Effects of *Bacillus subtilis* and *Bacillus licheniformis* on growth performance, immunity, short chain fatty acid production, antioxidant capacity, and cecal microflora in broilers

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**ABSTRACT** This study investigated the effects of dietary supplementation with *Bacillus subtilis* (B. *subtilis*) or *Bacillus licheniformis* (B. *licheniformis*) on growth performance, immunity, antioxidant capacity, short chain fatty acid (SCFA) production, and the cecal microflora in broiler chickens. In total, 360 male, 1-day-old Cobb 500 birds were randomly divided into 3 groups: the control group was fed a basal diet; the B. *subtilis* group was fed a basal diet supplemented with $1.5 \times 10^9$ CFU/kg B. *subtilis*; the B. *licheniformis* group was fed a basal diet supplemented with $1.5 \times 10^9$ CFU/kg B. *licheniformis*. Results showed that chickens supplemented with either B. *subtilis* or B. *licheniformis* had comparatively higher ($P < 0.05$) body weight and average daily gain, whereas no difference ($P > 0.05$) was observed in feed efficiency. Concentrations of serum IgA, IgY, and IgM, as well as anti-inflammatory IL-10 were significantly increased ($P < 0.05$), and proinflammatory IL-1β and IL-6 were significantly decreased ($P < 0.05$) by B. *subtilis* or B. *licheniformis* supplementation. Moreover, chickens fed with diets supplemented by either B. *subtilis* or B. *licheniformis* had greater antioxidant capacity, indicated by the notable increases ($P < 0.05$) in glutathione peroxidase, superoxide dismutase, and catalase, along with decrease ($P < 0.05$) in malondialdehyde. Compared to the control group, levels of SCFA, excluding acetic and propionic acid, in cecal content had improved ($P < 0.05$) by adding B. *licheniformis*, and significant increase ($P < 0.05$) in acetic and butyric acid was observed with B. *subtilis* supplementation. Microbial analysis showed that both B. *subtilis* or B. *licheniformis* supplementation could increase butyrate-producing bacteria such as *Alisteripes* and *Butyricicoccus*, and decrease pathogenic bacteria such as the *Synergistetes* and *Gamma-proteobacteria*. In summary, dietary supplemented with B. *subtilis* or B. *licheniformis* improved growth performance, immune status, and antioxidant capacity, increased SCFA production, and modulated cecal microbiota in chickens. Moreover, B. *licheniformis* was more effective than B. *subtilis* with the same supplemental amount.

**Key words:** probiotics, broiler, growth performance, immunity, and cecal microflora

**INTRODUCTION**

Growth-promoting antibiotics are commonly used worldwide to provide protection against disease and ultimately improve growth performance in poultry. However, due to the increasing challenges caused by the extensive use of antibiotics, including environmental pollution and the development of bacterial antibiotic resistance (Mehdi et al., 2018; Roth et al., 2019), growth-promoting antibiotics have been banned for use as feed additives, in Europe since 2006, the United States since 2014, and China since 2020. Therefore, seeking safe and effective antibiotic alternatives is of primary importance in the animal industry.

Probiotics are defined as live microorganisms that can have beneficial physiological effects on their host (Chauvyras-Durand and Durand, 2010; Santacroce et al.,...
Use of probiotics as an alternative to antibiotics in poultry diets, has gained considerable attention in the feed industry in recent years (Alagawany et al., 2018; Al-Khalafiah, 2018). Several studies have indicated that probiotics used in the poultry industry can regulate intestinal microbiota structure, enhance immunity to improve disease resistance, promote the digestion and absorption of nutrients, and ultimately improve growth and production performance (Cox and Dalloul, 2015; Khan et al., 2020; Tarradas et al., 2020). Mounting evidence suggests that among the probiotics, Bacillus subtilis (B. subtilis) and Bacillus licheniformis (B. licheniformis) have been recognized as safe for animal dietary use (EFSA, 2007) and can be effectively applied as alternatives to antibiotic growth promoters. Previous studies have shown that B. subtilis can improve the growth performance of heat-stressed broilers and enhance the recovery and restoration processes of damaged intestinal mucosa (Al-Fataftah and Abdelgader, 2014; Abdelgader et al., 2020). Furthermore, B. subtilis can compete with pathogens, balance intestinal microbiota, and enhance immunity in chickens (Elshaghabee et al., 2017; Abudabos et al., 2019; Guo et al., 2020). Similarly, B. licheniformis can enhance the growth performance of chickens (Liu et al., 2012) and maintain intestinal microbiota balance in broilers (Chen and Yu, 2020). Meanwhile, B. licheniformis also helps prevent necrotic enteritis in chickens by normalizing the ileal microbiota (Knap et al., 2010; Xu et al., 2018). Although ample studies have been conducted involving B. subtilis or B. licheniformis in broilers, comparative studies of these 2 probiotics from multiple perspectives, are limited. Therefore, the objective of the present study was to evaluate the effects of continuous dietary supplementation with B. subtilis or B. licheniformis, on growth performance, immunity, antioxidant capacity, short chain fatty acid (SCFA) production and cecal microflora in broilers.

MATERIALS AND METHODS

All animal experiments were conducted in accordance with the principles and specific guidelines presented in Guide for the Care and Use of Agricultural Animals in Research and Teaching (McGhile et al., 2010), and approved by the Research Center Institutional about Animal Care and Use Committee of Zhejiang Agricultural and Forestry University.

Experimental Design

A total of 360 male, 1-day-old (Cobb 500) broilers were randomly divided into 3 groups fed with different diets treatments. Each group consisted of 8 replicate pens with 15 chickens in per replicate. Dietary treatments included a basal diet (CON), a basal diet supplemented with 1.5 × 10^{8} CFU/kg of B. subtilis (BS), and a basal diet supplemented with 1.5 × 10^{8} CFU/kg of B. licheniformis (BL). The feed and water were available ad libitum. The basal diet was formulated to meet the nutritional requirements of broilers described by the NRC (1994), and the ingredient and chemical compositions of the basal diets used in this study are shown in Table 1. The feeding program consisted of 2 phases, namely starter phase (d 1 to 21) and finisher phase (d 22 to 42). The lighting program provided 23 h of light and 1 h of darkness, up to the end of the experiment. Room temperature was controlled at 32°C to 35°C for the first week and then thereafter reduced by 3°C to 5°C per week, to reach a final temperature of 26°C. The B. Subtilis (HJKC02) and the B. Licheniformis (HJDY01) strains were provided by Vegamax Biotechnology Co., Ltd. (Anji, Zhejiang, China).

Table 1. Composition and nutrient levels of the basal diet.

| Items                          | 1−21  | 22−42 |
|-------------------------------|-------|-------|
| Ingredients (air-dry basis,%)  |       |       |
| Corn                          | 56.33 | 57.4  |
| Soybean meal                  | 24.5  | 19    |
| Extruded soybean              | 5     | 4     |
| Corn distillers dried grains  | 5     | 8     |
| Corn gluten meal              | 2     | 3     |
| Soybean oil                   | 1.2   | 4.3   |
| Limestone                     | 1.3   | 1.3   |
| Fermented soybean meal        | 1.67  | 0     |
| Premix<sup>1</sup>            | 3     | 3     |
| Total                         | 100.00| 100.00|
| Nutrient levels               |       |       |
| Metabolizable energy (kcal/kg)| 2949  | 3148  |
| Crude protein (%)             | 20.6  | 18.6  |
| Crude fat                     | 4.9   | 8.0   |
| Lysine (%)                    | 1.17  | 0.99  |
| Methionine + Cysteine (%)     | 1.45  | 1.23  |
| Threonine + Tryptophan (%)    | 1.13  | 0.95  |
| Calcium (%)                   | 0.88  | 0.79  |
| Total phosphorus (%)          | 0.64  | 0.56  |

<sup>1</sup>Minimal vitamin levels per kg of diets: vitamin A (retinyl acetate), 1,500 IU; cholecalciferol, 200 IU; vitamin E (DL-α-tocopheryl acetate), 10 IU; riboflavin, 3.5 mg; pantothenic acid, 10 mg; niacin, 30 mg; cobalamin, 10μg; choline chloride, 1,000 mg; biotin, 0.15 mg; folic acid, 0.5 mg; thiamine 1.5 mg; pyridoxine 3.0 mg.

<sup>2</sup>Minimal mineral levels per kg of diet: Fe 80.00 mg; Cu 8.00 mg; Mn 60.00 mg; Zn 40.00 mg; I 0.18 mg; Se 0.15 mg.

Growth Performance

Body weight (BW) of broiler chickens was measured on d 1, 21, and 42 of the experiment, whereafter the average daily gain (ADG), average daily feed intake (ADFI), and the feed:gain ratio (F:G) for each cage were calculated for each phase. The individual cages were considered as the experimental unit.

Sample Collection

On d 42, 1 bird was chosen from each replicate. After weighing, a blood sample was collected from wing vein of each bird into a 10 mL tube. After centrifuging at 3,000 × g for 15 min at 4°C, serum samples were obtained and stored at −20°C for further study. Broilers were euthanized by cervical dislocation and immediately...
autopsied, where 2 to 3 g cecal contents were collected from each bird and stored at −80°C until analysis.

**Serum Immunoglobulin Content Analyses**

The serum IgA (Cat# ANG-E32004C), IgM (Cat# ANG-E32005C), IgY (Cat# ANG-E32209C), IL-1β (Cat# ANG-E32031C), IL-6 (Cat# ANG-E32013C), and IL-10 (Cat# ANG-E32011C) were measured using the chicken-specific ELISA kits obtained from Nanjing Aoqing Biotechnology Co., Ltd (Nanjing, Jiangsu, China).

**Serum Biochemical Indexes Content Analyses**

The serum biochemical indices, including malondialdehyde (MDA) (Cat# ANG-SH-10112), glutathione peroxidase (GSH-Px) (Cat# ANG-SH-10202), superoxide dismutase (SOD) (Cat# ANG-SH-10012), and catalase (CAT) (Cat# ANG-SH-10122) were assayed separately using specific kits (Nanjing Aoqing Biotechnology Co., Ltd, Nanjing, Jiangsu, China).

**SCFAs Measurement and Analysis**

Referring to the assay method of Yang et al. (2019), the concentration of SCFAs in cecal content was estimated by gas chromatography using a 7890B Network GC System and 7693 Automatic Liquid Sampler with G4513A injector (Agilent Technologies, Santa Clara, CA) equipped with a 30 m × 0.25 mm × 0.25 μm DB-FFAP Column (Cat# 122-3232, Agilent Technologies,), and a flame ionization detector. Briefly, 1 g of cecal content was dissolved in pure water. Following shock mixing and high-speed centrifugation (12,000 × g for 10 min at 4°C), the supernatant was extracted and mixed with 25% (m/v, 1:3) phosphoric acid. The mixed liquid was stabilized on ice for 35 min and filtered into the special injection bottles for machine detection.

**16S rRNA Sequencing and Analysis**

Cecal contents were collected for 16S rDNA sequence analysis after 42 days of feeding. The trademarked E.Z.N. A. Soil DNA Kit (Cat# D5625-01, Omega Bio-tek, Norcross, GA) was used to extract total bacterial DNA samples, which were stored at −80°C for further analysis. The V4 regions of 16S rRNA were amplified with primers 515F/806R (515F: 5′-GTGCCAGCMGCCGCGGTAA-3′, 806R: 5′-GGACTACVSGGG TATCTAAT-3′) using the Applied Biosystems GENEMAP 9700 thermocycler PCR system (Cat# 4413750, Thermo Fischer Scientific, Waltham, MA). PCR-amplification products were purified by 2% agarose gel electrophoresis, and the AXYGEN DNA Gel Extraction Kit (Cat# AP-GX-250G, Union City, CA) was used for recovery of DNA from the gels. Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on the Illumina MiSeq platform (PE300, Illumina, San Diego, CA) according to the standard protocols described by Majorbio Bio-Pharm Technology Co. Ltd. (Pudong, Shanghai, China).

Operational taxonomic units (OTUs) were generated at a 97% similarity threshold, and the taxonomy of each 16S rRNA gene sequence was analyzed using the Ribosomal Database Project (RDP) classifier algorithm against the database, at a confidence threshold of 70%. The microbiota diversity analysis included an alpha diversity component—Shannon, Chao, and Simpson, and observed species indices—and a beta diversity component in different samples. Weighted Unifase distances were visualized by principal component analysis (PCA) and principal co-ordinates analysis (PCoA).

Furthermore, linear discriminant analysis coupled with effect size (LEfSe) was performed to identify differential expression at species level between the groups of bacterial taxa and compared that with the relative abundance of the different taxa at species level of the bacterial taxa.

**Statistical Analysis**

Obtained data were analyzed by one-way ANOVA using IBM SPSS 22.0 software (IBM Corp., Armonk, NY), the results of which were expressed as mean ± SEM. Means were compared using the Tukey-Kramer test and statistical significance was determined at a value of P < 0.05. A histogram was created by GraphPad Prism version 7.0 (GraphPad Software Inc., San Diego, CA). For the analysis of 16S rRNA sequencing results, the relative abundance was calculated for each sample at different taxonomic levels, and the Kruskal-Wallis H test used to analyze the relative abundances at different taxonomic levels, between the groups included in the experiment. Outcomes of the significant difference tests of alpha (two-sided Student’s t test) and beta diversity (analysis of similarities) were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) bioinformatics pipeline.

### RESULTS

**Growth Performance**

The effects of *B. subtilis* or *B. licheniformis* supplementation on the growth performance of broilers are shown in Table 2. Results showed that supplementation with either *B. subtilis* or *B. licheniformis from d 1 to 42, significantly improved (*P < 0.05*) broiler BW, ADG, and ADFI, compared with broilers from the CON group. Notably, the increases in BW and ADG of BL group were higher (*P < 0.05*) than those of the BS group (*P < 0.05*) throughout the period from d 1 to 42. No significant differences were observed in F: G ratio (*P > 0.05*) of the 3 groups during the period from d 1 to 21, but the BL group had a lower F: G ratio than CON group (*P < 0.05*), during the starter phase.
**Immunoglobulins and Cytokines in Serum**

As showed in Figure 1, a significant increase ($P < 0.05$) in the levels of serum IgA, IgM, and IgY was observed in the BS and BL groups, in comparison to the CON group. Furthermore, levels of IgM and IgY in the BS and BL groups were significantly higher ($P < 0.05$) in the BL group than in BS group. As for the cytokines in serum, $B. subtilis$ or $B. licheniformis$ supplements significantly increased the concentration of anti-inflammatory factor IL-10 ($P < 0.05$), but decreased proinflammatory factors IL-1β and IL-6 ($P < 0.05$). Particularly, the serum IL-6 content of the BL group was significantly lower ($P < 0.05$) than that of BS group.

**Antioxidant Capacity**

The effects of $B. subtilis$ or $B. licheniformis$ treatment on antioxidant activities are shown in Figure 2. The levels of GSH-Px, SOD, and CAT were notably raised ($P < 0.05$) in the BS and BL groups compared to the CON group. Furthermore, the levels of MDA were notably lower ($P < 0.05$) in the BS and BL groups compared to the CON group. No significant difference was observed in antioxidant capacity between the BS and BL groups.

**SCFA Levels in Cecal contents**

Measured levels of SCFA, including acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid are presented in Figure 3. Results indicated that $B. subtilis$ supplementation improved ($P < 0.05$) acetic acid and butyric acid content, compared with the same parameters in the CON group. Furthermore, the levels of butyric acid, valeric acid, and isovaleric acid were upregulated ($P < 0.05$) after $B. licheniformis$ supplementation. However, no apparent effect was observed in propionic acid levels after administration of $B. subtilis$ or $B. licheniformis$ treatments.

**Diversity of Cecal Microbiota**

The changes in diversity of cecal microbiota are summarized in Figure 4. Based on the overlapping regions of the Venn diagram, 685 OTUs were shared between the CON, BS, and BL groups, in contrast, nonoverlapping regions showed far more unique OTUs in the BL group (n = 66) were far more than in the CON (n = 29) and BS (n = 15) group (Figure 4A). Alpha diversity analysis included the observed species, as well as Shannon, Chao, and Simpson indices, which were representative of the richness and diversity of the microbial community. Results indicated that diets containing $B. licheniformis$ presented with more significant diversity in cecal microbiota than $B. subtilis$ supplemented diets, whereas neither $B. subtilis$ nor $B. licheniformis$ supplementation appeared to affect community diversity in cecal microbiota, when compared to the CON group diet (Figures 4B–4D). However, as shown in Figures 4E and 4F, PCoA and PCA plots revealed a degree of diversity discrepancy in cecal microbiota communities between all groups, and especially between the BL and CON groups.

**Composition of Cecal Microbiota**

The relative abundance of OTUs of cecal microbiota was analyzed at different ranking levels from phylum to genus, to reflect the change in cecal microbiota community structure. As shown in Figure 5, at phylum level, the relative abundances of *Synergistota* and *Proteobacteria*, particularly, *c. Gammaproteobacteria* in the BS group, were lower than that in the CON group ($P < 0.05$; Figures 5A–5C, 5E). At genus level,
Butyricicoccus that always be considered as a butyric acid producer was increased more significantly \((P < 0.05)\) in the BS group than in CON and BL groups (Figure 5F). At spices level, the \(s\)-Bacteroides_barнесiae was increased significantly \((P < 0.05)\) in the CON group, compared with the BS group, and tended to be higher than that in the BL. Conversely, relative abundance of the dominant species \(s\)-gut_meta-genome in the BS group was distinctly higher \((P < 0.05)\), than in the CON group (Figures 5G–5I). Furthermore, the outcomes of LEfSe (linear discriminant analysis score = 3), indicated that 19 bacterial taxa were significantly more abundant \((P < 0.05)\) in the CON group, whereas only 14 taxa were overrepresented in the BS group \((P < 0.05)\);
Figure 4. The diversity of microbial community in cecal contents of broilers at d 42. (A) The Venn diagram summarizing the numbers of common and unique OTUs in the microflora community in cecal contents of broilers. (B–D) The shannon index, chao index, simpson reflecting species alpha diversity between groups. (E, F) The principal component analysis (PCA) and principal co-ordinates analysis (PCoA) reflecting species beta diversity within and between groups. CON = basal diet provided as the control; BS = basal diet supplemented with $1.5 \times 10^9$CFU/kg B. subtilis; BL = basal diet supplemented with $1.5 \times 10^9$CFU/kg B. licheniformis. Bars represent mean ± SD (n = 8). *Means different ($P < 0.05$), **means significant difference ($P < 0.01$).

Figure 5. The abundance of microbial community in cecal contents of broilers at d 42. (A, D, G) The top 10 relative abundance of microflora community between groups (level phylum, level genus, and level species) reveal by the histogram. (D–C, E, F, H, I) The bacteria with significant differences between groups (level phylum, level class, level genus and level species) indicate by histogram. CON = basal diet provided as the control; BS = basal diet supplemented with $1.5 \times 10^9$CFU/kg B. subtilis; BL = basal diet supplemented with $1.5 \times 10^9$CFU/kg B. licheniformis. Bars represent mean ± SD (n = 8). *Means different ($P < 0.05$), **means significant difference ($P < 0.01$).
Figure 6). Additionally, 6 bacterial taxa were more abundant in the CON group \((P < 0.05)\), whereas 11 taxa were overrepresented in the BL group \((P < 0.05; \text{Figure 6B})\).

**DISCUSSION**

With the growing interest in probiotics and their application in animal research, more and mounting supports the concept that dietary \(B.\ subtilis\) or \(B.\ licheniformis\) could promote the growth performance of chickens (Bader and Albin et al., 2012; Liu et al., 2012; Chen and Yu, 2020). Consistent with these studies, our results demonstrated that supplementation of broiler diets with \(B.\ subtilis\) or \(B.\ licheniformis\) improved growth performance by increasing the BW, ADG, ADFI, and reducing the F:G ratio, during the starter feeding phase. Moreover, other studies have shown that dietary \(B.\ subtilis\) supplementation or \(B.\ licheniformis\) can improve the growth parameters even under conditions of heat stress, immunological stress, or necrotic enteritis challenge (Al-Fatafah and Abdelqader, 2014; Gadde et al., 2017; Musa et al., 2019; Sokale et al., 2019; Abdelqader et al., 2020). One hypothesis is that improvements in growth performance may be related to beneficial metabolites produced by \(B.\ licheniformis\) or \(B.\ subtilis\), such as extracellular digestive enzymes, lysozyme, antifungal proteins, and varieties of antibiotics, amongst others (Kim et al., 2004; Sahu et al., 2008).

Another possibility is that administration of \(B.\ subtilis\) or \(B.\ licheniformis\) could enhance the broilers’ immunity (Dong et al., 2020; Guo et al., 2020) and regulate intestinal flora composition and metabolic function (Xu et al., 2018; Chen and Yu, 2020; Rodrigues et al., 2020). Additionally, our results showed that improvements in BW and ADG of the \(B.\ licheniformis\)-supplemented group, were significantly greater than those of the \(B.\ subtilis\)-supplemented group, at the same supplemental amount. The reason for this phenomenon requires further research, as few comparative studies are available that demonstrate the differences between \(B.\ subtilis\) or \(B.\ licheniformis\) activity.

Serum immunoglobulins, especially IgA, IgG (its avian counterpart, IgY), and IgM that is produced by B cells, act as important parameters that to reflect the humoral immune status of animals, which relates to their important roles in immune function and providing resistance against various infections (Carlier et al., 2016; Zhang et al., 2017; Balan et al., 2019). In previous studies, levels of IgY and IgA, or IgM in serum were increased in chickens fed with \(B.\ subtilis\) (Bai et al., 2017) or \(B.\ licheniformis\)-supplemented diets (Fazelnia et al., 2021). Our results concurred with previous research in which supplementation with \(B.\ subtilis\) or \(B.\ licheniformis\) significantly increased IgA, IgM and IgY concentrations in serum of broilers after 42 d, reflecting an improvement in immune function.

IL-1\(\beta\) and IL-6 are generally deemed as proinflammatory cytokines produced by classically activated macrophages1. Whereas IL-10, produced by alternatively activated macrophages2, is often considered an anti-inflammatory cytokine. In our research, the levels of IL-1\(\beta\) and IL-6 in serum were reduced and the level of IL-10 increased after \(B.\ subtilis\) or \(B.\ licheniformis\) supplementation. Gadde et al. (2017) and Guo et al. (2020) found that continual administration of \(B.\ subtilis\) following infection with \(E\). maxima or \(E\). coli, down-regulated the expression of IL-1\(\beta\) and IL-6 in the jejunum. Moreover, up-regulated expression of IL-2 and IL-10 in the ileum was observed in an unchallenged experiment (Park et al., 2020). The reason may be that \(B.\ subtilis\) or \(B.\ licheniformis\) could inhibit avian pathogenic growth and enhance functioning of the gut barrier (Wang et al., 2017; Medina et al., 2019; Wang et al., 2020). Interestingly, based on our results, \(B.\ licheniformis\) was more effective than \(B.\ subtilis\) in increasing serum immunoglobulins and decreasing the pro-inflammatory
cytokine, IL-6, further research is required to elucidate the exact, mechanism hereof.

Oxidative stress refers to a state of imbalance between antioxidants and free radicals, which could produce varieties of reactive oxygen species (ROS), in the form of hydroxyl free radicals and superoxide anions, in vivo. Excess ROS can damage the proteins, nucleic acids, and other biological macromolecules, and produce large amounts of MDA, which ultimately leads to tissue damage and the development of disease. However, the antioxidant enzymes, including SOD, glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPx), and CAT are produced simultaneously, to remove excess ROS and maintain a steady state of health (Maritim et al., 2003; Lauridsen, 2019). Oxidative stress in poultry can occur for many reasons, for example, due to heat stress, poor feed quality or pathogenic infection. Consequently, growth performance is reduced and meat quality could even be affected (Rehman et al., 2018; Zaboli et al., 2019; Wasti et al., 2020). According to previous research, supplementation of broiler diets with B. subtilis can enhance the activity of GSH, GR, GSH-Px, and SOD by increasing the antioxidant gene expression of nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase (HO-1), SOD, and GPx in the liver. (Bai et al., 2017). Furthermore, the findings of Zhao et al. (2020) revealed that MDA in the ileum was lower in a B. licheniformis-supplemented group, in which the activities of antioxidant enzymes, such as SOD, CAT, and total antioxidant capacity (T-AOC), and GSH were improved in the ileum, serum, or liver. In the present study, dietary B. subtilis or B. licheniformis were beneficial in improving antioxidant capacity of broilers by the enhancement of SOD, GPx and CAT, and a decrease in MDA. Nonetheless, research on the antioxidant effects of B. subtilis or B. licheniformis effect on antioxidant is still very limited, and the mechanism of antioxidant regulation remains to be further studied.

SCFAs are a group of saturated fatty acids with fewer than 6 carbon atoms. Apart from being the main energy source of colonocytes, there were an abundance of evidence shows that SCFAs play an important role in the maintenance of health and modulation of immune and inflammatory responses (van der Hee and Wells, 2021). Our results demonstrated that dietary addition of B. subtilis, and especially B. licheniformis, could increase the content of various SCFAs in cecal contents. In particular, the increase in butyric acid level was the most significant. Similar results have been observed in a study by Musa et al. (2019). Moreover, according to research by Parada et al. (2019) and Luu et al. (2020), the observed increase in immunoglobulins and changes in cytokines may be related to an increase in butyric acid production.

Numerous studies have revealed that the gut microbiota play an important role in host health status by absorbing nutrients, improving growth and metabolism, resisting harmful bacteria, and modulating the immune system to such an extent that its function is irreplaceable (Heiss and Olofsson, 2018; Pickard et al., 2017; Cheng et al., 2019). The cecum is the site of most abundant and concentrated intestinal flora in which the biological fermentation processes, especially the production of SCFAs, take place. Moreover, the gut microbiota can utilize or ferment feed in different ways and produce different metabolites (Tungland, 2018; Rychkik, 2020).

Our results obtained from PCA and PCoA conducted on cecal content revealed a degree of diversity discrepancy in cecal microbiota. Similar outcomes were achieved in studies by Ma et al. (2018) and Chen and Yu (2020). Furthermore, previous studies demonstrated that B. subtilis or B. licheniformis can inhibit the growth of pathogenic bacteria in vitro and in vivo, by producing certain antimicrobial peptides or inhibiting biofilm formation (Rivardo et al., 2009; Lin et al., 2017; Du et al., 2019; Guo et al., 2020). The Synergistetes, a group of gram-negative anaerobic organisms that can colonize in chicken cecal, and correlate with the fate of certain antibiotic resistance genes (Kubasova et al., 2019; Zhang et al., 2019), are reported opportunistic pathogens that have been detected frequently in cases of human disease such as cysts, abscesses, and oral cavity and dental diseases (Belibasakis et al., 2016; McCracken and Nathalia, 2020). In our study, the proportion of Synergistetes at phylum level, and even at genera level, trended lower in the BL group, and significantly lower in the BS group, than the CON group. Furthermore, the Gammaproteobacteria, which mainly includes certain pathogenic bacteria, such as Escherichia coli, and Salmonella enterica serovar Typhimurium, among others (Zhao and Houry, 2010), were observed in a downward similar trend to that of Synergistetes. This may also explain changes in immunoglobulin and cytokine levels in BS and BL groups. As one of the dominant intestinal bacteria in broilers, Bacteroides possesses strong ability to utilize amino acids and carbohydrates and could be related to immune system development (Zhou and Zhi, 2016; Singh, 2019). In previous studies, the genera Akrisipes and Butyricicoccus, which have been identified as butyrate producers in the gut and demonstrate good anti-inflammatory effects in human and animal experiments (Geirnaert et al., 2014; Eecckhaut et al., 2016; Wu et al., 2020), were observed in higher proportion in the BS group than the CON group. This may account for the increase in butyric acid level and the decrease in inflammatory cytokines, such as IL-1β and IL-6. Moreover, research has indicated that B. subtilis can induce a shift toward butyrate-producing bacteria in the broiler gut microbiome (Jacquier et al., 2019), which was consistent with our results. Per the outcome of LEfSe higher levels of bacteria associated with the production of SCFAs, such as the unclassified_f _Lachnospiraceae, g_Ruminococcus, and g_Faecalibacterium, amongst others (Antonissen et al., 2016; Blasco et al., 2020), were detected after B. subtilis or B. licheniformis supplementation. Considering these combined results, it
may explain the significant increase in level of the SCFAs.

In conclusion, supplementary *B. subtilis* or *B. licheniformis* significantly improved broiler growth performance, which may have been achieved by enhancing immune functions, modulating gut microbiota, and reducing the number of pathogens.

Moreover, based on our results, *B. licheniformis* was more effective than *B. subtilis* in immunomodulating and growth promotion of broilers.

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**DISCLOSURES**

The authors declare no conflict of interest.

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