Animal Nutrition 6 (2020) 152–159

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

Effects of benzoic acid, *Bacillus* coagulans and oregano oil combined supplementation on growth performance, immune status and intestinal barrier integrity of weaned piglets

Junning Pu, Daiwen Chen, Gang Tian, Jun He, Ping Zheng, Xiangbing Mao, Jie Yu, Zhiqing Huang, Junqiu Luo, Yuheng Luo, Bing Yu*

Key Laboratory for Animal Disease-Resistance Nutrition, Ministry of Education, Institute of Animal Nutrition, Sichuan Agricultural University, Chengdu, 611130, China

ARTICLE INFO

Article history:
Received 20 August 2019
Received in revised form
27 January 2020
Accepted 27 February 2020
Available online 31 March 2020

Keywords:
Benzoic acid
Bacillus coagulans
Oregano oil
Immune status
Intestinal barrier integrity
Piglets

ABSTRACT

This experiment was conducted to investigate the effects of benzoic acid, *Bacillus* coagulans and oregano oil combined supplementation on growth performance, immune status and intestinal barrier integrity of piglets. In a 26-d experiment, 25 piglets were randomly assigned to 5 treatments: 1) a basal diet, negative control (NC), 2) NC added with antibiotics, positive control (PC); 3) NC added with benzoic acid at 3,000 g/t and *Bacillus* coagulans at 400 g/t (AB); 4) NC added with benzoic acid at 3,000 g/t and oregano oil at 400 g/t (AO); 5) NC added with 3,000 g/t benzoic acid and *Bacillus* coagulans at 400 g/t and oregano oil at 400 g/t (ABO). On d 27, all piglets were euthanized to obtain jejunal mucosa to measure immune status and intestinal barrier integrity. Results showed that pigs fed AB diet increased the final body weight and average daily body weight gain and decreased the ratio of feed to gain compared with NC group ($P < 0.05$). Compared with NC group, AB, AO and ABO decreased serum tumor necrosis factor-α concentration and ABO decreased interleukin-1β concentration in serum and jejunal mucosa ($P < 0.05$). Compared with NC group, AB up-regulated mRNA expressions of sodium-glucose cotransporte1, claudin-1, occludin and mucin2 in jejunal mucosa and the populations of *Bifidobacterium* and *Bacillus* in cecal digesta ($P < 0.05$). Compared with NC group, ABO increased jejunal mucosal occludin mRNA abundance and *Bifidobacterium* population in cecal digesta, and decreased *Escherichia coli* population in cecal digesta ($P < 0.05$). Furthermore, AB and ABO increased *Bacillus* population in cecal digesta compared with PC group ($P < 0.05$). These results indicated that dietary AB supplementation could improve growth performance and intestinal barrier integrity of piglets when fed antibiotic-free diets, which was possibly associated with the improvement of immune status and intestinal microflora. Dietary ABO supplementation is also beneficial to improve immune status and intestinal barrier integrity and microflora of piglets.

1. Introduction

It is well known that early weaning is an effective way to maximize the whole herd production (Hao et al., 2015). However, early weaning is often associated with marked changes in the histology and biochemistry of the gastrointestinal tract of piglets, which resulted in digestive upset, diarrhea and retardation of growth performance (Che et al., 2012; Lauridsen, 2010). Antibiotics have been widely used as growth promoters and for therapeutic treatment of gastrointestinal disease in newly weaned piglets (Bhandari et al., 2008). However, the abuse of antibiotics in animal feeds not only leads to antibiotic residues in animal products and the environment, but also results in bacterial resistant to antibiotic and inhibition of innate immune function (Cromwell, 2002; Davis et al., 2007). Therefore, it is necessary to develop effective, safe, economical and environment-friendly alternatives to in-feed

* Corresponding author.
E-mail address: ybingtian@163.com (B. Yu).
Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2020.02.004

antibiotics. Previous studies have shown that organic acids, probiotics and essential oils could be used as substitutes for in-feed antibiotics for their benefits in decreasing the load of pathogenic bacteria in the gut (Aristimunha et al., 2016; Wang et al., 2013).

Some researchers have proposed that organic acid could improve growth performance and has antibacterial action primarily via decreasing pH values of the stomach and gut digesta, modulating microbial population, improving nutrients digestion, and other possible mechanisms (Mroz, 2005). Benzoic acid, as a kind of organic acid, was authorized to be used in pigs at the dose of 0.5% to 1.0% by European Union in 2003. Previous studies indicated that benzoic acid could improve growth performance and nutrient digestibility, and maintain intestinal microflora balance of weaned piglets (Guggenbuhl et al., 2007). The term probiotics has been defined as live microbial cell preparations or microbial cell components feed supplements, which beneficially improve growth performance by maintaining intestinal microbial balance and stimulating immune response of animals (Hill et al., 2014). *Bacillus* coagulans, as a kind of probiotic, not only has all the characteristics of lactic acid bacteria, but also has strong resistance to acid, high temperature, high pressure, and easy storage properties (Cavazzoni et al., 1998). Previous studies reported that *Bacillus* coagulans could improve growth performance, maintain intestinal microflora balance and improve immune response of animals (Zhou et al., 2010; Hung et al., 2012). Oregano oils, which are essential oils obtained from oregano plant by a steam distillation process, are comprised of more than 20 ingredients; of these ingredients, carvacrol and thymol are the most abundant (Vokou et al., 1993). It has been demonstrated that oregano oil could improve growth performance by improving antioxidant capacity and decreasing the number of pathogenic bacteria in the gut of pigs (Botsoglou et al., 2002; Zheng et al., 2009).

Based on their positive effects, benzoic acid, *Bacillus* coagulans and oregano oils are considered to be good potential alternatives for in-feed antibiotic growth promoter. However, the experimental results of these additives have varied widely and the effect of a single additive is limited. In recent years, organic acids, probiotics and essential oils combined supplementation in animal diets have attracted wide research interest due to their potential 'synergistic' and 'additive' benefits on growth performance (Basmacioglumalayoglu et al., 2016; Giannenas et al., 2016). However, little information is available on the effects of benzoic acid, *Bacillus* coagulans and oregano oils combined supplementation on growth performance, immune status and intestinal health of weaned piglets.

Therefore, the objective of this study was to determine the effects of benzoic acid, *Bacillus* coagulans and oregano oils combined supplementation on growth performance, immune status, intestinal barrier integrity and microflora of weaned piglets, and to evaluate their potential as an alternative to antibiotics growth promoters.

2. Materials and methods

The experiment was carried out in accordance with the Chinese guidelines for animal welfare and experimental protocol, and was approved by Sichuan Agricultural University Institutional Animal Care and Use Committee (Approval number: CD-SYXK-2017-015).

2.1. Benzoic acid, Bacillus coagulans and oregano oil products

Benzoic acid (VevoVitall) was purchased from DSM (China) Company Limited (purity, 99.9%); *Bacillus* coagulans was provided by Sanzheng Group (Kunming, China) at a density of 5×10^9 CFU/g; Oregano oil (free-flowing powder) was provided by Kemin Industries (Zhuhai, China), the major active components in the oregano oil were carvacrol and thymol (contained a minimum of 22 g/kg carvacrol and a minimum of 11 g/kg thymol). Defatted rice bran and silica were used as carriers.

2.2. Animals, diets and experimental design

A total of 25 weaned piglets [Duroc × (Landrace × Yorkshire)] with initial body weight (7.65 ± 0.38 kg) were weaned at 21 d of age and randomly assigned to 5 treatments ($n = 5$ piglets per treatment) based on their initial body weight. The dietary treatment groups were as follows: 1) corn-soybean basal diet (negative control, NC); 2) NC + colistin sulfate at 20 g/t + bacitracin zinc at 40 g/t (positive control, PC); 3) NC + benzoic acid at 3,000 g/t + *Bacillus* coagulans at 400 g/t (AB); 4) NC + benzoic acid at 3,000 g/t + oregano oil at 400 g/t (AO); 5) NC + benzoic acid at 3,000 g/t + *Bacillus* coagulans at 400 g/t + oregano oil at 400 g/t (ABO). The pigs were individually housed in metabolic cages (1.5 m × 0.7 m × 1.0 m) with woven wire flooring in a temperature-controlled nursery room (25 to 28 °C) during a 26-d experimental period. All pigs had ad libitum access to feed and water. The basal diet (Table 1) was formulated to meet or exceed the nutrient requirements recommended by the National Research Council (NRC, 2012). The experimental diets consisted of corresponding additive products replaced equivalent maize in the basal diet.

2.3. Growth performance and diarrhea incidence

The pigs were weighed individually at the start and the end of experimental period and the feed intake was recorded on daily basis. These values were used to calculate average daily body weight gain (ADG), average daily feed intake (ADFI) and the ratio of feed to gain (F:G). The feces of all pigs were observed daily throughout the study. Fecal consistency was scored as follows: 0, normal; 1, pasty; 2, semiliquid; and 3, liquid. Pigs with daily fecal consistency scores ≥2 were considered diarrhea (Yuan et al., 1998). Diarrhea incidence (%) = Number of diarrheal piglets × Diarrhea days/(Number of piglets × Test days) × 100.

2.4. Sample collection

Blood samples were collected from pigs via anterior vein into 10-mL vacuum tubes without anticoagulant on d 27 following an overnight fast. Samples were centrifuged at 3,500 × *g* for 10 min at 4 °C, and the serum samples were harvested and stored at −20 °C until further assay. After bled, all pigs were anesthetized by electric shock and then euthanized by exsanguinations. The small intestine was dissected from the mesentery and immediately placed on ice, approximately 20 cm of jejunal tissue sample was removed from the middle portion of jejunum and flushed with ice-cold saline to recover mucosa. Mucosa of jejunum was collected by scraping using a sterile glass microscope slide at 4 °C, and then the samples were rapidly frozen in liquid nitrogen and stored at −80 °C until analyses. At the same time, approximately 3 g of the digesta from the cecum were sampled into sterile tubes separately and immediately immersed in liquid nitrogen, then stored at −80 °C for microbial population determination.

2.5. Disaccharidase activities analysis

Frozen jejunal mucosa scrapings were weighed, thawed and homogenized (5 min) in 9 times the volume (wt/vol) of ice-cold physiologic saline. The mixtures were then centrifuged at 3,500 × *g* for 10 min at 4 °C, the supernatant was collected for disaccharidase activities examination. The total protein concentration of supernatant was determined by using the bicinchoninic acid (BCA) Protein Assay kit (Jiancheng Bioengineering Institute,

Table 1
Ingredients composition and nutrients level of the basal diet (%, as-fed basis).

Ingredients	Content	Nutrient level[1]	Content
Maize	29.80	Digestible energy, MJ/kg	14.72
Extruded maize	29.85	Crude protein	19.13
Fish meal	4.50	Calcium	0.75
Whey powder	6.00	Total phosphorus	0.56
Sucrose	3.00	Available phosphorus	0.37
Soybean meal	10.00	Lysine	1.30
Soybean protein concentrate	6.40	Methionine	0.41
Extruded soybean	6.70	Threonine	0.79
Soybean oil	1.70	Tryptophan	0.22
L-lysine-HCl (78%)	0.26		
L-threonine (98%)	0.02		
DL-methionine (98%)	0.09		
L-tryptophan (98%)	0.01		
Choline chloride	0.15		
Salt	0.20		
Limestone	0.60		
Dicalcium phosphate	0.37		
Vitamin premix[2]	0.05		
Mineral premix[3]	0.30		
Total	100.00		

[1] Nutrients levels were calculated values.
[2] The vitamin premix provided the following per kilogram of diets: vitamin A, 9,000 IU; vitamin D_3, 3,000 IU; vitamin E, 20 IU; vitamin K_3, 3.0 mg; vitamin B_1, 1.5 mg; vitamin B_2, 4.0 mg; vitamin B_6, 3.0 mg; vitamin B_{12}, 0.2 mg; niacin, 30 mg; pantothenic, 15 mg; folic acid, 0.75 mg; biotin, 0.1 mg.
[3] The mineral premix provided the following per kilogram of diets: Fe, 100 mg; Cu, 150 mg; Mn, 20 mg; Zn, 100 mg; I, 0.3 mg; Se, 0.3 mg.

Nanjing, China) according to the manufacturer's instructions. The activities of disaccharidases (maltase and sucrase) in the jejunal mucosal supernatant were measured by using Disaccharidase Assay Kit (Jiancheng Bioengineering Institute, Nanjing, China) combined with a UV-VIS Spectrophotometer (UV1100, MAPADA, Shanghai, China) according to the manufacturer's instructions. The activities of disaccharidases were presented as U/mg protein. One unit (U) of disaccharidase activity was defined as the amount of the enzyme that can hydrolyse 1μmol of substrate per minute under the standard assay conditions.

2.6. Serum and jejunal mucosal cytokines concentrations, and jejunal mucosal secretory immunoglobulin A concentration

In brief, 100 mg of jejunal mucosa was homogenized (5 min) in nine times the volume (wt/vol) of ice-cold PBS solution. The mixtures were then centrifuged at 3,500 × *g* for 15 min at 4 °C, the supernatants were collected and stored at −80 °C until further assay. The jejunal mucosal supernatant and serum were used to detect the tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10) and secretory immunoglobulin A (sIgA) concentrations with commercially available pig ELISA kits from Xinle Co. Ltd. (Shanghai, China) according to the manufacturer's instructions.

2.7. Total RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted from jejunal mucosa using TRIzol reagent (TaKaRa Biotechnology Co, Ltd, Dalian, China) following the manufacturer's instructions. The concentration and purity of RNA were analyzed spectrophotometrically (Beckman Coulter DU800; Beckman Coulter Inc.), considering the ideal absorbance ratio ($1.8 \leq A260/280 \leq 2.0$). The integrity of RNA was checked by electrophoresis on a 1.5% agarose gel. The RNA samples were reverse transcribed into cDNA using the PrimeScript RT reagent kit (TaKaRa Biotechnology Co, Ltd, Dalian, China) according to the manufacturer's instructions. The synthesis involved two steps: 37 °C for 15 min and 85 °C for 5 s. The cDNA was diluted 3-fold and used as a PCR template to evaluate gene expression. The primers were synthesized commercially by TaKaRa Biotechnology Co, Ltd. (Dalian, China), which are listed in Table 2. All qPCR reactions were done in triplicate on a QuanStudio 6 Flex Real-Time PCR System (Applied Biosystems), using SYBR Premix Ex Taq II (Tli RNaseH Plus; TaKaRa Biotechnology Co, Ltd, Dalian, China). Negative controls were performed in which water was substituted for complementary DNA. The amplification was performed in a final volume of 10 mL. This solution consisted of 5 mL SYBR Premix Ex Taq II (Tli RNaseH Plus), 0.2 mL ROX Reference Dye II, 0.4 mL forward primer, 0.4 mL reverse primer, 1 mL cDNA and 3 mL diethylpyrocarbonate-treated water. The cycling conditions were as follows pre-denaturation at 95 °C for 30 s and 40 cycles of denaturation at 95 °C for 5 s, annealing at the anneal temperatures (Table 2) for 30 s and extension at 72 °C for 60 s. A dissociation curve was constructed at the end of the reaction to ensure that only one amplification was formed. Target and house-keeping gene amplification efficiencies were calculated according to the specific gene standard curves that were generated from 10-fold serial dilutions, quantifying 6 concentrations. After verification that the primers amplified with an efficiency close to 100%, the results were analyzed using the $2^{-\Delta\Delta Ct}$ method, with porcine glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene as the housekeeping gene (Livak and Schmittgen, 2001).

2.8. DNA extraction and measurement of intestinal bacterial populations by qPCR

Bacterial DNA in the cecal digesta was extracted using the Stool DNA Kit (Omega Bio-Tek, Doraville, CA, USA) according to the manufacturer's instruction. All primers and probes (Table 3) were commercially synthesized from TaKaRa Biotechnology Co, Ltd. (Dalian, China). Briefly, the number of total bacteria was analyzed by qPCR using SYBR Premix Ex Taq reagents (TaKaRa Biotechnology Co, Lt., Dalian, China) and CFX-96 Real-Time PCR Detection System (BioRad Laboratories, Richmond, CA), and the numbers of *Bacillus, Lactobacillus, Escherichia coli* and *Bifidobacterium* were analyzed by qPCR using PrimerScript TM PCR kit (Perfect Real Time; TaKaRa Biotechnology Co, Ltd, Dalian, China) and CFX-96 Real–Time PCR Detection System (Bio-Rad Laboratories, Richmond, CA) as

Table 2
Primer sequences used for quantitative real-time PCR.

Primer	Primer sequence (5′ −3′)	Anneal temperature, °C	Product length, bp	GenBank accession No.
GAPDH	F: TGAAGGTCGGAGTGAACGGAT R: CACTTTGCCAGAGTTAAAAGCA	55.7	114	NM_001206359.1
SGLT1	F: AGAAGGGCCCCAAAATGACC R: TGTTCACTACTGTCCGCCAC	59	96	NM_001164021.1
GLUT2	F: TGGAATCAGCCAACCTGTTT R: ACAAGTCCCACCGACATGA	55.7	156	NM_001097417.1
PepT1	F: GCCAAAGTCGTCAAGTGC R: GGTCAAACAAAGCCCAGA	63.3	100	NM214347
Claudin-1	F: GCCACAGCAAGGTATGGTAAC R: AGTAGGGCACCTCCCAGAAG	59.0	140	FJ873109.1
Occludin	F: CTACTCGTCCAACGGGAAAG R: ACGCCTCCAAGTTACCACTG	59.0	158	NM_001163647.2
ZO-1	F: CAGCCCCCGTACATGGAGA R: GCGCAGACGGTGTTCATAGTT	59.0	114	XM_005659811
Mucin 1	F: GTGCCGCTGCCCACAACCTG R: AGCCGGGTACCCCAGACCCA	59.0	141	XM_001926883.4
Mucin 2	F: GGTCATGCTGGAGCTGGACAGT R: TGCCTCCTCGGGGTCGTCAC	59.0	181	XM_003122394.1

GAPDH = glyceraldehyde 3-phosphate dehydrogenase; *SGLT1* = sodium-glucose cotransporter 1; *GLUT2* = glucose transporter type 2; *PepT1* = oligopeptide transporter 1; *ZO-1* = zonula occludens 1.

Table 3
Primer and probe sequences used for quantitative real-time PCR.

Item	Primer and probe sequence (5′ −3′)	Anneal temperature, °C	Product length, bp
Total bacteria	F: ACTCCTACGGGAGGCAGCAG R: ATTACCGCGGCTGCTGG	57.9	200
Lactobacillus	F: GAGGCAGCAGTAGGGAATCTTC R: CAACAGTTACTCTGACACCCGTTCTTC P: AAGAAGGGTTTCGGCTCGTAAAACTCTGTT	53.0	126
Bifidobacterium	F: CGCGTCCGGTGTGAAAG R: CTTCCCGATATCTACACATTCCA P: ATTCCACCGTTACACCGGAA	57.9	121
Bacillus	F: GCAACGAGCGCAACCCTTGA R: TCATCCCCACCTTCCTCCGGT P: CGGTTTGTCACCGGCAGTCACCT	53.0	92
Escherichia coli	F: CATGCCGCGTGTATGAAGAA R: CGGGTAACGTCAATGAGCAAA P: AGGTATTAACTTTACTCCCTTCCTC	53.0	96

previously described (Chen et al., 2013). For the quantification of bacteria in the test samples, specific standard curves were generated by constructing standard plasmids as presented by Chen et al. (2013). In addition, bacterial copies were transformed ($\log_{10}$) before statistical analysis.

2.9. Statistical analysis

The data related to growth performance, disaccharidase activities, immune status, gene expression and intestinal bacteria were checked for normal distribution using the Shapiro–Wilk normality test of SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Then one-way ANOVA test was used to compare the difference of the normal distributed data among groups, followed by Duncan's multiple-range tests. Individual piglet served as the experimental unit. Results were presented as means ± standard errors (SE). Moreover, the Chi-square test was used to analyze diarrhea incidence. Differences were considered as significant at $P < 0.05$.

3. Results

3.1. Growth performance and diarrhea incidence

The results of growth performance and diarrhea incidence are shown in Table 4. Compared with NC group, pigs fed AB diet had greater final BW and ADG and lower F:G ratio ($P < 0.05$). However, no significant difference was observed on the ADFI and diarrhea incidence among treatments ($p > 0.05$).

3.2. Disaccharidase activities and nutrient transporter related gene expression in jejunal mucosa

As shown in Table 5, no significant difference was observed on the activities of jejunal mucosal maltase and sucrase among treatments ($P > 0.05$). Compared with NC group, dietary AB increased the mRNA abundance of SGLT1 in jejunal mucosa ($P < 0.05$).

3.3. Serum and jejunal mucosal cytokines concentrations, and jejunal mucosal sIgA concentration

As shown in Table 6, compared with NC group, dietary AB and AO decreased serum TNF-α concentration ($P < 0.05$). Meanwhile, lower serum TNF-α and IL-1β concentrations were observed in ABO group compared with NC group ($P < 0.05$). Furthermore, compared with NC and PC groups, dietary ABO decreased IL-1β concentration in jejunal mucosa ($P < 0.05$). However, no significant difference was observed on the concentrations of jejunal mucosal TNF-α, IL-6, IL-10 and sIgA among treatments ($P > 0.05$).

Table 4
Effects of dietary benzoic acid, *Bacillus* coagulans and oregano oil combined supplementation on growth performance and diarrhea incidence of weaned piglets.

Item	NC	PC	AB	AO	ABO
Initial BW, kg	7.61 ± 0.16	7.63 ± 0.18	7.70 ± 0.16	7.64 ± 0.22	7.66 ± 0.20
Final BW, kg	14.20 ± 0.77^{a}	15.32 ± 0.39^{ab}	16.42 ± 0.62^{b}	16.04 ± 0.77^{ab}	15.96 ± 0.81^{ab}
ADFI, g	436.59 ± 29.65	472.76 ± 23.25	515.14 ± 35.24	515.85 ± 38.76	522.17 ± 43.56
ADG, g	253.46 ± 25.84^{a}	295.77 ± 12.36^{ab}	335.38 ± 19.38^{b}	323.08 ± 25.19^{ab}	319.23 ± 28.82^{ab}
F:G	1.76 ± 0.09^{b}	1.60 ± 0.04^{ab}	1.54 ± 0.05^{a}	1.60 ± 0.05^{ab}	1.64 ± 0.07^{ab}
Diarrhea incidence, %	9.23	8.46	6.15	3.08	5.38

NC = negative control, a corn-soybean basal diet; PC = positive control, NC + colistin sulfate at 20 g/t + bacitracin zinc at 40 g/t; AB = NC + benzoic acid at 3,000 g/t + *Bacillus* coagulans at 400 g/t; AO = NC + benzoic acid at 3,000 g/t + oregano oil at 400 g/t; ABO = NC + benzoic acid at 3,000 g/t + *Bacillus* coagulans at 400 g/t + oregano oil at 400 g/t; BW = body weight; ADFI = average daily feed intake; ADG = average daily gain; F:G = the ratio of feed intake to gain.
[a, b] Within a row, values with different letter superscripts mean significant difference ($P < 0.05$).

Table 5
Effects of dietary benzoic acid, *Bacillus* coagulans and oregano oil combined supplementation on disaccharidase activities and nutrient transporter mRNA abundance in jejunal mucosa of weaned piglets.

Item	NC	PC	AB	AO	ABO
Maltase, U/mgprot	187.79 ± 18.48	197.84 ± 8.70	210.44 ± 11.03	207.15 ± 24.03	211.96 ± 28.95
Sucrase, U/mgprot	153.98 ± 43.55	173.35 ± 13.21	214.62 ± 33.59	166.29 ± 28.64	176.45 ± 31.90
SGLT1	1.00 ± 0.17^{a}	1.14 ± 0.27^{ab}	1.69 ± 0.27^{b}	1.31 ± 0.20^{ab}	1.61 ± 0.20^{ab}
GLUT2	1.00 ± 0.15	1.22 ± 0.33	1.65 ± 0.16	1.35 ± 0.31	1.42 ± 0.27
PepT1	1.00 ± 0.21	1.22 ± 0.33	1.52 ± 0.12	1.29 ± 0.26	1.50 ± 0.25

NC = negative control, a corn-soybean basal diet; PC = positive control, NC + colistin sulfate at 20 g/t + bacitracin zinc at 40 g/t; AB = NC + benzoic acid at 3,000 g/t + *Bacillus* coagulans at 400 g/t; AO = NC + benzoic acid at 3,000 g/t + oregano oil at 400 g/t; ABO = NC + benzoic acid at 3,000 g/t + *Bacillus* coagulans at 400 g/t + oregano oil at 400 g/t; *SGLT1* = sodium-glucose cotransporter1; *GLUT2* = glucose transporter type 2; *PepT1* = oligopeptide transporter 1.
[a, b] Within a row, values with different letter superscripts mean significant difference ($P < 0.05$).

Table 6
Effects of dietary benzoic acid, *Bacillus* coagulans and oregano oil combined supplementation on serum and jejunal mucosal cytokines concentrations and jejunal mucosal sIgA concentration of weaned piglets.

Item	NC	PC	AB	AO	ABO
Serum					
TNF-α, ng/L	203.49 ± 2.89^{b}	192.20 ± 8.48^{ab}	174.93 ± 12.78^{a}	169.44 ± 12.41^{a}	168.45 ± 8.27^{a}
IL-1β, ng/L	11.30 ± 0.83^{b}	10.00 ± 0.59^{ab}	9.66 ± 0.52^{ab}	9.44 ± 0.72^{ab}	8.23 ± 0.42^{a}
IL-6, ng/L	67.56 ± 4.62	60.44 ± 4.25	59.09 ± 3.53	59.43 ± 4.51	56.74 ± 3.25
IL-10, pg/mL	190.65 ± 17.02	207.18 ± 8.27	218.56 ± 4.71	200.32 ± 6.07	204.51 ± 12.12
Jejunal mucosa					
TNF-α, ng/L	96.67 ± 5.65	92.34 ± 10.53	82.49 ± 12.39	83.81 ± 11.66	70.68 ± 9.84
IL-1β, ng/L	14.11 ± 1.46^{b}	13.58 ± 1.22^{b}	11.85 ± 1.31^{ab}	12.08 ± 1.39^{ab}	8.51 ± 0.52^{a}
IL-6, ng/L	59.52 ± 7.77	58.40 ± 5.82	55.12 ± 7.17	54.04 ± 7.06	50.54 ± 7.57
IL-10, pg/mL	163.08 ± 18.02	181.53 ± 17.93	186.32 ± 15.74	173.42 ± 19.22	170.44 ± 19.37
sIgA, μg/mL	46.30 ± 2.39	55.31 ± 5.38	61.68 ± 11.38	55.04 ± 6.66	59.71 ± 2.98

NC = negative control, a corn-soybean basal diet; PC = positive control, NC + colistin sulfate at 20 g/t + bacitracin zinc at 40 g/t; AB = NC + benzoic acid at 3,000 g/t + Bacillus coagulans at 400 g/t; AO = NC + benzoic acid at 3,000 g/t + oregano oil at 400 g/t; ABO = NC + benzoic acid at 3,000 g/t + Bacillus coagulans at 400 g/t + oregano oil at 400 g/t; TNF-α = tumor necrosis factor-α; IL-1β = interleukin-1β; IL-6 = interleukin-6; IL-10 = interleukin-10; sIgA = secretion immunoglobulin A.
a, b Within a row, values with different letter superscripts mean significant difference (P < 0.05).

3.4. Barrier integrity related gene expression in jejunal mucosa

As shown in Table 7, compared with NC group, dietary AB increased the mRNA abundance of claudin-1, occludin and mucin2 in jejunal mucosa ($P < 0.05$). Meanwhile, higher occludin mRNA abundance was observed in ABO group compared with NC group ($P < 0.05$).

3.5. Bacteria populations in cecal digesta

The results of intestinal bacteria populations are shown in Table 8. Compared with NC group, dietary AB increased *Bifidobacterium* and *Bacillus* populations in cecal digesta ($P < 0.05$); dietary ABO increased *Bifidobacterium* population and decreased *E. coli* population in cecal digesta ($P < 0.05$). Furthermore, dietary AB and ABO increased *Bacillus* population in cecal digesta compared with PC group ($P < 0.05$).

4. Discussion

Weaning stress of piglets often causes a wide range of physiological responses, such as impaired intestinal metabolism and function, compromised immunity and decreased antioxidant capacity, which lead to depressed feed utilization efficiency, retarded growth, as well as increased morbidity and mortality (Hao et al., 2015). During last decades, as alternatives to antibiotics, benzoic acid, *Bacillus* coagulans and oregano oil have been used as effective supplements to improve growth performance and decrease diarrhea in piglets. Previous studies have shown that dietary 2,000 mg/kg benzoic acid +100 mg/kg thymol supplementation improved the

Table 7
Effects of dietary benzoic acid, *Bacillus* coagulans and oregano oil combined supplementation on barrier integrity related gene expression in jejunal mucosa of weaned piglets.

Item	NC	PC	AB	AO	ABO
Claudin-1	1.00 ± 0.15[a]	1.41 ± 0.20[ab]	1.70 ± 0.31[b]	1.39 ± 0.25[ab]	1.53 ± 0.22[ab]
Occludin	1.00 ± 0.14[a]	1.18 ± 0.16[ab]	1.52 ± 0.16[b]	1.36 ± 0.15[ab]	1.54 ± 0.14[b]
ZO-1	1.00 ± 0.17	0.80 ± 0.11	1.25 ± 0.29	1.17 ± 0.08	1.19 ± 0.18
Mucin1	1.00 ± 0.05	1.24 ± 0.07	1.27 ± 0.13	1.21 ± 0.19	1.25 ± 0.15
Mucin2	1.00 ± 0.15[a]	1.22 ± 0.09[ab]	1.41 ± 0.11[b]	1.19 ± 0.12[ab]	1.11 ± 0.07[ab]

NC = negative control, a corn-soybean basal diet; PC = positive control, NC + colistin sulfate at 20 g/t + bacitracin zinc at 40 g/t; AB = NC + benzoic acid at 3,000 g/t + *Bacillus* coagulans at 400 g/t; AO = NC + benzoic acid at 3,000 g/t + oregano oil at 400 g/t; ABO = NC + benzoic acid at 3,000 g/t + *Bacillus* coagulans at 400 g/t + oregano oil at 400 g/t; *ZO-1* = zona occludens 1.
[a, b] Within a row, values with different letter superscripts mean significant difference ($P < 0.05$).

Table 8
Effects of dietary benzoic acid, *Bacillus* coagulans and oregano oil combined supplementation on intestinal bacteria populations in cecal digesta of weaned piglets (log_{10}[copies/g]).

Item	NC	PC	AB	AO	ABO
Lactobacillus	8.40 ± 0.31	8.50 ± 0.18	8.42 ± 0.10	8.59 ± 0.10	8.78 ± 0.12
Bacillus	9.84 ± 0.09[ab]	9.71 ± 0.05[a]	10.16 ± 0.08[c]	9.93 ± 0.07[abc]	9.99 ± 0.12[bc]
Bifidobacterium	7.88 ± 0.09[a]	8.09 ± 0.11[ab]	8.19 ± 0.11[b]	8.08 ± 0.12[ab]	8.24 ± 0.09[b]
Escherichia coli	8.51 ± 0.09[b]	8.27 ± 0.23[ab]	8.14 ± 0.16[ab]	8.17 ± 0.18[ab]	7.86 ± 0.16[a]
Total bacteria	11.29 ± 0.03	11.40 ± 0.11	11.45 ± 0.10	11.39 ± 0.06	11.42 ± 0.08

NC = negative control, a corn-soybean basal diet; PC = positive control, NC + colistin sulfate at 20 g/t + bacitracin zinc at 40 g/t; AB = NC + benzoic acid at 3,000 g/t + *Bacillus* coagulans at 400 g/t; AO = NC + benzoic acid at 3,000 g/t + oregano oil at 400 g/t; ABO = NC + benzoic acid at 3,000 g/t + *Bacillus* coagulans at 400 g/t + oregano oil at 400 g/t.
[a, b, c] Within a row, values with different letter superscripts mean significant difference ($P < 0.05$).

growth performance and decreased diarrhea in weaned piglets (Hui et al., 2015). Papatsiros et al. (2011) reported that benzoic acid and *Bacillus* cereus var. toyoi combined supplementation improved ADG, decreased F:G and diarrhea in weaned piglets. Similarly, the results of the current study revealed that dietary benzoic acid and *Bacillus* coagulans combined supplementation significantly increased ADG and decreased F:G of weaned piglets. This may be due to the better gut characteristics including increased nutrient digestibility, healthy and stable intestinal microflora, and better intestinal barrier integrity.

The nutrient transporters in the small intestine are also very important to the nutrient utilization of animals and human. In the piglet small intestine, glucose absorption depends on 2 types of transport mechanisms: one is glucose transporter 2 (GLUT2), which serves as a facilitated diffusion system through lipid bilayers, and the other is SGLT1, which mediates Na+/glucose co-transport function as a secondary active transporter (Breves et al., 2007). Oligopeptide transporter 1 mainly transports dipeptides and tripeptides from the digestion of dietary proteins (Daniel, 2004). In the present study, dietary AB increased the mRNA abundance of SGLT1 in jejunal mucosa of piglets, thus resulting in the better nutrient absorption relative to piglets fed NC diet. The result is consistent with the improvement of growth performance in AB group. This may be associated with the changes in the intestinal barrier integrity.

Weaning stress has been reported to cause remarkable disturbances in intestinal barrier function (Smith et al., 2010). Disturbances in the intestinal barrier, characterized by increased intestinal permeability, results in translocation of luminal bacteria, toxins and antigens into the subepithelial tissues, affecting nutrient absorption (Blikslager et al., 2007). Therefore, an intact intestinal mucosal barrier is pivotal to ensure adequate provision of dietary nutrients to the whole body. The intestinal barrier can be commonly assessed by many indexes, such as the intestinal tight junction proteins and the mucus gel layer (Mao et al., 2011). Tight junction (TJ) proteins (occludin, claudin and intracellular plaque proteins [ZO and cingulin]), participate in tight junction structural integrity via binding to the act in cytoskeleton, are considered as major constituents of tight junctions and important regulators of paracellular permeability (Harhaj and Antonetti, 2004). In addition, intestine mucins, such as mucin1 and mucin2, are secreted by goblet cells, have been demonstrated to provide barrier protection against luminal virus, bacteria, and parasites invasions, thus playing important roles in regulating intestinal inflammation (Moughan et al., 2013). The present study showed that dietary AB increased the mRNA abundance of claudin-1, occludin and mucin2, and dietary ABO increased occludin mRNA level in jejunal mucosa of weaned piglets. These results indicated that dietary AB and ABO could ameliorate the weaning-associated intestinal damage by improving intestinal barrier integrity in weaned piglets.

As the crucial component of specific immunological responses, sIgA serves as the first line of defense in protecting the intestinal epithelium from enteric toxins and pathogenic microorganisms (Wittig and Zeitz, 2003). However, in the current study, dietary treatments did not affect on sIgA concentration in jejunal mucosa. A possible reason is that dietary benzoic acid, *Bacillus* coagulans and oregano oil combined supplementation are unable to stimulate sIgA secretion of the jejunal mucosa under normal feeding conditions. Furthermore, cytokine concentration is also widely used to assess the response to pathogens and antigens, both in experimental animal models and in human, as it can affect immune regulation and disease resistance (Wang et al., 2009). Previous studies have demonstrated that dietary benzoic acid, probiotics or essential oils supplementation enhanced immune response in early weaned pigs by modulating the production of inflammatory cytokines (IL-1β, IL-6, TNF-α and IL-10) (Lu et al., 2010; Ahmed et al., 2013; Li et al., 2008). Similar with previous studies, the current study also reported that dietary AB and AO supplementation decreased serum TNF-α concentration, dietary ABO decreased serum TNF-α and IL-1β concentrations. As indicated above, dietary AB, AO or ABO could modulate the immune status of weaned piglets by reducing the production of pro-inflammatory cytokines. Besides the important roles in immunity, cytokines also have important physiological and pathological effects on the intestinal barrier integrity. Pro-inflammatory cytokines such as TNF-α and IL-1β increase intestinal permeability by inducing disruption of tight junctions, whereas anti-inflammatory cytokines, including IL-10 and TGF-β, tend to protect the epithelial integrity (Alsadi et al.,

2009). In the present study, consistent with the decreased IL-1β concentration in jejunal mucosa, increased occludin mRNA abundance in ABO group was observed. However, no difference was observed on IL-10 concentration among treatments. These results indicated that dietary ABO may improve intestinal integrity partially by reducing pro-inflammatory stimulus, rather than enhancing the anti-inflammatory response.

Intestinal microflora is crucial for maturation of the immune system and development of normal intestinal morphology. Konstantinov et al. (2006) indicated that a healthy and stable microflora prevents the development of intestinal diseases and results in improved performance. Benzoic acid, *Bacillus* coagulans and oregano oil play an important role in stabilizing the intestinal ecosystem of animals, including increasing the number of health-promoting bacterial species (such as *Lactobacilli, Bacillus* and *Bifidobacteria*) and decreasing the number of potential pathogenic bacterial species (such as *E. coli*) (Diao et al., 2014; Lin et al., 2011; Zou et al., 2016). Our results demonstrated that dietary benzoic acid, *Bacillus* coagulans and oregano oil combined supplementation can improve intestinal microecological balance, which was evidenced by the improvement in *Bifidobacterium* or *Bacillus* populations, and the decrease in *E. coli* population of cecal digesta. Thus, dietary benzoic acid and *Bacillus* coagulans combined supplementation improve the intestinal microflora could also be an important reason that it could improve the growth performance of weaned piglets.

5. Conclusions

In conclusion, dietary AB supplementation could improve growth performance and intestinal barrier integrity of piglets when fed antibiotic-free diets, which was possibly associated with the improvement of immune status and intestinal microflora. Dietary ABO supplementation is also beneficial to improve immune status and intestinal barrier integrity and microflora of piglets.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgements

The authors would like to thank to Jingbo Liu, Yumei Zhao and Lingyan Jia for their collaboration during the experiment. The present study was supported by the National Key Research and Development Program of China (2018YFD0500605) and the Science and Technology Support Program of Sichuan Province (2014NZ0043, 2016NZ0006).

References

Ahmed ST, Hossain ME, Kim GM, Hwang JA, Ji H, Yang CJ. Effects of resveratrol and essential oils on growth performance, immunity, digestibility and fecal microbial shedding in challenged piglets. Asian Austral J Anim 2013;26:683–90.
Alsadi R, Boivin M, Ma T. Mechanism of cytokine modulation of epithelial tight junction barrier. Front Biosci 2009;14:2765–78.
Aristimunha PC, Rosa AP, Boemo LS, Garcez DC, Rosa DP, Londero A, et al. A blend of benzoic acid and essential oil compounds as an alternative to antibiotic growth promoters in broiler diets. J Appl Poultry Res 2016;25:455–63.
Basmacioglumalayoglu H, Ozdemir P, Bagriyanik HA. Influence of an organic acid blend and essential oil blend, individually or in combination, on growth performance, carcass parameters, apparent digestibility, intestinal microflora and intestinal morphology of broilers. Br Poultry Sci 2016;57:227–34.
Bhandari SK, Xu B, Nyachoti CM, Giesting DW, Krause DO. Evaluation of alternatives to antibiotics using an Escherichia coli K88+ model of piglet diarrhea: effects on gut microbial ecology. J Anim Sci 2008;86:836–47.
Blikslager AT, Moeser AJ, Gookin JL, Jones SL, Odle J. Restoration of barrier function in injured intestinal mucosa. Physiol Rev 2007;87:545–64.
Botsoglou NA, Floroupaneri P, Christaki E, Fletouris DJ, Spais AB. Effect of dietary oregano essential oil on performance of chickens and on iron-induced lipid oxidation of breast, thigh and abdominal fat tissues. Br Poultry Sci 2002;43:223–30.
Breves G, Kock J, Schröder B. Transport of nutrients and electrolytes across the intestinal wall in pigs. Livest Sci 2007;109:4–13.
Cavazzoni V, Adami A, Castrovilli C. Performance of broiler chickens supplemented with Bacillus coagulans as probiotic. Br Poultry Sci 1998;39:526–9.
Che L, Zhan L, Fang Z, Lin Y, Yan T, Wu D. Effects of dietary protein sources on growth performance and immune response of weanling pigs. Livest Sci 2012;148:1–9.
Chen H, Chen D, Michiels J, De SS. Dietary fiber affects intestinal mucosal barrier function by regulating intestinal bacteria in weaning piglets. Commun Agric Appl Biol Sci 2013;78:71.
Cromwell GL. Why and how antibiotics are used in swine production. Anim Biotechnol 2002;13:7–27.
Daniel H. Molecular and integrative physiology of intestinal peptide transport. Annu Rev Physiol 2004;66:361–84.
Davis ME, Brown DC, Baker A, Bos K, Dirain MS, Halbrook E, et al. Effect of direct-fed microbial and antibiotic supplementation on gastrointestinal microflora, mucin histochemical characterization, and immune populations of weanling pigs. Livest Sci 2007;108:249–53.
Diao H, Zheng P, Yu B, He J, Mao X, Yu J, et al. Effects of dietary supplementation with benzoic acid on intestinal morphological structure and microflora in weaned piglets. Livest Sci 2014;167:249–56.
Giannenas I, Doukas D, Karamoutsios A, Tzora A, Bonos E, Skoufos I, et al. Effects of Enterococcus faecium, mannan oligosaccharide, benzoic acid and their mixture on growth performance, intestinal microbiota, intestinal morphology and blood lymphocyte subpopulations of fattening pigs. Anim Feed Sci Technol 2016;220:159–67.
Guggenbuhl P, Séon A, Quintana AP, Nunes CS. Effects of dietary supplementation with benzoic acid (VevoVitall®;) on the zootechnical performance, the gastrointestinal microflora and the ileal digestibility of the young pig. Livest Sci 2007;108:218–21.
Hao R, Li Q, Zhao J, Li H, Wang W, Gao J. Effects of grape seed procyanidins on growth performance, immune function and antioxidant capacity in weaned piglets. Livest Sci 2015;178:237–42.
Harhaj NS, Antonetti DA. Regulation of tight junctions and loss of barrier function in pathophysiology. Int J Biochem Cell Biol 2004;36:1206–37.
Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 2014;11:506–14.
Hui D, Ping Z, Bing Y, He J, Mao X, Jie Y, et al. Effects of benzoic acid and thymol on growth performance and gut characteristics of weaned piglets. Asian Austral J Anim 2015;28:827–39.
Hung AT, Lin S-Y, Yang T-Y, Chou C-K, Liu H-C, Lu J-J, et al. Effects of Bacillus coagulans ATCC 7050 on growth performance, intestinal morphology, and microflora composition in broiler chickens. Anim Prod Sci 2012;52:874–9.
Konstantinov SR, Awati A, Williams BA, Miller BG, Jones PH, Stokes CR, et al. Postnatal development of the porcine microbiota composition and activities. Environ Microbiol 2006;8:1191–9.
Lauridsen C. Evaluation of the effect of increasing dietary vitamin E in combination with different fat sources on performance, humoral immune responses and antioxidant status of weaned pigs. Anim Feed Sci Technol 2010;158:85–94.
Li Z, Yi GF, Yin J, Sun P, Li D, Knight C. Effects of organic acids on growth performance, gastrointestinal pH, intestinal microbial populations and immune responses of weaned pigs. Asian Austral J Anim 2008;21:252–61.
Lin SY, Hung ATY, Lu JJ. Effects of supplement with different level of Bacillus coagulans as probiotics on growth performance and intestinal microflora populations of broiler chickens. J Anim Vet Adv 2011;10:111–4.
Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402–8.
Lu Z, Xu YQ, Liu HY, Lai T, Ma JL, Wang JF, et al. Evaluation of Lactobacillus rhamnosus GG using an Escherichia coli K88 model of piglet diarrhoea: effects on diarrhoea incidence, faecal microflora and immune responses. Vet Microbiol 2010;141:142–8.
Mao X, Zeng X, Qiao S, Wu G, Li D. Specific roles of threonine in intestinal mucosal integrity and barrier function. Front Biosci 2011;3:1192–200.
Moughan PJ, Rutherfurd SM, Balan P. Kiwifruit, mucins, and the gut barrier. Adv Food Nutr Res 2013;68:169–85.
Mroz Z. Organic acids as potential alternatives to antibiotic growth promoters for pigs. Adv Pork Prod 2005;16:169–82.
NRC. Nutrient requirements of swine. Eleventh Revised Edition. Washington, DC: The National Academies Press; 2012.
Papatsiros VG, Tassis PD, Tzika ED, Papaioannou DS, Petridou E, Alexopoulos C, et al. Effect of benzoic acid and combination of benzoic acid with a probiotic containing Bacillus cereus var. Toyoi in weaned pig nutrition. Pol J Vet Sci 2011;14:117–25.

Smith F, Clark JE, Overman BL, Tozel CC, Huang JH, Rivier JE, et al. Early weaning stress impairs development of mucosal barrier function in the porcine intestine. Am J Physiol-Gastr L 2010;298:G352–63.
Vokou D, Kokkini S, Bessiere JM. Geographic variation of Greek oregano (Origanum vulgare ssp. hirtum) essential oils. Biochem Systemat Ecol 1993;21:287–95.
Wang A, Yu H, Gao X, Li X, Qiao S. Influence of Lactobacillus fermentum I5007 on the intestinal and systemic immune responses of healthy and E. coli challenged piglets. Antonie Leeuwenhoek 2009;96:89–98.
Wang HF, Gao K, Wang C, Zhang WM, Liu JX. Effects of feeding bamboo vinegar and acidifier as an antibiotic substitute on the growth performance and intestinal bacterial communities of weaned piglets. Acta Agr Scand A-An 2013;63:143–50.
Wittig BM, Zeitz M. The gut as an organ of immunology. Int J Colorectal Dis 2003;18:181–7.
Yuan L, Kang SY, Ward LA, To TL, Saif LJ. Antibody-secreting cell responses and protective immunity assessed in gnotobiotic pigs inoculated orally or intramuscularly with inactivated human rotavirus. J Virol 1998;72:330–8.
Zheng Z, Tan JYW, Liu HY, Zhou XH, Xiang X, Wang KY. Evaluation of oregano essential oil (Origanum heracleoticum L.) on growth, antioxidant effect and resistance against Aeromonas hydrophila in channel catfish (Ictalurus punctatus). Aquaculture 2009;292:214–8.
Zhou X, Wang Y, Gu Q, Li W. Effect of dietary probiotic, Bacillus coagulans, on growth performance, chemical composition, and meat quality of Guangxi Yellow chicken. Poultry Sci 2010;89:588–93.
Zou Y, Xiang Q, Wang J, Peng J, Wei H. Oregano essential oil improves intestinal morphology and expression of tight junction proteins associated with modulation of selected intestinal bacteria and immune status in a pig model. BioMed Res Int 2016;2016:1–11.