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Antibiotic resistance, antimicrobial residues, and bacterial community diversity in pasture-raised poultry, swine, and beef cattle manures

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Abstract

Animal manure can be a source of antibiotic-resistant genes (**ARGs**) and pharmaceutical residues; however, few studies have evaluated the presence of ARG in pasture-raised animal production systems. The objective of this study was to examine changes in microbiome diversity and the presence of antibiotic residues (**ABRs**) on three farms that contained a diverse range of animal species: pasture-raised poultry (broiler and layer), swine, and beef cattle. Total bacterial communities were determined using 16S rRNA microbiome analysis, while specific ARGs (sulfonamide [**Sul**; *Sul*1] and tetracycline [**Tet**; *Tet*A]) were enumerated by qPCR (real-time PCR). Results indicated that the ARG abundances (*Sul*1 [$P < 0.05$] and *Tet*A [$P < 0.001$]) were higher in layer hen manures (16.5×10^{-4} and 1.4×10^{-4} µg kg^{-1}, respectively) followed by broiler chickens (2.9×10^{-4} and 1.7×10^{-4} µg kg^{-1}, respectively), swine (0.22×10^{-4} and 0.20×10^{-4} µg kg^{-1}, respectively) and beef cattle (0.19×10^{-4} and 0.02×10^{-4} µg kg^{-1}, respectively). Average fecal *Tet*A ABR tended to be greater ($P = 0.09$) for broiler chickens (11.4 µg kg^{-1}) than for other animal species (1.8 to 0.06 µg kg^{-1}), while chlortetracycline, lincomycin, and oxytetracycline ABRs were similar among animal species. Furthermore, fecal microbial richness and abundances differed significantly ($P < 0.01$) both among farms and specific species of animal. This study indicated that the microbial diversity, ABR, ARG concentrations, and types in feces varied from farm-to-farm and from animal species-to-animal species. Future studies are necessary to perform detailed investigations of the horizontal transfer mechanism of antibiotic-resistant microorganisms (**ARMs**) and ARG.

Key words: animal species, antibiotic resistance, genes, microbiome, pasture-raised animals

Introduction

Antibiotic residues (**ABRs**) that spread the environment through land application of livestock manure or compost could influence structure and function of microbial communities and stimulate the spread of antibiotic-resistant (**AR**) microorganisms (**ARMs**) and AR genes (**ARGs**). However, no regulations exist for concentration limits of antibiotics in manure, soil, or wastewater. A report from the U.S. Centers for Disease Control and Prevention (CDC, 2013) shows that drug-resistant infections continue to be a major concern (Thiele-Bruhn, 2003; Brandt et al., 2015; Menz et al., 2019).

Abbreviations

ABR	antibiotic residue
AR	antibiotic resistance
ARG	antibiotic-resistant gene
ARM	antibiotic-resistant microorganism
ASV	amplicon sequence variant
CEC	cation exchange capacity
CP	crude protein
DM	dry matter
EU	European Union
GI	gastrointestinal tract
HPLC	high-pressure liquid chromatography
OTU	operational taxonomic unit
PCoA	principal coordinated analysis
Sul	*Sul*1, sulfonamide
Tet	*Tet*A, tetracycline
WHO	World Health Organization.

Subtherapeutic (growth promoters) and therapeutic antibiotics (use to treat clinically ill animals) are administrated in commercial animal production systems (Thiele-Bruhn, 2003). A considerable quantity of the antibiotics administered are not adsorbed by the animals (17% to 80%) and are excreted through urine and feces (Halling-Sorensen et al., 1998; Montforts et al., 1999). Bacteria in an animal's gastrointestinal tract (**GI**) or in the environment after land application of manure exposed to ABR have been recognized as potential sources of antimicrobials and ARGs (Rothrock et al., 2016), which can affect environmental and animal health (Pruden et al., 2006; Heuer et al., 2011). We need to understand the environmental impact of antibiotic use in animal production; however, a broader knowledge of the microbiota community diversity, ABR, and ARG content in feces from different species of livestock and different production systems is needed. Several studies have shown that ARG increased in environments (e.g., soil and wastewater) where agricultural operations occur (Knapp et al., 2017) and in environments that receive animal manure (Shafiani and Malik, 2003; Luo et al., 2010). Following the lead of the United States, however, an environmental risk assessment of veterinary pharmaceuticals was prescribed in the European Union (**EU**) in 1998 with the EU directives 81/852/EEC and 92/18/EEC (council regulation) (EMEA, 1997).

Pasture-based domesticated animal production can strengthen small and mid-size farm communities across the United States (Conner et al., 2008; HFAC, 2019) and EU (Stampa et al., 2020). Pasture-raised animal products, such as meat, milk, and eggs, have increased consumer demand (Conner et al., 2008; Stampa et al., 2020). Little is known about the prevalence and characteristics of AR and ARG in the manure and soil. Experimental studies of cattle production systems usually find that cattle from conventional dairies harbor a greater occurrence of ARM compared to organic dairies or beef cattle operations; given that dairies usually use more antimicrobials (Zwald et al., 2004; Sato et al., 2005; Harvey et al., 2009). However, it is interesting to note that no significant difference in resistance to individual antimicrobial agents was observed between organic and conventional dairy farms in various studies (Ray et al., 2006; Noyes et al., 2016). In the face of developing AR and ARGs, it is important to examine reduced susceptibility of microorganisms below resistant breakpoints. Our knowledge of antimicrobial use among the farms in our study is limited to herd-level, farmer-reported antimicrobial agent use, so we were incompetent to observe the direct association between the amount of antimicrobial drug use and the ARM from these herds. This means that there may be a background population of ABR and exchange of ARG between organisms, which may contribute to AR and ARG presence on pasture-raised livestock and poultry farms (Singer et al., 2007). Melendez et al. (2010) collected samples from two pastured poultry farms (n = 164) and retail carcasses (n = 36) in Arkansas and found that *Salmonella* serotypes isolated from pasture-raised poultry farms (e.g., pens, feed, and water) exhibit AR and class I integrons (presence of an integrase gene [*intI*] and a proximal primary recombination site [*attI*]). Consequently, previous studies characterized ARM and ARG in native Nebraskan prairie soils (Durso et al., 2016) and organic livestock farms (Cadena et al., 2018). These works provided information on the most frequently detected ARG, which was for tetracycline (*Tet*).

The gut microbiome of livestock is complex, dynamic, and variable (Zhu et al., 2002; Ming et al., 2017). The characteristics of the gut microbiota may be explained by different host characteristics, environment, dietary compositions, and use of feed additives (Costa et al., 2017; Kers et al., 2018; Lourenco et al., 2019a). Antibiotics not only act on bacteria that cause infections but also affect the resident microbiota. A better understanding of the richness of ARM, ARGs, and ARGs associated with microbial diversity and pathogenicity in the animal gut will have a major role in reducing the contribution of animal production to this problem. Auffret et al. (2017) reported that 204 genes associated with ARM, colonization, communication, or pathogenicity functions were identified from 4,966 metagenomic genes from beef cattle. Same authors also reported that a high ratio of Proteobacteria to Firmicutes + Bacteroidetes ratio was confirmed as a good indicator for rumen dysbiosis and zoonotic pathogens. Furthermore, addition of plant tannins in the diets increased Firmicutes and Firmicutes/Bacteroidetes ratio in the rumen (Min et al., 2014a, 2014b; Carrasco et al., 2017), which improved average daily gain due to altered rumen fermentation (Min et al., 2019a, 2019b). All of these factors can have negative or positive effects on the overall health and production performance of cattle. Furthermore, Bacteroidetes and Firmicutes are the two prevailing bacterial phyla in the gut of humans, mice, and pigs (Ley et al., 2006; Guo et al., 2008), cattle and camels (Ming et al., 2017), and meat goats (Min et al., 2019a). This phylum is composed of many pathogenic bacteria such as *Escherichia coli*, and the richness of some of these adaptable pathogens is sensitive to dietary change (Bäumler and Sperandio, 2016). Most published studies have focused on either single animal species or indoor confinement systems. It is important to investigate the effects of pasture-raised animal species and different farms on fecal nutrient profiles, ABR, and fecal microbiome community diversity associated with ARG. The results from the present study represent a preliminary experiment that can begin to inform baseline AR/ARG monitoring studies in the future. The objective of this study was to determine the effects of various animal species and different farm on fecal ABR, microbiome changes, and ARG dynamics in the Southeastern region of the United States.

Materials and Methods

To effectively determine the environmental impact of antibiotic use in animal agriculture, a baseline of AR levels must first be determined. Background levels of AR in pasture-based "no antibiotics ever" poultry and livestock systems were determined

from: broiler chickens and layer hens, swine, and beef cattle. Animals were located on three farms within the southeastern United States (Table 1), with farm A being sampled once (n = 5) and farms B and C both being sampled twice (n = 10). This study was exempt from animal ethics approvals since the farmers managed the animals; thus, ethics approval was not required as per applicable institutional and national guidelines and regulations.

Farm Sites

This analysis was part of a longitudinal study conducted on 43 flocks of broiler chickens across 11 pastured poultry farms in the southeastern United States from March 2015 to November 2016. Five flocks from three farms were selected for this specific study because the farms also raised pastured layer hens, swine, and/or beef cattle (Table 1). All farms reared their pastured flocks/herds within specific areas of the farms cordoned off by temporary fencing, and the pastured were rotationally grazed. Rotation of flocks/herds depended on livestock species. Broilers and layers were reared in movable pens with temporary fences to allow for extended ranging. Poultry housing was relocated either daily (broiler chickens) or weekly (layer hens). Beef cattle were moved to fresh pasture three to four times a week and swine were moved two to three times weekly. Livestock breed and diet on pasture was at the discretion of the individual farmers and could not be controlled for within the experimental design. A brief description of the size, scale, and animal breed of each farm is contained in Table 1. Additionally, based on information obtained from the participating farmers prior to inclusion in the parent student, no antibiotics were used on farm for any animals during the study, or at any point prior to the study since they owned the farms (at least 5 yr for each participating farm).

Sample collection

Fresh fecal samples were collected from the area of the pasture they were currently grazing at the time of sampling. For each animal, their grazing area was divided into five areas, and within each area at least five fecal samples were combined by weight into a single sampling bag and homogenized by hand (≥25 g of fresh weight). This resulted in five replicate pooled samples for each animal for each sampling time. Due to availability of target animals on farm and ability to sample, farm A was only sampled once, while farms B and C were sampled twice during the experimental period. The participating farms were sampled between 2015 (June 17 and July 20) and 2016 (March 13, May 16, and June 08). Fecal samples were transported back to the laboratory on ice for all downstream analyses.

Chemical analysis

Analytical dry matter (**DM**) concentrations of fecal samples were determined by oven-drying at 105 °C for 24 h (AOAC, 1998). The fecal pH was determined after mixed with distilled water at 1:2.5 (w/v) solid-to-water ratio for 1 h (Huang et al., 2017). Total carbon (C) and total nitrogen (N), and crude protein (**CP**) were determined by extracting 10 g of sample with 100 mL distilled water (w/v 1:10) by 18-h end-over-end shaking, followed by membrane filtration of the supernatant using 0.45-µm cellulose acetate filters. The aqueous extracts were calorimetrically analyzed on a SEAL Auto-analyzer (Keeney and Nelson, 1982). Atomic absorption spectroscopy was used to determine concentrations of minerals in samples at the University of Georgia, Athens, GA (Paul et al., 2014).

Fecal ABRs

Samples were analyzed for the antibiotic substances shown in Table 4. Multiple ABRs from fecal samples were performed by the Water Sciences Laboratory (University of Nebraska-Lincoln) and quantified according to the modified methods described by Ho et al. (2012). One gram of wet sample was accurately weighed into a 15-mL centrifuge tube and spiked with 25 µL of phosphate-buffered saline (pH 7.4) solutions, 5 mL of extraction buffer (MeOH:acetonitrile:0.1 M ethylenediaminetetraacetic acid:0.2 M Mcilvaine buffer [pH 4.0]; 30:20:25:25 ratios) were added (Ho et al., 2012). The tube was vortexed for 30 s and then placed into an ultrasonic bath for 10 min. The tube was centrifuged at 1,800 × g for 10 min. The supernatant was then decanted into a clean 500-mL plastic bottle and the settled solid was extracted twice more. Decanted supernatant (20 mL) was diluted to 500 mL with ultrapure water. Finally, 250 µL of H_2PO_4 was added to adjust the pH to approximately 2.3. Prior to solid phase extraction, the extract was filtered through 0.45-µm nylon membrane filter paper (Whatman, United Kingdom) to remove particulate matter. The extract was then freeze-dried, resuspended in 5 mL of deionized water, and stored at −80 °C until analysis. The ABR samples were analyzed two farms only (no statistical different between farms) and then equally mixed from three farms into each animal species prior to analysis, due to the low ABR levels (or not detected). Analyses of selected antibiotics were performed using an Agilent 1260

Table 1. Comparison of the three all pastured "no antibiotic ever" farms

	Farm		
Item	Farm 1	Farm 2	Farm 3
Animal types	Layers, broilers, swine, beef cattle	Layers, broilers, swine, beef cattle	Layers, broilers, swine, beef cattle
Times sampled	1	2	2
Flock/herd size			
Broilers	>500	50	>500
Layers	>500	150	>500
Swine	35	15	25
Cattle	25	10	25
Breed information			
Broilers	Freedom Ranger	Cornish Cross	Freedom Ranger
Layers	Rhode Island Red	Rhode Island Red/Araucana	Rhode Island Red
Swine	Tamworth	Ossobaw	Tamworth
Cattle	South Poll	Dexter	South Poll

binary high-pressure liquid chromatography (**HPLC**) coupled to an Agilent 6410 Triple Quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). Antibiotics were separated using a HyPURITY C18 HPLC column 250 mm × 2.1 mm ID, 5 µM particle size (Thermo-Scientific, Waltham, MA) at a temperature of 50 °C (D'Alessio et al., 2019).

Microbiome analysis

To characterize the fecal microbiomes of pasture-raised animals, the Illumina MiSeq platform was used (Navas-Molina et al., 2013; Rothrock et al., 2014). Fecal samples (0.33 g wet weight) were weighed into a 2-mL lysing Matrix E tube (Fisher Scientific International, Inc., PA) and thoroughly mixed with 825 µL of sodium phosphate buffer and 275 µL of pre-lysis solution using a vortex for 15 s. The material was then centrifuged at 14,000 × *g* for 5 min. The supernatant was decanted by adding 700 µL of stool lysis buffer (ASL) and vortexed for 5 s. Samples were placed into a MPBio FastPrep 24 instrument (MP Biomedical, Santa Ana, CA), and homogenized at a speed of 6.0 m s^{-1} for 40 s. The homogenized samples were then centrifuged at 14,000 × *g* for 5 min and the supernatant transferred sterile 2-mL microcentrifuge tubes. The DNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA) using a Synergy 2 Microplate Reader (BioTek Instruments, Winooski, VT). Total 16S rDNA abundances (the V3/4 region of the 16S rRNA gene), an estimate of the total bacterial community contained within a sample, were PCR-amplified with primers containing MiSeq sequencing adapters and Golay barcodes and sequenced on the Illumina MiSeq platform (Caporaso et al., 2011, 2012), followed by sequence analysis using QIIME2 (https://docs.qiime2.org) ver. 2020.2 (Bolyen et al., 2019). All amplicon sequence variants (**ASVs**) were aligned using mafft via q2-alignment (Katoh et al., 2002) and phylogeny was constructed using q2-phylogeny with fasttree2 (http://www.microbesonline.org/fasttree) (Price et al., 2010). Taxonomy was assigned to ASVs using the q2-feature-classifier (https://github.com/qiime2/q2-feature-classifier) (Bokulich et al., 2018) classify-sklearn naïve Bayes taxonomy classifier against the Greengenes operational taxonomic unit (**OUT**) reference sequences (McDonald et al., 2012).

Antimicrobial resistance gene analysis

The ARG *Sul*1 and *Tet*A were selected for qPCR (real-time PCR) analyses of sulfonamide (**Sul**) and tetracycline (**Tet**) resistance, respectively. These genes have been closely associated with class I integrons responsible for transfer of ARGs between bacteria (Cadena et al., 2018). DNA extractions were carried out using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. The qPCR of *Sul*1 and *Tet*A were carried out using primers presented in Table 2. Primers were designed using AlleleID 7 (Premiere Biosoft, Palo Alto, CA) and were based on *Sul*1 and *Tet*A nucleotide reference sequences NG_048098.1 and NG_048153.1, respectively, from the Bacterial Antimicrobial Resistance Reference Gene Database (NCBI). Reaction mixtures for qPCR included 10 µL of 2× QuantiTect SYBR Green PCR kit (Qiagen, Carlsbad, CA), 300 nM of each primer, and 1.5 µL of extracted DNA for a 20 µL total reaction volume. Thermocycling conditions were the same for *Sul*1 and *Tet*A: 95 °C for 15 min; 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s; followed by a final melting from 65 to 95 °C, increased by 0.5 °C every 5 s. A standard curve was generated using serial dilutions (10^2 to 10^7 copies) of gBlocks (Integrated DNA Technologies, Coralville, IA) designed from 250 and 500 bp fragments of the *Sul*1 and *Tet*A genes, respectively (NCBI, 2019).

Statistical analysis

Statistical analyses were conducted using the GLM procedure of SAS (SAS Institute, Inc., Cary, NC), with the factors examined being different farms, animal species, and farms × animal species interactions. Data were presented as least-square means, together with the standard error of the mean. Results are reported at both $P \leq 0.05$ and $P \leq 0.01$ probability levels. Observed bacterial richness (*i.e.*, the number of distinct OTU), diversity indices (Hill et al., 2003; Oksanen et al., 2017), and the relative abundance of each OTU were analyzed by ANOVA using the GLM procedure of the SAS (Figure 1). Finally, UniFrac analysis (Lozupone and Knight, 2005) followed by weighted principal coordinated analysis (**PCoA**) characterized the diversity of select microbial populations. An unweighted distance-based analysis of molecular variance was used to assess the statistical significance of the spatial separation observed among various farms and animal species of the PCoA plots (Figure 2).

Results and Discussion

Chemical composition of pasture-raised animal manure

The average fecal properties across animal types and different farms are presented in Table 3. An average fecal sample in cattle contained low levels of DM, while a significant higher pH was detected in cattle fecal samples (7.6) compared to other animal species (6.2 to 7.4) which is similar to other studies (Huang et al., 2017; Muhsen and Al-Autaish, 2017). There was a significant interaction ($P < 0.01$) between farms and animal species for fecal pH. Fecal pH has a substantial impact on the sorption of ABR by changing the charge state of the antibiotics and sorbents (Figueroa et al., 2004; Chang et al., 2015). It should also be noted that under acidic conditions, like most other high-use antibiotics, tetracycline and oxytetracycline become negatively charged and can sorb to soil or clay minerals by anionic exchange or electrostatic adsorption (Figueroa and Mackay, 2005). However, under alkaline conditions, the majority of tetracycline is present in anionic form which causes electrostatic repulsion with the +/– of ABR with manure and soil C (organic matter–ABR complexes), reducing sorption (Figueroa et al., 2004; Figueroa and Mackay, 2005; Gu and Karthikeyan, 2005). Consequently,

Table 2. Sequences, target size, and melting temperature of primers used

Organisms or group	Target gene	Primer	Primer sequences (5′–3′)	T_m[1], °C
Sulfonamide (*Sul*) resistance	*Sul*1	*Sul*1-FW319	5′-CGA TCA GAT GCA CCG TGT T-3′	60
		*Sul*1-RV430	5′-CGC AGG GTC AGG AAA TCC-3′	
Tetracycline (*Tet*) resistance	*Tet*A	*Tet*A-FW767	5′-CAA CTT GTC GGA CAG GTG -3′	60
		*Tet*A-RV886	5′-GGC GAG TGA ATG CAG AAT-3′	

[1]T_m melting temperature.

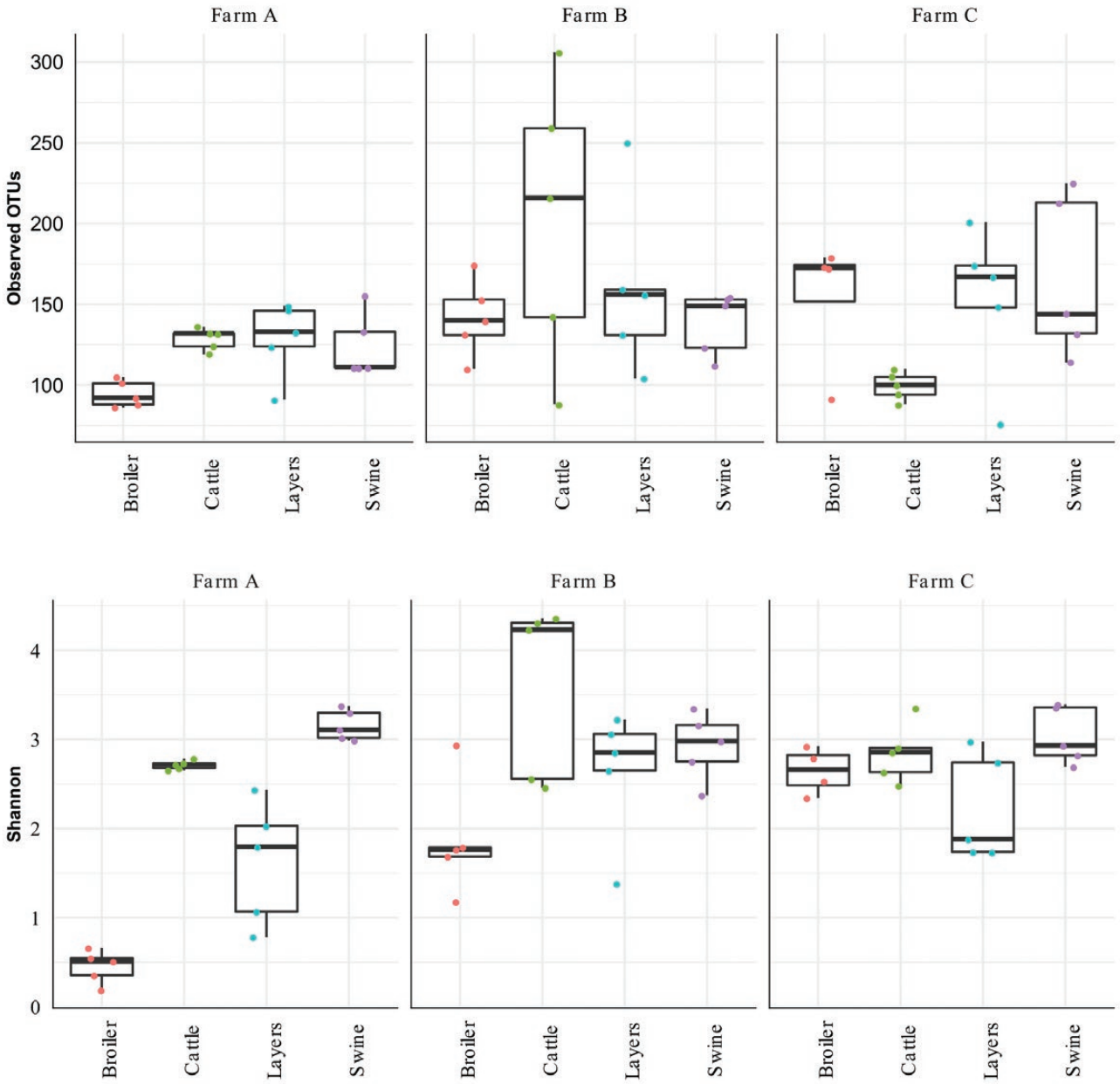

Figure 1. Alpha diversities (richness and abundance) among farms and livestock species (pairwise comparisons between species) in bacterial community compositions in broilers, cattle, layers, and swine fed mixed pasture-based diets. Richness and diversity were measured by OTUs and Shannon index, respectively. *P*-value: farm $P < 0.01$; animal species $P < 0.01$. Number of experimental samples used; $n = 5$ (one sampling time for farm A), $n = 10$ (two sampling times for farms B and C).

ABR sorption gradually decreases with pH increase due to a decreased cation exchange capacity (**CEC**) (Chang et al., 2015). In the present study, pH of cattle manure was higher ($P < 0.01$) than other animals studied (Table 3), with significant interactions between farms and livestock variables. Sorption of tetracycline, chlortetracycline, and oxytetracycline differ due to specific on soil properties, such as pH, clay content, soil type, CEC, anion exchange capacity, and organic C contents (Sassman and Lee, 2005). However, at present, K_d (solid–water sorption coefficients) values for antibiotics and other veterinary pharmaceuticals must be obtained experimentally (Sassman and Lee, 2005).

The primary factors that affect nutrient composition of livestock fecal matter are dependent on livestock type (ruminant vs. monogastric) and stage of growth and feeding management (ration, feed sources, quality of feeds, supplement use; Sheppard and Sanipelli, 2012; Huang et al., 2017; Ali et al., 2021). In the present study, feces from broiler chickens and layer hens contained high levels of N (1.0% to 1.3%) and potassium (K; 0.52% to 0.53%), compared to other species (Table 3) which is similar to other studies (Sheppard and Sanipelli, 2012; Huang et al. 2017). According to Sheppard and Sanipelli (2012), poultry manure (broilers and layers; 2.5% to 2.9%) exhibited greater K content than swine manure (3.42% to 8.32%), with the layer manures had the highest concentrations of most of the mineral elements (e.g., Ca, Cu, Mg, Mn, Mo, Na, P, Pb, etc.) compared to swine manure, demonstrating that a larger mineral nutrient input is

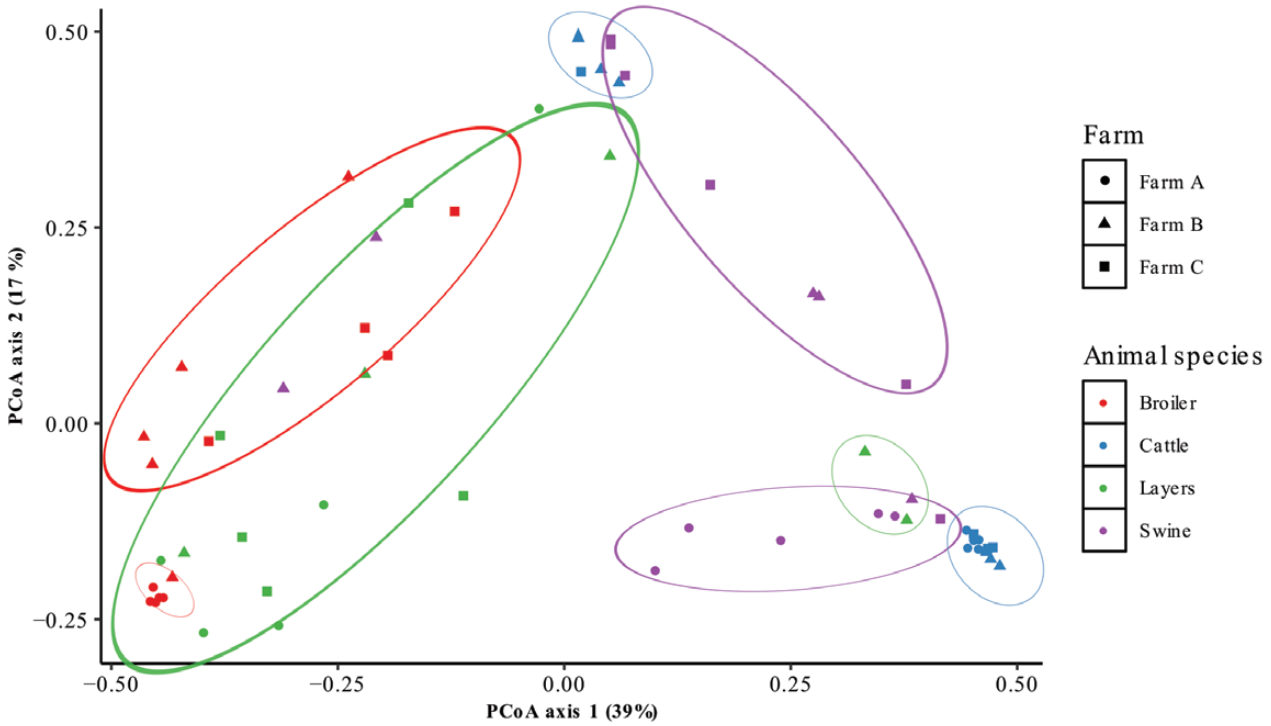

Figure 2. Principal co-ordinated analysis (PCoA) of 16S bacterial profiles (□-diversity indices) with pairwise comparisons between taxa from fecal collected from broilers, layers, swine, and cattle across the farms. *P*-value: farm $P < 0.01$; animal species $P < 0.01$. Number of experimental samples used; $n = 5$ (one sampling time for farm A), $n = 10$ (two sampling times for farms B and C).

required for layers. This is probably related to the high egg and especially egg-shell production that are both physiological sinks for mineral elements.

Cattle feces had significantly lower ($P < 0.01$) levels of total C, total N, calcium (Ca), K, phosphate (P), aluminum (Al), and chromium (Cr); however, cattle feces had a higher C:N ratio than other animals tested. These results are consistent with other data (Sheppard and Sanipelli, 2012; Huang et al., 2017). Their research reported a higher C/N ratio in cattle manure (10.7) than swine (8.0) and chicken (5.1) manures. The average C/N ratios for the broiler chickens (10.5:1) and layer hens (11.0:1) feces were lower ($P < 0.01$) than for cattle (33.2:1), but with a higher N content compared to others, likely due to diets containing high levels of C-rich cellulose and lignin (Kerr et al., 2006; MAFRD, 2015). The C/N ratio can be used as an index of fecal stability, as C/N ratios decrease during composting to values approaching 10:1 (Larney and Hao, 2007). Kerr et al. (2006) reported that increasing dietary cellulose increased manure C content as a percentage of nutrient intake. According to Sheppard and Sanipelli (2012), comparing manure concentrations of beef ($n = 20$) and dairy ($n = 30$), for every mineral element the concentrations for beef were lower than those of dairy. This is may be because beef cattle are fed more roughage and even lower quality feed than are dairy, with the result that more undigested organic matter is passed to the manure thus diluting the elemental concentrations. Beef cattle may also receive fewer mineral supplements.

Average amounts of fecal total C, iron (Fe), sulfate (S), Al, and Cr were greater ($P < 0.01$) for swine than for other animal species. There were significant interactions ($P < 0.01$) between farms and animal species for fecal total C, magnesium (Mg), S, copper (Cu), Zinc (Zn), and lead (Pb) concentrations, indicating that individual farm and animal type influenced fecal nutrient and elemental composition across animal types. Similar to other studies (Kerr et al., 2006; Larney and Hao, 2007; MAFRD, 2015), the present study showed that total C content varied significantly ($P < 0.01$) from 7.5% DM in cattle to 11.4% DM in swine, with a mean of 9.7% DM across all animal species. High total C in swine (11.4% DM) and broiler (10.8% DM) feces suggests high levels of undigested dietary carbohydrate than in feces from cattle and layer hens. Total fecal N and CP values were greater ($P < 0.01$) for broilers than for other animals, which has been related to the feeding of high CP diets and excess N excretion (Kerr et al., 2006; Spiehs et al., 2021).

Concentration of mineral elements in feces differed among animal species (Table 3). Generally, Ca and P were higher ($P < 0.01$) for layer hens than for other animals, but the Zn and Cu contents were greater ($P < 0.01$) for broiler chickens and swine than other animals, indicating that those animals are often fed Zn and Cu to support intestinal function (MAFRD, 2015). In the present study, the Pb content was greater ($P < 0.001$) for layer hens than other animals. Some trace elements such as cadmium (Cd), Pb, and mercury (Hg) concentrations have no biological function in plants and animals and application of these metals to soil has no positive effects on crops and can have problematic impacts when added in excess (Sheppard et al., 2009; MAFRD, 2015). Sheppard and Sanipelli (2012) studied about 60 elements in 124 manure or fecal samples from broilers, layers, turkey, swine, dairy, and beef operations in Manitoba, Canada. In general, same authors reported that the manure from young and growing animals often had greater trace element concentrations than the manure from grown animals. The fecal samples from beef cattle had lesser concentrations of trace elements than were conducted in the swine or poultry manures. Many

Table 3. Nutrients and elements composition in fecal samples from various livestock and poultry

	Farm A				Farm B				Farm C					*P*-value		
Item[1]	B	L	S	C	B	L	S	C	B	L	S	C	SEM	Farm	Anim.	INT
n	5	5	5	5	10	10	10	10	10	10	10	10	—	—	—	
DM	25.8^{b}	29.8^{ab}	31.3^{a}	11.1^{c}	26.4^{b}	43.9^{a}	21.8^{b}	15.9^{c}	22.6^{c}	28.0^{b}	36.0^{a}	19.4^{c}	3.29	0.05	0.01	0.10
pH	6.3^{c}	7.1^{b}	6.6^{c}	7.6^{a}	6.3^{c}	7.1^{bc}	8.2^{a}	7.5^{b}	6.1^{c}	7.6^{a}	7.4^{b}	7.7^{a}	0.22	0.01	0.001	0.01
Nutrient composition, % DM																
Total C	11.2^{a}	9.5^{b}	12.4^{a}	6.4^{c}	9.9^{a}	9.8^{a}	9.9^{a}	8.8^{b}	11.3^{a}	8.0^{b}	11.7^{a}	7.2^{b}	0.79	0.85	0.001	0.01
Total N	1.1^{a}	1.0^{a}	0.8^{ab}	0.3^{b}	1.6^{a}	1.0^{b}	0.4^{c}	0.3^{c}	1.1^{a}	1.0^{a}	0.8^{ab}	0.2^{b}	0.22	0.97	0.001	0.56
C/N ratio	10.8	10.7	14.7	22.0	9.3^{b}	12.1^{b}	25.9^{a}	27.0^{a}	11.3^{b}	10.2^{b}	14.4^{b}	50.4^{a}	6.21	0.43	0.001	0.14
Minerals, %																
Ca	0.68^{b}	1.33^{a}	0.50^{b}	0.12^{b}	0.38^{b}	2.21^{a}	0.39^{b}	0.17^{b}	0.66^{b}	1.97^{a}	0.43^{b}	0.22^{b}	0.174	0.46	0.001	0.13
Fe	0.01	0.13	0.22	0.04	0.04^{b}	0.11^{b}	0.42^{a}	0.03^{b}	0.02^{b}	0.04^{b}	0.18^{a}	0.03^{b}	0.059	0.16	0.001	0.47
K	0.42^{ab}	0.53^{a}	0.21^{b}	0.14^{b}	0.63^{a}	0.58^{a}	0.53^{a}	0.25^{b}	0.51^{a}	0.49^{a}	0.27^{ab}	0.18^{b}	0.050	0.01	0.001	0.30
Mg	0.11	0.12	0.15	0.08	0.09^{b}	0.18^{a}	0.14^{ab}	0.12^{b}	0.13^{b}	0.12^{b}	0.19^{a}	0.10^{b}	0.022	0.33	0.001	0.01
P	0.34^{a}	0.35^{a}	0.29^{a}	0.07^{b}	0.22^{b}	0.41^{a}	0.23^{a}	0.15^{b}	0.32^{a}	0.35^{a}	0.31^{a}	0.12^{b}	0.054	0.73	0.001	0.18
S	0.10^{a}	0.07^{ab}	0.08^{ab}	0.04^{b}	0.07^{b}	0.12^{a}	0.08^{b}	0.05^{b}	0.12^{a}	0.10^{ab}	0.09^{b}	0.06^{b}	0.001	0.01	0.001	0.01
Al	0.01^{c}	0.2^{b}	0.4^{a}	0.05^{c}	0.04	0.1^{b}	0.3^{a}	0.03^{c}	0.01^{b}	0.02^{b}	0.3^{a}	0.04^{b}	0.07	0.16	0.001	0.75
Zn	84.7^{b}	72.7^{b}	172.5^{a}	17.9^{c}	127.2^{a}	115.8^{ab}	67.9^{c}	31.2^{c}	252.5^{a}	122.2^{b}	137.4^{b}	16.9^{c}	29.01	0.01	0.001	0.001
Cr, ppm	1.0^{b}	3.2^{ab}	5.2^{a}	1.2^{b}	1.1^{b}	2.3^{ab}	4.4^{a}	1.0^{b}	^{b}1.2	3.2^{a}	3.1^{a}	1.0^{b}	0.97	0.67	0.001	0.73
Cu, ppm	8.8^{b}	6.5^{b}	19.4^{a}	18.7^{a}	21.1^{a}	12.8^{b}	10.1^{b}	5.0^{c}	16.9^{a}	8.3^{b}	17.6^{a}	5.0^{b}	1.85	0.67	0.001	0.01
Pb, ppm	5.6^{b}	7.6^{a}	5.6^{b}	5.6^{b}	1.9^{b}	10.3^{a}	6.0^{b}	4.1^{b}	0.4	0.5	1.2	0.5	1.07	0.001	0.001	0.01

[1]Anim. = animal. B, L, S, C = broilers, layers, swine, and cattle, respectively. INT = interaction between farm and animal species. Total C = total carbon, total N = total nitrogen, C/N ratio = carbon/nitrogen ratio. Number of experimental samples used; $n = 5$ (one sampling time for farm A), $n = 10$ (two sampling times for farms B and C).

[a–c]Means within row treatment within a farm or between farms (average only) with a different superscript differ at $P < 0.05$. Values without asterisks are not significantly different ($P < 0.05$).

trace elements, and especially the heavy metals, are sturdily retained by soils and can lead to problematic concentrations for animal or human consumption (Sheppard et al. 2009). Fitzgerald and Racz (2001) and Sheppard et al. (2009) reported that concentrations of some unwanted metals, such as Cd, nickel (Ni), Hg, and Pb, were associated with elements added as dietary supplements or for disease suppression, suggesting the Cd, Ni, and Pb were most possible pollutants in the mineral supplements. The occurrence of many of these undesirable elements in manure can be improved by altering the source of mineral supplements.

Antibiotic residues

Intensive animal agriculture has relied on antibiotics as feed additives to promote growth, disease treatment, and prevent diseases in healthy animals considered to be at risk and to enhance growth (Wegener et al., 1999; Boyd, 2001; Teillant et al., 2015). However, intensive feedlot cattle systems are restricted to the United States, the EU, Brazil, and Argentina (Millen et al. 2011). Yet, most antibiotics are still used for growth promotion and prophylaxis in intensive pig and poultry operations in much of the world (Acar et al., 2000; Teillant et al. 2015). ABRs used by the animal industry can enter the environment either directly by excretion from grazing animals or by the spreading of manure as crop fertilizer (Hamscher et al., 2002; Bergmann et al., 2011). There are also occurrences in surface water and ground water that can be recognized to veterinary uses (Hannappel et al., 2014; Bailey et al., 2015), but concentrations frequently drop into the very low levels of range (ng kg^{-1}) and thus are considerably lower than those found in soils (Burke et al., 2016). The most serious consequence of ABR entering the environment is the potential transfer of ARM to humans (Teillant et al., 2015). In addition, ABR may cause other various side effects, including allergies, reproductive disorders, immunopathological effects, mutagenicity, nephropathy (gentamicin), hepatotoxicity, and even carcinogenicity (Bacanli and Basaran, 2019).

The World Health Organization (**WHO**) list of antimicrobials of importance to human medicine contains 32 drug classes (260 individual drugs) listed as important, highly important, or critical for human medicine (WHO, 2011). Of the 260 drugs on the WHO list of antimicrobial agents important for human medicine, only 39 are suggested or recorded for use in cattle, swine, and poultry in the United States (Durso and Cook, 2014). Veterinary ABRs have been noticed frequently in livestock manure, surface water, and manure-amended soil (Martinez-Carballo et al., 2007; Harms and Bauer, 2011) and reached concentrations in soil varied from 2.56 μg kg^{-1} of sulfadimidine to 1,590.16 μg kg^{-1} of chlortetracycline (An et al., 2015). Numerous veterinary antibiotics are poorly adsorbed in animal gastrointestinal organs, subsequent in as much as 30% to 90% of the antibiotic compounds being defecated through feces or urine (Halling-Sorense et al., 1998; Alcock et al. 1999; Aust et al. 2008). In the present study, there was no significant different between farms, but chlortetracycline was the major ABR identified (Table 4). It was the highest in broiler manures (98.2 μg kg^{-1}), followed by swine (27.8 μg kg^{-1}), then cattle (21.7 μg kg^{-1}), and layer hens (8.6 μg kg^{-1}). There were no ABR detected for ractopamine, sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethizole, sulfamethoxazole, and trimethoprim in the feces of the four species of pasture-raised animals. Both chlortetracycline and tetracycline ABR tended to be greater (P = 0.10 to 0.09) in feces from broiler chickens than from layer hens, swine, and cattle. Lincomycin (0.05 to 0.85 μg kg^{-1}) and oxytetracycline (0.0 to 0.42 μg kg^{-1}) ABR were similar among animal species.

The greater chlortetracycline and lesser lincomycin, and oxytetracycline ABR detected in the manures of free-range poultry and grazing animals in this study was probably due to the several factors, such as antibiotic-containing manure used as a fertilizer or occur naturally in soils and water (D'Costa et al., 2006, 2007; Durso et al., 2012, 2016). A major reason for this finding may be that organic manure is commonly used as fertilizer in crop fields. The concentration of antibiotics in vegetable soil ranged from 0.29 to 1,590.16 μg kg^{-1}. Earlier studies indicated the manure applied to vegetable fields is often measured to be the most significant sources of antibiotics to soil (Hamscher et al. 2002; Brambilla et al. 2007; Karci and Balcioglu 2009). Likewise, studies investigated during the last two decades show that more than 40 different drugs can be found in river water, in groundwater, and even in drinking water sources from the nanogram per liter to the microgram per liter range (Jorgensen and Halling-Sorensen, 2000; Kummerer, 2001). Furthermore, An et al. (2015) reported that the concentrations of tetracyclines and sulfonamides including sulfadiazine, sulfamerazine, sulfadimidine, and sulfamethoxazole were in the range of the concentrations observed in different research areas, according to the previous literatures in manure, soil, and sludge. Additionally, Zho et al. (2010) reported that fecal samples collected from chickens (n = 54) and cows in confinement (n = 28) had oxytetracycline concentrations of 59,060 and 59,590 μg kg^{-1}, and chlortetracycline concentrations of 21,060 and 27,590 μg kg^{-1} for chickens and cattle, respectively. In a study conducted in China, pig manure (n = 30), chicken manure (n = 20), and soil samples (n = 30) had chlortetracycline, oxytetracycline, and tetracycline concentrations of 46,000, 29,000, and 23,000 μg kg^{-1}, respectively (Martinez-Carballo et al., 2007). Another study reported that the most frequently detected ABRs in pig manure were doxycycline, sulfadiazine, and lincomycin (Rasschaert et al., 2020). In the present study, feces from pasture-raised animals had a much lower average range of tetracycline-like ABR (0.96 to 98.2 μg kg^{-1}) when compared to average ranges of ABR in confinement animal farms in Austria (23,000 to 46,000 μg kg^{-1}) and China (21,06 to 59,590 μg kg^{-1}; Montforts et al., 1999; Martinez-Carballo et al., 2007; Zho et al., 2010). This phenomenon is mainly caused by the extensive production and overuse of antibiotics in China due to no restrictions on its use in animal feed (An et al., 2015).

ARG prevalence in fecal microbiota

AR and ARG challenge the effectiveness of antibiotic treatments for both humans and animals (Spellberg and Gilbert, 2014). Resistant microbes and ARG can circulate among humans, animals, food, water, and the environment. Because many antibiotics commonly used in subtherapeutic concentrations are identical or similar to antibiotics used in human medicine, the development of AMR and their transmission from animals to humans could reduce antibiotic effectiveness in humans (Marshall and Levy, 2011). The commonly identified ARG classes in domesticated animal manure include tetracycline (*tet*), sulfonamides (*sul*), β-lactams (*bla*), macrolide–lincosamide–streptogramin (*erm*), and fluoroquinolone (*fca*) (Qian et al., 2018; Hurst et al., 2019), which correspond to the major classes of antibiotics used by the animal industry (Durso and Cook, 2014; Checcucci et al., 2020). Of these five ARG classes, *tet* (10^2 to 10^{12} copies per gram) and *sul* (10^8 to 10^{11} copies per gram) are the most abundant ARG appearing in manure from confinement swine and poultry farms (He et al., 2020) along with organic farming operations in Nebraska, United States (Cadena et al., 2018). Substantial changes in the abundances of ARGs in

Table 4. ABR[1] profiles for the four domesticated animal fecal samples

Item, μg kg^{-1}	Farm A[2]	Farm B[2]	SEM	*P*-value
n	5	10	—	—
Chlortetracycline	60.2	59.11	36.64	0.98
Lincomycin	0.69	2.35	1.26	0.39
Oxytetracycline	0.98	0.01	0.40	0.13
Ractopamine	< LOD	< LOD	—	—
Sulfadiazine	< LOD	< LOD	—	—
Sulfadimethoxine	< LOD	< LOD	—	—
Sulfamethazine	< LOD	< LOD	—	—
Sulfamethizole	< LOD	< LOD	—	—
Sulfamethoxazole	< LOD	< LOD	—	—
Sulfathiazole	< LOD	< LOD	—	—
Tetracycline	1.97	2.53	1.17	0.83
Trimethoprim	< LOD	< LOD	—	—

Item	Animal type				SEM	*P*-value
	Broiler	Layer	Swine	Cattle		
n	25	25	25	25	—	—
Chlortetracycline	98.2	8.6	27.8	21.7	37.76	0.11
Lincomycin	0.85	0.05	0.70	2.92	1.49	0.19
Oxytetracycline	0.40	< LOD	0.42	< LOD	0.28	0.31
Ractopamine	< LOD	< LOD	< LOD	< LOD	—	—
Sulfadiazine	< LOD	< LOD	< LOD	< LOD	—	—
Sulfadimethoxine	< LOD	< LOD	< LOD	< LOD	—	—
Sulfamethazine	< LOD	< LOD	< LOD	< LOD	—	—
Sulfamethizole	< LOD	< LOD	< LOD	< LOD	—	—
Sulfamethoxazole	< LOD	< LOD	< LOD	< LOD	—	—
Sulfathiazole	< LOD	< LOD	< LOD	< LOD	—	—
Tetracycline	4.1	0.05	0.8	0.4	1.82	0.09
Trimethoprim	< LOD	< LOD	< LOD	< LOD	—	—

[1]Only ABR samples were equally mixed from three farms into each animal species prior to analysis, due to the low levels (or not detected) of ABRs detected. SEM = standard errors of the means. Number of experimental samples used; *n* = 5 (one sampling time for farm A), *n* = 10 (two sampling times for farms B and C).
[2]The limit of detection (< LOD).

livestock waste among livestock species have been observed (He et al., 2020), which may be due to varying antibiotic usage and dosing patterns. In this study, the average occurrence of *Sul*1 and *Tet*A ARG were the highest in the feces of layers (16.5×10^4 and 1.4×10^4 copies per gram), followed by broilers (2.9×10^4 and 1.7×10^4 copies per gram), swine (0.22×10^4 and 0.20×10^4 copies per gram), and beef cattle (0.19×10^4 and 0.02×10^4 copies per gram) (Table 5) which is similar to other studies (Yu et al., 2005; Selvam et al., 2012; Cheng et al., 2013).

All the three farms were positive for ARGs, demonstrating that ARGs are common in agricultural manure and soils, even in the absence of routine antibiotic drug or pesticide use. These data support other work done in organic farming operations examining ARGs in organic poultry, swine, and cattle production, where ARGs were also identified even when antibiotic drugs were not directed to animals (Stanton et al., 2011; Rothrock et al., 2016). It was not unexpected to identify *Sul*1 and *Tet*A ARGs at every farm sampled, as they occur naturally in soils, and have been detected in soils and water from around the globe (D'Costa et al., 2006, 2007; Allen et al., 2010; Durso et al., 2012, 2016). The abundance of total ARGs in untreated livestock waste varies from 10^6 to 10^{11} copies per gram dry weight or 10^6 to 10^{12} copies per mL (absolute abundance), and 10^{-3} to 10^{-1} copies per 16S ribosomal RNA (rRNA; relative abundance) (He et al., 2020). Similarly, Yu et al. (2005) and Cheng et al. (2013) reported that manure from poultry and swine samples contained greater abundance of *Sul* and *Tet* genes than cattle or sheep manure samples, respectively. Generally, chicken and swine waste show higher ARG abundances than cow waste (He et al., 2020). These variances may be as a result of the more intensive use of antibiotics on chicken (148 mg kg^{-1}) and swine (172 mg kg^{-1}) farms in comparison to cattle (45 mg kg^{-1}) farm (Van Boeckel et al., 2015).

There was a significant interaction ($P < 0.04$) between farm and animal species for *Sul*1, indicating that the *Sul*1 ARG was more prevalent in layer hen feces from farm 2 than from other animal species and farms 1 and 3. However, all farms were positive for at least three ARGs, demonstrating the prevalence of these genes in the feces of animals that did not routinely receive antibiotics. Similarly, Stanton et al. (2011) and Rothrock et al. (2016) reported that ARGs were positively detected in the feces of organically reared cattle, swine, and poultry, where ARGs were detected even when antibiotics were not administrated to animals (Cadena et al., 2018). These results are consistent with other studies (Allen et al., 2010; Durso et al., 2016). However, more research is needed to fully assess the prevalence of ARG in pasture-raised animal manure across farms, geophysical conditions (e.g., soil types), and seasons (e.g., weather).

Microbial community diversity

The alpha- (richness and abundance) and beta-diversities (variation of microbial communities between samples) of

Table 5. Bacterial phylum profiles and antimicrobial resistance genes (ARGs) for the four domesticated animal metagenome samples

Item[1]	Farm A				Farm B				Farm C					*P*-value		
	B	L	S	C	B	L	S	C	B	L	S	C	SEM	Farm	Anim	INT
n	5	5	5	5	10	10	10	10	10	10	10	10				
DNA concentration[2], ng μL^{-1}	20.2	34.1	36.0	23.7	21.9	24.2	34.9	22.1	21.7	14.2	16.9	19.9	5.64	—	—	—
Bacterial phylum																
Firmicutes (F)	98.9^{a}	83.8^{ab}	71.0^{b}	75.8^{b}	72.3^{a}	73.5^{a}	47.4^{b}	51.1^{b}	84.8^{a}	82.5^{a}	74.7^{b}	59.3^{c}	4.77	0.001	0.001	0.19
Bacteroidetes (B)	0.2^{d}	3.5^{c}	17.5^{b}	19.3^{a}	4.3^{c}	9.0^{c}	16.0^{b}	26.8^{a}	2.8^{b}	4.6^{b}	16.1^{ab}	23.1^{a}	3.52	0.37	0.001	0.95
Actinobacteria	0.3	1.3	1.4	0.1	6.9^{b}	3.3^{b}	17.6^{a}	8.3^{b}	3.4	2.6	2.4	0.5	2.56	0.001	0.21	0.12
Proteobacteria	0.5	10.6	0.7	0.4	16.1	7.8	14.9	7.9	8.4	6.8	2.9	13.2	3.64	0.03	0.90	0.12
F/B ratio[3]	1.0	1.0	0.7	0.7	0.9^{a}	0.8^{ab}	0.6^{b}	0.5^{b}	0.9^{a}	0.9^{a}	0.8^{ab}	0.6^{b}	0.07	0.01	0.01	0.91
ARGs, ×10^{4}																
*Sul*1	0.7	0.4	0.08	0.2	7.7^{b}	48.5^{a}	0.5^{c}	0.2^{c}	0.4	0.7	0.1	0.1	0.871	0.04	0.12	0.04
*Tet*A	2.6^{a}	1.4^{ab}	0.1^{b}	0.04^{b}	2.0^{a}	2.3^{a}	0.2^{b}	0.02^{b}	0.7	0.4	0.4	0.07	0.456	0.08	0.001	0.21

Average	Farm					Animal type					
	A	B	C	SEM	*P*-value	B	L	S	C	SEM	*P*-value
DNA concentration[2], ng μL^{-1}	28.5	25.8	18.2	2.82	—	21.3	24.2	29.3	21.9	3.76	—
Bacterial phylum											
Firmicutes (F)	82.4^{a}	61.1^{c}	75.3^{b}	2.29	0.01	85.3^{a}	80.0^{a}	64.4^{b}	62.1^{b}	3.06	0.01
Bacteroidetes (B)	10.1	14.0	11.6	1.69	0.18	2.4^{c}	5.7^{c}	16.5^{b}	23.0^{a}	2.28	0.001
Actinobacteria	0.8^{b}	9.0^{b}	2.2^{a}	1.30	0.01	3.6^{ab}	2.4^{b}	7.1^{a}	3.0^{ab}	1.73	0.05
Proteobacteria	3.0^{b}	11.7^{a}	7.8^{ab}	1.84	0.01	8.3	8.4	6.2	7.2	2.46	0.53
F/B ratio[3]	0.9^{a}	0.7^{b}	0.8^{ab}	0.05	0.01	1.0^{a}	0.9^{a}	0.7^{b}	0.6^{b}	0.05	0.01
ARGs, ×10^{4}											
*Sul*1	0.4^{b}	1.4^{a}	0.3^{b}	0.41	0.05	2.9^{b}	16.5^{a}	0.22^{b}	0.19^{b}	0.552	0.04
*Tet*A	1.1^{a}	1.1^{a}	0.4^{b}	0.24	0.02	1.7^{a}	1.4^{a}	0.2^{b}	0.02^{b}	0.32	0.001

[1]Anim. = animal. B, L, S, C = broilers, layers, swine, and cattle, respectively. INT = interaction between farm and animal species. *n* = number of experimental samples used. Sulfonamide resistance (*Sul*1; 10^{4}), tetracycline resistance (*Tet*A; 10^{4}). Number of experimental samples used; *n* = 5 (one sampling time for farm A), *n* = 10 (two sampling times for farms B and C).
[2]DNA concentration was measured before normalization for amplicon sequencing.
[3]Firmicutes/Bacteroides ratio = F/B ratio. Overall, 26 phyla were detected, but only four phyla were found in all animals as dominant phyla (cutoff: >1.0%).
[a–c]Means within row treatment within a farm or between farms (average only) with a different superscript differ at $P < 0.05$. Values without asterisks are not significantly different ($P < 0.05$).

bacterial communities were measured by OTUs, Shannon index, and PCoA analyses, respectively, among farms and animal species (Figures 1 and 2). Results indicated that observed OTU richness and Shannon index (the variety and abundance of species) differed significantly ($P < 0.01$) in samples collected from different farms and across animal species (Figure 1). Besides, the variety and abundance of bacterial species measured by the Shannon index, and the bacterial composition of the broiler chickens and layer hen feces were significantly different ($P < 0.01$) among farms. The number of OTU (bacterial richness) in the layer hen and swine feces was not different ($P > 0.10$) among farms, but OTUs in the broiler and cattle feces were significantly different ($P < 0.01$) among farms. This agrees with data of Wongsaroj et al. (2021), who reported that the greater dissimilarity in bacterial communities was found generally between the manures from different animal species (chicken, cattle, deer, swine, rabbits, and goats), while the slight variation in bacterial communities was found between the animal breeds (beef vs. dairy cattle, black-borne chickens vs. yellow-feather chickens), or the feeding diets (Pangola grass vs. Napier grass).

Furthermore, PCoA (multidimensional scaling; a similarity matrix) was conducted to compare the bacterial profiles among farms and animal types (Figure 2). As shown in Figure 2, PCoA axes 1 and 2 accounted for 39.0% and 17.0%, respectively. Fecal bacterial communities have a high degree of variability ($P < 0.01$; two distinct clusters across the farms) among farms and animal species (Figure 2) through PCoA axes 1 and 2, which indicated farm locations and animal species impacted the fecal microbial community in pasture-raised animals. Although this occurrence may have been noticed by researchers in previous scientific research studies, prior to our experiment, no formal report on the individual variations among animal species has been published. Results indicate that broiler, layer, swine, and cattle fecal samples have a wide degree of variability in microbial community composition across the pasture-raised, "no antibiotics ever" farms. Similar to our findings, a previous study followed the successional changes in the fecal microbiome of poultry and livestock and identified the effect of animal species, age, and dietary composition on PCoA clustering in manure from poultry (Lourenco et al., 2019a, b), beef cattle (Durso et al., 2010), dairy cattle (Mao et al., 2015), and swine (Guevarra et al., 2018).

When classified at the phylum level, there were 26 phyla observed but only four phyla had an abundance greater than 0.5% to 1.0% in one or more of the animal species (Table 5). Across animal species, Firmicutes (62.1% to 85.3%), Bacteroidetes (2.4% to 23.0%), Actinobacteria (2.4% to 7.1%), and Proteobacteria (6.2% to 8.3%) represented the major phyla in the fecal samples, which was consistent with previous reports (Mosites et al., 2017; Wongsaroj et al., 2021). Mosites et al. (2017) reported that the most abundant phyla among all samples (poultry, cattle, and human) were Firmicutes and Bacteroidetes, followed by Proteobacteria (56.4%, 27.7%, and 5.1% average abundance, respectively). Interestingly, Firmicutes were the most prevalent phyla in all animal species (62% to 85%), which is similar trend to other studies (Videnska et al., 2014; Ming et al., 2017). Regardless of diet, Firmicutes were the predominant bacterial phyla in the pasture-raised broiler chickens (63%; Lourenco et al., 2019a, b), Mongolian cattle (51.2%; Ming et al., 2017), camels (63%; Ming et al., 2017), feedlot cattle (65% to 70%; Bessegatto et al., 2017), and dairy cattle (60%; Hagey et al., 2019). This agrees with data of Mote et al. (2019), who reported a considerably larger number (up to 90%) of Firmicutes and Bacteroidetes phyla in steers grazing tall fescue (*Festuca arundinacea*). Results from the present experiment indicate that the microbial composition of feces from broiler and layer chickens were dominated by Firmicutes (85% to 80%) followed by Proteobacteria (8.3%). The ratio of Firmicutes to Bacteroidetes was a greater ($P < 0.01$) for broiler chickens than for other animal species. Bacteroidetes formed only 2.4% to 5.7% of the total fecal microbiota of broilers and layers, respectively, which is similar to what Videnska et al. (2014) reported. However, the Bacteroidetes (23.2%) population was greater ($P < 0.001$) in the feces from cattle than broilers (2.4%), layers (5.7%), and swine (16.5%) (Table 5). Bacteroidetes are increasingly regarded as specialists for the breakdown of organic matter (i.e., proteins and carbohydrates) (Thomas et al., 2011). Fecal Bacteroidetes were nearly 9-fold greater from cattle (23.0%) than broilers (2.4%), and this phylum had the highest abundance in the cattle manure (26.8%; Table 5), compared to other animals. In the current study, Proteobacteria were less variable (6.2% to 8.4%) and were the third most abundant phylum across the animal types. Actinobacteria were the fourth most prevalent phylum and varied across the animal types. Actinobacteria were more abundant in the layer hen feces (7.1%) than in feces from the other animal species (2.4% to 3.6%). Mosites et al. (2017) also reported that fecal samples from cattle were dominated by Firmicutes and Bacteroidetes, although a minor division of cow samples (13 out of 123) were dominated instead by Proteobacteria and Actinobacteria OTUs.

At the genus level, the most predominant were *Lactobacillus* (56.7%, 40.1%, 7.5%, and 0.2%), *Clostridium* (0.1%, 0.5%, 2.2%, and 1.5%), *Ruminococcus* (0.1%, 1.7%, 5.3%, and 16.9%), *Bacteroides* (0.5%, 2.2%, 0.5%, and 0.3%), *Faecalibacterium* (0.1%, 0.3%, 0.2%, and 0.01%), and unclassified bacteria derived from *Clostridiales* (1.3%, 1.1%, 3.6%, and 1.8%) in broiler, layer, swine, and cattle fecal samples, respectively. In mature chickens, *Lactobacillus* (35% to 60%) has been found to be the dominant bacterial genus in the GI (Gong et al., 2007; Xiao et al., 2017), which helps explain the results from the present study. Other studies have also found that the chicken GI was dominated by *Lactobacilli* and *Lactobacillales* (Bjer-rum et al., 2006; Dumonceaux et al., 2006; Mohd Shaufi et al., 2015). However, in the current study on grazing-based diets, the most abundance genera were *Ruminococcaceae* (16.9%). This result is supported by a previous study (Mote et al., 2019), indicating that *Ruminococcaceae* were core genera for pasture-raised cattle. Mote et al. (2019) reported that the relative abundances of both *Ruminococcaceae* and *Lachnospiraceae* families significantly increased in steers grazing tall fescue pasture over a 28-d period. Both bacterial families include cellulose- and hemicellulose-degrading bacteria and contribute to butyrate production (La Reau et al., 2016; La Reau and Suen, 2018). Butyrate is a main microbial fermentation product in the GI of humans and all animal species and contributes to the daily metabolizable energy requirement of ruminants and humans (Bergman, 1990). Production of volatile fatty acids, especially butyrate, in the gut microbiome is required for optimal health but is frequently limited by the lack of fermentable fiber in the diet (Baxter et al., 2019). These data provide insight into the composition of the core fecal microbiota and ARG of commercial farms and different animal types.

Conclusions

Given the diversity of alternative animal management styles, the results from this study cannot represent the AR landscape in all "no antibiotic ever" farming systems. Such a feat would take

multiple comprehensive surveys covering different geographical regions, which was beyond the scope of this preliminary study. Our study concluded that fecal samples from pasture-raised animals can serve as a reference for determining accurate target levels of ARG in animal production. This suggests that *Sul*1 and *Tet*A occurrence could serve as a valuable indicator of recent manure-borne resistance in the environment, and that there is possible advantage in monitoring this gene over time when manures are land-applied. They also deliver valuable evidence for studies examining the ecology of AR on farms and in fields. This study indicated that the microbial diversity, ABR, ARG concentrations, and types in feces varied from farm-to-farm and from animal species-to-animal species. Further controlled studies are needed to more fully understand the influence of both animal types and farm management practices (e.g., dietary components) on antibiotic resistomes (resistance genes and their precursors) and its interactions if these relationships are broadly applicable across different dimensional and sequential scales.

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Conflict of interest statement

The authors have no financial conflicts of interest to declare.

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