Effects of *Bacillus Coagulans* on growth performance, antioxidant capacity, immunity function, and gut health in broilers

Bing Zhang,[*,1] Haoran Zhang,[*,1] Yang Yu,[*] Ruiqiang Zhang,[*] Yanping Wu,[*] Min Yue,[†] and Caimei Yang[*,2]

[*]*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, Zhejiang Agriculture & Forestry University, Hangzhou 311300, China; and [†]College of Animal Science, Zhejiang University, Hangzhou 310058, China*

ABSTRACT This study evaluated the effects of *Bacillus coagulans* (***B. coagulans***) as an alternative to antibiotics on growth performance, antioxidant capacity, immunity function and gut health in broilers. A total of 480 one-day-old broilers were randomly divided into 3 treatments with 8 replicates comprising 20 broilers each. The experiment lasted 42 d. Treatments included: basal diet without antibiotics (NCO); basal diet supplemented with 75 mg/kg chlortetracycline (**ANT**); basal diet supplemented with 5×10^9 CFU/kg *B. coagulans* (**BC**). The *B. coagulans* enhanced body weight (**BW**) and average daily gain compared with the NCO group ($P < 0.05$). However, there were no significant differences in average daily feed intake and feed: gain ratio (**F: G**) among three groups ($P > 0.05$). The *B. coagulans* significantly increased catalase, superoxide dismutase, and glutathione peroxidase levels and reduced malondialdehyde levels ($P < 0.05$). The serum immunoglobulins (IgA, IgM, and IgY) were significantly higher in the BC group when compared to the NCO and ANT groups ($P < 0.05$). The *B. coagulans* also markedly reduced serum levels of proinflammatory factors (IL-1β, IL-6, and TNF-α) and enhanced anti-inflammatory factor (IL-10) concentrations compared with control group ($P < 0.05$). Moreover, compared with the control group, BC significantly inhibited serum xanthine oxidase activity ($P < 0.05$). The levels of acetic acid, propionic acid, butyrate, isobutyric acid and valerate in BC group were significantly increased on d 42 compared with the NCO and ANT groups ($P < 0.05$). Furthermore, BC significantly altered cecal microbiota by reducing *Desulfovibrio* and *Parasutterella*, and by increasing *Alistipes* and *Odoribacter* ($P < 0.05$, $P < 0.05$, $P < 0.001$, $P < 0.01$, respectively). In conclusion, dietary *B. coagulans*, when used as an alternative to antibiotics, improved body weight, average daily gain, antioxidant capacity, immunity function and gut health in broilers.

Key words: broiler, *Bacillus coagulans*, growth performance, immunity function, gut health

INTRODUCTION

Broilers are vulnerable to stress due to their small size, hypoplasia of organs, low immune function and other physiological conditions, resulting in high morbidity and mortality rates. Antibiotics have been used as feed additives in animal husbandry for their excellent therapeutic efficiency and growth-promoting traits (Yi et al., 2018).

However, their intensive use caused the generation of drug-resistant bacteria, antibiotic residues in food animals, and even environmental pollution (Zhou et al., 2019). Therefore, China has banned on adding antibiotics to livestock feed on July 1, 2020 (http://www.xmsyj. moa.gov.cn/zcjd/201907/t20190710_6320678.htm). The poultry industry needs nonantibiotic alternatives to maintain animal health and improve feed conversion rates. Therefore, research on and development of antibiotic substitutes, including probiotics, prebiotics, herbs, and exogenous enzymes, has attracted increased attention (Gadde et al., 2017). Presently, probiotics as a substitute for antibiotics are widely used in livestock and poultry farming.

Bacillus coagulans is a gram-positive, spore-forming, lactic acid producing bacillus that does not encode

enterotoxin (Jurenka, 2012). It possesses a protective, spore-like protein coating, which allows it to resist high temperature and survive stomach acid and bile salts, reach the small intestine, germinate, and multiply (Cavazzoni et al., 1998; Jurenka et al., 2012; Gu et al., 2015). Therefore, *B. coagulans*, as a potential probiotic, has been used in cattle, broilers, and weaned piglet production (Ripamonti et al. 2009; Zhou et al., 2010; Hung et al., 2012; Pu et al., 2020). Relevant studies have indicated that *B. coagulans* regulated intestinal microbe by increasing beneficial bacteria and reducing pathogenic bacteria in pig (Cavazzoni et al., 1998; Adami et al., 1999). Moreover, *B. coagulans* promoted growth performance and improved feed utilization rate in broilers by secreting enzyme protease, α-amylase, lipidase and xylanase, and generating vitamins and amino acids (Cavazzoni et al., 1998; Zhou et al., 2010; Wang et al., 2010). Besides adding *B. coagulans* to the diet of mammals' diminished intestinal inflammatory damage (Kalman et al., 2009). In vivo studies indicated that *B. coagulans* possessed antibacterial, antiviral, and antioxidant activity by regulating cytokines, potentiating phagocytosis, and suppressesing reactive oxygen species (**ROS**) in human model (Dolin, 2009; Sari et al., 2011). Moreover, in vitro experiments demonstrated that *B. coagulans* adjusted immune responses (Jensen et al., 2010), and generated bacteriocin, coagulin and *L*-lactic acids with extensive activity against various strains of *Salmonella, Coliform, Listeria monocytogenes*, and *Clostridium* species (Hyronimus et al., 1998; Le Marrec et al., 2000; Riazi et al., 2009; Honda et al., 2011; Ou et al., 2011; Abdhul et al., 2015; Gu et al., 2015). Therefore, *B. coagulans* might effectively abrogate livestock and poultry pathogens.

However, so far, no one has evaluated the effects of *B. coagulans* on intestinal mucosal barrier, volatile fatty acids (**VFAs**) and intestinal microflora in broilers. Accordingly, we focused on the effects of dietary *B. coagulans*, as one antibiotics alternative, on gut health in broilers.

MATERIALS AND METHODS
Animals, Diets, and Experimental Design

A total of 480 one-day-old female Arbor Acres broilers were randomly divided into three treatment groups, each with 8 replicates of 20 broilers. Broilers were grouped as follows: basal diet (control group; NCO group), basal diet supplemented with 75 mg/kg chlortetracycline (ANT group); basal diet supplemented with 5×10^9 CFU/kg *B. coagulans* (BC group). The *B. coagulans* was obtained from Vegamax Biotechnology Co., Ltd. (Huzhou, China). The experimental diets were fed for 42 days. The ingredients and nutrient levels of basal diets were formulated to meet the NRC (1994) nutrient requirements of broiler chickens (Table 1). Broilers were housed in the same coop with one cage for each repetition and food and water were given ad libitum. Broilers were kept under a commercial lighting and temperature schedule, and were vaccinated according to routine

Table 1. Composition and nutrient levels of the experimental basal diet (as-fed basis unless stated otherwise, %).

Items	1−21 d	22−42 d
Ingredients (%)		
Corn	61.80	65.60
Soybean meal	22.50	17.55
Extruded soybean	8.45	10.00
Import fish meal	3.00	3.00
$CaHPO_4$	1.66	1.45
Limestone	1.10	1.00
NaCl	0.32	0.30
DL-methionine (98%)	0.16	0.10
L-lysine HCL (78%)	0.01	
Vitamin-mineral premix[1]	1.00	1.00
Total	100.00	100.00
Nutrition levels		
Metabolizable energy (MJ/kg)	12.45	12.70
Crude protein (%)	21.00	19.20
Lysine (%)	1.15	0.95
Methionine (%)	0.54	0.44
Calcium (%)	0.99	0.89
Available phosphorus (%)	0.53	0.49

[1]Vitamin-mineral premix is provided for feed per kg: VA 1,500 IU, VB_1 1.5 mg, VB_6 3.0 mg, VB_{12} 0.01 mg, VD_3 200 IU, VE 10 IU, VK 0.5 mg, Biotin 0.15 mg, D-pantothenic acid 10 mg, Folic acid 0.5 mg, Nicotinic acid 30 mg, Trace elements Cu, Fe, Zn, Mn, Se, I are 8 mg, 80 mg, 40 mg, 60 mg, 0.15 mg, 0.18 mg, respectively. Calculated value based on the analyzed data of experimental diets.

methods. The present study was approved by the Animal Care and Use Committee of the Zhejiang Agriculture and Forestry University (Lin'an, China).

Growth Performance Evaluation

All broilers were individually weighed on days 1, 21 and 42. At the age of 21 and 42 days, the total feed consumption of each group was measured. The average daily gain (**ADG**), average daily feed intake, and the feed: gain ratio (**F: G**) were calculated.

Sample Collection

On d 21 and 42, one similar weight and healthy broiler was taken from 8 repetitions in each group (n = 8 per group) and slaughtered. Blood was collected and serum samples were acquired by centrifuging at 3,000 rpm for 15 minutes. After dissection, ileal mucosa was taken to measure the mRNA relative expression of Fitzpatrick. Colonic contents were collected for determination of intestinal flora. Above samples were conserved at −80°C for further analysis.

Serum Parameter Analysis

The ELISA kits for glutathione peroxidase (**GSH-Px**), superoxide dismutase (**SOD**), catalase (**CAT**), malondialdehyde (**MDA**), immunoglobulin A (**IgA**), immunoglobulin M (**IgM**), immunoglobulin Y (**IgY**), interleukin-1β (**IL-1β**), interleukin-6 (**IL-6**), interleukin-10 (**IL-10**), tumor necrosis factor-α (**TNF-α**), interferon-β (**INF-β**), blood NH_3 and xanthine oxidase (**XOD**) were purchased from the Nanjing Aoqing Biotechnology Co. Ltd. (Nanjing, China). The

Table 2. Sequences of the oligonucleotide primers used for quantitative real-time PCR.

Gene	GenBank accession number	sequence (5'-3')	Amplicon size (bp)
β-actin	NM.205518	Forward: TGCTGTGTTCCCATCTATCG	150
		Reverse: TTGGTGACAATACCGTGTTCA	
OCLN	NM_205128.1	Forward: AGCCCTCAATACCAGGATGTG	125
		Reverse: CGCTTGATGTGGAAGAGCTTG	
CLDN-1	NM_001013611.2	Forward: CACACCCGTTAACACCAGATTT	159
		Reverse: GAGGGGGCATTTTTGGGGTA	
TJP-1	XM_015278980.2	Forward: GGATGTTTATTTGGGCGGCT	153
		Reverse: CCATTGTTGCACTCTTGCCG	
MUC-2	JX284122.1	Forward: ATTGTGGTAACACCAACATTCATC	134
		Reverse: CTTTATAATGTCAGCACCAACTTCTC	

specific test steps were conducted strictly according to the instructions.

Total RNA Extraction and mRNA Quantification

According to the RNeasy mini kit instructions (Qiagen, Germantown, MD), total RNA was extracted from ileal mucosa tissue. Total RNA concentration and purity were measured by a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific). Following the manufacturer's instructions, genomic DNA contamination of RNA samples was erased with gDNA, and the Prime Script RT reagent kit (Takara Bio Inc., Beijing, China) was used for reverse transcription reaction. The cDNA was then diluted 10 times with RNase-free water for real-time PCR analysis. Quantitative real-time PCR was conducted using the Applied Biosystems 7500 Fast Real-Time PCR System and an SYBR Premix Ex Taq kit (Takara Bio Inc., Beijing, China). Reactions were performed in a 20.0 μL reaction mixture containing 10.0 μL of SYBR Premix Ex Taq (Takara Bio Inc., Beijing, China), 1.0 μL of cDNA, 0.5 μL of each primer (Table 2), and 7.0 μL of RNase-free water. Average gene expression relative to the β-actin endogenous control for each sample was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2002).

VFAs Analysis

Cecal VFAs were assayed by Gas Chromatography System (7890B, Agilent Technologies) according to previous studies (Yang et al., 2019). This analysis used a DB-FFAP, 30 m × 0.25 mm × 0.25 μm column (Agilent Technologies). Standards (acetic acid, propionic acid, butyrate, isobutyric acid, valerate, and isovalerate) and metaphosphoric acid were obtained from Aladdin Biochem Technology Co., Ltd. (Shanghai, China). The Gas Chromatography Analytical Conditions are listed as the follows: Carrier gas: N_2. Sample injection volume:1 μL. Split ratio: 25:1. The initial column temperature was 90°C; this was held for 2 min then increased by 10°C min^{-1} to 200°C and was held for 5 min. In study, 2 g cecal contents were weighed into a 10 mL centrifuge tube and 2 mL of pre-cooled deionized water was added. The cecal contents were mixed, centrifuged at 4°C for 10 min (10,000 rpm) after stood at 4°C for 30 min. Took 1 mL of supernatant, added 0.2 mL 25% metaphosphoric acid (w/v), mixed well, centrifuged at 4°C for 10 min (15,000 rpm) after ice bath for 30 min. Centrifuge again at 4°C at 15,000 rpm for 10 min, then took 500 μL supernatant into a sample bottle for gas chromatography analysis.

16S rRNA-Based Microbiota Analysis

The extraction of cecal microbial genomic DNA was performed using the QIAamp DNA stool Mini Kit (Qiagen GmbH, Hilden, Germany). The bacterial 16S rRNA were amplified using V3-V4 hypervariable region primer 338F (5'-ACTCCTACGGGAGGCACAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). After amplification and purification, the amplicons were pooled in equimolar and paired-end sequenced using an Illumina MiSeq platform (Illumina, San Diego) provided by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Lastly, alpha and beta diversity analyses were performed for differences in species composition between samples. Linear discriminant analysis (**LDA**) effect size (**LEfSe**) analysis was performed to identify differentially abundant bacterial taxa based on LDA score >2.0.

Statistical Analysis

Sorting out data with Excel (Microsoft); SPSS 25.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of the collected data. Data were analyzed using one-way ANOVA and presented as least squares means ± SEM. Differences among treatments were examined using Tukey's tests. Significance levels: $P < 0.05$ *; $P < 0.01$ **; $P < 0.001$ ***.

RESULTS

Growth Performance

The growth performance results are depicted in Table 3. Broilers supplemented with *B. coagulans* revealed higher BW and ADG ($P < 0.05$) than those fed basal diet. No significant difference in BW and ADG were found between the ANT and BC groups ($P > 0.05$). Dietary supplementation with *B. coagulans* had no effect on average daily feed intake and F: G during day 1 to 42 ($P > 0.05$).

Table 3. Effects of dietary supplementation of *B. coagulans* on growth performance in broilers.[1]

Items[2]	NCO	ANT	BC	SEM[4]	P value
BW, g					
1 d	37.89	37.77	37.13	0.16	0.125
21 d	740.25[b]	784.92[a]	771.78[a]	6.31	0.004
42 d	1,812.18[b]	1,987.47[a]	2,104.7[a]	42.09	0.007
ADG, (g/d)					
1−21 d	33.45[b]	35.58[a]	34.98[a]	0.30	0.004
22−42 d	51.04[b]	57.26[ab]	63.47[a]	1.90	0.017
1−42 d	42.25[b]	46.42[a]	49.23[a]	1.00	0.007
ADFI, g					
1−21 d	53.52	55.34	54.09	0.83	0.688
22−42 d	109.22	120.43	128.16	3.69	0.102
1−42 d	76.48	81.97	85.82	1.64	0.052
F: G					
1−21 d	1.61	1.55	1.55	0.02	0.408
22−42 d	2.09	2.06	1.97	0.05	0.665
1−42 d	1.78	1.75	1.70	0.02	0.169

[1]Data are expressed as mean ± SEM, n = 8. Values in the same column with different superscripts were significantly different (*P* < 0.05) while with same superscripts were insignificantly different (*P* > 0.05).
[2]Body weight (BW); Average daily gain (ADG); average daily feed intake (ADFI); Feed: gain ratio (F: G).
[3]NCO, broilers fed a basal diet; ANT, broilers fed a basal diet supplemented with 75 mg/kg chlortetracycline; BC, broilers fed a basal diet with 5×10^9 CFU/kg *B. coagulans*.
[4]Pooled SEM.

Antioxidant Activities

The effects of *B. coagulans* treatment on antioxidant activities of *B. coagulans* are shown in Table 4. Compared with basal diet group, the activity of GSH-PX, SOD, and CAT was significantly increased and the content of MDA was significantly decreased when *B. coagulans* was added to the diet of broilers (*P* < 0.05). Therefore, dietary *B. coagulans* supplementation improved the antioxidant capacity in broiler chickens when compared to control.

Serum Immunoglobulins and Inflammatory Factors

The effects of *B. coagulans* treatment on serum immunoglobulins and inflammatory factors levels are listed in

Table 4. Effects of dietary supplementation of *B. coagulans* on antioxidant activities in broilers.[1]

Items[2]	NCO	ANT	BC
GSH-PX (U/mL)	6.10 ± 1.03[b]	7.31 ± 0.94[ab]	9.65 ± 3.40[a]
SOD (U/mL)	12.40 ± 0.16[b]	13.12 ± 0.16[a]	13.53 ± 0.69[a]
CAT (U/mL)	9.64 ± 0.29[b]	10.76 ± 0.50[b]	13.02 ± 0.60[a]
MDA (nmol/mL)	7.97 ± 0.29[a]	7.61 ± 0.93[a]	6.30 ± 0.32[b]

[1]Data are expressed as mean ± SEM, n = 8. Values in the same column with different superscripts were significantly different (*P* < 0.05) while with same superscripts were insignificantly different (*P* > 0.05).
[2]Glutathione peroxidase (GSH-Px); Superoxide dismutase (SOD); Catalase (CAT); Malondialdehyde (MDA).
[3]NCO, broilers fed a basal diet; ANT, broilers fed a basal diet supplemented with 75 mg/kg chlortetracycline; BC, broilers fed a basal diet with 5×10^9 CFU/kg *B. coagulans*.

Table 5. Effects of dietary supplementation of *B. coagulans* on serum immunoglobulins and inflammatory factors in broilers.[1]

Items[2]	NCO	ANT	BC
IgA (ng/mL)	166.41 ± 9.94[b]	179.05 ± 8.05[b]	209.53 ± 9.91[a]
IgM (μg/mL)	3.07 ± 0.11[c]	3.75 ± 0.10[b]	4.53 ± 0.16[a]
IgY (ng/mL)	1.51 ± 0.07[c]	1.85 ± 0.04[b]	2.43 ± 0.08[a]
IL-1β (pg/mL)	92.77 ± 3.42[a]	85.79 ± 2.17[a]	64.18 ± 2.99[b]
IL-6 (pg/mL)	403.67 ± 8.29[a]	382.03 ± 6.57[ab]	369.62 ± 10.59[b]
IL-10 (ng/L)	17.27 ± 1.19[b]	19.82 ± 0.92[b]	27.01 ± 1.15[a]
TNF-α (ng/L)	39.29 ± 1.01[a]	40.97 ± 0.78[a]	34.96 ± 1.46[b]
IFN-β (pg/mL)	134.98 ± 12.52	149.07 ± 5.44	126.86 ± 6.39

[1]Data are expressed as mean ± SEM, n = 8. Values in the same column with different superscripts were significantly different (*P* < 0.05) while with same superscripts were insignificantly different (*P* > 0.05).
[2]Immunoglobulin A (IgA); Immunoglobulin M (IgM); Immunoglobulin Y (IgY); Interleukin-1β (IL-1β); Interleukin-6 (IL-6); Interleukin-10 (IL-10); Tumor necrosis factor-α (TNF-α); Interferon-β (INF-β).
[3]NCO, broilers fed a basal diet; ANT, broilers fed a basal diet supplemented with 75 mg/kg chlortetracycline; BC, broilers fed a basal diet with 5×10^9 CFU/kg *B. coagulans*.

Table 5. Compared with levels in the NCO and ANT groups, BC treatment improved the serum levels of IgA, IgM, and IgY of 42-day-old broilers (*P* < 0.05). The *B. coagulans* inhibited IL-1β, IL-6, and TNF-α (proinflammatory factors) when compared with those of the NCO group (*P* < 0.05). Meanwhile, *B. coagulans* enhanced IL-10 (an anti-inflammatory factor) compared with NCO and ANT groups (*P* < 0.05). The concentrations of INF-β (a pro-inflammatory factor) did not have statistically significant differences in different treatments (*P* > 0.05).

Nitrogen Metabolism

The effects of the *B. coagulans* on blood NH₃ and XOD activity are displayed in Table 6. Compared with the NCO group, the *B. coagulans* significantly downregulated serum XOD content (*P* < 0.05). The various groups were not significantly difference in blood NH₃ (*P* > 0.05).

Gene Expressions Related to Intestinal Barrier Function

The results of gene expressions related to intestinal barrier function are presented in Table 7. Compared

Table 6. Effects of dietary supplementation of *B. coagulans* on nitrogen metabolism in broilers.[1]

Items[2]	NCO	ANT	BC
NH₃ (μmol/L)	223.13 ± 10.73	236.38 ± 20.59	203.22 ± 12.20
XOD (U/L)	12.84 ± 0.63[a]	11.08 ± 0.66[ab]	9.63 ± 0.61[b]

[1]Data are expressed as mean ± SEM, n = 8. Values in the same column with different superscripts were significantly different (*P* < 0.05) while with same superscripts were insignificantly different (*P* > 0.05).
[2]Xanthine oxidase (XOD).
[3]NCO, broilers fed a basal diet; ANT, broilers fed a basal diet supplemented with 75 mg/kg chlortetracycline; BC, broilers fed a basal diet with 5×10^9 CFU/kg *B. coagulans*.

Table 7. Effects of dietary supplementation of *B. coagulans* on gene expressions related to intestinal barrier function in broilers.[1]

Items[2]	Treatments[3]		
	NCO	ANT	BC
OCLN	5.17 ± 1.32	10.23 ± 2.22	9.23 ± 1.74
CLDN-1	1.09 ± 0.17^{b}	1.88 ± 0.15^{ab}	1.52 ± 0.13^{a}
TJP-1	1.15 ± 0.11	1.69 ± 0.34	1.53 ± 0.35
MUC-2	0.91 ± 0.10	1.00 ± 0.17	0.95 ± 0.13

[1]Data are expressed as mean $\pm$ SEM, n = 8. Values in the same column with different superscripts were significantly different ($P < 0.05$) while with same superscripts were insignificantly different ($P > 0.05$).

[2]Occluding (OCLN); Claudin (CLDN-1); Tight junction protein-1 (TJP-1); Mucin-2 (MUC-2).

[3]NCO, broilers fed a basal diet; ANT, broilers fed a basal diet supplemented with 75 mg/kg chlortetracycline; BC, broilers fed a basal diet with 5×10^{9} CFU/kg *B. coagulans*.

with NCO and ANT groups, the supplementation of *B. coagulans* upregulated the mRNA expression of CLDN-1 in the ileum, but not significant ($P > 0.05$). Nevertheless, *B. coagulans* supplement didn't change the mRNA abundance of ileal OCLN, TJP-1, MUC-2 ($P > 0.05$).

Concentration of VFAs

The results of VFAs analysis are summarized in Table 8. At the 42 d of age, the concentrations of acetic acid, propionic acid, butyrate, isobutyric acid and valerate were significantly increased compared with NCO and ANT ($P < 0.05$). Adding *B. coagulans* to the diet might enrich the level of VFAs in the gut.

Microflora Structure in the Cecal Contents

We analyzed the changes of cecal microbiota of broilers. No obvious changes were found in Shannon and Simpson indices representing α-diversity among 3 groups (Figure 1A, B). However, β-diversity displayed as principal component analysis and principal coordinates analysis scatterplots demonstrated significant separation of BC group from NCO and ANT, despite interindividual variation ($P < 0.01$) (Figure 1C, D). Then, we examined the differences of intestinal flora at different taxonomic levels. Figure 1E, G exhibited the

Table 8. Effects of dietary supplementation of *B. coagulans* on VFAs in broilers.[1]

Items	Treatments[2]		
	NCO	ANT	BC
Acetic acid (μmol/g)	44.28 ± 4.64^{b}	32.90 ± 1.93^{c}	66.40 ± 2.30^{a}
Propionic acid (μmol/g)	9.38 ± 0.83^{b}	9.95 ± 0.28^{b}	14.21 ± 0.78^{a}
Butyrate (μmol/g)	0.12 ± 0.01^{c}	7.25 ± 0.76^{b}	15.17 ± 0.95^{a}
Isobutyric acid (μmol/g)	1.38 ± 0.13^{b}	0.69 ± 0.05^{c}	2.05 ± 0.19^{a}
Valerate (μmol/g)	1.57 ± 0.18^{a}	0.74 ± 0.06^{b}	1.94 ± 0.30^{a}
Isovalerate (μmol/g)	1.10 ± 0.24	0.72 ± 0.06	1.14 ± 0.30

[1]Data are expressed as mean $\pm$ SEM, n = 8. Values in the same column with different superscripts were significantly different ($P < 0.05$) while with same superscripts were insignificantly different ($P > 0.05$).

[2]NCO, broilers fed a basal diet; ANT, broilers fed a basal diet supplemented with 75 mg/kg chlortetracycline; BC, broilers fed a basal diet with 5×10^{9} CFU/kg *B. coagulans*.

compositions of gut microbiota at the phylum and genus levels in three groups. At phylum level, *Bacteroidetes* and *Firmicutes* were the most dominant bacterial phyla, followed by *Synergistota, Desulfobacteta* and *Proteobacteria* (Figure 1E). However, the relative abundance of these main phyla did not differ significantly ($P > 0.05$). One-way ANOVA analysis presented that *Synergistota, Desulfobacteta,* and *Proteobacteria* were strikingly different in abundance among three groups ($P < 0.001$, $P < 0.01$, $P < 0.001$, respectively), with decreased *Synergistota* and *Proteobacteria* abundance and improved *Desulfobacteta* abundance after BC treatment (Figure 1G). At genus level, *Bacteroides* and *Alistipes* were the two most dominant genera, followed by *unclassified-f-Lachnospiraceae, Feacalibacterium* and *Lactobacillus* (Figure 1F). Ternary plot indicated that BC and ANT treatments showed a higher proportion of *Alistipes*, while *Bacteroides* was the dominant genus in NCO group (Figure 1H).

Differences on genus level were illustrated in Figure 2A−D. *Alistipes* was significantly decreased by adding BC and ANT ($P < 0.05$, $P < 0.01$, respectively). The BC and ANT markedly reduced the richness of *Desulfobacteta* ($P < 0.05$, $P < 0.001$, respectively). A marked up-regulation of *Odoribacter* was induced by BC and ANT ($P < 0.001$, $P < 0.05$, respectively). Under BC and ANT treatments, the abundance of *Parasutterella* decreased ($P < 0.01$, $P < 0.001$, respectively). At genus level, LEfSe analysis exhibited that the abundance of *unclassified-f-Lachnospiraceae* and *Christensenellaceae-R-7-group* were up-regulated in the BC group, *Alistipes* and *Olsenella* were increased in the ANT group, and *Phascolarctobacterium* and *Synergistes* were relatively higher in the NCO group (Figure 2E).

DISCUSSION

Several recent studies have verified that probiotics can enhance livestock and poultry growth performance, such as *Bacillus* spp., *Lactobacillus* spp., and *Saccharomyces* spp. (Cao et al., 2019; Lokapirnasari et al., 2019; Massacci et al., 2019). As shown in Pu et al. (2020), a mixture of benzoic acid and *B. coagulans* enhanced the BW and ADG and reduced the F: G in pigs. Fitzpatrick (2013) presented that *B. coagulans*, as a probiotic, enhances chickens growth-related parameters. According to Khalique et al. (2020), necrotic enteritis-induced reduction in body weight gain was relieved by the addition of *B. coagulans* into broiler diets compared with the necrotic enteritis-infected birds. Similarly, our experimental results shown that 5×10^{9} CFU/kg *B. coagulans* supplementation significantly enhance broilers BW and ADG compared with NCO.

In the animal defense system, antioxidant enzymes are important factors in the fight against oxidative stress caused by xenobiotic (Wu et al., 2016). The GSH-PX, SOD, and CAT are the main enzymes in the antioxidant system, whose activities indirectly reflect the ability to scavenge ROS (Liu et al., 2020). In present study,
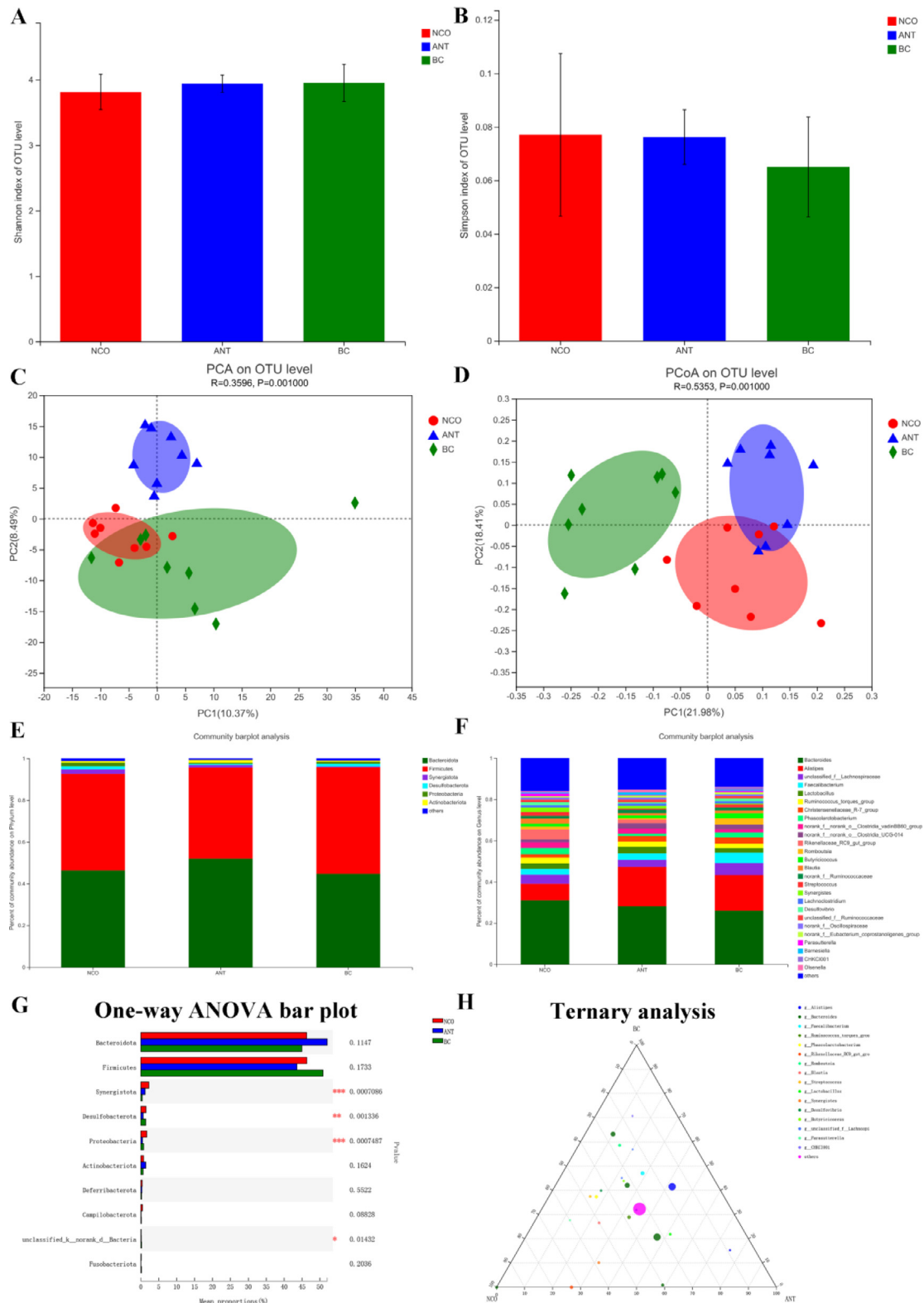
Figure 1. Analysis of the composition of cecal microbiota. (A, B) The Shannon and Simpson indexes reflecting α diversity. (C) Principal component analysis (PCA). (D) Principal coordinate analysis (PCoA) based on the bray curtis distance algorithm. (E, G) The microbiota composition on phylum level. The significance was analyzed by one-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (F) The microbiota composition on genus level. (H) Ternary phase diagram of the dominant genera.

broilers fed 5×10^9 CFU/kg *B. coagulans* increased GSH-PX, SOD and CAT activities, suggesting that the antioxidant capacity of broilers could be improved. Liu et al. (2020) also illustrated that MDA is a major

product of lipid peroxide degradation, reflecting the intensity and rate of lipid peroxides formation, as well as the extent of lipid peroxidation and free radical attack in cells. We observed a decrease in MDA with added *B.*
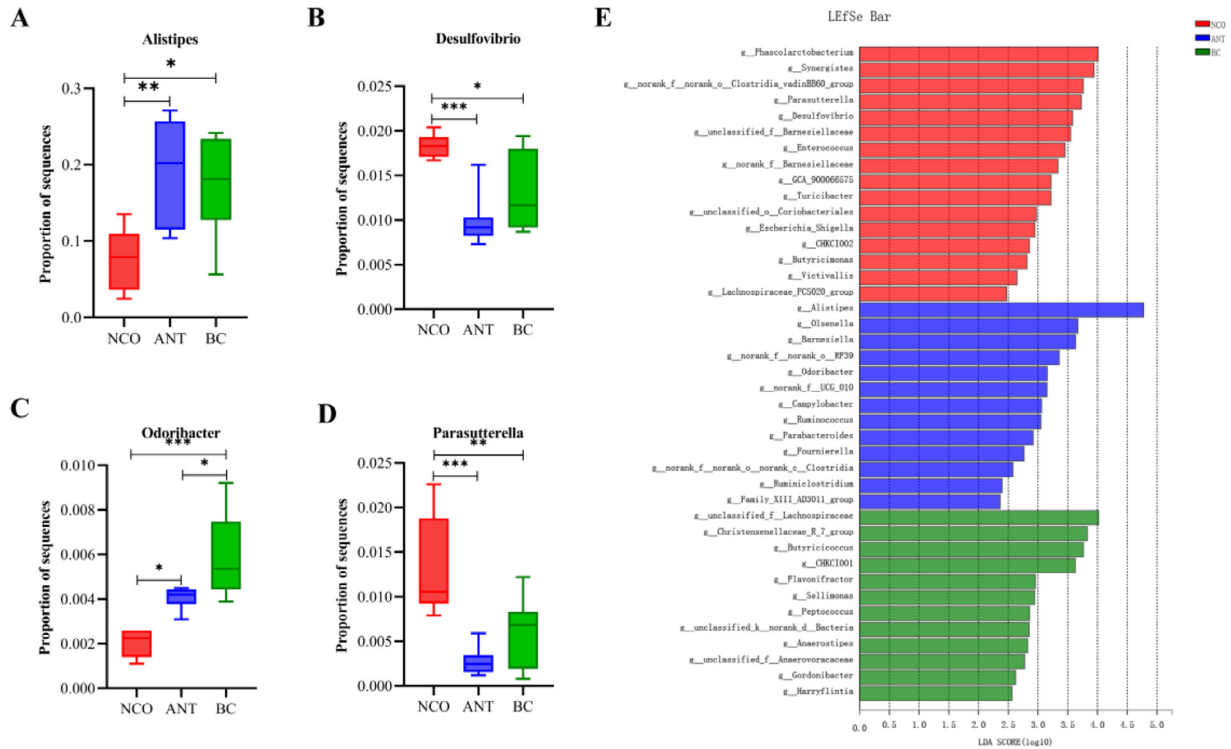
Figure 2. The significant genera and LEfSe analysis. (A, B, C, D) The significant genera among groups. Data were shown as Mean ± SEM and analyzed by one-way ANOVA Tukey test (N = 8 in each group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (E) Histogram of LDA scores for taxonomic biomarkers identified by LEfSe. LDA scores (log 10) >2 indicate enriched taxa in cases.

coagulans, implying that addition of *B. coagulans* effectively relieves the lipid peroxidation in chickens. As revealed by Lin et al. (2014), diet added *B. coagulans* significantly increased yellow broilers serum SOD and CAT activity, and remarkably reduced serum MDA concentration, which is comparable to our results. To sum up, *B. coagulans* can effectively protect the body from oxidative stress injury, which is also an important reason why it can improve the production performance of meat and poultry.

Immunoglobulins play a defensive role in vivo, and IgA, IgM and IgY are important immune factors in birds. The IgA participates in mucosal immune response, while IgM participates in the acute infection process (Bian et al., 2016). The IgY is the dominating type of antibodies produced by poultry and has the similar function as mammalian IgG (Haese et al., 2015). In this experiment, the supplementation of *B. coagulans* remarkably improved the content of IgA, IgM and IgY in broiler's serum. In addition, inflammatory factors drive the process of inflammation. Chen et al. (2019) showed that pivotal cytokines that mediate important inflammatory processes include IL-1β, IL-6, TNF-α and IL-10. We also found broilers treated with BC supplementation had a lower proinflammatory factors (IL-1β, IL-6, TNF-α), whereas had a higher anti-inflammatory factor (IL-10) in serum. The above results suggested that *B. coagulans* could be involved in the regulation of immune responses and processes. The results of the studies are consistent with the following conclusions: *B. coagulans* have immunoregulation in chickens, it

has already been stated by previous investigator Panda et al. (2005) and Bai et al. (2013). Similarly, in Fitzpatrick's study (2013), *B. coagulans* could enhance immunity and relieve intestinal inflammation in chickens.

The blood NH$_3$ concentration largely depends on the ammonia concentration in the gastrointestinal tract, and its level reflects the body's utilization of nitrogen. Decreased blood NH$_3$ concentration can also reduce its irritation and toxicity to the digestive tract and reduce the incidence of digestive tract diseases. Additionally, increased blood NH$_3$ concentration is often accompanied by oxidative stress. Sfarti et al. (2020) reported that excessive ROS production causes oxidative stress, thereby accelerating protein metabolism, increasing NH$_3$ concentration and MDA activity. In this study, *B. coagulans* supplementation had a tendency to reduce NH$_3$ in the serum. Adding *B. coagulans* can relieve oxidative stress, and reduce nitrogen emissions and digestive diseases. The XOD is related to the metabolism and production of uric acid (Chao et al., 2019). Uric acid can be transported into the blood stream, and finally excreted by reabsorption in the kidneys or intestines, or accumulated partly, causing tissue injury (Liu et al., 2019). Additionally, XOD is one of the main enzyme sources for the overproduction of ROS (Janyou et al., 2017). Excessive ROS generation leads to oxidative stress, which can trigger cell apoptosis and facilitate inflammation (Amornrit et al., 2015). The mechanism of gastrointestinal protection suggested by Singh et al. (2017) may be related to inhibiting XOD

activity and preventing oxidative stress in gastrointestinal tissues. Our experimental results revealed that *B. coagulans* addition in the diet can reduce the XOD activity. These findings mirrored that *B. coagulans* may relieve tissue damage caused by oxidative stress and uric acid deposition by reducing XOD activity, thus increasing antioxidant enzyme (GSH-PX, SOD, CAT) activities, reducing oxidase (MDA) activity and blood NH_3 concentration.

Gut permeability is critical for health, and strictly governed by the gut barrier, including tight junction-related proteins occluding (**OCLN**), claudin (**CLDN-1**), tight junction protein-1 (**TJP-1**), and mucus layer mainly composed of mucin-2 (**MUC-2**) (Capaldo et al., 2017). The luminal mucus layer composed of mucins, together with junctional proteins, acts as a defense against invasive pathogens (Daneshmand et al., 2020). Palamidi et al. (2018) suggested that the downregulation of tight junction protein and MUC-2 gene expression usually leads to severe ileum inflammation. Additionally, as reported by Ionelia et al. (2018), probiotics strengthen and maintain gut barrier by interacting with and increasing the expression of cellular junctional complex (adhesion or tight junction) proteins. Jäger et al. (2018) reported that *B. coagulans* played an important role in reducing intestinal mucosal damage caused by harmful intestinal bacteria and maintaining intestinal health of weaned piglets. In our present study, there was a tendency that *B. coagulans* increased expression of OCLN, CLDN-1, and TJP-1 and MUC-2 in the ileal mucosa. Ren et al. (2014) suggested that the disruption of intestinal barrier induced by methotrexate can be prevented by regulating OCLN and CLDN-1 through the cellular NF-κB signaling pathway. *B. coagulans* may be an inhibitor of NF-κB signaling pathway, but the mechanism of action remains unclear and needs further study.

The anaerobic microorganisms in the hindgut of livestock and poultry can ferment to produce a large amount of volatile short-chain fatty acids (**SCFA**), including acetic acid, propionic acid, and butyrate (Lourenço et al., 2019). The SCFA absorbed by the animal body can be used as an energy source for colonic epithelial cells or transported to various peripheral tissues for further metabolism (Wong et al., 2006). Filippo et al. (2010) pointed out that the increase in the diversity of microbial secretion enzymes will help produce a large amount of SCFA, which can provide more energy to the animal body, thereby helping to promote the improvement of animal production performance. This study found that the addition of *B. coagulans* to feed significantly increased the level of VFAs in the cecal content of broilers, indicating that *B. coagulans* promoted hindgut microbial metabolic activity and increased body weight. Furthermore, Balta et al. (2020) investigated that the concentration of VFAs produced in broilers intestine (cecum) is associated with stronger immunity. Several reports also highlighted the immunomodulatory capacities of SCFA (Lucas et al., 2018). Our study revealed that chickens supplemented with *B. coagulans* presented a significant improvement in the concentrations of cecal VFAs (acetic acid, propionic acid, butyrate, isobutyric acid and valerate) and serum immunoglobulins (IgA, IgM, and IgY), a significant reduction in inflammatory factors (IL-1β, IL-6, TNF-α). In summary, *B. coagulans* may promote the production of VFAs by regulating intestinal microflora, inhibit the activity of inflammatory mediators in the intestinal epithelium, and thereby inhibit the activation of NF-κB macrophages.

The intestinal flora has a dramatic metabolic potential and affects the nutritional status and health of the host (Rinttila et al., 2013). We found that gut microbiota is correlated with chicken BW and immunity in our previous study, (Zhang et al., 2020). In this paper, we found that BC improved the BW, ADG, and immunity of broilers via altering gut microbiota. Our findings indicated that BC significantly increased *Alistipes* and *Odoribacter*, and decreased *Desulfovibrio* and *Parasutterella*. In the paper of Li et al. (2018), *Alistipes* is a major SCFA producer. Moreover, a present report by Wu et al. (2020), *Alistipes* displayed good anti-inflammatory effectiveness in human and laboratory animals. Goker et al. (2011) reported that *Odoribacter* is a known SCFA producer. Sawin et al. (2015) reported that *Desulfovibrio* metabolize SCFA, which may be the cause of SCFA reduction. The microbiota produces SCFA that can provide maintenance energy (Beckers et al. 2017). Furthermore, bacteria produce SCFA that also alter signaling in the host immune system (Chan et al., 2019). In apparent correspondence with this observation, BC enhanced concentration of VFAs. Sun et al. (2019) suggested that *Parasutterella* may have a role in metabolic disorders. Accordingly, supplementation of BC can boost some beneficial bacteria and reduce some harmful bacteria.

In summary, *B. coagulans* improved BW and ADG, increased antioxidant capacity, might boost immunity by increasing the levels of immunoglobulin and anti-inflammatory factors and decreasing proinflammatory factors, and might enhance intestinal health by increasing the concentration of VFAs and improving intestinal microflora in broilers.

DISCLOSURES

There are no conflicts of interest to declare.

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