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Non Ruminant Nutrition

Effects of a blend of essential oils, medium-chain fatty acids, and a toxin-adsorbing mineral on diarrhea and gut microbiome of weanling pigs experimentally infected with a pathogenic *Escherichia coli*

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Abstract

A proprietary antimicrobial feed additive comprised of essential oils, medium-chain fatty acids, and a toxin-adsorbing mineral showed promising bacteriostatic and bactericidal effects in vitro. This study investigated the impacts of supplementing this blend on growth, gut microbiome, and enteric disease resilience in weaned pigs experimentally challenged with an enterotoxigenic *Escherichia coli* (**ETEC**). Thirty-six weanling pigs (6.88 ± 0.30 kg body weight) blocked by weight and gender were assigned to one of three dietary treatments: control or dietary supplementation with 0.25% or 0.50% of the antimicrobial blend. This study lasted 28 d with 7 d before and 21 d after the first ETEC inoculation (day 0). All pigs were orally inoculated with 10^{10} CFU F18 + ETEC/3-mL dose for 3 consecutive days. Growth performance data and diarrhea scores were recorded throughout the experiment. Fecal samples collected on days −7, 0, 7, and 21 post first inoculation (**PI**), and ileal digesta and mucosal tissue collected on day 21 PI were further analyzed for gut microbiome using 16S rRNA sequencing. All data, except for frequency of diarrhea and gut microbiome, were analyzed by ANOVA using the PROC MIXED of SAS. The chi-square test was used for analyzing frequency of diarrhea. Gut microbiome data were analyzed using QIIME2 and visualized using the R program. Dietary supplementation of 0.25% or 0.5% of the antimicrobial blend increased ($P < 0.05$) feed efficiency on days 14 to 21 PI of ETEC and reduced ($P < 0.05$) frequency of diarrhea during the study. Compared with the control group, adding 0.5% dietary antimicrobial blend increased ($P < 0.05$) relative abundance of Firmicutes but reduced ($P < 0.05$) Bacteroidetes and Proteobacteria in feces on day 7 PI. Pigs that received the antimicrobial blend also had higher ($P < 0.05$) relative abundance of *Lactobacillaceae*, but lower ($P < 0.05$) relative abundance of *Enterobacteriaceae* in feces on day 7 PI than pigs in control. In conclusion, supplementation of this antimicrobial blend at 0.5% reduced incidence of severe diarrhea in weaned pigs challenged with F18 ETEC and enhanced feed efficiency of weaned pigs at the last week of the experiment. Supplementation of this antimicrobial blend also modified the microbiota diversity in feces and ileal mucosa of weaned pigs.

Lay Summary

This experiment aims to investigate an antimicrobial blend consisting of essential oils, medium-chain fatty acids, and a toxin-adsorbing mineral on diarrhea, growth performance, and gut microbiome of newly weaned pigs experimentally infected with a pathogenic *Escherichia coli* (F18 *E. coli*). A total of 36 weaned pigs were randomly allotted to one of three dietary treatments: (1) a complex control diet that met the nutrient requirement of weaned pigs; (2) supplementing 0.25% of the antimicrobial blend; and (3) 0.50% of the antimicrobial blend. The experiment lasted 28 d with 7 d adaptation and 21 d after the first F18 *E. coli* inoculation. Results of this experiment demonstrate that supplementation of this antimicrobial blend enhanced disease resistance of weaned pigs, as indicated by reduced frequency of diarrhea during the entire experimental period. An improved feed efficiency was also observed in pigs supplemented with antimicrobial blend at the last week of the experiment. In addition, feces collected on day 7 post-*E. coli* inoculation contained relatively more *Lactobacillaceae* but less *Enterobacteriaceae* when pigs were supplemented with this antimicrobial blend. In conclusion, supplementation of antimicrobial blend could reduce diarrhea of *E. coli*-infected pigs and modify fecal microbiome of weaned pigs during the peak of *E. coli* infection.

Key words: antimicrobial feed additive, diarrhea, *Escherichia coli* challenge, microbiome, weaned pigs

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; BW, body weight; CFU, colony-forming unit; ETEC, enterotoxigenic *E. coli*; G:F, gain-to-feed ratio; INBW, initial body weight; OTU, operational taxonomic unit; PCoA, principal coordinate analysis; PCR, polymerase chain reaction; PI, postinoculation

Introduction

Weaning is generally accompanied with reduced feed intake, poor growth performance, and increased susceptibility to infectious diseases (Spreeuwenberg et al., 2001). Growth-promoting and prophylactic use of antibiotics has been an effective tool to control postweaning diarrhea and infectious

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diseases. However, as the pressure to lower antibiotic use increases (FDA GFI #213, 2016; Announcement of the Ministry of Agriculture and Rural People's Republic of China No. 194, 2019; Regulation (EU) 2019/6), strategies that can protect animal health and promote production performance have substantially increased importance in animal production.

Gut dysbiosis, mucosal atrophy, and impaired barrier functions are widely reported signs of gastrointestinal distress caused by weaning. Dietary strategies have been applied through feed or water in order to improve health and maximize production of weaned pigs (Liu et al., 2018). The candidates include but not limited to phytochemicals, short- and medium-chain fatty acids, certain micro minerals, functional amino acids, and pre- and probiotics (Liu et al., 2018; Xiong et al., 2019). These functional feed additives may regulate overall intestinal health and mitigate weaning-induced enteric distress via different modes of action. For instance, many phytochemicals were reported to possess a wide spectrum of antimicrobial activities and anti-inflammatory and immunomodulatory effects in vitro and in vivo (Hammer et al., 1999; Liu et al., 2012; 2013; 2014). Derivatives of short- and medium-chain fatty acids were shown to have strong antimicrobial activity against bacterial pathogens and serve as energy substrates for intestinal epithelial cells (Namkung et al., 2011; Kovanda et al., 2019), whereas certain minerals can bind to toxins released from the pathogens (Chi et al., 2017).

NeutraPath (Amlan International, Chicago, IL) is a formulated blend of functional feed additives consisting of essential oils (major active components are cinnamaldehyde and eugenol), hydrogenated fats (medium-chain fatty acids), and a thermally processed toxin-adsorbing mineral (bentonite). The formula showed potent bactericidal/bacteriostatic effects in vitro with a broad antibacterial spectrum against *Escherichia coli*, *Salmonella enterica*, *Clostridium perfringens*, and *Vibrio parahaemolyticus* (Wang et al., 2018) and demonstrated in vivo efficacy against *Clostridium perfringens*, *Salmonella* Heidelberg, and *Salmonella* Typhimurium infections in broiler chickens (Xue et al., 2018, 2019). Enterotoxigenic *Escherichia coli* (**ETEC**) is a prolific producer of a variety of enterotoxins such as heat-labile enterotoxin and the heat-stable enterotoxins (STa and STb), which play a key role in the pathogenesis of ETEC-associated swine diarrhea (Hartadi et al. 2020). The enterosorbent mineral can further disarm the pathogen by binding the ETEC-produced enterotoxins, thereby helping to prevent damage to the intestinal environment. Therefore, we hypothesized that the blend of essential oils, medium-chain fatty acids, and toxin-adsorbing minerals could enhance resilience to enteric pathogens and reduce diarrhea incidence in weaned pigs. The objective of the present experiment was to evaluate dietary supplementation of this antimicrobial blend on growth and diarrhea of weaned pigs experimentally infected with F18 ETEC. 16S rRNA sequencing analysis was also assessed to explore the impacts of this antimicrobial blend on intestinal microbiota diversity and community in disease-challenged pigs.

Materials and Methods

The experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of California, Davis (UC Davis, IACUC #20809).

Experimental design and animal management

A total of 36 piglets (21 to 24 d of age) with equal number of barrows and gilts (6.88 ± 0.30 kg body weight [**BW**]) were selected from the Swine Teaching and Research Center of UC Davis and used in this experiment. The sows and piglets used in the experiment did not receive vaccines against pathogenic *E. coli*, antibiotic injections, or antibiotics in creep feed. Before weaning, fecal samples from sows and all their piglets were tested and confirmed the absence of β-hemolytic *E. coli*. All piglets intended for use in the study were subject to genotyping for susceptibility to F18 ETEC based on the methods described previously in Kreuzer et al. (2013). Only genetically susceptible pigs were used in the experiment.

After weaning, all pigs were transferred to the Cole facility at UC Davis and were housed in individual pens (0.61 m × 1.22 m) for 28 d, including 7 d before and 21 d after the first ETEC inoculation. Pigs had free access to feed and water during the study. Animal rooms were equipped with fans and heaters to achieve the desired temperature for nursery pigs. A 12-h light cycle starting at 0730 h was automatically controlled in all animal rooms.

The study was conducted as a randomized complete block design. Pigs were blocked by initial body weight and sex and randomly assigned to one of three dietary treatments (n = 12/treatment). Pigs were fed a control diet based on corn and soybean meal supplemented with 0 (control), 0.25%, or 0.50% of the antimicrobial blend. The control diet was formulated to meet or exceed nutritional requirements of weaned pigs (Table 1). NeutraPath was provided by Amlan International (Chicago, IL). All experimental diets were provided in mash form and did not contain spray-dried plasma, antibiotics, or high levels of zinc oxide that exceeds recommendation. The experimental diets were formulated for two phases including phase 1 diets for weeks 1 and 2 and phase 2 diets for weeks 3 and 4. Pigs were fed with the experimental diets throughout the 28-d experimental period.

After 7-d adaptation, all pigs were orally inoculated with 10^{10} colony-forming unit (**CFU**)/3 mL F18 ETEC once daily from day 8 to day 10 of the study (day 0 to day 2 postinoculation [**PI**]). The F18 ETEC was originally isolated from a field disease outbreak by the University of Illinois Veterinary Diagnostic Lab (isolate number: U.IL-VDL # 05-27242). The strain expresses heat-labile toxin, heat-stable toxin b, and Shiga-like toxins. The dosage provided was shown to cause mild to watery diarrhea that was gradually recovered within 5 to 8 d after the first administration (Liu et al., 2013; Kim et al., 2019, 2021).

Data and sample collections

The experimental procedures were adapted from the previously published research (Liu et al. 2013; Kim et al. 2019). Briefly, fecal consistency was scored twice daily from day 0 to day 21 PI. The diarrhea score was daily assessed by two independent evaluators, using a 5-scale scoring system (1 = normal feces, 2 = moist feces, 3 = mild diarrhea, 4 = severe diarrhea, and 5 = watery diarrhea). The frequency of diarrhea was calculated as the percentage of the pig days with an average fecal consistency score ≥ 3 or ≥ 4 from day 0 to 21 PI. Pig BW and feeder weight were recorded at day −7 (beginning of the study), 0 (before inoculation), 7, 14, and 21 PI. Average daily gain (**ADG**), average daily feed intake (**ADFI**), and gain-to-feed ratio (**G:F**) were calculated for each period

Table 1. Ingredient compositions of control diets[1]

Ingredient, %	Phase I (weeks 1 and 2)	Phase II (weeks 3 and 4)
Corn	44.19	62.37
Dried whey	15.00	—
Soybean meal	18.00	28.00
Fish meal	8.00	3.00
Lactose	6.00	—
Soy protein concentrate	5.00	2.00
Soybean oil	2.00	2.00
Limestone	0.78	1.00
Monocalcium phosphate	—	0.60
l-Lysine·HCl	0.21	0.23
dl-Methionine	0.08	0.05
l-Threonine	0.04	0.05
Salt	0.40	0.40
Vitamin–mineral premix, Sow 6[2]	0.30	0.30
Total	100.00	100.00
Calculated energy and nutrient		
Metabolizable energy, kcal/kg	3,461	3,393
Net energy, kcal/kg	2,595	2,572
Crude protein, %	22.29	21.71
Arg,[3] %	1.25	1.30
His,[3] %	0.50	0.51
Ile,[3] %	0.84	0.79
Leu,[3] %	1.64	1.62
Lys,[3] %	1.35	1.23
Met,[3] %	0.44	0.37
Thr,[3] %	0.79	0.73
Trp,[3] %	0.23	0.23
Val,[3] %	0.92	0.87
Met + Cys,[3] %	0.73	0.69
Phe + Tyr,[3] %	1.49	1.52
Analyzed nutrient		
Dry matter, %	89.10	88.70
Crude protein, %	22.63	20.67
Total Ca, %	0.94	0.92
Total P, %	0.61	0.57

[1]Two additional diets were formulated by adding 0.25% or 0.50% of the antimicrobial feed additive to the control diet in each phase, respectively.
[2]Vitamin–mineral premix was provided by United Animal Health (Sheridan, IN). The premix provided the following quantities of vitamins and micro minerals per kilogram of complete diet: Vitamin A as retinyl acetate, 11,136 IU; vitamin D3 as cholecalciferol, 2,208 IU; vitamin E as dl-alpha tocopheryl acetate, 66 IU; vitamin K as menadione dimethylprimidinol bisulfite, 1.42 mg; thiamin as thiamine mononitrate, 0.24 mg; riboflavin, 6.59 mg; pyridoxine as pyridoxine hydrochloride, 0.24 mg; vitamin B12, 0.03 mg; d-pantothenic acid as d-calcium pantothenate, 23.5 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 20 mg as copper sulfate and copper chloride; Fe, 126 mg as ferrous sulfate; I, 1.26 mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganese sulfate; Se, 0.3 mg as sodium selenite and selenium yeast; and Zn, 125.1 mg as zinc sulfate.
[3]Amino acids are indicated as standardized ileal digestible AA.

from days –7 to 0, days 0 to 7 PI, days 7 to 14 PI, and days 14 to 21 PI. Fecal samples were collected from rectum using a fecal swap on day 0 (before inoculation), 2, 7, 14, and 21 PI. Fecal swabs were plated for bacterial culture to determine the percentage of β-hemolytic coliforms (Liu et al., 2013). Fecal samples collected on days –7, 0, 7, and 21 PI were also stored at –80 °C until analysis for gut microbiome. On day 21 PI, pigs were anesthetized after an intramuscular injection of Telazol/Ketamine/Xylazine mixture at the dose calculated based on body weight, then pigs were euthanized by an overdose of intracardiac injection of Fatal-Plus (390 mg/mL pentobarbital sodium solution, Vortech Pharmaceuticals, Ltd., Dearborn, MI). Ileal digesta and ileal mucosa were collected and immediately stored in liquid nitrogen for gut microbiome analysis.

Detection of β-hemolytic coliforms

Fecal samples were plated on MacConkey agar and Columbia Blood Agar containing 5% sheep blood to enumerate total coliforms and to identify β-hemolytic coliforms, respectively. Hemolytic colonies from the blood agar were subcultured on MacConkey agar for verification of lactose-fermenting bacteria, which develop flat pink colonies after bacteria culture. All plates were incubated at 37 °C for 24 h in an air incubator. Populations of both total coliforms and β-hemolytic coliforms on blood agar were assessed visually with a 8-scale scoring system (0 = no bacterial growth, 8 = very heavy bacterial growth). The score ratio of β-hemolytic coliforms to total coliforms was calculated. Questionable colonies were sub-subcultured on new MacConkey and blood agars to verify whether they were β-hemolytic ETEC by using triple sugar iron agar and lysine iron agar and to verify whether they were F18 + ETEC by means of a polymerase chain reaction (**PCR**; DebRoy and Maddox, 2001).

Gut microbiome in feces, ileal mucosa, and digesta

Bacterial DNA was extracted from approximately 100 to 150 mg of ileal mucosa, ileal digesta, and feces using the Quick-DNA fecal/soil microbe Kit (Zymo Research, Irvine, CA) following the manufacturer's instructions. Extracted bacterial DNA was quantified with Nanodrop Spectrophotometer (ThermoFisher Scientific, Waltham, MA) and diluted to 50 ng/μL. Then, the diluted DNA samples were amplified at variable region 4 of the 16S rRNA gene using PCR with the following conditions: initial denaturation at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 1 min, elongation at 72 °C for 1.5 min; and a final extension at 72 °C for 10 min. Each 25-μL PCR contained 2 μL of template DNA, 0.5 μL of barcoded forward primer, 0.5 μL (10 μmol/L) of reverse primer, 12.5 μL of GoTaq 2X Green Master Mix (Promega, Madison, WI), and 9.5 μL of nuclease free water. The forward primer was 515 F (5′-XXXXXXXX GTGTGCCAGCMGCCGCGGTAA-3′) with an 8 bp barcode (X) and Illumina adapter (GT), and the reverse primer was 806 R (5′-GGACTACHVGGGTWTCTAAT-3′; Caporaso et al., 2012). To reduce PCR bias, each sample was amplified in triplicate. The triplicate PCR products were pooled and subjectively quantified based on the brightness of the bands on a 2% agarose gel stained with SYBR safe (Invitrogen Co., Carlsbad, CA). All amplicons were then pooled at equal amounts. The pooled library was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and submitted to the UC Davis Genome Center DNA Technologies Core for 250bp paired-end sequencing on the Illumina MiSeq platform (Illumina, Inc. San Diego, CA).

The software saber (https://github.com/najoshi/saber) was used to demultiplex and remove barcodes from raw sequences. Sequences were then imported into Quantitative Insights Into

Microbial Ecology 2 (QIIME2; version 2020.8) for downstream filtering and bioinformatics analysis (Caporaso et al., 2010; Bolyen et al., 2019). Plugin q2-dada2 was used for quality control and constructing features. Taxonomic classification was assigned using the feature-classifier plugin trained with SILVA rRNA database 99% Operational Taxonomic Units (**OTU**), version 132 (Quast et al., 2013; Callahan et al., 2016).

Statistical analysis

Three pigs were removed from the study because of severe diarrhea and drastic drop in BW after ETEC inoculation. Among these pigs, one was from the group fed 0.25% antimicrobial blend and the other two from the group fed 0.50% of the antimicrobial blend. Normality of data was verified, and outliers were identified using the UNIVARIATE procedure (SAS Inst. Inc., Cary, NC). Outliers were identified and removed when values deviated from the treatment mean by more than three times the interquartile range. One outlier was removed from 0.25% antimicrobial blend group before data analysis. Data were analyzed by ANOVA using the PROC MIXED of SAS (SAS Institute Inc., Cary, NC). The statistical model included diet as the fixed effect, and pig nested in treatment and blocks were included as random effects. REPEATED statement was included in the model for variables measured over time. Least squares means were estimated for each treatment, and mean separation was performed using PDIFF option for variables significantly affected by main effect of treatment or interaction effect of treatment and time. Orthogonal contrasts were performed to analyze linear and quadratic dose responses of the antimicrobial blend on performance. Pig BW data were also analyzed with a two-way repeated-measures analysis of ANOVA by time and treatment using the PROC MIXED followed by Bonferroni post hoc test. The Chi-square test was used for analyzing frequency of diarrhea. Statistical significance and tendency were considered at $P < 0.05$ and $0.05 \leq P < 0.10$, respectively.

Data visualization and statistical analysis for microbiome were conducted using the R program (version 3.6.1). Two α-diversity indices, Chao1 and Shannon, were calculated using the phyloseq package. Relative abundance was calculated using the phyloseq package and visualized using ggplot2 package in R. Relative abundance data were aggregated at various taxonomical levels. Shapiro-Wilk normality test and Bartlett test were used to verify normality and constant variance, respectively, in α-diversity and relative abundance. Shannon index was analyzed using ANOVA with the statistical model including diets within different day or different types of sample as fixed effects. Significance in Chao1 index and relative abundance was observed using Kruskal–Wallis rank sum test followed by a Conover test for multiple pairwise comparison using the agricolae package. β-Diversity was calculated based on the Bray–Curtis dissimilarity (unweighted data) for principal coordinate analysis (**PCoA**). The homogeneity of multivariate dispersions was tested by the vegan package using the betadisper function, before the adonis function was used to calculate PERMANOVA with 999 replicate permutations. All gut microbiome-related data were pooled and served as control on day −7 before animals were assigned to their experimental diets.

Results and Discussion

Fecal β-hemolytic coliforms and severity of diarrheal illness

Postweaning diarrhea is a commonly occurring disease in the intensive pig production system, mainly affecting pigs during the first 1 to 2 weeks after weaning (Moeser and Blikslager, 2007; Rhouma et al., 2017). Postweaning diarrhea induced by pathogenic ETEC is characterized by discharge of watery feces, dehydration, a thin or unthrifty appearance, and sudden death of pigs (Fairbrother et al., 2005). The survey conducted by the USDA National Animal Health Monitoring Systems (NAHMS) reported that diarrhea caused by ETEC infection affected 32.1% to 45.5% of the medium-scale farms in the United States from the years 2000 to 2012 (Swine 2012). Therefore, an effective disease challenge model is an important tool to study pathogenesis and to evaluate preventive and therapeutic strategies. In the current study, weaned piglets were challenged with F18 ETEC, which is one of the leading causes for postweaning diarrhea in swine industry in the United States (Dubreuil, 2016). The same challenge model was shown to cause enteric infection and watery diarrhea in weaned pigs with the peak of infection occurring around 5 to 6 PI and most infected piglets were fully recovered within 14 PI (He et al., 2020a,b). Likewise, in the current study, β-hemolytic coliform was detected in fecal samples collected on days 2 and 7 PI (Figure 1), but not on day 0 (prior to ETEC challenge), suggesting that all pigs were successfully infected with F18 ETEC. The daily diarrhea score peaked from days 2 to 6 PI, the time of the worst diarrhea, after the first F18 ETEC inoculation (Figure 2), which is consistent with our previous studies wherein the same model of ETEC infection was used (Kim et al., 2019; He et al., 2020a,b).

On day 3 PI, the score was lower ($P < 0.05$) in 0.25% antimicrobial blend group compared with the control and 0.50% groups. Pigs supplemented with 0.25% or 0.50% antimicrobial blend had lower ($P < 0.05$) diarrhea score than control pigs on day 7 PI. Pigs that received 0.50% antimicrobial blend had lower ($P < 0.05$) diarrhea score compared with control on days 8 and 9 PI (Figure 2). Overall, pigs supplemented with antimicrobial blend had lower ($P < 0.05$) frequency of diarrhea (diarrhea score ≥ 3, 23.8% and 24.8%) than pigs in the control (32.6%), regardless of dose (Figure 3). Pigs that received 0.25% antimicrobial blend had lower ($P < 0.05$) frequency of diarrhea (diarrhea score ≥ 4; 9.8%) compared with control pigs (15.9%). Diarrhea score results suggest that both 0.25% and 0.50% of antimicrobial blend reduced incidence of diarrhea, while 0.25% of antimicrobial blend also reduced severity of diarrhea in ETEC-infected pigs. The enhanced disease resistance was also reported in previous researches, in which essential oils or clay was individually supplemented to weaned pigs infected with F18 ETEC (Song et al., 2012; Liu et al., 2013). In the present study, three pigs were removed from antimicrobial blend treatments (one pig from 0.25% group and two pigs from 0.50% group) after ETEC inoculation due to severe diarrhea and drastic drop in BW. These pigs were healthy and had normal growth rate and feed consumption at the adaption period. The removal was mainly due to the acute infection of ETEC and different responses of individual piglet on ETEC infection.

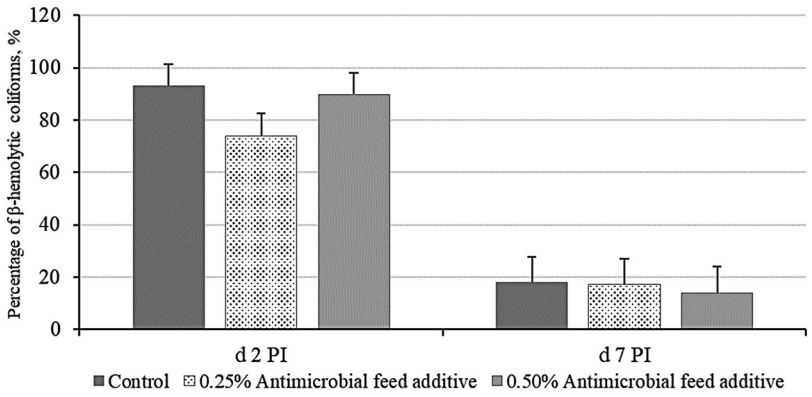

Figure 1. The percentage of β-hemolytic coliform in fecal samples of *Escherichia coli* challenged pigs fed diets supplemented with 0.25% or 0.50% of the antimicrobial feed additive. No β-hemolytic coliforms were observed in the fecal samples of pigs before *E. coli* challenge and days 14 and 21 postinoculation (PI). Each least squares mean represents 10–12 observations.

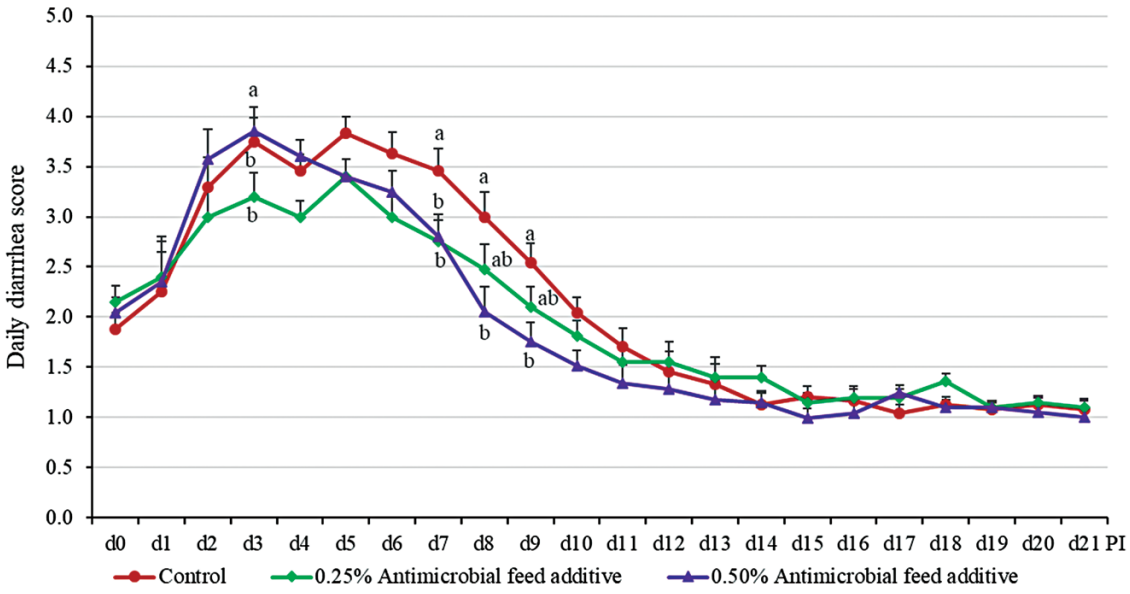

Figure 2. Daily diarrhea score of *Escherichia coli*-infected weaned pigs fed diets supplemented with 0.25% or 0.50% of the antimicrobial feed additive. Diarrhea score = 1, normal feces, 2, moist feces, 3, mild diarrhea, 4, severe diarrhea, 5, watery diarrhea. PI = postinoculation. [a,b]Means without a common superscript are different ($P < 0.05$). Each least squares mean represents 10–12 observations.

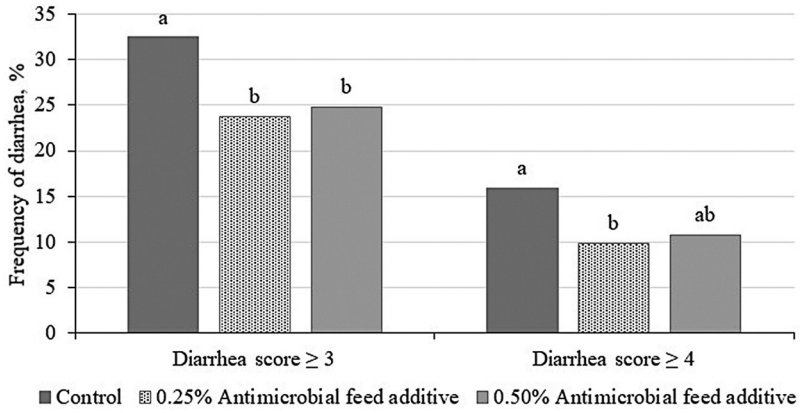

Figure 3. Frequency of diarrhea of *Escherichia coli*-infected weaned pigs fed diets supplemented with 0.25% or 0.50% of the antimicrobial feed additive from days 0 to 21 postinoculation. Frequency of diarrhea was calculated as the percentage of pig days with diarrhea score ≥ 3 or ≥ 4 in the total of pig days. [a,b]Means without a common superscript are different ($P < 0.05$).

Table 2. Growth performance of *Escherichia coli* challenged pigs fed diets supplemented with 0.25% or 0.50% of the antimicrobial feed additive

Item[1]	Control	0.25% the Antimicrobial feed additive	0.50% Antimicrobial feed additive	SEM	*P*-value		
					Diet	Linear	Quadratic
BW, kg							
INBW	6.89	6.86	6.88	0.30	0.96	0.93	0.78
Day 0 PI	7.73	7.84	7.75	0.47	0.87	0.93	0.62
Day 7 PI	8.31	8.55	8.78	0.93	0.76	0.47	0.99
Day 14 PI	11.65	12.05	12.19	1.45	0.78	0.51	0.86
Day 21 PI	16.23	17.02	16.93	1.64	0.70	0.50	0.64
ADG, g							
Day −7 to 0	120	141	128	32	0.75	0.77	0.50
Day 0 to 7 PI	82	119	161	76	0.57	0.30	0.96
Day 7 to 14 PI	477	516	494	82	0.74	0.73	0.50
Day 14 to 21 PI	654	713	678	39	0.53	0.64	0.32
Day 0 to 14 PI	280	313	326	75	0.65	0.38	0.83
Day 0 to 21 PI	368	382	402	43	0.71	0.41	0.92
ADFI, g							
Day −7 to 0	272	298	296	42	0.56	0.39	0.57
Day 0 to 7 PI	340	324	387	104	0.51	0.39	0.41
Day 7 to 14 PI	715	694	667	110	0.79	0.50	0.97
Day 14 to 21 PI	1,048	965	941	70	0.22	0.11	0.61
Day 0 to 14 PI	528	509	528	105	0.90	0.99	0.65
Day 0 to 21 PI	702	661	666	92	0.62	0.44	0.59
Gain:feed							
Day −7 to 0	0.43	0.47	0.41	0.075	0.78	0.85	0.50
Day 0 to 7 PI	0.16	0.33	0.14	0.23	0.73	0.94	0.44
Day 7 to 14 PI	0.69	0.77	0.74	0.048	0.53	0.49	0.40
Day 14 to 21 PI	0.64[b]	0.74[a]	0.74[a]	0.034	0.048	0.042	0.23
Day 0 to 14 PI	0.54	0.63	0.57	0.059	0.61	0.70	0.38
Day 0 to 21 PI	0.54	0.60	0.61	0.057	0.34	0.19	0.55

[1]INBW, initial body weight; BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; PI, post inoculation. Each least squares mean represents 10–12 observations.

Growth performance

The initial BW of pigs at day 7 were not different among treatment groups (Table 2).

Treatments did not affect BW, ADG, and ADFI throughout the experiment. Regardless of the dose, supplementation of the antimicrobial blend enhanced ($P = 0.048$) G:F ratio from days 14 to 21 PI. Supplementation of the antimicrobial blend improved ($P < 0.05$) pig growth longitudinally across the whole feed period regardless of dose (Figure 4). The overall growth rate of pigs supplemented with 0.25% and 0.50% antimicrobial blends was 258% and 261%, while the overall growth rate of pigs in control was 229%. Growth performance data in the present study indicated that pigs that received the antimicrobial blend had greater feed efficiency during the recovery period. The same antimicrobial blend (0.5% inclusion) was reported to improve growth rate of chickens that were challenged with *C. perfringens* (Xue et al., 2018). Many published research reported the modest positive impacts of essential oils (Sads and Bilkei, 2003; Blavi et al., 2016), medium-chain fatty acids (Lauridsen, 2020), and clays (Subramaniam and Kim, 2015; Liu et al., 2020) on weaned pig performance. However, there are limited research on the blend of those three components. In addition, the detection of small dietary effects on growth performance requires a large size of pig trial, which might include pig numbers far more than the disease challenge study as the present one.

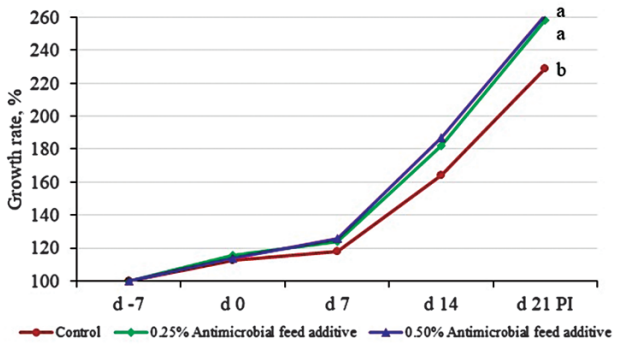

Figure 4. Growth kinetics of *Escherichia coli*-infected weaned pigs fed diets supplemented with 0.25% or 0.50% of the antimicrobial blend. Growth rate data were analyzed with a two-way repeated-measures ANOVA with Bonferroni's multiple comparison. [a,b]Growth curves without a common superscript were significantly different ($P < 0.05$) among treatments.

Fecal microbiota

The antimicrobial blend that was tested in the current study had shown strong bactericidal effects when tested in vitro against *Escherichia coli*, *Salmonella enterica*, *Clostridium perfringens*, and *Vibrio parahaemolyticus* (Wang et al., 2018). Administration of this antimicrobial blend also decreased intestinal colonization of *Salmonella* Heidelberg and *Salmonella* Typhimurium in broiler chickens (Xue et al., 2018, 2019). In the present study, 16S rRNA sequencing was performed to profile microflora in fecal samples collected at the beginning of the experiment, day 0 before ETEC inoculation, and days 7 and 21 PI, and ileal mucosa and ileal digesta collected at day 21 PI. A total of 3,831,509 qualified reads were obtained with a mean of 29,934 reads per sample. There were 4,215 OTUs identified in the current experiment.

The Chao1 index of fecal samples was lower ($P < 0.05$) on day −7, compared with samples collected on day 0, day 7 PI, and day 21 PI (Figure 5). No difference was observed in Shannon index in fecal samples collected from different days. There was a lack of significant treatment effect on the α-diversity of fecal samples at day 0 and day7 and 21 PI. For β-diversity (Bray–Curtis distance), compositional differences of fecal microbiota were observed among different days (Adonis, $P < 0.05$, $R^2 = 0.1813$; Figure 6). No separation of clusters was observed among dietary treatments within each time point. α-Diversity measures the richness or evenness of individual samples, while β-diversity indicates variability in community composition among samples. The results of α- and β-diversity suggest that the impacts of this antimicrobial blend on fecal microbiota diversity might be limited.

The profile of gut microbiota of pigs is affected by their health status, physiological states, housing environment, and ingredient and nutrition composition of the diet (Rist et al., 2013; Guevarra et al., 2018; Megahed et al., 2019). In the current study, Firmicutes was the most abundant phylum in feces in all sampling days followed by Bacteroidetes and Proteobacteria on day −7, day 0, and day 7 PI and Bacteroidetes and Actinobacteria on day 21 PI (Table 3; Supplementary Figure 1). In comparison to day −7, the relative abundance of Firmicutes was significantly decreased, while Bacteroidetes was increased in feces at the end of 1-week adaptation to the experimental diet

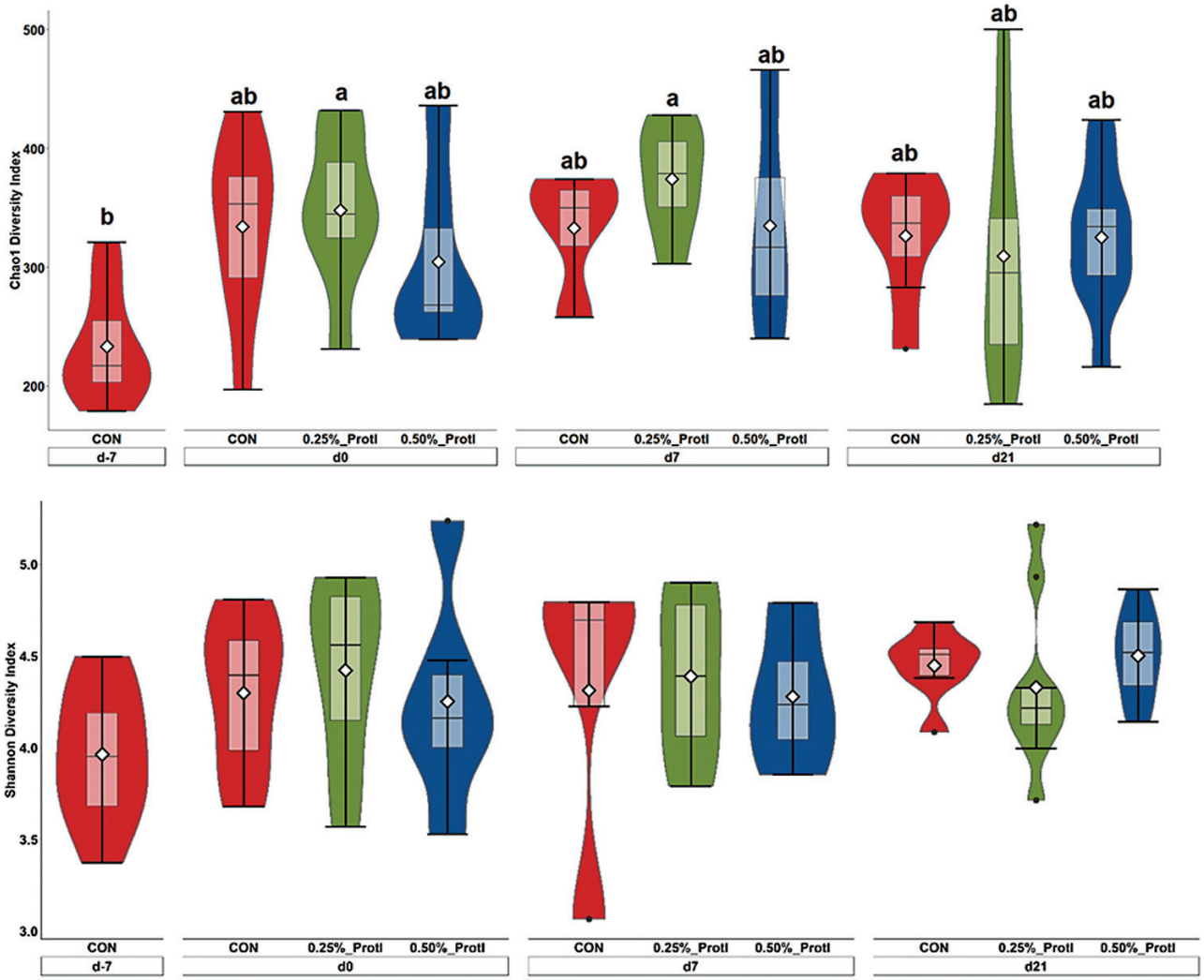

Figure 5. α-Diversity as indicated by Chao1 and Shannon in feces collected from enterotoxigenic *Escherichia coli* F18 challenged pigs fed diets supplemented with 0.25% or 0.50% of the antimicrobial feed additive at the beginning of the experiment, on day 0 before inoculation, on days 7 and 21 postinoculation. No difference was observed among treatments. CON = control; 0.25%_ProtI = 0.25% the antimicrobial feed additive; 0.50%_ProtI = 0.50% the antimicrobial feed additive. [a,b]Means without a common superscript are different ($P < 0.05$).

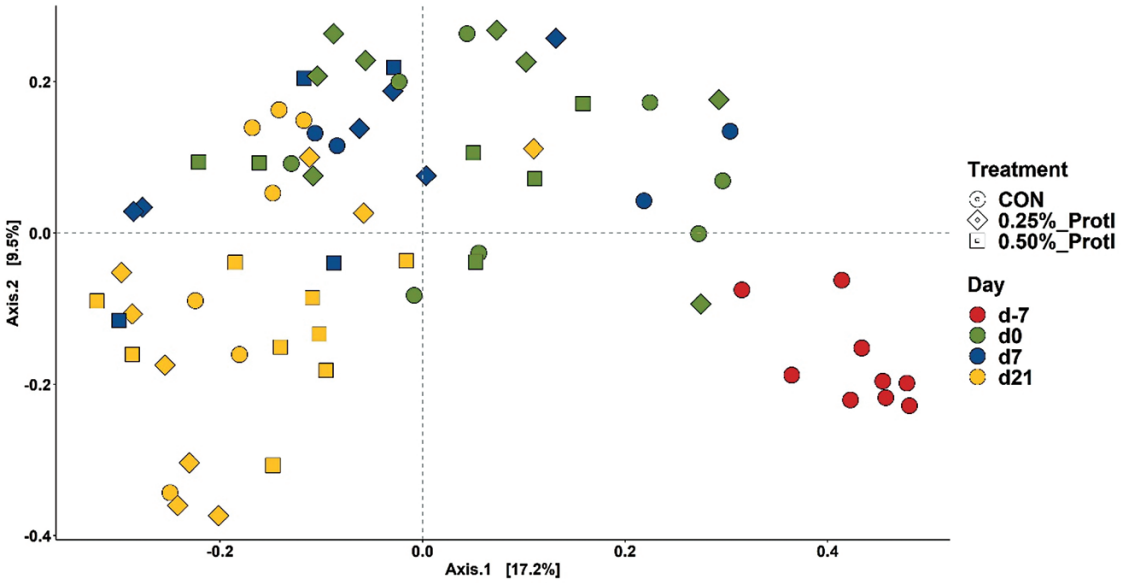

Figure 6. β-Diversity of fecal microbiota in enterotoxigenic *Escherichia coli* F18 challenged pigs at the beginning of the experiment, on day 0 before inoculation, on days 7 and 21 postinoculation. Data were analyzed by principal coordinate analysis (PCoA) based on the Bray–Curtis dissimilarity. Symbols indicate dietary treatments (CON = control; 0.25%_ProtI = 0.25% the antimicrobial feed additive; 0.50%_ProtI = 0.50% the antimicrobial feed additive), and colors indicate different dates. No difference was observed among treatments.

(day 0). At the family level, the relative abundance (%) of *Clostridiaceae1*, *Erysipelotrichaceae*, *Peptostreptococcaceae*, and *Bacteroidaceae* was decreased ($P < 0.05$), while the relative abundance of *Lachnospiraceae*, *Lactobacillaceae*, *Muribaculaceae*, *Prevotellaceae*, and *Succinivibrionaceae* was increased ($P < 0.05$) in the feces of weaned pigs at 7 d postweaning. The results are in agreement with findings from other studies wherein Firmicutes and Bacteroidetes were the most abundant phyla of the gut microbiome in pigs and most of influenced families are under the swine core gut microbiome (Frese et al., 2015; Li et al., 2020). The dramatic changes in fecal microbiome during the first week of weaning were not unexpected and were likely due to weaning from sow milk to a solid diet that consisted of mostly plant-based ingredients. For example, the relative abundance of several saccharolytic microbes was increased, including *Lactobacillaceae* that consume plant-derived mono- and di-saccharides and *Prevotellaceae* that may ferment plant-derived nonstarch polysaccharides (Ivarsson et al., 2014; Frese et al., 2015; Guevarra et al., 2018).

In the control group, we observed that pigs had enhanced ($P < 0.05$) relative abundance of Proteobacteria at the peak of F18 *E. coli* infection (data on day 7 PI). However, the relative abundance of Firmicutes was enhanced ($P < 0.05$), but the relative abundance of Bacteriodetes was reduced ($P < 0.05$) in feces of pigs at day 21 PI, when compared with pigs at day 0 before ETEC infection. Within each phyla, ETEC infection reduced ($P < 0.05$) the relative abundance of *Lactobacillaceae* but increased ($P < 0.05$) the relative abundance of *Enterobacteriaceae* on day 7 PI. Previously published research confirmed that ETEC infection could reduce pig appetite, disrupt intestinal functions, and induce intestinal inflammation, which may contribute to the imbalance of the microbiota (Zeng et al., 2017; Pollock et al., 2018; He et al., 2020a,b). ETEC are gram-negative bacilli of the family *Enterobacteriaceae* (Fairbrother et al., 2005). The desirable bacteria such as *Lactobacillus* decreased as pathogenic bacteria increased in the intestine of weaned pigs (Arguello et al., 2018).

The relative abundance of Firmicutes was higher ($P < 0.05$) and Bacteroidetes and Proteobacteria were lower ($P < 0.05$) in feces on day 7 PI, when pigs fed 0.50% of the antimicrobial blend were compared with pigs in the control group. Within the phylum Firmicutes, fecal samples of pigs that received 0.25% or 0.50% of the antimicrobial blend had higher ($P < 0.05$) relative abundance of *Lactobacillaceae* (20.28% and 18.10% vs. 8.83% under the entire kingdom) on day 7 PI than pigs in control (Table 3; Supplementary Figure 2). Supplementation of 0.50% antimicrobial blend also increased ($P < 0.05$) the relative abundance of *Lachnospiraceae* in feces of weaned pigs on day 7 PI, compared with control. On day 7 PI, dietary supplementation of this antimicrobial blend also reduced ($P < 0.05$) the relative abundance of *Bacteroidaceae* (Supplementary Figure 3) and *Enterobacteriaceae* (Supplementary Figure 4) in feces when compared with control, regardless of doses. Dietary supplementation of the antimicrobial blend did not affect relative abundance of any bacterial family within the phylum of Actinobacteria on days 7 and 21 PI, except that pigs in 0.50% antimicrobial blend group had greater ($P < 0.05$) relative abundance of *Bifidobacteriaceae* in feces than pigs in control on day 0 before ETEC infection (Table 3 and Supplementary Figure 5). Results of fecal microbiome indicated that supplementation of the antimicrobial blend for 7 d had limited impact on fecal microbiota in weaned pigs before ETEC infection, although *Bifidobacteriaceae* was enriched in feces of pigs supplemented with 0.50% antimicrobial blend. However, the enhanced *Lactobacillaceae* and reduced *Bacteroidaceae* and *Enterobacteriaceae* in feces at the peak of ETEC infection, indicating that supplementation of this antimicrobial blend could help to maintain the desirable bacteria in the intestine of ETEC-infected pigs, which may contribute to the concomitant decrease of pathogenic bacteria.

Table 3. The relative abundance (%) of top three enriched families in feces of enterotoxigenic *Escherichia coli* F18 challenged pigs fed diets supplemented with 0.25% or 0.50% of the antimicrobial feed additive

	Day −7	Day 0			Day 7			Day 21		
	Control	Control	0.25% Antimicrobial feed additive	0.50% Antimicrobial feed additive	Control	0.25% Antimicrobial feed additive	0.50% Antimicrobial feed additive	Control	0.25% Antimicrobial feed additive	0.50% Antimicrobial feed additive
Firmicutes	69.97^{ab}	59.95^{c}	64.48^{bc}	67.26^{abc}	46.12^{c}	70.91^{ab}	78.76^{a}	75.89^{a}	73.80^{a}	74.25^{a}
Christensenellaceae	3.95	3.60	4.06	1.73	3.03	2.45	3.08	2.12	3.73	1.92
Clostridiaceae1	18.79^{a}	0.56^{b}	2.70^{b}	0.64^{b}	0.69^{b}	1.06^{b}	0.67^{b}	1.88^{b}	1.67^{b}	5.06^{b}
Erysipelotrichaceae	3.31^{a}	0.66^{c}	1.36^{bc}	1.11^{bc}	0.41^{c}	0.65^{c}	0.85^{c}	1.96^{abc}	1.17^{c}	2.92^{ab}
Lachnospiraceae	8.56^{d}	11.66^{c}	14.27^{bc}	16.52^{abc}	11.95^{bcd}	18.00^{abc}	24.75^{a}	17.46^{ab}	14.34^{bc}	17.02^{ab}
Lactobacillaceae	5.59^{c}	20.34^{a}	18.63^{ab}	22.35^{a}	8.83^{c}	20.28^{ab}	18.10^{ab}	16.42^{abc}	11.24^{bc}	9.42^{c}
Peptostreptococcaceae	4.80^{a}	0.15^{c}	1.23^{bc}	0.14^{c}	0.63^{bc}	0.79^{bc}	1.53^{abc}	1.16^{ab}	1.24^{bc}	2.70^{ab}
Ruminococcaceae	18.85	13.35	13.74	13.80	13.85	14.64	17.08	18.31	16.46	18.80
Streptococcaceae	0.33^{cd}	0.26^{cd}	0.15^{d}	0.22^{cd}	0.27^{bcd}	5.89^{ab}	2.62^{abc}	5.88^{a}	9.14^{a}	5.65^{a}
Veillonellaceae	0.82^{b}	3.47^{ab}	4.97^{ab}	6.95^{a}	3.47^{ab}	4.97^{ab}	6.95^{ab}	7.61^{a}	12.31^{a}	8.16^{ab}
Bacteroidetes	10.82^{cd}	17.88^{a}	13.42^{abc}	14.17^{abcd}	18.87^{a}	14.04^{abcd}	8.49^{d}	12.87^{bcd}	14.64^{abcd}	14.19^{abcd}
Bacteroidaceae	3.98^{a}	0.72^{bc}	1.12^{bc}	0.88^{bc}	1.75^{ab}	0.83^{cd}	0.03^{de}	0.01^{e}	0.02^{e}	0.02^{e}
Muribaculaceae	0.72^{b}	4.42^{a}	2.28^{ab}	2.46^{ab}	1.04^{ab}	2.76^{ab}	1.55^{ab}	3.04^{ab}	1.49^{ab}	2.47^{ab}
Prevotellaceae	2.32^{b}	10.10^{a}	6.63^{ab}	9.00^{a}	12.80^{a}	8.21^{a}	5.82^{ab}	7.93^{a}	11.92^{a}	10.71^{a}
Rikenellaceae	2.10	2.05	2.44	1.50	1.18	1.46	0.53	1.78	1.16	0.93
Proteobacteria	5.52^{bc}	5.47^{bc}	5.80^{b}	4.91^{bc}	15.09^{a}	4.39^{bc}	2.37^{bc}	1.57^{bc}	0.86^{c}	1.46^{bc}
Burkholderiaceae	0.20	0.010	0.11	0.11	0.12	0.03	0.02	0.04	0.03	0.05
Desulfovibrionaceae	0.85	0.42	0.44	0.33	0.69	0.31	0.55	0.15	0.19	0.20
Enterobacteriaceae	4.12^{ab}	2.66^{b}	2.33^{b}	1.48^{b}	11.43^{a}	3.39^{b}	0.21^{b}	0.44^{b}	0.19^{b}	0.11^{b}
Succinivibrionaceae	0.18^{b}	2.08^{a}	2.76^{a}	2.94^{a}	2.26^{ab}	0.10^{ab}	0.79^{ab}	0.47^{ab}	0.33^{ab}	0.46^{ab}
Actinobacteria	1.98	1.04	0.88	2.29	0.53	0.88	1.46	2.01	3.41	2.89
Atopobiaceae	0.71	0.22	0.12	0.32	0.03	0.26	0.29	0.43	0.65	0.64
Bifidobacteriaceae	0.40^{c}	0.63^{bc}	0.65^{bc}	1.64^{a}	0.27^{c}	0.45^{bc}	0.79^{bc}	1.43^{ab}	2.54^{a}	2.08^{a}
Coriobacteriaceae	0.17	0.14	0.06	0.29	0.04	0.07	0.24	0.11	0.15	0.12
Eggerthellaceae	0.27	0.05	0.04	0.05	0.13	0.08	0.14	0.03	0.05	0.04

[a–c]Means without a common superscript are different ($P < 0.05$). Each mean represents five to nine observations.

Intestinal microbiota

The gastrointestinal tract of pigs varies longitudinally from proximal to distal and radially from mucosa to lumen. The variety creates a range of diverse microenvironments, which support distinct microbial diversity and structure (Zhao et al., 2015; Li et al., 2020). In the present study, regardless of dietary treatments, both Chao1 and Shannon indices of ileal mucosa and ileal digesta were significantly lower ($P < 0.05$) than those of fecal samples, suggesting the nature of less microbial diversity in ileal samples (Figure 7). β-Diversity (Bray–Curtis distance) presented as 2D PCoA plot (Adonis, $P < 0.05$, $R^2 = 0.2823$; Figure 8) showed clear separation among three types of samples, suggesting differences in compositions of microflora; the greatest dissimilarity was observed between ileal mucosa and day 28 fecal samples. It should be noted that ileal mucosa and digesta samples were distinctive in principal coordinate axis 2 but were similar in axis 1, whereas the opposite pattern was observed between ileal digesta and fecal samples. We speculate that differences in proximity to the mucosal layer along the radial direction may contribute to differentiating the mucosal microbiota from microbiota in ileal digesta, while the anatomical site where samples were collected in the GI tract may explain dissimilarity between ileal digesta and fecal samples. Adding the antimicrobial blend to the diet did not affect α- or β-diversity in any type of the samples. This observation was similar to the α- and β-diversity results in fecal samples, indicating that the impacts of this antimicrobial blend on the microbiota in ileum might be limited.

On day 21 PI, the top 4 phyla in ileal mucosa, ileal digesta, and feces were Firmicutes, Actinobacteria, Bacteroidetes, and Proteobacteria (Table 4; Supplementary Figure 6), and there was a lack of treatment effect on these most abundant phyla. The relative abundances of Firmicutes were the highest ($P < 0.05$) in ileal digesta followed by feces and the lowest in ileal mucosa, whereas relative abundance of Bacteroidetes ($P < 0.05$) followed the order of feces > ileal mucosa > ileal digesta. The relative abundance of Proteobacteria was higher ($P < 0.05$) in ileal mucosa than ileal digesta and feces. At the family level, *Christensenellaceae*, *Lachnospiraceae*, *Ruminococcaceae*,

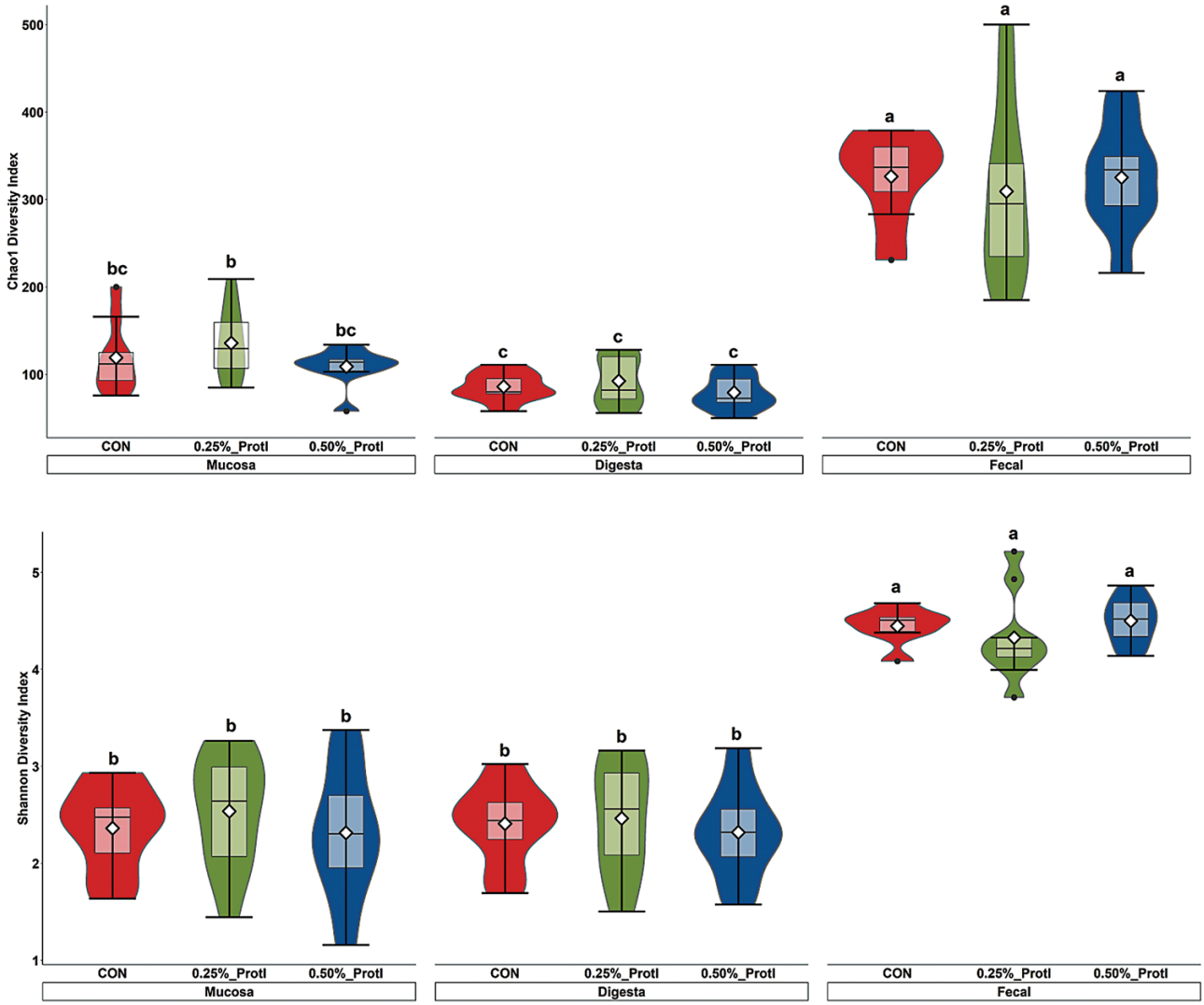

Figure 7. α-Diversity as indicated by Chao1 and Shannon in ileal mucosa, ileal digesta, and feces of enterotoxigenic *Escherichia coli* F18 challenged pigs fed diets supplemented with 0.25% or 0.50% the antimicrobial feed additive on day 21 postinoculation. No difference was observed among dietary treatments. CON = control; 0.25%_ProtI = 0.25% the antimicrobial feed additive; 0.50%_ProtI = 0.50% the antimicrobial feed additive. [a–c]Means without a common superscript are different ($P < 0.05$).

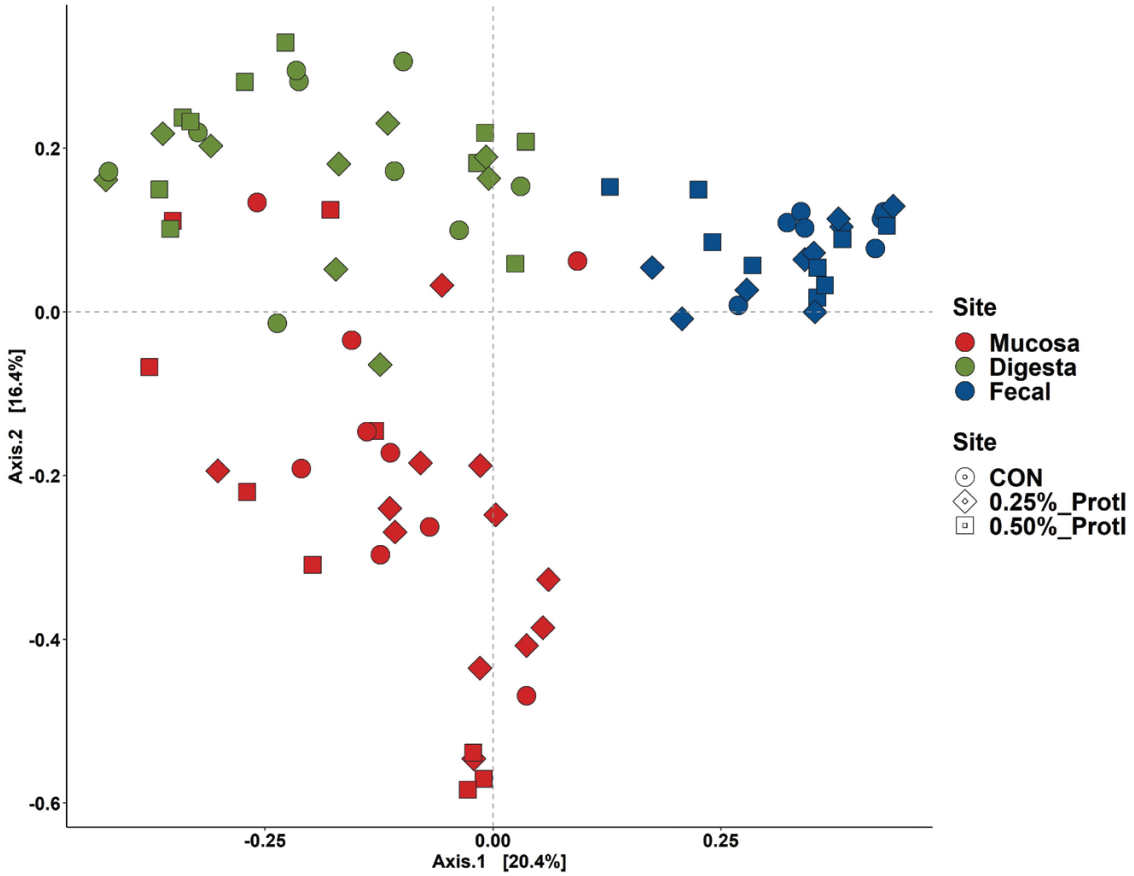

Figure 8. β-Diversity of microbiota in ileal mucosa, ileal digesta, and feces of enterotoxigenic *Escherichia coli* F18 challenged pigs on day 21 postinoculation. Data were analyzed by principal coordinate analysis (PCoA) based on the Bray–Curtis dissimilarity. Symbols indicate dietary treatments (CON = control; 0.25%_ProtI = 0.25% the antimicrobial feed additive; 0.50%_ProtI = 0.50% the antimicrobial feed additive), and colors indicate different intestinal segments.

Veillonellaceae, *Muribaculaceae*, *Prevotellaceae*, *Rikenellaceae*, and *Succiniyibrionaceae* accounted for higher ($P < 0.05$) abundance in feces than in ileal digesta, while the relative abundances of *Clostridiaceae1*, *Peptostreptococcaceae*, *Pasteurellaceae,* and *Bifidobacteriaceae* were lower ($P < 0.05$) in feces compared with ileal digesta (Table 4, Supplementary Figures 7–9). Compared with ileal digesta, ileal mucosa contained relatively lower ($P < 0.05$) abundance of *Lactobacillaceae*, but relatively higher ($P < 0.05$) abundance of *Ruminococcaceae*, *Muribaculaceae*, *Prevotellaceae*, *Rikenellaceae*, *Enterobacteriaceae*, *Pseudomonadaceae*, and *Succiniyibrionaceae*.

In contrast to the similar microbiome profiles between colon digesta and rectal feces, the remarkable difference in microbiota from ileal digesta compared with that in rectal feces suggested that luminal environment at small intestine (e.g., ileum) and colon modulate digesta microbiome differently (Gao et al., 2018; Pollock et al., 2019). Compared with the large intestine, the small intestine is characterized by relatively high oxygen levels and faster passage rate (He et al., 1999; Schwarz et al., 2002). In addition, the small intestinal lumen contains abundant simple carbohydrates that are shared and competed by the host and the bacteria (Zoetendal et al., 2012). However, the large intestine lumen comprises more complex carbohydrates that are mostly fermentable fibers and, to a less extent, nondigested starch. Therefore, differences in substrates, passage rate, and luminal environments may result in different fermentation processes. Although no difference was observed in α-diversity between ileal digesta and ileal mucosa, the differences in β-diversity and microbiota profile suggest the presence of mucosa-associated microbiota that is unique to the microbiota that reside within the lumen of ileum (Nava et al., 2011; Galley et al., 2014; Kelly et al., 2017). The α-diversity indices detect richness and evenness of an ecological community, as well as represent the uncertainty of species identified within a sample. The lack of difference in α-diversity indices is likely due to the existence of some rare genera in small population (Hill et al., 2003). Many studies revealed that oxygen tension also contributes to shaping the composition of mucosa-associated microbiomes (Kelly, 2001; Albenberg et al., 2014). Oxygen diffusion from the host capillary network creates a microenvironment within the mucosa that is more favorable by oxygen-tolerant microaerophilic species, such as, *Enterobacteriaceae* and *Pseudomonadaceae* (Albenberg et al., 2014). *Enterobacteriaceae* are a large family of gram-negative facultative anaerobes, which include *E. coli* and reside in the lower gut lumen and mucosa. A growing evidence suggest that the intestinal inflammation appears to provide a favorable environment for expansion of *Enterobacteriaceae* (Zeng et al., 2017). Thus, the differences in microbiota composition between ileal mucosa and digesta may be due to oxygen diffusion, substrate availability, and intestinal health status.

Table 4. The relative abundance (%) of top three enriched families in different segments of the intestine of enterotoxigenic *Escherichia coli* F18 challenged pigs fed diets supplemented with 0.25% or 0.50% of the antimicrobial feed additive

	Ileal mucosa			Ileal digesta			Feces		
	Control	0.25% Antimicrobial feed additive	0.50% Antimicrobial feed additive	Control	0.25% Antimicrobial feed additive	0.50% Antimicrobial feed additive	Control	0.25% Antimicrobial feed additive	0.50% Antimicrobial feed additive
Firmicutes	57.07^{bc}	45.92^{c}	55.41^{bc}	92.80^{a}	89.88^{a}	90.53^{a}	75.89^{b}	75.32^{b}	74.25^{b}
Christensenellaceae	0.03^{b}	0.02^{bc}	0.02^{bc}	0.01^{bc}	0.00^{c}	0.01^{bc}	2.12^{a}	2.83^{a}	1.92^{a}
Clostridiaceae1	13.82^{bc}	11.73^{bc}	22.99^{a}	20.82^{ab}	25.46^{a}	27.92^{a}	1.88^{d}	1.38^{d}	5.06^{cd}
Erysipelotrichaceae	0.66^{bc}	2.10^{ab}	0.38^{c}	1.68^{abc}	2.5^{a}	2.64^{ab}	1.96^{ab}	0.95^{bc}	2.92^{ab}
Lachnospiraceae	0.52^{b}	0.97^{b}	.047^{b}	0.70^{b}	1.31^{b}	0.36^{b}	17.46^{a}	15.03^{a}	17.02^{a}
Lactobacillaceae	13.62^{b}	6.94^{bc}	5.17^{c}	24.14^{a}	17.74^{ab}	29.58^{a}	16.42^{ab}	12.33^{bc}	9.42^{bc}
Peptostreptococcaceae	8.03^{ab}	3.79^{bc}	9.68^{ab}	11.41^{a}	16.90^{a}	12.85^{a}	1.16^{c}	0.87^{c}	2.70^{bc}
Ruminococcaceae	0.66^{b}	1.11^{b}	0.44^{b}	0.06^{c}	0.13^{c}	0.05^{c}	18.31^{a}	15.97^{a}	18.80^{a}
Streptococcaceae	17.46	13.02	12.90	30.69	20.79	14.13	5.88	10.18	5.65
Veillonellaceae	0.82^{c}	3.18^{bc}	1.07^{c}	2.64^{c}	3.87^{bc}	2.86^{c}	7.61^{ab}	13.57^{a}	8.16^{ab}
Bacteroidetes	1.42^{b}	3.08^{b}	1.15^{b}	0.05^{c}	0.14^{c}	0.08^{c}	12.87^{a}	14.92^{a}	14.19^{a}
Muribaculaceae	0.18^{b}	0.12^{b}	0.09^{b}	0.01^{c}	0.00^{c}	0.00^{c}	3.04^{a}	1.37^{a}	2.47^{a}
Prevotellaceae	1.17^{b}	2.84^{b}	0.94^{b}	0.04^{c}	0.14^{c}	0.08^{c}	7.93^{a}	12.87^{a}	10.71^{a}
Rikenellaceae	0.04^{c}	0.08^{b}	0.06^{bc}	0.00^{d}	0.00^{d}	0.00^{d}	1.78^{a}	0.64^{a}	0.93^{a}
Proteobacteria	9.49^{ab}	10.18^{a}	5.09^{abc}	1.51^{d}	3.97^{d}	4.03^{d}	1.57^{bcd}	0.82^{d}	1.46^{cd}
Burkholderiaceae	0.01^{ab}	0.04^{ab}	0.02^{bc}	0.00^{c}	0.00^{c}	0.00^{c}	0.04^{a}	0.02^{ab}	0.05^{a}
Enterobacteriaceae	6.02^{a}	4.08^{a}	2.34^{b}	0.05^{c}	2.65^{b}	3.69^{ab}	0.44^{bc}	0.21^{bc}	0.11^{bc}
Pasteurellaceae	2.02^{bc}	3.33^{a}	1.00^{bc}	1.45^{ab}	1.21^{ab}	0.32^{c}	0.00^{d}	0.00^{d}	0.00^{d}
Pseudomonadaceae	0.85^{a}	1.29^{a}	1.12^{a}	0.00^{b}	0.01^{b}	0.01^{b}	0.00^{b}	0.00^{b}	0.00^{b}
Succinivibrionaceae	0.27^{ab}	0.57^{a}	0.09^{b}	0.01^{c}	0.09^{b}	0.01^{c}	0.47^{a}	0.33^{ab}	0.64^{a}
Actinobacteria	2.11	1.92	2.24	5.41	5.28	5.22	2.01	3.40	2.89
Atopobiaceae	0.08^{b}	0.21^{ab}	0.21^{ab}	0.43^{ab}	0.74^{ab}	0.42^{ab}	0.43^{ab}	0.75^{a}	0.64^{ab}
Bifidobacteriaceae	1.86^{ab}	1.35^{b}	1.70^{ab}	4.91^{a}	4.46^{a}	4.70^{a}	1.43^{b}	2.48^{ab}	2.08^{ab}

$^{a-f}$Means without a common superscript are different ($P < 0.05$). Each mean represents 7–12 observations.

At the phylum level, supplementation of the antimicrobial blend did not affect Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria in ileal mucosa, ileal digesta, and feces on day 21 (Table 4 and Supplementary Figure 6). At the family level, pigs supplemented with 0.50% antimicrobial blend had higher ($P < 0.05$) relative abundance of *Clostridiaceae1*, but lower ($P < 0.05$) relative abundance of *Lactobacillaceae* and *Enterobacteriaceae* in ileal mucosa than pigs in control (Supplementary Figures 7 and 9). The ileal mucosa of pigs supplemented with 0.25% antimicrobial blend had higher ($P < 0.05$) relative abundance of *Rikenellaceae* than samples collected from control pigs (Supplementary Figure 8). Pigs supplemented with 0.25% antimicrobial blend had higher ($P < 0.05$) relative abundance of *Enterobacteriaceae* and *Succiniyibrionaceae* in ileal digesta than pigs in control. Pigs supplemented with 0.50% antimicrobial blend had greater ($P < 0.05$) relative abundance of *Enterobacteriaceae* and lower ($P < 0.05$) relative abundance of *Pasteurellaceae* in ileal digesta, compared with pigs in control.

Current results indicate that supplementation of high dose of the antimicrobial blend had remarkable impacts on the composition of the mucosa-associated microbiota in ileum of weaned pigs when they were fully recovered from *E. coli* infection. *Escherichia coli* infection induced postweaning diarrhea has more impact on the small intestine than the large intestine of weaned pigs because the ileum is the major colonization site of ETEC (Nagy and Fekete, 2005). In the present study, adding 0.50% of the antimicrobial blend to the diet enhanced the relative abundance of *Clostridiaceae1* in ileal mucosa. Previous studies suggest that some members of *Clostridiaceae1* could utilize oligosaccharides as their energy sources to promote mucus production and to maintain mucosa homeostasis (Macfarlane et al., 2001; Deplancke et al., 2002; Nava et al., 2011; Lopetuso et al., 2013). In combination with the reduced *Enterobacteriaceae* in ileal mucosa, results in the current study indicate that supplementation of the antimicrobial blend also shaped ileal mucosa to more favorable environment to support saccharolytic microbes.

Conclusions

Results of the present study confirmed that supplementation of the antimicrobial blend consisting of essential oils, hydrogenated fats, and a thermally processed toxin-adsorbing mineral could enhance disease resistance of weaned pigs by reducing the incidence and severity of diarrhea after *E. coli* challenge. Consistently, pigs fed with this antimicrobial blend also had greater feed efficiency during the recovery period. Weaning stress, nutrition changes, and ETEC infection all contributed to the fecal microbiome changes in weaned pigs. Supplementation of the antimicrobial blend significantly impacted the fecal microbiome during the peak of ETEC infection, as indicated by the enhanced relative abundance of *Lachnospiraceae* and *Lactobacillaceae* and reduced relative abundance of *Enterobacteriaceae*. Both changes suggest that supplementing this antimicrobial blend may help to maintain the nonpathogenic bacteria in the intestine of weaned pigs during the ETEC infection period. The impacts of the antimicrobial blend on ileal mucosa and digesta were limited when pigs were recovered from ETEC infection. Therefore, more sampling time points should be considered to explore the dynamic changes of ileal microbiota in ETEC-infected pigs in future research.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

Supplementary Figure 1. Stacked bar plot showing the relative abundance (%) of bacterial phyla in feces collected from enterotoxigenic *Escherichia coli* F18 challenged pigs at the beginning of the experiment, on day 0 before inoculation, on days 7 and 21 postinoculation. CON = control; 0.25%_Protl = 0.25% the antimicrobial feed additive; 0.50%_Protl = 0.50% the antimicrobial feed additive.

Supplementary Figure 2. Stacked bar plot showing the relative abundance (%) of Firmicutes in feces collected from enterotoxigenic *Escherichia coli* F18 challenged pigs at the beginning of the experiment, on day 0 before inoculation, on days 7 and 21 postinoculation. CON = control; 0.25%_Protl = 0.25% the antimicrobial feed additive; 0.50%_Protl = 0.50% the antimicrobial feed additive.

Supplementary Figure 3. Stacked bar plot showing the relative abundance (%) of Bacteroidetes in feces collected from enterotoxigenic *Escherichia coli* F18 challenged pigs at the beginning of the experiment, on day 0 before inoculation, on days 7 and 21 postinoculation. CON = control; 0.25%_Protl = 0.25% the antimicrobial feed additive; 0.50%_Protl = 0.50% the antimicrobial feed additive.

Supplementary Figure 4. Stacked bar plot showing the relative abundance (%) of Proteobacteria in feces collected from enterotoxigenic *Escherichia coli* F18 challenged pigs at the beginning of the experiment, on day 0 before inoculation, on days 7 and 21 postinoculation. CON = control; 0.25%_Protl = 0.25% the antimicrobial feed additive; 0.50%_Protl = 0.50% the antimicrobial feed additive.

Supplementary Figure 5. Stacked bar plot showing the relative abundance (%) of Actinobacteria in feces collected from enterotoxigenic *Escherichia coli* F18 challenged pigs at the beginning of the experiment, on day 0 before inoculation, on days 7 and 21 postinoculation. CON = control; 0.25%_Protl = 0.25% the antimicrobial feed additive; 0.50%_Protl = 0.50% the antimicrobial feed additive.

Supplementary Figure 6. Stacked bar plot showing the relative abundance (%) of bacterial phyla in ileal mucosa, ileal digesta, and feces of enterotoxigenic *Escherichia coli* F18 challenged pigs on 21 postinoculation. CON = control; 0.25%_Protl = 0.25% the antimicrobial feed additive; 0.50%_Protl = 0.50% the antimicrobial feed additive.

Supplementary Figure 7. Stacked bar plot showing the relative abundance (%) of bacterial family in Firmicutes phylum in ileal mucosa, ileal digesta, and feces of enterotoxigenic *Escherichia coli* F18 challenged pigs on 21 post-inoculation. CON = control; 0.25%_Protl = 0.25% the antimicrobial feed additive; 0.50%_Protl = 0.50% the antimicrobial feed additive.

Supplementary Figure 8. Stacked bar plot showing the relative abundance (%) of bacterial family in Bacteroidetes phylum in ileal mucosa, ileal digesta, and feces of enterotoxigenic *Escherichia coli* F18 challenged pigs on 21 post-inoculation. CON = control; 0.25%_Protl = 0.25% the antimicrobial feed additive; 0.50%_Protl = 0.50% the antimicrobial feed additive.

Supplementary Figure 9. Stacked bar plot showing the relative abundance (%) of bacterial family in Proteobacteria phylum in ileal mucosa, ileal digesta, and feces of enterotoxigenic *Escherichia coli* F18 challenged pigs on 21 post-inoculation. CON = control; 0.25%_Protl = 0.25% the antimicrobial feed additive; 0.50%_Protl = 0.50% the antimicrobial feed additive.

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Conflict of interest statement

H. Xue and S. L. Johnston are employees of Amlan International that funded this research.

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