Effects of multistrain probiotics on growth performance, apparent ileal nutrient digestibility, blood characteristics, cecal microbial shedding, and excreta odor contents in broilers

Z. F. Zhang* and I. H. Kim†[1]

*College of Life Science and Technology, Southwest University for Nationalities, Chengdu 610041, China; and †Department of Animal Resource and Science, Dankook University, Cheonan, Choongnam 330-714, South Korea

ABSTRACT This experiment was conducted to investigate the efficacy of *Lactobacillus acidophilus*, *Bacillus subtilis*, and *Clostridium butyricum* supplementation in broilers. A total of 400 one-day-old mixed sex Ross 308 broilers with an initial average BW of 46 ± 0.5 g were randomly allotted into 4 treatments with 5 replicate pens per treatment and 20 broilers in each pen for 35 d. Dietary treatments were (1) an antibiotic-free diet (CON), (2) CON + 5 mg/kg of avilamycin, (3) CON + 1×10^5 cfu of multistrain probiotics/kg of diet (P1), and (4) CON + 2×10^5 cfu of multistrain probiotics/kg of diet (P2). Broilers fed the P1 and P2 diets had greater BW gain than broilers fed the CON diet during d 22 to 35 ($P = 0.01$) and overall ($P = 0.02$). Feed conversion ratios in P1 and P2 were decreased ($P = 0.03$) compared with that in CON from d 22 to 35. Ileal digestibility of most essential amino acids, with the exception of His and Phe, were increased ($P < 0.05$) in P1 and P2 compared with CON. Serum IgA and IgM concentrations in P2 were higher ($P < 0.05$) than those in CON. The cecal *Lactobacillus* numbers were increased ($P = 0.02$), and the counts of *Escherichia coli* were decreased ($P = 0.03$) in P1 and P2 compared with CON. Dietary supplementation with multistrain probiotics decreased ($P < 0.05$) the excreta NH_3 content compared with the CON. In conclusion, dietary supplementation with multistrain probiotics improved broiler growth performance, ileal amino acids digestibility, and humoral immunity. Furthermore, the probiotics decreased the cecal numbers of *E. coli* and decreased the NH_3 content of excreta.

Key words: broiler, excreta odor content, growth performance, nutrient digestibility, probiotic

2014 Poultry Science 93:364–370
http://dx.doi.org/10.3382/ps.2013-03314

INTRODUCTION

The increasing resistance of pathogens to antibiotic growth promoters (**AGP**), as well as the levels of AGP residues in animal products and in the environment, has brought about a call for a worldwide AGP ban (van den Bogaard and Stobberingh, 2000). Live microbial additives (probiotics) have been serving as possible alternatives to AGP without clinical side effects in the last 15 y (Furrie et al., 2006).

Previous studies reported that dietary supplementation with *Lactobacillus*-, *Bacillus*-, and *Clostridium*-based probiotics could improve growth performance (Deniz et al., 2011), nutrient digestibility (Mountzouris et al., 2010), humoral immunity (Yang et al., 2012), meat quality (Mahajan et al., 2000), increase gastrointestinal lactobacilli counts (Lee et al., 2010a), as well as decrease coliforms numbers and the manure ammonia emission in broilers (Hassan and Ryu, 2012). However, inconsistent effects of probiotics are also reported, which are likely influenced by the probiotic strains used, administration dose, and diet composition (Zhang et al., 2013).

Sanders and Veld (1999) reported that multistrain probiotics may be more effective than single-strain probiotics, and could amplify the protective spectrum against microbial infections. It was also suggested that supplementation of 10^5 to 10^9 cfu/kg of probiotics in the diet could exert beneficial effects in broilers (Teo and Tan, 2007; Mountzouris et al., 2010). However, Huang et al. (2004) reported that a higher inclusion level did not always result in better performance in animals. Therefore, we hypothesized that 10^5 cfu of multistrain probiotics/kg of diet may exert beneficial effects in broilers. This study was conducted to investigate the effects of a mixture of *Lactobacillus acidophilus*, *Bacillus subtilis* DSM 17299, and *Clostridium butyricum* on broiler growth performance, amino acid digestibility, blood characteristics, excreta odor contents, as well as

Received May 14, 2013.
Accepted October 15, 2013.
[1]Corresponding author: inhokim@dankook.ac.kr

the numbers of *Lactobacillus* and *Escherichia coli* in cecal content.

MATERIALS AND METHODS

Probiotic Product

The probiotic product used in the present study was manufactured by a commercial company (Probion, Woogene B&G Co. Ltd., Seoul, South Korea). This product was composed of spray-dried spore-forming bacteria, which was guaranteed to contain 2×10^8 viable spores/kg of *L. acidophilus*, *B. subtilis* DSM 17299, and *C. butyricum*.

Birds and Experimental Treatments

All birds used in this trial were handled in accordance with the guidelines set forth by the Animal Care and Use Committee of Dankook University. Four hundred 1-d-old mixed sex Ross 308 broilers with an initial BW of 46 ± 0.5 g were obtained from a local commercial hatchery (Yang Ji Company, Cheonan, Choongnam, South Korea). All birds were raised in stainless steel pens of identical size (1.75 × 1.55 m) and were provided a 20-h light program. The temperature of the room was maintained at 33 ± 1°C for the first 3 d and decreased to 24°C until the end of the experiment. Broilers were randomly allotted into 4 treatments for 5 wk. Each treatment had 5 replicate pens of 20 broilers in each pen. The broilers were allowed ad libitum access to water and feed throughout the experimental periods.

Dietary treatments included an antibiotic-free diet (**CON**), 5 mg of avilamycin/kg of diet, 1×10^5 cfu of probiotic/kg of diet (**P1**), and 2×10^5 cfu of probiotic/kg of diet (**P2**). The diets were fed during the experiment in 2 phases, consisting of a starter phase from d 1 to 21 and a grower phase d 22 to 35, and were provided in mash form. Diets were formulated according to Ross (308) commercial management guide (Table 1). The CON diet was prepared weekly and no coccidiostat was added.

Bacterial Enumeration in Experimental Feed

Each feed in 1 mL of PBS was serially diluted from 10^{-1} to 10^{-7}. Dilutions were plated on selective agar media in duplicates for the enumeration of target bacteria. In particular, *Lactobacilli*, *Bacillus*, and *C. butyricum* counts were enumerated using de Man, Rogosa, Sharpe agar, blood agar plates, and clostridium basal medium (CBM) agar, respectively (Leuschner et al., 2003; Leuschner and Bew, 2003; Kong et al., 2011). The de Man, Rogosa, Sharpe agar and CBM agar plates were then incubated at 39°C for 48 h anaerobically, the blood agar plates were incubated at 37°C for 18 h aerobically, and colonies were counted. The average bacterial counts in the starter feed preparations were 1.07×10^5 and 2.11×10^5 cfu/kg in P1 and P2, respectively, whereas in the grower diet they were 1.12×10^5 and 2.09×10^5 cfu/kg in P1 and P2, respectively.

Chemical Analysis

Dietary DM (method 930.15), CP (method 968.06), crude fat without acid hydrolysis (method 920.39), calcium (method 984.01), phosphorus (method 965.17) were analyzed according to the procedures described by AOAC International (2005). Individual amino acid composition was measured using an Amino Acid Analyzer (Beckman 6300, Beckman Coulter Inc., Fullerton, CA) after 24-h of 6 *N*-HCl hydrolysis at 110°C (AOAC International, 2005). Performic acid was used before hydrolysis to oxidize Met and Cys to methionine sulfone and cysteic acid. Nitrogen was determined by a Kjectec 2300 Nitrogen Analyzer (Foss Tecator AB, Hoeganaes, Sweden). The gross energy was determined by measuring the heat of combustion in the samples using a Parr 6100 Oxygen Bomb Calorimeter (Parr instrument Co., Moline, IL).

Growth Performance and Coefficient Apparent Ileal Nutrient Digestibility

Broilers were weighed on a pen basis on d 0, 21 and 35, and feed consumption was recorded throughout the experiment. Body weight gain (**BWG**), feed intake,

Table 1. Ingredient composition and nutrient content of diets

Item	Starter[1]	Grower[1]
Ingredient, g/kg		
Maize	582.4	634.9
Corn gluten meal, 600 g of CP/kg	93.0	52.0
Soybean meal, 480 g of CP/kg	256.8	246.3
Soybean oil	36.7	37.3
Limestone	16.0	16.0
Dicalcium phosphate	5.0	4.5
L-Lys-HCl, 780 g/kg	1.8	1.0
DL-Met, 998 g/kg	1.8	1.5
Salt	2.5	2.5
Vitamin premix[2]	2.0	2.0
Trace mineral premix[3]	2.0	2.0
Calculated composition, MJ/kg		
ME	12.5	12.9
Analyzed composition, g/kg		
CP	214.9	197.9
Crude fat	48.4	50.3
Crude fiber	34.7	31.2
Lys	12.6	10.8
Met	6.0	5.1
Ca	10.2	9.3
P	8.7	7.1

[1]Starter diet was provided during d 0 to 21, whereas grower diet was provided during d 22 to 35.

[2]Supplied per kilogram of diet: 12,500 IU of vitamin A, 3,750 IU of vitamin D_3, 25 IU of vitamin E, 2.55 mg of vitamin K_3, 3 mg of B_1, 7.5 mg of B_2, 4.5 mg of vitamin B_6, 24 μg of vitamin B_{12}, 51 mg of niacin, 1.5 mg of folic acid, 0.2 mg of biotin, 13.5 mg of pantothenic acid, and 1,208 mg of choline chloride.

[3]Provided per kilogram of diet: 37.5 mg of Zn (as $ZnSO_4$), 37.5 mg of Mn (MnO_2), 37.5 mg of Fe (as $FeSO_4 \cdot 7H_2O$), 3.75 mg of Cu (as $CuSO4 \cdot 5H_2O$), 0.83 mg of I (as KI), and 0.23 mg of Se (as $Na_2SeO_3 \cdot 5H_2O$).

and feed conversion ratio were then calculated using this information.

From d 29 to 35, chromium oxide (Cr_2O_3) was added to the diets at a level of 2 g/kg as an indigestible marker for the determination of the nutrient coefficient apparent ileal digestibility (**CAID**; Sales and Janssens, 2003). On the last continuous 3 d (d 33, 34, and 35), 3 broilers per pen (15 broilers per treatment) were slaughtered by severing a jugular vein. The ileum was ligated and then separated from the rest of the gastrointestinal tract and, subsequently, the ileal digesta on the pen basis was immediately stored in sealed bags at −20°C and freeze-dried, after which they were finely grounded to be able to pass through a 1-mm screen and were then stored in a freezer at −20°C until analysis (Mountzouris et al., 2010). All the analytical methods were the same as described before. Chromium levels were determined via UV absorption spectrophotometry (Shimadzu, UV-1201, Japan) according to Williams et al. (1962). The apparent ileal nutrient digestibility was then calculated relative to the Cr_2O_3 concentration.

Blood Characteristics

At the end of the experiment, 5 broilers per pen (25 broilers/treatment) were bled via the wing vein and blood samples were collected into K_3 EDTA Vacuum tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). The white blood cell, red blood cell, and lymphocyte counts were analyzed using an automatic blood analyzer (ADVID 120, Bayer, Tarrytown, NY). The blood samples were then centrifuged (3,000 × *g*) for 15 min at 4°C (Becton Dickinson Vacutainer Systems). Serum IgG, IgM, and IgA were determined by double-antibody sandwich ELISA using commercial kits (Bethyl Laboratories, Montgomery, TX).

Cecal Microbial Shedding

At d 35, 15 broilers per treatment were killed by cervical dislocation. Ceca were ligated and cecal contents were immediately obtained and then placed on ice for transportation to the laboratory. The viable counts of *E. coli* and *Lactobacillus* were analyzed by the method of Wang and Kim (2011). Bacteria were plated in duplicates on MacConkey agar plates (Difco Laboratories, Detroit, MI) and *Lactobacillus* medium III agar plates (Medium 638, DSMZ, Braunschweig, Germany) to isolate *E. coli* and *Lactobacillus*, respectively. The *Lactobacillus* medium III agar plates were then incubated for 48 h at 39°C under anaerobic conditions. The MacConkey agar plates were incubated for 24 h at 37°C. The *E. coli* and *Lactobacillus* colonies were counted immediately after removal from the incubator.

Excreta Odor Contents

For analysis of NH_3 and H_2S contents in excreta, 300 g of fresh excreta samples from each pen were collected in plastic boxes (polyvinyl, 25 × 35 cm) in triplicates at the end of the experiment and fermented in an incubator (28°C) according to the method described by Cho et al. (2008). Concentrations of NH_3 and H_2S were measured within the range of 5.0 to 100.0 ppm (no. 3La, detector tube; Gastec Corp., Kanagawa, Japan) and 2.0 to 20.0 ppm (4LK, detector tube; Gastec Corp.) on d 1, 3, and 5 after fermentation.

Statistical Analysis

All data were analyzed as a completely randomized design by one-way ANOVA using the GLM procedure as outlined by SAS (SAS Institute Inc., Cary, NC) and the pen was used as the experimental unit. Before carrying out statistical analysis of the microbial counts, logarithmic conversion of the data was performed. Differences among treatment means were determined using the Duncan's multiple range test. Statements of statistical significance were based on $P < 0.05$.

RESULTS

Growth Performance and Mortality Rate

During the experiment, growth performance and mortality rate (data not shown) of broilers were not affected by dietary avilamycin (Table 2). During d 22 to 35, broilers fed P1 and P2 diets had greater ($P = 0.01$) BWG and lower ($P = 0.03$) feed conversion ratio than those fed CON diet. Overall, BWG was increased ($P = 0.02$) in P1 and P2 treatments compared with that in CON. No difference was observed in feed intake and mortality rate among treatments throughout the experimental periods.

Coefficient of Apparent Ileal Nutrient Digestibility

The CAID of DM, nitrogen, and energy were not affected by any dietary supplementation (Table 3). Broilers fed the P1 and P2 diets showed greater ($P < 0.05$) CAID of most essential amino acids, except His and Phe, compared with those fed the CON diet. The CAID of Ala and Gly was also increased ($P = 0.03$ and 0.02, respectively) in the P1 and P2 treatments compared with that of the CON treatment.

Blood Characteristics

The concentration of red blood cells, white blood cells, IgG, and lymphocyte was unaffected by avilamycin or probiotic supplementation (Table 4). Serum IgA concentration was increased ($P = 0.01$) in P2 compared with that in CON. Moreover, the P1 and P2 treatments showed higher ($P = 0.02$) IgM concentration than the CON treatment.

Table 2. The effect of a multistrain probiotic preparation on the growth performance of broilers[1]

Item	CON	ANT	P1	P2	SEM[2]	*P*-value[2]
BW gain, g						
d 0–21	928	941	943	942	29.5	0.32
d 22–35	986[b]	1,019[ab]	1,055[a]	1,064[a]	34.6	0.01
d 0–35	1,914[b]	1,960[ab]	1,998[a]	2,006[a]	25.7	0.02
Feed intake, g						
d 0–21	1,157	1,191	1,180	1,160	33.4	0.65
d 22–35	1,909	1,893	1,926	1,934	44.3	0.27
d 0–35	2,917	2,934	2,956	2,944	58.2	0.47
Feed conversion ratio						
d 0–21	1.25	1.27	1.25	1.23	0.068	0.22
d 22–35	1.94[a]	1.86[ab]	1.83[b]	1.82[b]	0.031	0.03
d 0–35	1.52	1.50	1.48	1.47	0.028	0.21

[a,b]Means in the same row with different superscripts differ ($P < 0.05$).

[1]CON = antibiotic-free diet; ANT = CON + 5 mg/kg of avilamycin; P1 = CON + 1×10^5 cfu of multistrain probiotics/kg of diet; P2 = CON + 2×10^5 cfu of multistrain probiotics/kg of diet.

[2]Each mean represents 5 replicate pens with 20 broilers per pen.

Table 3. The effect of a multistrain probiotic preparation on apparent ileal nutrient digestibility in broilers[1]

Item	CON	ANT	P1	P2	SEM[2]	*P*-value
DM	0.82	0.84	0.82	0.83	0.015	0.24
Nitrogen	0.80	0.81	0.81	0.81	0.012	0.85
Energy	0.82	0.82	0.82	0.82	0.015	0.36
Essential amino acid						
Arg	0.72[b]	0.74[ab]	0.77[a]	0.77[a]	0.013	0.04
Cys	0.91[b]	0.94[ab]	0.96[a]	0.96[a]	0.013	0.04
His	0.79	0.78	0.80	0.81	0.017	0.15
Ile	0.75[b]	0.77[ab]	0.80[a]	0.81[a]	0.018	0.02
Leu	0.81[b]	0.82[ab]	0.86[a]	0.87[a]	0.021	0.03
Lys	0.82[b]	0.84[ab]	0.86[a]	0.87[a]	0.015	0.02
Met	0.87[b]	0.93[a]	0.94[a]	0.94[a]	0.017	0.04
Phe	0.83	0.85	0.86	0.85	0.026	0.65
Thr	0.72[b]	0.77[ab]	0.81[a]	0.81[a]	0.024	0.02
Val	0.78[b]	0.80[ab]	0.82[a]	0.81[ab]	0.011	0.03
Nonessential amino acid						
Ala	0.70[b]	0.76[a]	0.76[a]	0.77[a]	0.023	0.03
Asp	0.83	0.84	0.86	0.84	0.018	0.31
Glu	0.89	0.90	0.87	0.88	0.019	0.17
Gly	0.90[b]	0.91[ab]	0.95[a]	0.95[a]	0.017	0.02
Pro	0.79	0.81	0.80	0.81	0.022	0.14
Ser	0.81	0.81	0.82	0.80	0.019	0.25
Tyr	0.88	0.89	0.90	0.88	0.013	0.74

[a,b]Means in the same row with different superscripts differ ($P < 0.05$).

[1]CON = antibiotic-free diet; ANT = CON + 5 mg/kg of avilamycin; P1 = CON + 1×10^5 cfu of multistrain probiotics/kg of diet; P2 = CON + 2×10^5 cfu of multistrain probiotics/kg of diet.

[2]Each mean represents 5 observations per treatment.

Table 4. The effect of a multistrain probiotic preparation on blood cell counts and plasma immunoglobulin concentration in broilers[1]

Item[2]	CON	ANT	P1	P2	SEM[3]	*P*-value
RBC, $10^6/\mu L$	2.1	2.3	2.2	2.3	0.08	0.32
WBC, $10^3/\mu L$	274	301	298	265	37.6	0.22
Lymphocyte,[4] %	76.9	70.2	80.4	75.6	9.45	0.18
IgG, mg/mL	0.192	0.193	0.193	0.192	0.001	0.95
IgA, mg/mL	0.153[b]	0.166[ab]	0.168[ab]	0.193[a]	0.010	0.01
IgM, mg/mL	0.218[b]	0.223[ab]	0.237[a]	0.235[a]	0.054	0.02

[a,b]Means in the same row with different superscripts differ ($P < 0.05$).

[1]CON = antibiotic-free diet; ANT = CON + 5 mg/kg of avilamycin; P1 = CON + 1×10^5 cfu of multistrain probiotics/kg of diet; P2 = CON + 2×10^5 cfu of multistrain probiotics/kg of diet.

[2]RBC = red blood cells; WBC = white blood cells.

[3]Each mean represents 5 replicate pens with 5 broilers per pen.

[4]Value presented as percentage of total white blood count.

Table 5. The effect of a multistrain probiotic preparation on cecal counts of *Lactobacillus* and *Escherichia coli* as well as the odor emission in excreta of broilers[1]

Item	CON	ANT	P1	P2	SEM[2]	*P*-value
Lactobacillus, $\log_{10}$ cfu/g	7.43[b]	7.55[ab]	7.88[a]	7.89[a]	0.214	0.02
Escherichia coli, $\log_{10}$ cfu/g	6.49[a]	6.25[ab]	5.92[b]	5.89[b]	0.187	0.03
NH_3, mg/m^3						
d 1	38.3[a]	35.2[ab]	32.4[bc]	31.0[c]	0.95	<0.01
d 3	56.2[a]	55.4[a]	48.3[b]	50.5[b]	1.06	0.01
d 5	82.5[a]	78.5[ab]	76.3[b]	77.4[b]	1.55	0.04
H_2S, mg/m^3						
d 1	1.6	1.7	1.4	1.6	0.24	0.89
d 3	2.3	2.4	2.1	2.2	0.16	0.24
d 5	3.5	3.8	3.6	3.3	0.25	0.90

[a–c]Means in the same row with different superscripts differ ($P < 0.05$).

[1]CON = antibiotic-free diet; ANT = CON + 5 mg/kg of avilamycin; P1 = CON + 1×10^5 cfu of multistrain probiotics/kg of diet; P2 = CON + 2×10^5 cfu of multistrain probiotics/kg of diet.

[2]Each mean represents 15 observations per treatment.

Cecal Microflora and Excreta Odor Contents

The cecal *Lactobacillus* numbers were increased ($P = 0.02$) in the P1 and P2 groups, and *E. coli* counts were decreased ($P = 0.03$) compared with those of the CON group (Table 5). Excreta NH_3 content in the P2 treatment was lowest ($P < 0.01$) among treatments at d 1, whereas the P1 treatment had a lower NH_3 concentration than that of the CON treatment. The excreta NH_3 concentration in the P1 and P2 treatments was decreased compared with those in the CON treatment at d 3 ($P = 0.01$) and 5 ($P = 0.04$). No difference between groups was observed with respect to the H_2S emission of excreta.

DISCUSSION

It has been suggested that probiotics can promote broiler performance by improving digestive function, modulating the intestinal microflora, enhancing immunomodulation, and enhancing broiler health (Gil de los Santos et al., 2005; Yang et al., 2012). In the current study, the addition of a mixture of *L. acidophilus*, *B. subtilis*, and *C. butyricum* (1×10^5 and 2×10^5 cfu/kg of diet) significantly improved the BWG and feed conversion ratio with no effect on feed intake throughout the finisher period, independent of the probiotic concentration in the diet. In agreement with our results, Huang et al. (2004) reported that dietary supplementation with 332 and 425 mg/kg of *L. acidophilus* significantly improved the BW and BWG of broilers compared with those of the control treatment. Zhang et al. (2012, 2013) found that the BWG was increased by the administration of 10^5 and 10^8 cfu/kg of a *Bacillus*-based probiotic. Yang et al. (2012) found that adding 2×10^7 or 3×10^7 cfu/kg of *C. butyricum* to the diet improved the ADG in broiler chickens. Similar positive effects were also reported by other researchers (Talebi et al., 2008; Zhou et al., 2010). However, Amerah et al. (2013) reported that dietary inclusion of a 3-strain *B. subtilis* at the level of 1.5×10^8 cfu/kg of feed improved the feed conversion ratio by reducing the feed intake without any beneficial effect on BWG in broilers. Other researchers also found no or minimal effect of probiotics on the growth performance of broilers (Mountzouris et al., 2007; Lee et al., 2010b; Zhang et al., 2011). This inconsistency might be attributed to the strains of probiotic, method of preparation, administration dosage, diet composition, bird age, and hygiene status (Mountzouris et al., 2007; Lee et al., 2010a; Zhang et al., 2012). Moreover, it is well accepted that avilamycin can be used as an AGP to compare the efficacy of probiotics (Mountzouris et al., 2010). In our study, the avilamycin group seems to exhibit a performance between those of the control group and the probiotic-fed group. Based on the low mortality rate and growth performance in CON, the health status and hygiene environment may lead to a comparable growth performance between control and avilamycin groups (Engberg et al., 2000). Furthermore, the dosage of avilamycin in this study was only half of the normal dose (5 vs. 10 mg/kg).

Previous studies have suggested that probiotics can improve nutrient uptake by changing the gastrointestinal bacterial community structure, and modifying mucin biosynthesis or degradation (Netherwood et al., 1999; Smirnov et al., 2005). Apata (2008) and Li et al. (2008) reported that the ileal apparent digestibility of energy, DM, and CP was improved by feeding broilers with diets containing probiotics. Contrary to their results, we did not find any difference in the apparent ileal digestibility of DM, nitrogen, and energy among treatments. However, in our study, probiotic treatments showed greater apparent ileal digestibility of most essential amino acids compared with CON. Li et al. (2008) reported that dietary supplementation with 0.2 to 0.6% probiotics (a mixture of yeasts and other microbes) increased the apparent digestibility of most amino acids by 6 to 11%, and found that the apparent digestibility of nutrients varied between 21- and 42-d-old broilers. The inconsistency may have been induced by the different age of broilers with different activities of digestive enzymes, secretions of endogenous amino acid, and bacterial metabolism (Snel et al., 2002). A probiotic may alter the synthesis and catabolism of amino acids in the

small intestine, resulting in changes in the amino acids in the ileal digesta (Wu, 1998). Therefore, we hypothesized that the improved amino acids ileal digestibility was related to the higher microbial activity in the ileum, which may lead to more bacterial degradation of amino acids for the probiotics treatments.

Plasma immunoglobulin concentrations can be used as a parameter to reflect the humoral immune status of animals because of their important roles in immune function. In the current study, the serum IgA and IgM concentrations were increased by administration of multistrain probiotics. Perdigon et al. (1995) reported that *L. acidophilus* increased the number of IgA-producing cells in a dose-dependent manner. Additional studies have reported that similar probiotic supplementation resulted in an enhancement of the broiler humoral immune response (Yang et al., 2012; Amerah et al., 2013). The bursa of Fabricius has an extremely vital role in the poultry immune system, and the weight of the bursa in broilers reflects the anatomical response to immune status (Willis et al., 2013). In the present study, the increased relative weight of bursa of Fabricius in P1 and P2 (data not shown) may also reflect the enhanced immune response. Moreover, it has been well documented that probiotics can enhance intestinal cell-mediated mucosal immunity, upregulation of heterophil bactericidal mechanisms, and can alter the expression of intestinal mucosal pro- and anti-inflammatory cytokine expression (Dalloul et al., 2003; Farnell et al., 2006; Chichlowski et al., 2007). With respect to the development of immune status, nutrients may be diverted from growth to immune cell development and function, which may explain why the nutrient digestibility was not affected by probiotics in the current study. However, further study is still warranted to confirm the positive effects of probiotics on humoral immunity beyond the purpose of our study.

It is known that supplementation of *Lactobacillus*-, *Bacillus*-, and *Clostridium*-based probiotics at the level of 10^6 to 10^9 cfu/kg of diet could fortify intestinal beneficial microorganisms, such as *Lactobacillus* and *Bifidobacterium*, and suppress the growth of potentially pathogenic bacteria, such as *Clostridium* and *E. coli* in broilers (Teo and Tan, 2007; Higgins et al., 2008; Mountzouris et al., 2010). In our study, we also confirmed that the dietary inclusion of 10^5 cfu/kg of probiotics could slightly increase the cecal *Lactobacillus* counts and decrease *E. coli* numbers compared with those of the control treatment. In turn, the improved intestinal microbial balance in the current study may explain the improved amino acid digestibility. Ng et al. (2009) suggested that probiotics may influence the intestinal microflora by facilitating antibody production, promoting epithelial barrier integrity, augmenting toll-like receptor signaling, and some other mechanisms.

Ammonia is a major aerial pollutant originating from livestock operations, and poultry is one of the principal contributors among domestic animals (Zhang et al., 2013). It has been reported that probiotics could reduce the levels of pollutants arising from animal manure by improving nutrient utilization, altering the intestinal microbiota ecosystem, and reducing the pH of manure (Ferket et al., 2002). Therefore, the reduction of excreta NH_3 may be considered as an improved ileal digestibility of amino acids and an increased intestinal *Lactobacillus* population. In agreement with our results, Hassan and Ryu (2012) reported that the dietary application of 10^7 cfu /kg of *Lactobacillus*- or *Bacillus*-based probiotics could reduce the NH_3 contents in the broiler excreta. Zhang et al. (2013) found also that dietary supplementation with *B. subtilis* UBT-MO_2 resulted in 26.5 and 37.9% lower excreta NH_3 and H_2S concentrations, respectively, compared with no supplementation.

In conclusion, dietary supplementation with 1×10^5 and 2×10^5 cfu/kg of *L. acidophilus*, *B. subtilis*, and *C. butyricum* could improve the growth performance and apparent ileal digestibility of most essential amino acids. Under the conditions of our study, probiotics could enhance the humoral immunity, beneficially modulate the cecal concentrations of *Lactobacillus* and *E. coli*, and decrease the concentration of ammonia in broiler excreta.

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