A Longitudinal Investigation of the Effects of Age, Dietary Fiber Type and Level and Injectable Antimicrobials on the Fecal Microbiome and Antimicrobial Resistance of Finisher Pigs

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LAY SUMMARY: Bacterial communities in the gut and of the feces are strongly influenced by a number of factors, particularly the age of the animal and the diet. In addition, antibiotic administration routinely used to treat bacterial diseases can also impact the community composition. A study with finisher pigs was conducted to evaluate age-related changes, effects of types, distiller's dried grains with solubles (DGGS) or. sugar beet pulp (SBP) and levels of dietary fiber, and injectable antibiotics on the fecal bacterial composition and antibiotic resistance in fecal bacteria. Fecal samples were collected from five pigs in each of the three dietary treatment groups, control diet with no supplement or supplemented with DDGS or SBP, on days 98, 110, 144 and 177 of age and on days 1 and 3 after the first injection of antibiotics, ceftiofur or penicillin G. Samples were analyzed to identify the bacterial community composition and prevalence of antibiotic resistance in fecal bacteria. Data generated suggested the overall bacterial composition changed with age and diet, and age appeared to have a greater impact than diet. Antibiotics had only a modest impact on the bacterial community and had minimum impact on antibiotic resistance of fecal bacteria.

TEASER TEXT: Age, diet and antimicrobial treatment can impact the overall hindgut microbiome with minimal impact on antimicrobial resistance in finisher pigs.

ABSTRACT: Age and diet are among the factors that influence the community composition of the fecal microbiome. Additionally, antimicrobial use can alter the composition of bacterial communities. An 86-d study with finisher pigs aimed to evaluate age-related dynamics (d 98-177 of age), effects of types and levels of dietary fiber, and injectable antimicrobials on the fecal microbiome and antimicrobial resistance (AMR) was conducted. A total of 287 pigs, housed in 36 pens, with 7 to 8 pigs per pen, fed a corn grain and soybean meal-based basal diet, formulated to contain 8.7% neutral detergent fiber (NDF), were randomly assigned to one of three treatments: 1. basal diet with no supplement, 2. basal diet supplemented with 20% distillers dried grains with solubles (DDGS) formulated to contain 13.6% NDF, or 3. basal diet supplemented with 14.5% sugar beet pulp (SBP) formulated to contain 13.6% NDF. Five finisher pigs from each treatment group were selected randomly, and fecal samples were collected on d 98, 110, 144, and 177 of age. In addition, fecal samples were collected from pigs that were injected intramuscularly ceftiofur hydrochloride or penicillin G on d 1 and 3 along with pen-mate untreated controls on d 1. Fecal samples were subjected to 16S rRNA amplicon-based microbiome analysis and culture methods to quantify the abundance of total and AMR coliforms and enterococci populations. The alpha diversity, such as species richness, increased with age, and the overall bacterial composition changed with age (P =0.001) and diet (P = 0.001). Diet-associated shifts in the specific bacterial taxa were observed. The richness, diversity, and evenness of bacterial taxa did not differ between pigs that were injected with ceftiofur versus their untreated pen mates or by dietary treatments, but differed in pigs that received penicillin G injection. Both antimicrobial treatments contributed to changes in the overall fecal bacterial composition at the genus level. Collectively, the data demonstrate that both age and the diet (control vs. DDGS-, control vs. SBP- or DDGS- vs. SBP-based diets) were associated with overall bacterial community composition and the impact of age on variations in fecal microbiome composition was greater

than the diet. Antibiotic treatment had minimal effect on bacterial diversity and relative abundance of taxa. Further, diets and antimicrobial treatment had minimal impact on the overall counts of AMR coliforms and enterococci populations in feces.

Key words: antimicrobials, antimicrobial resistance, dietary fiber, fecal microbiome, finisher pigs.

List of Abbreviations

ADFI: Average daily feed intake

ADG: Average daily gain

AMR: Antimicrobial resistance

ASV: Amplicon sequence variant

CFU: Colony-forming units

DDGS: Distiller's dried grains with solubles

ENT: *Enterococcus* agar

G/F: Gain to Feed ratio

MAC: MacConkey agar

NDF= Neutral detergent fiber

rRNA: Ribosomal RNA

SBP: Sugar beet pulp

INTRODUCTION

The composition of the gut microbial community is influenced by several factors, including the age of animals, diet, host immunity and genetics, and antimicrobial use (Looft et al., 2012; Relman, 2012; Scott et al., 2013). It has been suggested that gut microbial colonization is influenced by diet and progresses with the age of animals. Although microbial colonization of the pig intestine starts at birth and develops during the neonatal period to weaning time, the intake of solid feed after weaning has a significant impact on microbial community structure (De Rodas et al., 2018).

Swine are usually raised in confinement facilities, in farrow-finish or aggregated management systems, with the purpose of controlling infectious diseases. Historically, antimicrobials have been routinely provided therapeutically to individual animals to treat production-related diseases, such as respiratory, digestive, and joint diseases or metaphylactically to prevent the spread of infection (de Lange et al., 2010). The routine use of antimicrobials can have adverse effects on the normal and beneficial pig gut microbes and ultimately promote prevalence and spread of antimicrobial resistance (AMR) (Joyce et al., 2019), which can be transferred to humans directly through the food chain or through the environment via animal waste. Antimicrobial use has been shown to significantly increase overall AMR gene (i.e., resistome) abundance in growing pigs (Ghanbari et al., 2019). Thus, due to the growing concerns over AMR and with the removal antimicrobials as growth promotors since 2017 in the US (FDA, 2013), commercial swine producers have adopted practices to minimize or reduce and to reflect a more responsible use of ~~medically important~~ antimicrobials for the treatment and prevention of diseases in pigs (Zangaro, 2019). A study reported by Diana et al. suggested that removal of in-feed antimicrobials is possible with minor impact on animal performance and health, which can be addressed by the use of parenteral antibiotics and sound husbandry practices (Diana et al., 2019). However, such

practices can have negative impacts with pig health and with reductions in production performance, increased mortality in the finisher phase, and overall animal welfare issues (Parois et al., 2020); in particular, these have been seen with removal of in-feed prophylactic antimicrobials while allowing the continued use of parenteral antibiotics in swine. Thus, it is essential to find alternative strategies to improve pig performance and health for swine production. Diet modification that impacts gut microbiome could be an alternative strategy for swine producers to pursue.

The fiber component of the pig diet has been shown to be a significant factor in shaping the pig gut microbiome (Wang et al., 2019) and improving gut health and performance (Everts, 2010; Niu et al., 2019). Distillers dried grains with solubles (DDGS), a by-product of ethanol fuel production, and sugar beet pulp (SBP), a by-product of sugar beet processing, are widely available in the US to achieve a higher level of neutral detergent fiber (NDF) in weaned pig diets (Thomson et al., 2012; Li et al., 2020a). The fiber in DDGS is primarily insoluble, hence, less degradable than fiber in sugar beet, which is more soluble and degradable (Zhang et al., 2013; Li et al., 2020b). A recent study showed that DGGS and SBP-based diets increased the abundance of *Lactobacillus* species, which are beneficial commensal bacteria in the cecum and colon of pigs (Thomson et al., 2012). Inclusion of soluble fiber with carbohydrase supplementation has been shown to protect weaned pigs against enterotoxigenic *E. coli* (Li et al., 2020a). The study also showed that moderate inclusion of dietary fiber in the diet increased gut maturation and production performance of the piglet, with less incidence of diarrhea and less need for antimicrobial interventions (Hermes et al., 2009).

Despite the influence of dietary fiber on pig growth performance and health, there is limited information concerning how different levels and sources of dietary fiber impact the fecal microbiome community composition, and whether its effects interact with antimicrobial

resistant bacteria in the finisher pigs. In addition to the diet, age is another factor that can also modulate the gut microbiome composition (Chen et al., 2017) and occurrence of AMR in the fecal bacteria of pigs (Gaire et al., 2020); all while potentially interacting with dietary effects. However, information related to age-related dynamics, and the effects of diet with different levels and sources of fiber, and with or without concurrent antimicrobial treatments, on the fecal microbiome taxonomic composition and AMR of finisher pigs remains unclear, which could potentially signal a new and more sustainable alternative to antibiotics.

Therefore, we investigated the effects dietary fiber that differed in source (DDGS vs. SBP) type (soluble vs. insoluble) and level (8.7% NDF in control vs. 13.6% NDF in DGGS- or SBP-based diets) in a production pig cohort in the finisher phase. We evaluated, the effects of diets, age related dynamics and therapeutic antimicrobial treatments (ceftiofur hydrochloride or penicillin G) – and their interactions – on the fecal microbiome and phenotypic AMR in indicator bacterial (coliforms and enterococci) populations in finisher pigs. Our hypothesis was that there would be age-related dynamics and interacting effects of both the diet and antimicrobial treatments on the fecal microbiome and concentrations of AMR fecal bacteria, i.e., AMR coliforms and AMR enterococci.

MATERIALS AND METHODS

Animals, study design and diets

The study was performed at the Kansas State University Swine Teaching and Research Center, Kansas State University, Manhattan, Kansas. The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Kansas State University. A total of 287 pigs (DNA 600 × 241; initial BW 50.1 kg) were housed in a fully enclosed, environmentally regulated barn containing 36 pens. The pigs were previously used in an amino acid nutritional study and had not received any antibiotics in the feed or water. Pens

were equipped with a dry, two-space, single-sided feeder (Farmweld, Teutopolis, IL) and a cup waterer. Pigs were allowed *ad libitum* access to feed and water. Floor space allowance per pig was at 0.73 m^2. Pens were housed on a completely slatted concrete floor with a 1.3 m pit underneath for manure storage. An automatic feeding system (FeedPro; Feedlogic Corp., Wilmar, MN) was used to deliver and record daily feed additions to each pen. Each pen contained 7 or 8 pigs per pen with an equal number of barrows and gilts in each pen. Pigs were allowed ad libitum access to feed and water. Pens were randomly assigned to one of three dietary treatments: The basal diet was corn grain and soybean meal-based, and the three treatment groups were: 1. control diet with no supplement formulated to contain 8.7% neutral detergent fiber (NDF), basal diet supplemented with 20% distillers dried grains with solubles (DDGS) formulated to contain 13.6% NDF, or 3. basal diet supplemented with 14.5% sugar beet pulp (SBP) formulated to contain 13.6% NDF. The study duration was 86 days and the experimental diets were fed to the finisher pigs in three phases: phase 1 from d 91 to 109, phase 2 from d 110 to 143, and phase 3 from d 144 to 177 of age (market age). On d 177 of age, 86 d after feeding the experimental diets, final pen weights and individual weights were taken, and pigs were transported to a commercial packing plant. The diets were formulated and prepared at the Kansas State University O.H. Kruse Feed Technology Innovation Center (Manhattan, KS). The ingredients and chemical compositions of the three diets are detailed in Tables 1 and 2, respectively. Pigs and feeders were weighed to determine average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (G:F).

Longitudinal sampling of untreated pigs

Five pigs from each of the three dietary treatment groups (control, DDGS-based, and SBP-based diets) were randomly selected from different pens and sampled longitudinally during the finishing phase. Fecal samples were collected rectally from the same five pigs at

four age-points: at d 98 (a week after moving pigs into the finisher barn and the feeding of the experimental diet), 110, 144, and 177 (day of transportation for harvest) of age. These five pigs did not receive any antibiotics in the study. At each collection, approximately 50 g of fecal samples were placed in Whirl-Pak® bags (Nasco, Ft. Atkinson, WI) and transported to the laboratory. Fecal aliquots mixed with 50% glycerol were stored at -80°C for later analysis until DNA extraction, microbiome analysis, and quantification of AMR fecal bacteria (i.e., counts of coliforms and enterococci in the presence of antimicrobial) performed. A total 55 fecal samples were collected (Table 4).

Ceftiofur- and penicillin G-treated pigs

Pigs that exhibited clinical signs of respiratory infection, diarrhea, arthritis, or other production-related diseases indicative of bacterial infections were treated with ceftiofur hydrochloride (Excenel® RTU EZ, Zoetis Animal Health, Kalamazoo, MI; 50 mg/ml) injected intramuscularly (IM) at 4.4 mg/kg body weight (4 mL/45.4 kg BW of the 50 mg/mL product) or with procaine penicillin G at 1,500 U per 45.4 kg BW (Norocillin®, Norbrook Inc. Overland Park, KS). Injections were once a day for three consecutive days. Fecal samples were collected from ceftiofur hydrochloride- and penicillin G-treated pigs on d 1 and 3 of the treatment regimens, approximately 6 h (and no later than 8 h) after the injection. When pigs were not given the full 3-day treatment regimen due to rapid improved clinical signs, samples were collected only on d 1. Also, on d 1 of the treatment, a fecal sample was collected from an untreated pen-mate pig. A total of 40 fecal samples from ceftiofur-treated pigs along with untreated pen-mate control, and another 45 fecal samples from penicillin G treated pigs along with untreated pen-mate control were collected (Table 4). Fecal samples were transported to the laboratory and fecal aliquots mixed with 50% glycerol and stored at -

80°C until DNA extraction, microbiome and quantification of AMR coliforms and enterococci, were performed.

16s rRNA-based microbiome analysis

Fecal DNA Extraction

A total of 140 fecal samples 55 from longitudinal sampling of untreated pigs and 85 from ceftiofur or penicillin G along with untreated pen-mate control) were subjected for DNA extraction. Fecal DNA was extracted using the protocols published by Yu and Morrison (Yu and Morrison, 2004) and modified by Korte et al (Korte et al., 2020). Briefly, fecal samples were transferred into round-bottom tubes (2 mL) containing 800 µL of lysis buffer (4% w/v sodium dodecyl sulfate, 500 mM NaCl, and 50 mM EDTA), and a single steel bead (0.5 cm diameter). Samples were then heated at 70° C for 20 min with vortexing, then homogenized using a TissueLyser II (Qiagen, Venlo, The Netherlands) for 3 min at 30 per s, and then centrifuged at 5,000 × g for 5 min at room temperature. The supernatant was then transferred into an Eppendorf tube (1.5 mL; Thermo Fisher Scientific), and mixed with 200 µL of 10 mM ammonium acetate, incubated for 5 min on ice, and then centrifuged at 5,000 × g for 5 min. The supernatant (up to 750 µL) was mixed with an equal volume of isopropanol, incubated for 30 min on ice, and centrifuged at 16,000 × g at 4° C for 15 min. The recovered DNA pellet was washed and resuspended in 150 µL of Tris-EDTA. After the addition of proteinase-K (15 µL) and Buffer AL (200 µL) (DNeasy Blood and Tissue kit, Qiagen, Germany), samples were incubated at 70° C for 10 min. In each tube, 200 µL of 100% ethanol was added, vortexed, transferred to a spin column, and processed according to the manufacturer's instructions (Qiagen, Germany). The DNA concentration was

measured by fluorometry (Qubit 2.0, Life Technologies, Carlsbad, CA) using Quant-iT broad range (or high sensitivity) dsDNA reagent kits (Invitrogen, Carlsbad, CA).

16S rRNA gene sequencing

Extracted DNA was processed at the University of Missouri Metagenomics Center. The 16S rDNA amplicons (V4 region) were created with universal primers (U515F/806R) (Caporaso et al., 2011; Walters et al., 2011) against the V4 region (flanked by Illumina standard adapter sequences (Illumina Inc CA, USA). Oligonucleotide sequences are available at proBase (database of rRNA-targeted oligonucleotide probes and primers) (Loy et al., 2007). Dual-indexed forward and reverse primers were used in all reactions. Metagenomic DNA (100 ng) was used, and PCR was performed in 50 µL reactions with primers, dNTPs, and DNA polymerase. PCR plate was transferred to the thermocycler for amplification (98° C (3 min) + [98° C (15 s) + 50° C (30 s) + 72° C (30 s)] × 25 cycles+ 72° C (7 min). After amplification, amplicon pools (5 µL/reaction) were combined, mixed, and purified by adding Axygen Axyprep MagPCR clean-up beads (50 µL beads were thoroughly mixed with 50 µL amplicons) and incubated for 15 min at room temperature. The plate was placed on a magnetic stand for 5 min until the supernatant was cleared and then washed with 80% ethanol. The pooled amplicon was evaluated by using the Advanced Analytical Fragment Analyzer and quantified using Quant-iT HS dsDNA kits and diluted based on Illumina's standard protocol for sequencing (MiSeq Illumina).

Sequencing data processing and bioinformatic analysis

Microbiome analysis based on amplicon sequence variants (ASVs) was performed. The cutadapt (Martin, 2011) algorithm was used to remove the primers at the 5' end of forward reads. Read pairs were rejected, if either read in the pair did not match a 5' primer, and an error rate of 0.1 was allowed. Quality filtering, pairing, denoising, dereplication, and determination of ASV counts were performed with the Division Amplicon Denoising

Algorithm (DADA2) plugin (Callahan et al., 2016) in the QIMME2 platform. For quality trimming, forward and reverse reads were truncated to 150 bases; reads with more than two expected errors were discarded and reads with fewer than 249 or more than 275 nucleotides were discarded. Bacterial taxonomy was assigned to remaining sequences using the SILVA database v132 (Pruesse et al., 2007) using the classify-sklearn procedure. ASVs identified as something other than bacteria were removed from further analysis and normalized using the CSS method (Paulson et al., 2013) prior to downstream analysis.

Phenotypic AMR in fecal bacteria

Fecal samples were used to quantify coliforms and enterococci by spiral plating using an Eddy Jet 2 spiral plater (Neutech Group Inc., Farmingdale, NY, USA) as described previously (Chalmers et al., 2018). Fecal samples were diluted in phosphate-buffered saline (PBS) at a 1:10 ratio. The dilution was plated on MacConkey agar (MAC) (Remel, Thermo Scientific) to quantify total coliforms (i.e., without antimicrobial), and MAC supplemented with an antimicrobial drug to quantify AMR coliforms. Similarly, the dilution was plated on m-Enterococcus agar (ENT) (Remel, Thermo Scientific), to quantity total enterococci (i.e. without antimicrobial), and ENT supplemented with an antimicrobial drug to quantify AMR enterococci. The antimicrobials and their concentrations included in the agar are shown in Table 5. The plates were incubated at 37° C for 18 h for MAC and at 42° C up to 48 h for ENT. The coliform and *Enterococcus* colonies were counted, and viable counts of the bacteria were estimated as colony-forming units per gram (CFU/g) of feces, following the spiral-plater manufacturer's recommendations.

Statistical analysis

Performance data analysis

Average daily feed intake (ADFI), average daily gain (ADG) and gain to feed ratio were analyzed using the PROC MIXED procedure of SAS version 9.4 (SAS Institute, Inc., Cary, NC) with pen serving as the experimental unit. Results were considered significant $P \leq 0.05$ and marginally significant at $P \leq 0.10$.

Microbiome data analysis

To evaluate for any potential bias due to the difference in raw sequencing reads by study group or by diet, the number of raw reads generated from each sample were analyzed using generalized linear modeling (*glm* function, R). Alpha diversity, as captured by richness (observed), diversity (Shannon diversity index), and Pielou's evenness (Pielou, 1966) of taxa, was estimated from normalized counts using *phyloseq* in R (*estimate_richness* and *evenness* function). The associations of age, diet and their interaction (age x diet) with alpha diversity indices (outcome variables) were evaluated using linear mixed-effects models as implemented in the "*lme"* package in R software. The *lsmeans* function in R was used to perform pairwise comparisons when interactions were significant ($P < 0.05$). Similar analyses were performed for the ceftiofur- or penicillin G- treated group with explanatory variables (diet, treatment effects, and their interactions). Pig was entered as a random effect to account for the lack of independence between serial samples.

Non-metric multidimensional scaling (NMDS) ordination plots were made using the R package *vegan* (Jari Oksanen, 2019) on Hellinger transformed (Legendre and Gallagher, 2001) bacterial taxonomic counts data using the Bray-Curtis dissimilarity distance. NMDS plots were visualized using the ggplot2 package (Wickham, 2009). A two-dimension plot was used if the stress was less than 0.2. ASVs present in < 1% of samples were discarded to avoid potential bias before analysis. The permutational multivariate analysis of variance

(PERMANOVA) of the Bray-Curtis distance was performed using the *adonis* function in the R *vegan* package, with one or more of the explanatory variables (pig age, dietary treatment, antibiotics treatments effect). Individual pig identity was added to control for repeated measures. If significant results were observed, a pairwise comparison was performed using *pairwise.adonis* (Martinez, 2020) with Benjamin & Hochberg ("BH") corrected *P*-values. A dispersion analysis on the distance from the sample to the centroid of each group (age group, dietary treatment, or antibiotic treatment effect) was tested with the multivariate homogeneity of group dispersion test (*Betadisper* function in the *vegan* package in R) (Jari Oksanen, 2019) followed by Tukey's honest significance difference method.

The relative abundance of taxa for each sample was estimated by aggregating the taxa at corresponding phylum (phyla ≤ 0.3% were labeled as "others") and genus level (genera ≤ 1% labeled as "others") and stack bars were created to visualize each sample by age, dietary treatment, or antimicrobial treatments. Multivariate zero-inflated Gaussian models (*fitZig* function) were used to identify specific taxa abundances (phylum and genus level) significantly different between the age, diet, and antimicrobial treatments (Paulson et al., 2013). Random effect for the individual pig was included in the model using "useMixedModel" and "block". The pairwise comparison of each taxa abundance ($\log_2$ fold change) between the treatment groups (i.e., fixed effects) was performed using makeConstrast, Limma's package, *alpha* = 0.05, with Benjamin-Hochberg adjusted *P*-values (Benjamini and Hochberg, 1995; Ritchie et al., 2015). In addition, the difference in bacterial diversity (richness and Shannon diversity) by different levels of CP, ADF and NDF contents in diets were evaluated using linear mixed-effects models as implemented in the "*lme*" package in R software. The *lsmeans* function in R was used to perform pairwise comparisons when interactions were significant ($P < 0.05$).

Phenotypic AMR data analysis

The changes in the total (i.e., without antimicrobial) and AMR coliforms and enterococci counts (expressed as log10 CFU/g feces) by age, diet and antimicrobial treatment effect were analyzed using the zero-inflated Gaussian mixed models (*lme.zig* function) (Zhang and Yi, 2020) with pig as a random intercept to adequately control for repeated measures. *P*-values of models were extracted using the ANOVA function. If fecal samples did not have counts for a given antibiotic, the antibiotic was excluded from the analysis. The relationship between the counts of coliforms and enterococci in the presence of each antimicrobial (Table 5) and genus composition were determined using the *envit* function in vegan (Jari Oksanen, 2019) and using NMDS ordinations plots.

RESULTS

Analysis of feed samples showed that the measured NDF concentrations in the diets were lower than the formulated concentrations (7.1 vs. 8.7%, 10.9 vs 13.6% and 10.9 vs. 13.6% for control, DDGS and SBP diets, respectively) (Table 2). From d 91 to 177 d of age (86 d), there was no evidence of treatment difference for final BW, ADFI, and ADG (Table 3). Pigs fed DDGS diet had marginally lower ($P < 0.10$) gain to feed ratio compared to those fed the control or SBP diet.

Of the total 140 fecal samples, fifty-five fecal samples were from 5 pigs per diet at four age-points (98, 110, 144 and 177 d of age), except on 98 d of age, the number of samples collected were < 5 pigs per treatment (3 from the control, 4 from the DDGS-based and 3 from the SBP-based diet). None of the five pigs received any antibiotic injections. A total of 40 fecal samples were collected from the ceftiofur-treated (n=25; days 1 and 3) and untreated pen-mate control (n=15; day 1) pigs. Similarly, a total of 45 fecal samples were collected from the penicillin G-treated (n=30; days 1 and 3) and untreated pen-mate control (n=15;

day) pigs (Table 4). Metadata for each analyzed sample can be found in supplementary table S1.

Sequencing Summary

Amplicon sequencing of the V4 region of 16S rRNA gene in 140 samples yielded 15.6 million reads (mean: 111,354, median: 102,717, range: 58,777-167,794). After filtration, denoising, margining, and removal of chimeras, an average of 69% (range 59 - 77.2%) of input reads per sample were retained. The details of the sequencing summary along with metadata are presented in the supplementary table S1**.** Further, differences in raw reads among study groups and diets were evaluated using a generalized linear model. The average raw sequencing reads were significantly ($P < 0.0001$) higher in penicillin G-treated group than untreated age/diet group or ceftiofur-treated group. However, there was no significant difference in 16S rRNA sequencing reads among diets ($P = 0.363$). Based on these results, cumulative sum scaling (CSS) normalization to account for differences in read depth was used.

Overall ASV classification and taxonomic compositions

A total of 4,740 ASVs across 140 samples, after excluding ASVs classified as other than bacteria ("Archaea" and "Eukaryaota"), were identified. The taxa identified included 20 phyla, 28 classes, 44 orders, 87 families, 247 genera, and 122 species across all samples. The assignment of ASV to different taxonomic levels decreased with higher taxonomic resolution. Overall, 99.0%, 98.8%, 93.5%, 81.5%, and 51.7% were classified at phylum, class, order, family, genus, and species levels, respectively. Of the 20 phyla, Firmicutes (3,025 ASVs/64.4%), Bacteroidetes (744 ASVs/15.8%), Tenericutes (382 ASVs/8.1%), and Proteobacteria (175 ASVs/3.7%) contained high number of ASVs across all samples. Also, a

few ASVs in the phylum Fibrobacteres (5 ASVs/0.11%), which contains species that degrade plant cell wall-polysaccharides were identified. Across all samples, Firmicutes (1,633,045/67.0% total reads), Bacteroidetes (1,633,045/27.3% total reads), Tenericutes (51,697/2.1% total reads), Proteobacteria (29,013/1.2% total reads) were the most abundant phyla, accounting for 97.6% of the total bacterial community at the phylum level. Relative abundance of phyla at each age point, by dietary treatment, ceftiofur, and penicillin G-treated pigs (d 1 and 3) along with untreated pen-mates on d 1 are presented in Figs. 1, 2, respectively.

Similarly, at the family level, *Ruminococcaceae* (1,458 ASVs/32.9%), *Lachnospiraceae* (683 ASVs/15.4%), *Prevotellaceae* (321/7.2%), *Muribaculaceae* (214/4.8%) were the highest ASVs across all samples. *Ruminococcaceae* (396,578/16.5% total reads), *Veillonellaceae* (368,793/15.3% total reads), *Muribaculaceae* (330,672/13.7% total reads), *Prevotellaceae* (266,623/11.1% total reads), *Lachnospiraceae* (222,199/9.2% total reads), *Streptococcaceae* (215,560/8.9% total reads) and *Clostridiaceae 1* (200,021/8.31% total reads), *Peptostreptococcaceae* (50,167/2.08% total reads) were the most dominant families in relative abundance accounting for 85.17% of the total. At the genus level, a total of 3,074 ASVs were assigned to genera, of which 789 ASVs were either "uncultured", "uncultured bacterium", or "unidentified rumen bacterium RF39", etc. *Megasphaera* (240,057/10.4% total reads), *Streptococcus* (215,560/9.3% total), *Clostridium sensu stricto 1* (195,998/8.4% total), *Prevotella 9* (98,376/4.2%), *Dialister* (78,086/3.3% total) were the most abundant genera across all samples and across all treatment groups. Relative abundance of genera at each age point, by dietary treatment, and in ceftiofur-, and penicillin G-treated pigs (d 1 and 3) along with untreated pen-mates on d 1 are presented in supplemental Figs. S1, and S2, respectively.

When bacterial diversity by different levels of CP, ADF and NDF contents in diets were evaluated, there were significant difference (all $P < 0.001$) in bacterial richness and diversity (Shannon diversity; Supplementary Figure S7). For example, bacterial richness and diversity values were significantly higher in pigs fed CP at 15.3% compared to other CP levels (Supplementary Figure S7 A and D). Similarly, richness and diversity of bacterial community were also varied by the levels of ADF and NDF. In pigs fed the diet with 3.9% ADF, bacterial richness and diversity tended to be higher (Supplementary Figure S7 B and E). The richness and diversity of the bacterial community were significantly higher in pigs fed diet with 9.6% and 10.4% NDF compared to 6.8% level (Supplementary Figure S7 C and F).

Age-related dynamics and effects of dietary treatment on the fecal bacterial communities

The dynamics of the fecal microbiome of pigs fed the three diets differing in source, type and level of fiber sources were characterized with the 55 fecal samples collected on the four sampling days between d 98 through 177. Alpha diversity (within-sample diversity) metrics including observed richness, Shannon diversity, and evenness (Pielou's evenness index) of the fecal bacterial genera were influenced by the age (d 98, 110, 144, and 177) of the pigs receiving each of the three dietary treatments (Fig. 3). Overall, the bacterial richness, Shannon diversity, and evenness index increased from d 98 to 177 of age and significantly associated with pig age ($P < 0.001$). The richness, Shannon diversity, and evenness indices were relatively higher in feces of pigs fed DDGS- or SBP-based diets when compared to the control diet; however, the differences were not statistically significant ($P > 0.05$), suggesting age has a proportionally greater impact than diet on alpha diversity metrics. In addition, there was no interaction effect of age and diet ($P > 0.05$) on these diversity indices.

The non-metric multi-dimension scaling (NMDS) of the Bray-Curtis distances showed that age could lead to variation in the overall bacteriome composition (beta-diversity) of pigs

on various dietary treatments. Further, PERMANOVA results showed that age explained the largest source of variation in the microbial genera community compositions (R^2 = 36.20%, P < 0.001) with significant clustering in age groups except for d 98 vs. 144 (adjusted P=0.456) (Fig. 4, Table 6). Diet explained roughly 6% of total variation but had a statistically significant effect (PERMANOVA; R^2 = 6.10%, P < 0.001) in overall bacterial genera composition (Fig 4). The *post hoc* pairwise testing between the dietary treatments indicated that the bacterial community compositions significantly varied between the dietary treatments (control vs. DDGS diet, control vs. SBP diet, and DDGS vs. SBP diet). However, there was no evidence of any important interactions between age and diet (P = 0.587). The degree of variation in the bacteriome at the genus level among individual pigs was similar across age and diet (multivariate homogeneity test of group dispersion from centroid; P = 0.416, P = 0.573, respectively) (Table 6).

Firmicutes followed by Bacteroidetes were the most dominant phyla across all age points, but with slight variations between dietary treatments (Fig. 1). Across all diets, a decreasing trend of mean relative abundance of Firmicutes was observed with increased age of pigs (d 98: 71.7%, d 110: 69.3%, d 144: 69.9%, and d 177: 59.2%), while concomitant increasing trends of mean relative abundance of Bacteroidetes with age was observed (23 - 31%). Among the three diets, the relative abundance of Firmicutes was slightly higher in the control diet (69.6%) when compared to DDGS (66.8%) and SBP-based diets (65.1%); however, relative abundance of Bacteroidetes was slightly lower in pigs fed the control diet (24.1%) when compared to those fed the DDGS-based diet (26.8%) or the SBP-based diet (28.8%). There was little noteworthy shift in the relative abundance of phylum-based microbiomes based on dietary treatments.

The mean relative abundance of the genus *Clostridium sensu stricto* 1 increased with age (d 98: 6.0%, d 110: 8.7%, d 144: 10.7% and d 177: 10.9%); in contrast, *Megasphaera* and

Streptococcus decreased with age (d 98: 14.9% and 13.8%, d 110: 13.2% and 11.2%, d 144: 11.5% and 11.3%, and d 177: 2.6% and 7.9%, respectively). The abundance of *Clostridium sensu stricto* 1 was relatively higher in pigs fed the control diet (9.8%) or DDGS-based diet (~10.4%) compared to pigs fed the SBP-based diet (6.7%). In contrast, the genera *Megasphaera* and *Prevotella* 9 were most abundant in the SBP diet (13.4% and 6.5%, respectively) compared to the control diet (11.2% and 3.2%, respectively) and DDGS-based diet (8.0% and 4.2%, respectively) (Fig. S1). Further, individual taxa (at phylum and genus levels) that were differentially abundant when comparing pigs fed the control diet vs. DDGS- or SBP-based diet (for each age group) are presented in Table 7. On d 98, pigs fed the control diet had significantly higher abundances of the genera *Catenibacterium*, and *Holdemanella* compared to those that fed the SBP-based diet ($P < 0.05$). At the same age, abundance of phyla or genera were not different between control diet vs. DDGS-based or DDGS- vs. SBP-based diets. On d 110, none of the taxa were significantly different on any diets; however, at d 144, pigs fed DDGS-based diet had decreased genus *Coprococcus* 2 abundance compared to pigs fed the SBP-based diet ($P < 0.05$). On d 177, none of the taxa was differentially abundant in pigs fed control vs. DDGS-based diet, but lowered abundance of the species *Eubacterium ruminantium* and the phylum *Chlamydiae* were detected in pigs fed the control diet or DDGS compared to pigs fed the SBP-based diet (Supplementary Fig. S3). Analyses of differential abundance of taxa at each age-point by diet revealed higher abundance (at phylum and genus levels) with increasing age of pigs, regardless of the diet (Supplementary Fig. S4).

Fecal bacterial AMR by age and dietary group

Overall, 59.3% (95% CI: 54.6- 63.9%) and 46.7% (95% CI :42.4-50.9%) of fecal samples (n=55) collected from untreated pigs at d 98, 110, 144 and 177 carried coliforms and

enterococci resistant to at least one antimicrobial drug (1+ AMR), respectively. Temporally, there was variation among 1+ AMR coliforms (d 98: 62.5%, 95% CI: 50.9-73.08%; d 110: 54.1%, 95% CI 44.8-63.2%; d 144: 59.1% 95% CI: 49.8-68.04% and d 177: 62.5%, 95% CI 53.1-71.1%) and 1+ AMR enterococci (d 98: 5%, 95% CI: 44.7-64.9%; d 110: 37.3%, 95% CI 29.5-45.5%; d 144: 52.6%, 95% CI: 44.3-60.8% and d 177: 44.6%, 95% CI 36.5-52.9% with age of pigs. Likewise, 57.6% (95% CI: 49.1-65.8%), 57.8% (95% CI: 49.6-65.8%), and 62.5% (95% CI: 54.05-70.4%) samples carried 1+ AMR coliforms, while 51.6% (95% CI: 44.1-59.1%), 46.3% (95% CI: 39.6-53.6%), and 42.2% (95% CI: 34.9-49.7%) carried 1+ AMR enterococci across all ages in pigs fed control, DDGS- and SBP-based diets, respectively.

The total coliform counts (i.e., log10 CFU/g coliform without antimicrobial) changed ($P < 0.05$) with age (d 98 through d 177); however, these did not vary by dietary treatment. Coliform resistance to aminopenicillins, ceftriaxone, tetracyclines, macrolides, aminoglycosides, and sulfonamides did not vary with age or dietary treatment. However, there was a significant interaction effect between age and diet on coliform resistance to phenicols ($P < 0.001$). Further, a significant interaction between age and diet type on total fecal enterococci counts (i.e., enterococci without antimicrobials) was observed ($P < 0.05$) (Fig 5A). However, enterococci resistant to quinolone (nalidixic acid), tetracyclines, macrolides, and lincosamides did not change with age or dietary treatment (nor their interaction effects). However, there was a significant interaction between age and diet on enterococci resistant to penicillins and nitrofurans (Fig 5B). In addition, among pigs receiving the control diet, there was no significant association between AMR coliforms or enterococci with overall genera composition at any age-points while pigs fed either a DDGS- and or SBP-based diet, enterococci resistant to macrolides ($P = 0.059$), and enterococci resistant to

penicillins (P= 0.029) and quinolones (P=0.035) were associated with overall genera composition (Fig. 4 A-C, Supp. Table S2).

Effects of the injectable ceftiofur and dietary treatments on fecal bacterial community structure and fecal bacterial AMR

A total of 40 fecal samples were collected from the ceftiofur-treated (n=25) and untreated pen-mate control (n=15) pigs, which included 9 samples from pigs fed the control diet, 8 samples from pigs in each of DGGS or SBP dietary treatment group (Table 4). The alpha diversity metrics (richness, Shannon diversity, and evenness) of taxa did not differ between pigs that received ceftiofur treatment versus their untreated pen mates, either on d 1 or 3 of treatment or by dietary treatment (P > 0.05) (Fig. 6 A-C). Similarly, there was no significant interaction between diet and ceftiofur treatment concerning richness, diversity, and taxa evenness (Fig .7). Overall, treatment accounted the largest variation (PERMANOVA; R^2 = 17.3%, P = 0.002) followed by diet (R^2 = 12.9%, P = 0.01) for the bacterial genera composition. The *post hoc* pairwise testing revealed a significant difference in the bacterial community between untreated pen mates on d 1 vs. ceftiofur-treated pigs on d 1 (P = 0.021) and untreated pen mates on d 1 vs. ceftiofur-treated pigs on d 3 (P = 0.009). Similarly, a significant difference in the bacterial community was observed between DDGS vs. SBP-based diet (P = 0.018) (Table 6). However, there was no evidence of an interaction (P = 0.69) of diet fed to pigs and ceftiofur treatment on genera composition. The dispersion of the samples from the centroid of each group did not significantly vary by dietary treatment (P = 0.1187) or ceftiofur treatment (P = 0.052).

Across the three dietary groups, overall relative abundance of the phylum Bacteroidetes was relatively higher in untreated pen-mates (30.4%) than in ceftiofur-treated pigs on d 1 (27.02%), but similar to those treated on d 3 (29.3%), but with no noteworthy change in the

mean relative abundance of the phylum Firmicutes. However, the relative abundance of Bacteroidetes remained similar across all three dietary groups (control diet: 27.2%, DDGS-based diet: 26.2% and SBP-based diet: 28.7%) but with a slight variation in the mean relative abundance of Firmicutes (control: 68.2%, DDGS: 69.1% and SBP: 64.5%) (Fig. 2 A-C). At the genus level, mean relative abundance of *Clostridium sensu stricto* 1 was relatively similar between untreated pen mate control (9.7%) vs. ceftiofur-treated pigs on d 1 (8.2%) and d 3 (9.3%). In contrast, shifts in the relative abundance of *Megasphaera*; *Streptococcus*; and *Prevotella* 9 were consistently observed as being higher in untreated pen mates on d 1 than in ceftiofur-treated pigs on d 1 and 3. Similarly, the relative abundances of *Clostridium sensu stricto* 1 and *Streptococcus* were higher in the pigs fed the control diet (10.9% and 9.0%) compared to those fed DDGS-based (9.4% and 6.2%) and SBP-based diets (6.5% and 3.6%) (Fig. S2 A-C).

Differential abundance analysis (log_2 fold change) of the taxonomic assignments of the pig fecal microbiome revealed differences between the untreated pen mates and ceftiofur-treated pigs on both d 1 and 3 among pigs fed the three diets, and the effect was more apparent among pigs fed DDGS- and SBP-based diets compared to pigs fed the control diet (Table 7; Supp. Fig S5). For instance, in pigs fed the control diet, an increased abundance of the phylum *Chlamydiae* was observed in the feces of untreated pen mates on d 1 compared to ceftiofur-treated pigs on d 3 ($P < 0.05$); however, there was no difference in taxa abundance between either untreated pen-mates on d 1 vs. ceftiofur-treated pigs on the same day or between ceftiofur-treated pigs on d 1 vs. d 3. In contrast, among pigs on the DDGS diet, the abundance of phylum *Patescibacteria* and genus *Candidatus Saccharimonas* were increased in ceftiofur-treated pigs on d 1 compared to d 3 ($P < 0.05$). Interestingly, pigs fed SBP-based diet had a lower abundance of the phylum *Epsilonbacteraeota* in untreated pen mates on d 1 compared to the pigs treated with ceftiofur on the same day ($P < 0.05$). In contrast, an

increased abundance of the phylum *Chlamydiae* was detected in untreated pen mates when compared to ceftiofur-treated pigs on d 1 or 3. This suggests that exposure of pigs to ceftiofur may alter the abundance of certain taxa, and the effects are measurable on the same day of treatment.

Fecal bacterial AMR in ceftiofur-treated pigs

Among ceftiofur-treated pigs, (d 1 and 3) along with untreated pen mates on d 1, 58.7% (95% CI: 53.1-64.1%) and 55.8% (95% CI :50.5-61.0%) of samples (*n*=40) carried coliforms and enterococci resistant to at least one of the tested antimicrobial drugs (1+ AMR), respectively. Similarly, 56.6% (95% CI: 47.3-65.6%), 61.6% (95% CI: 52.3-70.3%) and 57.5% (95% CI: 45.9-68.4%) of pig fecal samples carried 1+ AMR coliforms and 51.1% (95% CI: 42.3-59.8%), 56.2% (95% CI: 47.4-64.8%), and 62.2% (95% CI: 51.38-72.2%) of pig fecal samples carried AMR enterococci in the untreated pen mates on d 1 and ceftiofur-treated pigs on d 1 and 3, respectively, across all three diets. Likewise, 50.8% (95% CI: 41.5-60.0%), 59.3% (95% CI: 48.8-69.2%), and 67.3% (95% CI: 57.4-76.2%) pig fecal samples carried 1+ AMR coliforms, and 58.5% (95% CI: 49.7-66.9%), 56.4 % (95% CI: 46.6-65.9%), and 52.13% (95% CI: 42.7-61.4%) carried 1+ AMR enterococci across all ages on control, DDGS- and SBP-based diets, respectively. Similarly, total coliform or coliform resistance to aminopenicillins, ceftriaxone, tetracyclines, macrolides, aminoglycosides, and sulfonamides did not vary significantly either by dietary treatment or ceftiofur treatment ($P > 0.05$) (Fig. 8). However, total enterococci significantly varied between control vs. SBP-based diet (P=0.03), but enterococci resistant to penicillins, quinolones, tetracyclines, macrolides, aminoglycosides, and lincosamides did not vary with either dietary treatment or ceftiofur treatment (Fig. 8). However, several antimicrobial drug classes associated with coliform and

enterococcal resistance were significantly associated with overall bacterial genera composition (Fig. 7, Supp. Table S3).

Effects of injectable penicillin G and dietary treatments on the fecal bacterial community structure and fecal bacterial AMR

A total of 45 fecal samples were collected from the penicillin G-treated (n=30) and untreated pen-mate control (n=15) pigs. Specifically, 5 pigs were sampled on each diet (control, DDGS- or SBP-based diets) on d 1 and 3 of the treatment regimen, along with 5 untreated pen-mates on d 1 of antibiotic injection on each diet (Table 4). The richness, Shannon diversity and evenness indices did not vary ($P > 0.05$) in penicillin G-treated pigs (d 1 and 3) compared to untreated pen-mate controls on d 1; however, the richness, Shannon diversity, and evenness differed by diet ($P = 0.018$, $P = 0.006$, and $P = 0.034$, respectively) with a significant difference observed between control diet compared to SBP-based diet. There was no interaction between diet and penicillin G treatment effect on richness, Shannon diversity, and evenness (Fig. 6 D-F).

Similar to ceftiofur treatment, the overall bacterial genera composition was impacted by penicillin G treatment (PERMANOVA; $R^2 = 9.9\%$, $P = 0.002$) when comparing untreated pen-mate control pigs on d 1 with penicillin G-treated pigs on d 3. Diets of the pigs explained the largest variation ($R^2 = 13.5\%$, $P = 0.003$) in overall microbial genera composition, with a significant difference between control vs. pigs fed SBP-based diet ($P = 0.003$) and pigs fed DDGS- vs. SBP-based diets ($P = 0.0045$) (Fig .9). However, no significant interaction ($P = 0.946$) was observed between dietary treatment and penicillin treatment effects on the bacterial genera composition. In addition, the dispersion of the samples from the centroid of each group significantly varied by treatment with penicillin G on d 3 vs. penicillin G on d 2

(difference: 0.048, $P = 0.023$); however, it remained the same by dietary treatment ($P = 0.397$) (Table 6).

Similarly, overall relative abundances of phyla *Bacteroidetes* and *Firmicutes* were similar in untreated pen-mates on d 1 compared to pigs treated with penicillin G on d 1 and 3 across the three diets (Fig. 2 D-F). At the genus level, mean relative abundance of *Clostridium sensu stricto* 1 was lower in untreated pen mate pigs on d 1 (6.0%) compared to pigs treated with penicillin-G on d 1 (6.5%) and d 3 (9.5%). Likewise, the relative abundances of *Clostridium sensu stricto* 1 and *Streptococcus* were higher in pigs fed the control diet (10.6% and 9.6%, respectively) compared to pigs fed DDGS- (6.3% and 8.3%, respectively) and SBP-based diets (5.1% and 6.1%, respectively) (Fig. S2). Differential abundance analysis also demonstrated that the observed changes were limited to the pigs that received the control diet (Table 7). For example, pigs fed the control diet and serving as untreated pen mates (d 1) had an increased abundance of phylum *Chlamydiae*, and genus *Streptococcus* and decreased abundance of genus *Terrisporobacter* compared to the control diet-fed pigs injected with penicillin G on d 1 or 3 ($P < 0.05$) (Supp Fig. S6).

Fecal bacterial AMR in penicillin G-treated pigs

Fully 62.8% (95% CI: 57.5-67.7%) and 54.0% (95% CI: 49.0-59.0%) of the penicillin G-treated (d 1 and 3, along with untreated pen mates on d 1), carried coliforms and enterococci resistant to at least one antimicrobial drug (1+ AMR) tested. Similarly, 60.0% (95% CI: 50.6-68.8%), 63.3% (95% CI: 54.0-71.9%) and 65.0% (95% CI: 55.7-73.4%) of samples carried 1+ AMR coliforms and 60.0% (95% CI: 51.2-68.3%), 48.1% (95% CI: 39.4-56.9%), and 54.0% (95% CI: 45.2-62.6%) of samples carried 1+ AMR enterococci in the untreated pen mates on d 1, and penicillin G-treated pigs on d 1 and 3, respectively, across all diet groups. Likewise, 64.1% (95% CI: 54.9-72.7%), 60.0% (95% CI: 50.6-68.8%), and 64.1% (95% CI:

54.9-72.7%) of fecal samples carried 1+ AMR coliforms, and 55.5% (95% CI: 43.8-61.2%), 52.5 % (95% CI: 43.8-61.2%), and 54.0% (95% CI: 45.2-62.6%) of samples carried 1+ AMR enterococci across all ages in control, DDGS- and SBP-based diet groups, respectively.

Total coliform counts and coliform resistance to aminopenicillins, ceftriaxone, tetracyclines, macrolides, and sulfonamides also did not change significantly with either dietary treatment or penicillin treatment ($P > 0.05$) (Fig. 10A). In contrast, total enterococci counts varied with dietary treatment (control vs. SBP-based diet, $P = 0.01$). Enterococci resistant to penicillins, tetracyclines, quinolones, macrolides, aminoglycosides, and lincosamides also did vary with either dietary treatment or penicillin G treatment or their interactions (Fig. 10B). Based on *envit* NMDS analysis, only coliform resistance to the sulfonamide drug class was significantly ($P < 0.05$) related to the overall bacterial genus composition in pigs on the DDGS-based diet (Fig. 9, Supp. Table 4). These results further suggest that penicillin G had a minimal impact on overall bacterial resistance.

DISCUSSION

Previous work has suggested that age, diet, and the use of antimicrobials can influence the swine gut microbial community (Ghanbari et al., 2019; Pollock et al., 2020). AMR occurrence is a natural phenomenon and bacteria have been evolving resistance mechanisms to naturally occurring antibacterials produced by other bacteria and fungi (Blair et al., 2015). Thus, these occurrences are not likely to be eliminated from gut bacteria in the natural system (D'Costa et al., 2011); therefore, alternative measures need to be evaluated to reduce the risk of AMR emergence and transmission from animal-to-animal and from animal-to-human, while increasing performance and health status of food animals. Because of the public health risk of AMR associated with the use of antimicrobials, the manipulation of the gut microbiome via diet modification would be an alternative strategy. One possible strategy is to

use dietary fiber in the feed, which is likely to have significant impact on the gut microbiome. The extent to which levels/sources of dietary fibers have desirable effects, both with and without concurrent antimicrobial treatments, during finishing phase of production pigs, is largely unknown. To address this question, dietary composition was modified to achieve different levels and sources of fibers to measure the age-related dynamics, diet effects and antimicrobial use on the fecal microbiome and phenotypic AMR on fecal bacteria. The two antibiotics used in the study were ceftiofur and penicillin G, which are common antibiotics used in finisher pigs in the United States.

Age significantly impacted bacterial diversity and taxonomic composition

Overall richness, Shannon diversity, and evenness of the fecal microbiome increased with pig's age from d 98 (a week after starting dietary treatments) until d 177 (day of shipment for slaughter) in pigs fed control, DDGS-, and SBP-based diets. This increased trend of richness and diversity indices with pig age is comparable to previously published studies (Kim et al., 2011; Lu et al., 2018; Wang et al., 2019; Arfken et al., 2020). Our results also suggested that gut microbial genera become more diverse, and presumably more stable, with age in growing pigs. Our analysis also revealed that the diet has minimal effects on the alpha diversity metrics, but overall bacterial genera community structure (beta-diversity) was impacted by age and diet; moreover, age explained greater variation than diet when measuring the taxonomic composition. Our findings also suggested that there was neither synergy nor antagonism of dietary fiber by pig age in affected microbial composition. Similar age-and-diet related clustering of the bacterial community was also reported in several previous studies of pigs (Frese et al., 2015; Wang et al., 2019; Arfken et al., 2020), while another study (Kraler et al., 2016) showed no differences in taxonomic composition related to diet modification (control diet, and with low and high fiber diets). The difference in results

suggests that microbial community could be impacted by age and diet variabilities, or there could be age or diet-specific effects on microbial taxa.

In our study, the most notable change in bacterial abundance with age was a decreased trend of mean relative abundance of phylum *Firmicutes* and a trend for increased phylum *Bacteroidetes*. Regardless of the diet, these two phyla accounted for more than 90% of the total sequences (Kim et al., 2011; Kim and Isaacson, 2015). Specifically, on d 98, pigs fed the control diet had significantly higher proportion of genera *Catenibacterium, and Holdemanella* compared to pigs that were fed the SBP-based diet; meanwhile, no differences between diets were detected on d 110. A similar diet-specific genus difference was noticed on d 144 with decreased *Coprococcus* 2 abundance in DDGS- compared SBP-fed pigs. Similarly, just prior to d 177, lower abundance of the species *Eubacterium ruminantium*, and the phylum *Chlamydiae* were observed in pigs fed the control diet and the DDGS-based diet than those fed SBP-based diet.

Dietary fiber is one of the major factors in shaping the pig gut microbiome (Wang et al., 2019). Zhang et al. (2016) reported that a moderate increase in fiber level (both soluble and insoluble fiber) in the diet influenced the gut microbial composition in piglets fed with different levels of fiber-containing diets compared to a control diet from d 7 to 22. In contrast, a study by (Kraler et al., 2016) showed no difference in taxonomic composition related to diet modification (with a control diet vs. low and high fiber diets). In our study, we utilized both soluble (i.e., basal diet supplemented with 20% DDGS) and insoluble fiber (i.e., diet supplemented with SBP). The overall bacterial community composition at the genus level was significantly different between control vs. SBP, and DDGS vs. SBP.

Diversity was not affected by antimicrobial injection, but the overall bacterial community composition varied with diet and antimicrobial treatment

Comparison of the alpha diversity metrics such as richness, Shannon diversity, and evenness indices revealed no significant difference between pigs that received ceftiofur treatment versus their untreated pen mates, either on the day of the treatment (d 1 and 3) or by diet type, demonstrating that within-sample bacterial diversity was not affected by antibiotic injection. Similar findings were reported by Pollock et al. (Pollock et al., 2020). In their study, higher levels of chlortetracycline and tylosin in the feed did not affect the fecal microbiome diversity in young pigs. Kalmokoff et al. (Kalmokoff et al., 2011) reported that the addition of either tylosin or virginiamycin to pig feed at 15 weeks of age did not affect the fecal bacterial composition (using 16S rRNA). Similarly, no impacts by subtherapeutic effects of chlortetracycline and tylosin on alpha diversity metrics of the fecal microbiome during all production phases of swine were reported by Holman and Chenier (Holman and Chenier, 2013). In contrast, among penicillin G-injected pigs, richness, Shannon diversity, and evenness did not vary by treatment, but there was a significant difference in alpha diversity metrics in pigs fed the control diet vs. SBP-based diet. The discrepancies between the ceftiofur and penicillin G suggests an antibiotic-specific changes in both duration and extent of the effect (Zeineldin et al., 2019).

In line with previous studies (Looft et al., 2014; Zhao et al., 2018), our results showed that both antibiotic treatment (ceftiofur and penicillin G) and diet impacted the overall bacterial genera composition (beta diversity), suggesting both antimicrobial treatments and diet may alter microbes in overall community composition. Recently, (Ruczizka et al., 2019) reported the effect of parenteral treatment of ceftiofur on the fecal microbiome in pigs from the suckling to growing stages (birth to d 97 of age), detected as early as 12 h postpartum. Ceftiofur is a commonly used antibiotic in pigs and is administered intramuscularly. Further,

it is effective for at least 158 h in plasma, but most of the drug is excreted as metabolites in the feces (Hornish and Kotarski, 2002; Ruczizka et al., 2019) and could impact the bacterial community both inside and outside the host. However, ceftiofur metabolite concentrations may depend on the day of the treatment (Gaire et al., 2021). Further, the effect of penicillin G treatment on the microbiome has also been documented in pigs (Zeineldin et al., 2019).

Of the ceftiofur-treated pigs, more differences were evident in the abundance of specific bacterial phyla or genera in pigs fed with the SBP-based diet compared to pigs fed the DDGS or control diet. Among the pigs fed the SBP-based diet, the abundance of phylum *Chlamydiae* was higher in untreated pen mates compared to ceftiofur-treated pigs on d 1 and 3. The phyla *Epsilonbacteraeota*, *Kiritimatiellaeota*, *Planctomycetes*, and *Spirochaetes* were lower in untreated pen mate controls compared to ceftiofur-treated pigs on d 1 and 3. Similarly, in the penicillin G-treated group, the only difference in taxa abundance was observed in pigs fed the control diet, where phylum *Chlamydiae* and genus *Streptococcus* abundance were higher in the untreated pen-mates than in penicillin G-treated pigs on d 1 and d 3. Taken together, our results suggest that antimicrobial-specific and diet effects affect specific microbial taxa.

Variability was observed in the abundance of fecal bacterial AMR with age of pigs, dietary treatment and antimicrobial injections

Several antimicrobial drug classes were tested to understand the overall ecological perspectives of AMR depending upon age, diet, and antimicrobial treatments. The culture-based methods were primarily used to study AMR in bacteria; that is, by isolation followed by susceptibility testing or counting of antibiotic-resistant bacteria. Importantly, current efforts to monitor AMR are primarily based on the culturing of indicator bacteria followed by phenotypic AMR determination (Munk et al., 2018). The measurement of resistance in

coliform bacteria population is commonly used in studies of the determination of AMR in fecal bacterial population. A previous study showed that housing and dietary changes could affect the prevalence of AMR by changing the total coliforms in the gastrointestinal tract pigs (Langlois, 1998) and calves (Hoyle et al., 2004). In the present study, variabilities in the abundance of AMR fecal coliforms and enterococci with the age of pigs (98-177 d of age) and among the pigs fed with different levels and sources of fiber. However, data suggest that diet had minimal effects in the abundance of phenotypic AMR in fecal coliforms and enterococci of finisher pigs (d 98 to 177), notwithstanding the major changes in abundance of fecal bacteria AMR between pigs injected with either ceftiofur or penicillin G on d 1 and 3 and untreated pen mate pigs. Further, variability in the abundance of phenotypic AMR in coliforms or enterococci was not generally affected by injectable ceftiofur or penicillin G. The total coliforms or enterococci (i.e., coliforms without antimicrobial drug) remained stable throughout the study period and across the dietary treatments. This could be related to the relatively short sampling time (d 1 and 3 of the treatment regimens), other factors affecting the entire farm, management, or environmental conditions (Mathew et al., 2003), or limited sample size of animals per diet and antimicrobial treatment to detect differences in AMR. These results suggest that the antibiotic injection is not the only factor that influences the prevalence of bacteria resistant to antibiotic(s) in pig feces.

CONCLUSIONS

Because of the growing concern over AMR among bacteria, alternative approaches to promote animal health and welfare, such as dietary manipulation associated with the level and source of fiber in the diet is a potential strategy. In addition to the diet, several factors such as animal age, antimicrobial use, and the environment could influence bacterial diversity and AMR in fecal bacteria. This study demonstrated that both age and diet (i.e., control vs.

DDGS-, control vs. SBP- or DDGS- vs. SBP-based diets) were associated with overall bacterial community composition, but had minimal effect on phenotypic AMR of the two common commensal fecal bacteria. Short-term administration of ceftiofur and penicillin G changed the overall bacterial composition (at the genus level) but had relatively minor effects on phenotypic AMR; meanwhile, some alterations were noticeable in specific taxa and antimicrobial resistance to certain drug classes. This knowledge can be of help to understand the effects of different levels and sources of dietary fiber, with or without antimicrobial treatments, on gut bacterial composition and fecal bacterial AMR. Further research with a larger number of pigs would help to fine tune the characterizations of microbiome composition and the burden of AMR among a wider range of microbes through metagenomic approaches; this, coupled with culture-based approaches should guide the interpretation of our findings and their future application.

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DISCLOSURES

This article was prepared while Victoriya Volkova was employed at Kansas State University. The opinions expressed in this article are the author's own and do not reflect the views of the National Institutes of Health, the Department of Health and Human Services, or the United States government. None of the authors has any conflict of interest with the publication of the study.

DATA AVAILABILITY STATEMENT

The raw sequence data generated during this study are available in the NCBI repository under BioProject PRJNA800791.

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Figure legends

Fig. 1. Stacked bar chart depicting the relative abundance of bacterial phyla in the feces collected at four age-points from finisher pigs fed A. corn grain and soybean meal-based diet formulated to contain 8.7% neutral detergent fiber (NDF) with no supplement, B. basal diet supplemented with 20% distillers dried grains with solubles (DDGS), formulated to contain 13.6% NDF, and C. basal diet supplemented with 14.5% sugar beet pulp, formulated to contain 13.6% NDF.

Fig. 2. Stacked bar chart of the relative abundance of individual phyla in the feces of finisher pigs fed one of three dietary treatments and receiving: A-C) no antibiotic (pen mate control) or ceftiofur hydrochloride injected intramuscularly on days 1 and 3, D-F) no antibiotic or penicillin G injected intramuscularly on days 1 and 3. The diets were corn grain and soybean meal-based diet formulated to contain 8.7% neutral detergent fiber (NDF) with no supplement (control), basal diet supplemented with 20% distillers dried grains with solubles formulated to contain 13.6% NDF (DDGS), and basal diet supplemented with 14.5% sugar beet pulp, formulated to contain 13.6% NDF (SBP).

Fig. 3. Age-dependent changes in alpha diversity (mean ± SE) of the fecal microbiome taxa (genus level) in feces from finisher pigs fed A. corn grain and soybean meal-based diet formulated to contain 8.7% neutral detergent fiber (NDF) with no supplement, B. basal diet supplemented with 20% distillers dried grains with solubles (DDGS), formulated to contain 13.6% NDF, and C. basal diet supplemented with 14.5% sugar beet pulp, formulated to contain 13.6% NDF. The richness (A), Shannon diversity (B), and evenness of fecal bacterial genera (C) from day 98 to day 177 of age. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, respectively.

Fig. 4. Beta diversity of the fecal microbiome taxa (genus level) in the feces collected at four age-points from finisher pigs fed corn grain and soybean meal-based diet formulated to contain 8.7% neutral detergent fiber (NDF) with no supplement (Control), basal diet supplemented with 20% distillers dried grains with solubles formulated to contain 13.6% NDF (DDGS), and basal diet supplemented with 14.5% sugar beet pulp, formulated to contain 13.6% NDF (SBP). Non-metric multidimensional scaling (NMDS) plots are based on the Bray-Curtis distances: A) by age and Control diet, B) by age and DDGS-based diet, C) by age and SBP-based diet, and D) by age across all diets. Relationship between overall microbial genera composition (by age and diet) and phenotypic AMR abundance in coliform and enterococcal populations with age of pigs receiving different diets are shown using multivariate approaches (*envit* function in R).Total coliforms with antimicrobials (EC), and coliforms resistant to aminopenicillins (EC.AMP), 3rd generation cephalosporins (EC.CEF), tetracyclines (EC.TET), macrolides (EC.MLIDE), aminoglycosides (EC. AMINO), sulfonamides (SUL), phenicols (EC.PHENICOLS); total enterococci (ENT) and enterococci resistant to quinolones (ENT.QUINO), lincosamides (ENT.LINCO), nitrofurans (ENT.NFURANS) and penicillins (ENT.PEN). AMR phenotypes that are significantly correlated with the overall microbiome are highlighted in red font and arrows.

Fig. 5. Abundance of: A) fecal coliforms, and B) enterococci ($\log_{10}$ CFU/g) growing in the presence of the clinical breakpoint concentration of a drug representing each antimicrobial

class and without antimicrobials. The data are for finisher pigs at 98, 110, 144, and 177 days of age receiving one of three diets. DDGS: basal diet supplemented with 20% distillers dried grains with solubles, formulated to contain 13.6% NDF; SBP: basal diet supplemented with 14.5% sugar beet pulp, formulated to contain 13.6% NDF.

Fig. 6. Alpha diversity of the fecal microbiome taxonomic composition (genus level) across the dietary treatments and antimicrobial treatments in finisher pigs. Richness, Shannon diversity, and evenness index in pigs receiving one of three dietary treatments and either no antimicrobial treatment or else treated with ceftiofur (A-C) or penicillin G (D-F). DDGS: basal diet supplemented with 20% distillers dried grains with solubles, formulated to contain 13.6% NDF; SBP: basal diet supplemented with 14.5% sugar beet pulp, formulated to contain 13.6% NDF. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, respectively

Fig. 7. Beta diversity of the fecal microbiome composition of pigs receiving one of three dietary treatments and antimicrobial drugs. Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis distances by ceftiofur-treatment group across diets: (A) Control diet, B) DDGS-based diet, C) SBP-based diet, and D) across all diets by ceftiofur-treated group. Relationship between overall microbial genera composition and phenotypic AMR abundance in coliform and enterococcal populations of pigs receiving different diets are shown using multivariate approaches (*envit* function in R). Total coliforms with antimicrobials (EC) and coliforms resistant to aminopenicillins (EC.AMP), 3rd generation cephalosporins (EC.CEF), tetracyclines (EC.TET), macrolides (EC.MLIDE), aminoglycosides (EC. AMINO), sulfonamides (SUL), phenicols (EC.PHENICOLS); total enterococci (ENT) and enterococci resistant to quinolones (ENT.QUINO), lincosamides (ENT.LINCO), nitrofurans (ENT.NFURANS) and penicillins (ENT.PEN). AMR phenotypes that are significantly correlated with the overall microbiome are highlighted in red font and arrows. DDGS: basal diet supplemented with 20% distillers dried grains with solubles, formulated to contain 13.6% NDF; SBP: basal diet supplemented with 14.5% sugar beet pulp, formulated to contain 13.6% NDF.

Fig. 8. Abundance of: A) fecal coliforms, and B) enterococci ($\log_{10}$ CFU/g) cultured in the presence of the clinical breakpoint concentration of a drug representing each antimicrobial class; specifically, for fecal coliforms: aminopenicillins, 3rd generation cephalosporins, tetracyclines, macrolides, aminoglycosides, sulfonamides, phenicols and without antimicrobials and for enterococci: penicillins, quinolones, tetracyclines, aminoglycosides, lincosamides, nitrofurans and without antimicrobials. The data are for finisher pigs receiving one of three diets and receiving either no antimicrobial treatment or else treated with ceftiofur for days 1 and 3 of the treatment regimens. DDGS: basal diet supplemented with 20% distillers dried grains with solubles, formulated to contain 13.6% NDF; SBP: basal diet supplemented with 14.5% sugar beet pulp, formulated to contain 13.6% NDF.

Fig. 9. Beta diversity of the fecal microbiome composition of pigs receiving one of three dietary treatments and antimicrobial drugs. Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis distances by penicillin G-treatment group across diets: (A) Control diet, B) DDGS-based diet, C) SBP-based diet, and D) across all diets by penicillin-treated

group. Relationship between overall microbial genera composition and phenotypic AMR abundance in coliform and enterococcal populations of pigs receiving different diets are shown using multivariate approaches (*envit* function in R). Total coliforms with antimicrobials (EC) and coliforms resistant to aminopenicillins (EC.AMP), 3rd generation cephalosporins (EC.CEF), tetracyclines (EC.TET), macrolides (EC.MLIDE), aminoglycosides (EC. AMINO), sulfonamides (SUL), phenicols (EC.PHENICOLS); total enterococci (ENT) and enterococci resistant to quinolones (ENT.QUINO), lincosamides (ENT.LINCO), nitrofurans (ENT.NFURANS) and penicillins (ENT.PEN). AMR phenotypes that are significantly correlated with the overall microbiome are highlighted in red font and arrows. DDGS: basal diet supplemented with 20% distillers dried grains with solubles, formulated to contain 13.6% NDF; SBP: basal diet supplemented with 14.5% sugar beet pulp, formulated to contain 13.6% NDF.

Fig. 10. Abundance of: A) fecal coliforms, and B) enterococci (log_{10} CFU/g) growing in the presence of the clinical breakpoint concentration of a drug representing each antimicrobial class; specifically, for fecal coliforms: aminopenicillins, 3rd generation cephalosporins, tetracyclines, macrolides, aminoglycosides, sulfonamides, phenicols and without antimicrobials and for enterococci: penicillins, quinolones, tetracyclines, aminoglycosides, lincosamides, nitrofurans and without antimicrobials. The data are for finisher pigs receiving one of three diets and receiving either no antimicrobial treatment or else treated with penicillin G for days 1 and 3 of the treatment regimen. DDGS: basal diet supplemented with 20% distillers dried grains with solubles, formulated to contain 13.6% NDF; SBP: basal diet supplemented with 14.5% sugar beet pulp, formulated to contain 13.6% NDF.

Table 1. Ingredient composition of the experimental diets fed to the finisher pigs (as-fed basis)

	Phase 1 (d 91 to 109 of age)			Phase 2 (d 110 to 143 of age)			Phase 3 (d 144 to 177 of age)		
Ingredient, %	Control[1]	DDGS[2]	SBP[3]	Control	DDGS	SBP	Control	DDGS	SBP
Corn	75.5	62.6	58.7	81.8	68.8	65.0	85.2	70.6	68.5
DDGS	-	20.0	-	-	20.0	-	-	20.0	-
Sugar beet pulp	-	-	14.5	-	-	14.5	-	-	14.5
Soybean meal, 47% crude protein	21.8	14.5	21.4	15.7	8.4	15.3	12.4	6.7	12.0
Choice white grease	-	0.20	2.80	-	0.25	2.85	-	0.40	2.75
Calcium carbonate	0.93	1.05	0.60	0.93	1.08	0.63	0.93	1.08	0.63
Monocalcium P (21% P)	0.55	0.25	0.63	0.40	0.10	0.45	0.35	-	0.38
Sodium chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
L-lysine-HCl	0.30	0.45	0.30	0.30	0.45	0.30	0.30	0.40	0.30
DL-methionine	0.07	0.03	0.11	0.03	-	0.08	0.02	-	0.06
L-threonine	0.09	0.09	0.12	0.10	0.10	0.13	0.11	0.09	0.14
L-tryptophan	0.01	0.03	0.02	0.02	0.04	0.02	0.02	0.03	0.03
L-valine	-	-	0.04	-	-	0.03	-	-	0.03
Trace mineral premix	0.15	0.15	0.15	0.13	0.13	0.13	0.10	0.10	0.10
Vitamin premix	0.15	0.15	0.15	0.13	0.13	0.13	0.10	0.10	0.10
Phytase[4]	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Total	100	100	100	100	100	100	100	100	100

[1]Control: Basal diet (corn grain and soybean meal-based) formulated to contain 8.7% neutral detergent fiber (NDF).

[2]DDGS: Basal diet supplemented with 20% distillers dried grains with solubles (DDGS), formulated to contain 13.6% NDF.

[3]SBP: Basal diet supplemented with 14.5% sugar beet pulp, formulated to contain 13.6% NDF.

[4]HiPhos 2700 (DSM Nutritional Products, Inc., Parsippany, NJ), providing 83.3 phytase units (FTU)/kg and an estimated release of 0.09% available P.

Table 2. Chemical analysis of experimental diets fed to finisher pigs (as-fed basis)[1]

Item	Phase 1 (d 91 to 109 of age)			Phase 2 (d 110 to 143 of age)			Phase 3 (d 144 to 177 of age)		
	Control[2]	DDGS[3]	SBP[4]	Control[2]	DDGS[3]	SBP[4]	Control[2]	DDGS[3]	SBP[4]
Dry matter	88.9	89.8	89.9	89.2	90.4	90.4	88.4	89.4	89.0
Crude protein	17.4	18.7	16.7	14.3	16.4	14.3	12.6	15.3	12.3
Crude fat	2.6	4.0	5.6	2.9	4.5	5.8	2.9	4.0	4.8
Acid detergent fiber	3.0	3.8	7.3	3.5	4.4	6.8	2.5	3.9	7.6
Neutral detergent fiber	6.8	10.6	11.1	7.8	12.6	11.3	6.7	9.6	10.4
Nitrogen free extract	63.7	60.2	59.0	66.7	62.4	61.4	68.0	63.9	63.1
Ash	3.8	4.4	4.8	3.5	4.2	5.1	3.4	3.7	4.4

[1]Analysis was performed by Ward Laboratories, Inc., Kearney, NE on pooled feed samples.
The basal diet was corn grain (75 to 85%) and soybean meal.
[2]Control: Basal diet (corn grain and soybean meal-based) formulated to contain 8.7% neutral detergent fiber (NDF).
[3]DDGS: Basal diet supplemented with 20% distillers dried grains with solubles (DDGS), formulated to contain 13.6% NDF.
[4]SBP: Basal diet supplemented with 14.5% sugar beet pulp, formulated to contain 13.6% NDF.

Table 3. Effects of dietary fiber type and level on growth performance of finishing pigs[1]

Item	Control[1]	DDGS[2]	SBP[3]	SEM	*P*-value
Body weight, kg					
Initial (d 91 of age; 0)	50.7	50.7	50.7	0.711	1.00
Final (d 177of age; 86)	132.1	130.3	131.0	0.856	0.328
Days 0 to 86					
Average daily feed intake, kg	2.79	2.77	2.74	0.034	0.625
Average daily gain, kg	0.94	0.92	0.93	0.014	0.228
Gain:feed	0.35	0.33	0.34	0.003	0.094

[1]Control: Basal diet (corn grain and soybean meal-based) formulated to contain 8.7% neutral detergent fiber (NDF).
[2]DDGS: Basal diet supplemented with 20% distillers dried grains with solubles (DDGS), formulated to contain 13.6% NDF.
[3]SBP: Basal diet supplemented with 14.5% sugar beet pulp, formulated to contain 13.6% NDF.

Table 4. Numbers of pigs sampled in the study

Treatment	Age at sampling, days	Dietary treatments			Total no. of fecal samples
		Control[1]	DDGS[2]	SBP[3]	
Diets	98	3	4	3	10
	110	5	5	5	15
	144	5	5	5	15
	177	5	5	5	15
Subtotal					55
Ceftiofur					
Ceftiofur-treated	Days 1 and 3	6	8	6	20
	Day 1 only[4]	3	-	2	5
Untreated pen-mate control	Day 1	6	4	5	15
Subtotal					40
Penicillin G					
Penicillin G-treated	Days 1 and 3	10	10	10	30
Untreated pen-mate control	Day 1	5	5	5	15
Subtotal					45
Total		48	46	46	140

[1]Control: Basal diet formulated to contain 8.7% neutral detergent fiber (NDF).
[2]DDGS: Basal diet supplemented with 20% distillers dried grains with solubles (DDGS), formulated to contain 13.6% NDF.
[3]SBP: Basal diet supplemented with 14.5% sugar beet pulp, formulated to contain 13.6% NDF.
[4]Pigs were only treated for 1-2 days when clinical improvement was seen; hence, only Day 1 sample was collected.

Table 5. Antimicrobials class and antimicrobial concentrations used in agar media to determine the phenotypic antimicrobial resistance

Bacteria/Media	Antimicrobial drug class	Antimicrobial used	Concentration (µg/mL)[1]
Coliforms/ MacConkey Agar	Aminoglycosides	Gentamicin (GEN)	16
		Streptomycin (STR)	32
	Cephalosporins	Ceftriaxone (AXO)	4
	Sulfonamides/folate pathway inhibitors	Sulfamethoxazole (SMX)	512
	Macrolides	Azithromycin (AZI)	32
	Beta lactams	Ampicillin (AMP)	32
	Phenicols	Chloramphenicol (CHL)	32
	Quinolones	Nalidixic acid (NAL)	32
	Fluoroquinolones	Ciprofloxacin (CIP)	1
		Enrofloxacin (ENR)	0.125[2]
	Tetracyclines	Tetracycline (TET)	16
Enterococcus spp. Enterococcosel agar	Aminoglycosides	Gentamicin (GEN)	500
		Streptomycin (STR)	1,024
	Lincosamides	Lincomycin (LIN)	8
	Macrolides	Erythromycin (ERY)	8
		Tylosin	32
	Nitrofurans	Nitrofurantoin (NIT)	128
	Beta lactams	Penicillin (PEN)	16
	Phenicols	Chloramphenicol (CHL)	32
	Quinolones	Nalidixic acid (NAL)	32[3]
	Fluoroquinolones	Ciprofloxacin (CIP)	4
		Enrofloxacin (ENR)	4
	Tetracyclines	Tetracycline (TET)	16

[1]Breakpoints based on Clinical Laboratory Standards Institute guidelines (CLSI, 2008) and National Antimicrobial Resistance Monitoring System
[2]European Committee on Antimicrobial Susceptibility Testing (epidemiological cutoff value)
[3]Adopted from *E. coli* break-point concentration

Table 6. Analysis of the effects of age, diet, antimicrobial treatment, and their interactions on the pig fecal microbial community composition (genus level) based on permutational multivariate analysis of variance **(**PERMANOVA**)** and beta-dispersion.

	PERMANOVA[1]		Beta-dispersion[2]
Untreated pigs	Overall	*Post-hoc analysis*	
Age (days)	R^2 = 0.362, P = 0.001	98 vs. 144 (R^2 = 0.134, P = 0.002); 98 vs. 177 (R^2 = 0.40, P = 0.001); 110 vs. 144 (R^2 = 0.172, P = 0.002); 110 vs. 177 (R^2 = 0.463, P = 0.001) and 144 vs. 177 (R^2 = 0.236, P = 0.001)	P = 0.416
Diet[3]	R^2 = 0.061, P = 0.001	Control vs. SBP (R^2 = 0.054, P = 0.022); DDGS vs. SBP (R^2 = 0.051, P = 0.026	P = 0.573
Age X Diet	R^2 = 0.069, P = 0.0587		
Ceftiofur-treated pigs			
Treatment[4]	R^2 = 0.173, P = 0.002	Untreated pen mate, Day 1 vs. Ceftiofur-treated, Day 1 (R^2 = 0.104, P = 0.021); Untreated pen mate, Day 1 vs. Ceftiofur-treated, Day 3 (R^2 = 0.246, P = 0.009);	P = 0.118
Diet[3]	R^2 = 0.128, P = 0.01	DDGS vs. SBP (R^2 = 0.157, P = 0.018)	P = 0.052
Treatment X Diet	R^2= 0.075, P = 0.69		
Penicillin G-treated pigs			
Treatment[4]	R^2 = 0.099, P = 0.002	Untreated pen mate, Day 1 vs. Penicillin G-treated, Day 3 (R^2 = 0.109, P = 0.009);	Penicillin-treated Day 3 vs. untreated pen-mate Day 1 (P = 0.023)
Diet[3]	R^2 = 0.134, P = 0.003	Control vs. SBP (R^2 = 0.1313, P = 0.003); DDGS vs. SBP (R^2 = 0.114, P = 0.0045)	P = 0.397
Treatment X Diet	R^2 = 0.055, P = 0.946		

[1]Permutational multivariate analysis of variance **(**Bray-Curtis distance**)** with 999 permutations of pair-wise comparisons of clustering of fecal microbial communities

[2]Analysis of variance (ANOVA) of distance **(**Bray-Curtis distance**)** to the centroid for evaluation of homogeneity of variance within each group

[3](Control: Basal diet formulated to contain 8.7% neutral detergent fiber (NDF); DDGS: Basal diet supplemented with 20% distillers dried grains with solubles (DDGS), formulated to contain 13.6% NDF; SBP: Basal diet supplemented with 14.5% sugar beet pulp, formulated to contain 13.6% NDF)

[4](Untreated pen mate, Day 1; Ceftiofur or Penicillin G treated Days 1 and 3)

Table 7. Log_2-fold change in abundance of fecal bacterial taxa (phylum or genus) for i) dietary treatments over the age of pigs from days 98, 110, 144, and 177 (untreated cohort study group), ii) treatment (untreated pen-mates day 1, ceftiofur- or penicillin G-treated pigs on days 1 and 3) across the three dietary treatments. The positive and negative log_2-fold changes indicated an increase and decrease in abundance of taxa for that group, respectively.

	Comparison	Differentially abundant taxa	Log_2 (fold change)	Mean abundance/expression	Adj. *P*-values
Untreated pigs					
Age (days)					
98	Control vs. DDGS	None	-	-	-
	Control vs. SBP	*g_Catenibacterium*	3.51	6.12	0.012
		g_Holdemanella	3.37	6.12	0.015
	DDGS vs. SBP	None	-	-	-
110	Control vs. DDGS	None	-	-	-
	Control vs. SBP	None	-	-	-
	DDGS vs. SBP	None	-	-	-
144	Control vs. DDGS	None	-	-	-
	Control vs. SBP	None	-	-	-
	DDGS vs. SBP	*g_Coprococcus 2*	-3.52	5.08	0.031
177	Control vs. DDGS				
	Control vs. SBP	*g_[Eubacterium] ruminantium group*	-3.91	4.38	0.04
	DDGS vs. SBP	p_Chlamydiae	-2.88	1.25	0.053
Ceftiofur-treated pigs					
Dietary treatment					
Control	Untreated pen mate day 1 vs. Ceftiofur-treated, day 1	None	-	-	-
	Untreated pen mate day 1 vs. Ceftiofur-treated, day 3	p_Chlamydiae	3.89	1.78	0.038
	Ceftiofur-treated day 1 vs. Ceftiofur-treated	None	-	-	-

	day 3				
DDGS	Untreated pen mate day 1 vs. Ceftiofur-treated, day 1	None	-	-	-
	Untreated pen mate day 1 vs. Ceftiofur-treated, day 3	None	-	-	-
	Ceftiofur-treated day 1 vs. Ceftiofur-treated day 3	*p_Patescibacteria*	2.80	3.29	0.001
		g_Candidatus Saccharimonas	2.80	3.29	0.037
SBP	Untreated pen mate day 1 vs. Ceftiofur-treated, day 1	p_Epsilonbacteraeota	-3.35	4.24	0.047
		p_Chlamydiae	3.04	2.06	0.047
	Untreated pen mate day 1 vs. Ceftiofur-treated, day 3	p_Chlamydiae	5.86	2.06	0.052
		p_Kiritimatiellaeota	-5.94	5.57	0.052
		p_Planctomycetes	-4.68	3.65	0.052
		p_Spirochaetes	-4.98	8.04	0.054
	Ceftiofur-treated day 1 vs. Ceftiofur-treated day 3	None	-	-	-
Penicillin G-treated pigs					
Dietary treatment					
Control	Untreated pen mate day 1 vs. Penicillin G-treated, day 1	p_Chlamydiae	4.91	1.63	0.02
	Untreated pen mate day 1vs. Penicillin G-treated, day 3	*g_Streptococcus*	3.41	11.99	0.013
		g_Terrisporobacter	-2.62	10.53	0.039

	Penicillin G-treated day 1 vs. Penicillin G-treated day 3	*g_Streptococcus*	3.47	11.99	0.01
DDGS- or SBP	Untreated pen mate day 1 vs. Penicillin G-treated, day 1	None	-	-	-
	Untreated pen mate day 1vs. Penicillin G-treated day 3	None	-	-	-
	Penicillin G-treated, day 1 vs. Penicillin G-treated day 3	None	-	-	-

Control: Basal diet (corn grain and soybean meal-based) formulated to contain 8.7% neutral detergent fiber (NDF)
DDGS: Basal diet supplemented with 20% distillers dried grains with solubles (DDGS), formulated to contain 13.6% NDF
SBP: Basal diet supplemented with 14.5% sugar beet pulp, formulated to contain 13.6% NDF)
None: none of the taxa abundance was significantly different
Taxa with initial p and *g* indicate phylum and genus, respectively.

Figure 1

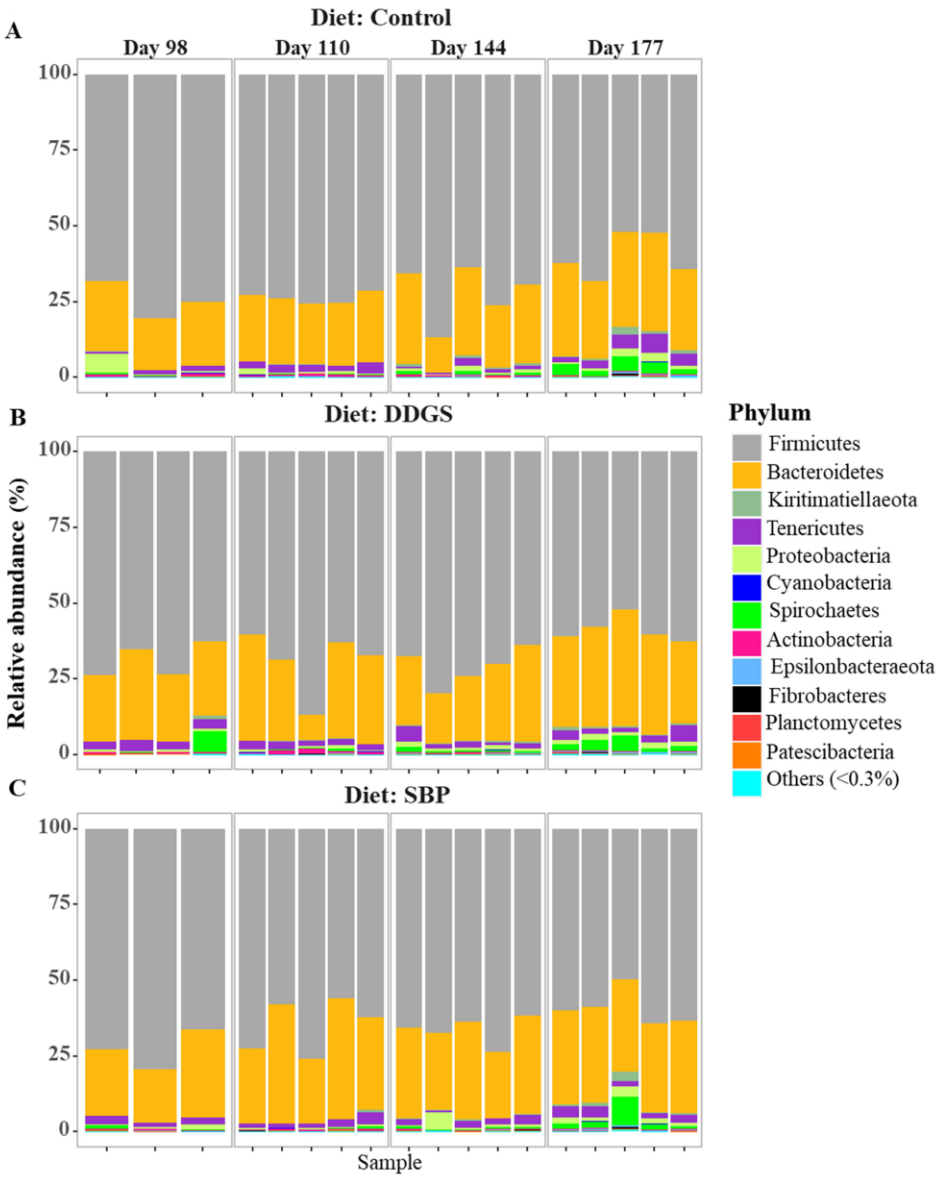

Figure 2

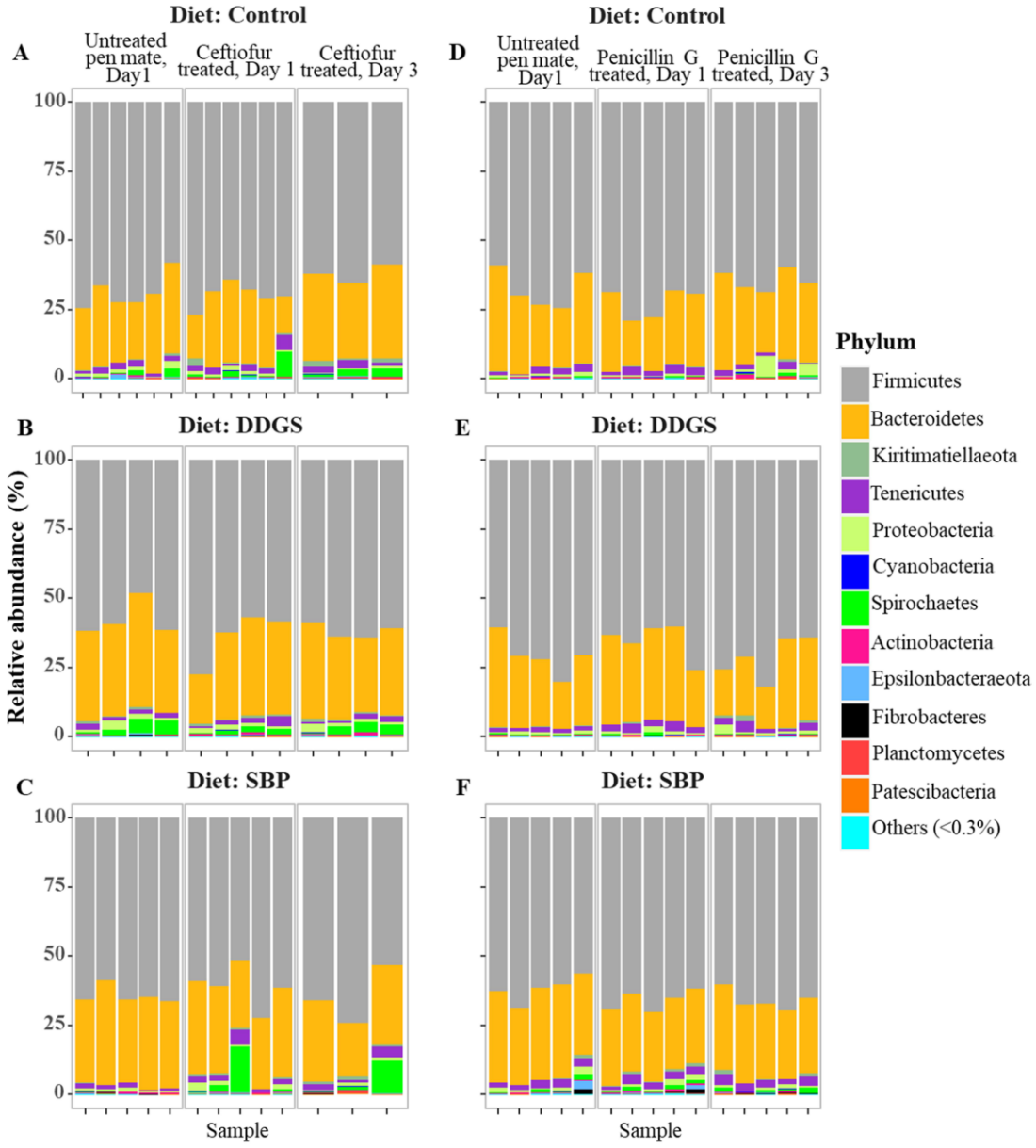

Figure 3

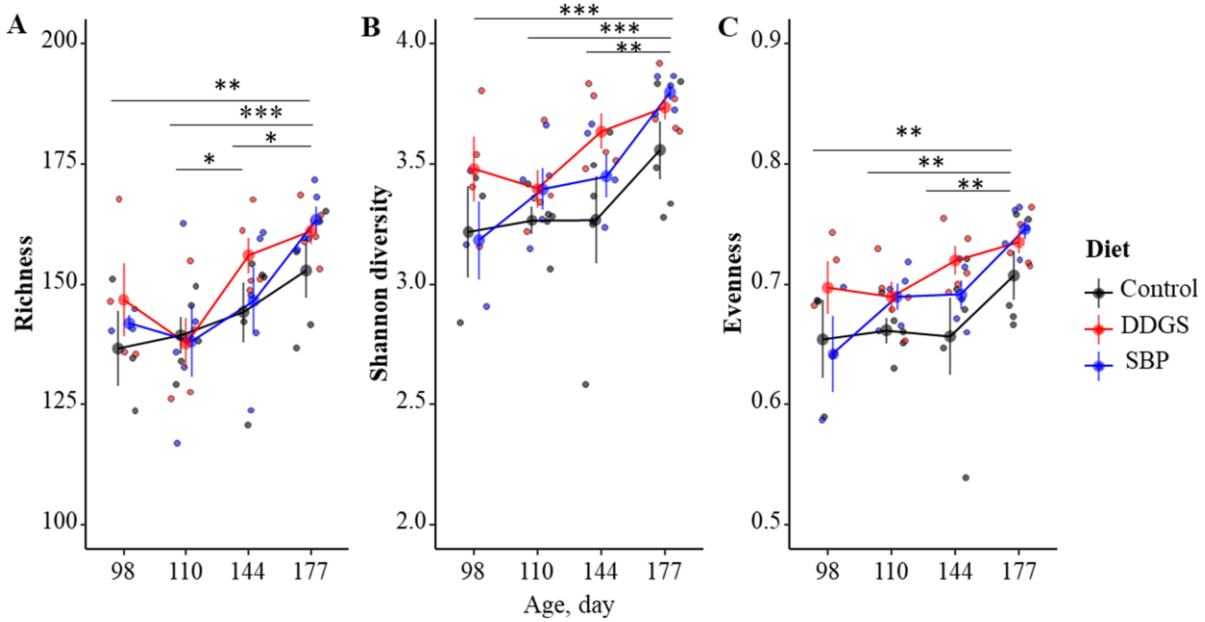

Figure 4

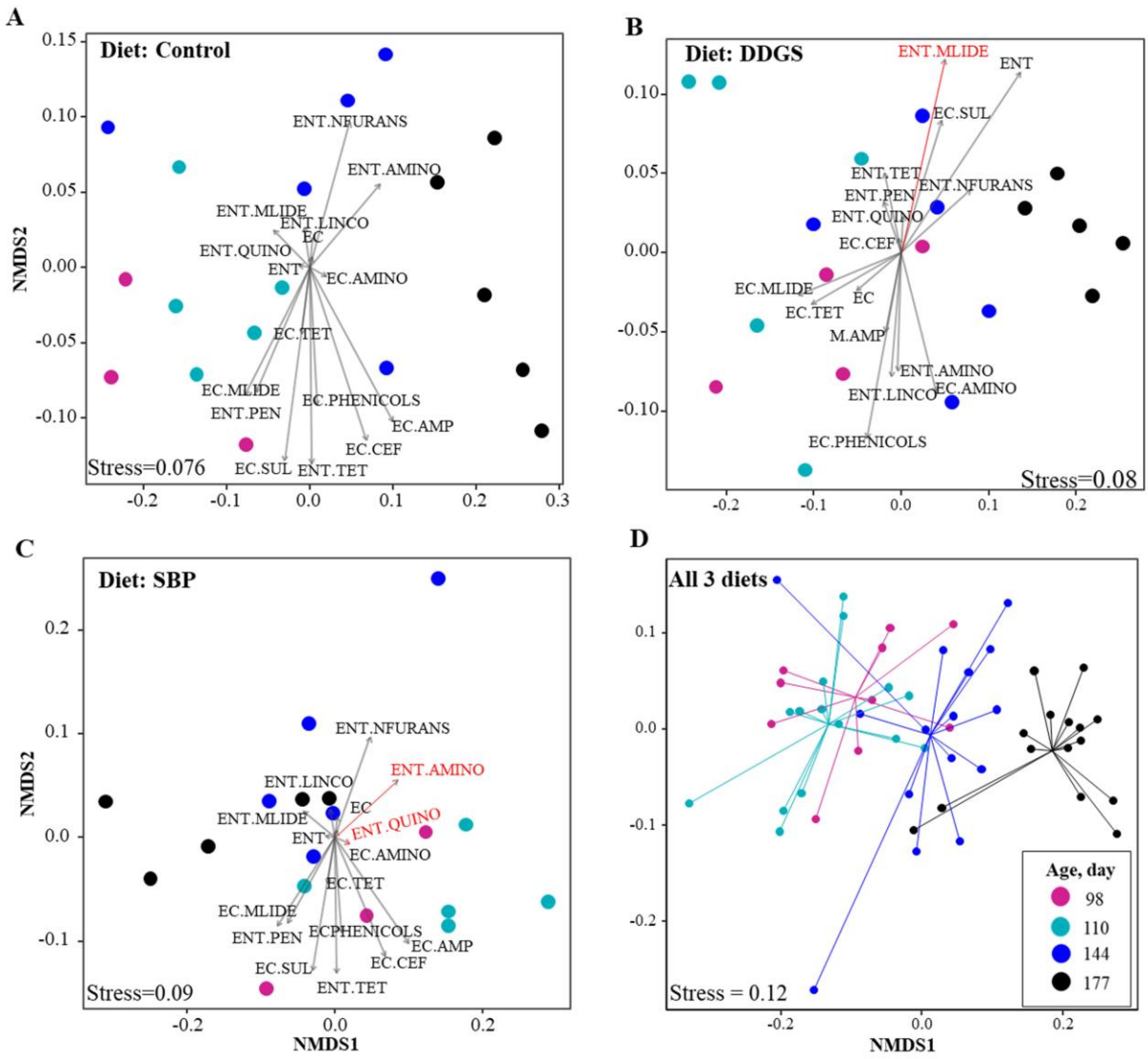

Figure 5

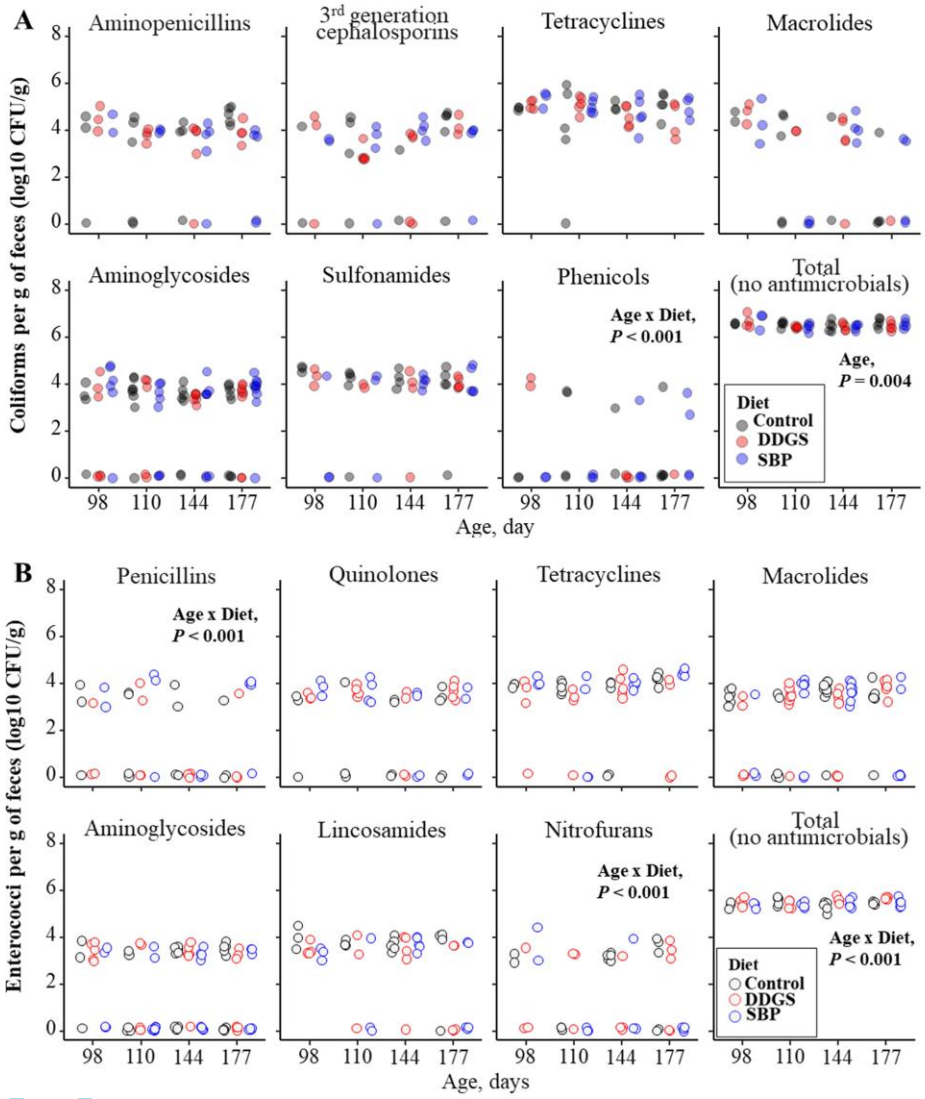

Figure 6

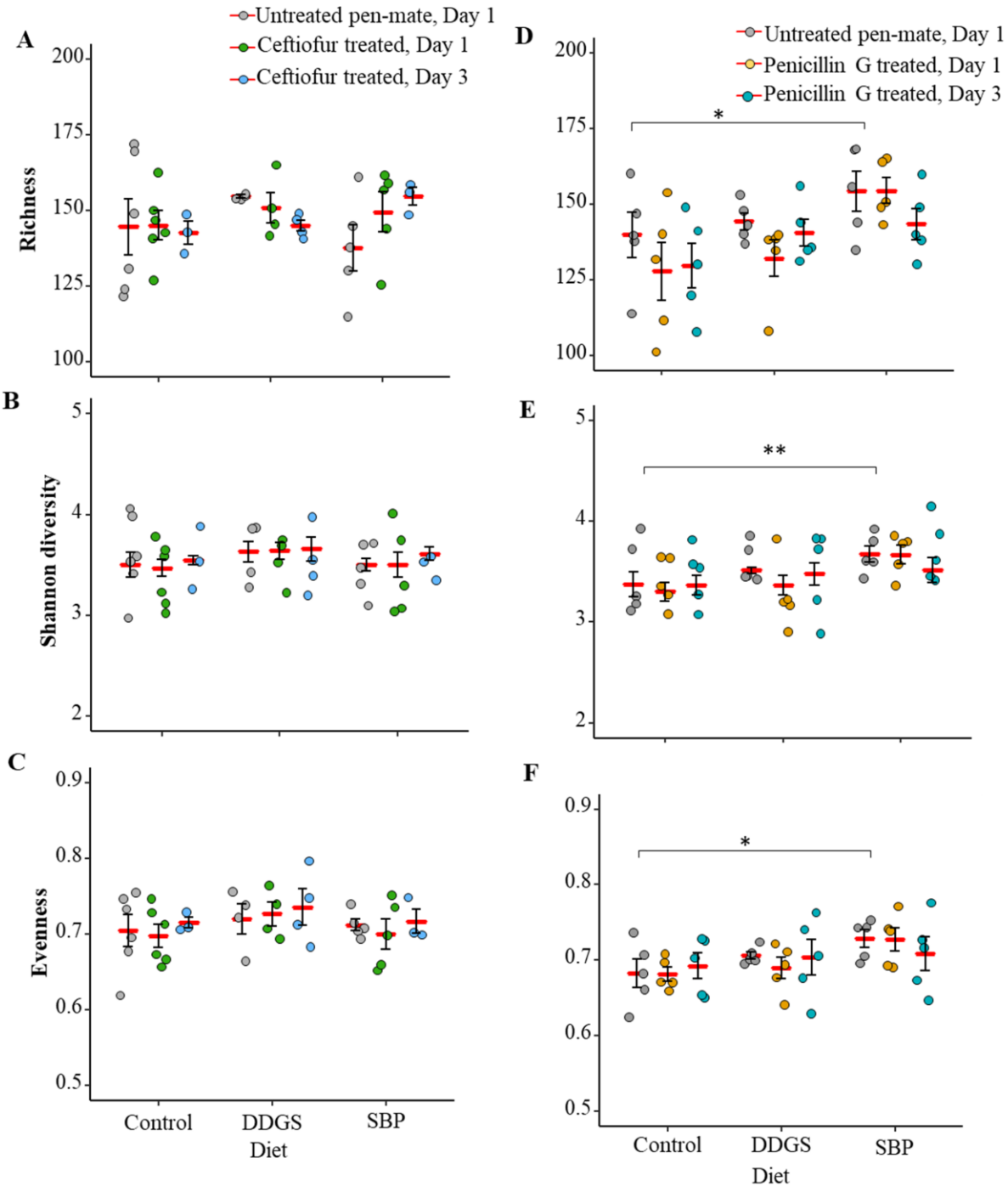

Figure 7

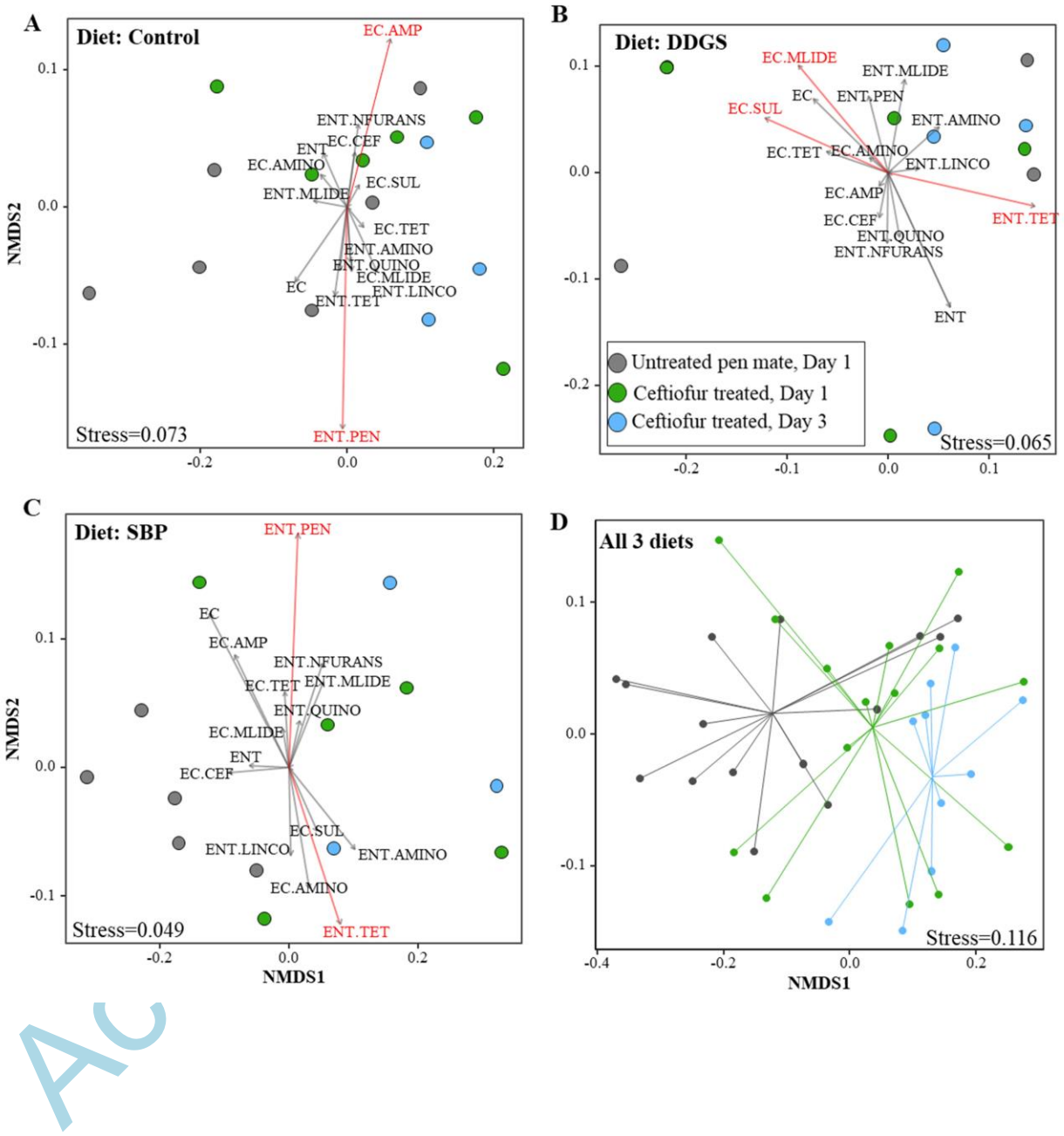

Figure 8

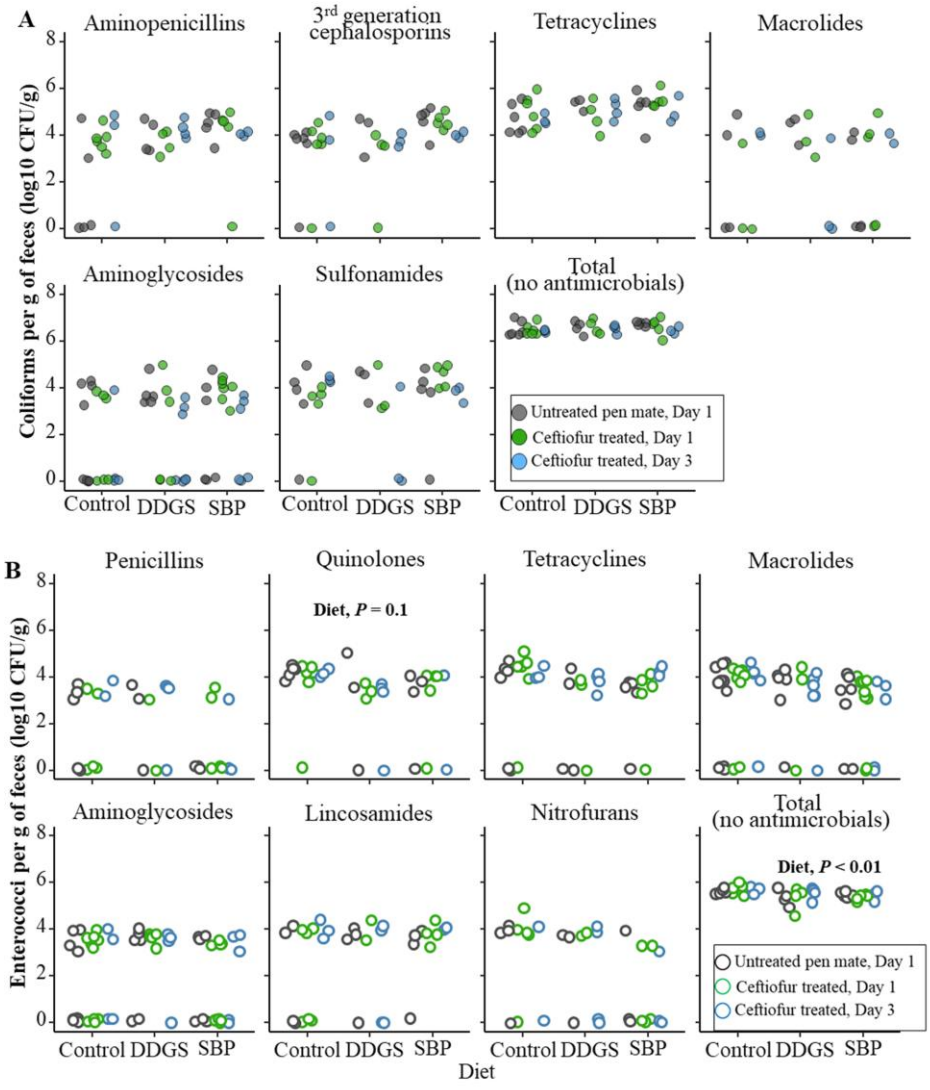

Figure 9

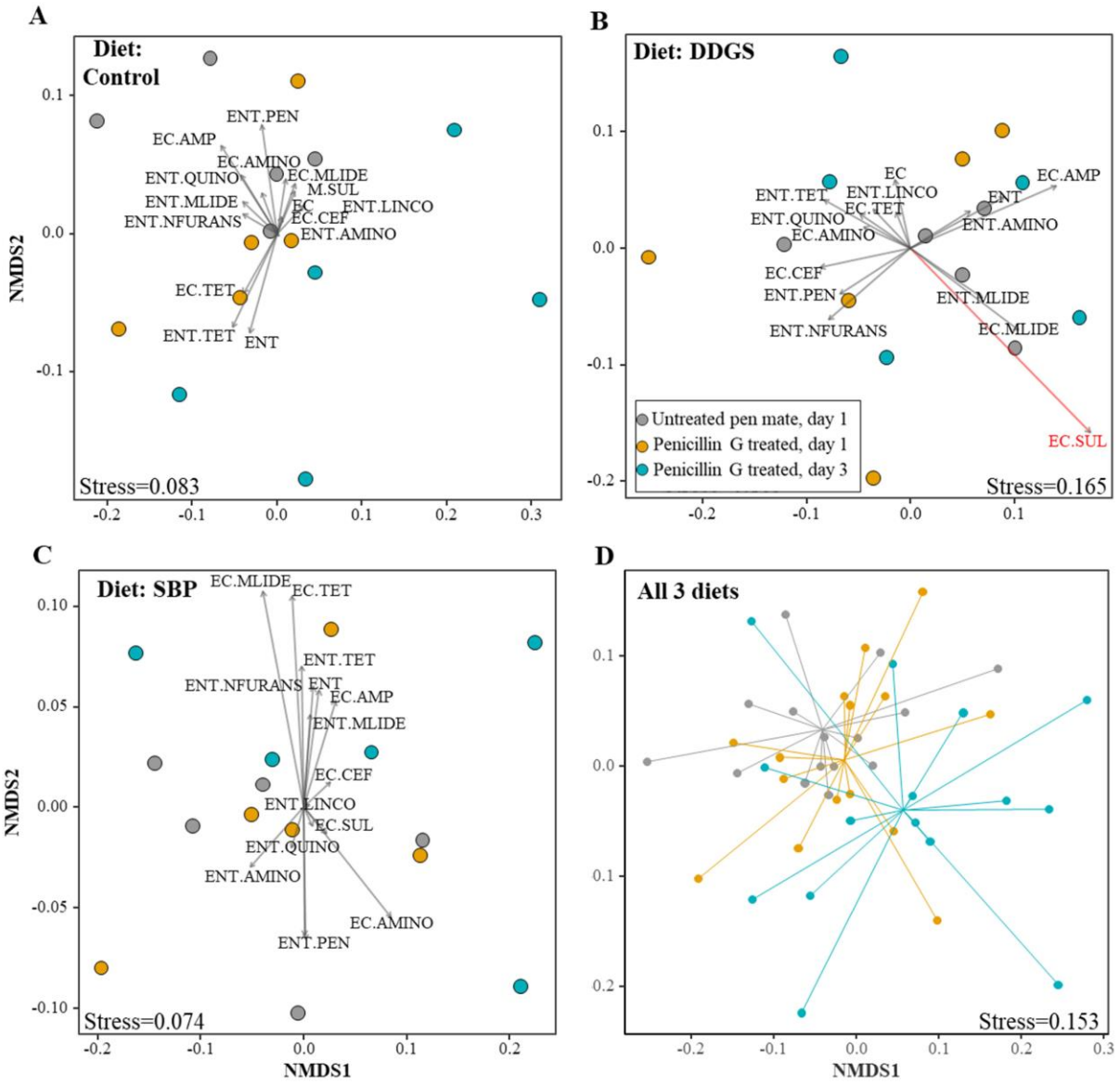

Figure 10