Screening lactic acid bacteria to manufacture two-stage fermented feed and pelleting to investigate the feeding effect on broilers

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ABSTRACT *Bacillus subtilis* var. *natto* N21 (BS) and different lactic acid bacteria were applied to produce two-stage fermented feeds. Broilers were fed these feeds to select the best fermented feed. The selected fermented feed was pelleted and investigated for its effects on growth performance, carcass traits, intestinal microflora, serum biochemical constituents, and apparent ileal nutrient digestibility. Trial 1 involved three hundred thirty-six 1-d-old broilers with equal numbers of each sex, randomly assigned into control, BS + *Bacillus coagulans* L12 (BBC), BS + *Lactobacillus casei* (BLC), BS + *Lactobacillus acidophilus* (BLA), BS + *Lactobacillus acidophilus* L15 (BLA15), BS + *Lactobacillus delbruekckii* (BLD), and BS + *Lactobacillus reuteri* P24 (BLR24) groups with 3 replicates per group. Trial 2 involved two hundred forty 1-d-old broilers with equal numbers of each sex, randomly assigned into control, BBC, and pelleted BS + *Bacillus coagulans* L12 fermented feed (PBBC) groups with 4 replicates per group. Trial 3 involved sixteen 21-d-old male broilers randomly assigned into control and PBBC groups with 4 replicates per group for a nutrient digestibility trial. The feed conversion ratio (FCR) in the BBC group was better than the control ($P < 0.05$), and the production efficiency factor (PEF) was the best. However, weight gain (WG), feed intake (FI), and PEF were the lowest in the BLD group ($P < 0.05$). The WG during 0 to 21 d and 0 to 35 d in the PBBC groups were higher than the control ($P < 0.05$). The relative weight of the proventriculus + gizzard in the BBC and PBBC groups were higher than the control ($P < 0.05$). The digestible amino acid content in the PBBC group increased significantly ($P < 0.05$). *Bacillus coagulans* L12 is the best lactic acid bacteria for second stage fermentation. PBBC improved broiler growth performance, which may be due to the higher digestible amino acid content, it has the potential to become a commercial feed.

Key words: broiler, lactic acid bacteria, pellet, two-stage fermented feed

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INTRODUCTION

Probiotic fermentation technologies enhance the concentration of probiotics, enzymes, metabolites, and may change some compounds into more effective components (Stanbury et al., 1995). Using probiotics for feed fermentation has been applied for many years (Cumby, 1986; Boguhn et al., 2006). Two-stage fermentation is a new fermentation technology, Chen et al. (2009) using *Bacillus subtilis* var. *natto* N21 (**BS**) which has high proteolytic capacity for 2 d aerobic feed fermentation in the first stage. *Saccharomyces cerevisiae* Y10 (**SC**), which has greater acidic capacity, is used for the 3 d anaerobic feed fermentation in the second stage. The two-stage BS + SC fermented feed improved broiler BW by 8.5 to 16.5%. This author used the same fermentation process, but replaced the lactic acid bacteria with *Bacillus coagulans* L12 (**BC**) in the second stage (Chang et al., 2007). Both BS + BC and BS + SC fermented feed improved broiler growth performance. The BS + BC fermented feed improvement effect was better than that of BS + SC. This result confirmed that changing the bacteria in the second stage could improve broiler growth performance.

Although two-stage BS + SC fermented feed improved broiler growth performance, its pH value was not low enough. The fermentation acidic capacity in feed may affect the feed quality and improve broiler growth performance. Added acid to feed can avoid moldy feed, improve feed conversion ratio, increase intestinal short chain fatty acids, decrease the stomach pH, and improve growth performance (Li et al.,1998; Partanen, 2001; Piva et al., 2007). If we selected a probiotic with

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higher acidic and reproductive capacity, the fermentation procedure would be shorter and the feed pH would be lower. *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbruekckii*, and *Lactobacillus reuteri* were the common probiotics used in the food and feed industry (Martinez-Cuesta et al, 2001; Olson and Aryana, 2008; Yu et al., 2008; Horiuchi and Sasaki, 2012).

Although two-stage fermented feed improved broiler growth, the wet type feed was difficult to apply to the poultry feed industry. Therefore, this study selected different lactic acid bacteria to manufacture the best two-stage fermented feeds. The selected fermented feed was then pelleted and investigated for its effects on broiler growth performance, carcass traits, intestinal microflora, serum biochemical parameters, and apparent ileal nutrient digestibility.

MATERIALS AND METHODS

Trial 1, the Effect of Inoculated Different Lactic Acid Bacteria in Second Stage Fermentation on 0 to 21 d Broiler Growth Performance

Probiotics and Fermented Feed Preparation BS and BC were selected from traditional food. *Lactobacillus acidophilus* L15 (**LA15**) and *Lactobacillus reuteri* P24 (**LR24**) were selected from chicken intestines. *Lactobacillus casei* (**LC**), *Lactobacillus acidophilus* (**LA**) and *Lactobacillus delbruekckii* (**LD**) were bought from the Food Industry Research and Development Institute (FIRDI, Taiwan). BS was incubated in Tryptone Soya Broth (**BD**) at 37°C in 150 rpm concave bottom-Erlenmeyer flask. BC was incubated in Tryptone Soya Broth at 37°C in 100 rpm Erlenmeyer flask. LA15, LR24, LC, LA, and LD were incubated in Lactobacilli MRS broth (BD) at 37°C in 100 rpm Erlenmeyer flask. After incubation the broth was centrifuged with 8,000 × *g* for 10 min, and the supernatant removed, with the same amount of sterile water added, and then shaken. This step was repeated 3 times to remove the medium.

Sterile water was added to the precipitates to dilute to 10^9 cfu/mL. The diluents ensured that the probiotics were higher than 10^9 cfu/mL using the plate culture method (BS and BC use Tryptone Soya agar (BD); LA15, LR24, LC, LA, and LD use Lactobacilli MRS agar (BD)).

The fermented feed preparation followed the description of Chen et al. (2009). The basal diet composition is shown in Table 1. Feed was supplemented with BS diluent (10^6 cfu/g of feed) and 10% water at 37°C for a 2 d aerobic fermentation in the first stage, then with BC, LA15, LR24, LC, LA or LD (10^6 cfu/g feed) and 13% water at 25 to 35°C for a 5 d anaerobic fermentation in the second stage. The fermented feed was dried using an oven. The feed moisture content was below 12%. All of the fermented feeds were made 3 batches.

Table 1. Composition of the basal diet.

Item	0 to 21 d	21 to 35 d
Ingredient, %		
Yellow corn, grain	44.11	51.65
Soybean oil	5.26	3.94
Full-fat soybean meal, 38%	13.38	16.43
Soybean meal, 44%	33.20	24.40
Dicalcium phosphate	1.61	1.39
Limestone, pulverized	1.51	1,37
Salts	0.33	0.33
DL-Methionine	0.41	0.17
Vitamin premix[1]	0.10	0.10
Mineral premix[2]	0.10	0.10
Total	100.00	100.00
Calculated value		
CP, %	23.0	21.0
ME, kcal/g	3,150	3,200
Calcium, %	0.95	0.85
Available phosphorus, %	0.47	0.42
Met + Cys, %	1.16	0.86
Lys, %	1.32	1.30

[1]Vitamin premix supplied per kilogram of diet: vitamin A, 3,000 IU; vitamin D3, 400 IU; vitamin E, 10 IU; vitamin K3, 1 mg; vitamin B1, 3.6 mg; vitamin B2, 5.4 mg; vitamin B6, 7.0 mg; Ca-pantothenate, 20.0 mg; niacin, 70 mg; biotin, 0.3 mg; folic acid, 1.1 mg; vitamin B12, 0.02 mg.

[2]Mineral premix supplied per kilogram of diet: Cu ($CuSO_4 \bullet 5H_2O$, 25.45% Cu), 8 mg; Fe ($FeSO_4 \bullet 7H_2O$, 20.09% Fe), 80 mg; Mn ($MnSO_4 \bullet H_2O$, 32.49% Mn), 60 mg; Zn (ZnO, 80.35% Zn), 40 mg; Se ($NaSeO_3$, 45.56% Se), 0.15 mg.

Bird Management and Experimental Design Three hundred thirty-six 1-d-old Arbor Acres broiler chicks with equal numbers of both sexes, were randomly assigned into control, BS + BC (**BBC**), BS + LC (**BLC**), BS + LA (**BLA**), BS + LA15 (**BLA15**), BS + LD (**BLD**), and BS + LR24 **(BLR24**) fermented feed groups with 3 replicates per group. The starter weights were 43.2 ± 4.2 g. The feeding trial was carried out for 21 d. Feed (Table 1) and water were provided ad libitum. Bird management followed the Arbor Acres broiler management manual (Arbor Acres, 2012). This experiment was conducted at National Chiayi University with the approval of the animal use committee.

Measurements and Analysis

Feed Physiological Characteristics (1) pH value: 1 g feed was added to 9 mL sterile water and then mixed. The pH value was measured using a portable pH meter (digital pH meter, Goodly, Taiwan). (2) The count of total lactic acid bacteria: 1 g feed was added to 9 mL sterile water and then mixed. The supernatants were diluted 10-fold with buffered peptone water. One hundred microliters of supernatant was smeared onto Lactobacilli MRS agar and incubated at 37°C with 13% CO_2 for 48 h.

Growth Performance Chicken BW and feed intake (**FI**) were recorded each week to calculate weight gain (**WG**), feed conversion ratio (**FCR**), and production efficiency factor (**PEF**) = (Survival rate (%) × BW (kg))/(age (d) × FCR) × 100. When the chickens died, the FI and BW were recorded, and the average WG and FCR calculated.

Trial 2, the Effect of Pelleted BBC Fermented Feeds on Broiler Growth Performance

Pelleted Fermented Feed Preparation The BBC fermentation was the same as in trial 1. After fermentation the feed was dried using an oven (65°C) for 7.5 h. Steam-pelleting was performed by heating the material with steam at 75°C for approximately 20 to 30 s in a pellet press conditioner. The feed was then pelleted using a pellet machine (Wan Der Ful Co., LTD., Taiwan, yield 180 kg/h) with a 3 mm die-hole diameter. The pelleted feed was cooled using an electric fan to reduce the moisture below 12%. The pelleted feed was ground before feeding chickens to facilitate chicken ingestion. All of the fermented feeds were made 3 batches.

Bird Management and Experimental Design Two hundred and forty 1-d-old Arbor Acres broiler chicks with equal numbers of both sexes were randomly assigned into control, BBC, and pelleted BBC feed (**PBBC**) groups with 4 replicates per group. The starter weights were 44.6 ± 5.1 g. The feeding trial was carried out for 35 d. Bird management was the same as in trial 1.

Measurements and Analysis

Feed Physiological Characteristics The pH value and the count of total lactic acid bacteria methods were the same as in trial 1. The count of Bacillus-like: 1 g feed was added to 9 mL sterile water and then mixed. The supernatants were diluted 10-fold with buffered peptone water. One hundred microliters of supernatant was smeared onto Tryptone Soya agar and incubated at 37°C for 24 h. The feed was diluted 10-fold (based on sample weight) with sterile water and vortexed. The samples were then centrifuged at 20,000 g for 20 min. Eight hundred μL of supernatant was added to 100 μL of trichloroacetic acid (1%) and 100 μL of crotonic acid (0.00158 m). The mixture was stored at room temperature overnight and then centrifuged at 20,000 g for 20 min. The supernatant was filtered with 0.45 μm filter. Fifty μL of filtered supernatant was used to determine the acetic acid, propionic acid, butyric acid, and lactic acid concentration using HPLC. HPLC column: Interaction ORH-801; the flow solution: 0.01 N H_2SO_4; flow rate: 0.05 mL/min; temperature: 35°C; detector: UV 210 nm.

Proximate Feed Analysis Moisture (method 930.15), ash (method 923.03), CP (method 990.03), calcium (method 927.02), and phosphorus (method 935.59) analyses were performed according to AOAC (1990) methods. The gross energy was measured with an adiabatic bomb calorimeter (model 356, Parr Instrument Company, Moline).

Feed Amino Acid Analysis Samples for amino acid analyses were hydrolyzed in 6 N HCl for 24 h at 110°C under a N atmosphere. Performic acid oxidation was carried out for the sulfur-containing amino acids Met and Cys before acid hydrolysis. Samples for Trp analysis were hydrolyzed using barium hydroxide (AOAC, 2000; method 982.30 E). The amino acids in the hydrolysate were subsequently determined using HPLC after post column derivation.

Growth Performance The measurements were carried out as in trial 1.

Carcass Traits Eight chicks each from the control, BBC, and PBBC groups were euthanized at 35 d of age to measure the weights of the liver, proventriculus + gizzard, intestine (from duodenum to rectum), abdominal fat (from the gizzard to celiac fat), breast (with bone and skin), and thigh (the fragment from femur to tibia, with bone and skin).

Digestive Tract Contents Ph The crop and cecum contents pH were measured using a portable pH meter (digital pH meter, Goodly, Taiwan).

Intestinal Tract Microflora Population The rectum contents were diluted 10-fold with buffered peptone water and vortexed for 2 min. The supernatant was preserved at −80°C. One hundred microliters of supernatant was smeared onto Plate count agar and MacConkey agar and incubated at 37°C for 24 h, to calculate the total aerobic and coliform bacteria, respectively. One hundred microliters of supernatant was smeared onto Plate count agar and Lactobacilli MRS agar and incubated at 37°C with 13% CO_2 for 48 h, to calculate the total lactic acid and anaerobic bacteria, respectively.

Lymphocyte Proliferation Assay The lymphocyte proliferation assay method was referred to in the description by Merendino et al. (1998). Blood samples were taken from the brachial vein of chickens at 35 d of age. The whole blood was collected with EDTA as an anticoagulant. The blood was centrifuged (1,000 g, 15 min) and plasma removed. RPMI-1640 was then added to the original blood volume. The sample solution was slowly added into Ficoll (the specific gravity was 1.077, 1/2 the original blood volume) in a tube and then centrifuged (250 g, 30 min) to remove the lymphocytes layer. One ml RPMI-1640 (no FBS) was added to the lymphocytes, mixed and placed into an ice bath. The cell activity was examined using the trypan blue dye exclusion test. The concentrations of the final suspensions were adjusted to 5×10^5 cell/mL with RPMI 1640. The lymphocytes were cultured in triplicate in 96 well microtiter plates (Costar 3599; Corning Inc, Corning, NY) at 37°C and 5% CO_2, for 24 h. The RPMI-1640 (as control, content 10% FBS, 10 μL), Lipopolysaccharide (**LPS**, 10 μg/mL, 10 μL), and phorbol myristate acetate (20 ng/mL) + Ionomycin (375 ng/mL) (**PMAION**, 10 μL) was immediately added and the cells were cultured in an incubator at 37°C in a 5% CO_2 atmosphere for 48 h. A 20 μL 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT, Sigma, St. Louis, MO) solution then was added for another 24 h at 37°C and 5% CO_2. After incubation, the 96 well microtiter plates were

Table 2. The effect of different second stage cultures on two-stage fermented feed physiological characteristics[1] (Trial 1).

		Control	Fermented feed[2]						SEM	*P*-value
			BBC	BLC	BLA	BLA15	BLD	BLR24		
pH	wet	6.42[a]	5.03[b]	4.46[d]	4.86[c]	4.85[c]	4.89[c]	4.82[c]	0.03	<0.0001
	dry	6.42[a]	4.99[b]	4.53[d]	4.91[b,c]	4.90[b,c]	4.91[b,c]	4.89[c]	0.03	<0.0001
Total lactic acid bacteria, log cfu/g feed	wet	3.65[e]	8.90[a,b]	8.28[d]	9.00[a]	8.57[c]	8.79[b]	8.45[c,d]	0.06	<0.0001
	dry	3.65[c]	8.75[a]	<5.00	5.81[b]	<5.00	<5.00	<5.00	0.02	<0.0001

[1]Data are means of 3 batches of each feed, each batch was tested in triplicate.
[2]BBC: fermented feed cultures were *Bacillus subtilis* var. *natto* N21 (BS) and *Bacillus coagulans* L12; BLC: fermented feed cultures were BS and *Lactobacillus case*; BLA fermented feed cultures were BS and *Lactobacillus acidophilus*; BLA15: fermented feed cultures were BS and *Lactobacillus acidophilus* L15; BLD: fermented feed cultures were BS and *Lactobacillus delbruekckii*; BLR24: fermented feed cultures were BS and *Lactobacillus reuteri* P24.
[a–e]Means in the same row with different superscripts are significantly different ($P < 0.05$).

centrifuged (900 × *g*, 10 min, 25°C) and the supernatant removed. To each well was added 200 μL alcohol (95%, content 20% DMSO) to dissolve the blue precipitate. The 96 well microtiter plates were centrifuged (250 × *g*, 10 min, 25°C), and 100 μL supernatant was transferred to a flat bottom 96 well microtiter plate. The optical density (**OD**) was measured at 570 nm using an automated ELISA reader (Model 680, Bio-Rad, St. Louis).

Serum Biochemical Constituents Blood samples were taken from the brachial-vein of chickens withdrawn from feed and water for 12 h at 35 d of age. After centrifuging (1,000 g, 15 min), serum was stored at −40°C for further analysis. Blood serum calcium, phosphorus concentrations and amylase, lactate dehydrogenase, glutamate oxaloacetate transaminase, creatine kinase, γ-glutamyl transpeptidase, and alkaline phosphatase activities were analyzed using an automatic blood chemical analyzer with Roche testing kits (Roche COBAS MIRA PLUS, Switzerland). Serum enzyme activity is defined as levels of international units (IU) per liter serum (Bergmeyer, 1983).

Trial 3, the Effect of Pelleted BBC Fermented Feeds on Broiler Apparent Ileal Amino Acid Digestibility

Probiotics and Fermented Feed Preparation Probiotics and fermented feed preparation were conducted the same as in trial 2.

Bird Management and Experimental Design Sixteen 21-d-old male Acres broiler chicks were assigned into control and PBBC group with 4 replicates per each. The chickens were housed in 30 cm × 25 cm × 40 cm cage. Feed and water were ad libitum. Bird management and approval of animal use protocol were the same as trial 1.

The metabolic trial began while the chicks were at 21-d-old. The feed adaptation period was 3 d. Feed mix with 0.3% Cr_2O_3 was then fed to the chicks for 4 d. Digesta samples were taken from the ileum using a distilled water wash bottle. The ileum segment was between Meckel's diverticulum and the ileal-caecal-colonic junction. The digesta pooled by cage. Digesta and feed samples were measured for CP, gross energy and amino acid content. Chromium was analyzed, after the samples were ashed at 600°C for 12 h in a muffle furnace, using inductively coupled plasma mass spectrometry (ICP-AES Vista, Varian, Palo Alto, CA) according to the method of AOAC (2005, method 985.01). The calculation formula is as follows: Apparent ileal digestibility (%) = [1 − (chromium in diet/chromium in ileal digesta) × (nutrient in ileal digesta/nutrient in diet)].

Statistical Analysis

Variances among the treatments were calculated using the GLM procedure (SAS Institute, 2008). Duncan's new multiple-range test was used to compare the means according to Steel and Torrie (1980).

RESULTS AND DISCUSSION

Trial 1

Fermented Feed Physiological Characteristics Table 2 presents the effect of different second stage cultures on two-stage fermented feed physiological characteristics. The pH value of all fermented feeds were lower than the control ($P < 0.05$). The BLC group presented the lowest pH, followed by the BLR24, BLA15, BLA, and BLD groups. The BBC group pH value was higher than the other fermented feed groups ($P < 0.05$). After drying, the feed pH exhibited a similar trend. The total lactic acid bacteria concentration for all fermented feeds reached 10^8 cfu/g with yogurt flavor. The results showed that all of the lactic acid bacteria grew successfully in the two-stage fermentation process. The total lactic acid bacteria concentration in the BLA group was the highest, followed by the BBC, BLD, BLA15, and BLR24 groups. The total lactic acid bacteria concentration in the BLC group was the lowest. After the drying process, the BBC group showed the highest total lactic acid bacteria level, followed by the BLA group. The

Table 3. The effect of different two-stage fermented feeds on broiler growth performance[1] (Trial 1).

	Control	Fermented feed[2]						SEM	*P*-value
		BBC	BLC	BLA	BLA15	BLD	BLR24		
				Weight gain, g/bird					
0 to 21 d	689[a]	749[a]	729[a]	746[a]	715[a]	564[b]	720[a]	26	0.0024
				Feed intake, g/bird					
0 to 21 d	988[a]	994[a]	1,001[a]	1,045[a]	997[a]	796[b]	999[a]	26	0.0002
				Feed conversion ratio, feed intake/weight gain					
0 to 21 d	1.43[a]	1.33[b]	1.37[a,b]	1.40[a]	1.39[a,b]	1.41[a]	1.39[a,b]	0.02	0.0854
Survival rate, %	97.9[a,b]	97.9[a,b]	100[a]	95.8[a,b]	95.8[a,b]	89.6[b]	95.8[a,b]	2.7	0.2662
PEF[3]	238[a]	278[a]	268[a]	259[a]	249[a]	184[b]	251[a]	16	0.0192

[1]Data are means of 3 pens of broilers with 16 broilers per pen.
[2]BBC: fermented feed cultures were *Bacillus subtilis* var. *natto* N21 (BS) and *Bacillus coagulans* L12; BLC: fermented feed cultures were BS and *Lactobacillus case*; BLA fermented feed cultures were BS and *Lactobacillus acidophilus*; BLA15: fermented feed cultures were BS and *Lactobacillus acidophilus* L15; BLD: fermented feed cultures were BS and *Lactobacillus delbruekckii*; BLR24: fermented feed cultures were BS and *Lactobacillus reuteri* P24.
[3]Production efficiency factor, PEF = (Survival rate (%) × BW (kg))/(age (d) × feed conversion ratio) × 100.
[a,b]Means in the same row with different superscripts are significantly different ($P < 0.05$).

other fermented feeds exhibited bacteria levels lower than 10^5 cfu/g.

The authors investigated the pH value and concentration of BC, LA, and LC in Lactobacilli MRS broth for 24 h. The pH values of BC, LA, LC were 4.20, 4.91, 4.32, and the concentrations were 1.8×10^9, 1.1×10^8, 3.6×10^9 cfu/g, respectively. The results shown in Lactobacilli MRS broth culture conditions, BC and LC presented the best acidic capability. LC was the most reproductive lactic acid bacteria. However, the BBC group pH value was higher than the other fermented feed groups in this trail, and the BLA group showed the highest total lactic acid bacteria concentration. The corn-soybean diet and Lactobacilli MRS broth fermentation conditions were different, which presented different fermentation characteristics. Using different lactic acid bacteria in second stage fermentation could affect the feed pH value. LC had the best acidic capability in two-stage fermentation because the BLC group had the lowest pH value. *Lactobacillus casei* has great lactic acid production ability, appropriate for making fermented milk or lactic acid (Kourkoutas et al., 2005). This view was consistent with trial 1. The lactic acid bacteria concentration for the BBC and BLA groups showed BC and LA had the best productive capacity in two-stage fermentation. Lactic acid bacteria produced lactic acid and acetic acid during fermentation. Acetic acid is a volatile organic acid that volatilizes at room temperature. After the drying process, all of the fermented feed groups exhibited significantly decreased lactic acid bacteria except the BBC group. *Bacillus coagulans* is the most commonly used probiotic bacteria in Europe and Asia (Sanders et al., 2003; Vecchi and Drago, 2006). Until 1974, *Bacillus coagulans* was classified as *Lactobacillus sporogenes*. Bergey's Manual (Buchanan and Gibbons, 1974) reclassified this bacterium as *Bacillus coagulans* because, although it shares taxonomic characteristics with the other Lactobacillus species such as producing lactic acid, none of the latter are spore forming (Sanders et al., 2001; Gandhi, 1994). The spores can resist high pressure, high temperature and low pH values (Palop et al., 1999). Therefore, the drying process has no significant effect on the lactic acid bacteria concentration in the BBC group.

Growth Performance Table 3 presents the effect of different two-stage fermented feeds on broiler growth performance. The BBC group had the best growth performance, with 7.0% improved FCR ($P < 0.05$) and 16.3% PEF. The BLD group had the lowest WG, FI, and PEF ($P < 0.05$).

The BBC group showed the best growth performance. Tang et al. (2008) indicated that BS + BC fermented feed improved 0 to 21-d-old broiler growth performance. This result was similar to this trial. BC has spore-forming capability that can withstand drying process. High level lactic acid bacteria concentration may be one of the mechanisms promoting broiler growth in the BBC group. The acidic capacity in BBC is lower than other fermented feed treatments, but it still decreased the pH value of feed. Fermented feed will affect the pH, microflora, fermented products, and decomposition of the substrate. It is not enough to evaluate the feeding effect of fermented feed only by the viable bacterial count and the acidic capacity. The mechanisms of improving the broiler growth performance in the BBC group need further study.

There was no significant difference on growth performance in the BLC, BLA, BLA15, and BLR24 groups. The WG and FCR in the BLD group were lower than in the control ($P < 0.05$). In this trial the fermented feed pH value was 4.53 to 4.99, and the BLD group was 4.91, therefore, the pH value did not cause poor growth in broilers. The lactic acid bacteria concentration in the BLD, BLC, BLA15, and BLR24 groups were below the detection value (lower than 10^5 cfu/g). Only the BLD group had significant negative effects on growth performance. Therefore, low lactic acid bacteria concentration in feed did not cause poor growth in broilers. We observed that the FI in BLD group was bad. *Lactobacillus delbruekckii* was used as a second fermentation

Table 4. The effect of pellet procedure on physiological characteristics of fermented feed[1] (Trial 2).

		Control	Fermented feed[2]		SEM	*P*-value
			BBC	PBBC		
pH	0 to 21 d	6.18[a]	4.71[b]	4.72[b]	0.01	<0.0001
	21 to 35 d	6.16[a]	4.74[b]	4.76[b]	0.02	<0.0001
Lactic acid, mg/g fee d	0 to 21 d	4.05[b]	20.43[a]	20.27[a]	0.18	<0.0001
	21 to 35 d	4.18[b]	20.5[a]	20.47[a]	0.17	<0.0001
Acetic acid, mg/g fee d	0 to 21 d	**	3.81	**		
	21 to 35 d	**	3.54	**		
propionic acid, mg/g fee d	0 to 21 d	**	**	**		
	21 to 35 d	**	**	**		
butyric acid, mg/g fee d	0 to 21 d	**	**	**		
	21 to 35 d	**	**	**		
Bacillus-like, log cfu/g fee d	0 to 21 d	3.35[b]	6.52[a]	6.52[a]	0.03	<0.0001
	21 to 35 d	3.35[b]	6.49[a]	6.52[a]	0.02	<0.0001
Total lactic acid bacteria, log cfu/g fee d	0 to 21 d	3.64[b]	8.11[a]	8.08[a]	0.04	<0.0001
	21 to 35 d	3.60[b]	8.27[a]	8.19[a]	0.03	<0.0001

**Not detected.
[1]Data are means of 3 batches of each feed, each batch was tested in triplicate.
[2]BBC: *Bacillus subtilis* var. *natto* N21 + *Bacillus coagulans* L12 fermented feed; PBBC: BBC was dried by oven at 65°C for 7.5 h, and then pelleted.
[a,b]Mean in the same row with different superscript are significantly different ($P < 0.05$).

probiotic might cause bad feed palatability, further decreasing 19.4% FI and 18.1% WG.

The lactic acid bacteria concentration and fermented feed pH value were not sufficient to explain the growth performance mechanisms in the BBC and BLD groups. The differences in probiotics and fermentation processes might affect growth performance. Although it was difficult to investigate the mechanisms of the different two-stage fermented feeds on broiler growth performance, we could select the best probiotic for two-stage fermented feed via the growth performance results. The BBC group showed the best growth performance in trial 1, therefore, we chose BC as the best second fermentation probiotic.

Trial 2

Fermented Feed Physiological Characteristics

Table 4 presents the pellet procedure effect on the physiological fermented feed characteristics. After fermentation the pH value was significantly reduced and the Bacillus-like and total lactic acid bacteria concentrations were significantly increased ($P < 0.05$). The main organic acids in two-stage fermented feed were lactic acid (20.4 to 20.5 mg/g feed) and acetic acid (3.54 to 3.81 mg/g feed). Propionic acid and butyric acid were below the detection value. The pelleted procedure significantly decreased the acetic acid content, but had no significant effects on the pH value and the Bacillus-like and total lactic acid bacteria concentrations ($P > 0.05$).

Bacillus coagulans has the ability to resist high temperatures. The author confirmed that heating BS and BC to 100°C for 10 min did not affect the survival rate. In trial 2 the heating time and temperature were lower than in these situations, so there was no effect on the BS and BC concentrations. The main organic acids in the fermented feed were lactic acid and acetic acid in this study. These results were similar to that of Chen et al. (2009), which indicated BS + SC fermented feed content for lactic acid (16.48 mg/g) and acetic acid (3.71 mg/g). The acetic acid was volatized at room temperature, so acetic acid was below the detection value. Therefore, the lactic acid was the main acidic source in dried fermented feed. The period of 0 to 21 d is an important period for chicken physiological development; we chose the 0 to 21 d period diet to determine proximate analysis, mineral content, and amino acid composition.

Proximate Analysis, Mineral Content, and Amino Acid Composition Table 5 presents the pellet procedure effect on fermented feed proximate analysis, mineral content, and amino acid composition. The results showed that Thr, Val, Ile, Leu, Phe, and non-essential amino acids in the BBC and PBBC groups were significantly higher than the control group ($P < 0.05$).

Microflora could change compounds into more effective components such as nitrogen-containing compounds, amino acids, peptides, or fatty acids during the fermentation process (Peralta et al., 2008). Shi et al. (2015) indicated that solid-state fermentation of rapeseed cake with *Aspergillus niger* significantly increased total amino acids by 24.3% and essential amino acids by 28.5%. The solid-state fermentation of cottonseed meal with *Candida tropicalis* increased total amino acids by 11.9% and essential amino acids by 12.0% (Khalaf and Meleigy, 2008). These reports were similar to present studies that the total amino acid and essential amino acids concentrations in the feed increased by 6.4% and 5.4% from two-stage fermentation. These statistics confirmed that two-stage fermentation could decompose the feed compounds and change the amino acid composition.

Feed pelleting is used to agglomerate smaller feed particles using mechanical pressure, moisture and heat (Abdollahi et al., 2013; Falk, 1985). The high pelleting

Table 5. The effect of pellet procedure on proximate analysis, mineral content, and amino acid composition of fermented feeds[1] (Trial 2).

	Control	Fermented feed[2]		SEM	*P*-value
		BBC	PBBC		
Proximate analysis					
Ash, %/DM	6.00	6.08	6.14	0.05	0.2724
CP, %/DM	26.3	26.5	25.8	0.2	0.2151
Gross energy, kcal/g/DM	4,730	4,702	4,702	10	0.1508
Calcium, %/DM	0.730	0.693	0.713	0.012	0.1899
Phosphorus, %/DM	0.720	0.727	0.723	0.008	0.8424
Amino acid composition, %/DM					
Total amino acid	19.4[b]	20.7[a]	21.0[a]	0.1	0.0003
Essential amino acid					
Thr	0.825[b]	0.875[a]	0.865[a]	0.006	0.0020
Val	0.853[b]	0.960[a]	0.985[a]	0.020	0.0070
Met	0.355	0.388	0.390	0.011	0.0848
Ile	0.730[b]	0.795[a]	0.800[a]	0.017	0.0434
Leu	1.73[b]	1.84[a]	1.85[a]	0.01	0.0006
Trp	0.680	0.715	0.713	0.011	0.0787
Phe	1.01[b]	1.10[a]	1.12[a]	0.01	0.0002
Lys	1.22	1.21	1.22	0.01	0.9143
His	0.575	0.575	0.565	0.006	0.4219
Arg	1.43	1.46	1.46	0.02	0.3786
Non-essential amino acid					
Asp	2.22[b]	2.33[a]	2.35[a]	0.02	0.0025
Ser	1.08[b]	1.12[a]	1.12[a]	0.01	0.0285
Glu	3.68[b]	4.00[a]	4.04[a]	0.03	0.0001
Pro	1.07[b]	1.16[a]	1.16[a]	0.01	0.0007
Gly	0.875[b]	0.945[a]	0.950[a]	0.009	0.0024
Ala	0.970[b]	1.055[a]	1.068[a]	0.005	<0.0001
Cys	0.185[b]	0.205[a]	0.205[a]	0.003	0.0080

[1]Data are means of 3 batches of each feed, each batch was tested in triplicate.
[2]BBC: *Bacillus subtilis* var. *natto* N21 + *Bacillus coagulans* L12 fermented feed; PBBC: BBC was dried by oven at 65°C for 7.5 h, and then pelleted.
[a,b]Mean in the same row with different superscript are significantly different ($P < 0.05$).

processing temperatures may also result in the marked degradation of heat-sensitive amino acids such as Cys, Lys, Arg, Thr, and Ser (Pickford, 1992; Silversides and Bedford, 1999; Papadopoulos, 1989).

Probiotics fermentation could decompose carbohydrates and proteins that may affect the feed proximate analysis results (Suganuma et al., 2007; Ward et al., 2006; Hong et al., 2004). However, the BS + SC two-stage fermentation process had no significant effects on feed proximate analysis (Chen et al., 2009). The results were similar in this trial. Camire et al. (1990) indicated that the pelleting process could not affect feed proximate analysis. This result agrees with the present study.

Growth Performance Table 6 presents the pelleted two-stage fermented feeds effect on broiler growth performance. The WG in the BBC and PBBC groups were higher than the control at 0 to 21-d-old ($P < 0.05$). The WG in the PBBC groups were higher than the control at 0 to 35-d-old ($P < 0.05$), and improved by 14.2% PEF.

In trial 1 the BBC group improved WG by 8.7% at 0 to 21-d-old. These results were similar to the improvements in WG for the BBC group in this trial. Tang et al. (2008) indicated that feeding BS + BC two-stage

Table 6. The effect of pelleted two-stage fermented feeds on broiler growth performance[1] (Trial 2).

	Control	Fermented feed[2]		SEM	*P*-value
		BBC	PBBC		
0 to 21 d					
Weight gain, g/bird	741[b]	781[a]	792[a]	6	0.0004
Feed intake, g/bird	1,001	1,047	1,030	14	0.1124
Feed conversion ratio, feed intake/weight gain	1.35	1.34	1.30	0.02	0.0921
21 to 35 d					
Weight gain, g/bird	1,117	1,130	1,187	30	0.3275
Feed intake, g/bird	1,984	1,927	1,960	38	0.5771
Feed conversion ratio, feed intake/weight gain	1.78	1.71	1.65	0.07	0.4598
0 to 35 d					
Weight gain, g/bird	1,858[b]	1,911[a,b]	1,981[a]	27	0.0510
Feed intake, g/bird	2,985	2,973	2,990	46	0.9657
Feed conversion ratio, feed intake/weight gain	1.61	1.56	1.50	0.04	0.2407
Survival rate, %	96.3	95.0	96.3	2.0	0.8743
PEF[3]	327	342	373	17	0.2354

[1]Data are means of 4 pens of broilers with 20 broilers per pen.
[2]BBC *Bacillus subtilis* var. *natto* N21 + *Bacillus coagulans* L12 fermented feed; PBBC: BBC was dried by oven at 65°C for 7.5 h, and then pelleted.
[3]Production efficiency factor, PEF = (Survival rate (%) × BW (kg))/(age (d) × feed conversion ratio) × 100.
[a,b]Means in the same row with different superscripts are significantly different ($P < 0.05$).

fermented feed could significantly increase broiler BW and FI at 0 to 21, 21 to 39, and 0 to 39-d-old. After oven drying the BS + BC fermented feed still improved broiler BW and FCR at 0 to 21, 21 to 39, and 0 to 39-d-old (You et al., 2008). The results from trial 1, trial 2 and these literatures showed that BBC fermented feed could improve broiler growth performance. The improvement effect remained after the drying procedure.

The feed industry uses feed pelleting technique to improve poultry WG and FCR. This technique increases the nutrient density per unit FI, decreases feed waste, and lowers the heat increment during digestion (Skoch et al., 1983; Behnke, 1994; Wondra et al., 1995; Medel et al., 2004). The pressure and heat in the pelleting procedure could degrade anti-nutrients, destroy the cell walls, and then improve nutrient utilization (Pickford, 1992; Silversides and Bedford, 1999; Medel et al., 2004). The WG in PBBC group was significantly higher than control by 6.6%. Although there were no significant difference with BBC group, the WG, FCR, and PEF in PBBC group improved by 3.7, 3.8, and 9.1%, respectively. The PBBC group not only has good growth performance, but also fit in with the pellet feed for the broiler industry. Therefore, the feeding value of PBBC group was better than BBC group.

Carcass Traits Table 7 presents the pelleted two-stage fermented feed effect on broiler carcass traits. The relative weight of the proventriculus + gizzard in the BBC and PBBC groups were significantly higher than the control ($P < 0.05$).

The proventriculus and gizzard are important digestive organs in poultry. In this trial, FI showed no significant difference in 0 to 35-d-old poultry among

Table 7. The effect of pelleted two-stage fermented feeds on broiler carcass traits[1] (Trial 2).

	Control	Fermented feed[2]		SEM	*P*-value
		BBC	PBBC		
	Relative weight, g/100 g of BW				
Dressing percentage	80.2	80.2	81.5	0.9	0.7233
Liver	2.27	2.22	2.22	0.10	0.9376
Intestine	5.47	5.48	5.61	0.25	0.7641
Proventriculus + gizzard	2.13[b]	2.56[a]	2.57[a]	0.11	0.0315
Abdominal fat	2.15	2.11	2.26	0.18	0.8491
Breast	21.3	21.6	21.7	0.6	0.9492
Thigh	21.2	21.2	21.6	0.3	0.6526

[1]Data are means of 4 pens of broilers, each pen was used 2 broilers.
[2]BBC: *Bacillus subtilis* var. *natto* N21 + *Bacillus coagulans* L12 fermented feed; PBBC: BBC was dried by oven at 65°C for 7.5 h, and then pelleted.
[a,b]Means in the same row with different superscripts are significantly different ($P < 0.05$).

Table 8. The effect of pelleted two-stage fermented feeds on crop and cecum pH value and rectum microflora in broiler[1] (Trial 2).

	Control	Fermented feed[2]		SEM	*P*-value
		BBC	PBBC		
	pH value				
Crop	4.85	4.78	4.83	0.10	0.9155
Cecum	6.33[b]	6.98[a]	6.57[a,b]	0.17	0.0459
	Rectum microflora, log cfu/g				
Total bacterial count	6.96	7.12	7.22	0.20	0.6181
Total anaerobic plate count	7.78	7.96	7.72	0.16	0.6684
E. coli	6.72	6.88	6.97	0.23	0.7032
Total lactic acid bacteria	8.11	8.25	8.09	0.18	0.8811

[1]Data are means of 4 pens of broilers, each pen was used 2 broilers.
[2]BBC: *Bacillus subtilis* var. *natto* N21 + *Bacillus coagulans* L12 fermented feed; PBBC: BBC was dried by oven at 65°C for 7.5 h, and then pelleted.
[a,b]Means in the same row with different superscripts are significantly different ($P < 0.05$).

treatments. This means that BBC fermented feed enhanced the relative weight of the proventriculus + gizzard, and that was not caused by FI. You et al. (2008) indicated that feeding BS + BC fermented feed significantly increased the relative proventriculus + gizzard weight and had no significant effect on FI, these results agreed with trial 2. These results confirmed that whether through the pelleting process or not, feeding two-stage fermented feed increased the relative weight of broiler proventriculus + gizzards.

The pelleting process converts the mashed diet into pellets. It also reduces the size of the mash particles, which affects the gastrointestinal digestive process (Amerah et al., 2007). A mash diet, in particular one that is coarsely ground, tends to remain longer in the gizzard, thus increasing the mechanical stimulation of this organ (Nir et al., 1994; Hetland and Svihus, 2001; Engberg et al., 2002). Numerous literatures reported heavier gizzard and longer intestine in mash-fed broilers compared to pellet fed birds (Mirghelenj and Golian, 2009; Abdollahi et al., 2011). However, the relative weight of proventriculus + gizzard and intestine in the BBC and PBBC groups showed no significant difference in trial 2. This may be due to the fermentation influenced the pellet effect on the relative weight of the proventriculus + gizzard weights. The liver is the main lipogenic organ in poultry. The presented study showed no significant effect on the relative weight of the liver, therefore, the abdominal fat also showed no significant effect. The WG in the PBBC group was significantly higher than the control, but there was no significant effect on the dressing percentage and the breast and thigh relative weights.

Intestinal pH and Microflora Table 8 presents the effect of pelleted two-stage fermented feeds on the crop and cecum pH values and the broiler rectum microflora. The cecum pH value in the BBC group was significantly higher than the control, followed by the PBBC group.

In many references, the feed was withdrawn from the chickens for 12 h before sacrifice to eliminate the digestive tract content (Sun et al., 2017; Ghosh et al., 2016; Yang et al., 2016). However, fasting eliminates digestive tract content and then affects the gastrointestinal pH value. Thus, You et al. (2008) tried to investigate the effects of dried BS + BC fermented feed on the gastrointestinal pH value of broilers under non-fasting conditions, and the results showed that BS + BC fermented feed significantly increased the cecum pH value. This is similar to trial 2. Feed fermentation may reduce the intestinal microbial available substrate, and this may affect the fermentation and pH in the cecum (van Winsen et al., 2002). Although the fermented feed increased cecum pH, it was still within the normal physiological range (5.8 to 6.8) (Huyghebaert, 2003).

La Ragione et al. (2001) reported that a single oral inoculum of 2.5×10^8 *Bacillus subtilis* spores was sufficient to suppress all aspects of *E. coli* O78:K80 infection. Riazi et al. (2009) found that *Bacillus coagulans* ATCC 7050 produced an antimicrobial protein. There was no significant effect on the rectum microflora in the BBC and PBBC group in this trial. This result agreed with Chen et al. (2009) that BS + SC fermented feed improved broiler growth performance, but had no significant effect on the intestinal microflora. We did not select the fermentation strains in this study based on their antibacterial or colonial ability. Therefore, the fermented feed groups had no significant effects on the intestinal microflora.

Peripheral Blood Lymphocyte Proliferation Table 9 presents the pelleted two-stage fermented feed effect on broiler peripheral blood lymphocyte proliferation. The pelleted fermented feed groups showed no significant differences in broiler peripheral blood lymphocyte proliferation at different mitogen stimulations ($P > 0.05$).

The probiotics have the potential to promote intestinal lymphoid tissue activity and intestinal immune state (Lin, 2004). The in vitro peripheral blood lymphocyte proliferation is one of the methods used for investigating the non-specific lymphocyte proliferation mechanism. This method separated the lymphocytes

Table 9. The effect of pelleted two-stage fermented feeds on broiler peripheral blood lymphocytes proliferation[1] (Trial 2).

	Control	Fermented feed[2]		SEM	*P*-value
		BBC	PBBC		
LPS/C[3]	1.06	1.05	1.01	0.03	0.5612
PMAION5/C[4]	0.83	0.84	0.86	0.05	0.9004

[1]Data are means of 4 pens of broilers, each pen was used 2 broilers.
[2]BBC: *Bacillus subtilis* var. *natto* N21 + *Bacillus coagulans* L12 fermented feed; PBBC: BBC was dried by oven at 65°C for 7.5 h, and then pelleted.
[3]Lymphocytes proliferation: Lipopolysaccharide (OD570 nm)/RPMI-1640 (OD570 nm).
[4]Lymphocytes proliferation: Phorbol myristate acetate + Ionomycin (OD570 nm) /RPMI-1640 (OD570 nm).

Table 10. The effect of pelleted two-stage fermented feeds on broiler serum biochemical constituents[1] (Trial 2).

	Control	Fermented feed[2]		SEM	*P*-value
		BBC	PBBC		
Amylase, U/L	681	653	1,100	226	0.3591
Glutamate oxaloacetate transaminase, U/L	266	288	286	12	0.5855
γ-glutamyl transpeptidase, U/L	24.3	19.9	20.8	1.4	0.1241
Lactate dehydrogenase, U/L	2,758[a]	2,195[a,b]	1,753[b]	184	0.0076
Creatine kinase, U/L	8,690	7,786	8,327	767	0.4976
Alkaline phosphatase, U/L	4,049	1,650	3,024	1,121	0.2173
Calcium, mg/dL	10.4	10.7	11.2	0.6	0.7661
Phosphorus, mg/dL	8.65	8.91	9.25	0.67	0.8849

[1]Data are means of 4 pens of broilers, each pen was used 2 broilers.
[2]BBC: *Bacillus subtilis* var. *natto* N21 + *Bacillus coagulans* L12 fermented feed; PBBC: BBC was dried by oven at 65°C for 7.5 h, and then pelleted.
[a,b]Mean in the row with different superscripts are significantly different ($P < 0.05$).

from the blood and then added mitogens (ex: LPS) to stimulate lymphocyte proliferation. The results showed that two-stage fermented feed did not affect the broiler non-specific lymphocyte proliferation.

Serum Biochemical Constituents Table 10 presents the pelleted two-stage fermented feed effect on broiler serum biochemical constituents. The serum lactate dehydrogenase activity in the PBBC group was significantly lower than the control ($P < 0.05$).

A healthy animal's serum biochemical constituents are in the normal range. The animal's physiological state and stress affect these parameters They can be used for animal health diagnosis (Boyd, 1988; Bai et al., 1996). Lactate dehydrogenase is widely distributed over egg, liver, heart, kidney, and muscle. Creatine kinase is a specific muscle and heart enzyme (Wang, 1992). Glutamate oxaloacetate transaminase is a pointer to liver damage (Tietz, 1995). Alkaline phosphatase is a specific cell membrane and endoplasmic reticulum enzyme (Wright and Plummer, 1974). While poultry grows rapidly, the bone cells break and the alkaline phosphatase activity in the blood will be higher (Bell and Freeman, 1971). The pancreas is the organ that has the highest amylase activity. Pancreatic function can be diagnosed using amylase. γ-glutamyl transpeptidase is one of the cell membrane enzymes, related to glutathione metabolism and the amino acid absorption in the glomerulus and small intestine (Kaplan and Pesce, 1996). γ-glutamyl transpeptidase is a poultry kidney specific enzyme.

The authors' previously study demonstrated that the BBC two-stage fermentation decomposed the large molecular feed proteins and degraded anti-nutrients such as glycinin or β-Conglycinin (data not shown). However, further study is needed to confirm whether the growth performance improvement and decrease in serum lactate dehydrogenase activity were caused by fermentation substrate decomposition. In addition, the pelleting process heat treatment may also reduce anti-nutrients. This may be one of the reasons lower lactate dehydrogenase activity was found in the PBBC group.

Table 11. The effect of pelleted two-stage fermented feeds on broiler apparent ileal digestibility of nutrient[1] (Trial 3).

	Control	PBBC[2]	SEM	*P*-value
CP, %	89.8	92.8	2.5	0.5068
Gross energy, %	83.2	83.5	2.7	0.9451
Total amino acid, %	90.2	92.8	3.4	0.6012
Essential amino acid, %				
Thr	87.7	90.6	4.8	0.6199
Val	87.0	91.2	4.4	0.5303
Met	91.1	93.7	3.0	0.5691
Ile	88.3	91.5	3.9	0.5945
Leu	89.7	92.4	3.5	0.6066
Trp	91.0	93.8	2.9	0.5230
Phe	90.5	93.2	3.2	0.5748
Lys	87.6	90.5	4.6	0.6708
His	92.2	93.6	2.7	0.7267
Arg	91.6	93.9	2.9	0.5975
Non-essential amino acid, %				
Asp	91.1	93.2	3.1	0.6552
Ser	91.2	93.6	3.7	0.5406
Glu	92.0	93.9	2.7	0.6416
Pro	91.0	93.7	3.0	0.4590
Gly	88.2	91.4	4.1	0.5988
Ala	88.4	91.7	3.9	0.5820
Cys	90.0	92.7	3.1	0.5565

[1]Data are means of 4 pens of broilers, each pen was used 2 broilers.
[2]PBBC: *Bacillus subtilis* var. *natto* N21 + *Bacillus coagulans* L12 fermented feed was dried by oven at 65°C for 7.5 h, and then pelleted.

Trial 3

Apparent Ileal Digestibility Table 11 presents the pelleted two-stage fermented feed effect on broiler apparent ileal nutrient digestibility. The apparent ileal CP, gross energy, and amino acid digestibility's in the PBBC group were higher than in the control, but it did not reach significant difference ($P > 0.05$).

The corn-soybean feed produced through the BS + SC two-stage fermentation process could significantly improve 21-d-old broiler gross energy retention (Chen et al., 2009). The corn-soybean feed produced through the BS + BC two-stage fermentation process could significantly improve 21-d-old broiler CP retention

Table 12. The digestible amino acid content of pelleted two-stage fermented feeds[1] (Trial 3).

	Control	PBBC[2]	SEM	*P*-value
Total amino acid, %/DM	17.5[b]	19.5[a]	0.1	0.0002
Essential amino acid, %/DM				
Thr	0.724[b]	0.784[a]	0.006	0.0017
Val	0.731[b]	0.898[a]	0.004	<0.0001
Met	0.323[b]	0.375[a]	0.008	0.0168
Ile	0.645[b]	0.732[a]	0.008	0.0017
Leu	1.55[b]	1.70[a]	0.01	0.0005
Trp	0.619[b]	0.689[a]	0.011	0.0207
Phe	0.91[b]	1.04[a]	0.01	0.0002
Lys	1.06[b]	1.12[a]	0.01	0.0038
His	0.530	0.529	0.006	0.8854
Arg	1.31[b]	1.42[a]	0.01	0.0005
Non-essential amino acid, %/DM				
Asp	2.02[b]	2.19[a]	0.02	0.0026
Ser	0.98[b]	1.04[a]	0.01	0.0093
Glu	3.38[b]	3.79[a]	0.02	0.0003
Pro	0.97[b]	1.13[a]	0.01	0.0017
Gly	0.772[b]	0.882[a]	0.008	0.0011
Ala	0.857[b]	0.990[a]	0.005	<0.0001
Cys	0.170[b]	0.190[a]	0.003	0.0032

[1]Data are means of 3 batches of each feed, each batch was tested in triplicate.

[2]PBBC: *Bacillus subtilis* var. *natto* N21 + *Bacillus coagulans* L12 fermented feed was dried by oven at 65°C for 7.5 h, and then pelleted.

[a,b]Mean in the row with different superscripts are significantly different ($P < 0.05$).

(Tang et al., 2008). However, the apparent ileal nutrient digestibility in trial 3 did not reach significant difference. The nutrient digestibility may not be a mechanism for improving growth performance in the PBBC group. Trials 2 confirmed that the PBBC had higher amino acid contents than control. Therefore, we further calculated the digestible amino acids content in the feed (Table 12). The results showed that the digestible amino acid contents in the PBBC group were significantly higher than in the control ($P < 0.05$) except for His. The PBBC group provided more digestible amino acid to broilers, and this may improve the growth performance.

CONCLUSION

Bacillus coagulans L12 is the best lactic acid bacteria for second stage fermentation. The PBBC feed improves broiler growth performance possibly due to the higher digestible amino acid content. It has potential to be a commercial feed.

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