Effects of probiotic *Clostridium Butyrate* on Performance and Immunocompetence and Digestive Function of poultry

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Abstract

The purpose of our research was to study the effects of dietary addition of *Clostridium butyricum* (CB) on performance, immunocompetence and digestive function of poultry. Using single factor experiment design, 720 one-day-old and healthy AA broilers were divided into 6 groups with 6 replicates in each group and 20 broilers in each replicate. Broilers in control group were fed a basal diet, while broilers of other experimental group were fed the basal diet supplemented with CB 250 mg/kg, 500 mg/kg, 750 mg/kg, 1000 mg/kg and 1250 mg/kg. The results showed as follows: The 1000mg/kg group had the best effect on immunity enhancement, with the thymus index, spleen index and bursa index increased by 18.38 % ($P < 0.05$), 16.00 % ($P < 0.05$) and 8.03 % ($P > 0.05$) in 1–21 days, and 6.45 % ($P < 0.05$), 12.92 % ($P < 0.05$) and 13.94 % ($P < 0.05$) in 22–49 days. The 1000 mg/kg group and 1250 mg/kg group had the best effect on improving the digestive function, with the VH/CD value increased by 27.56 % ($P < 0.05$) and 29.56 % ($P < 0.05$) in 1–21 days, and 29.48 % ($P < 0.05$) and 30.80 % ($P < 0.05$) in 22–49 days, with the number of harmful bacteria *Escherichia coli* and *Salmonella* decreased significantly ($P < 0.05$). These results indicated that the addition of CB in diets of broiler can improve the production performance, enhance immunity and improve digestive function, and the optimal dosage is 1000 mg/kg.

Key words: *Clostridium butyrate*, Broiler, Production performance, Immunocompetence.

Contents

1. Introduction	27
2. Materials and methods	28
3. Results and discussion	29
4. Conclusions	32
References	32

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1. Introduction

In recent years, antibiotics and synthetic antibiotics have been widely used in animal production as feed additives, which have played an active role in promoting animal growth, improving feed remuneration, preventing disease and improving product quality (Marshall & Levy, 2011).

But as a result of long-term large use, overuse and abuse, produced a series of negative effects, including to destroy the balance of intestinal flora, to increase bacterial resistance, to reduce the body's resistance, to cause food safety incidents by drug residues, to harm human health and to cause environmental pollution, etc. (Friedman, 2015; Daeseleire et al., 2016; Becattini et al., 2016), most countries in the world have formulated related regulations, restricted and prohibited the use of antibiotics and chemical synthesis of antimicrobial agents in feed (Watkins & Bonomo, 2016). In view of the severe restriction or total ban

on the use of antibiotics as growth promoters in poultry production, probiotics have been suggested as an alternative to antibiotics. Probiotics have been defined as viable microorganisms (bacteria or yeasts) that exhibit a beneficial effect on the health of the host when they are ingested. Several studies have shown that the addition of probiotics to the diets of broilers leads to improved performance (Jin et al., 1998).

Therefore, the research and development of new feed additives with the characteristics of both increased yield and increased efficiency and non-toxic, harmless and low residue is a common concern in the field of animal nutrition.

Probiotics are a group of active microorganisms that can bring benefits to host health when given to host animals in sufficient quantities (Zhang, 2019).

As a feed additive, probiotics can not only promote animal growth, improve immune regulation, maintain normal intestinal flora, regulate animal digestion and metabolism,

and reduce environmental pollution, but also have the advantages of no drug resistance, no residue, green and environmental protection, which is a new generation of green feed additives to replace antibiotics (Alagawany et al., 2018; Ni et al., 2018).

Currently, probiotics used as feed additives mainly include *Bacillus subtilis* (such as *Bacillus subtilis* and *Bacillus licheniformis*), *Lactobacillus* (such as *Bifidobacteria* and *Lactobacillus acidophilus*), *Saccharomycete* (such as *Saccharomyces cerevisiae* and petroleum yeast) and mildew (such as *Aspergillus oryzae* and *Aspergillus Niger*).

Tang Jiangwu et al., 2011 reported the effects of *Bacillus subtilis* on the intestinal flora and digestive enzyme activity of broilers, and Guo Xinyi et al., 2016 reported the effects of *Lactic acid bacteria* on the production performance, immune function and intestinal flora of broilers, the studies have shown that probiotics as a new type of microecologies has played a positive role in animal production, and shows broad prospects for development.

Clostridium butyricum (CB) is a bacillus, which was first discovered and reported by Dr. Mitsuaki miyagi of chiba medical university in Japan in 1933. CB has the function of promoting animal growth, regulating intestinal flora balance, enhancing body immunity and providing nutrition, etc., and has become a new micro-ecological feed additive (Liao et al., 2015; Cao et al., 2019).

In 2009, the Ministry of Agriculture of China approved the use of CB and its preparations as additives in feed, but its application research is still in its infancy, especially in the production of broiler, the application effect reports are different, and the appropriate dosage in broiler diet is still in great dispute (Zhang et al., 2011; Jia et al., 2016).

Based on the comprehensive comparison of previous studies, this study adopted a single factor test design to study the effects of CB on broiler performance, immunity and digestive function, so as to provide experimental basis for the rational use of CB in broiler production

2. Materials and methods

Chemicals and reagents. CB, live bacteria consent of 1×10^9 cfu/g, was obtained from Henan Jinbaihe Biotechnology Co., Ltd (Zhengzhou, China). RPMI-1640 cell culture medium was purchased from Gibco Company (USA). Concanavalin A (Con A) and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma Company (USA). Lymphocyte separation fluid was purchased from Shanghai Huajing Biotechnology Co., Ltd (Shanghai, China). Percoll cell stratification liquid was purchased from Pharmacia Company (USA). Newcastle disease virus (NDV) Lasota strain IV attenuated vaccine was purchased from Wuhan Zhongbo Biochemistry Co., Ltd (Wuhan, China). ND hem agglutination inhibition (HI) antigen was provided by Beijing Bodeansheng Trading Company (Beijing, China). Other reagents obtained from the market were analytically pure.

Experimental animals. 720 AA P broilers selected and mixed with male and female, with an initial average weight of 48.32 ± 1.2 g, were produced by Henan Hebi Bolong Breeding Poultry Co., Ltd.

Experimental design and diets. All procedures and experiments were conducted in accordance with the ethics of the local animal care committee, and were approved by the animal protection and use committee of the school of animal science and technology of Henan Institute of Science and

Technology (Xinxiang, China). Using a single factor test design, 720 AA broilers were randomly divided into one control group and five experimental groups, 120 chickens in each group, with 6 replicates and 20 chickens in each replicate. The trial period was 49d, divided into 1 to 21 days in the early stage and 22 to 49 days in the later stage. The control group chicks were fed with basal diets. Test 1, 2, 3, 4 and 5 groups chicks were fed with CB 250 mg/kg, 500 mg/kg, 750 mg/kg, 1000 mg/kg and 1250 mg/kg on the basis of basal diets, respectively. All chicks were offered the same basal diet, which was formulated to meet or slightly exceed the NRC (1994) broilers requirements for all nutrients. The composition and nutrient levels of the basal diet were shown in table 1.

Table 1

Composition and nutrition levels of the basal diets (Dry material basis)

Ingredient (% unless noted):	Days 1–21	Days 22–49
Corn	55.2	59.3
Soybean meal	36.7	33.2
maize oil	3.5	3.5
Limestone	1.1	1.3
Calcium hydrogen phosphate	1.9	1.25
Salt	0.35	0.35
Vitamin and mineral premix ¹	1.0	1.0
DL-Methione	0.2	0.07
Lysine	0.05	0.03
Nutrient level:		
Metabolisable energy (MJ/kg) ²	12.68	12.94
Crude protein (%)	21.48	18.98
Calcium (%)	0.86	0.81
Nonphytate phosphorus (%)	0.43	0.39

¹Supplied per kilogram of diet: Mn 80 mg, Zn 60 mg, Fe 80 mg, Cu 8 mg, I 0.35 mg, Se 0.15 mg, Vitamin A 3000 IU, Vitamin D 1250 IU, Vitamin E 15 IU, Vitamin K 2.2 mg, Vitamin B₁ 1.6 mg, Vitamin B₂ 2.8 mg, Vitamin B₆ 3.1 mg, Vitamin B₁₂ 0.01 mg, Niacin 40 mg, Folic acid 0.72 mg, Biotin 0.12 mg, Choline 1100 mg.

²The metabolic energy was calculated theoretically, and the remaining nutrients were determined.

Feeding and management. Feeding management of chicks shall refer to AA broiler feeding and management manual. Feeding methods: All chicks were raised in the same chicken house. Immunization procedures: The chicks were vaccinated against Newcastle disease virus (NDV) at 7 and 21 days of age with against NDV Lasota strain IV attenuated vaccine and against infectious bursal diseases virus (IBDV) at 14 days of age with a weak vaccine in drinking water. Temperature management: Light management: Constant light 24h per day was provided throughout the experimental period.

Sample collection and analyses. On Monday morning every week, the chicks in each treatment group were weighed following a 12h fast, body weight (BW), body weight gain (BWG) and feed intake (FI) were recorded, and average daily intake (ADG) and average daily feed intake (AFDI) and feed to gain ratio (FGR) were calculated.

Immunity. At 21 and 49 days of age, a total of 36 chicks were randomly selected from each repeat of the treatment group. They were slaughtered by jugular vein bloodletting. Thymus, spleen and bursa were separated and all connective tissues and fat were removed and the immune organ index

was calculated statistically using the following formula: Immune organ index(g/kg) = Immune organ weight (g)/bodyweight (kg). Blood samples were collected from wing vein of 36 randomly selected chicks, centrifuged at 5000 r/min for 5 min at 21 and 49 days of age to extract serum, which was used for the HI to detect the titers of serum NDV antibodies. The conversion rate of PLB was measured by MTT method as described by (Pascua-Maestro et al., 2018) for 36 randomly selected chicks with cardiac blood collection of 5 mL, heparin sodium anticoagulation, and isolated lymphocytes. The results were shown in the stimulation index (SI) as follows: SI = Con A stimulation group A_{570nm} /control group A_{570nm} .

Digestive function. With reference to the method described by (Rubio et al., 2010), 36 randomly selected chicks were slaughtered, 3cm of duodenal samples were collected, washed with 0.9 % normal saline, fixed with 10 % formalin solution for 24h, and stored in 70 % alcohol for histological examination. The fixed samples were dehydrated with ethanol step by step, embedded in paraffin, sectioned, stained with hematoxylin-eosin (H.E), and sealed with resin. The Villum height (VH) and the associated Crypt depth (CD) were observed and photographed with a 40-fold field of microscope, and the VH/CD ratio was calculated. 36 randomly selected chicks were slaughtered, the cecum of the same part was cut off, and the cecum contents were collected and put in an aseptic sample bag for storing and analyzed within 24 h. The cecal contents were diluted 10 fold from 10^{-2} to 10^{-9} with aseptic normal saline, then 0.1 mL of diluted samples were inoculated into selective agar for bacterial enumeration. *Escherichia coli* was incubated using Mac-

Conkey agar, and *Salmonella* was incubated using HE agar at 37.5 °C for 24 h. *Bifidobacterium* and *Lactobacillus* were incubated using TPY agar in an anaerobic incubator at 37.5h for 48h. Results were reported as log₁₀colony-forming units per gram of cecal contents by colony counting method (CCM) described by (Macdonald et al., 2017).

Statistical Analyses The experimental data were analyzed by one-Way ANOVA in SPSS 16.0 statistical software. The results were expressed as mean standard deviation. $P < 0.05$ was considered significant, while $P > 0.05$ was considered insignificant.

3. Results and discussion

Growth Performance. The results of the growth performance parameters were shown in Table 2. The effects of CB on ADG, the experimental groups compared with the control group, the test 4 group was the best with ADG increased by 6.98 % ($P < 0.05$) in 1–21 days, 5.17 % ($P < 0.05$) in 22–49 days, and 5.64 % ($P < 0.05$) in 1–49 days. The effect of CB on ADFI, the ADFI of each test group was somewhat higher than that of the control group in 1–21 days and in 22–49 days, but there was no significant effect ($P > 0.05$). The effects of CB on FGR, the experimental groups compared with the control group, the test 4 group was the best with FGR reduced by 1.68 % ($P < 0.05$) in 1–21 days, 3.63 % ($P < 0.05$) in 22–49 days, and 3.37 % ($P < 0.05$) in 1–49 days, respectively. This indicated that the addition of CB in the diet could improve the production performance of broilers, and the appropriate dosage was 1000 mg/kg.

Table 2

Effects of CB on performance of broilers (n = 6)

Groups	Days 1–21					Days 22–49				
	BWG (g)	ADG (g/d)	FI (g)	ADFI (g/d)	FGR	BWG (g)	ADG (g/d)	FI (g)	ADFI (g/d)	FGR
control	655.65 ± 14.47	31.22 ± 1.02	937.27 ± 10.21	45.15 ± 1.34	1.430 ± 0.08	1821.58 ± 13.44	65.06 ± 1.37	3911.25 ± 12.87	138.74 ± 1.64	2.147 ± 0.09
Test1	661.52 ± 16.33	31.50 ± 1.21	944.55 ± 9.89	46.31 ± 1.26	1.428 ± 0.07	1847.21 ± 16.52	65.97 ± 1.44	3927.21 ± 16.13	140.78 ± 1.86	2.126 ± 0.07
Test 2	675.17 ± 12.81 ^a	32.15 ± 1.47 ^a	961.36 ± 11.24	45.66 ± 1.12	1.424 ± 0.06 ^a	1861.20 ± 14.51 ^a	66.47 ± 1.21 ^a	3935.36 ± 16.88	141.66 ± 1.65	2.114 ± 0.09 ^a
Test 3	686.36 ± 18.54 ^a	32.68 ± 1.09 ^a	982.35 ± 11.46	46.71 ± 1.01	1.431 ± 0.08 ^a	1884.25 ± 13.11 ^a	65.87 ± 1.56 ^a	3954.53 ± 15.14	140.21 ± 1.36	2.099 ± 0.09 ^a
Test 4	701.38 ± 15.32 ^b	33.40 ± 1.35 ^b	986.37 ± 9.87	46.39 ± 1.25	1.406 ± 0.09 ^b	1915.64 ± 11.58 ^b	68.42 ± 1.42 ^b	3963.23 ± 18.10	140.16 ± 0.98	2.069 ± 0.08 ^b
Test5	692.55 ± 16.11 ^b	32.98 ± 1.53 ^b	979.65 ± 8.99	44.36 ± 1.32	1.415 ± 0.07 ^b	1901.31 ± 12.33 ^b	67.90 ± 1.36 ^b	3945.38 ± 15.41	136.57 ± 1.67	2.075 ± 0.06 ^b

Note: a, b means within the same column carrying same indicates no significant difference ($P > 0.05$), while different super scripts indicate significant difference ($P < 0.05$). The following is the same

Immunity. Immune organs index. The results were shown in Table 3. In 1–21 days, the experimental groups compared with the control group, the test 4 group was the best with thymus index, spleen index and bursa index increased by 18.38 % ($P < 0.05$), 16.00 % ($P < 0.05$) and 8.03 % ($P > 0.05$), respectively. In 22–49 days, the thymus

index, spleen index and bursa index were improved to some extent in test 4 group, which was 6.45 % ($P < 0.05$) 12.92 % ($P < 0.05$) and 13.94 % ($P < 0.05$), respectively. This indicated that the addition of CB in the diet could improve the immune organ index of broilers and thus improve the immunity of the body. The dosage should be 1000 mg/kg.

Table 3

Effects of CB on immune organ index of broilers (n = 6)

Groups	Days 1–21 (g)			Days 22–49 (g)		
	thymus index	spleen index	bursa index	thymus index	spleen index	bursa index
control	3.28 ± 0.17	0.75 ± 0.07	2.49 ± 0.17	3.41 ± 0.28	1.28 ± 0.12	1.65 ± 0.19
Test1	3.30 ± 0.16	0.80 ± 0.09	2.52 ± 0.16	3.37 ± 0.21	1.32 ± 0.13	1.72 ± 0.14
Test 2	3.41 ± 0.17	0.83 ± 0.07 ^a	2.58 ± 0.13	3.42 ± 0.25	1.35 ± 0.15	1.77 ± 0.17
Test 3	3.64 ± 0.17 ^a	0.85 ± 0.09 ^a	2.65 ± 0.15 ^a	3.54 ± 0.22 ^a	1.44 ± 0.11	1.87 ± 0.18 ^a
Test 4	3.85 ± 0.18 ^b	0.87 ± 0.08 ^b	2.69 ± 0.09 ^a	3.63 ± 0.27 ^b	1.47 ± 0.17 ^a	1.88 ± 0.19 ^a
Test5	3.81 ± 0.12 ^b	0.81 ± 0.09 ^b	2.66 ± 0.14 ^a	3.58 ± 0.21 ^b	1.45 ± 0.11 ^a	1.81 ± 0.14 ^a

NDV antibody titers and PBL proliferation. The results were shown in Table 4. Compared with the control group, the titers of NDV antibody in the all test groups were improved, in which the test 4 group was the largest with an increase of 5.46 % ($P > 0.05$) in 1–21 days and 4.27 % ($P > 0.05$) in 22–49 days. Therefore, the addition of CB in diet could increase the titers of NDV antibody in broilers,

but there was no significant difference ($P > 0.05$). The SI of each test group was higher than that of the control group, of which the test 4 group was the highest with an increase of 5.46 ($P < 0.05$) in 1–21 days and 12.79% ($P < 0.05$) in 22–49 days. This indicated that the addition of CB in diet could promote the proliferation of PBL in broilers, and the appropriate dosage was 1000 mg/kg.

Table 4

Effects of CB on NDV antibody titers and SI of broilers (n = 6)

Groups	Days 1–21		Days 22–49	
	NDV antibody titers	SI	NDV antibody titers	SI
control	4.76 ± 0.21	5.47 ± 0.18	5.15 ± 0.21	5.71 ± 0.21
Test1	4.77 ± 0.23	5.51 ± 0.19	5.22 ± 0.27	5.94 ± 0.27
Test 2	4.85 ± 0.26	5.49 ± 0.21	5.31 ± 0.28	6.11 ± 0.22
Test 3	4.99 ± 0.22	5.57 ± 0.27 ^a	5.32 ± 0.33	6.09 ± 0.28
Test 4	5.02 ± 0.27	5.76 ± 0.24 ^b	5.37 ± 0.28	6.44 ± 0.21 ^a
Test5	4.98 ± 0.26	5.87 ± 0.21 ^b	5.41 ± 0.31	6.51 ± 0.18 ^a

Digestive Function. Intestinal mucosa morphology. The results were shown in Table 5. The effects of CB on VH, the experimental groups compared with the control group, all the test groups were higher than the control group, with a significant difference ($P < 0.05$), among which the test 4 and 5 group showed a significant improvement, with an increase of 15.76 % ($P < 0.05$) and 19.57 % ($P < 0.05$) in 1–21 days, and an increase of 16.75 % ($P < 0.05$) and 17.86 % ($P < 0.05$) in 22–49 days, respectively. The effects of CB on CD, all the test groups were lower than the control group, except the test 1 and 2 group showed no significant difference ($P > 0.05$), the test 3, 4 and 5 group showed a significant difference ($P < 0.05$) with reduced by 6.43 % ($P < 0.05$), 7.27 % ($P < 0.05$), and 7.70 % ($P < 0.05$) in 1–

21 days, and 8.49 % ($P < 0.05$), 9.80 % ($P < 0.05$) and 9.858 % ($P < 0.05$) in 22–49 days, with no significant difference between the groups ($P > 0.05$). The effects of CB on VH/CD value, the VH/CD values of each test group were higher than those of the control group, except that the difference in group I was not significant ($P > 0.05$), and the other four groups were significantly different ($P < 0.05$), among them, the effects of test 4 and 5 group were obvious, which increased 27.56 % ($P < 0.05$) and 29.56 % ($P < 0.05$) in 1–21 days, and 29.48 % ($P < 0.05$) and 30.80 % ($P < 0.05$) in 22–49 days. Therefore, the addition of CB in diet can improve VH, reduce CD, improve VH/CD value, enhance digestibility, and the dosage of 1000 mg/kg was appropriate.

Table 5Effects of *Clostridium butyricum* on VH, CD and VH/CD value of broilers (n = 6)

Groups	Days 1–21			Days 22–49		
	VH(μm)	CD(μm)	VH/CD	VH(μm)	CD(μm)	VH/CD
control	1265.47 ± 102.24	90.11 ± 4.47	14.04 ± 1.56	1871.25 ± 118.52	112.76 ± 8.56	16.59 ± 2.41
Test1	1278.35 ± 111.37 ^a	90.20 ± 5.13	14.17 ± 1.24	1904.44 ± 108.21 ^a	110.63 ± 8.77	17.21 ± 2.22
Test 2	1323.84 ± 121.33 ^a	87.56 ± 5.41	15.12 ± 1.47 ^a	1969.61 ± 125.83 ^a	108.45 ± 9.87	18.16 ± 2.31 ^a
Test 3	1456.29 ± 123.34 ^a	84.32 ± 4.71 ^a	17.27 ± 1.38 ^a	2044.56 ± 112.35 ^a	103.19 ± 11.45 ^a	19.81 ± 2.24 ^a
Test 4	1496.37 ± 105.66 ^b	83.56 ± 5.69 ^a	17.91 ± 1.58 ^b	2184.69 ± 118.12 ^b	101.71 ± 12.15 ^a	21.48 ± 2.45 ^b
Test 5	1513.16 ± 113.55 ^b	83.17 ± 5.33 ^a	18.19 ± 1.62 ^b	2205.38 ± 121.57 ^b	101.65 ± 11.73 ^a	21.70 ± 2.37 ^b

Caecal microflora. The results were shown in Table 6. The experimental group compared with control group, the number of *Escherichia coli* decreased significantly in all the test groups, among which the test 4 and 5 group showed an obvious reduce, and reduced by 16.63 % ($P < 0.05$) and

15.57 % ($P < 0.05$) in 1–21 days, 16.94 % ($P < 0.05$) and 17.51 % ($P < 0.05$) in 22–49 days, respectively. The results of tests on *Salmonella* were consistent with that of *Escherichia coli*, the number of *Salmonella* in the test 4 and 5 group reduced by 17.95 % ($P < 0.05$) and 17.64 %

($P < 0.05$) in 1–21 days, 16.59 % ($P < 0.05$) and 16.90 % ($P < 0.05$) in 22–49 days, respectively.

The number of *Lactobacillus* increased significantly in all the test groups, among which the test 4 and 5 group showed an obvious increase, and increased by 12.02 % ($P < 0.05$) and 12.86 % ($P < 0.05$) in 1–21 days, 12.29 % ($P < 0.05$) and 13.01 % ($P < 0.05$) in 22–49 days, respectively. The results of tests on *Bifidobacterium* were consistent

with that of *Lactobacillus*, the number of *Bifidobacterium* in the test 4 and 5 group increased by 15.71 % ($P < 0.05$) and 16.69 % ($P < 0.05$) in 1–21 days, 17.54 % ($P < 0.05$) and 18.07 % ($P < 0.05$) in 22–49 days, respectively. The results showed that the addition of CB in diet could reduce the number of harmful bacteria, increase the number of beneficial bacteria, and improve the cecum flora. The dosage should be 1000 mg/kg.

Table 6

Effects of CB on cecal microflora of broilers (n = 6)

Groups	Days 1–21 (cfu/g)				Days 22–49 (cfu/g)			
	<i>E. coli</i>	<i>Salmonella</i>	<i>Lactobacillus</i>	<i>Bifidobacterium</i>	<i>E. coli</i>	<i>Salmonella</i>	<i>Lactobacillus</i>	<i>Bifidobacterium</i>
control	8.54 ± 0.17	6.35 ± 0.15	8.32 ± 0.15	7.13 ± 0.11	8.68 ± 0.15	6.39 ± 0.17	8.38 ± 0.10	7.47 ± 0.17
Test1	8.32 ± 0.11 ^a	6.28 ± 0.18 ^a	8.40 ± 0.12 ^a	7.21 ± 0.14 ^a	8.54 ± 0.17 ^a	6.25 ± 0.17 ^a	8.44 ± 0.13 ^a	7.52 ± 0.18 ^a
Test 2	7.78 ± 0.16 ^a	5.86 ± 0.17 ^a	8.74 ± 0.16 ^a	7.63 ± 0.12 ^a	8.11 ± 0.16 ^a	5.84 ± 0.16 ^a	8.85 ± 0.17 ^a	8.01 ± 0.23 ^a
Test 3	7.32 ± 0.18 ^a	5.45 ± 0.17 ^a	8.98 ± 0.15 ^a	7.91 ± 0.17 ^a	7.45 ± 0.19 ^a	5.53 ± 0.13 ^a	9.32 ± 0.26 ^a	8.54 ± 0.22 ^a
Test 4	7.12 ± 0.15 ^b	5.21 ± 0.15 ^b	9.32 ± 0.19 ^b	8.25 ± 0.16 ^b	7.21 ± 0.13 ^b	5.33 ± 0.21 ^b	9.41 ± 0.21 ^b	8.78 ± 0.27 ^b
Test5	7.21 ± 0.17 ^b	5.23 ± 0.18 ^b	9.39 ± 0.17 ^b	8.31 ± 0.19 ^b	7.16 ± 0.17 ^b	5.31 ± 0.24 ^b	9.47 ± 0.22 ^b	8.82 ± 0.19 ^b

Discussion

About the effects of CB on production performance of broilers. CB was an ideal probiotics discovered in recent years, which could improve animal production performance. (Chen et al., 2018; Wang et al., 2019) had shown that there were three aspects of the mechanism of action of CB, one was that the main product of CB in the intestinal tract was butyrate, which was the first energy source of intestinal cells, which was very important for the proliferation and repair of intestinal mucosal cells. The second was that butyrate could be used as an acid source to regulate the intestinal pH value, thus promoting the growth of beneficial bacteria, inhibiting the propagation of harmful bacteria, and maintaining the balance of intestinal microecology in animals. Third, CB could produce a large number of endogenous digestive enzymes in the process of animal proliferation, which was helpful to fully improve the digestive utilization rate of feed. Jia Conghui et al., 2016 reported that adding 800 mg/kg CB to the Ros308 broiler diet in the 42-day trial period, compared with the control group, the BW increased by 22.76 %, the ADG increased by 7.65 %, the ADFI increased by 7.37 %, and the FGR decreased by 2.20 %. The results of this study showed that ADG in each experimental group was higher than that in the control group, among which 1000 mg/kg had the best effect.

About the effects of CB on immunity of broilers. The main immune organs of broilers include thymus, spleen and bursa, and the immune organ index reflects the strength of immunity. He Ju et al., 2018 reported that CB can promote the growth and development of immune organs in broilers, and there is a certain quantitative effect relationship. Wang Junfeng et al., 2010 reported that adding 500 mg/kg CB to broiler diets increased the index of immune organs such as bursa bursa without significant changes to the later stage. Liu Tingting et al., 2012 showed that the spleen index of egg-laying chicks in the 1000 mg/kg addition group was significantly higher than that in the control group, and the optimal addition level of broilers was 1000 mg/kg, which was consistent with the results of this study.

The effects of CB on the titers of NDV antibody had not been reported yet. The results of this study showed that the effects of dietary supplementation with sufficient dose of

CB on the titers of NDV antibody were not significant, regardless of the results of 1–21 days in the early stage of the experiment, 22–49 days in the late stage of the experiment, and 1–49 days in the whole process. The effects of CB on the proliferation of PBL in broilers had not been reported yet. The results of this study showed that the addition of CB in diet could promote the proliferation of PBL in broilers. The effects were not obvious in the first 1–21 days, and the effects were obvious in the later 22–49 days. In addition, this study conducted experimental studies of CB on the three indexes of immune organ index, NDV antibody titers and PBL proliferation, without other indexes. Du Yunping et al., 2009 reported that the effects of CB on the prevention and treatment of broiler enteritis were better, and the fecal condition was improved obviously. Liu Xianhan et al., 2019 reported that adding 400 mg/kg CB to the diet of duck could increase the contents of IgA, IgG, C3, C4 in serum, and then increase the immune level of the body and strengthen the disease resistance.

About the Effects of CB on digestive function of broilers. The effects of CB on intestinal mucosa morphology in broilers were rare, but had been reported in other animal breeding studies. Pang Min et al., 2016 reported that dietary supplementation with CB 500mg/kg significantly reduced ileum CD and increased VH /CD value in weaned piglets. It was suggested that butyric acid, the main metabolite of CB, was the main nutrient for the regeneration and repair of intestinal epithelial tissues and played an important role in the regeneration and repair of intestinal epithelial tissues. Zheng Youxiu et al., 2018 reported that adding CB 500 mg/kg, 1000 mg/kg and 2000 mg/kg to the diet of weaned piglets was tested for 30 days, and the results showed that compared with the control group, jejunum VH increased by 35.71 %, 33.59 % and 47.36 %, and VH /CD value increased by 48.03 %, 19.69 %, and 22.83 %, respectively. CB could improve the intestinal structure of weaned piglets and enhance the digestive capacity. This indicated that the addition of CB in diet could increase the value of VH/CD and improve the digestibility of broilers, which was consistent with the results of this study.

Yi Zhonghua 2012 reported that CB could effectively regulate the flora balance of intestinal microecology by

rejecting substitution through competition and changing intestinal microenvironment to reduce the number of harmful bacteria, thus effectively reducing the diarrhea rate of animals and greatly increasing its survival rate. Gao Qi et al., 2012 had reported the mechanism of CB regulating intestinal flora. The optimum pH value of most probiotics in the gut was partial acidity, whereas most pathogenic bacteria were weakly acidic alkaline, butyric acid could fine-tune the pH value of intestinal environment, thus promoting the growth of probiotics and inhibiting the growth of harmful bacteria. The increase of probiotics will further compress the living space of harmful bacteria, which will aggravate the shedding and death of harmful bacteria and play a role in improving the community of bacteria. There were rare reports on the effects of CB on intestinal flora of broilers, and there were many studies and applications in pig breeding, and other animal breeding studies had been reported. Zhang Youxiu et al., 2018 reported that adding CB 500 mg/kg, 1000 mg/kg to weaned pig diets, the number of *Lactobacillus* and *Bifidobacterium* in cecum reached the highest level, and the number of *Escherichia coli* in cecum in the test group decreased by 23.18 % and 23.49 %, respectively, and *Salmonella* in cecum decreased by 16.56 % and 14.17 %, respectively, and the effect of adding CB 1000 mg/kg in weaned pig was better than that in the control group.

4. Conclusions

In summary, the results of this study showed that adding CB to the diet of broilers could improve the production performance, enhance immunity, improve intestinal mucosal morphology, regulate cecum flora, and improve digestive function. The optimal dosage was 1000 mg/kg.

Author's contributions

All authors participated in the study design, coordination and carried out data analyses. Xiaofei Wang, Yanzhao Xu participated and performed measurements, laboratory testing's and data collection. All authors read and approved the final manuscript. All authors contributed to the draft of the manuscript and discussed results. All authors gave final approval for publication.

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Conflict of interest

Author does not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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