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Bacillus subtilis-Fermented Products Ameliorate the Growth Performance, Alleviate Intestinal Inflammatory Gene Expression, and Modulate Cecal Microbiota Community in Broilers during the Starter Phase under Dextran Sulfate Sodium Challenge

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The aim of this study was to evaluate the effects of *B. subtilis*-fermented products (BSFP) on growth performance, intestinal inflammatory gene expression, and cecal microbiota community in broilers challenged with dextran sulfate sodium (DSS) in a 14-day experiment. A total of 32, 1-day-old male broiler chickens (Ross 308), were randomly divided into four groups of eight birds per group and reared individually ($n=8$). The treatments consisted of a control diet without supplementation and DSS challenge, control diet plus 1.5% DSS, control diet plus 1 g/kg BSFP in combination with 1.5% DSS, and control diet plus 3 g/kg of BSFP in combination with 1.5% DSS. The results showed that BSFP supplementation (1 and 3 g/kg) partially improved body weight and average daily gain in broilers under DSS challenge. Relative to DSS treatment alone, BSFP supplementation dose-dependently increased the body weight of broilers at 7 days of age, with the average daily gain being at 1 to 7 days of age. BSFP supplementation (1 and 3 g/kg) alleviated intestinal inflammatory gene expression in broilers under DSS challenge. The richness and evenness of bacterial species in cecal digesta increased in a dose-dependent manner in the groups treated with BSFP (1 and 3 g/kg) in combination with DSS challenge, compared with the control group. Unweighted principal coordinate analysis indicated distinct clusters separating the group treated with 3 g/kg of BSFP in combination with DSS challenge from the other three groups. The abundance of short-chain fatty acid-producing bacteria (genus *Ruminococcaceae_unclassified*) increased and that of mucin-degrading bacteria (genus *Ruminococcus torques group*) decreased in the cecal digesta of broilers fed 3 g/kg of BSFP, compared with the control group. In conclusion, BSFP supplementation dose-dependently improved growth performance, reduced gut inflammation, and regulated the cecal microbiota of broilers exposed to DSS challenge during the starter phase.

Key words: *Bacillus subtilis*, broiler, fermented product, dextran sulfate sodium, microbiota

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Introduction

Poultry products are one of the most important sources of protein for human consumption worldwide. However, chronic low-grade intestinal inflammation has a negative impact on poultry productivity by impairing nutrient utilization, resulting in severe economic losses. In the past, the use of antibiotics at low dosages as growth promoters (AGP) was widespread. Supplementation of AGP in feeds also reduces immunological stress and low-level inflammation, thereby improving growth in poultry (Roura *et al*., 1992; Niewold, 2007). However, the use of AGP in animal husbandry has been fully banned in the European Union since 2006 (Casewell *et al*., 2003). Therefore, there is an urgent need to find alternatives to AGP to prevent intestinal inflammation in broilers.

Chronic intestinal inflammation models for broilers have been proposed, such as nutritional stimulation and dextran sulfate sodium (DSS) (Menconi *et al*., 2015; Dal Pont *et al*., 2021). Dextran sulfate sodium, a heparin-like polysaccharide, is commonly used to induce enteric inflammation in rodents (Laroui *et al*., 2012). Administration of DSS in drinking water has been shown to disrupt the intestinal structure and

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induce necrotic enteritis in broilers (Menconi *et al*., 2015), causing body weight loss, diarrhea, and intestinal bleeding in broilers (Menconi *et al*., 2015; Kuttappan *et al*., 2016).

Proposed alternatives to AGP in poultry feeds include phytogenic feed additives, antimicrobial peptides, prebiotics, and probiotics (Abudabos *et al*., 2013; Abudabos *et al*., 2017; Suresh *et al*., 2018). The effect of phytogenics or probiotics is similar to that of the AGP in broilers under *Salmonella typhimurium* or *Clostridium perfringens* challenge during the starter phase (Abudabos *et al*., 2017, 2018). *Bacillus*-based probiotics have been shown to enhance immunity and alleviate lipopolysaccharide-induced (LPS)-induced immunological stress in broilers (Lee *et al*., 2010; Li *et al*., 2015; Gadde *et al*., 2017). Our previous findings demonstrated that *B. subtilis*-fermented products (BSFP), containing *B. subtilis* spores and the antibacterial cyclic lipopeptides derived from them, can prevent *C. perfringens*-induced necrotic enteritis and promote growth performance in broilers (Cheng *et al*., 2018; Horng *et al*., 2019). *B. subtilis*-derived antibacterial cyclic lipopeptides have inhibitory effects on LPS-induced inflammation (Kim *et al*., 2006; Zhang *et al*., 2015). Furthermore, BSFP also improve growth performance and modulates the gut microbiota of broilers under LPS challenge (Chen and Yu, 2021).

The gut microbiota regulates several physiological processes in poultry, such as nutrient utilization, gut morphology, and immune response (Diaz Carrasco *et al*., 2019). Disruption of intestinal microbiota reduces nutrient utilization and affects the immune response, leading to growth retardation in broilers (Dibner and Richards, 2005). DSS-induced inflammation not only causes intestinal dysfunction, but also disrupts the gut microbial composition (Kozhakhmetov *et al*., 2021). It has been demonstrated that DSS can induce microbial dysbiosis, and altered gut microbiota is associated with changes in the expression of inflammation-related genes (Håkansson *et al*., 2015; Shen *et al*., 2021). DSS has been used to imitate chronic gut inflammation caused by environmental factors, pathogens, and feed ingredients in the field in broilers (Menconi *et al*., 2015; Zou *et al*., 2018, 2019). Our previous study demonstrated that BSFP supplementation increases the abundance of beneficial microorganisms in the cecum of LPS-challenged broilers, and thus improves their growth (Chen and Yu, 2021). However, to the best of our knowledge, no studies have so far examined the effects of BSFP on the growth performance, intestinal inflammation, and cecal microbiota of broilers under DSS challenge.

Therefore, we hypothesized that BSFP supplementation can improve growth, alleviate intestinal inflammation, and normalize the gut microbiota in broilers under DSS challenge. Since the starter phase in broiler production is a crucial stage, the main objective of the present study was to investigate the effects of different levels of BSFP on growth performance, intestinal inflammation, and cecal microbiota of broilers under DSS challenge during the starter phase.

Materials and Methods

Animal Study

BSFP are commercially available feed additives (Life Rainbow Biotech, Yilan, Taiwan), and the concentration of *B. subtilis* spores and *B. subtilis*-derived antimicrobial cyclic lipopeptide (surfactin) in fermented products were 2×10^{13} CFU/g and 1.05 mg/g, respectively (Chen and Yu, 2021). The animal protocol was approved by the Institutional Animal Care and Use Committee of the National Ilan University (108-4). One-day-old healthy male broiler chickens (Ross 308) were obtained from a commercial hatchery. On day 1, 32 chicks with an average body weight of 43.9±2.25 g were randomly assigned to four treatments, with 8 birds per treatment. The birds were reared individually in stainless steel, temperature-controlled cages. Each cage was equipped with a nipple drinker connected to an independent reservoir. The experimental diets were (1) a basal diet with neither BSFP nor DSS as a control (C), (2) a basal diet plus 1.5% DSS in drinking water (D), (3) a basal diet plus 1 g/kg of BSFP in combination with 1.5% DSS in drinking water (LD), and (4) a basal diet plus 3 g/kg of BSFP in combination with 1.5% DSS in drinking water (HD). DSS (molecular weight 40,000; Bioman, New Taipei City, Taiwan) was added to the drinking water from days 3 to 13. The volume of drinking water to be provided to each bird was calculated based on age according to a previous study (Pesti *et al*., 1985). The diets were formulated to meet or exceed the requirements of the birds according to the National Research Council recommendations (NRC, Nutrient Requirements for Poultry, 1994, Table 1). No coccidiostats or antibiotics were included in the diets. Feed and water were provided *ad libitum* throughout the duration of the experiment. Room temperature was maintained between 32 and 34℃ from days 1 to 3, at 30℃ from days 4 to 7, and at 27℃ from days 8 to 14. The birds received continuous light for the first three days, and were then maintained under a 20 h light/4 h dark regime for the remainder of the study. Broilers were vaccinated by nose-drop administration with combined Newcastle disease-infectious bronchitis vaccines on day 4. The individual body weight and feed intake were recorded daily. The growth performance (average body weight, average daily gain, average daily feed intake, and feed conversion ratio) was calculated from 2 phases (days 1 to 7 and days 8 to 14). The mortality of broilers was monitored daily.

Small Intestinal Morphology Analysis

At the end of the experiment (day 14), 4 birds per group were randomly chosen (n=4) and euthanized by inhalation of carbon dioxide gas to collect the small intestine, as previously described (Chen and Yu, 2020). Briefly, fixed intestinal samples (duodenum, jejunum, and ileum) were prepared using paraffin embedding techniques. Samples were sectioned at a thickness of 5 μm (three cross-sections from each sample), placed on a glass slide, and stained with hematoxylin and eosin. Villus height and crypt depth were measured randomly for 30 villi per bird, using an Olympus CX43 microscope (Olympus Corporation, Tokyo, Japan).

Table 1. **Composition of Basal Diets**

Item	Day 1 to 14
Ingredients (as fed basis, %)	
Corn, yellow	47.08
Soybean meal	44.1
Fish meal	5.0
Limestone	2.0
Monocalcium phosphate	1.0
Salt	0.4
Choline chloride	0.02
DL-methionine	0.2
Vitamin premix[1]	0.1
Mineral premix[2]	0.1
Nutrient levels	
Metabolizable energy (kcal/kg)	3373.8
Crude protein (%)	23.0
Crude fat (%)	2.67
Lysine (%)	1.17
Methionine + Cystine (%)	0.85
Calcium (%)	1.39
Total phosphorus (%)	0.74

[1] Supplied per kg of diet: 1.8 mg all-trans-retinyl acetate, 0.02 mg cholecalciferol, 8.3 mg alpha-tocopheryl acetate, 2.2 mg menadione, 2 mg pyridoxine HCl, 8 mg cyanocobalamin, 10 mg nicotinamide, 0.3 mg folic acid, 20 mg D-biotin, and 160 mg choline chloride.

[2] Supplied per kg of diet: 32 mg Mn ($MnSO_4 \cdot H_2O$), 16 mg Fe ($FeSO_4 \cdot 7H_2O$), 24 mg Zn (ZnO), 2 mg Cu ($CuSO_4 \cdot 5H_2O$), 800 μg I (KI), 200 μg Co ($CoSO_4$), and 60 μg Se.

Gene Expression Analysis

At the end of the experiment, birds chosen for gene expression analysis were the same as those used for the small intestinal morphology analysis (n=4). The expressions of inflammatory genes (cyclooxygenase 2, inducible nitric oxide synthase, interleukin 1β, and interleukin 6) in intestinal samples were determined using quantitative polymerase chain reaction (qPCR). Total RNA from the small intestine was isolated using TRIzol reagent, according to the manufacturer's instructions (Thermo Scientific, Waltham, MA, USA). After quantification by spectrophotometry, RNA was reverse-transcribed using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). qPCR was carried out in triplicates using the iQ SYBR Green Supermix kit and Miniopticon Real-Time PCR Detection System (Bio-Rad). mRNA expression for each gene was calculated after normalization to 18S rRNA expression using the $2^{-\Delta\Delta Ct}$ method. The qPCR primers were as follows: 5-AAC ACA ATA GAG TCT GTG ACG TCT T-3 and 5-TAT TGA ATT CAG CTG CGA TTC GG-3 for cyclooxygenase 2 (*cox2*); 5-AGG CCA AAC ATC CTG GAG GTC-3 and 5-TCA TAG AGA CGC TGC TGC CAG-3 for inducible nitric oxide synthase (*iNOS*); 5-CGC TCA CAG TCC TTC GAC-3 and 5-TGA GCC TCA CTT TCT GGC-3 for interleukin 1β (*il-1*β); 5-AGG ACG AGA TGT GCA AGA AGT TC-3 and 5-TTG GGC AGG TTG AGG TTG TT-3 for interleukin 6 (*il-6*); 5-ATA ACG AAC GAG ACT CTG GCA-3 and 5-CGG ACA TCT AAG GGC ATC ACA-3 for 18S rRNA.

16S rRNA Sequencing and Analysis

Cecal digesta from broilers was freshly collected at the end of the experiment, and 4 birds per group were used for microbiota analysis (n=4). Total bacterial DNA from the cecum content was extracted using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The V3 and V4 hypervariable regions of the 16S rRNA genes were amplified using the 341F-805R primer (5-CCT ACG GGN GGC WGC AG-3 and 5-GAC TAC HVG GGT ATC TAA TCC-3) with the barcode. The PCR products were purified by 2% agarose gel electrophoresis, and the QIAquick Gel Extraction kit (QIAGEN, Germantown, MD, USA) was used to recover DNA from the gels. The library was constructed using TruSeq Nano DNA Library Prep kit (Illumina, San Diego, CA, USA). The constructed library was then sequenced using the paired-end method on the Illumina MiSeq platform, after being subjected to DNA quantification. The sequence reads were merged using Mothur software (version 1.39.5). The sequences were assembled using the QIIME 2 software (version 2017.4) after the chimeric sequences were removed using the USEARCH software (version 7.0.1001). Operational taxonomic units (OTUs) were generated at a 97% similarity threshold, and sequences less than two were removed from all samples. The obtained sequences were aligned to the Genomes Online Database (gold.jgi.doe.gov) to determine the phylogeny of the OTUs. Alpha diversity (richness and evenness) and phylogenetic assignment were assessed using QIIME 2 software and the naïve Bayesian classification method, respectively. Principal component analysis (PCA), principal coordinate analysis (PCoA) based on the unweighted and weighted UniFrac distance matrices, and co-occurrence networks of bacterial associations, were used to visualize the differences in microbiota among the groups using the R package (version 3.5.0 and version 1.7.13). PCA was used to compare the similarities in the relative abundances of bacteria at the phylum level between the groups. PCoA was used to compare the similarities in bacterial diversity based on the distance matrices using UniFrac. Unweighted (qualitative) PCoA is based on the presence or absence of bacterial species, and weighted (quantitative) PCoA accounts for the abundance of the observed bacterial species.

Statistical Analysis

Data were analyzed using one-way ANOVA through the general linear model procedure in the Statistical Analysis System Institute software (version 9.4, 2012; SAS Institute, Cary, NC, USA). Individual birds were considered experimental units. Statistical significance was set at $P<0.05$. If significant effects were found, individual means were compared using Tukey's honestly significant difference test. Linear and quadratic contrasts were used to determine the effects of different concentrations of BSFP on broilers. PCoA was performed based on UniFrac distances coupled with standard multivariate statistics. The relationship between abundant genera in broilers from different groups was analyzed using Pearson's correlation coefficient (r).

Results

Effects of BSFP on Growth Performance

The broilers were healthy during the experimental period. The effect of BSFP on the growth performance of broilers under DSS challenge is shown in Table 2. DSS challenge reduced the body weight at 7 and 14 days of age compared to the control group ($P<0.05$). There were no significant differences in body weight between the D and BSFP (1 and 3 g/kg) groups over the experimental period. Linear improvements in body weight were observed based on the inclusion level of BSFP under DSS challenge at 7 days of age ($P<0.05$). DSS challenge reduced the average daily gain at 1 to 7 days and 1 to 14 days, compared with the C group ($P<0.05$). No significant difference in the average daily gain was observed between the D and BSFP (1 and 3 g/kg) groups. Linear improvements in average daily gain were observed based on the inclusion level of BSFP under DSS challenge at 1 to 7 days ($P<0.05$). DSS challenge reduced the average daily feed intake at 1 to 7 days, compared to the C group ($P<0.05$). BSFP (1 g/kg) in combination with DSS challenge increased the average daily feed intake at 8 to 14 days and 1 to 14 days, compared with the C and D groups ($P<0.05$). There was a quadratic effect of BSFP inclusion on average daily feed intake at 8 to 14 days and 1 to 14 days ($P<0.05$). BSFP supplementation (1 g/kg) in combination with DSS challenge increased the feed conversion ratio at 8 to 14 days, compared with the C and D groups ($P<0.05$). BSFP supplementation (1 and 3 g/kg) in combination with DSS challenge increased the feed conversion ratio at 1 to 14 days, compared with the C group ($P<0.05$). The feed conversion ratio at 8 to 14 days and 1 to 14 days also showed quadratic effects as the inclusion level of BSFP increased in response to DSS challenge ($P<0.05$).

Effects of BSFP on Intestinal Morphology

No significant differences in intestinal morphology were observed among the groups (Table 3). There was a quadratic effect of BSFP inclusion on villus length in the duodenum of DSS-challenged broilers ($P<0.05$).

Effects of BSFP on Intestinal Inflammatory Gene Expression

The effects of BSFP on intestinal inflammatory gene expression in broilers are shown in Table 4. The *cox2*, *il-1β*, and *il-6* mRNA levels in the duodenum increased ($P<0.05$) in the D group, whereas BSFP supplementation (1 and 3 g/kg) reduced them. A linear and quadratic decrease in the *cox2*, *il-1β*, and *il-6* mRNA levels in the duodenum of DSS-challenged broilers was observed as the inclusion level of BSFP increased ($P<0.05$). DSS challenge increased the *cox2*, *iNOS*, *il-1β*, and *il-6* mRNA levels in the jejunum ($P<0.05$), whereas BSFP supplementation (1 and 3 g/kg) decreased them. A quadratic decrease in *cox2* and *iNOS* mRNA levels in the jejunum of DSS-challenged broilers was observed as the inclusion level of BSFP increased ($P<0.05$). The *il-1β* and *il-6* mRNA levels in the jejunum of DSS-challenged broilers decreased in the BSFP-supplemented groups (1 and 3 g/kg), showing both linear and quadratic effects ($P<0.05$). Similar to the jejunum, the expression of inflammatory genes in the ileum also increased ($P<0.05$) in the D group, whereas BSFP supplementation (1 and 3 g/kg) reduced it. A linear and quadratic decrease in the *iNOS* and *il-1β* mRNA levels in the ileum of DSS-challenged broilers

Table 2. **Effects of *B. Subtilis*-Fermented Products on the Growth Performance of Broilers under Dextran Sulfate Sodium Challenge**

							P value[2]	
	C[1]	D	LD	HD	SEM	*P* value	Linear	Quadratic
Body weight (g/bird)								
1 d	44.3	43.9	43.8	43.7	0.43	0.797	0.876	0.952
7 d	154.5^{a}	117.1^{b}	130.3^{ab}	141.5^{ab}	4.37	0.011	0.032	0.589
14 d	368.6^{a}	278.7^{b}	309.3^{ab}	315.0^{ab}	10.26	0.014	0.225	0.439
Average daily gain (g/d/bird)								
1−7 d	15.7^{a}	10.5^{b}	12.4^{ab}	14.0^{ab}	0.62	0.011	0.033	0.588
8−14 d	30.6	23.1	25.6	24.8	1.03	0.054	0.661	0.476
1−14 d	23.2^{a}	16.8^{b}	19.0^{ab}	19.4^{ab}	0.72	0.014	0.217	0.433
Average daily feed intake (g/d/bird)								
1−7 d	13.7^{a}	10.0^{b}	12.6^{ab}	12.4^{ab}	0.49	0.044	0.100	0.082
8−14 d	36.1^{b}	30.9^{b}	49.7^{a}	39.6^{ab}	1.89	0.001	0.315	<0.001
1−14 d	26.0^{b}	24.7^{b}	34.3^{a}	29.4^{ab}	0.98	<0.001	0.274	<0.001
Feed conversion ratio								
1−7 d	0.9	1.0	1.1	0.9	0.03	0.150	0.449	0.144
8−14 d	1.2^{b}	1.3^{b}	2.0^{a}	1.7^{ab}	0.1	0.009	0.297	0.027
1−14 d	1.1^{b}	1.5^{ab}	1.9^{a}	1.6^{a}	0.1	0.002	0.951	0.026

[1] C=control broilers without BSFP supplementation and DSS challenge; D=DSS-challenged broilers; LD=1 g/kg BSFP-treated DSS-challenged broilers; HD=3 g/kg BSFP-treated DSS-challenged broilers

[2] Data was analyzed using the results of D, LD, and HD groups.

[a−b] Means of a row with no common superscript are significantly different ($P<0.05$).

Table 3. **Effects of *B. Subtilis*-Fermented Products on the Small Intestine Morphology of Broilers under Dextran Sulfate Sodium Challenge**

								P value[2]	
		C[1]	D	LD	HD	SEM	*P* value	Linear	Quadratic
Duodenum	Villus length (μm)	1083.3	959.4	1234.0	1100.8	40.25	0.105	0.456	0.014
	Crypt depth (μm)	116.7	117.8	112.5	125.4	7.50	0.379	0.722	0.757
	Villus length: crypt depth	9.4	8.4	11.4	9.4	0.70	0.588	0.860	0.214
Jejunum	Villus length (μm)	434.4	473.4	415.8	573.6	32.26	0.945	0.263	0.341
	Crypt depth (μm)	80.2	68.8	84.7	75.9	4.85	0.705	0.787	0.355
	Villus length: crypt depth	5.5	7.1	5.6	7.7	0.58	0.593	0.639	0.338
Ileum	Villus length (μm)	470.1	395.3	393.3	400.9	19.95	0.228	0.918	0.949
	Crypt depth (μm)	102.8	81.4	95.5	105.7	6.30	0.345	0.261	0.746
	Villus length: crypt depth	4.6	5.4	4.1	4.0	0.31	0.444	0.209	0.388

[1] C＝control broilers without BSFP supplementation and DSS challenge; D＝DSS-challenged broilers; LD＝1 g/kg BSFP-treated DSS-challenged broilers; HD＝3 g/kg BSFP-treated DSS-challenged broilers
[2] Data was analyzed using the results of D, LD, and HD groups.

Table 4. **Effects of *B. Subtilis*-Fermented Products on the Intestinal Inflammatory Gene Expression of Broilers under Dextran Sulfate Sodium Challenge**

								P value[2]	
		C[1]	D	LD	HD	SEM	*P* value	Linear	Quadratic
Duodenum	*cox2*	0.9^{b}	8.5^{a}	2.6^{b}	1.9^{b}	0.91	＜0.001	0.010	＜0.001
	inos	0.8	5.7	1.8	1.3	0.72	0.050	0.063	0.064
	il-1β	0.9^{c}	5.4^{a}	3.1^{b}	0.8^{c}	0.58	＜0.001	＜0.001	0.008
	il-6	0.9^{b}	3.8^{a}	1.9^{b}	0.6^{b}	0.40	＜0.001	0.001	0.020
Jejunum	*cox2*	0.9^{b}	5.2^{a}	0.9^{b}	1.3^{b}	0.57	0.002	0.059	0.001
	inos	0.9^{b}	4.7^{a}	0.8^{b}	1.4^{b}	0.51	0.002	0.082	0.002
	il-1β	1.2^{b}	6.1^{a}	1.2^{b}	0.8^{b}	0.72	0.003	0.024	0.003
	il-6	1.0^{c}	4.4^{a}	2.4^{b}	1.5^{c}	0.41	＜0.001	0.001	0.006
Ileum	*cox2*	0.9^{b}	4.0^{a}	1.8^{b}	0.5^{b}	0.44	0.002	0.002	0.089
	inos	1.3^{b}	3.6^{a}	1.4^{b}	0.4^{b}	0.38	0.006	0.002	0.032
	il-1β	0.9^{b}	6.5^{a}	2.0^{b}	1.4^{b}	0.71	＜0.001	0.014	0.002
	il-6	0.8^{b}	7.2^{a}	2.4^{ab}	1.5^{ab}	0.97	0.031	0.076	0.106

[1] C＝control broilers without BSFP supplementation and DSS challenge; D＝DSS-challenged broilers; LD＝1 g/kg BSFP-treated DSS-challenged broilers; HD＝3 g/kg BSFP-treated DSS-challenged broilers
[2] Data was analyzed using the results of D, LD, and HD groups.
[a–c] Means of a row with no common superscript are significantly different ($P<0.05$).

was observed as the inclusion level of BSFP increased ($P<0.05$).

Effects of BSFP on Cecal Bacterial Diversity

The effects of BSFP on the cecal microbiota of broilers in response to DSS challenge are shown in Table 5. The cecal species richness (Chao1 and Fisher alpha) was reduced in the D and LD groups ($P<0.05$). A linear increase in cecal species richness of DSS-challenged broilers was observed as the inclusion level of BSFP increased ($P<0.05$). The cecal species evenness (Shannon and Enspie alpha) increased in the HD group ($P<0.05$). A linear increase in cecal species evenness in DSS-challenged broilers was observed as the inclusion level of BSFP increased ($P<0.05$). Principal component analysis (PCA) revealed that OTU composition among the groups was not well separated (Fig. 1A). Unweighted principal coordinate analysis (PCoA) revealed two major clusters: samples from C, D, and LD groups were clustered together in one group, whereas samples from HD group were clustered in another group (Fig. 1B). Weighted principal coordinate analysis (PCoA) also revealed two major clustering groups: samples from D, LD, and HD groups were clustered together in one group, while samples from C group were clustered in another group (Fig. 1C). A heatmap of the 35 most abundant genera in the cecal digesta is shown in Fig. 2. Differential bacterial community composition was observed between C D, LD, and HD groups, such as with respect to genera *Ruminococcaceae_unclassified, Escherichia-Shigella*, *Terrisporobacter*, *Clostridium sensu stricto 1*, and *Enterococcus*. A unique bacterial cluster, containing *Sellimonas*, *Lactobacillus*, and *Merdibacter*, was more abundant in the D group, whereas

Table 5. **Effects of *B. Subtilis*-Fermented Products on Bacterial Alpha Diversity in the Cecal Digesta of Broilers under Dextran Sulfate Sodium Challenge**

							P value[2]	
	C[1]	D	LD	HD	SEM	*P* value	Linear	Quadratic
Chao1	48.8[a]	37.5[c]	41.3[bc]	46.0[ab]	1.23	<0.001	<0.001	0.491
Fisher alpha	5.1[a]	3.8[c]	4.3[bc]	4.8[ab]	0.78	<0.001	<0.001	0.213
Shannon	2.6[bc]	2.4[c]	2.7[b]	3.1[a]	0.05	<0.001	<0.001	0.402
Enspie	3.4[b]	3.5[b]	4.4[a]	4.8[a]	0.16	0.001	0.009	0.147

[1] C=control broilers without BSFP supplementation and DSS challenge; D=DSS-challenged broilers; LD=1 g/kg BSFP-treated DSS-challenged broilers; HD=3 g/kg BSFP-treated DSS-challenged broilers
[2] Data was analyzed using the results of D, LD, and HD groups.
[a–c] Means of a row with no common superscript are significantly different ($P<0.05$).

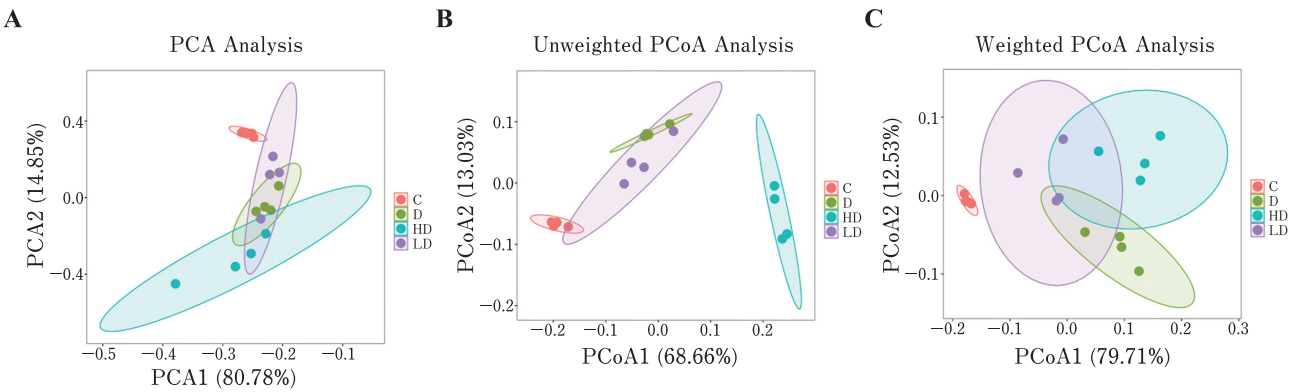

Fig. 1. **Analysis of the bacterial communities of cecal digesta.** (A) Principal component analysis of the cecal digesta of basal diet without treatment (C), basal diet plus dextran sodium sulfate (DSS) challenge (D), basal diet plus 1 g/kg of *Bacillus subtilis*-fermented products (BSFP) and DSS (LD), and basal diet plus 3 g/kg of BSFP and DSS (HD) ($n=4$). Principal coordinate analysis of (B) quantitative traits (unweighted UniFrac distances) and (C) qualitative traits (weighted UniFrac distances) of the cecal bacterial communities from C, D, LD, and HD ($n=4$).

another bacterial cluster, containing *Butyricicoccus*, was less abundant in the D group than in the other groups. Some genera (*Oscillibacter*, *Blautia*, and *[Ruminococcus] torques* group) were more abundant in the C group, whereas their abundance was reduced in the HD group. Similar bacterial community clusters, such as a cluster containing *Lachnospiraceae_unclassified* and *Ruminiclostridium 5*, were observed between the C and LD groups. Out of all four groups, microbial co-occurrence networks in the LD group were the most complicated (Fig. 3). In contrast, microbial co-occurrence networks were the simplest in the HD group (Fig. 3).

Effects of BSFP on Cecal Bacterial Taxonomic Composition

The effects of BSFP on bacterial taxonomy in the cecal digesta of broilers in response to DSS challenge is shown in Table 6. Relative to the C group, at the phylum level, the abundance of the phylum Firmicutes was reduced in the D, LD, and HD groups ($P<0.05$). The abundance of the phylum Proteobacteria increased in the D, LD, and HD groups, compared with the C group ($P<0.05$). At the genus level, the abundance of the genus *Ruminococcus torques group* in the D, LD, and HD groups was lower than that in the C group ($P<0.05$). BSFP supplementation at 3 g/kg further reduced the abundance of the genus *Ruminococcus torques group* in DSS challenged broilers, compared with the D and LD groups ($P<0.05$). A linear decrease in the abundance of the genus *Ruminococcus torques* in DSS-challenged broilers was observed as the inclusion level of BSFP increased ($P<0.05$). Relative to the C group, the abundance of the genus *Escherichia-Shigella* in the D, LD, and HD groups was higher than that in the C group ($P<0.05$). The abundance of the genera *Lachnospiraceae_unclassified* and *Butyricicoccus* in the D and HD groups decreased compared with other groups ($P<0.05$). In contrast, the abundance of the genus *Clostridium sensu stricto 1* in the D and HD groups was higher than in the other groups ($P<0.05$). The abundance of the genus *Ruminococcaceae_unclassified* in the HD group was higher than that in the other groups ($P<0.05$). A linear and quadratic increase in the abundance of the genus *Ruminococcaceae_unclassified* in DSS-challenged broilers was observed as the inclusion level of BSFP increased ($P<0.05$). Relative to the C group, the

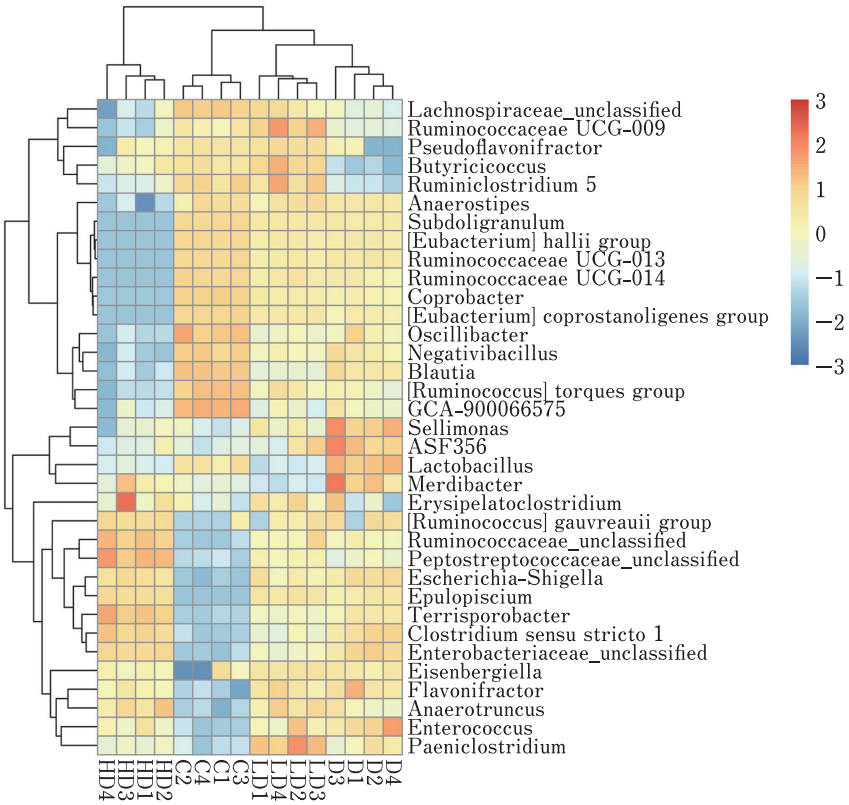

Fig. 2. **Taxonomic composition analysis of cecal digesta.** Heatmap showing the dominant 35 genera (y-axis) across different treatment groups (x-axis, basal diet without treatment (C), basal diet plus dextran sodium sulfate (DSS) challenge (D), basal diet plus 1 g/kg of *Bacillus subtilis*-fermented products (BSFP) and DSS challenge (LD), and basal diet plus 3 g/kg of BSFP and DSS challenge (HD), $n=4$).

abundance of the genus *Lactobacillus* in the D group increased ($P<0.05$). BSFP supplementation (1 and 3 g/kg) in combination with DSS challenge reduced the abundance of the genus *Lactobacillus* compared with the other groups ($P<0.05$). A linear decrease in the abundance of the genus *Lactobacillus* in DSS-challenged broilers was observed as the inclusion level of BSFP increased ($P<0.05$). The abundances of the genera *Enterococcus* and *Sellimonas* in the D group were higher than those in the other groups ($P<0.05$). A linear and quadratic decrease in the abundance of the genus *Sellimonas* in DSS-challenged broilers was observed as the inclusion level of BSFP increased ($P<0.05$).

Correlation Analysis of Bacterial Abundance Among the Dominant 10 Genera

The abundances of the genera *Butyricicoccus*, *Lachnospiraceae_unclassified*, and *Ruminococcus torques group* were positively correlated with each other (Fig. 4). The abundance of the genus *Butyricicoccus* was negatively correlated with the abundances of the genera *Enterococcus*, *Lactobacillus*, and *Sellimonas* (Fig. 4). The abundances of the genera *Enterococcus*, *Lactobacillus*, and *Sellimonas* were positively correlated with each other (Fig. 4).

Discussion

The gut microbiota is strongly shaped by the host environment, and developing a healthy gut microbiota can prevent inflammation and improve the growth of broilers (Pourabedin and Zhao, 2015). Intestinal inflammation disturbs microbial communities, resulting in intestinal microbiota dysbiosis (Lupp *et al*., 2007; Lobionda *et al*., 2019). DSS challenge can cause intestinal inflammation, body weight loss, diarrhea, and necrotic enteritis in poultry (Menconi *et al*., 2015; Kuttappan *et al*., 2016; Nii *et al*., 2020). In this study, the body weight and average daily gain were reduced in the D group, which is in agreement with a previous study (Nii *et al*., 2020). Dietary supplementation with *B. subtilis* has been shown to alleviate LPS-induced intestinal immunological stress, and improve intestinal barrier gene expression in broilers (Gadde *et al*., 2017). Furthermore, our recent study demonstrated that BSFP can improve the growth performance of broilers under LPS challenge (Chen and Yu, 2021). The body weight and average daily gain of broilers were partially improved by supplementation with BSFP in the present study. Interestingly, BSFP supplementation at 1 g/kg increased the average daily feed intake of DSS-challenged broilers at 8 to 14 days and 1

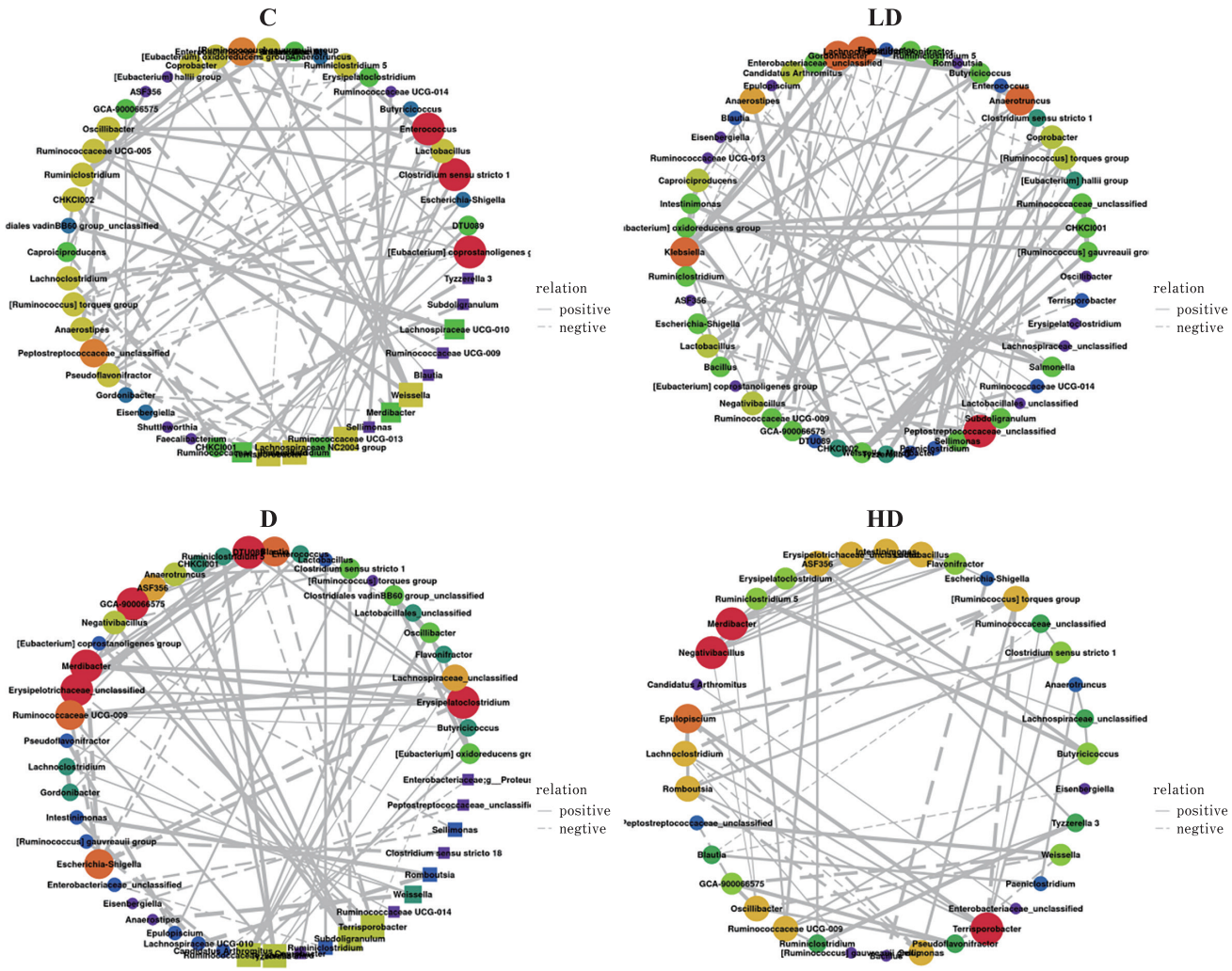

Fig. 3. **Microbial co-occurrence networks in the cecal digesta.** Co-occurrence networks showing the dominant 50 genera across different treatment groups (basal diet without treatment (C), basal diet plus dextran sodium sulfate (DSS) challenge (D), basal diet plus 1 g/kg of *Bacillus subtilis*-fermented products (BSFP) and DSS challenge (LD), and basal diet plus 3 g/kg of BSFP and DSS challenge (HD), $n=4$). The solid and dotted lines represent positive and negative correlations, respectively.

to 14 days of age, resulting in an increased feed conversion ratio. There is no clear explanation for this increase in average daily feed intake in the LD group. In this study, BSFP supplementation alleviated the expression of inflammatory genes in the small intestine of DSS-challenged broilers during the starter phase, which is in agreement with previous studies (Gadde *et al*., 2017; Chen and Yu, 2021). Taken together, the results indicate that BSFP supplementation can partially improve body weight and average daily gain of DSS-challenged broilers. DSS-induced intestinal inflammation in broilers was normalized by BSFP supplementation. Whether the beneficial effect of BSFP on anti-inflammation and growth performance of broilers under DSS challenge is still observed after a prolonged feeding period (market age of 35 days) remains to be confirmed in the future.

The gut morphology was not improved by supplementation with BSFP in DSS-challenged broilers in the present study. The gut microbiota may play a critical role in improving the growth performance of DSS-challenged broilers. Our previous study also demonstrated that BSFP can modulate the gut microflora composition of broilers under LPS challenge (Chen and Yu, 2021). The cecal species richness and evenness were not altered in response to LPS challenge in broilers, whereas LPS challenge in combination with BSFP supplementation dose-dependently reduced cecal species richness (Chen and Yu, 2021). In contrast, cecal species richness was reduced in response to DSS challenge in broilers, whereas BSFP supplementation at 3 g/kg increased the cecal species richness of DSS-challenged broilers. Similar to the LPS challenge, the cecal species evenness was not altered in response

Table 6. **Bacterial Taxonomy within the Cecal Digesta Of Broilers**

	Relative abundance (%)						*P* value[2]	
	C[1]	D	LD	HD	SEM	*P* value	Linear	Quadratic
Phylum								
Firmicutes	95.8[a]	83.0[b]	88.3[b]	83.6[b]	1.47	<0.001	0.870	0.751
Proteobacteria	1.5[b]	17.0[a]	11.7[a]	16.4[a]	1.72	<0.001	0.858	0.764
Genus								
Lachnospiraceae_unclassified	46.0[a]	25.5[b]	37.7[a]	21.3[b]	2.73	<0.001	0.261	0.370
Ruminococcaceae_unclassified	3.3[b]	12.0[b]	15.2[b]	30.4[a]	2.76	<0.001	<0.001	0.019
Ruminococcus torques group	23.6[a]	12.4[b]	14.7[b]	6.4[c]	1.65	<0.001	0.008	0.098
Escherichia-Shigella	1.4[b]	15.7[a]	11.2[a]	15.1[a]	1.60	<0.001	0.895	0.696
Clostridium sensu stricto 1	0.5[b]	8.8[a]	2.3[b]	10.8[a]	1.21	<0.001	0.262	0.293
Lactobacillus	3.7[b]	8.2[a]	0.7[c]	0.8[c]	0.80	<0.001	0.007	0.053
Enterococcus	0.4[b]	3.9[a]	2.3[ab]	1.7[ab]	0.45	0.041	0.087	0.075
Butyricicoccus	2.3[ab]	0.5[c]	3.6[a]	1.4[bc]	0.32	<0.001	0.731	0.545
Sellimonas	1.3[b]	2.7[a]	1.7[b]	1.4[b]	0.16	<0.001	0.002	0.009
Erysipelatoclostridium	0.8	1.1	1.5	2.0	0.24	0.329	0.251	0.390

[1] C=control broilers without BSFP supplementation and DSS challenge; D=DSS-challenged broilers; LD=1 g/kg BSFP-treated DSS-challenged broilers; HD=3 g/kg BSFP-treated DSS-challenged broilers
[2] Data was analyzed using the results of D, LD, and HD groups.
[a-c] Means of a row with no common superscript are significantly different ($P<0.05$).

to DSS challenge in broilers. However, BSFP supplementation (1 and 3 g/kg) increased cecal species evenness in DSS-challenged broilers. Interestingly, BSFP supplementation at 3 g/kg simplified microbial co-occurrence networks in the cecal digesta of DSS-challenged broilers. Simplified microbial occurrence networks generally indicate a low degree of interaction between beneficial and harmful bacteria. The beneficial bacterial population may be greater than the harmful bacterial population, and *vice versa*. In the present study, BSFP supplementation at 3 g/kg simplified microbial co-occurrence networks, and partially improved the growth performance of broilers under DSS challenge. Therefore, the simplified microbial co-occurrence networks in this group may benefit the health of broilers. Unweighted principal coordinate analysis also demonstrated distinct clusters separating the HD group from the other three groups, indicating that BSFP supplementation at 3 g/kg can significantly modify the cecal microbiota of broilers under DSS challenge. Collectively, these findings demonstrate that LPS and DSS challenge is unable to change the cecal species evenness, but differentially regulates the cecal species richness in broilers. Furthermore, BSFP supplementation differentially modulates cecal bacterial diversity in two *in vivo* inflammatory models in broilers. However, the detailed mechanism by which BSFP supplementation differentially modulates cecal bacterial diversity in response to LPS or DSS challenges remains to be elucidated.

The Firmicutes phylum is predominant in the gut of poultry, and is associated with the efficiency of energy harvesting in broilers (Hou *et al*., 2016). Decreased abundance of the phylum Firmicutes has been associated with inflammatory bowel disease (Magne *et al*., 2020). The phylum Proteobacteria contains many opportunistic pathogens, and an increase in its abundance has been found to be correlated to gut dysbiosis (Shin *et al*., 2015). In this study, the abundance of the phylum Firmicutes was decreased, and the abundance of the phylum Proteobacteria was increased, in the D, LD, and HD groups. These results indicate that DSS challenge can change the balance of cecal microbiota composition at the phylum level in broilers. At the genus level, the abundance of the genus *Escherichia-Shigella* increased in the D, LD, and HD groups. The genus *Escherichia-Shigella* consists of gram-negative pathogenic bacteria that have been reported to be negatively associated with health and growth performance in broilers (Shi *et al*., 2014; Han *et al*., 2021). In contrast, the abundance of the genus *Ruminococcus torques group* decreased in the D, LD, and HD groups. The bacteria of genus *Ruminococcus torques group* are able to degrade mucin in the gastrointestinal tract, decreasing gut barrier integrity (Malinen *et al*., 2010; De Cesare *et al*., 2017). The abundance of the genus *Escherichia-Shigella* in cecal digesta of broilers was negatively correlated with the abundance of the genus *Ruminococcus torques group* in this study, indicating that bacteria of genus *Escherichia-Shigella* may inhibit the growth of bacteria of genus *Ruminococcus torques group*. It has been demonstrated that bacteria of the family *Ruminococcaceae* are involved in short-chain fatty acid synthesis, and short-chain fatty acids can be used as an energy and carbon source for broilers (van Der Wielen *et al*., 2000; Xie *et al*., 2021). In this study, the abundance of the genus *Ruminococcaceae_unclassified* specifically increased in the HD group. Whether BSFP supplementation at 3 g/kg can promote short-chain fatty acid production in cecal digesta remains to be confirmed in the future. Bacteria of the genus *Sellimonas* are considered a potential biomarker of homeostasis gut recovery (Muñoz *et al*., 2020). In the present study, the abundance of the genus *Sellimonas* specifically increased in the D group, but fell back to normal levels when BSFP (1 and 3 g/kg) was supplied in the diet. Bacteria of the genus

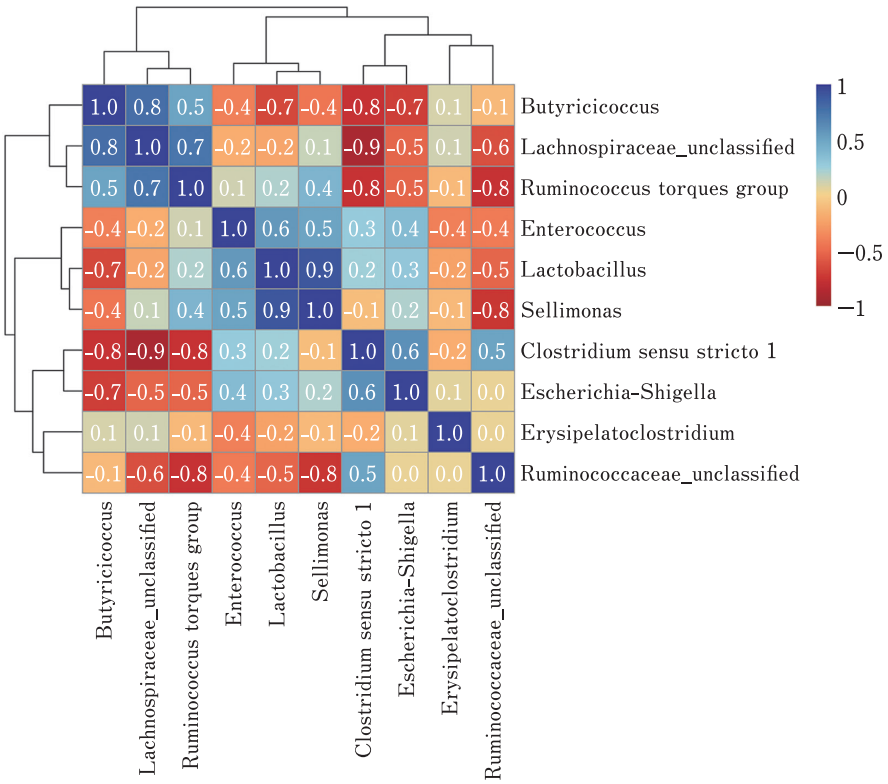

Fig. 4. **Correlation analysis of bacterial abundance among the dominant 10 genera.** Positive correlations are shown in blue, while negative correlations are shown in red. The values from +1 to −1 indicate the strength of the association.

Enterococcus are opportunistic pathogens, and are resistant to several antimicrobials in poultry (d'Azevedo *et al*., 2006; Medina Fernández *et al*., 2019). Furthermore, *Bacillus* strains have been shown to be effective at inhibiting pathogenic *Enterococcus* species *in vitro* (Medina Fernández *et al*., 2019). In this study, the abundance of genus *Enterococcus* was partially reduced under DSS challenge in combination with BSFP supplementation, which is in agreement with a previous study (Medina Fernández *et al*., 2019). The genus *Butyricicoccus* consists of butyric acid-producing bacteria with probiotic potential in broilers (Eeckhaut *et al*., 2016). Butyric acid produced by bacteria of the genus *Butyricicoccus* has been reported to inhibit the growth of pathogenic bacteria such as *Salmonella* and *Clostridium perfringens* in broilers (Timbermont *et al*., 2010). The abundance of the genus *Butyricicoccus* was specifically reduced in the D group, but returned back to normal levels when BSFP (1 g/kg) was supplied in the diet in the present study. This finding is in agreement with the results of Keerqin *et al*. (2021), who observed that *Bacillus subtilis* supplementation increased the abundance of the genus *Butyricicoccus* in the cecal digesta, and improved the health of broiler chickens under necrotic enteritis conditions. Taken together, these results indicate that BSFP supplementation can restore gut microbiota dysbiosis in broilers during the starter phase under DSS challenge.

It has been reported that *B. subtilis*-derived antimicrobial cyclic lipopeptides are effective at inhibiting LPS-induced inflammation *in vitro* (Zhang *et al*., 2015). *B. subtilis*-derived antimicrobial cyclic lipopeptides also exhibit antibacterial activity against common enteric pathogens, such as *C. perfringens*, *Brachyspira hyodysenteriae*, and *Eimeria tenella* (Horng *et al*., 2019; Yu *et al*., 2021). *B. subtilis* spores are able to germinate in the gastrointestinal tract of broilers, exerting immunomodulatory and health-promoting effects through the production of antimicrobial cyclic lipopeptides, and competitive exclusion of pathogens (Latorre *et al*., 2014; Gadde *et al*., 2017). BSFP contain both *B. subtilis* spores and its antimicrobial cyclic lipopeptides, which may contribute to the protective effects on broilers under DSS challenge. Thus, the potential mechanisms proposed on the basis of this study are as follows: (1) *B. subtilis*-derived antimicrobial cyclic lipopeptides promote immunomodulation in the gut mucosal immune system, (2) *B. subtilis*-derived antimicrobial cyclic lipopeptides inhibit enteric pathogen growth, and (3) *B. subtilis* spores regulate gut microbiota by competitive exclusion or production of antimicrobial lipopeptides.

In conclusion, we demonstrated for the first time that BSFP supplementation partially improved the body weight of DSS-challenged broilers at 7 and 14 days of age. Broiler body weight at 7 days of age and average daily gain at 1 to 7 days of age increased linearly with increasing BSFP levels. BSFP supplementation dose-dependently normalized intestinal in-

flammation and cecal bacterial diversity in broilers under DSS challenge. Whether altered gut microbiota caused by BSFP has a direct positive effect on health and growth remains to be confirmed in the future.

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Author contributions

Conceptualization, Yu-Hsiang Yu; methodology, Jiun-Yu Chen and Yu-Hsiang Yu; validation, Jiun-Yu Chen and Yu-Hsiang Yu; formal analysis, Jiun-Yu Chen and Yu-Hsiang Yu; investigation, Jiun-Yu Chen and Yu-Hsiang Yu; resources, Yu-Hsiang Yu; data curation, Yu-Hsiang Yu; writing-original draft preparation, Yu-Hsiang Yu; writing-review and editing, Yu-Hsiang Yu; supervision, Yu-Hsiang Yu; funding acquisition, Yu-Hsiang Yu. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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