Original research article

Delivery routes for probiotics: Effects on broiler performance, intestinal morphology and gut microflora

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ABSTRACT

Four delivery routes, via, feed, water, litter and oral gavage, were examined for their efficacy in delivering a novel probiotic of poultry origin, Lactobacillus johnsonii, to broilers. Seven treatments of 6 replicates each were allocated using 336 one-day-old Cobb broiler chicks. The treatments consisted of a basal diet with the probiotic candidate, L. johnsonii, added to the feed, and three treatments with L. johnsonii added to the drinking water, sprayed on the litter, or gavaged orally. In addition, a positive control treatment received the basal diet supplemented with zinc-bacitracin (ZnB, 50 mg/kg). The probiotic strain of L. johnsonii was detected in the ileum of the chicks for all four delivery routes. However, the addition of L. johnsonii as a probiotic candidate did not improve body weight gain, feed intake and feed conversion ratio of broiler chickens raised on litter during the 5-week experimental period regardless of the route of administration. The probiotic treatments, regardless of the routes of delivery, affected ($P < 0.05$) the pH of the caecal digesta and tended ($P = 0.06$) to affect the pH of the ileal digesta on d 7, but the effect disappeared as the birds grew older. All probiotic treatments reduced the number of Enterobacteria in the caeca on d 21, and tended ($P < 0.054$) to reduce it in the ileum and caeca on d 7 and in the ileum on d 21 compared with the controls. The probiotic also tended to increase the number of lactic acid bacteria and lactobacilli in the ileum and caeca on d 7, but this trend was not evident on d 21. The trend appeared most pronounced when the probiotic was delivered orally or via litter. The probiotic also decreased ($P < 0.05$) the population of Clostridium perfringens rapidly from an early age to d 21 in the caeca, leading to a 3-fold decrease in the number of C. perfringens between d 7 and 21. It also showed that the probiotic treatment presented the lowest number of C. perfringens in the caeca. Delivery of the probiotic through feed, water and litter increased ($P < 0.01$) the weight of the pancreas on d 21, but the probiotic did not affect other morphometric parameters of the gut. Furthermore, the probiotic did not affect the pH and the concentrations of short chain fatty acids and lactic acid in either the ileum or caeca.

1. Introduction

Probiotics display numerous health benefits beyond providing basic nutritional advantages. Probiotic products consisting of beneficial microflora can help to establish and maintain the balance of the intestinal microflora in commercial broilers. However, selecting a probiotic microorganism that has beneficial effects in broiler chickens requires an extensive search for the optimum candidate, and one which will perform under practical conditions. Inoculating one-day-old chicks with competitive exclusion (CE) cultures or more classical probiotics serves as an effective model for determining the modes of action and efficacy of these microorganisms. Because of the susceptibility of one-day-old chicks to infection, this practice is also of commercial importance. By using this model, a number of probiotics have been shown to reduce colonization and shedding of Salmonella and Campylobacter (Netherwood et al., 1999; Fritts et al., 2000). However, one of the

key factors determining their efficacy in practical use is stability during storage, delivery and feed processing.

There are many different methods for administering probiotic preparations to broiler chickens: through feed, water, gavage (including droplet or inoculations), spray or litter, but adding to feed is the most commonly used method in poultry production.

Introducing probiotics through drinking water, into the crop by tube and syringe, with crumbles, or by spraying on bird environment and litter had no effects on the survival rate of bacteria (Gardiner et al., 2000; Morelli, 2000; Corcoran et al., 2004). The feed-type probiotic products rarely produce optimum results in pelletized diets usually fed to broilers (Nguyen et al., 1988; Scheuerman, 1993). Kozasa (1986) found that two probiotic bacteria incorporated into crumbles, successfully survived the duration of the experiment. Also, Gould and Hurst (1969) reported that spores of bacillus are well known for being able to survive high temperatures. Thus, the best natural solution to the challenge of stability in direct-fed microbial products is to use spore-forming beneficial strains of microbes or fed as crumbles (Crawford, 1979). However, Seuna et al. (1978) showed that the viability of the organisms rapidly declined, especially in chlorinated water when bacteria via the drinking water rather than gavage compared.

The literature suggests that spray application of probiotic cultures, either on the environment of the birds or on the litter material seems to be an effective way of administering probiotic cultures (Blankenship, 1992), whilst according to Nurmi and Rantala (1973) intubation into the crop is perhaps the most satisfactory method for delivering a precise dose of probiotics to the animal.

The aim of this study was to determine the efficacy of administering a probiotic strain of Lactobacillus johnsonii which chosen by antimicrobial activities showed the best resistant in promoting growth performance, intestinal morphology and gut microflora in broiler chickens.

2. Materials and methods

2.1. Probiotic strains

The bacterial strain used in this experiment was selected using the antagonistic activity assay described by Teo and Tan (2005).

A pure L. Johnsonii isolate was grown in MRS broth overnight (at 39°C) and harvested by centrifugation at 4,420 × g for 15 min (Induction Drive Centrifugation, Beckman Model J2-21M, Beckman Instruments Inc., Palo Alto, California, USA). It was re-suspended in phosphate-buffered solution (PBS, pH 7.4) and mixed by constant mechanical stirring (Heidolph MR 3001K stirrer, Heidolph Instruments GmbH & Co., Schwabach, Germany) for 10 min. This pre-mixture of PBS probiotic solution was added to feed, drinking water, or was gavaged orally. The quantities of MRS broth and premix phosphate-buffered solution (PBS, solution used were calculated by determining the bacterial concentration needed for the experiment. In this study, the concentration of the probiotic candidate, L. johnsonii, supplied via different routes was: feed delivery $> 10^6$ cfu/gram of feed samples; oral delivery $> 10^8$ cfu/mL of BPS solution; litter delivery $> 10^8$ cfu/mL of PBS spray solution and water delivery $> 10^6$ cfu/mL of water sample.

Representative feed, water, and litter samples of each treatment batch were tested for bacterial concentrations weekly on d 1 and 7. Ten grams (or millilitres) of samples were dissolved in 90 mL of peptone water (Oxoid, CM0009) and 10-fold dilutions were performed in Hungate tubes with 9mL of peptone water. The numbers of lactic acid bacteria in the samples were determined on MRS agar (Oxoid, CM0361) inoculated with 0.1 mL of diluted sample and after anaerobic incubation at 39°C for 48 h.

2.2. Bird husbandry

A total of 336 one-day-old male Cobb broiler chicks, which were vaccinated against Marek's disease, infectious bronchitis, and Newcastle disease, were obtained from a local hatchery (Baiada hatchery, Kootingal, NSW, Australia) and randomly allocated to 42 cages in four-tier floor pens (600 × 600 × 300 mm dimension, with a floor space of 0.36 m²/cage) sit on sawdust litter in climate-controlled rooms. Each of the 7 dietary treatments was randomly assigned to 6 cages with 8 birds per cage (except for the water treatment group which needed to be in line in order to be serviced by the same water pipe that supplied the water containing the probiotics). At d 21, birds were transferred to slide-in cages (800 × 740 × 460 mm) in an environmentally controlled room.

The room temperature was gradually decreased from 33°C on d 1 to 24°C on d 21. Eighteen hours of light was provided per day throughout the trial, excluding d 1 to 7 during which 23 h of light was provided. Relative humidity was between 65 and 70%. Each cage was equipped with a feeding trough placed outside and had water pipes providing drinking nipples inside. Feed and water were provided ad libitum.

2.3. Experimental treatments

2.3.1. The diet and treatments

The basal diets (starter and finisher) were based on corn, wheat and soybean meal as shown in (Table 1), and fed as a one-phase mash feed to avoid inactivation of the probiotic. Seven 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[1]	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

[1] Vitamin and mineral premix (Ridley Agriproducts Pty Ltd., Tamworth, NSW) contained the following minerals in milligrams per kilogram of diet: vitamin A (as all-trans retinol), 12,000 IU; cholecalciferol, 3,500 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.

were provided as three diet batches during the first three weeks for starter as follows: 1) the negative control, litter delivery, negative oral gavage and probiotic oral gavage treatment groups were provided with the basal diet; 2) the positive control treatment was provided with the antibiotic, zinc-bacitracin (ZnB, 50 mg/kg) added; and 3) the feed supplementary treatments groups (starter feed) included an overnight culture of *L. johnsonii*. 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 the different treatments. The experimental diet with the probiotic candidate was mixed weekly and supplied for the first three weeks.

All treatments received the same basal finisher diet once the birds were transferred to slide-in cages, and growth performance was measured weekly. Feed was provided ad libitum. The delivery routes of experimental treatments are shown in (Table 2).

2.3.2. Delivery via feed

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 4,420 × g for 15 min (Induction Drive Centrifugation, Beckman Model J2-21M, Beckman Instruments Inc., Palo Alto, California, USA), resuspended in PBS (pH 7.4) and mixed into a premix with the basal diet for 10 min using a miniature mixer. This pre-mixture 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).

2.3.3. Delivery via drinking water

For the first three weeks, drinking water was supplied through pipes (nipples drinker installed) connected to a 20-L drum. A small pump (low power, Aqua One maxi series power head, Kongs International Co., Ltd, China) was installed to constantly agitate the water. The water containing the probiotic was prepared daily and supplied for the first three weeks in probiotic water treatment groups. After three weeks the birds were transferred to slide-in cages and drinking water was supplied in troughs placed outside the cages. Water was provided ad libitum.

2.3.4. Litter application

The sawdust used as litter for this experiment was selected from commercial products produced by Bellsouth Pty. Ltd., Australia. The lactic acid bacterial concentration was determined using an MRS agar plate display. The sawdust contained a low number of lactic acid bacteria before use ($< 10^2$ cfu/g of sawdust). The probiotic solution (PBS, pH 7.4 containing $> 10^6$ cfu/mL of *L. johnsonii*) was sprayed on litter daily for the first three weeks for the litter treatment groups.

2.3.5. Oral gavage

L. johnsonii cultures were resuspended into PBS solution (pH 7.4) which contained approximately 10^8 cfu/mL. Each bird received 1 mL of PBS mixed solution on d 1, 2, 4, 6 and 14; the birds in the negative control group received 1 mL of PBS solution (pH 7.4) on the same days.

2.3.6. Sample collection and processing

Feed leftovers and birds were weighed on a weekly basis for calculation of average feed intake (FI) and body weight. Mortality was recorded when it occurred and feed conversion ratio (FCR; feed intake/weight gain) was corrected for mortality. Three birds on d 7 and two birds on d 21, from each cage were randomly selected and killed by cervical dislocation. The abdominal cavity was opened and visceral organs were weighed.

The weights of the empty gizzard, the duodenum, jejunum and ileum were recorded individually. The weights of the pancreas, liver, spleen, and bursa were also measured and recorded individually. The contents of the gizzard, ileum and caeca were collected in plastic containers, and stored at −20°C until volatile fatty acids (VFA) analysis was performed. A 2-cm piece of the proximal ileum was flushed with ice-cold phosphate-buffered saline (PBS saline) at pH 7.4 and fixed in 10% formalin for gut morphological measurements. One gram (approximately) each of ileal and caecal fresh digesta was transferred individually into 15 mL MacCartney bottles containing 10 mL of anaerobic broth for bacterial enumeration using the methods described in Section 2.3.8

2.3.7. Digesta pH, VFA analysis and gut morphology

Intestinal pH was measured immediately after death and excision of viscera. 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 short-chain fatty acids (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).

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.

2.3.8. Enumeration of intestinal bacteria and isolation of lactobacilli

A 10 mL aliquot of anaerobic broth was homogenized for 2 min in CO_2-flushed plastic bags using a bag mixer (Interscience, St. Norm, France) immediately after sample collection. The 10-fold increment serial dilution technique was conducted according to Miller and Wolin (1974). One millilitre of the homogenized suspension was then transferred into 9 mL of anaerobic broth and

Table 2
Experimental treatments via different delivery routes.[1]

Treatment & routes	NC	PC	Feed	Water	Litter	Oral-NC	Oral-Pro
Feed	Basal	Basal + Antibiotic	Basal + Pro	Basal	Basal	Basal	Basal
Water	Tap	Tap	Tap	Tap + Pro	Tap	Tap	Tap
Litter	Sawdust	Sawdust	Sawdust	Sawdust	Sawdust + Pro	Sawdust	Sawdust
Antibiotic	Non	ZnB, 50 mg/kg	Non	Non	Non	Non	Non
Oral gavage	Non	Non	Non	Non	Non	PBS	Pro-*L. Johnsonii*

[1] Dietary treatments: NC, negative control, with no additives added to the basal feed, water and litter; PC, positive control, with the antibiotic, zinc-bacitracin (ZnB, 50 mg/kg) added in feed; Oral-NC, negative control, with no additives added to the basal feed, water and litter, orally inoculated with phosphate-buffered solution (PBS) solution. Other treatments, with probiotic (Pro) *L. johnsonii* delivery by oral gavage, feed, water and litter, respectively.

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 d. 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 *Clostridium perfringens* (*Cp*) 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).

Twenty pure colonies were randomly collected from the highest dilution Rogosa agar plates from the oral gavage treatment groups (negative and probiotic). The bacterial isolates were transferred to MRS broth individually and aerobically incubated at 39°C for 24 h. The amplification of bacterial colonies was collected in Eppendorf tubes (2.5 mL) and stored at −20°C for further DNA analysis.

2.3.9. Extraction of genomic DNA

Forty bacterial colonies, 20 colonies from each treatment were randomly picked from Rogosa agar plates (ileum, most of colonies from the highest dilution and some from different dilutions) from the oral inoculation treatment and negative control oral inoculation treatment on d 7. Using a sterile toothpick, cells from a single (pure) colony were used to individually inoculate 10 mL of MRS broth in screw cap tubes. The cells were grown at 39°C for 24 h. The supernatant (about 8 mL) was discarded and 1.5 mL of broth containing the bacterial cells were transferred into Eppendorf tubes. The bacterial cells were harvested by centrifugation (5,000 × *g*, 5 min) in an Eppendorf centrifuge (Eppendorf 5,415D, Eppendorf AG, Hamburg, Germany). The supernatant was removed and the cells were re-suspended in 1.0 mL of TES buffer (0.05 M Tris, 0.05 M NaCl, 0.005 M EDTA, pH 8.0), before being centrifuged again (5,000 × *g*, 5 min) and the supernatant discarded. After washing the pellet cells were stored at −20°C for 24 h to improve lysis. The pellet was then again resuspended into 0.5 mL of TES buffer (same as above) with 5 μL of lysozyme (10 mg/mL, freshly prepared) added and incubated at 37°C for 30 min. Subsequently, 5 μL each of proteinase K (10 mg/mL) and RNase (10 mg/mL) were added and mixed by vortex (VM1 vortex mixer, Stansens, Mt. Waverley VIC, 3149, Australia) and incubated at 65°C for 1 h. After the above steps lysis was finally achieved by the addition of 50 μL 24% (wt/vol) sodium dodecyl sulphate (SDS), followed by incubation for another 10 min at 65°C, with the suspension clearing as the cells lyse. The lysed suspension was then cooled and the cells were subjected to bead beating with 0.5 g of glass-beads (0.5 mm of diameter) cell disruption media in a mini bead-beater (Disruptor Genie, Scientific Industries Inc., New York, USA) at 5,000 × *g* for 5 min. The precipitation and purification of DNA were carried out using the DNeasy Tissue kit (Qiagen Pty. Ltd., Doncaster, VIC, Australia) according to manufacturer instructions after recovering the supernatants.

2.3.10. PCR amplification of 16-23S rDNA

The primers used in this experiment for PCR amplification are listed in Table 3. The method was according to Guan et al. (2003), Mikkelsen et al. (2003), Vidanarachchi (2006) and as reported as lactobacillus 16-23S rDNA (16S rRNA gene and the entire 16S-23S rRNA intergenic region) analysis with modifications. The reaction mixture (50 μL) contained a 0.01 mM deoxynucloside triphosphate (dNTP), 1.5 nM MgCl₂, 1.1 Unit Taq (*Thermus aquaticus*) DNA polymerase supplied with the 10 × PCR buffer (all from Fisher Biotec, West Perth, WA, Australia), 10 pmol both forward and reverse primers (Proligo Australia Pty. Ltd., Lismore, NSW, Australia) and 2.0 μL purified template DNA. The reaction mixtures were amplified in an Eppendorf PCR Thermal Cycler (MasterCycler, Eppendorf AG, Hamburg, Germany) under the following conditions: initial cycle of 1 min denaturation at 95°C, followed by 30 cycles of 30 s denaturation at 95°C, 30 s of annealing at 57°C and 45 s elongation at 72°C with a final extension of 10 min at 72°C. Amplified PCR products were electrophoresed on a 1% agarose gel containing 5 μL of GelStar nucleic acid gel stain (BioWhittaker Molecular Application, Rochland, ME, USA), viewed by UV transillumination and digitized on an Infinity CN – 3000 Gel Documentation System (Vilber Lourmat, Cedex, France). The formulation of the master mixture is listed in Table 4.

2.3.11. Amplified Ribosomal DNA Restriction Analysis (ARDRA) of 16-23S rDNA

The amplified 16-23S rDNA intergenic spacer regions of lactobacillus isolates were digested with the restriction endonuclease HaeIII enzymes (restriction enzyme isolated from *Haemophilus aegptius*) according to the manufacturer's instructions (New England BioLabs, Brisbane, QLD, Australia). HaeIII restriction enzyme recognizes and cleaves directly the centre of the 5′ … GG/CC … 3′, 3′ … CC/GG … 5′ DNA sequence. Restriction digestion was carried out for 2 h at 37°C in 40 μL final volume containing 4 μL 10 × buffer, 15 μL PCR grade water, 1 μL enzyme (10 U/μL) and 20 μL of amplified PCR product. Restriction digestion products were electrophoretically resolved in a 2% agarose gel containing 5 μL of GelStar nucleic acid gel stain (BioWhittaker Molecular Applications, Rockland, ME, USA) for 4 h at 90 V and band patterns were viewed by UV transillumination and digitized on Infinity CN – 3000 Gel Documentation System (Vilber Lourmat, Cedex, France). Infinity Capture version 12.6 for Windows software was used for image analysis.

2.4. Statistical analysis

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

2.5. Animal ethics

Health and animal husbandry practices complied with the 'Australian code of the care of animal for scientific purposes' (NHMRC, 2004). The Animal Ethic Committee of the University of

Table 3
Primers used for amplification for 16-23S r DNA (from Guan et al., 2003; Mikkelsen et al., 2003; Vidanarachchi et al., 2006).

Primer	Direction	Nucleotide sequence (5′ to 3′)
Lb 16a	Forward	GTG CCT AAT ACA TGC AAG TCG
23-1B	Reverse	GGG TTC CCC CAT TCG GA

Table 4

Formulation of reaction mixture for PCR amplification of 16-23S rDNA (from Mikkelsen et al., 2003).

Composition	Concentration	Volume, μL
Master mixture		
Deoxynucleoside triphosphate (dNTP)	2.0 nmol/μL	5.0
Taq DNA polymerase	5.5 U/μL	0.2
$MgCl_2$	25 nM	6.0
PCR buffer	10×	5.0
Forward primer – Lb16a	5 pmol/μL	2.0
Reverse primer – 23-1B	5 pmol/μL	2.0
PCR grade water	–	27.8
Total volume of master mixture for each sample		48
DNA crude extracts		2.0
Total reaction mixture for each sample		50

New England approved the experiments in this study (authority number: AEC07/016).

3. Results

3.1. Growth performance

Body weight gain (BWG), FI and FCR were not affected by different delivery methods of probiotic supplementation (Table 5). The oral gavage tended ($P = 0.3$) to give higher BWG than the negative control groups.

3.2. Organ weights, intestinal pH and SCFA concentrations

The relative weight of the pancreas was significantly increased ($P < 0.01$) at d 21 with oral gavage giving the heaviest pancreas (Table 6). There were no effects of diet on the relative weights of visceral organs, including the small intestine.

The probiotic treatments, regardless of the routes of delivery, affected ($P < 0.05$) the pH of the caecal digesta and tended ($P = 0.06$) to affect the pH of the ileal digesta on d 7, but the effect disappeared as the birds grew older (Table 6). Although there were numerically higher concentrations of lactic acid in the ileal digesta and succinic acid in the caecal digesta compared with the negative

controls, these were not statistically significant. Furthermore, the trend diminished as the birds grew older (Table 7).

3.3. Bacterial populations in intestinal digesta

The probiotic treatment groups had significant effects on the bacterial count in the caecal digesta with the number of *Enterobacteria* decreasing ($P < 0.05$) on d 7 and 21. The probiotic treatments tended ($P = 0.08$) to reduce the number of *Enterobacteria* in the ileum on d 7. However, it did not affect the counts of total anaerobic bacteria, LAB, lactobacilli and *C. perfringens* in the digesta of the ileum and caeca either at d 7 or at d 21. Furthermore, the number of *Enterobacteria* in the ileal digesta at d 21 was not affected (Table 8).

The number of the LAB was the highest in the ileal digesta in the oral gavage treatment (8.23; 7.52) and litter treatment (8.24; 7.58) on d 7 and 21, respectively. They were also highest in the caecal digesta for the oral gavage treatment (8.91) on d 21. The lactobacillus population was greatest in the caecal digesta for the oral gavage treatment on d 7 (9.30) and d 21 (8.81), and in the ileal digesta for the oral gavage treatment the lactobacillus population reached its peak (7.61) at d 21.

3.4. Intestinal histomorphology

The effects of different treatments on villus height, crypt depth and villi:crypt ratio of ileum on d 7 and 21 are shown in Table 9. Results show that the probiotic candidate *L. johnsonii* did not significantly influence ileal morphology of broiler chickens when administered by different delivery routes, compared with the positive and negative control treatments.

3.5. Amplified Ribosomal DNA Restriction Analysis of 16–23s rDNA

Forty isolates tentatively assigned to different groups of *Lactobacillus* spp. are listed in Table 10. The isolates were tentatively identified as *L. crispatus* and *L. salivarius* by Vidanarachchi (2006) who used the Amplified Ribosomal DNA Restriction Analysis (ARDRA) method for *Lactobacillus* spp. analysis. The *L. johnsonii* group was tentatively identified by comparing patterns from a pure culture used for oral inoculation. This pure culture was identified by Vidanarachchi (2006) using the sequences of 16S rRNA gene (Gen Bank accession No. AE017198) (Fig. 1). The result showed that *L. johnsonii* was detected from the oral inoculation

Table 5

The effects of delivering *L. johnsonii* via different routes on the performance of broilers.[1]

Item	Treatments[2]							SE	*P*-value
	NC	PC	Oral – NC	Feed	Water	Litter	Oral – Pro		
Day 1 to 7									
BWG, g/bird	157	158	156	155	156	157	158	1.57	0.87
FI, g/bird	156	157	154	157	155	160	160	2.88	0.67
FCR	0.995	0.998	0.988	1.010	0.995	1.017	1.015	0.02	0.94
Day 1 to 21									
BWG, g/bird	854	874	854	851	856	867	862	9.22	0.54
FI, g/bird	1,201	1,222	1,201	1,198	1,202	1,216	1,210	9.27	0.48
FCR	1.407	1.398	1.407	1.410	1.403	1.403	1.405	0.01	0.67
Day 1 to 35									
BWG, g/bird	1,797	1,816	1,794	1,800	1,792	1,792	1,824	11.44	0.31
FI, g/bird	2,899	2,935	2,891	2,908	2,883	2,899	2,952	27.05	0.55
FCR	1.623	1.617	1.622	1.636	1.619	1.637	1.634	0.02	0.99
Mortality, %	6.25	4.17	4.17	2.08	6.26	4.17	8.33	–	–

BWG = body weight gain; FI = feed intake; FCR = feed conversion ratio

[1] Values are means ($n = 6$) and standard error of means (SE).

[2] Dietary treatments: NC, negative control, with no additives added to the basal feed, water and litter; PC, positive control, with the antibiotic, zinc-bacitracin (ZnB, 50 mg/kg) added in feed; Oral-NC, negative control, with no additives added to the basal feed, water and litter, orally inoculated with PBS solution; Other treatments, with probiotic (Pro) *L. johnsonii* delivery by oral gavage, feed, water and litter, respectively.

Table 6
Relative weights (% BW) of organs from broilers given a probiotic via different routes.[1]

Item	Treatment[2]							SE	P-value
	NC	PC	Oral – NC	Feed	Water	Litter	Oral – Pro		
Day 7									
Liver	5.33	4.59	5.62	5.09	4.74	5.62	5.37	0.43	0.5071
Spleen	0.09	0.08	0.10	0.08	0.06	0.08	0.09	0.01	0.3327
Pancreas	0.35	0.34	0.45	0.40	0.40	0.38	0.37	0.04	0.6937
Bursa	0.15	0.12	0.13	0.12	0.16	0.17	0.13	0.02	0.2055
Gizzard	4.76	4.25	4.79	4.49	4.84	4.30	4.19	0.24	0.2467
Duodenum	2.07	1.69	1.79	2.00	2.09	1.90	2.03	0.16	0.5204
Jejunum	2.71	2.43	2.77	2.72	2.63	2.68	2.87	0.21	0.8536
Ileum	2.02	1.72	1.74	1.80	1.84	2.02	2.01	0.17	0.6834
Day 21									
Liver	3.23	3.35	3.34	3.28	2.98	3.28	3.43	0.36	0.1328
Spleen	0.09	0.08	0.07	0.09	0.09	0.07	0.08	0.01	0.4059
Pancreas	0.30[a]	0.27[a]	0.25[b]	0.24[b]	0.32[c]	0.30[a]	0.37[d]	0.02	0.0077
Bursa	0.16	0.16	0.12	0.17	0.19	0.15	0.16	0.02	0.3899
Gizzard	2.48	2.54	2.81	2.36	2.44	2.53	2.22	0.13	0.1144
Duodenum	1.17	1.04	1.27	1.27	1.24	1.17	1.22	0.08	0.4325
Jejunum	1.90	1.62	1.74	1.86	1.78	1.64	1.78	0.08	0.1842
Ileum	1.23	1.01	1.13	1.07	1.06	1.14	1.15	0.08	0.6000

[a,b,c,d] Means within the same row with no common superscripts differ significantly (P < 0.05).

[1] Values are means (n = 6) and standard error of means (SE).

[2] Dietary treatments: NC, negative control, with no additives added to the basal feed, water and litter; PC, positive control, with the antibiotic, zinc-bacitracin (ZnB, 50 mg/kg) added in feed; Oral-NC, negative control, with no additives added to the basal feed, water and litter, orally inoculated with PBS solution; Other treatments, with probiotic (Pro) L. johnsonii delivery by oral gavage, feed, water and litter, respectively.

treatment and also showed high numbers (8/20) of probiotic candidate colonies in the oral gavage groups in 20 randomly selected isolates. However, no L. johnsonii strains were found in the negative control group.

The results showed that two genotypic L. johnsonii patterns (300-bp, 500-bp) were present in the ARDRA test (Fig. 1). They are clearly differentiated from other patterns on the test. There are three patterns with L. crispatus (250-bp, 500-bp and 700-bp), two patterns with L.

Table 7
Digesta pH and organic acids concentrations (μmol/g) on d 7 and 21.[1]

Item	Treatments[2]							SE	P-value
	NC	PC	Oral – NC	Feed	Water	Litter	Oral – Pro		
Day 7									
Gizzard									
pH	3.06	3.01	3.08	2.95	3.15	3.09	3.02	0.09	0.7866
Ileum									
pH	6.71	6.64	6.67	6.84	6.97	6.79	6.51	0.10	0.0600
Formic acid	0.34	0.29	0.31	0.46	0.39	0.36	0.58	0.31	0.9451
Acetic acid	1.68	1.35	1.54	1.73	1.61	1.59	1.67	0.52	0.7956
Lactic acid	3.03	3.46	4.37	4.32	5.41	3.49	3.87	2.57	0.8351
Caeca									
pH	6.19[b]	6.08[c]	6.13[b]	6.13[b]	6.56[a]	5.71[a]	6.11[c]	0.14	0.0158
Acetic acid	57.51	52.32	58.53	47.97	61.27	55.69	52.27	6.79	0.8769
Propionic acid	2.83	2.45	2.26	3.11	2.49	3.91	2.89	0.34	0.1021
Butyric acid	14.11	14.41	13.43	13.02	13.87	14.19	14.54	0.87	0.8801
Succinic acid	2.12	2.25	2.68	3.41	2.69	2.76	2.91	0.59	0.7708
Day 21									
Gizzard									
pH	2.75	2.67	2.48	3.04	2.64	2.94	2.69	0.19	0.4784
Ileum									
pH	6.96	7.04	6.72	6.91	6.70	6.82	6.98	0.15	0.5746
Formic acid	0.48	0.39	0.53	0.53	0.32	0.51	0.45	0.24	0.5671
Acetic acid	2.41	2.57	2.49	2.76	2.34	2.55	2.70	0.67	0.8317
Lactic acid	7.24	6.77	9.41	6.91	7.18	8.51	8.76	3.21	0.6270
Caeca									
pH	5.77	5.86	5.62	5.87	5.77	5.89	5.86	0.15	0.8511
Acetic acid	57.41	69.24	49.71	64.28	61.49	55.06	58.12	12.34	0.3745
Propionic acid	4.57	4.49	3.89	3.76	4.72	4.28	4.51	0.89	0.6841
Butyric acid	12.64	11.47	12.38	13.16	11.78	12.68	12.97	3.54	0.7680
Succinic acid	1.08	1.24	1.29	1.31	1.27	1.09	1.11	0.38	0.8620

[a,b,c] Means within the same row with no common superscripts differ significantly (P < 0.05).

[1] Values are means (n = 6) and standard error of means (SE).

[2] Dietary treatments: NC, negative control, with no additives added to the basal feed, water and litter; PC, positive control, with the antibiotic, zinc-bacitracin (ZnB, 50 mg/kg) added in feed; Oral-NC, negative control, with no additives added to the basal feed, water and litter, orally inoculated with PBS solution; Other treatments, with probiotic (Pro) L. johnsonii delivery by oral gavage, feed, water and litter, respectively.

Table 8
Bacterial counts (lg cfu/g) in the digesta of birds on d 7 and 21.[1]

Item	Treatments[2]							SE	P-value
	NC	PC	Oral –NC	Feed	Water	Litter	Oral – Pro		
Day 7									
Ileum									
Total anaerobes	8.28	8.08	8.49	7.69	8.16	8.26	8.10	0.23	0.383
LAB	8.07	8.18	8.71	8.16	8.27	8.24	8.23	0.29	0.801
Lactobacilli	7.72	8.03	8.05	8.00	7.80	7.85	7.97	0.28	0.967
Enterobacteria[3]	6.27	6.14	6.17	5.69	5.45	6.72	5.94	0.28	0.084
C. perfringens	3.87	3.71	3.85	3.73	3.96	3.96	3.50	0.25	0.856
Caeca									
Total anaerobes	10.26	10.14	10.02	10.33	10.43	10.00	10.32	0.16	0.385
LAB	9.69	9.50	9.54	9.54	9.61	9.41	9.58	0.17	0.947
Lactobacilli	8.82	8.52	9.22	8.96	9.22	8.96	9.30	0.28	0.457
Enterobacteria[3]	9.33	9.25	9.51	9.13	9.14	9.31	8.76	0.15	0.054
C. perfringens	8.14	7.41	8.11	7.68	7.75	7.76	7.76	0.22	0.250
Day 21									
Ileum									
Total anaerobes	6.78	6.93	6.52	7.39	7.52	7.24	7.55	0.35	0.291
LAB	7.47	7.01	7.36	7.37	7.21	7.58	7.52	0.17	0.232
Lactobacilli	7.30	6.86	7.16	7.41	7.36	6.96	7.61	0.23	0.106
Enterobacteria[3]	6.19	5.68	5.97	5.58	5.83	5.78	5.33	0.26	0.380
C. perfringens	4.42	4.55	4.35	4.19	4.15	4.82	4.63	0.34	0.791
Caeca									
Total anaerobes	8.92	8.70	8.80	8.80	9.01	8.78	9.15	0.17	0.548
LAB	8.45	8.29	8.61	8.75	8.63	8.50	8.91	0.19	0.370
Lactobacilli	8.31	8.17	7.79	8.35	8.31	8.21	8.81	0.26	0.223
Enterobacteria[3]	8.16[a]	8.02[a]	8.08[a]	7.60[c]	7.82[b]	7.93[b]	7.59[c]	0.14	0.040
C. perfringens	5.36	4.83	5.26	4.66	4.44	4.83	4.83	0.41	0.708

[a,b,c] Means within the same row with no common superscripts differ significantly ($P < 0.05$).

[1] Values are means ($n = 6$) and standard error of means (SE).

[2] Treatments: NC, negative control, with no additives added to the basal feed, water and litter; PC, positive control, with the antibiotic, zinc-bacitracin (ZnB, 50 mg/kg) added in feed; Oral-NC, negative control, with no additives added to the basal feed, water and litter, orally inoculated with PBS solution; Other treatments, with probiotic (Pro) *L. johnsonii* delivery by oral gavage, feed, water and litter, respectively.

[3] *Enterobacteria* are coliform and lactose negative enterobacteria.

salivarius (200-bp, 500-bp), and one or four patterns with the unidentified strains (350-bp, 300-bp, 400-bp, 500-bp and 700-bp).

4. Discussion

4.1. Delivery routes and growth performance

A well-accepted method to quickly introduce a commensal microflora in chicks is through the administration of probiotics. Probiotic strains have been administrated in feed (Jin et al., 2000; Kalavethy et al., 2003) and water (Timmerman et al., 2006). Many reports have demonstrated that probiotics improve the growth performance and feed efficiency, and are potentially able to enhance nutrient absorption in broiler chickens. However, spraying of litter with probiotics is a method that has not been widely reported in poultry management. On the other hand, administering probiotics in drinking water is generally reported to result in a smaller increase in average daily gain compared with administering them via feed (Jin et al., 2000; Kalavethy et al., 2003). Compared to probiotics delivered via drinking water or compared with a negative control treatment, *L. johnsonii*, delivered as a feed supplement, did not significantly affect growth performance or feed conversion between d 1 and 21 in broiler chickens (Pelicano et al., 2004). They also observed that FI was slightly

Table 9
Ileal morphometry of broilers on d 21 and 35.[1]

Item	Treatments[2]							SE	P-value
	NC	PC	Oral – NC	Feed	Water	Litter	Oral – Pro		
Day 7									
Villus height, μm	603	593	589	574	583	605	579	37.29	0.532
Crypt depth, μm	110	98	103	117	106	107	103	6.25	0.741
Villi:crypt ratio	5.48	6.05	5.72	4.91	5.50	5.65	5.62	0.57	0.312
Muscle depth, μm	278	256	268	267	255	259	283	14.24	0.231
Day 21									
Villus height, μm	795	803	827	793	759	782	798	47.38	0.178
Crypt depth, μm	122	135	132	127	130	129	136	8.92	0.615
Villi:crypt ratio	6.52	5.95	6.27	6.24	5.84	6.06	5.87	0.74	0.236
Muscle depth, μm	311	302	291	285	272	298	307	16.36	0.347

[1] Values are means ($n = 6$) and standard error of means (SE).

[2] Dietary treatments: NC, negative control, with no additives added to the basal feed, water and litter; PC, positive control, with antibiotic, zinc-bacitracin (ZnB, 50 mg/kg) added in feed; Oral-NC, negative control, with no additives added to the basal feed, water and litter, orally inoculated with PBS solution; Other treatments, with probiotic (Pro) *L. johnsonii* delivery by oral gavage, feed, water and litter, respectively.

Table 10
Distribution of major genotypic groups of lactobacilli isolates from ileum of broiler on d 7.[1]

Isolates ID	Treatment[2]	DT	ARDRA patterns	Tentative distribution
L. johnsonii	Origin		150-bp, 300-bp, 500-bp	*L. johnsonii*
7-Ileum-5	Oral – NC	5	250-bp, 500 bp, 700 bp	Unidentified *Lactobacillus* sp.
7-Ileum-5	Oral – NC	5	300-bp, 400-bp, 500-bp, 700-bp	Unidentified *Lactobacillus* sp.
7-Ileum-5	Oral – NC	5	150-bp, 200 bp, 500 bp	Possibly *L. crispatus*
7-Ileum-5	Oral – NC	5	250-bp, 350-bp, 500-bp, 600-bp	Possibly *L. salivarius*
8-Ileum-5	Oral – NC	5	250-bp, 350-bp, 500-bp, 600-bp	Possibly *L. salivarius*
8-Ileum-5	Oral – NC	5	250-bp, 350-bp, 500-bp, 600-bp	Possibly *L. salivarius*
9-Ileum-6	Oral – NC	6	350-bp	Unidentified *Lactobacillus* sp.
9-Ileum-6	Oral – NC	6	250-bp, 350-bp, 500-bp, 600-bp	Possibly *L. salivarius*
10-Ileum-5	Oral – NC	5	150-bp, 200 bp, 500 bp	Possibly *L. crispatus*
10-Ileum-5	Oral – NC	5	350-bp	Unidentified *Lactobacillus* sp.
10-Ileum-5	Oral – NC	5	250-bp, 350-bp, 500-bp, 600-bp	Possibly *L. salivarius*
10-Ileum-5	Oral – NC	5	200-bp, 500-bp, 600-bp	Unidentified *Lactobacillus* sp.
11-Ileum-6	Oral – NC	6	150-bp, 200 bp, 500 bp	Possibly *L. crispatus*
11-Ileum-5	Oral – NC	5	350-bp	Unidentified *Lactobacillus* sp.
11-Ileum-5	Oral – NC	5	150-bp, 200 bp, 500 bp	Possibly *L. crispatus*
12-Ileum-6	Oral – NC	6	300-bp, 400-bp, 500-bp, 700-bp	Unidentified *Lactobacillus* sp.
12-Ileum-6	Oral – NC	6	250-bp, 350-bp, 500-bp, 600-bp	Possibly *L. salivarius*
12-Ileum-5	Oral – NC	5	250-bp, 350-bp, 500-bp, 600-bp	Possibly *L. salivarius*
12-Ileum-5	Oral – NC	5	150-bp, 200 bp, 500 bp	Possibly *L. crispatus*
12-Ileum-5	Oral – NC	5	300-bp, 400-bp, 500-bp, 700-bp	Unidentified *Lactobacillus* sp.
31-Ileum-5	Oral – Pro	5	150-bp, 300-bp, 500-bp	Possibly *L. johnsonii*
31-Ileum-5	Oral – Pro	5	150-bp, 300-bp, 500-bp	Possibly *L. johnsonii*
31-Ileum-5	Oral – Pro	5	250-bp, 350-bp, 500-bp, 600-bp	Possibly *L. salivarius*
31-Ileum-4	Oral – Pro	4	250-bp, 350-bp, 500-bp, 600-bp	Possibly *L. salivarius*
31-Ileum-4	Oral – Pro	4	300-bp, 400-bp, 500-bp, 700-bp	Unidentified *Lactobacillus* sp.
32-Ileum-6	Oral – Pro	6	250-bp, 350-bp, 500-bp, 600-bp	Possibly *L. salivarius*
32-Ileum-5	Oral – Pro	5	150-bp, 300-bp, 500-bp	Possibly *L. johnsonii*
32-Ileum-5	Oral – Pro	5	150-bp, 300-bp, 500-bp	Possibly *L. johnsonii*
33-Ileum-6	Oral – Pro	6	150-bp, 300-bp, 500-bp	Possibly *L. johnsonii*
33-Ileum-6	Oral – Pro	6	250-bp, 350-bp, 500-bp, 600-bp	Possibly *L. salivarius*
33-Ileum-6	Oral – Pro	6	150-bp, 200 bp, 500 bp	Possibly *L. crispatus*
34-Ileum-5	Oral – Pro	5	350-bp	Unidentified *Lactobacillus* sp.
34-Ileum-5	Oral – Pro	5	150-bp, 300-bp, 500-bp	Possibly *L. johnsonii*
34-Ileum-5	Oral – Pro	5	150-bp, 200 bp, 500 bp	Possibly *L. crispatus*
35-Ileum-5	Oral – Pro	5	200-bp, 500-bp, 600-bp	Unidentified *Lactobacillus* sp.
35-Ileum-5	Oral – Pro	5	250-bp, 350-bp, 500-bp, 600-bp	Possibly *L. salivarius*
36-Ileum-6	Oral – Pro	6	150-bp, 300-bp, 500-bp	Possibly *L. johnsonii*
36-Ileum-5	Oral – Pro	5	150-bp, 300-bp, 500-bp	Possibly *L. johnsonii*
36-Ileum-5	Oral – Pro	5	350-bp	Unidentified *Lactobacillus* sp.
36-Ileum-5	Oral – Pro	5	350-bp	Unidentified *Lactobacillus* sp.

DT = dietary treatment; ARDRA = amplified ribosomal DNA restriction analysis.

[1] Pure isolates were randomly selected from the ileum.

[2] Oral-NC, negative control, with no additives added to the basal feed, water and litter, orally inoculated with PBS solution; Oral - Pro, with probiotic (Pro) *L. johnsonii* delivery by oral gavage.

higher when a probiotic containing *L. reuteri* and *L. johnsonii* had been administered, but giving via feed or drinking water did not present different effects on growth performance and gut microbial composition in broilers.

The results of this study showed that different routes for administering *L. johnsonii* did not significantly influence the parameters of growth performance. The probiotic, when given via oral inoculation, achieved the highest weight gain (1,824 g) and FI (2,952 g) during the 35 d of the experiment, but these were not statistically significant. It is not uncommon that the use of *L. johnsonii* as a probiotic does not markedly improve bird performance (Maiorka et al., 2001; Murry et al., 2006). It is evident that probiotics such as *L. johnsonii* are effective in controlling pathogens (Cho et al., 2000; La Ragione et al., 2004) although growth enhancement by probiotics has also been reported (Schneitz, 2005).

4.2. Effects of delivery routes on organ weights and gut development

The probiotic did not affect the relative weights of intestinal tracts of broilers after 21 d of feeding. Jin et al. (1998) demonstrated that the probiotic supplement lactobacillus does not have an effect on organ weights and intestinal weight. Similar results were observed by Huang et al. (2004) who supplemented either *L. casei* or *L. acidophilus* with or without cobalt in the diets of broiler chickens.

The relative (to body weight) weights of the liver, spleen, and bursa of broilers were not affected by the probiotic *L. johnsonii* administrated by different delivery routes. However, delivery of the probiotic through feed, water and litter increased the pancreas weight on d 21. The reason(s) for this increase is not known.

The relative weights of the key organs can often be used as an indicator of changes in the morphology of the gut. The results of ileal morphology from the current study show that probiotic supplementation did not influence villus height, crypt depth and villi:crypt ratio compared with control treatments on d 7 and 21. Additives such as probiotics are regarded as modifying agents of the intestinal wall thickness due to the elimination of prejudicial bacteria (Rosen, 1995), thus germ-free birds have lighter intestinal tracts than birds originating from commercial farms (Coates et al., 1981). In an investigation on the impact of antibiotics on the organs of broilers Jong et al. (1985) reported physical alterations in the structure of the intestine, leading to a reduction in the intestinal weight. Henry et al. (1987) speculated that a decrease in the intestinal mass may result in less utilization of nutrients by the mucosa, sparing nutrients for the birds. However, neither antimicrobials (Loddi et al., 2004) nor probiotics (Pedroso,
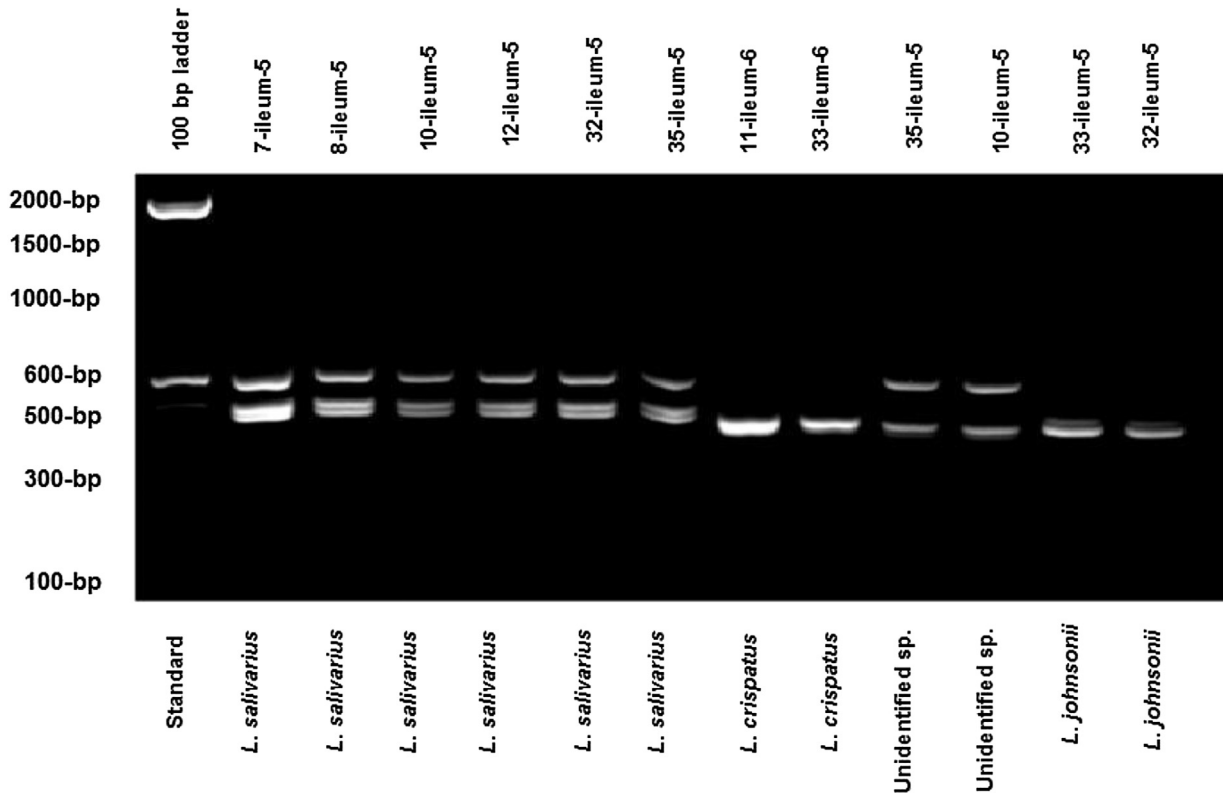
Fig. 1. Results for ARDRA analysis for 40 isolates from ileum of broiler chicken on d 7 (paret).

1999) produced significant changes in the micro-structure of the intestine of birds.

4.3. Bacterial populations, intestinal pH and SCFA concentrations

The present results show that *Enterobacteria* and *Lactobacilli* are the most important groups of bacteria in the ileum and caeca during the early life of the chicks. The number of *Enterobacteria* starts to decrease from d 7 to 21 whereas that of lactobacilli decreases progressively from d 7 to 21. This result is supported by Van der Wielen et al. (2000) who reported that, after a decline in the early life of broilers, the number of *Enterobacteria* and *Lactobacilli* stabilized after 3 weeks of age.

Direct-fed microbials are known to benefit the host animal by improving its intestinal microflora balance (Fuller, 1998). The current study showed that the number of *Enterobacteria* decreased in the caeca and ileum significantly in probiotic treatment groups compared with control treatments. This is may indicate that the *Enterobacteria* group was inhibited by the dominant probiotic group. Thus, with the establishment of *L. johnsonii* in the gastro-intestinal tract (GIT) of the birds, the enterobacterial population was outcompeted and the equilibrium of the gut microflora in the ileum and caeca was restored. This result, supported by those of Salminen and Wright (1993), demonstrates that *Lactobacillus* spp. exert a direct influence on enterobacterial colonization and it is tempting to describe the observed effects in such a manner. Vahjen et al. (1998) also indicated that a high lactobacillus population competitively excluded other members of the intestinal microflora of broilers, which displayed a slow rise in numbers in the ileum on d 21 followed by a rather sharp decline (up to ten-fold) on d 28. The number of enterobacteria in the ileum followed the same declining trend.

One of the mechanisms by which CE occurs is through the production of SCFA by the dominating microflora. This study shows the presence of high concentration of acetic and lactic acids in the ileum, and butyric and succinic acids in the caeca in the probiotic treatment groups compared with control groups on d 7 and 21. This may mean that *Enterobacteria* are more susceptible to SCFA than lactobacilli. Indeed, Van der Wielen et al. (2000) demonstrated that an increasing concentration of SCFA caused a gradual decrease in the proliferation rate of *Enterobacteria*, but not that of the lactobacilli.

C. perfringens is a ubiquitous bacterium present in the chicken gut that causes necrotic enteritis when the conditions are right for the organism (Kocher, 2003). Necrotic enteritis is estimated to cost the global broiler industry US$2 billion per annum (Keyburn et al., 2006). The current study examined the effect of supplemental *L. johnsonii* on the number of *C. perfringens* in the ileum and the caeca. The population of *C. perfringens* decreased rapidly from an early age to d 21 in the caeca, leading to a 3-fold decrease in the number of *C. perfringens* between d 7 and 21. It also showed that the probiotic treatment presented the lowest number of *C. perfringens* in the caeca (7.76 vs. 8.14 on d 7; and 4.83 vs. 5.36 on d 21). This finding is consistent with previous research (Olnood et al., 2015a or b or c) showing that *L. johnsonii*, used as a feed supplement, resulted in lower populations of *C. perfringens* in the caeca compared to the negative control on d 35 (3.67 vs. 4.24). This seems to suggest that the probiotic used in the current study may be used to alleviate the risk associated with the proliferation of *C. perfringens* in the gut, which predisposes broiler flocks to economically devastating disease of necrotic enteritis.

4.4. Probiotic candidates dominant in the gut

The microbial community of the GIT ultimately reflects the coevolution of microorganisms with their animal host and the diet adopted by the host (Drasar and Barrow, 1985). In chickens, the diet and the environment affect the microbial status of the GIT. Dirty litter and other management parameters affect the microbial composition of the chickens both directly by providing a continuous source of bacteria and indirectly by influencing the physical condition and

defence of the birds (Apajalahti et al., 2003). Changes in the composition of the animal's microflora can have beneficial or detrimental effects on the health, growth, and maturation of the host animal (Hill, 1982) Lu et al. (2003) analysed the composition of the bacterial flora in the ileum and caeca of broilers by the percent G + C profiling sequencing of 1,230 clones from a 16S rDNA community DNA library. Their results showed that *Lactobacillus* species were most abundant at 68.5% of the total sequences and *L. acidophilus*, *L. salivarius*, *L. crispatus*, *L. delbrueckii*, *L. reuteri* and *L. aviarius* were the dominant strains of lactobacilli in the ileum and caeca of chickens. Their results also indicated that *L. johnsonii* was not a dominant bacterial species in the intestinal tract of a normal chicken. Also (Dumonceaux et al., 2006) analysed the microbiota in the caeca of broilers on d 47. Their results demonstrated that the most commonly recovered sequences were lactobacilli that accounted for more than 65% of the total isolates. *L. salivarius*, and *L. crispatus* were the predominant lactobacilli in the caecal microflora and only three sequences (*L. salivarius*, *L. buchneri* and *L. crispatus*) were found in both the small intestine and the caeca.

A single dose of bacteria inoculated to newly hatched chicks can change digestal communities (Apajalahti et al., 1998). The results of this study show that *L. johnsonii* colonies were not detected in 20 of the ileal isolates in the negative control groups. This may indicate that *L. johnsonii* isolates (8/20), which were found in the oral inoculation treatment, had become dominant strains in the composition of lactobacilli in the ileum of broilers.

5. Conclusions

The novel probiotic candidate *L. johnsonii* was dominant in the intestinal tract of broiler chickens in the treatment groups. This was detected by 16-23S rDNA ARDRA patterns which also confirmed the influence of *L. johnsonii* on the gastrointestinal microfloral composition and notably the associated decrease in enterobacterial colonization in the ileum of broiler chicken between 1 and 21 d of age.

The delivery of the probiotic via drinking water, in feed, by litter application or oral gavage did not improve bird performance during the experimental period. Furthermore, there were no statistically significant differences between the various methods of delivery on the gut microflora, but individual oral application showed best regarding the reduction of *Enterobacteria* numbers in trial. The probiotic decreased the number of *Enterobacteria* and *C. perfringens*, a finding which may be regarded as a key attribute of probiotic application in poultry diets.

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