Effects of *Bacillus subtilis* and coccidiosis vaccine on growth indices and intestinal microbiota of broilers

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ABSTRACT Avian coccidiosis is the most serious parasitic disease in the poultry industry. Therefore, the aim of the current study was to explore the effects of *B. subtilis* and live coccidiosis vaccine alone or in combination on the production performance and anticoccidiosis, as well as the dynamic changes of intestinal microbial community. Nine hundred ninty Mahuang chickens were randomized into 4 preimmune groups including control group, coccidiosis vaccine immunization group; *B. subtilis* administration group and a group that was administrated a combination of live coccidiosis vaccine and *B. subtilis* group. Intestinal mucosal scraps collected from all these experimental groups at the age of 8 d and 15 d for microbial community 16S rRNA gene sequencing. At the age of 25 d, 30 broilers from each preimmune group were randomly assigned to a subgroup infected with *Eimeria* spp. and renamed as CI, V-CI BS-CI, and VBS-CI group. The production performance was monitored at the age of 25 d, 35 d, 45 d, and 55 d for the rest broilers from each pre-immune group. Otherwise, in the *Eimeria* spp. challenge stage, intestinal mucosal scraps collected for microbial community sequencing, while duodenum, jejunum, and cecum collected for pathological examination after sacrifice at the age of 32 d. In addition, the oocysts per gram of feces (**OPG**) and intestinal lesion score of broilers after *Eimeria* spp. challenge were also counted. Overall, the probiotics and coccidiosis vaccine resulted in the significantly improvement of the production performance. Otherwise, the intestinal lesion score and OPG after *Eimeria* spp. infection was significantly decreased in the VBS-CI group ($P < 0.05$). Moreover, these protective effects may also be closely related to genus such as *Romboutsia, Blautia*, and *Butyricococcus*, as well as microbiota functions like the quorum sensing pathway. According to these results, a combination of *B. subtilis* and coccidiosis vaccines can improve performance and provide additional protection against *Eimeria* spp. infection.

Key words: *Bacillus subtilis*, coccidiosis vaccine, growth performance, histopathology, Intestinal microbiota

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INTRODUCTION

The poultry industry is currently the fastest growing meat industry in the world. Currently, the major challenge that the poultry industry faces is disease control (metabolism, nutrition, and infectious diseases). More specifically, avian coccidiosis, a host-specific parasitic disease caused by *Eimeria,* is a major poultry infectious disease that causes huge economic losses, especially in intensive management systems. These losses are related to the increase in drug costs and the decline in flock performance (Blake et al., 2020; El-Shall et al., 2022). Control of coccidiosis is more economically important than other poultry diseases, such as Newcastle disease, Marek's disease, infectious bronchitis, pullorum, and chicken anemia (Quiroz-Castañeda and Dantán-

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González, 2015). The negative impact on performance stems from excessive coccidia circulation and intestinal damage, which results in reduced nutrient absorption, low calorie conversion, and poor growth.

Due to the high incidence and mortality of coccidiosis in poultry, broilers receive anticoccidiosis drugs throughout their lifespan. However, incorrect use of sulfa quinoxaline, monensin, and other ionophores can cause parasites to develop drug resistance and poor performance in birds (Chapman et al., 2010). In view of these shortcomings, vaccines are generally considered a safer option. Live attenuated vaccines or non-attenuated live vaccines (virulent coccidia strains) are increasingly used in the production of layers and breeders and some broilers (Titilincu et al., 2008). In addition, vaccine efficacy is highly dependent on the *Eimeria* strain contained in the vaccine since *Eimeria* strains are geographically diverse. However, this immunization method can also lead to a decline in early growth performance and may increase the susceptibility of chicks to secondary infections, such as necrotizing enteritis. Therefore, reducing side effects of live vaccine is an urgent problem to be solved in the prevention and control of chicken coccidiosis.

Probiotics are viable, nonpathogenic microorganisms capable of maintaining the normal gastrointestinal microbiota. Previous research exhibits that supplementing broiler chicken with probiotics can increase the activity of beneficial bacteria in the gastrointestinal tract, stimulate immunity, enhance the activity of digestive enzymes, reduce ammonia production, neutralize enterotoxins, and regulate cellular and humoral immunity (Memon et al., 2021). In animal husbandry, *Bacillus* spp. has important application value as a probiotic in feed due to its ability in producing spores. The spores of *Bacillus* retain strong environmental adaptability as they were able to withstand and survive in the intestines of animals with various digestive enzymes and acidic environments. It can similarly survive in the external environment that is subjected to drastic changes in temperature (Kimelman and Shemesh, 2019; Deng et al., 2020). Previous studies shown that adding *Bacillus* probiotics to feed can control experimental *Eimeria* infections, promote growth, and reduce adverse reactions caused by *Eimeria* vaccination (Giannenas et al., 2012; Wang et al., 2019).

The intestinal microbiota is composed of more than 2,000 metagenomic (**MGS**) bacteria that are distributed throughout the gastrointestinal tract. Many functions such as metabolism of nutrients in the diet, fiber fermentation, short-chain fatty acid (**SCFA**) production, vitamin production, barrier function and tight junction regulation, antibacterial compound secretion, and immune regulation were attributed to the intestinal microbiota (Cai et al., 2020). The microbial metabolites released by the gut microbiota circulate may have an effect on function of other organs and systems in the body. Previously, Chen et al. found that in chickens infected with *E. tenella*, the abundance of probiotics such as *Lactobacillus*, *Faecalibacterium*, *Ruminococcaceae* UCG-013, *Romboutsia*, and *Shuttleworthia* were decreased in the intestinal microflora; whereas the opportunistic pathogens *Enterococcus* and *Streptococcus* increased in abundance over time in response to the infection (Chen et al., 2020). However, Rami A. Dalloul et al. have discovered that *Lactobacillus*-based probiotics are able to reduce cellular immune responses and reduce fecal oocyst shedding (Dalloul et al., 2003; Kasornpikul et al., 2009). Therefore, chicken coccidia might also increase host inflammation through intestinal microflora regulation. However, it was still unclear whether *Bacillus*-based probiotics involve the restoration of the diversity of the intestinal microflora.

Based on these findings, we hypothesized that the combined use of live attenuated vaccines and *B. subtilis* may be beneficial to control *Eimeria* spp. infection in chickens. In this study, we explore the effect of use of *B. subtilis* probiotics and live attenuated vaccines as feed supplements alone or in combination in the growth performance, intestinal damage and changes in the composition and function of the intestinal microflora in chicken.

MATERIALS AND METHODS

Material Preparation

One day old Mahuang chickens were purchased from Chaozhou farm and raised in a coccidia-free environment. Chickens were ad libitum fed with conventional poultry feed produced by Shantou Wenfa Feed Industry Co., Ltd., which does not contain anti coccidia drugs and antibiotics.

The sporulation and purification procedure of chicken coccidia oocysts were performed as previously described in our published article (Qi et al., 2021). In addition, the quadrivalent live vaccine for chicken coccidiosis (*E. tenella* ETGZ strain, *E. necatrix* ENHZ strain, *E. acervulina* EAGZ strain, and *E. maxima* EMPY strain) were preserved and prepared by the Institute of Animal Health, Guangdong Academy Agricultural Sciences.

The commercial probiotics of *B. subtilis* (5.0×10^9 colony forming units [CFU]/g) was purchased from Qingdao Vland Biotech Group Co., Ltd.

All the animal procedures carried out in the present study were in accordance with the guidelines of the Animal Ethics Committee (No. PT-2020012) of Institute of Animal Health, Guangdong Academy of Agricultural Sciences.

Experimental Design

Nine hundred ninty Mahuang chickens were randomly divided into 4 groups, including control group (CON group, N = 270), *B. subtilis* pretreatment group (BS group, N = 240), coccidiosis vaccine immunization group (V group, N = 240) and coccidiosis vaccine and *B. subtilis* co-pretreatment group (VBS group, N = 240). Chickens in both V and VBS groups were inoculated with chicken coccidiosis tetravalent live

vaccine by drinking water at the age of 3 d with 1,700 oocysts/chicken. Thereafter, chickens in BS group and VBS group were fed with *B. subtilis* supplement at the age of 3 to 6 days and 10 to 13 d, respectively, with the dosage of 0.25 g/kg feed (1.25×10^9 CFU/kg feed).

Eimeria *Challenge*

After the administration of individual treatments mentioned above, 30 Mahuang chickens at the age of 25 d were randomly selected from each treatment group and orally infected with 1 mL/bird of *Eimeria* spp. sporulated oocysts (3.4×10^4 sporulated oocysts/mL). These infected chickens taken out from CON, V, BS, and VBS group were redefined as CI (Coccidia Infection), V-CI, BS-CI, and VBS-CI group respectively. Additionally, another 30 chickens were selected from Mahuang chickens CON group and administrated with the same procedure without *Eimeria* spp. to ensure there was no environment source of *Eimeria* spp. contamination or cross contamination.

Growth Performance

After Mahuang chickens feeding with live chicken coccidiosis vaccine and *B. subtilis* alone or in combination, the body weight and feed intake were recorded at day 1, day 25, day 35, day 45, and day 55 respectively. Chicken body weight gain (**BWG**), feed consumption (**FC**), and feed conversion ratio (**FCR**) were then calculated. All parameter acquisition and calculations refer to previous research (Yi et al., 2018).

Lesion Score

Seven days after Mahuang chickens infected with *Eimeria* spp. (32 d of age), chickens in CI, V-CI, BS-CI, and VBS-CI group were euthanized, and the duodenum, jejunum, and cecum were collected. Intestinal lesion score was then obtained using the method from Williams, R. B. (Williams and Catchpole, 2000).

Oocysts per Gram of Feces

On the 5th to the 7th day (30−32 d of age) after the *Eimeria* spp. challenge, a fresh excrement sample from the isolator was collected and homogenized. Five grams of standardized excrement from each test group was added to 45 mL of saturated saline for homogenization. Following this, the McMaster counter was performed to count the oocysts. The number of oocysts per gram (**OPG**) in feces was then calculated according to the method described by Ghasemi et al. (2010).

Histopathology Evaluation

On the 7th day after infection, 4 chickens from each group were randomly selected, and their intestines (duodenum, jejunum, and cecum) were collected and fixed in 10% buffered neutral formalin solution. The intestinal samples were then prepared by paraffin sectioning with hematoxylin and eosin stain; the lesion was then observed via microscopic observation.

16S rRNA Sequencing and Data Analysis

Four chickens were randomly selected from each group for euthanasia at the 8th and 15th day of the immunization treatment, and the 7th day after the challenge test (32 d of age). The intestinal contents were collected and stored at −80°C after quick freezing with liquid nitrogen. The samples were then subjected to E.Z.N.A. Stool DNA Kit (Omega Bio-Tek, Norcross, GA) to extract bacterial DNA. For subsequent 16s analysis, we used 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) to amplify the 16S rRNA gene V3-V4 hypervariable region, and the amplified product was subjected to QIAquick PCR purification kit (Germany Qiagen) for purification. The purified PCR products were directly used for 16S rRNA sequencing using the Illumina MiSeq paired-end (**PE**) 300 sequencing platform (Illumina Inc., Shanghai, China).

The raw data obtained by sequencing was processed by the DADA2 pipeline of Quantitative Insights into Microbial Ecology 2 (**QIIME2**) version 2020.11 for data cleaning, denoising, and cluster analysis. In the ASV cluster analysis, SILVA database version 132 was used as a reference. The R version 4.1.0 was then utilized to analyze sequencing data leveling, microflora diversity, microflora structure, and microflora function of the data. The sequencing results were then leveled by using the rarefy_even_depth function of the phyloseq version 1.36.0 package. Bacterial diversity which includes alpha diversity and beta diversity, was calculated using the alpha function of the microbiome version 1.14.0 package and the ordinate function of the phyloseq package, respectively. The bacterial colony structure, which includes bacterial colony composition and dominant factor analysis was determined by linear discriminant analysis effect size (**LEfSe**) using the microeco version 0.6.0 package. Tax4Fun version 1.1.5 package was used to analyze the function of the microbiota.

Statistical Analysis

Here we sample one-way ANOVA followed by Tukey's honest significant differences (**HSD**) post-hoc test method to analyze the difference in production performance and clinicopathological parameters at each time point. All statistical analyses were performed using the Social Scientists (SPSS) version 19 (SPSS Inc.) and GraphPad Prism version 6 (GraphPad, CA). Results in tables are presented as leas square means with standard error of mean (**SEM**). Significance was taken at a P value of <0.05.

RESULTS

B. subtilis *Improved Growth Performance in Combination of Coccidiosis Vaccine*

The effects of coccidiosis vaccine and *B. subtilis* alone or in combination on the performance of broilers are listed in Table 1. Compared with other experimental groups, feeding *B. subtilis* alone effectively improved the BWG in all the age of days tested ($P < 0.05$). In addition, the combined use of coccidiosis vaccine and *B. subtilis* significantly increased BWG at 55 d of age compared to administration of coccidiosis vaccine alone and the control group ($P < 0.05$). After 35 d of age, the feed intake was reduced in the VBS group in comparison to both V and CON group, however, the difference was not significant ($P > 0.05$). In addition, the feed conversion ratio in the VBS group (2.05 ± 0.06) was significantly lower than CON group (2.41 ± 0.03) and V Group (2.51 ± 0.10) ($P < 0.05$) at the age of 55 d. Therefore, feeding *B. subtilis* alone can effectively improve the BWG of broilers, and administration of coccidiosis vaccine and *B. subtilis* in combination showed positive effect in improving the growth performance of broilers.

Administration of B. subtilis *and Coccidiosis Vaccine Alleviated Fecal Oocyst Discharge and Intestinal Injury in Broilers Challenged with* Eimeria *spp*

The intestinal tissue lesion score and oocysts number after *Eimeria* spp. challenge in different treatment groups are listed in Table 2. Compared to the CI group, the lesion score of cecum, jejunum, and duodenum in BS-CI group showed no significant difference ($P > 0.05$). Conversely, the lesion scores of all the intestinal portions in V-CI group were significantly reduced compared to that of the CI group ($P < 0.05$). Interestingly, in the VBS group, the lesion scores of cecum, jejunum, and duodenum were reduced to 0.64 ± 0.18, 0.52 ± 0.14, and 0.32 ± 0.14 respectively, indicated that the combined feeding of coccidiosis vaccine and *B. subtilis* could prevent and control the damage of the three intestine regions caused by *Eimeria* spp. Moreover, the OPG in the VBS-CI (11.43 ± 2.22) was significantly lower than that of the CI group ($P < 0.05$). Therefore, combined feeding of coccidiosis vaccine and *B. subtilis* can effectively prevent intestinal damage and fecal oocysts output caused by *Eimeria* spp.

Histological sections of jejunum, cecum and duodenum obtained after the challenge test were produced and observed under a microscope. In the control group that was not treated with *Eimeria* spp., no histopathological changes or necrosis were observed in the three intestinal segments (Figure 2A). Coccidian oocysts can be seen in both jejunum and cecum sections in all the coccidia infection groups (Figure 2B–E). On the other hand, shedding of villi epithelial cells, and the redistribution of lymphoid tissue nodules can be observed in groups without *B. subtilis* or immune coccidiosis vaccine. However, the epithelial integrity of the 3 intestinal segments in the combined application of *B. subtilis* and coccidiosis vaccine was relatively intact compared to the CI group (Figure 2E). Based on our result, the combined application of *B. subtilis* and chicken coccidiosis vaccine have been shown to effectively protect the integrity of the intestinal barrier.

Alpha and Beta Diversity of Intestinal Microbial Constitution

A total of 2,801,994 reads were collected from 52 samples aged 8, 15, and 32 days, with an average of 53,884 ± 1,724 (mean ± SEM) reads per sample. After processing the raw data to Qiime2 Data2, an average of 19,546 ± 1,203 (mean ± SEM) clean reads per sample

Table 1. Effect of administration of *Bacillus subtilis* and coccidiosis vaccine on growth performance of spotted-brown chicken.

Traits	Treatments[1]				*P*-value[2]
	CON	BS	V	VBS	
Body weight gain (g/bird)					
25 days	367.67 ± 8.09^{b}	449.00 ± 6.35^{a}	335.00 ± 7.51^{b}	354.00 ± 2.89^{b}	8.37E−06
35 days	733.00 ± 8.14^{b}	854.00 ± 4.04^{a}	707.00 ± 9.81^{b}	708.00 ± 5.20^{b}	1.34E−06
45 days	1,037.33 ± 8.19^{c}	1,198.00 ± 3.46^{a}	1,059.00 ± 10.39^{bc}	1,079.00 ± 5.77^{b}	1.43E−06
55 days	1,427.33 ± 8.11^{d}	1,663.00 ± 6.93^{a}	1,447.00 ± 6.93^{c}	1,526.00 ± 2.31^{b}	2.12E−08
Feed intake (g/bird)					
25 days	674.69 ± 6.51	719.92 ± 47.75	649.96 ± 18.57	694.66 ± 23.08	4.03E−01
35 days	1,401.49 ± 5.28^{b}	1,923.28 ± 46.24^{a}	1,510.49 ± 20.59^{b}	1,365.12 ± 16.50^{b}	1.54E−06
45 days	2,385.22 ± 29.71^{b}	2,851.61 ± 36.23^{a}	2,555.91 ± 103.30^{ab}	2,180.22 ± 89.57^{b}	7.29E−03
55 days	3,440.23 ± 61.03	3,721.17 ± 164.79	3,631.83 ± 133.91	3,138.12 ± 89.32	3.58E−02
Feed conversion (g feed/g gain)					
25 days	1.83 ± 0.06^{ab}	1.60 ± 0.08^{b}	1.95 ± 0.10^{a}	1.96 ± 0.05^{a}	3.15E−02
35 days	1.91 ± 0.02^{b}	2.25 ± 0.04^{a}	2.14 ± 0.06^{a}	1.93 ± 0.01^{b}	5.53E−04
45 days	2.30 ± 0.01	2.38 ± 0.11	2.42 ± 0.12	2.02 ± 0.07	4.94E−02
55 days	2.41 ± 0.03^{a}	2.23 ± 0.09^{ab}	2.51 ± 0.10^{a}	2.05 ± 0.06^{b}	1.24E−02

Means within a row with no common superscript letter are significantly different ($P \leq 0.05$).

[1]Treatments: CON: chicken without administration of *B. subtilis* and coccidiosis vaccine; BS: chicken administrated with *B. subtilis*; V: chicken fed with coccidiosis vaccine; VBS: chicken administrated with coccidiosis vaccine and *B. subtilis*.

[2]*P* value in overall treatment at different ages.

[a–c]Means not sharing a common superscript differ significantly ($P < 0.05$).

Table 2. Lesion score in intestinal segment and oocyst per gram of spotted-brown chicken challenged with *Eimeria* species.

Traits	Treatments[1]					*P*-value[2]
	CON	V-CI	CI	BS-CI	VBS-CI	
Lesion scores						
Cecum	0.00 ± 0.00^{c}	0.80 ± 0.12^{b}	2.04 ± 0.14^{a}	1.36 ± 0.10^{ab}	0.64 ± 0.18^{bc}	2.33E−15
Jejunum	0.00 ± 0.00^{c}	0.68 ± 0.10^{b}	1.44 ± 0.14^{a}	1.36 ± 0.15^{ab}	0.52 ± 0.14^{bc}	1.49E−12
Duodenum	0.00 ± 0.00^{c}	0.60 ± 0.10^{b}	1.40 ± 0.12^{a}	1.44 ± 0.13^{a}	0.32 ± 0.14^{bc}	8.88E−16
Oocyst per gram ($\times 10^6$ oocysts/g)	ND[3]	15.71 ± 0.92^{b}	89.43 ± 2.75^{a}	32.29 ± 4.05^{ab}	11.43 ± 2.22^{b}	7.42E−05

Means within a row with no common superscript letter are significantly different ($P \leq 0.05$).

[1]Group: CON: chicken without administration of *B. subtilis* and coccidiosis vaccine or *Eimeria sp.* challenge; V-CI: chicken administrated with coccidiosis vaccine and challenged with *Eimeria* spp.; CI: chicken only challenged with *Eimeria sp.*; BS-CI: chicken fed with *B. subtilis* and then challenged with *Eimeria* spp.; VBS-CI: chicken administrated with *B. subtilis* and coccidiosis vaccine, and then challenged with *Eimeria* spp. Data presented as mean ± SEM. SEM: standard error of the mean.

[2]Overall treatment *P*-value.

[3]ND = none detected.

[a–c]Means not sharing a common superscript differ significantly ($P < 0.05$).

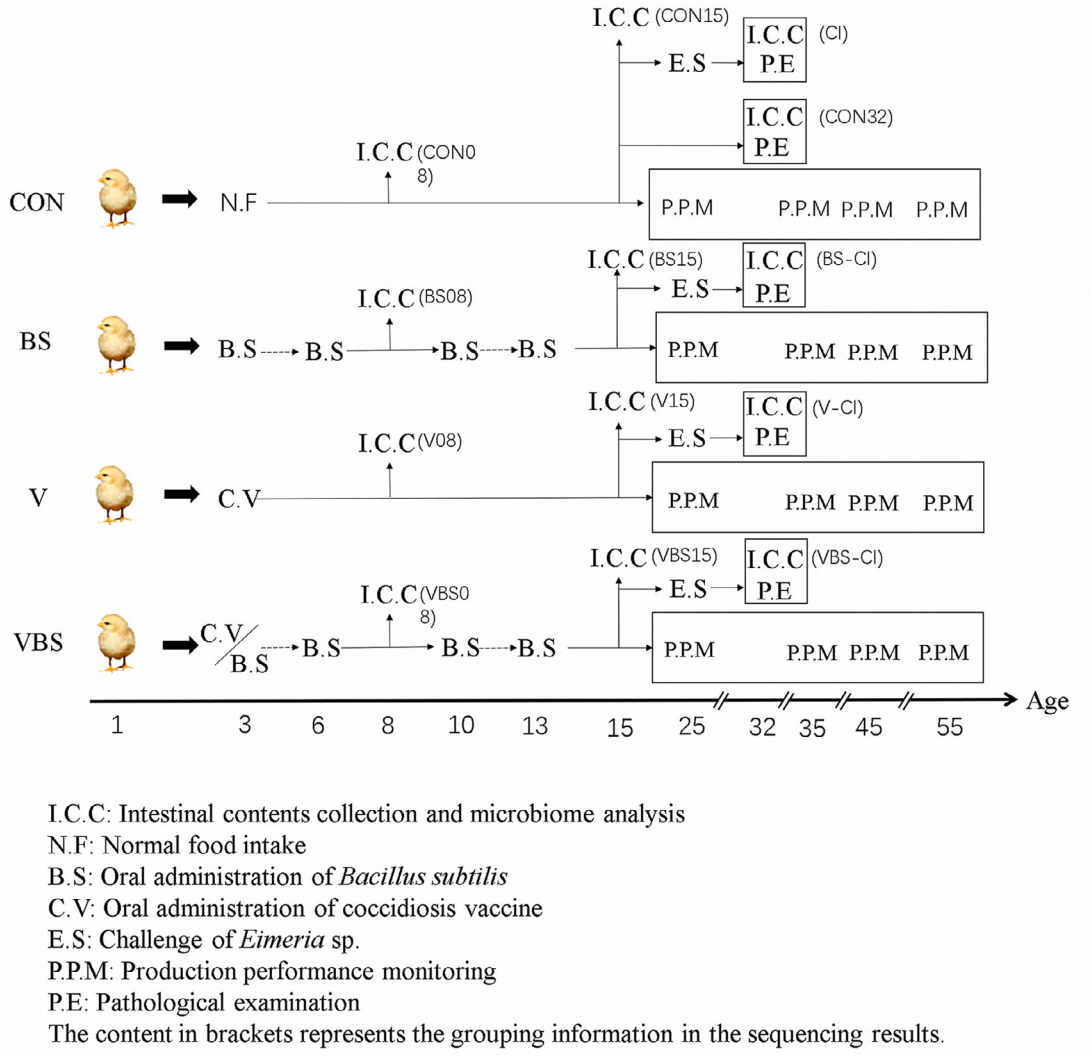

Figure 1. Schematic presentation of the experimental trial. The broilers were divided into four experimental groups. At the third day of age, BS group and V group were orally administrated with *Bacillus subtilis* and coccidiosis vaccine separately, while VBS group was simultaneously orally administrated with the combination of *Bacillus subtilis* and coccidiosis vaccine. In VBS or BS group, *B. subtilis* was supplied for 8 d, including 3 to 6 d of age and 10 to 13 d of age respectively. In the meantime, chickens in the control group (CON group) were orally administrated with PBS as negative control. Intestinal contents were collected at 8 and 15 d of age for microbiome 16S rRNA sequencing. Production performance including body weight gain and feed intake was monitored at 25, 35, 45, and 55 d of age. In addition, in order to compare the protective effects of different immune strategies on avian coccidia, 30 experimental animals in each group were randomly selected at 25 d of age and then challenged with *Eimeria* spp. At 32 days of age, chickens were sacrificed, the intestinal tissue and intestinal content samples were collected for pathological examination and microbiome analysis respectively. The corresponding relationship of the group set at the infection experiment with immunization experiment: V group, BS group, VBS group corresponded to V-CI group, BS-CI group, and VBS-CI group, respectively.

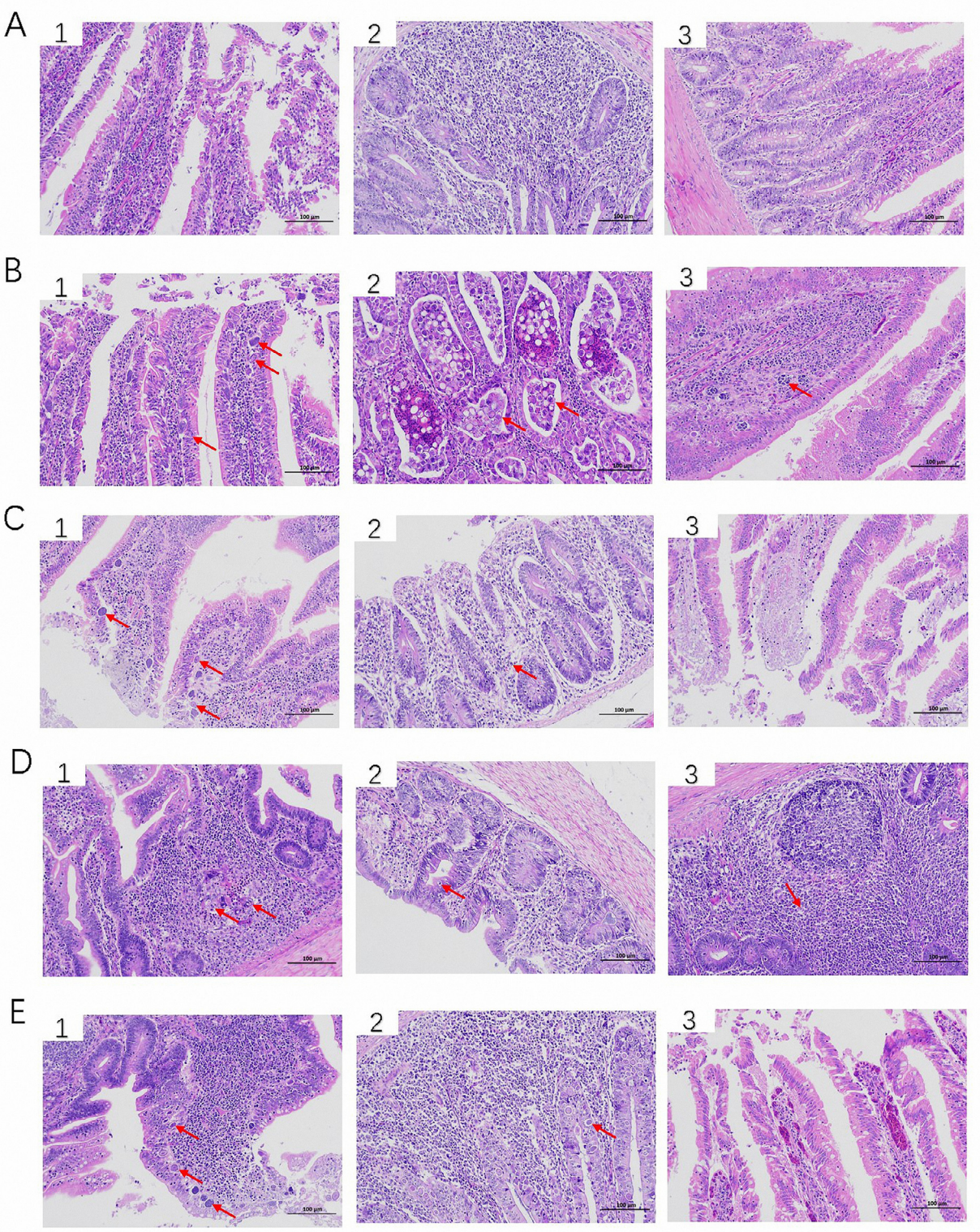

Figure 2. Histological analysis of the effects of *B. subtilis* and chicken coccidial vaccine on avian coccidiosis in vivo. This result described the intestinal histopathological changes from control group (A), V-CI group (B), CI group (C), BS-CI group (D), and VBS-CI group (E). The red arrow represented the observation of coccidia oocysts. The intestinal tissue section results of each test group included jejunum tissue (1), cecum tissue (2) and duodenum (3). Samples were collected from the day 32 of age. Scale bar—100 μm.

were obtained. An average of 14,975 reads and 876 ASVs per sample was obtained ultimately. After rarefaction curve analysis, the species richness corresponding to the data volume of each sample had reached a plateau, the subsequent bacterial diversity and functional analysis were performed.

In order to evaluate the impact of different pretreatments and infection tests on the diversity (i.e., alpha-diversity) within the microbial samples, the observed Taxa, Chao1 index, Shannon diversity and inverse Simpson diversity of each sample were calculated. At

the same time, the alpha-diversity difference analysis was carried out for the experimental groups of different ages. Among them, the corresponding results of the 8-day, 15-day, and 32-day-old chickens from challenge test stages are exhibited in Figures S2, S3, and S4, respectively. After the Kruskal-Wallis difference analysis, there was no significant difference in the results of the alpha diversity parameters of the samples in the test group at each age stage ($P > 0.05$). In addition, the diversity (beta-diversity) among microbial samples at each age was assessed based on the Principal Coordinate Analysis (**PCoA**) and Nonmetric Multidimensional Scaling (**NMDS**) 2 dimensionality reduction sorting analysis methods, in which the similarities and differences between the community composition of the experimental group at different age stages were explored (Figure S5).

Effect of B. subtilis *and Coccidiosis Vaccine on Structure of Intestinal Microorganisms*

At the phylum level, *Firmicutes, Bacteroidetes,* and *Actinobacteria* were the three main phyla (Figure 3A). At the genus level, *Lactobacillus* and Unclassified *Lachnospiraceae* dominate the microflora (Figure 3B). Overlap analysis of ASVs was further performed, and the results showed that 64 ASVs were co-existed among all experimental groups at 8 d of age, whereas it increased to 69 ASVs at 15 d of age (Figure S6). At the same time, a heat map of the top 50 abundance ASVs shows that there was a significant gap among the groups (Figure 4A). To determine specific bacterial taxa as biomarkers for each experimental treatment group, based on LEfSe method, the relative abundance of different bacterial taxa related to the most abundant phylum in each group classified by LDA scores. The results represented in Figure 4B, C, and D illustrated the differential abundance distributions of dominant bacterial taxa between the V group, BS group, and VBS group as compared to that of the CON group at the 15-day-old stage. Upon feeding *B. subtilis* or immunizing with coccidiosis vaccine, there was an increase in the relative abundance of the genera including *Blautia, Ruminococcus torques* group, *Lachnoclostridium*, and *Senllimonas* (Figure 4B and C). Moreover, the VBS group was relatively enriched with genera such as *Staphylococcus, Lactobacillus*, and *Enterococcus*, compared with CON group. *Lactobacillus aviarius* and *Lactobacillus reuteri* were the most dominant species in the VBS group (Figure 4D). And *L. aviarius* was also the dominant species in the V group.

Different Structure of Intestinal Microorganisms in Preimmunity Broilers Challenged With Eimeria *spp*

In order to further analyze the difference in gut microbiome composition and structure of different immune strategies after challenging *Eimeria* spp., phyloseq package was applied to analyze the differences at ASV and Genus level. Compared with the control group, the 2 strategies of immunizing coccidiosis vaccine alone and feeding *B. subtilis* alone significantly increased the relative abundance of ASV_466 (*Ruminococcaceae* UCG 005) after challenge treatment, while significantly reducing the relative abundance of ASV_68 (*Bacteroides*), ASV_408 (*Flavonifractor*), ASV_525 (*Ruminococcaceae* GCA.900066225) and ASV_636 (*Ruminococcaceae* UCG 014) ($P < 0.05$) (Figure 5A and C). Among them, ASV_525 was also significantly down-regulated in the VBS-CI group (P <0.05 vs. CON group) (Figure 5D). In addition, the VBS-CI group also significantly up-regulated ASV_149 (*Romboutsia*), ASV_198 (*Lactobacillus*), ASV470 (*Butyricicoccus*), ASV_864 (*Blautia*), and reduced the relative abundant of ASV_101 (*Alistipes*) and ASV_346 (*Faecalicoccus*). However, none of the above ASVs were significantly regulated by CI group. And CI group specifically and significantly increased the relative abundant of ASV_204 (*Lactobacillus*), ASV_246 (*Enterococcus*), ASV_248 (*Enterococcus*), and ASV_511 (*Eubacterium coprostanoligenes* group) ($P < 0.05$ vs. CON group) (Figure 5B).

Predicted Functions of Intestinal Microbiota

Microbial function was of great significance for explaining the biological significance of changes in microflora. Here we used both the immune treatment test and the attack test to predict microbial function. Among them, at KEGG Level 1, all test groups account for the largest proportion of metabolism-related functions (Figure S6A). At the same time, at KEGG Level 3, the first 40 high-abundance signal pathways were compared with heat maps. The results showed that the abundance levels of different signal pathways in different test groups were different (Figure S6B). We compared the microbial functions of the attack test, and 39 signal pathways were significantly different (Figure 6A). Compared with the control group, *Eimeria* spp. infection was dominant in the functional genes of Glycolysis/Gluconeogenesis pathway and sucrose metabolism pathway. These signaling pathways do not appear after feeding subtilis spores or immune coccidiosis vaccine alone (Figure 6B, C and D). Interestingly, these signaling pathways were also significantly enriched signaling pathways after the combined application of *B. subtilis* and coccidiosis vaccine and re-attack treatment ($P < 0.05$) (Figure 6E). However, the experimental group that combined *B. subtilis* and coccidiosis vaccine re-attack treatment also had a larger number of functional genes in the quorum sensing pathway ($P < 0.05$), but this signaling pathway was not the dominant signaling pathway during *Eimeria* spp. infection.

DISCUSSION

Vaccination has been used to prevent chicken coccidiosis in the poultry industry in the past 50 y. However,

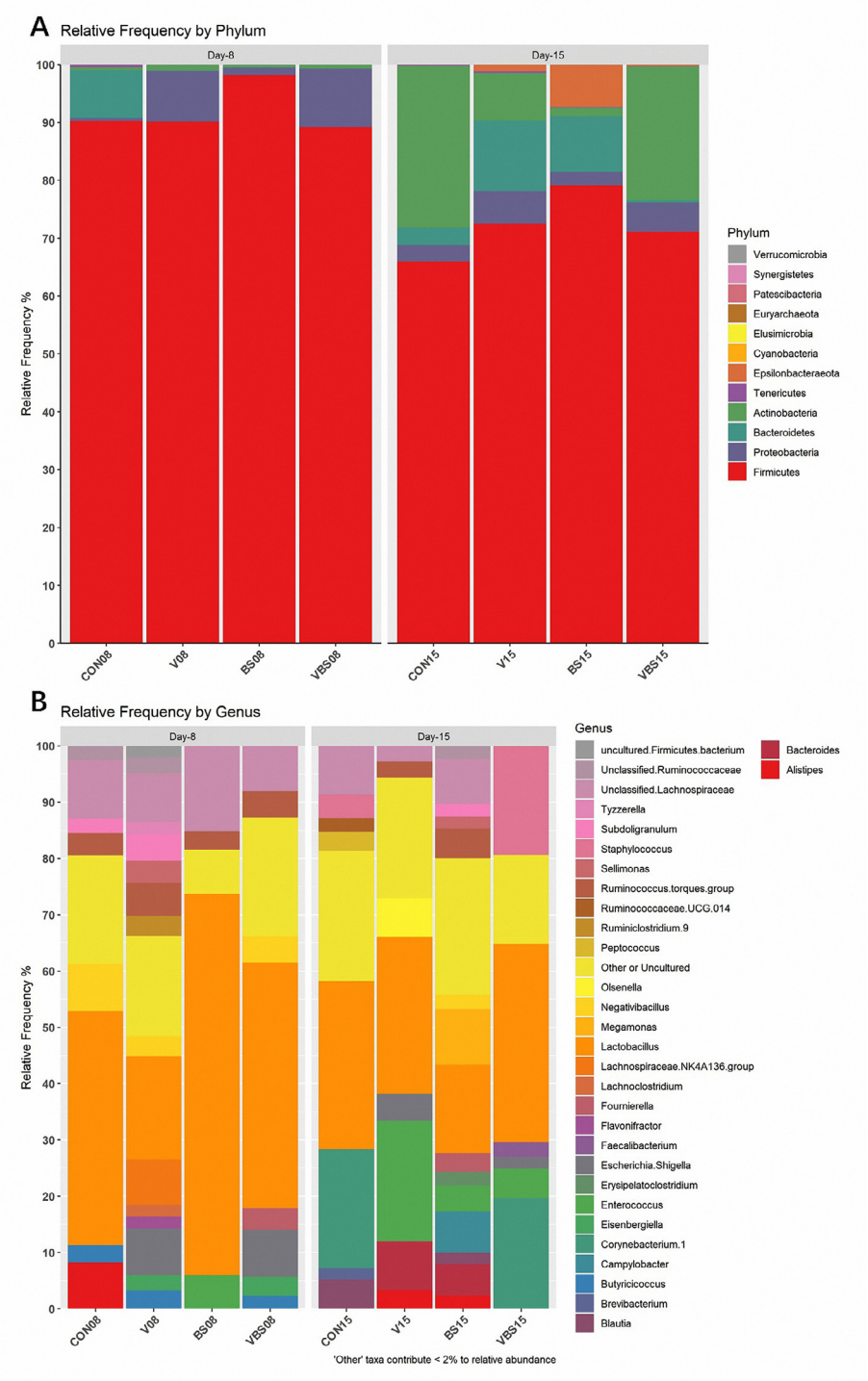

Figure 3. Microbial community composition of various experimental groups. The distribution and abundance of bacterial phylum (a) and bacterial genus (b) in various immune groups at different ages were determined by ggplot 2 packages (version 3.3.5) in rstudio (version 1.4.1717). CON08, V08, BS08, and VBS08 represent the gut microbiota analysis of the CON, V, BS, and VBS group at 8 days of age. Correspondingly, CON15, V15, BS15 and VBS15 were sequencing results from 15 d of age.

several studies have reported that coccidiosis vaccination shows negative impact on the growth performance of the chickens (Gautier et al., 2020; Bafundo et al., 2021). In the early stages of growth (days 1−21), the live oocysts of the vaccine may replicate in the host, leading to mild subclinical coccidiosis, which is related to reduced intestinal epithelial absorption area, malabsorption, and inflammation. This study aims to explore the effects of *B. subtilis* and chicken coccidia live vaccines alone and in combination on broiler performance and resistance to *Eimeria* spp. infection, as well as the dynamic changes of the intestinal microbial community.

In this study, the combined use of *B. subtilis* and coccidiosis vaccine significantly increased BWG and decreased FI and FCR values. The results showed that the addition of *B. subtilis* helped to combat the growth performance impairment associated with inoculation

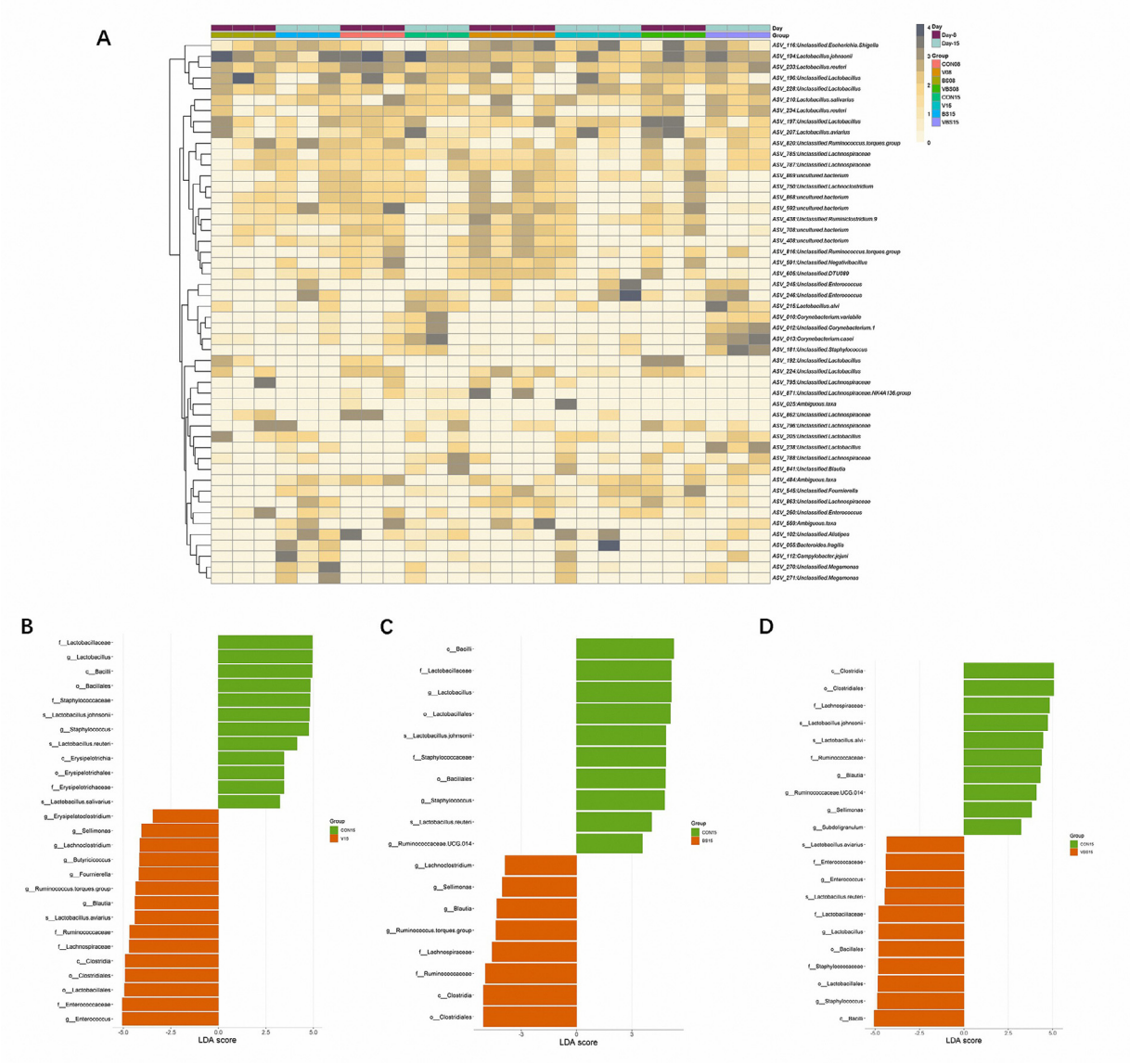

Figure 4. Clustering and difference analysis results at ASV level of each group in immune treatment study. (A) Top 50 ASVS of relative abundance for cluster analysis built by plot_taxa_heatmap function. Otherwise, linear discriminative analysis (LDA) effect size (lefse) analysis was performed to analyze the effect of immunoprophylactic treatment of *B. subtilis* and coccidiosis vaccine on the intestinal flora of test animals at different days of age. (B)−(D) are the lefse analysis of VBS15, V15, and BS15 group in comparison to CON15 group respectively. The horizontal bar represents the effect size of each taxon. The length of the bar represents the log10 transformed LDA score, indicated by the vertical dashed line. The threshold of the log LDA score for discriminating features was set at 2.0. The relative abundance was statistically significantly changed ($P < 0.05$). The bacterial taxon written next to the horizontal line were segmented by underlining, and the finer taxa are species. Lefse analysis was done with the microeco version 0.6.0.

with live chicken coccidia vaccine in broilers. *Eimeria* infection is shown to cause damage to the host's intestinal mucosa and intestinal cells during the progression of its life cycle. Severe damage led to malabsorption of nutrients and subsequent performance degradation. In addition, parasitic infections result in the shift of nutrient resource allocation from growth to immune response, which may lead to a decrease in growth performance. However, Yang et al. found that feeding *B. subtilis* alone had a significant recovery effect on the BWG reduction of coccidia infection in chickens (Yang et al., 2021). However, the immunization effect of the combined administration of *B. subtilis* and coccidiosis vaccine in broiler has not yet been reported. Miranda et al. previously reported that after the co-administration of PoultryStar (*Bifidobacterium animalis, Lactobacillus salivarius*, and *Enterococcus faecium*) and coccidia vaccine, the FI and FCR of broiler chickens were significantly lower than those immunized with live coccidia vaccine alone (Ritzi et al., 2016). Significant reductions in OPG and Lesion scores were also observed in the combined administration of *B. subtilis* and coccidiosis vaccine. The lower the lesion score, the higher the effective utilization and absorption rate of intestinal nutrition, and the higher the effective utilization rate of feed. Therefore, our results are consistent with these perspectives. Otherwise, the pathological sections of different intestinal tracts were also compared and analyzed. The results showed that the combined administration of *B. subtilis* and coccidiosis vaccine immunization strategy

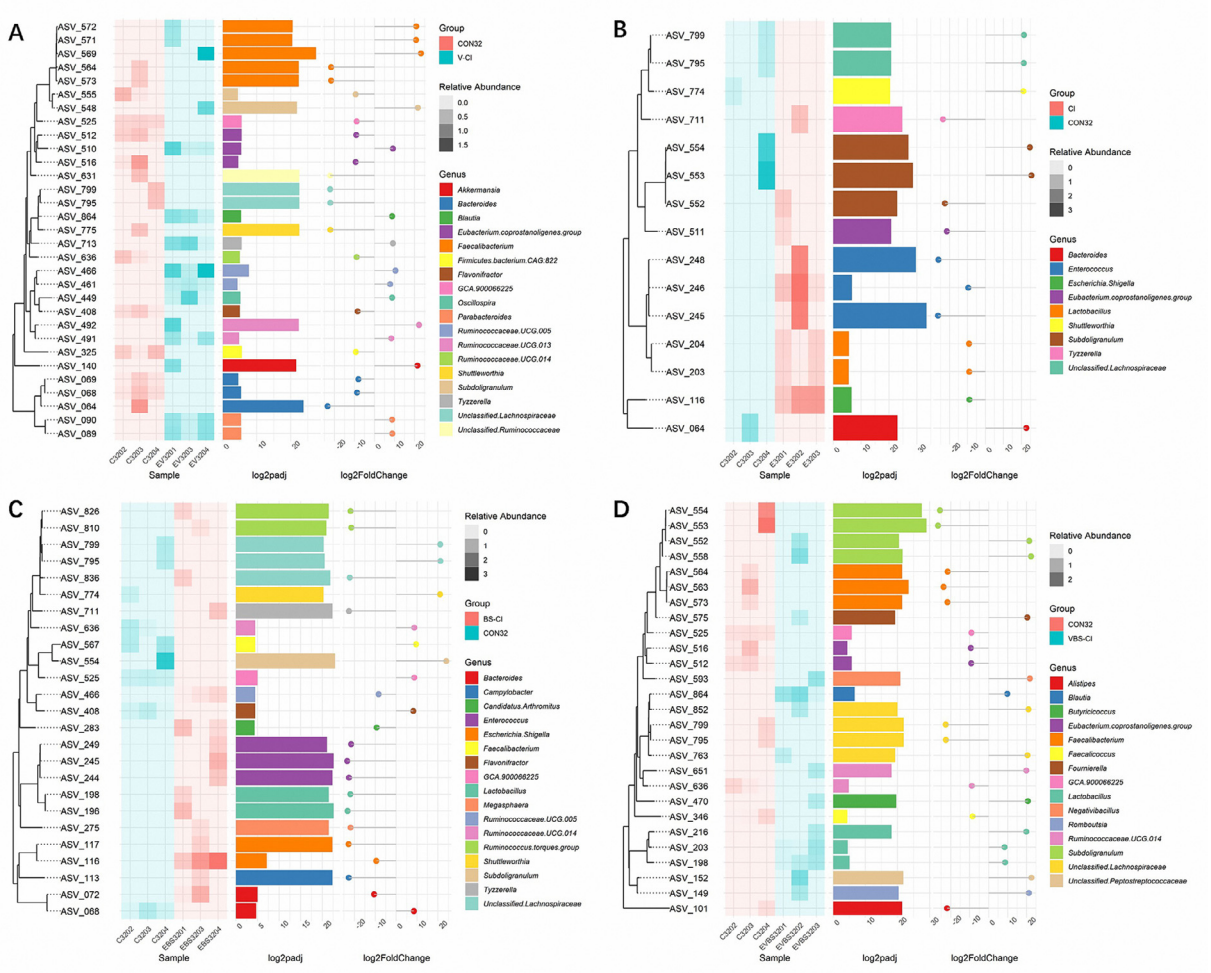

Figure 5. Differential abundance of intestinal microbiota between different experimental groups. (A)−(D) were the flora difference analysis results of the VBS-CI, BS-CI, CI, and V-CI in comparison to the control group CON32 respectively. Here, the phylogenetic relationship of ASV and the abundance difference of ASV correspond to adjusted *P*-value, fold change result and gene details. In which *P*-value and fold change data were transformed by log base 2 (log2). The above difference analysis performed with nyankomicro package (https://github.com/xvtyzn/nyankomicro), which was a derivative of the DESeq2 package (version 1.34.0). In the calculation process, DESeq function parameter settings: test = "Wald", fitType = "parametric". And deseq2_tree needed to set alpha to 0.01.

did not observe oocysts, which is consistent with the significant reduction of OPG results. Due to the immune strategy effectively inhibits the colonization of chicken coccidia, it prevents the increase of oocysts in the intestine. In addition, the results of the pathological section also showed that under the combined immunization strategy, the epithelial cells did not fall off significantly, and the intestinal integrity of the chicken remains normal (Figure 2E).

Furthermore, the combined application of *B. subtilis* and coccidiosis vaccine also regulates the structure of chicken intestinal microbial community. Trillions of microorganisms live in the intestines of chickens, which benefit the host in many ways, including the regulation the immune response. However, there was limited information on the role of the chicken gut microbiota and its response to vaccination under different feeding regimens. Recent studies have also shown that functional additives reduce the impact of coccidiosis challenges on the broiler microbiota (Vieira et al., 2020). In the preimmunization test, combined application of *B. subtilis* and coccidiosis vaccine resulted in a significant increase in the relative abundance of *Staphylococcus, Lactobacillus*, and *Enterococcus*, along with *L. aviarius* and *L. reuteri* species ($P < 0.05$) (Figure 4B). Among them, *Lactobacillus* and Enterococcus were also the dominant bacterial genera while broiler immuned with coccidiosis vaccine alone. *Lactobacillus* and *Enterococcus* belong to the common probiotic genus (Gaggìa et al., 2010). Previous study showed that the intake of *Lactobacillus*-derived feed products increased intestinal TNF-α, IL-12, IFN-γ, and IL-2 expression, which resulting a reduction of oocysts in chicken (Chaudhari et al., 2020; Vieira et al., 2020). *Enterococcus*, especially *E. faecium*, aided in increasing the abundance of beneficial bacteria and regulating intestinal flora. Supplementation with *E. faecium* could increase cytokines such as IL-1β, IL-6, IL-10, and IFN-γ, which in turn reduced the severity of intestinal injury (Madlala et al., 2021). However, our results also found that the relative abundance of *L. reuteri* was significantly increased in the VBS group. Intestinal colonization and growth of *L. reuteri* was likely to favor

Figure 6. Differential analysis of functional prediction of gut bacterial communities modulated by different treatments. (A) The relative abundance results of different bacterial communities in the challenge treatment group. Among them, the representative signal pathway used trans_diff function (method = "rf"), and plot built by plot_diff_abund function. (B)−(E) The bar graphs showed the differences between the groups in the control of intestinal flora function by different immune treatments at the age of 32 d. The result was performed by plot_lefse_bar function in microeco package. The parameters needed to be set in the analysis process as follows, alpha = 0.05, LDA_score = 2.

colonization and growth of other beneficial lactic acid bacteria, resulting in a greater diversity of *Lactobacillus* species. In addition, it also inhibited the overproduction of pathogenic microorganisms such as *Klebsiella, Chryseobacterium, Citrobacter, Aeromonas, Acinetobacter*, and *Campylobacterales* (Nakphaichit et al., 2011). Thus, the increased relative abundance of *Lactobacillus* and *Enterococcus*, especially *L. reuteri*, were the key factor for the combined application of B. subtilis and coccidiosis vaccine immunization strategies to safeguard intestinal health.

As part of this study, we also examined the changes in the coccidia flora after the above immune boost. Among them, the combined application of *B. subtilis* and coccidiosis vaccine significantly up-regulated *Romboutsia, Blautia, Butyricicoccus* and *Lactobacillus*, as well as

down-regulated the relative abundant of *Ruminococcaceae* GCA.900066225, *Alistipes* and *Faecalicoccus*. Romboutsia played a role in intestinal glucose digestion (Qiao et al., 2018), was largely responsible for maintaining host health status, and might be a highly effective candidate biomarker for intestinal health (Chen et al., 2020). Otherwise, Wen et al. found a relatively high abundance level of *Romboutsia* and significantly higher egg-laying efficiency in chickens (Wen et al., 2021). *Butyricicoccus*, a potential active ingredient in probiotic formulations and a producer of short chain fatty acids, was associated with better growth and development in broilers when its relative abundance in the cecum was high (Zhou et al., 2021). And *Lactobacillus* genus was able to increase the expression level of immune cytokines in the intestinal tract, which contributed to clearing the oocysts in the intestine, consistent with the statistical results of fecal oocysts. Furthermore, Elham et al. found that broiler chickens fed probiotic *Bacillus* spp. also reduced the relative abundance of *Ruminococcaceae* species GCA900066225 in the intestine (Soumeh et al., 2021). Some studies also found that the relative abundance of *Alistipes* and *Ruminococcaceae* decreased when chickens supplemented *Lactobacillus* (Baldwin et al., 2018). In addition, *Alistipes* was a relatively new bacterial genus, isolated from medical clinical samples and closely related to some human diseases (Ty et al., 2022). However, its mechanisms within the microbiome were still not entirely understood (Parker et al., 2020). Otherwise, the function of *Faecalicoccus* in the broiler was still unclear, but patients with Crohn's disease tended to have higher abundance of *Faecalicoccus* (Forbes et al., 2018). Therefore, *B. subtilis* and coccidiosis vaccine jointly resisted avian coccidiosis mainly by regulating the intestinal flora and creating a micro-ecological environment suitable for the growth of beneficial microorganisms, and then reducing intestinal lesions and increasing the immune protection effect.

Changes in the gut microbiota may lead to altered microbial metabolism. In the present study, Tax4Fun was used to predict the function of the microbiome. Among them, the unique high abundance signaling pathway in the experimental group that was re-attacked with *B. subtilis* and coccidiosis vaccine was the quorum sensing pathway. Previous study also confirmed that while host produces an immune response against parasites, such as *Leishmania*, it relies on the quorum sensing pathway (Postat et al., 2018). Therefore, the combined immunization strategy may also generate an immune response to chicken coccidia through the quorum sensing pathway, thereby activating relevant immune cells and immune molecules to participate in anticoccidial infection behavior.

CONCLUSION

In the current study, the application of *B. subtilis* alone or the combined application of *B. subtilis* and chicken coccidia live vaccine display significant improvement on the performance of broiler chickens; nonetheless only the second strategy can effectively achieve the anticoccidial effect. In addition, the analysis results of the intestinal microbial community structure further revealed that the above-mentioned immune strategy achieved anticoccidial mainly through the regulation of *Romboutsia, Blautia, Butyricicoccus* and other bacterial genera, as well as the quorum sensing pathway. The relevant results of this study can provide a theoretical basis for further exploration of more chicken coccidiosis vaccines and other combined probiotics prevention and control strategies.

UNCITED FIGURE

Figure 1

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Data Availability Statement: The raw sequencing data for bacterial communities have been submitted to the National Center of Biotechnology Information (NCBI) Sequence Read Archive under accession number PRJNA814845.

Author contributions: Nanshan Qi and Mingfei Sun conceived and designed the research. Haiming Cai and Shenjun Luo analyzed the data and wrote the manuscript. Qingfeng zhou, Zhuanqiang Yan, Qihong Liu, Zhen Kang and Jianfei Zhang conceived and designed the research. Shenquan Liao, Juan Li, Minna Lv, Xuhui Lin, Junjing Hu, and Shuilan Yu performed the experiments and analyzed the data. Mingfei Sun and Nanshan Qi supervised the project and revised the manuscript. All authors contributed to the article and approved the submitted version.

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DISCLOSURES

Qingfeng Zhou and Zhuanqiang Yan are employed by Wen's Group Academy, Wen's Foodstuffs Group Co., Ltd. Qihong Liu is employed by Jiangsu HFQ Biotechnology Co., Ltd. Zhen Kang are employed by Qingdao Vland Biotech Group Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2022.102091.

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