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Research Article

Probiotic Effects of *Bacillus subtilis* on Growth Performance and Intestinal Microecological Balance of Growing-to-Finishing Pigs

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The present study investigated the growth performance, immune status, gut morphology, and gut microflora modulation in growing-to-finishing pigs ($n = 72$; 24 pigs/group) after dietary supplementation with *Bacillus subtilis* (*B. subtilis*; two groups (experiment 1 (E1) and experiment 2 (E2): 63 and 98 days, respectively) and control (Ct, basal diet). The results revealed that both 1–98 d and 1–63 d groups significantly improved growth performance, including an increase in midtest body weight (MBW) ($P = 0.0114$), final body weight (FBW) ($P < 0.0001$), and average daily gain (ADG) ($P < 0.0001$) and a decrease in the ratio of feed to gain (F/G) ($P < 0.0001$). There was an increase in serum IgG and SOD levels after supplementation with *B. subtilis* ($P = 0.0074$). Furthermore, *B. subtilis* potentiated the integrity of intestinal morphology (villus height (VH) ($P < 0.0001$) and villus height/crypt depth (VH/CD) ($P = 0.0009$)) in growing-to-finishing pigs. The LEfSe analysis identified 11 and 13 biomarkers in the fecal samples of the 1–63 d group and 1–98 d, respectively. The gut microflora alterations of growth-finishing pigs suggested that dietary *B. subtilis* could promote gut health by altering the relative abundances of different bacterial communities. A correlation analysis showed that *B. subtilis* could regulate the functional network of the intestine microflora and their interactions with its host. Taken together, dietary *B. subtilis* supplementation had a positive influence on the growth performance, gut health, and the composition of gut microorganisms, suggesting that *B. subtilis* can be used as a functional probiotic candidate for application in the production of growing-to-finishing pigs. *Practical Applications*. The addition of probiotics in the diet can modulate intestinal health, improve the digestibility of nutrients, and thus, help improve the nutrient utilization and production performance of pigs. It is one of the ideal substitutes for antibiotics. This study will help us understand the function of *B. subtilis* in the regulation of pig intestinal health, so as to provide scientific basis for the rational use of probiotics in the future pig industry.

1. Introduction

The use of antibiotics as feed additives is increasingly restricted due to the side effects such as drug resistance and drug residues caused by adding antibiotics to feed. Moreover, due to the abuse of antibiotics, there are antibiotic residues in pork as well as drug-resistant bacteria [1, 2]. Since 2020, the addition of antibiotics to feed has been completely prohibited in China. However, this move is accompanied by a series of problems, such as decreased animal performance, a decrease in feed conversion efficiency, and an increase in the incidence of certain diseases [3]. Natural feed additives that improve animal health and productivity are still of growing interest. It has been proven that probiotics, prebiotics, and synbiotics are effective alternatives to antibiotics [4, 5].

Gut homeostasis and metabolism depend on microbial colonization [6, 7]. Intestinal health can be improved by probiotics, which are alive microbial agents that can control

gut pathogens and enhance gut microbiota composition [8, 9]. Recent studies have demonstrated that aerobic and endospore-forming bacteria such as *Bacillus* are good candidates for probiotics because of their thermostability and low pH resistance. Their easy mixing into solid feed allows them to germinate in the gastrointestinal tract [10]. It is known that *B. subtilis* promotes lactic acid bacteria growth, improves nutrient digestion and absorption, strengthens immunity, and enhances growth performance by modulating gut microbiota [11, 12]. For instance, the combination of two strains of *B. subtilis* isolated from pigs with high performance demonstrated promising performance benefits for nursery pigs, reducing F/G ratio by up to 5% [13]. In weaned pigs, the addition of *B. subtilis* KN-42 not only promoted growth but also suppressed diarrhea rate, perhaps by reducing *E. coli* abundance [14, 15]. Other studies revealed the defensive mechanisms of beneficial *B. subtilis* against enterotoxigenic *E. coli* (ETEC) in young piglets by observing that piglets that received *B. subtilis* probiotic had improved goblet cell function and gut integrity, thereby ameliorating ETEC-induced enteritis [16, 17]. Tang et al. demonstrated that *B. subtilis* DSM 32315 supplemented diet could promote the growth of weaned piglets by modulating intestinal morphology, hindgut microflora composition, and maintaining the gut barrier integrity [18].

Growing-to-finishing pigs have not been investigated extensively on intestinal health caused by *B. subtilis*, and there have been no relevant systematic studies. We hypothesized that dietary supplementation of *B. subtilis* has beneficial effects on growth properties and health status by improving nutrient utilization and intestinal microbiota balance. Therefore, the aim of the present study was to evaluate the effects of *B. subtilis* on the growth performance and gut microflora of growing-to-finishing pigs and investigated whether *B. subtilis* could improve health to provide valuable information and promote the utilization of *B. subtilis*.

2. Materials and Methods

2.1. Pig Management. In our research, all pigs were examined and deemed healthy by a veterinarian. All pigs were housed in a disinfected clean room under the same hygienic conditions and equipped with 20 pens (3.0 m × 3.2 m). There was a stainless steel self-feeder (Jinan York Agricultural and Animal Husbandry Equipment Co., Ltd.) and a nipple drinker (Dezhou Xinbaijia Animal Husbandry Equipment Co., Ltd) included in each pen. Throughout the experiment, the pigs had free access to feed and water. All animal care and treatment procedures were approved by the Animal Ethics Committee of Shandong Agricultural University, China, and performed following the committee's guidelines and regulations (Approval No. 2004006).

2.2. Experimental Procedures. The probiotic used in this study were purchased by B&B KOREA Co., Ltd (Seoul, Korea). The *B. subtilis* product was added at a concentration of 5.40×10^9 colony-forming unit (CFU) per gram *B. subtilis* powder. The animal trial was carried out at Mouping Pig Farm (Yantai City, Shandong Province, China). Seventy-two ((Landrace × Yorkshire) × Duroc) three-crossbred weaned pigs (10.12 ± 0.27 kg body weight) were selected according to age and weight and allocated into three dietary groups. Each group consisted of six pens, and each pen contained four piglets (half male and half female). The three dietary groups consisted of the basal diet (Ct), basal diet supplemented with 2 g/kg probiotic *B. subtilis* for 63 days (E1), and basal diet supplemented with 2 g/kg probiotic *B. subtilis* for 96 days (E2). There were two phases of feeding the diets in mash form, a growing phase from 10 to 65 kg body weight and a finishing phase from 66 to 95 kg body weight. In Table 1, the diets were formulated to meet or exceed the nutrition requirements of the National Research Council [19]. As a replacement for corn, the probiotic was added to the diet.

2.3. Sampling and Sample Processing. On the mornings of day 98 after an overnight fast, blood samples were collected from the external jugular vein into coagulation accelerator tubes followed by centrifugation at 2500 rpm for 15 min at 4°C to obtain serum.

A 22-G needle was used to inject Telazol (2.5 mg/kg), ketamine (1.25 mg/kg), and xylazine in one intramuscular injection to sedate all pigs at the end of the trial. An 18-G needle was used to administer sodium pentobarbital (1 mL/4.5 kg) intracardially to euthanize the pigs. In order to examine cecum morphology, 3 cm of cecum tissue was flushed with physiological saline to remove chyme and collected into a 50 ml centrifuge tube containing 10% neutral formalin solution.

For 16sRNA analysis, fecal samples in each replicate were collected from two randomly selected pigs (one female and one male) on day 98 by rectal palpation with a sterile cotton swab and then pooled and stored at −80°C.

2.4. Laboratory Analysis

2.4.1. Growth Performance Analysis. Pig's body weight was measured individually on 0, 63, and 98 days of the treatment, and daily feed consumption was recorded for each pen. We calculated the average daily feed intake (ADFI), average daily gain (ADG), and feed efficiency (F/G).

2.4.2. Serum Parameter Analysis. Regent kits for total antioxidant capacity (T-AOC), malondialdehyde (MDA), and superoxide dismutase (SOD) were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Serum concentrations of immunoglobulin were measured by using immunoglobulin ELISA kits (Shanghai, China). The optical density was measured at 450 nm, and all concentrations were calculated using a standard curve ($n = 6$). In accordance with the use instructions, each parameter was strictly analyzed.

2.4.3. Intestinal Morphology Analysis. Formalin-fixed duodenal tissues were embedded in paraffin, and paraffin sections (5 ml) were sliced with a microtome and mounted on glass slides. Dewaxed sections were hydrated and stained

Table 1: Ingredients and chemical composition of basal diets (as-fed basis; E1 and E2).

	Experimental diets					
	Phase I (day 1 to day 63)			Phase II (day 64 to day 98)		
	Ct	E1	E2	Ct	E1	E2
Ingredients (%)						
Corn	67.00	67.00	67.00	65.00	65.00	65.00
Soybean meal	20.00	20.00	20.00	15.00	15.00	15.00
Broken rice	—	—	—	10.00	10.00	10.00
Wheat bran	7.50	7.50	7.50	2.00	2.00	2.00
Rice bran meal	—	—	—	4.00	4.00	4.00
Fish meal	1.50	1.50	1.50	—	—	—
Probiotic	—	—	+	—	+	+
Premix	4.00	4.00	4.00	4.00	4.00	4.00
Total	100.00	100.00	100.00	100.00	100.00	100.00
Nutrient content						
Dry matter (%)	87.88	87.88	87.88	87.93	87.93	87.93
Crude protein (%)	17.22	17.22	17.22	14.67	14.67	14.67
Crude fiber (%)	5.8	5.8	5.8	7.2	7.2	7.2
Lysine (%)	1.15	1.15	1.15	0.86	0.86	0.86
Methionine (%)	0.28	0.28	0.28	0.24	0.24	0.24
Digestible energy (MJ/kg)	13.08	13.08	13.08	13.42	13.42	13.42
Calcium (%)	0.68	0.68	0.68	0.66	0.66	0.66
Available phosphorous (%)	0.39	0.39	0.39	0.35	0.35	0.35
Threonine (%)	0.64	0.64	0.64	0.54	0.54	0.54
L-Leucine (%)	0.62	0.62	0.62	0.53	0.53	0.53
Methionine + cysteine (%)	0.56	0.56	0.56	0.50	0.50	0.50

Note. Each kg of premix contains: vitamin A 200000 IU, vitamin D 4000 IU, vitamin E 450 mg, vitamin K 35 mg, vitamin B 140 mg, vitamin B 2100 mg, vitamin B 12350 g, biotin 1.0 mg, pantothenic acid 250 mg, copper 3500 mg, iron 2500 mg, and manganese 1000 mg, zinc 2000 mg.

with hematoxylin and eosin after dewaxing with xylene. Light microscopes coupled with image processing software were used to observe the morphology of three intact villi-crypt units in each sample (Image J 1.8.0). A ratio of villus height to crypt depth (VH/CD) was calculated based on measurements of villus height (VH) and crypt depth (CD).

2.4.4. Fecal Microbiota Analysis. In accordance with the manufacturer's instructions, microbial DNA was extracted from stool samples using the HiPure Stool DNA Kits (Magen, Guangzhou, China). NanoDrop was used to verify DNA concentration, and 1% agarose gels were used to assess DNA quality. PCR (95°C for 5 min, followed by 30 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 7 min using primers 341F: CCTACGGGNGGCWGCAG; 806R: GGACTACHVGGGTATCTAAT) was used to amplify the 16S rDNA V3-V4 region of the ribosomal RNA gene. Amplicons were extracted from 2% agarose gels, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions and quantified using QuantiFluor-ST (Promega, USA). Following purification, amplicons were sequenced on the Illumina MiSeq PE250 platform, and bioinformatics analysis was conducted by Gene Denove Co., Ltd. (Guangzhou, China).

2.4.5. Bioinformatics Analysis. Raw reads were further filtered using FASTP (version 0.18.0) to obtain high-quality clean reads. With a minimum overlap of 10 bp and a mismatch error rate of 2%, paired-end clean reads were merged as raw tags using FLASH (version 1.2.11) [20].To obtain high-quality clean tags, noisy sequences of raw tags were filtered under specific conditions [21]. UPARSE (version 9.2.64) pipeline was used to cluster clean tags into operational taxonomic units (OTUs) of ≥97% similarity. To obtain effective tags for further analysis, all chimeric tags were removed using the UCHIME algorithm [22]. Within each cluster, the tag sequence with the highest abundance was selected as the representative sequence.

Biomarker features in each group were screened by LEfSe software (version 1.0). Alpha and beta diversity measures were calculated in QIIME (version 1.9.1). Reconstruction of unobserved states (PICRUSt) (version 2.1.4) was used to infer the KEGG pathway analysis of the OTUs. Based on the R project psych package (version 1.8.4), we calculated the Spearman correlation coefficient between environmental factors and genus.

2.5. Calculation and Statistical Analysis. For 16S sequencing data, statistical analysis of the intestinal microbial composition was performed using QIIME and GraphPad Prism 9.0 (GraphPad Software, United States). Alpha index comparison between groups was calculated by Welch's *t*-test in R project Vegan package (version 2.5.3). The significance of differences between three groups was computed by Turkey's HSD test.

The PROC GLM procedures of SAS 9.2 software (SAS Institute Inc., USA) were used to analyze all data except intestinal microbiota in a randomized complete block

design, and the experimental unit was the pen. The statistical model for growth performance, intestinal morphology, and immune responses of pigs included the effects of dietary supplementation as a fixed effect. A one-way analysis of variance (ANOVA) was used to determine the significance of differences between groups followed by Duncan's multiple range test. The data are expressed as mean ± standard error of the mean. A–d letters indicate statistical significance ($P < 0.05$) of differences; different letters within a column show a significant difference between the means.

3. Results

3.1. Fattening Performance. As presented in Table 2, days 1–63, 64–98, and 1–98 for ADG, ADFI, and F/G, dietary *B. subtilis* supplementation obviously increased midtest body weight (MBW), final body weight (FBW), and ADG (in the growing and growing-to-finishing period) while decreased the ADFI (in the fattening period) and F/G (in the whole growing-to-fattening period), compared with the basal diet ($P < 0.001$).

3.2. Serum Immune Parameters and Antioxidant Indicators. To determine the effects of *B. subtilis* on the health status of finishing pigs, we tested IgA, IgG, and IgM levels in serum (Table 3). In the treatments with supplementation of *B. subtilis*, significant difference analysis showed an obvious increasing effect of the probiotic on IgG ($P = 0.0074$), but there was no difference in serum IgA and IgM between different groups ($P > 0.05$).

As shown in Table 4, dietary *B. subtilis* affected serum antioxidant status. Serum SOD concentration was higher in both two *B. subtilis*-treated groups ($P = 0.0074$), whereas MDA content in the serum was decreased by dietary *B. subtilis*. In contrast, T-AOC activity did not differ between different groups ($P > 0.05$).

3.3. Duodenum Morphological Structure. The VH and CD in the duodenum of growing-to-finishing pigs were examined to evaluate the effect of *B. subtilis* on intestinal morphology traits (Table 5). Intestinal morphology analysis indicated that VH ($P < 0.0001$) and VH/CD ($P = 0.0009$) presented positive responses to *B. subtilis*.

3.4. Fecal Microbial Diversity. A total of 1814102 effective tags were obtained from 9 samples (feces; $n = 3$), and OTUs were achieved 97% similarity (Table S1). All rarefaction curves approached a saturation plateau, indicating that all microbial diversity information could be captured in the current analysis with adequate depth (Figure 1(a)). Figure 1(b) shows that added *B. subtilis* had a higher number of OTUs, and three groups shared 428 of the fecal microbiota.

Among the three groups, there were no significant differences in Sob, Chao1, Simpson, and Shannon indices when comparing alpha-diversity of bacterial communities ($P > 0.05$, Figure 2(a)). Beta diversity was determined using unweighted UniFrac, and PCoA was performed. As shown in Figure 2(b), the microbial communities of each sample were separated into three groups. Additionally, ANOSIM analysis revealed that group variation was greater than sample variation within a group ($R = 0.942$, $P = 0.004$, Figure 2(c)).

3.5. Fecal Microbial Community. The prevalence and relative abundance of the bacterial phyla and genera are presented in Tables S2 and S3, respectively. A detailed analysis of the bacterial composition of the three groups revealed that the predominant phyla were *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* for all treatments (Figure 3(a)). When we examined taxa with a relative abundance >0.1%, six distinct phyla were identified (Figure 3(b)). *B. subtilis* 98 days-fed treatment obviously increased the relative abundance of *Proteobacteria* ($P = 0.0073$), *Epsilonbacteraeota*, and *Planctomycetes* compared to the Ct group, and decreased that of *Spirochaetes* ($P = 0.0356$). The relative abundance of *Patescibacteria* in the *B. subtilis* 63 days-fed group was significantly higher than both *B. subtilis* 98 days-fed ($P = 0.0225$) and Ct groups ($P = 0.0028$), whereas *Firmicutes* abundance was lower than other dietary treatments ($P = 0.0280$).

The clustering was performed from the both top 20 bacterial taxa and groups to generate heat maps (Figure 4(a)). And the relative abundance of *Lactobacillus*, *Bacillus*, *Terrisporobacter*, and *Streptococcus* was counted respectively (Figure 4(b)). Compared to *B. subtilis* 63 days-fed and Ct group, the relative abundance of *Bacillus* was obviously increased in *B. subtilis* 98 days-fed group ($P = 0.0079$). The relative abundance of *Lactobacillus* was higher in the *B. subtilis* 63 days-fed group compared with the other two groups ($P = 0.0127$). Moreover, the relative abundance of *Terrisporobacter* was lower in the dietary *B. subtilis* supplementation groups ($P = 0.0032$), while the *Streptococcus* was higher than in the Ct groups ($P = 0.0076$).

3.6. Fecal Microbial Function. The detailed changes in the fecal microorganisms of growing-to-finishing pigs fed the same level of probiotics under different treatments were performed using LEfSe. Using the LDA score, the contributions of the microorganisms to the differences among the groups were evaluated. The results showed that 30 bacterial biomarkers were identified in Figure 5. The Carnobacteriaceae, Atopostipes, Lactobacillales, Anaerococcus, Terrisporobacter, Peptostreptococcaceae, Methanobacteria, Euryarchaeota, Methanobacteriales, Archaea, Methanobacteriaceae, Methanobrevibacter, and Ruminococcaceae were found in the feces of the B. subtilis 98 days-fed group..

We predicted the functional differences of fecal microbiota that were altered by *B. subtilis* treatment using PICRUSt. Ten KEGG pathways were enriched (7 increased and 3 decreased) in the *B. subtilis* 98 days-fed group and twelve were enriched (10 increased and 2 decreased) in the *B. subtilis* 63 days-fed group compared to Ct group (Figure 6). Amino acid metabolism, metabolism of cofactors and

Table 2: Effects of dietary probiotic (*B. subtilis*) on the growth performance in growing-to-finishing pigs.

Variables	Time	Treatments			*P* values
		Ct	E1	E2	
IBW (kg)	Day 1	10.27 ± 0.30	9.72 ± 0.24	10.38 ± 0.28	0.1927
MBW (kg)	Day 63	55.42 ± 2.41^{b}	63.77 ± 2.62^{a}	65.55 ± 1.28^{a}	0.0114
FBW (kg)	Day 98	80.84 ± 1.32^{c}	90.56 ± 1.54^{b}	95.20 ± 1.77^{a}	<0.0001
	Day 1 to 63	0.71 ± 0.03^{b}	0.85 ± 0.04^{a}	0.87 ± 0.01^{a}	0.0047
ADG (kg)	Day 64 to 98	0.80 ± 0.04	0.80 ± 0.05	0.83 ± 0.06	0.8481
	Day 1 to 98	0.72 ± 0.01^{b}	0.82 ± 0.02^{a}	0.87 ± 0.02^{a}	<0.0001
	Day 1 to 63	1.59 ± 0.09	1.46 ± 0.08	1.52 ± 0.08	0.5516
ADFI (kg)	Day 64 to 98	2.90 ± 0.04^{a}	2.62 ± 0.04^{b}	2.67 ± 0.05^{b}	<0.0001
	Day 1 to 98	2.05 ± 0.09	1.87 ± 0.08	1.92 ± 0.08	0.2809
	Day 1 to 63	2.23 ± 0.12^{a}	1.72 ± 0.09^{b}	1.74 ± 0.09^{b}	0.0006
F/G	Day 64 to 98	3.63 ± 0.05^{a}	3.27 ± 0.05^{b}	3.21 ± 0.06^{b}	<0.0001
	Day 1 to 98	2.84 ± 0.12^{a}	2.26 ± 0.09^{b}	2.22 ± 0.09^{b}	<0.0001

Data were mean ± SEM of six pens per treatment ($n = 6$). Within a row, means without a common superscript different differ ($P < 0.05$). Seventy-two weaned pigs (10.12 ± 0.27 kg body weight) were allocated into three groups and were, respectively, given the basal diet (Ct), 2 g/kg probiotic *B. subtilis* for 63 days (E1), and 2 g/kg probiotic *B. subtilis* for 96 days (E2); IBW, initial body weight; MBW, midtest body weight; FBW, final body weight; ADG, average daily gain; ADFI, average daily feed intake; F/G, ratio of feed to gain.

Table 3: Effects of dietary probiotic (*B. subtilis*) on the serum biochemical and immunological indicators in growing-to-finishing pigs.

Variables	Treatments			*P* values
	Ct	E1	E2	
IgA (μg/ml)	343.11 ± 64.98	458.53 ± 26.01	468.68 ± 46.45	0.1700
IgG (mg/ml)	5.19 ± 0.63^{b}	11.27 ± 0.55^{a}	10.12 ± 1.85^{a}	0.0074
IgM (mg/ml)	11.33 ± 0.32	13.37 ± 1.71	12.32 ± 1.34	0.5436

Data are mean ± SEM of six pigs (half male and half female) per treatment ($n = 6$). Within a row, means without a common superscript different differ ($P < 0.05$). Seventy-two weaned pigs (10.12 ± 0.27 kg body weight) were allocated into three groups and were respectively given the basal diet (Ct), 2 g/kg probiotic *B. subtilis* for 63 days (E1), and 2 g/kg probiotic *B. subtilis* for 96 days (E2); IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M.

Table 4: Effects of dietary probiotic (*B. subtilis*) on the serum antioxidant indicators in growing-to-finishing pigs.

Variables	Treatments			*P* values
	Ct	E1	E2	
T-AOC (μmol Trolox/ml)	0.37 ± 0.04	0.48 ± 0.02	0.47 ± 0.06	0.1761
SOD (U/ml)	159.13 ± 15.26^{b}	278.14 ± 41.57^{a}	270.58 ± 29.52^{a}	0.0074
MDA (nmol/ml)	10.60 ± 0.86^{a}	7.56 ± 0.76^{b}	7.64 ± 1.12^{b}	0.0626

Data are mean ± SEM of six pigs (half male and half female) per treatment ($n = 6$). Within a row, means without a common superscript different differ ($P < 0.05$). Seventy-two weaned pigs (10.12 ± 0.27 kg body weight) were allocated into three groups and were respectively given the basal diet (Ct), 2 g/kg probiotic *B. subtilis* for 63 days (E1), and 2 g/kg probiotic *B. subtilis* for 96 days (E2); T-AOC, total antioxidant capacity; SOD, superoxide dismutase; MDA, malondialdehyde.

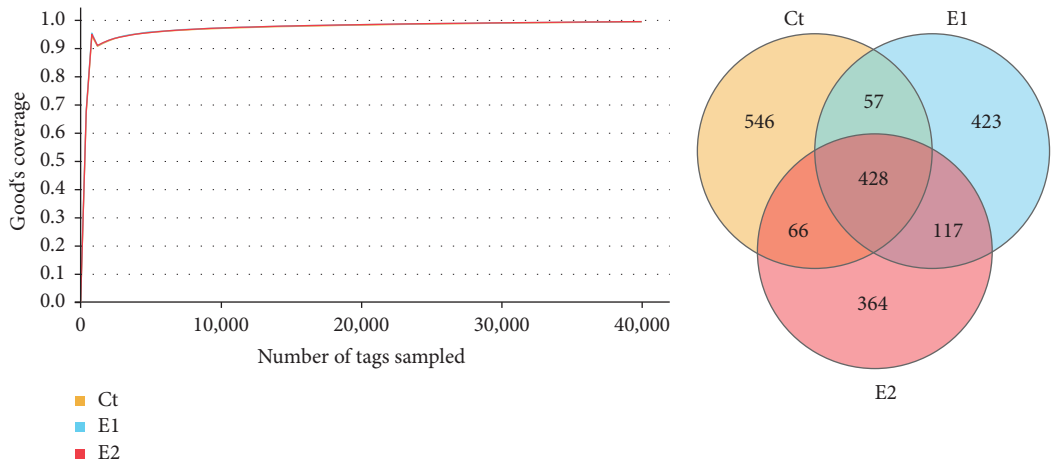
Table 5: Effects of dietary probiotic (*B. subtilis*) on the intestinal morphology in growing-to-finishing pigs.

Variables	Treatments			*P* values
	Ct	E1	E2	
VH (mm)	0.38 ± 0.01^{c}	0.52 ± 0.01^{b}	0.59 ± 0.02^{a}	<0.0001
CD (mm)	0.13 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.3790
VH/CD	2.97 ± 0.16^{b}	4.11 ± 0.19^{a}	4.30 ± 0.24^{a}	0.0009

Data are mean ± SEM of six pigs (half male and half female) per treatment ($n = 6$). Within a row, means without a common superscript different differ ($P < 0.05$). Seventy-two weaned pigs (10.12 ± 0.27 kg body weight) were allocated into three groups and were, respectively, given the basal diet (Ct), 2 g/kg probiotic *B. subtilis* for 63 days (E1) and 2 g/kg probiotic *B. subtilis* for 96 days (E2); VH, villus height; CD, crypt depth.

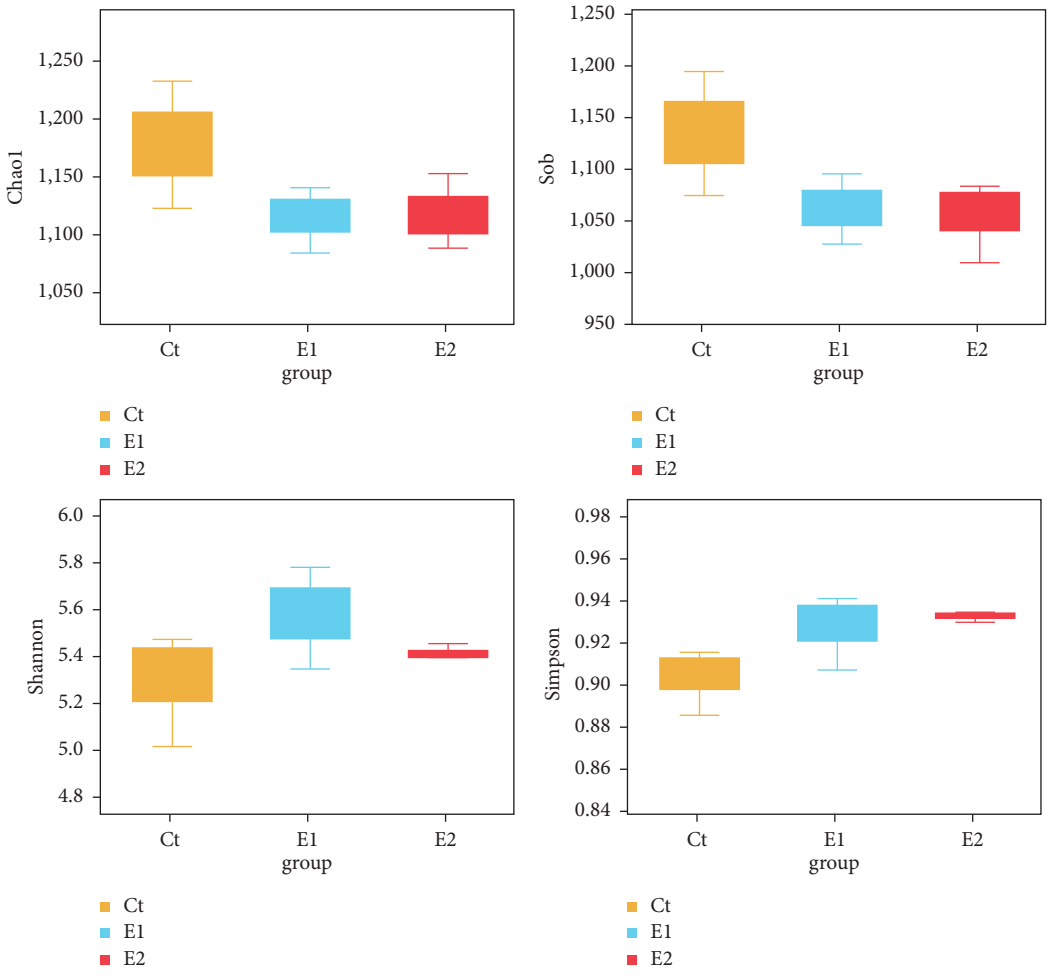
vitamins, and lipid metabolism were highly represented in both the groups. Meanwhile, the *B. subtilis* 63 days-fed group enhanced cell motility and environment adaptation compared to the *B. subtilis* 98 days-fed group.

3.7. Correlation Analysis. Spearman's correlation analysis (Figure 7) showed a positive correlation between serum SOD levels and *Bacillus* ($P = 0.0298$), *Lactobacillus* ($P = 0.0358$), *Acinetobacter* ($P = 0.0246$), and *Streptococcus* ($P = 0.0199$),



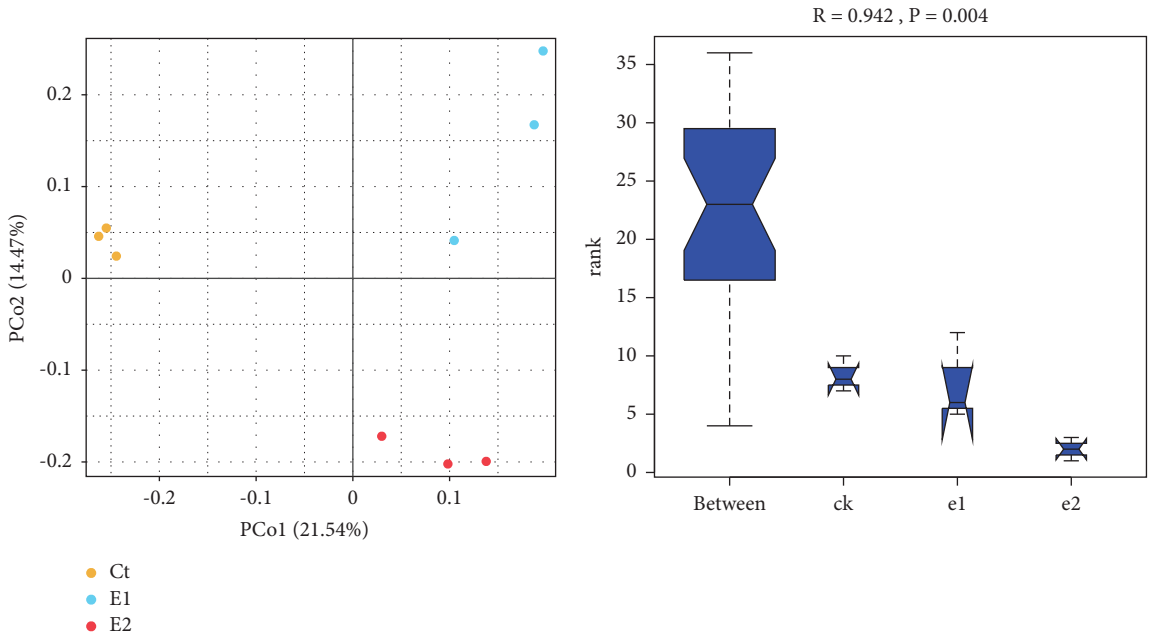
(a) (b)

Figure 1: Numbers of the fecal OTUs in the three dietary groups: (a) rarefaction curves of Good's coverage reached saturation in different groups; (b) Venn diagram of OTUs of fecal microbiota in different groups of growing-to-finishing pigs.



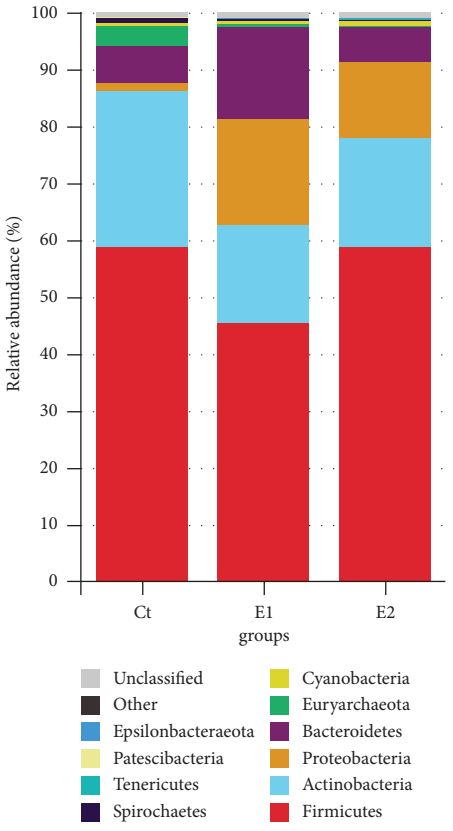
(a)

Figure 2: Continued.



(b) (c)

Figure 2: Effects of dietary probiotic (*B. subtilis*) on biodiversity of fecal microbiota of growing-to-finishing pigs: (a) an alpha-diversity index box graph was established based on Chao1, Sob, Shannon, and Simpson indexes; (b) PCoA plot based on the weighted UniFrac metric; (c) ANOSIM analysis of all groups. An R value close to 1.0 indicates more differences between the groups ($n = 3$) than within the group. $P < 0.05$ indicates statistical significance.



(a)

Figure 3: Continued.

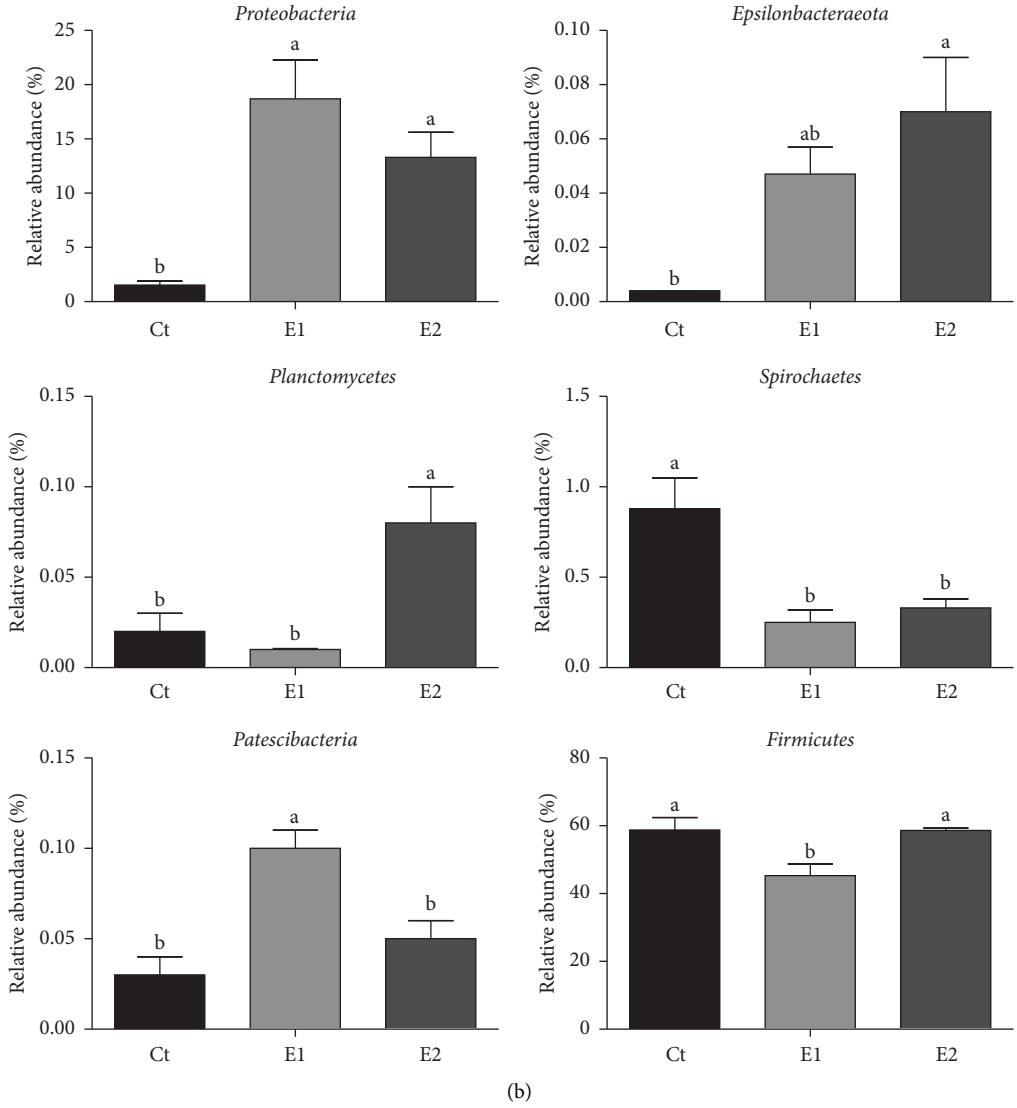


Figure 3: Relative abundance of the fecal microbiota at the phylum level in different groups based on the 16S rDNA gene sequence: (a) the stack-column of the fecal microbiota from different groups at the phylum level; (b) the relative abundance of *Proteobacteria*, *Epsilonbacteraeota*, *Planctomycetes*, *Spirochaetes*, *Patescibacteria*, and *Firmicutes* were expressed as mean ± SEM. Different lowercase letters indicate significant differences at $P < 0.05$.

and correlated negatively with the relative abundance of *Prevotellaceae_NK3B31_group* ($P = 0.0159$) and *Atopostipes* ($P = 0.0424$). The relative abundance of *Methanobrevibacter* was positively correlated with MDA content in the serum ($P = 0.0358$), and the relative abundance of *Bacteroides* ($P = 0.0096$), *Ignatzschineria* ($P = 0.0159$), and *Oblitimonas* ($P = 0.0199$) was positively correlated with IgG level in the serum. Moreover, the relative abundance of *Bacteroides* ($P = 0.0053$), *Ignatzschineria* ($P = 0.0096$), *Kurthia* ($P = 0.0298$), and *Oblitimonas* ($P = 0.0298$) was negatively correlated with serum MDA content, and the relative abundance of *Methanobrevibacter* ($P = 0.0246$) and *Clostridium sensu stricto 1* ($P = 0.0159$) was negatively correlated with serum IgG level.

4. Discussion

Many studies have been published on the positive effects of probiotics on the growth performance and health of pigs. Nevertheless, most studies have examined the effectiveness of *Bacillus* supplementation in weaned pigs and growing pigs.

The present study demonstrated that growing-to-finishing pigs fed *B. subtilis* had obviously increased ADG while decreased the F/G in the 98 days-fed group. Therefore, it significantly stimulates the efficiency of digestion and metabolism of nutrients. A previous research reported that dietary *B. subtilis* Kn-42 could increase ADG and feed efficiency throughout the entire treatment period [14]. Zhang

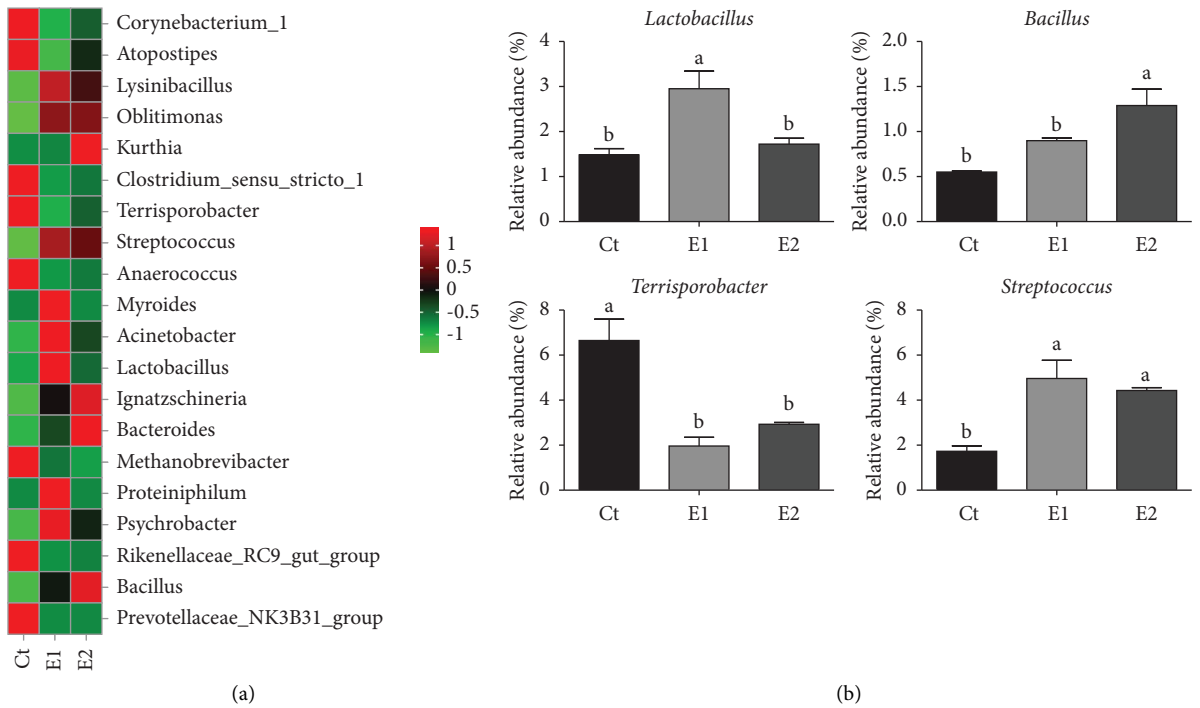
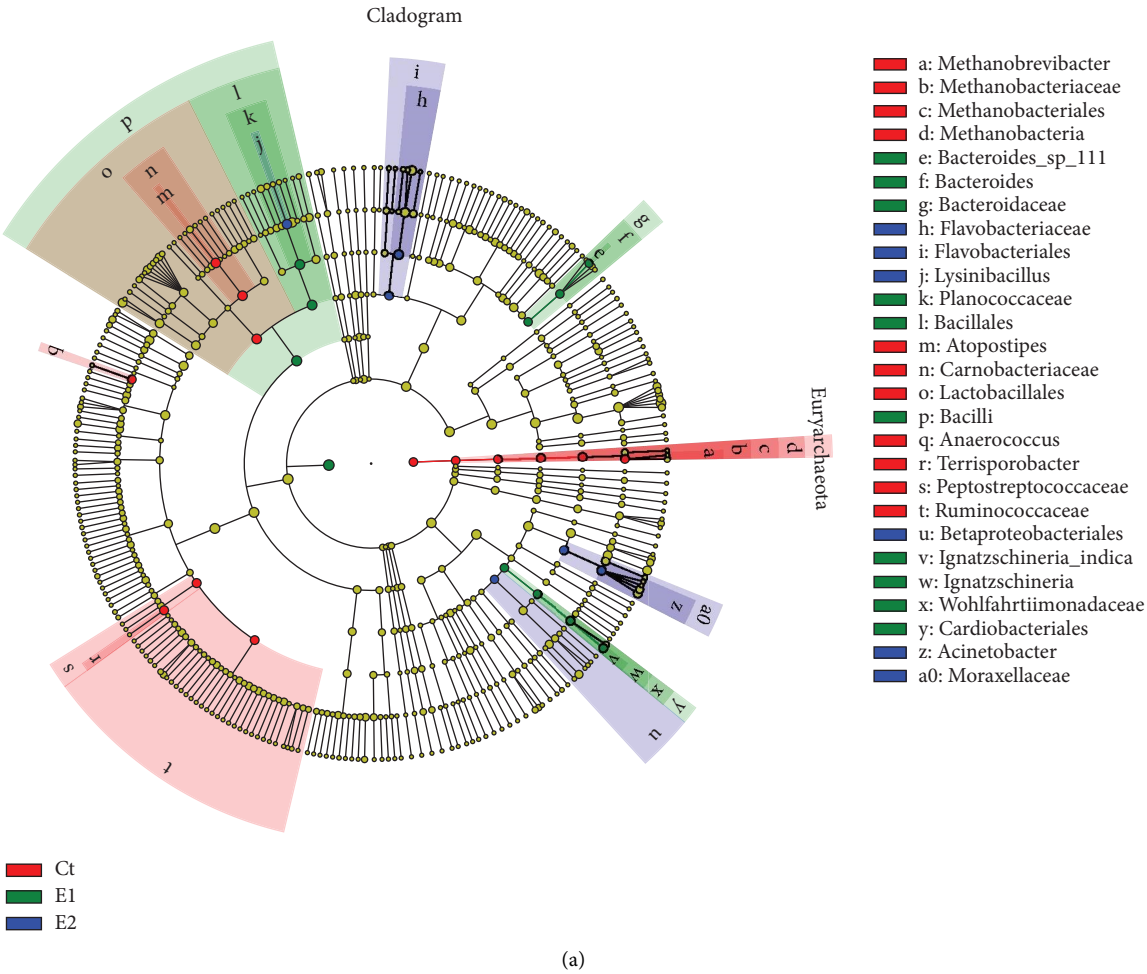
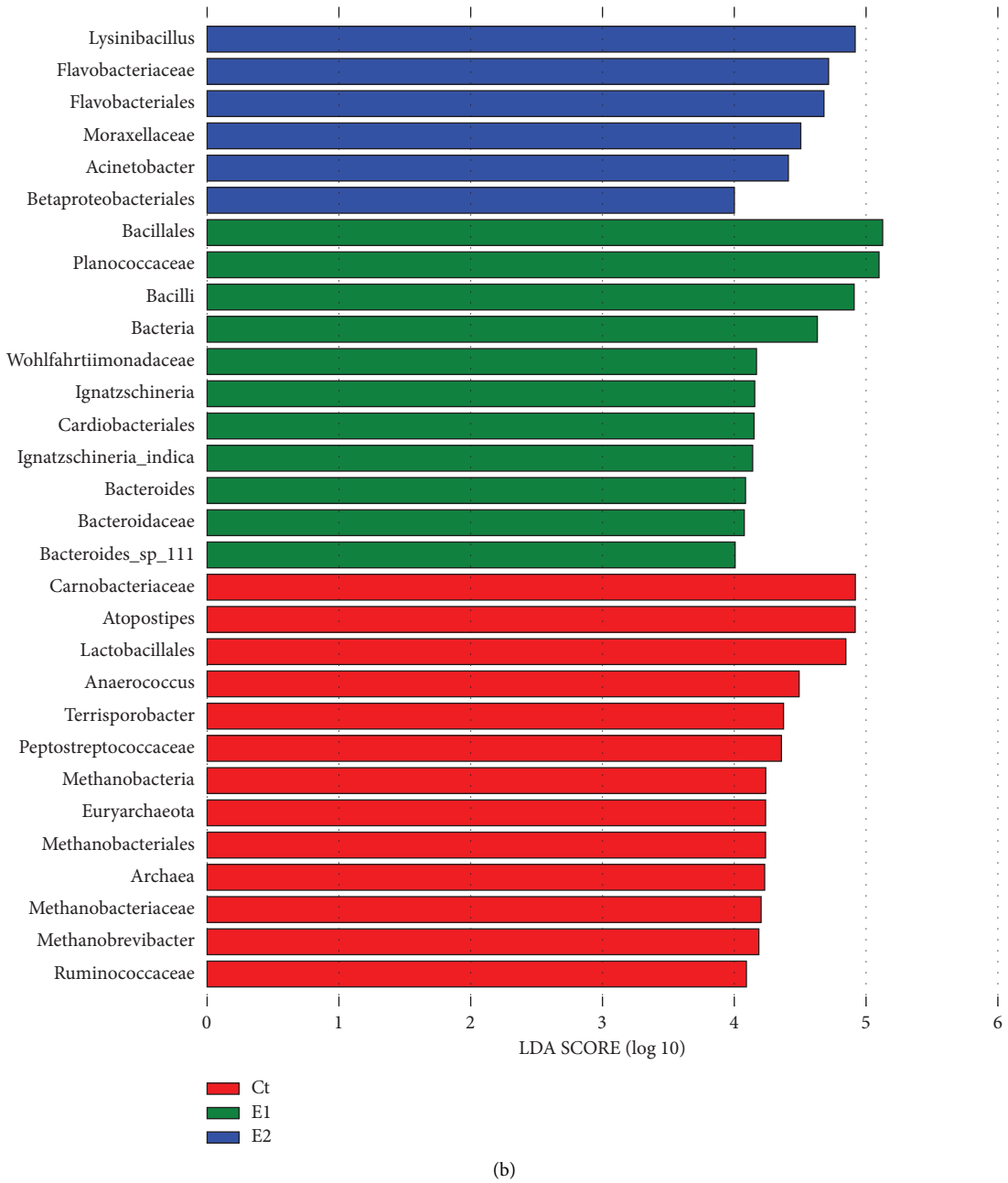


Figure 4: Relative abundance of the fecal microbiota at the genus level in different groups based on the 16S rDNA gene sequence: (a) bar graph of the top 20 genera from samples; (b) the relative abundance of *Lactobacillus*, *Bacillus*, *Terrisporobacter*, and *Streptococcus* were expressed as mean ± SEM. Values within a row with no common superscripts differ significantly ($P < 0.05$).



(a)

Figure 5: Continued.



(b)

Figure 5: The main taxa of bacteria that were different in different groups: (a) cladogram of the main taxa of microbiota that were different on the basis of LEfSe analysis: (b) LEfSe analysis (taxa with LDA score > 4). Color code: red represents significantly different taxa, with their highest relative abundance in Ct; green represents significantly different taxa, with their highest relative abundance in E1; blue-green represents significantly different taxa, with their highest relative abundance in E2.

et al. demonstrated that piglets fed the diet containing probiotics had increased ADG and G: F comparing to that receiving the diet without probiotics ($P < 0.05$) [23]. However, some former researches failed to observe such positive effects [15, 24]. Disparities in *Bacillus*' effects on pig growth can be attributed to a variety of factors, including diet formula and different species, dose, and pig age.

In the present study, the improved growth performance is likely to have been induced by maintaining intestinal microbiota balance and promoting intestinal development. Similarly, Lee et al. found that weanling pigs fed *B. subtilis* supplemented diets improved digestibility of dry matter (DM), N, and gross energy [25]. Pigs fed probiotics tended to have improved apparent total tract digestibility (ATTD) of crude protein (CP) [26]. Additionally, some previous studies also demonstrated that pigs supplemented with *Bacillus*-based probiotics showed improved nutrient digestibility [27, 28]. Yang et al. argued that compound-supplemented *B. subtilis* yb-114,246 and *B. licheniformis* yb-214,245 improved the activities of chymotrypsin, lipase, and amylase in the digestion of chicken small intestines [29]. Based on these

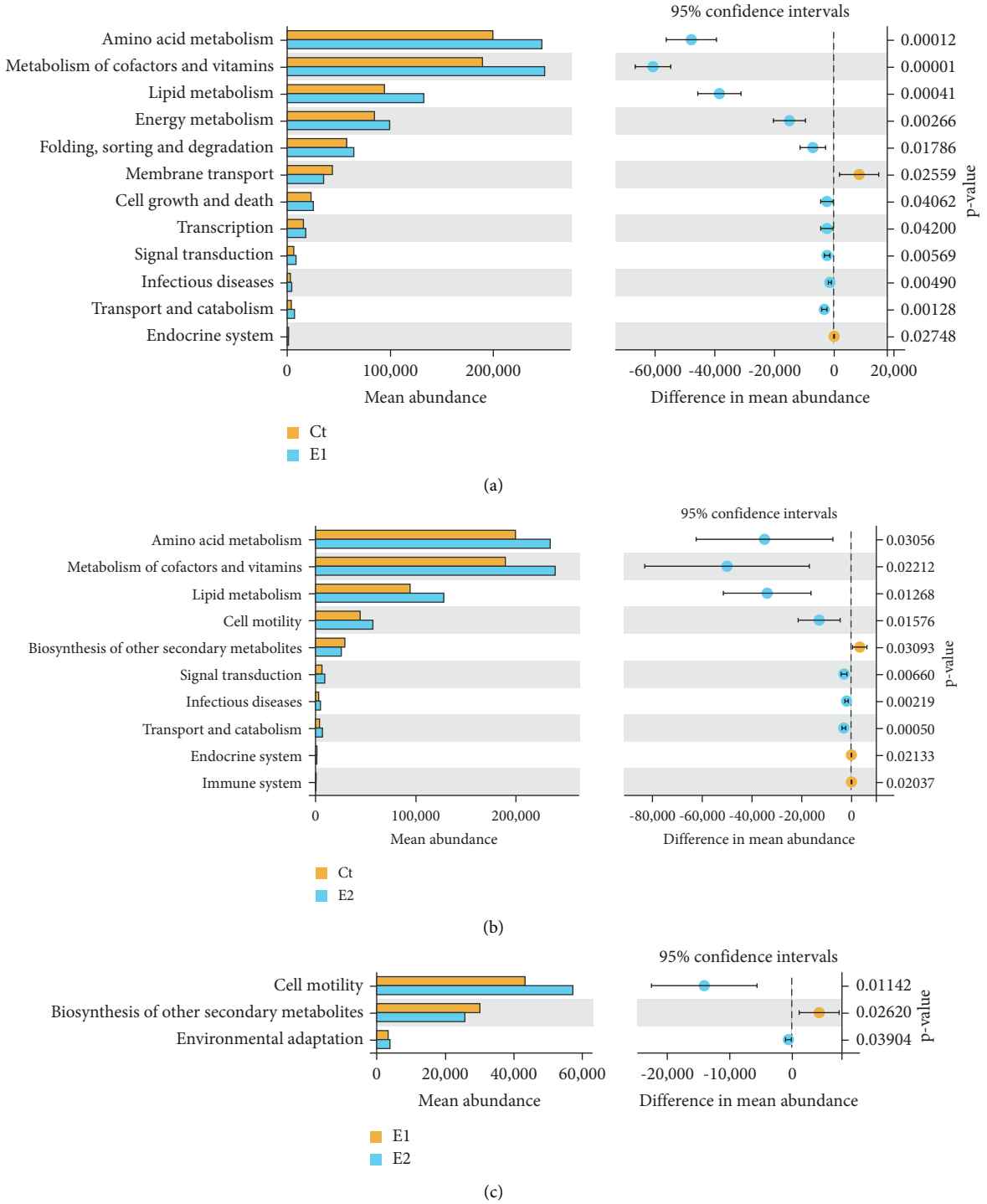


Figure 6: PICRUSt functional profiles of fecal microbiota communities under different treatments based on the KEGG pathway analysis. Function differences were observed between *B. subtilis* treatments and the Ct group. (a)–(c) Functional abundance of (a) Ct vs E1, (b) Ct vs E2, and (c) E1 vs E2 (Welch's test).

results, it can be explained that improved growth rate of pigs may be a result of a tendency to nutrient digestibility. Accordingly, feeding dietary *B. subtilis* supplements to growing-to-finishing pigs could improve their growth performance. The exact mechanism will be revealed in future studies with *B. subtilis*.

In vivo, IgG, a major glycoprotein in serum and an antibody in the immune system, has the longest residence time of any serum immunoglobulin component. Increased IgG concentration reflects better immune response and health in lactating sows. The cell barrier of *Bacillus* is composed of dextran, which serves as an immune stimulant

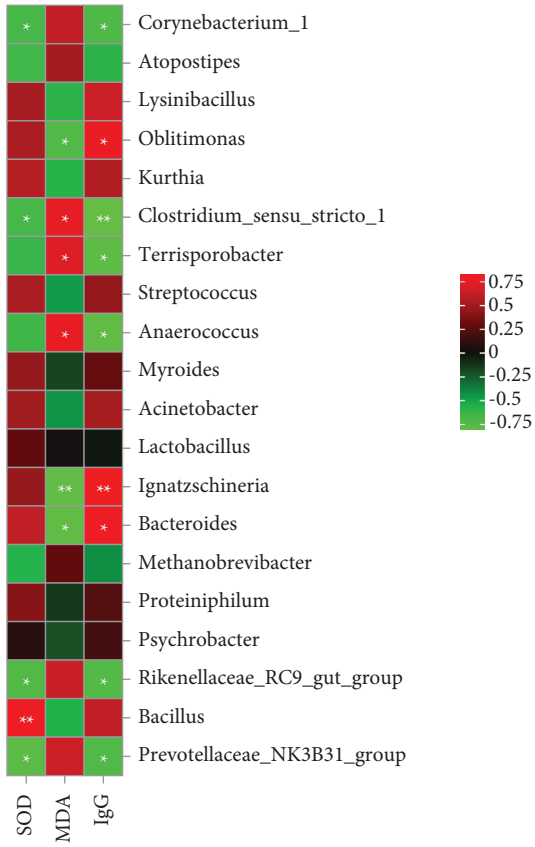


FIGURE 7: Correlation analysis between the microbiota and variables. Each column represents a factor, and each row represents a classification. Color (red to blue) indicates the correlation (positive to negative). $^{*}P < 0.05$, $^{**}P < 0.01$ represent the correlation strength.

[30]. This was in agreement with the finding of Samolinska, who also observed significant differences ($P < 0.05$) were found in the IgG level of growing pigs between the control group (15.6 mg/ml) and groups receiving the probiotic supplementation (17.4 mg/ml) [31]. Wang et al. similarly proved that diets supplemented with the combination of *B.subtilis* and *E. faecium* promoted the serum IgG level of sows [32]. There are many previous studies which have also shown that serum IgG, IgM, and IgA of broilers and mice showed improved and diversified status by 1.0×10^9 CFU/day *B. subtilis* or 4.0×10^9 CFU/m^2*B. amyloliquefaciens*, regulating the immune response [33, 34]. These results demonstrated that *Bacillus* has a positive impact on improving the immune capacity. In our research, dietary B. subtilis did not affect IgA and IgM levels, but significantly increased IgG level, which plays a key role in antibody-mediated defense [35]. Our results indicate that *B. subtilis*' immunomodulatory effect on growing-to-finishing pigs was reflected in elevated levels of IgG and the consequent effects on various stresses and health status may have contributed to the improved growth performance. Probiotics have an immunomodulatory effect by releasing cytokines, such as interferons (IFNs), transforming growth factors (TGFs), and ILs [36]. Moreover, probiotics increase gut barrier function by influencing cytokine production [37]. Probiotics, however, have strain-specific effects on immunomodulating cytokines. Therefore, it is necessary to further explore the effect of *B. subtilis* on serum cytokines of laying hens.

Under normal physiological conditions, the production and clearance of reactive-oxygen species in animals are in dynamic equilibrium. However, under many exogenous or endogenous stimuli, reactive-oxygen and reactive-nitrogen species can be overproduced and lead to oxidative stress. A marker of oxidative stress, MDA is one of the products of polyunsaturated fatty acid peroxidation in the cells [38]. In response to severe oxidative stress, enzymes are regulated and expressed by a defense system. Superoxide anion radicals can be converted into H_2O_2 by SOD [39]. Notably, our experiment indicated that dietary treatment with *B. subtilis* effectively increased the SOD activity in serum. In terms of growth performance, these features are beneficial [40]. Similarly, a recent study of wean piglets demonstrated that *B. subtilis* ASAG 216 addition could counteract DON-induced oxidative by increasing SOD activity, as well as by decreasing content of MDA [41]. In addition, Zhao et al. concluded that probiotics may regulate the redox status of the host through the actions of their antioxidant enzymes (serum: SOD, CAT, and T-AOC, GSH; ileum: SOD, CAT, and T-AOC; liver: SOD, CAT) and gut microbiota [42]. Previous research has summarized that antioxidant enzymes (SOD, MnSOD, CAT, GSH, and GSH-Px) are the first line of defense against oxidative stress [43]. Our data confirmed that *B. subtilis* had an antioxidant effect on growing-to-finishing pigs, suggesting that it could be an alternative to antibiotic growth promoters, and also be used as an effective antioxidant probiotic.

The duodenum is the part of the small intestine that has the strongest function of nutrient digestion and absorption, and the villi and intestinal glands are more developed. The gut surface area is mainly associated with gut mucosal structure, such as intestinal villus morphology, which determines the nutrient absorption capacity of the intestine. With a longer VH, the intestine has a larger absorption area, and nutrients can be absorbed more readily. The increase in CD indicates that the intestinal mucosa's villi have atrophied and are less able to absorb nutrients [44]. In the present research, the effect of dietary *B.subtilis* on intestinal morphology was evaluated. Similarly, previous researches reported a positive influence on gut histomorphology in pigs that consume *Bacillus*-based probiotics [43, 45]. This finding suggested that dietary *B. subtilis* could promote more favorable mucosal structure and larger absorption areas of luminal villous, thereby promoting better intestinal development of growing-to-finishing pigs. The above-mentioned evidences indicated that better intestinal development might be related to greater absorptive capacity and nutrient digestibility caused by dietary *B. subtilis* treatment [25].

The gut microbes contribute to the digestion and absorption of nutrients by maintaining the structure and function of the intestine [46]. As a novel biomarker presenting health and metabolic abilities, microbial community diversity is highly associated with host health [47]. Cohering with our results, dietary *B. subtilis* supplementation did not

change the fecal microbial richness or diversity of wean pigs compared to the basal diets [48, 49]. This suggests that increasing intestine microbiota diversity may not be a prerequisite for potential beneficial effects. PCoA analysis exhibited that community structures of pigs fed basal diets were obviously different from those of pigs fed basal diets supplemented with *B. subtilis*. Moreover, the unique OTUs identified by Venn diagrams in our research suggest that dietary *B. subtilis* supplementation may result in new gut microbiota in growing-to-finishing pigs. Some researchers also demonstrated that *B. subtilis* intervention could alter gut microflora structure and improve livestock health [18, 50]. Additional studies are demanded for various doses and probiotic *Bacillus* strains that produce obvious changes in the gut microbiota between groups.

Similar to the former researches on pigs, *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* were the top four abundant phyla in the present study [51, 52]. Research in wean pigs found that the supplementation of *B. subtilis*-based probiotic increased the fecal *Proteobacteria* and decreased *Spirochaetes* [26], which was similarly found in our study. *B. subtilis* may regulate intestinal microflora composition and promote intestinal health, based on these findings. In the fecal microbiota of *B. subtilis*-treated pigs, probiotic bacteria like *Lactobacillus* and *Streptococcus* abundance were significantly increased, which was consistent with the correlation analysis results in our study. Studies on feeding *Bacillus* sp. during late gestation and lactation show an increase in beneficial bacteria, primarily *Lactobacillus* sp., and a decrease in pathogenic bacteria, including *C. perfringens* and *E. coli* [53], which was in line with the results of our study. According to previous studies, *B.subtilis* C-3102 can increase *Lactobacillus* sp. in feces of sows and decrease *Clostridium* sp. in feces of the progeny [54]. *Clostridium* has previously been implicated in dietary fiber metabolism [55]. Lower *Clostridium* abundance in *B. subtilis* groups indicates that a diet containing *B. subtilis* was more effective at utilizing short-chain fatty acids (SCFAs) by growing-to-finishing pigs compared to a diet lacking it. *Lactobacillus* has been well studied for enhancing intestinal barrier function, balancing intestinal microbiota, and modulating innate immunity, thereby improving host health [56]. Our findings are similar to those of Kornegay and Risley [57], who reported that dietary *Bacillus*-based multimicrobe increased the fecal *Lactobacillus* abundance and decreased *coliform* abundance in weaning pigs. In addition, the administration of *B. subtilis* DSM32315 alters intestinal bacterial composition, increasing *Lactobacillus* and *Bifidobacterium* [18]. According to Giang, dietary *Bacillus* supplementation had no effect on the count of fecal *Lactobacillus* in growing-to-finishing pigs [58]. Based on these inconsistent results, it appears that probiotics may influence fecal microbiota in relation to pig growth phases. What is noteworthiness is that products of bacteriocins and bacteriocin-like inhibitory substances have been identified in a number of *B. subtilis* strains. Most bacteriocins produced by *B. subtilis* bacteria actively inhibit the growth of pathogenic bacteria [59, 60]. The external bacteriostatic test indicates bacteriocin of *B. subtilis* has inhibitory activity against Gram-negative pathogenic bacteria (*E. coli* and *S. typhimurium*) and Gram-positive pathogenic bacteria (*S. aureus*) [61, 62]. In contrast, we did not find an effect of the dietary addition of *B. subtilis* on the bacteria showed above in growing-to-finishing pigs, which might be attributed to the probiotics strains, concentration of probiotics, hygiene status, and individual differences.

Most notably, further analysis of LEfSe confirmed obviously modified taxa and introduced a better understanding of how dietary interventions can improve health of growing-to-finishing pigs. We conducted PICRUSt to estimate the metabolic changes caused by *B. subtilis* supplementation based on the functional differences in microbiota. In our study, dietary supplementation with *B. subtilis* could upregulate the expression of microbiota genes involved in amino acid metabolism, metabolism of cofactors and vitamins, and lipid metabolism of growing-to-finishing pigs. It has also been confirmed by Wallace that amino acids and ammonia are the preferred nitrogen sources for most intestinal microbes [63]. Consistent with a previous study, dietary *B. subtilis* DSM 32315 could adjust metabolic pathways related to gut microbiota [64]. There was an increase in the number of KEGG pathways related to amino acid metabolism, showing enhanced digestion and absorption of proteins. Different tissues require vitamins and cofactors to maintain homeostasis and biotransform nutrients into energy [65]. As an important producer of vitamins, the gut microbiota plays a critical role in the health of animals [66]. Cui et al. also confirmed that *B. subtilis* regulates lipid metabolism via altering the proportion of *Bacteroidetes* and *Firmicutes* in the intestine [67]. Therefore, our results indicate that *B. subtilis* supplementation could accelerate intestinal microbiota maturation through various metabolic pathways, but further research is needed to clarify specifics.

Correlation analysis showed the relationship between pig intestine microbiota and serum biochemical indexes, indicating the potential impact of diets *B. subtilis* on pig production and immune status. Using spearman rant test, we found abundances of *Bacillus*, *Lactobacillus*, and *Streptococcus* were positively related to serum SOD activity and were stimulated in growing-to-finishing fed *B. subtilis*. The bacteria that were negatively correlated with the body status, including *Atopostipes*, *Methanobrevibacter*, and *Clostridium sensu stricto 1*, indicated that these bacteria and the secondary metabolites by these bacteria produced were harmful for health. Since metabolites can pass through the intestinal lumen and exchange small molecules with the host's mucosal surfaces, the gut microbiota plays a crucial role in host immune development and function. The specific mechanisms by which *B. subtilis* increases host health via its effects on the gut microbiota need to be explored, despite the correlation that we found between gut microbiota and health. Overall, dietary *B. subtilis* enriched the bacteria with high antioxidation level, which may enhance its potential as an antioxidant source.

Of course, probiotics also encountered some problems in pig production and application. First of all, in terms of its action mechanism, the current trend is to use compound

probiotics for nutritional regulation, and its regulation mechanism becomes more complex. Therefore, there is still a long way to go to explore the mechanism of probiotics on pig intestinal health. Second, in terms of application, the production standards of some probiotic preparations are not perfect, the activity of probiotics is unstable or even low, and the preservation time to maintain its activity is short. At the same time, the price is too high, which is not conducive to large-scale promotion and use. Therefore, in actual production and application, it is urgent to develop and improve the production standard of probiotics, improve the stability of its activity, and extend its effective storage time. Moreover, the low stomach acid environment in pigs prevented probiotics from effectively colonizing in their intestines, limiting the use of some probiotics. Although there are many problems, with the in-depth study on the mechanism of probiotics, the effective application of probiotics in pig production is bound to be a trend, which will also bring huge economic benefits to pig production.

5. Conclusion

In summary, under the conditions of this research, a diet rich in *B. subtilis* could enhance the physical barrier function of the intestine, modify antioxidant properties and immunity, and induce a healthier microbiota composition of growing-to-finishing pigs, which may further promote growth performance and improve the profitability of pork producers. Major effects on FBW and VH were observed during the entire grow-to-finish periods suggesting that dietary *B. subtilis* supplementation is more suitable for continuous feeding for 98 days. More studies will be further conducted to confirm this potential and to further explore the underlying mechanisms.

Data Availability

All raw data were deposited in the SRA of the NCBI (https://www.ncbi.nlm.nih.gov/sra) under accession numbers SRX 17419181 to SRX 17419189.

Ethical Approval

All animal care and treatment procedures were approved by the Animal Ethics Committee of Shandong Agricultural University, China, and performed following the Committee's guidelines and regulations (Approval No. 2004006).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Table S1: The tags and OTUs information of different samples based on the 16S rDNA gene sequence. Table S2: The relative abundance (top 10) of the bacterial at phylum level of different samples. Table S3: The relative abundance (top 10) of the bacterial at genus level of different samples. (*Supplementary Materials*)

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