Title

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Journal

Journal of Animal Science, 98(9)

ISSN

0021-8812

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Publication Date

2020-09-01

DOI

10.1093/jas/skaa290

Peer reviewed

AMERICAN SOCIETY OF ANIMAL SCIENCE

Journal of Animal Science, 2020, Vol. 98, No. 9, 1–13

doi:10.1093/jas/skaa290
Advance Access publication September 2, 2020
Received: 24 July 2020 and **Accepted:** 27 August 2020
Non Ruminant Nutrition

NON RUMINANT NUTRITION

Bacillus subtilis: a potential growth promoter in weaned pigs in comparison to carbadox

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Abstract

The study was conducted to investigate the efficacy of a probiotic *Bacillus subtilis* strain on growth performance, diarrhea, systemic immunity, and intestinal health of weaned pigs experimentally infected with an enterotoxigenic *Escherichia coli* and to compare the efficacy of *B. subtilis* with that of carbadox. Weaned pigs (n = 48, 6.17 ± 0.36 kg body weight [**BW**]) were individually housed in disease containment rooms and randomly allotted to one of four dietary treatments: negative control (**NC**, control diet without *E. coli* challenge), positive control (**PC**, control diet with *E. coli* challenge), and supplementation of 50 mg/kg of carbadox (antibiotic growth promotor [**AGP**]) or 2.56×10^9 CFU/kg of *B. subtilis* probiotics (**PRO**). The experiment lasted for 28 d with 7 d before and 21 d after the first *E. coli* inoculation. Fecal and blood samples were collected on days 0, 3, 7, 14, and 21 post inoculation (**PI**) to analyze β-hemolytic coliforms and complete blood cell count, respectively. Diarrhea score was recorded daily for each pig to calculate the frequency of diarrhea. All pigs were euthanized at day 21 PI to collect jejunal and ileal mucosa for gene expression analysis. Pigs in AGP had greater ($P < 0.05$) BW on days 7, 14, and 21 PI than pigs in PC and PRO groups. Supplementation of PRO enhanced pigs' BW on day 21 PI compared with the PC. *Escherichia coli* F18 challenge reduced ($P < 0.05$) average daily gain (**ADG**) and feed efficiency from day 0 to 21 PI, while supplementation of carbadox or PRO enhanced ADG and feed efficiency in *E. coli* F18-challenged pigs from day 0 to 21 PI. Pigs in AGP and PRO groups had reduced ($P < 0.05$) frequency of diarrhea throughout the experiment and fecal β-hemolytic coliforms on day 7 PI than pigs in the PC. Pigs in PRO had greater ($P < 0.05$) gene expression of *CLDN1* in jejunal mucosa than pigs in the PC. Supplementation of carbadox or PRO reduced ($P < 0.05$) the gene expression of *IL6* and *PTGS2* in ileal mucosa of *E. coli*-infected pigs compared with pigs in the PC. Pigs in the PRO group had lower ($P < 0.05$) white blood cell number and neutrophil count, and serum haptoglobin concentration on day 7 PI, and less ($P < 0.05$) monocyte count on day 14 PI, compared with PC. In conclusion, supplementation of probiotic *B. subtilis* could enhance disease resistance and promote the growth performance of weaned pigs under disease challenge conditions. The potential mechanisms include but not limited to enhanced gut barrier integrity and local and systemic immune responses of weaned pigs.

Key words: *Bacillus subtilis*, diarrhea, *Escherichia coli*, gut health, immunity, weaned pigs

Abberviations

ADFI	average daily feed intake
ADG	average daily gain
AGP	antibiotic growth promotor
Baso	basophil
BW	body weight
CLDN	claudin
DS	diarrhea score
Eos	eosinophil
ETEC	enterotoxigenic E. coli
G:F	gain-to-feed ratio
HCT	packed cell volume
HGB	hemoglobin
IL	interleukin
IL1B	interleukin-1 beta
Lym	lymphocyte
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
Mono	monocyte
MPV	mean platelet volume
MUC	mucin
NC	negative control
Neu	neutrophil
OCDN	occludin
PC	positive control
PI	post inoculation
PRO	probiotics
qRT-PCR	quantitative real-time PCR
RBC	red blood cell
RDW	red cell distribution width
SCFA	short-chain fatty acids
WBC	white blood cell
ZO	zonulae occludens

Introduction

In the swine industry, weaning is the most challenging time that has significant bearings on animal welfare and growth performance. At weaning, piglets are immediately imposed onto several nutritional, environmental, and psychosocial stressors that predispose them to diseases, such as postweaning diarrhea, which adversely impact their growth and survival at this vulnerable stage. According to the National Animal Health Monitoring System (2012), the postweaning mortality ratio in weanling pigs is generally around 2.7% to 3.9%. However, during an acute outbreak, it was reported that the mortality ratio rose to 7% and reached as high as 20% to 30% in some severe cases (Amezcua et al., 2002). It is well understood now that postweaning diarrhea is primarily caused by an *Escherichia coli* pathotype referred to as enterotoxigenic *E. coli* (**ETEC**) (Fairbrother et al., 2005). To maintain health and improve production performances of animals, antibiotic growth promoters (**AGP**s) were widely used in the swine diet in the past few decades. However, this practice of using in-feed antibiotics are now considered to contribute to the spread of antibiotic-resistant pathogens in both livestock and humans, raising significant concerns for public health (Van Boeckel et al., 2015). Since January 2017, FDA regulations prohibit the use of in-feed antibiotics as growth promoters in livestock and poultry (FDA, 2013). Therefore, the use of other bioactive components as alternatives to antibiotics will be needed to maintain pig health and productivity, especially during postweaning periods.

Different technologies have been developed to improve the health of weanling pigs, either by modulating the microbial ecology in the digestive tract or by ensuring the proper function of the immune system (Pettigrew, 2006). Probiotics, also known as direct-fed microbials, are live microorganisms, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001). Probiotics are categorized into three main groups, namely *Bacillus* spp., lactic acid-producing bacteria, and yeast (Stein and Kil, 2006). Compared with other candidates for probiotics, the spore-forming *Bacillus* spp. have been known for their ability to endure some harsh environmental conditions and be stable and viable after feed processing and long-term storage, making them a more suitable candidate for feed additives (Elshaghabee et al., 2017). Previous research has shown that supplementation of probiotics could improve the gut health of pigs by modifying gut microflora, which inhibits the growth of pathogens, enhances immune regulation and response, increases nutrient availability and digestibility, and ultimately improves growth performance (Prescott et al., 2005; Cho et al., 2011; Giang et al., 2011; Kenny et al., 2011). Furthermore, dietary probiotics *Bacillus subtilis* was shown to be able to reduce the incidence and severity of diarrhea in nursery pigs (Bhandari et al., 2008; Hu et al., 2014; Luise et al., 2019). Although ETEC F18 is one of the most dominant strains of ETEC that is responsible for around 33.9% to 43.1% of postweaning diarrhea in weanling pigs (Francis, 2002; Luppi et al., 2016; Do et al., 2019), there is limited research on the mechanisms of dietary probiotics *Bacillus* spp. against ETEC F18 infection. In addition, there is limited research on comparing the health and growth performance benefits of *B. subtilis* with those of AGPs. Therefore, the objective of this research was to determine the impacts of a new probiotic strain *B. subtilis* DSM 25841 compared with an AGP, carbadox, on growth performance, diarrhea, intestinal health, and systemic immunity of weaned pigs experimentally infected with ETEC F18.

Materials and Methods

Animals, housing, experimental design, and diet

The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC # 19322) of the University of California, Davis. A total of 48 weanling pigs (21 d old; 6.17 ± 0.36 kg) with an equal number of barrows and gilts were used in this experiment. The eight sows and piglets used in this experiment did not receive *E. coli* vaccines, antibiotic injections, or antibiotics in creep feed. Before weaning, fecal samples were collected from sows and all their piglets destined for this experiment to verify the absence of β-hemolytic *E. coli*. The *E. coli* F18 receptor status in all piglets was also tested based on the previously described methods (Kreuzer et al., 2013). All pigs used in this experiment were susceptible to *E. coli* F18 infection and free of *E. coli* F18 prior to the experiment. After weaning, all pigs were transferred to the Cole facility at the University of California, 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 *E. coli* challenge. All pigs had free access to feed and water. Animal rooms were equipped with fans and heaters to achieve the desired thermoneutral zone for nursery pigs. The light period was provided for 12 h starting from 0730 hours.

Pigs were randomly assigned to one of four treatments in a randomized complete block design with weight within sex and litter as the blocks and individual pig as the experimental unit. There were 12 replicates per treatment. The treatments included: 1) negative control (**NC**), control diet without *E. coli* challenge, 2) positive control (**PC**), control diet with *E. coli* challenge, 3) AGP, control diet supplemented with carbadox at 50 mg/kg and with *E. coli* challenge, and 4) probiotics (**PRO**), control diet supplemented with a *B. subtilis* at 2.56×10^9 colony forming units (CFU)/kg diet and with *E. coli* F18 challenge. All diets were based on corn, dried whey, soybean meal, and fish meal and met the current estimates for nutrient requirements of nursery pigs (Table 1; (NRC, 2012). The experimental diets were fed to pigs as a two-phase feeding program with weeks 1 and 2 as phase 1 and weeks 3 and 4 as phase 2. Spray-dried plasma, antibiotics, and zinc oxide were not included in the diets.

Pigs were housed in individual pens for 28 d, including 7 d before and 21 d after the first *E. coli* F18 challenge. In the *E. coli* challenge treatments, all pigs were orally inoculated with an *E. coli* F18 for three consecutive days from day 0 postinoculation (**PI**). The *E. coli* F18 were isolated from a field disease outbreak by the University of Illinois Veterinary Diagnostic Lab (isolate number: U.IL-VDL # 05-27242). The *E. coli* F18 were provided at 10^{10} CFU per 3 mL dose in phosphate-buffered saline and they express heat-labile toxin, heat-stable toxin b, and Shiga-like toxins. This dose causes mild diarrhea based on our previous research (Liu et al., 2013; Kim et al., 2019a, 2019b).

Clinical observations and sample collections

The procedures for this experiment were adapted from the methods of Liu et al. (2013) and Kim et al. (2019a, 2019b). During the experiment, clinical observations (diarrhea score [**DS**] and alertness score) were recorded twice daily starting from day 0 PI. The DS 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 (≥3, %) was calculated by the formula: [(pig days with DS of 3 or higher) ÷ (total pig days)] × 100 (Liu et al., 2013). The frequency of diarrhea (≥4, %) was also calculated using the same formula with the pig days with DS of 4 or higher. 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 during the experiment (data not shown).

Pigs and feeders were weighed on the day at weaning, day 0 before inoculation, and days 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 interval from day –7 to 0, 0 to 7 PI, 7 to 14 PI, and 14 to 21 PI. Fecal samples were collected from the rectum of all pigs on day 0 before inoculation and on days 3, 7, 14, and 21 PI using cotton swabs for the detection of β-hemolytic coliforms (Liu et al., 2013; Kim et al., 2019a). Blood samples were collected from the jugular vein of all pigs with or without ethylenediaminetetraacetic acid to yield whole blood and serum, respectively, before *E. coli* challenge (day 0), and on days 2, 7, 14, and 21 PI.

All pigs were euthanized at the end of the experiment. Before euthanization, pigs were anesthetized with 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 body weight (**BW**) was used to euthanize each pig. Four 3-cm segments from the duodenum, the middle of the jejunum, ileum (close to the ileocecal junction), and distal colon were collected and fixed in Carnoy's solution (ethanol, chloroform, and glacial acetic acid, 6:3:1 v/v/v) for histology

Table 1. Ingredient compositions of experimental diets[1]

Ingredient, %	Control, phase I	Control, phase II
Corn	44.41	57.27
Dried whey	15.00	10.00
Soybean meal	18.00	22.00
Fish meal	10.00	7.00
Lactose	6.00	—
Soy protein concentrate	3.00	—
Soybean oil	2.00	2.00
Limestone	0.56	0.70
L-Lysine·HCl	0.21	0.23
DL-Methionine	0.08	0.05
L-Threonine	0.04	0.05
Salt	0.40	0.40
Vit–mineral, Sow 6[2]	0.30	0.30
Total	100.00	100.00
Calculated energy and nutrient		
Metabolizable energy, kcal/kg	3,463	3,429
Net energy, kcal/kg	2,601	2,575
Crude protein, %	22.27	20.80
Arg,[3] %	1.23	1.15
His,[3] %	0.49	0.47
Ile,[3] %	0.83	0.76
Leu,[3] %	1.62	1.55
Lys,[3] %	1.35	1.23
Met,[3] %	0.45	0.39
Thr,[3] %	0.79	0.73
Trp,[3] %	0.23	0.21
Val,[3] %	0.91	0.84
Met + Cys,[3] %	0.74	0.68
Phe + Tye,[3] %	1.45	1.38
Ca, %	0.80	0.70
Total P, %	0.68	0.59
Digestible P, %	0.47	0.37
Analyzed nutrient, as-is		
Dry matter, %	90.70	89.90
Crude protein, %	23.13	21.30
Acid detergent fiber, %	7.26	9.35
Neutral detergent fiber, %	2.54	3.60
Ca, %	0.96	0.88
P, %	0.71	0.59

[1]In each phase, two additional diets were formulated by adding probiotics or carbadox to the control diet, respectively. The dose for probiotics was 2.56×10^9 CFU/kg diet. The dose for carbadox was 50 mg/kg diet.
[2]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 were indicated as standardized ileal digestible AA.

analysis. Intestinal mucosa samples were collected from the middle of the jejunum AND ileum for gene expression analysis. Briefly, approximately 10-cm intestinal samples were opened longitudinally and gently rinsed with phosphate-buffered saline to remove luminal content. The mucosa sample was collected by gently scraping the sample with a glass slide and immediately snap-froze in liquid nitrogen (Kim et al., 2019b).

Bacterial translocation

Mesenteric lymph nodes were aseptically collected and then pooled within the pig, grounded, diluted, and plated on blood agar for measurement of total bacteria, and the results were expressed as CFU per milligram of lymph node (Swildens et al., 2004). Spleen samples were analyzed in the same way as mesenteric lymph nodes for bacterial translocation.

Detection of β-hemolytic coliforms

Briefly, fecal samples were plated in Columbia Blood Agar with 5% sheep blood to identify hemolytic coliforms, which can lyse red blood cells (**RBC**s) surrounding the colony. Fecal samples were also plated on MacConkey agar to enumerate total coliforms. 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 and 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 agars to verify if they were β-hemolytic *E. coli* by using triple sugar iron agar and lysine iron agar and to verify if they were F-18^{+} *E. coli* by means of a polymerase chain reaction (DebRoy and Maddox, 2001).

Complete blood count and measurement of serum TNF-α and haptoglobin

Whole blood samples were analyzed by the Comparative Pathology Laboratory at the University of California, Davis, for total and differential blood cell count. A multiparameter, automated programmed hematology analyzer (Drew/ERBA Scientific 950 FS Hematological Analyzer, Drew Scientific Inc., Miami, FL) was used for the assay to optimally differentiate porcine blood. Serum samples were analyzed for TNF-α (R&D System Inc., Minneapolis, MN) and haptoglobin (GenWay Biotech Inc., San Diego, CA). All samples were analyzed in duplicate including standard and control. The intra-assay coefficients of variation for TNF-α and haptoglobin were 3.6% and 2.7%, respectively. The inter-assay coefficients of variation for TNF-α and haptoglobin were 9.2% and 6.2%, respectively.

Intestinal morphology

The fixed intestinal tissues were embedded in paraffin, sectioned at 5 μm, and stained with high iron diamine and alcian blue (Kim et al., 2019b). The slides were photographed by an Olympus BX51 microscope at 10×, 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 surrounded area 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 Kim et al. (2019b) and Deplancke and Gaskins (2001).

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 RNA extraction procedural guidelines provided by the reagent manufacturer. The complementary deoxyribonucleic acid (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. To check for DNA and RNA quality, absorption measurements were made on a Thermo Scientific NanoDrop 2000 Spectrophotometer (Thermo Scientific, Inc., Waltham, MA). The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of approximately 1.8 was accepted for DNA; a ratio of approximately 2.0 is accepted as for RNA. The mRNA expression of mucin-2 (***MUC2***), zona occludens-1 (***ZO-1***), claudin-1 (***CLDN1***), and occludin (***OCDN***) in jejunal mucosa and cyclooxygenase-2 (*PTGS2*), tumor necrosis factor-alpha (*TNFA*), interleukin-1 beta (***IL1B***), and interleukin-6 (***IL-6***) in ileal mucosa was analyzed by qRT-PCR. Data normalization was accomplished using beta-actin (*ACTB*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as housekeeping genes. Primers were designed based on published literature and commercially synthesized by Integrated DNA Technologies, Coralville, IA. 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 quantification of genes compared with negative control (Livak and Schmittgen, 2001).

Statistical analysis

The normality of data was verified using the UNIVARIATE procedure of SAS. All data (except frequency of diarrhea) were analyzed using the Proc Mixed of SAS (SAS Inst. Inc, Cary, NC). The statistical model included treatment as the fixed effect and blocks (sex and BW) as random effects, and with individual pig as the experimental unit. Treatment means were calculated using the LSMEANS statement, and means were separated using 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

All animals were healthy before *E. coli* F18 challenge. A total of six pigs were removed from the whole data set due to health issues after *E. coli* infection or as outliers, including three pigs from the PC group and three pigs from the AGP group. Compared with pigs in the NC group, *E. coli* F18 infection in the PC group had decreased ($P < 0.05$) BW on days 14 and 21 PI, decreased ($P < 0.05$) ADG from day 14 to 21 and 0 to 21 PI, decreased ($P < 0.05$) ADFI from day 14 to 21 PI, and decreased ($P < 0.05$) G:F from day 0 to 7, 7 to 14, and 0 to 21 PI (Table 2). Compared with pigs in the PC group, pigs in the AGP group had increased ($P < 0.05$) BW on days 7, 14, and 21 PI, increased ($P < 0.05$) ADG from day 0 to 7, 7 to 14, 14 to 21, and 0 to 21 PI, increased ($P < 0.05$) ADFI from day 7 to 14, 14 to 21, and 0 to 21 PI, and increased ($P < 0.05$) G:F from day 0 to 7 and 0 to 21 PI. Compared with pigs in the PC group, pigs in the PRO group had greater ($P < 0.05$) final BW at day 21 PI, greater ($P < 0.05$) ADG and G:F ratio from day 0 to 21 PI. Compared with AGP, pigs supplemented with PRO had lower ($P < 0.05$) BW on days 7 and 21 PI, lower ($P < 0.05$) ADG and ADFI from day 0 to 7

Table 2. Growth performance of *E. coli*-challenged pigs fed diets supplemented with antibiotics or probiotics

Item[1]	NC	PC	AGP	PRO	SEM	*P*-value
BW, kg						
Day –7	6.19	6.14	6.22	6.14	0.36	0.89
Day 0 PI	7.03	7.17	7.28	7.04	0.43	0.78
Day 7 PI	8.78^{b}	8.53^{b}	9.80^{a}	8.56^{b}	0.48	<0.05
Day 14 PI	12.55^{ab}	10.56^{c}	13.84^{a}	12.07^{bc}	0.7	<0.05
Day 21 PI	17.23^{ab}	13.69^{c}	18.79^{a}	16.46^{b}	0.99	<0.01
ADG, g						
Day –7 to 0	119	146	151	112	19.6	0.43
Day 0 to 7 PI	250^{b}	212^{b}	359^{a}	218^{b}	28.5	<0.01
Day 7 to 14 PI	540^{ab}	459^{b}	594^{a}	501^{ab}	52.7	0.08
Day 14 to 21 PI	668^{a}	466^{b}	718^{a}	628^{a}	44.2	<0.01
Day 0 to 21 PI	486^{ab}	347^{c}	558^{a}	449^{b}	36.0	<0.01
Overall	394^{ab}	296^{c}	452^{a}	365^{bc}	27.2	<0.01
ADFI, g						
Day –7 to 0	217	241	231	208	16.7	0.43
Day 0 to 7 PI	374^{b}	435^{ab}	497^{a}	403^{b}	22.6	<0.05
Day 7 to 14 PI	746^{ab}	687^{b}	895^{a}	751^{ab}	66.4	0.10
Day 14 to 21 PI	1,070^{b}	822^{c}	1,306^{a}	1,029^{b}	76.6	<0.01
Day 0 to 21 PI	730^{b}	647^{b}	899^{a}	728^{b}	57.5	<0.01
Overall	602^{b}	543^{b}	731^{a}	598^{b}	46.0	<0.01
G:F						
Day –7 to 0	0.56	0.61	0.67	0.53	0.072	0.54
Day 0 to 7 PI	0.68^{ab}	0.49^{c}	0.73^{a}	0.54^{bc}	0.059	<0.05
Day 7 to 14 PI	0.72^{a}	0.59^{b}	0.68^{ab}	0.67^{ab}	0.039	0.07
Day 14 to 21 PI	0.62	0.61	0.56	0.61	0.03	0.50
Day 0 to 21 PI	0.66^{a}	0.53^{b}	0.63^{a}	0.62^{a}	0.023	<0.01
Overall	0.65^{a}	0.55^{b}	0.63^{a}	0.62^{a}	0.023	<0.05

[1]Each least-squares mean represents 9 to 12 observations.
[a–c]Means without a common superscript are different ($P < 0.05$).

and 0 to 21 PI, and lower ($P < 0.05$) G:F from day 0 to 7 PI. However, no difference was observed in G:F from day 0 to 21 PI between the PRO and AGP groups.

Diarrhea score

Pigs in the PC group had greater ($P < 0.05$) daily DS from day 4 to 9 PI than pigs in the NC group. Daily DS of pigs in AGP and PRO groups from day 4 to 9 PI were lower ($P < 0.05$) compared with pigs in the PC group, and no difference was observed between AGP and PRO groups (Figure 1). Pigs in the PC group had greater ($P < 0.05$) daily DS on days 11 and 12 PI compared with the other treatments. No difference in daily DS between AGP and PRO was observed. *Escherichia coli* F18 challenge increased ($P < 0.05$) the frequency of diarrhea (DS ≥ 3) of pigs in the PC group to approximately 33.2%, compared with pigs in the NC (11.7%; Figure 2). The frequency of diarrhea (DS ≥ 4) of pigs in the PC group (14.9%) was slightly greater ($P < 0.05$) than that of pigs in the NC group (0.62%), AGP group (8.66%), and PRO group (9.57%). No difference in the frequency of diarrhea between AGP and PRO was observed.

Fecal culture and bacterial translocation

No β-hemolytic coliforms were identified in fecal samples of pigs in the NC group before or after the *E. coli* inoculation. No β-hemolytic coliforms were identified in fecal samples of pigs in the PC, AGP, and PRO groups before the *E. coli* inoculation and on days 14 and 21 PI. Pigs in the AGP and PRO group had reduced ($P < 0.05$) percentage of β-hemolytic coliforms in fecal samples on day 7 PI, compared with pigs in the PC group (Figure 3). No difference in β-hemolytic coliforms between AGP and PRO was observed. No difference was observed in bacterial counts in both lymph nodes and spleen samples among four treatments (Figure 4).

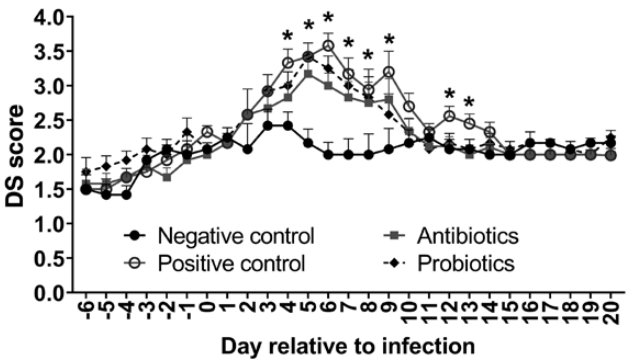

Figure 1. Daily fecal score of *E. coli*-infected weaned pigs fed diets supplemented with antibiotics or probiotics. DS = 1, normal feces; 2, moist feces; 3, mild diarrhea; 4, severe diarrhea; and 5, watery diarrhea. *$P < 0.05$, indicating DS were different among treatments. Each least-squares mean represents 9 to 12 observations.

Intestinal morphology

On day 21 PI, pigs in the PC group had less ($P < 0.05$) sialomucin percentage (% villi and crypt area) in the duodenum and greater ($P < 0.05$) goblet cell number in colon crypt than noninfected pigs in the NC group (Table 3). Pigs in the AGP group had increased ($P < 0.05$) villi height and sialomucin percentage in duodenum and increased ($P < 0.05$) villi height in ileum compared with pigs in the PC group. No difference was observed in the intestinal

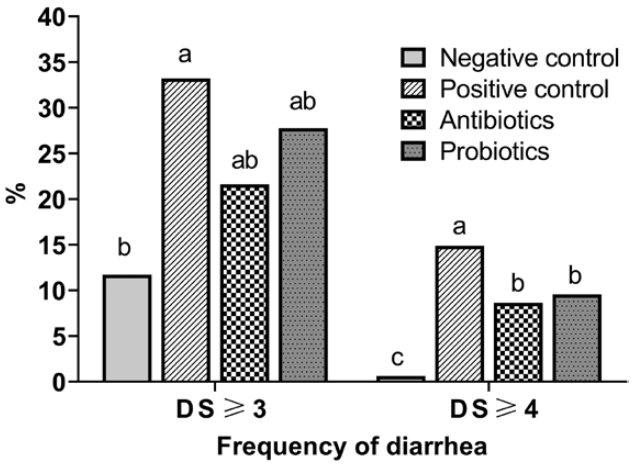

Figure 2. Frequency of diarrhea of *E. coli*-infected weaned pigs fed diets supplemented with antibiotics or probiotics. Frequency of diarrhea was calculated as the percentage of pig days with DS ≥ 3 or ≥ 4 in the total of pig days. Each least-squares mean represents 9 to 12 observations. [a-c]Means without a common superscript are different ($P < 0.05$).

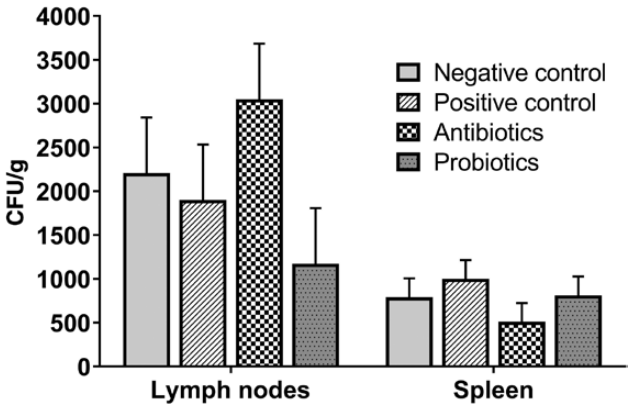

Figure 4. Bacterial counts (CFU/g) in lymph node and spleen of *E. coli*-infected weaned pigs fed diets supplemented with antibiotics or probiotics on day 21 PI. Each least-squares mean represents 9 to 12 observations. No difference was observed in bacterial counts in lymph node and spleen among different treatments.

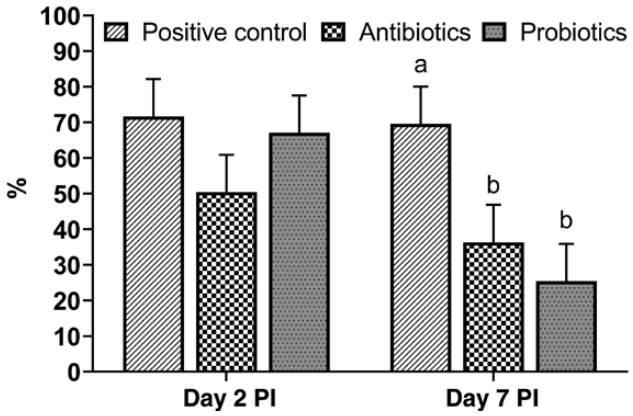

Figure 3. The percentage (%) of β-hemolytic coliform in fecal samples of *E. coli*-challenged pigs fed diets supplemented with antibiotics or probiotics. No β-hemolytic coliforms were observed in the fecal samples of pigs before *E. coli* challenge and days 14 and 21 PI. Each least-squares mean represents 9 to 12 observations. [a,b]Means without a common superscript are different ($P < 0.05$).

morphology of pigs between PRO and PC groups. Pigs in the PRO group had shorter ($P < 0.05$) villi height, lower ($P < 0.05$) sialomucin percentage in villi, and smaller ($P < 0.05$) crypt area of duodenum than pigs in the AGP group. However, no other differences were observed in intestinal morphology in jejunum, ileum, and colon of *E. coli*-infected pigs between the PRO and AGP groups.

Intestinal barrier and innate immunity

No difference was observed in the mRNA expression of *MUC2*, *ZO1*, and *OCLN* in jejunal mucosa among four treatment groups (Figure 5). Pigs in the PRO group had greater ($P < 0.05$) gene expression of *CLDN1* in jejunal mucosa than pigs in the NC and PC groups. Pigs in the PC group had greater ($P < 0.05$) mRNA expression of *IL6* and *PTGS2* in ileal mucosa than pigs in the NC group (Figure 6). Pigs in the AGP and PRO groups had reduced ($P < 0.05$) gene expression of *IL6* and *PTGS2* in the ileal mucosa compared with pigs in the PC group. The mRNA expression of *IL6*, *PTGS2*, and *IL1B* was not different in the ileal mucosa of pigs supplemented with AGP or PRO, whereas pigs in the PRO group expressed more ($P < 0.05$) *TNFA* in ileal mucosa than pigs in the AGP group.

Systemic immunity and RBC profile

No difference was observed in white blood cell (**WBC**) profile and serum haptoglobin concentration among four treatment groups on day 0 before *E. coli* inoculation (Table 4). Compared with pigs in the NC group, *E. coli* infection in the PC group increased ($P < 0.05$) WBC number on days 2, 7, and 14 PI, increased ($P < 0.05$) neutrophil count on days 2 and 7 PI, and increased ($P < 0.05$) lymphocyte and monocyte number on day 14 PI, and decreased ($P < 0.05$) neutrophil-to-lymphocyte ratio on days 14 and 21 PI. Pigs in the AGP group had decreased ($P < 0.05$) WBC number and neutrophil counts on days 7 and 14 PI and decreased ($P < 0.05$) lymphocyte and monocyte number on day 14 PI, compared with pigs in the PC group. Pigs in the AGP group had decreased ($P < 0.05$) serum TNF-α concentration on days 2 and 14 PI and decreased ($P < 0.05$) serum haptoglobin concentration on day 14 PI, compared with pigs in the PC group. Pigs in the AGP group had lower ($P < 0.05$) serum TNF-α than pigs in the PRO group on days 0, 2, 14 PI. Pigs in the PRO group had lower ($P < 0.05$) WBC number and neutrophil count, and serum haptoglobin concentration on day 7 PI, and less ($P < 0.05$) monocyte count on day 14 PI compared with pigs in the PC group.

Compared with pigs in the NC group, *E. coli* infection in the PC group increased ($P < 0.05$) RBC number on days 7 and 14 PI and decreased ($P < 0.05$) mean corpuscular volume (**MCV**) and mean corpuscular hemoglobin (**MCH**) on days 14 and 21 PI (Table 5). Pigs in the AGP group had lower ($P < 0.05$) RBC number on day 2 PI, greater ($P < 0.05$) MCV on days 7, 14, and 21 PI, and greater ($P < 0.05$) MCH on days 2, 7, 14, and 21 PI than pigs in the PC group. Pigs in the PRO group had lower ($P < 0.05$) RBC number on days 2 and 7 PI, greater ($P < 0.05$) MCV on days 14 and 21 PI, and greater ($P < 0.05$) MCH on days 2, 7, 14, and 21 PI than pigs in the PC group. No difference was observed in the RBC profile between the PRO and AGP groups.

Table 3. Intestinal morphology of weaned pigs on day 21 post *E. coli* inoculation

Item[1]	NC	PC	AGP	PRO	SEM	*P*-value
Duodenum						
Goblet cell number, per villi	27.78	21.48	25.25	27.72	2.60	0.38
Villi height, μm	405.37[ab]	335.02[b]	473.99[a]	375.08[b]	28.79	<0.05
Crypt depth, μm	147.48	136.65	154.37	156.73	16.20	0.69
Villi height:crypt depth	3.15	2.69	3.30	2.58	0.33	0.35
Sialomucin, % of villi and crypt area	4.51[a]	2.98[b]	4.84[a]	3.61[b]	0.36	<0.01
Sulfomucin, % of villi and crypt area	6.80	5.71	6.84	6.77	0.48	0.35
Jejunum						
Goblet cell number, per villi	15.03	16.09	14.46	16.72	1.51	0.71
Villi height, μm	417.38	406.38	401.56	428.13	20.65	0.81
Crypt depth, μm	138.28	141.84	115.16	115.92	14.03	0.38
Villi height:crypt depth	3.45	3.48	3.75	3.94	0.40	0.71
Sialomucin, % of villi and crypt area	2.54	2.94	2.97	2.60	0.27	0.64
Sulfomucin, % of villi and crypt area	5.56	5.94	5.06	4.98	0.46	0.46
Ileum						
Goblet cell number, per villi	20.92	17.82	20.59	20.31	1.87	0.72
Villi height, μm	309.85[ab]	281.73[b]	351.49[a]	321.12[ab]	17.31	0.081
Crypt depth, μm	108.94	113.25	117.59	126.32	7.38	0.35
Villi height:crypt depth	3.05	2.55	3.09	2.62	0.22	0.11
Sialomucin, % of villi and crypt area	4.09	3.27	3.87	3.68	0.32	0.40
Sulfomucin, % of villi and crypt area	7.95	6.95	6.89	7.48	0.46	0.40
Colon						
Goblet cell number, per crypt	26.72[c]	38.26[ab]	42.10[a]	32.42[bc]	2.97	<0.01
Crypt depth, μm	371.68	405.51	414.66	350.87	26.53	0.28
Sulfomucin, % of villi and crypt area	13.38	11.44	15.03	11.22	1.12	0.12

[1]Each least-squares mean represents 9 to 12 observations.
[a–c]Means without a common superscript are different ($P < 0.05$).

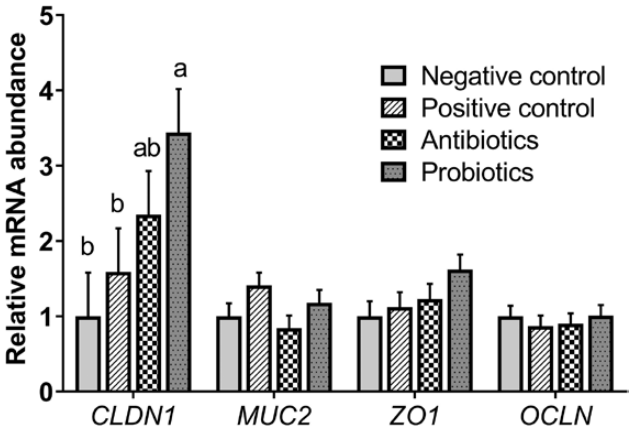

Figure 5. Relative mRNA abundance of *CLDN1*, *MUC2*, *ZO1*, and *OCLN* in the mid-jejunal mucosa of *E. coli*-infected weaned pigs fed diets supplemented with antibiotics or probiotics on day 21 PI. Each least-squares mean represents 9 to 12 observations. [a,b]Means without a common superscript are different ($P < 0.05$).

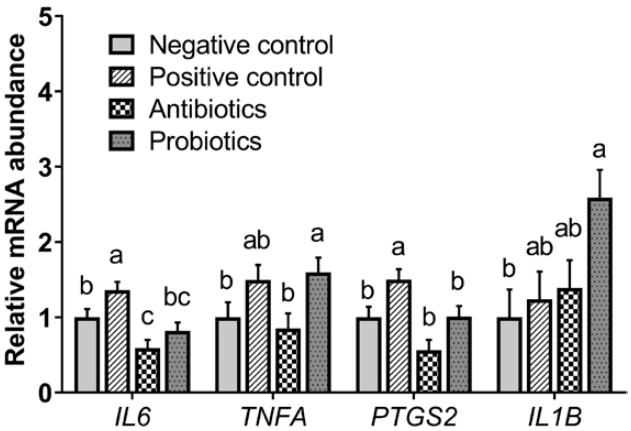

Figure 6. Relative mRNA abundance of *IL6*, *TNFA*, *PTGS2*, and *IL1B* in the ileal mucosa of *E. coli*-infected weaned pigs fed diets supplemented with antibiotics or probiotics on day 21 PI. Each least-squares mean represents 9 to 12 observations. [a–c]Means without a common superscript are different ($P < 0.05$).

Discussion

Results from the present study demonstrated that supplementation of *B. subtilis* improved growth performance, reduced severity of diarrhea, and alleviated systemic inflammation of *E. coli* F18-challenged pigs. These findings agree with our previously published research using the same *E. coli* strain and *B. subtilis* (DSM 25841) strain, in which the growth performance and health status of weanling pigs were improved in piglets fed the *B. subtilis* probiotics (Kim et al., 2019b). The difference between the two studies was that the current study had a longer experimental period (21 d after first *E. coli* inoculation) compared with Kim et al. (2019b), which was only 11 d post *E. coli* inoculation. Both experiments confirmed that supplementation of *B. subtilis* at the dose of 2.56×10^9 CFU/kg enhanced growth and disease resistance of weaned pigs. Although in the present study pigs supplemented with *B. subtilis* probiotic did not have superior growth performance compared with pigs supplemented with carbadox, the comparison of these two groups has shown that *B. subtilis* probiotic could be an alternative to antibiotics to improve postweaning pigs' growth performance and health.

Postweaning diarrhea by ETEC is a common issue in the swine industry. The disease is typically seen in early weaned

Table 4. Total and differential WBCs, and serum haptoglobin and TNF-α in *E. coli*-infected weaned pigs fed diets supplemented with probiotics

Item[1]	NC	PC	AGP	PRO	SEM	*P*-value
Day 0 before inoculation						
WBC, $10^3/\mu L$	10.72	11.37	11.11	10.79	1.44	0.97
Neu, $10^3/\mu L$	5.45	6.17	6.7	5.69	1.11	0.65
Lym, $10^3/\mu L$	4.41	4.33	3.55	4.19	0.40	0.35
Mono, $10^3/\mu L$	0.69	0.74	0.75	0.82	0.09	0.77
Eos, $10^3/\mu L$	0.135	0.097	0.082	0.074	0.039	0.58
Baso, $10^3/\mu L$	0.037	0.026	0.028	0.019	0.011	0.32
Neu:Lym	1.23	1.51	1.81	1.53	0.18	0.16
Serum						
Haptoglobin, mg/mL	1.10	1.14	0.98	1.17	0.20	0.92
TNF-α, pg/mL	117.90^{a}	92.29^{ab}	75.32^{b}	112.14^{a}	16.15	<0.05
Day 2 PI						
WBC, $10^3/\mu L$	10.81^{b}	14.39^{a}	11.93^{ab}	12.64^{ab}	1.01	<0.05
Neu, $10^3/\mu L$	5.88^{b}	8.45^{a}	6.83^{ab}	7.06^{ab}	0.75	<0.05
Lym, $10^3/\mu L$	4.04	4.97	4.21	4.7	0.37	0.30
Mono, $10^3/\mu L$	0.78	0.8	0.77	0.76	0.19	0.99
Eos, $10^3/\mu L$	0.092	0.124	0.105	0.091	0.025	0.79
Baso, $10^3/\mu L$	0.018	0.037	0.027	0.015	0.012	0.21
Neu:Lym	1.59	1.81	1.66	1.61	0.22	0.86
Serum						
Haptoglobin, mg/mL	0.99	1.09	1.21	0.91	0.32	0.83
TNF-α, pg/mL	110.05^{a}	96.11^{a}	74.09^{b}	94.47^{a}	8.52	<0.01
Day 7 PI						
WBC, $10^3/\mu L$	14.13^{b}	18.10^{a}	14.55^{b}	14.64^{b}	1.12	0.08
Neu, $10^3/\mu L$	7.86^{b}	10.01^{a}	7.71^{b}	7.87^{b}	0.72	0.07
Lym, $10^3/\mu L$	5.29	6.95	5.74	5.94	0.57	0.29
Mono, $10^3/\mu L$	0.84	0.88	0.89	0.72	0.10	0.57
Eos, $10^3/\mu L$	0.109	0.208	0.181	0.081	0.060	0.41
Baso, $10^3/\mu L$	0.024	0.044	0.03	0.027	0.008	0.34
Neu:Lym	1.56	1.53	1.38	1.38	0.14	0.73
Serum						
Haptoglobin, mg/mL	0.90^{ab}	1.65^{a}	0.85^{ab}	0.66^{b}	0.31	0.078
TNF-α, pg/mL	79.11	87.08	73.92	81.89	14.30	0.93
Day 14 PI						
WBC, $10^3/\mu L$	12.00^{bc}	15.58^{a}	10.38^{c}	13.66^{ab}	0.80	<0.01
Neu, $10^3/\mu L$	6.43^{a}	7.46^{a}	4.57^{b}	7.17^{a}	0.64	<0.01
Lym, $10^3/\mu L$	4.72^{b}	7.09^{a}	5.02^{b}	5.74^{ab}	0.49	<0.05
Mono, $10^3/\mu L$	0.73^{b}	0.97^{a}	0.75^{b}	0.66^{b}	0.10	<0.05
Eos, $10^3/\mu L$	0.100	0.104	0.072	0.066	0.027	0.071
Baso, $10^3/\mu L$	0.022	0.024	0.017	0.018	0.006	0.74
Neu:Lym	1.46^{a}	1.13^{bc}	0.95^{c}	1.30^{ab}	0.17	<0.01
Serum						
Haptoglobin, mg/mL	0.63^{ab}	1.10^{a}	0.41^{b}	1.06^{a}	0.18	<0.05
TNF-α, pg/mL	70.79^{a}	60.70^{a}	32.03^{b}	56.71^{a}	6.14	<0.01
Day 21 PI						
WBC, $10^3/\mu L$	9.79^{a}	8.10^{ab}	6.16^{b}	9.12^{a}	0.94	<0.05
Neu, $10^3/\mu L$	5.83^{a}	4.25^{ab}	2.64^{b}	5.03^{a}	0.56	<0.01
Lym, $10^3/\mu L$	3.46	3.27	2.99	3.50	0.40	0.71
Mono, $10^3/\mu L$	0.42	0.49	0.47	0.53	0.08	0.46
Eos, $10^3/\mu L$	0.055	0.054	0.023	0.043	0.014	0.26
Baso, $10^3/\mu L$	0.014	0.009	0.002	0.012	0.004	0.11
Neu:Lym	1.78^{a}	1.32^{bc}	0.90^{c}	1.40^{ab}	0.14	<0.01
Serum						
Haptoglobin, mg/mL	0.55	0.71	0.15	0.66	0.14	0.11
TNF-α, pg/mL	41.63	81.64	67.77	48.17	12.78	0.11

[1]Neu, neutrophil; Lym, lymphocyte; Mono, monocyte; Eos, eosinophil; Baso, basophil. Each least-squares mean represents 9 to 12 observations.

[a–c]Means without a common superscript are different ($P < 0.05$).

pigs at 3 to 10 d after weaning and can spread rapidly within a herd. In some cases, the morbidity rates could be 80% to 90%, and mortality rates reach as high as 30%. Moreover, the pigs survived generally suffer from reduced growth rates, which inevitably result in economic losses to the swine producers (Radostits et al., 2006). Carbadox is an oxidative DNA-damaging agent, which

Table 5. RBC profile in *E. coli*-infected weaned pigs fed diets supplemented with probiotics

Item[1]	NC	PC	AGP	PRO	SEM	*P*-value
Day 0 before inoculation						
RBC, 10^6/μL	6.65	6.66	6.55	6.61	0.14	0.94
HGB, g/dL	9.79	9.93	9.95	10.22	0.25	0.42
HCT, %	30.13	29.92	30.45	31.12	0.80	0.53
MCV, fL[2]	45.45^{ab}	45.02^{b}	46.98^{a}	47.16^{a}	0.98	0.08
MCH, pg	14.77	14.97	15.38	15.51	0.33	0.14
MCHC, g/dL	32.53	33.23	32.71	32.9	0.37	0.58
RDW, %	22.87	23.23	22.12	22.58	0.80	0.60
Platelets, 10^3/μL	362	469	470	433	55.7	0.20
MPV, fL[2]	10.57^{a}	9.78^{b}	10.56^{a}	10.34^{ab}	0.39	0.08
Total protein, g/dL	4.92	4.66	4.77	5.01	0.13	0.26
Day 2 PI						
RBC, 10^6/μL	6.53^{ab}	6.80^{a}	6.03^{c}	6.34^{bc}	0.15	<0.01
HGB, g/dL	9.75	9.85	9.36	9.73	0.21	0.41
HCT, %	29.2	29.96	27.7	29.04	0.65	0.15
MCV, fL[2]	44.77	44.12	46.1	45.79	0.63	0.12
MCH, pg	14.94^{bc}	14.52^{c}	15.59^{a}	15.38^{ab}	0.25	<0.05
MCHC, g/dL	33.39	32.88	33.82	33.56	0.33	0.28
RDW, %	22.89	23.49	22.51	22.85	0.83	0.72
Platelets, 10^3/μL	337	378	367	414	49.5	0.72
MPV, fL[2]	10.53	9.51	10.16	9.72	0.58	0.11
Total protein, g/dL	5.04	4.98	4.88	5.12	0.10	0.47
Day 7 PI						
RBC, 10^6/μL	5.98^{b}	6.66^{a}	6.13^{ab}	6.01^{b}	0.21	0.07
HGB, g/dL	8.82	9.76	9.76	9.37	0.28	0.12
HCT, %	26.92	29.23	28.68	27.28	0.76	0.15
MCV, fL[2]	45.12^{ab}	43.98^{b}	47.21^{a}	45.51^{ab}	0.88	<0.05
MCH, pg	14.89^{bc}	14.70^{c}	16.00^{a}	15.67^{ab}	0.38	<0.01
MCHC, g/dL	32.98^{b}	33.38^{ab}	33.99^{ab}	34.39^{a}	0.40	0.06
RDW, %	23.17	22.83	23.7	22.37	0.63	0.46
Platelets, 10^3/μL	457	440	470	382	58	0.69
MPV, fL[2]	10.24	9.95	10.34	10.13	0.51	0.93
Total protein, g/dL	4.76	4.93	4.86	4.8	0.20	0.93
Day 14 PI						
RBC, 10^6/μL	5.77^{b}	6.35^{a}	6.12^{ab}	6.16^{ab}	0.16	0.10
HGB, g/dL	9.26	9.38	10.08	9.78	0.29	0.25
HCT, %	28.39	28.67	30.88	30.01	0.75	0.11
MCV, fL[2]	49.28^{a}	45.30^{b}	50.44^{a}	49.07^{a}	0.97	<0.01
MCH, pg	16.13^{a}	14.82^{b}	16.46^{a}	16.05^{a}	0.33	<0.01
MCHC, g/dL	32.71	32.6	32.62	32.69	0.35	0.99
RDW, %	26.77	24.22	25.5	25.82	1.00	0.17
Platelets, 10^3/μL	491	591	482	478	48.7	0.37
MPV, fL[2]	10.37	9.37	10.01	9.94	0.36	0.27
Total protein, g/dL	4.69	4.72	4.67	4.76	0.12	0.96
Day 21 PI						
RBC, 10^6/μL	5.63	5.86	5.35	5.59	0.16	0.25
HGB, g/dL	9.05	8.72	8.75	8.83	0.28	0.68
HCT, %	27.98	26.48	26.8	26.8	0.71	0.44
MCV, fL[2]	49.60^{ab}	45.47^{c}	50.36^{a}	47.85^{b}	0.92	<0.01
MCH, pg	16.08^{a}	15.05^{b}	16.38^{a}	15.80^{ab}	0.47	<0.05
MCHC, g/dL	32.37	32.95	32.63	33.01	0.44	0.49
RDW, %	23.14	23.23	22.67	22.73	0.60	0.85
Platelets, 10^3/μL	441	541	401	504	50	0.22
MPV, fL[2]	9.56	9.02	8.98	9.12	0.40	0.43
Total protein, g/dL	5.1	4.91	4.82	4.96	0.16	0.60

[1]HGB, hemoglobin; HCT, packed cell volume; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; MPV, mean platelet volume. Each least-squares mean represents 9 to 12 observations.
[2]fL, femtolitre (10^{-15} liters).
[a–c]Means without a common superscript are different ($P < 0.05$).

can interfere with DNA synthesis and cause the breakdown of chromosome in many gram-negative bacteria including *E. coli* (Suter et al., 1978; Cheng et al., 2015). Historically, it was shown that feeding carbadox at subtherapeutic concentration (55 mg/kg) could effectively improve the growth performance of weanling pigs (Blair and Shires, 1981; Roof and Mahan, 1982; Yen and Pond, 1993). Moreover, carbadox supplementation at subtherapeutic concentration also greatly promoted the growth of weanling pigs that were exposed to high levels of antigens (Stahly et al., 1997). In agreement with these studies, results from the present study have demonstrated that carbadox was consistently effective in promoting the growth of weanling pigs and protecting them against *E. coli* F18 infection.

Similarly, supplementation of *B. subtilis* in the present study was also effective in enhancing the growth performance of *E. coli* F18-challenged pigs. However, the *B. subtilis* may exert its beneficial effects on these weanling pigs through different mechanisms, such as modulation of host immune responses, enhancement of the expression of tight junction protein, and production of antimicrobials (Stein, 2005; Hu et al., 2014; Lee et al., 2014). In the current study, pigs supplemented with *B. subtilis* or carbadox had a lowered frequency of diarrhea (9.57% and 8.66%) compared with pigs in the PC (14.89%), indicating both *B. subtilis* and carbadox had enhanced disease resistance of these pigs. In consistence with clinical signs' results, pigs supplemented with *B. subtilis* and carbadox had less β-hemolytic coliforms compared with pigs in the PC, indicating lowered *E. coli* F18 shedding in pigs' feces throughout the infection period or a faster exclusion of *E. coli* F18 compared with pigs in the PC. Taken altogether, both the antibiotic and probiotic supplementations could help pigs to recover sooner from the ETEC F18 infection.

Our previously published research has shown that oral inoculation of *E. coli* F18 could induce systemic inflammation of weaned pigs by increasing total WBC counts, neutrophils, and lymphocytes with the peak of inflammation at approximately day 5 to 6 PI (Liu et al., 2013). In the current study, both carbadox and *Bacillus* probiotic groups had reduced counts of neutrophil and lymphocytes PI, indicating alleviated inflammation status of these pigs. It is also worth noting that a significant PI reduction of MCH and MCV was observed in pigs from the PC group. Although levels of these two blood parameters are within the reference interval, it may reflect an anemic state of these ETEC-infected pigs, which may result from reduced absorption of iron from the duodenum (Ganz, 2013; Cooper et al., 2014). The exact mechanism of this reduction in MCH and MCV may need further investigation; nonetheless, both carbadox and *Bacillus* probiotic maintained their levels as balanced as the negative control.

It is long recognized that immunologically challenged animals have reduced growth performance as a result of changes in behavioral and metabolic activities induced by inflammatory responses (Johnson, 1997). Endotoxin lipopolysaccharides from *E. coli* induce the production of proinflammatory cytokine TNF-α, which mediate shifts in carbohydrate, protein, and lipid metabolism in support of the immune system and thus antagonize growth (Klasing and Johnstone, 1991; Johnson, 1997). Moreover, circulating concentrations of acute phase proteins such as haptoglobin also increase during the initial acute phase response (Chen et al., 2003). In the present study, pigs supplemented with carbadox had reductions in TNF-α levels throughout the experimental period. This finding demonstrated the capacity of carbadox in alleviating gut inflammation and protecting the pigs from ETEC infection, possibly by reducing the total bacterial population in the gut (Allen and Stanton, 2014). It may also be a result of antibiotics accumulation in the inflammatory cells, thus inhibiting the inflammatory response of the animals (Niewold, 2007). In contrast, *Bacillus* probiotic had lowered haptoglobin levels but not TNF-α levels PI. Despite our ETEC challenge, this unchanged serum TNF-α level may be due to confined cytokine production within inflamed gut tissue. In addition, it was previously reported that the serum TNF-α levels in disease-challenged pigs may be related to the route of inoculation (Balaji et al., 2000; Lee et al., 2012).

Besides the important function of digestion and absorption of nutrients, the intestinal epithelium serves as a physical barrier separating internal mucosal tissue from the external environment. On the intestinal epithelium, the apical areas of adjacent epithelial cells are joined by intercellular tight junctions consisting of several multiprotein junctional complexes, namely zonulae occludens (ZO)-1, OCDNs, and CLDNs (Buckley and Turner, 2018). The tight junctions form the physical barrier and regulate the paracellular movement of substances across the intestinal epithelium. Thus, it is of great importance to maintain the integrity of intestinal structure and barrier function. With ETEC infection and its heat-stable toxin b secretion, loss of absorptive epithelial cells, alteration of cellular structure, and shortening of villi (11% to 19%) are generally observed in the small intestine of pigs (Rose et al., 1987; Dubreuil, 2008). ETEC infection also causes impairment to the barrier function by delocalizing ZO-1 and decreasing expression of occludin, leading to increased permeability of the gut and the consequent intestinal inflammation (Roselli et al., 2007; Ngendahayo Mukiza and Dubreuil, 2013). In the current study, disruption in gut morphology was not observed when comparing the infected and noninfected pigs, this is likely due to a fast turnover rate of intestinal epithelial cells and that the majority of pigs were fully recovered from ETEC infection at 21 PI. However, the carbadox group had greater villi height at the end of the study, demonstrating a growth-promoting effect through an uncompromised nutrient absorption in the gut. Previously, Looft et al. (2014) reported that the administration of carbadox increased the relative abundance of *Prevotella*, *Roseburia*, and *Faecalibacterium* in the gut of nursery pigs, and it was indicated that this shift in microbiota population may increase the production of short-chain fatty acids (**SCFA**), especially acetate, propionate, and butyrate, which are beneficial to pigs' intestinal health and energy utilization (Bedford and Gong, 2018). Thus, in the present study, the enhanced growth performance by carbadox supplementation may result from not only a reduced intestinal inflammation but also a possible shift in the microbial population and the beneficial effects of SCFA. On the other hand, supplementation of *B. subtilis* probiotic did not improve the gut morphology of ETEC-infected pigs but did enhance the mRNA expression of CLDN-1 among all the tight junction proteins in jejunal mucosa. CLDN-1 is a ubiquitously distributed tight junction protein in body tissues, and it plays a general role in epithelial barrier function. Consistently, Kim et al. (2019b) also reported that the same *B. subtilis* probiotic strain increased the CLDN-1 mRNA expression in ileal mucosa of ETEC-infected pigs on day 11 PI. Taken altogether, results from the present study and from the study of Kim et al. (2019b) may indicate a persistent effect of this strain of *B. subtilis* in improving the gut barrier function of weaned pigs. However, it is worth noting that the upregulation of CLDN-1 mRNA expression may not correspond to an increase in the production of CLDN-1 protein. Furthermore, the localization of tight junction proteins to the apical membrane may be regulated, as the translated CLDN proteins may serve as a

tight junction protein reservoir during the remodeling of the junctional protein complex (Garcia-Hernandez et al., 2017). Future research will be needed to confirm the effects of *B. subtilis* on improving tight junction protein productions. The exact mechanism of the increase of CLDN-1 in pigs supplemented with *B. subtilis* remains unclear, but studies have shown that under pathological conditions, barrier function is mediated by various proinflammatory cytokines, such as interferon-γ, TNF-α, and IL-1β (Wang et al., 2005; Al-Sadi et al., 2008). In the present study, the expression of IL-6 and PTGS2 was reduced, and they were reported to have effect on the expression of tight junction proteins; however, whether they played a role in the regulation of CLDN-1 will need further investigation (Fredenburgh et al., 2011; Suzuki et al., 2011).

Results from the present study demonstrated that supplementation of carbadox and *B. subtilis* alleviated the severity of diarrhea caused by *E. coli* F18 infection and enhanced the growth performance of weaned pigs. Although the growth performance of pigs fed *B. subtilis* probiotic is less prominent compared with pigs fed carbadox, an enhanced disease resistance reflected by an attenuated systemic and intestinal inflammation and improved gut barrier integrity were observed. Future research will consider incorporating metagenomics to provide more insight into the effects of this *B. subtilis* on nursery pigs' gut microbial community. Metabolomics on serum, fecal, and mucosal samples may also help provide a more comprehensive analysis of the effects of *B. subtilis* on nursery pigs' health and growth performance.

Supplementary Data

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

Supplementary Table S1. Gene-specific primer sequences and PCR conditions[1]

Acknowledgments

We would like to thank the National Pork Board (#18-081) and Animal Health Formula Funds (CALV-AH-045) for the financial support of this research. Appreciation is also expressed to the joint laboratories of Dr. Edward R. Atwill and the Western Institute for Food Safety and Security for assistance with the *E. coli* F18 inoculum.

Conflict of interest statement

The authors disclose that there was no conflict of interest.

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