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Effects of *Clostridium butyricum* on Growth Performance, Nitrogen Metabolism, Intestinal Morphology and Cecal Microflora in Broiler Chickens

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Abstract: A total of 600, 1 day old male Lingnan Yellow broiler chickens were used to investigate the effects of *Clostridium butyricum* (*C. butyricum*) on growth performance, nitrogen metabolism, intestinal morphology and cecal microflora in broiler chickens. The birds were randomly assigned into 5 treatments (6 replicate pens per treatment with 20 birds per pen) and fed the same antibiotic-free basal diets during a 42 days feeding experiment. The treatments were as follows: no addition (Control), 2.5×10^7 cfu *C. butyricum* kg^{-1} of diet (CB1), 5×10^7 cfu *C. butyricum* kg^{-1} of diet (CB2), 1×10^8 cfu *C. butyricum* kg^{-1} of diet (CB3) and 10 mg colistine sulfate kg^{-1} of diet (Antibiotic). Compared with the control birds, birds fed either CB1 or CB2 or antibiotic diet had greater ($p<0.05$) Body Weight (BW) on day 21 and 42 and higher ($p<0.05$) Average Daily Gain (ADG) from day 1-42. Birds fed *C. butyricum* or antibiotic diet had lower ($p<0.05$) Feed-to-Gain ratio (F:G) than the control birds from day 1-42. Dietary *C. butyricum* decreased ($p<0.05$) the concentration of serum Uric Acid (UA) compared with the control diet on day 21. Supplementation with CB2 or CB3 decreased ($p<0.05$) serum ammonia concentration compared with the control diet on day 21 and 42. Birds fed *C. butyricum* diet had higher ($p<0.05$) ileal villus height than the control birds on day 21 and 42. Birds fed CB2 or CB3 diet had lower ($p<0.05$) ileal crypt depth than the control birds on day 21 and 42. Supplementation with CB1 or CB3 decreased ($p<0.05$) the population of cecal *Escherichia coli* (*E. coli*) compared with the control on day 21. Birds fed *C. butyricum* diet had higher ($p<0.05$) population of cecal Bifidobacterium on day 21 and birds fed CB2 diet had higher ($p<0.05$) cecal Bifidobacterium on day 42 compared with the control birds. Supplementation with CB1 or CB2 increased ($p<0.05$) the number of cecal Lactobacillus on day 21 and supplementation with CB2 increased ($p<0.05$) Lactobacillus on day 42 compared with the control or antibiotic groups. The results indicate that supplementation with *C. butyricum* promotes growth performance, modulates nitrogen metabolism improves intestinal morphology and balances cecal microflora in broiler chickens.

Key words: *Clostridium butyricum*, growth performance, nitrogen metabolism, intestinal morphology, cecal microflora, broiler chickens

INTRODUCTION

Antibiotics have been used to improve growth and protect bird health in the poultry industry for many years (Harms *et al*., 1986; Bedford, 2000; Engberg *et al*., 2000; Awad *et al*., 2009). However, the over use of antibiotics has caused severe problems such as drug residues (Burgut, 1991), antibiotic resistance (Gustafson and Bowen, 1997; Jin *et al*., 1998; Nayak and Kenney, 2002) and disorder of intestinal microflora (Zeyner and Boldt, 2006). Hence, scientists are working to find alternatives to antibiotics. Probiotics are live microbial feed supplements which beneficially affect the host animal by improving its microbial balance (Afrc, 1989), therefore they are perceived as a potential substitute for antibiotics. *Clostridium butyricum* (*C. butyricum*), a butyric-acid producing gram-positive anaerobe found in soil and intestines of healthy animals and humans (Finegold *et al*., 1983) has been approved for human clinical use since 1968 in Japan (Takahashi *et al*., 2004). More experiments were conducted to investigate the effects of *C. butyricum* on infection of *Escherichia coli*, mucosal immunity, gastrointestinal microflora in mice (Murayama *et al*., 1995; Takahashi *et al*., 2004; Kong *et al*., 2011). Song *et al*. (2006) found that supplementation with *C. butyricum* could mediate the Humoral Immune Response (HIR) and improve growth performance in *Miichthys miiuy*. Recently, a few trials showed that administration of

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C. butyricum had beneficial influences on meat quality and gut health in broiler chickens (Yang *et al*., 2010; Zhang *et al*., 2011). Markovic *et al*. (2009) reported that direct feed microbial supplementation on diets resulted in an increase in villus height and width as well as a decrease in crypt depth in all parts of the intestines in broiler chickens. Barnes and Impey (1974) and Yeo and Kim (1997) found that some types of bacteria exhibited hydrolytic activities of Uric Acid (UA) in the chicken intestine. Lactobacilli supplement decreased the concentration of environmental volatile ammonia produced from amino acid degradation (Chang and Chen, 2003). However to the knowledge, there are no published reports on the effects of *C. butyricum* on intestinal mucosa morphology and nitrogen metabolism in broiler chickens. The present experiment was conducted to investigate the effects of *C. butyricum* on growth performance, nitrogen metabolism, intestinal morphology and cecal microflora in broiler chickens.

MATERIALS AND METHODS

Animals and experimental design: All of the procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University. A total of 600, 1 day old male Lingnan Yellow broiler chickens were randomly assigned into 5 treatments (6 replicate pens per treatment and 20 birds per pen). The Lingnan Yellow broiler chicken is famous for its meat quality in China. The birds were obtained from a commercial hatchery (Charoen Pokphand Group, Hangzhou, China) and raised in wired cages. The ambient temperature of barn was kept at 32°C in the1st week and gradually decreased (3°C week^{-1}) to atmospheric temperature. Diets and fresh water were supplied *ad libitum*. The experimental treatments were as follows: basal diet without addictives (control), basal diet addicted with 2.5×10^7 cfu *C. butyricum* kg^{-1} of diet (CB1), 5×10^7 cfu *C. butyricum* kg^{-1} of diet (CB2), 1×10^8 cfu *C. butyricum* kg^{-1} of diet (CB3), basal diet addicted with 10 mg colistine sulfate kg^{-1} of diet (Antibiotic). The basal diets of growing phase (day 1-21) and finishing phase (day 22-42) met the recommendation of Chinese Nutrient Requirements for Broiler Chickens (ZB B43005-86). The dietary ingredients and compositions of nutrient were shown in Table 1.

Bacterial preparation: This *strain* of *C. butyricum* was provided by Zhejiang Huijia Biological Technology Co., Ltd., China (HJCB998) and grew anaerobically in liquid fermentation tank at 37°C for 48 h. After centrifugation, the cells were harvested and dried using spray drying technology. Then, the powder of *C. butyricum* was added to the basal diet.

Table 1: Ingredients and nutrient composition of diets[1]

Ingredient (%)	Starter (1-21 days)	Finisher (22-42 days)
Corn	63.60	64.70
Soybean meal	27.50	27.40
Fishmeal[2]	5.00	2.00
Rapeseed oil[3]	0.00	2.00
NaCl	0.30	0.30
Calcium phosphate	1.10	1.40
Limestone	1.20	1.10
DL-Methionine	0.30	0.10
Vitamin-mineral premix[4]	1.00	1.00
Calculated nutrition composition		
ME (Mcal kg^{-1})	2.90	3.00
Crude protein (%)	21.05	19.01
Lysine (%)	1.13	0.92
Methionine + cysteine (%)	0.91	0.71
Ca (%)	1.01	0.90
Total P (%)	0.65	0.62
Available P (%)	0.45	0.44

[1]Nutrient level of the diets was based on Chinese nutrient requirements for broiler chicken (ZB B43005-86); [2]Crude protein content is 62.5% and metabolizable energy is 2.79 Mcal kg^{-1}; [3]Metabolizable energy is 8.8 Mcal kg^{-1}; [4]Supplied per kilogram of diet: vitamin A (retinyl acetate), 1,500 IU; cholecalciferol, 200 IU; vitamin E (DL-α-tocopheryl acetate), 10 IU; riboflavin, 3.5 mg; pantothenic acid, 10 mg; niacin, 30 mg; cobalamin, 10 μg; choline chloride, 1,000 mg; biotin, 0.15 mg; folic acid, 0.5 mg; thiamine 1.5 mg; pyridoxine 3.0 mg; Fe, 80 mg; Zn, 40 mg; Mn, 60 mg; I, 0.18 mg; Cu, 8 mg; Se, 0.15 mg

Growth performance: Chickens were weighed on day 21 and 42 to determinate BW and ADG. Feed intake per pen was measured throughout the experiment to calculate F:G.

Sample collection: On day 21 and 42, 6 birds from each treatment group were randomly selected and blood samples were collected from the wing veins. The birds were then killed via CO_2 inhalation to collect intestinal samples. Blood samples were allowed to clot at 4°C and centrifuged at 3000×g for 10 min and serums were harvested and stored at -20°C until they were analyzed. Two 1 cm segments of ileum were collected immediately after slaughter. The segment located a distal segment about 5 cm proximal to the ileocecal junction. The cecum was ligated with light twine and collected in sterile bags.

Serum uric acid and ammonia: The concentrations of serum UA and ammonia were measured by spectrophotometer using the kits purchased from Jiancheng Biological Engineering Research Institute, Nanjing, China.

Ileal morphology analysis: Each segment was flushed with a 0.9% salt solution, fixed with 4% formaldehyde-phosphate buffer for 48 h. The following process consisted of dehydration (Leica TP1020), clearing and impregnation with wax. The Formalin-fixed paraffin-embedded tissues were embedded into Leica EG1160, fixed upon Rotary Microtome (Leica HI1210) and cut in thickness of 5 um. The tissue segments were baked with

Roast Piece of Machine (Leica HI1220) at 38°C for 24 h. Then, the slides were dyed with hematoxylin and eosin in the dyeing machine (Leica Autostain BRXL) and covered by cover slides. Ten longest cecal villus and relevant crypt were selected and measured with Olympus AX70 microscope (Olympus Corporation, Tokyo, Japan). Images were analyzed using image software Qwin. In addition, villus height was measured from the villus-crypt junction to the tip of villus and crypt depth was measured from the root of villus to lamina propria.

Cecal microflora: The excreta samples of cecum were aseptically suspended in buffered 1% peptone water (1:9 w/v) and diluted with germ-free saline (pH 7.0) which were serial decimal prepared. The bacterial species were incubated at 37°C and numbered on selective agars as follows: *E. coli* was incubated using MacConkey agar for 48 h; Bifidobacterium and Lactobacillus were incubated using Briggs liver agar and Man-Rogosa-Sharpe agar, respectively for 48 h in the anaerobic incubator. All agar mediums were purchased from Land Bridge Technology, Beijing, China.

Statistical analysis: One-way ANOVA was performed using SPSS 15.0 Software (SPSS Inc., Chicago, IL). Differences among means of the dietary treatment were compared using LSD. An alpha value of 0.05 was used to assess significance among means.

RESULTS AND DISCUSSION

Growth performance: Birds fed either *C. butyricum* or antibiotic diet had greater ($p<0.05$) BW on day 21 and birds fed CB1 or CB2 or antibiotic diet had greater ($p<0.05$) BW on day 42 compared with those in control (Table 2). Supplementation with *C. butyricum* or antibiotic improved ($p<0.05$) ADG compared with the control diet from day 1-21. Birds fed either CB1 or CB2 or antibiotic diet had higher ($p<0.05$) ADG than the control birds from day 1-42. Birds fed either *C. butyricum* or antibiotic diet had lower ($p<0.05$) F:G than the control birds from day 1-42.

Serum uric acid and ammonia: The data showed that supplementation with *C. butyricum* decreased ($p<0.05$) serum UA concentration compared with the control diet on day 21 (Table 3). Birds fed *C. butyricum* had lower ($p<0.05$) serum ammonia concentration compared with the control birds on day 21. Supplementation with CB2 or CB3 reduced ($p<0.05$) serum ammonia concentration compared with the control diet on day 42.

Table 2: Effects of *Clostridium butyricum* on growth performance in broiler chickens[1]

Items	Age (days)	Control	CB1	CB2	CB3	Antibiotic	SEM	p-values
BW (g)	1	42.10	42.30	42.20	42.20	42.60	0.23	0.970
	21	387.80^{b}	444.70^{a}	431.40^{a}	427.60^{a}	425.00^{a}	5.65	0.001
	42	1327.50^{b}	1414.30^{a}	1402.30^{a}	1385.50^{ab}	1428.50^{a}	12.10	0.041
ADG (g)	1-21	16.50^{b}	18.50^{a}	19.20^{a}	18.40^{a}	18.20^{a}	0.27	0.001
	22-42	44.70	46.20	46.20	45.60	47.80	0.44	0.307
	1-42	30.60^{b}	32.40^{a}	32.70^{a}	32.00^{ab}	33.00^{a}	0.29	0.045
F:G	1-21	1.75	1.65	1.68	1.71	1.67	0.01	0.137
	22-42	2.31	2.22	2.21	2.23	2.24	0.01	0.179
	1-42	2.15^{a}	2.06^{b}	2.06^{b}	2.08^{b}	2.08^{b}	0.01	0.030

^{a,b}Within the same row; Means with different superscript letters differ significantly ($p<0.05$); [1]Each mean represents 6 birds. Control = Basal diet; CB1 = 2.5×10^{7} cfu *C. butyricum* kg^{-1} of diet; CB2 = 5×10^{7} cfu *C. butyricum* kg^{-1} of diet; CB3 = 1×10^{8} cfu *C. butyricum* kg^{-1} of diet; Antibiotic = 10 mg colistin sulfate kg^{-1} of diet

Table 3: Effects of *Clostridium butyricum* on the concentrations of serum uric acid and ammonia in broiler chickens[1]

Items	Age (days)	Control	CB1	CB2	CB3	Antibiotic	SEM	p-values
UA (mg mL^{-1})	21	439.00^{a}	378.00^{bc}	354.00^{c}	371.00^{bc}	426.00^{ab}	9.95	0.016
	42	466.00	431.00	429.00	414.00	448.00	8.42	0.365
Ammonia (μmol mL^{-1})	21	0.38^{a}	0.33^{b}	0.31^{b}	0.33^{b}	0.34^{ab}	0.01	0.046
	42	0.44^{a}	0.38^{ab}	0.33^{b}	0.32^{b}	0.40^{a}	0.01	0.002

$^{a-c}$Within the same row; Means with different superscript letters differ significantly ($p<0.05$); [1]Each mean represents 6 birds.Control = Basal diet; CB1 = 2.5×10^{7} cfu *C. butyricum* kg^{-1} of diet; CB2 = 5×10^{7} cfu *C. butyricum* kg^{-1} of diet; CB3 = 1×10^{8} cfu *C. butyricum* kg^{-1} of diet; Antibiotic = 10 mg colistin sulfate kg^{-1} of diet

Table 4: Effects of *Clostridium butyricum* on ileal morphology in broiler chickens[1]

Items	Age (days)	Control	CB1	CB2	CB3	Antibiotic	SEM	p-values
Villus height (μm)	21	473^{d}	486^{c}	651^{b}	737^{a}	439^{e}	13.01	0.001
	42	596^{c}	627^{b}	811^{a}	736^{a}	506^{d}	4.49	0.001
Crypt depth (μm)	21	142^{b}	126^{c}	116^{d}	105^{e}	166^{a}	2.92	0.001
	42	165^{b}	163^{b}	149^{c}	152^{c}	179^{a}	2.97	0.001

$^{a-e}$Within the same row; Means with different superscript letters differ significantly ($p<0.05$); [1]Each mean represents 6 birds.Control = Basal diet; CB1 = 2.5×10^{7} cfu *C. butyricum* kg^{-1} of diet; CB2 = 5×10^{7} cfu *C. butyricum* kg^{-1} of diet; CB3 = 1×10^{8} cfu *C. butyricum* kg^{-1} of diet; Antibiotic = 10 mg colistin sulfate kg^{-1} of diet

Ileal morphology: Birds fed *C. butyricum* had higher ($p<0.05$) ileal villus height than the control birds on day 21 and 42 (Table 4). Dietary *C. butyricum* reduced ($p<0.05$) ileal crypt depth compared with the control diet on day 21. Supplementation with CB2 or CB3 decreased ($p<0.05$) crypt depth compared with serum UA concentration of the control or antibiotic diet on day 42.

Cecal microflora: Birds fed CB1 or CB3 had less ($p<0.05$) population of *E. coli* in cecal content compared with the control birds on day 21 (Fig. 1). Birds fed *C. butyricum* diet had greater ($p<0.05$) counts of cecal Bifidobacterium on day 21 compared with the birds fed control or antibiotic diet (Fig. 2). The number of cecal Lactobacillus in birds fed CB1 or CB2 was greater ($p<0.05$) than that of the birds fed control or antibiotic diet on day 21 (Fig. 3). Birds fed CB2 diet had greater ($p<0.05$) counts of Bifidobacterium and Lactobacillus in cecal content than the birds fed control or antibiotic diet on day 42.

For a long time, *C. butyricum* has been used to prevent the disturbances of intestinal microfora and cure the antibiotic-associated diarrhea in human (Ito *et al*., 1997; Seki *et al*., 2003). *C. butyricum* used as a probiotic has valuable influences on the improvement of growth performance in *Miichthys miiuy* (Song *et al*., 2006), regulation of immune system in mice (Murayama *et al*., 1995), perfection of intestinal morphological structure in broiler chickens (Zhang *et al*., 2011) and modulation of intestinal microflora in mice (Kong *et al*., 2011). Moreover, Han *et al*. (1984) and Yang *et al*. (2010) indicated that *C. butyricum* could enhance the growth of broiler chickens which were similar with other species of probiotics (Jin *et al*., 1998; Timmerman *et al*., 2006; Apata,

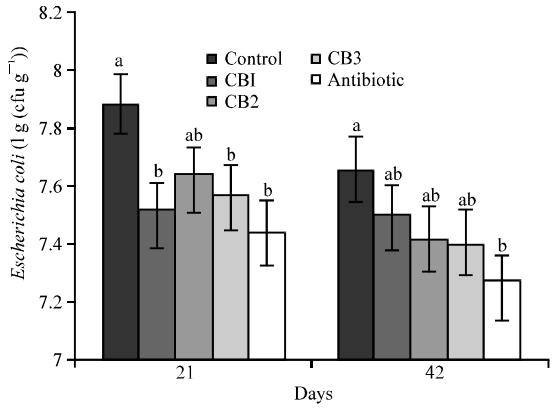

Fig. 1: Effects of *Clostridium butyricum* on cecal *E. coli* in broiler chickens. [a-c]Each bar represents mean for 6 birds per treatment ±SE. Within the same day, bars with different letters differ significantly ($p<0.05$). [1]Each mean represents 6 birds. Control = basal diet; CB1 = 2.5×107 cfu *C. butyricum* kg^{-1} of diet; CB2 = 5×107 cfu *C. butyricum* kg^{-1} of diet; CB3 = 1×108 cfu *C. butyricum* kg^{-1} of diet; Antibiotic = 10 mg colistin sulfate kg^{-1} of diet

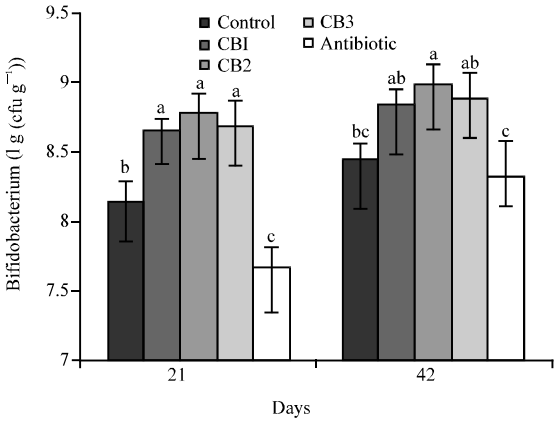

Fig. 2: Effects of *Clostridium butyricum* on cecal Bifidobacterium in broiler chickens; [a, b]Each bar represents mean for 6 birds per treatment ±SE. Within the same day, bars with different letters differ significantly ($p<0.05$); [1]Each mean represents 6 birds. Control = basal diet; CB1 = 2.5×10^7 cfu *C. butyricum* kg^{-1} of diet; CB2 = 5×10^7 cfu *C. butyricum* kg^{-1} of diet; CB3 = 1×10^8 cfu C. butyricum kg^{-1} of diet; Antibiotic = 10 mg colistin sulfate kg^{-1} of diet

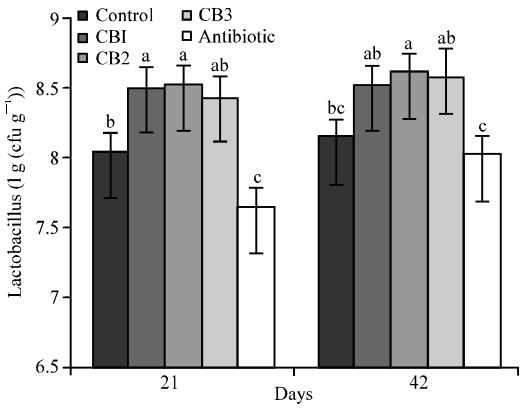

Fig. 3: Effects of *Clostridium butyricum* on cecal Lactobacillus in broiler chickens. [a-c]Each bar represents mean for 6 birds per treatment ±SE. Within the same day, bars with different letters differ significantly ($p<0.05$). [1]Each mean represents 6 birds. Control = basal diet; CB1 = 2.5×10^7 cfu *C. butyricum* kg^{-1} of diet; CB2 = 5×10^7 cfu *C. butyricum* kg^{-1} of diet; CB3 = 1×10^8 cfu *C. butyricum* kg^{-1} of diet; Antibiotic = 10 mg colistin sulfate kg^{-1} of diet

2008; Czerwinski *et al*., 2010; Lin *et al*., 2011). In the present study, the results demonstrated that a diet supplemented with 2.5×10^7 cfu *C. butyricum* kg^{-1} or 5×10^7 cfu *C. butyricum* kg^{-1} increased growth performance in broilers. The reason why *C. butyricum* promotes growth performance still remains unknown. Nakanishi *et al.* (2003) found that *C. butyricum* could produce large amounts of short chain fatty acids such as butyrate and acetate which stimulated colonic sodium and fluid absorption and exerted proliferative effects on colonocyte. researchers indicate that *C. butyricum* promoted growth because short chain fatty acids produced by *C. butyricum* are important energy resources to increase the animal growth. We all know that poultry production faces the global environmental challenges, especially the nitrogen emissions to the atmosphere (Powers and Angel, 2008). Furthermore, numerous trials on dietary crude protein content and amino acid composition were conducted to address nitrogen losses in poultry (Ferguson *et al*., 1998; Angel *et al*., 2006; Applegate *et al*., 2008; Powers and Angel, 2008). Kim and Kim (1992) found that probiotics used in animal feeds as growth promotions could suppress the urea hydrolysis and subsequently reduce ammonia production in GI tract. However, Shareef and Al-Dabbagh (2009) reported that supplementation with *Saccharomyces cerevisiae* had no effect on uric acid level in broiler chickens. Similarly, Yeo and Kim (1997) found that dietary probiotic decreased urease activities in the small intestinal contents in young chickens. Potrikus and Breznak (1980) reported that a group of nitrogen Streptococcus and Citrobacter promoted the anaerobic degradation of uric acid in the termite gut. The data showed that supplementation with *C. butyricum* decreased the concentration of serum uric acid and ammonia in broiler chickens. The results indicated that *C. butyricum* may play a considerable role in nitrogen metabolish in broiler chickens. Future research needs to be conducted to explore the mechanism of *C. butyricum* on nitrogen metabolism in broiler chickens.

Research projects showen that intestinal villus height and crypt depth are tightly related to the nutrient absorption and performance of hosts (Caspary, 1992; Dunham *et al*., 1993; Shamoto and Yamauchi, 2000; Samanya and Yamauchi, 2002; Xu *et al*., 2003). Awad *et al.* (2009) showed that the probiotics resulted in an increase in villus height and a decrease in crypt depth of intestinal mucosa of broilers. Chichlowski *et al.* (2007) reported that feeding of micro-direct microflora increased jejunal villus height and decreased crypt depth compared with the control group. In the present study, supplementation with *C. butyricum* improved ileal villus length and decreased crypt depth. Researchers found that butyric acid produced from *C. butyricum* could provide energy for the epithelial growth (Topping and Clifton, 2001; Yang *et al*., 2010). Zhang *et al.* (2011) found that dietary inclusion of *C. butyricum* with diets increased jejunal villus height and the relative length of caecum in broilers and theresults were consist with what they have found. The results suggest that dietary supplementation with *C. butyricum* benefited ileal morphology in broilers. In chickens, cecum has the maximum number of microflora in gastrointestinal tract and is the most common site infected with enteric pathogens (Jozefiak *et al*., 2004). Moreover, the administration of probiotics modulates the gut micro-ecology conditions by suppressing harmful microorganisms and favoring the beneficial bacterial (Biernasiak and Slizewska, 2009; Lin *et al*., 2011). Kuroiwa *et al.* (1990) reported that *C. butyricum* M 588 inhibited the growth of Vibrio cholerae O1. Takahashi *et al.* (2004) reported that *C. butyricum* MIYAIRI strain 588 had preventive and therapeutic effects on *Escherichia coli* O157:H7 infection by inhibiting the growth in gnotobiotic mice. In the current experiment, *C. butyricum* inhibited the growth of cecal *E.coli*, Salmonellas and promoted the growth of Bifidobacterium and Lactobacillus. Kong *et al.* (2011) reported that a certain dose of *C. butyricum* increased the population of Lactobacillus and Bifidobacterium compared with the normal group in mice after day 14 and this was consist with the data. The possible explanations could be that dietary *C. butyricum* produces large amount of short chain fatty acids which promoted the growth of beneficial bacteria (Zhang *et al*., 2011) and *C. butyricum* produces bacteriocin which inhabit the growth of harmful microorganisms (Clarke and Morris, 1976; Nakanishi and Tanaka, 2009).

CONCLUSION

The results of the present experiment indicated that dietary *C. butyricum* improves growth performance and the appropriate levels may be 2.5×10^7 or 5×10^7 cfu kg^{-1}. *C. butyricum* used as probiotics can modulate nitrogen metabolism, perfect intestinal morphology and balance the cecal microflora in broiler chickens.

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