

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

Yinglei Xu,<sup>\*,1</sup> Yang Yu,<sup>\*,1</sup> Yuanyuan Shen,<sup>\*</sup> Qing Li,<sup>\*</sup> Junhong Lan,<sup>\*</sup> Yanping Wu,<sup>\*</sup> Ruiqiang Zhang,<sup>\*</sup> Guantian Cao,<sup>†</sup> and Caimei Yang<sup>\*,2</sup>

<sup>\*</sup>Key Laboratory of Applied Technology on Green-Eco-Healthy Animal Husbandry of Zhejiang Province, Zhejiang Provincial Engineering Laboratory for Animal Health and Internet Technology, College of Animal Science and Technology College of Veterinary Medicine, Zhejiang Agriculture & Forestry University, Hangzhou 311300, China; and <sup>†</sup>College of Standardisation, China Jiliang University, Hangzhou 310018, China

**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-1 $\beta$  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 *Alis-tipes* and *Butyricicoccus*, and decrease pathogenic bacteria such as the *Synergistetes* and *Gammaproteobacteria*. 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

2021 Poultry Science 100:101358

<https://doi.org/10.1016/j.psj.2021.101358>

## 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 (Chaucheyras-Durand and Durand, 2010; Santacroce et al.,

© 2021 The Authors. Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Received May 8, 2021.

Accepted June 22, 2021.

<sup>1</sup>These authors contribute equally.

<sup>2</sup>Corresponding author: [yangcaimei2012@163.com](mailto:yangcaimei2012@163.com)

2019). 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-Khalaifah, 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 Abdelqader, 2014; Abdelqader 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* (Mcglone 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  $1.5 \times 10^9$  CFU/kg of *B. subtilis* (BS), and a basal diet supplemented with  $1.5 \times 10^9$  CFU/kg of *B. licheniformis* (BL). The feed and water were available

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

Items	Ages (d)	
	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 with solubles	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>1,2</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- $\alpha$ -tocopheryl acetate), 10 IU; riboflavin, 3.5 mg; pantothenic acid, 10 mg; niacin, 30 mg; cobalamin, 10  $\mu$ g; 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 0.18mg; Se 0.15 mg.

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).

### 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  $3,000 \times g$  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  $-80^{\circ}\text{C}$  until analysis.

### Serum Immunoglobulin Content Analyses

The serum IgA (Cat# ANG-E32004C), IgM (Cat# ANG-E32005C), IgY (Cat# ANG-E32209C), IL-1 $\beta$  (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  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$  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  $\times$   $g$  for 10 min at  $4^{\circ}\text{C}$ ), 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  $-80^{\circ}\text{C}$  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 GENEAMP 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  $\times$  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 Uniface 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  $\pm$  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.

**Table 2.** Effects of dietary supplementation of *B. subtilis* or *B. licheniformis* on growth performance in broilers.

Items	Treatments <sup>1,2</sup>			SEM	P-value
	NCO	BS	BL		
Body weight, g/bird					
1 d	37.89	37.94	37.24	0.26	0.156
21 d	758.46	802.22	803.14	9.84	0.072
42 d	1828.94 <sup>c</sup>	2069.88 <sup>b</sup>	2174.37 <sup>a</sup>	37.63	0.001
Average daily gain, g/bird					
1-21 d	34.31	36.39	36.47	0.47	0.069
22-42 d	50.98 <sup>c</sup>	60.54 <sup>b</sup>	65.30 <sup>a</sup>	1.58	0.001
1-42 d	42.64 <sup>c</sup>	48.38 <sup>b</sup>	50.88 <sup>a</sup>	0.89	0.001
Average daily feed intake, g/bird					
1-21 d	55.93	57.30	57.07	0.66	0.689
22-42 d	108.54 <sup>b</sup>	124.26 <sup>a</sup>	129.02 <sup>a</sup>	2.50	0.001
1-42 d	77.58 <sup>b</sup>	85.00 <sup>a</sup>	87.97 <sup>a</sup>	1.20	0.001
Feed: Gain ratio, (g:g)					
1-21 d	1.63 <sup>a</sup>	1.57 <sup>ab</sup>	1.56 <sup>b</sup>	0.01	0.001
22-42 d	2.13	2.05	1.98	0.40	0.156
1-42 d	1.82	1.76	1.73	0.02	0.107

<sup>a,b</sup>Mean with different superscripts in the same row differ significantly ( $P < 0.05$ ).

<sup>1</sup>NCO = basal diet provided as 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*.

<sup>2</sup>The mean represent results from 8 replicate cages per treatment.

## 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 serum 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-1 $\beta$  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

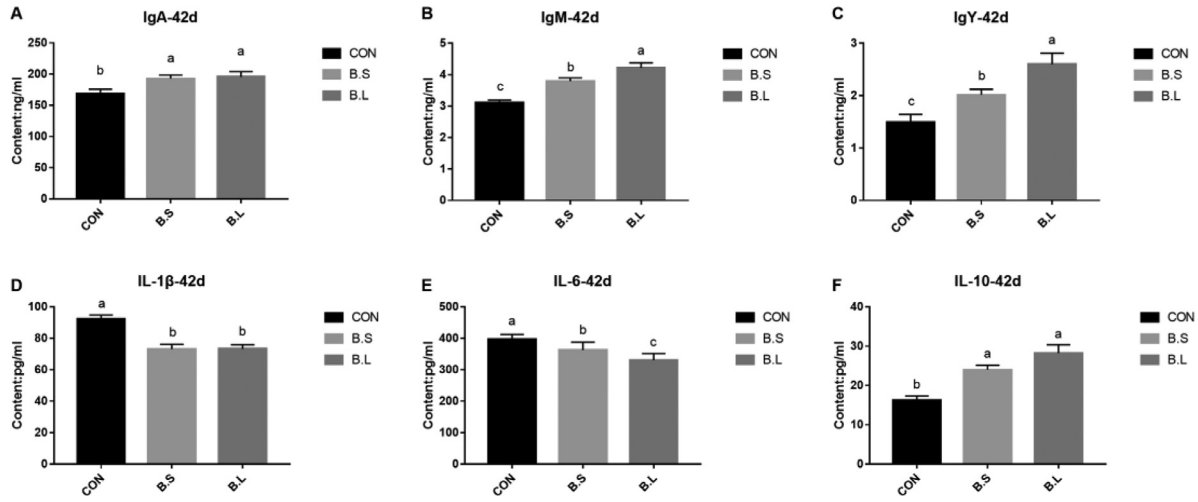
effect was observed in propionic acid levels after administration of *B. subtilis* or *B. licheniformis* treatments.

## Diversity of Cecal Microbiota

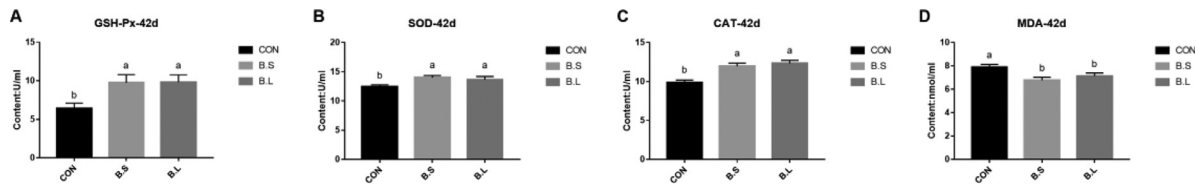
The changes in diversity of cecal microbiota are summarized in [Figure 4](#). Based on the overlapping regions of the Venn diagram, 685 OTUs were shared between the CON, BS, and BL groups, in contrast, nonoverlapping regions showed far more unique OTUs in the BL group ( $n = 66$ ) were far more than 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,



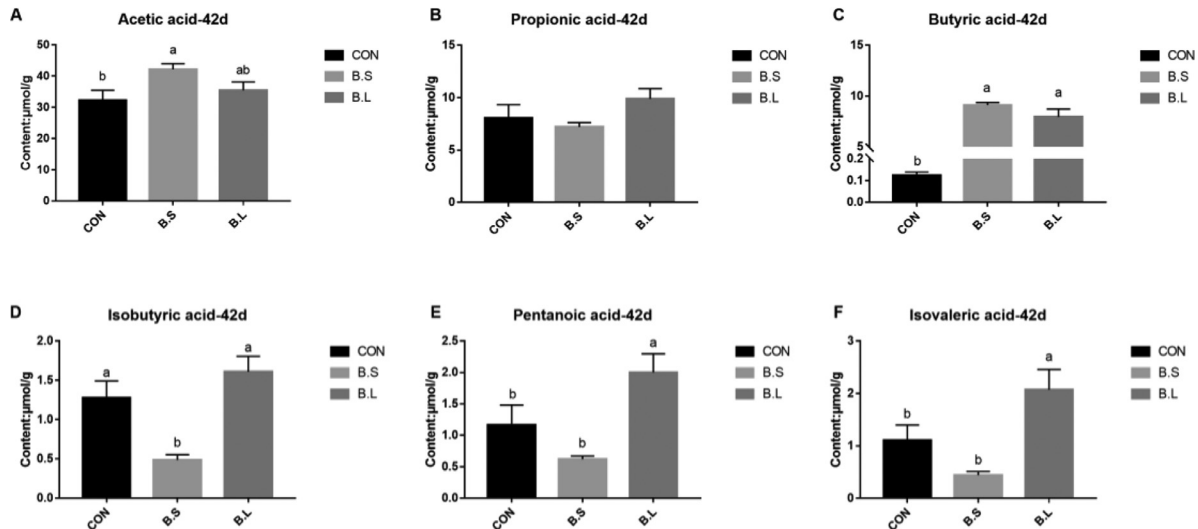
**Figure 1.** The effects of *B. subtilis* or *B. licheniformis* supplement on serum immunoglobulins (A–C) and cytokine (D–F) of broiler chickens at d 42. Levels of immunoglobulins (A–C) and cytokine (D–F) in the serum at d 42. CON = basal diet provided as the control; BS = basal diet supplemented with  $1.5 \times 10^9$ CFU/kg *B. subtilis*; BL = basal diet supplemented with  $1.5 \times 10^9$ CFU/kg *B. licheniformis*. Bars represent mean  $\pm$  SD (n = 8). Different lowercase letters (a, b) above bars represent significantly different means ( $P < 0.05$ ).



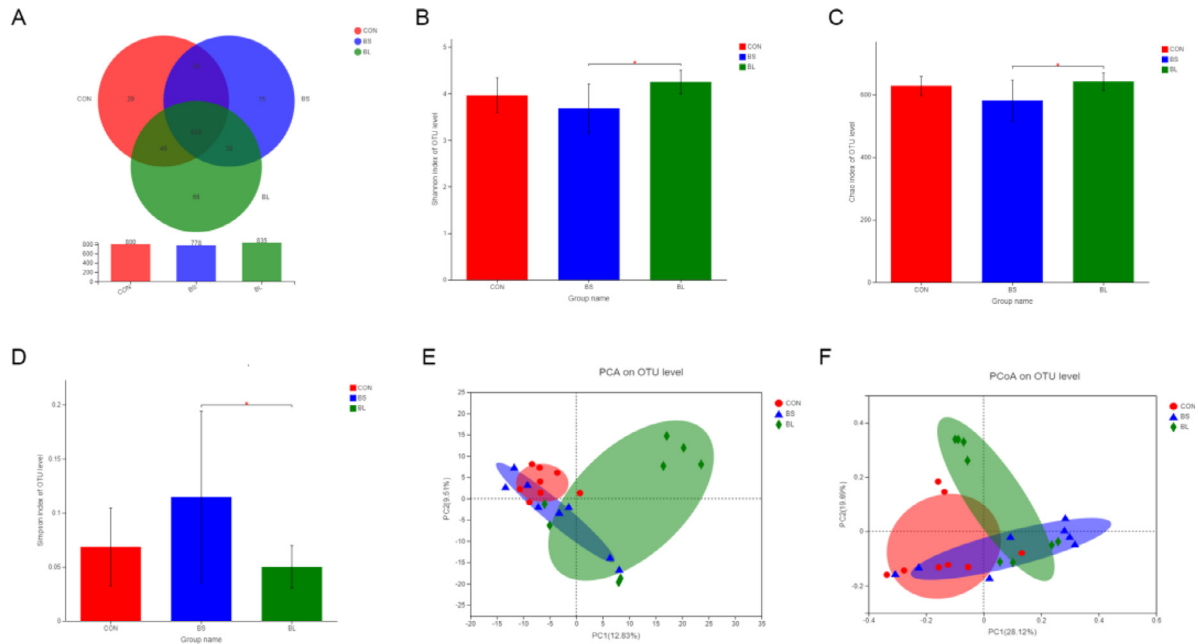
**Figure 2.** The effects of *B. subtilis* and *B. licheniformis* supplement on serum antioxidant activity of broilers at d 42. CON = basal diet provided as the control; BS = basal diet supplemented with  $1.5 \times 10^9$ CFU/kg *B. subtilis*; BL = basal diet supplemented with  $1.5 \times 10^9$ CFU/kg *B. licheniformis*. Bars represent mean  $\pm$  SD (n = 8). Different lowercase letters (a, b) above bars represent significantly different means ( $P < 0.05$ ).

*Butyricoccus* 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 species level, the *s\_Bacteroides\_barneisae* 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

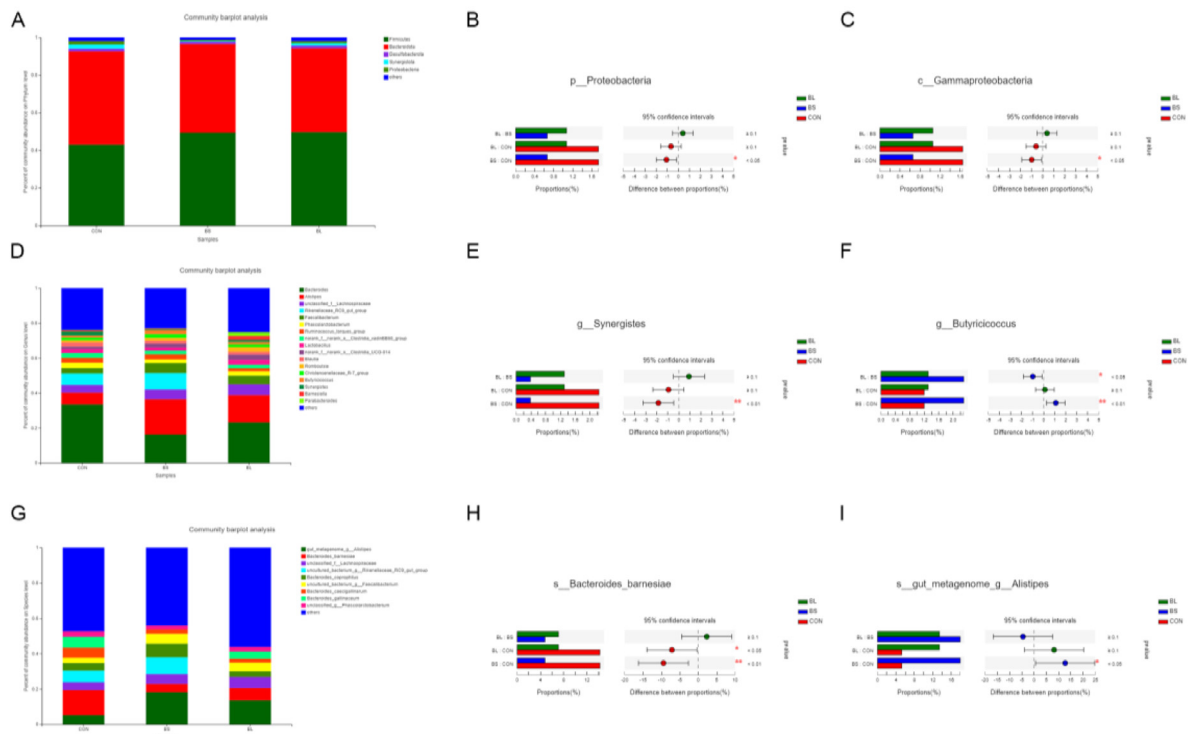
abundance of the dominant species *s\_gut\_meta-genome* in the BS group was distinctly higher ( $P < 0.05$ ), than in the CON group (Figures 5G–5I). Furthermore, the outcomes of LEfSe (linear discriminant analysis score = 3), indicated that 19 bacterial taxa were significantly more abundant ( $P < 0.05$ ) in the CON group, whereas only 14 taxa were overrepresented in the BS group ( $P < 0.05$ ;



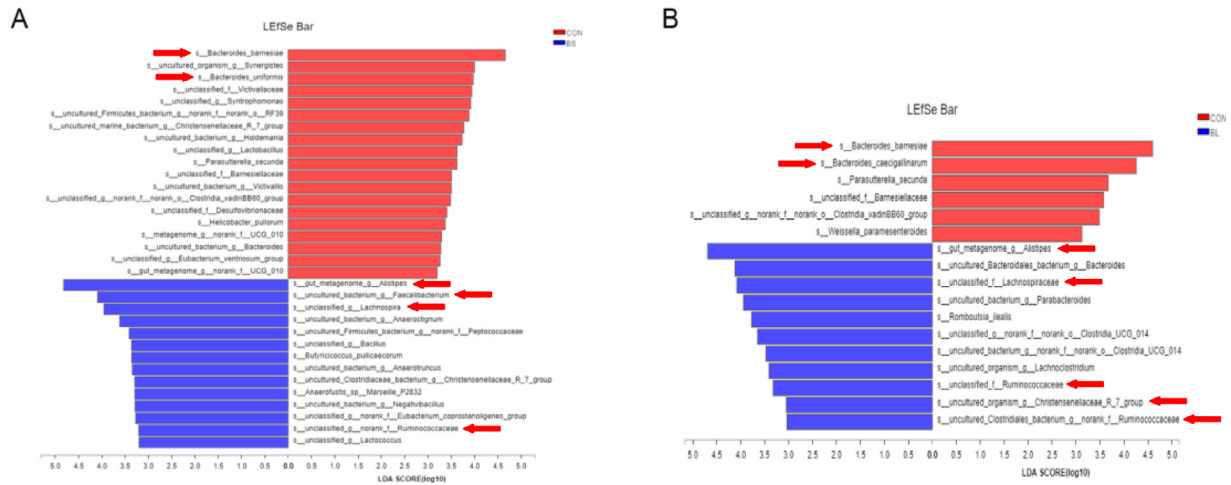
**Figure 3.** The effects of *B. subtilis* or *B. licheniformis* supplement on the short chain fatty acid in cecum contents of broiler chickens at d 42. CON = basal diet provided as the control; BS = basal diet supplemented with  $1.5 \times 10^9$ CFU/kg *B. subtilis*; BL = basal diet supplemented with  $1.5 \times 10^9$ CFU/kg *B. licheniformis*. Bars represent mean  $\pm$  SD (n = 8). Different lowercase letters (a, b) above bars represent significantly different means ( $P < 0.05$ ).



**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, chao1 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  $\pm$  SD (n = 8). Bars represent mean  $\pm$  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 (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  $\pm$  SD (n = 8). Bars represent mean  $\pm$  SD (n = 8). \*Means different ( $P < 0.05$ ), \*\*means significant difference ( $P < 0.01$ ).



**Figure 6.** Taxa that were significantly differentially represented between groups were examined by linear discriminant analysis coupled with effect size (LEfSe) using the default parameters (LDA score = 3). (A) Taxa that were significantly differentially represented between CON and BS. (B) Taxa that were significantly differentially represented between CON and BL. 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*. Red arrow means a significant difference ( $P < 0.05$ ) between the two groups.

Figure 6A). Additionally, 6 bacterial taxa were more abundant in the CON group ( $P < 0.05$ ), whereas 11 taxa were overrepresented in the BL group ( $P < 0.05$ ; 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*-supplement 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 (Carrier 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 *Eimeria maxima* or *Escherichia 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; Rychlik, 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 *Alistipes* 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; Eeckhaut 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-1 $\beta$  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.

## ACKNOWLEDGMENTS

This work was supported by the Zhejiang Provincial Key Research and Development Program (No.2019C02051; No.2020C02032), Zhejiang Provincial Leading Innovation and Entrepreneurship Team Project (No.2020R01015), and the National Key Research and Development Program Intergovernmental International Innovation Cooperation Project (No.2018YFE0112700). Zhejiang Provincial Key Agricultural Research Institute Project on Green Animal Health Products (No. 2021Y30004)

## DISCLOSURES

The authors declare no conflict of interest.

## REFERENCES

- Abdelqader, A., M. Abuajamieh, F. Hayajneh, and A. R. Al-Fataftah. 2020. Probiotic bacteria maintain normal growth mechanisms of heat stressed broiler chickens. *J. Therm. Biol.* 92:102654.
- Abudabos, A. M., H. A. A. Alhourri, I. A. Alhidary, M. A. Nassan, and A. A. Swelum. 2019. Ameliorative effect of *Bacillus subtilis*, *Saccharomyces boulardii*, oregano, and calcium montmorillonite on growth, intestinal histology, and blood metabolites on Salmonella-infected broiler chicken. *Environ. Sci. Pollut. Res. Int.* 26:16274–16278.
- Alagawany, M., M. E. Abd El-Hack, M. R. Farag, S. Sachan, K. Karthik, and K. Dhama. 2018. The use of probiotics as eco-friendly alternatives for antibiotics in poultry nutrition. *Environ. Sci. Pollut. Res. Int.* 25:0611–10618.
- Al-Fataftah, A. R., and A. Abdelqader. 2014. Effects of dietary *Bacillus subtilis* on heat-stressed broilers performance, intestinal morphology and microflora composition. *Anim. Feed Sci. Technol.* 198:279–285.
- Al-Khalaifah, H. S. 2018. Benefits of probiotics and/or prebiotics for antibiotic-reduced poultry. *Poult. Sci.* 97:3807–3815.
- Antonissen, G., V. Eeckhaut, D. K. Van, L. Onrust, F. Haesebrouck, R. Ducatelle, R. J. Moore, and I. F. Van. 2016. Microbial shifts associated with necrotic enteritis. *Avian. Pathol.* 45:308–312.
- Bader, J., A. Albin, and U. Stahl. 2012. Spore-forming bacteria and their utilisation as probiotics. *Benef. Microbes.* 3:67–75.
- Balan, P., K. Han, and P. J. Moughan. 2019. Impact of oral immunoglobulins on animal health—a review. *Anim. Sci. J.* 90:1099–1110.
- Bai, K., Q. Huang, J. F. Zhang, J. T. He, L. L. Zhang, and T. Wang. 2017. Supplemental effects of probiotic *Bacillus subtilis fmbJ* on growth performance, antioxidant capacity, and meat quality of broiler chickens. *Poult. Sci.* 96:74–82.
- Belibasakis, G. N., J. Mir-Mari, P. Sahrman, I. Sanz-Martin, P. R. Schmidlin, and R. E. Jung. 2016. Clinical association of spirochaetes and synergistetes with peri-implantitis. *Clin. Oral. Implants. Res.* 27:656–661.
- Blasco, L., M. Kahala, E. Tampio, M. Vainio, S. Ervasti, and S. Rasi. 2020. Effect of inoculum pretreatment on the composition of microbial communities in anaerobic digesters producing volatile fatty acids. *Microorganisms* 8:581.
- Carlier, F. M., Y. Sibille, and C. Pilette. 2016. The epithelial barrier and immunoglobulin A system in allergy. *Clin. Exp. Allergy.* 46:1372–1388.
- Chaucheyras-Durand, F., and H. Durand. 2010. Probiotics in animal nutrition and health. *Benef. Microbes.* 1:3–9.
- Chen, Y. C., and Y. H. Yu. 2020. *Bacillus licheniformis*-fermented products improve growth performance and the fecal microbiota community in broilers. *Poult. Sci.* 99:1432–1443.
- Cheng, H. Y., M. X. Ning, D. K. Chen, and W. T. Ma. 2019. Interactions between the gut microbiota and the host innate immune response against pathogens. *Front. Immunol.* 10:607.
- Cox, C. M., and R. A. Dalloul. 2015. Immunomodulatory role of probiotics in poultry and potential in ovo application. *Benef. Microbes.* 6:45–52.
- Dong, Y. X., R. Li, Y. Liu, L. Y. Ma, J. H. Zha, X. B. Qiao, T. J. Chai, and B. Wu. 2020. Benefit of dietary supplementation with *Bacillus subtilis* *BYS2* on growth performance, immune response, and disease resistance of broilers. *Probio. Antimicrob. Proteins.* 12:1385–1397.
- Du, Y. P., Z. C. Xu, G. L. Yu, W. Liu, Q. F. Zhou, D. H. Yang, J. Li, L. Chen, Y. Zhang, C. Y. Xue, and Y. C. Cao. 2019. *Bacillus subtilis*-A newly isolated strain named WS-1 inhibited diarrhea and death caused by pathogenic in newborn piglets. *Front. Microbiol.* 10:1248.
- Eeckhaut, V., J. Wang, P. A. Van, F. Haesebrouck, M. Joossens, G. Falony, J. Raes, R. Ducatelle, and I. F. Van. 2016. *Butyricicoccus pullicaecorum*—the probiotic reduces feed conversion and protects from potentially harmful intestinal microorganisms and necrotic enteritis in broilers. *Front. Microbiol.* 7:1416.
- EFSA. 2007. Introduction of a qualified presumption of safety (QSP) approach for assessment of selected microorganisms referred to EFSA - opinion of the scientific committee. *EFSA J* 5:87:1–16.
- Elshaghabee, F. M. F., N. Rokana, R. D. Gulhane, C. Sharma, and H. Panwar. 2017. *Bacillus* as potential probiotics: status, concerns, and future perspectives. *Front. Microbiol.* 8:1490.
- Fazelnia, K., J. Fakhraei, H. M. Yarahmadi, and K. Amini. 2021. Dietary supplementation of potential probiotics *Bacillus subtilis*, *Bacillus licheniformis*, and *saccharomyces cerevisiae* and synbiotic improves growth performance and immune responses by modulation in intestinal system in broiler chicks challenged with *Salmonella typhimurium*. *Probio. Antimicrob. Proteins.* undefined: undefined.
- Gadde, U. D., S. T. Oh, Y. S. Lee, E. Davis, N. Zimmerman, T. Rehberger, and H. S. Lillehoj. 2017. Dietary *Bacillus subtilis*-based direct-fed microbials alleviate LPS-induced intestinal immunological stress and improve intestinal barrier gene expression in commercial broiler chickens. *Res. Vet. Sci.* 114:236–243.
- Geirnaert, A., A. Steyaert, V. Eeckhaut, B. Debruyne, J. B. A. Arends, I. F. Van, N. Boon, and D. W. T. Van. 2014. *Butyricicoccus pullicaecorum*, a butyrate producer with probiotic potential, is intrinsically tolerant to stomach and small intestine conditions. *Anaerobe* 30:70–74.
- Guo, M. J., M. T. Li, C. C. Zhang, X. R. Zhang, and Y. T. Wu. 2020. Dietary administration of the *Bacillus subtilis* enhances immune responses and disease resistance in chickens. *Front. Microbiol.* 11:1768.
- Heiss, C. N., and L. E. Olofsson. 2018. Gut microbiota-dependent modulation of energy metabolism. *J. Innate. Immun.* 10:163–171.
- Jacquier, V., A. Nelson, M. Jali, L. Rhayat, K. S. Brinch, and E. Devillard. 2019. *Bacillus subtilis* 29784 induces a shift in broiler gut microbiome toward butyrate-producing bacteria and improves intestinal histomorphology and animal performance. *Poult. Sci.* 98:2548–2554.
- Khan, S., R. J. Moore, D. Stanley, and K. K. Chousalkar. 2020. The gut microbiota of laying hens and its manipulation with prebiotics and probiotics to enhance gut health and food safety. *Appl. Environ. Microbiol.* 86:e00600–e00620.
- Kim, Y., J. Y. Cho, J. H. Kuk, J. H. Moon, J. I. Cho, Y. C. Kim, and K. H. Park. 2004. Identification and antimicrobial activity of phenylacetic acid produced by *Bacillus licheniformis* isolated

- from fermented soybean, Chungkook-Jang. *Curr. Microbiol.* 48:312–317.
- Knapp, I., B. Lund, A. B. Kehlet, C. Hofacre, and G. Mathis. 2010. *Bacillus licheniformis* prevents necrotic enteritis in broiler chickens. *Avian Dis.* 54:931–935.
- Kubasova, T., M. Kollarcikova, M. Crhanova, D. Karasova, D. Cejkova, A. Sebkova, J. Matiasovicova, M. Faldynova, F. Sisak, V. Babak, A. Pokorna, A. Cizek, and I. Rychlik. 2019. Gut anaerobes capable of chicken caecum colonisation. *Microorganisms.* 7:597.
- Lauridsen, C. 2019. From oxidative stress to inflammation: redox balance and immune system. *Poult. Sci.* 98:4240–4246.
- Lin, Y. C., S. Xu, D. Zeng, X. Q. Ni, M. J. Zhou, Y. Zeng, H. S. Wang, Y. Zhou, H. Zhu, K. C. Pan, and G. Y. Li. 2017. Disruption in the cecal microbiota of chickens challenged with *Clostridium perfringens* and other factors was alleviated by *Bacillus licheniformis* supplementation. *PLoS One.* 12:e0182426.
- Liu, X. L., H. Yan, L. Lv, Q. Q. Xu, C. H. Yin, K. Y. Zhang, P. Wang, and J. Y. Hu. 2012. Growth performance and meat quality of broiler chickens supplemented with *Bacillus licheniformis* in drinking water. *Asian-Australas. J. Anim. Sci.* 25:682–689.
- Luu, M., H. Monning, and A. Visekruna. 2020. Exploring the molecular mechanisms underlying the protective effects of microbial SCFAs on intestinal tolerance and food allergy. *Front. Immunol.* 11:1225.
- Ma, Y. B., W. W. Wang, H. J. Zhang, J. Wang, W. M. Zhang, J. Gao, S. G. Wu, and G. H. Stahl. 2018. Supplemental *Bacillus subtilis* DSM 32315 manipulates intestinal structure and microbial composition in broiler chickens. *Sci. Rep.* 8:15358.
- Maritim, A. C., R. A. Sanders, and J. B. Watkins. 2003. Diabetes, oxidative stress, and antioxidants: a review. *J. Biochem. Mol. Toxicol.* 17:24–38.
- McGlone, J., S. Ford, F. Mitloehner, T. Grandin, P. Ruegg, C. Stull, G. Lewis, J. Swanson, W. Underwood, J. Mench, T. Mader, S. Eicher, P. Hester, J. Salak-Johnson, and M. Galyean. 2010. Pages 102-120 in Chapter 9 Guide for the Care and Use of Agricultural Animals in Research and Teaching (3th Rev ed.). Poultry. Federation of Animal Science Societies, Champaign, IL.
- McCracken, B. A., and G. M. Nathalia. 2020. Phylum Synergistetes in the oral cavity: a possible contributor to periodontal disease. *Anaerobe* 2020:102250.
- Mehdi, Y., M. P. Létourneau-Montminy, M. L. Gaucher, Y. Chorfi, G. Suresh, T. Rouissi, S. K. Brar, C. Côté, A. A. Ramirez, and S. Godbout. 2018. Use of antibiotics in broiler production: global impacts and alternatives. *Anim. Nutr.* 4:170–178.
- Medina, F. S., M. Cretenet, and M. Bernardeau. 2019. In vitro inhibition of avian pathogenic *Enterococcus cecorum* isolates by probiotic *Bacillus strains*. *Poult. Sci.* 98:2338–2346.
- Musa, B. B., Y. Duan, H. Khawar, Q. Z. Sun, Z. Z. Ren, M. M. A. Elsidig, I. H. R. Abbasi, and X. J. Yang. 2019. *Bacillus subtilis* B21 and *Bacillus licheniformis* B26 improve intestinal health and performance of broiler chickens with *Clostridium perfringens*-induced necrotic enteritis. *J. Anim. Physiol. Anim. Nutr. (Berl).* 103:1039–1049.
- NRC (National Research Council). 1994. Nutrient Requirements of Poultry (9th Rev ed.). Natl. Acad. Press, Washington, DC.
- Parada, Venegas D., L. F. M. K. De, G. Landskron, M. J. González, R. Quera, G. Dijkstra, H. J. M. Harmsen, K. N. Faber, and M. A. Hermoso. 2019. Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. *Front. Immunol.* 10:277.
- Park, I., Y. Lee, D. Goo, N. P. Zimmerman, A. H. Smith, T. Rehberger, and H. S. Lillehoj. 2020. The effects of dietary *Bacillus subtilis* supplementation, as an alternative to antibiotics, on growth performance, intestinal immunity, and epithelial barrier integrity in broiler chickens infected with *Eimeria maxima*. *Poult. Sci.* 99:725–733.
- Pickard, J. M., M. Y. Zeng, R. Caruso, and G. Núñez. 2017. Gut microbiota: role in pathogen colonization, immune responses, and inflammatory disease. *Immunol. Rev.* 279:70–89.
- Rehman, Z. U., C. C. Meng, Y. J. Sun, A. Safdar, R. H. Pasha, M. Munir, and C. Ding. 2018. Oxidative stress in poultry: lessons from the viral infections. *Oxid. Med. Cell Longev.* 2018:5123147.
- Rivardo, F., R. J. Turner, G. Allegrone, H. Ceri, and M. G. Martinotti. 2009. Anti-adhesion activity of two biosurfactants produced by *Bacillus spp.* prevents biofilm formation of human bacterial pathogens. *Appl. Microbiol. Biotechnol.* 83:541–553.
- Rodrigues, D. R., W. Briggs, A. Duff, K. Chasser, R. Murugesan, C. Pender, S. Ramirez, L. Valenzuela, and L. Bielke. 2020. Cecal microbiome composition and metabolic function in probiotic treated broilers. *PLoS One.* 15:e0225921.
- Roth, N., A. Käsbohrer, S. Mayrhofer, U. Zitz, C. Hofacre, and K. J. Domig. 2019. The application of antibiotics in broiler production and the resulting antibiotic resistance in *Escherichia coli*: a global overview. *Poult. Sci.* 98:1791–1804.
- Rychlik, I. 2020. Composition and function of chicken gut microbiota. *Animals (Basel)* 10:103.
- Sahu, M. K., N. S. Swarnakumar, K. Sivakumar, T. Thangaradjou, and L. Kannan. 2008. Probiotics in aquaculture: importance and future perspectives. *Indian J. Microbiol.* 48:299–308.
- Santacroce, L. G., I. A. Charitos, and L. Bottalico. 2019. A successful history: probiotics and their potential as antimicrobials. *Expert. Rev. Anti. Infect. Ther.* 17:635–645.
- Singh, R. P. 2019. Glycan utilisation system in *Bacteroides* and *Bifidobacteria* and their roles in gut stability and health. *Appl. Microbiol. Biotechnol.* 103:7287–7315.
- Sokale, A. O., A. Menconi, G. F. Mathis, B. Lumpkins, M. D. Sims, R. A. Whelan, and K. Doranalli. 2019. Effect of *Bacillus subtilis* DSM 32315 on the intestinal structural integrity and growth performance of broiler chickens under necrotic enteritis challenge. *Poult. Sci.* 98:5392–5400.
- Tarradas, J., T. Núria, E. Esteve-Garcia, and J. Brufau. 2020. The control of intestinal inflammation: a major objective in the research of probiotic strains as alternatives to antibiotic growth promoters in poultry. *Microorganisms* 8:148.
- Tungland, B. 2018. Short-chain fatty acid production and functional aspects on host metabolism. *Hum. Microbi. Health Dis* 2018:37–106.
- van der Hee, B., and J. M. Wells. 2021. Microbial regulation of host physiology by short-chain fatty acids. *Trends Microbiol.* 29:700–712.
- Wang, Y., W. Du, K. Lei, B. K. Wang, Y. Y. Wang, Y. S. Zhou, and W. F. Li. 2017. Effects of dietary *Bacillus licheniformis* on gut physical barrier, immunity, and reproductive hormones of laying hens. *Probio. Antimicrob. Proteins* 9:292–299.
- Wang, Y. Y., C. N. Heng, X. H. Zhou, G. T. Cao, L. Jiang, J. S. Wang, K. X. Li, D. C. Wang, and X. A. Zhan. 2020. Supplemental *Bacillus subtilis* DSM 29784 and enzymes, alone or in combination, as alternatives for antibiotics to improve growth performance, digestive enzyme activity, anti-oxidative status, immune response and the intestinal barrier of broiler chickens. *Br. J. Nutr.* 125:494–507.
- Wasti, S., N. Sah, and B. Mishra. 2020. Impact of heat Stress on poultry health and performances, and potential mitigation strategies. *Animals (Basel)* 10:1266.
- Wu, M., S. J. Yang, S. Zi. Wang, Y. Cao, R. Zhao, X. Y. Li, Y. W. Xing, and L. T. Liu. 2020. Effect of berberine on atherosclerosis and gut microbiota modulation and their correlation in high-fat diet-fed apoE<sup>-/-</sup> mice. *Front. Pharmacol.* 11:223.
- Xu, S., Y. C. Lin, D. Zeng, M. J. Zhou, Y. Zeng, H. S. Wang, Y. Zhou, H. Zhu, K. C. Pan, B. Jing, and X. Q. Ni. 2018. *Bacillus licheniformis* normalize the ileum microbiota of chickens infected with necrotic enteritis. *Sci. Rep.* 8:1744.
- Yang, C. M., L. L. Zhang, G. T. Cao, J. Feng, M. Yue, Y. L. Xu, B. Dai, Q. J. Han, and X. Q. Guo. 2019. Effects of dietary supplementation with essential oils and organic acids on the growth performance, immune system, faecal volatile fatty acids and microflora community in weaned piglets. *J. Anim. Sci.* 97:133–143.
- Zaboli, G., X. Huang, X. Feng, and D. U. Ahn. 2019. How can heat stress affect chicken meat quality? - a review. *Poult. Sci.* 98:1551–1556.
- Zhang, J. Y., T. D. Lu, P. H. Shen, Q. W. Sui, H. Zhong, J. B. Liu, J. Tong, and Y. S. Wei. 2019. The role of substrate types and substrate microbial community on the fate of antibiotic resistance genes during anaerobic digestion. *Chemosphere* 229:461–470.
- Zhang, X. Y., R. A. Calvert, B. J. Sutton, and K. A. Doré. 2017. IgY: a key isotype in antibody evolution. *Biol. Rev. Camb. Philos. Soc.* 92:2144–2156.

- Zhao, B. Y., and W. A. Houry. 2010. Acid stress response in enteropathogenic gammaproteobacteria: an aptitude for survival. *Biochem. Cell Biol.* 88:301–314.
- Zhao, Y., D. Zeng, H. S. Wang, X. D. Qing, N. Sun, J. Xin, M. Luo, A. Khaliq, K. C. Pan, G. Shu, B. Jing, and X. Q. Ni. 2020. Dietary probiotic *Bacillus licheniformis* H2 enhanced growth performance, morphology of small intestine and liver, and antioxidant capacity of broiler chickens against *Clostridium perfringens*-induced subclinical necrotic enteritis. *Probio. Antimicrob. Proteins* 12:883–895.
- Zhou, Y. T., and F. C. Zhi. 2016. *Bacteroides* lower level of in the gut microbiota is associated with inflammatory bowel disease: a meta-analysis. *Biomed. Res. Int.* 2016:5828959.