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The Effects of Probiotic *Lactobacillus reuteri* Pg4 Strain on Intestinal Characteristics and Performance in Broilers

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ABSTRACT : This study was conducted to evaluate the feasibility of using *L. reuteri* Pg4, a strain isolated from the gastrointestinal (GI) tract of healthy broilers, as a probiotic. In preliminary *in vitro* studies the Pg4 strain was proven capable of tolerating acid and bile salts, inhibiting pathogenic bacteria and can adhere to intestinal epithelial cells. The probiotic properties were then evaluated on the basis of the broiler's growth performance, intestinal microbial population and cecal volatile fatty acid and lactic acid concentrations under conventional feeding. Dietary supplementation of dried *L. reuteri* Pg4 decreased significantly feed intake in grower chickens and improved significantly the feed conversion by 5% in a 0-6 weeks feeding period compared with the control group. The *Lactobacillus* counts in the crop, ileum, and cecum of the probiotic group were higher than in the control group. The *L. reuteri* Pg4 strain was traceable in the GI tract of probiotic supplemented chicks and showed capability of survival in the intestine for a protracted period. The probiotic group had a higher lactic acid concentration and lower pH value in the cecum than the control chicks. Probiotic supplement also affected the histology of the intestinal mucosa of chicks. The present findings demonstrated that *L. reuteri* Pg4 possesses probiotic characteristics and it is suggested, therefore, that the organism could be a candidate for a new probiotic strain. (**Key Words** : Broiler, Growth Performance, Intestine, Probiotic)

INTRODUCTION

Animal agriculture relies heavily on antibiotics, both for disease treatment and growth promotion. Mounting public concerns of antibiotic resistance include bans on sub therapeutic antibiotic usage in Europe. There is also a possibility in the near future in other regions of the world of increasing pressure to eliminate antibiotic additives in feed (Reid and Friendship, 2002). A number of antibiotic substitutes to improve animal health and productivity have been developed in recent decades. Probiotics, defined as direct feed micro-organisms or microbial cell preparations, are beneficial to the host animal by improving its intestinal microbial balance, thus offering greater potential for the antibiotic replacement (Fuller, 1989; Rambaud et al., 1993). Sufficient evidence has demonstrated the effectiveness of probiotics in improving growth performance, reducing diarrhea and enhancing the immune system (Reid and Friendship, 2002; Patterson and Burkholder, 2003; Koenen et al., 2004; Khaksefidi and Rahimi, 2005). In contrast, the reports from Watkins and Kratver (1983; 1984) show that the oral administration of high numbers of different *Lactobacillus* strains or introducing commercial *Lactobacillus* in drinking water did not have any significant effects on broiler growth or feed intake. Jin et al. (1998) demonstrated the failure of probiotic cultures to enhance chicken production has been attributed to the inability of micro-organisms to colonize or survive in the gastrointestinal tract, and their inability to antagonize or competitively exclude the pathogenic bacteria. Therefore, the ideal micro-organism for probiotic use must be able to overcome potential hurdles, such as the low pH of the stomach, and the presence of bile acids in the intestines. It must also provide competition against the adhesion of gastrointestinal pathogens to the intestinal mucosa while producing organic acids and/or bacteriocin to inhibit pathogen growth, which can then establish itself and flourish in the intestine (Kailasapathy and Chin, 2000; Amit-Romach et al., 2004). Species currently being used in probiotic preparations are *Lactobacillus*, *Streptococcus*, *Bacillus*, and yeast (Jones, 1991). Of the lactic acid bacteria species, members such as the genera *Lactobacillus*, are normal inhabitants in the intestine and commonly used in

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most probiotic preparations (Tannock et al., 1999).

The strain of *Lactobacillus reuteri* Pg4 was isolated from the gastrointestinal tract of healthy broilers. At first, this strain was evaluated for probiotic characteristic: tolerance to the acid and bile of intestinal digesta and adhesion to intestinal epithelial cells in an *in vitro* study. The second study is a feeding trial to investigate the effects of *L. reuteri* Pg4 administration on growth performance, gastrointestinal populations of lactobacilli, intestinal mucosa histology, and the concentrations of organic acids in broiler chickens digesta. The micro-organism adherence to the cecal mucosa was also examined using scanning electron microscopy (SEM).

MATERIALS AND METHODS

The bacteria

Excreta collected from healthy chickens were diluted with sterilized phosphate buffered saline (PBS). It was then inoculated in de Man-Rogosa Sharpe medium (MRS, Merck, Darmstadt, Germany) and incubated at 37°C for 48 h. A colony of Gram-positive, Catalase-negative, acid producing, rod shaped bacteria were selected by microscopy. Further assays were conducted to determine the specific probiotic characteristics. The selected strain was analyzed using the API Kit (API 50 CHL, bioMerieux, France) with API LAB plus software (Ver. 3.3.3; bioMerieux, France) and based on the 16S ribosomal RNA sequence (Song et al., 2000) to identify the bacterial strain. The selected bacterial strain, *Lactobacillus reuteri*, was nominated as *L. reuteri* Pg4. The isolated bacterial strain was stored at -70°C before use.

Experiment 1: Specific probiotic characteristic analysis

Tolerance to acid and bile salts : For testing, *Lactobacillus* was sub-cultured in MRS broth overnight at 37°C. After centrifugation (7,000 rpm, 5 min), the pellets were re-suspended in adjusted pH 2.60 PBS and incubated at 37°C for 1 and 3 h. The resulting suspension was then inoculated into a MRS medium for the total vial count. In order to test the *Lactobacillus* resistance to bile salts, after being treated with acid the surviving cells were collected by centrifugation and washed once with PBS. They were re-suspended (1%) into MRS broth, with and without 0.3% (W/V) oxgall (Bile, Sigma B-3883), and incubated at 37°C for 2 and 6 h according to the method described by Gilliland and Walker (1990). The percentage of bile tolerance was assayed by comparing the viable counts on MRS medium. Each analysis was performed in triplicate

Antibacterial activity of the selected strains : Three pathogenic strains of *E. coli* (CCRC 10675), *S. typhimurium* I50 and *S. typhimurium* 29E were obtained from the Cultural Collection Research Center in Hsing Chu, Taiwan, as indicator strains. For testing, the bacteria were spread onto nutrient agar with 10^7 and 10^{12} cfu/ml, placed in a sterilized stainless steel ring. 100 μl of the spent supernatant from the overnight *Lactobacillus* culture was poured in and incubated at 37°C for 14-15 h. The radial diameter of the transparent zone was measured as antibacterial activity (Rammelsberg and Radler, 1990).

The adhesive capability to the epithelial cells : The method of Annika et al. (1983) was used for epithelial cell preparation. Segments of ileum and crop from broiler chickens were opened and rinsed with PBS. Epithelial cells were scrapped off with the edge of a microscope slide and suspended in PBS. The cell suspension was examined microscopically to ensure the total removal of all adhered bacteria. The cell suspension concentration was approximately 10^4-10^5 cells/ml. The test *Lactobacillus* culture in MRS broth was centrifuged and the cell pellets were re-suspended in PBS. The suspension contained 1×10^8 cfu/ml of the lactic acid bacteria. One ml of this bacterial suspension was mixed with 1 ml of the epithelial cell suspension described above. The mixture was rotated in a tube at 20 rev/min at 37°C for 30 min. Adhesion was determined using Gram stained preparation observation under phase contrast microscopy.

Experiment 2: Feeding trial

Preparation of Lactobacillus reuteri Pg4 powder : *Lactobacillus reuteri* Pg4 was inoculated into MRS broth at 1% (v/v) and incubated at 37°C for 24 h. Afterwards, skim milk powder and glycerol, which were used as cryoprotectors, were added to the incubated culture at a final concentration of 50% (w/v) and 1% (w/v) respectively. The *Lactobacillus* culture preparation was then lyophilized and stored at 4°C until later required. The bacterial count of the *L. reuteri* Pg4 powder was approximately 10^8 CFU/g.

Animals and experimental design : A total of 240 day-old Arbor Arces breed broilers were weighed individually and assigned at random to eight pens of 30 chicks, with equal pens of males and females. The experiment was also randomized with two dietary treatments and four replicates. The dietary treatments were a basal diet (antibiotic-free, control group), and with supplementation 0.1% *L. reuteri* Pg4 powder (probiotic group). The detailed composition of the basal diet, which is formulated base on NRC (1994) recommendations, is presented in Table 1. The dietary CP and ME were 22% and 3,100 Kcal/kg for the grower (0-3 weeks), and were 20% and 3,000 Kcal/kg for the finisher (4-6 weeks). During the experimental period, the chickens were placed in floor pens with 0.17 m^2/bird in an open-sided house, under natural conditions with an average temperature of 28.6°C, and 22 h of incandescent per day.

Table 1. The composition of experimental diet for 0-6 week-old broilers

Items	Periods	
	0-3 weeks	4-6 weeks
	g/kg	
Corn	545.9	588.0
Soybean meal (44%)	247.6	272.0
Full fat soybean meal	88.0	82.0
Fish meal	46.0	9.5
Soybean oil	38.5	15.0
Monocalcium phosphate	11.0	11.5
Limestone (pulverized)	16.0	16.0
Salt	2.5	2.5
DL-methionine	1.1	0.5
Vitamin premix[1]	0.5	0.5
Mineral permix[2]	2.5	2.5
Total	1,000	1,000
Calculated value	%	
Crude protein	22.00	20.00
Crude fiber	3.48	3.58
ME (kcal/kg)	3,100	3,007
Lysine	1.24	1.10
Methionine	0.50	0.38
Calcium	1.0	0.9
Available phosphorus	0.47	0.40
Total phosphorus	0.71	0.65
Analysed value		
Crude protein	21.50	19.70
Ether extract	11.50	9.75
Crude fiber	4.02	4.13

[1] Supplied per kg of diet: Vit. A 15.000 IU; Vit. D_3 3.000 IU; Vit. E 30 mg; Vit. K_3 4 mg; Riboflavin 8 mg; Pyridoxine 5 mg; Vit. B_{12} 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.

Feed in a mash form and drinking water were provided *ad libitum*. The individual broiler weights were measured at the ages of 21 and 42 days. Feed consumption for each pen was recorded weekly. Four chickens from each treatment group were sacrificed on days 1, 3, 7, 14, 28 and 42 for measurement of the *Lactobacillus* count in the digesta. The volatile fatty acids (VFA) and lactate concentration were measured at 14, 28, and 42 days, microbial adhesion was observed at 14 days of age of the tested chickens, and the intestinal histology was measured at 42 days.

Viable counts of Lactobacillus in the gastrointestinal tract : On days 1, 3, 7, 14, 28 and 42, the crop, ileum and cecal contents of the broilers were collected, serially diluted with PBS, and inoculated onto MRS medium. The plates were incubated under anaerobic conditions (95% CO_2 and 5% O_2) at 37°C until colonies appeared (usually within 48 h). The results are expressed as logarithmic colony forming units (log CFU) per gram of wet weight of gastrointestinal contents.

Identification of Lactobacillus strain from the intestine : The isolates of lactobacilli on MRS medium were identified by direct colony PCR using the species-specific primers, Lisreu (5' TCTTAAAACTTATAACCTATAAGACG 3') and Reu540r (5' CCTAAACAATCAAAGATTGTCTG 3'), designed from nucleotide sequences of the 16S-23S rRNA intergenic spacer region of *L. reuteri* Pg4 (Tannock et al., 1999). All PCR reactions were performed in a total volume of 10 μl. The PCR mixture contained 0.4 μl primers (Lisreu and Reu540r, 50 pmole/μl each primer), 1 μl dNTP (2 mM, dNTP), 1 μl $MgCl_2$ (2 mM), 1 μl 10X *Taq* buffer, 0.1 μl *Taq* DNA polymerase and 6.5 μl distilled water. Lactobacilli colonies grown in a MRS medium were randomly chosen and each was mixed with the PCR mixture. PCR reactions were carried out for an initial denaturation step at 94°C or 10 minutes, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 48°C for 40 sec and extension at 72°C for 50 sec, then a final extension step at 72°C for 8 min. The PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide (0.5 μg/ml) and visualized with a Kodak photo system.

Determination of volatile fatty acids, lactate and pH value of cecal content : The cecal contents of four broilers on days 14, 28, and 42 from each group were collected for analysis of the concentrations of VFA and lactate according to the methods of Parker and McMillan (1976) and Marsili et al. (1981), respectively. The pH values of the cecal contents were directly measured using the glass electrode of a portable pH meter (Sentex TS-1, Taiwan).

Intestinal histology and observation of the microbial adhesion on the cecal mucosa by Scanning Election Micrograph : At the age of 42 days, four chicks in each group were randomly selected and sample sections taken (3 cm in length) from middle regions of the ileum and cecum for intestinal histology observation according to the method of Yu et al. (1999).

Cecal samples with an area of approximately 1 cm^2 were taken from 14-day-old chickens and the microbial adhesion on the cecal mucosa observed by SEM according to the method of Jin et al. (1997).

Statistical analysis

Analysis of variance was calculated using the general linear model procedure (GLM) of the Statistical Analysis Systems (1999). Duncan's new multiple range tests was used to compare the treatment means according to Steel and Torrie (1984).

RESULTS

Specific characteristics of *L. reuteri* Pg4 strain

Tolerance to acidic and bile salts : For simulating the GI conditions, the surviving cells from the acid treatment

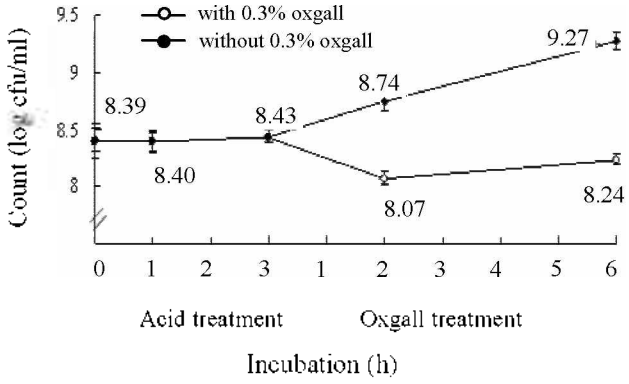

Figure 1. Survival count of *L. reuteri* Pg4 after incubation at pH 2.6 and oxgall.

were re-incubated in the medium with oxgall. The acidic treatment did not significantly affect the count. After 6 h of incubation in oxgall, the survival rate of the *L. reuteri* Pg4 strain was 88.2%; The count of Lactobacillus with or without oxgall was 8.24 and 9.27 cfu/ml, respectively (Figure 1).

Inhibitory capability on bacteria : Testing of the Pg4 strain displayed an inhibitory growth effect on all of the three-indicator strains as shown in Table 2. Results indicated that the suspension of the Pg4 culture to inhibit strains of *Sal. typhimurium* I50, *E. coli* (CCRC 10657) and *Sal. typhimurium* 29E was higher than when compared with the control bacterial strain of *L. acidophilus* LCP1 strain, which was isolated from a commercial probiotic product.

Table 2. Inhibitory activity of *Lactobacillus* strains cultural supernatant to pathogenic bacteria

Pathogenic bacteria concentration	*L. reuteri* Pg4	*L. acidophilus* LCP1[2]
Sal. typhimurium I50		
10^{12} cfu/ml	++[1]	++
10^{7} cfu/ml	+++	+++
E. coli (CCRC 10675)		
10^{12} cfu/ml	++	+++
10^{7} cfu/ml	+++	+++
Sal. typhimurium 29E		
10^{12} cfu/ml	++	++
10^{7} cfu/ml	+++	+++

[1] "–+", 10-15 mm clearance zone; "++–" ≥ 15 mm clearance zone (n = 3).
[2] *L. acidophilus* LCP1 strain isolated from a commercial probiotic product.

Adhesion to epithelium cells : Figure 2 presents a microscopic photograph (400×) of the adhesion of Pg4 and control (LCP1) strains to the crop (1a) and intestinal (1b) epithelium of chickens. Pg4 prominently and firmly adhered to the prepared crop and intestinal epithelial cells, whereas LCP1 did not demonstrate prominent adherence to these cells. This result was similar to our related study, which had revealed the tested *L. reuteri* Pg4 strain adhered efficiently to both porcine gastric mucin and chicken small intestinal mucus (Liu et al., 2005).

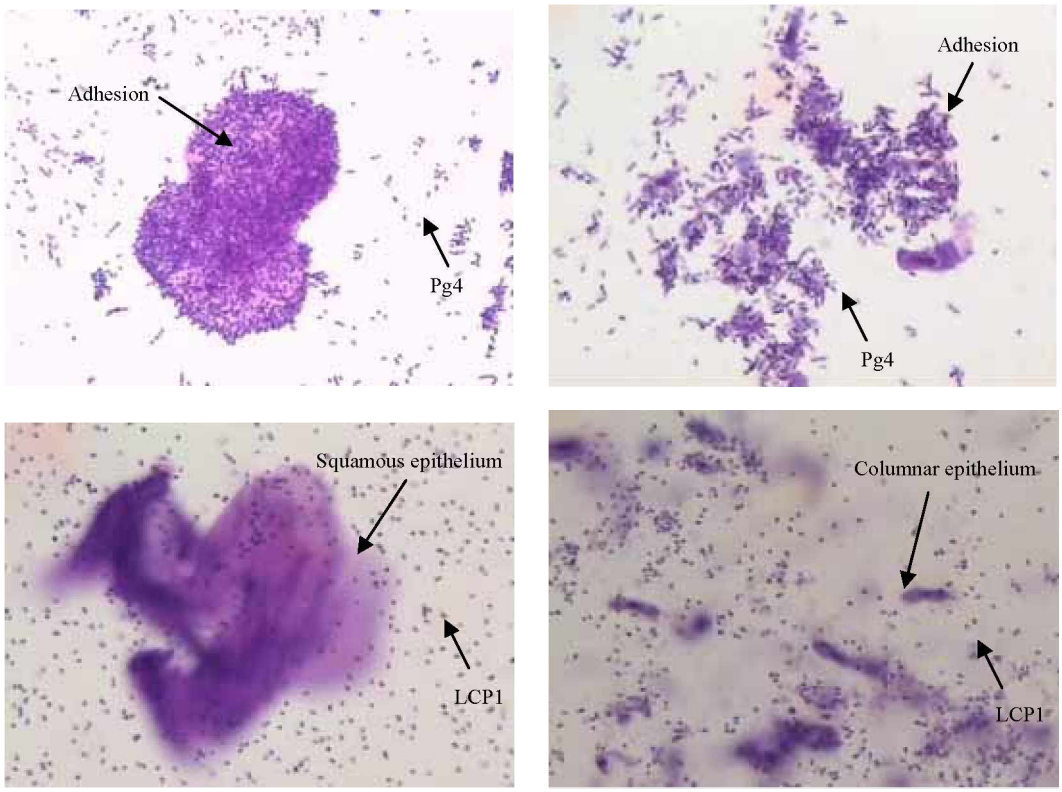

Figure 2. Adhesion of *L. reuteri* Pg4 and LCP1 strains to the crop (left) and intestinal epithelium (right) of broilers (magnified 400×).

Table 3. Effects of *Lactobacillus reuteri* Pg4 supplementation on the performance of broilers

Item	Control group	Probiotic group
0-3 weeks		
Body weight gain (g/day/bird)	39.86[1]±2.90	40.26[2]±1.24
Feed intake (g/day/bird)	57.43±1.20[a]	55.52±0.72[b]
Feed conversion(intake/gain)	1.65±0.03	1.59±0.05
4-6 weeks		
Body weight gain (g/day/bird)	63.57[3]±6.62	64.24[4]±4.43
Feed intake (g/day/bird)	122.14±8.71	117.10±4.90
Feed conversion (intake/gain)	2.01±0.06	1.91±0.08
0-6 weeks		
Body weight gain (g/day/bird)	51.74±4.76	52.28±2.76
Feed intake (g/day/bird)	89.81±2.57	86.31±2.45
Feed conversion (intake/gain)	1.74±0.08[a]	1.65±0.04[b]

[a, b] Means within same rows with different superscript are significantly different ($p<0.05$).

[1, 2, 3, 4] Mean±SD (n = 102, 101, 99, 98).

Effect of *Lactobacillus reuteri* Pg4 supplementation on broiler performance

During the growing or finishing periods, the probiotic inclusion did not significantly affect the body weight gain, feed intake, and feed conversion ($p>0.05$), but significantly decreased the feed intake in the growing period ($p<0.05$). The *Lactobacillus* supplement did produce a modest improvement in the weight gain and feed intake, however significantly improved the feed conversion rate by 5% ($p<0.05$) during 0-6 week period, as shown in Table 3.

Distribution of *Lactobacillus* count in the intestine

Table 4 demonstrates that the probiotic supplement significantly increased lactobacilli populations in the crop on days 3 and 7 and in the ileum on day 1, but did not significantly influence the lactobacilli populations in the crop and ileum of the 6-week old broilers. On the other hand, the lactobacilli populations in the cecum of the probiotics-supplemented broilers were significantly higher than the control group on days 1, 7, and 42.

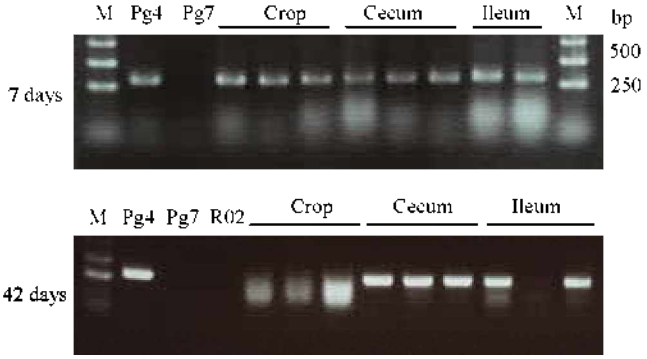

Figure 3. PCR products obtained from the colonies isolated from the intestinal digesta of broilers in probiotic group at 7 and 42 days of age. M: 1 kb DNA ladder; Pg4: *L. reuteri* Pg4 strain; Pg7, R02: negative control *Lactobacillus* strain; Crop, Cecum, Ileum: Colonies isolated from the crop, cecum and ileum of broilers in the probiotic group.

Identification of *Lactobacillus* strain from the intestine

To confirm the survival of Pg4 strain in the GI tract of chicks in a conventional feeding environment, the lactobacilli isolated from the broiler's gastrointestinal tract were identified by direct colony PCR using species-specific primer pair, which was designed from the nucleotide sequences of the 16S-23S rRNA intergenic spacer region of *L. reuteri* Pg4 and to amplify a 345 bp product. As demonstrated in Figure 3, most of the *Lactobacillus* colonies isolated from the crop, ileum and cecum of the broilers in the probiotic group showed the expected 345-bp amplified fragment in agarose gel electrophoresis. This is also true in the stock strain of *L. reuteri* Pg4, which served as a positive control. On the other hand, *L. brevis* R02 and *L. reuteri* Pg7, which served as negative controls, did not show this expected fragment. Therefore, it is likely that *L. reuteri* Pg4 strains are able to survive in the intestine of broilers under a conventional feeding regime.

Concentrations of VFA and lactic acid and pH level of cecal content

Probiotic supplementation did not significantly affect

Table 4. Supplementation effects of Lactobacillus reuteri Pg4 on Lactobacillus count in different segments of broiler gastrointestinal tract at different ages

Age (days)	Crop		Ileum		Cecum	
	C[1]	T	C	T	C	T
	log cfu/g					
1	8.5[2]±0.55	9.2±0.15	7.2±0.16[b]	8.6±0.61[a]	7.4±0.35[b]	8.6±0.96[a]
3	8.5±0.85[b]	9.7±0.50[a]	7.7±0.57	8.1±0.59	9.6±0.24	9.7±0.87
7	7.6±0.18[b]	9.0±0.68[a]	7.6±0.71	8.2±0.53	8.8±0.44[b]	10.2±0.89[a]
14	8.4±0.60	8.5±0.23	7.3±0.44	7.7±0.58	6.7±0.47	7.4±0.21
28	8.9±0.20	9.6±0.44	7.5±0.97	8.1±0.72	7.7±0.58	8.6±0.23
42	8.3±0.37	9.1±0.25	7.2±0.55	7.4±0.60	7.2±0.51[b]	8.8±0.49[a]

[a, b] Means within same rows in the same segment with different superscript are significantly different ($p<0.05$).

[1] C = Control group; T = Probiotic group. [2] Mean±SD (n = 4).

Table 5. Supplementation effects of *Lactobacillus reuteri* Pg4 on the concentrations of volatile fatty acid, lactic acid and pH value of cecal digesta in broilers

Item	Control group	Probiotic group
2nd weeks		
Total VFA (μmole/g)	86.9[1]±6.69	60.1±7.70
Lactic acid (μmole/g)	34.9±1.97	33.7±1.07
pH	5.5±0.08	5.6±0.33
4th weeks		
Total VFA (μmole/g)	61.0±11.21	97.3±9.73
Lactic acid (μmole/g)	83.6±11.24	89.2±16.96
pH	6.3±0.06	5.9±0.29
6th weeks		
Total VFA (μmole/g)	63.0±18.60	71.4±15.37
Lactic acid (μmole/g)	51.8±13.60[b]	115.7±17.73[a]
pH	6.3±0.16[a]	5.8±0.19[b]

[a, b] Means within same rows with different superscript are significantly different ($p<0.05$).
[1] Mean±SD (n = 4).

the cecal total VFA concentrations, but showed a trend toward higher concentration at the 4 and 6 weeks of age. Supplementation also did not significantly influence the cecal pH value and lactic acid concentrations at 2 and 4 weeks of age, but significantly decreased the pH value and increased lactic acid concentration ($p<0.05$) at 6 weeks of age (Table 5). These results corresponded with our previous trial, showing that chicks inoculated with a cecal culture from healthy chickens had a higher trend of lactic acid concentrations and lower pH in the cecum than in non-inoculated analogs (Yu et al., 1999).

Intestinal histology and microbial colonization

The villus height, villi perimeter in the ileum, and mucosa height in the cecum were significantly greater for the 42 day-old broilers treated with the probiotic as shown in Table 6.

Figure 4 demonstrates the SEM micrographs of microbial colonization adhering to the cecal mucosa of 14-day-old chicks. The *Lactobacillus* supplemented chicks showed more colonized microbes on the mucosa. The aggregation of the cocci or rod-shaped colonized microbes in the supplemented chicks was also shown more clearly in the magnified SEM micrograph (Figure 2F, 5,000×). Conversely, scattered colonized microbes occurred only in small numbers on the mucosa surface of the control chicks (Figure 2C). These results were similar to the findings of Jin et al. (1997) who reported the same pattern with SEM examinations of the epithelial surfaces of jejunal, ileal and cecal mucosa in chicks treated with fecal extract of M98-5 medium.

DISCUSSION

Lactobacillus used as probiotic adjuncts are commonly

Table 6. Effects of *Lactobacillus reuteri* Pg4 strain supplementation on the mucosa histology of the ileum and cecum of 6 week-old broilers

Item	Control group	Probiotic group
Ileum		
Villous height (μm)	759[1]±206[b]	884±246[a]
Villous perimeter (μm)	1,787±535[b]	2,143±581[a]
Crypt depth (μm)	142±28	191±71
Muscle layer (μm)	190±63	203±28
Cecum		
Mucosa height (μm)	321±55[b]	360±30[a]
Muscle layer (μm)	281±36	291±36

[a, b] Means within same rows with different superscript are significantly different ($p<0.05$).
[1] Mean±SD (n = 4).

delivered in a food and/or feed systems. Therefore, probiotic bacteria should be resistant to the digestion process in the stomach and intestinal tract (Chou and Weimer, 1999). Prior to reaching the intestinal tract, the probiotic bacteria must survive transit through the stomach where secretions of gastric acid represent a primary defense mechanism against the majority of ingested micro-organisms (Strompfova et al., 2004). The probiotic bacteria subsequently enter the upper intestinal tract where bile is secreted. After traveling through this harsh environment, the probiotic bacteria colonize in the epithelium of the lower intestinal tract. Thus, probiotic bacteria must be able to tolerate stomach acid and bile, attach to the epithelium, and grow in the lower intestinal tract before they can start providing any health benefits. In an *in vitro* study, we observed that *L. reuteri* Pg4 displayed a resistance to acidic conditions and contact with bile salts. This experiment found that *L. reuteri* Pg4 survived in the crop, ileum and cecum of broilers after oral administration in powder form, indicating that the *L. reuteri* Pg4 strain could survive transit through the stomach and the intestine for a protracted period. It may then colonize by its adhesive ability. This could prove important to the microbial ecology of the intestinal environment.

Volatile fatty acids arise from the fermentation of carbohydrates and/or the fermentation by the indigenous anaerobic microflora in the GI tract. Therefore, VFA concentration can be an index of the anaerobic-organism population (Savage, 1977). The VFA may actually provide benefits to the host animal: they act as metabolic fuel for epithelium cells, and possess bactericidal properties against organisms such as *Salmonella* and *E. coli* (Corrier et al., 1990; Thompson and Hinton, 1997). In present study, the probiotic group showed a trend towards increased cecal VFA concentration, but did not reach significant levels. Lactic acid, produced mainly by saccharolytic bacteria such as *Lactobacillus*, *Enterococcus*, and *Bifidobacteria* during the fermentation of carbohydrates, may serve to protect the animal from pathogenic bacteria by decreasing the pH of

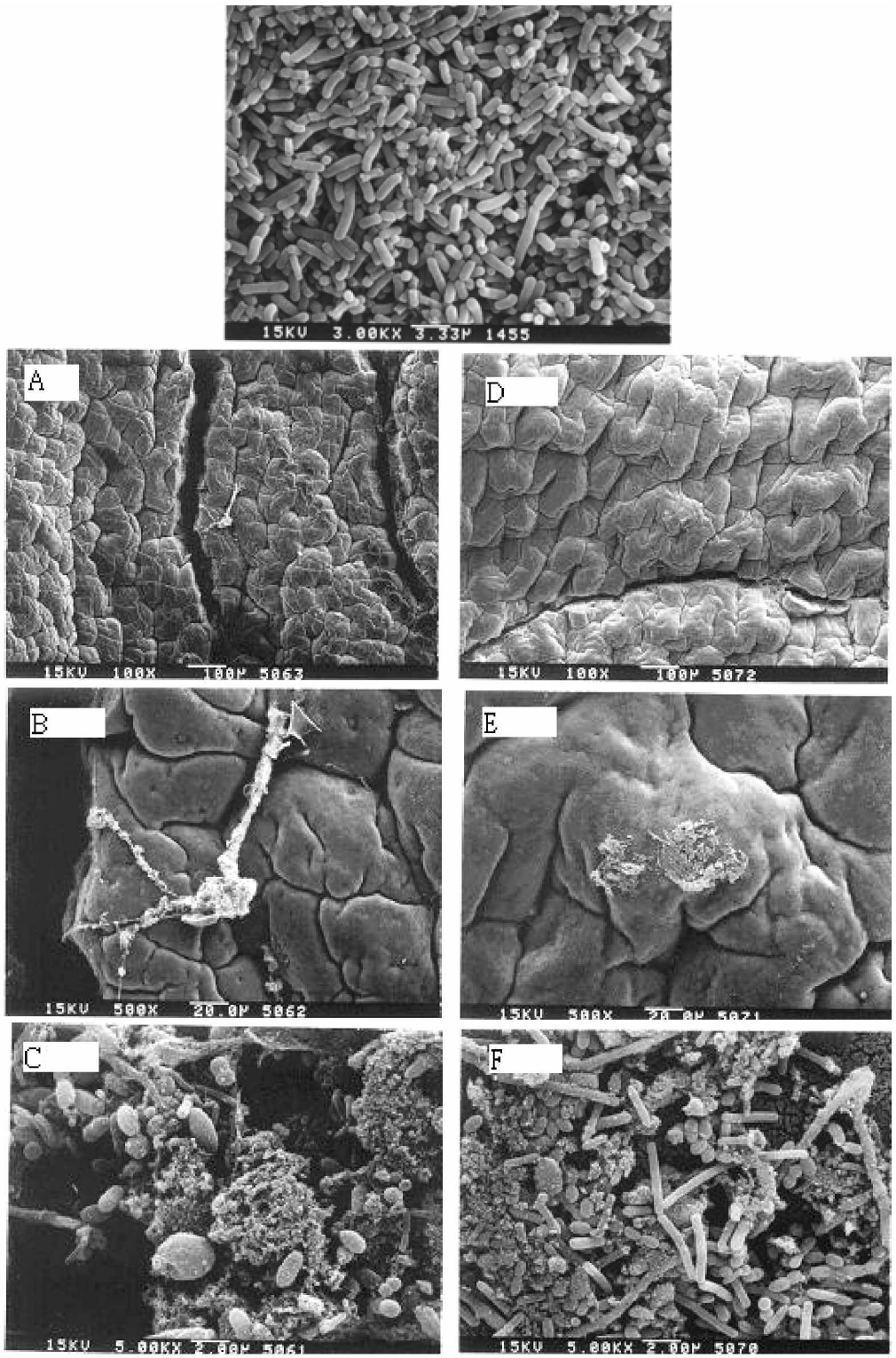

Figure 4. Scanned electron micrograph of micro-flora adhering to the cecal mucosa of a 14-day-old chicken in control group (A, B, C) and probiotic group (D, E, F). Bars = 100 μm (A and D); 20 μm (B and E); 2 μm (C and F). Top: *L. reuteri* Pg4 strain. Arrow: micro-flora adhering to cecal mucosa.

the hind-gut, thus impeding the growth of the unfavorable bacteria (Barrow, 1992). In an *in vitro* study, the pH value of the culture of *L. reuteri* Pg4 after 24 h of incubation was 4.14. Therefore, the inhibitory activity on the tested pathogenic bacteria might be partially attributed to a decrease in environmental pH. Sakata et al. (2003) reported that probiotic bacteria actually increase the production rates of VFA, lactic acid, and occasionally succinic acids due to

the increase in the breakdown of indigestible carbohydrates. Fuller (1989) suggested that a density of 10^7 CFU/g of *Lactobacillus* can effectively suppress other bacteria in the GI tract due to decreased lumen pH from the production of lactic acid. Thompson and Hinton (1997) indicated that the lowered pH in the intestine could increase the concentration of undissociated acid molecules. The VFA diffuses into the bacterial cell in undissociated form, resulting in reduction of intracellular pH and anion accumulation. In this study, we found that the lactobacilli populations and lactic acid concentrations in the cecum of broilers in the probiotic group were significantly higher but with a lower pH than those in the control group at the finishing period ($p<0.05$). Thus, it reflected that oral administration of *L. reuteri* Pg4 powder may benefit in establishing a healthy condition of the GI tract.

It has been demonstrated that villus height and crypt depth are a direct representation of the gut function and health. Uni et al. (1995) suggested that an increase in villus height might also indicate a greater absorption area and vice versa. The mucosa enzyme activity per mass of intestine is closely associated with the number of enterocytes per villi in chickens. Therefore, more digestive enzymes and absorption activities have been noted in the longer villi. Conversely, under unfavorable GI environment such as disease that caused abnormal changes in villus height or crypt depth and led to a reduction in nutrient absorption. In this study, *L. reuteri* Pg4 supplementation may improve the gut health in balancing the micro flora and then increasing the villous height and crypt depth, thus further improving the performance of chickens. Alternatively, insignificant improvements were obtained in the supplemented chicks during the finisher period.

In the probiotic group, we observed a microbia broad colonization on the intestinal mucosa, probably *Lactobacillus*. Conversely, scattered colonized microbes occurred only in small numbers on the mucosa surface of the control chicks. The colonization of a dense mat of micro flora appears to play an important role in the protection of chickens against pathogenic bacterial infection. We therefore conclude that dietary supplementation of the *L. reuteri* Pg4 strain in chicks can improve the colonization of lactobacilli. In this situation it is assumed that the benefits derived is a result of the organism growing, and displays beneficial effects on the gut health.

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