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Non ruminant nutrition

The Effects of *Bacillus subtilis QST713* and β-mannanase on growth performance, intestinal barrier function, and the gut microbiota in weaned piglets

Junchen Liu,[†] Xiangyuan Ma,[†] Yong Zhuo,[†] Shengyu Xu,[†] Lun Hua,[†] Jian Li,[†] Bin Feng,[†,] Zhengfeng Fang,[†] Xuemei Jiang,[†] Lianqiang Che,[†] Zeyuan Zhu,[‡] Yan Lin,[†,1] and De Wu[†,1,]

[†]Institute of Animal Nutrition, Key Laboratory for Animal Disease-Resistance Nutrition of China, Ministry of Education, Sichuan Agricultural University, Chengdu, Sichuan 611130, China
[‡]Elanco Animal Health, Mutiara Damansara, Selangor, Malaysia

[1]Corresponding author: wude@sicau.edu.cn

Abstract

We investigated the effects of different *Bacillus subtilis QST713* doses and a *B. subtilis QST713* and β-mannanase mix on growth performance, intestinal barrier function, and gut microbiota in weaned piglets. In total, 320 healthy piglets were randomly assigned to four groups: 1) control group (basal diet), 2) BS100 group (basal diet plus 100 mg/kg *B. subtilis QST713*), 3) BS200 group (basal diet plus 200 mg/kg *B. subtilis QST713*), and 4) a BS100XT group (basal diet plus 100 mg/kg *B. subtilis QST713* and 150 mg/kg β-mannanase). The study duration was 42 d. We showed that feed intake in weaned piglets on days 1 to 21 was increased in group BS100 ($P < 0.05$), and that the feed conversion ratio in group BS100XT animals decreased throughout the study ($P < 0.05$). In terms of microbial counts, the BS100XT group showed reduced *Escherichia coli* and *Clostridium perfringens* numbers on day 21 ($P < 0.05$). Moreover, no significant α-diversity differences were observed across all groups during the study ($P > 0.05$). However, principal coordinates analysis indicated clear separations in bacterial community structures across groups (analysis of similarities: $P < 0.05$) on days 21 and 42. Additionally, *E-cadherin*, *occludin*, and *zonula occludens-1* (*ZO-1*) expression in piglet feces increased ($P < 0.05$) by adding *B. subtilis QST713* and β-mannanase to diets. Notably, this addition decreased short-chain fatty acid concentrations. In conclusion, *B. subtilis QST713* addition or combined *B. subtilis QST713* plus β-mannanase effectively improved growth performance, intestinal barrier function, and microbial balance in weaned piglets.

Lay Summary

The use of antibiotics in pig farming raises serious concerns in terms of antibiotic resistance. Consequently, alternative approaches such as probiotics, including *Bacillus subtilis*, and enzymes such as β-mannanase, have been proposed to improve pig health and performance. In particular, *B. subtilis* improves gut microbiota and reduces the prevalence of harmful bacteria such as *Escherichia coli* and *Clostridium perfringens*. Similarly, β-mannanase enhances feed digestibility and improves nutrient use in pigs. Thus, combined *B. subtilis* and β-mannanase may provide synergistic effects toward pig performance and gut health. In this study, we showed that adding *B. subtilis* to a weaned piglet diet improved feed intake, while a *B. subtilis* and β-mannanase mix reduced feed conversion ratios in weaned piglets.

Key words: *Bacillus subtilis*, β-mannanase, combined *Bacillus subtilis* and β-mannanase, gut microbiota, weaned piglets

Abbreviations: ADFI: average daily feed intake; BW: body weight; cDNA: complementary DNA; DNA: deoxyribonucleic acid; DR: diarrhea rate; OTUs: operational taxonomic units; PCoA: principal coordinate analysis; PCR: polymerase chain reaction; RNA: ribonucleic acid; SID: standardized ileal digestibility

Introduction

Antibiotics are highly beneficial when treating bacterial disease in livestock and poultry, and also for promoting growth performance via feed supplementation (Li et al., 2017a; Wang et al., 2020; Ruvalcaba-Gómez et al., 2022). However, their prolonged use causes residual issues that significantly impact surrounding environments and human health (Mohr, 2016; Hutchings et al., 2019; Roth et al., 2019). Consequently, investigating antibiotic alternatives is vital when improving animal growth performance and immune function.

Organic acids, enzymes, probiotics, and essential oils are promising alternatives to antibiotics in animal feed (Gadde et al., 2017; Gresse et al., 2017; Omonijo et al., 2018). *Bacillus* species have excellent probiotic potential as spores are resistant to heat and other environmental factors, such as pH and pressure, which preserve viability during gastrointestinal transit and stability during feed pelleting, storage, and handling (Ruiz Sella et al., 2021). *Bacillus subtilis* maintains a balanced intestinal microbiota by inhibiting harmful bacteria growth and promoting beneficial bacteria proliferation, thereby improving production performances and immunity levels in livestock and poultry. For instance, *B. subtilis* addition to weaned piglet diets enhances intestinal integrity and antioxidant capacity in lactating piglets and also increases the abundance and structure of jejunal bacteria (Yun et al., 2021). Chickens supplemented with either *B. subtilis* or *Bacillus*

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licheniformis have higher body weights and increased average daily gain (**ADG**) (Xu et al., 2021).

β-Mannan is a hemicellulose molecule in soybean meal (**SBM**), sesame meal, and palm meal, and is non-conducive to nutrient absorption and utilization, especially considering its high content in SBM. The antitrophic effects mediated by β-mannan are primarily due to ineffective immune responses which cause energy and nutrient wastage, thus reducing maximum growth potential in piglets (Vangroenweghe et al., 2021). Previous studies reported that dietary β-mannanase supplementation improved the dietary energy efficiency of corn–SBM in laying hens and potentially reduced the dietary cost of laying hens (Wu et al., 2005). Another study reported that β-mannanase improved growth performances in weaned piglets and growing-finishing pigs (Pettey et al., 2002). While *B. subtilis* and β-mannanase combinations may have synergistic effects on pig performance and gut health, data in this area are lacking.

Therefore, to address this knowledge gap, we investigated the effects of different *B. subtilis QST713* doses and a *B. subtilis QST713* and β-mannanase mix on the growth performance, intestinal barrier function, and gut microbiota in weaned piglets.

Materials and Methods

Animal studies

Study protocols were reviewed and approved by the Animal Care and Use Committee of Sichuan Agricultural University. In total, 320 healthy crossbred piglets (Duroc × Landrace × Yorkshire, body weight (**BW**) = 7.00 ± 0.50 kg) were weaned at 25 d old and fed a corn–SBM-based diet. After a 3-d adaptation period, piglets were randomly assigned to one of four groups: 1) control group (basal diet), 2) BS100 group (basal diet plus 100 mg/kg *B. subtilis QST713* (Baymix Grobig BS; Elanco, Shanghai, China), 3) BS200 group (basal diet plus 200 mg/kg *B. subtilis QST713*), and 4) a BS100XT group (basal diet plus 100 mg/kg *B. subtilis QST713* and 150 mg/kg β-mannanase (Hemicell XT; Elanco). Groups contained eight replicates with 10 piglets/replicate. The study lasted 42 d. Pigs were fed over two phases (phase 1, days 28 to 49 and phase 2, days 50 to 71). Basal diet composition and nutrient levels met nutritional requirements for weaned piglets, which were established by the National Research Council (Tables 1 and 2). The nutrient content per kilogram of feed in the four groups was similar in both phases. In phase 1, the control group had a net energy (**NE**) content of 2,463 kcal/kg, while the enzyme-treated group had a NE content of 2,448 kcal/kg, which equated to a 15-kcal/kg reduction. In phase 2, the control group had an NE content of 2,452 kcal/kg, while the enzyme-treated group had an NE content of 2,412 kcal/kg, equating to a 40-kcal/kg reduction. The Baymix Grobig BS and Hemicell XT feeds used in this study were provided by Elanco Animal Health. Baymix Grobig BS dry material contained *B. subtilis* at 1.0×10^{10} CFU/g, while Hemicell XT dry material contained β-mannanase at 3.60×10^{8} U/kg.

The study was conducted at a cooperative commercial pig farm at Sichuan Agricultural University, and specific feeding and safety production management schedules were conducted in accordance with university regulations. Pandemic prevention measures were also implemented according to standard procedures, which included keeping pens clean and well-ventilated at all times. Free access to feeding and drinking was provided, and piglets were fed three times a day (0800, 1400, and 2000 hours).

Growth performance and health status

Throughout the study, individual piglet weights were recorded on days 1, 21, and 42, and feed consumption was monitored daily in all pens. At 1 to 21, 22 to 42, and 1 to 42 d periods, we calculated ADG, average daily feed intake (**ADFI**), and feed conversion ratios (**FCR**; feed consumed/weight gain). Piglet health status was evaluated using a three-grade fecal consistency scoring system; 1 indicated firm and dry feces, 2 indicated thick and fluid feces, and 3 indicated watery feces. Diarrhea rates (DR) (%) were calculated by dividing the total number of piglets with diarrhea (during specific periods) by the number of piglets (in specific periods) and multiplying by 100.

Sample collection

On days 1, 21, and 42, two piglets from each replicate were selected for fresh fecal sampling. Fresh feces were placed into storage tubes pre-filled with RNALater (Beyotime, Shanghai, China) buffer and tubes filled with STE (Solarbio, Beijing, China) and glycerol (20%) buffer. RNALater tubes were stored overnight at 4 °C, transferred to −20 °C, and finally moved to −80 °C for long-term storage. Tubes containing STE and glycerol (20%) buffer were stored in liquid nitrogen and transferred to −80 °C for long-term storage.

16S rRNA sequencing and bioinformatics

Microbial genomic DNA was extracted from samples using a Soil DNA Kit (OmegaBio tek, Norcross, GA, US) following manufacturer's instructions. DNA concentrations and quality were determined using a Nano-Drop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and DNAs were stored at −80 °C until required. Forward 338F (ACTCCTACGGGAGGCAGCAG) and reverse 806R (GGACTACHVGGGTWTCTAAT) primers were used to amplify the V3 to V4 region of bacterial *16S rRNA*. Paired-end reads from Illumina sequencing (Illumina, San Diego, CA, USA) were initially assembled based on overlap relationships, and then sequence quality was controlled and filtered. After distinguishing samples, sequences underwent operational taxonomic unit (**OTU**) clustering analysis at 97% similarity thresholds, followed by taxonomic classification analyses.

Quantitative real-time PCR

E-cadherin, occludin, and *zonula occludens-1* (*ZO-1*) expression levels were quantified using primers from previous studies (He et al., 2017; Yun et al., 2021). Total RNA was isolated from fecal samples using the Gene JET RNA Purification Kit (ThermoFisher). RNA concentrations and quality were measured using a Nano-Drop 2000 spectrophotometer (ThermoFisher Scientific). After concentration normalization, RNA was reverse transcribed to cDNA using the EasyScript All in One First Strand cDNA Synthesis SuperMix kit for qPCR (TransGen Biotech, China) and samples were stored at −20 °C. Target genes were detected using fluorescence quantitative PCR using GoTaq q-PCR Master Mix (Promega, Madison, WI, USA). Amplification primers are listed (Table 3). β-Actin was used as a housekeeping gene and relative target gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Composition and nutritional levels in study diets

Items	Phase 1		Phase 2	
Ingredients, %	Control	Hemicell XT[b]	Control	Hemicell XT[b]
Corn	24.84	25.38	39.61	38.94
Crushed rice	15.00	15.00	15.00	15.00
Extruded corn	10.00	10.00	5.00	5.00
Wheat flour	12.50	10.00	11.80	11.80
Soybean oil	0.50	0.50	0.50	0.00
Extruded soybean	5.00	6.10	3.00	2.70
Soybean meal 46%	11.90	14.50	16.50	19.60
Low protein whey powder	6.00	6.00	0.00	0.00
Fish meal	5.00	3.00	2.00	0.00
Glucose	2.50	2.50	2.50	2.50
Sugar	2.50	2.50	0.00	0.00
Calcium citrate	1.00	1.00	0.00	0.00
Limestone	0.280	0.425	0.531	0.540
Calcium phosphate	0.000	0.000	1.076	1.307
Sodium chloride	0.376	0.430	0.350	0.400
L-Lysine HCl	0.692	0.709	0.613	0.636
L-Threonine	0.260	0.265	0.205	0.211
DL-Methionine	0.116	0.134	0.103	0.120
L-Tryptophan	0.052	0.050	0.000	0.032
Zinc oxide 72%	0.20	0.20	0.00	0.00
Choline chloride 50%	0.20	0.20	0.12	0.12
Antioxidant	0.02	0.02	0.01	0.01
Phytase	0.01	0.01	0.01	0.01
Sodium butyrate	0.05	0.05	0.05	0.05
Premix[a]	1.00	1.00	1.00	1.00
Hemicell XT[b]	0.000	0.015	0.000	0.015
Baymix Grobig BS[c]	0.00	0.01	0.00	0.01

[a]Premix is provided per kilogram of diet: vitamin A (11,000 IU), vitamin D3 (2,500 IU), vitamin E (40 IU), vitamin K3 (4 mg), vitamin B1 (2 mg), vitamin B2 (6 mg), vitamin B12 (0.05 mg), biotin (0.2 mg), niacin (50 mg), pantothenic acid (20 mg), iron (100 mg as ferrous sulfate), copper (150 mg as copper sulfate), manganese (60 mg as manganese oxide), zinc (100 mg as zinc oxide), iodine (0.3 mg as potassium iodide), and selenium (0.3 mg as sodium selenite).
[b]Hemicell XT dry feed contains β-mannanase at 3.60×10^8 U/kg.
[c]Baymix Grobig BS dry feed contains *Bacillus subtilis* at 1.0×10^{10} CFU/g.

Short-chain fatty acid analysis

After thawing, samples were homogenized and approximately 0.3 g was added to a 2-mL centrifuge tube. Then, 1.2 mL ultra-pure water was added, the mixture incubated for 30 min, and then centrifuged at 10000 *g* for 15 min. Next, 1 mL supernatant was mixed with 0.2 mL of 25% (w/v) metaphosphoric acid solution and 23.3 µL of 210 mmol/L crotonate solution and incubated at 4°C for 30 min. Tubes were then centrifuged at 8000 *g* for 10 min, after which, 0.3 mL supernatant was added to 0.9 mL of a chromatogen-methanol mixture (1:3 dilution) and recentrifuged at 8000 *g* for 5 min. Finally, the supernatant was filtered through a 0.22 µm filter membrane into a 1.5 mL tube. Short-chain fatty acids (**SCFAs**) were analyzed by gas chromatography (Agilent Inc., Palo Alto, CA, USA) using 1 µL of supernatant.

B. subtilis QST713 qPCR

Total microbial DNA was extracted and purified using the Soil DNA kit (OmegaBio tek) and DNAs were stored at –80 °C. A *B. subtilis QST713* gene sequences was cloned into a pUC57 vector (Sangon Biotech, Shanghai, China) and used to generate a standard curve. The *QST713* primer, recommended in previous studies (Table 3; Mendis et al., 2018), was used to quantify general microbial DNA from feces and specific DNA from plasmids.

Microbial count analyses

A 1-g fecal sample was suspended in 9 mL distilled water and centrifuged for 10 min at 12,000 × *g* at 4 °C. The supernatant was multiply diluted and inoculated onto *Escherichia coli* chromogenic media (Hopebio, Qingdao, China), *Salmonella* chromogenic media (Hopebio), and *Clostridium perfringens* assay media (Hopebio). Plates were incubated at 37 ± 1 °C for 24 h and *E. coli* and *Salmonella* colonies were counted, while *C. perfringens* plates were anaerobically incubated at 37 ± 1 °C for 24 h and colonies were counted.

Statistical analysis

Growth performance, SCFA, fecal gene expression, and *B. subtilis QST713* quantification data were analyzed using

Table 2. Nutritional level in study diets

Items	Phase 1		Phase 2	
Nutrient level[a]	Control	Hemicell XT[b]	Control	Hemicell XT[b]
CP, %	17.50	17.50	17.00	17.00
Net energy, kcal/kg	2,463	2,448	2,452	2,412
SID[c]-Lys, %	1.35	1.35	1.23	1.23
SID-Met, %	0.39	0.39	0.36	0.36
SID-Thr, %	0.80	0.80	0.73	0.73
SID-Trp, %	0.22	0.22	0.20	0.20
SID-Arg, %	0.97	0.97	1.00	1.00
Calcium (Ca), %	0.65	0.65	0.70	0.70
Total phosphorus (TP), %	0.67	0.67	0.60	0.60
Sodium (Na)	0.25	0.25	0.25	0.25
Crude fat (EE), %	4.08	4.08	3.84	3.13
Crude fiber (CF), %	2.98	2.98	3.02	3.18
Crude ash, %	5.43	5.43	4.97	5.05

[a]Nutrient levels are calculated values.
[b]Hemicell XT dry feed contains β-mannanase at 3.60×10^8 U/kg.
[c]Standardized ileal digestible.

Table 3. Study primers

Item	Primers	Accession number
β-Actin	GGCGCCCAGCACGAT	DQ845171.1
	CCGATCCACACGGAGTACTTG	
Occludin	CAGTGGTAACTTGGAGGCGT	NM_001163647.2
	CCGTCGTGTAGTCTGTCTCG	
ZO-1[a]	CAGCCCCCGTACATGGAGA	XM_021098896.1
	GCGCAGACGGTGTTCATAGTT	
E-cadherin	CAAACGGCCATTTCAGCTTCA	NM_001163060.1
	GTCACCTTGGTGGACAGCTT	
QST713	GACGTATGGATACACCTCTTTAAT	MG720020.1
	CCAAATTCCTCAGAAGAGAGAG	

[a]Zonula occludens-1.

one-way analysis of variance in SPSS 24.0 software (SPSS, Inc., Chicago, IL, USA). Multiple comparisons were conducted using Tukey tests and a $P < 0.05$ value was considered statistically significant. Microbial counts, α-diversity indices, and the relative species abundance of gut microbial communities were analyzed using Kruskal–Wallis tests, while β-diversity was analyzed using principal coordinates analysis (**PCoA**) based on Bray-Curtis distances at OTU levels. Differential bacterial taxa across groups were identified using linear discriminant analysis (**LDA**) and linear discriminant analysis effect-size (**LEfSe**) approaches.

Results

Growth performance

The effects of *B. subtilis QST713* and β-mannanase supplementation on growth performance in weaned piglets are shown (Table 4). On days 1 to 21, piglets in the BS100 group had a higher ($P < 0.05$) ADFI when compared with controls. During the overall study period (1 to 42 d), FCR was lower ($P < 0.05$) in the BS100XT group when compared with controls. As indicated (Table 5), the BS100XT group effectively reduced feed costs by replacing expensive protein sources and reducing NE. In phase 1, feed costs were decreased by 26.24 Chinese Yuan (CNY)/ton (3.65$), while in phase 2, costs decreased by 67.84 CNY/ton (9.45$). Furthermore, on days 1 to 21 and 1 to 42, piglets in BS100 and BS200 groups had lower ($P < 0.05$) DRs when compared with controls.

Fecal SCFAs

The effects of *B. subtilis QST713* and β-mannanase supplementation on fecal SCFA concentrations are shown (Table 6). On day 1, no significant differences in acetate, propionate, butyrate, valerate, or total SCFA levels were observed across treatments. On day 21, the BS100 group had lower butyrate levels when compared with controls ($P < 0.05$), while the BS100XT group had lower propionate, butyrate, valerate, and total SCFA levels when compared with controls ($P < 0.05$). On day 42, the BS100 group had lower acetate, butyrate, valerate, and total SCFA levels when compared with controls ($P < 0.05$), and the BS200 group had lower acetate and

Table 4. The effect of dietary *Bacillus subtilis QST713* and β-mannanase supplementation on growth performance in weaned piglets

Items	Control	BS100	BS200	BS100XT	SEM	*P*
1 d BW (kg)	7.00	7.05	7.06	7.02	0.04	0.951
21 d BW (kg)	10.53	10.94	10.54	10.58	0.08	0.183
42 d BW(kg)	19.64	20.24	19.24	20.51	0.18	0.054
Days 1 to 21						
ADFI (g/d)	363.24^{b}	401.61^{a}	378.21^{ab}	349.21^{b}	6.23	0.005
ADG (g/d)	168.46	185.45	165.68	169.28	3.39	0.153
FCR	2.16	2.17	2.29	2.07	0.04	0.190
DR (%)	6.08^{a}	3.28^{b}	3.56^{b}	3.77^{ab}	0.36	0.015
Days 21 to 42						
ADFI(g/d)	733.99^{ab}	772.30^{ab}	688.27^{b}	794.12^{a}	14.92	0.041
ADG(g/d)	433.73^{ab}	442.90^{ab}	414.20^{b}	472.83^{a}	7.11	0.023
FCR	1.69	1.74	1.66	1.68	0.01	0.198
DR (%)	3.70	2.51	1.91	2.80	0.24	0.056
Days 1 to 42						
ADFI (g/d)	546.48	583.93	532.78	548.39	8.10	0.132
ADG (g/d)	301.09	314.17	289.94	321.05	4.45	0.056
FCR	1.81^{a}	1.86^{a}	1.84^{a}	1.71^{b}	0.02	0.005
DR (%)	4.90^{a}	2.89^{b}	2.74^{b}	3.32^{ab}	0.26	0.006

Data were expressed as the mean and SEM (n = 8). Mean values with different letters indicate significant differences (P < 0.05). BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion ratio; DR: diarrhea rate.

Table 5. Feed price (CNY/ton) across treatment groups

Feeding phase	Control	BS100	BS200	BS100XT
Feed price (CNY/ton)				
Phase 1	4,652.20	4,670.20	4,688.20	4,625.96
Phase 2	3,864.76	3,882.76	3,900.76	3,796.92
Overall	4,258.48	4,276.48	4,294.48	4,211.44

total SCFA levels when compared with controls (P < 0.05). The BS100XT group had lower valerate levels when compared with controls (P < 0.05).

Fecal microbial counts

The effects of *B. subtilis QST713* and β-mannanase supplementation on *E. coli* and *C. perfringens* are shown (Figure 1). On days 1 and 42, no significant differences in bacterial levels were identified between treatments. However, on day 21, a significant decrease (P < 0.05) in bacterial levels was observed in the BS100XT group when compared with controls. Additionally, no *Salmonella* was detected in any treatment group during the study period.

Fecal microbiota composition

Community richness was evaluated using Abundance-based Coverage Estimator (ACE) and Chao1 indices, while community diversity was evaluated using Shannon and Simpson indices (Table 7). We observed no significant differences in α-diversity indices across groups during the study. β-Diversity indices among groups were represented by PCoA based on Bray-Curtis distances (Figure 2) and compared using analysis of similarities (ANOSIM; Table 8). We observed no significant differences between groups on day 1, however, on day 21, the BS100XT group (P = 0.008) showed a distinct cluster that was markedly separated from controls. Similarly, on day 42, BS100 (P = 0.086), BS200 (P = 0.008), and BS100XT groups (P = 0.071) formed clusters distinct to controls.

Next, we taxonomically assessed microbial composition in weaned piglets (Tables 9 and 10) and observed that *p_Firmicutes* and *p_Bacteroidetes* were dominant in the fecal microbiome over the study duration, and constituted 90% of the total bacteria in piglets. Dietary *B. subtilis QST713* and β-mannanase supplementation had limited effects on these populations. On day 42, relative *p_Actinobacteriota* abundance increased in the BS200 group when compared with controls (P < 0.05). At the family level, *f_Muribaculaceae* in the BS100XT group was significantly lower when compared with the BS100 group on day 21. To further investigate differences among groups, we identified key species as putative microbiological markers at different levels in groups using LefSe (Figure 3). Lower p_Desulfobacterota, *g_Lachnospiraceae_NK4B4_group*, and *g_Chlamydia* proportions were observed in BS100 and BS100XT groups when compared with controls. Moreover, *g_Paludicola* was significantly expanded in the BS100XT group, while *g_Erysipelotrichaceae_UCG-009* and *g_Olsenella* were significantly expanded in the BS100 group.

Fecal gene expression

The effects of *B. subtilis QST713* and β-mannanase supplementation on fecal barrier gene expression in weaned piglets are shown (Table 11). On day 21, the BS100XT group showed significantly up-regulated (P < 0.05) *ZO-1* and *E-cadherin* levels, while the BS100 group exhibited significantly up-regulated (P < 0.05) *ZO-1* levels when compared with controls. On day 42, the BS100 group showed significantly up-regulated (P < 0.05) *occludin* levels, while the BS100XT group exhibited significantly up-regulated (P < 0.05) *E-cadherin* levels when compared with controls.

Table 6. The effects of dietary *Bacillus subtilis QST713* and β-mannanase supplementation on fecal short-chain fatty acids (mg/g) in weaned piglets

Items	Control	BS100	BS200	BS100XT	SEM	*P*
1 d						
Acetate	1.30	1.32	1.18	1.31	0.07	0.868
Propionate	0.61	0.58	0.52	0.53	0.03	0.693
Butyrate	0.42	0.34	0.32	0.40	0.03	0.644
Valerate	0.23	0.21	0.17	0.22	0.01	0.378
Total SCFAs	2.99	2.93	2.69	2.82	0.14	0.885
21 d						
Acetate	4.67	4.50	4.61	4.10	0.09	0.112
Propionate	2.33^{a}	2.16^{a}	2.18^{a}	1.80^{b}	0.05	0.001
Butyrate	1.86^{a}	1.47^{bc}	1.65^{ab}	1.22^{c}	0.06	<0.001
Valerate	0.61^{a}	0.50^{ab}	0.60^{a}	0.43^{b}	0.02	0.001
Total SCFAs	10.30^{a}	9.44^{ab}	9.88^{a}	8.20^{b}	0.20	0.001
42 d						
Acetate	6.23^{a}	4.79^{b}	4.61^{b}	5.14^{ab}	0.17	0.005
Propionate	3.08	2.46	2.44	2.65	0.11	0.119
Butyrate	2.32^{a}	1.53^{b}	1.68^{ab}	1.79^{ab}	0.10	0.040
Valerate	0.82^{a}	0.55^{b}	0.66^{ab}	0.57^{b}	0.34	0.027
Total SCFAs	13.83^{a}	10.18^{b}	10.30^{b}	11.14^{ab}	0.43	0.009

Data were expressed as the mean and SEM (n = 16). Mean values with different letters indicate significant differences (P < 0.05). SCFA: short-chain fatty acid.

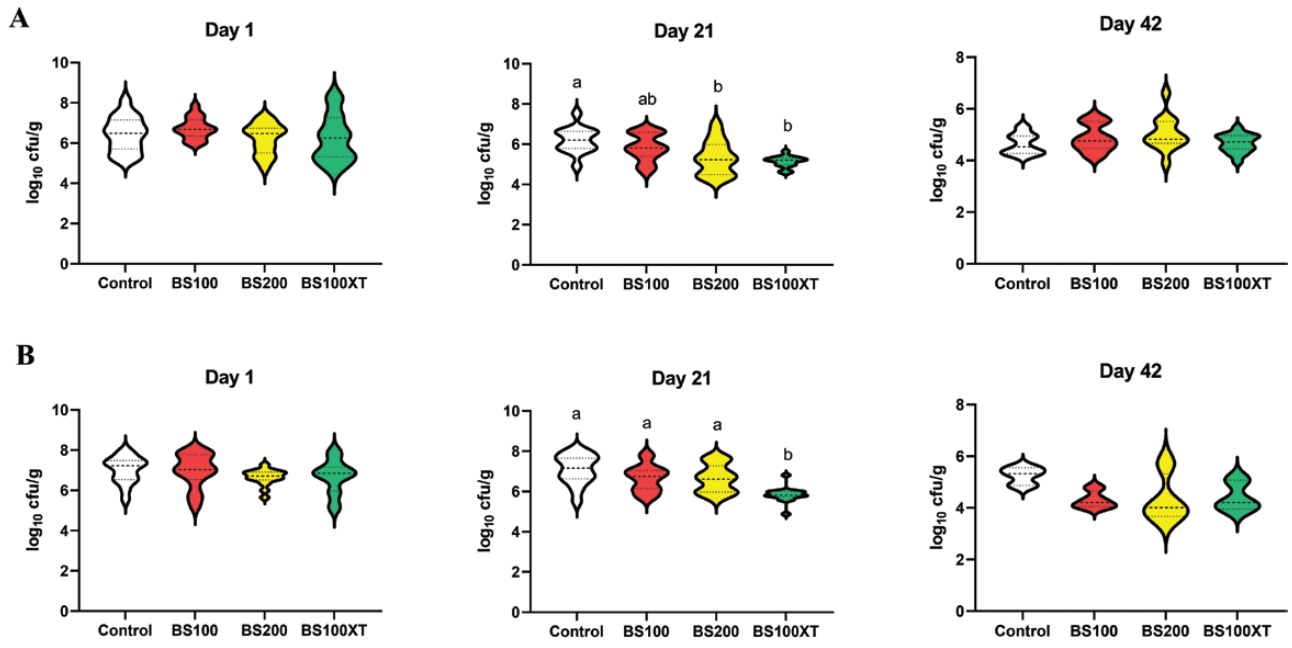

Figure 1. The effects of dietary *Bacillus subtilis QST713* and β-mannanase supplementation on *Escherichia coli* and *Clostridium perfringens* levels in weaned piglets (n = 16). Different lower-case letters indicate significant differences in treatment groups (P < 0.05). (A) *E. coli* data on different days; (B) *C. perfringens* data on different days.

Fecal *B. subtilis* QST713 quantities

As indicated (Figure 4), no experimental groups showed detectable *B. subtilis QST713* levels on day 1, similar to controls. On day 21, significantly higher *B. subtilis QST713* levels were observed in BS200 group feces when compared with BS100 and BS100XT groups (P < 0.05). On day 42, BS100, BS200, and BS100XT groups showed no significant differences with respect to *B. subtilis QST713* levels (P > 0.05).

Discussion

Pig growth performance is critical during swine production as it directly impacts profitability and competitiveness in the industry. In this study, the ADFI in the BS100 group was higher when compared with controls on days 1 to 21. Previous research reported that adding *B. subtilis* to weaned piglet diets improved their weight, ADG, and reduced DRs (Tian et al., 2020). Evidence has also suggested that combined *B.*

Table 7. The effects of dietary *Bacillus subtilis QST713* and β-mannanase supplementation on α-diversity levels in weaned piglets

Items	Control	BS100	BS200	BS100XT	SD	*P*
1 d						
ACE	480.30	521.70	539.80	516.10	101.29	0.27
Chao1	481.50	532.50	546.80	516.50	103.71	0.20
Shannon	3.65	3.86	3.68	3.54	0.46	0.11
Simpson	0.08	0.06	0.08	0.09	0.04	0.20
21 d						
ACE	648.50	636.70	648.10	613.30	88.70	0.83
Chao1	658.30	642.00	662.40	622.30	92.55	0.76
Shannon	4.04	4.02	4.01	3.92	0.40	0.82
Simpson	0.08	0.07	0.08	0.09	0.05	0.68
42 d						
ACE	620.00	603.40	599.60	646.40	83.10	0.32
Chao1	626.60	614.00	608.20	658.20	85.78	0.28
Shannon	2.97	3.26	3.38	3.13	0.61	0.34
Simpson	0.25	0.19	0.16	0.22	0.10	0.10

Data were expressed as the mean and SD (n = 16). Mean values with different letters indicate significant differences ($P < 0.05$).

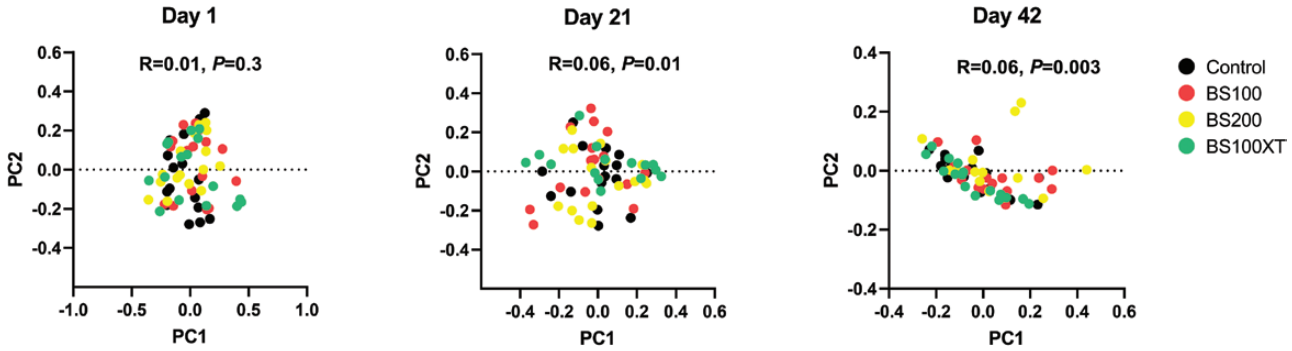

Figure 2. The effects of dietary Bacillus subtilis QST713 and β-mannanase supplementation on β-diversity levels in weaned piglets (n = 16). A, B, and C show principal component analysis score plots for gut microbiota on days 1, 21, and 42, respectively.

Table 8. Inter-group β-diversity comparisons using ANOSIM

Time	Control vs. BS100	Control vs. BS200	Control vs. BS100XT	BS100 vs. BS200	BS100 vs. BS100XT	BS200 vs. BS100XT	*P*
1 d	0.152	0.190	0.476	0.507	0.277	0.678	0.300
21 d	0.183	0.150	0.008	0.147	0.025	0.065	0.010
42 d	0.086	0.008	0.071	0.050	0.027	0.043	0.003

subtilis and *plantarum* increased ADFI and ADG in weaned piglets aged 14 to 28 and 28 to 42 d (Liu et al., 2022). Thus, these observations suggest positive *B. subtilis* effects on piglet growth performance.

Additionally, supplementing diets with β-mannanase to degrade dietary β-mannan fibers was reported to restore and improve swine performance (Vangroenweghe et al., 2021). In our study, from days 1 to 42, the FCR in the BS100XT group was lower when compared with other groups. Throughout the study, feed costs for the BS100XT group were lower when compared with the control. Specifically, feed costs/ton were reduced by 26.24 CNY in phase 1, and even more significantly by 67.84 CNY in phase 2. The BS100XT group used more SBM, had reduced fishmeal, and lowered energy supply, which lowered feed prices. Previous research indicated that adding β-mannanase to corn–SBM diets, without reducing energy, reduced FCR in weaned piglets (Pettey et al., 2002). Another study found that supplementing β-mannanase to a group with 40 kcal lower NE maintained similar production performances as high-energy diets, but with no FCR differences (Sánchez-Uribe et al., 2022). Therefore, we hypothesize that reduced FCR in the low-energy group (β-mannanase and *B. subtilis QST713* mix) was possibly due to the synergistic effects of the mix, or possibly because energy concentrations in our feed formula were insufficient. Overall, adding a *B. subtilis QST713* and β-mannanase mix improved growth performances in weaned piglets and reduced feed costs.

In recent years, several studies have highlighted the significant role of SCFAs in maintaining swine gut health, particularly butyrate, propionate, and acetate, which are major SCFAs

Table 9. The effects of dietary *Bacillus subtilis QST713* and β-mannanase supplementation on phylum levels (top five/period) in weaned piglets

Items	Control	BS100	BS200	BS100XT	SD	*P*
1 d						
p__Firmicutes	79.85	79.36	81.15	78.16	9.50	0.86
p__Bacteroidota	10.82	13.09	14.88	14.00	8.55	0.54
p__Actinobacteriota	2.06	2.05	1.47	2.38	1.91	0.37
p__Synergistota	2.93	1.51	0.54	1.77	3.30	0.13
p__Proteobacteria	1.46	1.42	1.04	1.60	1.81	0.81
21 d						
p__Firmicutes	67.07	63.12	68.41	67.79	10.29	0.36
p__Bacteroidota	29.63	31.96	28.2	28.58	10.24	0.63
p__Spirochaetota	1.56	3.27	1.87	2.15	2.33	0.19
p__Actinobacteriota	0.95	0.94	1.04	1.11	0.83	0.95
p__Proteobacteria	0.45	0.29	0.25	0.13	0.42	0.12
42 d						
p__Firmicutes	84.52	80.26	82.43	85.12	7.77	0.31
p__Bacteroidota	12.58	16.12	12.11	12.01	7.37	0.45
p__Actinobacteriota	1.00^{b}	1.79^{ab}	2.88^{a}	0.83^{b}	1.40	0.02
p__Spirochaetota	1.13	0.72	1.27	1.23	1.10	0.27
p__Cyanobacteria	0.22	0.52	0.34	0.34	0.48	0.35

Data were expressed as the mean and SD (n = 16). Mean values with different letters indicate significant differences ($P < 0.05$).

Table 10. The effects of dietary *Bacillus subtilis QST713* and β-mannanase supplementation on bacterial family levels (top five/period) in weaned piglets

Items	Control	BS100	BS200	BS100XT	SD	*P*
1 d						
f__Oscillospiraceae	18.38	17.41	24.69	18.62	10.18	0.47
f__Lachnospiraceae	8.26	12.47	11.94	10.28	7.12	0.39
f__Christensenellaceae	7.13	10.6	10.53	6.14	7.84	0.29
f__Ruminococcaceae	7.20	8.67	7.26	6.58	5.83	0.68
f__Eubacterium_coprostanoligenes_group	5.39	7.23	5.86	7.80	6.38	0.75
21 d						
f__Clostridiaceae	25.29	20.7	22.66	24.53	10.11	0.45
f__Prevotellaceae	17.15	17.03	15.92	17.52	10.37	1.00
f__Oscillospiraceae	9.80	8.56	8.53	10.61	4.16	0.20
f__Lachnospiraceae	7.34	9.34	7.8	8.33	3.86	0.97
f__Muribaculaceae	7.66^{ab}	9.83^{a}	8.52^{ab}	6.12^{b}	3.51	0.03
42 d						
f__Clostridiaceae	47.92	41.91	34.76	45.67	13.40	0.07
f__Peptostreptococcaceae	7.66	7.68	8.43	8.26	3.62	0.92
f__Muribaculaceae	6.43	6.77	5.03	5.94	3.20	0.40
f__Lachnospiraceae	5.13	5.56	6.84	5.15	2.74	0.61
f__Oscillospiraceae	4.94	4.72	5.39	6.92	2.95	0.51

Data were expressed as the mean and SD (n = 16). Mean values with different letters indicate significant differences ($P < 0.05$).

generated during microbial fermentation of non-digestible fibers (Duncan et al., 2004; Vasquez et al., 2022). As SCFAs are dietary fiber fermentation products, their concentrations are highly dependent on bacterial community composition. Some studies identified specific families within the Clostridiales order (*Firmicutes*) which produced butyrate, including *Lachnospiraceae* (*Coprococcus*, *Eubacterium*, *Anaerostipes*, and *Roseburia*), *Ruminococcaceae* (*Faecalibacterium* and *Subdoligranulum*), and *Erysipelotrichaceae* (*Holdemanella*) (Martin-Gallausiaux et al., 2021). However, propionate is predominantly produced by *Bacteroidetes* and some *Firmicutes*, such as *Negativicutes* (*Veillonella* and *Phascolarctobacterium*) (Martin-Gallausiaux et al., 2021). Humans with inflammatory bowel disease, irritable bowel syndrome, type 2 diabetes, obesity, autoimmune disorders, and also cancer patients often experience decreased numbers of bacteria that produce SCFAs (Markowiak-Kopec and Slizewska, 2020). In our study, *B. subtilis QST713* and β-mannanase dietary

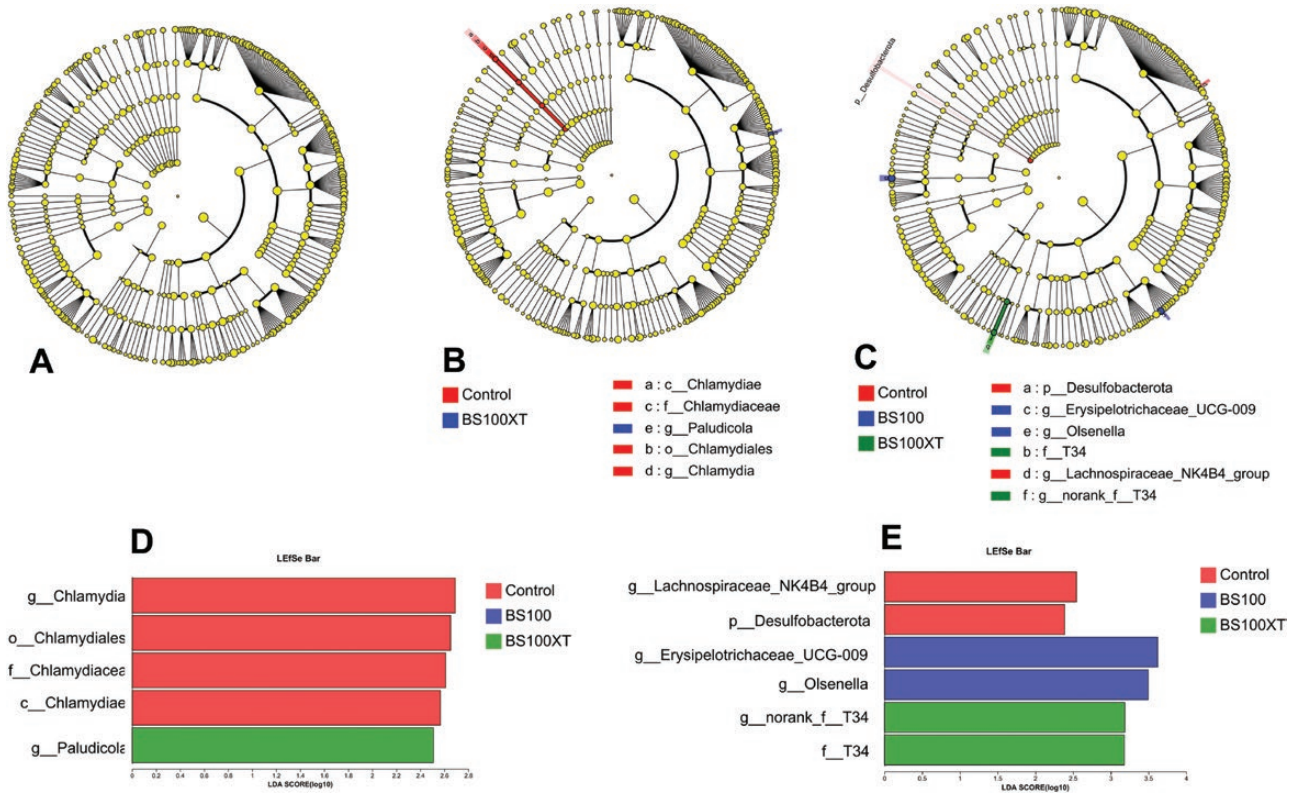

Figure 3. Linear discriminant analysis effect-size (LEfSe) of gut microbiota (n = 16). A, B, and C show cladogram plots of LEfSe analyses on days 1, 21, and 42, respectively. D and F show histograms of linear discriminant analysis (LDA) scores (threshold: 2.0) on days 21 and 42, respectively.

Table 11. The effects of dietary *Bacillus subtilis QST713* and β-mannanase supplementation on fecal barrier gene expression in weaned piglets

Items	Control	BS100	BS200	BS100XT	SEM	*P*
1d						
Occludin	1.10	1.05	0.77	0.85	0.06	0.142
ZO-1	1.10	0.50	0.75	0.92	0.17	0.663
E-cadherin	1.32	0.76	0.52	0.90	0.12	0.103
21d						
Occludin	1.36	1.14	1.28	2.03	0.13	0.066
ZO-1	1.03^{b}	2.37^{ab}	1.96^{ab}	2.85^{a}	0.22	0.026
E-cadherin	1.11^{c}	3.59^{ab}	2.69^{bc}	5.12^{a}	0.36	0.001
42d						
Occludin	1.00^{b}	1.50^{a}	1.10^{ab}	1.14^{ab}	0.06	0.022
ZO-1	1.17	1.79	1.35	0.95	0.13	0.135
E-cadherin	1.21^{b}	1.51^{b}	1.70^{b}	4.98^{a}	0.41	0.002

Data were expressed as the mean and SEM (n = 16). Mean values with different letters indicate significant differences ($P < 0.05$).

addition reduced SCFA concentrations, but we observed no changes in the bacteria responsible for SCFA production. This possibly indicated that reduced SCFA concentrations were attributable to rapid absorption by the host via the colonic epithelium or utilization by other gut microbiota communities (Loh et al., 2006; Ringel-Kulka et al., 2015; Shin et al., 2021). This observation was possibly linked to substrate availability, intestinal transit time, and cooperative or competitive interactions between different microbiota species (Macfarlane and Macfarlane, 2003).

E. coli, *C. perfringens*, and *Salmonella* are significant causes of bacterial diarrhea in piglets (Fairbrother et al., 2005; Tran et al., 2018; Posthaus et al., 2020). Traditional methods preventing diarrhea episodes in piglets involve supplementing antibiotics to diets, but such long-term and excessive use can cause bacterial resistance and generate antibiotic residues in animal products. In recent years, probiotics have been extensively proposed as antibiotic substitutes when treating piglet diarrhea (Su et al., 2022). We showed that the BS100XT group had reduced *E. coli* and *C. perfringens* counts when compared with controls, while the BS200 group had only reduced *E. coli* counts. Previous studies also demonstrated that probiotics reduced *E. coli* and *C. perfringens* counts while increasing *Lactobacillus*

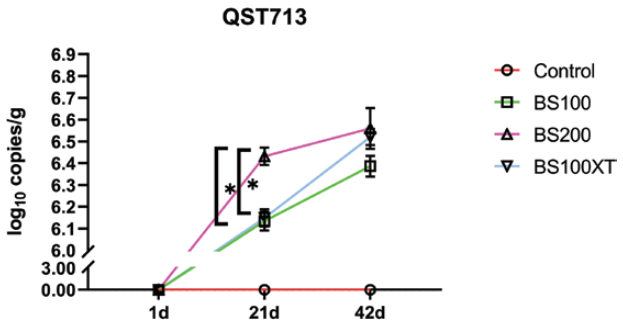

Figure 4. The effects of dietary *Bacillus subtilis QST713* and β-mannanase supplementation on *Bacillus subtilis QST713* levels in weaned piglets. Data were expressed as the mean ± standard error of the mean (n = 16), * P < 0.05.

levels (Baker et al., 2013; Starke et al., 2013). Therefore, probiotics may be advantageous as antibiotic substitutes in preventing and treating piglet diarrhea.

Increasingly, the evidence now suggests that gut microbiota composition is closely related to host health (Shreiner et al., 2015; Saffouri et al., 2019). Therefore, we examined if *B. subtilis QST713* and β-mannanase supplementation altered fecal microbiota composition in weaned piglets, and thus affected growth and development. We observed no significant differences in α-diversity indices between groups. However, β-diversity analyses showed a significant separation in bacterial community structures between experimental and control groups. Therefore, *B. subtilis QST713* and β-mannanase addition to weaned piglet diets altered gut microbiota structure and diversity. Further, ANOSIM β-diversity analyses identified significant differences in bacterial community structures between BS100 and BS100XT groups. Previous research reported that β-mannanase exerted no effects on the gut microbiota (Genova et al., 2023). Therefore, we speculated that β-mannanase may have impacted *B. subtilis QST713*. *Firmicutes* and *Bacteroidetes* were the two dominant phyla in our study, consistent with previous research (Hu et al., 2016). *Bacteroidetes* abundance was non-significantly higher in the BS100 group when compared with controls. Previous research also identified positive correlations between the ADFI in weaned piglets and *Bacteroidetes* (Ban-Tokuda et al., 2017), which may account for higher ADFI levels in the BS100 group. A recent study reported that pigs with high *Prevotella* abundance tended to have lower feed efficiency, suggesting that *Prevotella* may serve as a potential biomarker for decreased feed efficiency (Niu et al., 2015). However, in our study, no correlations were identified between FCR in the BS100XT group and *Prevotella*, possibly because animals obtained more energy from β-mannan breakdown from SBM via β-mannanase, and also decreased FCR did not rely on microbial changes. *Actinobacteria* were significantly higher in the BS200 group when compared with controls, while *f_Muribaculum* was significantly lower in the BS100XT group when compared with the BS100 group. Previous studies reported that a high proportion of *Actinobacteria* was associated with inflammatory bowel disease and colon cancer (Frank et al., 2007; Brim et al., 2017). This may be one of the reasons for the poor production performance of the BS200 group. However, *f_Muribaculum* was also positively correlated with pro-inflammatory cytokines (Reinoso Webb et al., 2018). LEfSe showed that when compared with controls, both BS100 and BS100XT groups exhibited lower Desulfobacteria and *Chlamydia* proportions in the gut. *Chlamydia* and *E. coli* are common pathogens in humans and pigs and contribute to different diseases (Schautteet and Vanrompay, 2011). Desulfobacteria are associated with intestinal immunity and microbiota dysbiosis (Xiao et al., 2021). Additionally, the BS100XT group showed a higher proportion of *g_Paludicola*, the function of which remains unclear (Li et al., 2017b). The BS100 group showed higher *g_Erysipelotrichaceae_UCG-009* and *g_Olsenella* proportions - the former bacteria belongs to the *Erysipelotrichaceae* family, and both *Lactobacillus* and *Erysipelotrichaceae* strains may help maintain intestinal mechanical barrier function (Bian et al., 2020). *Olsenella* is also considered a beneficial bacterium (Oh et al., 2021). Therefore, *B. subtilis QST713* and β-mannanase addition altered gut microbial community structures and reduced harmful bacteria in the gut. However, no dominant bacteria enrichment or harmful bacteria reductions were observed in the BS200 group, which may be related to poor production performance.

The intestinal barrier is composed of complex connections, including epithelial cells, tight junctions (including occludin, claudin, and ZO-1), adhesion junctions (including E-cadherin and catenins), gap junctions, and desmosomes (Ukena et al., 2007). Tight junction protein expression reflects connection levels between epithelial cells and intestinal barrier integrity functions (Deng et al., 2022). It was reported that modulating protein functions in epithelial cells, in particular by increasing *E-cadherin, occludin,* and *ZO-1* mRNA levels, could underpin new therapies for diseases involving altered epithelial barrier functions (Yang et al., 2015). In our study, the BS100 group showed significantly increased *occludin* expression in pig feces, while the BS100XT group showed significantly increased *ZO-1* and *E-cadherin* expression levels. These observations were consistent with previous research showing that *B. subtilis* supplementation improved intestinal barrier integrity by upregulating *E-cadherin, occludin,* and *ZO-1* expression in the colon (Ding et al., 2021). Another study reported that the *B. subtilis* treatment of pig epithelial cells *in vitro* upregulated *occludin* and *ZO-1* expression (Gu et al., 2014). Therefore, *B. subtilis* QST713 addition or combination with β-mannanase upregulated tight junction protein expression, thereby enhancing intestinal barrier integrity. The gut microbiota has crucial roles maintaining intestinal barrier function. Previous studies reported that probiotics such as *Lactobacillus plantarum* and *Bacillus subtilis* increased tight junction protein expression in piglet intestinal epithelial cells, thereby strengthening intestinal barrier function (Tian et al., 2020; Liu et al., 2022). Therefore, *B. subtilis* QST713 addition or combination with β-mannanase may enhance intestinal barrier function, which may be related to changes in the gut microbiota. The intestinal barrier has crucial roles maintaining general health by preventing harmful substances from penetrating intestinal tissue (Scapigliati, 2013). When the barrier is compromised, increased intestinal permeability may lead to pathogen, toxin, and antigen infiltration, leading to negative effects on nutrient absorption (Lv et al., 2022). Therefore, improved intestinal barrier function can positively impact production performances in post-weaning piglets. However, in this study, the BS200 group failed to increase occludin, ZO-1, and E-cadherin expression levels, which may be one reason why this group exhibited poor production performances.

On day 1 of our study, no fecal *B. subtilis QST713* was detected in all piglet groups. However, on days 21 and 42, high fecal *B. subtilis QST713* levels were detected in piglets when fed diets containing *B. subtilis QST713*, while the strain was not present in controls. These results indicated successful *B. subtilis QST713* colonization in experimental piglet intestines. Additionally, on day 21, *B. subtilis QST713* expression levels in the BS200 group were significantly higher when compared with BS100 and BS100XT groups, possibly due to higher *B. subtilis QST713* doses in this group. This further confirmed successful *B. subtilis QST713* colonization in piglet intestines. Interestingly, we observed differences in *B. subtilis QST713* expression levels in feces between BS100 and BS100XT groups on day 42, with higher expression levels in the BS100XT group. Therefore, we speculate that as the study progressed, β-mannanase in the BS100XT group increased *B. subtilis QST713* expression levels in intestines, which suggested potential β-mannanase synergistic effects toward *B. subtilis QST713*.

Conclusions

From our findings, *B. subtilis QST713* (100 mg/kg) addition or combined with β-mannanase effectively improved growth performance, intestinal barrier function, and the microbiota balance in weaned piglets. Moreover, adding a *B. subtilis QST713* and β-mannanase mix to corn–SBM diets reduced the requirements for expensive protein diets for weaned pigs. Even with a reduction of 15 kcal/kg NE in phase 1 and 40 kcal/kg NE in phase 2, improved feed efficiency was achieved. Therefore, the *B. subtilis QST713* and β-mannanase combination were economically cheaper. However, the high-dose group showed no advantages in production performance, intestinal barrier function, and microbiota changes, which were possibly due to various factors. Future research should be conducted to determine the optimal dosages. It is worth noting that while we did not directly identify synergistic effects between *B. subtilis QST713* and β-mannanase, we identified mutual influences between them, which provides a reference point for future studies.

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Conflict of interest statement

The authors declare no real or perceived conflicts of interest.

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