Effects of dietary *Bacillus amyloliquefaciens* CECT 5940 supplementation on growth performance, antioxidant status, immunity, and digestive enzyme activity of broilers fed corn-wheat-soybean meal diets

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ABSTRACT This experiment was conducted to investigate the effects of dietary supplementation with *Bacillus amyloliquefaciens* CECT 5940 (**BA-5940**) on growth performance, antioxidant capacity, immunity, and digestive enzyme activity of broiler chickens. A total of 720 one-day-old Arbor Acres male broiler chicks (average body weight, 45.87 ± 0.86 g) were randomly allocated to 5 treatments of 8 replicates with 18 chicks in each replicate. Broilers in the control group were fed a corn-wheat-soybean basal diet, the other 4 groups were fed the same basal diet supplemented with 500, 1,000, 1,500, or 2,000 mg/kg Ecobiol (1.27×10^9 CFU/g BA-5940) for 42 d, respectively. Broilers fed diets supplemented with BA-5940 showed a quadratic response ($P < 0.05$) of average daily gain (**ADG**) and feed to gain ratio (**F:G**) during d 22 to 42 and d 0 to 42. The glutathione peroxidase (**GSH-Px**) and superoxide dismutase (**SOD**) activities in serum and liver, and total antioxidant capacity (**T-AOC**) in liver of broilers on d 42 increased linearly ($P < 0.05$) with increasing levels of BA-5940, while malondialdehyde (**MDA**) level in serum decreased linearly ($P < 0.05$). Concentrations of serum immunoglobulin (**Ig**) A and IgM on d 21, and IgM on d 42 increased linearly ($P < 0.05$) as BA-5940 levels increased. Supplementation with increasing levels of BA-5940 linearly decreased serum tumor necrosis factor-α (**TNF-α**) levels on d 21 and 42, while increased interleukin (**IL**)-10 concentration (linear, $P < 0.05$) on d 21. Meanwhile, the levels of IL-1β, IL-6, and TNF-α in the mucosa of jejunum and ileum were decreased (linear, $P < 0.05$) on d 42 as dietary supplementation of BA-5940 increased. Additionally, supplementation with BA-5940 also increased the activities of amylase (linear, $P < 0.01$), lipase (linear, $P < 0.05$) and chymotrypsin (linear, $P < 0.01$) in jejunal digesta, and lipase (linear, $P < 0.05$) in ileal digesta of broilers on d 42. To summarize, inclusion of BA-5940 in corn-wheat-soybean meal-based diet improved growth performance of broilers through improving antioxidant capacity, immunity, and digestive enzyme activity. Based on the results of this study, $1.1{-}1.6 \times 10^9$ CFU/kg BA-5940 is recommended for supplementation in broiler diets.

Key words: antioxidant capacity, *Bacillus amyloliquefaciens*, broiler, digestive enzyme, immunity

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INTRODUCTION

Broilers are vulnerable to many stressors or diseases in the intensive and large-scale production of poultry farming (Humam et al., 2020). Probiotics as feed additives can relieve many challenges of broilers under complex commercial conditions and have safety characteristics such as lack of residual and toxic side effects (Al-Khalaifah, 2018). *Bacillus* species form heat-resistant spores, which can survive at low pH of the gastrointestinal tract and germinate within the intestinal tract (Elshaghabee et al., 2017). It also can be stored long-term at room temperature in the form of freeze-dried powder without lowering viability and efficacy (Cutting, 2011). Once germinated in vivo, they can produce various enzymes in the gut to improve nutritional digestion (Li et al., 2019). So far, many kinds of *Bacillus* spp. have been authorized for utilization in broiler diets as direct-fed microbials, such as *Bacillus subtilis, Bacillus coagulans, Bacillus licheniformis,* and *Bacillus amyloliquefaciens* (Ortiz et al., 2013; Elshaghabee et al., 2017).

The *Bacillus amyloliquefaciens* (**BA**) is a very promising *Bacillus* spp. for broilers. Studies have shown that dietary inclusion of different BA strains, such as BA-KB3 (1,000−2,000 mg/kg), BA-DFM ($0.75{-}1.5 \times 10^8$

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CFU/g), BA-LFB112 ($1-100 \times 10^7$ CFU/g), BA-H57 (2.48×10^8 CFU/g) or BA-TL (4×10^6 CFU/g) improved the growth rate, feed intake, or feed conversion ratio of broilers (Ahmed et al., 2014; Lei et al., 2015; Wei et al., 2017; Shini et al., 2020; Hong et al., 2021). However, some studies reported that dietary inclusion of 1.3×10^9 CFU/g BA-KU801 (An et al., 2008), 1×10^6 CFU/g BA-L (Jerzsele et al., 2012), or 1×10^5 CFU/g BA-SC06 (Wang et al., 2021) did not affect the growth performance of broilers. The above results of studies on BA were inconsistent, which may be since BA efficacy should rely on the characteristics of target strains and supplementation level, and the composition of diets, etc. *Bacillus amyloliquefaciens* CECT 5940 (**BA-5940**) is a safe strain for chickens approved by EFSA (2008). De Oliveira et al. (2019) reported that the addition of BA-5940 improved F:G, reduced *Clostridium perfringens* count in ileal content, improved carcass and breast meat yield of broilers challenged with *Eimeria spp.* and *Clostridium perfringens*. Gharib-Naseri et al. (2020;2021) reported that supplementation with BA-5940 increased serum IgA, IgM, and IgG concentrations, and improved digestibility of cystine, valine, and lysine of broilers under necrotic enteritis challenge. Previous studies on BA-5940 mainly focus on growth performance, carcass trait, digestibility, and immune globulin of broilers under challenging conditions. However, the effects of BA-5940 on antioxidant capacity, cytokines level, and digestive enzyme activity of broilers remain unclear, especially under commercial broiler production conditions.

We hypothesized that dietary BA-5940 can improve growth performance of broilers through improving antioxidant capacity, immunity, and digestive enzyme activities. Therefore, the purpose of this study was to evaluate the efficacy of BA-5940 in broiler under commercial production conditions by determining growth performance, antioxidant capacity, immunity, and digestive enzyme activities. Also, the suitable addition level of BA-5940 in the broiler diet be investigated.

MATERIALS AND METHODS

The experimental procedures were approved by the China Agricultural University Animal Care and Use Committee (Beijing, China; NO. AW30801202-1-1). This study was performed on the Fengning Research Unit of China Agricultural University (Hebei, China).

Bacillus Amyloliquefaciens

Bacillus amyloliquefaciens CECT 5940 used in this experiment was a commercial product (Ecobiol) provided by Evonik China Co., Ltd (Beijing, China) in freeze-dried powder form containing spores and calcium carbonate as the carrier. The spores count of BA-5940 in Ecobiol was 1.27×10^9 CFU/g determined using the spread plate method.

Experimental Birds and Design

A total of 720 one-day-old Arbor Acres (**AA**) male broiler chicks with an average weight of 45.87 ± 0.86 g were bought from Arbor Acres Poultry Breeding Company (Beijing, China), and allocated randomly to 5 treatments with 8 replicates and 18 chicks per replicate. The experiment was conducted using a completely randomized design. The control group was fed the corn-wheat-soybean meal basal diet, and the else groups were fed the same basal diet supplemented at the expense of limestone with 500, 1,000, 1,500, or 2,000 mg/kg Ecobiol for 42 d, respectively.

Diet and Management

The corn-wheat-soybean meal diet was formulated according to the nutritional requirements of broilers (NRC, 1994) without any antimicrobial growth promoters. Tables 1 and 2 showed the compositions and nutritional levels of basal diets, respectively. From d 0 to 21, broilers were fed with starter diets (mash feed), and then grower diets were provided until the end of this experiment. All chicks were raised in wire-floored cages in an environmentally controlled room and had free access to feed and water. During the first week, the temperature was controlled at 33°C and then decreased gradually by 3°C per week until 24°C. A 24 h light lighting program was applied during the first 3 d, and then a 23 h light to 1 h dark lighting program was used until d

Table 1. Ingredient composition of basal diets (%, as fed basis).

Ingredient (%)	Starter phase (0−21 d)[b]	Grower phase (22−42 d)
Corn	41.38	39.91
Soybean meal (48% CP)	25.32	22.82
Wheat	20.00	20.00
Soybean oil	3.14	4.97
Corn distillers dried grains with solubles (25% CP)	3.00	3.00
Rapeseed meal (39% CP)	2.00	5.00
Dicalcium phosphate	1.63	1.34
Limestone	1.41	1.18
Baking soda	0.54	0.51
NaCl	0.03	0.08
L-lysine-HCl (78.8%)	0.42	0.28
DL-methionine (99%)	0.33	0.25
L-threonine (98.5%)	0.21	0.12
Choline chloride (60%)	0.09	0.04
Premix [a]	0.50	0.50
Total	100.00	100.00

[a]Premix provided per kg of diet: vitamin A, 9,000 IU; vitamin D_3, 3,000 IU; vitamin E, 24 mg; vitamin K_3, 1.8 mg; vitamin B_1, 2.0 mg; riboflavin, 5 mg; vitamin B_6, 3.0 mg; vitamin B_{12}, 0.1 mg; nicotinic acid, 40 mg; calcium pantothenate, 15 mg; folic acid, 1 mg; biotin, 0.05 mg; iron, 80 mg; copper, 20 mg; zinc, 90 mg; manganese, 80 mg; iodine, 0.35 mg; selenium, 0.3 mg.

[b]The experimental diets were supplemented with 0, 500, 1,000, 1,500, or 2,000 mg/kg Ecobiol in the basal diet for starter or grower phase of broiler, respectively. Ecobiol was added to the diets at the expense of limestone.

Table 2. Analyzed nutrient level and *Bacillus amyloliquefaciens* CECT 5940 spores counts of experimental diets (%, as fed basis).

[a]	Ecobiol (mg/kg)				
Item	0	500	1,000	1,500	2,000
Starter diets (0−21 d)					
Metabolizable energy (MJ/kg)	12.76	12.76	12.76	12.76	12.76
Crude protein	21.36	20.81	20.51	21.12	20.69
Calcium	0.97	0.93	0.95	0.95	0.95
Total phosphorus	0.71	0.72	0.72	0.72	0.73
Non-phytate phosphorus	0.45	0.45	0.45	0.45	0.45
Lysine	1.44	1.33	1.35	1.37	1.41
Methionine+Cysteine	1.18	1.12	1.15	1.17	1.12
Bacillus amyloliquefaciens CECT 5940 spores counts (CFU/g)	<100	5.60×10^5	1.44×10^6	2.68×10^6	2.73×10^6
Grower diets (22−42 d)					
Metabolizable energy (MJ/kg)	13.18	13.18	13.18	13.18	13.18
Crude protein	20.49	20.47	20.52	20.45	20.85
Calcium	0.82	0.87	0.85	0.81	0.84
Total phosphorus	0.65	0.64	0.64	0.63	0.65
Non-phytate phosphorus	0.40	0.40	0.40	0.40	0.40
Lysine	1.26	1.34	1.25	1.27	1.28
Methionine+Cysteine	0.91	0.91	0.97	0.93	0.94
Bacillus amyloliquefaciens CECT 5940 spores counts (CFU/g)	<100	8.50×10^5	1.46×10^6	1.99×10^6	3.30×10^6

[a]Metabolizable energy and non-phytate phosphorus were calculated values using NRC (1994) values; others were analyzed values.

42. All chicks were vaccinated with Newcastle disease vaccine by eye-drop and nose-drop on d 7 and vaccinated with Newcastle disease vaccine or infectious bursal disease vaccine by drinking water on d 14, 21, 28. Body weight (**BW**) and feed intake (**FI**) per replicate were recorded on d 21 and 42 to calculate average daily gain (**ADG**), average daily feed intake (**ADFI**), and feed to gain ratio (**F:G**) of broilers for the periods d 0 to 21, d 22 to 42, and the overall experiment.

Sample Collection

On d 21 and d 42, eight birds (1 bird per replicate, n = 8 per group) close to the average weight were selected for blood sampling from the wing vein of broilers by wing vein puncture with disposable blood collecting needle. The blood samples were centrifuged at 3,000 *g* for 15 min, and serums were stored at −20°Cfor further study. After that, the selected birds were killed by severing the jugular vein, and the samples of liver, mucosa, and digesta in the mid of jejunum and ileum were collected immediately on d 42. All samples were put in liquid nitrogen immediately for quick freezing and then stored at −20°C for further analysis.

Sample Analysis

Diets were analyzed for crude protein (Method 990.03), calcium and total phosphorus (Method 985.01), methionine, cysteine, and lysine (Method 982.30) according to the methods of the Association of Official Analytical Chemists (AOAC, 2005). The spore count of BA-5940 in each diet was determined using the spread plate method. The frozen liver and mucosa were homogenized with 0.9% physiological saline, and then the homogenate was centrifuged at 5,000 *g* for 15 min, and the supernatant was collected. The total antioxidant capacity (**T-AOC**), superoxide dismutase (**SOD**) and glutathione peroxidase (**GSH-Px**) activities, and malondialdehyde (**MDA**) levels in serum and liver were measured with detection kits of Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's protocol. The immunoglobulin A (**IgA**), immunoglobulin G (**IgG**), and immunoglobulin M (**IgM**) concentrations in serum, and the activities of amylase, lipase, trypsin, and chymotrypsin in digesta of jejunum and ileum were also measured with detection kits of Nanjing Jiancheng Bioengineering Institute. The interleukin-10 (**IL-10**), interleukin-6 (**IL-6**), interleukin-1β (**IL-1β**), and tumor necrosis factor-α (**TNF-α**) levels in serum and mucosa of jejunum and ileum were measured with detection kits of Beijing Kang Jia Hong Yuan Biological Technology Co. Ltd., (Beijing, China) according to the manufacturer's protocol.

Statistical Analysis

The experimental unit for performance data was the cage while it was 1 bird per cage for the other response measurements taken. The linear and quadratic effects of the BA-5940 levels were evaluated using orthogonal polynomial contrasts performed by SAS 9.2 (2009). A quadratic regression fitting curve model was performed using GraphPad Prism 7 to evaluate the optimal addition level of Ecobiol in the diets. The difference was deemed significant when the *P*-value was less than 0.05.

RESULTS

Growth Performance

Table 3 showed that ADG and F:G of broilers showed a quadratic response ($P < 0.05$) to the increasing of dietary BA-5940 supplementation level during d 22−42 and d 0 −42. Whereas ADFI was not affected by dietary BA-5940 level. According to quadratic fit model of dietary Ecobiol supplementation level and ADG (y = -1.891×10^{-6}

Table 3. Effects of dietary Ecobiol supplementation on growth performance of broilers[1].

Item[2]	Ecobiol (mg/kg)					SEM	P value	
	0	500	1,000	1,500	2,000		Linear	Quadratic
0–21 d								
ADG (g/d)	37.08	36.41	37.26	37.33	36.04	0.42	0.393	0.215
ADFI (g/d)	47.56	46.43	47.06	47.66	46.50	0.56	0.619	0.964
F:G (g/g)	1.28	1.28	1.26	1.28	1.29	0.01	0.713	0.231
22–42 d								
ADG (g/d)	78.41	80.73	82.29	81.14	79.20	1.39	0.656	0.038
ADFI (g/d)	132.56	134.59	132.79	134.37	132.25	1.86	0.886	0.484
F:G (g/g)	1.69	1.67	1.62	1.66	1.67	0.02	0.405	0.039
0–42 d								
ADG (g/d)	57.75	58.57	59.78	59.23	57.62	0.72	0.860	0.019
ADFI (g/d)	89.24	89.52	89.75	90.10	88.53	0.88	0.761	0.285
F:G (g/g)	1.55	1.53	1.50	1.52	1.54	0.01	0.514	0.021

[1]Data are the means of 8 replicates per treatment with 18 birds per replicate.
[2]Abbreviations: ADG, average daily gain; ADFI, average daily feed intake; F:G, feed to gain ratio.

$x^2 + 3.863 \times 10^{-3} x + 57.56$, $R^2 = 0.91$) or F:G ($y = 3.202 \times 10^{-8} x^2 - 6.919 \times 10^{-5} x + 1.548$, $R^2 = 0.85$) from d 0 to 42, the best dietary inclusion level of Ecobiol is 1,020 mg/kg and 1,080 mg/kg, and the optimal inclusion level of Ecobiol range is 800.41−1,242.43 mg/kg and 913.97−1,246.86 mg/kg, respectively.

Antioxidant Capacity

As shown in Table 4, dietary BA-5940 supplementation increased T-AOC (linear, $P < 0.01$) in serum of broilers on d 21, but there were no effects on serum GSH-Px and SOD activities, and MDA level ($P > 0.05$). As dietary BA-5940 supplemental level increased, the activities of GSH-Px and SOD in serum and liver, and T-AOC in liver increased linearly ($P < 0.05$), while MDA level in serum decreased linearly ($P < 0.05$) on d 42.

Serum Immunoglobulins

The effects of dietary BA-5940 on serum immunoglobulin parameters of broilers are shown in Table 5. With the increasing of dietary BA-5940 supplementation level, serum concentrations of IgA and IgM on d 21 as well as IgM on d 42 increased linearly ($P < 0.05$). However, there was no significant difference in the levels of IgG among all treatments ($P > 0.05$) on both d 21 and 42.

Serum and Intestinal Mucosa Cytokines

Effects of dietary Ecobiol supplementation on cytokines level of serum and intestinal mucosa in broilers are shown in Table 6. The IL-6 (linear, $P < 0.01$) and TNF-α (linear, $P < 0.05$) levels in serum were decreased, while IL-10 level was increased (linear, $P < 0.01$) on d 21 with increasing levels of BA-5940. Dietary BA-5940 supplementation reduced the contents of serum IL-1β (linear, $P < 0.01$) and TNF-α (linear, $P < 0.01$) on d 42; IL-1β and IL-10 levels showed a quadratic response ($P < 0.05$) as the level of BA-5940 inclusion increased. Additionally, the levels of IL-1β, IL-6, and TNF-α in the mucosa of jejunum and ileum were decreased (linear, $P < 0.05$) as dietary supplementation of BA-5940 increased. However, the contents of IL-10 in the mucosa of jejunum and ileum were not affected ($P > 0.05$) by BA-5940 levels.

Table 4. Effects of dietary Ecobiol supplementation on antioxidant capacity of broilers[1].

Item [2]	Ecobiol (mg/kg)					SEM	P value	
	0	500	1,000	1,500	2,000		Linear	Quadratic
D 21								
Serum								
GSH-Px (U/mL)	484.95	495.15	485.68	505.34	518.81	32.77	0.457	0.773
SOD (U/mL)	129.09	134.30	136.44	137.95	134.83	4.10	0.251	0.267
T-AOC (mmol/L)	0.28	0.30	0.34	0.32	0.31	0.01	0.005	0.002
MDA (nmol/mL)	4.36	4.18	3.99	3.80	4.13	0.29	0.377	0.362
D 42								
Serum								
GSH-Px (U/mL)	515.39	530.91	532.76	561.58	598.89	26.70	0.025	0.485
SOD (U/mL)	128.16	133.86	130.54	139.94	143.20	4.84	0.024	0.668
T-AOC (mmol/L)	0.30	0.29	0.34	0.30	0.32	0.01	0.280	0.368
MDA (nmol/mL)	3.69	3.64	3.15	2.55	2.88	0.39	0.036	0.661
Liver								
GSH-Px (U/mg)	220.28	194.43	280.80	441.15	415.47	33.26	<0.001	0.554
SOD (U/mg)	185.21	202.61	205.21	297.70	226.06	19.31	0.007	0.231
T-AOC (mmol /mg)	71.11	67.60	80.75	90.67	93.84	6.11	0.001	0.661
MDA (nmol/mg)	2.08	1.91	1.94	1.61	1.93	0.17	0.273	0.337

[1]Data are the means of 8 replicates per treatment.
[2]Abbreviations: GSH-Px, glutathione peroxidase; MDA, malonaldehyde; SOD, superoxide dismutase; T-AOC, total antioxidant capacity.

Table 5. Effects of dietary Ecobiol supplementation on serum immunoglobulin content of broilers (g/L)[1].

Item[2]	Ecobiol (mg/kg) 0	500	1,000	1,500	2,000	SEM	*P* value Linear	Quadratic
D 21								
IgG	7.08	7.95	7.05	7.88	7.71	0.38	0.325	0.803
IgA	0.85	0.95	0.92	1.16	1.02	0.05	0.003	0.275
IgM	0.76	0.75	0.82	0.85	0.84	0.04	0.019	0.762
D 42								
IgG	8.03	8.10	8.57	7.67	8.64	0.47	0.597	0.805
IgA	0.91	1.07	1.02	0.97	1.08	0.05	0.100	0.514
IgM	0.72	0.75	0.92	0.85	0.90	0.04	<0.001	0.163

[1]Data are the means of 8 replicates per treatment.
[2]Abbreviations: IgG, immunoglobulin G; IgA, immunoglobulin A; IgM, immunoglobulin M.

Intestinal Digestive Enzyme

The effects of dietary Ecobiol supplementation on the digestive enzyme activities of broilers on d 42 are presented in Table 7. With the increasing of dietary BA-5940 supplementation level, the activities of amylase (linear, $P < 0.01$), lipase (linear, $P < 0.05$) and chymotrypsin (linear, $P < 0.01$) in jejunal digesta, and lipase (linear, $P < 0.05$) in ileal digesta were increased. In addition, the activities of amylase in jejunal digesta, and trypsin in ileal digesta showed a quadratic response ($P < 0.05$) to increasing levels of BA-5940. However, there were no linear or quadratic effects of BA-5940 on the activities of trypsin in digesta of jejunum, and amylase and chymotrypsin in digesta of ileum ($P > 0.05$).

DISCUSSION

Growth Performance

Studies have shown that dietary supplementation with BA exhibited a positive effect on the growth performance of broilers via regulating intestinal and immune system development, secreting multiple enzymes, and increasing feeding frequency (Abdel-Azeem, 2013; Wei et al., 2017; Du et al., 2018a). However, various studies reported different effects of BA on growth performance of broilers. An et al. (2008) reported that dietary supplementation with 500 to 2,000 mg/kg BA-KU801 (1.3×10^9 CFU/g) did not affect ADFI and F:G of Ross broilers fed corn-soybean diets. While dietary supplementation with 1,000-2,000 mg/kg BA-KB3 improved ADG, ADFI, and F:G of Ross 308 broilers (Ahmed et al., 2014), and 30 to 60 mg/kg BA (2.5×10^9 CFU/g) improved ADG and F:G of AA broilers (Lei et al., 2015) fed a corn-soybean meal basal diet. Shini et al. (2020) reported that dietary addition of BA-H57 (2.48×10^8 CFU/g feed) increased ADG and improved F:G of Ross 308 broilers challenged with *Clostridium perfringens* fed a wheat-soybean diet. Previous studies on BA-5940 have shown that the addition of BA-5940 (1.0×10^6 CFU/g of feed) improved F:G of Cobb 500 broilers challenged with *Eimeria* and *Clostridium perfringens* fed a corn-soybean meal diet (De Oliveira et al., 2019), and improved BWG, FI, and F:G of Ross 308 broilers fed wheat-soybean meal diets under necrotic enteritis challenge (Gharib-Naseri et al., 2021).

Table 6. Effects of dietary Ecobiol supplementation on cytokines level of broilers[1].

Item[2]	Ecobiol (mg/kg) 0	500	1,000	1,500	2,000	SEM	*P* value Linear	Quadratic
D 21								
Serum (pg/mL)								
IL-1β	31.45	30.65	29.75	28.44	30.22	0.86	0.094	0.150
IL-6	61.92	53.73	52.17	51.59	52.96	2.08	0.004	0.014
TNF-α	108.60	108.85	92.32	96.17	100.29	3.83	0.021	0.058
IL-10	31.96	36.99	41.82	38.69	41.13	1.89	0.002	0.072
D 42								
Serum (pg/mL)								
IL-1β	33.18	30.45	27.35	27.47	28.79	1.23	0.005	0.019
IL-6	62.91	61.84	61.89	55.15	60.98	1.80	0.072	0.306
TNF-α	111.38	104.50	104.66	89.93	99.38	4.02	0.005	0.245
IL-10	29.60	34.15	34.71	35.71	33.44	1.54	0.067	0.028
Jejunum (pg/mg)								
IL-10	21.37	24.89	24.70	20.24	23.24	1.67	0.864	0.403
IL-1β	32.74	23.17	25.03	23.43	26.24	1.22	0.002	<0.001
IL-6	129.73	107.02	93.69	104.31	109.88	5.04	0.012	<0.001
TNF-α	144.30	128.30	131.73	117.44	132.79	5.23	0.048	0.028
Ileum (pg/mg)								
IL-10	22.86	21.07	24.20	25.49	21.73	1.55	0.662	0.327
IL-1β	42.92	31.68	25.43	28.98	28.88	1.93	<0.001	<0.001
IL-6	154.22	131.38	118.78	104.93	118.32	5.87	<0.001	0.003
TNF-α	162.63	146.53	133.52	127.31	115.68	6.37	<0.001	0.514

[1]Data are the means of 8 replicates per treatment.
[2]Abbreviations: IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IL-10, interleukin-10.

Table 7. Effects of dietary Ecobiol supplementation on digestive enzyme activity of broilers on d 42[1].

Item	Ecobiol (mg/kg)					SEM	*P* value	
	0	500	1,000	1,500	2,000		Linear	Quadratic
Jejunum								
Amylase (U/mg protein)	3.25	3.97	4.37	4.74	4.59	0.17	<0.001	0.008
Lipase (U/g protein)	23.30	23.85	49.88	45.34	36.48	6.92	0.036	0.065
Chymotrypsin (U/mg protein)	9.02	12.66	12.79	13.28	14.67	1.18	0.003	0.351
Trypsin (U/mg protein)	5,457.52	7,097.37	6,339.53	5,311.48	6,449.46	730.47	0.932	0.644
Ileum								
Amylase (U/mg protein)	3.71	3.60	4.21	4.85	4.20	0.38	0.075	0.469
Lipase (U/g protein)	22.62	21.02	21.54	26.07	32.71	3.69	0.032	0.165
Chymotrypsin (U/mg protein)	6.49	5.57	4.14	5.10	6.48	1.18	0.896	0.123
Trypsin (U/mg protein)	3,156.53	5,502.37	5,311.72	4,794.89	4,405.85	582.32	0.337	0.012

[1]Data are the means of 8 replicates per treatment.

Under commercial production conditions, the present study showed that BA-5940 supplementation improved ADG and F:G of AA broilers fed a corn-wheat-soybean meal diet, which confirmed that ADG and F:G can be improved by BA-5940 supplementation even in unchallenged conditions at different types of diets. These disparities results among studies may be attributable to multiple factors, including bacterial strains and dose, diet composition, and experimental bird species and health status. Besides, according to the quadratic regression curve of ADG and F:G, the optimal inclusion level of Ecobiol range is 900 to 1,250 mg/kg. Based on the above results, dietary inclusion of $1.1-1.6 \times 10^9$ CFU/kg diet BA-5940 to broilers is recommended.

Antioxidant Status

The SOD and GSH-Px are important antioxidant enzymes that can scavenge free radicals and relieve oxidative damage, while MDA is the indicator of lipid peroxidation used as a key indicator of oxidative stress (Zhou et al., 2020a). An in vitro study found that the extracellular polysaccharides isolated from BA decreased MDA concentration and improved SOD activity in HepG2 cells to relieve oxidative stress induced by H_2O_2 (Yang et al., 2015). Previous studies also reported that dietary inclusion of BA improved SOD and GSH-Px activities and T-AOC, decreased MDA levels of broilers or piglets (Li et al., 2015; Wang et al., 2017; Wang et al., 2018). The results of the present study showed that dietary BA-5940 supplementation effectively improved the antioxidant ability, and relieve lipid peroxidation of broilers by improving the activities of SOD and GSH-Px, and reducing MDA levels. The possible mechanisms are as follows: BA treatment might activate the Nrf2/Keap1 signaling pathway which further increases the expression of the antioxidant enzyme gene and activity (Wang et al., 2019), and the antioxidant metabolites produced by BA also exhibited strong antioxidant potential, such as antioxidant peptide (Rahman et al., 2018).

Immunity

Serum Ig plays a crucial role in immune function to prevent various infections in broilers (Bai et al., 2016). It has been shown that the inclusion of BA in diet improved immunity of broilers or mice through increasing serum IgA, IgG, or IgM levels (Ahmed et al., 2014; Cao et al., 2019). In this study, dietary BA-5940 supplements improved serum IgA and serum IgM levels of broilers, which are similar to the above findings. These results indicate that dietary BA enhanced humoral immunity of broilers by elevating immunoglobulins concentration, which may be due to that BA treatment improved the activity and number of T cells and B cells which enhanced the synthesis of Ig (Jiang et al., 2004).

Excessive production of proinflammatory cytokines IL-1β, IL-6, and TNF-α can aggravate inflammation, whereas anti-inflammatory cytokines play a key role in relieving inflammation, such as IL-10 (Li et al., 2005). Previous studies showed BA treatment reduced the levels of IL-1β, IL-6, and TNF-α, while increased the contents of IL-10 in serum, jejunum, or liver of laying hens and piglets (Du et al., 2018b; Zhou et al., 2020b). Our study showed that adding BA-5940 to diets enhances the immune system and relieve inflammation of broilers by improving IL-10 level in serum, and reducing the levels of IL-1β, IL-6, and TNF-α in serum and small intestine, which is similar to the results reported by Li et al. (2018) in piglets. This may be due to that BA consumes a lot of free oxygen to multiply in the intestines, which improves the growth and multiply of anaerobic probiotics such as *lactic acid bacteria* and *bifidobacteria*, and inhibits pathogens colonization (Lei et al., 2015; Tang et al., 2018; Neveling and Dicks, 2021). Further studies are needed to determine the number of bacteria or using 16S rRNA sequencing assay to support this speculation. BA can also produce surfactins, and bacteriocins with bactericidal activity such as subtilisin to inhibit inflammation of the gut (Lisboa et al., 2006; Li et al., 2018). Additionally, the inclusion of probiotics may initiate both immune and intestinal development, thereby preventing leaky gut issues that may initiate inflammatory processes (Ahmadi et al., 2020). The specific mechanism by which BA-5940 reduced proinflammatory markers in this study remains to be elucidated.

Digestive Enzyme Activity

The intestinal digestion and absorption of nutrients are closely related to digestive enzyme activity

(Saeed et al., 2019). A study in vitro showed that BA could produce various digestive enzymes, such as xylanase, β-glucanase, and amylase (Farhat-Khemakhem et al., 2018). The BA can also improve the digestive enzyme activity of intestinal tract chyme after being ingested by animals. Research on pigs indicates that dietary BA supplementation increased the activities of trypsin, amylase, and lipase in the chyme of duodenum and jejunum of growing-finishing pigs and piglets (Hu et al., 2018; Cao et al., 2020). However, reports on the effect of BA on the digestive enzyme activity of broilers are very limited. Wang (2015) reported that dietary BA could improve the activities of trypsin, amylase, and lipase in the duodenal chyme of broilers. Furthermore, dietary supplementation with BA increased the digestibility of amino acid, crude protein, and dry matter of broilers (Lei et al., 2015; Gharib-Naseri et al., 2021), which may be due to that BA improved digestive enzyme activities. In the current study, supplementation with BA-5940 improved the activities of amylase, lipase, and chymotrypsin in the jejunum of broilers. Therefore, it could be assumed that the digestion and utilization of nutrients in diets could be increased then the growth performance is improved by supplementation BA. The increase of digestive enzyme activities may be due to the secretion of BA, and the stimulation of endogenous enzyme synthesis by BA in the intestines (Hu et al., 2018; Cao et al., 2020). Additionally, dietary BA also may improve enzyme levels by perfecting intestinal morphology and integrity (Lei et al., 2015).

CONCLUSIONS

In conclusion, adding *Bacillus amyloliquefaciens CECT 5940* to the corn-wheat-soybean meal diet in this study improved the growth performance of broilers which may be the result of improving antioxidant capacity, immunity, and intestinal digestive enzyme activities of broilers. Dietary inclusion of $1.1-1.6 \times 10^9$ CFU/kg diet to broilers is recommended.

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DISCLOSURES

No conflicts of interest.

REFERENCES

Abdel-Azeem, M. 2013. Do probiotics affect the behavior of turkey poults? J. Vet. Med. Anim. Health. 5:144–148.

Ahmadi, S., S. Wang, R. Nagpal, B. Wang, S. Jain, A. Razazan, S. P. Mishra, X. Zhu, Z. Wang, K. Kavanagh, and H. Yadav. 2020. A human-origin probiotic cocktail ameliorates aging-related leaky gut and inflammation via modulating the microbiota/taurine/tight junction axis. JCI Insight 5:e132055.

Ahmed, S. T., M. M. Islam, H. S. Mun, H. J. Sim, Y. J. Kim, and C. J. Yang. 2014. Effects of *Bacillus amyloliquefaciens* as a probiotic strain on growth performance, cecal microflora, and fecal noxious gas emissions of broiler chickens. Poult. Sci. 93:1963–1971.

Al-Khalaifah, H. 2018. Benefits of probiotics and/or prebiotics for antibiotic-reduced poultry. Poult. Sci. 97:3807–3815.

An, B. K., B. L. Cho, S. J. You, H. D. Paik, H. I. Chang, S. W. Kim, C. W. Yun, and C. W. Kang. 2008. Growth performance and antibody response of broiler chicks fed yeast derived β-glucan and single-strain probiotics. Asian Austral. J. Anim. 21:1027–1032.

Association of Official Analytical Chemists (AOAC). 2005. Official Methods of Analysis. 18th ed AOAC, Washington DC.

Bai, K., Q. Huang, J. Zhang, J. He, L. Zhang, and T. Wang. 2016. Supplemental effects of probiotic *Bacillus subtilis* fmbJ on growth performance, antioxidant capacity, and meat quality of broiler chickens. Poult. Sci. 96:74–82.

Cao, G., K. Wang, Z. Li, F. Tao, Y. Xu, J. Lan, G. Chen, and C. Yang. 2019. *Bacillus amyloliquefaciens* ameliorates dextran sulfate sodium-Induced colitis by improving gut microbial dysbiosis in mice model. Front. Microbiol. 9:3260.

Cao, X., L. Tang, Z. Zeng, B. Wang, Y. Zhou, Q. Wang, P. Zou, and W. Li. 2020. Effects of probiotics *Basc06* on intestinal digestion and absorption, antioxidant capacity, microbiota composition, and macrophage polarization in pigs for fattening. Front. Vet. Sci. 7:570593.

Cutting, S. M. 2011. *Bacillus* probiotics. Food microbiol 28:214–220.

De Oliveira, M. J. K., N. K. Sakomura, J. C. de Paula Dorigam, K. Doranalli, L. Soares, and G. da Silva Viana. 2019. *Bacillus amyloliquefaciens* CECT 5940 alone or in combination with antibiotic growth promoters improves performance in broilers under enteric pathogen challenge. Poult. Sci. 98:4391–4400.

Du, R., S. Jiao, Y. Dai, J. An, J. Lv, X. Yan, J. Wang, and B. Han. 2018a. Probiotic *Bacillus amyloliquefaciens* C-1 improves growth performance, stimulates GH/IGF-1, and regulates the gut microbiota of growth-retarded beef calves. Front. Microbiol. 9:2006.

Du, W., H. Xu, X. Mei, X. Cao, L. Gong, Y. Wu, Y. Li, D. Yu, S. Liu, and Y. Wang. 2018b. Probiotic *Bacillus* enhance the intestinal epithelial cell barrier and immune function of piglets. Benef. Microbes 9:743–754.

Elshaghabee, F. M., N. Rokana, R. D. Gulhane, C. Sharma, and H. Panwar. 2017. *Bacillus* as potential probiotics: status, concerns, and future perspectives. Front. Microbiol. 8:1490.

European Food Safety Authority (EFSA). 2008. Safety and efficacy of Ecobiol® (*Bacillus amyloliquefaciens*) as feed additive for chickens for fattening. EFSA J 6:773.

Farhat-Khemakhem, A., M. Blibech, I. Boukhris, M. Makni, and H. Chouayekh. 2018. Assessment of the potential of the multienzyme producer *Bacillus amyloliquefaciens* US573 as alternative feed additive. J. Sci. Food Agric. 98:1208–1215.

Gharib-Naseri, K., J. C. de Paula Dorigam, K. Doranalli, S. Kheravii, R. A. Swick, M. Choct, and S. B. Wu. 2020. Modulations of genes related to gut integrity, apoptosis, and immunity underlie the beneficial effects of *Bacillus amyloliquefaciens* CECT 5940 in broilers fed diets with different protein levels in a necrotic enteritis challenge model. J. Anim. Sci. Biotechnol. 11:104.

Gharib-Naseri, K., J. C. P. Dorigam, K. Doranalli, N. Morgan, R. A. Swick, M. Choct, and S. B. Wu. 2021. *Bacillus amyloliquefaciens* CECT 5940 improves performance and gut function in broilers fed different levels of protein and/or under necrotic enteritis challenge. Anim. Nutr. 7:185–197.

Hong, Y., Y. Cheng, L. Guan, Z. Zhou, X. Li, D. Shi, and Y. Xiao. 2021. *Bacillus amyloliquefaciens* TL downregulates the ileal expression of genes involved in immune responses in broiler chickens to improve growth performance. Microorganisms 9:382.

Hu, S., X. Cao, Y. Wu, X. Mei, H. Xu, Y. Wang, X. Zhang, L. Gong, and W. Li. 2018. Effects of probiotic *Bacillus* as an alternative of antibiotics on digestive enzymes activity and intestinal integrity of piglets. Front. Microbiol. 9:2427.

Humam, A. M., T. C. Loh, H. L. Foo, W. I. Izuddin, E. A. Awad, Z. Idrus, A. A. Samsudin, and N. M. Mustapha. 2020. Dietary supplementation of postbiotics mitigates adverse impacts of heat stress on antioxidant enzyme activity, total antioxidant, lipid peroxidation, physiological stress indicators, lipid profile and meat quality in broilers. Animals 10:982.

Jerzsele, A., K. Szeker, R. Csizinszky, E. Gere, C. Jakab, J. Mallo, and P. Galfi. 2012. Efficacy of protected sodium butyrate, a protected blend of essential oils, their combination, and *Bacillus amyloliquefaciens* spore suspension against artificially induced necrotic enteritis in broilers. Poult. Sci. 91:837–843.

Jiang, H. Q., M. Thurnheer, A. Zuercher, N. Boiko, N. Bos, and J. Cebra. 2004. Interactions of commensal gut microbes with subsets of B-and T-cells in the murine host. Vaccine 22:805–811.

Lei, X., X. Piao, Y. Ru, H. Zhang, A. Péron, and H. Zhang. 2015. Effect of *Bacillus amyloliquefaciens*-based direct-fed microbial on performance, nutrient utilization, intestinal morphology and cecal microflora in broiler chickens. Asian Austral. J. Anim. 28:239–246.

Li, C.l., J. Wang, H. J. Zhang, S. G. Wu, Q. R. Hui, C. B. Yang, R. J. Fang, and G. H. Qi. 2019. Intestinal morphologic and microbiota responses to dietary Bacillus spp. in a broiler chicken model. Front. Physiol. 9:1968.

Li, J. J., Y. L. Guo, and Y. J. Yang. 2005. Enhancing anti-inflammatory cytokine IL-10 may be beneficial for acute coronary syndrome. Med. Hypotheses 65:103–106.

Li, Y., H. Zhang, Y. Chen, M. Yang, L. Zhang, Z. Lu, Y. Zhou, and T. Wang. 2015. *Bacillus amyloliquefaciens* supplementation alleviates immunological stress and intestinal damage in lipopolysaccharide-challenged broilers. Anim. Feed Sci. Tech. 208:119–131.

Li, Y., H. Zhang, W. Su, Z. Ying, Y. Chen, L. Zhang, Z. Lu, and T. Wang. 2018. Effects of dietary *Bacillus amyloliquefaciens* supplementation on growth performance, intestinal morphology, inflammatory response, and microbiota of intra-uterine growth retarded weanling piglets. J. Anim. Sci. Biotechno. 9:22.

Lisboa, M. P., D. Bonatto, D. Bizani, J. A. Henriques, and A. Brandelli. 2006. Characterization of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* isolated from the brazilian atlantic forest. Int. Microbiol. 9:111–118.

National Research Council (NRC). 1994. Nutrient Requirement of Poultry. 9th ed. National Academy Press, Washington, DC.

Neveling, D. P., and L. M. Dicks. 2021. Probiotics: an antibiotic replacement strategy for healthy broilers and productive rearing. Probio. Antimicro. 13:1–11.

Ortiz, A., P. Yánez, M. I. Gracia, and J. J. Mallo. 2013. Effect of probiotic Ecobiol on broiler performance. 19th European Symposium on Poultry Nutrition.

Rahman, M. S., Y. H. Choi, Y. S. Choi, M. B. Alam, S. H. Lee, and J. C. Yoo. 2018. A novel antioxidant peptide, purified from *Bacillus amyloliquefaciens*, showed strong antioxidant potential via Nrf-2 mediated heme oxygenase-1 expression. Food Chem 239:502–510.

Saeed, M., T. Ayasan, M. Alghawany, M. Hack, and A. K. Patra. 2019. The role of ß-mannanase (hemicell) in improving poultry productivity, health and environment. Braz. J. Poult. Sci. 21:1–8.

SAS Institute. Inc. 2009. SAS/STAT 9.2 User's Guide. SAS Institute Inc., Cary, NC.

Shini, S., D. Zhang, R. Aland, X. Li, P. Dart, M. Callaghan, R. Speight, and W. Bryden. 2020. Probiotic *Bacillus amyloliquefaciens* H57 ameliorates subclinical necrotic enteritis in broiler chicks by maintaining intestinal mucosal integrity and improving feed efficiency. Poult. Sci. 99:4278–4293.

Tang, R. Y., Z. L. Wu, G. Z. Wang, and W. C. Liu. 2018. The effect of *Bacillus amyloliquefaciens* on productive performance of laying hens. Ital. J. Anim. Sci. 17:436–441.

Wang, B., Y. Zhou, L. Tang, Z. Zeng, L. Gong, Y. Wu, and W. F. Li. 2021. Effects of *Bacillus amyloliquefaciens* Instead of antibiotics on growth performance, intestinal health, and intestinal microbiota of broilers. Front. Vet. Sci. 8:679368.

Wang, S. Q. 2015. Pages 22−23 in study on feeding effects of Bacillus amyloliquefaciens on broilers. MSc thesis, Henan Agr. Univ. Zhengzhou, China.

Wang, Y., Y. Wang, H. Xu, X. Mei, L. Gong, B. Wang, W. Li, and S. Jiang. 2018. Direct-fed glucose oxidase and its combination with *B. amyloliquefaciens* SC06 on growth performance, meat quality, intestinal barrier, antioxidative status, and immunity of yellow-feathered broilers. Poult. Sci. 97:3540–3549.

Wang, Y., Y. Wu, B. Wang, X. Cao, A. Fu, Y. Li, and W. Li. 2017. Effects of probiotic *Bacillus* as a substitute for antibiotics on antioxidant capacity and intestinal autophagy of piglets. AMB Express 7:52.

Wang, Y., Y. Wu, B. Wang, H. Xu, X. Mei, X. Xu, X. Zhang, J. Ni, and W. Li. 2019. *Bacillus amyloliquefaciens* SC06 protects mice against high-fat diet-induced obesity and liver injury via regulating host metabolism and gut microbiota. Front. Microbiol. 10:1161.

Wei, X., X. Liao, J. Cai, Z. Zheng, L. Zhang, T. Shang, Y. Fu, C. Hu, L. Ma, and R. Zhang. 2017. Effects of *Bacillus amyloliquefaciens* LFB112 in the diet on growth of broilers and on the quality and fatty acid composition of broiler meat. Anim. Prod. Sci. 57:1899–1905.

Yang, H., J. Deng, Y. Yuan, D. Fan, Y. Zhang, R. Zhang, and B. Han. 2015. Two novel exopolysaccharides from *Bacillus amyloliquefaciens* C-1: antioxidation and effect on oxidative stress. Curr. Microbiol. 70:298–306.

Zhou, J., X. Ao, Y. Lei, C. Ji, and Q. Ma. 2020a. *Bacillus subtilis* ANSB01G culture alleviates oxidative stress and cell apoptosis induced by dietary zearalenone in first-parity gestation sows. Anim. Nutr. 6:372–378.

Zhou, Y., S. Li, Q. Pang, and Z. Miao. 2020b. *Bacillus amyloliquefaciens* BLCC1-0238 Can effectively improve laying performance and egg quality via enhancing immunity and regulating reproductive hormones of laying hens. Probiotics. Antimicro. 12:246–252.