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Effects of Multi-Strain Probiotics Combined with *Gardeniae fructus* on Intestinal Microbiota, Metabolites, and Morphology in Broilers

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This study was conducted to investigate the effects of a multi-strain probiotic combined with *Gardeniae fructus* on the growth performance, intestinal microbiota composition and metabolites, and intestinal morphology of broiler chickens. The dietary treatments included the basal diet without any antimicrobials (C), the basal diet supplemented with 10 ppm avilamycin (A), the basal diet supplemented with 0.1% multi-strain probiotics powder containing *Lactobacillus acidophilus* LAP5, *L. fermentum* P2, *L. casei* L21, and *Pediococcus acidophilus* LS (1×10^7 CFU/g) (P), and the basal diet supplemented with a mixture of 0.1% multi-strain probiotics and 0.05% herbal medicine *G. fructus* (PH). The results showed no significant differences in growth performance across all groups. A denaturing gradient gel electrophoresis analysis indicated that the groups PH, P, and A exhibited an increase in the similarity coefficients of their intestinal microbial populations. The real-time polymerase chain reaction (PCR) analysis showed that the relative concentrations of *Firmicutes* and *Lactobacillus* in the cecum and *Bifidobacterium* spp. in the ileum were higher in the groups PH, P, and A than in group C, and the diet supplemented with multi-strain probiotics combined with *G. fructus* decreased the concentrations of cecal *Escherichia* spp. and *Clostridium perfringens*. The broilers fed with multi-strain probiotics combined with *G. fructus* showed a significant increase ($P<0.05$) in the cecal short-chain fatty acids (total SCFA, acetic acid, and butyric acid) compared to the other groups. The treatment with antibiotics, multi-strain probiotics, or multi-strain probiotics combined with *G. fructus* increased the villus height/crypt depth ratio in the ileum of broilers. In conclusion, the supplementation of multi-strain probiotics combined with *G. fructus* was beneficial to the intestinal microflora composition, metabolites, and morphology in broilers.

Key words: broiler, denaturing gradient gel electrophoresis, *Gardeniae fructus*, multi-strain probiotics, quantitative real-time PCR

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Introduction

Owing to the prohibition of subtherapeutic antibiotic usage in animal feed, the interest in finding alternatives to antibiotics in feed has increased. It is well known that the feed additives, including probiotics, phytogenics, organic acids, and essential oils, which could be used as potential alternatives to antibiotics, might improve gut health and growth performance (Jayaraman *et al*., 2013). Probiotics, defined as live non-pathogenic microorganisms, are beneficial to a host when present in sufficient numbers (Fuller 1989). Several studies have reported the effects of probiotics on the growth performance, nutrient digestibility, and intestinal morphology of poultry animals (Luo *et al*., 2013; Saleh *et al*., 2015). Herbal medicines are well-known feed additives used in the animal industry (Wang *et al*., 1998) and, in particular, are added in the feed to replace original antibiotics in the post-antibiotic period. Researchers have reported that traditional herbal medicines can enhance the productive performance of poultry animals, improve their gastrointestinal health, and strengthen their immune system against pathogenic invasion (Jung *et al*., 2010; Saleh *et al*., 2014, 2017). *Gardeniae fructus* is well known for enhancing protection against oxidative damage (Tseng *et al*., 1995), improving the cytotoxic ability of immune cells (Jagadeeswaran *et al*., 2000), and acting as an anti-bacterial modulator (Chang *et al*., 2013). Besides, the diet supplementation with *G. fructus* can eliminate splenic and intestinal *Salmonella choleraesuis* in the *Salmonella*-challenged mice (Chang *et al*., 2013).

In our related study, we found that the combination of *Lactobacillus* and *Scutellariae radix* and *G. fructus* enhanced the immunity against *Salmonella* infection in swine and broilers (Chang *et al*., 2013; Hsu *et al*., 2016). In addition, it

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was hypothesized that diet supplementation with both Chinese herbs and probiotics could improve intestinal microflora and act as a novel feed additive strategy. The probiotic strains, *Lactobacillus acidophilus* LAP5 (Tsai *et al*., 2005), *L. fermentum* P2 (Lin *et al*., 2007), *Pediococcus acidophilus* LS, and *L. casei* L21, were used in this study. Previous studies showed that the probiotic strains *L. acidophilus* LAP5 and *L. fermentum* P2 were acid- and bile-tolerant and were able to adhere to the cultured human intestinal cell lines (Tsai *et al*., 2005; Lin *et al*., 2007).

The primary aim of this study was to elucidate the effect of the combination of multi-strain probiotics, *L. acidophilus* LAP5, *L. fermentum* P2, *L. casei* L21 and *P. acidophilus* LS, with herbal medicine (*G. fructus*) on the growth performance, intestinal microflora, gut morphology, and cecal short-chain fatty acids (SCFA) of broilers.

Materials and Methods

Preparation of Herbal Extracts

The *Gardeniae fructus* herbal material was purchased from Ko Da Pharmaceutical Co., Ltd., Taoyuan, Taiwan. The plant materials were finely powdered and extracted using distilled water at 100℃ for 1 h (water: plant=10:1, w/v). The insoluble matter was removed by filtration, and the filtrate was concentrated in vacuum and lyophilized to yield a residue. The percentages of indicator compounds in the herbal materials were confirmed using a high-performance liquid chromatogram by Ko Da Pharmaceutical Co., Ltd. The average concentration of geniposide, an important component of *G. fructus*, was 40.15 mg/g. The herbal material of *G. fructus* was pulverized to a fine powder and passed through an 80-mesh sieve. The finely powered herbal material was used for the broiler chickens model.

Bacterial Strains and Culture Conditions

The probiotic strains, including *L. acidophilus* LAP5 (Tsai *et al*., 2005), *L. fermentum* P2 (Lin *et al*., 2007), *P. acidophilus* LS, and *L. casei* L21, were isolated in our laboratory and referred to as LAB strains. These LAB strains were cultured in the deMan-Rogosa-Sharpe (MRS) broth (Merck, Darmstadt, Germany) for 24 h at 37℃. After centrifugation at 3000×g for 10 min, the bacterial cells were washed twice with sterilized phosphate buffered saline (PBS) (pH 7.2). The multi-strain probiotics mixed in a ratio of 1:1 were used in the chicken experiments. Subsequently, the LAB culture preparation was lyophilized and stored at −20℃ until required later. The bacterial count of the LAB strain powder was 10^{10} CFU/g.

Experimental Birds and Housing

The experiment was conducted at the National Chung Hsing University, Taiwan, and the experimental protocol for animal use was approved by the Animal Care and Use Committee. A total of 400 1-day-old broiler chickens (Ross 308) were evenly divided by gender and randomly allocated to four treatments, each of which had four replicates/pens and 25 birds/pen (totaling 100 birds or 50 males and 50 females per treatment). The initial average body weight of the birds in different pens was similar (average 46.0 to 46.5 g/bird approximately). The temperature of the room was maintained at 33±1℃ for the first 3 d and then decreased to 27±1℃ until the end of the experiment. The broilers were allowed access to water and feed ad libitum throughout the experimental periods.

Diets and Experimental Treatments

All birds were offered the same antibiotic-free basal diets. The treatments were as follows: the basal diet without supplementation (C), the basal diet supplemented with 10 mg avilamycin/ kg (A), the basal diet supplemented with 0.1% multi-strain probiotics *L. acidophilus* LAP5, *L. fermentum* P2, *L. casei* L21, and *P. acidophilus* LS (final weight of the feed at 1×10^{7} CFU/g) (P), and the basal diet supplemented with 0.1% multi-strain probiotics (1×10^{7} CFU/g) and 0.5% herbal medicine *G. fructus* (PH) of the total feed. The birds were fed the starter diets from d 1 to d 21 (starter phase) and finisher diets from d 22 to d 35 (finisher phase). The basal diet was formulated to meet the nutrient needs suggested by the National Research Council (NRC 1994; Table 1). Both starter and finisher diets were mixed in the mash feed. The feed intake, body weight, and feed conversion rate (FCR) were recorded and calculated at d 21 and d 35. The growth performance of four replicate pens was averaged, regardless of the sex of birds.

Sample Collection

The digestive tracts were sampled from the birds at 35 d of age. Twenty birds (five birds per cage) were selected randomly from each treatment group and killed by cervical dislocation after a 12-h feed withdrawal. The ileum and cecum were collected and kept on ice after dissection. The digesta samples were immediately collected from the lumen of the ileum and cecum. The equal amounts of ileal or cecal digesta samples (200 mg) from the five birds within each replicate (cage) at each sampling (35 d) were pooled for DNA extraction and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). The samples were stored in a microcentrifuge tube (Eppendorf) at −80℃ for bacterial genomic DNA extraction.

DNA Extraction

DNA was extracted from the digesta samples by using the QIAamp Fast DNA Stool Mini Kit (Qiagen Inc., Germany), according to the manufacturer's recommendations. The amount of DNA extracted was determined by measuring the absorbance with a spectrophotometer at 260 nm. The DNA was stored at −20℃ until use.

PCR-DGGE Analysis

The PCR amplification of the total bacterial community DNA was performed using the primers HDA1-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3′; GC-clamp in boldface) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′) (Walter *et al*., 2000). The PCR conditions and mixture were described by Chang *et al*. (2011). The amplification program consisted of preheating at 94℃ for 4 min 30 s and 35 cycles of denaturation at 94℃ for 30 s, annealing at 56℃ for 30 s, and extension at 68℃ for 1 min, followed by the final extension step at 68℃ for 7 min.

Table 1. **Ingredients and chemical composition of the experimental diets**

Ingredient	Starter diet (1–21 days)	Finisher diet (22–35 days)
	g/kg	
Corn, yellow	472.6	5108.0
Soybean meal (CP 44%)	345.2	295.9
Full fat soybean meal (CP 34%)	100	100
Soybean oil	35.1	45.0
Monocalcium phosphate	18.6	16.6
Calcium carbonate	16.1	13.4
L-Lysine-HCl	3.8	3.2
DL-Methionine	2.0	1.3
NaCl	3.8	3.8
Choline-Cl	0.8	0.8
Vitamin premix1	1	1
Mineral premix2	1	1
Total	1000	1000
Calculated nutrient value		
ME, kcal/ kg	3050.1	3175.3
Crude protein, %	23	21
Calcium, %	1.05	0.90
Total Phosphorus, %	0.76	0.70
Available Phosphorus, %	0.50	0.45
Lysine, %	1.43	1.25
Methionine + Cystein, %	1.07	0.96

[1] Supplied per kg of diet: Vit. A 15,000 IU; Vit. D3 3,000 IU; Vit. E 30 mg; Vit. K 34 mg; Riboflavin 8 mg; Pyridoxine 5 mg; Vit. B12 25 μg; Ca-pantothenate 19 mg; Niacin 50 mg; Folic acid 1.5 mg; Biotin 60 μg.
[2] Supplied per kg of diet: Co ($CoCO_3$) 0.255 mg; Cu ($CuSO_4 \cdot 5H_2O$) 10.8 mg; Fe ($FeSO_4 \cdot H_2O$) 90 mg; Zn (ZnO) 68.4 mg; Mn ($MnSO_4 \cdot H_2O$) 90 mg; Se (Na_2SeO_3) 0.18 mg.

After visual confirmation of the PCR products on a 2% agarose gel, DGGE was performed using a Dcode Mutation Detection System (Bio-Rad Lab. Hercules, CA, USA) as described by the manufacturer. The PCR amplicons were electrophoresed in 8% (w/v) polyacrylamide gels (acrylamide: bisacrylamide＝37.5:1) with a 35 to 55% gradient of denaturant increasing in the direction of electrophoresis (100% denaturant is 7M urea and 40% deionized formamide) (Sigma-Aldrich, St. Louis, MO, USA). The bacterial PCR products (V3 region of 16S rRNA) were loaded in each line, and electrophoresis was performed in 1X TAE buffer at 60℃ under 80 V for 16 h. The gels were stained with SybrGold (1:10,000 dilution) (Invitrogen, San Diego, USA) and viewed using a UV image analysis system (Major Science, Taiwan). The similarities of PCR-DGGE profiles were analyzed with Gel Compar® Π Quick Guide Version 6.5 (Applied Maths), using the Dice function that is based on the appearance of DGGE DNA bands. The bands were manually assigned in the software and compared using a positional tolerance of 5% with manual correction wherever required. The DGGE patterns from the ileal and cecal samples were compared in separate analyses. A distance matrix was calculated using the Dice, and the dendrograms were constructed from this matrix using the unweighted pair group mean average (UPGMA). The degree of similarity was represented by a similarity coefficient.

Identification of Bacteria by Cloning and Sequencing

The DGGE bands of interest were excised aseptically from the DGGE gels into 1X PCR buffer, rinsed twice, and then incubated overnight at 4℃ in 1X PCR buffer and 0.1% Triton X-100. One microliter of the eluent was used for the subsequent PCR amplification, using the HDA primers and reaction conditions as described earlier. The PCR amplicons were then separated on a second DGGE gel as described earlier. The DNA bands comigrating with the original bands in the adjacent lanes were isolated from the gel, reamplified with the primers containing no GC-clamp, and cloned into the vector, pCR® 4-TOPO®, using the TOPO TA cloning Kit (Invitrogen) according to the manufacturer's instructions. The recombinant plasmid was transformed into *E. coli* TOP 10. The clones from each DGGE band were randomly selected and sequenced using the M13 primer with an ABI PRISM 377 Automated DNA Sequencer (Applied Biosystems, Foster City, CA). The retrieved sequences were compared with the National Center for Biotechnology Information (NCBI) GenBank database (http://blast.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST) algorithm. When a cloned sequence matched several database sequences, only the sequence with the greatest similarity to a distinct species/genus was selected as the closest se-

Table 2. **Primers used to quantify 16S rDNA in real-time PCR reactions**

Target	Primer Sequence (5′ → 3′)[a]	Genomic DNA standard	Reference
Firmicutes phylum	F: ATG TGG TTT AAT TCG AAG CA R: AGC TGA CGA CAA CCA TGC AC	*Lactobacillus farciminis* BCRC 14043^T	Queipo-Ortuño *et al.*, 2013
Bacteroidetes phylum	F: CAT GTG GTT TAA TTC GAT GAT R: AGC TGA CGA CAA CCA TGC AG	*Bacteroides vulgatus* ATCC 8482^T	Queipo-Ortuño *et al.*, 2013
Lactobacillus spp.	F: AGC AGT AGG GAA TCT TCC A R: CAC CGC TAC ACA TGG AG	*Lactobacillus farciminis* BCRC 14043^T	Rinttilä *et al.*, 2004
Bifidobacterium spp.	F: TCG CGT CYG GTG TGA AAG R: CCA CAT CCA GCR TCC AC	*Bifidobacterium longum* subsp. *infantis* BCRC 14602^T	Rinttilä *et al.*, 2004
Escherichia spp.[b]	F: GTT AAT ACC TTT GCT CAT TGA R: ACC AGG GTA TCT AAT CCT GT	*Escherichia coli* BCRC 10675^T	Wise *et al.*, 2007
Clostridium perfringens	F: ATG CAA GTC GAG CGA KG R: TAT GCG GTA TTA ATC TYC CTT T	*Clostridium perfringens* ATCC 13124^T	Rinttilä *et al.*, 2004

[a] F means forward, R means reverse
[b] The targeted *Escherichia* spp. contained the genera of *E. coli*, *Hafnia alvei*, and *Shigella*

quence relative (Sun *et al.*, 2013).

Real-Time Quantitative PCR Analysis

A real-time quantitative real-time polymerase chain reaction (qPCR) was carried out using Power SYBR Green PCR Master Mix (Applied Biosystem, UK) and an AB Step One Real-Time PCR System (Applied Biosystem). The amplification reaction was performed in a final volume of 20 μL containing 10 μL of 2X SYBR Green PCR Master Mix (Applied Biosystems), 2 μL of primer (1 μL each of forward and reverse primers), 1 μL of template, and 7 μL of the PCR-grade water. The qPCR data were analyzed using the absolute quantification method (Sun *et al.*, 2013). The amplification program started with denaturing at 95℃ for 30 s, and cycled 40 times with denaturation at 95℃ for 5 s, annealing at 60℃ for 20 s, and extension at 72℃ for 30 s. The fluorescence detection was carried out at the extension step for each cycle. All reactions were performed in triplicate. Subsequently, the specificity of PCR products was checked by performing a melting-curve analysis with continuous fluorescence measurements at every 0.5℃ increase in temperature, starting from 72 to 95℃. The specificity of PCR products was also checked by running the samples on a 2% agarose gel. The 16S rDNA genes of *Lactobacillus* spp., *Bifidobacterium* spp., *Escherichia* spp., *Clostridium perfringens* as well as the members of the phyla *Firmicutes* and *Bacteroidetes* were amplified using the gene-specific primers (Table 2). The 16S rDNA genes of *L. farciminis* BCRC 14043^T, *Bifidobacterium longum* subsp. *infantis* BCRC 14602^T, *E. coli* BCRC 10675^T, *C. perfringens* ATCC 13124^T, and *Bacteroides vulgatus* ATCC 8482^T were amplified and gel purified to construct the standard curves with a 10-fold dilution series. For each group, a partial 16S rRNA gene sequence was amplified with the real-time-PCR primers described above, and subsequently cloned into the pMD18-T vector (Takara Bio Inc., Shiga, Japan). The plasmid was purified using a commercial kit (Zymo Research, Irvine, CA, USA), and its concentration was determined using a spectrophotometer (NanoDrop). With the molecular-weight data of the plasmid and insert sequences, its copy number (g/molecule) was calculated using the equation by Whelan *et al.* (2003).

Short-chain Fatty Acid Analysis

For the determination of short-chain fatty acids (SCFA), including acetate, propionate, butyrate, isobutyrate, isovalate, and n-valerate, 1 g of cecal content was mixed with 4 mL of 25% metaphosphoric acid. The samples were centrifuged at 10000×g for 20 min, and the supernatants were filtered using 0.45-μm filters (Minisart® NML Syringe Filters 16555-K Sartorius). The analysis of SCFA was performed by gas chromatography Clarus® 580 (PerkinElmer, MA, USA) using the Nukol™ fused silica capillary column (30 m×0.25 mm ×0.25 μm; Supelco, MO, USA). The SCFA standard mixes (Supelco) were used as standard solutions.

Morphometric Analysis of the Small Intestine

At the end of the experiment (d 35), one bird per replicate cage from each treatment (a total of 4 birds/treatment) was randomly selected and sacrificed. During the necropsy, the jejunum (from the pancreatic loop to Meckel's diverticulum) and ileum (from Meckel's diverticulum to the ileo-ceco-colic junction) were removed. The 3-cm long segments were taken from the center of each part and fixed in 10% buffered formalin (pH 7.2) overnight for conducting morphometric assays under light microscopy (Yu *et al.*, 1999).

Statistical Analysis

The statistical analysis of the data was performed by analysis of variance (ANOVA) tests for completely randomized designs using the generalized linear model (GLM) procedure of the SAS software program (Statistical Analysis System, ver. 8.1, SAS Institute Inc., Cary, NC, USA). Significant statistical differences among various treatment group means were determined using the Tukey's honestly significant difference (HSD) test. The effects of the experimental diets on response variables were considered to be significant at $P < 0.05$.

Results

Productive Parameters

The growth performance of broilers was evaluated on d 35.

All broilers were healthy and had no disease symptoms during the experimental period. No significant differences were found among the treatments for body weight, feed intake, weight gain, and feed conversion ratio at any time point. The cumulative d-35 performance data were summarized as follows: feed intake (2986, 2981, 2994, and 2988 g), weight gain (2092, 2121, 2111, and 2123 g), and feed conversion ratio (1.44, 1.41, 1.42, and 1.41) in the control, antibiotic, multi-strain probiotics, and multi-strain probiotics combined with *G. fructus* groups, respectively.

Bacterial Communities

To study the effects of the dietary inclusion of multi-strain probiotics combined with herbal medicine (*G. fructus*) on the ileal and cecal bacterial communities of broiler chickens, the intestinal contents were analyzed by PCR-DGGE. The DGGE patterns of ileum and cecum are shown in Figures 1A and 2A, respectively. The 35-d-old chickens in the control, antibiotic, probiotics, and probiotics combined with *G. fructus* treatments showed different DGGE profiles of the similarity coefficients of the ileal (90.8, 85.7, 93.8, and 92.3 %) and cecal patterns (79.9, 90.9, 91.3, and 91.5%) (Figures 1B and 2B). Interestingly, the DGGE banding patterns of the probiotics combined with *G. fructus* treatment were more homogeneously distributed than those of the control chickens in cecum. The predominant DGGE bands (marked with numbers in Figures 1A and 2A) were excised and re-amplified to identify species in the sample, as shown in Table 3. The sequence similarity of each band was ≧ 97% (except 95% for band 14) as compared with that available in the GenBank database. As shown in Figures 1A and 2A, the results presented major differences in bands 2, 7, 11, and 15 among all treatments. The groups PH and P promoted *L. oris* (11), whereas group C increased *C. jejuni* (2). In addition, the treatment with PH increased *L. crispatus* (7), whereas that with P promoted *Bacteroides* sp. (15).

Intestinal Microbiota Composition

A qPCR-based method was used to determine the population of *Firmicutes*, *Bacteroidetes*, *Escherichia* spp., *Bifidobacterium*, *Lactobacillus*, and *C. perfringens* in the intestinal contents of chickens (Table 4). In the bacterial phyla, the abundance of *Firmicutes* in the ileum and cecum showed a significant increase in the probiotics combined with *G. fructus* (PH), probiotics (P), and antibiotics (A) groups compared with the control groups. In the ileum, a significant increase was observed in the *Bacteroidetes* population ($P<0.05$) in the P group compared with the C and A groups. The populations of *Lactobacillus* in the cecal contents of broiler chickens from PH-, P-, and A-supplemented groups were significantly ($P<0.05$) higher than those of the control group. All supplemented dietary treatments (A, P, and PH) significantly ($P<0.05$) increased the number of bifidobacteria in the ileum in broiler chickens at 35 d of age when compared to the control group. The birds fed with the diets supplemented with antibiotics (A) and probiotics combined with *G. fructus* (PH) had significantly ($P<0.05$) lower populations of *Escherichia* spp. in the cecum than those fed with the control diet. At 35 d of age, the birds fed with the diets A and PH showed significantly ($P<0.05$) lower *C. perfringens* populations compared to the control birds.

SCFA Analysis

The effects of the dietary treatments on the cecal SCFA concentrations of broilers are shown in Table 5. The broiler chickens fed with the probiotics combined with *G. fructus* (PH) diet had significantly ($P<0.05$) higher total SCFA, acetic acid, and butyric acid concentrations when compared to the other treatment groups, but no significant difference was observed in the concentration of propionic acid compared to the probiotic group.

Intestinal Morphology

The effects of the diet supplementation of antibiotics, probiotics, and probiotics combined with *G. fructus* on the intestinal morphology of broilers after 35 d are presented in Table 6. With regard to the morphology of the jejunum, similar villus height was observed in either group. However, the PH group showed significantly higher villus height/crypt depth than the other groups ($P<0.05$). The chickens fed with antibiotics, probiotics, and probiotics combined with *G. fructus* groups had higher ileum villus height than the control groups. The PH groups also had higher ileum villus height/crypt depth as compared with the corresponding control group ($P<0.05$). Moreover, the ileal crypt depth and villus height/crypt depth showed no significant differences among the antibiotic, probiotics, and probiotics combined with *G. fructus* groups ($P>0.05$).

Discussion

In general, the consumption of the multi-strain probiotics combined with *G. fructus* did not affect the growth performance parameters of broilers in this study. Similarly, Salehimanesh *et al*. (2016) reported that 0.09% multi-strain probiotics (*L. casei*, *L. acidophilus*, *B. thermophilum*, and *Enterococcus faecium*) did not affect the growth performance in broilers at 42 d. In addition, Shams Shargh *et al*. (2012) pointed out that adding the mixture of *L. acidophilus*, *L. plantarum*, *L. rhamnosus*, *L. bulgaricus*, *Streptococcus thermophilus*, *Aspergillus oryzae*, *B. bifidum*, *E. faecium*, and *Candida pintolepesii* (6.0×10^7 CFU/g) had no influence on the growth performance in broilers at d 42. The application of 1.5 or 3% yarrow (*Achillea millefolium L.*), a kind of herbal medicine, in diets had no effects on the feed intake, weight again, and feed conversion ratio of broiler chickens (Yakhkeshi *et al*., 2012). However, Saleh *et al*. (2013) found that supplementation with a probiotic mixture that included *Aspergillus awamori* and *Saccharomyces cerevisiae*, increased body-weight gain and improved feed conversion in broiler chickens. Hossain *et al*. (2012) reported that adding a blend of 0.5% *Alisma canaliculatum* and mixed probiotics (*L. acidophilus*, *L. plantarum*, *E. faecium*, *B. subtilis*, *B. coagulans*, and *S. cerevisiae*) to the diet could increase the growth performance of broilers. These inconsistencies might be due to the differences in probiotic species, herbal medicine, diets, feed additive dosage, and rearing conditions. In addition, with the absence of the pathogen challenge or infection, the broiler chickens supplemented

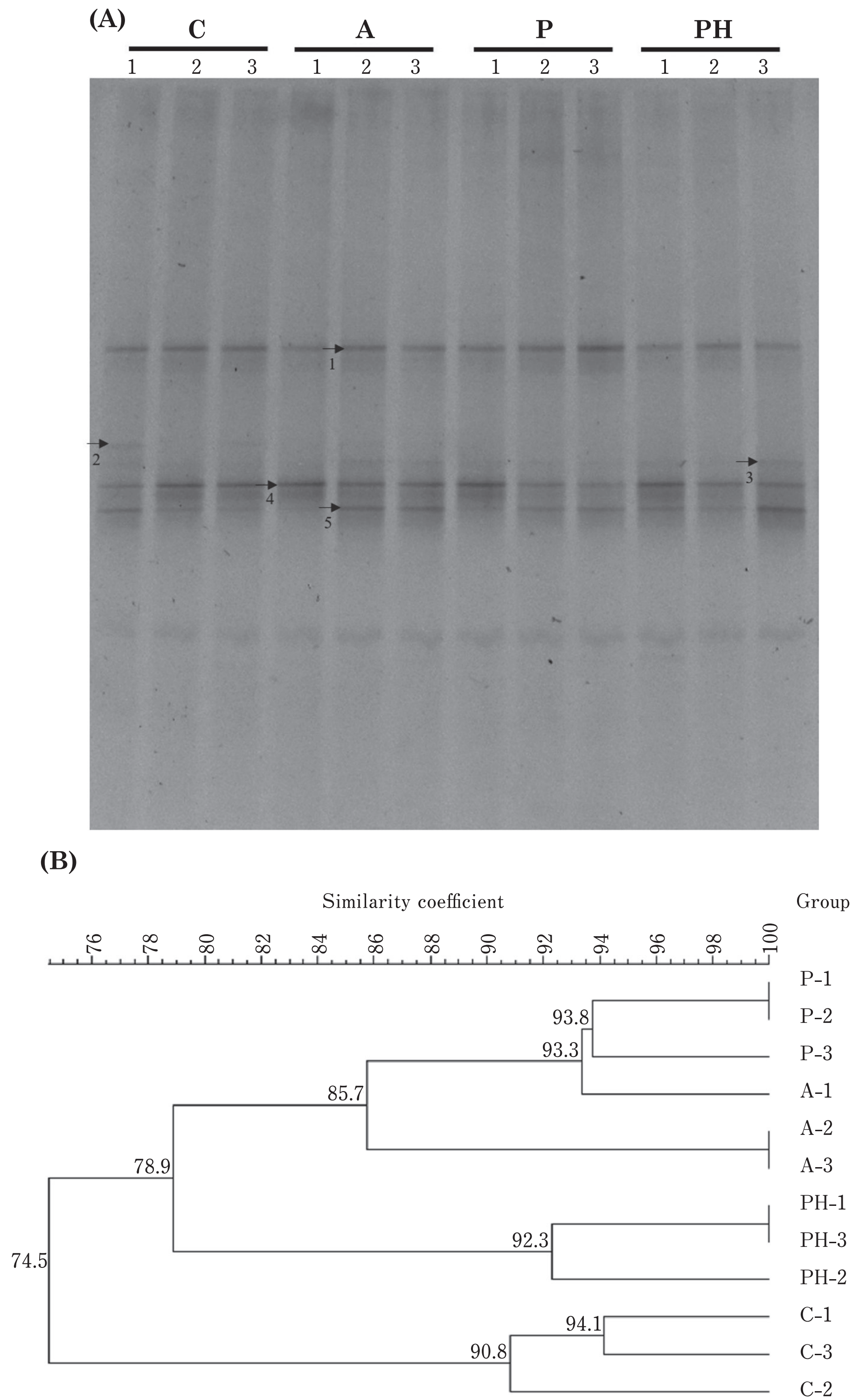

Fig. 1. **Bacterial microbiota in the ileum of broilers at 35 d of age.** C=basal diet; A=supply with 10 mg/kg of avilamycin; P=supply with 0.1% multi-strain probiotics; PH=supply with 0.1% multi-strain probiotics combined with 0.5% *G. fructus.* (A) polymerase chain reaction denaturing gradient gel electrophoresis (DGGE). Bands 1 to 7 refer to the corresponding clones in Table 3. (B) Dendrogram representing the relatedness of the PCR-DGGE profiles of ileum samples.

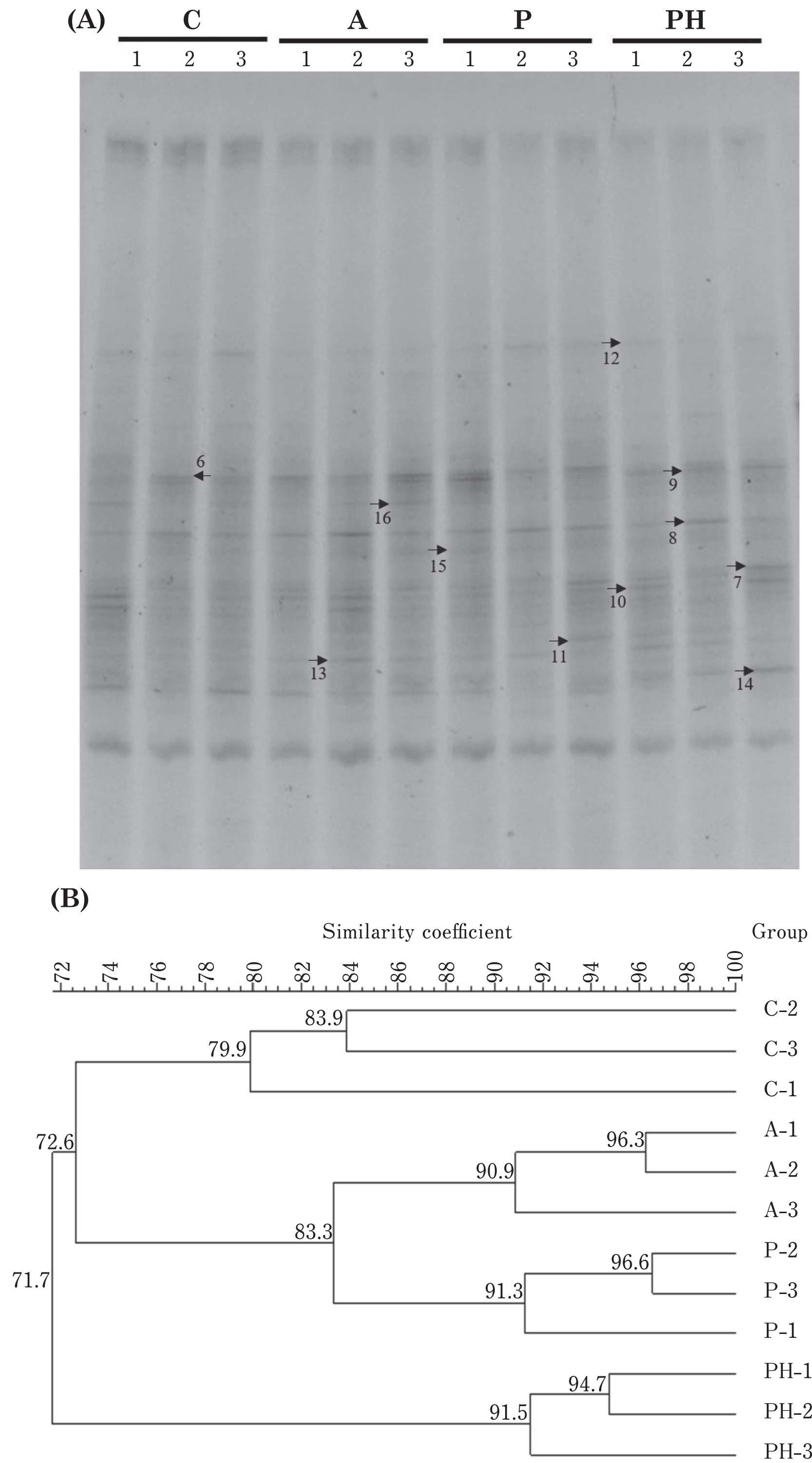

Fig. 2. **Bacterial microbiota in the cecum of broilers at 35 d of age.** C=basal diet; A=supply with 10 mg/kg of avilamycin; P=supply with 0.1% multi-strain probiotics; PH=supply with 0.1% multi-strain probiotics combined with 0.5% *G. fructus*. (A) polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Bands 8 to 19 refer to the corresponding clones in Table 3. (B) Dendrogram representing the relatedness of the PCR-DGGE profiles of cecum samples.

Table 3. **16S ribosomal RNA gene sequences of strong DNA bands from the ileum and cecum of 35-day-old broilers detected by denaturing gradient gel electrophoresis[1]**

Band number[2]	NCBI[3] accession number	Sequence size (bp)	Closest sequence relative[4]	Sequence similarity (%)
Ileum				
1	LC369503	174	Uncultured bacterium clone VDRD42BIO43 (JN021907.1)	100
2	LC369505	175	*Campylobacter jejuni* OD267 strain (CP014744.1)	99
3	LC369506	174	Uncultured bacterium clone B4-377(KF494521.1)	100
4	LC369507	200	*Lactobacillus crispatus* BC1 strain (AB976542.1)	100
5	LC369509	200	*Lactobacillus aviarius* subsp. *araffinosus* (LC071826.1)	99
Cecum				
6	LC369510	174	*Helicobacter pullorum* 3758-94 strain (KJ534305.1)	99
7	LC369511	199	*Lactobacillus crispatus* Marseille-P1443 strain (LT223588.1)	100
8	LC369512	199	Uncultured bacterium G-W-A05 clone (AB506204.1)	100
9	LC369513	177	*Bacterium* sp. NLAE-zl-C231 (JQ608310.1)	98
10	LC369514	174	Uncultured bacterium N-7 clone (JQ248083.1)	100
11	LC369515	201	*Lactobacillus oris* MAB23 strain (AF375889.1)	100
12	LC369516	194	*Bacteroides dorei* 54034 strain (KP944150.1)	100
13	LC369517	174	*Bacteroidales* bacterium ARUP UnID 176 strain (JQ259372.1)	98
14	LC369518	175	Uncultured bacterium WD5_aak40b02 clone (EU510727.1)	95
15	LC369520	194	*Bacteroides* sp. HGA0138 (JX519759.1)	97
16	LC369521	175	Uncultured bacterium 47 clone (GU060383.1)	99

[1] Determined by sequence comparison by the Basic Local Alignment Search Tool (BLAST) analysis. Ileal and cecal digesta samples were collected from the chickens at 35 d of age.
[2] Band numbers correspond to those in Figures 1A and 2A.
[3] NCBI=National Center for Biotechnology Information.
[4] The bacterial relatives were the closest BLAST matches of the named organisms deposited in GenBank.

Table 4. ***Firmicutes, Bacteroidetes, Lactobacillus* spp., *Escherichia* spp., *Clostridium perfringens* and *Bifidobacterium* spp. populations in the ileum and cecum of the 35-day-old broilers by quantitative real-time PCR[1]**

Item	Experimental diets				SEM	P value
	C	A	P	PH		
Firmicutes	----log^{10} of copy number/g DNA extract----					
Ileum	7.83^c	8.58^a	8.18^b	8.53^a	0.04	0.001
Cecum	8.22^b	8.60^a	8.57^a	8.73^a	0.08	0.044
Bacteroidetes	----log^{10} of copy number/g DNA extract----					
Ileum	5.02^a	5.17^a	3.15^b	4.31^{ab}	0.32	0.022
Cecum	7.00	7.01	6.76	6.76	0.11	0.344
Lactobacillus spp.	----log^{10} of copy number/g DNA extract----					
Ileum	4.72	5.21	4.73	5.09	0.24	0.515
Cecum	5.79^b	6.42^a	6.36^a	6.44^a	0.09	0.027
Escherichia spp.	----log^{10} of copy number/g DNA extract----					
Ileum	4.22	4.22	3.72	4.14	0.25	0.564
Cecum	4.12^a	3.42^b	3.81^{ab}	3.41^b	0.12	0.028
Clostridium perfringens	----log^{10} of copy number/g DNA extract----					
Ileum	4.03^a	3.52^b	3.65^{ab}	3.68^{ab}	0.09	0.108
Cecum	4.30^a	3.45^b	3.97^{ab}	3.39^b	0.21	0.061
Bifidobacterium spp.	----log^{10} of copy number/g DNA extract----					
Ileum	4.26^b	5.20^a	5.31^a	5.23^a	0.07	0.001
Cecum	5.17	5.35	5.45	5.32	0.18	0.817

[1] C=basal diet; A=basal diet + 10 mg/kg of avilamycin; P=basal diet + 0.1% multi-strain probiotics; PH=basal diet + 0.1% multi-strain probiotics + 0.5% *G. fructus*
[a, b] Means with different superscripts in each row were significantly different ($P < 0.05$).
Each value represents the mean of four replicates with four birds in each replicate

Table 5. **Effect of a diet comprising the multi-strain probiotics combined with *Gardeniae fructus* preparation on the short-chain fatty acid (μmole/g) profile in the cecal contents of 35-day-old broilers[1]**

Item	Experimental diets				SEM	P value
	C	A	P	PH		
	---SCFA, (μmole/g)---					
Total SCFA	24.17^{b}	25.11^{b}	24.01^{b}	29.81^{a}	0.57	0.0031
Acetic acid	12.27^{b}	11.84^{b}	11.90^{b}	13.91^{a}	0.36	0.0407
Propionic acid	3.88^{b}	4.01^{b}	5.20^{a}	5.84^{a}	0.25	0.0116
Butyric acid	5.30^{b}	6.79^{b}	3.91^{c}	7.08^{a}	0.30	0.0039
Isobutyric acid	0.66	0.54	0.71	0.69	0.03	0.1046
Isovaleric acid	1.04	0.95	1.08	1.12	0.05	0.3478
n-Valeric acid	1.02	0.98	1.21	1.17	0.08	0.3895

[1] C=basal diet; A=basal diet + 10 mg/kg of avilamycin; P=basal diet + 0.1% multi-strain probiotics; PH=basal diet + 0.1% multi-strain probiotics + 0.5% *G. fructus*

[a, b] Means with different superscripts in each row were significantly different ($P<0.05$).

Each value represents the mean of four replicates with four birds in each replicate

Table 6. **Effect of a diet comprising the multi-strain probiotics combined with *Gardeniae fructus* preparation on intestinal morphology of 35-day-old broilers[1]**

Item	Experimental diets				SEM	P value
	C	A	P	PH		
Jejunum						
Villus height, (μm)	1459	1443	1404	1488	21.0	0.1427
Crypt depth, (μm)	167	168	186	172	4.5	0.2654
Villus height/Crypt depth	7.07^{b}	6.50^{b}	7.03^{b}	8.11^{a}	0.2	0.0007
Ileum						
Villus height, (μm)	825^{c}	1050^{a}	972^{b}	940^{b}	13.2	0.0001
Crypt depth, (μm)	160	176	162	157	4.8	0.3495
Villus height/Crypt depth	5.34^{b}	6.14^{a}	$5.84^{a,b}$	6.42^{a}	0.2	0.0265

[1] C=basal diet; A=basal diet + 10 mg/kg of avilamycin; P=basal diet + 0.1% multi-strain probiotics; PH=basal diet + 0.1% multi-strain probiotics + 0.5% *G. fructus*

SEM=standard error of the mean.

[a,b,c] Means with different superscripts in each row were significantly different ($P<0.05$).

Each value represents the mean of 16 replicates (One bird per replicate×four replicates per treatment×four measurements per section).

with probiotics and herbs might not significantly influence their growth performance (Gunal *et al*., 2006; Shams Shargh *et al*., 2012).

DGGE is extremely sensitive for detecting the dominant bacteria, which constitute up to 1% of the total bacterial community (Zoetendal *et al*., 2004). In the present study, *Heliobacter pullorum* and *Bacteriodes* sp. were found in all treatments. *Heliobacter pullorum*, a related bacterium, is a common inhabitant of the ceca and large intestine of the asymptomatic broiler chickens (Atabay *et al*., 1998). The bacteria in the *Bacteriodes* genus are Gram-negative bacteria that utilize plant glycans as their main energy sources (Martens *et al*., 2008). Moreover, the members of the *Bacteriodes* genus are one of the predominant anaerobic bacterial groups found in the chicken ceca (Lan *et al*., 2006). Based on the DDGE results, the broilers treated with PH and P might show no effect on these pathogens.

However, among different bands in the DGGE results, *C. jejuni* was not found in the ileum of the A, P, and PH groups, but was present in the ileum of the control group. Campylobacteriosis is an infection caused by *Campylobacter* species, most commonly *C. jejuni*. It is one of the most common bacterial infections of humans, often caused by contaminated food. It sometimes induces an inflammation in the blood, leading to negative effects, including diarrhea, dysentery syndrome, cramps, fever, and pain (Cean *et al*., 2015). In contrast, Johansen *et al*. (2007) reported that salinomycin did not influence the *C. jejuni* counts in the ceca of a *C. jejuni*-challenged broiler. These differences might attribute to various antibiotic and pathogen challenges.

On the other hand, the results of real-time PCR showed that the PH treatment increased the number of *Firmicutes*, but did not influence the *Bacteroidetes* population. The *Firmicutes* and *Bacteroidetes* are the two most abundant bacterial phyla in the cecum of broilers (Threlfall *et al*., 2000). The importance of these two phyla has been high-

lighted in host metabolism. An increased ratio of *Firmicutes*/*Bacteroidetes* has been shown to be associated with obesity in humans and mice due to an increase in the energy harvesting capacity of bacterial species in the *Firmicutes* phylum (Turnbaugh *et al*., 2006; Turnbaugh *et al*., 2009). We also found that the relative number of *Lactobacillus* species increased in these three treatments (PH, P and A). Besides, the treatments with P, PH, and A increased the number of *Bifidobacterium* spp. in the ileum and decreased the number of *E. coli* and *C. perfringens* in the ceca of broilers. The modulation of intestinal microbiota in the P and PH treatments might be attributed to supplementation with a mixture of probiotics in broiler diets. Kim *et al*. (2012) found that supplementation with a probiotic mixture that included *L. acidophilus*, *Bacillus subtilis*, and *S. cerevisiae* decreased the amounts of *Clostridiu*m spp. and coliforms in broiler chickens. Zhang *et al*. (2014) also pointed out that the addition of a mixture of 2×10^8 viable spores/kg of *L. acidophilus*, *B. subtilis*, and *C. butyricum* resulted in increased *Lactobacillus* and decreased *E. coli* counts in the cecum. *Lactobacillu*s is one of the predominant bacteria in chicken digestive tracts that can prevent diarrhea and intestinal infection (Edelman *et al*., 2002; Strompfová *et al*., 2006). Generally, lactobacilli can produce antibacterial factors, including hydrogen peroxide, organic acids, and bacteriocins, which might act synergistically to suppress the proliferation of enteric pathogens *in vivo* (Lima *et al*., 2007). Besides, both lactobacilli and bifidobacteria are able to limit the growth of pathogens, such as *E. coli*, *Salmonella*, and *C. perfringens*, through the production of bacteriocins or volatile fatty acids and competing with pathogens for attachment sites on the intestinal surface (Grilli *et al*., 2009; Tejero-Sariñena *et al*., 2013; Mookiah *et al*., 2014).

Similar to our results, Mountzouris *et al*. (2007) showed that the addition of the mixture of lactobacilli in diets had the same effects as antibiotics on increasing the cecal *Bifidobacterium* spp. counts. Though we did not observe higher levels of beneficial bacteria in the PH group compared to the P or A groups, the former could improve the intestinal microbial structure compared to the control group. Therefore, it is suggested that a mixture of probiotics or probiotics combined with herbal medicine could potentially replace antibiotic supplementation in broiler diets. Likewise, Chang *et al*. (2013) confirmed that *G. fructus* and two probiotics (*L. acidophilus* LAP5 and *L. reuteri* PG4) decreased the fecal and intestinal numbers of *Salmonella* in the *Salmonella*-challenged swine. Hsu *et al*. (2016) also found that supplementation with the probiotics (*L. acidophilus* LAP5 and *L. reuteri* PG4) combined with *G. fructus* could decrease the number of *Salmonella* in the intestine and feces of the *Salmonella enterica* serovar Typhimurium-challenged broilers.

In the present study, the multi-strain probiotics combined with *G. fructus* not only improved the intestinal bacterial balance, but also regulated the concentration of intestinal SCFA and morphology. High levels of total SCFA, acetic acid, and propionic acid produced in the PH treatment was probably due to an increase in the amount of fermented *Lactobacillus* and *Bifidobacterium* species in the intestine of broilers. The SCFA are not only efficiently absorbed by the colonic mucosa, but are also responsible for the reduction of pathogens in the ceca by creating a low-pH intestinal environment that inhibits the viability and growth of pathogenic bacteria (Van Der Wielen *et al*., 2000). The SCFA, including acetic, propionic, and butyric acids, provide energy to the intestinal epithelial cells. In addition, the SCFA were considered to be the main factors for stimulating the development of intestinal mucosa (Von Engelhardt *et al*., 1998). Apart from the intestinal SCFA, the *Lactobacillus* species might strengthen the mucosal barrier by enhancing the production of mucin, which is an important component defending against intestinal pathogens. To conclude, in the PH treatment, large populations of *Lactobacillus* and *Bifidobacterium* were associated with increased cecal SCFA and improvement of intestinal morphology. With healthier gut morphology, a host might further prevent pathogen invasion and stabilize intestinal microbiota conversely.

Conclusion

Diet supplementation with a mixture of multi-strain probiotics and *G. fructus* increased the total SCFA concentration in the cecum, thereby improving intestinal development and integrity in broilers. Moreover, the combined treatment enhanced the beneficial bacteria (*Lactobacillus* and *Bifidobacterium*) counts and inhibited pathogenic bacteria (*E. coli* and *C. perfringens*). We suggest that the multi-strain probiotics combined with *G. fructus* will be beneficial to the intestinal microflora composition and metabolites and intestinal morphology in broilers.

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