Effects of dietary supplementation of probiotic, Clostridium butyricum, on growth performance, immune response, intestinal barrier function, and digestive enzyme activity in broiler chickens challenged with Escherichia coli K88

Ling Zhang¹,², Lingling Zhang², Xiu’an Zhan¹, Xinfu Zeng³, Lin Zhou¹, Guangtian Cao¹, An’guo Chen¹ and Caimei Yang²*

Abstract

Background: Colibacillosis caused by enterotoxigenic Escherichia coli (E. coli) results in economic losses in the poultry industry. Antibiotics are usually used to control colibacillosis, however, E. coli has varying degrees of resistance to different antibiotics. Therefore the use of probiotics is becoming accepted as an alternative to antibiotics. In this study, we evaluated the effects of Clostridium butyricum (C. butyricum) on growth performance, immune response, intestinal barrier function, and digestive enzyme activity in broiler chickens challenged with Escherichia coli (E. coli) K88.

Methods: The chickens were randomly divided into four treatment groups for 28 days. Negative control treatment (NC) consisted of birds fed a basal diet without E. coli K88 challenge and positive control treatment (PC) consisted of birds fed a basal diet and challenged with E. coli K88. C. butyricum probiotic treatment (CB) consisted of birds fed a diet containing 2 × 10⁷ cfu C. butyricum/kg of diet and challenged with E. coli K88. Colistin sulfate antibiotic treatment (CS) consisted of birds fed a diet containing 20 mg colistin sulfate/kg of diet and challenged with E. coli K88.

Results: The body weight (BW) and average day gain (ADG) in the broilers of CB group were higher (P < 0.05) than the broilers in the PC group overall except the ADG in the 14-21 d post-challenge. The birds in CB treatment had higher (P < 0.05) concentration of tumor necrosis factor-α (TNF-α) at 3 and 7 d post-challenge, and higher (P < 0.05) concentration of interleukin-4 (IL-4) at 14 d post-challenge than those in the PC treatment group. The concentration of serum endotoxin in CB birds was lower (P < 0.05) at 21 d post-challenge, and the concentrations of serum diamine oxidase in CB birds were lower (P < 0.05) at 14 and 21 d post-challenge than in PC birds. Birds in CB treatment group had higher (P < 0.05) jejunal villi height than those in PC, NC, or CS treatment at 7, 14, and 21 d post-challenge. In comparison to PC birds, the CB birds had lower (P < 0.05) jejunal crypt depth during the whole experiment. The birds in CB or CS treatment group had higher (P < 0.05) activities of amylase and protease at 3, 7, and 14 d post-challenge, and higher (P < 0.05) activity of lipase at 3, 7 d post-challenge than PC birds.

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Conclusions: In all, these results indicate that dietary supplementation with *C. butyricum* promotes immune response, improves intestinal barrier function, and digestive enzyme activities in broiler chickens challenged with *E. coli* K88. There is no significant difference between the *C. butyricum* probiotic treatment and the colistin sulfate antibiotic treatment. Therefore, the *C. butyricum* probiotic may be an alternative to antibiotic for broiler chickens.

Keywords: Broiler chickens, *Clostridium butyricum*, Digestive enzyme activity, *Escherichia coli* K88, Growth performance, Immune response, Intestinal barrier

Background
Colibacillosis caused by enterotoxigenic *Escherichia coli* (*E.coli*) is a serious infection that results in huge economic losses in the poultry industry worldwide [1–4]. Although antibiotics are usually used to control colibacillosis, various reports have demonstrated that pathogenic *E. coli* has varying degrees of resistance to different antibiotics [5, 6]. Additionally, resistance genes extended-spectrum beta-lactamases (ESBL) and/or plasmid-mediated Amp-C beta-lactamases (Amp-C) in commercial *E. coli* may pose a human health hazard. [7] Therefore, there is an urgent need to identify sustainable alternatives to antibiotics for animal production.

The use of probiotics in the poultry industry is quickly becoming accepted as a potential alternative to antibiotics for use as growth-promoters, and in some cases, for control of specific enteric pathogens [8–15].

*Clostridium butyricum* (*C. butyricum*) is a butyric-acid producing Gram-positive anaerobe found in soil and intestines of healthy animals and humans. *C. butyricum* increases the concentrations of n-butyric acid in caecaldigesta of birds [16], and butyric acid is of particular importance because of its nutritional properties for epithelial cells and pathogen inhibitory effects in the gut [17]. *C. butyricum* also survives at low pH and high temperature, which renders it a good feed additive [18]. Previous studies demonstrated that *C. butyricum* promoted growth performance [16, 19–21], balanced intestinal microflora [16, 17, 19, 20], improved intestinal morphology [16, 19], stimulated the immune system [19, 20], improved meat quality and fatty acid profiles [21–23], and influenced the digestive tract [23] in broiler chickens. In addition, *C. butyricum* prevented *E.coli*-induced intestinal disorders through inhibiting *E.coli* viability and mediating *E.coli*-induced apoptosis [24]. However, there are few published reports on the effects of *C. butyricum* on *E.coli*-challenged animals. The present study was conducted to investigate the effects of *C. butyricum* on the immune response, intestinal barrier function, and digestive enzyme activities in broiler chickens challenged with *E. coli* K88.

Methods

Ethics statement
All procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

Birds, diets, and experimental design

Three hundred and sixty 1-d-old male Cobb broiler chickens purchased from a commercial hatch (Charoen Pokphand Group, Haining, China) were randomly assigned to four treatment groups. Negative control treatment (NC) consisted of birds fed a basal diet without challenging with *E. coli* K88. Positive control treatment (PC) consisted of birds fed a basal diet and orally challenged with 0.5 mL *E. coli* K88 (2 × 10⁸ cfu/mL) on d 7. The *C. butyricum* treatment (CB) probiotic group consisted of birds fed a diet containing 2 × 10⁷ cfu *C. butyricum*/kg of diet and orally challenged with 0.5 mL *E. coli* K88 (2 × 10⁸ cfu/mL) on d 7. Colistin sulfate treatment (CS) antibiotic group consisted of birds fed a diet containing 20 mg colistin sulfate/kg of diet and orally challenged with 0.5 mL *E. coli* K88 (2 × 10⁸ cfu/mL) on d 7. Each treatment consisted of 6 replicate pens with 15 birds per pen. Birds in NC treatment were housed in one room, while the birds in other three *E. coli*-challenged treatment groups were housed in another room to prevent cross-contamination. The two rooms were of the same configuration and the previous growth studies revealed no significant contamination room effects.

Chickens were placed in the wire cages and all birds were offered the same antibiotic-free basal diets and provided ad libitum access to water and diet. The nutrient levels of the diets met the NRC (1994) broiler recommendations (Table 1). The temperature was adjusted to 32 °C in the first week and gradually lowered to 25 °C.

The *C. butyricum* strain (HJCB998) was obtained from Zhejiang Huijia Biological Technology Ltd., Anji, China. The probiotic strain was grown anaerobically in a liquid fermentation tank at 37 °C for 48 h, and then the cells were harvested by centrifugation and dried by spray-drying technology. Colistin sulfate was obtained from Zhejiang Qianjiang Biochemical Ltd., Haining, China.
Table 1 The composition and nutrients of basal dietα

| Ingredient                | Content, % | Chemical composition | Content |
|---------------------------|------------|----------------------|---------|
| Corn                      | 55.23      | CP, %                | 20.90   |
| Soybean meal              | 30.67      | ME, Mcal/kg          | 3.00    |
| Wheat shorts              | 4.00       | Calcium, %           | 1.00    |
| Fish mealb               | 3.00       | Total P, %           | 0.65    |
| Soybean oilc              | 2.90       | Available P, %       | 0.45    |
| DL-Methionine             | 0.27       | Methionine + cysteine, % | 0.90     |
| NaCl                      | 0.27       | Lysine, %            | 1.05    |
| Limestone                 | 1.33       | Calcium phosphate    | 1.33    |
| Calcium phosphate         | 1.33       |                      |         |
| Vitamin-mineral premixd   | 1.00       |                      |         |

αNutrient level of the diets was based on NRC (1994)
bCrude protein content is 62.5% and metabolizable energy is 2.79 Mcal/kg
cMetabolizable energy is 8.8 Mcal/kg

Oral challenge

The E. coli K88 strain was obtained from College of Animal Sciences, Zhejiang University (Hangzhou, China) and grown at 37 °C. The birds in different treatments were fed the corresponding diet for the first 6 d. On d 7, birds in PC, CB, and CS treatments were orally fed with 0.5 mL (2 × 10⁸ cfu/mL) E. coli K88 inoculants by using a polyethylene tube attached to a syringe. The birds in NC treatment were administered the same amount of sodium chloride solution as control.

Sample collection

Birds were weighed individually at 3, 7, 14, and 21 d post-challenge to evaluate BW and ADG. Feed consumption and feed-to-gain ratio could not be determined because of an indeterminate amount of feed wastage.

Six birds per treatment (1 bird per pen) were randomly selected for sample collection at 3, 7, 14, and 21 d post-challenge. Blood samples were taken from the wing vein and centrifuged (3,000 x g, 10 min) at 4 °C, and then the serum was harvested and stored at -20 °C until analysis. The birds were then killed by CO₂ inhalation and the jejunal mucosa was weighed out, diluted into 4.5 mL of 0.9 % salt solution, and centrifuged at 6,000× g for 15 min. The homogenate was kept on sterile ice and the supernatant was harvested into 1.5 mL sterile microcentrifuge tubes. The concentrations of interleukin-4 (IL-4) and tumor necrosis factor-α (TNF-α) were respectively measured using IL-4 (1042-09) and TNF-α (1041-09) ELISA kits (GBD Ltd, USA) specific for chicken.

Serum endotoxin and diamine oxidase

The concentrations of serum endotoxin were measured using a limulus amoebocyte lysate (LAL)-based kit (LAL QCL-1000 kit, Lonza, Walkersville, MD). The samples were heated for 10 min at 70 °C. Internal control for recovery calculation was included in the assessment. Standards and samples were incubated for 10 min at 37 °C with LAL and then for another 6 min with colorimetric substrate. The reaction was stopped with 25 % acetic acid and then the absorbance was read at 405 nm. Diamine oxidase (DAO) activity (1 ml) was examined by a spectrophotometric assay. The DAO standard (batch number D7876-250) was purchased from Sigma.

Jejunal morphology analysis

Jejunal segments were flushed with a 0.9 % salt solution, and then fixed with 10 % formaldehyde-phosphate buffer for 48 hours. The formalin-fixed, paraffin-embedded tissues were embedded into Leica EG1160, fixed upon Rotary Microtome (Leica RM2153) and then cut to a thickness of 6 μm. The tissue segments were dehydrated with Leica H11220. Slides were stained with hematoxylin and eosin (H&E; Leica Autostain BRXL) and covered by cover slides. Images were analyzed using software Qwin. Then the 10 longest jejunal villi and lowest jejunal crypts were measured with Olympus AX70 microscope (Olympus Corporation, Tokyo, Japan) and the mean value was calculated. The villus height was measured from the villus-crypt junction to the tip of villus, whereas crypt depth was measured from the root of villus to the lamina propria.

Digestive enzyme activities

Amylase, lipase, and protease were analyzed using the corresponding kit provided by Jiancheng Bioengineering Institute (Nanjing, China). In brief, the jejunal mucosa was transferred into sterilized tubes containing 10 mL PBS (7.4 pH), then ultrasonic treatment was applied for 4 min to dissociate the tissues. The later procedure was accomplished by centrifugation (5,000 x g for 25 min). Then the supernatant was used to determine the enzymatic activities following the manufacturer's instructions.
Table 2 Effects of Clostridium butyricum on growth performance in broilers

| Items | Age of (post-ch) | Experimental treats | Statistics |
|-------|-----------------|---------------------|------------|
|       |                 | NC                  | PC         | CB         | CS         | SEM        | P-value   |
| BW, g | 3d              | 351.71a             | 319.83b    | 342.23a    | 339.95a    | 3.588      | <0.01     |
|       | 7d              | 401.16a             | 354.00b    | 402.71a    | 398.81a    | 4.479      | <0.01     |
|       | 14d             | 747.33ab            | 649.00c    | 774.83a    | 738.16b    | 11.161     | <0.01     |
|       | 21d             | 1283.5a             | 1064.8b    | 1265.8a    | 1275.2a    | 26.491     | <0.01     |
| ADG, g| 3-7d            | 12.36b              | 8.54c      | 15.12a     | 14.71a     | 0.651      | <0.01     |
|       | 7-14d           | 49.45a              | 42.14b     | 53.16a     | 48.47a     | 1.117      | <0.01     |
|       | 14-21d          | 76.60               | 59.40      | 70.13      | 76.72      | 2.831      | 0.089     |
|       | 3-21d           | 51.76a              | 41.38b     | 51.30a     | 51.95a     | 1.365      | <0.01     |

*Means in the same row with different superscript letters differ significantly (P < 0.05)

Table 3 Effects of Clostridium butyricum on jejunal mucosa cytokines in broilers

| Items | Age of (post-ch) | Experimental treats | Statistics |
|-------|-----------------|---------------------|------------|
|       |                 | NC                  | PC         | CB         | CS         | SEM        | P-value   |
| TNF-α, ng/L | 3d              | 53.80a              | 48.88b     | 76.66a     | 65.09ab    | 7.19       | 0.030     |
|        | 7d              | 65.29ab             | 53.88b     | 69.32a     | 61.51b     | 4.72       | 0.040     |
|        | 14d             | 63.39               | 50.22      | 62.92      | 61.23      | 6.88       | 0.220     |
|        | 21d             | 50.56               | 35.28      | 54.46      | 56.92      | 6.92       | 0.110     |
| IL-4, ng/L | 3d              | 52.52               | 50.9       | 68.7       | 67.51      | 5.78       | 0.140     |
|        | 7d              | 68.23a              | 52.16b     | 62.91ab    | 57.03ab    | 3.55       | 0.030     |
|        | 14d             | 70.65a              | 50.46b     | 69.79a     | 59.78ab    | 4.26       | 0.010     |
|        | 21d             | 52.64               | 42.59      | 56.38      | 57.09      | 6.41       | 0.350     |

*Means in the same row with different superscript letters differ significantly (P < 0.05)

Statistical analysis
One-way ANOVA was performed using SPSS 16.0 Software. Mean values of treatment groups were compared using Duncan’s multiple range test with P < 0.05 considered statistically significant.

Results
Birds in the PC (positive control) treatment group had less (P < 0.05) BW than the NC (negative control), CB (Clostridium butyricum), and CS (colistin sulfate) birds from 3 to 21 d post-challenge (Table 2). There were no significant differences among the BW of NC, CB, and CS groups. The birds of CB group had higher ADG than those fed the PC diet during 3-7, 7-14, and 3-21 d post-challenge. No significant differences in BW and ADG were observed among the birds of NC, CB, and CS groups except that the ADG of CB and CS broilers was higher than the broilers of the NC groups in 3-7 d post-challenge.

Birds in CB treatment had higher (P < 0.05) concentration of jejunal mucosa TNF-α than those in NC or PC treatment at 3 d post-challenge, and higher (P < 0.05) concentration of TNF-α than PC birds at 7 d post-challenge (Table 3). There was no significant difference in the concentration of TNF-α between CB and CS birds during the whole experiment. In comparison to PC birds, CB birds had greater (P < 0.05) concentration of jejunal mucosa IL-4 on 14 d post-challenge. No significant differences were observed in the concentration of IL-4 among CB, NC, and CS treatments during the whole experiment.

The E. coli challenge significantly increased the concentration of serum DAO during the entire experimental period. Birds in CB treatment had lower (P < 0.05) concentration of serum DAO than those in PC treatment at 14 and 21 d post-challenge. No significant differences were found in the concentration of serum DAO between CB and CS treatment group during the course of the experiment.

Birds fed CB had higher (P < 0.05) jejunal villi height than PC, NC, or CS birds at 7, 14, and 21 d post-challenge (Table 5). In comparison to the broilers in PC treatment, broilers in CB treatment had lower (P < 0.05) jejunal crypt depth throughout the experiment. Birds fed CS had lower (P < 0.05) jejunal crypt depth compared to PC birds at 7, 14, and 21 d post-challenge.

The E. coli challenge significantly decreased the activity of jejunal mucosa amylase; however, broilers fed with CB or CS had increased (P < 0.05) amylase activity.

The E. coli challenge significantly increased the concentration of serum endotoxin during the experiment. Birds in CB treatment had lower (P < 0.05) concentration of serum endotoxin at 7 d post-challenge compared to PC birds. There were no significant differences in the concentrations of serum endotoxin between CB and CS treatment during the whole experiment. The E. coli challenge significantly increased the concentration of serum DAO during the entire experimental period. Birds in CB treatment had lower (P < 0.05) concentration of serum DAO than those in PC treatment at 14 and 21 d post-challenge. No significant differences were found in the concentration of serum DAO between CB and CS treatment group during the course of the experiment.

Birds fed CB had higher (P < 0.05) jejunal villi height than PC, NC, or CS birds at 7, 14, and 21 d post-challenge (Table 5). In comparison to the broilers in PC treatment, broilers in CB treatment had lower (P < 0.05) jejunal crypt depth throughout the experiment. Birds fed CS had lower (P < 0.05) jejunal crypt depth compared to PC birds at 7, 14, and 21 d post-challenge.

The E. coli challenge significantly decreased the activity of jejunal mucosa amylase; however, broilers fed with CB or CS had increased (P < 0.05) amylase activity.
compared with broilers in the PC treatment group from 3 to 14 d post-challenge (Table 5). No significant differences were found in the activity of lipase between CB and CS treatments during the whole experiment. Compared with PC treatment, the activity of lipase in the CB and CS treatments had higher (P < 0.05) activity of lipase from 3 to 14 d post-challenge. There were no significant differences in the activity of protease among the four treatment groups at 21 d post-challenge. Moreover, there were no significant differences between CB and NC treatments in the activity of lipase at 3 and 7 d post-challenge, but those two treatment groups had higher (P < 0.05) activity of lipase than PC treatment; and no significant differences in the activity of lipase among the four treatments at 14 and 21 d post-challenge.

Discussion

Many reports have showed that probiotics can promote growth performance and improve nutrient utilization efficiency in chickens [6, 25–27], although other studies have also reported that probiotics have no effect on growth performance [28–30]. In contrast, *Clostridium butyricum* is a probiotic that has been shown to improve growth performance and nutrient utilization efficiency in chickens [20, 21, 31] although Zhang et al. reported that *C. butyricum* had no effect on broiler performance [16]. In this study we showed that *C. butyricum* improved the BW and ADG of chickens challenged with *E. coli* K88 compared with broilers in the PC group, and the broilers in the CB group showed no significant differences compared to the CS groups on the BW and ADG overall.

Previous reports had shown that probiotics stimulate the immune response [20, 27, 32–34]. Specifically, *C. butyricum* has been shown as capable of influencing the host immune system by modulating cytokine expression [19, 35–37]. *C. butyricum* could induce the sensitization of the host by increasing pro-inflammatory cytokines such as IL-8, IL-6, and TNF-α, and provide beneficial effects to the host by synthesizing the immunosuppressive cytokines such as IL-10 [19, 36, 37]. IL-4 and TNF-α were also secreted by an intracellular signaling cascade in the immune response to *C. butyricum* [38]. Huang et al. [39] reported that *Bacillus* induced TNF-α in spleens and mesenteric lymph nodes of mice [39]. Lee et al. [30] showed that IL-4 transcripts were increased by *B. subtilis* strains LSSAO1, Bs278, and Avicor in broiler chickens [30]. However, Fujiiwa et al. [28] reported that supplementation with *Bacillus subtilis* var. natto fermented soybean did not affect IL-4 gene expression in spleens in broiler chickens [28]. In the present study, chickens fed with *C. butyricum* had higher concentrations of TNF-α and IL-4. This indicated

### Table 4 Effects of *Clostridium butyricum* on the concentrations of serum LPS and DAO in broilers

| Items         | Age of (post-ch) | Experimental treatments | Statistics |
|---------------|------------------|-------------------------|------------|
| Endotoxin, EU/mL | 3d               | NC 0.460b 0.378a 0.704* 0.734a 0.025 < 0.01 |            |
|               | 7d               | PC 0.455b 0.640a 0.586a 0.578a 0.027 < 0.01 |            |
|               | 14d              | CB 0.327b 0.413* 0.335* 0.347* 0.024 0.070 |            |
|               | 21d              | CS 0.252a 0.380* 0.304* 0.332* 0.023 < 0.01 |            |
| DAO, U/mL     | 3d               | NC 2.559b 8.823a 7.493a 8.056a 0.498 < 0.01 |            |
|               | 7d               | PC 1.570* 8.649* 7.121a 7.496a 0.617 < 0.01 |            |
|               | 14d              | CB 1.250a 6.254a 4.194b 4.201b 0.527 < 0.01 |            |
|               | 21d              | CS 0.819b 3.952a 2.419b 3.060ab 0.424 < 0.01 |            |

*Means in the same row with different superscript letters differ significantly (P < 0.05)

1 Each mean represents 6 birds. NC = birds fed a basal diet without challenged with *E. coli* K88; PC = birds fed a basal diet and challenged with *E. coli* K88.

CB = birds fed a basal diet including 2 × 10⁷ CFU *C. butyricum*/kg of diet and challenged with *E. coli* K88; CS = birds fed a basal diet including 20 mg colistin sulfate/kg of diet and challenged with *E. coli* K88.

2 The days after challenging

### Table 5 Effects of *Clostridium butyricum* on Jejunum morphometry in broilers

| Items         | Age of (post-ch) | Experimental treatments | Statistics |
|---------------|------------------|-------------------------|------------|
| Villi height, m | 3d               | NC 264.35 259.81 275.71 253.01 5.69 < 0.01 |            |
|               | 7d               | PC 267.65bc 287.54a 346.75a 254.41c 7.91 < 0.01 |            |
|               | 14d              | CB 429.41b 433.6b 531.09a 407.26b 9.37 < 0.01 |            |
|               | 21d              | CS 429.41b 433.6b 531.09a 407.26b 9.37 < 0.01 |            |
| Crypt depth, m | 3d               | NC 50.22a 47.01ab 33.68c 42.9b 2.08 < 0.01 |            |
|               | 7d               | PC 50.06b 60.3a 39.44c 38.2c 2.17 < 0.01 |            |
|               | 14d              | CB 66.07b 73.96a 56.51c 59.95c 1.87 < 0.01 |            |
|               | 21d              | CS 82.5b 115.46a 84.04b 76.03b 3.29 < 0.01 |            |

*Means in the same row with different superscript letters differ significantly (P < 0.05)

1 Each mean represents 6 birds. NC = birds fed a basal diet without challenged with *E. coli* K88; PC = birds fed a basal diet and challenged with *E. coli* K88.

CB = birds fed a basal diet including 2 × 10⁷ CFU *C. butyricum*/kg of diet and challenged with *E. coli* K88; CS = birds fed a basal diet including 20 mg colistin sulfate/kg of diet and challenged with *E. coli* K88.

2 The days after challenging
that *C. butyricum* influenced the immune response in broiler chickens challenged with *E. coli* K88.

Lipopolysaccharide (LPS) is an integral component of the outer cell membranes of Gram-negative bacteria which are shed from the bacteria when cell lysis occurs [40]. *E. coli* K88 produces LPS [41], which induced endotoxic shock by triggering the systemic inflammatory response [42–45]. The endotoxins from LPS induce a degenerative morphology and the destruction of lymphocytes in birds [46]. Moreover, endotoxin is associated with intestinal permeability [47–49]. When gut permeability is increased, the endotoxin will translocate from the gut into circulation. Ait-Belgnaoui et al. [48] reported that *Lactobacillus farcininis* treatment prevented stress-induced peripheral endotoxin in rats [48]. DAO is localized mainly in the small intestinal mucosa, particularly near the tips of villi and reflects small intestinal integrity and maturity [50–52]. Intestinal mucosal damage causes leakage of DAO from small intestinal villus tips into the circulation, so DAO is an index of intestinal mucosal barriers [52–54]. Zhang et al [35] reported that the serum level of DAO in allergic mice was markedly higher than that in control mice [35]. Synbiotic therapy prevented HR-related decrease of intestinal integrity that was indicated by the reduction in serum DAO activity [55] and returned serum DAO activities to normal levels after hepatectomy in humans [56]. Zhou et al. reported that *Lactobacillus plantarum* significantly lowered the plasma DAO activities in the bile duct ligation rat model [57]. Sun et al [58] reported that probiotics relatively decreased the levels of LPS and DAO in rats compared with the cardiopulmonary bypass (CPB)-operated ones [58]. In this study, dietary supplementation of *C. butyricum* decreased serum endotoxin and DAO in *E. coli* K88-challenged birds. Our results indicated that CB benefits the intestinal barrier function in broiler chickens challenged with *E. coli* K88.

The length of villi and the depth of crypt are the important morphological parameters, and are considered as indicators of optimal intestinal functions. The previous studies showed that dietary supplementation with probiotics increased the villus height and villus height: crypt depth ratio, decreased the crypt depth in broiler chickens [59–64]. Our previous research also showed that birds fed *C. butyricum* had higher ileal villus height and lower ileal crypt depth [31]. The present study showed that dietary supplementation with CB increased the jejunal villus height and decreased the crypt depth in broiler chickens challenged with *E. coli* K88. The current result demonstrated that *C. butyricum* improved the structure and function of intestinal mucosa in *E. coli* infected condition.

Amylase, lipase, and protease play very important roles in the digestion of nutrient materials. Reports on the efficacy of probiotics on the digestive enzymes have been varied. Rajput et al. [34, 65] reported that *Saccharomyces boulardii* supplementation increased the activity of lipase, but had no significant improvement in amylase and trypsin in the jejunum of broiler chickens [65]. Wang and Gu [26] reported that the probiotic *Bacillus coagulans* NJ0516 increased the activities of protease and amylase but had no effect on the activities of lipase in

| Table 6 Effects of *Clostridium butyricum* on digestive enzyme activities in broilers | Items | Age of (day post-ch) | Experimental treats | Statistics |
|---|---|---|---|---|
| Amylase, U/mgprot | 3d | NC | 0.94a | 0.058 <0.01 |
| | | PC | 0.33c |
| | | CB | 0.70b |
| | | CS | 0.74ab |
| | 7d | NC | 0.96a |
| | | PC | 0.45b |
| | | CB | 0.88a |
| | | CS | 0.83a |
| | 14d | NC | 0.80a |
| | | PC | 0.60b |
| | | CB | 0.87a |
| | | CS | 0.78a |
| | 21d | NC | 0.91a |
| | | PC | 0.75b |
| | | CB | 0.79ab |
| | | CS | 0.85ab |
| Protease, U/mgprot | 3d | NC | 106.86a |
| | | PC | 54.66c |
| | | CB | 76.92b |
| | | CS | 80.10b |
| | 7d | NC | 103.76a |
| | | PC | 65.29b |
| | | CB | 93.32a |
| | | CS | 89.39a |
| | 14d | NC | 98.17a |
| | | PC | 67.04b |
| | | CB | 93.60a |
| | | CS | 91.46a |
| | 21d | NC | 130.76 |
| | | PC | 109.06 |
| | | CB | 133.03 |
| | | CS | 125.01 |
| Lipase, U/mgprot | 3d | NC | 190.87ab |
| | | PC | 105.14c |
| | | CB | 154.02b |
| | | CS | 205.53a |
| | 7d | NC | 193.84a |
| | | PC | 106.65b |
| | | CB | 186.09a |
| | | CS | 155.90ab |
| | 14d | NC | 183.76 |
| | | PC | 167.93 |
| | | CB | 205.40 |
| | | CS | 195.76 |
| | 21d | NC | 194.07 |
| | | PC | 177.85 |
| | | CB | 197.87 |
| | | CS | 203.41 |

*Means in the same row with different superscript letters differ significantly (P < 0.05)

1Each mean represents 6 birds. NC = birds fed a basal diet without challenged with *E. coli* K88; PC = birds fed a basal diet and challenged with *E. coli* K88. CB = birds fed a basal diet including 2 × 10⁷ CFU *C. butyricum* /kg of diet and challenged with *E. coli* K88. CS = birds fed a basal diet including 20 mg colistin sulfate/kg of diet and challenged with *E. coli* K88

2The days after challenging
broilers [26]. However, de Lima et al. [29] reported that the addition of the probiotic Bacillus subtilis in the diet did not affect digestive enzymes activities in broiler chickens [29]. The present study indicated that dietary supplementation of C. butyricum promoted digestive enzyme activities in broiler chickens challenged with E. coli K88. This was likely due to C. butyricum-induced protection of the intestinal integrity by inhibiting the activities of E. coli K88 and LPS, so augmented the activities of these enzymes. It is also possible that C. butyricum is capable of directly producing digestive enzymes in the gut of animals.

**Conclusion**

The results of our study indicated that E. coli K88 challenge lowered the BW and ADG, decreased the intestinal barrier function and digestive enzyme activities, but dietary supplementation of C. butyricum reversed these observations and promoted the immune response, improved intestinal barrier function and digestive enzyme activities in broiler chickens challenged with E. coli K88. Our results suggest that including C. butyricum in poultry diets has the potential for rearing healthier birds. There was no significant difference between the C. butyricum probiotic treatment and the colistin sulfate antibiotic treatment on the effect of growth performance, immune response, intestinal barrier function, and digestive enzyme activity in broiler chickens challenged with Escherichia coli. Therefore, the C. butyricum probiotic may be an alternative to antibiotic for animal production.

**Abbreviations**

C. butyricum: Clostridium butyricum; E. coli K88: Escherichia coli K88; NC: negative control treatment; PC: positive control treatment; CB: C. butyricum probiotic treatment; CS: Colistin sulfate antibiotic treatment; BW: body weight; ADG: average daily gain; TNF: tumor necrosis factor; IL-4: interleukin-4; DAO: Diamine oxidase.

**Competing interests**

The authors have declared that no competing interests exist.

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