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Dietary supplementation of *Bacillus subtilis* influenced intestinal health of weaned pigs experimentally infected with a pathogenic *E. coli*

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Abstract

Background: There is growing evidence to support the beneficial effects of supplementing direct-fed microbials (DFM) on performance, health status, and immune responses of weaned pigs. Therefore, the objective of this study was to investigate dietary supplementation of *Bacillus subtilis* (DSM 25841) on growth performance, diarrhea, gut permeability and immunity of weaned pigs experimentally infected with a pathogenic F-18 *Escherichia coli* (*E. coli*).

Results: The F18 *E. coli* infection reduced ($P < 0.05$) growth performance and intestinal villi height, whereas increased ($P < 0.05$) diarrhea and transcellular and paracellular permeability in the jejunum compared with non-challenged control. Supplementation of *Bacillus subtilis* linearly enhanced average daily gain of *E. coli* infected pigs from d 0 to 5 post-inoculation (PI) ($P < 0.05$) and d 0 to 11 PI ($P = 0.058$). Supplementation of high dose of *Bacillus subtilis* reduced ($P < 0.05$) both transcellular and paracellular permeability on d 5 and d 11 PI compared with the *E. coli* infected pigs fed with control diet. *E. coli* infection up-regulated ($P < 0.05$) the mRNA expression of *SLC5A10* (soluble carrier family 5 member 10) and *MUC2* (mucin 2) on d 5 PI, but down-regulated ($P < 0.05$) expression of *SLC5A10, MUC2*, and *CLDN1* on d 11 PI in jejunal mucosa when pigs were fed with the control diet. Supplementation of *Bacillus subtilis* linearly up-regulated ($P < 0.05$) the mRNA expression of *CFTR* and *ZO1* on d 5 PI and *SLC5A10* and *MUC2* on d 11 PI in jejunal mucosa of *E. coli* infected pigs. In addition, *E. coli* infection increased ($P < 0.05$) the mRNA expression of several immune genes (*IL1A, IL1B*, and *IL7* on d 5 PI, and *IL1B, IL6, IL7*, and *TNF* on d 11 PI) in the ileal mucosa of weaned pigs. Inclusion of *Bacillus subtilis* to control diet linearly down-regulated gene expression of *IL1A* on d 5 PI ($P = 0.07$) and IL6 on d 11 PI ($P < 0.05$) in ileal mucosa of *E. coli* infected pigs.

Conclusions: Supplementation of *Bacillus subtilis* (DSM 25841) enhanced growth rate and improved gut barrier function of weaned pigs experimentally infected with a pathogenic *E. coli*.

Keywords: *Bacillus subtilis*, Growth rate, Gut barrier function, Intestinal inflammation, Pathogenic *Escherichia coli*, Weaned pigs

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Background

Post-weaning diarrhea accounts for tremendous economic losses in the swine industry due to mortality and morbidity, weight loses, and cost of medication [1, 2]. Enterotoxigenic *Escherichia coli* (*E. coli*) infection is still one of the most important causes of post-weaning diarrhea in pigs. In past decades, antibiotics have been used as a powerful component to prevent post-weaning diarrhea due to *E. coli* infection. However, use of in-feed antibiotics for production purposes in the livestock industry was completely banned in USA and EU [3], which is increasing remarkably the challenges of keeping pigs healthy, especially in the post-weaning period. Thus, any reliable strategy that could enhance disease resistance and production of weaned pigs will yield substantial benefits to the industry [4, 5].

Direct-fed microbials (DFM), also known as probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [6]. Direct-fed microbials are categorized into 3 main groups, including lactic acid-producing bacteria, yeast, and *Bacillus* [5, 7]. Growing evidence supports that the inclusion of DFM bringing various health benefits to weaned pigs by modulating gut microbiota. The benefits may include but are not limited to the inhibition of pathogen growth, improvement of nutrient digestibility and growth performance, as well as enhancement of the immunity [8–11]. In comparison to other types of DFM, *Bacillus*-based DFM have obvious advantages because they are spore-forming. This specific characteristic makes them thermostable for feed storage and processing (i.e., pelleting and extrusion) and successful survival at low pH in the stomach [12, 13]. The potential benefits of *Bacillus* spp. supplementation on performance, gut health, and immunity have been reported in healthy weaned pigs or *E. coli* challenged pigs [14–16]. However, there is limited research focusing on the impacts of *Bacillus subtilis* on performance and disease resistance of weaned pigs infected with F18 *E. coli*, which is one of major *E. coli* strains responsible for post-weaning diarrhea. Therefore, the objectives of this study were to determine the effects of a novel, specially selected strain of *Bacillus subtilis* (DSM 25841) on diarrhea and performance of weaned pigs experimentally infected with F18 *E. coli*, and to explore the potential modes of action of *Bacillus*-based DFM by investigating gut permeability, intestinal morphology, and immune responses of weaned pigs.

Materials and methods

Animals, housing, experimental design, and diet

A total of 48 weanling pigs (crossbred; initial body weight (BW): 6.73 ± 0.77 kg) with an equal number of gilts and barrows were selected from the Swine Teaching and Research Center of the University of California, Davis and used in this study. The sows and piglets used did not receive *E. coli* vaccines, antibiotic injections, or antibiotics in creep feed. Before weaning, feces were collected from sows and all their piglets destined for this study to verify the absence of β-hemolytic *E. coli*. The F18 *E. coli* receptor status was also tested in the piglets based on the methods of Kreuzer et al. [17]. All pigs used in this study were susceptible to F18 *E. coli*. After weaning, all pigs were randomly assigned to one of four dietary treatments in a randomized complete block design with body weight within sex and litter as the blocks and pig as the experimental unit. There were 12 replicate pigs per treatment. Pigs were individually housed (pen size: 0.61 m × 1.22 m) in an environmental control unit at Teaching and Research Animal Care Services at University of California, Davis for 19 days, including 7 days before and 11 days after the first *E. coli* challenge (d 0). The piglets had *ad libitum* access to feed and water. Environmental enrichment was provided for each pig. The light was on at 07:00 and off at 19:00 h daily in the environmental control unit.

The 4 dietary treatments included: 1) Negative control: control diet, without *E. coli* challenge; 2) Positive control: control diet, with *E. coli* challenge; 3) Low dose *Bacillus subtilis* (DSM 25841): control diet plus 1.28×10^9 CFU of *Bacillus subtilis*/kg feed, with *E. coli* challenge; 4) High dose *Bacillus subtilis*: control diet plus 2.56×10^9 CFU of *Bacillus subtilis*/kg feed, with *E. coli* challenge (Table 1). Spray-dried plasma, antibiotics, and high levels of zinc oxide exceeding recommendation and normal practice were not included in the diets. The experimental diets were fed to pigs throughout the study duration. After 7 days adaptation, all pigs were orally inoculated with 3 mL of F18 *E. coli* for 3 consecutive days from d 0 post-inoculation (PI). The F18 *E. coli* were originally isolated from a field disease outbreak by the University of Illinois Veterinary Diagnostic Lab (isolate number: U.IL-VDL # 05–27,242). The F18 *E. coli* expressed heat labile toxin (LT), heat stable toxin b (STb), and shiga-like toxin (SLT-2). 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} CFU per 3 mL dose in phosphate buffer saline (PBS). This dose caused mild diarrhea in the current study, which is consistent with our previous published research [18–20].

Clinical observations and sample collections

The procedures for this study were adapted from previous research methods [20]. Clinical observations (diarrhea score and alertness score) were recorded twice daily throughout the study. The diarrhea score of each pig was assessed visually each day by 2 independent

Table 1 Ingredient compositions of experimental diets[a]

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
Vit-mineral, Sow 6[b]	0.30
Total	100.00
Calculated energy and nutrients, as-fed	
Metabolizable energy, kcal/kg	3487
SID Lysine, %	1.35
SID Methionine, %	0.44
SID Threonine, %	0.79
SID Tryptophan, %	0.23
SID Methionine and Cysteine, %	0.74
SID Leucine, %	1.68
SID Isoleucine, %	0.86
SID Valine, %	0.95
Analyzed nutrients, % as-fed	
Crude protein, %	23.05
Ca, %	1.04
Total P, %	0.75

[a]Two additional diets were formulated by adding low (1.28×10^9 CFU/kg) or high dose (2.56×10^9 CFU/kg) of *Bacillus subtilis* (DSM 25841) to the control diet, respectively

[b]Provided the following quantities of vitamins and micro minerals per kilogram of complete diet: Vitamin A as retinyl acetate, 11136 IU; vitamin D_3 as cholecalciferol, 2208 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 B_{12}, 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

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 3 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 alertness score 1 throughout the study, so data were not reported. Pigs were weighed on weaning day, d 0 before inoculation, and d 5, and 11 PI. Feed intake was recorded throughout the study. Average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (gain:feed) was calculated for each interval from d − 7 to 0, d 0 to 5 PI, and d 5 to 11 PI. Fecal samples were collected from the rectum of the 48 pigs (12 pigs/treatment) on d 0 before inoculation, d 2 and 5 PI and from the rectum of the 24 pigs (6 pigs/treatment) on d 8 and 11 PI using a fecal loop for the detection of β-hemolytic coliforms [18, 20]. Twenty-four pigs (3 barrows and 3 gilts from each treatment) were euthanized on d 5 PI near the peak of infection, and the remaining pigs were euthanized at the end of the study (d 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 previous published research using same *E. coli* strain and inoculation dose [18–20]. 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. Fresh jejunal samples were collected in the middle of the jejunum and stored in Krebs solution for gut permeability analysis. Jejunal mucosa (the middle of jejunum) and ileal mucosa (close to the ileocecal junction) were collected and immediately stored in liquid nitrogen for gene expression analysis. Briefly, approximately 10 cm intestinal samples were opened longitudinally and gently rinsed with PBS to remove luminal content. Mucosa samples were collected by gently scraping samples with glass slides. Three 3-cm segments from the duodenum, middle of the jejunum, and the ileum (10 cm from 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.

Detection of β-hemolytic coliforms

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 sub-cultured 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. Questionable colonies were

sub-sub-cultured 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 to verify if they were F-18+ *E. coli* using PCR [21].

Gut permeability analysis with Ussing chamber

The procedures for gut permeability analysis followed previously published methods [22]. Tissues were mounted in an Ussing Chamber (Physiological Instruments, San Diego, CA) after being stripped of the longitudinal muscle and opened along the mesenteric border. The chamber exposed the tissue surface area (0.5 cm^2) to 2.5 mL of oxygenated Krebs-mannitol (10 mmol/L) and Krebs-glucose (10 mmol/L) at 37 °C on the luminal and serosal sides, respectively. After a 30-min period of equilibration, short circuit current and conductance were measured. Transcellular and paracellular permeability were determined by measuring the flux of horseradish peroxidase (HRP) and FITC-4000 (FD-4) across the jejunal mucosa, respectively. HRP (0.5 mg) and FD-4 (1 mg) were added to the mucosal chamber and 200 μL of sample was collected from the serosal chamber every 30 min for 1 h. To maintain a constant volume within the chambers, an equivalent volume of Krebs-glucose solution was replaced at each sample point. O-dianisidine peroxidase substrate was used to detect HRP at absorbance 450 nm. Concentration of FD-4 was measured via fluorescence at excitation of 485 nm and emission of 538 nm.

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 scanned by the NanoZoomer Digital Pathology System (Hamamatsu Co., Bridgewater, NJ) and all measurements were conducted in the associated slide-viewing software (NDP.view; Hamamatsu Co.) and 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 Deplancke and Gaskins [23] and Almeida et al. [19].

Quantitative real-time PCR

Total RNA were extracted from jejunal and ileal mucosa samples that were collected on d 5 and 11 PI as in previously described [24]. The RNA quality and quantity were assessed by Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). First-strand cDNA was produced from 1 μg of total RNA per sample with SuperScript III First-Strand Synthesis SuperMix for quantitative real time-PCR (qRT-PCR) kit (Invitrogen; Carlsbad, CA) in a total volume of 20 μL. The mRNA expression of cystic fibrosis transmembrane conductance regulator (*CFTR*), claudin 1 (*CLDN1*), interferon gamma (*IFNG*), interleukin-1 alpha (*IL1A*), interleukin 1 beta (*IL1B*), interleukin 6 (*IL6*), interleukin-7 (*IL7*), mucin 2 (*MUC2*), occludin (*OCLN*), cyclooxygenase 2 (*PTGS2*), soluble carrier family 5 member 10 (*SLC5A10*), tumor necrosis factor alpha (*TNF*), zonula occludens-1 (*ZO-1*) in ileal mucosa were 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 (Additional file 1: Table S1). The qRT-PCR reaction conditions followed previously published research [24]. The $2^{-\Delta\Delta CT}$ method was used to analyze relative expression of genes compared with negative control [25].

Statistical analysis

Normality of data were verified and outliers were identified for all data expect for gut permeability and frequency of diarrhea using the UNIVARIATE procedure (SAS Inst. Inc., Cary, NC). However, no outliers were detected and removed from the dataset. For growth performance, diarrhea score, gut permeability, and gene expression, data were analyzed by ANOVA using the PROC MIXED of SAS in a randomized complete block design with pig as the experimental unit. The statistical model included treatment 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. Contrast statements were used to test linear and quadratic effects of *Bacillus subtilis* by comparing with the positive control. The chi-squared test was used for analyzing frequency of diarrhea. Statistical significance and tendency were considered at $P < 0.05$ and $0.05 \leq P < 0.10$, respectively.

Results

Growth performance, diarrhea score, β-hemolytic coliforms

No difference was observed in the initial BW of pigs among dietary treatments (Table 2). Compared with negative control pigs, pigs in the positive control group had reduced BW on d 5 PI ($P < 0.05$), lower ADG and ADFI from d 0 to 5 PI ($P < 0.05$), lower ADG from d 0 to 11 PI ($P = 0.057$), and less gain:feed from d 0 to 11 PI ($P = 0.084$). Supplementation of *Bacillus subtilis* linearly increased body weight ($P = 0.086$) on d 5 PI, ADG from d 0 to 5 PI ($P < 0.05$) and from d 0 to 11 PI ($P = 0.058$), compared with the positive control. No differences were observed in ADG, ADFI, and gain:feed ratio among treatment groups from d 5 to 11 PI. No differences were

Table 2 Growth performance of weaned pigs fed diets supplemented with *Bacillus subtilis*

Item[d]	*E. coli* challenge				SEM	*P*-value		
	Negative control	Positive control	Low dose *Bacillus subtilis*	High dose *Bacillus subtilis*		Diet	Lin.[e]	Quad.[e]
BW, kg								
d – 7[f]	6.76	6.76	6.64	6.73	0.23	0.73	0.82	0.32
d 0[f]	7.98	7.78	7.84	7.91	0.59	0.87	0.61	0.99
d 5 PI[g]	9.52[a]	8.41[b]	8.83[ab]	9.06[ab]	0.60	**0.039**	0.086	0.76
d 11 PI[g]	12.93	11.02	11.92	12.56	1.67	0.14	0.084	0.86
ADG, g								
d – 7 to 0[f]	174	146	167	168	77.33	0.83	0.50	0.72
d 0 to 5 PI[f]	308[a]	125[c]	193[bc]	230[ab]	40.79	**< 0.01**	0.036	0.71
d 5 to 11 PI[g]	426	373	435	452	129.5	0.73	0.31	0.73
d 0 to 11 PI[g]	416[a]	272[b]	340[ab]	375[ab]	74.45	0.057	0.058	0.71
ADFI, g								
d – 7 to 0[f]	308	311	303	271	57.66	0.74	0.33	0.73
d 0 to 5 PI[f]	572[a]	365[b]	386[b]	423[ab]	58.29	**< 0.01**	0.21	0.85
d 5 to 11 PI[g]	637	585	566	735	96.61	0.24	0.095	0.24
d 0 to 11 PI[g]	617	507	459	603	48.76	0.11	0.16	0.13
G:F								
d – 7 to 0[f]	0.58	0.42	0.50	0.57	0.171	0.13	0.051	0.99
d 0 to 5 PI[f]	0.52	0.35	0.45	0.52	0.078	0.32	0.11	0.82
d 5 to 11 PI[g]	0.67	0.61	0.74	0.60	0.150	0.47	0.90	0.14
d 0 to 11 PI[g]	0.68[a]	0.52[b]	0.71[a]	0.59[ab]	0.127	0.084	0.36	**0.034**

[a-c]Means without a common superscript are different ($P < 0.05$); bold *P* values denote statistical significance at the $P < 0.05$
[d]*BW* body weight, *ADG* average daily gain, *ADFI* average daily feed intake, *G:F* gain:feed, *PI* post inoculation
[e]Linear and quadratic effects of adding *Bacillus subtilis* to the control diet in pigs infected with F18 *E. coli*
[f]Each least squares mean represents 12 observations
[g]Each least squares mean represents 6 observations

observed in pig BW, ADG, ADFI, and feed efficiency between negative control and high dose *Bacillus subtilis* diet throughout the study, with the exception that pigs in the high dose *Bacillus subtilis* diet had less ($P < 0.05$) ADFI from d 0 to 5 PI compared with the pigs in negative control group.

Compared with the negative control, F18 *E. coli* challenge increased ($P < 0.05$) daily diarrhea score from d 2 to 10 PI (Fig. 1), enhanced periodically diarrhea score from d 0 to 5 PI and d 5 to 11 PI in weaned pigs. The frequency of diarrhea (percentage of pig days with diarrhea score ≥ 3) was 14.81% in negative control, 53.70% in positive control, 45.37% in low dose *Bacillus subtilis*, and 49.07% in high dose *Bacillus subtilis* group, respectively. *E. coli* challenge enhanced ($P < 0.05$) frequency of diarrhea in weaned pigs, but supplementation of *Bacillus subtilis* did not affect diarrhea score and frequency of diarrhea, compared with the positive control.

No β-hemolytic coliforms was detected in the feces of pigs in the negative control group (Fig. 2). Pigs in the positive control, low dose and high dose *Bacillus subtilis* group had higher ($P < 0.05$) percentage of β-hemolytic coliforms in feces, compared with the negative control. No difference was observed in fecal β-hemolytic coliforms in pigs among positive control, low dose and high dose *Bacillus subtilis* groups, however, supplementation of *Bacillus subtilis* linearly increased ($P < 0.05$) the percentage of fecal β-hemolytic coliforms on d 5 and 11 PI, compared with the positive control.

Gut permeability and intestinal morphology

F18 *E. coli* challenge increased ($P < 0.05$) transcellular and paracellular permeability in the jejunum of weaned pigs if positive control was compared with negative control, whereas supplementation of high dose of *Bacillus subtilis* reduced ($P < 0.05$) jejunal permeability on d 5 and d 11 PI, compared with the positive control (Fig. 3). Compared with the negative control, *E. coli* challenge reduced ($P < 0.05$) jejunal villi height and villi area on d 5 PI and reduced ($P < 0.05$) duodenal villi height and crypt depth on d 11 PI (Table 3). However, supplementation of *Bacillus subtilis* did not affect the intestinal morphology, compared with the positive control.

E. coli challenge also reduced ($P < 0.05$) goblet cell number in duodenal villi and reduced ($P < 0.05$) sialomucin area (%) compared with the negative control (Additional file 1:

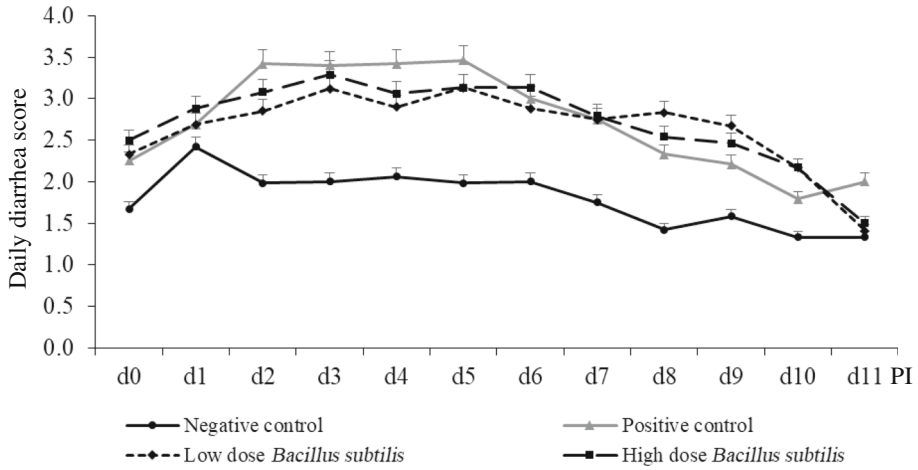

Fig. 1 Daily diarrhea score of weaned pigs fed diets supplemented with *Bacillus subtilis*. Diarrhea score = 1, normal feces, 2, moist feces, 3, mild diarrhea, 4, severe diarrhea, 5, watery diarrhea. Diarrhea score was lower ($P < 0.05$) in pigs fed the negative control diet, compared with all other diets from d 2 to d 10 post-infection (PI). No differences were observed in pigs fed with the positive control diets and the two diets supplemented with *Bacillus subtilis*. Each least squares mean represents 12 observations from d 0 to d 5 PI. Each least squares mean represents 6 observations from d 6 to d 11 PI

Table S2). Supplementation of *Bacillus subtilis* linearly increased ($P < 0.05$) sialomucin area along the crypts of duodenum on d 5 PI. No differences were observed in goblet cell number, sialomucin and sulfomucin in different intestinal segments among treatments on d 11 PI.

Gene expression in intestinal mucosa

E. coli infection up-regulated ($P < 0.05$) the mRNA expression of *SLC5A10* and *MUC2* in jejunal mucosa on d 5 PI, but down-regulated ($P < 0.05$) the mRNA expression of *SLC5A10*, *MUC2*, and *CLDN1* in jejunal mucosa on d 11 PI if positive control was compared with negative control (Table 4). Supplementation of high dose *Bacillus subtilis* up-regulated ($P < 0.05$) the mRNA expression of *CFTR* and *ZO1* on d 5 PI and *SLC5A10* expression on d 11 PI, but reduced ($P < 0.05$) *MUC2* gene expression in jejunal mucosa on d 5 PI, compared with the positive control. Supplementation of low dose *Bacillus subtilis* down-regulated ($P < 0.05$) *MUC2* gene expression on d 5 PI but increased ($P < 0.05$) *MUC2* gene expression in jejunal mucosa on d 11 PI, compared with the positive control. No differences were observed

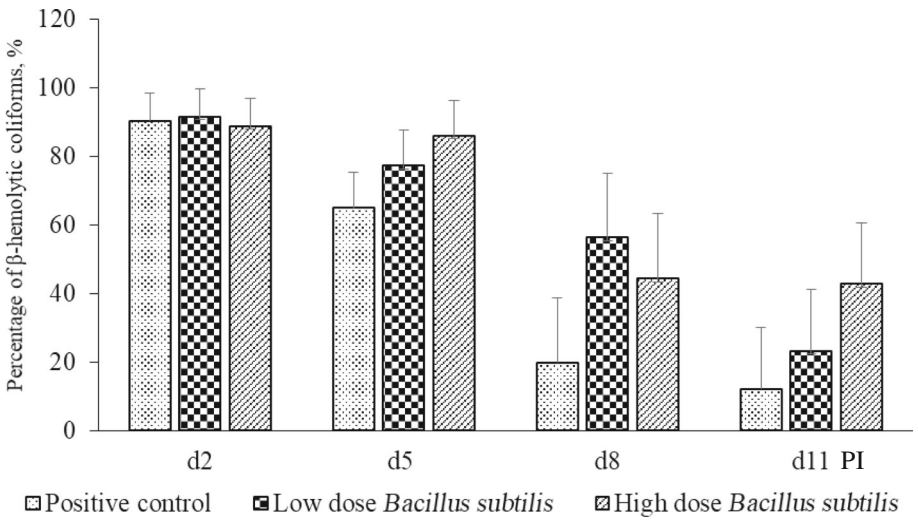

Fig. 2 The percentage of β-hemolytic coliforms in the feces of weaned pigs fed diets supplemented with *Bacillus subtilis*. No β-hemolytic coliforms was detected in the feces of weaned pigs in the negative control group. Supplementation of *Bacillus subtilis* linearly increased ($P < 0.05$) percentage of β-hemolytic coliforms (%) in the feces of weaned pigs on d 5 and d 11 post-inoculation (PI). Each least squares mean represents 12 observations on d 2 and 5 PI, whereas each least squares mean represents 6 observations on d 8 and 11 PI

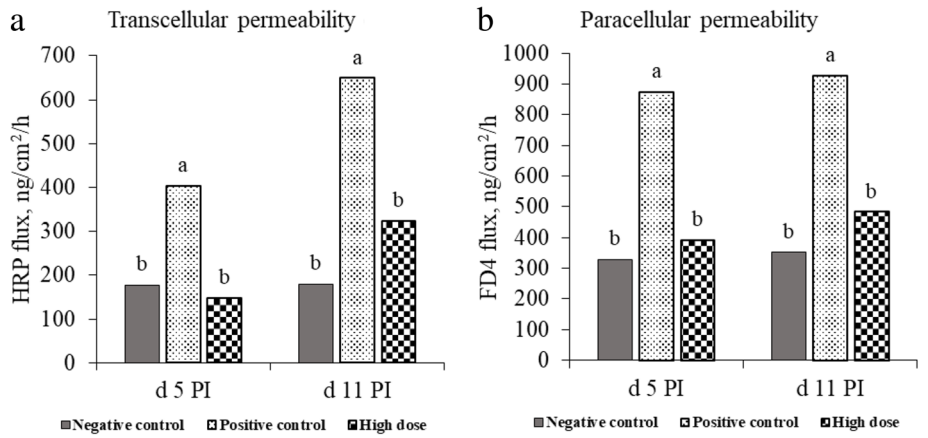

Fig. 3 Gut permeability of weaned pigs fed diets supplemented with *Bacillus subtilis*. **a** Transcellular permeability, **b** Paracellular permeability. The observations for east square mean were: 4 observations in negative control and 6 observations for positive control and high dose *Bacillus subtilis*, respectively. [a,b]Means without a common superscript are different ($P < 0.05$)

in the mRNA expression of *OCLN* in jejunal mucosa of weaned pigs among treatments on d 5 and 11 PI.

E. coli challenge up-regulated ($P < 0.05$) the gene expression of *IL1A*, *IL1B*, and *IL7* in ileal mucosa on d 5 PI and up-regulated ($P < 0.05$) the gene expression of *TNF*, *IL1B*, *IL6*, and *IL7* in ileal mucosa on d 11 PI if positive control was compared with negative control (Table 5). No differences were observed in the mRNA expression of immune genes among pigs in the positive control and *Bacillus subtilis* supplemented groups, except that supplementation of *Bacillus subtilis* linearly reduced ($P < 0.05$) *IL6* gene expression in ileal mucosa of *E. coli* challenged pigs on d 11 PI.

Discussion

F18 *E. coli*-induced diarrhea is a common cause of morbidity and mortality in weaned pigs [26]. Results revealed in the current study indicate that supplementation of *Bacillus subtilis* improved growth rate, reduced gut permeability, and may modify intestinal health of weaned pigs experimentally challenged with F18 *E. coli*. These findings are in agreement with previous published research, showing an improvement of growth performance and health status in piglets fed different strains of *Bacillus subtilis* [27–29]. The potential mechanisms related to those benefits may include but are not limited to: 1) supplementation of *Bacillus subtilis* (DSM 25841) improved gut integrity by enhancing gut barrier function and reducing gut permeability in the jejunum; 2) supplementation of *Bacillus subtilis* regulated intestinal immunity of weaned pigs; 3) inclusion of *Bacillus subtilis* modulated the gut microbiome and their metabolites. The current experiment was focused more on the first potential mechanism.

In the present study, pigs in *E. coli* challenge groups had increased frequency of diarrhea and β-hemolytic coliforms in their feces, reduced intestinal villi, and increased gut permeability after F18 *E. coli* infection, compared with pigs in the negative control group. These observations are consistent with previously published research using the same *E. coli* strain [18–20, 30]. The clear clinical signs and symptoms indicated the pigs were successfully infected with F18 *E. coli.* The *E. coli* inoculum that was used in the current study expressed LT, STb, and SLT-2 toxins, which could cause villus atrophy, leaky gut, and intestinal inflammation in young pigs [20, 24, 31]. The disrupted intestinal morphology, such as reduced villus height, could decrease nutrient absorption and impair growth performance of pigs as observed in the present study [32]. Interestingly, we observed that one of sodium-dependent transporters, *SLC5A10,* was overexpressed in the jejunum at the peak day of F18 *E. coli* infection (d 5 PI), but then was down-regulated during the recovery period of *E. coli* infection (d 11 PI). The *SLC5A10* encodes SGLT5, a member of the sodium/glucose transporter family. This family also contains sodium-dependent glucose transporter 1, which serves as major water pumps in the small intestine responsible for the daily intake of fluid from the normal intestine [33, 34]. It has been revealed that enteropathogenic *E. coli* infection could rapidly inactivate SGLT1 function in Caco-2 cells *in vitro*, which has been considered as another potential mechanism for the rapid onset of severe watery diarrhea caused by enteropathogenic *E. coli* [35]. However, the degree of sodium-dependent glucose transporter 1 inactivation *in vivo* could be impacted by many factors, including bacterial inoculum size, infection state, host age, host/bacterial genotypes, and even mucosal inflammatory response [35,

Table 3 Intestinal morphology of weaned pigs fed diets supplemented with *Bacillus subtilis*

Item[c]	*E. coli* challenge				SEM	*P*-value		
	Negative control	Positive control	Low dose *Bacillus subtilis*	High dose *Bacillus subtilis*		Diet	Lin.[d]	Quad.[d]
d 5 PI								
Duodenum								
Villi height, μm	464	433	437	428	19.73	0.57	0.86	0.80
Crypt depth, μm	319	294	313	301	19.08	0.79	0.81	0.52
Villi height:Crypt depth	1.46	1.49	1.40	1.44	0.058	0.74	0.56	0.37
Villi width, μm	150	161	138	136	12.85	0.43	0.15	0.47
Villi area, mm^2	0.066	0.058	0.059	0.058	0.004	0.59	0.99	0.82
Jejunum								
Villi height, μm	446[a]	361[b]	370[b]	401[ab]	33.58	**0.038**	0.18	0.69
Crypt depth, μm	259	236	230	237	23.78	0.50	0.93	0.71
Villi height:Crypt depth	1.74	1.54	1.62	1.70	0.123	0.49	0.26	0.99
Villi width, μm	110	109	110	108	3.41	0.98	0.88	0.74
Villi area, mm^2	0.054[a]	0.038[b]	0.039[b]	0.041[b]	0.004	**0.042**	0.60	0.91
Ileum								
Villi height, μm	375	372	355	359	17.71	0.56	0.43	0.45
Crypt depth, μm	206	222	219	213	8.90	0.46	0.39	0.85
Villi height:Crypt depth	1.82	1.68	1.61	1.68	0.054	0.082	0.95	0.36
Villi width, μm	115	114	117	109	6.37	0.85	0.58	0.53
Villi area, mm^2	0.042	0.041	0.037	0.038	0.003	0.37	0.45	0.33
d 11 PI								
Duodenum								
Villi height, μm	511[a]	402[b]	439[b]	436[b]	32.75	**0.015**	0.28	0.46
Crypt depth, μm	388[a]	330[b]	314[b]	319[b]	39.50	**0.012**	0.62	0.58
Villi height:Crypt depth	1.33	1.23	1.41	1.37	0.077	0.17	0.091	0.14
Villi width, μm	159	163	142	139	9.53	0.086	**0.039**	0.31
Villi area, mm^2	0.077	0.073	0.064	0.079	0.019	0.84	0.74	0.43
Jejunum								
Villi height, μm	448	405	385	441	25.80	0.14	0.22	0.16
Crypt depth, μm	269	252	248	254	14.51	0.76	0.93	0.78
Villi height:Crypt depth	1.70	1.61	1.55	1.74	0.086	0.40	0.28	0.24
Villi width, μm	122	111	112	115	4.40	0.31	0.55	0.77
Villi area, mm^2	0.054	0.045	0.042	0.052	< 0.01	0.077	0.17	0.16
Ileum								
Villi height, μm	405	344	391	369	28.86	0.17	0.38	0.16
Crypt depth, μm	229	203	237	224	13.30	0.32	0.27	0.16
Villi height:Crypt depth	1.77	1.70	1.67	1.65	0.088	0.62	0.65	0.94
Villi width, μm	128	120	122	126	6.26	0.92	0.53	0.95
Villi area, mm^2	0.047	0.040	0.047	0.045	< 0.01	0.47	0.35	0.31

[a,b]Means without a common superscript are different ($P < 0.05$); bold *P* values denote statistical significance at the $P < 0.05$
[c]Each least squares mean represents 6 observations. *PI* post-inoculation
[d]Linear and quadratic effects of adding *Bacillus subtilis* to the control diet in pigs infected with F18 *E. coli*

36]. There are limited research reported on *SLC5A10* status in the small intestine of weaned pigs because it is not the major glucose transporter in the small intestine. More research may be needed to explore the specific roles of *SLC5A10* and other members in the same sodium/glucose cotransporter family in F18 *E. coli* infection.

Table 4 The relative mRNA expression of genes in jejunal mucosa of weaned pigs fed diets supplemented with *Bacillus subtilis*

Item[c]	*E. coli* challenge				SEM	*P*-value		
	Negative control	Positive control	Low dose *Bacillus subtilis*	High dose *Bacillus subtilis*		Diet	Lin.[d]	Quad.[d]
d 5 PI								
CFTR	1.00[b]	0.99[b]	1.36[ab]	2.57[a]	0.43	**< 0.05**	**< 0.05**	0.52
CLDN1	1.00	0.60	1.41	1.81	0.62	0.45	0.09	0.73
MUC2	1.00[b]	2.58[a]	0.87[b]	1.37[b]	0.27	0.08	**< 0.05**	**< 0.05**
OCLN	1.00	1.14	1.17	1.65	0.33	0.33	0.08	0.47
SLC5A10	1.00[b]	2.41[a]	1.69[ab]	1.82[ab]	0.37	**< 0.05**	0.33	0.38
ZO1	1.00[b]	0.78[b]	1.33[ab]	2.15[a]	0.45	**< 0.05**	**< 0.05**	0.77
d 11 PI								
CFTR	1.00	0.60	0.81	0.87	0.22	0.65	0.41	0.80
CLDN1	1.00[a]	0.49[b]	0.65[ab]	0.61[ab]	0.15	**< 0.05**	0.56	0.57
MUC2	1.00[a]	0.39[b]	0.88[a]	0.76[ab]	0.21	**< 0.05**	**< 0.05**	**< 0.05**
OCLN	1.00	0.64	1.33	1.24	0.31	0.52	0.23	0.33
SLC5A10	1.00[a]	0.29[b]	0.62[ab]	0.86[a]	0.16	**< 0.05**	**< 0.05**	0.77
ZO1	1.00	0.68	1.10	0.78	0.24	0.62	0.78	0.24

[a,b]Means without a common superscript are different ($P < 0.05$); bold *P* values denote statistical significance at the $P < 0.05$
[c]Each least squares mean represents 6 observations. *PI* post-inoculation. *CFTR* cystic fibrosis transmembrane conductance regulator; *CLDN1* Claudin 1; *MUC2* Mucin-2; *OCLN* Occludin; *SLC5A10* soluble carrier family 5 member 10; *ZO1* Zonula occludens-1
[d]Linear and quadratic effects of adding *Bacillus subtilis* to the control diet in pigs infected with F18 *E. coli*

Table 5 The relative mRNA expression of genes in ileal mucosa of weaned pigs fed diets supplemented with *Bacillus subtilis*

Item[c]	*E. coli* challenge				SEM	*P*-value		
	Negative control	Positive control	Low dose *Bacillus subtilis*	High dose *Bacillus subtilis*		Diet	Lin.[d]	Quad.[d]
d 5 PI								
IFNG	1.00	1.25	1.14	0.75	0.26	0.44	0.14	0.60
IL1A	1.00[b]	1.98[a]	1.47[ab]	1.13[ab]	0.28	**< 0.05**	0.07	0.82
IL1B	1.00[b]	4.40[a]	2.79[ab]	3.25[a]	0.82	**< 0.05**	0.28	0.24
IL6	1.00	0.99	1.00	1.10	0.16	0.95	0.62	0.83
IL7	1.00[b]	4.83[a]	2.97[ab]	3.42[a]	1.10	**< 0.05**	0.20	0.22
MUC2	1.00	1.53	2.04	1.69	0.32	0.23	0.76	0.37
PTGS2	1.00	1.07	1.12	1.00	0.05	0.29	0.35	0.19
TNFA	1.00[b]	1.92[ab]	1.24[ab]	2.06[a]	0.31	**< 0.05**	0.78	0.07
d 11 PI								
IFNG	1.00	2.33	1.41	1.35	0.68	0.50	0.28	0.56
IL1A	1.00	1.92	1.69	1.20	0.47	0.52	0.32	0.82
IL1B	1.00[b]	3.32[a]	1.59[ab]	3.83[a]	0.99	**< 0.05**	0.73	0.13
IL6	1.00[b]	1.89[a]	0.72[b]	0.64[b]	0.22	**< 0.05**	**< 0.05**	0.09
IL7	1.00[b]	3.56[a]	2.37[ab]	4.00[a]	1.00	**< 0.05**	0.78	0.27
MUC2	1.00	1.61	2.30	2.35	0.54	0.27	0.35	0.64
PTGS2	1.00	1.19	1.12	1.14	0.06	0.26	0.64	0.61
TNFA	1.00[b]	3.46[a]	1.94[ab]	3.42[a]	1.24	**< 0.05**	0.97	0.12

[a,b]Means without a common superscript are different ($P < 0.05$); bold *P* values denote statistical significance at the $P < 0.05$
[c]Each least squares mean represents 6 observations. *PI* post-inoculation, *IFNG* Interferon gamma; *IL1A* Interleukin-1 alpha, *IL1B* Interleukin 1 beta, *IL6* Interleukin 6, *IL7* Interleukin-7, *MUC2* Mucin 2, *PTGS2* Cyclooxygenase 2, *TNF* Tumor necrosis factor alpha
[d]Linear and quadratic effects of adding *Bacillus subtilis* to the control diet in pigs infected with F18 *E. coli*

As one of many potential candidates to partially replace in-feed antibiotics, *Bacillus subtilis* have attracted much attention as they are thermostable during feed processing and they are able to deliver their potential benefits to the small/large intestine after surviving at low pH in stomach [5]. The reported effects of *Bacillus subtilis* on the incidence of diarrhea in weaned pigs are inconsistent. As an example, Bhandari et al. [37] and Hu et al. [15] revealed that addition of *Bacillus subtilis* reduced diarrhea of weaned pigs either in a K88 *E. coli* challenge study or in a normal housing condition, but this was not the case in the research reported by Giang et al. [38]. Although supplementation of *Bacillus subtilis* did not reduce frequency of diarrhea in *E. coli* challenged pigs in the present study, it did enhance growth rate and feed efficiency of weaned pigs after *E. coli* infection. These observations clearly indicate that addition of *Bacillus subtilis* promoted weaned pig performance probably through other mechanisms. A follow-up experiment is being conducted to explore the correlation between gut permeability and overall diarrhea score by adding more sampling points and extending experimental period.

Tight junction proteins, such as ZO1, Occludin, and Claudin, are critical in the maintenance of intestinal integrity and barrier function [39]. Growing evidence suggests that the expression of intestinal tight junction proteins is oppositely correlated with gut permeability [40]. Thus, the first potential mode of action for *Bacillus subtilis* supplementation was that it may enhance intestinal integrity and reduce nutrient loss of weaned pigs during bacterial infection [41, 42]. In the present study, it has been confirmed that supplementation of *Bacillus subtilis* reduced transcellular and paracellular permeability and enhanced gene expression of *ZO1* in the jejunum of *E. coli* infected pigs. Similar findings were also reported *in vitro* by Gu et al. [43], in which *Bacillus subtilis* decreased permeability of tight junction and improved expression of *ZO-1* and *occludin* in a porcine epithelial cell line. *Bacillus subtilis* was also observed to enhance the gene expression of tight junction proteins in mice with inflammatory bowel disease [44]. Cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated anion channel, is very important in regulating the secretion of chloride and bicarbonate ions during *E. coli* infection [45, 46]. It has also been reported that the up-regulation of *CFTR* and its mediated bicarbonate ion secretion was highly involved in bacterial killing and host defense mechanism [47]. In the current experiment, supplementation of *Bacillus subtilis* enhanced *CFTR* expression in jejunal mucosa of *E. coli* infected pigs on d 5 PI, indicating this *Bacillus* strain may enhance host defense that was concomitant with better performance. However, the regulatory role of CFTR has to be confirmed in future research.

Mucins are secreted by goblet cells and are classified into neutral, sulfate (sulfo-), and acidic (sialo-) mucins [48]. Goblet cell number and mucin production may be impacted by intestinal infections as a protective mechanism [49]. In the present study, *E. coli* infected pigs had reduced goblet cell number in duodenum on d 5 PI during the peak of infection, but this was not the case on d 11 PI as pigs recovered from the infection. Although no differences were observed in mucin percentage in the crypts of the jejunum and ileum based on histological analysis, pigs infected with F18 *E. coli* had increased *MUC2* gene expression during the peak of infection but decreased *MUC2* expression during the recovery period. These observations confirmed the role of mucin as the first line of host response against F18 *E. coli* infection [50]. The effects of *Bacillus subtilis* on intestinal mucin production are limited, with the exception that supplementation of *Bacillus subtilis* increased the percentage of sialomucin in the duodenum of weaned pigs on d 5 PI. However, supplementation of *Bacillus subtilis* oppositely impacted *MUC2* expression in jejunal mucosa of *E. coli* infected pigs compared with positive control, and the mRNA expression of *MUC2* gene in jejunal mucosa of weaned pigs was not different among *Bacillus subtilis* groups compared with the negative control. These results indicate that pigs supplemented with *Bacillus subtilis* may have less severe *E. coli* infection compared with pigs in the positive control.

Supplementation of *Bacillus*-based DFM have been reported to enhance intestinal mucosa immunity in both pre-weaning and post-weaning pigs, by regulating the population of intraepithelial lymphocytes and/or the production of secretory IgA [51–53]. In the present study, supplementation of *Bacillus subtilis* had limited effects on the expression of several immune genes involved in intestinal inflammation except that *IL6* expression was down-regulated by feeding *Bacillus subtilis* on d 11 PI. In addition, pigs supplemented with low dose *Bacillus subtilis* had similar mRNA expression of several other inflammatory mediators (i.e. *TNF*, *IL1B*, and *IL7*) in the ileal mucosa, compared with pigs that were not infected with *E. coli*. Results of *MUC2* and immune gene expression suggest that pigs supplemented with *Bacillus subtilis* may have less severe of intestinal inflammation induced by *E. coli* infection, comparison with infected pigs fed with the control diet. However, more measurements are suggested in the future research to confirm this potential effects, for example, including an additional sampling point (i.e. d 2 PI) after *E. coli* inoculation to focus on exploring intestinal immunity, or analyzing protein concentrations of inflammatory mediators in ileal mucosa of weaned pigs.

Other potential mechanisms may also be related to the enhanced growth rate by feeding *Bacillus subtilis*. As an

example, *Bacillus*-based DFM may enhance growth performance of weaned pigs by improving digestibility of energy and nutrients [29, 54]. In particular, *Bacillus*--based DFM may increase fiber degradation in the intestinal tract of pigs [11, 55]. *Bacillus*-based DFM supplementation may also increase the population of beneficial microorganisms in the intestinal tract, which compete nutrients and attachment sites with pathogens, therefore, reducing the proliferation of pathogens [56]. Further research is necessary to examine the potential modes of action listed here.

Conclusion

Dietary intervention on weaned pig intestinal health and performance is complicated by targeting different areas, which includes but is not limited to strengthening gut integrity and enhancing nutrient digestion and absorption, a balanced intestinal immunity, and a favorable intestinal microflora. It is important to keep in mind that it is difficult to cover the majority of areas with one single dietary change. Results of this study indicate that in feed supplementation of *Bacillus subtilis* (DSM 25841) enhanced the growth rate of F18 *E. coli* infected pigs by enhancing gut integrity and decreasing gut permeability. However, no clear reduction of diarrhea and fecal β-hemolytic coliforms was observed in pigs supplemented with *Bacillus subtilis*. Feeding *Bacillus subtilis* may also impact intestinal inflammation of *E. coli* infected pigs. In conclusion, the present study indicates that supplementation of *Bacillus subtilis* in animal feed could improve gut barrier function and modify immunity of weaned pigs, which may further promote weaned pig growth performance and increase profitability of pork producers as the use of antibiotics in feed is restricted. The current study has demonstrated the great potential of *Bacillus subtilis*, more research will be further conducted to confirm this potential and to further explore the underlying mechanisms.

Additional file

Additional file 1: Table S1. Gene-specific primer sequences and PCR conditions[1]. **Table S2.** Goblet cell number in the small intestine and relative amounts of sulfo- and sialomucin area (%) of weaned pigs fed diets supplemented with *Bacillus subtilis (DOCX 22 kb)*

Abbreviations

ACTB: β-actin; ADFI: Average daily feed intake; ADG: Average daily gain; CFTR: Cystic fibrosis transmembrane conductance regulator; CLDN1: Claudin 1; DFM: Direct-fed microbials; *E. coli*: *Escherichia coli*; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IFNG: Interferon-gamma; IL: Interleukin; LT: Heat-labile toxin; mRNA: Messenger RNA; MUC2: Mucin 2; OCLN: Occludin; PBS: Phosphate buffer saline; PI: Post-inoculation; PTGS2: Cyclooxygenase 2; SGLT1: Sodium-dependent glucose transporter 1; SLC5A10: Soluble carrier family 5 member 10; ST: Heat-stable toxin; STL-2: Shiga-like toxin; TNF: Tumor necrosis factor alpha; ZO1: Zona occludens-1

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Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Authors' contributions

The contributions of the authors were as follows: KK conducted the animal work and most of the laboratory work and wrote most of the manuscript. YH, XX, and AE. helped to conduct animal trial and part of the laboratory work. XL, HR, ERA, EM, and JJ provided labs and staffs for helping the animal work and part of the laboratory work and helped to revise the manuscript. YL was the principle investigator. She designed the experiment, oversaw the development of the study and wrote the last version of the manuscript. The authors declare no conflicts of interest. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of California, Davis. The study was conducted at the Teaching and Research Animal Care Services (TRACS) P building at the University of California, Davis.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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