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Original research article

Novel probiotics: Their effects on growth performance, gut development, microbial community and activity of broiler chickens

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ABSTRACT

A total of 294 one-day-old Cobb broiler chickens were used to investigate the effects of four *Lactobacillus* strains on gut microbial profile and production performance. The six dietary treatments, each with 7 replicates were: (i) basal diet (negative control); (ii) one of four strains of *Lactobacillus* (tentatively identified as *Lactobacillus johnsonii*, *Lactobacillus crispatus*, *Lactobacillus salivarius* and an unidentified *Lactobacillus* sp.) and (iii) basal diet with added zinc-bacitracin (ZnB, 50 mg/kg). Results showed that the addition of probiotic *Lactobacillus* spp. to the feed did not significantly improve weight gain, feed intake and feed conversion rate (FCR) of broiler chickens raised in cages during the 6-week experimental period, but tended to increase the number of total anaerobic bacteria in the ileum and caeca, and the number of lactic acid bacteria and lactobacilli in the caeca; and to significantly increase the small intestinal weight (jejunum and ileum). Furthermore, all 4 probiotics tended to reduce the number of *Enterobacteria* in the ileum, compared with the control treatments. The probiotics did not affect the pH and the concentrations of short chain fatty acids (SCFA) and lactic acid in both the ileum and caeca.

1. Introduction

The use of probiotics has become a field of science, medicine and business that is growing rapidly. In agricultural science, probiotic, prebiotics, feed enzymes and organic acids, have been seen as potential alternatives to in-feed antibiotics (IFA) (Choct, 2002).

The addition of either pure *Lactobacillus* cultures or mixtures of lactobacilli and other bacteria to broiler diets has produced variable results. Kalavathy et al. (2003) found an improvement in body weight gain (BWG) and feed conversion ratio (FCR) of broilers fed a mixture of different *Lactobacillus* strains from 1 to 42 days of age. A consistent improvement in BWG of chickens fed a culture of *Lactobacillus* has also been reported (Awad et al., 2009). Feeding broiler chickens up to 6 weeks of age with a diet containing a single strain of *Lactobacillus acidophilus* or a mixture of lactobacilli significantly improved BWG and FCR (Jin et al., 1998a). Cao et al. (2013) found that supplementation the broiler diets with a single strain of *Lactobacillus* (*Enterococcus faecium*) significantly improved the BW and BWG compared to the control. However, Ashayerizadeh et al. (2011) did not find any significant difference in the performance of chickens fed on diets containing a mixture of *Lactobacillus* cultures and other bacteria, compared with a non-supplemented diet. Variation in the effects of probiotics on growth performance of broiler chickens may be attributed to the differences in the strains of bacteria used as the dietary supplements.

In the present study, the effects of four strains of *Lactobacillus* spp. on pH, the concentrations of short chain fatty acids (SCFA) and lactic acid, and growth performance of broiler chickens were investigated; the populations of total anaerobic bacteria, lactic acid bacteria, *Lactobacilli*, *Enterobacteria* and *Clostridium perfringens* in gut environment were detected.

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2. Materials and methods

2.1. Probiotic strains

A total of 235 *Lactobacillus* isolates were tested using an antagonistic activity assay as described by Schillinger and Lucke (1989), Teo and Tan (2005), and the four strains of *Lactobacillus* isolates were selected as probiotic candidates by largest inhibition zone appearance with indicator pathogenic strains of *C. perfringens* and *Escherichia coli*. These four strains of *Lactobacillus* were tentatively identified as *Lactobacillus johnsonii, Lactobacillus crispatus*, *Lactobacillus salivarius* and one unidentified *Lactobacillus* sp.

All the strains were kept at −20 °C in de Man, Rogosa, Sharpe (MRS) broth (Oxoid, CM0359) with 40% glycerol. The culture medium used for growth was MRS agar (Oxoid, CM0361). The overnight culture of each *Lactobacillus* isolate was used as a feed additive probiotic candidate after anaerobic incubation at 39 °C for 24 h.

2.2. Experimental design and bird management

A total of 294 one-day-old male Cobb broiler chickens vaccinated against Marek's disease, infectious bronchitis, and Newcastle disease were randomly assigned to 6 diets each with 7 replicates with 7 birds per replicate. Chickens were reared in multi-tiered brooder cages placed in a climate-controlled room up to 21 d, and then the birds were transferred to a metabolic cage room to 35 d. Feed and water were provided ad libitum. The room temperature was gradually decreased from 33 °C on d 1 to 24 °C on d 35. Eighteen hours of light was provided per day throughout the trial, excluding d 1 to 7 during which 23 h of light was provided. Each cage was equipped with a feeding and water trough placed outside and also an excreta collection tray. The commercial starter and finisher diets was formulated by Ridley AgriProducts (Tamworth, NSW, Australia) as shown in (Table 1) and fed as a one-phase mash feed to avoid inactivation of the probiotics. Four strains of *Lactobacillus* (No 1286 tentatively identified as *L. johnsonii*, No 709 tentatively identified as *L. crispatus*, No 697 tentatively identified as *L. salivarius* and No 461 unidentified *Lactobacillus* sp.) were selected as probiotic candidates and added to the feed to make up four different treatments. Two control treatments were also included, a negative control, with no additives and a positive control treatment with the antibiotic, zinc-bacitracin (ZnB, 50 mg/kg), added. The experimental diets with the probiotic candidates were mixed weekly. The individual strains were grown in MRS broth contained 5 g/L of yeast extract (powder, Oxoid, LP0021) and 20 g/L of glucose, for overnight (at 39 °C) and harvested by centrifugation at 4420× *g* for 15 min (Induction Drive Centrifugation, Beckman Model J2-21M, Beckman Instruments Inc., Palo Alto, California, USA), resuspended in phosphate buffered saline (PBS, pH 7.4) and mixed into a premix with the basal diet for 10 min using a miniature mixer. This premixture of product with feed (1 kg) was then transferred into a larger mixer (total capacity 300 kg) where the final volume of the weekly feed batch was prepared. The mixer equipment was thoroughly cleaned between the mixing of different treatments by using a vacuum cleaner and a wash diet (basal feed).

2.3. Probiotic bacterial concentrations in feed samples

Representative feed samples of each feed batch were tested for bacterial concentrations on d 1, 3, and 7 of each week during the experimental period. Ten grams of sample feed were dissolved in 90 mL of peptone water (Oxoid, CM0009) and 10-fold dilutions were performed in Hungate tubes with 9 mL of peptone water. The numbers of lactic acid bacteria in the feed samples were determined on de MRS agar inoculated with 0.1 mL of diluted sample and after anaerobic incubation at 39 °C for 48 h.

Representative samples from all experimental feeds were tested as above for bacterial concentrations before being added to the probiotic candidates to make up six different treatments.

Table 1
Ingredient composition and calculated chemical composition of basal diets (as-fed basis).

Item	1 to 3 weeks (Starter)	4 to 6 weeks (Finisher)
Ingredient, g/kg		
Wheat	262.0	214.0
Sorghum	350.25	400.2
Mung beans	100.0	100.0
Tallow in mixer	32.5	34.0
Sunflower meal		25.0
Canola meal	60.0	60.0
Cottonseed meal		50.0
Soybean meal	157.0	81.5
Limestone B10	15.5	16.0
Kynofos/Biofos MDCP	11.5	11.0
Salt	1.75	1.5
Sodium bicarbonate	2.0	2.0
Choline Chloride (75%)	0.6	0.6
DL-Methionine	2.1	1.3
L-Lysine scale 3	2.1	0.4
L-Threonine	0.2	
Vitamin and mineral premix[a]	2.5	2.5
Calculated chemical composition, g/kg		
ME, MJ/Kg	12.26	12.39
Crude protein	200.02	190.00
Crude fibre	35.17	43.14
Crude fat	52.16	54.47
Lys	11.49	8.98
Met + Cys	8.32	7.37
Ca	9.73	9.79
Available phosphorous	6.50	6.71
Na	1.62	1.65
Cl	2.19	1.75

[a] Vitamin and mineral premix contained the following: vitamin A (as *all-trans* retinol), 12,000 IU; cholecalciferol, 3500 IU; vitamin E (as D-α-tocopherol), 44.7 IU; vitamin B_{12}, 0.2 mg; biotin, 0.1 mg; niacin, 50 mg; vitamin K_3, 2 mg; pantothenic acid, 12 mg; folic acid, 2 mg; thiamine, 2 mg; riboflavin, 6 mg; pyridoxine hydrochloride, 5 mg; D-calcium pantothenate, 12 mg; Mn, 80 mg; Fe, 60 mg; Cu, 8 mg; I, 1 mg; Co, 0.3 mg; and Mo, 1 mg.

2.4. Sample collection and processing

Feed leftovers and birds were weighed on a weekly basis for calculation of average feed intake and body weight. Mortality was recorded when it occurred and FCR (feed intake/weight gain) was corrected for mortality. On d 21 and 35, two birds from each cage were randomly selected and killed by cervical dislocation. The abdominal cavity was opened and visceral organs were weighed. The weight and the length of the full small intestine and then the empty weight of each intestinal segment were recorded.

The contents of the gizzard were collected into plastic containers. An approximately 2 cm piece of the proximal ileum was flushed with ice-cold PBS at pH 7.4 and fixed in 10% formalin for morphological measurements. The contents of the ileum and caeca were collected, and then stored at −20 °C until volatile fatty acids (VFA) analysis was performed.

2.5. Enumeration of intestinal bacteria

About 1 g of fresh digesta samples from the ileum and caeca were transferred into 15 mL MacCartney bottles containing 10 mL of anaerobic broth. The suspension was homogenized for 2 min in CO_2-flushed plastic bags using a bag mixer (Interscience, St. Norm, France) and serially diluted in 10-fold increments in anaerobic

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broth according to the technique of Miller and Wolin (1974). One millilitre of the homogenized suspension was then transferred into 9 mL of anaerobic broth and serially diluted from 10^{-1} to 10^{-5} (for the ileal samples) or 10^{-1} to 10^{-6} (for the caecal samples). From the last three diluted samples, 0.1 mL each was plated on the appropriate medium (10 mL) for enumeration of microbial populations.

Total anaerobic bacteria were determined using anaerobic roll tubes containing 3 mL of Wilkins-Chalgren anaerobe agar (Oxoid, CM0619) incubated at 39 °C for 7 days. Lactic acid bacteria were enumerated on MRS agar (Oxoid, CM0361) incubated in anaerobic conditions at 39 °C for 48 h. Coliforms and lactose-negative *Enterobacteria* were counted on MacConkey agar (Oxoid, CM 0007) incubated aerobically at 39 °C for 24 h as red and colourless colonies, respectively. Lactobacilli were enumerated on Rogosa agar (Oxoid, CM 0627) after anaerobic incubation at 39 °C for 48 h. Numbers of *C. perfringens* were counted on Tryptose-Sulfite-Cycloserine and Shahidi-Ferguson Perfringens agar base (TSC & SFP) (Oxoid, CM0587 OPSP) mixed with egg yolk emulsion (Oxoid, SR0047) and Perfringens (TSC) selective supplement (Oxoid, SR0088E) according to the pour-plate technique, where plates were overlaid with the same agar after spreading the inoculums and incubated anaerobically at 39 °C for 24 h. All plates were incubated in the anaerobic cabinet (Model SJ-3, Kalter Pty. Ltd., Edwardstown, SA, Australia) and bacterial number counted using colony counter (Selby, Model SCC100, Biolab Australia, Sydney, NSW, Australia).

2.6. Gut histomorphology

Tissue samples were collected from the proximal ileum and flushed with buffered saline and fixed in 10% neutral buffered formalin for histomorphological analysis. Samples were embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. Sample sections were captured at 10× magnification using a Leica DM LB microscope (Leica Microscope GmbH, Wetzlar, Germany) and morphometric indices were determined as described by Iji et al. (2001). Each sample was measured in 15 vertically, well-oriented, intact villi, muscle depth and crypts photomicrographs of a stage micrometer recorded at 5× magnification.

2.7. Digesta pH, VFA, lactic acid and succinic acid analyses

Intestinal pH was measured immediately after death and excision of viscera at d 21 and 35. The pH of ileal and caecal contents was determined by the modified procedure of Corrier et al. (1990). After thawing at room temperature, the concentrations of SCFA and lactic acid of each digesta sample from the ileum and caeca were measured using gas chromatography (Varian CP-3800. Netherlands) according to the method described by Jensen et al. (1995).

2.8. Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) (StatGraphics Plus version 5.1 – Professional Edition, Manugistics Inc., Rockville, Maryland, USA) and the differences between mean values were identified by the least significant difference (LSD). Differences between treatments were deemed to be significant only if the P value was <0.05. All results were expressed as means. Bacterial counts were transformed to $\log_{10}$ values.

2.9. Animal ethics

The Animal Ethics Committee of the University of New England approved this study (authority number AEC 06/093). Health and animal husbandry practices complied with the 'Australian code of the care of animals for scientific purposes' issued by the National Health and Medical Research Council (NHMRC, 2004).

3. Results

3.1. Lactic acid bacterial (LAB) concentration in feed samples

The experimental diets were prepared weekly. The concentration of LAB reached 8.57 lg cfu/mL (the highest) when the probiotic candidates were re-suspended in PBS solution (Table 2). Furthermore, the high concentrations of LAB (>5.04 lg cfu/g feed, the highest being 6.83 lg cfu/g feed) were observed from the probiotic treatments compared with the negative and positive control treatments.

The concentration of LAB in feed decreased as each feeding week progressed (probiotic-containing diets were freshly made on a weekly basis, typically at the beginning of the week).

3.2. Gross response

There were no significant ($P > 0.05$) effects on BWG, feed intake (FI) or FCR when the probiotic candidates were added into the feed during the 6-week experimental period (Table 3). Although there were no major differences in mortalities in the different treatments (range 0 to 7.14%).

3.3. Visceral organ weights

Probiotics increased ($P < 0.01$) the relative weight of the jejunum and ileum in 21-day-old chickens (Table 4), as well as that of the ileum in 42-day-old birds compared with controls. The weights of liver, spleen, pancreas, bursa, gizzard and duodenum were not affected by the treatments.

Table 2
Lactic acid bacteria count (lg cfu/g) in feed samples from experimental diets during 1 to 42 d.

Item	Diet					
	NC	PC	Iso461	Iso69	Iso709	Iso1286
PBS solution	–	–	8.32	8.20	8.30	8.57
Week 1						
1st day	4.04	3.75	6.64	6.23	6.68	6.73
3rd day	4.13	3.89	5.74	5.53	5.49	5.36
7th day	4.21	3.93	5.88	5.56	5.29	5.20
Week 2						
1st day	3.99	3.69	6.75	6.37	6.74	6.70
3rd day	4.01	3.58	6.67	5.68	5.49	5.47
7th day	4.09	3.83	5.83	5.12	5.37	5.34
Week 3						
1st day	4.09	3.93	6.83	6.53	6.81	6.71
3rd day	3.83	4.09	5.94	5.26	5.52	5.39
7th day	3.99	3.98	5.58	5.33	5.04	5.16
Week 4						
1st day	4.13	3.96	6.58	6.57	6.53	6.67
3rd day	4.04	3.90	5.83	5.20	5.60	5.49
7th day	3.75	4.16	5.84	5.37	5.32	5.26
Week 5						
1st day	3.90	4.03	6.76	6.53	6.56	6.65
3rd day	4.23	3.92	5.72	5.72	5.43	5.52
7th day	3.45	3.89	5.68	5.51	5.21	5.32
Week 6						
1st day	3.87	3.68	6.55	6.51	5.56	6.68
3rd day	3.77	3.83	5.82	5.38	5.81	5.47
7th day	4.09	3.92	5.62	5.40	5.37	5.26

PBS = phosphate buffered saline; NC = negative control, with no additives added to the basal feed; PC = positive control, with the antibiotic, zinc-bacitracin (ZnB, 50 mg/kg) added; Iso461 = isolate treatments, with probiotic No. 461 unidentified *Lactobacillus* sp; Iso697 = isolate treatments, with probiotic No. 697 *L. salivarius*; Iso709 = isolate treatments, with probiotic No. 709 *L. crispatus*; Iso1286 = isolate treatments, with probiotic No. 1286 *L. johnsonii* added to the feed, respectively.

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Table 3
Body weight gain (BWG), feed intake (FI), feed conversion rate (FCR) and mortality of broiler chickens during d 1 to 42.

Item	Diet						SE	*P*-value
	NC	PC	Iso461	Iso697	Iso709	Iso1286		
BWG, g/bird								
d 1-21	811	827	791	809	821	826	10.4	0.173
d 22-42	1423	1485	1466	1465	1469	1432	52.9	0.954
d 1-42	2334	2412	2357	2375	2389	2358	53.8	0.903
FI, g/bird								
d 1-21	1211	1220	1190	1208	1233	1228	14.9	0.309
d 22-42	2889	2970	2859	2915	2938	2907	77.4	0.870
d 1-42	4038	4125	4007	4061	4109	4079	82.7	0.752
FCR, g feed intake/g weight gain								
d 1-21	1.44	1.42	1.44	1.43	1.45	1.43	0.02	0.895
d 22-42	2.03	2.00	1.95	1.99	2.00	2.03	0.04	0.896
d 1-42	1.73	1.71	1.70	1.71	1.72	1.73	0.02	0.914
Mortality, %								
d 1-42	7.14	3.57	0.00	3.57	0.00	5.36	–	–

SE = standard error of means; NC = negative control, with no additives added to the basal feed; PC = positive control, with the antibiotic, zinc-bacitracin (ZnB, 50 mg/kg) added; Iso461 = isolate treatments, with probiotic No. 461 unidentified *Lactobacillus* sp; Iso697 = isolate treatments, with probiotic No. 697 *L. salivarius*; Iso709 = isolate treatments, with probiotic No. 709 *L. crispatus*; Iso1286 = isolate treatments, with probiotic No. 1286 *L. johnsonii* added to the feed, respectively.
Each value represents the mean of 7 replicates.

3.4. Intestinal pH and SCFA concentrations

The probiotic treatments did not affect the intestinal pH (Table 5). As expected, the pH changed from acidic to alkaline from the proximal to the distal regions of the gastrointestinal tract (GIT), with a slight reversal of the trend in the caeca. Thus, at 3 weeks of age, digesta pH was 3.19, 7.45 and 6.67 in the gizzard, ileum and caeca, respectively. The corresponding values at 5 weeks of age were 3.06, 8.11 and 6.96. It was also observed that pH values in the ileum and caeca were generally higher in older birds (35 days of age) than younger birds (21 days of age).

The concentrations of VFA, formic acid and lactic acids did not differ in any part of the intestine.

Table 4
Relative organ weights (% body weight) of broiler chickens on d 21 and 35.

Item	Diet						SE	*P*-value
	NC	PC	Iso461	Iso697	Iso709	Iso1286		
Day 21								
Liver	3.05	3.03	2.95	2.97	3.12	3.06	0.14	0.955
Spleen	0.07	0.07	0.07	0.08	0.09	0.08	0.01	0.562
Pancreas	0.25	0.19	0.25	0.24	0.23	0.25	0.02	0.119
Bursa	0.13	0.13	0.14	0.14	0.14	0.14	0.01	0.929
Gizzard	1.72	1.90	1.96	1.95	1.87	2.08	0.17	0.759
Duodenum	0.96	0.95	1.03	1.13	0.99	1.05	0.08	0.665
Jejunum	1.37b	1.44b	1.67a	1.67a	1.59a	1.74a	0.08	0.009
Ileum	0.84c	0.88c	1.99a	1.07b	0.98b	1.29a	0.06	0.001
Day 35								
Liver	2.21	2.50	2.38	2.42	2.53	2.57	0.115	0.285
Spleen	0.08	0.09	0.07	0.11	0.10	0.10	0.01	0.039
Pancreas	0.18	0.20	0.19	0.19	0.20	0.20	0.01	0.727
Bursa	0.18	0.18	0.18	0.19	0.19	0.20	0.01	0.970
Gizzard	2.17	2.10	2.09	2.20	2.20	2.24	0.11	0.893
Duodenum	0.91	0.87	0.88	0.88	0.89	0.93	0.04	0.859
Jejunum	2.44	2.36	2.56	2.62	2.64	2.66	0.09	0.163
Ileum	1.29d	1.44b	1.46bc	1.45b	1.56a	1.51ac	0.06	0.048

SE = standard error of means; NC = negative control, with no additives added to the basal feed; PC = positive control, with the antibiotic, zinc-bacitracin (ZnB, 50 mg/kg) added; Iso461 = isolate treatments, with probiotic No. 461 unidentified *Lactobacillus* sp; Iso697 = isolate treatments, with probiotic No. 697 *L. salivarius*; Iso709 = isolate treatments, with probiotic No. 709 *L. crispatus*; Iso1286 = isolate treatments, with probiotic No. 1286 *L. johnsonii* added to the feed, respectively.
Each value represents the mean of 7 replicates.
a,b,c,d Means within a row not sharing a common online letter are significantly different ($P < 0.05$).

3.5. Bacterial populations in GIT

The experimental diets did not affect the count of total anaerobic bacteria, LAB, *Lactobacilli*, *Enterobacteria* and *C. perfringens* in the digesta of the gizzard, ileum and caeca of birds at 21 days of age, except that the anaerobes and LAB tended to be higher in birds fed probiotics. At d 35, the number of *Enterobacteria* in the gizzard varied significantly ($P < 0.05$), with Iso697 and Iso1286 giving a lower count than the controls. The same was true in the caeca where all the isolates reduced enterobacterial counts, compared with the negative control (Table 6).

3.6. Intestinal tract morphology

The effects of different dietary treatments on villus height, crypt depth, muscle depth and villi:crypt ratio of the ileum on d 21 and 35 are shown in Table 7. The dietary treatments had no significant effect on villus height, crypt depth and muscle depth either on d 21 or 35. When the ratios of villus height to crypt depth were compared, a significantly higher ($P < 0.05$) ratio was obtained in the ileum of chickens fed diets containing probiotics on both d 21 and 35.

4. Discussion

4.1. Growth performance

All the birds were in very good health during the experimental period of 6 weeks, and dietary supplementation with probiotics resulted in numerically higher BWG compared to the negative control group. There was no significant effect on growth performance of broiler chickens when the probiotic candidates were administered via feed. These results were in line with those of Huang et al. (2004) who supplemented either *Lactobacillus casei* or *L. acidophilus* with or without cobalt in the diets of broiler chickens. There have also been several studies in which no positive results were found when broilers were fed with probiotic supplements. For example, Watkins and Kratzer, 1984; Maiolino et al., 1992; Panda et al., 2000 did not find any significant difference in the BWG of chickens given feed containing host-specific probiotics (KTM, 74/1 and 59), *L. acidophilus* and *Streptococcus faecium* compared with those given a non-supplemented diet.

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Table 5
The pH and organic acids (μmol/g) in gizzard, ileum and caeca digesta on d 21 and 35 of birds fed on experimental diets.

Item	Diet						SE	*P*-value
	NC	PC	Iso461	Iso697	Iso709	Iso1286		
Day 21								
Gizzard								
pH	3.44	3.13	3.10	3.20	3.25	3.03	0.12	0.201
Ileum								
pH	7.33	7.52	7.50	7.78	7.37	7.17	0.20	0.385
Formic acid	0.47	0.35	0.24	0.47	0.37	0.39	0.22	0.982
Acetic acid	2.32	2.58	2.46	2.69	2.50	2.64	0.45	0.993
Lactic acid	6.8	6.16	10.40	6.80	7.23	8.16	4.17	0.983
Caeca								
pH	6.50	6.39	6.86	6.75	6.89	6.60	0.20	0.445
Acetic acid	60.39	58.26	44.23	36.71	61.46	52.03	8.99	0.324
Propionic acid	3.10	2.73	2.11	3.64	3.23	2.16	0.65	0.503
Butyric acid	14.55	12.14	11.00	11.84	15.10	13.48	1.51	0.356
Succinic acid	2.53	4.73	4.28	2.84	3.06	6.87	1.95	0.631
Iso-SCFA	3.34	6.21	1.49	10.94	1.36	4.77	4.51	0.624
Day 35								
Gizzard								
pH	2.99	3.06	2.99	3.13	3.10	3.08	0.14	0.979
Ileum								
pH	8.03	8.3	7.91	8.02	8.16	8.22	0.14	0.357
Formic acid	0.98	2.14	1.18	0.76	1.49	0.74	0.45	0.258
Acetic acid	2.11	2.42	2.21	1.74	2.36	1.67	0.40	0.682
Lactic acid	3.76	2.39	2.19	3.49	4.75	1.79	0.95	0.253
Caeca								
pH	7.23	6.88	6.95	6.88	6.89	6.92	0.20	0.793
Acetic acid	67.67	56.29	46.97	48.89	63.51	63.78	8.09	0.397
Propionic acid	5.26	4.45	3.52	3.71	3.13	3.85	0.96	0.682
Butyric acid	11.18	9.46	11.46	9.52	12.46	13.71	2.24	0.735
Succinic acid	4.68	3.00	5.11	3.48	4.09	4.19	1.63	0.953
Iso-SCFA	1.45	1.02	1.42	0.83	1.00	0.61	0.57	0.871

SE = standard error of mean; NC = negative control, with no additives added to the basal feed; PC = positive control, with the antibiotic, zinc-bacitracin (ZnB, 50 mg/kg) added; Iso461 = isolate treatments, with probiotic No. 461 unidentified *Lactobacillus* sp; Iso697 = isolate treatments, with probiotic No. 697 *L. salivarius*; Iso709 = isolate treatments, with probiotic No. 709 *L. crispatus*; Iso1286 = isolate treatments, with probiotic No. 1286 *L. johnsonii* added to the feed, respectively; SCFA = short-chain fatty acid.
Each values represents the mean of 7 replicates.

On the other hand, there are numerous studies that report positive effects of various probiotics on bird performance. For example, BWG of broiler was improved by a culture of *L. acidophilus* (Jin et al., 2000), and by a single strain of *Lactobacillus* (*E. faecium*) (Cao et al., 2013) or a mixture of *Lactobacillus* (Jin et al., 1998b; Kalavathy et al., 2003). The magnitude of improvement depends on the type of probiotics added and the conditions under which they are used. It was reported by Mohan et al. (1996) that the BWG could range from 5 to 9% higher and FI 2% lower when chickens were fed with probiotic supplements.

Variation in the effects of probiotics on growth performance of broiler chickens may be attributed to differences in the strains of bacteria used as the dietary supplements. Several health benefits, resulting from improved digestion, have been claimed for both *Lactobacillus* spp. and *Bifidobacterium* spp. At the nutritional level, they increase the digestibility of fermented milk products in humans (Deeth and Tamine, 1981) and increase the bioavailability of calcium, iron, copper, phosphorus, zinc and manganese in rats (McDonough et al., 1983). Furthermore, Yeo and Kim (1997) reported that feeding a diet containing a probiotic (*L. casei*) significantly increased average intake of broiler chickens during the first 3 weeks but not during 4 to 6 weeks of age. Chickens gained more weight as a result of mixing *L. salivarius* with another two *Lactobacillus* spp. in their diets (Lan et al., 2003). Yeo et al. (2008) reported that *L. johnsonii* improved growth performance significantly, acting as an antimicrobial addition in feed for broiler chickens.

In the current study, strains of *L. johnsonii, L. crispatus*, *L. salivarius* and one unidentified *L.* sp. tended to improve BWG, FI and FCR in broiler chickens. It is viewed that the effects of probiotics on the growth performance, feed conversion or production of farm animals are, even in specific situations, not consistent enough to consider their use due to economic considerations (Veldman, 1992). The current study was based on a laboratory scale experiment under clean conditions, which may have masked any growth promoting effect of the probiotics. Another possibility is the concentration of the probiotics in the diet. In the current study, the concentration of the probiotic candidates in the experimental feed was around 10^6 cfu/g of feed, which were few folds lower than is usually recommended as the inclusion rate (10^8 cfu/g of products) of commercial probiotic feed additives. This was due to the limited fermentation capacity for amplification of the probiotic candidates in the current study. It is possible that higher concentrations of the probiotic candidates in the feed may exert a more profound positive response on growth performance, especially if the infection pressure from pathogenic bacteria, such as *C. perfringens*, is high. However, this needs to be investigated in future studies.

4.2. Organ weights and intestinal histomorphology

In the current study, the relative weights of the major digestive and immune organs were not affected by probiotic treatments compared with the controls. However, probiotic supplementation significantly increased the relative weight of the jejunum and ileum on d 21 and that of the ileum on d 42. Such findings have been reported in the literature. For example, Pedroso et al. (2003) added *Lactobacillus reuteri* and *L. johnsonii* into drinking water and reported a significant increase in intestinal weight in 21-day-old broilers. The mechanism by which this occurs is not known as the effect of probiotics on organ weights in animals is equivocal. Thus, Jin et al. (1998a) and Guan et al. (2003) found that supplementation

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Table 6
Effects of experimental diets on bacterial counts (lg cfu/g) in digesta of birds on d 21 and 35.

Item	Diet						SE	*P*-value
	NC	PC	Iso461	Iso697	Iso709	Iso1286		
Day 21								
Gizzard								
Total anaerobes	6.61	6.12	5.42	6.52	6.33	6.49	0.33	0.161
LBA	6.51	6.16	5.83	6.37	6.24	6.49	0.32	0.680
Lactobacilli	6.28	5.98	5.77	6.37	6.03	6.43	0.33	0.681
Enterobacteria[a]	3.10	3.29	2.95	3.37	3.23	3.22	0.12	0.223
C. perfringens	3.05	3.06	2.95	3.11	3.23	3.03	0.07	0.175
Ileum								
Total anaerobes	7.91	7.40	7.24	8.10	8.19	8.45	0.37	0.190
LBA	7.91	7.68	7.49	8.05	8.28	8.42	0.37	0.491
Lactobacilli	7.87	7.16	7.45	8.00	8.12	8.43	0.45	0.399
Enterobacteria[a]	5.39	5.50	4.08	4.78	4.39	4.67	0.40	0.121
C. perfringens	3.47	3.49	3.10	3.52	4.09	3.26	0.27	0.210
Caeca								
Total anaerobes	8.96	9.28	9.39	8.87	9.10	9.29	0.14	0.079
LBA	8.98	9.36	9.44	9.22	9.22	9.42	0.12	0.086
Lactobacilli	8.88	9.26	9.25	9.12	9.23	9.35	0.12	0.158
Enterobacteria[a]	8.09	8.22	8.23	7.83	7.93	8.20	0.19	0.590
C. perfringens	4.25	4.04	5.13	4.85	4.35	4.36	0.38	0.365
Day 35								
Gizzard								
Total anaerobes	7.19	6.90	7.07	6.76	6.79	6.16	0.37	0.458
LAB	7.05	6.91	7.23	6.76	6.93	6.39	0.32	0.556
Lactobacilli	7.19	7.04	7.15	6.91	6.98	6.20	0.32	0.277
Enterobacteria[a]	3.39b	3.87a	3.52a	2.96c	3.32b	3.16c	0.19	0.040
C. perfringens	3.01	3.05	2.99	2.99	3.00	3.03	0.04	0.791
Ileum								
Total anaerobes	7.83	7.64	7.91	7.77	8.01	7.84	0.21	0.876
LAB	7.70	7.54	7.95	7.61	7.99	7.56	0.24	0.669
Lactobacilli	7.79	7.66	7.87	7.68	7.90	7.50	0.25	0.864
Enterobacteria[a]	5.03	4.18	4.40	3.86	5.13	4.06	0.41	0.190
C. perfringens	3.08	3.09	3.01	3.13	3.19	3.11	0.06	0.446
Caeca								
Total anaerobes	9.07	9.21	9.18	9.22	9.17	9.22	0.14	0.973
LAB	9.26	9.17	9.24	9.14	9.23	9.22	0.15	0.992
Lactobacilli	9.09	9.12	9.15	9.17	9.15	9.22	0.17	0.997
Enterobacteria[a]	8.11a	7.21c	7.60b	7.46b	7.96a	7.09c	0.26	0.040
C. perfringens	4.24	3.87	4.32	3.71	3.49	3.67	0.39	0.619

SE = standard error of means; NC = negative control; PC = positive control, with the antibiotic, zinc-bacitracin (ZnB, 50 mg/kg) added; Iso461 = isolate treatments, with probiotic No. 461 unidentified *Lactobacillus* sp; Iso697 = isolate treatments, with probiotic No. 697 *L. salivarius*; Iso709 = isolate treatments, with probiotic No. 709 *L. crispatus*; Iso1286 = isolate treatments, with probiotic No. 1286 *L. johnsonii* added to the feed.
Each value represents the mean of 7 replicates.
a,b,c Means within a row not sharing same online letter are significantly different ($P < 0.05$).
[a] Enterobacteria are coliform and lactose negative Enterobacteria.

Table 7
Effects of experimental diets on the ileal morphometry.

Item	Diet						SE	*P*-value
	NC	PC	Iso461	Iso697	Iso709	Iso1286		
Day 21								
Villus height, μm	723	770	745	773	754	781	66.43	0.332
Crypt depth, μm	129	134	124	124	126	127	7.45	0.176
Villi/crypt ratio	5.61b	5.75b	6.01a	6.23a	5.98a	6.15a	0.89	0.032
Muscle depth, μm	278	302	289	267	298	285	17.26	0.423
Day 35								
Villus height, μm	789	825	827	894	871	938	21.30	0.870
Crypt depth, μm	138	143	136	144	139	149	11.20	0.365
Villi/crypt ratio	5.72c	5.77c	6.09a	6.21ab	6.27ab	6.29ab	0.62	0.017
Muscle depth, μm	367	421	384	362	409	396	19.21	0.587

SE = standard error of means. NC = negative control, with no additives added to the basal feed; PC = positive control, with the antibiotic, zinc-bacitracin (ZnB, 50 mg/kg) added; Iso461 = isolate treatments, with probiotic No. 461 unidentified *Lactobacillus* sp; Iso697 = isolate treatments, with probiotic No. 697 *L. salivarius*; Iso709 = isolate treatments, with probiotic No. 709 *L. crispatus*; Iso1286 = isolate treatments, with probiotic No. 1286 *L. johnsonii* added to the feed, respectively.
Each value represents the mean of 7 replicates.
a,b,c Means within a row not sharing a common online letter are significantly different ($P < 0.05$).

of broiler diets with lactobacilli did not affect the weight of the intestine.

On the other hand, probiotics appear to influence the microstructure of the gut more consistently. The current study showed that probiotics significantly affected villus height to crypt depth ratio in the ileum compared with control diets. This indicates that the absorptive function in the ileum of these chickens was higher compared with control treatments. Iji et al. (2001) found that, at

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d 21, the ileal villi were significantly longer in chickens fed a less viscous diet although they were not different during the first 7 days of the experiment. The intestine can change its surface area by growing in length, and/or by increasing or decreasing the height of its villi when probiotics are supplied in the diet. Shortening and fusion of villi will result in loss of surface area for digestion and absorption of food (van Dijk et al., 2002), whereas the converse is true with longer villi and shallower crypts (Chiou et al., 1996).

The GIT has the ability to adapt or to react morphologically to changing conditions such as altered diet (Huisman et al., 1990; van der Klis and Van der voorst, 1993). Of course, it is well-known that dietary probiotics lead to marked changes in the gut microflora, often favouring the host. The influence of probiotics on the gut microflora will be discussed in the following section.

4.3. Bacterial populations in GIT and bacterial activities

The current study demonstrates that *Enterobacteria* make up only a minor proportion of the ileal and caecal microflora in broilers on the sampling days (d 21 and 35). Probiotic supplementation reduced the population of *Enterobacteria* in the ileum and caeca compared to the control groups. This is in agreement with the findings of Mulder et al. (1997) who reported that inoculation with a probiotic strain of *L. reuteri* significantly reduced the number of *Enterobacteria* in broiler chickens. A similar finding was presented by Ln et al. (2003) with a mixture of *L. acidophilus/gallinarum*, *Lactobacillus agilis*, *L. salivarius*, and *Lactobacillus* spp.

Probiotics, such as *L. crispatus*, *L. salivarius* and *L. johnsonii*, have antimicrobial activities against *Enterobacteria* (Garriga et al., 1998; Pascual et al., 1999; Veldman, 1992; Van der Wielen et al., 2002). Cao et al. (2013) reported that broiler chickens fed diets supplemented with *Lactobacilli* spp. were more resistant to the pathogenic effects of *E. coli*. The antimicrobial effects of probiotics come from the VFA and other organic acids such as lactate and succiniate produced (Thompson et al., 1998; Kubena et al., 2001) and through the production of bacteriocins and phage-displayed peptides (Ingham et al., 2003; Joerger, 2003; Sakai et al., 2006). The probiotic candidates used in the current study tended to increase the number of lactic acid bacteria and lactobacilli in the ileum and caeca on d 21. Furthermore, all probiotic candidates, except Iso461, tended to increase the concentration of acetic and lactic acids in the ileum compared with the control treatments. Other potential antimicrobial agents such as bacteriocins and decencies were not measured in the current study.

Although the population of lactobacilli was larger in the ileal and caecal contents of the treatment groups fed probiotic supplements, the current study does not demonstrate an improvement in growth performance of birds. The impact of lactobacilli on animal health and performance is controversial. Whilst many *Lactobacillus* spp. act via a number of mechanisms, including competitive exclusion, to reduce the number of pathogens in the GIT, leading to improvement in bird performance (Jin et al., 1998ab; Schneits and Hakkinen, 1998), other species seem to be neutral in their effects on birds performance (Gunal et al., 2006). The metabolic activity of common lactobacilli results in the production of end-products such as lactate, succinate, H_2, CO_2 and CH_4 and SCFA, acetate, propionate and butyrate, as well as bacterial biomass. It was shown by De Vries and Stouthammer (1968) that most of the VFA formed by intestinal bacteria are absorbed and metabolized by the birds, thus contributing to host energy requirements (Fooks and Gibson, 2002). However, it is possible that the competition for nutrients by a large number of lactobacilli in the GIT of birds may offset some or all of the beneficial effects of probiotics on nutrient digestibility and absorption. This hypothesis will require investigation in the future.

5. Conclusion

Four *Lactobacillus* probiotic candidates had no adverse on the general health status of broiler chickens and altered the gut microflora of birds resulting in a reduction in the number of entrobacteria in the ileum and an increase in the weight of the jejunum and the ileum. However, there were no other significant effects of these probiotics on the growth performance and gut development of birds, due probably to the hygienic experimental conditions of the current study.

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