Effects of the Probiotics Supplementation in Diet on Intestinal Microflora Ecosystem in Broilers

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***Abstract*—The study was conducted to evaluate the effects of multi-strain probiotics and antibiotic on the intestinal ecosystem in broilers. One hundred and eighty 1-day-old broilers (Ross 308) were randomly allocated into 3 dietary treatments including the control (corn-soybean meal, basal diet), and basal diet supplemented with 0.1% probiotics (*Lactobacillus acidophilus* LAP5、*L. casei* L21、*L. fermentum* P2 and *Pediococcus acidilactici* LS) or 10 ppm antibiotic (avilamycin) for 3 weeks feeding period. Nine birds were sacrificed at 1, 2 and 3 weeks of age from each group to analyze the intestinal ecosystem by using the real time PCR combined with denaturing gradient gel electrophoresis (DGGE). The DGGE banding profiles showed that the similarity of microbial community was higher level in probiotic group compared to the control and antibiotic groups, and it have more accordant to bacterial similarity and enhance clustering effect compared with other groups in cecum. The results of real-time PCR revealed that the population of Lactobacilli in the cecum of probiotic group was significantly higher than control and antibiotic groups ($P<0.05$). The population of *Bifidobacterium* in cecum was significantly increased, while the population of *Clostridium perfringens* was decreased in probiotic group at 2 and 3 weeks. In conclusion, diets supplemented with multi-strain probiotics could increase the population of beneficial bacteria (including Lactobacilli and *Bifidobacterium*) as well as decrease the number of *C. perfringens*; hence probiotics can improve the homogeneity of microbial community inhabited in the cecum of broilers.**

***Index Terms*—probiotic, DGGE, real time PCR.**

I. Introduction

Intestinal bacteria of healthy animals could be maintained for a long time in a stable dynamic equilibrium. However, the composition of microflora is affected by external and internal factors, including: the host's intestinal environment, microbial factors, interaction between strains and dietary factors [1]. Monitoring the intestinal microflora may be done using a variety of techniques, including traditional bacterial culture, among the current various methods of molecular biology. Due to the complex intestinal microflora composition and many strains having a symbiotic relationship, microflora cannot be fully detected using traditional culture. Therefore, the development in recent years of many applications of molecular techniques of culture-independence plays a very important role in the study of intestinal bacterial flora [2]. Chen et al. indicate that using the DGGE methods can facilitate full screening; they discuss the intestinal strains and show how the distribution results of the intestinal microflora can be rapidly understood [3]. Furthermore, the PCR-DGGE technique is largely used to analyze the complex environmental flora, such as: soil microflora [4], marine microflora [5], river microflora [6] and intestinal microflora [7].

Antibiotics have been widely used in poultry feeds, at first to control disease, but subsequently, subtherapeutic levels of antibiotics have been used to increase growth rates and improve feed efficiency. However, the recent European Union ban on the prophylactic use of in-feed antibiotics has escalated the search for alternatives for use within the poultry industry. The probiotic may be a possible way to improve intestinal and animal performance in the absence of antibiotic growth promoters.

The distribution of bacterial species in the intestine of broiler will be measured using PCR-DGGE combined with the 16s rDNA clone library. The diversity of enteric bacteria communities between broilers with various growth rates will be surveyed, and the correlation among microbiota, growth rate and intestinal development will be investigated. In addition, the effects of probiotics and antibiotics on the intestinal bacteria community, intestinal histology, energy utilization and growth performance in the broilers will be studied. In accordance with the results, the effect of probiotics as compared to the effects of antibiotics on the optimum modulation of intestinal microflora in improving the health and growth of the broiler will be revealed.

II. Materials and Methods

A. Birds and Experimental Treatments

One hundred and eighty 1-d-old male Ross 308 broilers were obtained from a local commercial hatchery. Broilers were vaccinated on d 1 for Marek, infectious bronchitis, and Newcastle disease and were randomly allocated in 3 experimental treatments for 3 wk. Housing and care of the birds conformed to Faculty of Animal Science and Aquaculture guidelines. The experimental treatments received a corn-SBM basal diet (BD) and depending on the addition were labeled as follows: BD-no other addition (Control), BD containing a probiotic concentration of 10^8 cfu/kg of diet (Probiotic), and BD

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containing avilamycin at 10.0 mg/kg of diet (Antibiotic). Probiotic contains primarily *L. acidophilus* LAP5、*L. casei* L21、*L. fermentum* P2 and *P. acidilactici* LS along with other genera of bacteria. On d 7, 14, and 21, 9 chickens from each group are then sacrificed. The intestinal region of the crop, ileum and cecum was collected using an aseptic technique. All samples were kept on ice and processed immediately after dissection. The mixture of crop, ileum and cecum contents and wall-associated bacterial samples from each of chickens was maintained at −20°C until use. The samples were subsequently used for further investigations determination of microbial concentration, and DNA extraction.

B. DNA Extraction

DNA was extracted from the crop, ileum and cecum contents. An amount of 2.0 ml of caecal material suspended in ethanol was centrifuged at 10,000 x g for 1 min. The supernatant was discarded and the sample was washed with BPW and centrifuged at 10,000 x g for 1 min. The washing step was repeated. Finally, the DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen Inc., Hilden, Germany). The extraction was carried out in accordance with the instructions of the manufacturer, with an additional step of lysozyme treatment, which was added to the procedure before the use of Inhibit EX tablets. An amount of 140 µl of a 10 mg/ml solution of lysozyme (Sigma- Aldrich, St. Louis, MO, USA) in Tris-EDTA buffer (10:1 mM), pH 8, was added to each extraction tube and the samples were incubated at 37 ℃ for 60 min. The DNA was eluted in 100 µl buffer AE (Qiagen). All DNA samples were stored at -20 ℃ until further processing.

C. PCR Amplification with HDA (Universal 16S rDNA) Primers

PCR amplifications of total bacterial community DNA were 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 ; GCclamp in boldface) and HDA2 (5'-GTA TTA CCG CGG CTG CTG GCA C-3'). The thermocycling programme was: 94 ℃ for 20 s ; 25 cycles of 94 ℃ for 30 s, 56 ℃ for 30 s, and 68 ℃ for 1 min; and finally 68 ℃ for 7 min [8]. PCR was performed in 0.2 ml tubes with a 2720 Thermal Cycler (Applied Biosystems) and a reaction mixture as previously described [9] The PCR products were confirmed by electrophoresis on a 2% agarose gel containing 0.1 µg/ml HealthView nucleic acid stain (Genomics,Taiwan) and viewed by UV transillumination.

D. DGGE Analysis

DGGE was performed with the Dcode universal mutation detection system (Bio-Rad) using 16 cm by 16 cm by 1 mm gels. The 8% polyacrylamide gels (ratio of acrylamide: bisacrylamide, 37.5:1) (Bio-Rad) contained a 30 to 55% gradient of urea and formamide (Fluka, Sigma-Aldrich, St. Louis, MO, USA) increasing in the direction of electrophoresis, which was run at 80 V and 60 ℃ for 16 h. The gels were stained with SybrGold (1:10,000 dilution) and viewed by UV transillumination [9]. The intestinal bacterial community profiles were compared using the GelCompar II Quick Guide (Version 6.5; Bio-Rad). Initially, the DGGE gels were normalized by means of the DGGE markers used, and the software conducted a band search according to a 5% minimum profiling and a 10% grey-zone interval. Subsequently, all bands were checked manually. The comparisons were based on the Dice similarity coefficient and the un-weighted pair group method using arithmetic averages (UPGMA) for clustering.

E. Bifidobacterium and Bacteroides Abundance Analysis by Real-Time Quantitative PCR

The colonizations of *C. perfringens*, *Bifidobacterium*, and *Lactobacillus spp.* were analyzed by real-time PCR. Sample genomic DNA was used as a template for PCR amplification using SYBR Green PCR technology (Applied Biosystems, Foster City, CA) and an ABI 7500 real-time PCR instrument (Applied Biosystems). Species-specific 16S rRNA primers were used for the *C. perfringens* [F:5'ATGCAAGTCGAGCGAY-3'] and [R:5'-TATGCGGTATTAATCTYCCTTT-3']; [10], the *Bifidobacterium* subgroup [F:5'-GGGTGGTAATGCCGGATG-3'] and [R:5' TAAGCCATGGACTTTCACACC-3']; [11], and the *Lactobacillus* spp. [F:5'-AGCAGTAGGGAATCTTCCA-3'] and [R: 5'-CACCGCTACACATGGAG-3']; [10]. Amplification was performed in 20 µL containing 10 µL of 2× SYBR Green PCR Master Mix (Applied Biosystems), 2 µL of primer (1 µL of forward and 1µL of reverse in each), 1 µL of template, and 7 µL of PCR-grade water. Bacteria copy numbers were generated from a standard curve prepared from purified plasmid clones of the 16S rRNA gene from *Bifidobacterium*, *Lactobacillus* and *C. perfringens*. Gene copy number was calculated from the concentration of the extracted plasmid DNA clone assuming $7.8x10^6$, $9.6x10^6$ and $2.1x10^9$ g/bp.

F. Statistical Analysis

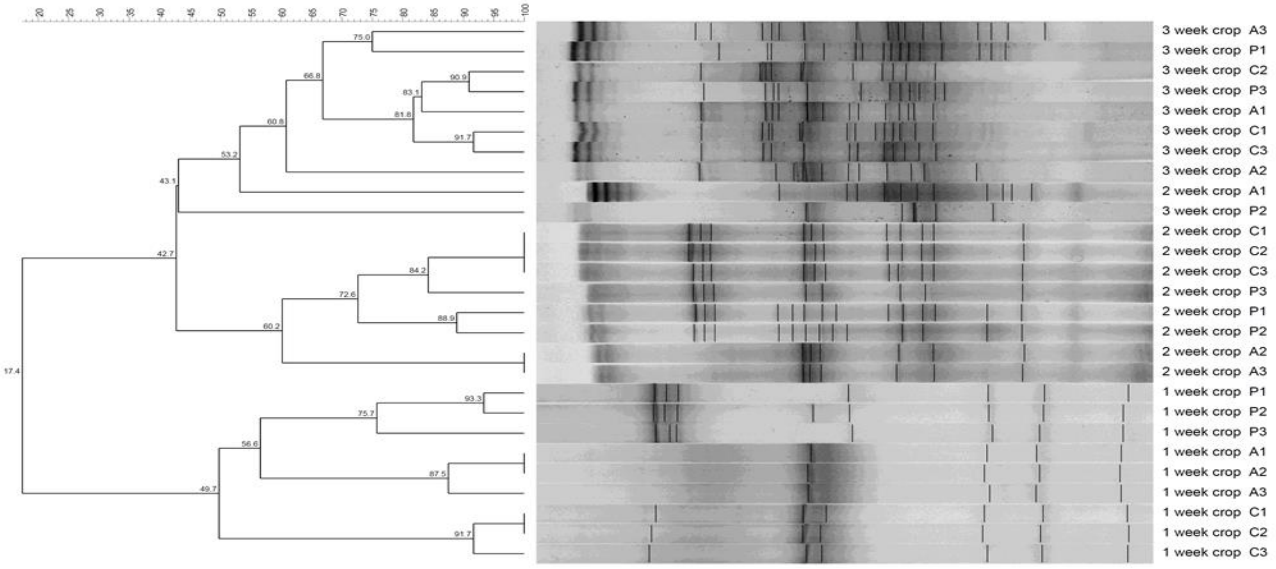
One-way ANOVA was used to determine the significant ($P < 0.05$) difference between the groups tested with individual chickens representing an experimental unit. Statistic analysis was carried out using SAS (version 8, SAS Institute Inc., Cary, NC).

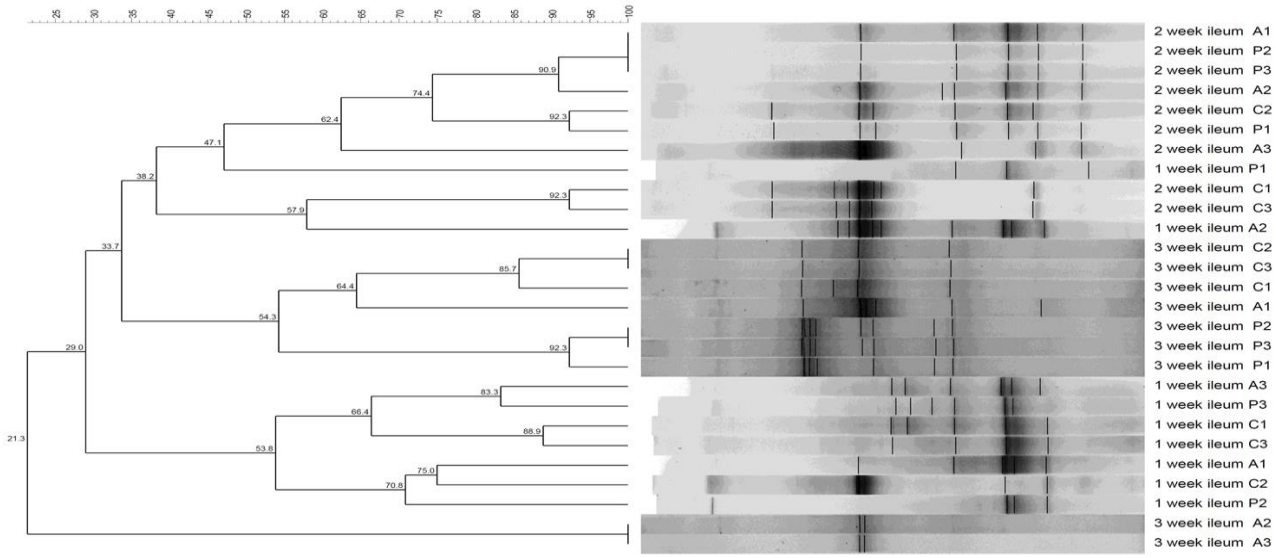
III. Results and Disscution

The DGGE banding profile showed that the similarity of microbial community in caecum was higher in probiotic group compared with control and antibiotic groups, and it have more accordant to bacterial similarity and enhance clustering effect in cecum, compared to the other two groups.

The populations of lactobacilli, Bifidobacterium, and C. perfringens in the crop, ileum, and cecum are shown in Table I-Table III. The assay showed that the numbers of Bifidobacterium, and C. perfringens 16S rRNA gene copies both in the control and treatment groups showed

an apparent fluctuation from weeks 1-2 although there were no obvious changes in the density. The population of *Bifidobacterium* in cecum was significantly increased, while the population of *C. perfringens* was decreased in probiotic group at 2 and 3 weeks. In the cecum, the probiotics treatment showed significantly higher the Lactobacillus levels than did the antibiotic and control groups.





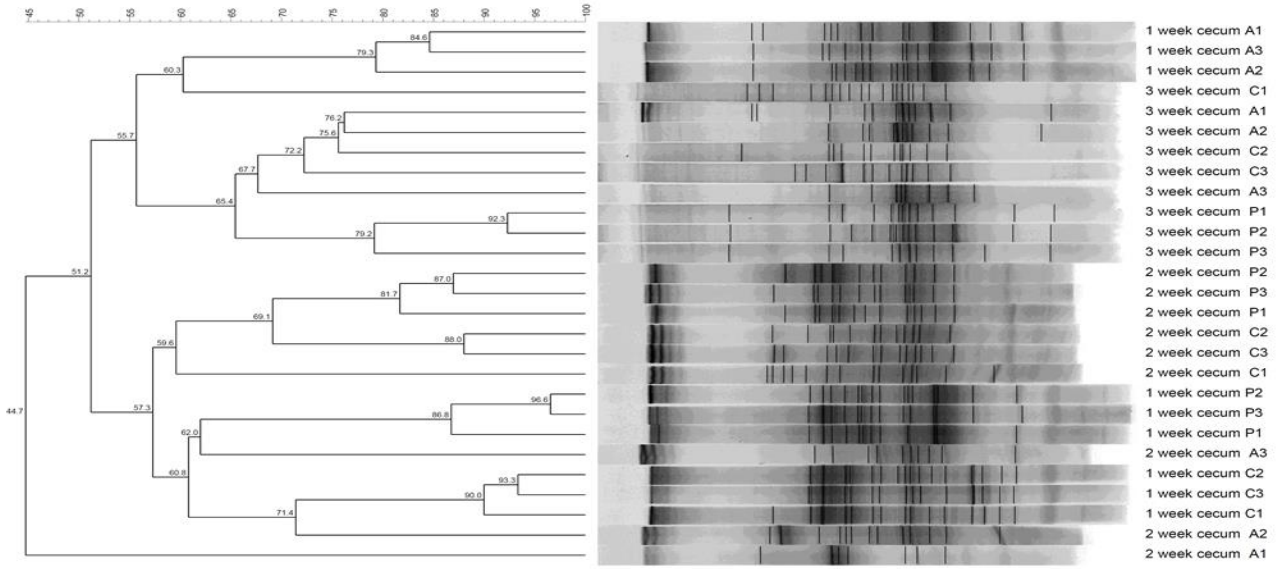


TABLE I. MICROBIAL POPULATIONS IN THE SMALL INTESTINAL CONTENT OF BROILER CHICKENS *C. PERFRINGENS*, *BIFIDOBACTERIUM*, AND *LACTOBACILLUS* IN CROP SAMPLES WERE ANALYZED BY REAL-TIME PCR

age (week)	Experimental diets			
	Control	Probiotic	Antibiotic	SEM
	Lactobacillus group copy number (copies/ml)			
1	17600	13467	16880	1410
2	42000 [ab]	46850 [a]	34220 [b]	1530
3	23933 [b]	43500 [a]	19767 [b]	5628
	Bifidobacterium sp. copy number (copies/ml)			
1	42.74	37.99	6.88	12
2	80	70.93	77.31	4.37
3	29.87	15.18	20.17	4.29
	C. perfringens copy number (copies/ml)			
1	0.26	0.54	0.21	0.12
2	3.91	4.21	2.68	2.64
3	1730	2246.7	2070	400

Probiotic: *L. acidophilus* LAP5, *L. casei* L21, *L. fermentum* P2 and *P. acidilactici* LS; Antibiotic: 10 ppm avilamycin.
[a,b] Mean in the same rows without same superscript are significantly different (P<0.05).

TABLE II. MICROBIAL POPULATIONS IN THE SMALL INTESTINAL CONTENT OF BROILER CHICKENS *C. PERFRINGENS*, *BIFIDOBACTERIUM*, AND *LACTOBACILLUS* IN ILEUM SAMPLES WERE ANALYZED BY REAL-TIME PCR

age (week)	Experimental diets			
	Control	Probiotic	Antibiotic	SEM
	Lactobacillus group copy number (copies/ml)			
1	4305	1940	3180	1596
2	37833	34000	42000	9650
3	31945	33500	30300	6267
	Bifidobacterium sp. copy number (copies/ml)			
1	26.28	46.38	39.11	11
2	32	41.15	32.01	2.29
3	42.79	54.47	42.49	5.16
	C. perfringens copy number (copies/ml)			
1	2.48	1.16	0.51	0.95
2	4.28	5.73	6.45	0.63
3	585	341.3	344.9	143

Probiotic: *L. acidophilus* LAP5, *L. casei* L21, *L. fermentum* P2 and *P. acidilactici* LS;
Antibiotic: 10 ppm avilamycin.
[a,b] Mean in the same rows without same superscript are significantly different (P<0.05).

TABLE III. MICROBIAL POPULATIONS IN THE SMALL INTESTINAL CONTENT OF BROILER CHICKENS *C. PERFRINGENS*, *BIFIDOBACTERIUM*, AND *LACTOBACILLUS* IN CECUM SAMPLES WERE ANALYZED BY REAL-TIME PCR

Age (week)	Experimental diets			
	Control	Probiotic	Antibiotic	SEM
	Lactobacillus group copy number (copies/ml)			
1	44200 [b]	181500 [a]	41600 [b]	8776
2	342905 [c]	553655 [a]	449700 [b]	1430
3	413963 [b]	592000 [a]	450060 [b]	21558
	Bifidobacterium sp. copy number (copies/ml)			
1	3460	2285	2680	457
2	34900 [b]	178000 [a]	13527 [b]	30632
3	23800 [b]	174000 [a]	53767 [b]	15139
	C. perfringens copy number (copies/ml)			
1	3.19	1.68	2.5	1.39
2	4715	4915	3445	2148
3	16850	13515	11193	1046

Probiotic: *L. acidophilus* LAP5, *L. casei* L21, *L. fermentum* P2 and *P. acidilactici* LS; Antibiotic: 10 ppm avilamycin.
[a,b] Mean in the same rows without same superscript are significantly different (P<0.05).

The probiotic induced a statistically significant increase in the microbiota diversity as deduced from the DGGE analysis. In other aspects, the probiotic was more effective, namely in the reduction of coliforms and the increased *Bifidobacterium* together with *Lactobacillus* [12]. The use of probiotics to prevent *C. perfringens* colonization and NE displays potential as strains including *Bacillus subtilis* PB6 [13] and *Lactobacillus* spp. [14] have been found to prevent *C. perfringens* growth in vitro. Few bacterial strains have successfully demonstrated the capacity to prevent the development of NE in vivo; however, *L. johnsonii* FI9785 has been reported to prevent *C. perfringens* colonization in pathogen-free broilers [15].

IV. Conclusion

In conclusion, diets supplemented with multi-strain probiotics could increase the population of beneficial bacteria (including *Lactobacillus* and *Bifidobacterium* strains) as well as decrease the number of *C. perfringens*; hence probiotics can improve the homogeneity of microbial community inhabited in the cecum of broilers.

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