Original Research Article

Effect of *Saccharomyces boulardii* and *Bacillus subtilis* B10 on gut microbiota modulation in broilers

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A B S T R A C T

The gut microbiota plays important roles in animal overall health and productiveness. Balancing host gut microbiota by probiotics has been documented. Our previous study showed that *Saccharomyces boulardii* (*Sb*) and *Bacillus subtilis* B10 (*Bs*) significantly improve growth performance and modulate the intestinal histomorphology in broilers. To increase the knowledge regarding *Sb* and *Bs*, this study investigated the effects of these 2 probiotic strains on the gut microbiota in broilers. Three hundred 1-day-old Sanhuang broilers (Chinese cross breed) were randomly divided into 3 groups, each group with 5 replications ($n = 20$). The control group (CK) was fed a basal diet containing an antibiotic (virginiamycin, 20 mg/kg) and the other 2 groups received *Sb* and *Bs* (1×10^8 cfu/kg of feed) in addition to the basal diet. After 72 d of treatment, pyrosequencing revealed that the bacterial communities varied along the section of intestinal tract in the control and *Bs* groups, but not in the *Sb* group. No difference in microbial diversity was observed among 3 groups. The major phyla observed along the GI tract of broilers (particularly in the duodenum and cecum) were Firmicutes, Bacteroidetes, Proteobacteria, and Verrucomicrobia, which were considered potentially growth performance-related. Bacteroidetes, Proteobacteria, and Verrucomicrobia were observed at a much higher abundance in the jejunums and ileums of the *Sb* group ($P < 0.05$). In addition, the jejunal microbial communities formed 3 different clusters at either the genus level or the category of metabolism among the groups, based on the principal component analyses. These data indicated that *Sb* and *Bs* can modulate the microbial ecosystem, and subsequently enhance the health status of broilers.

1. Introduction

The gastrointestinal (GI) tract is home to a complex and dynamic microbial ecosystem — a so-called 'microbiota superorganism', which contains hundreds of microbe species (Apajalahti et al., 2004; Wei et al., 2013; Zoetendal et al., 1998). The genomes of these intestinal microbes form a microbiome that by far outnumbers the host's genome (Cisek and Binek, 2014). Consequently, these gut microbes play a key role in host energy metabolism and immune functions and greatly contribute to a wide range of processes involved in gastrointestinal development, including regulation of intestinal epithelial proliferation (Forder et al., 2007), vitamin synthesis and ion absorption, carbohydrate and protein fermentation (Hamer et al., 2012), bile acid biotransformation (Degirolamo et al., 2014; Ridlon et al., 2006), protection against pathogens and immune system modulation (Guarner, 2006; Guarner and Malagelada, 2003; Noverr and Huffnagel, 2004; Round and Mazmanian, 2009).

Bacterial colonization of the animal gut by environmental microbes begins immediately at birth or after hatching. The

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composition of the gut microbiota is modulated by numerous extrinsic factors such as age, diet, medication, and stress (Claesson et al., 2011; De La Cochetiere et al., 2008; Louis et al., 2007; O'Toole and Claesson, 2010). Additionally, balance among the gastrointestinal microbial communities is crucial for host health maintenance. Disturbances of the gut microbiota, also known as dysbiosis, have often been associated with several diseases including inflammatory bowel diseases (Frank et al., 2011), diabetes (Cani et al., 2008), obesity (Ley et al., 2006), fatty liver (Dumas et al., 2006), and anxiety (Neufeld et al., 2011). The gut microbiota composition is readily changeable (Jia et al., 2008); consequently, this plasticity favors the development of gut microbiota-targeted therapies such as antibiotics, prebiotics, and probiotics. Antibiotics in feed have been successfully utilized since the 1950s for growth promotion during food-animal production (Dibner and Richards, 2005; Gaskins et al., 2002). However, antibiotic resistance among bacterial pathogens and antibiotic residues in animal products has garnered global interest in limiting antibiotic use in animal agriculture (Seal et al., 2013). Probiotics are live microorganisms that provide beneficial effects to the host when adequately administered. Researchers have shown that probiotic bacteria have a variety of beneficial effects, including counteraction of dysbiosis, promotion of gut health and homeostasis, promotion of growth enhancement of immune defenses and protection of the host from infection by pathogens (Aureli et al., 2011; Cisek and Binek, 2014).

Poultry has become one of the most prominent sources of animal protein worldwide; therefore, the gut microbiome of chicken is a major interest of investigators attempting to improve the growth, health and food safety of poultry (Kohl, 2012; Oakley et al., 2013; Wise and Siragusa, 2007). Our previous study showed that *Saccharomyces boulardii* (*Sb*) and *Bacillus subtilis* B10 (*Bs*) significantly improve the growth performance and modulate the morphology of the intestine (the intestinal villus height, width, and goblet cell number are increased in the *Sb* and *Bs* groups) in broilers (Rajput et al., 2013a). Accordingly, we speculated that the administration of these 2 probiotic strains might yield a common or similar gut microbial community to that induced by virginiamycin treatment or even a more optimal gut microbial structure than virginiamycin in broilers. In addition, there is little information available concerning the effect of these 2 probiotic strains on the gut microbiota in broilers. Therefore, this study is aimed to interpret the beneficial effects of the 2 probiotic strains in the gut microbiota perspective.

2. Materials and methods

Procedures involving animals were performed in according to the guideline of the declaration of Zhejiang Animal Center at the Institute of Medical Science, and approved by the Ministry of Livestock, Zhejiang University, Hangzhou, China.

2.1. Bacteria and yeast

Bacterial strains *Sb* and *Bs* used here were isolated and identified by the Institute of Feed Sciences, Zhejiang University, China. *S. boulardii* was cultured in yeast peptone dextrose broth (Oxoid, Basingstoke, UK) in aerobic conditions at 30 °C for 24 h, and *B. subtilis* in Luria-Bertani broth (Oxoid) for 12 h. After cultivation, the yeast and bacterial were collected by centrifugation (6,000 × g for 5 min at 4 °C). The pellets were washed twice with PBS (pH 7.4) and resuspended in sterile water at a final concentration of 1.0×10^9 cfu/mL. The prepared mixture was added into the basal diet (Table 1) and maintained at 1×10^8 cfu/kg.

Table 1
Ingredients (as-fed basis) and calculated composition of the basal diet (%).

Item	1 to 35 d	36 to 72 d
Ingredients		
Corn	55.90	61.60
Soybean meal	31.00	27.00
Wheat shorts	3.00	4.00
Imported fish meal	5.00	2.00
Rapeseed oil	1.50	2.00
Salt	0.30	0.30
Dicalcium phosphate	1.20	1.00
Limestone	1.00	1.00
DL-Met	0.10	
Lysine		0.10
Premix[1]	1.00	1.00
Total	100.00	100.00
Calculated composition		
ME, MJ/kg	12.78	13.05
Crude protein	22.86	19.14
Lys	1.07	0.98
Met + Cys	0.86	0.72
Ash	7.38	6.41
Ca	0.93	0.91
Total phosphorus	0.64	0.56

[1] Each kilogram of premix compound contained: vitamin A, 7,000 IU; vitamin D_3, 2,500 IU; vitamin E, 30 mg; vitamin K_3, 1 mg; vitamin B_1, 1.5 mg; vitamin B_2, 4 mg; vitamin B_6, 2 mg; vitamin B_{12}, 0.02 mg; niacin, 30 mg; folic acid, 0.55 mg; pantothenic acid, 10 mg; biotin, 0.16 mg; choline chloride, 400 mg; Cu, 20 mg; Fe, 70 mg; Mn, 100 mg; Zn, 70 mg; I, 0.4 mg and Se, 0.5 mg.

2.2. Animals

Three hundred 1-day-old Sanhuang broilers (a Chinese cross breed) were randomly divided into 3 groups, each group with 5 replications ($n = 20$). The control group (CK) was fed a basal diet (Table 1) supplemented with an antibiotic (virginiamycin, 20 mg/kg), whereas broilers in *Sb* and *Bs* groups were fed the basal diet supplemented with *Sb* and *Bs* (1×10^8 cfu/kg of feed) for 72 d.

2.3. Sample collection

After the 72-d feeding treatment, 10 broilers from each replicate of each treatment group were killed by administering lethobarb (0.5 mL/bird) intravenously and then weighed before sample collection. Duodenum, jejunum, ileum and cecum were collected aseptically. The contents of each section of the gut (duodenum, jejunum, ileum, and cecum) were isolated. And, the contents of each section of the gut of each replicate from different treatment groups were pooled and stored at −70 °C for microbiota analysis.

2.4. DNA extraction and pyrosequencing

For each treatment group, 3 pool samples of each section of the gut were selected randomly to use for microbiota analysis. Total bacteria DNA from each sample (200 mg) was extracted using a DNA Isolation Kit (Tiangen, Beijing, China). Sequencing was performed at Tongji-SCBIT Biotechnology Co. Ltd. Shanghai, China. Briefly, DNA was amplified using the conserved primers 341F (5′-XXXXXXXCCTACGGGAGGCAGCAG-3′) and 534R (5′- ATGAGCT-GATTACCGCGGCTGCT-3′), which targets the V3 region of the 16S rRNA, with the forward primer containing a 7-bp barcode unique to each sample. The PCR was performed with the following condition: 94 °C for 5 min (94 °C for 40 s, 55 °C for 30 s, and 72 °C for 30 s) × 30 cycles. The PCR products were purified using a gel extraction kit (Axygen Scientific Inc., USA). The concentration was measured with a UV−vis spectrophotometer (NanoDrop ND1000, USA) and then adjusted to 50 ng/μL for each sample. Finally, equal amounts of DNA

from each sample were mixed together and sequenced by Tongji-SCBIT Biotechnology Co., Ltd. (Shanghai, China) using the 454 Life Sciences/Roche GS-FLX sequencing system (Roche Applied Science, Penzburg, Germany).

Sequences obtained through 454 pyrosequencing were then filtered by QIIME software (QIIME version 1.9.0) with default parameters (Caporaso et al., 2010; Zhang et al., 2014). Low-quality or ambiguous reads were discarded, and primer sequences and barcodes were trimmed from the 5′ region. The operational taxonomic unit (OTU) clustering pipeline UPARSE was used to select OTU at 97% similarity. Diversity between the samples (Shannon index) was also analyzed by QIIME. The final taxonomic assignment was based on the consensus identification for each OTU. A multivariate data analysis was performed using METAGENassist (http://www.metagenassist.ca/METAGENassist/faces/Home.jsp), a web server tool that assigns probable microbial functions based on taxonomy (16 S ribosomal subunit) as described by Arndt et al. (2012) and Badri et al. (2013). Principal component analyses (PCA) and the identification of significant features were performed for all treatments combined.

2.5. Statistical analyses

Data were represented as means ± SEM and analyzed with SPSS 16.0. The intergroup variation was tested with paired-samples t-test, followed by Fisher's least significant difference test among the groups. P-value $\leq$ 0.05 is considered significant.

3. Results

3.1. Microbial diversity along the broiler intestinal tract

Shannon index was used to evaluate the microbial ecological diversity of each sample (Fig. 1). The results revealed that the ileal and jejunal samples had much lower diversity, whereas the cecal and duodenal samples had much higher Shannon index values (Fig. 1). B. subtilis B10 and Sb administrations did not affect the microbiota diversity along the GI tract compared with the control, except in the duodenums of the Bs group, the microbiota diversity was lower than that of the control (Fig. 1, $P < 0.05$).

3.2. Changes in bacterial community structure along the broiler intestinal tract

The bacterial community structure varied among different anatomical regions along the chicken intestinal tract (Figs. 2 and 3). Taxonomically, 19 different bacterial phyla were identified in the broiler intestinal tract (Fig. 2). The major bacterial phyla identified were Bacteroidetes, Firmicutes, Proteobacteria, and Verrucomicrobia. Only Firmicutes, Proteobacteria, and Verrucomicrobia were found in all sections of the intestine in all treatment groups (Fig. 2). Firmicutes was dominant along the broiler intestinal tract and with much higher abundance in the jejunum and ileum than in the duodenum and cecum (Fig. 2). However, Actinobacteria were much enriched in the duodenum and jejunum, and the relative abundance of Bacteroidetes was much higher in the duodenum and cecum than in the jejunum and ileum (Fig. 2, $P < 0.01$). Based on the PCA, we observed that the bacterial community among the duodenum, ileum, and jejunum formed into 2 different clusters in control (Fig. 4A), whereas in the Bs group, the bacterial community of the cecal was separated from those of the ileum and jejunum (Fig. 4B). Interestingly, no significant difference was observed in the bacterial communities along the intestinal tract in the Sb group (Fig. 4C).

At the genus level, Akkermansia, Bacteroides, Oscillospira, Prevotella, and Ruminococcus were all enriched in the duodenum and cecum (Fig. 3). However, Lactobacillus was enriched in the jejunum and ileum, and the abundance of Lactobacillus was much higher in the jejunum and ileum than in the duodenum and cecum (Fig. 3, $P < 0.05$).

3.3. Effect of probiotics on the microbial structure along the intestinal tract

The effects of probiotics on the microbial community structure in the chicken intestinal tract were identified at the phylum (Fig. 2) and genus (Fig. 3) by pyrosequencing analyses. The microbial community structure after the probiotic treatments were mainly changed at the genus levels in the duodenum and jejunum compared with the control group (Fig. 3). However, the microbial community structure in the ileum and cecum was relative stable among the 3 groups (Fig. 3).

For microbial communities of digesta in the duodenum of broilers, no significant difference was observed among 3 groups at phylum level base on PCA (Fig. 5A); However, at the genus levels,
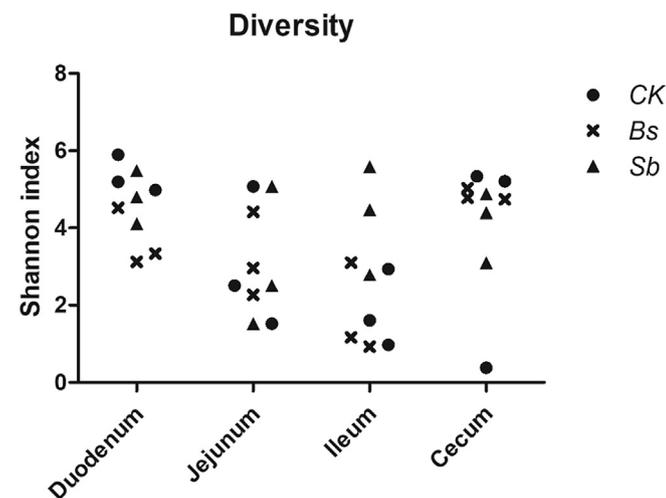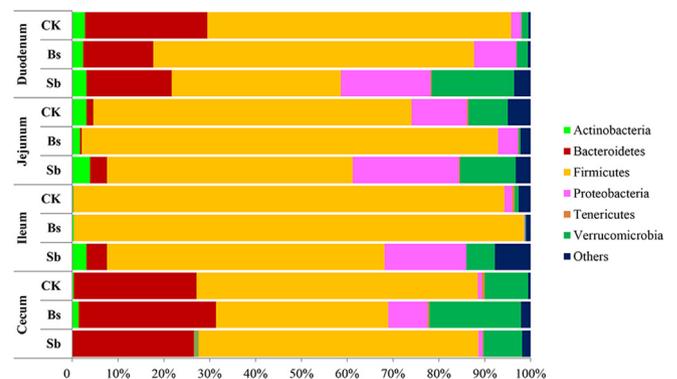

Fig. 1. Shannon diversity of each sample from different treatment groups. CK: birds fed the basal diet supplemented with virginiamycin; Bs: birds fed the basal diet supplemented with Bacillus subtilis B10; Sb: birds fed the basal diet supplemented with Saccharomyces boulardii.

Fig. 2. Relative abundance of bacterial phyla present in the intestinal tract in different treatment groups revealed by pyrosequencing. CK: birds fed the basal diet supplemented with virginiamycin; Bs: birds fed the basal diet supplemented with Bacillus subtilis B10; Sb: birds fed the basal diet supplemented with Saccharomyces boulardii.

Fig. 3. Relative abundance of bacterial genus present in the intestinal tract in different treatment groups revealed by pyrosequencing. CK: birds fed the basal diet supplemented with virginiamycin; *Bs*: birds fed the basal diet supplemented with *Bacillus subtilis* B10; *Sb*: birds fed the basal diet supplemented with *Saccharomyces boulardii*.
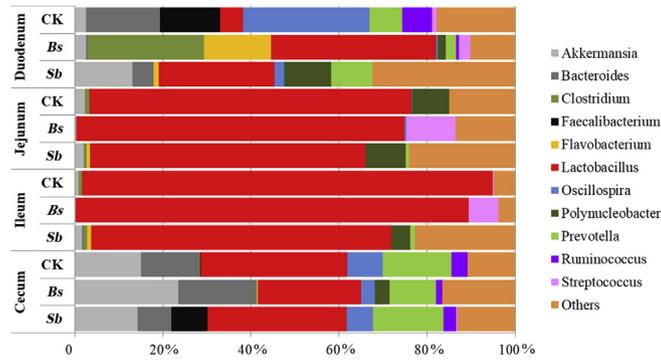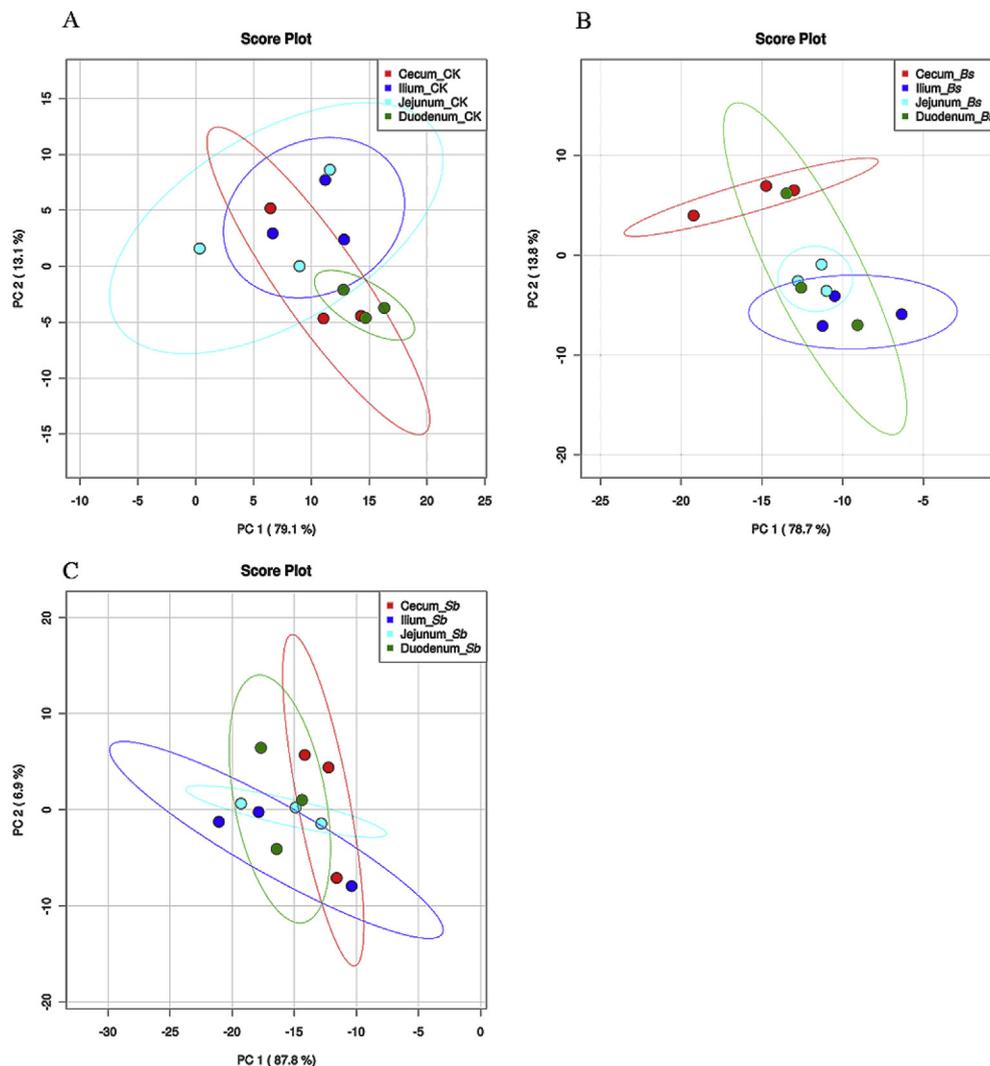

Fig. 4. Microbial community analyzed by principal component (PC) analyses at phyla levels. (A) CK: birds fed the basal diet supplemented with virginiamycin; (B) *Bs*: birds fed the basal diet supplemented with *Bacillus subtilis* B10; (C) *Sb*: birds fed the basal diet supplemented with *Saccharomyces boulardii*.

the microbial communities in the *Bs* and control groups formed 2 different clusters in the duodenum (Fig. 6A). The abundance of *Lactobacillus* in the *Bs* and *Sb* groups was much higher than that of the control group (Fig. 3, $P < 0.05$), and the abundance of *Clostridium* in the *Bs* group was also higher than those of the control

and *Sb* groups (Fig. 3, $P < 0.05$). However, the abundance of *Bacteroides* and *Oscillospira* in the *Bs* and *Sb* groups was lower than that of the control group (Fig. 3, $P < 0.05$).

In the jejunum, the microbial community structure was similar to that of the duodenum at the phylum level. According to the
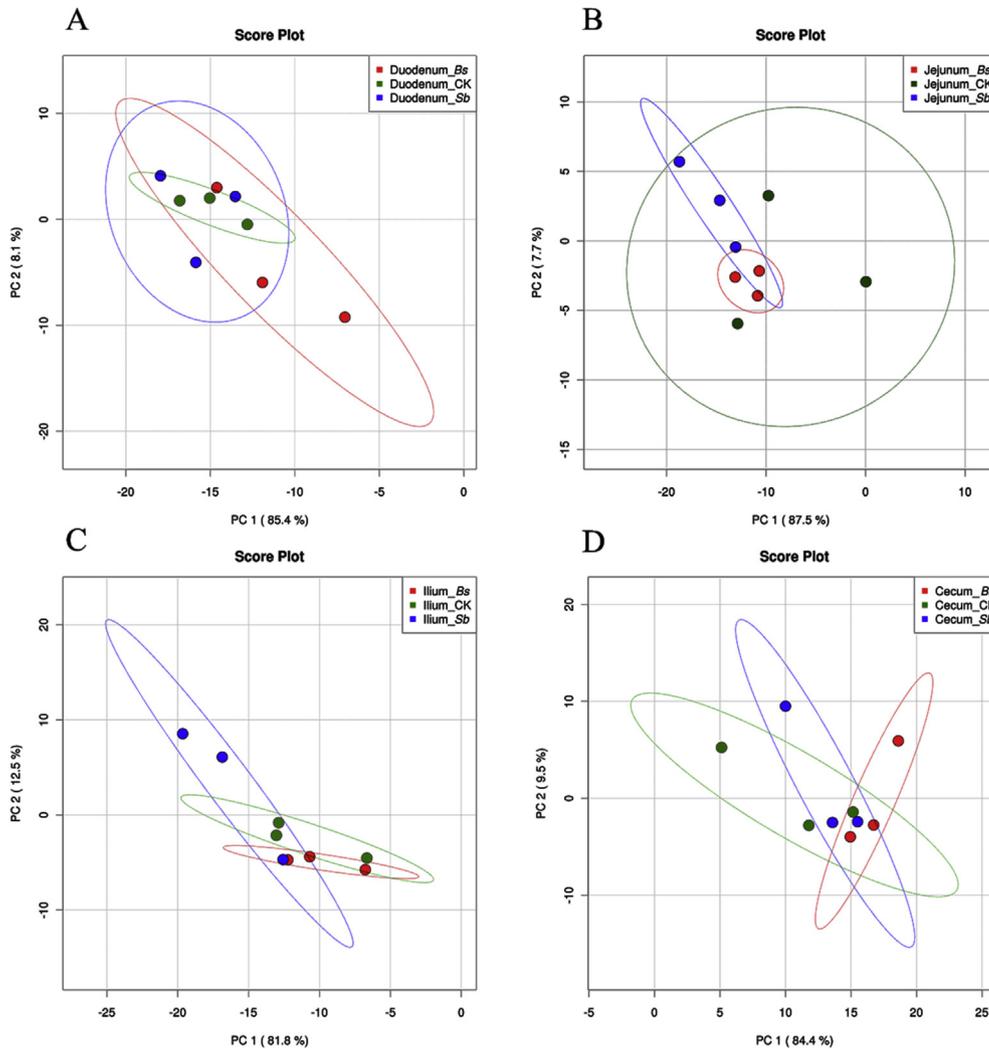
Fig. 5. Gut microbiome sequencing data of treatments and controls analyzed by principal component (PC) analyses at phyla level (A) duodenum, (B) jejunum, (C) ileum and (D) cecum CK: birds fed the basal diet supplemented with virginiamycin; *Bs*: birds fed the basal diet supplemented with *Bacillus subtilis* B10; *Sb*: birds fed the basal diet supplemented with *Saccharomyces boulardii*.

PCA, there was no significant difference between the probiotic treatment and control groups at the phylum level (Fig. 5B). However, at the genus level, the microbial communities among the 3 groups formed 3 different clusters in the jejunum (Fig. 6B). At the genus level, the microbial diversity was increased, and the abundance of other bacteria was also increased at the expense of *Lactobacillus* in the *Sb* treatment group compared with the control and *Bs* treatment groups (Fig. 3B). The abundance of *Streptococcus* was higher in *Bs* group than those in the control and *Sb* groups (Fig. 3, $P < 0.05$).

In the Ileum and cecum, the bacterial community structure was not much different and relatively stable among the 3 groups (Figs. 2 and 3). In the Ileum, no significant difference was observed among the 3 treatment groups based on PCA at phylum or genus level (Figs. 5C and 6C). In the cecum, no significant difference was observed based on PCA in phyla levels (Fig. 5D). Moreover, neither *Bs* administration nor *Sb* administration changed the cecum microbial community at the genus level compared with the control (Fig. 3), and the same results were obtained by the PCA (Fig. 6D).

3.4. Clustering of the bacterial community based on the category of metabolism

The OTU were assigned from taxonomic to phenotypic mapping using the METAGENassist webserver tool for multiple phenotype (about 21) categories classified based on habitat, metabolism, oxygen requirements, energy source, and other factors. Based on the category of metabolism, the PCA revealed that the microbial community of the 3 treatment groups formed into 3 different clusters in the jejunum and ileum (Figs. 7B and C). However, no difference was observed in either the cecum or duodenum (Figs. 7A and D). Notably, we also observed different microbial communities in different sections of the intestinal tract, based on the metabolism category analyses (Fig. 8). The microbial communities in the ileum and jejunum were separated from the cecum and duodenum in the control group (Fig. 8A). In the *Bs* group, the bacterial community in the cecum differed from those in the ileum and jejunum (Fig. 8B). Whereas in the *Sb* group, the bacterial community in the cecum differed from those in the jejunum and duodenum (Fig. 8C).
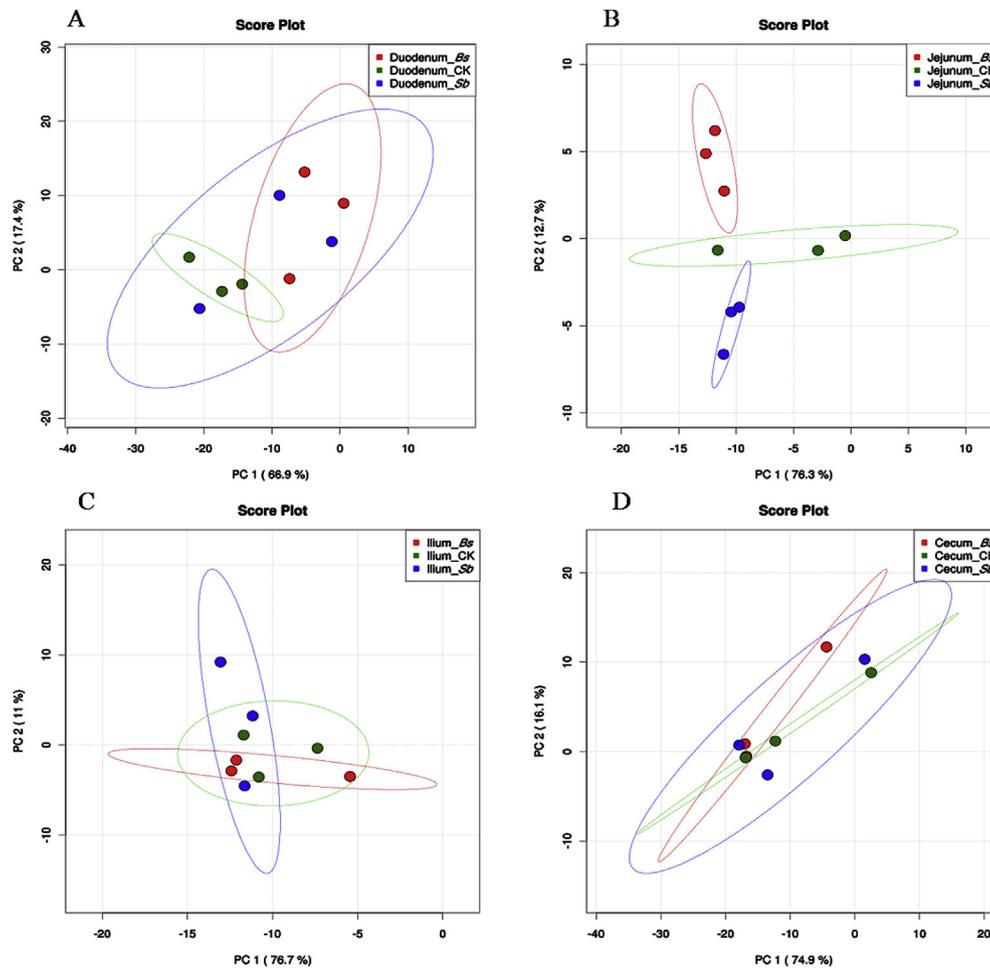
Fig. 6. Gut microbiome sequencing data of treatments and controls analyzed by principal component (PC) analyses at genus level (A) duodenum, (B) jejunum, (C) ileum, and (D) cecum. CK: birds fed the basal diet supplemented with virginiamycin; *Bs*: birds fed the basal diet supplemented with *Bacillus subtilis* B10; *Sb*: birds fed the basal diet supplemented with *Saccharomyces boulardii*.

4. Discussion

The intestinal microbiota has an enormous metabolic potential and affects the host's state of health and nutrition (Rinttila and Apajalahti, 2013). Manipulation of the intestinal microbiota is a way to improve animal health and growth performance. Antibiotics are considered to modulate the microbial community within the GI tract, which subsequently promotes growth promotion and reduces disease occurrence when administered at subtherapeutic levels (Angelakis et al., 2013; Danzeisen et al., 2011). Virginiamycin is one of the antibiotic growth promoters that are widely used in agricultural animal production (Dumonceaux et al., 2006; Miles et al., 1984). However, antibiotics resistance and health problems make the trend to ban the use of them in animal agriculture. Our previous research indicated that *Bs* or *Sb* administration resulted in a higher body weight in broilers compared with virginiamycin treatment (Rajput et al., 2013a). This paper is aimed to interpret growth promotion effects (as observed in our previous study) of the 2 probiotic strains in the gut microbiota perspective.

The bacterial communities of different sections of the GI tract are markedly different, and it has been suggested that they should be considered separate ecosystems (van der Wielen et al., 2002). Moreover, the microbial densities and diversities vary in the different sections of GI tract, being maximal in the ceca of broilers,

where fermentation is most active, reviewed by Yeoman and White (2014). Our results in this study also support this point of views. The microbial diversity in duodenum and cecum was much higher than that in the jejunum and ileum in all treatment groups. In addition, *Bs* or *Sb* administration did not affect gut microbial diversity compared with the control (except in the duodenums of *Bs* treatment group). What is more, according to the PCA, the microbial communities from different sections of the GI tract trended to be separated from each other, except in the *Sb* treatment group. The microbiota were parallel among the sections of the GI tract after the *Sb* administration, but the mechanism was not clear.

The ability to balance the host gut microbiota with probiotics has been documented. Chicken performance is linked to the gut microbiota. According to Torok's research, there are 8 common performance-linked OTU identified within both the ileum and cecum of chickens based on 16S rRNA gene sequences, which belong to the phyla Firmicutes, Bacteroidetes, Proteobacteria, and Verrucomicrobia (Torok et al., 2011). Interestingly, our results showed that the major phyla of Firmicutes, Bacteroidetes, Proteobacteria, and Verrucomicrobia were observed along the GI tract in all groups, especially in the duodenum and cecum. Furthermore, Bacteroidetes, Proteobacteria, and Verrucomicrobia were observed much higher abundance in the jejunum and ileum of the *Sb* group compared with the control and *Bs* groups. When
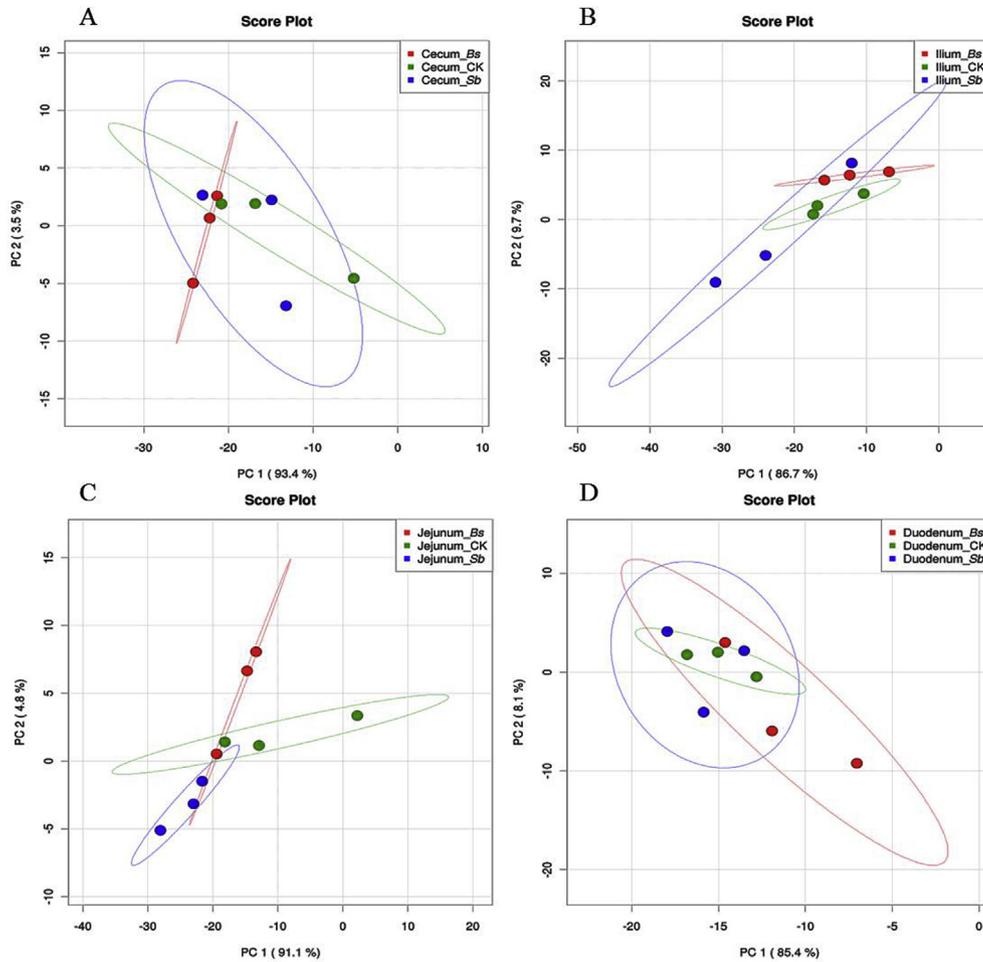
Fig. 7. Gut microbiome sequencing data of treatments and controls analyzed by principal component (PC) analyses base on the category of metabolism (A) cecum, (B) ileum, (C) jejunum, and (D) duodenum. CK: birds fed the basal diet supplemented with virginiamycin; *Bs*: birds fed the basal diet supplemented with *Bacillus subtilis* B10; *Sb*: birds fed the basal diet supplemented with *Saccharomyces boulardii*.

the sequences were classified further into the genus levels, our present results were also similar to those of Torok et al. (2011). At the genus level, we found that *Akkermansia*, *Bacteroides* and *Lactobacillus*, which were performance-related genera (Torok et al., 2011) observed in the duodenum (except for *Lactobacillus* in the control and *Bacteroides* in the *Bs* group) and cecum at a much higher abundance compared with other intestinal sections. A low abundance of *Akkermansia* and *Bacteroides* was in the jejunum and cecum, whereas *Lactobacillus* was enriched in the jejunum and ileum, which was consistent with previous studies (Stanley et al., 2012; van der Hoeven-Hangoor et al., 2013). In addition, we also observed a very high abundance of *Prevotella* in the duodenum and cecum among the 3 treatment groups. *Prevotella* has been shown to be the dominant genus in both ruminants and swine (Kim et al., 2011; Stevenson and Weimer, 2007). Recently, Kang et al. (2013) demonstrated significantly lower abundances of the genera *Prevotella*, *Coprococcus,* and unclassified *Veillonellaceae* in samples from autistic children. This finding indicated that *Prevotella* may play an important role in animal behavior and health.

The gut microbiota plays an important role in host metabolism and nutrient absorption. The microbes help break down and digest the food ingested by the host. In ruminant livestock, the gut microbiota are required to fulfill approximately 70% of

the animal's daily energy requirements (Flint and Bayer, 2008). The small intestine, which consists of the duodenum, jejunum, and ileum, is the compartment where most of the digestion and absorption of nutrients occurs (Renner, 1965). Previous studies showed that the activities of jejunal digestive enzymes (Na$^+$ K$^+$ ATPase, lipase and γ-glutamyl transpeptidase [γGT]) were significantly increased in the *Sb* treatment group, and the activity of γGT in the *Bs* treatment group also significantly increased compared with the control (Rajput et al., 2013b). Moreover, the villus height and width in the jejunum and ileum increased after *Sb* administration (Rajput et al., 2013a). Interestingly, our results showed that the microbial community in jejunum significantly differed among the *Bs*, *Sb*, and control groups at the genus level based on the PCA, forming 3 different clusters. The same result was also observed when it was analyzed base on the category of metabolism by PCA. These findings indicated that *Sb* and *Bs* administrations, especially that of *Sb*, could enhance food digestion and nutrient absorption by regulating or optimizing the gut microbial community composition, subsequently improve growth performance.

In conclusion, the results of the present study revealed that *Sb* and *Bs* can modulate a healthier microbial ecosystem, subsequently enhance the health status of broilers, and eventually improve growth performance.
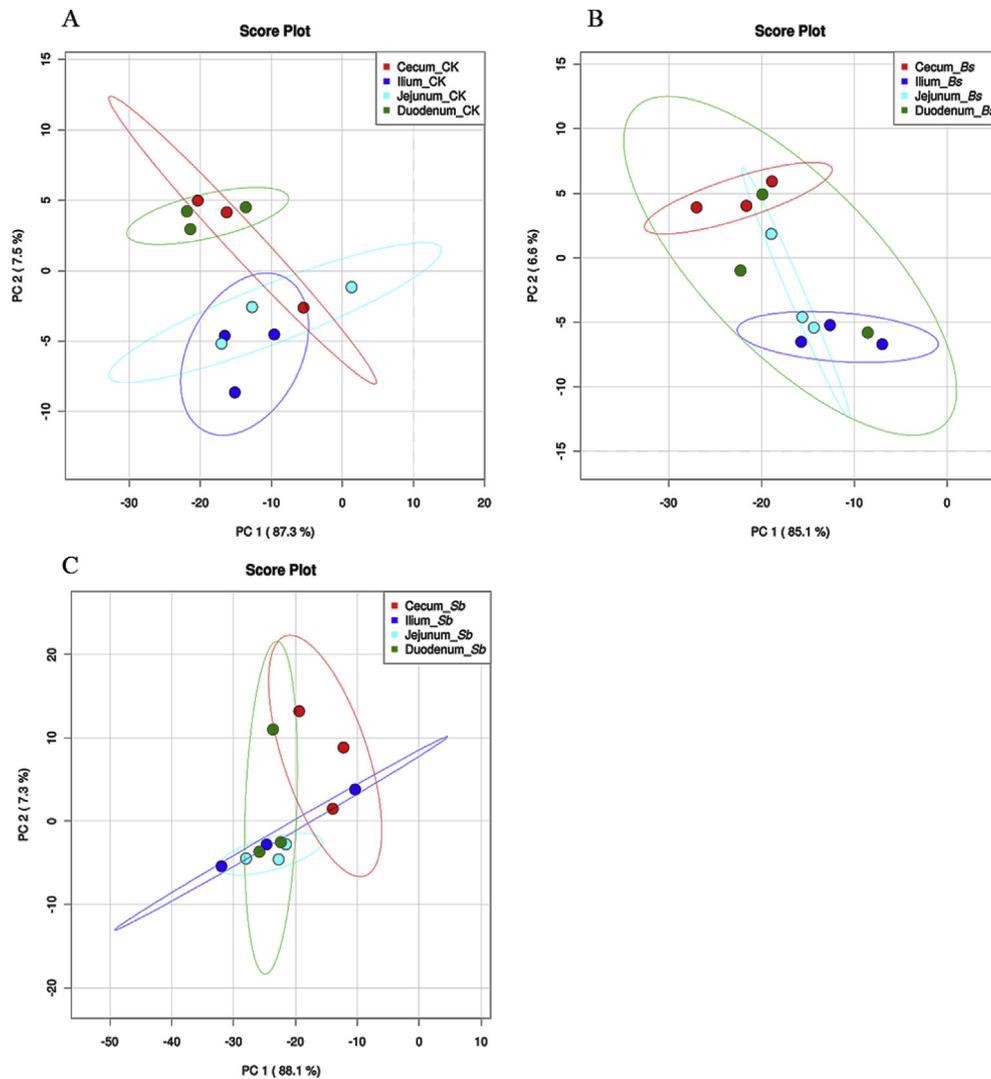
Fig. 8. Microbial community analyzed by principal component (PC) analyses based on metabolism. (A) CK: birds fed the basal diet supplemented with virginiamycin; (B) *Bs*: birds fed the basal diet supplemented with *Bacillus subtilis* B10; (C) *Sb*: birds fed the basal diet supplemented with *Saccharomyces boulardii*.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aninu.2018.03.004.

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