-Kramer test. Significant differences between media with K and without K were calculated with Student *t*-test. Probability values of $p<0.05$ were considered significant. Each sample was measured in triplicate.

# RESULTS

## Detection of Cs-137 in the Skeletal Muscle, Feces, Urine, and Ruminal Contents

The concentration of Cs-137 in the skeletal muscle (Bq/kg) was 639.1 (female, $n = 10$) and 536.8 (male, n=5) in Kawauchi village, and 2705.4 (female, $n = 5$) and 2962.9 (male, $n = 3$) in Tomioka town. There was no significant difference in Cs-137 concentration in male and female animals in the two geographic regions. In eight of these animals, fecal samples were also taken, and in some of these, urine and ruminal samples were also obtained. The respective concentrations of Cs-137 in these animals are shown in **Table 2**. The Cs-137 concentration in the skeletal muscle was higher than that in the feces for some samples. However, the Cs-137 concentration in the feces from A1012 was 9.5 times higher than that in the skeletal muscle. A1012 was located in Tomioka town, a highly contaminated area located at a distance of 3 km from FNPP. Likewise, the Cs-137 concentration in the feces from B51 (located 5 km from FNNP) was 4.6 times higher than that in the skeletal muscle. In this study, we obtained only three samples in feces, urine and ruminal contents because condition of samples was different. In particular, urine and ruminal contents were not contained in the bladder and rumen, at the time of sampling. A high deposition of Cs-137 was observed in the feces and this was higher than that in the ruminal contents and urine (**Table 2**). The Cs-137 concentration was 2–53 times higher in the feces than in the ruminal contents. In addition, cattle housed in non-contaminated areas did not have detectable Cs-137 levels (6). Radioactivity Cs-137 concentration was about 2,300 and 2,700 Bq/kg in the soil open area, and 3,000 and 3,700 Bq/kg in grass (Japanese pampas grass) with grazed marks in Kawauchi village. In Inobuta sampled in Tomioka town, feces were significantly higher than skeletal muscle and stomach contents (**Table 3**, $p < 0.01$).

## Uptake of Cs-137 by Intestinal Bacteria

To examine Cs-137 uptake by intestinal bacteria, we chose to use common bacterial strains found in the bovine intestine.

**TABLE 3 |** Cs-137 concentration in Inobuta.

| | Cs-137 concentration (Bq/kg) |
|---|---|
| Skeletal muscle | 1100.0 ± 200.0* |
| Feces | 5464.3 ± 2923.8 |
| Stomach contents | 996.0 ± 648.6* |

*Concentration of Cs-137 are shown in mean ± standard deviations.*
*N. D, Non data.*
**Significantly different from the feces. Significant difference at the 99% confidence level, using the Tukey-Kramer test.*

(24). Cs-137 was detected from both the bacterial suspension and the medium (**Table 4**). The uptake ratio was calculated as the radioactivity in the bacterial suspension divided by the total radioactivity (bacterial suspension plus medium) and multiplied by 100. It was observed that the bacterial suspension had a higher Cs-137 dose than the medium. Although each bacterium took up Cs-137, the uptake ratio was different among different species, with *B. vulgatus* showing the highest value. The significant differences have been presented in **Table 4**. In the results of blank test, the amount of Cs-137 extracted by water was less compared to the bacterial uptake.

## Inhibition of Cs-137 Uptake

The Cs-137 uptake ratio for all the strains, except *B. longum*, was significantly lower in the supplemented medium than in the non-supplemented BHI ($p < 0.01$) (**Table 4**). In contrast, *B. longum* showed an increase of Cs uptake after the addition of K. The $K^+$ concentration were about 1,500 ppm (**Table 5**).

# DISCUSSION

Obtained results showed that activity concentration in feces were higher than in muscle. Therefore, the excretion of Cs-137 via feces is important for discussion on the dynamics of radionuclide uptake in the body. In addition, bacterial uptake of Cs-137 was examined in this study. This uptake model showed that intestinal bacteria take up Cs-137. It is well-known that there are a huge number of viable bacteria in feces. It was reported that bacterial biomass is a major component of organic substances in the feces (32). Therefore, it is possible that the uptake of Cs-137 by intestinal bacteria is related to its high distribution in the feces.

It has been demonstrated that the concentration of Cs-137 in feces is higher than ruminal contents (**Table 2**). This result was obvious because the bulk of the ruminal contents will lighten through the digestive process. As a result, the concentration of Cs-137 in the ruminal contents would be lower. Furthermore, it is possible that Cs-137 in the ruminal contents is transferred to the blood in the process of digestion. Moreover, it is also likely that this result was caused by the intestinal bacteria taking up Cs-137, which was then secreted to the intestinal tract and subsequently excreted through the feces. It does not have to consider the possibility of returning Cs-137 to the intestine via bile. Leggett et al. showed that biliary secretion represents only a few percent of the total percent of Cs-137 in liver (14). In conclusion, it is obvious that the fecal route is the most important pathway of Cs-137 elimination from cattle body. As seen in **Table 2**, the concentration of Cs-137 in the feces was higher than that in urine, again showing the contribution of fecal discharge of the radionuclide. In this study, we specifically collected samples from the Japanese black breed, which generally discharges daily about 30 and 20 kg of feces and urine, respectively (Unpublished data). The amount of discharge in livestock fluctuates according to body weight, types of livestock and feed, and breeding form, among other parameters. Generally, these values are used in the scale calculations of feces and urine processing facilities. Therefore, it was thought that the amount of discharge and the Cs-137 concentration in feces are higher than that of urine, indicating that feces eliminate Cs-137 from the body more efficiently than urine. In a previous study, urine was concluded as the main route for Cs-137 discharge from the body because of its water-soluble characteristics (14). In a previous study, urine was concluded as the main route for Cs-137 discharge from the body because of its chemical characteristics, which is in contrast with these results. This suggest that feces have a higher contribution than urine in this regard.

In this study, we examined the radioactivity in skeletal muscle and feces. Radioactive Cs concentration in organs is dependent on the feeding conditions and the geographic location of cattle (33). Cattle used for sampling were born before the FNPP accident occurred. These cattle have been exposed radioactive substances until they are euthanized. Furthermore, in our previous study we have showed the radioactivity concentration of Cs in the soil and grass (6). In Tomioka town, Cs-137 concentration was 10,000 ∼ 25,000 Bq/kg in the soil (http://www.maff.go.jp/j/wpaper/w_maff/h23_h/trend/part1/sp/sp_c2_2_02.html) and 1,000 ∼ 10,000 Bq/kg in the grass (Unpublish data), respectively. The Cs-137 concentration in soil and grass in Tomioka town was higher than that in Kawauchi village. The Cs-137 concentration of grass was found have high radioactivity concentration and this was eaten by the cattle in dairy. Therefore, it was thought that radioactive Cs was accumulated in cattle body. In addition, Cs-137 concentration of feces was higher than skeletal muscle in Inobuta (mixed kind; pig and wild boar) (**Table 3**). High distribution of Cs-137 was also shown monogastric animals and in Tomioka town. Moreover, it has been reported that the fecal route is an important route in other ruminants (lambs and ewes) and shown to be approximately equal to urinary excretion for radioactive Cs (34). The study of this paper revealed that concentration of Cs-137 in feces was higher than in muscle and urine. Therefore, the results of this paper are consistent with previous studies. Furthermore, it was suggested that feces contribute to the excretion of Cs-137 in ruminants.

It was thought that intestinal bacteria were able to trap Cs-137 and the uptake ratio was different among the species (**Table 3**). Thus, even though intestinal bacteria are related to the intake and discharge of Cs-137, their uptake ratio differs depending on the strain type. The uptake of Cs-137 with K occurs through the $K^+$ pump located in the bacterial cell membrane (14). The element Cs is homologous to K, and hence, both exhibit similar behavior. Therefore, bacteria take up Cs-137 in the same way they do for K. The *Bacteroides* species and *C. ramosum* showed high uptake

**TABLE 4 |** Uptake of Cs-137 by intestinal bacteria.

| Group | Cs-137 concentration; BHI ($\times 10^3$ nBq/mg) | | | Cs-137 concentration; BHI added $K_2HPO_4$ ($\times 10^3$ nBq/mg) | | |
|---|---|---|---|---|---|---|
| | Bacteria | Medium eliminated bacteria | Uptake ratio (%) | Bacteria | Medium eliminated bacteria | Uptake ratio (%) |
| *B. longum* | 5.98 ± 1.4 | 9.84 ± 0.8 | $37.8 \pm 3.1^{b}$ | 159.5 ± 4.2 | 108.4 ± 2.8 | $59.5 \pm 0.4^{a*}$ |
| *C. perfringens* | 9.92 ± 2.0 | 12.1 ± 3.6 | $45.0 \pm 2.8^{b}$ | 27.3 ± 1.6 | 103.1 ± 3.4 | $21.0 \pm 0.6^{b*}$ |
| *C. ramosum* | 9.46 ± 1.0 | 3.19 ± 1.5 | $74.8 \pm 1.1^{a}$ | 28.9 ± 1.3 | 102.3 ± 3.5 | $22.0 \pm 0.5^{bc*}$ |
| *B. fragilis* | 9.26 ± 1.0 | 3.80 ± 1.6 | $70.9 \pm 1.2^{a}$ | 32.7 ± 1.9 | 100.9 ± 3.1 | $24.5 \pm 0.6^{c*}$ |
| *B. vulgatus* | 11.0 ± 1.4 | 2.55 ± 1.9 | $81.2 \pm 1.2^{a}$ | 29.6 ± 1.6 | 103.1 ± 3.4 | $22.3 \pm 0.4^{bc*}$ |
| Medium only | 2.7 ± 0.1 | 105.5 ± 9.7 | 2.6 ± 0.1 | 3.8 ± 0.4 | 110.7 ± 3.6 | 3.4 ± 0.3 |

*Data are presented as the mean ± SD. Statistical significances were calculated by two-way analysis of variance (Strains ×Concentration). The main effect for both Strains and Concentration and the interaction are significant [Strains $F_{(4, 20)} = 37.10$, $p < 0.01$, Concentration $F_{(1, 20)} = 1094.37$, $p < 0.01$, Interaction $F_{(4, 20)} = 243.10$, $p < 0.01$].*Show the significant differences compared with group of media without added potassium ($p < 0.01$). Significant differences were calculated with Student t-test. a, b, c. The same letters represent the no significant differences at 99% confidence level, compared among five bacterial strains in the groups of media with or without added K, respectively. Significant differences were calculated with Turkey-Kramer test.*

**TABLE 5 |** Confirmation of $K^+$ concentration.

| Medium | $K^+$ concentration (ppm) |
|---|---|
| BHI | 223.3 ± 3.3 |
| GAM | 903.3 ± 3.3 |
| BHI added $K_2HPO_4$ | 1566.7 ± 33.3 |
| GAM added $K_2HPO_4$ | 1466.7 ± 33.3 |

*Data are presented as the mean ± SD.*

ratios, whereas that of *B. longum* was low. The reason for this result is unclear, but this could be related to the structure of the bacterial surface layers since *Bacteroides* species are Gram-negative, whereas *Clostridium* and *Bifidobacterium* species are Gram-positive. In addition, it was reported by Kato et al. (35), that *Bacteroidetes* bacteria, especially *Flavobacterium* spp. appear to have significant tolerance to high concentrations of $Cs^+$ *in vitro*. Therefore, in this result, it was thought that *B. fragilis* and *B. vulgatus* were able to accumulate Cs-137 actively. Moreover, because Cs is homologous to K, the requirement of K in the metabolic system could be related to the uptake of Cs-137. More examination is needed to clarify the reasons behind these results.

The Cs-137 uptake ratio by some strains was significantly lower in the supplemented medium than in the non-supplemented BHI (**Table 4**). These results indicated that the uptake of Cs-137 could be inhibited in most strains by increase of the K concentration in the medium. In addition, the growth in the supplemented medium showed no significant difference compared with the non-supplemented medium (data not shown). This further suggests that the uptake of Cs-137 is related to that of K. Since the uptake of Cs-137 is inhibited by K, contamination by Cs-137 depends on the K concentration in the body. In another study, Cs-137 uptake was also found to be inhibited by $K^+$s in soil microbes in a dose-dependent manner (31). It is interesting to note that the Cs-137 uptake by *B. longum* significantly increased due to the addition of K; the reason for this remains to be elucidated.

It is thought that potassium ion is an essential element for bacteria. Actually, K is required for the activity of the ribosome and a lot of enzymes (36). However, it has not been reported the relationship between intestinal bacteria and potassium ion. Recent study, *Lactobacillus rhamnosus* JB-1 demonstrated that $K^+$ uptake in the intestine contribute to the beneficial effect for allergic and other inflammatory disorders (37). This study suggested that $K^+$ uptake contribute to the health of host. In this study, we demonstrated that intestinal bacteria contribute to Cs-137 excretion for host. $K^+$ transporter help to excrete Cs-137. It was suggested that $K^+$ transporter of intestinal bacteria also show beneficial effects.

In conclusion, we demonstrated that intestinal bacteria contribute to the elimination of Cs-137 from the body of cattle. The data of this study provides little information because it was in a limited condition, hence larger studies with higher number of animals will be required to make this statement in the further study. During the digestive process, Cs-137 was not only absorbed in blood but also taken up by intestinal bacteria and subsequently discharged via the fecal route. However, there is also a possibility that intestinal bacteria maintain Cs-137 in the intestinal tract. It is important to consider that many factors influence uptake of $Cs^+$ *in vivo*. As a result, feces contribute to the excreting of Cs-137 from cattle. In future studies, we plan to examine the mechanism of Cs-137 elimination in detail and the possibility of eliminating this radionuclide using other bacteria.

## DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

Each experimental protocol was approved by the Institutional Ethics Commissions for Animal Research at Tohoku University.

Our collection of organs from the euthanized cattle was in collaboration with the units of veterinary doctors of both the Livestock Hygiene Service Centre (LHSC) of Fukushima Prefecture and the Ministry of Agriculture, Forestry and Fisheries, Japan. The veterinary doctors of the LHSC euthanized the cattle in strict accordance to procedures laid out by the Regulation for Animal Experiments and Related Activities at Tohoku University (Regulation No. 122). The cattle owners could be identified on the ear tag of each animal, and their informed consent was obtained by the veterinary doctors of Fukushima Prefecture.

## AUTHOR CONTRIBUTIONS

KS, KK, RS, YK, TS, HS, HidY, TF, JK, YA, JN, YU, HirY, MF, and EI performed the experiments (e.g., sample collection, measurement of the radionuclide activity in the tissues, bacterial experiments. KS and EI designed the research. KS, JN, YK, and EI wrote the manuscript.

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# Differential Effects of Bacitracin Methylene Disalicylate (BMD) on the Distal Colon and Cecal Microbiota of Young Broiler Chickens

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Antibiotics have been used extensively for growth promotion in poultry, along with other food production animals, as well as therapeutically to treat infectious diseases. However, with concerns over selection for drug antibiotic resistant bacteria the practice of using subtherapeutic doses of antibiotics is under increased scrutiny. Consequently, we assessed the impact of the commonly used antibiotic bacitracin methylene disalicylate (BMD) on the gastrointestinal microbiota of chickens. For this we administered therapeutic doses of BMD as a feed additive and 16s rRNA gene amplicon sequencing to measure changes in taxonomic abundance on the distal colon and cecal microbiota of young broiler chickens. While BMD treatment was found to impact the abundance of selected taxa and overall beta diversity, significant changes were, in general, limited to the colon of the treated birds. Selected taxa at the phylum, class, and genus levels that were most impacted were identified. The composition of the cecum remained relatively stable in BMD-treated animals. As poultry production practices seek alternatives to growth promoting antibiotic feed additives, manipulation of the gastrointestinal microbiota holds promise. These results suggest that targeting the cecum may offer a means to promote changes to the microbiota that maximize the benefits for the hosts.

**Keywords: poultry, microbiota, antibiotic, 16S rRNA, growth promoting antibiotics**

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## INTRODUCTION

Along with therapeutic use, antibiotics are well-established for their ability to promote growth through improved weight gain and feed efficiency in livestock (1), including in broiler chickens (2). In poultry, bacitracin methylene disalicylate (BMD) is commonly used for growth promotion (3). Compared to other growth promoting antibiotics, such as virginiamycin, BMD is a relatively narrow spectrum antibiotic that targets primarily gram-positive bacteria, including *Streptococci*, *Staphylococci*, *Clostridia*, *Fusobacterium*, and *Actinomyces*. BMD interferes with protein synthesis and cell wall production and induces cell lysis in these microorganisms (4, 5). The antibiotic is not well-absorbed by the intestine and therefore primarily acts on bacteria in the gastrointestinal (GI) tract of the animals through delivery as a feed additive (6).

Feeding chickens low doses of BMD benefited the birds, including increased villus height throughout the small intestine and improved digestion of dietary components that correlated with increased body weight and feed consumption (7).

BMD is also used as to treat and prevent necrotic enteritis caused by *Clostridium perfringens* (8, 9), which is a cause of significant economic loss in the poultry industry (10). While antibiotic growth promoters make important contributions to the overall efficiency of livestock production, they are also not without their concern as sub-therapeutic doses used are also associated with selection and spread of drug resistant bacterial pathogens (11, 12). Concerns over widespread use of antibiotics in agriculture has prompted a ban on their use in the European Union with increased scrutiny for their use in the United States (13).

Given the importance of poultry for human nutrition and the food animal industry world-wide, emphasis has been placed on characterizing the chicken microbiome as a means to improve our understanding of antibiotic growth promotion and to identify alternative strategies that do not select for drug resistant bacteria (14–18). Toward this, numerous studies, representing a variety of methods, have assessed the impact of antibiotic treatment on the microbiota of poultry (7, 9, 19–32). In general, these studies have shown that growth-promoting antibiotics can have significant effects on the structure and function of the microbiota colonizing the GI tract. As to be expected with a list of wide-ranging studies, there are few bacterial taxa that are consistently altered by antibiotics that can explain their growth promoting activities since the composition and activity of the chicken microbiome is highly dependent on environmental conditions, feed composition and method of assessment of the microbioal communities. Interestingly, however, chicken microbiota studies have revealed that BMD, along with other growth-promoting antibiotics, can deplete species of *Lactobacillus*, as well as other probiotic species (32, 33). This observation has led to the suggestion that a reduction in bile-salt hydrolase activity encoded by many of these bacteria may contribute to growth promotion by reversing the negative effects on fat metabolism of these enzymes (34). Clearly additional studies are needed to better understand how changes to the microbiota by low-dose antibiotics contribute to animal growth enhancement.

To further out understanding of how the growth promoting feed additive BMD impacts the chicken microbiota, we have focused on distinguishing between the effects of the antibiotic on cecal vs. colon bacterial populations. These two compartments of the chicken digestive tract are colonized with distinct microbial communities (35, 36). Also, while metabolism and adsorption of macronutrients occurs primarily in the colon, fermentation of complex polysaccharides occurs primarily in the cecum (14, 15, 36). Because of these spatial and functional differences, we sought to determine the extent to which BMD impact the microbiota of the distinct compartments of the GI tract. For this, we conducted 16s rRNA gene amplicon taxonomic profiling of the microbiota of the distal colon and cecum from young broiler birds using therapeutic doses (8, 37) of the antibiotic to accentuate differences in microbial composition in the GI tract.

# MATERIALS AND METHODS

## Animal Model and Housing

This study was carried out with the approval of the Iowa State University Institutional Animal Care and Usage Committee under protocol number 6-11-7167-G. The design followed a necrotic enteritis model, however, no pathogens were administered to the chickens and only antimicrobial feed additives were added to the experimental group.

Approximately 30 day old jumbo Cornish/Rock broiler chicks were obtained from Welp Hatchery (Bancroft, IA) and housed in pens created by tying two 32″ × 8′ × 1/8″ (81 cm × 2.45 m × 0.3 cm) pegboards together to form a circle. This circular pen was divided into three equally sized areas with similar pegboard material. Each pen was bedded using ∼3″ (7.5 cm) of wood shavings. Heat lamps were made available for each pen. One two-gallon, galvanized waterer and one galvanized metal feeder were supplied to each pen.

Groups of 15 birds were housed in the pens described. On days 1–7, each group received 1 kg of a low-protein chick starter (LPF) once a day. On days 8–10, each group was given 1 kg of a high protein feed (HPF) once a day. On days 11–18, the control group remained on the same HPF feed while the challenge group received the HPF supplemented with BMD (200 g/ton). On these days, each group was fed 1 kg HPF with or without BMD twice a day. On day 19, the chicks were euthanized and samples were collected. Distal colon and cecal contents were collected and stored at −80°C until total DNA was isolated.

## DNA Isolation

Total genomic DNA was extracted using the PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA). The manufacturer's protocol was followed with the exception of the initial vortexing step, which was extended to 20 min to thoroughly homogenize the samples. Purified genomic DNA extracts were quantified using a Quibt 2.0 Fluorometer (Life Technologies, Carlsbad, CA), and DNA stored at −20°C in the provided 10 mM Tris buffer.

## Sequencing and Analysis

PCR amplification of the variable regions 3–5 of the 16S rRNA gene was done using region specific primers 357F (CTCCTACGGGAGGCAGCAG)−926R (CCGTCAATTCMTTTRAGTTT). Amplicon sequencing was performed at the Research and Testing Laboratory (Lubbock, TX) using the Roche 454 Titanium platform.

The resulting DNA sequences were analyzed using QIIME (Quantitative Insights into Microbial Ecology) (38). The reads were first demultiplexed and binned per sample. The reads were also quality filtered during this step to remove poor quality sequences using default quality filtering values. Denoising of the 454 reads was performed using Denoiser (39). Chimeric sequences were identified using USEARCH and removed (40, 41). The remaining sequences were clustered into OTUs at 97% similarity using USEARCH and the open reference OTU picking strategy in QIIME. Sequences were aligned to the Greengenes (13_5) rRNA sequence core reference database using PyNAST (42, 43). Taxonomic assignments were made using the RDP

Classifier 2.2 at a 97% similarity to the Greengenes reference database (44). Phylogenetic trees were built using FastTree 2.1.3 (45). Alpha and beta diversity, analysis of similarity (ANOSIM) and Adonis tests were generated using QIIME. PCoA plots were generated using Emperor in QIIME (46). Mann–Whitney *U*-tests were performed on taxonomic summaries using a custom R script (R Project) developed at the Institute of Genome Sciences at the University of Maryland-Baltimore. Sequence reads have been submitted to NCBI's short read archives.

# RESULTS

## Alpha and Beta Diversity

A total of 79,670 sequences were analyzed using QIIME. After filtering based on quality scores, 73,529 sequences corresponded to 619 OTUs with an average of 2,298 ± 1,335 sequences per sample.

**Figure 1** summarizes the alpha diversity measurements used to determine the extent to which BMD altered the composition of the microbiota. This included measurements of observed OTUs (1A), Faith's phylogenetic diversity (1B), Shannon (1C), and Simpson (1D) alpha diversity indices to compare treatment groups. Non-parametric two-sample *t*-tests were used to identify significant differences among the groups. While significant differences in OTUs were not observed among the groups by the Simpson and Shannon diversity metrics (data not shown), differences were noted by Faith's phylogenetic diversity (**Table 1**).

Within the BMD treated group, the distal colon showed higher phylogenetic diversity compared to the cecal samples ($p = 0.036$). The phylogenetic diversity of the colon of the BMD treated birds was also greater than the same samples from the control group ($p = 0.006$). This could indicate the BMD treatment is causing a few OTUs to become depleted hence allowing for other

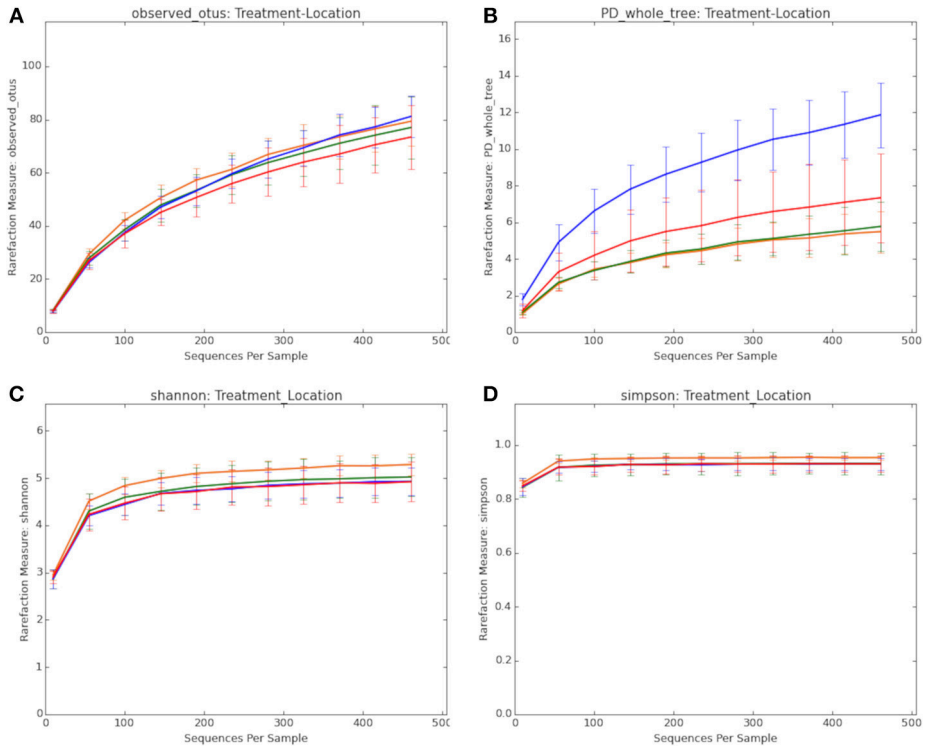


**FIGURE 1 |** Comparison of the composition of the microbiota in treatment groups. **(A)** Observed OTUs. **(B)** Faith's phylogenetic diversity. **(C)** Shannon alpha diversity index. **(D)** Simpson alpha diversity index. Treatment groups include: BMD treated, cecum (red line); BMD treated, distal colon (blue line); Control, cecum (orange line); Control, distal colon (green line).

**TABLE 1 |** Significant differences among the treatment and control groups as assessed by Faith's phylogenetic diversity non-parametric two-sample *t*-tests.

| Group 1 | Group 2 | Group 1 mean | Group 1 std | Group 2 mean | Group 2 std | *t* statistic | *p*-value |
|---|---|---|---|---|---|---|---|
| Control-cecum | BMD-cecum | 5.48 | 1.13 | 7.32 | 2.41 | −1.80 | 0.66 |
| BMD-cecum | BMD-colon | 7.33 | 2.41 | 11.86 | 1.76 | −3.89 | 0.04 |
| Control-cecum | Control-colon | 5.48 | 1.13 | 5.77 | 1.32 | −0.43 | 1 |
| BMD-colon | Control-colon | 11.86 | 1.76 | 5.76 | 1.32 | 7.30 | 0.01 |

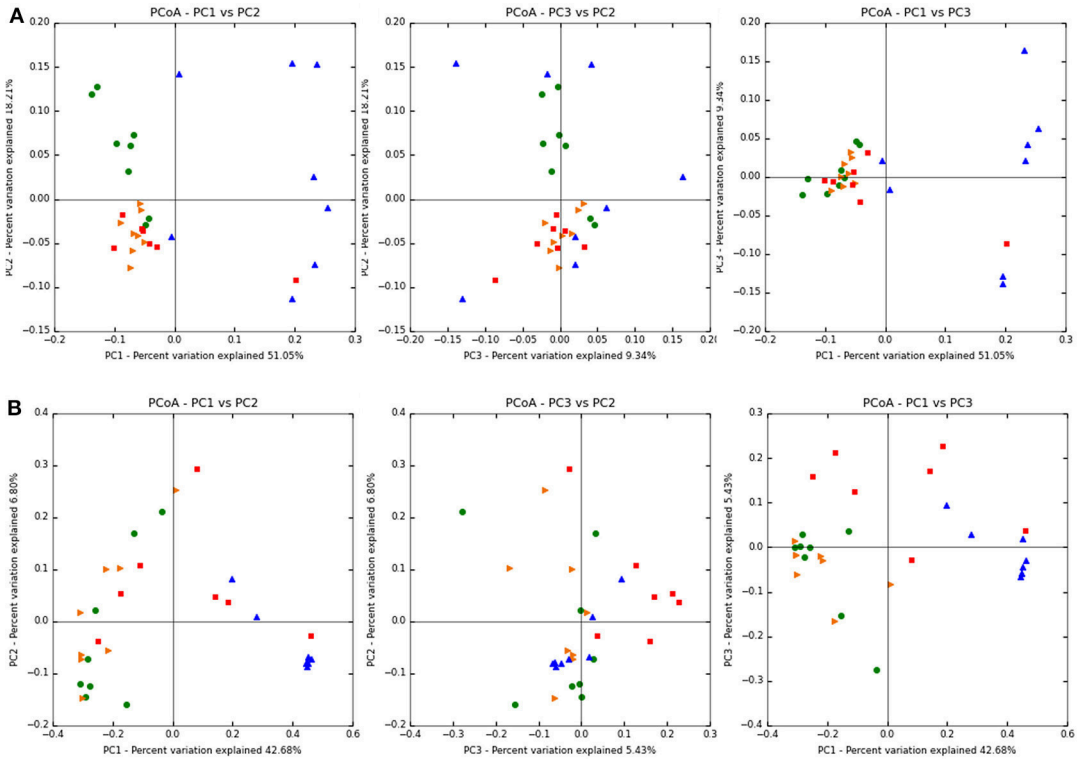


**FIGURE 2 |** Unifrac PcoA plots of the treatment groups. **(A)** Weighted **(B)** unweighted. Treatment groups include: BMD treated, cecum (red squares); BMD treated, distal colon (blue triangles); Control, cecum (orange triangles); Control, distal colon (green circles).

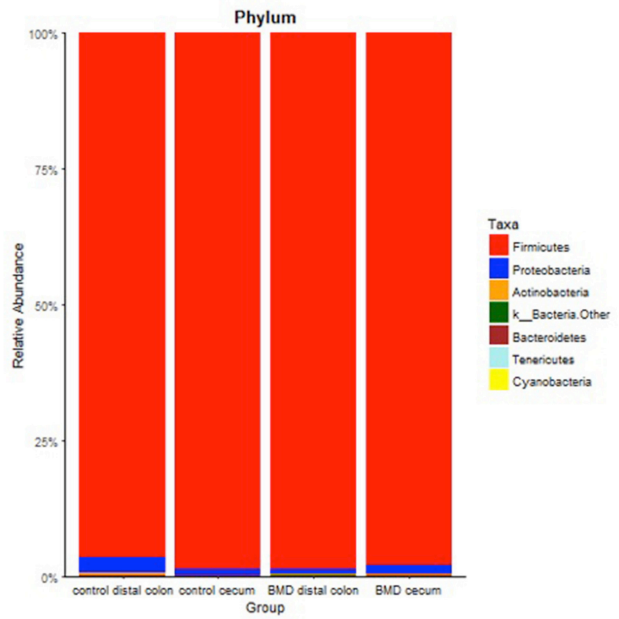


**FIGURE 3 |** Relative abundance at the phylum level for each of the treatment groups. Specific phyla are shown in the key on the right.

unique OTUs to establish or increase in their proportion within the community.

**Figure 2** shows the weighted (abundance considered) Unifrac PcoA beta diversity plots of the treatment groups (**Figure 2A**), while **Figure 2B** shows the unweighted (abundance independent) plots. In the weighted Unifrac PCoA analysis, cecal samples from both treated and control groups showed greater similarity than samples from the colon. Conversely, the colon samples of the control and BMD groups showed greater variability, with less distinct clustering. Also, the control samples from the colon clustered closer to each other and also closer to the cecal samples than the BMD colon samples.

The unweighted Unifrac PCoA plots showed that treatment influenced the dissimilarity of the samples more than GI tract location as samples in each group were not clustered as tightly as the weighted PCoA. Analysis of similarity (ANOSIM) and Adonis tests were performed on the weighted and unweighted Unifrac distances obtained from the beta diversity workflow in QIIME. The ANOSIM test based on both treatment and GI location resulted in a $p$-value of 0.001 and a test statistic of 0.379 for weighted and a $p$-value of 0.001 and test statistic of 0.556 for unweighted Unifrac analysis. These metrics indicated that the grouping based on the variables of treatment and GI location is weak (i.e., the differences can be explained by randomness). Adonis tests also based on both treatment and GI

**TABLE 2 |** Bacterial taxa with significant differences in abundance in pairwise comparisons between treatment groups as determined by Mann–Whitney *U*-tests.

| Taxonomic rank | Treatment Groups[a] | | | | Taxa with significant differences | Mann–Whitney *p* value |
|---|---|---|---|---|---|---|
| | Control | | BMD | | | |
| | Co | Ce | Co | Ce | | |
| Phylum | | | | | *Proteobacteria* | 0.043 |
| Class | | | | | *Bacilli* | 0.019 |
| Class | | | | | *Clostridia* | 0.007 |
| Genus | | | | | *Lachnospiraceae* | 0.008 |
| Genus | | | | | *Oscillospira* | 0.008 |
| Genus | | | | | *Erysipelotrichaceae* cc_115 | 0.034 |
| Genus | | | | | *Enterococcus* | 0.009 |
| Genus | | | | | *Peptostreptococcaceae* | 0.035 |
| Genus | | | | | *Lachnospiraceae* | 0.049 |

[a]*Co, colon; Ce, cecum. Filled cells show pairwise comparisons associated with significant differences in taxonomic abundance.*

location resulted in a $p$-value of 0.001 and an $R^2$ value of 0.441 for weighted and a $p$-value of 0.001 and $R^2$ value of 0.404 for unweigthed Unifrac analysis.

## Relative Abundance

Differences in relative abundance among all the treatment groups at different taxonomic levels were assessed using Mann–Whitney U-tests. **Figure 3** shows the taxonomic summary for each group at the phylum level and **Table 2** shows the $p$-values for the Mann–Whitney U-tests for phylum level differences. As evident, the dominant phylum for each treatment group in both the distal colon and cecum was *Firmicutes* (96.5–98.7%), with other phyla including *Proteobacteria* (0.9–3.0%), *Actinobacteria* (0.1–0.5%), and *Bacteroidetes* (∼0.1%).

At the class level (**Table 2**), *Bacilli* were depleted in the cecum of the control fed group compared to the distal colon site ($p = 0.01865$) and *Clostridia* were enriched in the cecum of the control birds compared to the distal colon ($p = 0.007$). **Figure 4** shows the relative abundance of the classes from each treatment group. The *Firmicutes Clostridia* and *Bacilli* were the dominant class (77.7–94.5 and 2.0–15.6%, respectively), with the remaining classes including *Erysipelotrichi* (1.5–3.2%), *Gammaproteobacteria* (0.9–2.9%), and *Actinobacteria* (0.1–0.5%). The *Clostridia* appeared to comprise a greater relative abundance in the cecum (84.3–94.5%) of both treatment groups compared to the distal colon (60.2–87.2%). There were no significant differences between the cecal samples of the control and BMD treated groups or the cecal samples and the distal colon samples of the BMD group.

Selected genera were also significantly altered by the BMD treatment (**Table 2**). Specifically, two genera, *Oscillospira* and an unnamed member within the *Erysipelotrichaceae* family (cc_115), were depleted in the distal colon of the BMD supplemented group compared to the distal colon of the control group ($p = 0.008$ and 0.034, respectively). Conversely, an unknown genus in the family *Lachnospiraceae* was enriched in the distal colon of the BMD treated group compared to the distal colon of the control group ($p = 0.008$). The same microorganism was also depleted in the cecum compared to the colon of the BMD

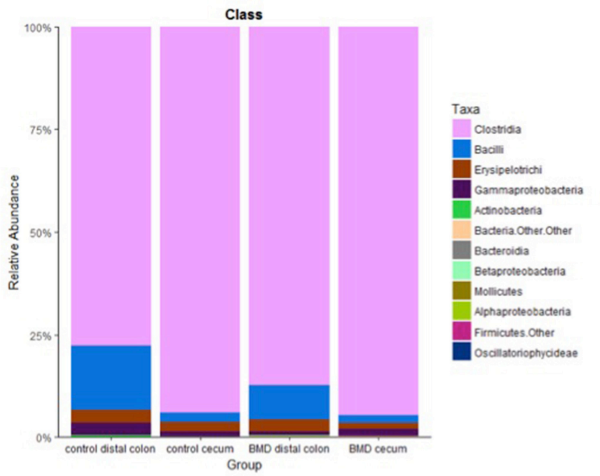


**FIGURE 4 |** Relative abundance at the class level for each of the treatment groups. Specific classes are shown in the key on the right.

treated birds ($p = 0.049$). Only an unknown genus in the family *Peptostreptococcaceae* was depleted in the cecum of the BMD treated group compared to the cecum of the control group ($p = 0.034$). **Figure 5** summarizes the genera that comprised each of the treatment groups and reveals that no one genus dominated in abundance.

# DISCUSSION

Consistent with previous studies, the distal GI tracts of the birds surveyed here were dominated by *Firmicutes* (**Figure 3**), which included the classes *Clostridia* and *Bacilli*. Members of the phyla *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Tenericutes* were also detected (47–49). The differences in the distribution of the microbiota were more evident at the class and genus level with the greatest changes observed in the distal colon in the BMD treated birds. Faith's phylogenetic diversity revealed an

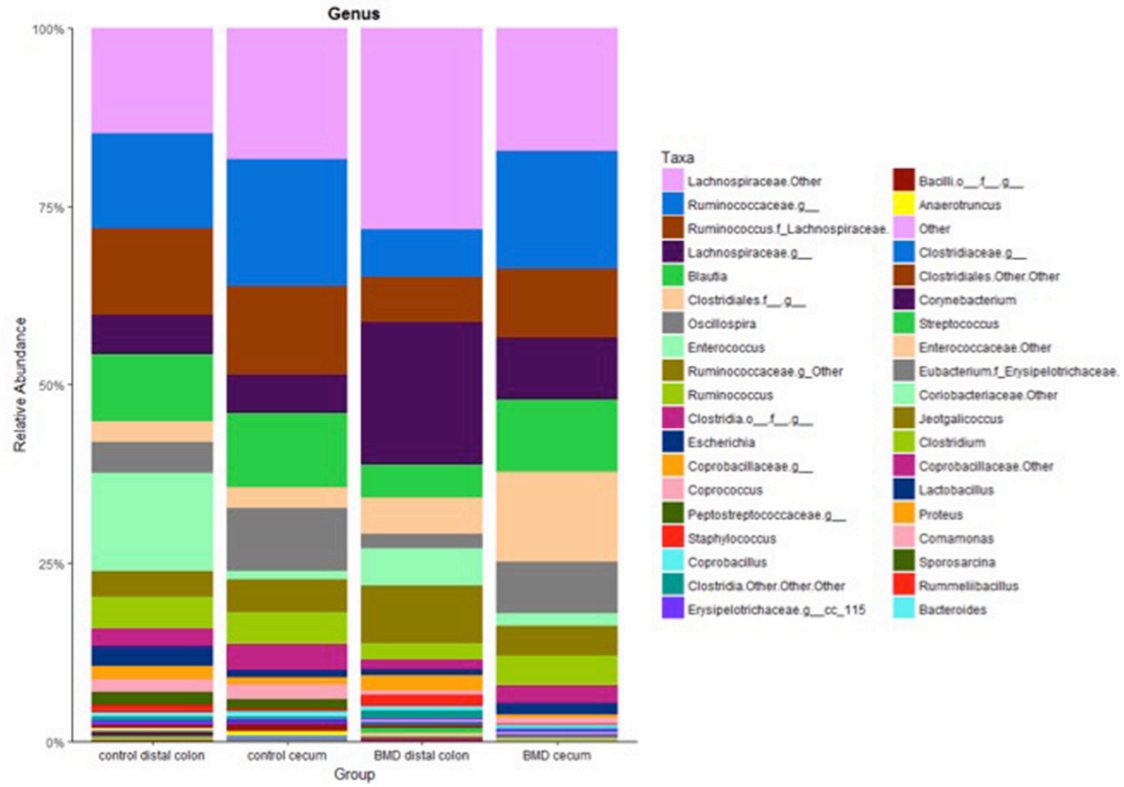


**FIGURE 5 |** Relative abundance at the genus level for each of the treatment groups. Specific genera are shown in the key on the right.

increase in diversity in the colon of the BMD treated birds. One explanation for this is that the antibiotic treatment depleted some gram-positive species that allowed others to expand in their place without significantly altering the total number of OTUs observed within the samples. In general, the composition of cecal samples showed more similarity than samples recovered from the distal colon for both the control and BMD treated birds. There were also fewer changes between the cecal samples of the control and BMD groups compared to the distal colon samples. The observed differences in the microbial populations and relative stability of the cecum is consistent with previous studies comparing cecal populations and feces in broiler chickens (36), and indicates that the cecal microbiota is buffered to some extent from the antimicrobial effects of BMD. This likely holds true for other feed additives in chickens (31, 50).

Individual variation was more also evident in the distal colon samples than the cecal samples. Bird to bird variation is not uncommon in chickens and may be explained by the immediate environment having significant impact on how domestic fowl acquire their microbiota over maternal sources as is observed in most mammals. Chicks hatched in commercial settings are typically not exposed to the hen's microbiota post hatching, therefore colonization depends on environmental factors and could be affected by the surroundings, litter management practices, and contact with other chicks (50, 51).

As cited in the introduction, there have been several studies showing that sub-therapeutic doses of antibiotics can alter the microbiota. To enhance these effects, we used a BMD dose considered to be therapeutic designed to reduce potential pathogens during outbreaks of GI diseases such as necrotic enteritis (8, 37). At this dosage, the Phylum *Proteobacteria* was reduced ($p = 0.04$) in the distal colon of the BMD treated birds when compared to the control. BMD treatment also decreased *Oscillospira, Peptostreptococcaceae*, and an unknown *Erysipelotrichaceae* in the colon of the birds, while only *Peptostreptococcaceae* was depleted in the cecum of the BMD group.

Between treatment groups, the control samples clustered closer together in the Unifrac PCoA plots compared to the BMD groups. The individual variation among birds, as well as variability in feed consummation may have contributed to the lack of tight clustering in the BMD groups. "Pecking order" among the birds may also influence feed (and BMD) consumption within the groups (52).

In general, few OTUs were significantly different between the control and BMD treated group in the distal colon. While the more proximal GI has greater susceptibility to antibiotics than the distal GI, this may also indicate the bacteria in the distal colon of the chicks have a higher proportion of bacteria that are resistant to bacitracin (22, 53, 54).

These results have implications for development of new strategies as use of antibiotics for growth promotion is being phased out of commercial use. Specifically, selective manipulation of the microbiome through alternative supplementation is growing in interest as an alternative to antibiotics (55, 56). This can include the use of beneficial bacteria as probiotics, prebiotic supplementation, phytobiotics, or enzymes (30). Antibiotic alternatives can confer resistance to colonization of pathogens through competitive inhibition, decreasing pH of the GI tract, or by contributing to overall animal health through immune modulation (55, 57, 58).

Probiotics can consist of one or more Gram-positive bacteria, such as *Lactobacillus*, *Enterococcus*, or *Bacillus*, as well as multiple strains of the same species. For example, Nisbet found administration of 29 microorganisms from ceca of older, *Salmonella* free chicks protected 1-day old chicks from various enteric pathogens (59, 60). Probiotics also have potential to replace growth-promoting antibiotics as evidenced by studies showing broilers fed *Bacillus subtilis* daily had increased weight gain and improved feed conversion ratios than the control animals (58). Additional benefits of probiotics include enhanced production of short chained fatty acids (butyrate, acetate, and proprionate) in broilers fed non-digestible carbohydrates or oligosaccharides that are fermented by members of the microbiota, as well as enhanced protection and antimicrobial production (30). Prebiotics can increase adaptive immune responses when administered *in ovo* and aided in intestinal development in newly hatched chicks (61).

The relative stability of the chicken microbial community in the cecum compared to the distal colon may prove to be beneficial to production practices that seek to exploit the microbiome to enhance production. For example, modern poultry production typically prevents contact between chicks and older birds. This means the chicks are exposed to environmental bacteria rather than those associated with a healthy bird. Cecal transplants, or beneficial bacteria sourced from the cecum, could be used as "seeds" for post hatched chicks. While more studies are needed, the cecal microbiota presents itself as a potential source for colonizing newly hatched chicks with a healthy, chicken specific community that can speed GI development and help prevent diseases such as necrotic enteritis.

## ETHICS STATEMENT

This study was carried out with the approval of the Iowa State University Institutional Animal Care and Usage Committee under protocol number 6-11-7167-G.

## AUTHOR CONTRIBUTIONS

AP designed and conducted the experiments, analyzed the data, and wrote the manuscript. GP assisted with data analysis and helped write the manuscript.

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# Applications of Microbiome Analyses in Alternative Poultry Broiler Production Systems

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While most of the focus on poultry microbiome research has been directed toward conventional poultry production, there is increasing interest in characterizing microbial populations originating from alternative or non-conventional poultry production. This is in part due to the growing general popularity in locally produced foods and more specifically the attractiveness of free-range or pasture raised poultry. Most of the focus of microbiome characterization in pasture flock birds has been on live bird production, primarily on the gastrointestinal tract. Interest in environmental impacts on production responses and management strategies have been key factors for comparative microbiome studies. This has important ramifications since these birds are not only raised under different conditions, but the grower cycle can be longer and in some cases slower growing breeds used. The impact of different feed additives is also of interest with some microbiome-based studies having examined the effect of feeding these additives to birds grown under pasture flock conditions. In the future, microbiome research approaches offer unique opportunities to develop better live bird management strategies and design optimal feed additive approaches for pasture flock poultry production systems.

**Keywords: pasture flock, microbiome, feed additive, preharvest, gastrointestinal tract**

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## INTRODUCTION

Pasture flock or free-range raised poultry continues to be a popular market option for retail poultry products for a variety of reasons including the attractiveness of being locally produced and sold for retail (1–4). As the production of naturally-raised poultry either as pasture flock or free-range grown chickens increases, consideration of factors such as environmental impact and food safety concerns have to be taken into account (4–6). This impact may include changes in nitrogen and phosphorus levels as well as antimicrobial runoff and pathogen contamination (6, 7). However, small poultry producer operations both in the U.S. and internationally are highly diverse in management styles and present challenges for making uniform recommendations (8, 9). Given this diverse range of management approaches, food safety problems can be somewhat unpredictable, and coupled with the more restrictive nature of mitigation options, present a challenge for restricting the prevalence of foodborne pathogens (7, 10). This combined with favorable public opinion regarding free-range livestock production, represents a dilemma for food safety risk management (11).

Foodborne pathogens that have been associated with free-range birds either in preharvest production or from retail birds include *Campylobacter*, *Listeria*, and *Salmonella* (12–19). Consequently, more focus is being directed toward developing acceptable methods for controlling and reducing the levels of prevalence of these pathogens on retail poultry products. One of the primary targets is live bird production where limiting foodborne pathogen establishment is certainly a driver but improving bird health and reducing mortalities are also important considerations. Along these lines, feed additives such as probiotics and prebiotics are attractive as they represent generally acceptable management practices and have been demonstrated to be at least somewhat effective in conventional poultry production (20–23). These issues have become more critical with the removal of antibiotics from conventional production. However, as with conventional live bird production, it has also become clear that designing optimal feed amendment approaches requires a better understanding of the avian gastrointestinal tract (GIT) system microbial ecology as well as the complexities associated with responses to the alteration of feed and feed amendments (24). With the emergence of sequence based assessment of microbial communities, it has now become possible to develop a much more comprehensive assessment of individual members of the microbial GIT communities. The objective of this review is to examine and discuss the use of these approaches for gaining a better understanding of the microbial populations in pasture or free-range poultry production.

## PASTURE FLOCK PRODUCTION–GENERAL CONCEPTS

Poultry production in the early 20th century was historically characterized by small flock type farms with poultry viewed as supplemental income in a mixed food animal operation dependent upon multiple sources of revenue (3, 25–27). As commercial poultry production progressed over the 20th century and into the 21st century, the size of flocks increased dramatically with nutrition and breeding technologies advancing and the industry becoming vertically integrated to the point of large commercial flocks dominating the poultry meat market (25). However, a new market phenomenon has emerged in the past few years in the rise of locally grown free-range or pasture flock raised birds with on-farm processing (28, 29). While chicken can be marketed as either natural or organic, natural labeled poultry products generally outsell their organic counterparts (1).

Organically produced foods are much more rigorously regulated with requirements in place for all aspects of production and processing while natural poultry and other meats are only regulated from a post-processing side (1). Pasture raised poultry, when considered within a broad category, is defined as a production system where birds are raised outdoors in some sort of small, moveable, ventilated pen arrangement (16, 30, 31). Management of these pens in terms of frequency of rotation in a pasture, protection from predators, types of housing, and other requirements are described in detail by Fanatico (32). Housing can either be moveable or fixed with portable housing being easily moved without causing injury to the birds (16, 30). Given the diverse nature of these types of practices, several potential challenges exist for achieving consistent production levels to meet market demands over a period of time. Issues considered important for small producers may vary depending on the geographical region. For example, in a Minnesota-based survey, Jacob et al. (8) concluded that extension programs were needed by small scale antibiotic-free flock producers for feed and pasture choice, waste disposal, pre-slaughter feed withdrawal, and marketing. When Hilimire (33) surveyed California pasture flock growers, the primary issues identified by these farmers were predation of birds and feed costs.

On-farm poultry processing using mobile poultry processing units (MPPUs) has become an attractive means for pasture flock growers to process their birds in preparation for the market (29). The mobile characteristic of these processing units offer distinct advantages for rural small flock producers where the nearest processing facilities may be located at distances that preclude ready access (3). This is also consistent with the conclusion by Angioloni et al. (29) that MPPUs and on-farm processing setups cost more to purchase initially, but once established, enable a lower processing cost compared to off-farm alternatives. In addition, wastewater originating from on-farm processing and MPPUs generate lower total Kjeldahl nitrogen and total phosphate than conventional processing (6). While individual requirements for construction of MPPUs may vary from state to state, most are on some type of either open or enclosed wheeled trailer that houses most of the standard components of poultry processing, including kill cones, pickers, evisceration tables, chill tanks, and hand washing sinks (3). Similar to organic poultry production, the range of available sanitizers remains somewhat limited for pasture flock processing compared to conventionally produced poultry, and further research is needed to identify more antimicrobials that would not only be effective against foodborne pathogens but acceptable for MPPU application and economical for the producer (10, 34). This is due in part to the need to limit foodborne pathogen contamination originating from birds entering processing as well as from cross contamination events that may occur during processing (16).

## PASTURE FLOCKS AND FOODBORNE PATHOGENS

There are several unique and particularly challenging aspects of pasture flock poultry production including maintaining bird health, reducing mortalities, and limiting foodborne pathogen prevalence. Of the issues associated with pasture poultry flocks, foodborne pathogen occurrence has probably been the most extensively studied. Foodborne pathogens have been isolated from all phases of pasture poultry production from live bird production, during processing, and from birds marketed at retail. Most of the focus has been on *Salmonella* and *Campylobacter*, but other foodborne pathogens such as *Listeria* have been isolated as well. Milillo et al. (15) using an analytical profile index of *Listeria*, *sigB* allelic typing, and *hlyA* PCR tests found that both *Listeria monocytogenes* and *Listeria innocua*, including hemolytic

*L. innocua*, could be isolated from the cecal and environmental (grass/soil) samples of pasture flock birds. Locatelli et al. (35) isolated *Listeria* spp. from fecal and soil samples in 15 % of the pasture fock poultry farms they surveyed and identified *L. innocua*, *L. monocytogenes*, and *L. welshimeri*.

Bailey and Cosby (12) isolated *Salmonella* from 31% of 135 free-range carcasses and 25% of 53 all-natural carcasses of birds that had not received meat, poultry meal, or antibiotics. Scheinberg et al. (36) saw similar prevalence for *Salmonella* when they examined whole chickens from farmers' markets and reported that 28 and 90% were positive for *Salmonella* spp. and *Campylobacter* spp., respectively. In a pasture flock surveillance study, Melendez et al. (14) identified 59 *Salmonella* isolates from pens, feed, water, and insect traps at the farm, as well as from retail carcasses obtained from a local natural foods store and a processing plant. When the *Salmonella* isolates were serotyped, the majority were *S.* Kentucky at 53%, *S.* Enteritidis (24%), *S.* Barelly (10%), *S.* Mbandaka (7%), *S.* Montevideo (5%), and *S.* Newport (2%). Interestingly, despite originating from antibiotic-free production systems, all isolates were resistant to sulfixasole and novobiocin, some were resistant to additional antibiotics, and most contained class I integrons. Additionally, a study by Rothrock et al. (37) found antibiotic resistance from poultry and environmental isolates of *E. coli*, *Salmonella*, *Campylobacter*, and *Listeria* from 15 all-natural, antibiotic-free, pasture flocks taken from six farms in the southeastern U.S. Using the NARMS antibiotic sensitivity protocols, Rothrock et al. (37) observed that levels of antibiotic resistance tended to remain consistent throughout the farm-to-fork chain. However, they also found that there appeared to be individual farm-level effects, shown by *Salmonella* only being isolated from three farms, and that high levels of antibiotic resistance in one genus did not correlate to resistance in others, highlighting the need to design specific assessments for resistance in public health studies. Other characteristics of foodborne pathogens may be influenced by conditions associated with free-range production systems. For example, Hanning et al. (13) characterized *Campylobacter* isolates from pasture flock farms, retail, and processing facilities over an 8 month time period and observed that the prevalence between conventional and pasture flock retail birds was similar. However, when they sequenced the short variable region of the *flaA* locus (*flaA* SVR) to genotype *C. jejuni* isolates, they noted that the genetic diversity of the *flaA* SVR genotypes increased from the farm to the carcass in pasture flock birds when compared with conventional poultry.

Some of the prevalence of foodborne pathogens in small-scale and pasture raised birds could be related to rearing conditions. Indeed, Lupatini et al. (38) demonstrated that organic farming increased taxonomic and phylogenetic richness, diversity, and heterogeneity of soil microbial consortia when compared to conventional farming. Tangkham et al. (39) tracked *Campylobacter* appearance on a weekly basis in eggshells, live birds, feed, and the drinking water in the rearing environments of small broiler operations where birds were raised either in open-air housing or environmentally controlled housing. They concluded that vertical transmission from eggs was not a factor but did note an increase in *Campylobacter* spp. in birds raised in open-air housing compared to those from environmentally-controlled housing. Li et al. (40) reported an increase in the incidence of *Salmonella* and *C. jejuni* recovered from the ceca of birds processed in a MPPU after being raised on built-up litter when compared to birds reared on clean shavings. The method of processing may not be a factor. Trimble et al. (17) compared pasture raised broilers processed on farm sites, a small U.S. Dept. of Agriculture inspected facility, and a MPPU pilot plant facility and concluded that birds generally were contaminated with *Salmonella* and/or *Campylobacter* regardless of the type of facility. However, Li et al. (40) reported that post-chill application of antimicrobials can successfully reduce *Campylobacter* and *Salmonella* carcass contamination. Reduction of foodborne pathogens during the processing of pasture flock birds may be important, not only for decreasing levels on the retail birds but also for reducing pathogen loads in the processing waste disposal onto the soil (18).

## MICROBIAL ECOLOGY IN PASTURE FLOCK POULTRY– GENERAL CONCEPTS

As pasture flock poultry production continues to grow to meet market demand, increased efforts will be needed to develop systematic approaches to improve the microbial safety of the product during all phases of the farm-to-fork continuum. This remains a challenge due to the diversity in locations of farms and management practices. This is important because part of the development for more optimal antimicrobials as well as control measures during the grow-out of pasture flock birds requires an understanding of the microbial ecology not only of the GIT in the bird, which can harbor high levels of foodborne pathogens, but also during processing of the bird into meat products. Non-pathogenic indigenous microbial communities in the avian GIT can influence the ability of the respective pathogen to colonize and become established (41). Feed amendments such as probiotics and prebiotics can alter or shift the GIT microbial population to become more of a barrier to pathogen colonization while other agents such as antimicrobial chemicals and biological agents such as bacteriophages can decrease pathogen populations already established (42–44). The concern during processing is not just the presence of pathogens but the general bacterial load that, depending upon the quantity and type of organisms present, can decrease retail shelf life of the processed bird. In short, better characterization of the microbial populations is needed to develop a comprehensive approach to targeting microbial populations present in these different phases of pasture flock production.

Classic culture-based microbiology has been insightful for some aspects of microbial ecology in food animal systems. However, limitations in the recovery of representative viable organisms may lead to under representing certain microbial populations such as with strict anaerobes in the GIT. This has resulted in an incomplete picture of the impact of feed amendments such as probiotics (45). More recently, next generation sequencing (NGS) technologies have become routine and the opportunity to characterize a microbial population

in its entirety without relying on culturing is now possible. Based on the sequencing of the 16S rRNA gene, microbial taxa can be identified as function of operational taxonomic units (OTUs) and differences in the microbial community from independent samples or sampling sites can be compared as a function of microbiome composition (46). Improvements in computer program pipelines and bioinformatic tools offers in-depth analyses of microbiomes and delineation of specific factors that may be influential on overall microbial communities as well as individual members of the respective communities and potential integrative networks among groups of organisms (46–48). Microbiome analysis techniques have certainly been used extensively to study poultry GIT responses to different treatments and to a lesser degree poultry processing microbial populations (49, 50). However, much less research has been done on microbiome analyses with pasture flock birds even though the differences in the microbial communities would likely offer an opportunity to delineate specific patterns based on the respective microbial consortia profiles and potentially predict outcomes in response to changes such as general dietary modification or inclusion of specific feed additives. In the following sections the microbiome research that has been done with pasture flock poultry will be discussed.

## POTENTIAL FACTORS THAT INFLUENCE GIT MICROBIOMES IN PASTURE FLOCK BIRDS

Based on conventional poultry production studies, the GIT microbiome composition and intestinal function of the bird is influenced by several factors, some more obvious than others. Factors which impact the diversity of the microbiota in the bird and GIT function can originate from diet, stocking density, geographical location, feed additives such as, bird age, bird environment, and pathogen presence among other less well defined factors (51–56). Given the diverse nature of pastures with potential differences in forages as well as exposure to a wide range of environmental conditions, it would not be surprising that the birds' GIT microbial populations might also reflect this diversity. Likewise, differences in the length of growth cycles, utilization of slower growing bird breeds, stocking density, and potential contact with wildlife could be influential as well.

While these factors may potentially influence the microbiome diversity in pasture raised birds, only minimal research has been conducted with birds from these types of production systems. The majority of poultry GIT microbiome work has focused on birds raised under conventional management practices and any conclusions pertaining to pasture flock birds need to be extrapolated from the outcome of these studies. Some factors such as exposure to wildlife would be considerably different than conventional poultry production systems but much less is known on wildlife microbiomes. Hird (57) has pointed out that captivity alters the microbiome and that the birds yield highly diverse microbiomes. However, some of the work that has been done with wild bird species may have potential relevance. For example, when Bodawatta et al. (58) sequenced the GIT of New Guinean passerine bird species, they noted a dietary influence with more microbial diversity detected in the omnivore species than in the insectivore species, with insectivore GITs consisting mainly of lactic acid bacteria. Since pasture flock birds would have access to a variety of insects, it would be of interest to compare their GIT taxonomy with wild birds that consume insects as a proportion of their diet. Teyssier et al. (59) observed that during the later stages of nestling development of the Great Tit (*Parus major*) passerine bird, the nest environment impacted the composition of the GIT. Along these lines, in the domesticated Peking duck, Best et al. (60) demonstrated that GIT populations were different in aviary-raised ducks vs. barn-raised birds.

Diet differences between conventionally raised poultry and pasture flock birds may also be a distinguishing influence on the GIT microbiota, particularly if low nutrient diets are used to ensure slower growth in pasture flock birds (61). Even when diets are quite similar, differences between GIT microbiota may still be observed. For example, when de Greeff et al. (62) compared jejunal gene expression in layer hens fed either conventional or organic diets of an otherwise identical composition, they detected differences in the expression of 49 genes, including those associated with cholesterol synthesis and immunological processes. In addition, pasture flock birds have access to a much wider variety of food sources such as insects and forages in addition to the formulated diets provided. Whether fiber intake occurs from the forages present on pasture presents another unknown. Low fiber diets have been shown to alter gizzard function and have been touted as a means to maintain proper GIT function and improve overall bird performance (63, 64). There is likely an impact on the GIT microbiota as well. The cecal microbiota of layer hens and chicks have been demonstrated to be capable of fermenting fiber sources such as alfalfa, causing subsequent effects on the GIT microbiota (65–70).

Age and breed of bird are likely factors as well. Kers et al. (71) concluded that host related factors of sex, age and breed exhibited considerable impact on GIT microbial populations with differences in microbial community composition between layer and meat-type chickens. Lumpkins et al. (72) compared Athens Canadian Random Bred (ACR) broilers with modern multipurpose bird strains and high yield bird strains and detected differences in bird performance, GIT measurements, and the GIT microbial consortia between the ACR birds and the modern bird strains. It is likely that these effects may also be observed in pasture flock raised birds as well. For example, Hanning et al. (73) observed differences in body weight responses to fiber or prebiotic supplemented diets between Naked Neck slow-growing birds vs. Cornish White Rock cross fast-growing broilers reared under pasture flock conditions. Age and development of the avian GIT also appears to greatly influence GIT microbiome composition. *In vitro* cecal incubations using inocula sourced from birds of different ages support the impact of age on microbiome composition and ability to inhibit *Salmonella* introduced into the incubation (74, 75). Future studies will need to be conducted specifically with pasture flock birds to delineate the relative levels of influence that age vs. breed have on the development of the GIT microbiota to establish a baseline for

additional comparisons with variables such as impact of diet and environment.

## FEED ADDITIVES AND PASTURE FLOCK GIT MICROBIOMES

Given the environmental stresses and other challenges associated with pasture flock poultry production, choices in feed additives and dietary modulators are an important consideration to improve bird health, reduce mortalities, and limit foodborne pathogen establishment. Several feed additives have been suggested over the years that could potentially be used in pasture flock and/or organically raised poultry and replace antibiotics in conventional poultry production. These include bacteriophages, botanical products, organic acids, probiotics and prebiotics, and others (10, 22, 34, 76–82). Most of these feed additives have only been suggested as potential agents for use in alternative poultry production and have only had minimal research conducted with pasture flock poultry operations. However, some pasture flock research has been conducted with prebiotic supplementation that determined GIT microbial population responses.

Prebiotics are compounds, usually complex carbohydrates, which cannot be directly utilized but can be fermented by GIT bacteria, particularly members that are considered beneficial to the host such as bifidobacteria and lactic acid bacteria (21–23, 78, 83–85). Considerable emphasis has been placed on prebiotics as one of the candidates to replace antibiotics in conventional poultry production but there have only been a few isolated studies on pasture flock poultry (22, 23). Initial research conducted on pasture flock poultry and prebiotics focused primarily on the supplementation of commercial probiotics and their impact on bird performance and meat quality characteristics. After feeding probiotics and prebiotics from bacterial and yeast sources to free-range broilers, Pelícia et al. (86) reported lower mortalities and greater weight gain in birds fed the bacterial-based prebiotic, while both bacterial and yeast-based probiotics and prebiotics improved carcass yield when compared to control birds. However, some of the responses may be poultry breed dependent as well as specific for a particular type of prebiotic or dietary supplement. Hanning et al. (73) reported that free-range raised fast-growing Cornish Cross White Plymoth Rock broilers fed diets supplemented with the prebiotic fructooligosaccharide (FOS) exhibited a higher final body weight (8 weeks) while slow-growing Naked Neck free-range birds on a fiber source (plum fiber) had greater final body weight gains.

With advances in molecular techniques for microbial identification and characterization, more recent pasture flock studies have included in-depth analyses of the GIT microbiota. Park et al. (87) examined the response of Naked Neck chicks fed commercial yeast-based prebiotics while being raised in pasture pens that were moved twice a week. They did not detect differences in feed conversion ratios, live bird body weights, or post-processing body weights, however the commercial prebiotics did decrease cecal *Campylobacter* populations. Using a PCR-based denatured gradient gel electrophoresis (DGGE) method to compare cecal microbial populations from birds fed different commercial prebiotic treatments, Park et al. (87) also found that the prebiotic cecal populations were more related within their respective groups than control bird cecal populations. When individual bands were excised from the DGGE bands and sequenced, *Bacteroides slaanitronis* was identified in all treatment groups while *Barnesiella ciscericola* and *Firmicutes* were detected only in the prebiotic treatment group ceca. The authors concluded that DGGE could be useful in easily detecting shifts in cecal populations from prebiotic usage despite the limitations in the technique.

Development of NGS techniques for routine microbial population characterization based on 16S rRNA gene comparisons have greatly improved the ability to conduct comprehensive in-depth GIT microbiome analyses (24, 46). Park et al. (88) used an Illumina MiSeq platform based on the V4 region of the 16S rRNA gene to identify cecal populations in free-range birds fed two commercial yeast cell wall-based prebiotic compounds. Diversity differences among the treatments were relatively minimal with the two products resulting in different levels of OTUs, one similar to that of control birds and the other yielding lower numbers of OTUs. When microbial population diversities were compared among the groups, the two prebiotic fed groups and the control group cecal populations were distinctly clustered on unweighted principal coordinated analysis (PCoA) Unifrac plots. Taxonomic analyses revealed a somewhat minimal impact by both prebiotics at the phyla level, although one of the yeast prebiotics did lead to an increase in *Proteobacteria* and *Cyanobacteria* OTUs compared to the other treatments while increased OTUs of *Firmicutes* were detected in the control diet fed bird ceca. At the genus level, one of the yeast cell prebiotics led to an increase in *Faecalibacterium*. Overall it appeared that microbiome analyses could successfully detect differences in cecal microbial populations from pasture flock poultry fed different prebiotic containing diets even when the prebiotic sources were derived from similar commercial sources.

The impact of age on microbiome population composition could also be shown through 16S rDNA analysis. Park et al. (89) utilized microbiome sequencing to compare cecal microbiota populations from chickens with plum fibers, FOS, or GOS feed additives and found cecal populations to be impacted by their respective treatments. As the plum fiber and FOS fed birds aged (2–6 weeks), Shannon diversity indices increased while the total number of OTUs did not increase appreciably for control birds. However, when phylogenetic clustering for each treatment was compared, bird age had a much greater impact on clustering patterns than that from the corresponding treatments. Analysis based on correlations with metadata found that host age and developmental stages were the key contributors to microbial community diversity. The influence of age on GIT microbial composition has also been reported by Cui et al. (90) in young vs. older hens in both caged and free-range birds. Further analyses breakdown of OTUs revealed that the genus *Alistipes* increased with age across all bird groups and could be a potential predictive indicator for age, weight, and *Campylobacter* populations. *Lactobacillus intestinalis* was also predictive for *Campylobacter* as well as the presence of FOS, GOS, and plum fiber in birds at 2 weeks of age. Clearly age is a major driver

of changes in microbial diversity to the point of masking other factors; however, this could be different depending on the stage of bird development. It would be interesting to examine the changes in diversity during the first 2 weeks of age in birds when different dietary amendments are introduced to determine whether age is still a predominant factor.

## FUTURE DIRECTIONS

As pasture flock poultry markets continue to grow in popularity, there will be an increasing need to develop systematic approaches for optimizing management practices to reduce mortalities, improve health, and limit pathogens. The introduction of feed additives that are considered acceptable by the both producers and consumers offers a means to potentially achieve some of these goals. However, more research is needed to assess and develop consistent baseline patterns of production responses that could be viewed as some sort of standard for evaluating newly developed feed additives. To accomplish this, factors such as breed differences, environmental impact, and dietary management would have to be considered. As a part of this evaluation, the impact on bird responses due to changes in the GIT microbiota is emerging as an important factor as well. With the introduction of more advanced and cheaper sequencing methods, in-depth assessment of GIT microbial responses has now become a reality. In the limited set of studies conducted thus far, it appears that age is one of the more important factors impacting microbiome diversity development. Whether dietary amendments such as prebiotics can also have an impact will need additional studies with a broader spectrum of prebiotic compounds and more frequent incremental sampling to delineate age vs. treatment influence on the microbiome.

There are other opportunities to apply microbiome analyses to pasture flock poultry production operations. While there has been a focus on studying the pasture flock birds, much less has been done to determine the impact of these free-range birds on their surrounding environments. Rothrock et al. (37) found that the antibiotic profiles of soil samples exhibited similar rates of antibiotic resistance as that from fecal samples from pasture flocks of birds, demonstrating the potential impact they may have. Public health studies, particularly those that focus on antimicrobial levels, must account for the role of poultry flocks on the environment (91). This could be a critical issue to consider given the placement of pens in pastures and the exposure of the soil and fresh water sources to these flocks with microbial populations shared by pasture flock birds potentially influenced by the presence of these birds. Whether the density of pasture flock birds would be sufficient to produce similar alterations in the soil microbiota remains to be determined, but it is conceivable that the frequency of moving pens vs. remaining in one place for an extended period of time may impact the soil in proximity of the pen.

Microbiome analyses could potentially also be informative for the assessment of pasture flock poultry processing. Deciding optimal sanitizers and antimicrobials is dependent on microbial profiling, usually done with combinations of non-selective and selective culture plating to enumerate the respective spoilage and foodborne pathogen microbial populations. Microbiome mapping has been done with conventional poultry processing and proven to be useful for following shifts in microbial populations during the various processing stages (50, 92). In the course of conducting the sequencing analyses and taxa identification, potential indicator microorganisms have been identified that offer predictable baselines for intervention evaluations. It is anticipated that microbiome approaches could be applied to pasture flock processing to achieve similar outcomes.

Improvements in sequencing technologies such as further development of long read sequencing platforms such as the Oxford Nanopore and Pacific Bioscience sequencers along with further development of fourth generation sequencing technologies offer opportunities for deep sequencing of microbial communities both in the GIT as well as in poultry processing microbial communities (93). Along with improved sequencing resolution data analyses will become more sophisticated with advanced statistical power to achieve correlations and network construction of the microbial communities and elucidate host genome wide-microbiome relationships (94). This could prove to be particularly important with the diverse chicken breeds used in pasture flock operations. Likewise, identification of non-pathogenic indicator organisms reflective of pathogens and other factors both in live bird production and processing may be more likely. This would allow for improved prediction of feed additive and antimicrobial strategies. Finally, as sequencing technologies and bioinformatics become more advanced it may become possible to link microbial population patterns back to the live bird flock prior to slaughtering and use this information to optimize sanitizer applications to retain fresh product shelf life.

## AUTHOR CONTRIBUTIONS

ZS, MR, and SR wrote and edited the manuscript. All authors significantly contributed to the work of this review.

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REVIEW
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# Formaldehydes in Feed and Their Potential Interaction With the Poultry Gastrointestinal Tract Microbial Community–A Review

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As antibiotics continue to be phased out of livestock production, alternative feed amendments have received increased interest not only from a research standpoint but for commercial application. Most of the emphasis to date has focused on food safety aspects, particularly on lowering the incidence of foodborne pathogens in livestock. Several candidates are currently either being examined or are already being implemented in commercial settings. Among these candidates are chemical compounds such as formaldehyde. Formaldehyde has historically been used to inhibit *Salmonella* in feeds during feed processing. Currently, there are several commercial products available for this purpose. This review will cover both the historical background, current research, and prospects for further research on the poultry gastrointestinal tract and feeds treated with formaldehyde.

**Keywords: formaldehyde, feed, poultry, *Salmonella*, gastrointestinal tract**

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## INTRODUCTION

Treatment of animal feeds has always been considered a critical component to food animal management to prevent the formation of mycotoxins and other biological contributors to contamination and feed quality decline during storage (1). There have been numerous research studies and applications for various chemicals to be added to animal and poultry feeds during feed processing, and these have been well-documented in published review articles over the past few decades (1–5). Not surprisingly much of the focus for the application of chemical additives particularly in poultry feeds has been directed toward limiting *Salmonella* in the feed (2–7). Research studies have ranged from the assessment of feed contamination at the feed mill to bird feeding trials and involved both natural *Salmonella* contamination and inoculation of a marker *Salmonella* strain.

In order for a particular chemical feed additive to possess commercial attractiveness to be promoted for routine use in animal and poultry feeds, several criteria essential to meet this demand would have to be considered. Some of these specifically for feed antimicrobials have been outlined previously (3) but would still apply in a general sense. Effectiveness in the presence of a high organic load that is characteristic of a typical mixed feed and/or individual feed ingredient would be a must. The effective dose would have to be safe in the target animal and not result in undesired residues in animal products.

The relative cost to be applied to large bulk quantities of feed would also need to be of a commercial scale level of utility as well as ease of application and minimal damage to milling equipment. Governmental regulatory approval both domestically in the United States and internationally for use in animal diets should be in place. The worker safety during application in the feed mill and post-milling, delivery to the farm and use at the farm would have to be established.

In this review a discussion of *Salmonella* occurrence in feeds will be described in brief, followed by discussion of one of the more prominent and widespread used chemical group of compounds, namely, aldehydes with the primary focus on formaldehyde/formalin in terms of antimicrobial mechanism(s) and efficacy as feed additives in the poultry gastrointestinal tract. Finally, future directions for application and improving efficacy will be discussed.

## FORMALDEHYDE—NATURAL OCCURRENCE AND BIOLOGICAL APPLICATIONS

In general aldehydes are relatively ubiquitous in the environment originating not only as a natural compound but as an intermediary endogenous product in biological metabolism and other processes as well-generation from automobile exhaust gases and indoor environments from sources such as building materials and furniture (8–10). The chemistry and pathways for their formation have been extensively discussed in a review by O'Brien et al. (8) and will not be discussed in detail in the current review. Numerous aldehydes including formaldehyde are detectable in a wide range of foods including fruits, vegetables, meat, cheese, and seafood (8, 11, 12). Aldehydes can be detected in the air, feed, tissue, and feces via personal monitors, spectrophotometric measurement of color reaction between tissue distillate, and chromatographic-sulphuric acid reaction, respectively (13). They can be formed as volatile aldehydes during cooking, particularly from edible oils, auto oxidation of unsaturated fatty acids, odor compounds emanating from rancid high-fat foods, and occurring as products from the storage of beer (12). Aldehydes and ketones are known to increase during milk thermal processing and storage of milk powder, resulting in changes in flavor and milk powder porosity (14). Formaldehyde in foods is released in the stomach and absorbed into the bloodstream where it is metabolized to formic acid by the red blood cells. Formic acid is further metabolized to carbon dioxide and water (15, 16). The metabolic half-life of formaldehyde is 60 to 90 s. This route of metabolism may be similar for other aldehydes. Aldehydes are also an important set of useful compounds for industrial processes such as flavors, fragrances, and pharmaceutical precursors. In addition, efforts have been made to genetically modify microorganisms to accumulate sufficient quantities for commercial purposes (17, 18).

Formaldehyde can serve as a fixative preventing cell autolysis and reacting with proteins, lipids and nucleic acids (19–21). The interaction of formaldehyde with peptides has been characterized by Metz et al. (17) as occurring via formation of either methylol groups, Schiff bases, or methylene bridges. Methylol and Schiff base modifications are considered reversible whereas methylene bridge products are stable and can lead to cross-linking of protein chains (17, 22–24). The type of bond formed between formaldehyde and protein/ amino acids is dependent on the reaction conditions (25).

The reaction of formaldehyde with aqueous solutions of crystalline amino acids (98:2 ratio) at 24°C resulted in the formation of a compound (described as an adduct) exhibited antimicrobial activity against *E. coli* and *Salmonella* (26). Only lysine, arginine, histidine and asparagine were reported to form the adduct. The bond between lysine and formaldehyde was found to be reversible and was broken by distillation in a mildly acidic solution suggesting a methylol or Schiff base linkage. This is consistent with the findings of Alexander et al. (25) that reported that methylol derivatives of formaldehyde and amino groups are unstable and dissociate under mildly acidic conditions. Additional research by Barlow (27) and Rude et al. (28) indicate formaldehyde added to fishmeal or corn amended with crystalline lysine under mild reaction conditions (ambient temperature) does not affect availability at the 3 kg/ton level.

The ability of formaldehyde to form methylene bridges and cross-link protein was first utilized to improve the elasticity of wool. Intensive research has been conducted in this area and various reaction conditions utilized. Reaction conditions required to cross-link amino have been found to be dependent on the ratio of formaldehyde to protein, reaction temperature, reaction time and pH (25). Theis and Jacoby (29) first reported that protein could be cross-linked by formaldehyde when a 3:2 ratio of amino acid to formaldehyde was incubated at 60°C for 30 min, but that at a 3:1 ratio of amino acid, the bond was reversible. Other researchers have utilized higher reaction temperatures (up to 100°C), longer reaction times (up to 24 h) and higher formaldehyde to protein ratios to form a cross-linked protein (25, 30). However, the interaction of formaldehyde with proteins may be somewhat more complex and variable compared to isolated peptides. For example, formaldehyde peptide cross-linking has been examined in more detail by Toews et al. (31) who reported that some regions within proteins are more susceptible to formaldehyde cross-linking than other regions of the respective proteins, and the variation in three dimensional structures of proteins dictate relative reactivities to formaldehyde (31).

Regardless of the exact mechanism(s) in which proteins are cross-linked, exposure of proteins to formaldehyde results in decreased water sensitivity, and increased resistance to chemical and enzyme exposure (22). This has been used for several practical applications in biology. Historically, formaldehyde has been used as a tissue fixative for clinical sample preservation that ensures stability for several years (32, 33). The ability to modify proteins has been taken advantage in the process of inactivating bacterial toxins and viruses for generating vaccines (17). Formaldehyde has also been implemented as a means to stabilize and retain intact whole cells, particularly bacteria. This has been used to preserve a consistent set of rumen bacterial cells to serve as an agent for immunization in layer hens to generate egg yolk polyclonal antibodies (34). Fixation of bacterial

cells harvested after growth in large scale growth vessels and subsequent addition of thimerosol allowed for extended frozen storage of whole intact cells without the growth of bacterial contaminants until they could be used to immunize hens (35).

Using formaldehyde to stabilize bacterial cells has benefits for other types of studies where retaining intact whole cells may be critical. For example, formalin solutions have also been used to harvest and preserve rumen bacterial cells after continuous culture growth studies for cell dry weight determinations (36–38). Isaacson et al. (36) incorporated formalin fixation as part of the recovery process due to concerns over cell lysis occurring during the centrifugation and washing steps of mixed cultures that could impact the accuracy of dry weight estimates of rumen bacterial populations from continuous cultures. They concluded that the addition of formalin did not alter the dry weight results appreciably to impact the interpretation of the dry cell data. In a more recent study, Baker et al. (39) used formalin solutions to preserve pathogenic *Escherichia coli* strains for use in flow cytometry detection. In their study, there was a need to standardize an immuno-based flow cytometry analyses with known quantities of particular pathogenic *E. coli* pure culture isolates to serve as standards before assessing food samples. In this particular study, they demonstrated that formalin preserved sets of *E. coli* could be spiked into ground beef samples, recovered, quantified by both quantitative polymerase chain reaction and flow cytometry, and demonstrated that the two methods did not statistically differ from each other. They concluded that formalin fixed solutions of pathogenic *E. coli* could serve as internal standards for calibrating flow cytometry-based assays by providing stable known quantities of *E. coli* cells.

## FORMALDEHYDE—POULTRY APPLICATIONS AND ANTIBACTERIAL MECHANISM(S)

Given the ability of formaldehyde to interact with macromolecules and serve as a fixative agent for bacterial cells it is not surprising the formaldehyde would be a potential antimicrobial compound. Glutaraldehyde-based chemicals have been used for sterilization in clinical settings such as dental, medical and veterinary surgical facilities (40). Glutaraldehydes have also been employed as disinfectant sprays in broiler and animal housing and livestock transportation vehicles for limiting bacterial and viral contamination (41–45). Formaldehyde fumigation has been used for eggshell surface decontamination, but hazard concerns have motivated research for alternative methods that are as effective as formaldehyde in reducing bacterial loads even though formaldehyde remains one of the more effective antibacterials that are available (46–52). While it has been noted by Carrique-Mas et al. (53) that there are concerns regarding the safety of formaldehyde to humans, in order to reduce occupational exposure, formaldehyde is applied in an enclosed system [mixer/enclosed auger; USDA (54)]. In a recent risk assessment, the European Food Safety Authority indicated that formaldehyde would not be considered a risk to humans when employed as an animal nutrition product, but anyone handling the product should avoid exposure to the respiratory tract, skin, and eyes (3, 55, 56).

Historically only limited microbial data responses mostly based on culture methods have been generated for evaluating aldehyde disinfectants in poultry operations (46–52). Consequently, microbial profiling is confined to which media is used, the respective selective processes, and the segment of the microbial population capable of forming visible colonies. Now that microbiome sequencing has become routine, more comprehensive microbial community profiling has become possible to conduct a comparative assessment of disinfectant treatments on microbial populations such as those that inhabit poultry houses. For example, Jiang et al. (45) compared different disinfectant sprays and reported that glutaraldehyde not only reduced overall airborne bacterial contamination in empty broiler houses but based on 16S rDNA sequencing using an Illumina HiSeq sequencer, decreased the number of detectable phyla by nearly half (from 32 phyla to 17 phyla) compared to the non-disinfected house. Phyla diversity was even more substantially decreased (6 phyla detected) when a disinfectant mixture (aldehyde, alcohol, and quaternary ammonium salt) was used leading the authors to suggest a much broader antibacterial spectrum for the disinfection mixture. In future studies, it would be of interest to conduct metagenomic profiling to determine the frequency of antibacterial resistance genes in these microbial populations that are specific to certain disinfectants being implemented routinely.

Formaldehyde was first utilized in the animal feed sector as a mold inhibitor for the preservation of high moisture corn (57). Formaldehyde has also been used extensively as a feed chemical antimicrobial to reduce *Salmonella* and improve general bacterial hygiene in feeds [**Figure 1**[1], (3, 4, 53)]. In general, potential cell targets of formaldehyde include the spore cores of bacterial spores, the cell walls of bacteria, and the amino groups of fungi (58, 59). The antimicrobial activity of glutaraldehyde and formaldehyde is believed to be elicited primarily by both the formation of a Schiff base product and irreversible cross-linking of proteins, RNA, and DNA in bacteria and of proteins in feeds (3, 4, 26, 53, 58, 59).

Unlike some of the other feed additive acids that have been used over the years, little bacteriological work has been conducted to determine mechanisms of formaldehyde exposure on *Salmonella*. Temcharoen and Thilly (60) examined toxic and mutagenic effects of formaldehyde in a mutant *Salmonella* Typhimurium test strain that lacked either membrane translocation or phosphoribosyl-transferase. The basic concept in using the *Salmonella* tester strain ($his^+$ revertant of an Ames *Salmonella* tester strain) is that if a particular compound is mutagenic then the histidine auxotrophic version of the tester strain will revert to a version that no longer requires histidine and can grow on media plates without histidine supplementation (61). Based on their results, Temcharoen and Thilly (60) concluded that formaldehyde was toxic and mutagenic to the *S.* Typhimurium tester strain and

[1]Formaldehyde targets were based upon (58).

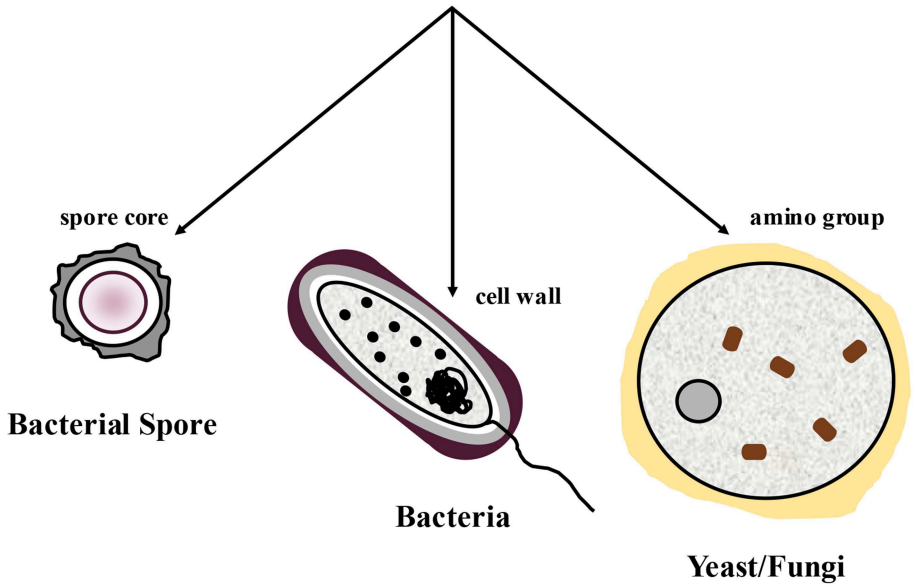


**FIGURE 1 |** The impact of the use of formaldehyde on bacterial spores, bacteria, and yeast/fungus by targeting key components.

the minimum concentration required to induce mutagenicity was 0.167 mM. They hypothesized that formaldehyde may lead to mutations either by direct interaction with the bacterial cell's DNA, or reacting with amino groups, simple amines, amino acids, nucleic acids, or proteins to form mutagenic product(s).

As of date, there is no clear evidence linking the use of formaldehyde in poultry operations to the expression of resistance factors in *Salmonella*. For example, when *Salmonella* isolates exposed to different disinfectants including formaldehyde in Danish broiler houses were characterized by Gradel et al. (62) for minimum inhibitory concentrations (MIC), no clear-cut association could be detected among serovar persistences, tendencies to persist, or use of a particular disinfectant. Likewise, *S.* Enteritidis isolates from egg-laying flocks where a quaternary ammonium-formaldehyde disinfectant was used also did not exhibit alterations in susceptibility/resistance responses (63). This again proved to be true in *Salmonella* isolates known to be persistent in a fish feed plant (64) where even though these isolates had been exposed to a commercial organic acid- formaldehyde mixture they were no more resistant to disinfectants than *Salmonella* isolates from other sources. In feed applications as a chemical antimicrobial additive, formaldehyde is unlikely to directly interact with *Salmonella* cells in a fashion similar to the pure culture *Salmonella* incubations conducted by Temcharoen and Thilly (60) as described above. Instead, it is much more likely to chemically interact with the proteins present in feeds upon exposure and potentially affecting bird performance.

## POULTRY FEEDS AND MICROBIAL CONTAMINATION

Biological contamination of feeds by organisms has always been considered a complex issue with numerous factors influencing levels and types of organisms likely to be present on a particular feed or feed ingredient at any given time or location as previously discussed (1, 3, 65–67). Although few conclusions can be drawn, the microbial composition associated with animal and poultry feeds can be quite diverse (1, 3, 68). Prokaryotes, bacteriophage, fungi, and yeast have all been identified in feeds and in some cases isolated from a wide range of feeds (1–3, 69–72). Detecting particular patterns or critical factors that dictate specific bacterial and/or non-bacterial populations associated with feed remains elusive. Indeed, factors such as environmental conditions during storage and subsequent feeding to animals, storage time, and feed treatments would be expected to contribute to the final composition of a feed or feed ingredient but to what degree and what other factors may be involved remains unknown. As molecular techniques develop, it is conceivable that such methods could be employed to begin comprehensive studies that establish signature populations in the feed that do correlate with certain influential factors and potentially identify which factors are most critical to certain feed processing operations.

Among the bacterial contaminants potentially present in animal and poultry feeds several organisms would also be considered foodborne pathogens that could cause disease in humans. These include *Salmonella*, *Clostridium perfringens*, *Clostridium botulinum*, and *Listeria*, some of which have been more frequently identified with feed than others

(2, 3, 73–77). Of the foodborne pathogens isolated from feeds, foodborne *Salmonella* serovars have received the most attention particularly with poultry feeds and feed ingredients and remain an issue for all aspects of vertically integrated poultry operations (6, 78–82). *Salmonella*-contaminated feed certainly has to be considered a potential risk factor for salmonellosis.

Poultry feed has been known to be a source of *Salmonella* since 1948 (83). In integrated operations, *Salmonella* control typically begins at the breeder level (84). Snoeyenbos (85) reported that the transmission of *Salmonella* in breeder eggs occurred with sufficient frequency to require control measures for *Salmonella* at the breeder and multiplier level. Wilding and Baxter-Jones (86) estimated that colonization of one breeder/multiplier by *Salmonella* might affect 65 broilers. Shapcott (87) reported that the presence of *Salmonella* in breeder feed might impact the transmission to broiler chicks. After implementing a rigorous program for the control of *Salmonella* at the breeder farm, the hatchery and the feed mill, both the broiler and breeder operations were *Salmonella* negative for >3 years. However, in June of 1980, a single breeder feed tested positive for *Salmonella* Sofia. Within 1 year, 100% of the flocks tested positive for *S*. Sofia. Jenson and Rosales (88) reported that 80% of the *Salmonella* serotypes found in breeder feed might be detected weeks later in breeder birds or their offspring.

The significance of *Salmonella* in feed and animal produce is less understood. There are many vectors for *Salmonella* transmission to poultry and animal produce, including breeders, hatchery, farm, feed mill, and the processing plant. Morris et al. (89) first discussed *Salmonella* in feed and its association with processing plant contamination. Of 12 serotypes of *Salmonella* isolated from the processing plant, six isolates were also present in feed. Only *S*. Montevideo isolates displayed a relationship in the frequency of detection between the feed mill and processing plant. Lahellec and Collins (90) reported that 8 of 16 serotypes of *Salmonella* isolated from the processing plant were found in feed. In a 3 year study of a large integrated broiler operation, McKenzie and Bains (91) observed that *Salmonella* in broiler carcasses displayed a 100% correlation with *Salmonella* in feed ingredients and grains. In Europe where *Salmonella* contamination rates of feed are low (<2%), Davies et al. (92) used a slightly different approach to determine if *Salmonella* in feed was associated with processing plant contamination. During a 2 year study, samples of dust and residues from feed mills of two large integrated broiler operations were analyzed for *Salmonella*. Corry et al. (93) compared isolated serotypes from feed to those present at the processing plant and found that 55% of *Salmonella* isolates from the processing plant originated from the feed. The connection of potential for salmonellosis to feed has been made in other ways as well. As an illustration of this particular point, Bucher et al. (94) characterized *Salmonella* isolates from chicken nuggets, strips, and pelleted broiler feed and concluded that *Salmonella* strains isolated from broiler feed were indistinguishable from isolates recovered from packaged raw, frozen chicken nuggets, and strips. Similar observations have been noted in commercial egg operations. Shirota et al. (95, 96) reported that both the frequency and the serotypes of *Salmonella* in feed were correlated to the frequency and serotypes of *Salmonella* in eggs (58% of egg isolates was identical to feed isolates). The authors of these studies concluded that *Salmonella* contamination of carcasses and egg contamination could be significantly reduced by minimizing the incidence of *Salmonella* in the feed. This would suggest that *Salmonella* possesses the capability of being transmitted from feed production, broiler growout/egg production, poultry processing and eventually retail establishments.

As a result of the widespread prevalence of *Salmonella* spp. in the environment and its capacity for survival under relatively harsh conditions such as increases in temperature (97–99) it is not surprising that *Salmonella* would come in contact with different stages of feed production all the way from cereal grain harvesting to feed milling and in turn lead to cross-contamination in places such as feed mills (3, 6, 78, 80, 81, 100–105). It is clear that better tracking methods will be needed to pinpoint ultimate origins for particular *Salmonella* spp., but this will be somewhat of a challenge given the high number of serovars that have been identified. Likewise, this makes developing effective control measures difficult due to the complexity of *Salmonella* occurrence in all phases of feed production and the range of potential *Salmonella* serovars that could be contaminants.

## FORMALDEHYDE—FEED STUDIES

Given the effectiveness of formaldehyde as a general sanitizer, it is not surprising that there would be interest in applying it as an antimicrobial treatment for poultry feeds. Duncan and Adams (106) examined the use of formaldehyde gas as a potential treatment to fumigate feeds and eliminate *Salmonella* loads using chick starter, fish meal, and meat and bone meal artificially contaminated with *S.* Senftenberg as their test model. They initially tested a commercial acid-based blended product containing propionic acid, isopropyl alcohol, and phosphoric acid but found this to be relatively ineffective at reducing *S.* Senftenberg levels in the various feeds. Following this experiment, they formaldehyde fumigated contaminated feed samples at 37°C and 60% relative humidity in a forced-draft incubator. They concluded that 5 min of formaldehyde fumigation was adequate and that the maximum fumigant penetration was <2.54 cm, but at least 1.91 cm and effective depth was increased to over 5 cm for 500 gm samples if they were continuously mixed.

While formaldehyde fumigation applications were initially tested, formaldehyde liquid solutions that could be incorporated/mixed directly into the feed matrices were examined as potential chemical feed additives to feeds as a means to reduce *Salmonella* contamination. Moustafa et al. (107) artificially contaminated commercial poultry with *S.* Typhimurium after the feeds had initially been sterilized via autoclaving. They concluded that a 40 % formaldehyde solution applied at a rate of 10 L/ton resulted in complete reduction of *S.* Typhimurium within the first hour of treatment while only 94% reduction was achieved with a 5 L/ton rate during this same application time frame. More recently,

Sbardella et al. (108) examined the effect of a 3.0 g per kg formaldehyde-propionic acid blend on natural bacterial populations in pig feed and reported reductions in natural populations of the enterobacteria populations. Based on these studies it appeared that formaldehyde solutions could be directly added to feeds and once mixed into the feed were effective in substantially reducing *Salmonella* contamination.

Studies on the residual activity of formaldehyde treated feed/ingredients to prevent recontamination by *Salmonella* was first reported by Barlow et al. (27). Fishmeal was treated with a formaldehyde-based product at 2 kg/ton and subsequently challenged with 200–500 cfu/g of *S.* Senftenberg and the time required to kill *Salmonella* determined. At 2 kg/ton, 5 to 9 days were required. When fishmeal was treated with 3 kg/ton and challenged with 1,500 to 2,000 cfu/g, all *Salmonella* was eliminated within the first 24 h. A similar study was conducted by Primm (109) using a mixed culture of *Salmonella* serotypes and higher challenge rates. At a challenge rate of 3,400 cfu/g, no *Salmonella* was detected at 3 kg/ton. The 3 kg/ton failed to protect the feed at challenge rates of >34,000 cfu/g.

## FORMALDEHYDE COMPARATIVE STUDIES WITH OTHER FEED ADDITIVES

Commercially, there are several chemical options for treatment of feeds to control *Salmonella* as described in several reviews published over the years (1–7). From a management perspective it is important to be able to compare various sanitizers to identify either single compounds or combinations that are optimal for the particular conditions they are being applied. Along these lines, studies have been conducted over the years to directly compare feed additive organic acid blends with formaldehyde. In early work Smyser and Snoeyenbos (110) compared 12 different compounds as antimicrobials for *Salmonella* when these compounds were added to meat and bone meal (MBM). Several acids and non-acid antimicrobials were examined including among others, acetic acid, oleic acid, propionate salts, benzoic, sorbic, methylparaben, formalin at 0.05, 0.1, 0.12, and 0.2 % (w/w) and some commercial blends. A nalidixic acid resistant *S.* Infantis strain was used as the marker strain to inoculate the samples set at a moisture level in the MBM to support *Salmonella* growth. Plate enumerations were conducted beginning at 2 to 3 days post-inoculation and subsequently continued for anywhere from 1 to 2 weeks afterwards. All compounds except formalin at levels >0.1 % failed to prevent *S.* Infantis growth. The authors noted that while initial declines in *S.* Infantis occurred for many of the additives, the pH of the feed mixtures also became alkaline over time with spoilage ensuing.

Smyser and Snoeyenbos (110) commented that from their previous work that most of these compounds including formalin had minimal effect on *Salmonella* in MBM when added to the MBM matrix with a much lower moisture content. This would suggest that water activity is an important component for ensuring optimal antimicrobial activity. In a more recent study, Carrique-Mas et al. (53) used a spray application of a *Salmonella* inocula to a feed matrix to compare the respective efficacies of four different commercial organic acid (various combinations of formic, propionic, and sorbic acids) and formaldehyde-based feed additives in either fishmeal or MBM. The *Salmonella* inocula (*S.* Enteritidis, *S.* Typhimurium, *S.* Senftenberg, and *S.* Mbandaka) were sprayed onto the feed matrix accompanied by mixing, subsequently allowed to incubate over time followed by recovery for pre-enrichment. A critical outcome of the research results noted by the authors was that the carryover of the antimicrobials into the recovery media in turn appeared to "mask" and/or reduce the population recovery levels of the inoculated *Salmonella* and thus led to an overestimation of the antimicrobial effect due to decreased levels of *Salmonella* surviving in the recovery media. To counter this masking effect, the authors employed antimicrobial neutralizing antagonists such as histidine for formaldehyde or sodium hydroxide for organic acids to the pre-enrichment media to neutralize artifactual antimicrobial decreases resulting from the respective feed additive to add. One of the formaldehyde-based treatments elicited less masking and more efficacy against *Salmonella* with no differences among the serovars. Clearly, as more feed studies are done, caution will need to be exercised to avoid *Salmonella* methodology misinterpretations occurring from masking regardless of the antimicrobial used. This will mean that some quantitative methodology validation will need to be conducted to ensure that the results represent the *Salmonella* populations originally present in the feed matrix after treatment of the feeds. This may not only be a concern for *Salmonella* but may need to be considered for all non-*Salmonella* bacterial population enumerations to avoid artificial selection by masking in either the dilutions or the plating media.

Other factors for optimizing feed treatments to control *Salmonella* may be influential as well. Carrique-Mas et al. (53) pointed out that the timing of when a feed additive is applied could be important as they and others (111) have noted that pretreatment with organic acids and formaldehyde prior to inoculation of *Salmonella* results in a more rapid decline in bacterial populations suggesting that pretreated feeds may be more resistant to subsequent contamination. This has practical significance as the potential for *Salmonella* cross contamination during milling is considered a concern. This is illustrated in a study by Jones and Richardson (80) where they detected *Salmonella* recontamination originating from dust in the feed mill. This led them to conclude that potential cross contamination between areas of the mill operation are possible and must be taken into account as part of control strategy for *Salmonella* feed contamination. Even if *Salmonella* levels in feed are initially decreased during milling, risk of exposure to *Salmonella* remains. For example, Jones (81) concluded that thermal processes such as pelleting could reduce *Salmonella* levels, but recontamination could occur post-pelleting and suggested that the addition of chemical disinfectants could diminish potential recontamination.

There are strategies that can be utilized to limit recontamination. To this point, Cochrane et al. (112) examined post rendering chemical treatment of rendered feed ingredients by comparing a wide range of feed additives including a medium chain fatty acid (MCFA) blend (caproic, caprylic, and capric

acids) with an organic acid blend (lactic, formic, propionic, and benzoic acids), an EO blend (garlic oleoresin, tumaric oleoresin, capsicum oleoresin, rosemary extract, and wild oregano), sodium bisulfate, and a commercial formaldehyde product. They initially treated the rendered protein feed ingredients (feather meal, blood meal, MBM, and poultry by-product meal) with the corresponding feed additive followed by spray inoculation with a *S.* Typhimurium strain. They observed that the feed ingredient matrix impacted *Salmonella* persistence as similar populations were recovered from both blood meal and MBM and, in turn, were higher than the populations enumerated from feather meal and poultry by-product meal. Out of all the products examined, they concluded that the MCFA blend and the formaldehyde commercial product were the most effective in preventing *S.* Typhimurium post processing contamination, but time and feed matrix type were all factors in reducing *S.* Typhimurium levels.

In summary, formaldehyde is an effective control agent for limiting *Salmonella* in feeds but when and where to apply it to achieve maximum efficacy needs to be standardized. This can be accomplished by developing a more complete picture of the microbial ecology of feed production (3). Understanding the microbial ecology of the feed mill as well as the feed ingredient and mixed feed matrices could potentially be helpful not only for *Salmonella* tracking but general microbial contamination that occurs in feed processing. While many of these non-*Salmonella* organisms are probably not deleterious to animals and/or humans their presence could be indicative of the impact of processing environmental conditions on the feed prior to being fed to the animal.

Application of next-generation sequencing (NGS) technologies would offer a more complete profile of the microbial population and depending on the bioinformatics analysis identify core feed microbial populations that align with certain characteristics including feed type, feed mill location, individual processing steps in the feed mill (such as before and after pelleting). These identified populations could also serve as indicator organism(s) for the likelihood of occurrence of *Salmonella.* This may be important if *Salmonella* occurs relatively infrequently in feeds and/or is dramatically reduced after antimicrobial treatments. Therefore, if based on natural contamination, screening of antimicrobials for control of *Salmonella* would be more difficult and identification of indicator organisms that are more frequent and parallel *Salmonella* behavior would have utility for routine testing.

## FORMALDEHYDE, FEED DIGESTIBILITY, AND POTENTIAL INTERACTION WITH THE POULTRY GIT

Knowing the core feed microbial populations may be helpful not only for establishing effectiveness of antimicrobial treatments such as formaldehyde in the feed matrix but would also enhance understanding of the GIT microbial population response to formaldehyde treated feed as it enters the GIT. In most poultry studies, emphasis has been placed on antibacterial activities in either the feed matrix or the subsequent impact on *Salmonella* occurrence in the GIT of birds consuming treated feed. As more is becoming understood about the avian microbiome it is becoming possible to establish relationships between diets, dietary components and the specific responses of the avian microbial community. While this relationship has not been explored extensively with formaldehyde treated feeds there is indirect evidence of potential impact on the poultry GIT based on poultry performance and digestibility studies.

Wales et al. (4) concluded that formaldehyde, when applied as an antimicrobial feed additive, has not been generally shown as a cause of adverse responses in animals. However, Ricke (3) suggested given the dynamics of GIT digestion and microbial responses that a more detailed impact of formaldehyde on dietary protein availability for the concentrations of formaldehyde used as a feed antimicrobial treatment may also need to be considered. As more studies are conducted to examine the utility of formaldehyde as a chemical antimicrobial for poultry feed application, more specific nutritional responses such as amino acid and protein availability for digestion and absorption should also be taken into account in the overall determination of optimal concentrations to be used for antimicrobial applications.

Spears et al. (113) evaluated the impact of soybean meal treated with 0, 3, 6 or 9 kg/ton of formaldehyde (37%) on the performance of broiler chicks through 10 days of age. No adverse effects on body weight gain, feed consumption or feed conversion were observed at the 3 kg/ton treatment. At 6 kg/ton, feed intake was adversely affected. Spratt (57) reported no negative effect of high moisture corn diets treated with 2.5 kg/ton of formaldehyde (37%) in broilers (6 wks) or pullets (6 wks) or laying hens (20–33 wks). In more recent trials with broilers (114, 115), white layers (116), and brown layers (117), consumption of feed treated with a formaldehyde-based product (33% formaldehyde) at 2 to 3 kg/ton was not observed to negatively affect performance. The effect of higher levels of formaldehyde in feed has been evaluated in broilers and cockerels (118, 119). At 2.5 and 5 kg/ton of formaldehyde (37% solution), no adverse effects were reported. Feeding poultry 10 kg of formaldehyde/ton depressed feed intake, reduced body weight gain, and caused ulceration of the crop/gastrointestinal tract.

Barlow (27) was the first to examine the digestibility of formaldehyde in fishmeal destined for aquaculture. Fishmeal was treated with 0, 2, 4, or 6 kg/ton and fed to mink (test animal digestibility trials in aquaculture). No negative effects on protein digestibility occurred at levels up to 6 kg per ton. In digestibility trials in broilers, FBP treated feed/soybean meal was not observed to significantly affect protein digestibility broilers when fed at 2 kg/ton in both non-cecectomized broilers (115) and cecectomized broilers (120). Ironically, in both studies, protein digestibility was numerically improved but was not significantly different ($P > 0.05$). However, in both studies, it was not disclosed if the feed was subjected to pelleting thus the possibility of cross-linking of formaldehyde and amino acids at high reaction temperatures was not addressed. Jones et al. (121) conducted a study in which feed was treated with 3 kg/ton of a formaldehyde-based product and subjected to extreme pelleting conditions (86°C for 5.5 min). Feed was subsequently fed to cecectomized roosters (20 replicates/treatment), and

amino acid digestibility determined. Formaldehyde was observed to not impact amino acid digestibility except for arginine (<1% reduction in digestibility).

While poultry performance and digestibility have been determined for birds fed formaldehyde treated feeds very little is known about the GIT microbial responses to these feeds. The lack of general influence on performance and digestibility would suggest that minimal impact occurs on the poultry GIT microbial populations except for the higher levels of formaldehyde when bird performance effects are noted. However, this does not rule out specific GIT microbial population responses in birds fed formaldehyde treated feeds. Historically, it was difficult to discern more subtle GIT microbial responses to differences in diets due to the limitations associated with culture methods for recovery of representative GIT microbial populations, particularly the more strict anaerobic GIT microorganisms. Development of molecular identification approaches such as NGS has made total GIT microbial populations much more accessible. As 16S rDNA sequencing methodologies for poultry microbiomes continue to advance it should be possible to achieve more indepth resolution for specific poultry GIT microbial population responses to formaldehyde treated feeds. Even when differences in overall poultry performance or digestibility responses are not detectable in the presence of formaldehyde treated feed, it is still possible that shifts in GIT could occur in response to changes in individual dietary components such as free amino acids and/or proteins. This response could vary depending on the particular GIT compartment in the bird such as the crop at the beginning of the GIT vs. the ceca at the terminal of the GIT. Not only are the microbial populations distinct in each of these GIT compartments but the lumen environment, pH, and metabolite composition are likely to be different as well (122–124). Application of metabolomics and transcriptomics may further reveal poultry GIT microbial responses even when detectable changes in GIT microbial populations composition do not occur.

## CONCLUSIONS

While a fairly wide range of chemical, physical and biological agents have been examined and in some cases commercially applied over the years as feed additives, formaldehyde remains one of the more frequently used from a commercial standpoint. It is considered effective as a feed additive, but it also may possess different antimicrobial mechanism(s) against *Salmonella* and other organisms such as GIT indigenous bacteria. However, its activity in the GIT, once consumed by the bird, may be different as well. It is conceivable since formaldehyde may bind directly to feed proteins that perhaps it is more stable in the GIT and therefore is more likely to reach the lower parts of the tract. It would be interesting to conduct studies on labeled formaldehyde similar to the work done by Hume et al. (125) with labeled propionate to determine whether that is indeed the case.

Formaldehyde can react with several different amino acids including the epsilon-amino group of lysine, the primary amide groups of asparagine and glutamine, the sulphydryl group of cysteine among others (23). This differential reactivity for particular amino acids could account for some of the variation seen in feed protein additive studies and the interaction with the GIT microbial community as proteins become modified with formaldehyde linkages and potentially present unique targets for protein hydrolysis by GIT microorganisms. In conclusion, the introduction of microbiome sequencing and bioinformatic tools should help to sort out some of the microbial ecology complexities associated with formaldehyde treated feeds both in the feed itself as well as once it is consumed by the bird.

## AUTHOR CONTRIBUTIONS

All authors significantly contributed to the work of the current review. SR wrote the review with the assistance from DD and KR.

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# Microbiota-Related Changes in Unconjugated Fecal Bile Acids Are Associated With Naturally Occurring, Insulin-Dependent Diabetes Mellitus in Dogs

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Diabetes mellitus (DM) in humans has recently been associated with altered intestinal microbiota. The consequences of intestinal dysbiosis, such as increased intestinal permeability and altered microbial metabolites, are suspected to contribute to the host inflammatory state and peripheral insulin resistance. Human diabetics have been shown to have changes in bile acid (BA) metabolism which may be detrimental to glycemic control. The purpose of this study was to examine BA metabolism in dogs with naturally-occurring, insulin-dependent DM and to relate these findings to changes in the intestinal microbiota. A prospective observational study of adult dogs with a clinical diagnosis of DM ($n = 10$) and healthy controls (HC, $n = 10$) was performed. The fecal microbiota were analyzed by 16S rRNA gene next-generation (Illumina) sequencing. Concentrations of fecal unconjugated BA (fUBA) were measured using gas chromatography and mass spectrometry. Analysis of bacterial communities showed no significant difference for any of the alpha-diversity measures between DM vs. HC dogs. Principal coordinate analysis based on unweighted Unifrac distance metric failed to show significant clustering between dog groups ($ANOSIM_{Unweighted}$: $R = 0.084$; $p = 0.114$). However, linear discriminate analysis effects size (LEfSe) detected differentially abundant bacterial taxa ($\alpha = 0.01$, LDA score >2.0) on various phylogenetic levels. While *Enterobacteriaceae* was overrepresented in dogs with DM, the proportions of Erysipelotrichia, *Mogibacteriaceae*, and *Anaeroplasmataceae* were increased in HC dogs. Dogs with DM had increased concentration of total primary fUBA compared to HC dogs ($p = 0.028$). The concentrations of cholic acid and the cholic acid percentage of the total fUBA were increased ($p = 0.028$ and $p = 0.035$, respectively) in the feces of DM dogs relative to HC dogs. The levels of lithocholic acid (both absolute value and percentage of the total fUBA) were decreased ($p = 0.043$ and $p < 0.01$, respectively) in DM dogs vs. HC dogs. Results indicate that dogs with DM have both intestinal dysbiosis

and associated fUBA alterations. The pattern of dysbiosis and altered BA composition is similar to that seen in humans with Type 2 DM. The dog represents a novel large animal model for advancing translational medicine research efforts (e.g., investigating pathogenesis and therapeutics) in DM affecting humans.

**Keywords: bile acids, diabetes mellitus, microbiota, lipopolysaccharide, dog, T2DM**

## INTRODUCTION

The intestinal microbiota is increasingly recognized as a pivotal environmental factor contributing to development of metabolic diseases in humans, including obesity, insulin resistance and type 2 diabetes mellitus (T2DM) (1–3). Microbes in the gut play an important role in metabolic disturbances by increasing energy extraction from ingested foods, regulating host metabolism and generating low-grade intestinal inflammation. High throughput 16S rRNA sequencing of the gut microbiota of *ob/ob* mice found that their obesity was associated with significant shifts in relative abundance of select bacterial taxa (e.g., Bacteroidetes decreased while Firmicutes were increased) vs. lean controls (4). These same investigators demonstrated that the proportion of Bacteroidetes in obese humans was decreased as compared to intestinal populations in lean humans (5). However, other studies aimed at evaluating altered gut microbial composition and its association with human diabetes have produced conflicting results (3, 6–8).

The intestinal microbiota also influences metabolism of bile acids (BA). Cholic acid and chenodeoxycholic acid are primary BA synthesized from cholesterol in the liver. Following ingestion of a fat- and protein-rich meal, primary BA travel down the intestines where they are then modified by anaerobic bacteria into different secondary BA, primarily deoxycholic and lithocholic acids. Accumulating evidence demonstrates that BA are important signaling molecules regulating hepatic glucose metabolism via farsenoid X receptor (FXR)-mediated pathways (9–11). Primary BA are also involved in energy metabolism due to their interaction with G protein coupled BA receptor (TGR-5) activation and release of glucagon-like peptide-1 (GLP-1) (10, 12). As compared to non-obese healthy subjects, the fasting serum of obese humans contains decreased primary BA but increased secondary BA concentrations (13). Whether similar microbiota-related changes in the bile acid profile are associated with naturally occurring, insulin-dependent diabetes mellitus (DM) in dogs has not been previously investigated.

With this study, we evaluated the fecal unconjugated bile acid (fUBA) profiles of diabetic vs. healthy control dogs, and hypothesized that diabetic dogs would have perturbations in fecal bile acids similar to those reported in humans with T2DM, including alterations in their intestinal microbiota.

## MATERIALS AND METHODS

### Ethical Animal Use

The collection and analysis of blood and fecal samples from healthy dogs and dogs with spontaneous DM were previously approved by the Iowa State University Institutional Animal Care and Use Committee. Written informed consent was obtained from all owners of healthy and DM dogs enrolled in this trial (IACUC Log number: 9-14-7859).

### Animals and Enrollment

Dogs with naturally occurring, insulin-dependent DM ($n = 10$) and healthy control dogs (HC, $n = 10$) were enrolled from the hospital population at the ISU Lloyd Veterinary Medical Center (2014–2016). Dogs with DM were diagnosed on the basis of historical polyuria-polydipsia, change in appetite, and weight loss accompanied by supportive laboratory abnormalities including sustained hyperglycemia, hypercholesterolemia, and glucosuria (14). Dogs with diabetes were enrolled if they were >2 years of age and weighed >6 kg, were fasted overnight (12 h minimum) prior to diagnostic sampling, had no discernible other diseases (including bacterial urinary tract infection with results confirmed by urine culture/susceptibility testing) and received no medications (including antibiotics) within 3 weeks of presentation.

Control dogs were between 2 and 9 years of age and judged to be healthy on the basis of history and normal physical examination. Additionally, HC dogs could not have received antibiotics for a period of 6 months prior to diagnostic sampling nor had any other medications administered other than prophylactic flea/tick/heartworm preventatives. All HC dogs were fasted for at least 12 h before samples were obtained.

The majority ($n = 7$) of DM dogs were fed a low-fat weight reducing diet[1] and three DM dogs were fed commercial maintenance rations. In these instances, dogs with DM were fed low-fat, high-fiber diets to combat obesity and to facilitate insulin regulation (15). Control dogs were fed either a commercial maintenance ration ($n = 5$), commercial elimination diet ($n = 2$), or a low-fat weight reducing diet[1,2] ($n = 3$). None of the dogs had a history of antibiotic administration for at least 6 months prior to sample collection.

### Sample Collection

Blood was collected from both groups of dogs in the mornings by routine venipuncture using the jugular or cephalic veins. Serum was separated quickly within 15 min of collection and archived at −80°C until laboratory analysis. Fecal samples were obtained by digital extraction following phlebotomy in most (17/20) dogs. In some instances, clients brought in fresh fecal samples (contained in a plastic bag) which had been voided naturally during the morning of

[1] Hill's prescription diet w/d.
[2] Purina Pro Plan Veterinary Diet OM.

diagnostic sampling. In these instances, feces were maintained chilled by refrigeration (<4 h) until archived at −80°C for later analysis.

## DNA Isolation and Sequencing of 16S rRNA Genes

Total bacterial DNA was extracted from canine stool samples (10 DM and 10 control dogs) using a MoBio Power soil DNA isolation kit (MoBio Laboratories, USA) following the manufacturer's instructions. DNA concentration and quality in the extracts was determined using a NanoDrop 1000 spectrophotometer. Sequencing of the V4 region of the 16S rRNA gene primers 515F (5′-GTGCCAGCMGCCGCG GTAA-3′) to 806R (5′-GGACTACVSGGGTATCTAAT-3″) was performed using Illumina sequencing at the MR DNA laboratory (www.mrdnalab.com, Shallowater, TX, USA).

The obtained sequences were processed and analyzed using QIIME v 1.9 (16) as previously described (17), and were uploaded to Sequence Read Archive at NCBI with accession number SRP122536. Briefly, sequence data was first demultiplexed, quality filtered using the default settings in QIIME, chimeras were filtered from the sequence set using USEARCH against the 97% clustered representative sequences from the Greengenes v 13.8 database, while remaining sequences were clustered into Operational Taxonomic Units (OTUs) by using an open reference approach in QIIME (18). Prior to downstream analysis, sequences assigned as chloroplast, mitochondria, and low abundance OTUs, containing <0.01% of the total reads in the dataset were removed. All samples were rarefied to 97,980 sequences for even depth of analysis. Alpha diversity measures included Chao1, Shannon diversity, and observed OTUs (observed species). Beta diversity was evaluated with the phylogeny based UniFrac distance metric and visualized using Principal Coordinate Analysis (PCoA) plots. Bray-Curtis dissimilarity was also calculated from the data set.

## Fecal Unconjugated Bile Acids

The fecal unconjugated bile acids (fUBA) quantified were cholic acid (CA), chenodeoxycholic acid (CDCA), lithocholic acid (LCA), deoxycholic acid (DCA), and ursodeoxycholic acid (UDCA). For the identification and quantification of unconjugated bile acids, the protocol was adapted and modified from methods previously described (19, 20). Unconjugated CA, CDCA, LCA, DCA, and UDCA were purchased from a commercial supplier (Sigma-Aldrich, St. Louis, MO). Deuterated internal standards CA-$d_4$ and LCA-$d_4$ were purchased from CDN Isotopes (Quebec, Canada). Hydrochloric acid (37% American Chemical Society reagent), hexane [for high-performance liquid-chromatography (HPLC)], 1-butanol for HPLC, and derivatization agent (Supelco's® Sylon HTP HMDS + TCMS + Pyridine, 3:1:9 Kit) were used for preparation of trimethylsilyl ether (TMS) and butyl ester bile acid derivatives.

Naturally voided fecal samples were collected from healthy dogs and dogs with DM. Approximately 0.5 g of wet feces was aliquoted into a tube (5 mL, 57 × 15.3 mm, polypropylene, Sarstedt, Nümbrecht, Germany) using a spatula (Smart Spatula, USA Scientific, Ocala, FL). Fecal samples were kept frozen at −80°C and then lyophilized overnight (Labconco FreeZone 2.5 Plus, Kansas City, MO). Samples were then pulverized and aliquoted using a spatula (Smart Spatula, USA Scientific, Ocala, FL) into disposable glass centrifuge tubes (5 mL, Kimble-Chase, Rockwood, TN). Aliquots of 10–15 mg of lyophilized feces were used, and concentrations of bile acids were later back calculated according to the precise weight of each aliquot. A total volume of 200 μL of butanol containing the internal standards CA-$d_4$ and LCA-$d_4$ was added to each fecal sample. Twenty microliters of HCl were then added for a final volume of 220 μL and vortexed for 30 s. Samples were then capped and incubated at 65°C for 4 h. Next, samples were evaporated under nitrogen gas until dryness at 65°C for ∼25 min. Two-hundred microliters of TMS-derivatization agent were then added to the sample and incubated at 65°C for 30 min. Following incubation, samples were again evaporated under nitrogen gas until dryness at 65°C (∼25 min). Samples were then resuspended in 200 μL of hexane, vortexed briefly then centrifuged for 10 min at 3,200 rcf. A 100 μL aliquot was transferred to a GC/MS vial insert (250 μL glass with polymer feet, Agilent, Santa Clara, CA) and the vial was capped for further downstream analysis.

**TABLE 1** | Patient characteristics.

| Group | Age (years) | Sex | Weight (kg) | BCS (0–9) |
|---|---|---|---|---|
| **A. PATIENT DEMOGRAPHICS** | | | | |
| Healthy | 6 | F | 23 | 6 |
| Healthy | 9 | F | 17 | 6 |
| Healthy | 8 | F | 11 | 4 |
| Healthy | 8 | M | 7 | 5 |
| Healthy | 5 | F | 5 | 6 |
| Healthy | 6 | F | 6 | 6 |
| Healthy | 4 | M | 13 | 7 |
| Healthy | 3 | M | 23 | 3 |
| Healthy | 3 | M | 30 | 6 |
| Healthy | 2 | M | 37 | 5 |
| Diabetic | 10 | F | 3 | 6 |
| Diabetic | 8 | M | 7 | 6 |
| Diabetic | 8 | F | 15 | 8 |
| Diabetic | 8 | F | 10 | 2 |
| Diabetic | 14 | M | 8 | 7 |
| Diabetic | 13 | F | 12 | 9 |
| Diabetic | 6 | F | 45 | 6 |
| Diabetic | 8 | F | 35 | 3 |
| Diabetic | 8 | M | 32 | 4 |
| Diabetic | 11 | M | 14 | 7 |

| Parameter | Healthy dogs | Diabetic dogs |
|---|---|---|
| **B. CANINE GROUP COMPARISONS** | | |
| No. of females/no. of males | 5/5 | 6/4 |
| Mean age (years) | 5.4 | 9.4* |
| Mean weight (kg) | 8.0 | 17.4 |
| Mean BCS (0–9) | 5.4 | 5.8 |

*$p < 0.05$; BCS, body condition score.

Gas chromatography (GC) and mass spectrometry (MS) was used (6890N and 5975 inert Mass Selective Detector, Agilent, Santa Clara, CA). The instrument was equipped with an autosampler (7683 Series, Agilent, Santa Clara, CA). A capillary column (DB-1ms Ultra Inert, Agilent, Santa Clara, CA) was used with the following dimensions: length: 30 m, diameter: 0.250 mm, film: 0.25 μm. A 20:1 split ratio was utilized after a 1 μL sample injection with an inlet temperature of 250°C. After injection, oven temperature was held at 150°C for 1 min, then ramped at 21°C per minute to a final temperature of 276°C then held at that temperature for 21 min. Post-data acquisition, the oven was heated to 325°C for 3 min for post-run column cleaning. Helium was used as the carrier gas at a nominal flow rate of 1 mL/min. Flow varied slightly to maintain a retention time lock of cholestane-$d_4$ set to elute at 11.4 min. Mass spectral data was analyzed using ChemStation (Agilent's Enhanced Data Analysis in MSD version D.02.002.275). Use of this assay demonstrating perturbations in fUBA in dogs with chronic enteropathy has been recently reported (21).

## Serum Lipopolysaccharide (LPS) Concentration

An LPS test was performed to quantitate the production of Gram-negative bacterial endotoxin associated with low-grade intestinal inflammation in insulin-dependent states of DM dogs (22, 23). Serum samples from 10 control and 10 DM dogs were analyzed for their concentration of LPS using the LAL

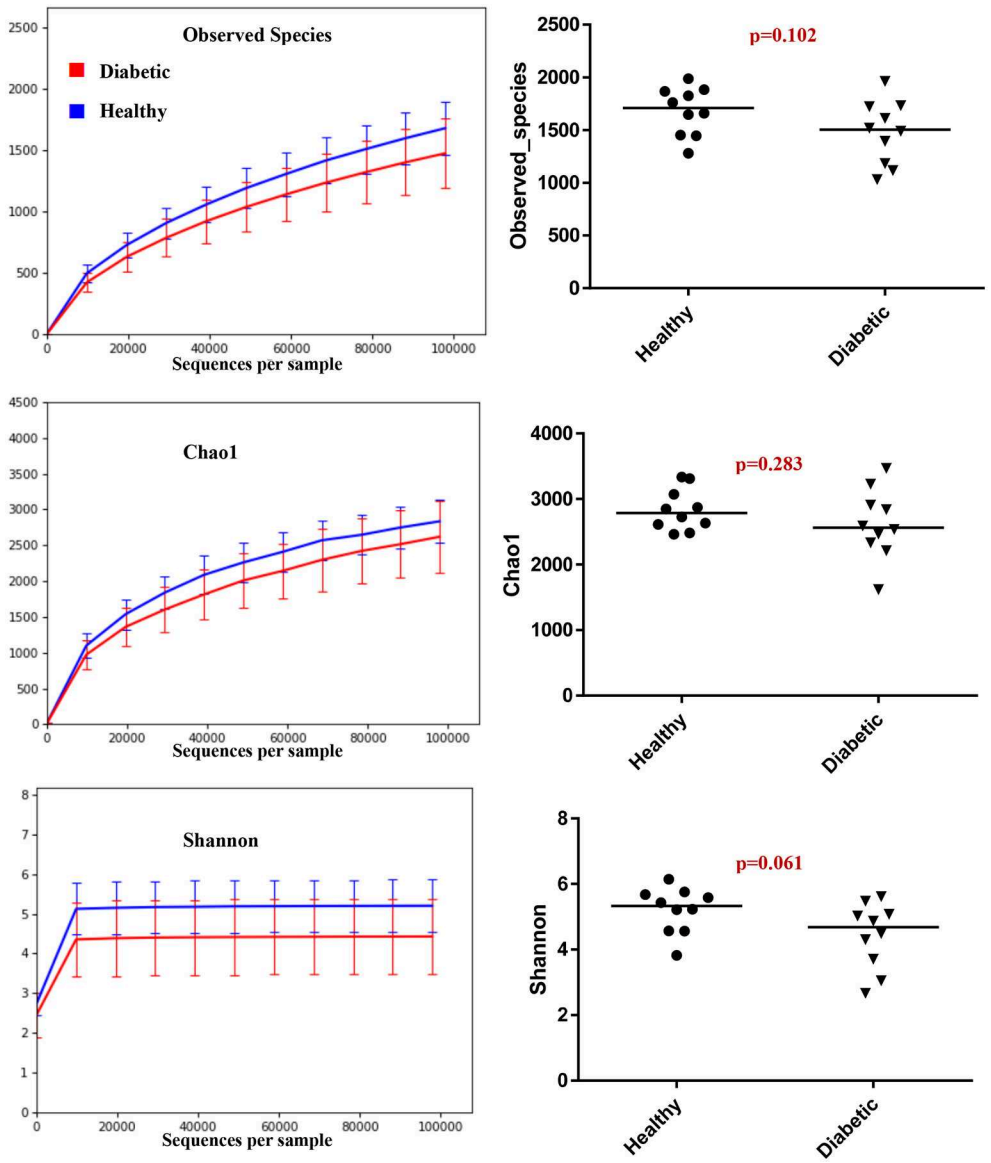


**FIGURE 1 |** Summary of alpha diversity measures.

Chromogenic Endotoxin Quantification Kit according to the manufacturer's instructions.

## Statistical Analysis

Normality was tested using Shapiro-Wilk for all measurements (or variables) in the fUBA dataset. When the assumption did not hold, the non-parametric Mann-Whitney test was used for comparison of the groups (R software version 3.5.1, R Foundation for Statistical Computing, Vienna, Austria, and JMP 10, SAS software Inc.). A Fisher's Exact Test was used to test for proportions when evaluating confounding factors, such as sex distribution between healthy control (HC) dogs and dogs with DM. A statistical software package (GraphPad Prism version 5.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com) was used for generating graphs.

For sequence data, linear discriminant analysis effect size (LEfSe) was used to elucidate bacterial taxa different between groups. LEfSe was used in the Galaxy workflow framework with the parameters set at $\alpha = 0.01$, LDA score = 2.0. Mann-Whitney test (JMP Pro 11, SAS software Inc.) were performed and adjusted for multiple comparison using a Benjamini-Hochberg procedure with a false discovery rate (FDR) at each taxonomic level. ANOSIM (analysis of similarity) test within PRIMER 6 software package (PRIMER-E Ltd., Luton, UK) was used to analyze significant differences in microbial communities between both dog groups.

The Mann-Whitney test was performed to compare differences in LPS values between the two dog groups. For all statistical analyses, a $p$-value $< 0.05$ was considered significant.

# RESULTS

The patient demographic data for the canine cohorts is presented in **Table 1**. In brief, gender, body weight, and body condition score were comparable for diabetic and control groups. For diabetic dogs, 1 dog was newly diagnosed with DM while the remaining 9 dogs were receiving parenteral insulin and considered to have poorly regulated DM based on persistent clinical signs, results of blood (sustained hyperglycemia) and urinalysis (marked glucosuria) testing. None of the enrolled animals was ketoacidotic based on the absence of ketonuria and metabolic acidosis on laboratory analysis.

The different diets fed to HC and DM dogs were dictated by their overall health status and individual owner preference. For example, the majority ($n = 7$) of DM dogs were obese (i.e., body condition score [BCS] $> 5$) and therefore were placed on low-fat, high-fiber (i.e., standard) weight-reducing commercial rations. Those DM dogs having normal body condition ($n = 3$) were fed canine commercial-derived maintenance rations which do not promote weight loss when fed at recommended levels. The HC dogs having normal body condition ($n = 5$) were also fed commercial maintenance rations while 3 HC dogs having an obese phenotype were fed low-fat weight-reducing diets. Two HC dogs with past histories of adverse food reaction were currently fed commercial elimination diets at the time of enrollment.

Analysis of bacterial communities showed no significant difference for any of the alpha-diversity measures between DM dogs vs. HC dogs (**Figure 1**). Principal coordinate analysis (PCA) based on unweighted Unifrac distance metric did not reveal significant clustering between dog groups (ANOSIM $_{\text{Unweighted}}$: $R = 0.084$, $p = 0.114$; ANOSIM$_{\text{weighted}}$: $R = 0.074$, $p = 0.131$; **Figure 2**). Calculation of Bray-Curtis dissimilarity was significant (i.e., $R = 0.132$, $p = 0.038$). PCA based on unweighted Unifrac distance metric was compared using ANOSIM to address confounding factors, such as administration of antibiotics, body condition score, diet, concurrent health issues, and sexual status. None of these comparisons reached statistical significance between comparison groups. However, linear discriminate analysis effects size (LEfSe) detected differentially abundant bacterial taxa ($\alpha = 0.01$, LDA score $>2.0$) on various phylogenetic levels (**Figure 3**). While the family *Enterobacteriaceae* was overrepresented in dogs with DM, the proportions of the class Erysipelotrichia, and families *Mogibacteriaceae*, and *Anaeroplasmataceae* were increased in HC dogs. Similarly, on a

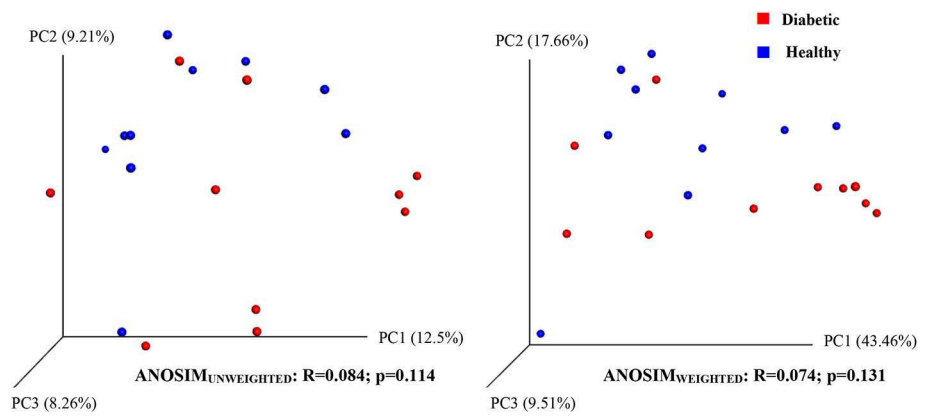


**FIGURE 2 |** Principal coordinate analysis (PCoA) plot showing clustering of microbial communities from feces of healthy and diabetic dogs (red = diabetic, blue = healthy). The microbiome (beta diversity) of healthy dogs did not differ from that of diabetic dogs (ANOSIM; $p = 0.114$).

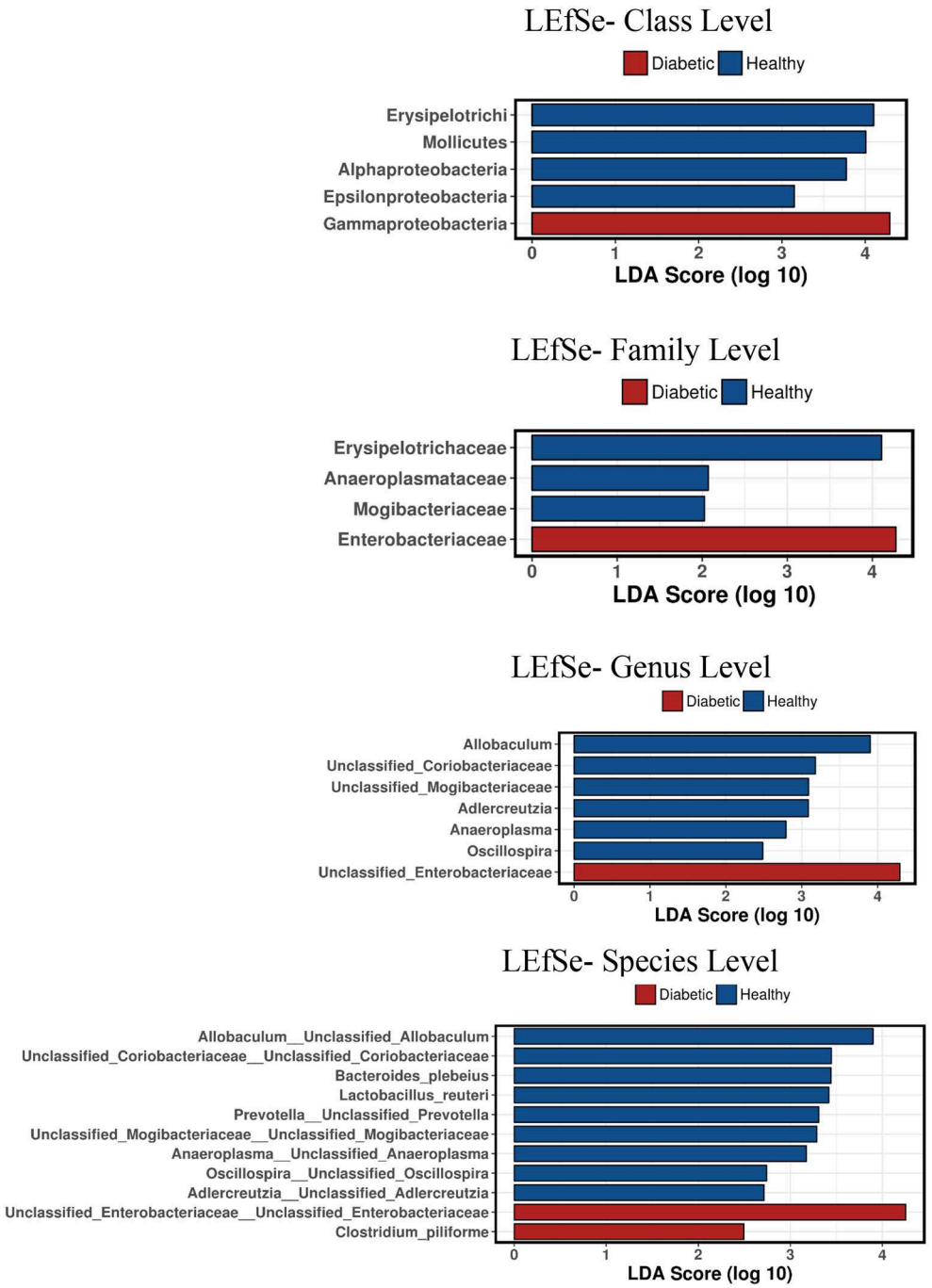


**FIGURE 3 |** Linear discriminant analysis effect size (LefSe) of bacterial taxa and their association with different canine groups. Only LefSe values >2 are shown.

species level, the abundance of an unclassified species belonging to *Enterobacteriaceae* family was most strongly associated with dogs having DM, while the abundance of *Lactobacillus reuteri* and *Bacteroides plebeius* were most strongly associated with HC dogs. Univariate analysis confirmed these findings, as the same bacterial groups were significantly ($p < 0.05$) altered (**Supplemental Table 1**).

Analysis of serum LPS concentrations showed that DM dogs had increased ($p = 0.0187$) circulatory levels of LPS vs. healthy dogs (**Figure 4**).

Dogs with DM had increased concentration of total primary fUBA compared to HC dogs ($p = 0.028$; **Table 2**). The concentrations of CA and the CA percentage of the total fUBA were increased ($p = 0.029$ and $p = 0.036$, respectively) in the

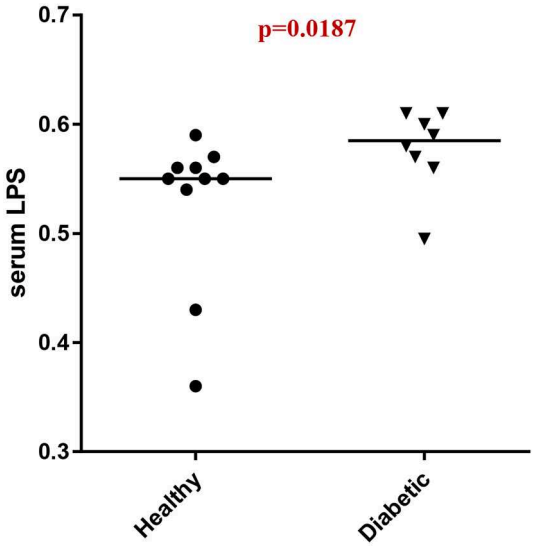


**FIGURE 4 |** Serum LPS concentrations (EU/ml) in healthy controls (HC) vs. dogs with diabetes mellitus (DM).

**TABLE 2 |** Fecal bile acid parameters between cohorts.

| Bile acid parameter | *p*-Value |
|---|---|
| Cholic acid | 0.1053 |
| Chenodeoxycholic acid | 0.3027 |
| Lithocholic acid | 0.0072 |
| Deoxycholic acid | 0.0657 |
| Ursodeoxycholic acid | 0.3385 |
| Total BA | 0.6063 |
| Total primary BA | 0.0976 |
| Total secondary BA | 0.0772 |
| Secondary to primary ratio | 0.0429 |
| Primary to secondary ratio | 0.0429 |
| Cholic acid % of Total | 0.0510 |
| Chenodeoxycholic acid % of Total | 0.8829 |
| Lithocholic acid % of Total | 0.0036 |
| Deoxycholic acid % of Total | 0.2538 |
| Ursodeoxycholic acid % of Total | 0.1851 |
| Total primary BA % of Total | 0.0429 |
| Total secondary BA% of Total | 0.0429 |

*Shaded value indicates a fUBA of $p < 0.05$ indicating significant difference between canine groups. fUBA, fecal unconjugated bile acid.*

feces of DM dogs relative to HC dogs. The levels of LCA (both absolute value and percentage of the total fUBA) were decreased ($p = 0.043$ and $p = 0.01$, respectively) in DM dogs vs. HC dogs (**Figure 5**).

## DISCUSSION

Alterations in gut microbiota composition have been linked to the development of human metabolic diseases, including both T1DM and T2DM (2, 3, 24, 25). The physiologic interplay between the intestinal microbiota and BA metabolism indicates that dysbiosis may be accompanied by altered BA homeostasis, thereby contributing to the metabolic dysregulation seen in DM. Dogs develop naturally-occurring, insulin-dependent DM which may also be associated with dysbiosis and altered bile acid metabolism, suggesting that they may serve as a clinically relevant model for investigating human disease.

We have now demonstrated that canine insulin-dependent DM is associated with changes in the composition of the intestinal microbiota evident from the family to species levels. In performing LEfSe analysis, we detected numerous bacterial taxa that were differentially abundant (e.g., LDA score >2.0) between dog groups. This included the relative abundance of family *Enterobacteriaceae* which was overrepresented while the abundance of subclass Erysipelotrichia, and of families *Mogibacteriaceae*, and *Anaeroplasmataceae* were underrepresented in DM dogs as compared to HC dogs. In support of our findings, other animal studies have shown that type 2 diabetic mice harbor reduced abundance of Bacteroides-related bacteria that are linked to endotoxemia-induced inflammation. Of interest, separate studies involving T2DM patients have shown differences in the relative abundance of select bacterial groups including the Bacteroidetes, Proteobacteria, and *Clostridia* spp (3, 26). While differences in gut microbial composition of human T2DM patients were observed between these different studies, one consistent finding was a decreased abundance of butyrate-producing bacteria (3, 27–29). This inconsistency regarding which bacteria are significantly altered in T2DM was later explained by the administration of metformin, a commonly administered glucose lowering drug (30).

It is now well-recognized that changes in the gut microbiota composition contribute to the development of metabolic endotoxemia (2, 24, 31). Specific compositional changes, caused by enrichment of Gram-negative species, may modulate host inflammatory activity through increased lipopolysaccharide (LPS) absorption. Importantly, the DM dogs in our study were observed to have increased abundance of Gammaproteobacteria and elevated serum LPS concentrations, similar to humans with T2DM (3). The LPS component of Gram negative bacteria acts as an immunodominant antigen which binds to toll-like receptor 4 (TLR-4) to trigger immune system activation (32–34) which has been previously reported in dogs (35). Interestingly, compared to HC dogs, serum LPS concentrations in DM dogs of the present study were elevated supporting the presence of metabolic endotoxemia. While we anticipated even higher levels of LPS to be present in DM dogs, the magnitude of serum elevation may have been influenced by the fact that most (7/10) DM dogs were being fed low-fat, weight reducing rations at the time of trial enrollment (36).

Bile acids have been previously investigated for their role in metabolic homeostasis and also for their well-known functions in lipid digestion (37). Distinct patterns of altered BA metabolism have been observed in rodent models and humans with T2DM, including increased fasting taurine-conjugated BA (T-BA) and post-prandial total BA responses (38, 39), increased urinary BA excretion (40), perturbations in serum BA metabolomic

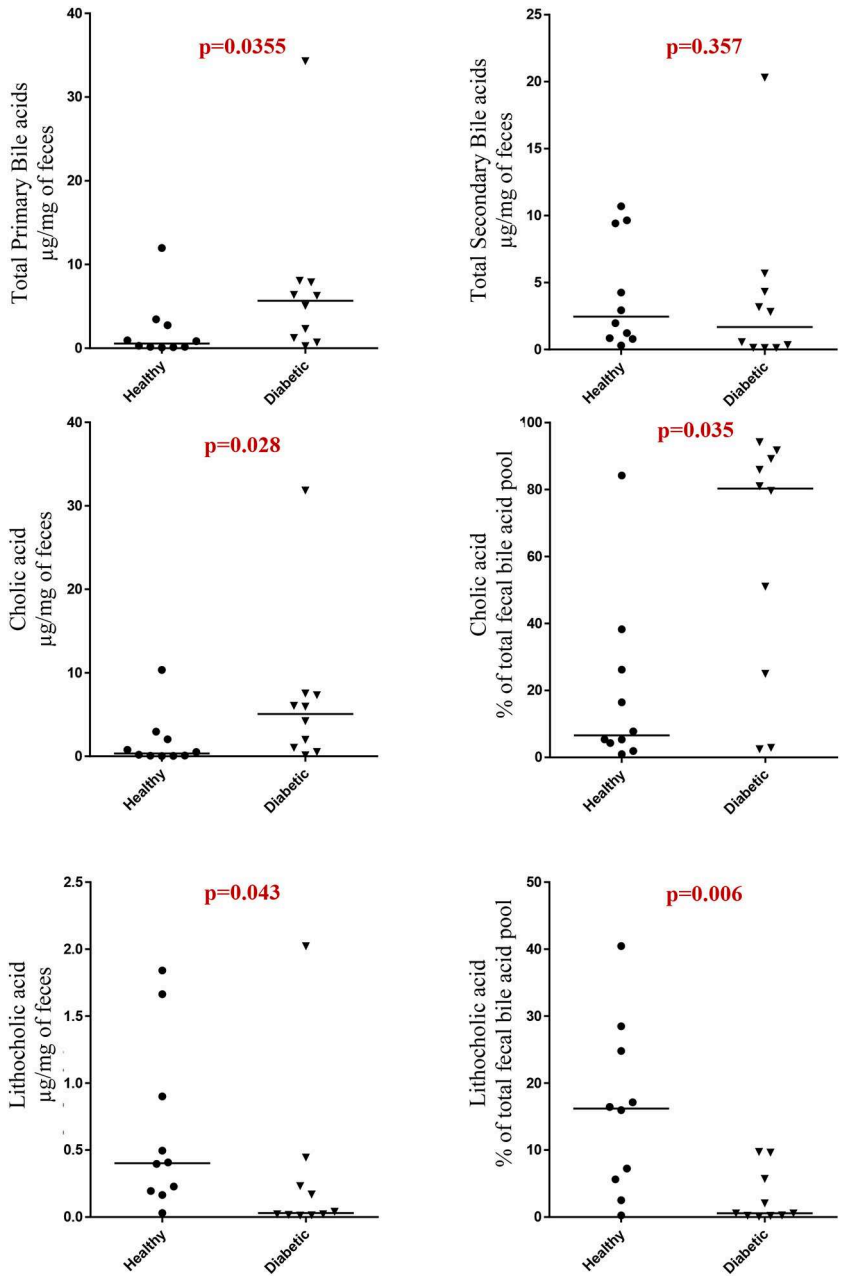


**FIGURE 5 |** Comparison of primary and secondary fecal unconjugated bile acids in canine cohorts.

signature (41), and changes in bile acid metabolism present in non-obese, spontaneously diabetic (NOD) mice (42). In our study, we observed changes in both primary and secondary fUBA in insulin-dependent DM dogs, characterized by increased total primary fUBA with increased concentrations of CA and the CA percentage of the total fUBA, while the levels of LCA (both absolute value and percentage of the total fUBA) were decreased in DM dogs vs. HC dogs. Whereas, our data show similarities (43) and differences (13) to studies performed in humans, they all imply a distinct link between impaired glucose homeostasis and altered BA pool composition in diabetic-susceptible individuals.

The relationship between host-microbiota interactions and bile acids is complex and bidirectional. Microbial metabolism of BA involves a number of reactions including BA deconjugation by species having bile salt hydrolase (BSH) activity, 7-dehydroxylation of primary BA into secondary BA, and the generation of iso-BA (i.e., oxo- or keto-BA) by bacteria containing hydroxysteroid dehydrogenases (HSDHs) (44–50). As a result of altered BA composition, different BA modulate

downstream signaling events through activation of receptors FXR and TGR5 in metabolically active tissues (51). In contrast, BA can modulate composition of the gut microbiota through their direct antimicrobial actions (i.e., destroying bacterial membranes by means of detergent properties) and indirectly through FXR activation which promotes transcription of antimicrobial (i.e., iNOS, IL-18) products (52). The ingestion of high fat diets may also impact host health by modulating BA and fecal microbiota composition to cause dysbiosis and local (gut mucosal) and systemic inflammation (53, 54).

Surprisingly, data investigating the role of gut microbiota in modulating the circulating BA pool in diabetic humans is sparse. A single study has analyzed the expression of microbiota-derived bile acid modification genes in humans with inflammatory bowel disease and diabetes (55). It was shown that Firmicute-derived bile salt hydrolase (BSH) genes and other BA modification genes were significantly reduced in the feces of T2DM patients relative to healthy controls. Similarly, other reports in animal models and humans with T2DM have shown the significance of gut microbial BSH genes in promoting positive physiologic changes and alteration in the overall BA pool (38, 56, 57). In one recent untargeted metabolomic study involving diabetic dogs, the primary and secondary bile acids, taurochenodeoxycholic acid, taurodeoxycholic acid, and tauroursodeoxycholic acid, were significantly lower in dogs with diabetes as compared to healthy dogs (58). While we observed reduced abundance of Firmicutes in the feces of our DM dogs, analysis of microbial-derived BSH or other BA modulating genes was not performed in this study.

There are only few published studies investigating canine BA profiles in health and disease. Most recent data suggest a relevant role for altered BA in canine chronic enteropathies. Honneffer et al. (59) utilized an untargeted metabolomic approach to identify several bile acid metabolites that were altered in the feces of dogs diagnosed with idiopathic inflammatory bowel disease (IBD). In a follow-up study to this report, Guard et al. (60) performed longitudinal assessment of microbial dysbiosis, fUBA concentrations and clinical disease activity in dogs with IBD that were treated with glucocorticoids. In that study, secondary fUBA were significantly decreased in IBD dogs and were accompanied by fecal dysbiosis and increased disease activity. While both fecal concentrations and the percentage of secondary fUBA increased post-treatment in IBD dogs, fecal microbial imbalances persisted in spite of resolution of clinical disease activity. Similarly, humans with IBD may have decreased proportions of fecal secondary BA without significantly altered primary BA (61) and increased primary BA are reported in people with diarrhea-predominant irritable bowel syndrome (62).

The ingestion of a high-fat diet (i.e., 75% of total energy requirement supplied by dietary fat) by healthy dogs has resulted in changes in fecal BA concentrations (63). In this pilot study, transitioning from a commercial dry food to a high-fat/low-fiber ration during a 7 weeks dietary trial increased fecal concentrations of the secondary BA DCA and UDCA. The investigators noted that there was significantly higher relative abundance of an OTU in the family *Clostridiaceae* (i.e., *Clostridia hiranonis*) which is particularly adept at converting primary BA to secondary BA. The consequences of this and other diet-associated alterations of the fecal microbiome and BA metabolome in dogs will require future adequately powered studies. There are some limitations to this study. First, the number of patients evaluated in both dog groups was relatively small and a longitudinal study design evaluating changes in microbiota and BA in response to treatment should be performed in the future. Trial enrollment of dogs with DM proved challenging due to the frequent use of antimicrobials in these patients (for suspicion of bacterial urinary tract infection or other unrelated causes) that reduced our sample size. Second, both cohorts were not fed a standardized diet which may have influenced microbial composition and the production of secondary BA. Third, we report data only for fUBA (but not conjugated BA) which may not provide a complete picture of BA dysmetabolism present in dogs with DM. That said, we are unaware of other published methods, beyond those described in the present study, to measure fecal BA concentrations in healthy and diseased dogs.

In conclusion, our results indicate that both intestinal dysbiosis and altered fecal BA levels are present in dogs with naturally occurring, insulin-dependent DM. Diabetic dogs demonstrate increased fecal primary (CA) BA and decreased levels of secondary (LCA) BA. The patterns of microbial imbalance and impaired BA homeostasis bear strong similarity to T2DM in humans. The dog represents a novel large animal model for advancing translational medicine research efforts (e.g., investigating pathogenesis and therapeutics) in DM affecting humans.

## AUTHOR CONTRIBUTIONS

AJ, BG, AR, and JoS contributed to research design and implementation. AJ, BG, AR, JaS, GR, and LC performed the experiments. AJ, BG JaS, JM, LC, YS, and RP contributed to data analysis. AJ, BG, JM, YS, JaS, and RP prepared the manuscript. AJ, BG, JaS, JM, JoS, KA, JL, and RP reviewed and edited the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2019.00199/full#supplementary-material

**Supplemental Table 1 |** Percentage of bacterial taxa.

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# A Microbiomic Analysis of a Pasture-Raised Broiler Flock Elucidates Foodborne Pathogen Ecology Along the Farm-To-Fork Continuum

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While conventionally grown poultry continues to dominate the U. S. poultry industry, there is an increasing demand for locally-grown, "all natural" alternatives. The use of next generation sequencing allows for not only the gross (e.g., community structure) but also fine-scale (e.g., taxa abundances) examination of these complex microbial communities. This data provides a better understanding of how a pasture flock's microbiome changes throughout the production life cycle and how that change in microbial ecology changes foodborne pathogens in alternative poultry production systems. In order to understand this ecology better, pooled broiler samples were taken during the entire flock life cycle, from pre-hatch gastrointestinal samples ($N = 12$) to fecal samples from the brood ($N = 5$), and pasture ($N = 10$) periods. Additional samples were taken during processing, including skin and feather rinsates ($N = 12$), ceca ($N = 12$), and whole carcass rinses ($N = 12$), and finally whole carcasss rinsates of final products ($N = 3$). Genomic DNA was extracted, 16S rDNA microbiome sequencing was conducted (Illumina MiSeq), and microbiomes were analyzed and compared using QIIME 1.9.1 to determine how microbiomes shifted throughout production continuum, as well as what environmental factors may be influencing these shifts. Significant microbiome shifts occurred during the life cycle of the pasture broiler flock, with the brood and pasture fecal samples and cecal samples being very distinct from the other pre-hatch, processing, and final product samples. Throughout these varied microbiomes, there was a stable core microbiome containing 13 taxa. Within this core microbiome, five taxa represented known foodborne pathogens (*Salmonella*, *Campylobacter*) or potential/emerging pathogens (*Pseudomonas, Enterococcus, Acinetobacter*) whose relative abundances varied throughout the farm-to-fork continuum, although all were more prevalent in the fecal samples. Additionally, of the 25 physiochemical and nutrient variables measured from the fecal samples, the carbon to nitrogen ratio was one of the most significant variables to warrant further investigations because it impacted both

general fecal microbial ecology and *Campylobacter* and *Enterococcus* taxa within the core fecal microbiomes. These findings demonstrate the need for further longitudinal, farm-to-fork studies to understand the ecology of the microbial ecology of pasture production flocks to improve animal, environmental, and public health.

**Keywords: microbiome, pastured poultry, *Salmonella*, *Campylobacter*, ecology**

## INTRODUCTION

The gastrointestinal tract (GIT) of poultry hosts a complex and dynamic bacterial microbiota (1), and these microbial communities can directly affect animal, environmental, and public health (2, 3). Studies have shown that environmental factors such as hatchery hygiene levels (4), housing (5), and production system (6, 7), litter quality and management (8, 9), and climate and geographical locations (10, 11) can significantly influence poultry GIT microbiota and the diversity demonstrate the dynamics of GIT microbial ecology. Additionally, the poultry GIT microbiome can serve as a reservoir for zoonotic pathogens like *Campylobacter*, *Salmonella*, and *Acinetobacter* spp. (12). Therefore, investigations into the dynamics of poultry microbiomes are understood throughout the entire farm-to-fork continuum.

Early in the poultry production chain, the colonization of the GIT of newly hatched chicks is a combination of the hen and hatchery environment during the pre-hatch phase (13). The GIT microbiome diversity of the very young chick (0–1 weeks old) increases gradually and with significant population variability compared to older mature birds (14), even within the same farm or flock (15, 16). While zoonotic pathogen colonization can occur at any stage of the farm-to-fork continuum, the lack of a mature GIT microbial ecology makes newly-hatched chicks susceptible (17). The source of these pathogens that colonize juvenile birds are not only from the surrounding farm environment (18, 19), but also from the other chickens within a flock (20, 21). These observations suggest that the environmental influences that drive the GIT microbiome diversification and establishment of these birds early in life have a lasting effect throughout the pre-harvest and grow-out periods. The poultry microbiome and resident zoonotic pathogens such as *Salmonella* and *Campylobacter* can be transferred from the farm pre-harvest environment to the post-harvest processing environment, and ultimately, the consumer (22–24). Therefore, it is important to attempt to understand these longitudinal dynamics from farm-to-fork.

A recent study attempted to take a farm-to-fork approach to poultry (23); however, there was no direct link between the pre-harvest, post-harvest, and final product samples analyzed, and the focus was on conventionally grown poultry. While conventionally grown chicken account for the majority of the poultry products produced in the U.S., consumers are increasingly concerned with the safety and welfare of poultry produced within conventional systems (25–27). This has resulted in an increased commercial demand for alternatively grown poultry products (28). Within the state of Georgia, which is the largest conventional poultry producing state in the U.S., 97% of the respondents of an online survey stated that they were very supportive of organic or all-natural poultry products that are locally grown on small farms. Furthermore, respondents would consider considering shifting their poultry purchases from conventional sources even when prices for pasture flocks reached $5.00 a pound (29). One alternative production system that is growing in popularity is pasture-raised poultry, which requires flocks to have continual access to fresh pasture and the outdoor environment on a daily basis (27, 30, 31). There is a limited amount of research available regarding the overall microbial community and the resulting foodborne pathogen dynamics within this production system [see (32) for a recent review].

Therefore, to better understand the dynamics of general microbial populations and foodborne pathogens within GIT communities, a single pastured-raised broiler flock was followed throughout the entire production continuum. To accomplish this, samples were collected from the flock during the pre-hatch, pre-harvest (brood, pasture), processing, and to the final product. Then, 16S rDNA microbiome sequencing was performed using the Illumina MiSeq platform. The data was analyzed with QIIME and comparisons were made between the microbiomes of various sample types (GIT, feces, ceca, carcass rinses) and stages along the farm-to-fork continuum. By comparing these microbiomes within sample type, not only among sample types and stages, but also to physiochemical data collected during the pre-harvest live production period, environmental influences of these general and pathogenic communities could be potentially elucidated, which could be used to better understand the drivers of these bacterial community throughout the broiler's life before reaching the consumer.

## MATERIALS AND METHODS

### Hatchery Sample Collection

A commercial broiler hatchery in the southeastern U.S. provided all of the eggs for this study. The broilers used for this study were a Cobb 500 cross. Once the eggs were set in the commercial hatchery, eggs ($n = 25$ total) were collected at four time points: (1) 1 week after set, (2) 2 weeks after set, (3) after *in ovo* immunization (2.5 weeks after set), and (4) one-day post-hatch. All necropsies throughout the course of the pre-hatch component of the study were performed at the University of Georgia Poultry Disease and Research Center (Athens, GA, USA) and all work was covered under Institutional Animal Care and Use Committee (IACUC) number A2010 11-568-Y1-A0.

At each sampling time, necropsies were performed to aseptically remove the embryonic gastrointestinal tracts (GIT) from each egg. Eggs were removed from the 37°C incubator, placed in a Type II biosafety cabinet (BSC), sprayed with 0.4%

Bioguard (Neogen Corp, Lansing MI, USA), and allowed to dry prior to sampling. Once the embryos were dry, sterile forceps were used to crack the egg at the air cell end. The egg shell was discarded, the embryos removed from the shell with sterile forceps and the embryos were euthanized by cervical dislocation (CD). Embryos were pooled in groups of seven into a sterile 110 $mm^3$ petri dish and sampled. The abdominal cavity of the embryos was opened with sterile scissors and the intestines were removed with sterile forceps. The GIT samples from each group of seven embryos were placed into a small filtered stomacher bag (Seward Laboratory Systems, Inc., Davie, FL).

For the post-hatch sample collection, an extra set of eggs were collected from the commercial hatchery, the eggs were placed into hatching baskets by breeder flock, and were then set in a single stage Natureform Hatcher (NatureForm Hatchery Technologies, Jacksonville, FL) and allowed to hatch at the University of Georgia facility. Chicks were removed from the hatcher, placed in ventilated transport containers and transported to the lab. For each group, chicks were euthanized by CD and placed on sterile 110 $mm^3$ petri dishes inside the BSC. The GIT samples were collected and pooled as described above.

Each pooled GIT sample was weighed and sterile 1x phosphate-buffered saline (PBS) was added to pooled GIT samples (3:1; 1x PBS volume: GIT mass) to ensure enough homogenate was available for all analytical needs. The pooled GIT samples were homogenized via stomaching (Seward Laboratory Systems, Inc.) on max speed for 60 s. Two 0.5 mL aliquots per sample were placed into separate FastPrep Lysing Matrix A tubes (MP Biomedicals, Solon, OH, USA), and all tubes were then frozen at −20°C until DNA extraction.

## Brood and Pasture Sample Collection

After the post-hatch GIT samples were collected, a set of 50 1-day old chicks were transported in chick carriers to a small pastured poultry homesteading farm ∼3 acres in size in north-central Georgia. The facility collectively rears the broilers with pastured layer hens, pastured guinea hens, dairy goats, a small flock of sheep, as well as housing a small swine herd on an adjacent, but completely separate, plot of land. The swine herd and sheep flock had <5 animals throughout the course of the study. While the above animals were grown for agricultural purposes, the homesteading farm also housed one horse, one cow, and one goat within the same pasture during pastured broiler live production.

Chicks were brooded through 3 weeks of age in two groups of 25 chicks housed within separate 80-gallon plastic totes with wood chip bedding. Chicks were given food and water *ad libitum*, and fresh bedding was overlaid over old bedding (deep litter method) every day. The bedding was completely removed and replaced weekly. Since the totes were kept within the farmer's house, no heat lamps were required during the brooding stage. For the first week post-hatch, all accessible fecal samples were aseptically scrapped from the liners at the bottom of the chick carriers and pooled into a single initial fecal sample. Weekly fecal samples were collected from week 1 to week 3 post-hatch, and all observable fresh fecal samples were removed from both of the totes and pooled into a single sample for that sampling point, with care being given to remove as much bedding material as possible from the sample.

By 4 weeks of age, the chickens were moved to mobile pens on the farm pasture. The mobile pens house 25 birds per pen, had a foot print of ∼72 $ft^2$ (6 × 12 ft), and contained a waterer, feeder, and roosting bars. The mobile pens were covered by plastic tarps to provide some environmental protection, these pens were moved daily to fresh pasture, and during the day the broilers were given access to pasture outside of the pen. Birds were fed and watered *ad libitum* and were not physically handled unless necessary for their safety or protection. The birds were grown this way on pasture until 16 weeks of age. Through week 8, fresh fecal samples were collected on a weekly basis. After week 8, sampling occurred every other week until 16 weeks of age when the birds were processed. For fecal sampling, after the mobile pens were moved for the day, all fresh fecal samples from the previous mobile pen area were collected and pooled into a single broiler fecal sample for that time point. During sampling, any fecal samples that could be identified as belonging to another animal species on the farm (horse, cow, goat, layer, guinea hen) within the area the broilers were currently being reared were also collected and processed in the same manner as the broiler feces, described below.

For all fecal samples, pooled fecal samples were placed on ice at the farm and transported back to the laboratory. Pooled fecal samples were weighed into three separate 0.5 g subsamples, and each of these subsamples were placed into separate FastPrep Lysing Matrix E tubes (MP Biomedicals), and all tubes were then frozen at −20°C until DNA extraction.

## Processing and Final Product Sample Collection

At 16 weeks of age, after a 24-h feed withdrawal, the broilers were moved individually to the processing area on the farm. Broilers were culled via exsanguination using "kill cones," and post-bleed out the head, feet, and wing tips were removed. The farmer completely removed the skin and feathers from the carcass, and then the entire viscera was subsequently removed. Removed skin with feathers were placed into individual sterile plastic bags containing 250 mL of 10 mM PBS and shaken vigorously manually for 1 min to produce a skin with feather rinse (SFR) sample. The rinsate was then poured into a filtered stomaching bags (Seward Laboratory Systems, Inc., Davie, FL). For each carcass, ceca were aseptically removed at the cecal tonsil juncture and placed into sterile, filtered stomaching bags.

Carcasses were rinsed using non-chlorinated well water and placed on ice until all carcasses were processed, which acted as the chilling step. The average time from kill cone to chilling was 12 min per bird per farmer, so with two farmers processing birds, the entire flock was processed in ∼5 h. The processed and chilled carcasses were moved into the house and rinsed internally and externally with in a dilute vinegar solution. The next step is termed the post-processing whole carcass rinse (P-WCR). For these sample collections, chilled carcasses were placed into individual sterile plastic bags containing 100 mL 10 mM PBS and shaken vigorously manually for 1 min, with the resulting

rinsate being placed in sterile filtered stomaching bags and stored on ice for transportation to the lab. The carcasses were then wrapped using food grade plastic wrap and placed at 4°C for 24-h. At that time, the carcass was considered the final product that the farmer provides to the customers using a customer-supported agriculture (CSA) model. Final product whole carcass rinse (FP-WCR) samples were created using the protocol described above for the P-WCR samples on those carcasses.

All SFR, cecal, P-WCR, and FP-WCR samples were transported back to the lab on ice and processed within 2 h post-collection. Cecal samples were diluted 1:3 using 10 mM PBS, while no buffer addition was needed for the three rinsate samples. All samples were homogenized for 60 s at the maximum setting and 0.5 mL of each sample was placed into separate FastPrep Lysing Matrix E tubes (MP Biomedicals), and all tubes were subsequently frozen at −20°C until DNA extraction.

## DNA Extraction, Microbiome Sequencing, and Data Analysis

Genomic DNA was extracted from the GIT, feces, ceca, SFR, P-WCR, and FP-WCR samples using a hybrid extraction method optimized for poultry samples (33). In short, 1 mL of Qiagen ASL buffer (Qiagen, Valencia, CA, USA) was added to each Lysing Matrix sample tube and vortexed at the maximum setting for 1 min, followed by a more thorough homogenization using the FastPrep 24 (MP Biomedicals) at 6.0 m/s for 45 s. After centrifugation (14,000° g for 10 min), supernatant was removed, added to a sterile 2 mL tube, and incubated at 95°C in a water bath for 5 min. At this point, all samples were processed using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) using the QIAcube robotic workstation (Qiagen) and the stool pathogen detection protocol. After the automated extraction and purification steps, the two extracted aliquots for each pooled sample were combined in 100 mL sterile molecular grade water using Vacufuge™ Plus (Eppendorf, Hauppage NY, USA), and the DNA concentration in each sample was determined spectrophotometrically using the Take3® plate in conjunction with the Synergy H4 multimode plate reader (BioTek, Winooski, VT, USA).

Library construction and sequencing were performed by the Earth Microbiome Project Laboratory at the U.S. Department of Energy, Argonne National Laboratory (Argonne, IL). In short, the hypervariable V4 domain of bacterial 16S rDNA gene was amplified using the F515 (5′-CACGGTCGKCGGCGCCATT-3′) and R806 (5′-GGACTACHVGGGTWTCT AAT-3′) primer set with each primer containing Illumina adapter sequences (Illumina, Inc., San Diego, CA) and the reverse primer containing the Golay barcodes to facilitate multiplexing (34). Raw reads were obtained by using the Illumina MiSeq platform.

A total of 17,700,915 raw reads were generated and processed by the QIIME v1.9.1 (Quantitative Insights Into Microbial Ecology) pipeline (35). Forward and reverse sequence reads were merged according to the fastq-join parameter within the *join_paired_ends.py* command. Quality filtering and library splitting according to the Golay barcode sequences were performed on the merged sequences with *split_library_fastq.py* script (-q 19, all other parameters were default) and resulted in a total of 13,419,288 sequences with an average of 74,139 sequences per sample. Sequences were chimera checked against the Greengenes 13_8 database (36) and clustered into Operational Taxonomic Units (OTUs) according to their sequence similarity (97%) using the usearch option (37) with *pick_otus.py* script (-m usearch, all other parameters were default). A representative sequence for each OTU was selected with *pick_rep_set.py* script (default parameters) and used for taxonomic assignment using UCLUST and the Greengenes 13_8 database (36) with *assign_taxonomy.py* (default parameters). Sequences were aligned (*align_seqs.py script*, default parameters) using PyNAST (38) and filtered (*filter_alignment.py*, default parameters). A phylogenetic tree was subsequently produced with the *make_phylogeny.py* script (with default settings and FastTree program). This pipeline resulted in a total of 1,106,557 sequences were obtained with an average of 52,693 sequences per sample for further analysis. Overall, a total of 1,789 unique OTUs were identified across all samples. The raw sequence and metadata files have been deposited in the MG Rast public database and is accessible with the MG-Rast ID mgm4844877.3.

Alpha diversity was used to describe the microbial richness, evenness and diversity within samples using the Chao1, Equitability, and Shannon metrics. Significant differences in alpha diversity parameters were tested between the sample types and different stages using the *compare alpha diversity.py* script. Beta diversity was determined using the Bray-Curtis distance to measure the dissimilarity between samples. Principal

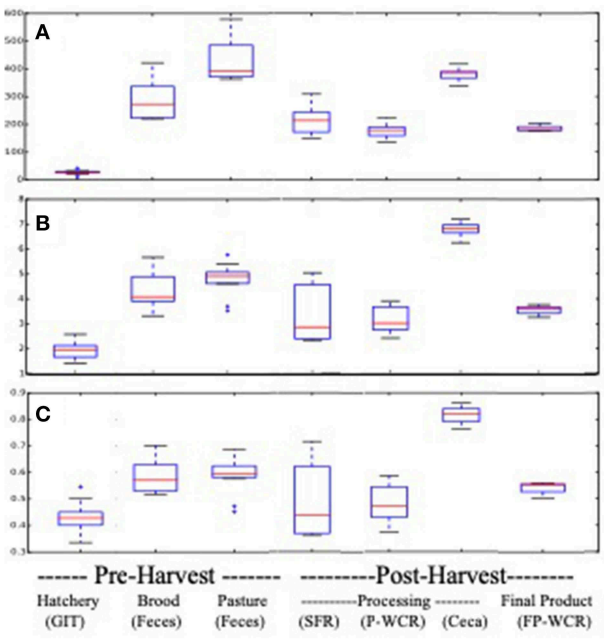


**FIGURE 1 |** Alpha-diversity boxplots for microbiomes from different sample types and stages along the farm-to-fork continuum of a pasture-raised broiler flock. **(A)** Comparison of richness based on the chao1 metric. **(B)** Comparison of diversity based on the Shannon Diversity metric. **(C)** Comparison of evenness based on the equitability metric.

coordinate analysis (PCoA) of the Bray-Curtis distance was performed to determine the change in the community structure using the vegan package v2.3-0 (39) in R software v3.2.1. Whole bacterial community composition was examined using non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarities with the *metaMDS* function. The function *envfit* was used to calculate the regression statistic for fecal physiochemical variables on ordination scores at a $p \leq 0.05$. Two different non-parametric analysis methods including analysis of similarities (ANOSIM) and permutation multivariate analysis of variance (PERMANOVA) were used to examine whether there were significant differences in community structures between the different sample types collected throughout the study and also between the different stages of the farm-to-fork continuum. The Bray-Curtis distance was used for the ANOSIM and PERMANOVA analyses in QIIME using *compare_category.py*. Core microbiome analyses were performed using the *compute_core_microbiome.py script* using and minimum fraction for core score of 0.75 (OTU must be in at least 75% of samples).

Using qPCR, total bacteria [16S rDNA gene; (40), *Salmonella* spp. (*invA*) (41), *Campylobacter jejuni* (*hipO*) (42), and *Listeria monocytogenes* (*hylA*) (43)]. All DNA extractions analyzed with qPCR were performed on Mastercycler® ep Realplex s2 and s4 thermocycling machines (Eppendorf) in 20 μL reaction mixture was prepared using 10 μL of 2x PerfeCTa qPCR ToughMix, ROX (Quanta BioSciences, Gaithersburg, MD, USA) and 5 μL template of 1:10 diluted sample (containing 10–15 ng genomic DNA) following the previously published protocols. The PCR amplification efficiency and detection sensitivity were determined by using a series of 10-fold dilutions of standards ($10^8$-$10^1$ copies per reaction) created from purified plasmids for the target gene. Target gene copy number was determined using Mastercycler ep Realplex software (Eppendorf).

## Fecal Physiochemical Analysis

The moisture content of the fecal samples was determined by drying overnight at 65°C and calculating the difference between the wet and dried weights of the litter. Fecal pH and electrical conductivity (EC) were determined using an Orion Versa Star Advanced Electrochemistry Meter (ThermoScientific) using 1:5 dilutions in distilled water. Fecal samples were submitted to the University of Georgia Soils Testing Laboratory for Total Carbon, Total Nitrogen, and elemental (Al, As, B, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Si, Zn) composition.

# RESULTS AND DISCUSSION

## Gross Microbiome Changes Throughout the Farm-To-Fork Continuum

Pasture flock broiler microbiomes significantly changed throughout the farm-to-fork continuum. Cecal microbiomes possessed the richest, most diverse, and most even communities from all of the assayed samples, although brooder and pasture fecal microbiomes had equivalent richness (**Figure 1**). Conversely, the hatchery GIT samples possessed the least rich,

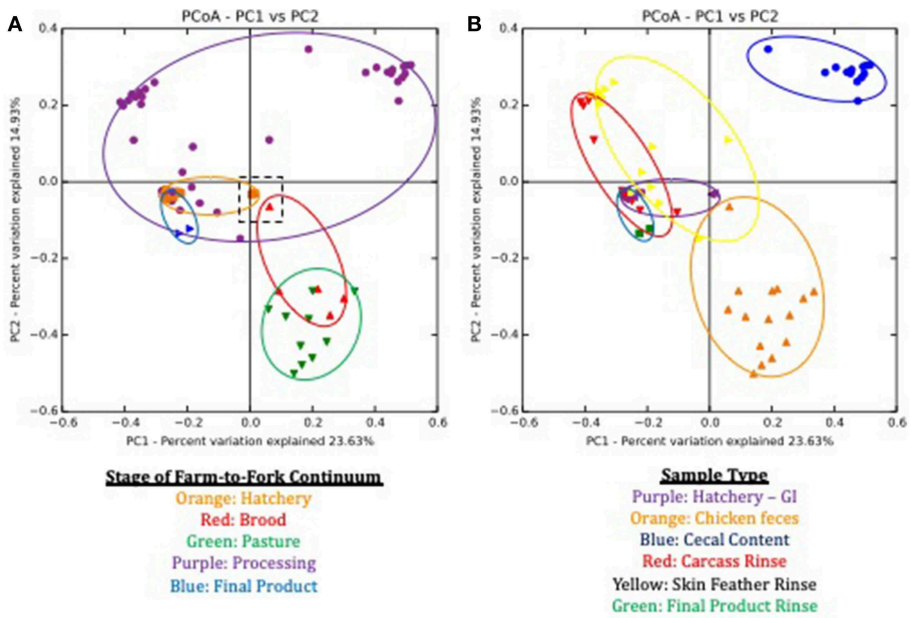


**FIGURE 2 |** Principle Coordinate Analysis (PCoA) plots based on Bray Curtis dissimilarities of microbiomes during the lifespan of a pasture-raised broiler flock. **(A)** Sample separation based on stage of farm-to-fork continuum, with each stage being assigned a different color. Symbols represent different samples from a given stage, and the ovals encompass the area of the graph that covers all of the samples for a given stage. The dashed black box in the middle of the graph highlights the GIT and feces samples that occur within the first day post-hatch. **(B)** Sample separation based on the sample type, with each sample being assigned a different color. Symbols represent different samples from a given sample type, and the ovals encompass the area of the graph that covers all of the samples for a given sample type.

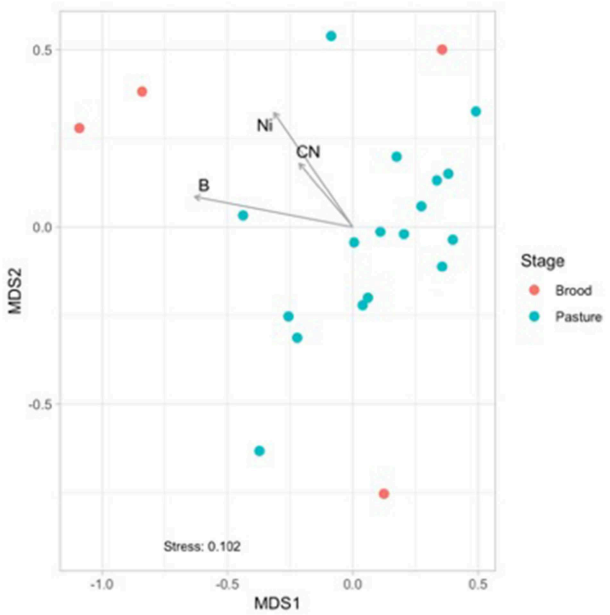


**FIGURE 3 |** Non-metric multidimensional scaling (NMDS) based on Bray Curtis dissimilarities of broiler fecal microbiomes from the brood and pasture stages. The broiler age (in weeks) is overlaid on the different points within the graph, with 1A–3 representing brood fecal microbiomes and 4A–16 representing pasture fecal microbiomes. Significant physiochemical parameters (B, C:N ratio, Ni; $p < 0.01$) were fitted onto the NMDS plot using the envfit function in the VEGAN package.

diverse and even communities. Hatchery GIT samples were significantly ($p < 0.05$) lower than any other sample collected aside from only the FP-WCR sample in terms of evenness. The general trend for all α-diversity estimates was, from highest to lowest, was ceca > pasture feces > brood feces > FP-WCR > SFR > P-WCR > GIT.

Beta-diversity estimates, based on the Bray-Curtis dissimilarity matrix, also showed distinct communities at each stage of the farm-to-fork continuum (**Figure 2A**) and sample type (**Figure 2B**). Both farm-to-fork stage ($p = 0.001$; $R^2 = 0.675$) and sample type ($p = 0.001$; $R^2 = 0.391$) significantly affected the resulting microbiomes according to ANOSIM analyses. When focusing on the stage of the continuum (**Figure 3**), microbiomes from all stages were tightly clustered, other than those microbiomes from the processing stage, which encompassed the upper half of the diagram (purple symbols and outline). While there were no major differences between the hatchery and post-harvest (processing, final product) microbiomes in terms of β diversity, the fecal samples (brood, pasture) formed a discrete cluster separate from those samples.

To better understand the large variability within the processing microbiomes, clustering was performed based on sample type (**Figure 2B**). Sample type clustering showed that the cecal microbiomes (blue symbols and outline) were discretely clustered compared to the SFR (yellow) and P-WCR (red) microbiomes. The two processing rinse sample types clustered closely with the final product rinse (green) and the GIT (purple) microbiomes. These findings are generally consistent with previous studies showing greater diversity and richness in fecal microbiomes compared to post-harvest rinses (23). That data is significantly different microbiomes when comparing pre-harvest (fecal, litter) with intestinal samples (ileum, cecum) both in chickens (44, 45) and turkeys (24), although none of these studies were able to directly link the fecal and post-harvest samples within the same flock as done in this present study.

It is interesting to note the shift in microbiomes between the hatchery and the brooding stage. While the GIT and fecal (brood and pasture) microbiomes generally clustered together, there were a set of outlier samples for both that clustered near each other (**Figure 2A**, dashed box). The microbiomes in this box represent the GIT samples 1-day post-hatch (orange) and the fecal samples from 1-day old chicks in the brooder box (red). By 1 week of age in the brood box, the fecal microbiomes shift significantly and are clustered with all subsequent fecal microbiomes. There is also a significant shift in total bacterial concentrations in these samples, as assessed by targeted qPCR. One-day post-hatch, the GIT 16S rDNA copy number (5.22 $\log_{10}$ copies) significantly increased compared to pre-hatch levels (1.45 $\log_{10}$ copies). The 1-day post-hatch fecal samples exhibited a significantly lower 16S rDNA (5.69 $\log_{10}$ copies) compared to the rest of the brood or pasture fecal samples (6.98 and 7.34 $\log_{10}$ copies). Stable, mature gut microbiomes have been previously shown to develop at various times throughout the broilers' life, ranging from 3 to 6 weeks of age in cecal microbiomes of conventionally-grown broilers (16, 46, 47), but this shift toward a stable microbiome occurred earlier in the present study using the pasture-raised model. This indicates that the shift toward a mature gut microbiome as assessed by fresh feces can be established very early, and this has implications for any attempts to modify or modulate the broiler gut microbiome to improve performance and health through the use of pre- or probiotics, as discussed elsewhere (32, 48–51). This data suggests that application of these products needs to occur immediately post-hatch or potentially even *in ovo* within the hatchery before the stable, mature gut microbiome develops (during the first week of life).

## Potential Environmental or Management Drivers of Fecal Microbiomes

Physiochemical data was collected from the brood and pasture samples to see if they had any potential effects on the fecal microbiomes using non-metric multidimensional scaling (NMDS) analyses (**Figure 3**). When only considering the fecal samples, there was a separation between the brood (red) and pasture (blue) samples, and three physiochemical parameters were found to be significantly correlated to the brood fecal microbiomes: boron ($p = 0.048$; $R^2 = 0.387$), nickel ($p = 0.043$; $R^2 = 0.554$), and carbon to nitrogen (C:N) ratio ($p = 0.012$; $R^2 = 0.432$). These three variables had no effect on the pasture fecal microbiomes, so it appears that during the first month of life brood microbiomes are significantly influenced by the concentrations of boron, nickel, and the

C:N ratio within the feces. This data reinforces that the relatively stable mature gut microbiome is formed early after hatch.

Considering the environmental exposure of these pastured flocks to other animal species on this multi-purpose farm, and the coprophagic nature of broilers, the question arises as to whether

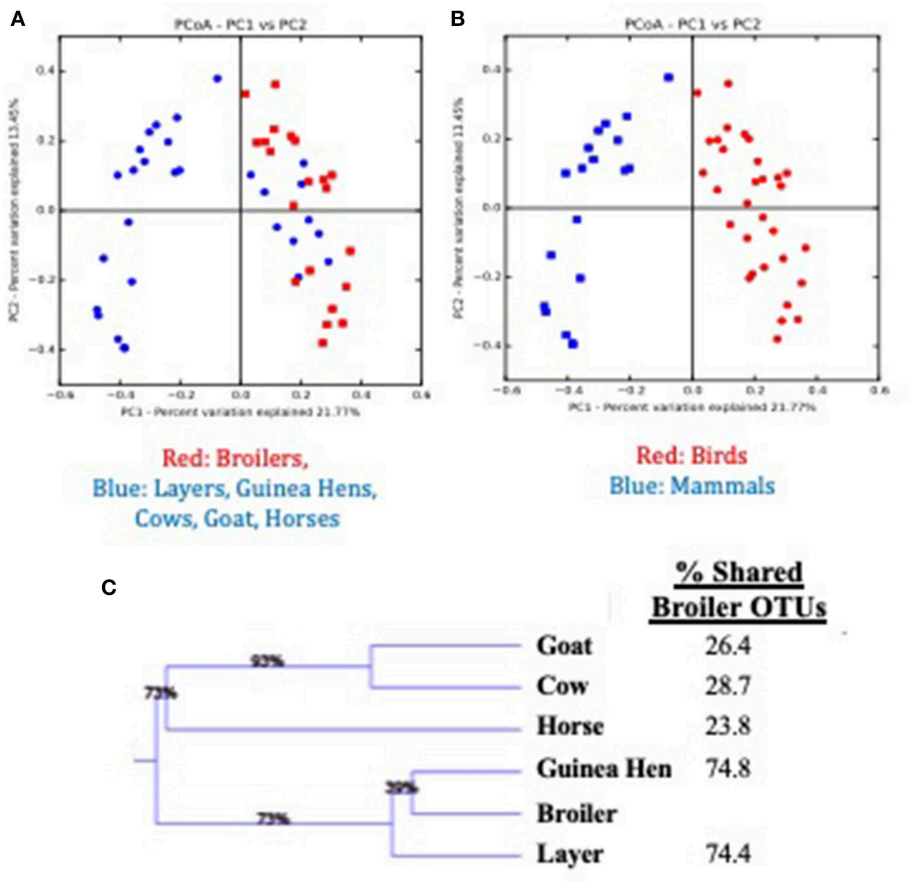


**FIGURE 4 |** Comparison of feces from multiple animal species present during the pre-harvest (live production) period for a pasture-raised broiler flock. **(A)** Principle Coordinate Analysis (PCoA) plot based on Bray Curtis dissimilarities comparing broiler feces (red) to all other feces (blue) recovered on pasture during live production. **(B)** Principle Coordinate Analysis (PCoA) plot based on Bray Curtis dissimilarities comparing bird feces (Broilers, Layers, Guinea Hens; red symbols) to mammal feces (Cow, Goat, Horse; blue symbols) recovered on pasture during live production. **(C)** WPGMA comparison of fecal microbiomes from different animals, with the final column describing the percent of OTUs shared with the broiler microbiome.

**TABLE 1 |** Relative abundances of major phyla-level taxa for microbiomes from different sample types and stages along the farm-to-fork continuum of a pasture-raised broiler flock[1,2].

| | Hatchery (GIT) (%) | Brood (Feces) (%) | Pasture (Feces) (%) | Processing (SFR) (%) | Processing (Ceca) (%) | Processing (P-WCR) (%) | Final Product (FP-WCR) (%) |
|---|---|---|---|---|---|---|---|
| Actinobacteria | 1.39 | 4.16 | 6.32 | 5.49 | 3.50 | 1.11 | 1.60 |
| Cyanobacteria | 2.27 | 0.02 | 0.02 | 3.96 | 0.18 | 6.28 | 2.80 |
| Firmicutes | $10.20^{B}$ | $57.64^{A}$ | $68.26^{A}$ | $12.80^{B}$ | $61.34^{A}$ | $6.64^{B}$ | $16.73^{B}$ |
| Proteobacteria | $85.76^{A}$ | $28.72^{B}$ | $23.08^{B}$ | $74.25^{A}$ | $5.12^{B}$ | $84.81^{A}$ | $76.80^{A}$ |
| Bacteroidetes | $0.18^{B}$ | $7.96^{B}$ | $1.85^{B}$ | $2.85^{B}$ | $21.89^{A}$ | $0.70^{B}$ | $1.53^{B}$ |
| Euryarchaeota | $0.00^{B}$ | $0.04^{B}$ | $0.05^{B}$ | $0.02^{B}$ | $2.87^{A}$ | $0.00^{B}$ | $0.00^{B}$ |
| Tenericutes | $0.00^{B}$ | $0.40^{B}$ | $0.02^{B}$ | $0.05^{B}$ | $2.11^{A}$ | $0.05^{B}$ | $0.00^{B}$ |

*[1]Information in parentheses in the top row indicates the sample type (GIT, gastrointestinal tract; SFR, Skin & Feather Rinse; P-WCR, Processing Whole Carcass Rinse; FP-WCR, Final Product Whole Carcass Rinse).*

*[2]Superscript letters next to the a-diversity estimates indicated significantly different values for a single metric across a row, based on mean separation of ANOVA using $p < 0.05$ significance level.*

the presence of other animals on the farm impact the broilers raised on these pastures. To assess possible broiler microbiome effects, fecal samples from all animals raised on the pasture during the broiler's lifetime (horses, cows, goats, layers, guinea hens) were collected weekly (if present in the current broiler sampling site) and the fecal microbiomes for all animals were compared (**Figure 4**). When comparing the broilers to all other animals based in Bray-Curtis dissimilarity matrix of β-diversity (**Figure 4A**), the other animal fecal microbiomes (blue) generally clustered separately from the broiler fecal microbiomes (red), although there was some clustering of non-broiler with the broiler microbiomes. The identities of these similar non-broiler microbiomes were found to be other bird species (layers, guinea hens), with distinct clustering of microbiomes found between bird and mammal species on the farm (**Figure 4B**; red and blue symbols, respectively). Weighted Pair Group Method with Arithmetic Mean (WPGMA) analyses revealed that mammal fecal microbiomes only shared 24–29% of the OTUs with the broilers, while the other bird species shared ∼75% of the OTUs with broiler fecal microbiomes (**Figure 4C**). While there have been studies that have described the impact that the pasture-raised management model has on biosecurity (52–54) and on the prevalence or abundance of foodborne pathogens (27, 55–57), this data suggests that rearing broilers concomitantly with other mammal species does not significantly affect their gut microbiomes, potentially due to the rapid establishment of a mature broiler gut microbiome.

## Multi-Level Taxonomic Microbiome Changes

There were significant phyla-level differences between the various microbiomes across the farm-to-fork continuum (**Table 1**). Firmicutes and Proteobacteria accounted for >85% of all OTUs for nearly all sample types, with Firmicutes significantly dominating the brood feces, pasture feces, and the cecal microbiomes and Proteobacteria significantly more abundant in the hatchery, SFR, P-WCR, and FP-WCR communities. Firmicutes and Proteobacteria only account for about 66.5% of the OTUS in the cecal samples, which exhibited significantly higher abundances of Bacteriodetes, Euryarchaeota, and Tenericutes compared to all the other samples collected in the study. The phyla are consistent with other studies and meta-analyses of poultry pre-harvest (23, 33), intestinal (45, 58), and processing (59, 60) microbiomes.

To simplify the genus-level taxa shifts throughout the farm-to-fork continuum (which contained 430 total taxa), the core poultry microbiome from all samples was determined. To accomplish this, OTUs that were present in 50 or 75% of all samples were identified. There were 105 taxa consistent across 50% core microbiome, and in most cases these OTUs were found in at least two sample types and/or stages throughout the farm-to-fork continuum (**Table 2**). The Hatchery samples did not possess any core OTUs unique to those GIT samples, whereas 20% of the core OTUs were unique to only the cecal samples. The only other stage to have >4% unique OTUs was the brood feces (11.1%). Refinement of the core microbiome focused on those OTUs that were present in at least 75% of all samples (**Figure 5**). The more stringent core microbiome consisted of 13 groups representing three phyla: Actinobacteria (*Corynebacterium*), Firmicutes (*Rummelbacillus, Enterococcus, Lactobacillus, Leuconostocaceae* unclassified, *Leuconostoc, Clostridiales* unclassified), and Proteobacteria (*Campylobacter, Enterobacteriaceae* unclassified, *Enterobacter, Salmonella, Acinetobacter, Pseudomonas*). Definite shifts in this core microbiome were observed throughout the farm-to-fork

**TABLE 2 |** Shared (found in at least two sample types) and Unique (found in only one sample type) OTUs found within the core microbiome found in at least 50% of the different sample types and stages along the farm-to-fork continuum of a pasture-raised broiler flock[a].

| | Shared OTUs (%) | Unique OTUs (%) |
|---|---|---|
| Hatchery (GIT) | 100.00 | 0.00 |
| Brood (Feces) | 87.60 | 11.10 |
| Pasture (Feces) | 93.40 | 4.00 |
| Processing (SFR) | 99.35 | 0.00 |
| Processing (Ceca) | 80.00 | 18.60 |
| Processing (P-WCR) | 99.10 | 0.72 |
| Final Product (FP-WCR) | 96.30 | 3.20 |

[a]*Information in parentheses in the first column indicates the sample type (GIT, gastrointestinal tract; SFR, Skin & Feather Rinse; P-WCR, Processing Whole Carcass Rinse; FP-WCR, Final Product Whole Carcass Rinse).*

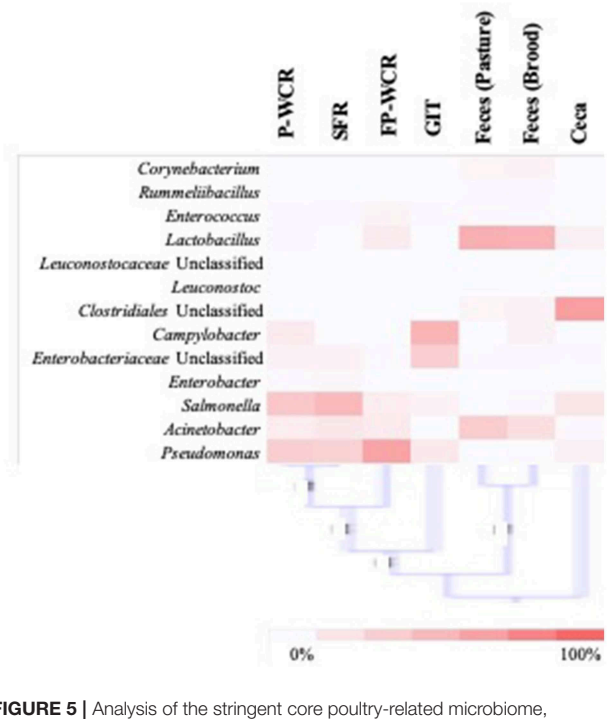


**FIGURE 5 |** Analysis of the stringent core poultry-related microbiome, representing 13 taxa that were present in 75% of all samples along the farm-to-fork continuum of a pasture-raised broiler flock. The distribution of these taxa within each sample type/stage of the farm-to-fork continuum are shown by the heatmap (with higher concentrations denoted by darker red color), and WPGMA below the heatmap indicating the relatedness of the stringent core microbiomes within the different sample types/stages.

**TABLE 3 |** Relative abundances of the stringent core microbiome taxa (OTUs present in 75% of all samples) within the core and total microbiomes from different sample types and stages along the farm-to-fork continuum of a pasture-raised broiler flock[a,b].

| | Hatchery (GIT) | | Brood (Feces) | | Pasture (Feces) | |
|---|---|---|---|---|---|---|
| | % Core[b] | % Total[c] | % Core | % Total | % Core | % Total |
| *Corynebacterium* | 0.00 | 0.00 | 5.65 | 1.39 | 4.26 | 3.11 |
| *Rummelibacillus* | 0.00 | 0.00 | 2.48 | 1.02 | 1.99 | 3.16 |
| *Enterococcus* | 1.59 | 1.27 | 2.54 | 0.87 | 2.97 | 5.87 |
| *Lactobacillus* | 0.00 | 0.00 | 48.57 | 31.62 | 51.46 | 39.86 |
| *Leuconostocaceae* Unclassified | 0.00 | 0.00 | 1.15 | 0.13 | 0.48 | 0.28 |
| *Leuconostoc* | 0.00 | 0.00 | 0.53 | 0.02 | 0.02 | 0.01 |
| *Clostridiales* Unclassified | 0.17 | 0.14 | 5.69 | 2.08 | 4.24 | 1.74 |
| *Campylobacter* | 48.26 | 41.12 | 6.31 | 0.06 | 0.48 | 0.21 |
| *Enterobacteriaceae* Unclassified | 30.39 | 23.61 | 2.67 | 0.57 | 2.09 | 1.21 |
| *Enterobacter* | 0.00 | 0.00 | 0.20 | 0.00 | 0.01 | 0.01 |
| *Salmonella* | 6.01 | 5.18 | 2.79 | 0.04 | 0.07 | 0.04 |
| *Acinetobacter* | 0.79 | 0.65 | 19.63 | 6.49 | 31.90 | 19.92 |
| *Pseudomonas* | 12.78 | 14.64 | 1.78 | 4.71 | 0.03 | 0.09 |
| Total | 100.00 | 86.61 | 100.00 | 49.00 | 100.00 | 75.50 |

| | Processing (SFR) | | Processing (Ceca) | | Processing (P-WCR) | | Final Product (FP-WCR) | |
|---|---|---|---|---|---|---|---|---|
| | % Core | % Total | % Core | % Total | % Core | % Total | % Core | % Total |
| *Corynebacterium* | 0.21 | 0.77 | 0.15 | 0.01 | 0.15 | 0.22 | 0.01 | 0.27 |
| *Rummelibacillus* | 0.04 | 0.03 | 0.27 | 0.03 | 0.21 | 0.18 | 0.86 | 0.69 |
| *Enterococcus* | 2.11 | 1.43 | 0.72 | 0.06 | 1.45 | 0.88 | 4.83 | 3.70 |
| *Lactobacillus* | 0.92 | 0.98 | 6.83 | 1.88 | 2.31 | 3.32 | 10.33 | 9.54 |
| *Leuconostocaceae* Unclassified | 0.02 | 0.01 | 0.19 | 0.01 | 0.01 | 0.01 | 0.02 | 0.01 |
| *Leuconostoc* | 0.01 | 0.01 | 0.13 | 0.01 | 0.01 | 0.00 | 0.01 | 0.01 |
| *Clostridiales* Unclassified | 0.50 | 0.24 | 62.96 | 6.12 | 0.01 | 0.01 | 0.09 | 0.07 |
| *Campylobacter* | 1.07 | 1.00 | 0.90 | 0.06 | 10.91 | 10.75 | 0.86 | 1.68 |
| *Enterobacteriaceae* Unclassified | 5.86 | 4.82 | 2.36 | 0.35 | 6.50 | 6.14 | 2.12 | 2.18 |
| *Enterobacter* | 4.05 | 4.04 | 1.24 | 0.21 | 3.01 | 3.29 | 0.64 | 0.59 |
| *Salmonella* | 44.68 | 35.09 | 14.80 | 2.15 | 35.63 | 31.07 | 9.59 | 7.38 |
| *Acinetobacter* | 12.93 | 8.13 | 3.30 | 0.22 | 9.85 | 7.86 | 11.09 | 9.64 |
| *Pseudomonas* | 27.60 | 16.22 | 6.16 | 0.45 | 29.96 | 21.39 | 59.55 | 47.51 |
| Total | 100.00 | 72.79 | 100.00 | 11.57 | 100.00 | 85.12 | 100.00 | 83.28 |

[a] *Information in parentheses in the top row indicates the sample type (GIT, gastrointestinal tract; SFR, Skin & Feather Rinse; P-WCR, Processing Whole Carcass Rinse; FP-WCR, Final Product Whole Carcass Rinse).*

[b] *Represents the relative abundance of each taxa within the stringent core microbiome including OTUs present in 75% of all samples (13 total taxa).*

[c] *Represents the relative abundance of each taxa within the total microbiome without excluding OTUs based on presence in a set percentage of samples (430 taxa).*

continuum, with the Firmicutes members being more prevalent in the fecal and cecal samples and the Proteobacteria being more abundant in the hatchery, processing, and final product samples (**Table 3**). The core microbiomes of the rinsate samples (SFR, P-WCR, FP-WCR) were more similar, with two rinsates collected during processing and the fecal core microbiomes from the brood and pasture identified as the most similar via WPGMA analyses (**Figure 5**). The only other longitudinal broiler microbiome study in the literature also detected *Corynebacterium, Lactobacillus, Campylobacter*, and *Enterobacter* in the core microbiome of fecal, litter, carcass rinse, and weep samples (23), although these samples were not collected from the same flock (pre-harvest, carcass rinse, and weep samples were all collected from different sources at different times). The difference in core microbiomes between that study and this one is likely due to the samples being collected from conventional-based poultry management systems.

The WPGMA findings align with what was observed for the total microbiomes (**Figure 2**), and the cecal microbiomes were found to be very unique compared to all other microbiomes. The 13 taxa of the 75% core microbiome represented ∼50% or more of the total microbiome of the other six stages per sample types, representing an average of ∼75% of the total microbiome. However, these 13 taxa only accounted for ∼12% of the total cecal microbiome, making this microbiome more unique (**Table 3**). In combination with the fact that 50% core microbiomes contained 20% unique OTUs, these results demonstrate the

very unique bacterial communities contained within this part of the poultry GIT. Other studies based on conventionally grown birds have demonstrated that cecal microbiomes are unique from other poultry-related microbiomes collected from the farm (23, 61) or from other sections of the gastrointestinal tract (24, 62). Considering the cecum is a common sample target for food safety research, this data suggests that the uniqueness of ceca microbial ecology needs to be considered. The survival and persistence of potential pathogens within the very unique cecal environment may not correlate with pathogen survival in different microbial communities throughout the farm-to-fork continuum (**Figures 2**, **5**). According to the data from the current study, it is possible that post-processing carcass rinses (P-WCR) may represent a better proxy for what is found on the final product (FP-WCR) compared to the ceca.

## Specific Focus on Foodborne Pathogens

Due to the increased access to the environment and other farm animals in the pastured poultry management system (32, 63), there is a hypothesis that this exposure would increase the prevalence of foodborne pathogens within pasture-raised flocks. One of the most interesting outcomes of the stringent core microbiome analysis above was the inclusion of known foodborne pathogens (*Campylobacter, Salmonella*) and genera that could potentially possess foodborne pathogen species (*Pseudomonas, Enterococcus*) or considered emerging pathogens (*Acinetobacter*). While much of the food safety-related work in poultry is focused on the post-harvest environments (processing, final product), the above data (**Figure 5**, **Table 3**) demonstrates that these zoonotic bacterial pathogens are consistent members of the poultry microbiome. The persistence of these pathogens are consistent from the pre-hatch through the post-processing environments to the consumer's kitchens. Therefore, these five foodborne pathogen taxa within the total microbiomes were specifically investigated throughout the entire lifetime of this pasture-raised broiler flock (**Figure 6**).

While each of these taxa were present in all stages and/or sample types, their relative abundance within the total microbiomes of each stage and/or sample type shifted dramatically (**Figure 6A**). The most abundant zoonotic taxa in total microbiomes for each stage of the farm-to-fork continuum were: *Campylobacter* in the hatchery GIT samples (41.1%); *Acinetobacter* in the brood and pasture fecal samples (6.5 and 19.9%, respectively); *Salmonella* in the processing SFR, ceca, and P-WCR samples (35.1, 2.1, 31.1%, respectively); and *Pseudomonas* in the FP-WCR samples (47.5%). *Enterococcus* was present throughout the study but was never the dominant zoonotic taxa and always represented <0.5% of the total microbiomes for any sample. Finding these known or potential foodborne pathogens as endemic taxa within the core poultry-related microbiome has definite implications for the use of future intervention strategies to reduce these zoonotic populations. Focusing on a single stage of the farm-to-fork continuum (typically the processing environment) may only result in a partially efficacious intervention, since these pathogens can thrive at all stages of the management system. These findings, while they do not indicate virulence, do highlight the need to take a more systems-based approach to intervention strategies that look deeper into the dynamics of the specific zoonotic serotypes/species throughout the farm-to-fork continuum to determine whether broader or more targeted strategies are needed, and at what stage they are going to be most effective.

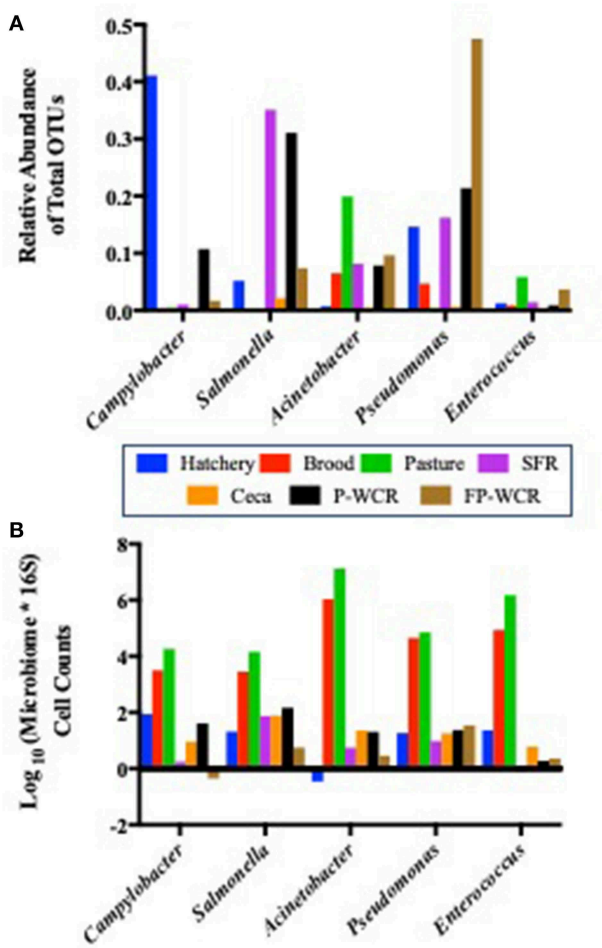


**FIGURE 6 |** Prevalence of five core zoonotic taxa within the total microbiomes from different sample types and stages along the farm-to-fork continuum of a pasture-raised broiler flock. **(A)** Relative abundances of the five core zoonotic taxa within the total microbiomes (430 total taxa). **(B)** $Log_{10}$-transformed quantified microbiome cell counts of the five core zoonotic taxa, based on multiplying the microbiome relative abundance data by the total bacterial counts for each sample according to 16S qPCR analysis.

While microbiome analysis allows for an assessment of bacterial communities and its individual members, it only does so pseudo-quantitatively by determining each taxa's relative abundance to the whole bacterial population within those samples. This must be considered when looking at the data within **Figure 6A**, where known poultry pathogens such as *Campylobacter* and *Salmonella* represent 1.7 and 7.4% of the final product microbiomes, respectively, but only 0.04 and 0.06% of the fecal microbiomes. Therefore, qPCRs were performed to quantify the total bacterial populations in the farm-to-fork

**TABLE 4 |** qPCR quantification ($log_{10}$-transformed) of total bacteria and foodborne pathogens (*Salmonella, Campylobacter jejuni, Listeria monocytogenes*) from different sample types and stages along the farm-to-fork continuum of a pasture-raised broiler flock[a,b].

| | Pre-harvest | | | Post-harvest | | | |
|---|---|---|---|---|---|---|---|
| | Hatchery | Brood | Pasture | Processing | | | Final Product |
| **Target (gene)** | **GIT** | **Feces** | **Feces** | **SFR** | **Ceca** | **P-WCR** | **FP-WCR** |
| Total Bacteria (16S) | 2.39 ± 1.72 | 6.72 ± 0.66 | 7.34 ± 0.46 | 1.61 ± 0.94 | 3.89 ± 0.70 | 2.11 ± 0.73 | 1.68 ± 0.50 |
| *Salmonella* spp. (invA) | 0.10 ± 0.12 | 0.24 ± 0.13 | 0.22 ± 0.34 | 0.42 ± 0.15 | 0.52 ± 0.10 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| *Campylobacter jejuni* (hipO) | 1.43 ± 0.21 | 1.72 ± 0.56 | 1.74 ± 0.95 | 0.16 ± 0.23 | 0.11 ± 0.35 | 1.30 ± 1.21 | 0.00 ± 0.00 |
| *Listeria monocytogenes* (hylA) | 0.19 ± 0.40 | 0.17 ± 0.33 | 0.73 ± 0.82 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |

[a] *Information in the third row indicates the sample type (GIT, gastrointestinal tract; SFR, Skin & Feather Rinse; P-WCR, Processing Whole Carcass Rinse; FP-WCR, Final Product Whole Carcass Rinse).*

[b] *Values represent the average ± standard deviation for replicate samples for each sample type (N = 12, 5, 10, 12, 12, and 3 for the GIT, Brood Feces, Pasture Feces, SFR, Ceca, P-WCR, and FP-WCR, respectively).*

**TABLE 5 |** Correlation of pathogenic taxa within the stringent core microbiome (OTUs present in 75% of all samples) to elemental concentrations within pre-harvest (brood, pasture) fecal samples of a pasture-raised broiler flock[a,b,c].

| | Total C (%) | Total N (%) | C:N Ratio | Ca (ppm) | K (ppm) | Mg (ppm) | Mn (ppm) | Na (ppm) | P (ppm) | S (ppm) | Si (ppm) | Zn (ppm) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Campylobacter* | 0.627 | 0.464 | **0.0172** (*0.486*) | 0.355 | 0.554 | 0.541 | 0.665 | 0.321 | 0.641 | 0.575 | 0.646 | 0.616 |
| *Salmonella* | 0.407 | 0.447 | 0.355 | 0.160 | 0.277 | 0.312 | 0.469 | 0.176 | 0.398 | 0.285 | 0.218 | 0.431 |
| *Acinetobacter* | **0.0122** (*0.521*) | **0.0184** (*0.479*) | 0.653 | **0.0196** (*0.435*) | **0.009** (*0.510*) | **0.0152** (*0.461*) | **0.0496** (*0.333*) | **0.0188** (*0.440*) | **0.044** (*0.347*) | **0.0162** (*0.454*) | **0.0357** (*0.370*) | **0.0396** (*0.359*) |
| *Pseudomonas* | 0.549 | 0.490 | 0.463 | 0.662 | 0.600 | 0.767 | 0.866 | 0.422 | 0.775 | 0.498 | 0.892 | 0.866 |
| *Enterococcus* | 0.548 | 0.341 | **0.0428** (*0.382*) | 0.705 | 0.434 | 0.556 | 0.496 | 0.291 | 0.510 | 0.564 | 0.955 | 0.389 |

[a] *Physiochemical and nutrient variables that did not have any significant correlations to bacterial taxa are not included in this table.*

[b] *Information in parentheses in the top row indicates units of concentration per gram of feces.*

[c] *Values represent p-values of the correlation analysis, with the significant correlations ($p < 0.05$) bolded. The $R^2$ values for the significant correlations are provided in italics within parentheses below the p-value.*

continuum samples, as well as specific foodborne pathogens (*Salmonella, C. jejuni,* and *L. monocytogenes*; **Table 4**).

To determine if the *Campylobacter* and *Salmonella* are numerically more abundant in the final product or just in the FP-WCR samples, the total bacterial populations for each sample were determined by 16S qPCR (40). Using the qPCR $C_T$ values (16S copies per PCR) and the relatively abundance values from the microbiome data, a cell count was determined (**Figure 6B**). The quantification of the microbiome data shows that all five pathogens taxa within the core microbiome were significantly ($p < 0.001$) higher in the brood and fecal samples compared to all hatchery, processing, and final product samples. For all taxa, the calculated cell count was lower than 1 log in the FP-WCR samples (brown bar). This shift in increased prevalence in the fecal samples is directly due to the fact that the brood and pasture fecal samples possessed much larger bacterial densities (6.7 and 7.3 $log_{10}$ 16S copies/qPCR, respectively) compared to the hatchery (2.6 $log_{10}$ 16S copies/qPCR), processing (1.6, 3.9, 2.1 $log_{10}$ 16S copies/qPCR for SFR, ceca, and P-WCR, respectively), and final product samples (1.7 $log_{10}$ 16S copies/qPCR). It appears that while *Campylobacter* and *Salmonella* represent a larger portion of the FP-WCR microbiome, numerically the populations were ∼3 logs higher in the fecal samples than in the final product samples. There are numerous potential biases with this quantification of the microbiome data, including the use of different 16S rDNA primer sets for the qPCR and microbiome data. Additionally, the 16S copy number can range from 1 to 7 depending on the bacteria, which can also bias the data; however, that bias should be relatively equal for all samples and the resultant trends should be accurate.

Since all five pathogenic taxa within the core microbiome were more prevalent in the fecal samples, correlations to physiochemical (pH, EC, moisture), and nutrient data (total C, total N, C:N ratio, elements) were performed to determine any potential environmental drivers of their relative abundances within these fecal microbiomes (**Table 5**). Just under half (12 of 25) of the measured environmental variables showed significant correlation to relative abundances of any of the pathogenic core taxa. Of the five zoonotic core taxa, two were not significantly correlated to any measured environmental variables (*Salmonella*, *Pseudomonas*), while *Campylobacter* and *Enterococcus* showed a significant correlation to only C:N ratio. Previous poultry-related microbiome work has not shown any associations between *Campylobacter* to other bacterial microbiome taxa (23), but this

current study shows that C:N ratio is significantly correlated to not only *Campylobacter* and *Enterococcus*, but also was shown to have a significant effect of the β-diversity distribution of the total fecal microbiomes (**Figure 3**). Additionally, *Acinetobacter* was significantly correlated to 11 of the 25 environmental variables, with $R^2$ values ranging from 0.333 to 0.521. These data provide insight into potential physiochemical variables that effect foodborne pathogen abundance during the on-farm production stage of pastured broiler management, which could potentially lead to the development of pre-harvest mitigation strategies if these parameters can be modulated in the broiler gut.

Multiple studies have shown that alternative poultry production management systems, including pasture-raised, can reduce *Salmonella* prevalence compared to conventional systems in pre-harvest samples (55, 64), but results can vary in the post-harvest environments (65–68). *Campylobacter* was typically the most prevalent of the zoonotic pathogens recovered from alternative poultry management systems, with *Campylobacter* found within various pre-harvest and post-harvest/final product samples (54, 55, 66–68), with counts higher in farm samples but prevalence being higher in processing/final product samples (23, 56). Both of these pathogens represented important members of the farm-to-fork core microbiome of this current study, and their abundance varied along the farm-to-fork continuum. Having higher pre-harvest counts, but higher prevalence in post-harvest samples has been previously reported for *Campylobacter* in conventional (23) and pastured poultry management systems (56), although no data was available for *Salmonella* or the other three zoonotic core taxa from this study. Given the importance of *Acinetobacter* species as a potentially drug resistance pathogen in clinical settings (12), it is important to further elucidate the environmental drivers of *Acinetobacter* relative abundance within the broiler farm environment, since it is a relatively uninvestigated reservoir for this emerging pathogen.

## CONCLUSIONS

While there are significant shifts in the poultry microbiome from the pre-hatch to the final product stage, there was a core microbiome that was present throughout the entire farm-to-fork continuum of this pastured-raised broiler flock. Investigations of these microbiomes revealed several important findings that need to be further investigated, including (1) the relatively rapid (by 1 week of age) development of stable gut microbiome in these broilers, as evidenced by the fecal microbiomes; (2) the uniqueness of the cecal microbiome and the cecal environment and how poorly it correlates to the final product microbiome (and what implications that may have for food safety-related research); and (3) the presence of known pathogens (*Salmonella, Campylobacter*) and potential/emerging pathogens (*Pseudomonas, Enterococcus, Acinetobacter*) in the core microbiome found throughout the farm-to-fork continuum, which underlines the importance of understanding these pathogens from a longitudinal pre-harvest and post-harvest perspective. It should be noted that these results are only from a single small pasture-raised flock, but on-going research has expanded to 10 more farms and 40 more flocks over 4 years, and preliminary assessments of the data support the three major findings presented above. Therefore, these findings demonstrate the need for further longitudinal, farm-to-fork studies to understand the ecology of these bacteria to develop better abatement/intervention strategies to improve animal, environmental, and public health in alternative, as well as conventional, poultry production systems.

## ETHICS STATEMENT

Live animals were managed and handled solely by the collaborating farmer, including the butchering process. Laboratory technicians only handled environmental samples (feces, soil) and poultry samples post-mortem (ceca, rinses).

## AUTHOR CONTRIBUTIONS

MR designed and executed the study, analyzed the data, and wrote the manuscript. AL, AC, and JG helped in the analysis of the microbiome (AL, AC) and pathogen specific (JG) data. KH assisted in the initial design of the experiment. SR and KF assisted in the construction and revision of the manuscript and provided analytical support for the microbiome analysis.

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# Respiratory Dysbiosis in Canine Bacterial Pneumonia: Standard Culture vs. Microbiome Sequencing

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It is unknown how the respiratory microbiome influences and is influenced by bacterial pneumonia in dogs, as culture of lung samples and not microbial sequencing guides clinical practice. While accurate identification of pathogens are essential for treatment, not all bacteria are cultivable and the impact of respiratory dysbiosis on development of pneumonia is unclear. The study purposes were to (1) characterize the lung microbiome in canine bacterial pneumonia and compare deviations in dominant microbial populations with historical healthy controls, (2) compare bacteria identified by culture vs. 16S rDNA sequencing from bronchoalveolar lavage fluid (BALF) culture-, and (3) evaluate similarities in lung and oropharyngeal (OP) microbial communities in community-acquired and secondary bacterial pneumonia. Twenty BALF samples from 15 client-owned dogs diagnosed with bacterial pneumonia were enrolled. From a subset of dogs, OP swabs were collected. Extracted DNA underwent PCR of the 16S rRNA gene. Relative abundance of operational taxonomic units (OTUs) were determined. The relative abundance of bacterial community members found in health was decreased in dogs with pneumonia. Taxa identified via culture were not always the dominant phylotype identified with sequencing. Dogs with community-acquired pneumonia were more likely to have overgrowth of a single organism suggesting loss of dominant species associated with health. Dogs with secondary bacterial pneumonia had a greater regional continuity between the upper and lower airways. Collectively, these data suggest that dysbiosis occurs in canine bacterial pneumonia, and culture-independent techniques may provide greater depth of understanding of the changes in bacterial community composition that occur in disease.

**Keywords: microbiota, pneumonia, respiratory, dog, bronchoalveolar lavage, culture, sequencing, dysbiosis**

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## INTRODUCTION

Canine bacterial pneumonia is a common respiratory disorder, occurring as primary disease process, or secondary to aspiration, viral infections (1, 2), immunodeficiency, or a nosocomial event (3). In both humans and companion animals, bacterial pneumonia can be life-threatening making prompt diagnosis and targeted treatment essential. Diagnostic approaches to identify the causative

agents have traditionally relied on *ex vivo* culture from carefully collected airway lavage [e.g., bronchoalveolar lavage fluid (BALF)]. This method is predicated on the belief that the deep airways are largely free of bacteria and any growth on selective media represents aberrant colonization. The recent development of culture-independent molecular techniques has revealed that in humans (4), cats (5), dogs (6), sheep (7), and likely other host species, the healthy lungs harbor low biomass microbial populations seeded via direct extension from upper airway communities, repeated microaspiration, and inhalation of bacteria in air (8). Moreover, these culture-independent methods have reinforced that a lack of cultivable organisms does not necessarily indicate a sterile environment (9). Collectively, such findings suggest that sequencing methods might have clinical utility in the identification of microbes associated with bacterial pneumonia. Toward that end, the current study compared the results of traditional culture-based methods and a targeted sequencing approach applied to 20 BALF samples collected from 15 dogs affected with bacterial pneumonia in a referral veterinary hospital setting.

Canine bacterial pneumonia is categorized as either community-acquired pneumonia (CAP) or secondary bacterial pneumonia (SBP) based on the etiology, clinical presentation, and patient history. As the name implies, CAP is typified by known contagious pathogens, such as *Bordetella bronchiseptica* and *Streptococcus equi subspecies zooepidemicus* and is often seen in dogs with a history of acute onset clinical signs following exposure to reservoirs of infectious agents, such as shelters, boarding facilities, and dog parks (10, 11). Secondary bacterial pneumonia, on the other hand, occurs as a sequela to a predisposing anatomic or physiological condition, such as megaesophagus, laryngeal paralysis, or ciliary dyskinesia (12), and the microbes recovered in a diagnostic sample are often not primary contagious pathogens, *per se*. Rather, dysfunction of the upper respiratory tract or gastrointestinal tract allows or facilitates increased translocation of material to the lower airways and/or prevents effective microbial clearance, leading to the hypothesis that the lower and upper airway microbiota would be more similar in cases of SBP relative to cases of CAP. To address this question, oropharyngeal (OP) swabs were collected from a subset of dogs alongside BALF samples and the compositional similarity of OP and BALF microbiota was evaluated in the context of clinical diagnoses and predisposing anatomic factors.

# MATERIALS AND METHODS

## Experimental Design

The current study was performed prospectively at the University of Missouri Veterinary Health Center (VHC), a referral and primary care veterinary hospital located in Columbia, MO, USA. All dogs contributing samples to the current study presented to the VHC with clinical signs related to bacterial pneumonia between August 2016 and December 2017. Bronchoscopic examination and diagnostic collection of BALF were performed as part of their standard care. Peripheral blood was also collected at presentation for hematologic and serum chemistry analyses. Dogs were then diagnosed with bacterial pneumonia based on clinical signs associated with septic suppurative inflammation or a positive aerobic or anaerobic culture result of BALF, and categorized by type of pneumonia based on the history, clinical signs and other diagnostic findings. Dogs were of various breeds and ages; a table showing the range of patient demographics is provided in **Table 2**.

## Sample Collection

Anesthetic protocols were performed at the discretion of a board certified veterinary anesthesiologist. Samples were collected as previously described (5). Briefly, after induction for anesthesia, while avoiding the rest of the oral cavity, a sterile swab was used to vigorously rub the caudodorsal aspect of the oropharynx, from a subset of patients (see **Table 1**). The swab was added to 800 μL lysis buffer adapted from Yu and Morrison (13). Dogs were initially intubated using sterile endotracheal tubes. Control samples were obtained by running a 10 ml aliquot of sterile saline through the endoscope channel before its use. Immediately prior to the bronchoscopy, the endotracheal tube was replaced with a sterile red rubber catheter to provide oxygen and the endoscope was passed directly through the larynx into the tracheobronchial tree. BALF collection was performed by instilling one or two 20 mL aliquots of sterile saline through the channel of a sterile bronchoscope when wedged in an airway. All dogs provided one BALF sample with the following exceptions: dog I provided one sample on 1/12/2017 (I1) and two samples from the left and right lung lobes in on 11/2016 (I2 and I3, respectively), dog M provided samples on 11/4 and 12/20 of 2016 (M1 and M2), and dog G provided samples on 11/11, 12/1, and 12/21 of 2016 (G1, G2, and G3, respectively). Following collection of BALF, samples were split to provide a minimum of 1 mL of material to the University of Missouri Veterinary Medical Diagnostic Laboratory for culture on Blood agar and MacConkey agar plates for aerobic cultures, and chocolate agar plates for anaerobic cultures. All aerobic samples were incubated at 35°C, and anaerobic cultures were incubated at 35°C with 95% air and 5% $CO_2$ for 24–36 h. The laboratory does not culture *Mycoplasma* spp. in large part due to challenging growth requirements. Bacterial isolates were Gram-stained and identified with conventional biochemical reactions (14), the Automated Sensititre AP-80 or AP 90 for aerobic bacteria or the MALDI-TOF identification system (Matrix Assisted Laser Desorption/Ionization-Time of Flight: Bruker Daltonics, Inc. 40 Manning Road, Manning Park, Billerica, MA 01821). Aerobic susceptibility testing was performed with the Sensititre Micro-Broth (Thermofisher Scientific 12076 Santa Fe Drive, Lenexa, KS 66215) dilution minimal inhibitory concentration system. Up to 30 mL of the remaining BALF material was promptly centrifuged, and the resulting pellet was frozen and maintained at −80°C until DNA extraction was performed.

**Abbreviations:** BALF, bronchoalveolar lavage fluid; CAP, community-acquired pneumonia; OUT, operational taxonomic unit; PCoA, principal coordinate analysis; RA, relative abundance; SBP, secondary bacterial pneumonia.

**TABLE 1 |** Comparison of culture and targeted sequencing results for lower airways (BALF) and relative abundance of predominant OTU in BALF found in upper airways.

| | BALF | | | | | OP |
|---|---|---|---|---|---|---|
| **Dog** | **Culture results** | **Closest 16S rRNA match** | **RA (%)** | **16S rRNA > 10% RA** | **RA (%)** | **RA (%)** |
| A* | *Streptococcus canis* | *Streptococcus canis* | 99.30 | *Streptococcus* spp. | 99.30 | 10.88 |
| B* | *Bordetella bronchiseptica* | Not detected | | *Mycoplasma canis* PG14 | 53.30 | 7.67 |
| | | | | *Mycoplasma* sp. VJC358 | 44.60 | 1.72 |
| C* | *Enterococcus faecalis* | Not detected | | *Mycoplasma* sp. | 99.60 | 7.87 |
| | *Enterococcus hirae* | Not detected | | | | |
| | *Lactobacillus* sp. | *Lactobacillus* sp. | <0.01 | | | |
| | *Corynebacterium* sp. | *Corynebacterium* sp. | <0.01 | | | |
| | *Pseudomonas* sp. | *Pseudomonas* sp. | <0.01 | | | |
| D | *Streptococcus canis* | *Streptococcus* sp. | 96.40 | *Streptococcus* sp. | 96.40 | |
| E | *Bordetella bronchiseptica* | Not detected | | *Ureaplasma* sp. | 25.90 | |
| | | | | *Mycoplasma* sp. | 15.40 | |
| | | | | *Pseudomonas* sp. | 13.30 | |
| | | | | *Achromobacter xylosoxidans* | 11.40 | |
| | *Brevundimonas vesicula* | *Brevundimonas* sp. | 6.80 | *Bradyrhizobium* sp. T92 | 10.50 | |
| F | *Staphylococcus schleiferi* | *Staphylococcus* sp. | 0.20 | *Acinetobacter* sp. | 37.90 | |
| | | | | *Rhizobium* sp. | 20.60 | |
| | | | | *Brevundimonas* sp. | 11.20 | |
| | *Bacillus* sp. | Not detected | | *Bradyrhizobium* sp. T92 | 10.40 | |
| G1* | *Acinetobacter junii* | *Acinetobacter* sp. | 24.30 | *Acinetobacter* sp. | 24.49 | 3.90 |
| | *Acinetobacter johnsonii* | | | | | |
| | *Lactobacillus salivarius* | *Lactobacillus salivarius* | 1.30 | Family *Beijerinckiaceae* | 26.40 | 0.001 |
| | *Klebsiella pneumoniae* | *Klebsiella* sp. Z1 | <0.01 | | | |
| G2* | *Escherichia coli* | *Esherichia-Shigella* | 2.80 | *Bacteroides* sp. | 17.23 | 10.96 |
| | | | | *Pseudomonas putida* | 13.7 | 0.03 |
| G3* | *Streptococcus canis* | *Streptococcus canis* | 14.30 | *Prevotella* sp. (COT 298) | 31.10 | 15.21 |
| | | | | *Streptococcus canis* | 14.30 | 6.68 |
| | *Escherichia coli* | *Escherichia-Shigella* | 10.80 | *Escherichia-Shigella* | 10.80 | 15.33 |
| H | *Escherichia coli* | *Escherichia-Shigella* | 1.70 | *Acinetobacter* sp. | 25.90 | |
| | | | | *Agrobacterium* sp. Emb7 | 13.80 | |
| I1* | *Staphylococcus pseudintermedius* | *Staphylococcus pseudintermedius* E140 | 74.50 | *Staphylococcus pseudintermedius* E140 | 74.50 | 1.52 |
| | *Pseudomonas putida* | *Pseudomonas* sp. | 1.50 | | | |
| I2 (L) | *Achromobacter xylosoxidans* | *Achromobacter xylosoxidans* | 6.20 | *Acinetobacter* sp. | 31.30 | |
| | | | | *Rhizobium* sp. | 18.60 | |
| I3 (R) | *Pseudomonas aeruginosa* | *Pseudomonas aeruginosa* | 92.00 | *Pseudomonas aeruginosa* | 92.00 | |
| | *Achromobacter xylosoxidans* | *Achromobacter xylosoxidans* | 5.30 | | | |
| J | *Bacteroides fragilis* (anaer.) | *Bacteroides* sp. | 8.40 | *Pseudomonas* sp. | 20.30 | |
| | | | | *Brevundimonas* sp. | 12.70 | |
| | | | | *Bradyrhizobium* sp. T92 | 12.50 | |
| | | | | *Acinetobacter* sp. | 10.30 | |
| K | No growth | | | *Pseudomonas putida* | 71.90 | |
| L | *Pseudomonas alcaligenes* | *Pseudomonas* sp. | 8.50 | *Pseudomonas putida* | 30.20 | |
| | | | | *Alloprevotella* sp. | 11.90 | |
| M1* | *Klebsiella pneumoniae* | *Klebsiella* sp. Z1 | 34.80 | *Klebsiella* sp. Z1 | 34.80 | 51.89 |
| | | | | *Acinetobacter* sp. | 13.10 | 0.32 |
| M2* | *Haemophilus parainfluenza* | *Haemophilus* sp. (COT 326) | 6.40 | *Prevotella* sp. (COT 298) | 25.88 | 32.13 |
| | | | | *Bacteroides* sp. | 15.77 | 12.48 |
| N* | *Achromobacter* sp. | *Achromobacter xylosoxidans* | 3.80 | *Bradyrhizobium* sp. T92 | 57.10 | 1.08 |
| | *Ochrobactrum anthropi* | Not detected | | | | |
| | *Chryseobacterium* sp. | *Chryseobacterium* sp. | 2.30 | | | |
| O* | No growth | | | *Pasteurella* sp. | 50.20 | 0.84 |
| | | | | *Pantoea* sp. | 17.00 | 0.02 |

*Asterisks indicate dogs providing paired BALF samples and OP swabs.*

**TABLE 2 |** Patient demographics related to samples included in the current analysis.

| Dog | Breed | Sex | Age | Wt. (kg) | Type of pneumonia |
|---|---|---|---|---|---|
| A* | Siberian husky | MC | 8 years | 24.5 | CAP |
| B* | Great Dane | F | 6 months | 25 | CAP |
| C* | Great Dane | F | 6 months | 32.4 | CAP |
| D | Giant Schnauzer | FS | 1 year | 25 | CAP |
| E | Bulldog | MC | 4 months | 8 | CAP |
| F | Great Dane | MC | 5 years | 71 | CAP |
| G* | Mixed | MC | 1 year | 13.5 | SBP; megaesophagus; AP |
| H* | Mastiff | FS | 10 months | 30 | SBP; upper airway obstruction; AP |
| I* | Mixed | MC | 8 years | 8.8 | SBP; chronic lower airway disease |
| J | Chesapeake Bay retriever | FS | 4 years | 31.7 | SBP; chronic lower airway disease |
| K | Mixed | FS | 1 year | 10.7 | SBP; pyothorax |
| L | Maltese | MC | 6 years | 9.8 | SBP: tracheal FB; AP |
| M* | Welsh corgi | MC | 2 years | 8.6 | SBP; tongue myopathy; AP |
| N* | Mixed | FS | 10 years | 13 | SBP; UES achalasia; AP |
| O* | Border collie | MC | 12 years | 22 | SBP; laryngeal paralysis; AP |

*MC, male castrated; FS, female spayed; CAP, community acquired pneumonia; SBP, secondary bacterial pneumonia; AP, aspiration pneumonia; FB, foreign body; UES, upper esophageal sphincter; Letters designated with *, paired OP and BALF samples.*

## DNA Extraction

To maximize yields, DNA was first extracted using a manual nucleic acid precipitation, followed by resuspension of DNA in buffer and purification using DNeasy kits (Qiagen) according to manufacturer's instructions with minor modifications. Briefly, BALF was first centrifuged at 5,000 × g for 10 min at room temperature, followed by removal of the supernatant and resuspension in 800 μL lysis buffer as adapted from Yu and Morrison (13). Samples were then incubated at 70°C for 20 min with periodic mixing and centrifuged as before. Next, 10 mM ammonium acetate (200 μL) was added to the supernatant and samples were incubated on ice for 5 min, before centrifugation at 5,000 × g for 10 min at room temperature. Up to 750 μL of the supernatant was then mixed with an equal volume of chilled isopropanol and incubated on ice for 30 min. Samples were then centrifuged at 16,000 × g for 15 min at 4°C. Precipitated nucleic acids were then washed with 70% ethanol, resuspended in 150 μL Tris-EDTA (10 mM Tris and 1 mM EDTA), and processed according to the DNeasy kit's manufacturer's instructions, with the following modification. Instead of eluting in the AE buffer provided with the kits, DNA was eluted in the comparable, but EDTA-free, EB buffer (Qiagen). Yields were determined via fluorometry (Qubit 2.0) using Qubit dsDNA BR assays (Life Technologies, Carlsbad, CA). Samples were stored at −20°C until library preparation was performed.

## 16S rRNA Library Preparation and Sequencing

Library construction and sequencing were performed at the University of Missouri DNA Core facility, as previously described (6). Briefly, 16S rRNA amplicons were generated via amplification of the V4 region of the 16S rRNA gene using single-indexed universal primers (U515F/806R) (15, 16) flanked by Illumina standard adapter sequences. Following amplification, products were pooled for sequencing using the Illumina MiSeq platform and V2 chemistry with 2 × 250 bp paired-end reads.

## Informatics

Assembly, annotation, and binning of DNA sequences were performed at the University of Missouri Informatics Research Core facility. Contiguous DNA sequences were assembled using FLASH software (17) and removed if found to be short after trimming for a base quality <31. Qiime v1.9.1 software (18) was used to perform *de novo* and reference-based chimera detection and removal, and remaining contiguous sequences were assigned to operational taxonomic units (OTUs) via *de novo* OTU clustering and a criterion of 97% nucleotide identity. Taxonomy was determined for selected OTUs using BLAST against the SILVA database (19, 20). Principal coordinate analyses (PCoA) were performed using ¼ root-transformed OTU relative abundance data in PAST 3.17 (21). Metrics of richness and α-diversity were determined based on a rarefied dataset subsampled to a uniform read count of 2,289 reads per sample using beta_diversity_through_plots.py, available at http://qiime.org/scripts/beta_diversity_through_plots.html.

## Statistical Analysis

Distribution of read counts in experimental and control samples was first tested for normality using the Shapiro-Wilk method, and differences in read count were then determined using a Mann-Whitney rank sum test due to non-normality, implemented in SigmaPlot 13.0. Differences in β-diversity between BALF and OP swab communities were determined using one-way permutational multivariate analysis of variance (PERMANOVA), implemented in Past 3.18 (21). To evaluate the similarity between OP and BALF bacterial communities in the context of SBP vs. CAP, time-matched OP and BALF samples were collected from a subset of patients (designated with a * in **Table 2**) and the intra-subject similarities between the OP and BALF communities

in patients with SBP and CAP were visualized and tested for significance (between disease type) via principal coordinate analysis (PCoA) and one-way PERMANOVA, respectively. In both analyses (i.e., PCoA and PERMANOVA), comparisons were performed using both unweighted (i.e., Jaccard) and weighted (i.e., Bray-Curtis) metrics. Briefly, the Jaccard similarity is based on the agreement between two samples with regard to the proportion of shared taxa while the Bray-Curtis similarity also accounts for agreement between two samples with regard to the relative abundance of shared taxa. In all cases, significance was established as $p < 0.05$.

## RESULTS

Twenty BALF samples were collected from 15 different dogs that met the enrollment criteria, i.e., a diagnosis of bacterial pneumonia based on clinical signs and associated with BALF septic suppurative inflammation or a positive culture result. Eighteen of those 20 samples had positive bacterial cultures, from which ten of 18 (56%) yielded one bacterial isolate, and eight of 18 (44%) yielded between two and five isolates.

Sequencing of 16S rRNA amplicon libraries generated from BALF and control fluid flushed through the bronchoscopes resulted in significantly different ($p = 0.004$) mean (± SEM) read counts of 31,524 (± 7,663) and 4,728 (±1,576) reads per sample, respectively. Additionally, seven BALF samples returning read counts within two standard deviations of the mean read counts generated from control samples showed generally good overall agreement with culture results indicating that the data were still meaningful. In contrast to the number of taxa identified based by culture, the DNA detected in patient BALF samples represented between 22 and 185 distinct operational taxonomic units (OTUs). Thus, while traditional culture methods provide evidence of live and cultivable bacteria in a sample and allow antimicrobial susceptibility testing, 16S rRNA sequencing provides a more comprehensive profile of taxa present in a sample, whether or not they are viable or cultivable. Additionally, mean ± SD numbers of unique OTUs in BALF from dogs with CAP were significantly lower than those with SBP (26 ± 16 and 82 ± 30 OTUs, respectively; $p = 0.0002$).

In many cases, there was remarkable agreement between the two methods, particularly in instances of a marked overgrowth of a dominant taxon. Specifically, samples from dogs A and D were both culture positive for *Streptococcus canis* alone, and 16S rRNA sequencing detected *S. canis* or *Streptococcus* sp. at 99.3 and 96.4% relative abundance (RA), respectively (**Table 1**). Similarly, samples I1, I3, and M1 were culture positive for *Staphylococcus pseudintermedius* and *Pseudomonas putida* (I1), *Pseudomonas aeruginosa* and *Achromobacter xylosoxidans* (I3), and *Klebsiella pneumoniae* (M1), with 16S sequencing detecting *S. pseudintermedius* (74.5% RA) and *P. putida* (1.5% RA), *P. aeruginosa* (92.0% RA) and *A. xylosoxidans* (5.3% RA), and *Klebsiella* sp. Z1 (34.8% RA), respectively in the same samples.

In the majority of the remaining samples, taxa detected via culture were also detected via sequencing but not as the dominant phylotype. In samples I2, M2, G1, G2, G3, H, J, L, F, and N, the dominant taxon determined by sequencing was not cultured despite many of the detected species being readily cultivable. In the aforementioned group of samples, cultured bacteria represented from <0.01 to 24.3% of the DNA detected via 16S rRNA sequencing (median 2.55% RA) or, in four of the eighteen culture-positive cases (22.2%), were not detected at all in the sequencing data (i.e., *Bordetella bronchiseptica* in samples F and H, *Enterococcus* spp. in sample G, and *Ochrobacterium anthropi* in sample N).

In cases of SBP, we hypothesized that the same factors predisposing the dog to pneumonia would facilitate greater translocation of upper airway microbes to the lung, relative to what occurs in CAP. PCoA analysis revealed complete separation of BALF and OP samples in dogs with CAP, and substantial overlap between BALF and OP samples in dogs with SBP (**Figure 1**). Accordingly, PERMANOVA detected a significant difference between samples sites when based on the Jaccard similarity ($p = 0.0003$; $F = 3.21$), but not Bray-Curtis ($p = 0.15$; $F = 1.32$), indicating that the BALF and OP communities differ based on the presence or absence of certain taxa, but not with regard to the relative abundance of shared taxa. Factoring in individual variability, the intra-subject similarity between BALF and OP communities was low in the three cases of CAP from whom OP samples were collected (i.e., dogs A–C), regardless of the similarity index used (**Figure 2**). In contrast, samples from severely dysphagic dogs with confirmed aspiration pneumonia (e.g., dogs G and M) evinced a high degree of compositional similarity between BALF and OP microbiota (**Figure 3**). It is worth noting that, despite the apparent dissimilarity between BALF and OP swabs in samples from dogs diagnosed with CAP, the dominant taxa detected in the lower airways were consistently detected in the matched upper airway samples, albeit at a much lower relative abundance. Relative abundance (RA) of the dominant taxon is another metric used to describe resident bacterial communities in CAP and SBP. In 4/6 dogs with CAP, and in 1/9 dogs with SBP, there was near eradication of the microbial diversity in the lower airways, with predominant OTUs found in RAs between 92.4 and 99.9%.

## DISCUSSION

Methods complementary to traditional laboratory-based culture of BALF in dogs with bacterial pneumonia, such as 16S rRNA sequencing, are useful to understand the complex relationship between pathogen and resident microbes. In this study, cultures of BALF, used by clinicians to provide insight into underlying pathogens, identified between zero and five bacterial species in dogs with bacterial pneumonia. This was in stark contrast to DNA sequencing that revealed rich microbial communities ranging from 22 to 185 distinct OTUs. Dysbiosis was appreciated by increases in the relative abundance of specific taxa, and interlinked with this change, a decrease in the predominating taxa found in healthy dog lungs (6). In the majority of BALF samples, the cultured bacteria were present in the sequencing data, despite variation between dogs as to whether the dominant taxa determined by culture was identical to

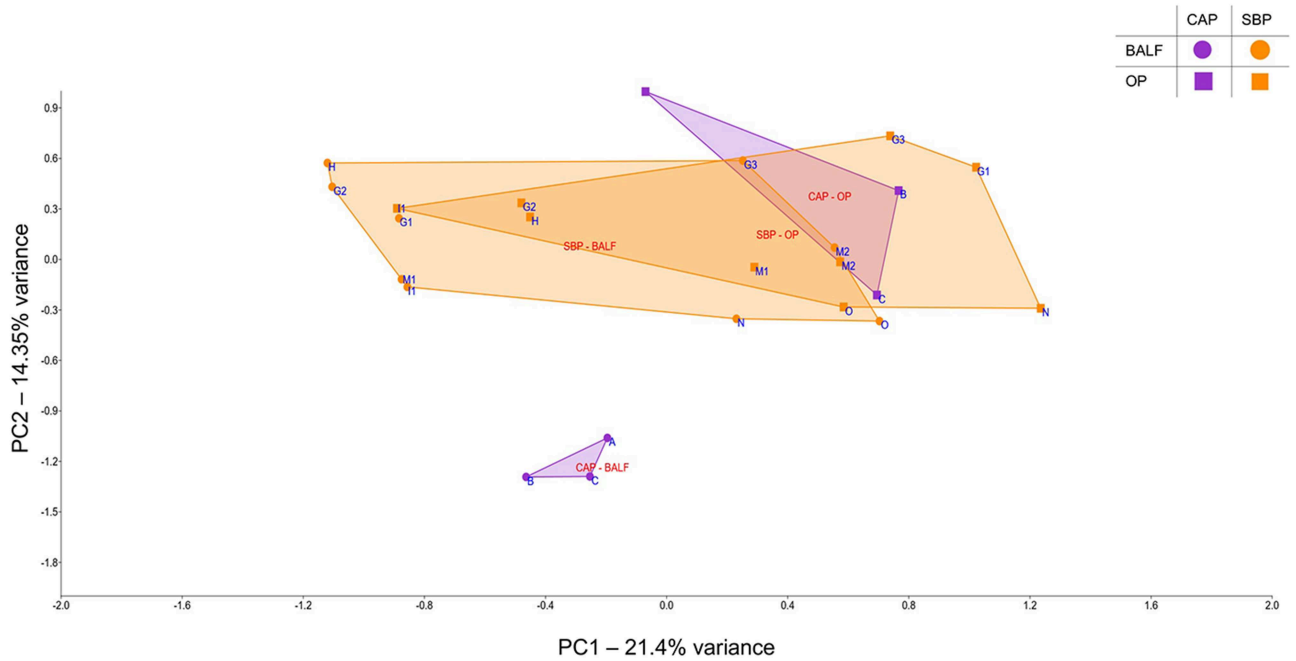


**FIGURE 1 |** Principal component analysis of samples from bronchoalveolar lavage fluid (BALF) and oropharyngeal swabs (OP), for a select number of cases of community-acquired pneumonia (CAP) and secondary bacterial pneumonia (SBP); circles represent BALF, squares represent OP, SBP samples are in orange and CAP samples are in purple.

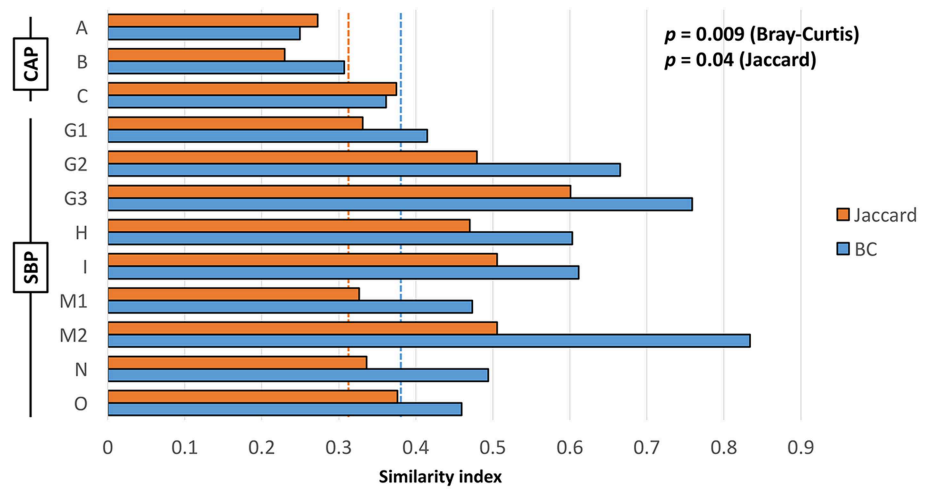


**FIGURE 2 |** Intra-subject Jaccard (orange) and Bray-Curtis (blue) similarity between BALF and OP microbiota in cases of community-acquired pneumonia (CAP) and secondary bacterial pneumonia (SBP); dotted lines indicate mean inter-subject similarity between all samples. Results of Student's *t*-test shown on chart.

the dominant taxa based on sequencing. This phenomenon has also been described in mechanically ventilated patients in which 75% of organisms identified via culture were the most abundant organisms identified via sequencing (22). An advantage of molecular techniques is their enhanced ability to provide insight as to how microbial communities change in disease and allow comparisons in regional differences in populations (i.e., upper vs. lower airways). Bacterial populations present in the lungs during pneumonia depend on the clinical scenario with differences between dogs with CAP and SBP. In the former, there was generally a stark loss of microbial diversity and replacement with a predominant taxon. In the latter, with secondary risk factors for pneumonia, such as laryngeal or esophageal dysfunction, lower airway communities are likely heavily derived from those present in the upper airways.

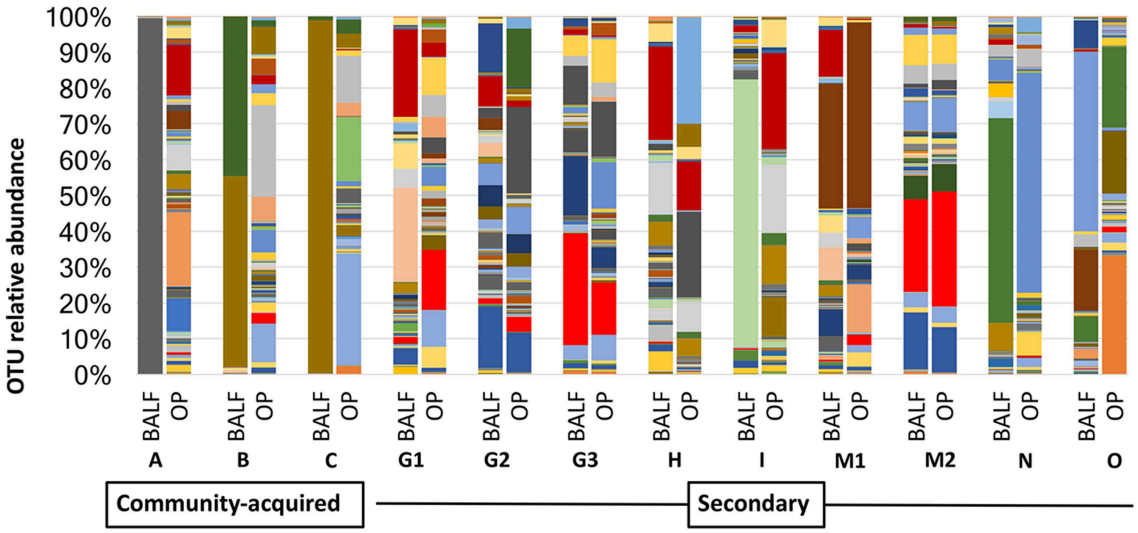


**FIGURE 3 |** Comparison of relative abundance in BALF and OP between community acquired and secondary bacterial pneumonia.

In a previous analysis of the lung microbiota of 16 healthy dogs at the same institution (6), the most abundant taxa were *Acinetobacter johnsonii, Brevundimonas diminuta* and members for the family *Pseudomonadaceae* [mean (range) % RA 19.81 (13.89–26.72), 22.48 (17.36–26.67), and 29.6 (18.57–34.94), respectively]. The remainder of the taxa identified in healthy dogs were present at a RA of 2.73% or less. This is in stark contrast to the findings in this study, where *Acinetobacter*, *Brevundimonas*, and members of the family *Pseudomonadaceae* were present between 10 and 50% RA in 9/20 samples, suggesting respiratory dysbiosis is a common component of canine bacterial pneumonia.

In dogs with bacterial pneumonia, cultures of BALF would seemingly support a single dominant or very small number (<5) of lung pathogens; however, results of DNA sequencing underscore the complexity of microbial communities in the lung, even in the presence of infection. Mean numbers of distinct OTUs identified in BALF of dogs with CAP (23) and SBP (82) were similar to a prior study of the healthy canine lower respiratory tract microbiome (6). Thus, richness (overall numbers of distinct OTUs), while variable in pneumonia, was discordant with the paucity of cultivable bacteria. Similarly, in a study evaluating respiratory microbial communities of mechanically ventilated patients with pneumonia, 12 out of 56 patients (21%) had positive cultures. These patients had a significant decrease in microbial richness compared to culture negative subjects there was a wide range in terms of richness (22). In patients that developed pneumonia after receiving lung transplantation also demonstrated a decrease in microbial richness (24). Most of the microbes found via sequencing are not cultivable using standard techniques, making it challenging to discern the larger picture of microbial interactions within the lung. It is unclear how many uncultured or as yet unidentified lung pathogens are responsible for bacterial pneumonia, but study of the respiratory microbiome will be critical moving forward.

The fact that, in 4 of 6 dogs with CAP, the predominant OTUs represented between 92.4 and 99.9% of recovered DNA suggests overgrowth of these organisms and reflect dramatic changes from that reported in healthy dogs (6). It has been proposed that disease is associated not just with pathogens gained, but with resident species that are lost and that a greater risk of repopulation with virulent organisms may be more likely with disappearance of dominant species associated with health (25). In dogs diagnosed with CAP, microbial diversity was abolished relative to dogs with SBP.

With regard to those bacteria identified via standard culture but not found in the sequencing data, *Bordetella bronchiseptica* is a common and important canine respiratory pathogen causing CAP that is also as a commensal organism in healthy, asymptomatic dogs (23, 26). It is unclear as to why *B. bronchiseptica* or closely related taxa were not detected in the 16S rRNA dataset. While the SILVA database used to annotate the current data does contain multiple sequences specific to other *Bordetella* spp., it is worth noting that the 16S rRNA sequence of *Achromobacter* and *Bordetella* spp. share a high degree of homology, and *A. xylosoxidans* was sequenced in both samples. It is also possible that the *Enterococcus* and *Bacillus* species identified via culture were a result of contamination, as these organisms are considered to be ubiquitous. There are clinical implications to identification of organisms on culture that are not present in the sample according to targeted sequencing. In large part, results of standard culture techniques are relied upon for information to guide treatment. This could potentially lead to incorrect antibiotic selection.

Conversely, there are also limitations to the utility of molecular approaches, the most apparent of which is the inability to distinguish viable and dead bacteria using standard 16S rRNA sequencing methods. The fact that many of the bacterial species found in the sequencing data were also cultured indicates that at least some of the bacteria present in the lungs are viable, and even

if DNA detected in BALF represents bacteria that have been killed by the immune defenses of the lung (e.g., pulmonary alveolar macrophages), it does not obviate an influence of those bacteria on airway health. Without a viable isolate, it is also not possible to perform antimicrobial susceptibility testing on taxa identified via sequencing. Moreover, whether applying traditional culture-based or culture-independent molecular techniques, there is an inherent risk of contamination and identification of false positives. However, while a single CFU can theoretically be detected on culture, the competitive nature of 16S rRNA sequencing (i.e., an overabundance of DNA amidst a limited number of binding substrates on the sequencer flow cell) renders single CFUs as background noise and inadvertent contaminants will not be detected as dominant taxa in the data. Lastly, clinicians are limited by our still nascent appreciation of these microbial communities. Until there is a better understanding of the lung microbiota in the context of both health and disease, it will be challenging to incorporate sequencing data into clinical decisions.

The development of targeted sequencing techniques highlights limitations associated with standard culture. The belief that the lower airways are sterile is still perpetuated in textbooks; however, the notion of lung sterility has been refuted by a number of studies following the first culture-independent report of the healthy human lung microbiome (27). Healthy dogs and other species have diverse and dynamically changing microbial communities inhabiting specific ecologic niches in the lower airways in the absence of clinical evidence of infection (6, 28–31). The composition of the lung microbiome depends on microbial immigration into the airways, microbial elimination and the relative reproduction rates of host microbial communities determined by regional growth conditions (32). Immigration is influenced by local mucosal extension from the upper airways that lack a physical barrier separating them from the lower airways, microaspiration, and inhalation of bacteria from ambient air (8, 33). Protective reflexes, such as cough, mucociliary function, and innate and adaptive mucosal immune responses affect elimination (8). Although bacterial pneumonia has been regarded as resulting from invasion and growth of a pathogen in the lungs, recent work suggests a primary driver of disease is disruption of homeostasis of the complex microbial ecosystem (34). The upper respiratory tract has been called the gatekeeper to respiratory health, wherein "colonization resistance" is provided by local bacterial communities preventing establishment of mucosal infections capable of spreading to the lower respiratory tract. Thus, while the airway microbiome has the capacity to blunt growth of pathogenic species during states of equilibrium, dysbiosis of upper airways in humans has been linked to CAP (35), SBP (36), and ventilator associated pneumonia (22). In the subset of dogs with paired OP and BALF samples, 3 dogs had CAP, and 8 had SBP. The dogs with CAP were more likely to have a single OTU predominate. In contrast, dogs with SBP were more likely to have $\geq$2 OTUs. It is interesting to note that in several cases (G3, M1, M2), one of the OTUs found in BALF at a relative abundance of $>$10% was also present in the OP at an even greater relative abundance. Taking into consideration an underlying process disrupting homeostasis in SBP, it was not surprising that dogs with SBP had more similar microbial compositions of the upper and lower airways (i.e., more evident regional continuity) and greater diversity than dogs with CAP.

While traditional culture methods provide evidence of live and cultivable bacteria in a sample and allow antimicrobial susceptibility testing, 16S rRNA sequencing provides a more comprehensive profile of taxa present in a sample, whether or not they are viable or cultivable. Additionally, these data highlight that just as lack of growth does not imply a sterile environment, identification of a particular organism with either approach does not imply that this organism is the causative agent of disease (37). Lack of growth could also be related to the presence of fastidious organisms, such as *Mycobacterium* that require specific media or growth conditions using standard methods as it has been documented in people with pneumonia (38) Bacterial culture with sensitivity testing guides treatment of clinical infections and thus will still play a key role in therapy of bacterial pneumonia. However, culture-independent techniques may provide greater depth of understanding of the changes in microbial composition that occur in bacterial pneumonia. These methods could allow for identification of pathogens that may not be readily cultivable, help discriminate true pathogens from colonizing bacteria (37) and provide insight into potential treatment strategies that restore balance toward a microbial population associated with health.

## CONCLUSIONS

In comparing standard culture and targeted sequencing techniques to identify organisms found in BALF of dogs with bacterial pneumonia, we demonstrated discrepancies between these techniques in terms of presence or absence of predominating taxa and numbers of unique bacteria. Dysbiosis of the respiratory microbiome is a key feature of canine pneumonia, with decreased relative abundance of bacterial community members found in health. Additionally, there appears to be greater regional continuity between the upper and lower airways in dogs with SBP. While much more commonly observed in dogs with CAP than SBP, obliteration of microbial diversity with evidence of overgrowth of one organism was noted in one-third of the dogs in this study. This may suggest that loss of dominant species associated with health could underlie disease pathology. Clinical application of DNA sequencing may be employed if culture is negative in dogs with compatible clinical signs and septic suppurative BALF cytology, or if targeted antimicrobial therapy against the cultivable bacteria fails to produce disease resolution. Future studies aimed at restoring a dysbiotic airway microbiome in canine bacterial pneumonia are warranted.

## DATA AVAILABILITY STATEMENT

All sequence data have been deposited in the NCBIA Sequence Read Archive (SRA) under the BioProject accession number: PRJNA510415.

## ETHICS STATEMENT

All studies were performed in accordance with the Guide for the Use and Care of Laboratory Animals, and were approved by the University of Missouri Institutional Animal Care and Use Committee (MU IACUC protocol # 8240).

## AUTHOR CONTRIBUTIONS

AV-P participated in the conception and design of the study, sample collection, DNA extraction, data interpretation, and co-authored the manuscript. AE participated in the conception and design of the study, contributed resources, interpreted sequence data, and co-authored the manuscript. CR participated in conception and design of the study, sample collection, data interpretation, and co-authored the manuscript. HR assisted with DNA extraction, sample collection, and study coordination. All authors read and approved the final manuscript.

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