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Non Ruminant Nutrition

NON RUMINANT NUTRITION

Trace amounts of antibiotic exacerbated diarrhea and systemic inflammation of weaned pigs infected with a pathogenic *Escherichia coli*

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Abstract

The experiment was conducted to investigate the effects of trace amounts of antibiotic on growth performance, diarrhea, systemic immunity, and intestinal health of weaned pigs experimentally infected with an enterotoxigenic *Escherichia coli*. Weaned pigs (n = 34, 6.88 ± 1.03 kg body weight [**BW**]) were individually housed in disease containment rooms and randomly allotted to one of the three dietary treatments: nursery basal diet (**CON**) and two additional diets supplemented with 0.5 or 50 mg/kg carbadox to the nursery basal diet (**TRA** or **REC**), respectively. The experiment lasted 18 d with 7 d before and 11 d after the first *E. coli* inoculation. The *E. coli* F18 inoculum was orally provided to all pigs with a dose of 10^{10} colony-forming unit (CFU)/3 mL for three consecutive days. Fecal and blood samples were collected on day 0 before inoculation and days 2, 5, 8, and 11 postinoculation (**PI**) to test the percentage of β-hemolytic coliforms in total coliforms and complete blood cell count, respectively. Sixteen pigs were euthanized on day 5 PI, whereas the remaining pigs were euthanized at the end of the experiment to collect the jejunal and ileal mucosa and mesenteric lymph node for gene expression and bacterial translocation, respectively. Pigs in REC had greater ($P < 0.05$) final BW and lower ($P < 0.05$) overall frequency of diarrhea compared with pigs in the CON and TRA groups. Pigs in TRA had the lowest ($P < 0.05$) average daily gain and feed efficiency from day 0 to 5 PI, highest ($P < 0.05$) percentage of β-hemolytic coliforms in fecal samples on days 2 and 5 PI, and greatest ($P < 0.05$) bacterial colonies in mesenteric lymph nodes on day 11 PI compared with pigs in the CON and REC groups. Pigs in TRA had the greatest ($P < 0.05$) neutrophils on day 5 PI and higher ($P < 0.05$) white blood cell counts and lymphocytes than other groups on day 11 PI. Pigs in TRA had the greatest ($P < 0.05$) serum C-reactive protein on days 2 and 5 PI and serum tumor necrosis factor-α on day 5 PI, compared with pigs in the CON and REC groups. Pigs fed REC had increased ($P < 0.05$) mRNA expression of *zona occludens-1* (*ZO-1*) and occludin (*OCDN*) and reduced ($P < 0.05$) interleukin-1 beta (*IL1B*), interleukin-6 (*IL6*), and tumor necrosis factor-alpha (*TNFA*) in ileal mucosa on day 5 PI, compared with the CON, whereas TRA upregulated ($P < 0.05$) mRNA expression of *IL1B*, *IL6*, and cyclooxygenase-2 (*COX2*) in the ileal mucosa on day 11 PI, compared with the REC. In conclusion, trace amounts of antibiotic may exacerbate the detrimental effects of *E. coli* infection on pig performance by increasing diarrhea and systemic inflammation of weanling pigs.

Key words: carbadox, diarrhea, enterotoxigenic *Escherichia coli*, gut health, immunity, weaned pigs

Abbreviations

ADFI	average daily feed intake
ADG	average daily gain
cDNA	complementary DNA
CFU	colony-forming unit
CON	the complex nursery basal diet (control)
ETEC	enterotoxigenic *E. coli*
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
PI	postinoculation
qRT-PCR	quantitative real-time polymerase chain reaction
REC	addition of 50 mg/kg carbadox (recommended dose of antibiotic) to the basal diet
STEC	Stx-producing *Escherichia coli*
Stx	Shiga toxins
TNF	tumor necrosis factor
TRA	addition of 0.5 mg/kg carbadox (trace amounts of antibiotic) to the basal diet

Introduction

Postweaning diarrhea causes remarkable economic losses in the swine industry due to increased mortality and morbidity, lowered growth rate, and cost of medication (Fairbrother et al., 2005; Nagy and Fekete, 2005). The disease is mainly caused by enterotoxigenic *Escherichia coli* (ETEC), in which *E. coli* F18 is one of the predominant strains in the United States (Nagy and Fekete, 1999). In the past decades, in-feed antibiotics have been commonly used in the swine industry to suppress or inhibit certain microorganisms' growth, prevent diarrhea, and enhance growth performance and productivity (Cromwell, 2002). However, trace amounts of antibiotics and antibiotics resistance and their possible adverse effects have been recognized as a global health concern, which has now been escalated as one of the top public health challenges (Marshall and Levy, 2011).

After the administration of in-feed antibiotics, a certain amount of antibiotics may be excreted through urine and feces (Campagnolo et al., 2002). Although the exact amounts of antibiotics excretion from livestock animals are difficult to estimate, the presence of trace levels of antibiotics occur in different environmental compartments, such as surface water, soil, air, and dust at various concentrations (Thiele-Bruhn, 2003; Hernando et al., 2006). For instance, trace concentrations of antibiotics in surface water or wastewater and soil generally range from few μg/L to thousands mg/L, or from few ng/kg to mg/kg, respectively (Kay et al., 2005; Michael et al., 2013; Novo et al., 2013; Hou et al., 2015). Substantial quantities of several antibiotics (e.g., tylosin and various tetracyclines) have also been detected in the dust of a swine farm (Hamscher et al., 2003). These trace amounts of antibiotics might cause adverse health effects on animals and humans, including toxicity, mutagenicity, carcinogenicity, reproductive disorders, allergy, and transfer of antibiotic-resistant bacteria to humans (Nisha, 2008). Moreover, the pharmacokinetics of trace amounts of antibiotics may affect animal and human infectious diseases (Craigmill et al., 2018). Growing evidence demonstrated that the intake of trace amounts of antibiotics might delay the growth and development of young animals and humans and slow down the recovery from diseases (Lee et al., 2001; Daghrir and Drogui, 2013; Jayalakshmi et al., 2017). Especially, young animals that are highly susceptible to diseases are more sensitive to trace amounts of antibiotics due to their immature immune system and gut microbiota community (Martin et al., 2010). However, limited studies have evaluated the impacts of trace amounts of antibiotics and underlying mechanisms on young pigs, particularly under disease-challenged conditions. Therefore, the objective of this study was to investigate the impacts of trace amounts of antibiotic on growth performance, diarrhea, intestinal health, and systemic immunity of weanling pigs experimentally infected with *E. coli* F18.

Materials and Methods

Animals, housing, experimental design, and diet

The protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of California, Davis (IACUC #19322). The study was conducted at the Cole Facility at the University of California, Davis. A total of 34 weanling pigs (crossbred; initial BW: 6.88 ± 1.03 kg) with an equal number of gilts and barrows were used in this study. They were randomly selected from the Swine Teaching and Research Center at the University of California, Davis. The eight sows (multiparous with parity from two to four) and their piglets used in this experiment did not receive *E. coli* vaccines, antibiotic injections, or antibiotics in creep feed. Before weaning, feces were collected from sows, and all their piglets were destined for this study to verify the absence of β-hemolytic *E. coli*. The *E. coli* F18 receptor status was also tested based on the methods of Kreuzer et al. (2013), and all piglets used in this study were susceptible to *E. coli* F18.

After weaning, all pigs were randomly assigned to one of the three dietary treatments in a randomized complete block design with BW within sex and litter as the blocks and pig as the experimental unit. Pigs were individually housed (pen size: 0.61 × 1.22 m) in environmental control rooms at the Cole Facility at the University of California, Davis for 18 d, including 7 d before and 11 d after the first *E. coli* challenge (day 0). The piglets had ad libitum access to feed and water. Environmental enrichment was provided for each pig. The light was on at 0700 hours and off at 1900 hours daily in the environmental control rooms.

The three dietary treatments included: 1) the complex nursery basal diet (control; CON), 2) addition of 0.5 mg/kg carbadox (trace amounts of antibiotic; TRA) to the basal diet, or 3) addition of 50 mg/kg carbadox (recommended dose of antibiotic; REC) to the basal diet. There were 12 replicate pigs in CON, 9 replicate pigs in TRA, and 13 replicate pigs in REC. The carbadox used in this experiment was Mecadox 10 (Phibro Animal Health Corporation, Teaneck, NJ). Spray-dried plasma, other antibiotics except carbadox, and high levels of zinc oxide exceeding recommendation and normal practice were not included in the diets. All diets were formulated to meet pig nutritional requirements (Table 1; NRC, 2012) and provided as mash form throughout the experiment.

After 7 d of adaptation, all pigs were orally inoculated with 3 mL of *E. coli* F18 using a disposable Luer-lock syringe for three consecutive days from day 0 postinoculation (PI). The *E. coli* F18 was originally isolated from a field disease outbreak by the University of Illinois Veterinary Diagnostic

Lab (isolate number: U.IL-VDL # 05-27,242). The *E. coli* F18 expresses heat-labile toxin, heat-stable toxin b, and Shiga-like toxin. The inoculums were prepared by the Western Institute for Food Safety and Security at the University of California, Davis, and were provided at 10^{10} colony-forming unit (CFU) per 3 mL dose in phosphate-buffered saline. This dose caused mild diarrhea in the current study, which is consistent with our previous published researches (Liu et al., 2013; Kim et al., 2019a, 2019b).

Clinical observations and sample collections

The procedures for this study were adapted from previous research methods of Liu et al. (2013) and Kim et al. (2019a, 2019b). Clinical observations (diarrhea score and alertness score) were recorded twice daily throughout the study. The diarrhea score of each pig was assessed each day visually by two independent evaluators, with the score ranging from 1 to 5 (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 a diarrhea score 4 or greater. The alertness score of each pig was assessed visually with a score from 1 to 3 (1 = normal, 2 = slightly depressed or listless, and 3 = severely depressed or recumbent). All pigs had an alertness score 1 throughout the study; therefore, data are not reported.

Pigs were weighed on weaning day (day −7), day 0 before inoculation, day 5, and 11 PI. Feed intake was recorded throughout the study. Average daily gain (ADG), average daily feed intake, and feed efficiency (gain:feed) was calculated for each interval from day −7 to 0, day 0 to 5 PI, and day 5 to 11 PI. Fecal samples were collected from the rectum of all pigs throughout the experiments using a fecal loop or cotton swap on days 2, 5, 8, and 11 PI to test for β-hemolytic coliforms and the percentage of β-hemolytic coliform to total coliforms (Liu et al., 2013; Kim et al., 2019a, 2019b). Sixteen pigs (six pigs in CON, four pigs in TRA, and six pigs in REC) were randomly selected and euthanized on day 5 PI near the peak of infection, and the remaining pigs (six pigs in CON, five pigs in TRA, and seven pigs in REC) were euthanized at the end of the experiment (day 11 PI) that was the recovery period of the infection. The selection of necropsy time was based on the results of clinical observations and immune response parameters that were reported in previously published research using the same *E. coli* strain and inoculation dose (Kim et al., 2019a, 2019b).

Before euthanasia, pigs were anesthetized with a 1-mL mixture of 100 mg Telazol, 50 mg ketamine, and 50 mg xylazine (2:1:1) by intramuscular injection. After anesthesia, intracardiac injection with 78 mg sodium pentobarbital (Vortech Pharmaceuticals, Ltd., Dearborn, MI) per 1 kg of BW was used to euthanize each pig. Three 3-cm segments from the duodenum, the middle of the jejunum, and the ileum (10 cm close to the ileocecal junction) were collected and fixed in Carnoy's solution (ethanol, chloroform, and glacial acetic acid, 6:3:1 v/v/v) for intestinal morphology analysis. Blood samples were collected from the jugular vein of all pigs with or without EDTA to yield whole blood and serum, respectively, before *E. coli* challenge (day 0) and on days 2, 5, and 11 PI. Serum samples were collected and immediately stored at −80 °C before further analysis. Mesenteric lymph nodes were aseptically collected and then pooled within the pig, ground, diluted, and plated on brain heart infusion agar for measurement of total bacteria, and the results were expressed as CFU per gram of lymph node (Almeida et al., 2013; Garas et al., 2016). Spleen samples were analyzed in the same way as mesenteric lymph nodes for bacterial translocation.

Table 1. Ingredient compositions of experimental diets[1]

Ingredient, %	Control diet
Corn	44.51
Dried whey	15.00
Soybean meal	14.00
Fish meal	10.00
Soy protein concentrate	7.00
Lactose	6.00
Soybean oil	2.00
Limestone	0.56
L-Lysine·HCl	0.15
DL-Methionine	0.06
L-Threonine	0.02
Salt	0.40
Vitamin–mineral, Sow 6[2]	0.30
Total	100.00
Calculated energy and nutrient	
Metabolizable energy, kcal/kg	3,487
Net energy, kcal/kg	2,615
Ile,[3] %	0.86
Leu,[3] %	1.68
Lys,[3] %	1.35
Met,[3] %	0.44
Thr,[3] %	0.79
Trp,[3] %	0.23
Val,[3] %	0.95
Met + Cys,[3] %	0.74
Analyzed nutrients, % as-fed	
Crude protein, %	25.0
Ca, %	1.19
Total P, %	0.80

[1]Two additional diets were formulated by adding 0.5 mg/kg or 50 mg/kg Carbadox, respectively.
[2]The vitamin-mineral Sow 6 was provided by United Animal Health (Sheridan, IN). It 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 amino acids.

Detection of β-hemolytic coliforms

The detection of β-hemolytic and total coliforms was based on Song et al. (2012) and Liu et al. (2013). Briefly, fecal samples were plated on Columbia Blood Agar with 5% sheep blood to identify hemolytic coliforms, which can lyse red blood cells surrounding the colony. Fecal samples were also plated on MacConkey agar to enumerate total coliforms. Hemolytic colonies from the blood agar were subcultured on MacConkey agar to confirm that they were lactose-fermenting bacteria and flat pink colonies.

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 score from 0 to 8 (0 = no bacterial growth, 8 = very heavy bacterial growth). The ratio of scores of β-hemolytic coliforms to total coliforms was calculated (Song et al., 2012; Liu et al., 2013). Questionable colonies were sub-subcultured on new MacConkey and blood agar plates to verify if they were β-hemolytic *E. coli* by using triple sugar iron agar and lysine iron agar, and then verify if they were F18-positive *E. coli* using polymerase chain reaction (**PCR**; DebRoy and Maddox, 2001).

Complete blood count

Whole blood samples were used for measuring total and differential blood cell counts by Comparative Pathology Laboratory at the University of California, Davis. A multiparameter, automated programmed hematology analyzer (Drew/ERBA Scientific 950 FS Hematological Analyzer, Drew Scientific Inc., Miami, FL) was used for the assay to differentiate porcine blood optimally.

Measurements of serum cytokine and acute-phase proteins

Serum samples were analyzed for a pro-inflammatory cytokine (tumor necrosis factor-α [TNF-α]; R&D System Inc., Minneapolis, MN) and acute-phase proteins (C-reactive protein and haptoglobin; GenWay Biotech Inc., San Diego, CA) using porcine-specific ELISA kits. All samples were analyzed in duplicate, including standard and control. Briefly, to analyze serum TNF-α, standard, control, and samples were plated to the wells with a coated monoclonal antibody specific for each measurement of cytokine. After 2 h of incubation, any unbound substances were washed away with a diluted washing solution, and an enzyme-linked polyclonal antibody specific for the tested cytokine was added to the wells to sandwich the cytokine immobilized during the first incubation. Another 2 h of incubation was followed by a wash to remove any unbound antibody-enzyme reagent, then a substrate solution was added to the wells, and color developed in proportion to the amount of the cytokine bound in the initial step. A stop solution was added to all wells to stop the development of color prior to the measurement of color intensity at 450 nm with a correction wavelength set at 540 nm using a plate reader (BioTek Instruments, Inc., Winooski, VT). Concentrations of each cytokine in the tested samples were calculated based on a standard curve. Similar procedures have been used to analyze serum C-reactive protein and haptoglobin as those described above, except pretreatment of samples and incubation times. The intra-assay coefficients of variation for TNF-α, C-reactive protein, and haptoglobin were 6.2%, 4.1%, and 2.7%, respectively. The inter-assay coefficients of variation for TNF-α, C-reactive protein, and haptoglobin were 10.0%, 5.6%, and 6.2%, respectively. The results of TNF-α, C-reactive protein, and haptoglobin were expressed in picograms, micrograms, or milligrams per milliliter based on the standard curves.

Intestinal morphology

The fixed intestinal tissues were embedded in paraffin, sectioned at 5 μm, and stained with high iron diamine and alcian blue. The slides were photographed by an Olympus BX51 microscope at 100× magnifications, and all measurements were conducted in the image processing and analysis software (Image J, NIH). Fifteen straight and integrated villi and their associated crypts and surrounding area per slide were selected to analyze villi height, crypt depth, the number of goblet cells per villus, and cross-sectional area of sulfo- and sialomucin as described by Deplancke and Gaskins (2001) and Kim et al. (2019b).

Intestinal barrier and innate immunity

Jejunal and ileal mucosa samples were analyzed for gene expression by quantitative real-time PCR (qRT-PCR). Briefly, approximately, 100 mg of mucosa sample was homogenized using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA). Then, total RNA was extracted following the RNA extraction procedural guidelines provided by the reagent manufacturer. The RNA quality and quantity were assessed by Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). The complementary DNA (cDNA) was produced from 1 μg of total RNA per sample using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA) in a total volume of 20 μL. The mRNA expression of mucin-2 (*MUC2*), claudin-1 (*CLDN1*), zona occludens-1 (*ZO-1*), and occludin (*OCDN*) in jejunal mucosa and interleukin-1 beta (*IL1B*), interleukin-6 (*IL6*), tumor necrosis factor-alpha (*TNFA*), and cyclooxygenase-2 (*COX2*) in ileal mucosa were analyzed by qRT-PCR. Data normalization was accomplished using beta-actin (*ACTB*), and ribosomal protein L4 (*RPL4*) as housekeeping genes. Primers were designed based on published literature and commercially synthesized by Integrated DNA Technologies, Coralville, IA (Kim et al., 2019b). All primers were verified prior to qRT-PCR (Supplementary Table S1). The qRT-PCR reaction conditions followed the published research (Liu et al., 2014; Kim et al., 2019b). The $2^{-\Delta\Delta CT}$ method was used to analyze the relative expression of genes compared with control (Livak and Schmittgen, 2001).

Statistical analysis

The normality of data was verified, and outliers were identified using the UNIVARIATE procedure (SAS Inst. Inc., Cary, NC). Outliers were identified and removed as values that deviated from the treatment mean by more than three times the interquartile range. All data were analyzed by ANOVA using the PROC MIXED of SAS (SAS Institute Inc., Cary, NC) in a randomized complete block design with the pig as the experimental unit. The statistical model included diet as the main effect and blocks as random effects. Treatment means were separated by using the LSMEANS statement and the PDIFF option of PROC MIXED. The Chi-square test was used for analyzing the frequency of diarrhea. Statistical significance and tendency were considered at $P < 0.05$ and $0.05 \leq P < 0.10$, respectively.

Results

Growth performance, diarrhea score, and β-hemolytic coliforms

No difference was observed in initial BW and day 0 BW of pigs among dietary treatments (Table 2). Pigs fed REC had greater ($P < 0.05$) BW on day 5 PI than pigs in the TRA and had greater ($P < 0.05$) final BW than pigs in the CON and TRA groups. Pigs fed TRA had the lowest ($P < 0.05$) ADG and feed efficiency from day 0 to 5 PI compared with pigs in the other groups.

Compared with pigs in the CON and TRA groups, pigs fed REC had lower ($P < 0.05$) average diarrhea score from day 0 to 5 PI and day 5 to 11 PI and frequency of diarrhea (Table 3; Figure 1). Pigs in

Table 2. Growth performance of ETEC F18-challenged pigs fed diets supplemented with antibiotics

	Diet				
Item[1]	CON	TRA	REC	SEM	*P*-value
BW, kg					
Day –7	6.79	6.72	6.89	0.27	0.94
Day 0	8.56	8.93	8.68	0.39	0.85
Day 5 PI	10.54[ab]	10.16[b]	11.89[a]	0.41	0.08
Day 11 PI	14.29[b]	13.89[b]	17.06[a]	0.52	<0.05
ADG, g					
Day –7 to 0	251	314	284	31.76	0.50
Day 0 to 5 PI	409[a]	232[b]	489[a]	72.59	<0.05
Day 5 to 11 PI	608	651	675	44.38	0.37
ADFI, g					
Day –7 to 0	425	449	349	48.20	0.29
Day 0 to 5 PI	620	527	648	48.53	0.36
Day 5 to 11 PI	856	833	897	44.09	0.24
G:F					
Day –7 to 0	0.61	0.74	0.81	0.072	0.24
Day 0 to 5 PI	0.64[a]	0.37[b]	0.73[a]	0.099	<0.01
Day 5 to 11 PI	0.70	0.79	0.69	0.050	0.42

[1]ADFI, average daily feed intake; PI, postinoculation. Each least squares mean represents 9 to 13 observations, except day 5 to 11 that has 5 to 7 observations.
[a,b]Within a row, means without a common superscript differ ($P < 0.05$).

Table 3. Diarrhea score and frequency of diarrhea of ETEC F18-challenged pigs fed diets supplemented with antibiotics

	Diet				
	CON	TRA	REC	SEM	*P*-value
Diarrhea score					
Day 0 to 5 PI[1]	2.87[a]	2.62[a]	1.94[b]	0.26	<0.01
Day 5 to 11 PI[2]	2.59[a]	2.74[a]	1.48[b]	0.34	<0.05
Pen days	120	84	105		
Frequency of diarrhea[3]	29.17[a]	26.19[a]	7.62[b]	—	<0.05

[1]Each least squares mean represents 9 to 13 observations. PI, postinoculation.
[2]Each least squares mean represents 5 to 7 observations.
[3]Frequency = number of pen days with fecal score ≥ 4.
[a,b]Within a row, means without a common superscript differ ($P < 0.05$).

the TRA group had a higher ($P < 0.05$) percentage of β-hemolytic coliforms in feces on days 2 and 5 PI compared with the CON and REC, but there were no differences observed on days 8 and 11 PI among the treatments (Table 4).

Systemic immunity and red blood cell profile

Pigs fed REC had greater ($P < 0.05$) lymphocyte counts but lower ($P < 0.05$) neutrophils to lymphocytes ratio on day 0 before *E. coli* inoculation (Table 5). Pigs fed TRA had greater ($P < 0.05$) neutrophils and neutrophils to lymphocytes ratio, but lower ($P < 0.05$) lymphocytes and monocytes on day 2 PI, compared with pigs in the REC group. Pigs in the TRA had lower ($P < 0.05$) monocytes on 11 PI, but greater ($P < 0.05$) white blood cell number and lymphocytes on day 11 PI, in comparison to pigs in the REC group. No differences were observed in the white blood cell profiles between pigs in the TRA group and CON group, with the exception that pigs in the TRA group still had higher ($P < 0.05$) white blood cell counts than pigs in the CON group on day 11 PI.

Pigs fed TRA had the greatest ($P < 0.05$) serum C-reactive protein on days 2 and 5 PI, and serum TNF-α on day 5 PI, compared with pigs in the CON and REC groups. Pigs in the REC group had lower ($P < 0.05$) serum haptoglobin than pigs in the other groups on days 2, 5, and 11 PI.

Before *E. coli* inoculation (day 0), pigs in the REC group had the lowest ($P < 0.05$) mean corpuscular volume (**MCV**) and total platelets but higher ($P < 0.05$) total protein among all dietary treatments (Table 6). Pig fed TRA had higher ($P < 0.05$) packed cell volume on days 2 and 5 PI but lower ($P < 0.05$) mean corpuscular hemoglobin (**MCH**) and mean corpuscular hemoglobin concentration (**MCHC**) on day 5 PI compared with pigs in the REC group. Pigs fed REC also enhanced ($P < 0.05$) MCHC and total protein concentration in the whole blood of pigs on day 11 PI in comparison to pigs in the TRA group. No differences were observed in the red blood cell profiles between pigs in the TRA group and the CON group, with the exception that the TRA group had higher ($P < 0.05$) MCV and MCH than pigs in the CON group on day 2 PI.

Bacterial translocation

No difference was observed in total coliforms in the spleen among three dietary treatments on days 5 and 11 PI (Figure

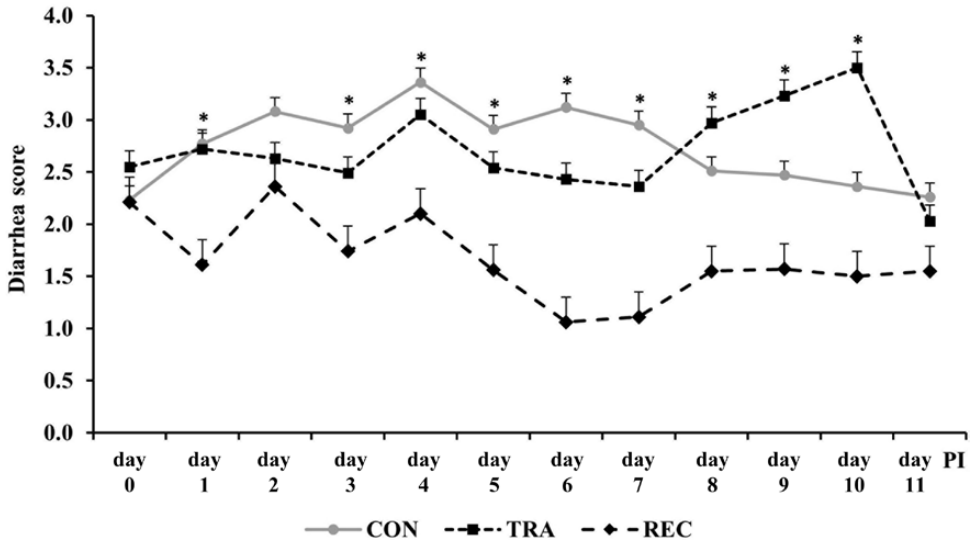

Figure 1. Daily diarrhea score of ETEC F18-challenged pigs fed diets supplemented with antibiotics. Diarrhea score = 1, normal feces; 2, moist feces; 3, mild diarrhea; 4, severe diarrhea; and 5, watery diarrhea. Each least squares mean from day 0 to 5 postinoculation (PI) represents 9 to 13 observations. Each least squares mean from day 6 to 11 PI represents 5 to 7 observations. *Significant differences were observed among dietary treatments: $P < 0.05$.

Table 4. The percentage (%) of β-hemolytic coliform to total coliforms in feces of ETEC F18-challenged pigs fed diets supplemented with antibiotics

	Diet				
	CON	TRA	REC	SEM	*P*-value
Day 2 PI[1]	68.86[b]	88.36[a]	37.88[c]	5.42	<0.01
Day 5 PI[1]	58.97[b]	80.16[a]	12.42[c]	4.97	<0.01
Day 8 PI[2]	15.97	18.85	3.18	5.59	0.27
Day 11 PI[2]	6.52	14.61	3.72	4.67	0.32

[1]Each least squares mean represents 9 to 13 observations.
[2]Each least squares mean represents 5 to 7 observations.
[a–c]Within a row, means without a common superscript differ ($P < 0.05$).

2). Pigs in the TRA group had the highest ($P < 0.05$) bacterial translocation in lymph nodes on day 11 PI compared with pigs in the other groups.

Intestinal morphology

On day 5 PI, pigs fed REC increased ($P < 0.05$) villi height and villi height-to-crypt depth ratio in both the duodenum and ileum and enhanced ($P < 0.05$) villi height-to-crypt depth ratio in jejunum compared with pigs in the CON and TRA groups (Table 7). Pigs fed with REC had a greater ($P < 0.05$) villi area than pigs in the CON group. Pigs fed TRA had greater ($P < 0.05$) sulfo- and sialomucin area in the jejunum than pigs in the REC and had greater ($P < 0.05$) sulfomucin area in the ileum than pigs in the other two treatments. On day 11 PI, pigs in REC had a higher ($P < 0.05$) sialomucin area in the jejunum. Pigs fed REC also had the greatest ($P < 0.05$) villi height and villi height-to-crypt depth ratio in both the jejunum and ileum and biggest ($P < 0.05$) sialomucin area in the ileum among three treatments. No difference was observed in the intestinal morphology of pigs between the CON and TRA groups.

Intestinal barrier and innate immunity

Pigs supplemented with REC had increased ($P < 0.05$) mRNA expression of *ZO-1* and *OCDN* on day 5 PI compared with pigs in the CON group (Figure 3). Pigs in the TRA group downregulated ($P < 0.05$) the mRNA expression of *MUC2* on day 11 PI compared with the REC. No differences were observed in the mRNA expression of *CLDN1* in jejunal mucosa of weaned pigs among treatments on days 5 and 11 PI.

Pigs in REC had reduced ($P < 0.05$) mRNA expression of *IL1B*, *IL6*, and *TNFA* in ileal mucosa on day 5 PI, compared with the CON (Figure 4). Pigs fed TRA upregulated ($P < 0.05$) mRNA expression of *IL1B*, *IL6*, and *COX2* in ileal mucosa on day 11 PI compared with the REC.

Discussion

ETEC)is one of the major causes of postweaning diarrhea mortality in pigs. This disease mainly occurs in early weaning pigs (3 to 4 wk of age), and symptoms usually appear between 3 and 10 d after weaning (Nagy and Fekete, 2005). Carbadox is one of the most common antibiotics and is widely used in the U.S. swine industry to control enteric diseases and to promote the growth of nursery pigs (USDA, 2015). Previous studies have supported that the supplementation of carbadox at the subtherapeutic concentration (50 to 55 mg/kg) improved growth performance and enhanced disease resistance of weanling pigs (Roof and Mahan, 1982; Yen and Pond, 1993;

Table 5. Total and differential white blood cells and serum inflammatory markers in ETEC F18-challenged pigs fed diets supplemented with antibiotics

	Diet				
Item[1]	CON	TRA	REC	SEM	*P*-value
Day 0 before infection					
WBC, $10^3/\mu L$	15.24	15.20	16.35	0.91	0.48
Neu, $10^3/\mu L$	7.89	8.07	7.56	0.71	0.80
Lym, $10^3/\mu L$	6.39^b	6.09^b	7.77^a	0.37	<0.05
Mono, $10^3/\mu L$	0.66	0.76	0.73	0.09	0.72
Eos, $10^3/\mu L$	0.24	0.18	0.20	0.05	0.73
Baso, $10^3/\mu L$	0.057	0.037	0.080	0.02	0.37
Neu:Lym	1.28^a	1.39^a	0.97^b	0.01	<0.05
TNF-α, pg/mL	120.83	130.90	76.69	46.18	0.29
C-reactive protein, μg/mL	13.60	9.90	8.08	2.69	0.37
Haptoglobin, mg/mL	1.100	0.920	0.944	0.17	0.58
Day 2 PI					
WBC, $10^3/\mu L$	19.90	23.15	18.99	1.58	0.25
Neu, $10^3/\mu L$	11.44^{ab}	15.04^a	8.73^b	1.31	<0.05
Lym, $10^3/\mu L$	6.88^b	6.68^b	8.59^a	0.47	<0.05
Mono, $10^3/\mu L$	1.09^{ab}	0.84^b	1.37^a	0.14	0.09
Eos, $10^3/\mu L$	0.39	0.48	0.22	0.08	0.13
Baso, $10^3/\mu L$	0.109^a	0.089^{ab}	0.036^b	0.02	0.09
Neu:Lym	1.96^a	2.26^a	0.96^b	0.25	<0.01
TNF-α, pg/mL	113.09	125.94	81.54	26.13	0.21
C-reactive protein, μg/mL	20.43^{ab}	24.01^a	13.14^b	2.55	<0.05
Haptoglobin, mg/mL	1.321^{ab}	1.860^a	1.062^b	0.19	<0.05
Day 5 PI					
WBC, $10^3/\mu L$	18.05	17.52	20.95	1.99	0.50
Neu, $10^3/\mu L$	9.28	9.67	9.61	1.68	0.97
Lym, $10^3/\mu L$	7.93	7.03	8.89	0.80	0.35
Mono, $10^3/\mu L$	0.73^b	0.89^{ab}	1.25^a	0.16	0.07
Eos, $10^3/\mu L$	0.12^b	0.24^{ab}	0.54^a	0.13	<0.05
Baso, $10^3/\mu L$	0.049	0.056	0.070	0.01	0.56
Neu:Lym	1.14^{ab}	1.56^a	1.08^b	0.17	0.08
TNF-α, pg/mL	91.77^{ab}	138.50^a	29.10^b	22.03	0.09
C-reactive protein, μg/mL	21.61^{ab}	23.58^a	15.34^b	2.65	0.08
Haptoglobin, mg/mL	1.822^a	1.664^a	0.771^b	0.29	<0.01
Day 11 PI					
WBC, $10^3/\mu L$	15.64^b	21.24^a	15.58^b	1.67	<0.05
Neu, $10^3/\mu L$	8.70	11.82	7.33	1.38	0.10
Lym, $10^3/\mu L$	6.40^{ab}	8.41^a	5.57^b	0.77	<0.05
Mono, $10^3/\mu L$	0.64^b	0.77^b	1.51^a	0.19	<0.01
Eos, $10^3/\mu L$	0.17	0.19	0.24	0.07	0.71
Baso, $10^3/\mu L$	0.060	0.062	0.043	0.02	0.70
Neu:Lym	1.46	1.50	1.12	0.22	0.53
TNF-α, pg/mL	82.71	104.68	58.30	26.89	0.76
C-reactive protein, μg/mL	20.93	16.86	15.32	2.81	0.28
Haptoglobin, mg/mL	0.807^a	0.784^a	0.247^b	0.13	<0.05

[1]WBC, white blood cell; Neu, neutrophil; Lym, lymphocyte; Mono, monocyte; Eos, eosinophil; Baso, basophil; PI, postinoculation. Each least squares mean represents 9 to 13 observations, except day 11 PI that has 5 to 7 observations.
[a,b]Within a row, means without a common superscript differ ($P < 0.05$).

Stahly et al., 1997; Harper and Estienne, 2002; He et al., 2020). The exact mechanism of action of carbadox is not known, but it has been suggested that virulence activity was disabled by interfering DNA synthesis in Gram-negative bacteria, including *E. coli* (Das, 1984; Cheng et al., 2016).

In the present study, *E. coli* F18 infection increased the frequency of diarrhea and the percentage of β-hemolytic coliforms in fecal samples of control pigs. These observations were closely consistent with our previous studies in which the same *E. coli* strain and dose were used to inoculate pigs (Liu et al., 2013; Kim et al., 2019a, 2019b; He et al., 2020). The results of the current experiment have confirmed that pigs supplemented with recommended dose of antibiotic had lower diarrhea rates and enhanced growth performance than pigs in control, indicating subtherapeutic dose of carbadox could protect pigs against *E. coli* F18 infection. Consistently, pigs supplemented with 50 mg/kg carbadox had the lowest percentage of β-hemolytic coliforms in feces among all treatments after *E. coli* infection, suggesting less *E. coli* F18 may be shed in the intestine of these pigs. Taken altogether, the supplementation of recommended dose of antibiotic reduced diarrhea severity and accelerated the recovery of pigs from *E. coli* F18 infection.

Neutrophils play an important role as a first-line defense against bacterial infection, whereas lymphocytes provide

Table 6. Red blood cell profiles in ETEC F18-challenged pigs fed diets supplemented with antibiotics

	Diet				
Item[1]	CON	TRA	REC	SEM	P-value
Day 0 before infection					
RBC, 10^6/μL	7.16	6.49	7.04	0.22	0.17
HGB, g/dL	9.80	9.57	9.99	0.24	0.55
HCT, %	31.63	30.60	30.36	0.69	0.43
MCV, fL[2]	44.65[ab]	47.19[a]	43.34[b]	1.16	0.08
MCH, pg	13.87	14.74	14.26	0.42	0.35
MCHC, g/dL	31.02[b]	31.33[ab]	32.92[a]	0.51	<0.05
RDW, %	26.57	25.92	25.72	1.10	0.84
Platelets, 10^3/μL	420[b]	564[a]	305[b]	39.79	<0.01
MPV, fL[2]	14.42	9.74	9.60	3.41	0.53
Total protein, g/dL	4.70[ab]	4.47[b]	4.90[a]	0.09	<0.05
Day 2 PI					
RBC, 10^6/μL	6.68[a]	6.14[ab]	6.00[b]	0.21	0.09
HGB, g/dL	9.36	9.28	9.38	0.27	0.95
HCT, %	29.81[a]	29.74[a]	26.14[b]	0.80	<0.01
MCV, fL[2]	45.26[ab]	48.20[a]	43.42[b]	1.29	<0.05
MCH, pg	14.18[b]	15.11[ab]	15.65[a]	0.40	<0.05
MCHC, g/dL	31.51	31.30	36.08	0.62	<0.01
RDW, %	26.71	25.88	27.06	1.31	0.80
Platelets, 10^3/μL	379	432	459	34.72	0.30
MPV, fL[2]	9.37	9.92	9.88	0.34	0.35
Total protein, g/dL	4.65	4.70	4.74	0.11	0.83
Day 5 PI					
RBC, 10^6/μL	5.98	6.55	5.76	0.44	0.39
HGB, g/dL	8.36	8.94	9.04	0.67	0.23
HCT, %	29.82[a]	31.24[a]	26.69[b]	0.94	<0.05
MCV, fL[2]	46.65	47.68	46.18	1.43	0.57
MCH, pg	14.08[b]	14.13[b]	16.29[a]	0.33	<0.01
MCHC, g/dL	30.34[b]	29.59[b]	35.51[a]	0.59	<0.01
RDW, %	28.37	26.44	29.17	1.16	0.33
Platelets, 10^3/μL	479	539	498	45.95	0.71
MPV, fL[2]	9.17	9.57	9.66	0.33	0.47
Total protein, g/dL	4.60	4.45	4.72	0.16	0.49
Day 11 PI					
RBC, 10^6/μL	6.36	6.29	6.24	0.12	0.81
HGB, g/dL	10.09	9.77	10.26	0.23	0.42
HCT, %	30.31	29.44	29.60	0.96	0.52
MCV, fL[2]	48.30	48.13	45.86	1.26	0.35
MCH, pg	15.90	15.48	16.42	0.25	0.16
MCHC, g/dL	32.89[b]	31.24[b]	36.45[a]	0.76	<0.05
RDW, %	26.85	26.99	28.32	1.39	0.60
Platelets, 10^3/μL	427	431	314	49.05	0.26
MPV, fL[2]	9.34	9.56	8.28	0.41	0.19
Total protein, g/dL	4.32[b]	4.16[b]	5.13[a]	0.12	<0.01

[1]RBC, red blood cell; HGB, hemoglobin; HCT, packed cell volume; RDW, red cell distribution width; MPV, mean platelet volume; and PI, postinoculation. Each least squares mean represents 9 to 13 observations, except day 11 PI that has 5 to 7 observations.
[2]fL, femtolitre (10^{-15} liters).
[a,b]Within a row, means without a common superscript differ ($P < 0.05$).

specific cellular and humoral immune responses. Their ratio is commonly used as a biomarker to determine the severity of systemic inflammation (Gordon-Smith, 2009). Our previously published research confirmed that *E. coli* F18 inoculation could induce systemic inflammation of weaned pigs by increasing total white blood cell counts and neutrophils (Liu et al., 2013). In the current study, pigs supplemented with 50 mg/kg carbadox had the lowest neutrophils to lymphocytes ratio among all treatments during the peak of *E. coli* infection, indicating an alleviated inflammation status of these pigs. Consistent with the results of white blood cell profiles, pigs supplemented with recommended dose of antibiotic had the lowest inflammatory indicators in serum throughout the experiment. Similarly, Kiarie et al. (2008) reported that carbadox supplementation reduced intestinal inflammatory mediators induced by *E. coli* K88 in weanling pigs. Moreover, it has been revealed that feeding carbadox at the subtherapeutic concentration (50 mg/kg) prevented an increase of fecal *E. coli* populations in weanling pigs (Looft et al., 2014). These observations demonstrated the efficacy of carbadox alleviating gut inflammation in pigs against *E. coli* F18, possibly by reducing bacterial growth and metabolism, prophage induction, and potential transduction of bacterial

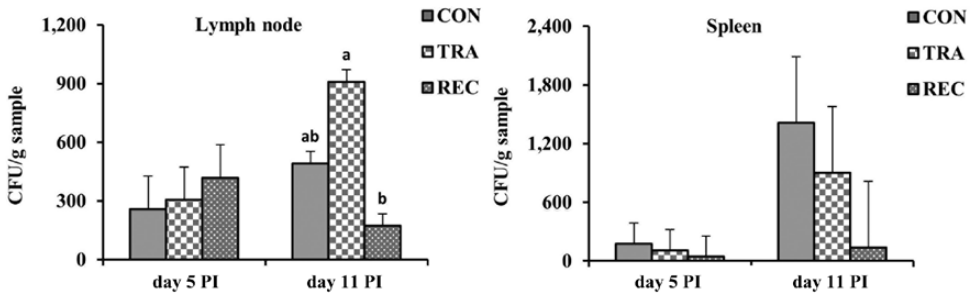

Figure 2. Total coliforms in mesenteric lymph nodes and spleen of ETEC F18-challenged pigs fed diets supplemented with antibiotics. [a,b]Means without a common superscript differ ($P < 0.05$). Each least squares mean represents four to seven observations.

fitness genes in swine gut bacterial communities (Johnson et al., 2017). Anti-inflammatory effects of in-feed antibiotics could also be partially explained by the accumulation of antibiotics in inflammatory cells, which could enhance the intracellular killing of bacteria and inhibit part of immune responses (Niewold, 2007).

Heat-stable toxin b secreted by ETEC could cause intestinal morphological lesions, such as loss of villus absorptive cells and villus atrophy. The lesions are responsible for poor growth by impaired nutrient absorption (Rose et al., 1987; Dubreuil, 2008). In the present study, the supplementation of recommended dose of antibiotic improved small intestinal morphology, as indicated by the increased villus height-to-crypt depth ratio in pigs than those in control. These findings are in agreement with previously published research, in which an improvement of intestinal morphology was observed in healthy weaned pigs or *E. coli* K88-challenged pigs fed with carbadox (55 mg/kg), respectively (Owusu-Asiedu et al., 2003; Oliver and Wells, 2013). ETEC infection could also induce a defect of intestinal barrier function by downregulating the expression of tight junction proteins (Dubreuil, 2017; Kim et al., 2019b). As a consequence, bacterial translocation could be enhanced due to reduced intestinal integrity (Lessard et al., 2009; Almeida et al., 2013). In the current study, the supplementation of recommended dose of antibiotic enhanced the mRNA expression of *ZO-1* and *OCDN* in jejunal mucosa and downregulated mRNA expression of several inflammatory mediators (i.e., *TNFA, IL1B*, and *IL6*) on day 5 PI. Consistently, pigs supplemented with recommended dose of antibiotic had lower total bacterial translocation from the intestinal lumen to mesenteric lymph nodes. These results indicated that the supplementation of subtherapeutic dose of antibiotic helped to maintain the normal intestinal integrity and immune functions, therefore enhancing disease resistance and performance of *E. coli*-challenged pigs.

The supplementation of recommended dose of antibiotic may also enhance beneficial bacteria and their metabolites, which support energy utilization and overall intestinal integrity. As an example, Looft et al. (2014) revealed that the administration of carbadox (50 mg/kg) increased the relative abundance of *Prevotella*, *Roseburia*, and *Faecalibacterium* in feces of nursery pigs, and these bacteria are closely related to short-chain fatty acids production in the large intestine. Especially, *Prevotella* produces acetate, and *Roseburia* and *Faecalibacterium* produce butyrate by consuming acetate (Köhler et al., 2000; Louis et al., 2014). Short-chain fatty acids have been shown to support energy utilization in pigs (Rossi et al., 2010) and to inhibit the colonization and growth of pathogenic *E. coli* (Shibata et al., 2017). Thus, further analysis on gut microbiota and their metabolites will be needed to identify the impacts of recommended dose of antibiotic on the modification of intestinal microbiota in weaned pigs challenged with ETEC.

The results from the present study demonstrated that pigs fed with trace amounts of antibiotic (0.5 mg/kg) had opposite results in comparison to the recommended dose of antibiotic. Pigs in the trace amounts of antibiotic group had the lowest growth rate among all treatments, especially during the peak infection period post-*E. coli* F18 challenge. Likewise, the supplementation of trace amounts of antibiotic carbadox did not alleviate the incidence of diarrhea compared with pigs in control. In consistence with the results of clinical signs, pigs supplemented with trace amounts of antibiotic had the highest percentage of β-hemolytic coliforms in feces on days 2 and 5 PI among all treatments. These observations indicated that more *E. coli* F18 shed in pigs' intestines throughout the peak infection period or the exclusion of *E. coli* F18 from the gut was slower in pigs fed trace amounts of antibiotic than pigs in the control and recommended dose of the antibiotic group. Taken altogether, dietary supplementation of trace amounts of antibiotic may delay the recovery of pigs from *E. coli* F18 infection.

We also observed that pigs supplemented with trace amounts of antibiotic had the highest neutrophils to lymphocytes ratio during the peak infection period (day 0 to 5 PI) and still had greater white blood cell counts during the recovery period (day 6 to 11 PI) than pigs supplemented with recommended dose of antibiotic. These results indicate that pigs in the trace amounts of the antibiotic group were still in elevated systemic inflammatory status induced by *E. coli* F18, which may explain the delayed recovery and poor growth rate of those pigs. Consistently, pigs in the trace amounts of the antibiotic group had the greatest serum TNF-α and C-reactive protein during the peak infection period and had the highest haptoglobin concentration throughout the postinoculation period compared with the other groups. Growing evidence suggests that trace concentrations of antibiotics could act as a signaling molecule to stimulate conversing the prodrug to a toxic compound (Romero et al., 2011; Cheng et al., 2016). Exposure to trace amounts of antibiotics may also trigger special bacterial responses, which further disrupt the native intestinal microbiota and induce the secretion of inflammatory markers, followed by the infection (Fajardo and Martínez, 2008; Buffie and Pamer, 2013). Though the interactions between trace amounts of antibiotic and *E. coli* F18 may need further investigation, it is clear that trace amounts of antibiotic exacerbated the intestinal and systemic inflammation status of *E. coli*-challenged pigs. The detrimental impacts of trace amounts of antibiotic on systemic inflammation of challenged pigs are possibly

Table 7. Intestinal morphology of ETEC F18-challenged pigs fed diets supplemented with antibiotics

	Diet				
Item[1]	CON	TRA	REC	SEM	P-value
Day 5 PI					
Duodenum					
Villi height, µm	386[b]	404[b]	498[a]	20.47	0.02
Crypt depth, µm	272	285	206	23.61	0.14
Villi height:Crypt depth	1.47[b]	1.42[b]	2.44[a]	0.11	<0.01
Villi width, µm	130	137	143	5.60	0.35
Villi area, µm^2	55,360[b]	63,640[ab]	77,530[a]	5,094	0.06
Goblet cell number, per villi	30.28	34.02	37.70	2.26	0.15
Sulfomucin area, % of villi area	9.00	9.29	7.56	0.99	0.53
Sialomucin area, % of villi area	5.42	5.90	4.34	0.64	0.34
Jejunum					
Villi height, µm	376	414	447	28.44	0.27
Crypt depth, µm	200	217	157	20.47	0.16
Villi height:Crypt depth	1.94[b]	1.84[b]	2.89[a]	0.17	<0.01
Villi width, µm	102	107	111	6.83	0.43
Villi area, µm^2	46,860	60,280	55,380	6,947	0.49
Goblet cell number, per villi	15.31	17.94	18.42	3.29	0.80
Sulfomucin area, % of villi area	6.77[ab]	8.54[a]	4.75[b]	0.67	0.02
Sialomucin area, % of villi area	2.08[ab]	2.61[a]	1.67[b]	0.29	0.06
Ileum					
Villi height, µm	325[c]	357[b]	401[a]	8.20	<0.01
Crypt depth, µm	179	191	157	13.80	0.29
Villi height:Crypt depth	1.87[b]	1.89[b]	2.57[a]	0.13	<0.01
Villi width, µm	113	98.6	110	6.74	0.44
Villi area, µm^2	36,810	40,140	44,480	3,576	0.13
Goblet cell number, per villi	19.14	21.3	18.97	1.80	0.68
Sulfomucin area, % of villi area	7.57[b]	12.47[a]	5.33[c]	0.71	<0.001
Sialomucin area, % of villi area	2.74	3.28	1.78	0.45	0.18
Day 11 PI					
Duodenum					
Villi height, µm	467	526	523	17.35	0.08
Crypt depth, µm	232	253	221	10.78	0.23
Villi height:Crypt depth	2.04	2.11	2.38	0.11	0.16
Villi width, µm	150	147	147	5.26	0.86
Villi area, µm^2	70,680	76,710	73,070	5,083	0.69
Goblet cell number, per villi	31.85	30.70	35.24	1.80	0.29
Sulfomucin area, % of villi area	7.32	8.49	7.94	0.91	0.71
Sialomucin area, % of villi area	2.92[b]	2.67[b]	5.96[a]	0.77	0.03
Jejunum					
Villi height, µm	396[b]	430[ab]	474[a]	13.80	0.01
Crypt depth, µm	199	200	174	8.81	0.14
Villi height:Crypt depth	2.03[b]	2.18[b]	2.75[a]	0.12	0.002
Villi width, µm	110	107	115	3.93	0.38
Villi area, µm^2	50,450	56,230	54,650	3,111	0.47
Goblet cell number, per villi	15.89	20.52	18.05	1.63	0.24
Sulfomucin area, % of villi area	6.96	7.91	6.44	0.70	0.45
Sialomucin area, % of villi area	1.72	2.50	2.33	0.24	0.13
Ileum					
Villi height, µm	355[b]	335[b]	429[a]	12.36	<0.01
Crypt depth, µm	162	163	169	11.22	0.87
Villi height:crypt depth	2.26[ab]	2.10[b]	2.54[a]	0.14	0.12
Villi width, µm	110	121	105	5.52	0.27
Villi area, µm^2	37,660	48,260	43,220	3,636	0.22
Goblet cell number, per villi	22.86	20.29	20.51	2.21	0.70
Sulfomucin area, % of villi area	6.61	6.68	5.41	0.63	0.33
Sialomucin area, % of villi area	2.61[ab]	1.77[b]	3.32[a]	0.31	0.04

[1]Each least squares mean represents 4 to 7 observations. PI, postinoculation.
[a–c]Within a row, means without a common superscript differ ($P < 0.05$).

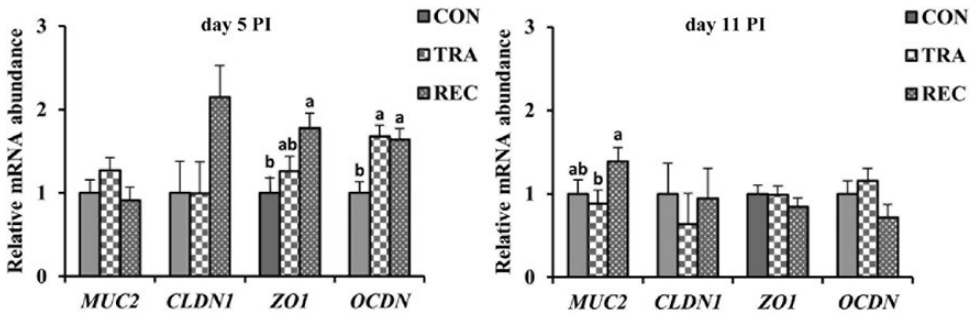

Figure 3. Gene expression profiles in jejunal mucosa of ETEC F18-challenged pigs fed diets supplemented with antibiotics. [a,b]Means without a common superscript differ ($P < 0.05$). Each least squares mean represents four to seven observations.

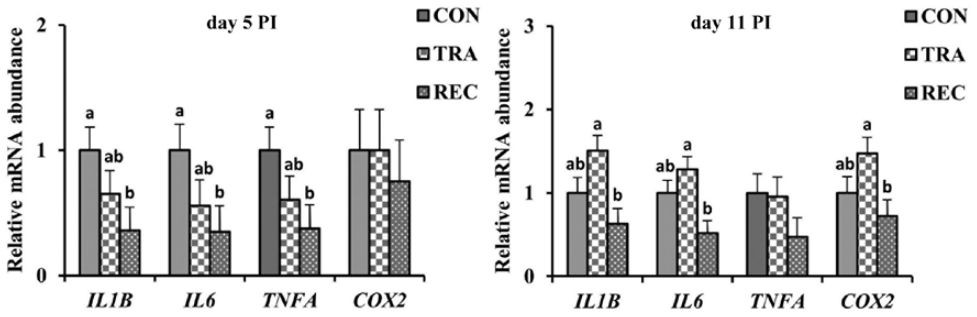

Figure 4. Gene expression profiles in ileal mucosa of ETEC F18-challenged pigs fed diets supplemented with antibiotics. [a,b]Means without a common superscript differ ($P < 0.05$). Each least squares mean represents four to seven observations.

related to toxin production. Shiga toxins (Stx) released by pathogenic *E. coli* could cause systemic inflammation, therefore increasing inflammatory cytokines (Tarr et al., 2005). Previous in vitro studies reported that antibiotics lower than minimal inhibitory (subinhibitory) concentrations enhanced Stx production from Stx-producing *E. coli* (STEC; Grif et al., 1998; Matsushiro et al., 1999). Consistently, Köhler et al. (2000) also reported that the supplementation of a subinhibitory concentration of carbadox increased the release of Stx phages from STEC, which may contribute to increase and spread STEC in the intestine. Moreover, STEC could utilize hemoglobin as an iron source for their proliferation and virulence production (Law and Kelly, 1995). In the present study, pigs supplemented with trace amounts of antibiotic had lower MCH and MCHC during the peak of infection compared with recommended dose antibiotic. Both MCH and MCHC are common blood parameters to reflect iron concentration in red blood cells (Archer and Brugnara, 2015). These results also support that pigs supplemented with trace amounts of antibiotic may contain relatively high level of *E. coli* F18 in their intestinal tract. Hence, analyzing the amount of Stx and Stx-converting phage particles is suggested to confirm these potential consequences in future research.

The supplementation of trace amounts of antibiotic did not affect intestinal morphology and innate immunity of pigs compared with control diet. These observations further confirmed that trace amounts of antibiotic did not exhibit beneficial impacts on intestinal development, thus not promoting animal growth as well. However, exposure to trace concentrations of antibiotic may exert adverse effects on disease resistance of weaned pigs through modulating of gut microbiota. Previous research has shown that the supplementation of subtherapeutic penicillin altered gut microbiota in mice, which also induced metabolic and immunologic changes (Cox et al., 2014). Especially, low-dose penicillin downregulated differentiation, activation, adhesion, recruitment, and quantity of immune cells in the ileum (Cox et al., 2014). Therefore, further research is necessary to examine how the trace amounts of antibiotic exposure impacts gut microbiota and their interactions with the pigs under *E. coli* F18 disease challenge and to explore how the changes correlate to growth performance and the overall intestinal health.

In conclusion, the current study demonstrated that the supplementation of recommended dose of antibiotic enhanced disease resistance and promoted the growth of weaned pigs challenged with a pathogenic *E. coli* F18. These observations are consistent with the literature and confirmed the importance of antibiotics in disease treatment and prevention in animal production systems. However, trace amounts of antibiotic, which could represent potential exposure to traceable antibiotics in reality, exacerbated the severity of diarrhea of *E. coli*-challenged weaned pigs. The pigs grew slower and had delayed recovery from *E. coli* infection compared with pigs without antibiotic supplementation. The deterioration of disease was likely due to the increased severity of systemic inflammation of pigs in the trace amounts of the antibiotic group. Therefore, more nutrients were used for immune responses instead of supporting animal growth. Further research will consider to repeat the *E. coli* challenge study with a larger number of weaned pigs in order to confirm the detrimental effects of trace amounts of antibiotics in animal feed. The analysis of gut microbiota and host metabolism changes is also needed to understand the mechanisms.

Supplementary Data

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

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Conflict of interest statement

The authors disclose that there was no conflict of interest.

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