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Importance of Farm Environment to Shape Poultry-Related Microbiomes Throughout the Farm-to-Fork Continuum of Pasture-Raised Broiler Flocks

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The poultry farm environment plays a key role in the microbial colonization of chickens during production, which shapes what enters the processing and final retail environments. The aim of this study was to evaluate the effect of the farm environment on the microbial composition of pasture-raised broilers using a combined cultural and microbiomic farm-to-fork. To achieve this, two nearby pastured poultry farms raised small flocks of Freedom Ranger broilers obtained from the same hatchery flock and fed the same diet throughout live production. The major differences between the two farms were the physical farm environment, the method of feather removal during processing (scalding vs. skinning), and the storage conditions of carcasses before customers received final product (refrigeration vs. freezing). Microbiomes were compared from fecal and soil samples (live production), ceca (processing), and whole carcass rinses (processing, final product) to determine what effect the physical farm environment had on the poultry-related microbiomes. Overall, microbiomes in feces ($p < 0.04$), soil ($p < 0.02$), and ceca ($p < 0.02$) samples from farm 1 harbored a higher taxonomic richness than farm 2. Beta-diversity analysis demonstrated significant differences between the broiler microbiomes of the two farms for samples collected at the live production ($p < 0.04$) and processing stages ($p < 0.01$), but not for final product carcass rinses. At the early live production stage (~3 weeks old), fecal microbiomes from farm 1 were positively correlated to aluminum, iron, manganese, silicon and zinc concentrations in feces but not fecal microbiota from farm 2. At the late live production stage (~12 weeks old), fecal microbiomes from both farms were no longer correlated to mineral content of feces but were negatively correlated to fecal pH. Given that the farm environment itself was the major difference, the results show that even when raising the same breed fed the same diet, poultry farms have their own ecology that shape the composition of the poultry-related microbiomes. Therefore, it is vital that future work focuses on elucidating the farm environmental variables that have the greatest influence on these microbiomes, thus allowing for eventual targeted interventions to better manage these microbial populations to benefit animal, environmental, and public health.

Keywords: pastured poultry, farm-to-fork, microbiome, ecology, *Campylobacter*

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INTRODUCTION

Microbial communities associated with live poultry are of major importance because they directly impact animal health, food safety and public health (Dupont, 2007; EFSA European Centre for Disease Prevention Control, 2015). Lately, the ecology of the gastrointestinal tract (GIT) microbiome has been specifically studied due to its key role in poultry production performance (Stanley et al., 2014; Apajalahti and Vienola, 2016). The composition of gut microbiota is known to affect many host functions relative to the growth and development of chicken, including nutrient utilization and gut epithelium nourishment (Pan and Yu, 2014) and also to influence the gut immune system and consequently resistance against microbial infections (Schokker et al., 2017).

The GIT of poultry hosts a complex and dynamic bacterial microbiota (Zhu et al., 2002). Considerable variation in poultry GIT microbial community composition has been observed both within and across studies (Stanley et al., 2013; Waite and Taylor, 2014); consequently, it's difficult to define the normal gut microbiota composition and to compare results between the different studies. While a part of this microbial variation may be attributed to technical factors such as sampling procedures, DNA extraction, the choice of PCR primers and corresponding genomic region to be sequenced and the sequencing platforms used (Laukens et al., 2015), the variation in microbial composition may also be explained by different host characteristics and environmental factors (Kers et al., 2018). Poultry-intrinsic host factors identified to influence the composition of intestinal microbiota include birds age (Ballou et al., 2016; Pedroso et al., 2016), sex (Torok et al., 2013; Zhao et al., 2013), type/breed (Videnska et al., 2014; Kim et al., 2015), and GIT regions (Yeoman et al., 2012). Of the external factors that influence the microbiota composition, the diet composition (Pan and Yu, 2014; Walugembe et al., 2015) and the use of feed additives (Videnska et al., 2013; Costa et al., 2017) have been well studied. Environmental factors such as the hygiene levels within the hatcheries (Stanley et al., 2013), the type of housing (Ludvigsen et al., 2016) and production system (Bjerrum et al., 2006; Xu et al., 2016), the litter quality and management (Torok et al., 2009; Dumas et al., 2011) as well as the climate and geographical locations (Videnska et al., 2014; Zhou et al., 2016) are also known to have an effect on the intestinal microbiota.

In the poultry production chain, newly-hatched chicks already have a GIT microbiota at the pre-hatched phase acquired directly from the mother or from the environment surrounding the eggs (Roto et al., 2016). During the first week of chick's life, the diversity of GIT microbiota increases gradually to reach the hundreds to thousands of distinct GIT taxa found in market age adults (Crhanova et al., 2011; Danzeisen et al., 2011; Ballou et al., 2016). In young birds, a high variation is observed in individual microbiota composition compared to older birds from the same flock (Crhanova et al., 2011; Ballou et al., 2016). This observation suggests that chicks might initially be randomly colonized by bacterial species present in their close environment during their early life (from 0 to 20 days), leading to a great individual diversity in microbiota composition which tend to converge in older individuals from the same flocks.

The chicken GIT microbiome commonly contains several taxa capable of causing significant illnesses in humans, most importantly *Campylobacter* and *Salmonella*. Pathogen colonization of live poultry can occur at all stages of the production. Newly-hatched chicks are more susceptible to pathogen colonization because they lack mature gut microbiota or feed in the alimentary tract (Nisbet et al., 1993). This colonization occurs by horizontal transfers between the farm environment and chickens (Patriarchi et al., 2011; Hermans et al., 2012) and also among chickens in a flock (Shanker et al., 1990; Byrd et al., 1998).

The surrounding environment of young chickens at the hatchery, during the transport to the farm or at the farm, has a decisive impact on the composition of microbial communities, including pathogenic bacteria, that colonize their intestinal tracts. Data describing the impact of environmental factors on poultry microbiome are scarce and no study to date has investigated the effect of the farm environment as a whole on the microbiota composition of broilers. Therefore, we investigated how the global effect of the farm environment, including management practices, influenced the microbiota of pasture-raised broilers along the production chain. Two all pasture-raised, antibiotic free farms located in the north-central Georgia raised the same breed of broiler with the same diet and a variety of samples were recovered along the entire farm-to-fork continuum. Illumina MiSeq-based 16S rRNA-based sequencing was used to compare the bacterial microbiomes between these two farms from soil and feces samples collected at the live production stage; ceca and carcass rinses samples collected during the processing step; and carcass rinse obtained from final product, to better elucidate the potential environmental and management drivers of the microbial ecology of pastured broilers.

MATERIALS AND METHODS

Flock Management

This study was conducted in two pasture-raised broiler farms located in the north-central Georgia, United States from March to June 2014. One day old Freedom Ranger type chicks were purchased from the same hatchery flock (Freedom Ranger Hatchery, Reinholds, PA, USA) and were randomly split in two groups of 50 individuals between farm 1 and farm 2. While many similar management practices were used by both farms, the major differences are listed in **Table 1**. During the brooding period (0–∼3.5 weeks of age), chicks at both farms were reared under heat lamps with bedding material composed of wood shavings (with fresh shaving added weekly on top of the existing bedding) and chicks were fed with the DuMOR Chick Starter/Grower 20% (D20). At ∼3.5 weeks of age, young broilers were transferred to pasture and were fed with the DuMor 16% Crumbles (D16) under modified Salatin-type chicken tractor housing systems (2 houses per farm with ∼25 birds per house). During live production, broilers were provided feed and water *ad libitum*, and the chicken tractors were moved onto a fresh piece of pasture every day. As

TABLE 1 | Major management differences between the two pastured broiler flocks.

		Farm 1	Farm 2
Live production	Layers on farm	Yes	No
	Cattle on farm	No	No
	Swine on farm	Yes	No
	Goats on farm	Yes	No
	Organic Vegetable Production	No	Yes
	Age to pasture (weeks)	4	3
Processing	Defeathering Method	Skin Removal	Scalding
	Scalder temperature (°C)	–	82
	Chilling method	Air	Water
	Water source	–	Public
	Water chlorinated?	–	Yes
Final product storage	Storage time (days)	1	13
	Storage temperature (°C)	4	−20

broilers increased in size, plastic temporary fencing was placed around the tractors to allow for increased grazing area, and this fencing was moved daily with the houses. At ∼13 weeks of age, broilers were processed on-farm for both farms, but farm 1 de-feathered the carcasses by removing the skin (no scalding step used) and air chilled the carcasses, while farm 2 used a traditional scalding step followed by de-feathering using an automated picker (leaving the skin on the carcass) and water chilled the carcasses. Final products as well have a different storage period and temperature (see **Table 1**).

Sample Collection and Preparation

Soil and feces samples were collected from the pasture where the flock was currently residing at the time of sampling. Samplings occurred three times during grow-out: (i) within a few days of being placed in the pasture (**T1**), (ii) halfway through their time on pasture (**T2**), and (iii) on the day the flock was processed (**T3**). At each sampling time, the pasture area was divided into five separate sections, and five subsamples in each section were pooled into a single sample for each section (a total of five soil samples and five feces samples were collected on each sampling day). Soil samples were collected from the surface (0–7 cm) with sterile scoops, and feces samples were collected from fresh droppings on the soil surface. Gloves and scoops were changed between sample types and between sampling areas.

During the on-farm processing day, ceca and carcass rinse samples were collected. Upon evisceration, cecal sacs from five carcasses were removed and placed into a single sampling bag to create a pooled sample. A total of five pooled samples ($n = 5$) was created. Gloves and scissors were changed between each pooled sample. Prior to packaging and storage of the carcasses for the consumer, each of the 25 carcasses were placed in sterile plastic bags, rinsed with 100 ml of 10 mM phosphate-buffered saline (PBS) and vigorously shaken for 1 min. Whole carcass rinses (WCR) from five carcasses were pooled together in a filtered stomacher bag creating five pooled samples ($n = 25$). Carcasses were returned to the farmer to be packed and stored according to the usual procedure applied at the farm. Upon receiving the final product, the procedure described above was repeated to obtain the final product WCR samples.

All fecal, soil, cecal, and WCR samples were transported back to the laboratory on ice and processed within 2 h of collection. To prepare the environmental samples for homogenization, 3 g (feces, soil) or 5 ceca were combined within filtered stomacher bags (Seward Laboratory Systems, Inc., Davie, FL), and diluted 1:3 using 10 mM phosphate-buffered saline (PBS). For the WCR, 100-ml of 10 mM PBS were added to each carcass within the storage bag, and the bags were vigorously shaken for 60 s. Five WCR were pooled into a single filtered stomaching bag, and this was repeated a total of 5 times ($N = 25$ carcass rinses). No further dilution in 10 mM PBS was required for the WCR samples. All samples were homogenized for 60 s and these homogenates were used for all downstream cultural isolations, and the cecal homogenates and WCR rinsates were used for DNA extraction.

Cultural Isolation Methods

Salmonella spp.

As a pre-enrichment step, the stomached homogenates remained in the filtered stomacher bags and incubated overnight at 35°C. Two different enrichments broths were used to isolate *Salmonella* spp. from these environmental samples: Tetrathionate (TT; Becton-Dickinson, Sparks, MD) broth and Rappaport-Vassiliadis (RV; Becton Dickinson) media. After overnight incubation at 42°C in both of these enrichment broths, 1 loopful from each enrichment broth was spread on two different differential media: Brilliant Green Sulfa with novobiocin (BGS; Becton Dickinson) agar and xylose lysine tergitol-4 (XLT-4; Becton Dickinson) agar. These plates were incubated overnight at 35°C, and on each plate, 3 *Salmonella*–like colonies per subsample were picked and confirmed using triple sugar iron agar (TSI; Becton-Dickinson) and lysine iron agar fermentation (LIA; Becton-Dickinson) using an incubation period of 18–24 h at 35°C. Final confirmation of suspect TSI/LIA isolates was performed using *Salmonella* polyvalent O antiserum agglutination (Becton-Dickinson), using manufacturer's specifications. Positive salmonellae were serogrouped using individual *Salmonella* poly O antisera for O groups A through I, following the Kauffman-White scheme (Popoff and Le Minor, 1997).

Campylobacter spp.

Campylobacter spp. were isolated using a selective (Cefex) and non-selective (Campycheck) method. For the selective media method, the recovery of *Campylobacter* spp. from homogenized samples was performed as previously described (Stern et al., 1992). Initially, 100 μL of homogenized suspension was removed, plated onto Campy-Cefex agar, and subsequently incubated at 42 ± 1°C for 36–48 h in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2). Putative *Campylobacter* spp. colonies were enumerated, and up to 5 colonies per sample were sub-cultured on Brucella agar supplemented with 10% laked horse blood (BAB plates) for isolation and incubated as previously described. For the Campycheck method (Lastovica and Le Roux, 2001), homogenized samples were applied to non-selective plates using a filtration technique and grown in a hydrogen-enriched atmosphere. Briefly, a 50 mm, 0.6 μm mixed

cellulose ester filter (Whatman, Schleicher & Schuell; Dassel, Germany) was aseptically placed in the center of a Brucella agar plate supplemented with *Campylobacter* growth supplement SR84 (Oxoid/Remel, Lenexa, KS) and 10% laked horse blood (Oxoid/Remel). Four 50 μL aliquots of homogenized fecal sample were applied at distinct locations on the filter and allowed to sit at room temperature for 15 min. The filter was aseptically removed, using sterile tweezers, and the plate placed in a ZipTop bag that was subsequently filled with an atmosphere of 7.5% H_2, 2.5% O_2, 10% CO_2, and 80% N_2 and incubated at 37°C for 48 h. Several putative *Campylobacter* spp. colonies were sub-cultured on Brucella agar supplemented with 10% laked horse blood (BAB plates) and subsequently stored at −80°C in 16% glycerol stocks for further identification and analyses.

Listeria spp.

As a pre-enrichment step, the stomached homogenates remained in the filtered stomacher bags and was incubated overnight at 35°C. This pre-enrichment was followed by two subsequent enrichments in UVM Modified Listeria Enrichment Broth (UVM, Becton-Dickinson) and Fraser Broth (Becton-Dickinson), both requiring an overnight incubation period at 30°C. One loopful of the Fraser's enrichment was streaked for isolation of Listeria-selective agar (Becton-Dickinson). These plates were incubated overnight at 30°C, and on each plate, 3 *Listeria*–like colonies per positive subsample were picked and confirmed as *Listeria* using the appropriate BAX PCR assay (DuPont, Wilmington, DE). *Listeria* species and *L. monocytogenes* serovars of *Listeria*-like colonies were determined using multiplex-PCR as described previously (Locatelli et al., 2017b).

Escherichia coli

Recovery of *E. coli* was performed by spreading 1 mL of the homogenates onto Petrifilm *E.coli*/Coliform Count Plates (3M, St. Paul, MN) and incubated overnight at 37°C. Blue colonies with associated gas production indicative of *E. coli* and all blue and red colonies with entrapped gas were counted as coliforms, and up to 5 *E.coli* colonies per sample were isolated and used for further characterization.

All quantifiable cultural data (*E. coli,* and *Campylobacter* isolated on CEFEX) were normalized via log10-transformation prior to any statistical comparisons. A one-way ANOVA followed by Tukey's *post hoc* test was carried out to compare the *E. coli* and *Campylobacter* counts (mean log10 CFU/ml) between the 2 farms for each sample type.

Fecal and Soil Physiochemical Analysis

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

DNA Extraction and Quantification

DNA extractions were performed on 0.33 g of feces, 0.33 g of soil, and 0.5 ml of cecal homogenate and 0.5 ml of WCR. DNA was extracted from samples according to a semi-automated hybrid DNA extraction protocol previously described (Rothrock et al., 2014). This method was a combination of a mechanical method using the FastDNA Spin Kit for Feces (MP Biomedicals, Solon, OH, USA) and an enzymatic method based on the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA). DNA purification was performed using the DNA Stool—Human Stool—Pathogen Detection Protocol of the QIAcube Robotic Workstation. After purification, 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).

Illumina MiSeq Library Construction and Analyses

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 rRNA gene was amplified using the F515 (5′-CACGGTCGKCGGCGCCATT-3′) and R806 (5′-GGACTACHVGGGTWTCT AAT-3′) primer set with each primer containing Illumina adapter regions (Illumina, Inc., San Diego, CA) and the reverse primer containing the Golay barcodes to facilitate multiplexing (Caporaso et al., 2011). Raw reads were obtained by using the Illumina MiSeq platform. A total of 3,297,242 raw sequence reads were generated and processed by the QIIME v1.9.1 (Quantitative Insights Into Microbial Ecology) pipeline (Caporaso et al., 2010b). Quality filtering and library splitting according to the Golay barcode sequences were performed on the R1 read (*split_library_fastq.py* script, default parameters). Sequences were chimera checked against the gold.fa database (http://drive5.com/uchime/gold.fa) and clustered into Operational Taxonomic Units (OTUs) according to their sequence similarity (97%) using the usearch option (Edgar, 2010) 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 (using the most_abundant method for picking, all other parameters were default) and used for taxonomic assignment using UCLUST and the Greengenes 13_8 database (Desantis et al., 2006) with *assign_taxonomy.py* (default parameters). Sequences were aligned (*align_seqs.py script*, default parameters) using PyNAST (Caporaso et al., 2010a) 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). Among the 96 samples analyzed, 5 were removed because they were composed of <100 reads. Finally, a total of 2,557,191 sequences (average of 28,101 sequences/sample) were obtained for further analysis. Overall, among all samples, a total of 1,939 unique OTUs were identified. For all subsequent analyses, sequences were analyzed according

to the sample type (feces, soil, ceca, processing carcass rinse and final product carcass rinse).

Alpha diversity was used to describe the microbial richness, evenness and diversity within samples using the Chao1, Equitability and Shannon metrics, respectively. For each sample type, significant differences in alpha diversity parameters were tested between the 2 farms using the *compare alpha diversity.py* script. To determine β-diversity, the Bray-Curtis distance was used to measure the dissimilarity based on the rarefied OTU table. Data visualization and statistical tests were performed in R (v3.4.3) using the vegan package v2.4-5. Whole bacterial community composition was examined using nonmetric multidimensional scaling (NMDS) of Bray-Curtis dissimilarities with the *metaMDS* function. The function *envfit* was used to calculate the regression statistic for fecal and soil physiochemical variables on ordination scores at a p-value $\leq$ 0.01. Analysis of similarities (ANOSIM) was used to examine the significant differences in community structures between the 3 sampling times for feces and soil samples and between the 2 farms for each sample type. A nonparametric Kruskal-Wallis test with the Dunn's *post hoc* test was carried out to compare the mean relative abundance of each taxa (phylum and genus) between the 2 farms at each sampling time and for each sample type.

RESULTS AND DISCUSSION

Poultry Microbiome at the Live Production Stage

The effect of the farm environment on the pastured poultry microbiome was first evaluated at the live production stage by investigating the bacterial community structure in fecal and pasture soil samples collected at the beginning of their time on pasture (∼4 weeks old; T1), at ∼8 weeks of age (T2) and on the day of processing (∼12 weeks old; T3). Overall, feces samples in farms 1 and 2 were dominated by *Firmicutes* (70.5 vs. 69.7%), *Proteobacteria* (15.1 vs. 25.8%), *Actinobacteria* (6.7 vs. 2.4%), and *Bacteroidetes* (5.2 vs. 1.9%), which are all phyla that are known to dominate chicken fecal microbiota (Singh et al., 2012; Videnska et al., 2013; Locatelli et al., 2017a). Fecal and soil community composition, based on β-diversity using the Bray-Curtis dissimilarity distances, significantly varied over time for both farms ($p < 0.01$, **Figure 1**). As a result, bacterial composition data from the three sampling times were treated independently. The farm environment had a significant effect on fecal microbiome at all three sampling times (**Figure 1A**), with the farm effect being the strongest during T1 ($R = 1$, $p = 0.02$) and T2 ($R = 0.916$, $p = 0.01$) and being the weakest (but still significant) at T3 ($R = 0.381$, $p = 0.04$) according to ANOSIM analyses. Fecal α-diversity was not as greatly affected by farm environment though, with only one of the three metrics showing significant differences (richness; **Table 2**) but without a consistent trend between farms (greater for farm 1 at T1 and T2, greater for farm 2 at T3). As expected, soil microbiomes were significantly different between the 2 farms at T1 ($R = 0.59$, $p = 0.012$), T2 ($R = 1$, $p = 0.009$), and T3 ($R = 0.926$, $p = 0.007$) (**Figure 1B**), and like with the fecal microbiomes, there were some significant differences between farms for α-diversity metrics, but no consistent trends (richness higher for farm 1 at T1, T3, but diversity and evenness greater for farm 2 at T2; **Table 2**).

To determine if measured physiochemical or management data could account for these significantly different fecal communities between these farms, non-metric multidimensional scaling (NMDS) analyses were performed. At T1 and T2, fecal microbiomes from farm 1 were significantly positively correlated to aluminum (Al, $R^2 = 0.337$, $p = 0.003$), iron (Fe, $R^2 = 0.304$, $p = 0.003$), manganese (Mn, $R^2 = 0.557$, $p = 0.001$), silicon (Si, $R^2 = 0.375$, $p = 0.002$) and zinc (Zn, $R^2 = 0.428$, $p = 0.002$) concentrations, whereas no such significant correlations were observed for farm 2 (**Figure 1C**). This may be explained by the higher content of these five nutrients in feces from farm 1 as compared to farm 2 (**Table 3**). Fecal microbiomes of the market age broilers (T3) from farm 1 and farm 2 tended to converge (**Figure 1A**), confirming the moderate effect of the farm environment for this sampling time according to the ANOSIM analysis. The flock age exhibited the strongest correlation on the bacterial community ($R^2 = 0.711$, $p = 0.001$), which is consistent with previous reports describing the effect of chicken development stage on the composition of intestinal microbiota (Crhanova et al., 2011). Fecal pH decreased with increasing chicken age and was negatively correlated to bacterial community of both farms ($R^2 = 0.4$, $p = 0.001$) (Ilhan et al., 2017; Siegerstetter et al., 2018).

It has been reported that the deficiency or biofortification of micronutrients such as zinc (Reed et al., 2015) or iron (Reed et al., 2017) in chicken diet has led to significant changes in the composition of the gut microbiota. In our study, broilers have been fed with the same diet, so the difference in fecal micronutrient load should be linked to another source. Considering pastured poultry supplement their nutritional needs via foraging, pasture soils could represent a possible source of these micronutrients. In fact, soil samples from farm 1 had higher concentrations of Fe, Mn, and Zn (Al and Si not measured) than farm 2 (**Table 3**) and may have influenced the concentration of micronutrients absorbed by chickens as they continuously take up elements from the surrounding environment during their growth cycle. This hypothesis may be supported by the fact that soil nutrient concentrations from farm 1 had significant positive correlations to the soil microbiomes, especially at T2, while no such correlations were observed for farm 2 (**Figure 1D**). The most significant correlations based on the R^2 values for farm 1 were calcium (Ca, $R^2 = 0.797$, $p = 0.001$), total carbon and total nitrogen (TC, TN, $R^2 = 0.722$, $p = 0.001$), sodium (Na, $R^2 = 0.716$, $p = 0.001$), and chromium (Cr, $R^2 = 0.711$, $p = 0.001$). Although different from the nutrients that strongly correlated to fecal microbiomes, these results indicate that higher concentrations of these nutrients in the feces and soil of pastured poultry farms have the potential to shape the β-diversity of those microbial populations.

Not only did the farm environment effect the overall fecal microbiomes throughout live production, but it also significantly affected individual taxa within those microbiomes. Fecal samples from farm 1 exhibited a higher number of OTUs assigned at

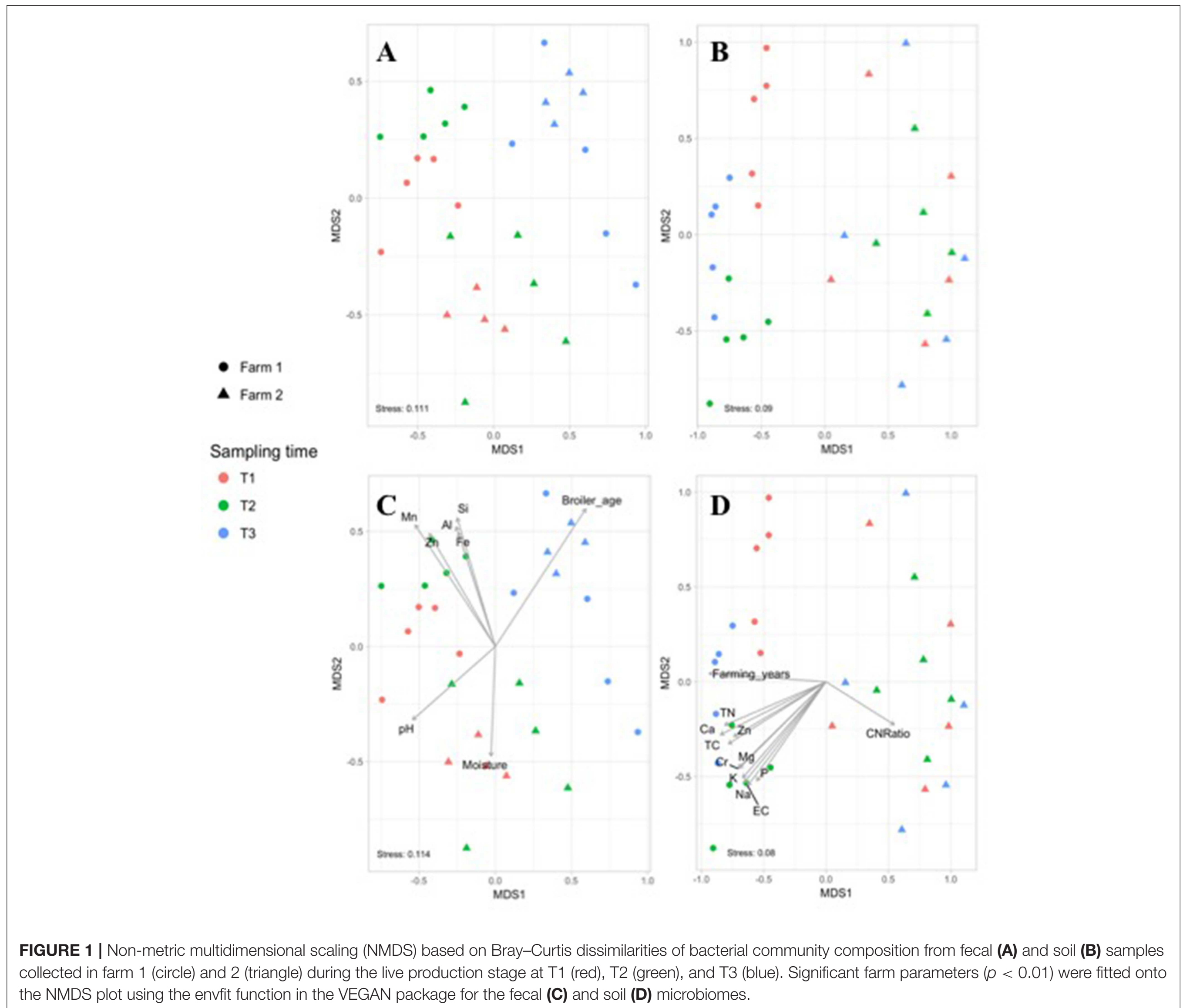

FIGURE 1 | Non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarities of bacterial community composition from fecal **(A)** and soil **(B)** samples collected in farm 1 (circle) and 2 (triangle) during the live production stage at T1 (red), T2 (green), and T3 (blue). Significant farm parameters ($p < 0.01$) were fitted onto the NMDS plot using the envfit function in the VEGAN package for the fecal **(C)** and soil **(D)** microbiomes.

both the phylum (mean relative abundance 12 vs. 6; Kruskal-Wallis test, $p = 0.046$) and the genus (mean relative abundance 84 vs. 65; Kruskal-Wallis test, $p = 0.043$) level compared to farm 2. Significant differences in phyla between farms 1 and 2 were observed at T1 [Firmicutes (84.2 vs. 93.8%), Actinobacteria (3.4 vs. 0.2%)], T2 (Bacteroidetes (10.9 vs. 0.3%) and Actinobacteria (6.1 vs. 366 0.2%)], and at T3 [Bacteroidetes (1.5 vs. 5.5%)]. At the genus level, fecal microbiomes of farm 1 harbored a higher number of total and unique (present only in farm 1 fecal microbiomes) OTUs compared than farm 2 ($p = 0.037$ and 0.046, respectively), but have an equivalent number of dominant (≥1% relative abundance) OTUs ($p = 0.658$). The two farms shared 32, 34.6, and 32.5% of their total OTUs and 43.8, 57.1, and 66.7% of their dominant OTUs at T1, T2, and T3, respectively. While *Lactobacillus* and *Escherichia* OTUs were dominant for both farms at all three sampling times, significant differences were observed between farms in dominant genera-level OTU distribution throughout live production, although the number of significantly different dominant OTUs decreased as the birds aged (**Table 4**).

To see if these significant differences in fecal microbiome taxa could be a result of the physical farm environment, fecal OTUs unique to each farm were compared to unique soil OTUs from the same farm (**Figure 2A**). Shared unique OTUs found only in the feces and soil of farm 1 represented 22.5% (T1), 24.2% (T2), and 28.1% (T3) of the unique farm 1 fecal OTUs, while shared unique farm 2 OTUs represented 25, 33.3, and 47.9% of the unique farm 2 fecal OTUs (for T1, T2, and T3, respectively). When the cross comparisons were made (farm 1 unique feces OTUs vs. farm 2 unique soil OTUs; farm 2 unique feces OTUs vs. farm 1 unique soil OTUs), very few unique OTUs were shared (**Figure 2B**). This

TABLE 2 | Comparison of α-diversity indices of microbiomes throughout the farm-to-fork continuum from two pastured poultry flocks[a].

Stage (sample type)	Sampling time[b]	Richness (Chao1)			Evenness (Equitability)			Diversity (Shannon)		
		Farm 1	Farm 2	*p*-value[c]	Farm 1	Farm 2	*p*-value	Farm 1	Farm 2	*p*-value
Pasture (feces)	T1	666.78	522.83	***0.02***	0.61	0.61	*0.919*	5.49	5.22	*0.355*
	T2	591.48	478.95	***0.04***	0.66	0.62	*0.288*	5.72	5.201	*0.217*
	T3	423.33	515.68	***0.03***	0.64	0.67	*0.357*	5.27	5.6	*0.216*
Pasture (soil)	T1	740.27	366.92	***0.001***	0.79	0.67	*0.364*	7.18	5.44	*0.066*
	T2	496.01	409.47	*0.13*	0.67	0.78	***0.008***	5.6	6.46	**0.010**
	T3	441.57	291.21	***0.01***	0.78	0.69	*0.096*	6.31	5.33	*0.060*
Processing (ceca)		568.29	449.09	***0.02***	0.822	0.809	*0.389*	7.303	6.981	*0.082*
Processing (WCR)		274.86	224.24	*0.08*	0.62	0.761	***0.008***	4.673	5.445	***0.007***
Final product (WCR)		237.26	302.31	*0.214*	0.575	0.705	*0.11*	4.31	5.44	*0.116*

[a]*Values for α-diversity indices are given as mean of 5 distinct area samples (Pasture) collected on farm 1 and 2 at each sampling time or 5 pooled samples from 5 broilers (Processing, Final Product). The QIIME 1.9.1 estimate used to determine each index are listed in the parentheses in the top row.*
[b]*Pasture sampling times were ∼4 weeks of age (T1), ∼8 weeks of age (T2), and ∼12 weeks of age (T3).*
[c]*Bolded values indicate significant differences between the two farms based on pairwise comparisons using a p < 0.05 significance level.*

TABLE 3 | Nutrient concentrations (ppm) from fecal and soil samples at three timepoints from two pastured poultry broiler flocks[a].

Sample type	Nutrient[b]	Sampling time								
		T1 (4 weeks of age)			T2 (8 weeks of age)			T3 (12 weeks of age)		
		Farm 1	Farm 2	*p*-value[c]	Farm 1	Farm 2	*p*-value	Farm 1	Farm 2	*p*-value
Feces	Al	525.78	271.83	0.063	6294	192.04	**0.003**	546.58	2527.8	**0.049**
	Fe	222.46	165.8	0.372	4489.6	91.48	**0.004**	358.22	1450.45	0.061
	Mn	73.23	30.55	**0.001**	95.13	36.86	**0.001**	44.66	46.00	0.890
	Si	452.54	203.53	**0.01**	1549.02	164.88	**0.003**	330.10	948.80	0.055
	Zn	62.00	23.35	**<0.001**	82.72	39.15	**0.003**	49.70	35.15	0.194
Soil	Fe	24.81	37.81	0.092	30.99	11.06	**<0.001**	23.41	17.46	0.602
	Mn	71.49	29.58	**0.005**	76.71	17.99	**<0.001**	59.41	39.13	0.091
	Zn	28.20	7.57	**<0.001**	55.66	4.45	**<0.001**	25.87	6.42	**0.003**

[a]*Concentrations from are given as mean of 5 distinct area samples collected on farm 1 and 2 at each sampling time*
[b]*Al, aluminum; Fe, iron; Mn, manganese; Si, silicon; Zn, zinc.*
[c]*Bolded values indicate significant differences between the two farms based on pairwise comparisons using a p < 0.05 significance level.*

observation indicates that there is a much stronger link in microbiome OTU composition between environmental samples within the same farm compared to samples between farms, further supporting the effects that the farm environment can have on the pastured poultry microbiome during live production. Overall phyla and genus level OTU comparisons for the live production, processing and final product samples can be found in **Supplementary Tables 1–3**, respectively.

Poultry Microbiome at the Processing Stage

The effect of farm environment was evaluated on the cecal and post-processing, pre-storage whole carcass rinse (WCR) microbiome samples collected during on-farm processing for both farms. Cecal microbiomes were strongly affected by the farm environment (ANOSIM, $R = 1$, $p = 0.01$) as shown by the NMDS (**Figure 3A**; blue symbols) with farm 1 representing a significantly richer populations (chao1, $p = 0.02$) distributed among significantly more phyla (mean relative abundance 13 vs. 9; Kruskal-Wallis test, $p = 0.009$) compared to farm 2 (**Table 2**). In terms of taxa distribution, *Firmicutes* were the most abundant bacterial phylum in all cecal samples, representing 53.0 and 73.8% of taxa in farms 1 and 2, respectively, followed by *Bacteroidetes, Proteobacteria,* and *Tenericutes*. The prevalence and abundance of these phyla have been commonly found within broiler ceca (Danzeisen et al., 2011; Xu et al., 2016; Zhou et al., 2016; Costa et al., 2017). Of the major phyla, only an unassigned OTU group abundance (10 vs. 10.03% for farms 1 and 2, respectively) were not significantly different between farms. At the genus level, 101 total OTUs were identified, including 58 shared between farms 1 and 2. Farm 1 harbored a higher number of dominant (mean relative abundance 20 vs. 14; Kruskal-Wallis test, $p < 0.014$) OTUs than farm 2. The top-5 genera represented ∼50% of the total taxa and was composed

TABLE 4 | Mean relative abundance (%) of dominant genus-level taxa (>1% of total OTUs) of fecal microbiomes at three timepoints from two pastured poultry broiler flocks[a,b].

Taxa	Farm 1	Farm 2	
T1 (4 WEEKS OF AGE)			
Lactobacillus	41.06	49.95	
SMB53	14.54	9.30	
Clostridium	0.44	11.00	*
*Bacillales*_Unclassified	7.54	1.28	*
*Clostridiales*_Unclassified	4.14	3.93	
Escherichia	1.26	4.80	
*Clostridiaceae*_Unclassified	0.52	4.75	*
*Lactobacillales*_Unclassified	4.08	0.18	*
*Lachnospiraceae*_Unclassified	1.54	1.93	
Acinetobacter	2.56	0.00	*
Bacteroides	2.54	0.00	*
Candidatus Arthromitus	1.84	0.65	
Lachnospiraceae_[Ruminococcus]	1.24	1.25	
*Micrococcaceae*_Unclassified	1.98	0.00	*
Turicibacter	0.38	1.63	*
T2 (8 WEEKS OF AGE)			
Lactobacillus	22.16	41.72	
Streptococcus	17.04	2.86	*
Escherichia	1.10	15.38	
SMB53	6.62	8.58	
Enterococcus	5.06	3.80	
Bacteroides	6.34	0.06	*
*Planococcaceae*_Unclassified	3.46	2.44	
Acinetobacter	2.42	2.42	
*Clostridiales*_Unclassified	2.18	2.64	
*Lactobacillales*_Unclassified	0.92	3.76	*
*Enterobacteriaceae*_Unclassified	0.26	3.36	*
*Bacteria*_Unclassified	3.26	0.20	*
*Clostridiaceae*_Unclassified	1.06	2.14	
*Lachnospiraceae*_Unclassified	2.22	0.86	
Corynebacterium	2.44	0.00	*
Arthrobacter	2.40	0.02	*
Staphylococcus	2.10	0.06	*
T3 (12 WEEKS OF AGE)			
Acinetobacter	8.06	37.80	*
Lactobacillus	23.58	4.63	
Escherichia	20.58	5.18	
Turicibacter	0.66	10.15	
Enterococcus	5.92	2.45	
Corynebacterium	3.58	3.78	
*Bacillales*_Unclassified	4.22	1.28	
*Enterobacteriaceae*_Unclassified	4.32	1.00	
SMB53	4.14	0.80	*
*Planococcaceae*_Unclassified	3.54	1.35	
Arthrobacter	1.88	2.13	
*Clostridiaceae*_Unclassified	2.80	0.78	
*Comamonadaceae*_Unclassified	0.04	3.35	*
*Lactobacillales*_Unclassified	1.16	1.48	
*Clostridiales*_Unclassified	1.62	0.85	
Sphingobacterium	0.70	2.00	
Trichococcus	0.44	2.33	*
Paenibacillus	1.70	0.33	
Solibacillus	0.24	2.03	*

[a] *Relative abundances from are given as mean of 5 distinct area samples collected on farm 1 and 2 at each sampling time.*
[b] *A * symbol in the final column indicates significant differences ($p < 0.05$) for that taxa between farm 1 and farm 2.*

of *Akkermansia*, *Clostridiales, Ruminococcaceae, Oscillospira,* and *Bacteroides* for farm 1 and *Oscillospira*, *Ruminococcaceae*, *Ruminococcus Faecalibacterium*, and *Clostridiales* for farm 2, and significant differences were found between 70% of the dominant cecal OTUs (**Table 5**). The cecal OTU distributions for these two pastured poultry farms were globally different from other studies focusing on conventional broiler production (Oakley et al., 2014; Oakley and Kogut, 2016; Xu et al., 2016; Zhou et al., 2016); however, some dominant genera identified across farms 1 and 2 such as *Ruminococcus, Fecalibacterium*, *Bacteroides,* and *Lachnospiraceae* have also been reported to be abundant in ceca previously (Danzeisen et al., 2011; Oakley et al., 2014; Sergeant et al., 2014; Oakley and Kogut, 2016; Costa et al., 2017). This indicates that here is a core broiler ceca microbiome, regardless of production management system, although more research will need to be performed to better define this core broiler cecal microbiome.

Thirty-four and Nine OTUs were unique to cecal samples from farm 1 and farm 2, respectively. In farm 1, 79.4 and 52.9% of the unique cecal OTUs were also identified in fecal and soil samples, respectively; only 14.7% of the unique cecal OTUs were not found before the processing stage and were likely acquired during processing. In farm 2, 55.5% of the unique OTUs in ceca were found for both the fecal and soil samples, while 11% of these OTUs were absent in the pre-harvest/live production samples. Although broiler cecal and fecal samples are known to have different phylogenetic distributions of sequences (Oakley and Kogut, 2016), our study suggests a conservation of certain OTUs across the live production and processing environments that could potentially constitute a unique biosignature for the farm environment.

Microbiomes from post-processing, pre-storage WCR samples significantly differed between farms (ANOSIM, $R = 0.842$, $p = 0.008$; **Figure 3**, red symbols). A greater number of dominant OTUs was observed in processing WCR samples from farm 2 along with an overall higher microbial equitability ($p = 0.008$) and diversity ($p = 0.007$) compared to farm 1 (**Table 2**). The processing WCR microbiomes from both farms shared the same dominant phyla, with *Proteobacteria* (89.2 vs. 63.7%), *Firmicutes* (5.4 vs. 28.4%), *Cyanobacteria* (2.1 vs. 2.4%), *Bacteroidetes* (1.7 vs. 2.0%), and *Actinobacteria* (1.2 vs. 2.0%) accounting for more than 98% of all sequences. The qualitative and quantitative composition of the dominant phyla associated

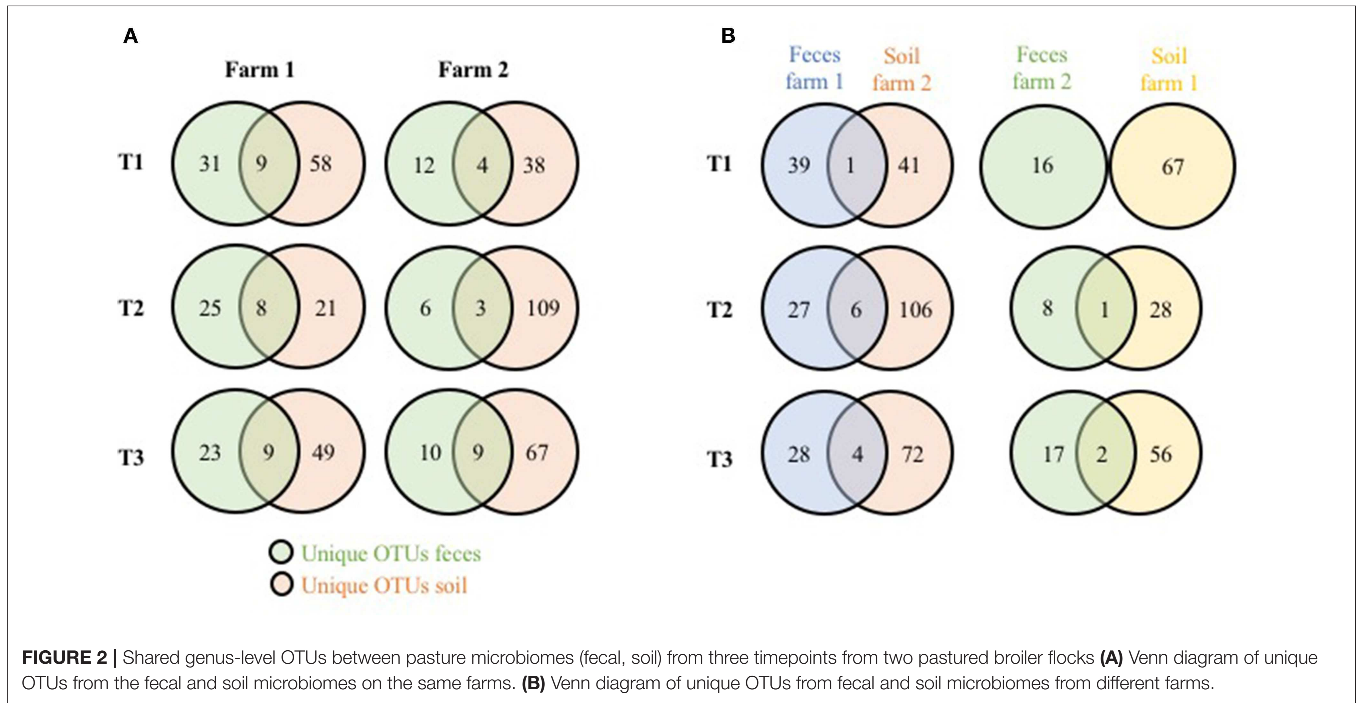

FIGURE 2 | Shared genus-level OTUs between pasture microbiomes (fecal, soil) from three timepoints from two pastured broiler flocks **(A)** Venn diagram of unique OTUs from the fecal and soil microbiomes on the same farms. **(B)** Venn diagram of unique OTUs from fecal and soil microbiomes from different farms.

FIGURE 3 | Non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarities of bacterial community composition from cecal (blue) and pre-storage WCR (red) samples collected in farm 1 (circle) and 2 (triangle) during the on-farm processing stage.

TABLE 5 | Mean relative abundance (%) of dominant genus-level taxa (>1% of total OTUs) of processing (ceca, WCR) and final product (WCR) microbiomes from two pastured poultry broiler flocks[a,b].

Taxa	Farm 1	Farm 2	
CECA			
Akkermansia	14.98	0.28	*
Bacteroidales	4.38	0.30	*
Bacteroides	4.86	0.10	*
Campylobacter	2.40	0.23	*
Christensenellaceae	0.94	1.10	
Clostridiales	3.82	3.70	
*Clostridiales*_Unclassified	9.16	6.08	*
Clostridium	1.22	0.85	
Coprococcus	1.68	0.30	*
Cyanobacteria_YS2	1.52	0.68	
Faecalibacterium	2.92	6.40	*
*Firmicutes*_Unclassified	3.62	4.63	
Lachnospiraceae	2.66	0.68	
*Lachnospiraceae*_Unclassified	0.34	1.23	*
Oscillospira	6.36	15.83	*
Phascolarctobacterium	3.00	0.55	*
Ruminococcaceae	1.20	4.10	*
*Ruminococcaceae*_Unclassified	8.18	10.45	*
Ruminococcus	3.40	10.23	*
Tenericutes_RF39	1.28	5.98	*
PROCESSING WCR			
Acinetobacter	10.20	18.76	*
Campylobacter	17.74	10.22	*
Enterobacter	2.90	11.28	*
*Enterobacteriaceae*_Unclassified	14.40	2.24	*
Escherichia	35.06	1.98	*
Lactobacillus	0.94	6.00	*
*Planococcaceae*_Unclassified	0.24	7.76	
*Pseudomonadaceae*_Unclassified	0.06	4.52	*
Pseudomonas	3.46	4.76	
Stenotrophomonas	2.26	3.46	
FINAL PRODUCT WCR			
Escherichia	38.00	11.52	
Campylobacter	22.17	18.78	
Acinetobacter	13.83	7.32	*
*Enterobacteriaceae*_Unclassified	10.73	6.80	
*Streptophyta*_Unclassified	0.03	9.68	*
Brevibacillus	0.00	7.24	*
Enterococcus	3.10	2.52	
Enterobacter	2.13	2.62	
Lactobacillus	0.13	3.44	*
Pseudomonas	0.17	3.08	*
Pantoea	4.37	0.24	*
Ochrobactrum	0.63	1.38	
Stenotrophomonas	0.00	1.66	*
Lactococcus	0.00	1.60	

[a] *Relative abundances from are given as mean of 5 pooled samples from 5 broilers during processing and final product stages.*

[b] *A * symbol in the final column indicates significant differences (p < 0.05) for that taxa between farm 1 and farm 2.*

with the processing WCR microbiomes agreed with previous findings (Kim et al., 2017; de Cesare et al., 2018). Among the 15 phyla identified across all processing WCR samples, only the relative abundance of *Proteobacteria* and *Firmicutes* significantly differed between the two farms. A total of 153 OTUs were assigned at the genus level, including 60 OTUs shared by processing WCR microbiomes from both farms. Among the 11 dominant OTUs shared between farms, the distribution and relative abundances significantly differed based on the farm (**Table 5**), although it should be noted that two of the top-3 genera within the processing WCR microbiomes for both farms are emerging or known foodborne pathogens (*Acinetobacter* and *Campylobacter*).

While these results indicate that the farm environment significantly influences processing microbiomes, it must be noted that the on-farm processing strategies differed in two main ways: (1) defeathering process and (2) chilling method. Farm 1 defeathered carcasses via removal of the skin and chilled the carcasses via air chilling, while farm 2 defeathered carcasses via scalding followed by automated picking and chilled the carcasses via immersion chilling. We expect that the defeathering method has a greater effect on the WCR samples compared to the cecal samples (due to the quick scalding process), and the chilling method to only effect the WCR samples (since the ceca are removed prior to chilling). Indeed, bacterial populations recovered from broiler carcass rinse samples with or without skin differ according to the chicken parts (Berrang et al., 2001). Additionally, the comparison of chilling methods used in commercial poultry facilities can lead to significant differences in pathogenic bacterial incidence and specific population concentrations within carcass rinse samples (Sanchez et al., 2002; Berrang et al., 2008). It should be noted that in the current study, for both farms, more OTUs were shared exclusively between the processing WCR microbiomes and soil microbiomes (17 and 19 OTUs for farm 1 and 2, respectively) than between the processing WCR and fecal microbiomes (1 and 6 OTUs for farm 1 and 2, respectively) (**Figure 4**). This suggests that the pastured poultry farm environment farm environment, specifically the pasture soil, has a lasting effect on the processing WCR microbiomes, regardless of processing management strategies.

Poultry Microbiome in Final Product

While there were significant differences in the microbiomes between the farms from the live production and processing stages, final product WCR microbiomes were more uniform between farms. Although the number of OTUs assigned at the phyla (Kruskal-Wallis test, $p = 0.021$) and genera ($p = 0.024$) level were higher on farm 2, the species richness ($p = 0.214$), evenness ($p = 0.11$), and diversity ($p = 0.116$) did not significantly vary between the two farms (**Table 2**). Additionally, there were no significant differences in the β-diversity of the final product WCR microbiomes between farms (ANOSIM, $R = 0.415$, $p = 0.093$). Among the 16 phyla identified among all final product WCR samples, no significant differences were observed between farms 1 and 2. The final product WCR microbiomes were dominated by three genera, *Escherichia, Campylobacter* and *Acinetobacter*, representing 74.0 and 37.6%

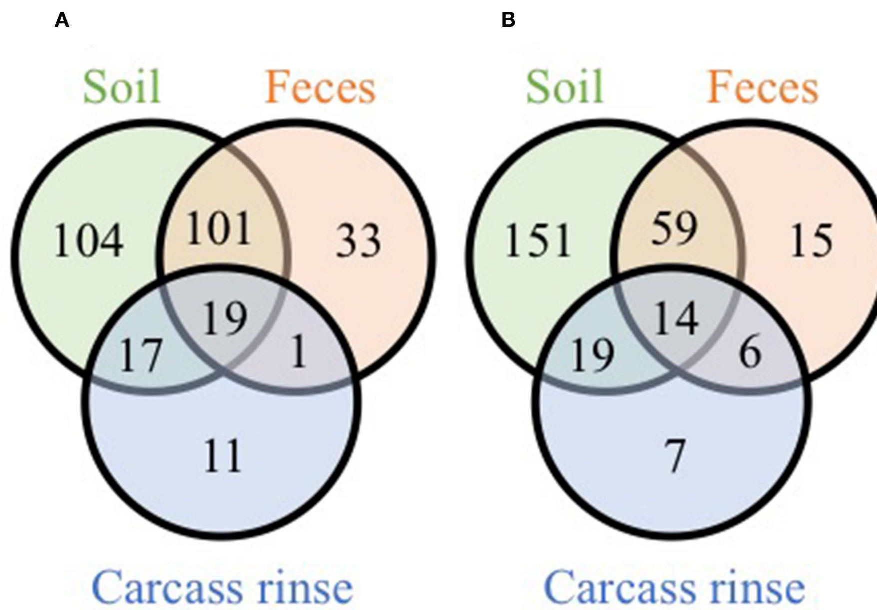

FIGURE 4 | Venn diagrams of shared genus-level OTUs from soil, fecal and pre-storage WCR microbiomes from farm 1 **(A)** and farm 2 **(B)**.

of the total OTUs recovered for farms 1 and 2, respectively (**Table 5**). These three genera were also the major genera in the processing WCR microbiomes. Unlike the processing WCR microbiomes where significant differences between farms were found for ∼73% of the dominant genus-level taxa, only 50% of the dominant genus-level taxa from the final product WCR microbiomes were significantly different between farms. This finding was unexpected, not only given the differences between the microbiomes from these two farms at all stages up to this point, but the fact that the final products from both farms were stored under different conditions based on each farm's typical practices: Farm 1 stored the carcasses for 1 day at 4°C while farm 2 stored the carcasses at −20°C for 2 weeks. The storage temperature (refrigeration vs. freezing) has been previously shown to have a variable effect on bacterial populations (Bailey et al., 2000). Generally, bacterial populations on carcasses increased significantly during a storage of 7 days at 4°C (Bailey et al., 2000; Hinton et al., 2004; Pradhan et al., 2012), while remaining unchanged or declined for a storage at freezing temperatures below −18°C (Bailey et al., 2000; Georgsson et al., 2006). Regardless of all the diverse live production, processing, and final product handling conditions, very few differences were observed between farms for the final product WCR microbiomes at either the gross community (α and β-diversity estimates) or individual taxa levels, potentially indicating that there is a stable microbiome for pastured broiler products that needs to be further investigated in future studies.

Occurrence of Putative Pathogens

Due to the increased access to the environment and other farm animals in the pastured poultry management system (Siemon et al., 2007; Park et al., 2013), there is a hypothesis that this exposure would increase the prevalence of foodborne pathogens within pasture-raised flocks. But does the farm environment itself have any effect on the presence of known and emerging pathogens, or indicators? To address this question, taxa related to putative foodborne pathogens (*Salmonella, Campylobacter, Listeria*) and an indicator organism (*Escherichia coli*) were specifically analyzed and compared with cultural data (enumeration and/or enrichment) targeting these same four organisms. OTUs associated to the genus *Escherichia* were found in all samples of both farms (**Table 6**), with relative abundances being higher in the farm 2 preharvest samples (feces, soil), but significantly higher for farm 1 postharvest (ceca, processing WCR, final product WCR) samples. *Escherichia* has been commonly reported genera for poultry-associated samples (Oakley et al., 2014; de Cesare et al., 2018). The ubiquitous nature of *E. coli* throughout the farm-to-fork continuum was confirmed culturally, although cell counts were higher for all farm 1 samples other than the ceca, unlike what was observed in the non-quantitative microbiome relative abundance data. While there is no direct correlation between the microbiome and cell count data, it should be noted that a similar trend was observed in the final product WCR samples, where the estimate for farm 1 was approximately 3 times higher than farm 2.

Of the three targeted foodborne pathogens, only OTUs related to *Campylobacter* were found within the pastured poultry-related microbiomes (**Table 6**). Like *Escherichia, Campylobacter* OTUs were found in all sample types for both farms, with the highest values found in the final product WCR samples. In general, relative abundances of *Campylobacter* were higher

TABLE 6 | Putative foodborne pathogen (*Campylobacter, Salmonella, Listeria*) and indicator (*Escherichia*) levels in different sample types throughout the farm-to-fork continuum from two pastured broiler flocks, measured by microbiome, direct cell counts, and enrichments[a].

		***Campylobacter* spp.**			***Salmonella* spp.**			***Listeria* spp.**			***Escherichia***		
	Sample type	**Farm 1**	**Farm 2**	***p*-value[b]**	**Farm 1**	**Farm 2**	***p*-value**	**Farm 1**	**Farm 2**	***p*-value**	**Farm 1**	**Farm 2**	***p*-value**
Microbiome relative abundance (% total OTUs)	Feces	0.14	0.04	*0.103*	0	0	*na*	0	0	*na*	7.65	8.98	*0.781*
	Soil	0.00	0.16	***0.001***	0	0	*na*	0	0	*na*	1.10	2.50	***0.026***
	Ceca	2.40	0.23	*0.263*	0	0	*na*	0	0	*na*	0.02	0.002	***0.030***
	Processing WCR	17.74	10.22	***0.025***	0	0	*na*	0	0	*na*	3.06	1.98	***<0.001***
	Final product WCR	22.17	18.78	*0.311*	0	0	*na*	0	0	*na*	38.00	11.52	***<0.001***
Cell counts (log10 CFU/mL)	Feces	2.6	0.0	***0.001***	–	–		–	–		7.1	6.9	*0.078*
	Soil	1.6	0.0	***0.014***	–	–		–	–		6.3	3.4	***<0.001***
	Ceca	6.2	0.0	***<0.001***	–	–		–	–		5.0	6.0	***<0.001***
	Processing WCR	2.3	0.0	***0.042***	–	–		–	–		2.9	2.5	*0.108*
	Final product WCR	0.7	0.0	***0.027***	–	–	–	–	–		3.3	1.3	***<0.001***
Enrichment (# samples +)[c]	Feces	15 (100)	11 (73.3)	*0.100*	1 (6.7)	0 (0)	*>0.99*	5 (33.3)	13 (86.7)	***0.008***	–	–	
	Soil	9 (60)	7 (46.7)	*0.724*	0 (0)	0 (0)	*>0.99*	10 (66.7)	5 (33.3)	***0.008***	–	–	
	Ceca	5 (100)	0 (0)	***0.008***	0 (0)	1 (20)	*>0.99*	2 (40)	5 (100)	*0.167*	–	–	
	Processing WCR	2 (40)	0 (0)	*0.444*	0 (0)	0 (0)	*>0.99*	0 (0)	0 (0)	*>0.99*	–	–	
	Final product WCR	0 (0)	0 (0)	*>0.99*	0 (0)	0 (0)	*>0.99*	3 (60)	4 (80)	*>0.99*	–	–	

[a]*Values are given as mean of 5 distinct area samples (feces, soil) collected on farm 1 and 2 at each sampling time or 5 pooled samples from 5 broilers (ceca, processing WCR, final product WCR)*

[b]*Bolded values indicate significant differences between the two farms based on pairwise comparisons using a $p < 0.05$ significance level. For the Microbiome and Cell Count data, the student's t-test was used to compare farms, while the Fisher's exact test was used for the Enrichment data.*

[c]*The value in the parentheses represents the percentage of samples positive ($n = 15$ for feces, soil, $n = 5$ for ceca, processing WCR, and final product WCR).*

in farm 1, although it was only significantly higher in the processing WCR samples. *Campylobacter* spp. (mostly *C. jejuni* and *C. coli*) are present in nearly all birds at up to 10^7 CFU g^{-1} in the chicken intestine (Stern et al., 1995) and were evidenced in sequencing results from samples collected along the poultry production chain (Oakley et al., 2014; Park et al., 2016). Unlike the microbiome data, *Campylobacter* was only recovered from farm 1 samples culturally using selective CEFEX agar, although enrichment using a non-selective media allowed for the recovery for *Campylobacter* from the fecal and soil farm 2 samples but with a significantly lower prevalence that recovery from farm 1 ($p = 0.011$). These disparate results indicate a complex *Campylobacter* ecology throughout the pastured poultry farm-to-fork continuum but given the consistently higher prevalence on farm 1 for all microbiome and cultural estimates, farm environment appears to influence *Campylobacter* colonization and prevalence within pastured poultry.

No sequences were assigned to the *Salmonella* or *Listeria* genera in this study (**Table 6**). The low prevalence of *Salmonella* was confirmed culturally, with only a single *Salmonella* isolate recovered for both farms. Generally, *Salmonella* is a minor component of the poultry-associated microbiota (Oakley et al., 2014; de Cesare et al., 2018). However, the occurrence of *Salmonella* in samples collected from pasture farms to retail carcasses in this study is low compared to others (Melendez et al., 2010; Álvarez-Fernández et al., 2012; Lee et al., 2016). Unlike *Salmonella*, *Listeria* was recovered via enrichments from numerous samples, and while there were some significant differences in prevalence in the preharvest samples (**Table 6**; farm 1 soil, farm 2 feces), overall farm environment did not have a significant effect on *Listeria* prevalence ($p = 0.205$). The overall prevalence of *Listeria* spp. along the different production stages is consistent with previous reports (Sakaridis et al., 2011; Rothrock et al., 2017). While prevalence was unaffected, farm environment did majorly affect the species recovered, since all isolates from farm 1 were *L. innocua* while all isolates from farm 2 were *L. monocytogenes* serogroup 1/2a-3a. It has been recently reported that *Listeria* species recovery from pastured poultry farms can be potentially affected by culture conditions (Locatelli et al., 2017b), so it is possible that the different environmental conditions on both farms (such as the fertilization of pasture with organic fertilizer for organic vegetable production on farm 2) could equally influence the *Listeria* species on these farms.

CONCLUSION

Although both farms in this study raised the same chicken breed fed the same diet, each pastured poultry farm possessed their own ecology that shapes the structure and composition of the poultry-related microbiomes throughout the farm-to-fork continuum. Significant differences were observed in terms of not only α- (richness, diversity, evenness) and β- (Bray Curtis PCoA) diversity estimates between the two farms for the different sample types, but also in the taxa distribution within those samples. Farm environmental also significantly affected the presence of foodborne pathogens within these microbiomes, both quantitatively (*Campylobacter*) and in terms of recovered species (*Listeria*). The pasture soils from both farms significantly affected the OTU composition and nutrient composition of the live production fecal samples, highlighting the need for a better understanding of farm-level ecological dynamics inherent within pastured poultry management systems and its effect on poultry-related microbiomes. Future studies focusing on these farm-level environmental drivers of overall and foodborne pathogen microbial ecology will allow us to better manage these microbial populations to benefit animal, environmental and public health.

ETHICS STATEMENT

We did not handle any live birds or sample anything from live birds. We sampled soil and fecal droppings during live production, and only sampled ceca once the birds were butchered/processed by the farmers themselves. The farmers were responsible for the management and care of the birds during live production and they processed/butchered the birds on their own farms. We did not sample until the birds were already butchered. Therefore, no IACUC was required

AUTHOR CONTRIBUTIONS

MR: designed the study. MR and AL: analyzed the data and wrote 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/fsufs.2019.00048/full#supplementary-material

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