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Effects of In Ovo Infusion of Probiotic Strains on Performance Parameters, Jejunal Bacterial Population and Mucin Gene Expression in Broiler Chicken

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In ovo infusion, Jejunum, Microbial population, qPCR, Probiotic.

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ABSTRACT

The objective of the present study was to evaluate the effects of *in ovo* infusion of probiotic strains (*Bacillus subtilis, Enterococcus faecium,* and *Pediococcus acidilactici*) on jejunal microbial population and mucin gene expression in broiler chicken. In a completely randomized design, 0.5 ml of mediums containing 10^7 cfu of different probiotic strains, was administered into amniotic fluid of the 480 Cobb fetus (day 18 of incubation), with four treatments, five replicates with twenty four eggs each. For mucin gene expression, samples from the jejunum were taken on day 21 of incubation and day 3 post-hatch. Microbial profile was determined for total lactobacillus and *E. coli* by sampling jejunal contents on days 1 and 3 of age. Expression of the mucin gene in the jejunum was higher ($p<0.05$) in chicks that received *Bacillus subtilis* in comparison with the control group. Infusion of the probiotic strains had no effect on jejunal *E. coli* and lactic acid bacteria populations on day 1 post-hatch ($p>0.05$). There were no significant differences among treatments for performance parameters at different periods and the whole period. It was concluded that infusion of probiotic bacteria during the late of incubation has no effect on feed intake, gain and feed conversion ratio, but has a positive effect on mucin gene expression in the jejunum. The best probiotic strain for mucin gene expression was *Bacillus subtilis* and for beneficiary bacteria colonization was *Bacillus subtilis* and *Pediococcus acidilactici*.

INTRODUCTION

The application of probiotics as an alternative of antibiotics in non-ruminant rations has become necessary. Crawford (1979) reported that probiotics could increase the population of beneficial bacteria and reduce the growth of pathogenic flora in the gastrointestinal tract. Fuller (1989) concluded that probiotics are suitable alternatives for antibiotics as the latter have residues in meat, increase the resistant bacteria and resulted in imbalance of normal microflora. The action modes of probiotics, which affect gut function and health in poultry, include maintaining a normal micro-flora and beneficial microbial population by competitive exclusion and antagonism (Fuller, 1989), and improving gut mucin composition and amount (Tsirtsikos *et al*., 2012).

Modern poultry production excludes the contact between chick and the hen. Sterzo *et al*., (2005) reported that bacteria present in the hatchery determine the colonization of beneficial or pathogenic bacteria. Cukrowska *et al*. (2002) showed that pathogens can be lodged in the chick´s intestine and colonize in the first contact with microbes in the hatchery, so it is necessary to inoculate probiotic bacteria before the chick and hen can be in contact.

The majority of gastrointestinal tract is covered by a viscoelastic mucous gel layer that acts as a protective barrier against potential physical and chemical hazards in the luminal environment (Dharmani *et al*., 2009). The mucins as main component of the mucus layer, are produced and secreted by goblet cells. Development of the small intestinal mucus-secreting cells in chicks occurs in the late embryonic and immediate post-hatch period. The Mucin-producing cells were present in the small intestine with no differentiation between various sections from 17 days of incubation and produce only acidic mucin (Uni et al., 2003). After hatching, an increase in the goblet cells density production, neutral and acidic mucin was observed from duodenal to ileal axis (Uni et al., 2003).

Fuller (1989) and Tsirtsikos *et al*. (2012) reported potential link between gut mucin composition and gut microflora described earlier in broiler chicks. Uni & Ferket (2004) and Tako *et al*. (2004) reported that intra-amniotic nutrient injection accelerated small intestine development and had an enhanced effect on the function of enterocytes.

In the poultry industry, exclusion of the contact between chick and the hen causes a delay in gut colonization with desirable microorganisms. This exclusion exposes chicks to risk of colonization with pathogenic bacteria present in the hatchery (Cukrowska *et al*., 2002). Thus, injection of probiotic bacteria intra egg may be an alternative to microbiota acquisition by chicks before hatching and may reduce or avoid the gastrointestinal colonization by pathogens. Three species of bacteria, *Bacillus subtilis, Enterococcus faecium,* and *Pediococcus acidilactici*, are naturally occurring microbiota in the intestine of birds and common in commercial probiotic products. Information concerning the effects of *in ovo* infusion of these probiotics on beneficial and pathogenic bacterial population and mucin gene expression in the intestine of chicks, is limited. Therefore, the main objective of this study was to evaluate the effects of different probiotic strains as *in ovo* infusion on the performance parameters, mucin gene expression and bacterial population in the jejunum of broiler chicken at the pre and post-hatch periods.

MATERIAL AND METHODS

Fertile Cobb chicken eggs were obtained from a commercial hatchery from 38 weeks old breeding flock. Eggs were incubated in single-stage incubators under the same condition of 37.6 °C and 60% relative humidity, while being turned once per hour. On day 17 of incubation, eggs with live fetus (no: 480, average weight of 58 ± 1.1 g) were selected and weighed. In a completely randomized design, eggs were assigned to four experimental groups with five replicates of each twenty four eggs. Infusion of probiotic strains was done for 3 hours in a plastic tent constructed in front of setter doors, with two heaters at 36 °C and 55% relative humidity. The four treatment groups that received *in ovo* 0.5 ml of sterile distilled water or probiotic mediums (10^7 cfu) into the amniotic fluid were: 1) sterile distilled water as control group, 2) B*acillus subtilis*, 3), *Enterococcus faecium* and 4) *Pediococcus acidilactici*. B*acillus subtilis* was obtained from Iranian TaqGen Company (Tehran, Iran), *Enterococcus faecium* was obtained from Biochem Company (Lohne, Germany) and *Pediococcus acidilactici* was obtained from Lallemand Inc. (Paris, France).

The *in ovo* infusion procedure was performed as described by Tako *et al*. (2004). Solution was injected with a suitable needle inserted into the amniotic fluid, which was identified by candling. After injection, the hole in the egg shell was sealed with cellophane tape, and eggs were placed in hatching trays. Chicks were raised for 42 days and fed Cobb standard rations (Table 1). Chickens management (water, feed, bed material,

Table 1 – Ingredients and compositions of rations

Ingredients (%)	Intake period		
	Starter (days 1-10)	Grower (days 11-28)	Finisher (days 29-42)
Corn grain	55.06	60.95	62.59
Soybean meal (46% CP)	37.05	31.52	29.13
Soybean oil	3.51	3.50	4.35
Calcium Carbonate	0.95	0.91	0.92
Di-calcium phosphate	1.92	1.78	1.80
Salt	0.27	0.24	0.22
DL- Methionine	0.28	0.24	0.21
L-Lysine	0.12	0.13	0.07
L-Threonine	0.10	0.08	0.07
Vitamin-mineral premix[1]	0.160	0.50	0.50
Sodium bicarbonate	0.15	0.15	0.15
Analysis results of nutrients			
AMEn (kcal/kg)	3035	3108	3180
CP (%)	21	19	18
Ca (%)	0.9	0.84	0.84
Available Phosphorus (%)	0.45	0.42	0.42
Met + Cys	0.88	0.80	0.74
Digestible Lys (%)	1.18	1.05	0.95
Digestible Thr (%)	0.77	0.69	0.65

[1] Premix contain: 4400000 IU/kg of Vit. A, 2000000 IU /kg of Vit. D_3, 30000 IU /kg of Vit. E, 1200 mg/kg of Vit. K (Menadione), 1200 mg/kg of B_1, 3200 mg/kg of B_2, 24000 mg/kg of Nicotinic Acid, 6000 mg/kg of Pantothenic Acid, 1600 mg/kg of B_6, 60 mg/kg of Biotin, 800 mg/kg of Folic Acid, 6 mg/kg of B_{12}; 6400 mg/kg of Copper, 500 mg/kg of Iodine, 16000 mg/kg of Iron, 48000 mg/kg of Manganese, 120 mg/kg of Selenium, 40000 mg/kg of Zinc.

light program and pen environment) were the same in all groups and were based on Cobb 500 broiler chickens (Cobb Manual Guide, 2012). Chickens were weighed in the final of starter (day 10) and finisher (day 42) to estimate their growth. To determine daily feed intake by each pen, the uneaten feed was discarded and fresh feed was replaced in feeders at the end of each day. Feed conversion ratio was calculated in the mentioned periods.

On day 21 of incubation and day 3 of age, one chick per replicate (5 eggs or 5 chicks for each treatment) were randomly selected. Eggs were opened and fetus or chicks were anesthetized with diethyl ether. Immediately, small intestine was removed and flushed with NaCl (150 mmol/l) to remove the contents. Samples of jejunum (2 cm) were taken at the midpoint between the entry of the bile duct and Meckel's diverticulum and immediately stored in liquid nitrogen for messenger RNA (mRNA) extraction. Jejunum was selected as it is the main section of absorption and is the middle of the small intestine.

On days 1 and 3 of age, two chicks per replicate were randomly selected, anesthetized with diethyl ether and jejunum removed and placed in ice and used for microbial assays.

After sectioning and preparation of the samples, RNA samples were extracted according to the method described by Moghaddam *et al.* (2011). After extraction, RNA purity was determined by calculating the ratio of the absorbance readings at 260 and 280 nm. Additionally, the quality of RNA was assessed by visualization of distinct bands after gel electrophoresis with ethidium bromide staining. A quantity of 1ug of each RNA sample was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcriptase kit (Bioneer Co., Seoul, South Korea). The 20 µL cDNA synthesis reaction contained in addition to the RNA template, 1 µl oligodT (CinnaGen, Iran), 1 µl of 10 mM dNTP (CinnaGen, Iran), 1 µl of Random hexamer (CinnaGen, Iran), 2 µl of 10X MMuLV and 0.5 µl Enzyme M-MLV (Moloney murine leukemia virus). Nuclease-free water was added bringing the reaction up to final volume (20 µl). The mixture was incubated for 1 hour at 42 °C (Moghaddam *et al*., 2011). The resulting cDNA was stored at -20 °C prior to use.

Real time PCR was performed for mucin-2 and reference genes at a final volume of 20 µl. This volume contained of 100 ng cDNA, 12.5 µl SYBR® Green Real-Time PCR Master Mixes (Applied Bio System), 1 µl of primers 10 mmol/µl and 4.5 µl distilled water. Amplification of the mucin-2 gene was performed for 40 cycles, which consisted of an initial activations step (95 °C, 5 min), denaturation cycle (95 °C, 15s) and combined annealing and extension (60 °C, 60s). The GAPDH reference gene was amplified at 40 cycles under the same conditions in a different tube. After each run, preparation of standard curve was performed by serial dilution of pooled cDNA from samples. The sequences of the primers used for expression analysis were Mucin chicken F: 5'-CAGCGTTAACACAGGGCTTA-3', Mucin chicken R: 5'-GCAGCAGACGTTGAT CTCAT-3', GAPDH Chicken F: 5'-TCTCTGGCAAAGTCCAAGTG-3', GAP-DH Chicken R: 5'- TGCCCATTGATCACAAG TTT-3'. The relative changes in gene expression were analyzed by using the $2^{-\Delta\Delta Ct}$ method. Results are expressed as fold-change relative to the control group (Livak & Schmittgen, 2001).

The populations of *Escherichia coli* and lactic acid bacteria in jejunal contents were estimated as cfu per gram. In sterile condition, sterilized PBS (99 ml) was added (1:100) to 1 g of jejunal content, and then subsequent dilutions prepared. *E. coli* was cultured on MacConkey agar (Merck, Germany) at 37 °C for 24 hours, and the presence of *E. coli* then determined. Lactic acid bacteria were enumerated on MRS (Merck, Germany) agar after incubation under anaerobic condition for 72 hours at 37 °C (Witkamp and Olson, 1963).

The statistical normality of all data were tested in MINITAB® software (confidence level=95%). Then treatments analyzed by ANOVA procedure using the GLM procedure of SAS® software. When significant differences among means were found, means were separated using Duncan's Multiple Comparison test (α=5%) for post hoc multiple comparisons.

RESULTS

Hatchability of different treatments was 98.5, 96, 95.5 and 95% for control and three infused groups, respectively. There were no significant differences among treatments for hatchability.

The effects of *in ovo* infusion of different probiotic strains on the mucin gene expression on day 21 of incubation and day 3 of post-hatch are presented in Table 2. On both days of measurements, a significant difference ($p<0.05$) was found among treatments. Infusion of *Bacillus subtilis* induced mucin gene expression 4 and 3.7 folds higher than the control group in day 21 of incubation and day 3 of post-hatch, respectively. Infusion of *Pediococcus acidilactici*

increased the gene expression, but its effect was lower than *Bacillus subtilis*.

Table 2 – Effects of *in ovo* infusion of different probiotic strains on the expression of mucin gene in the jejunum of pre- and post-hatch chicks

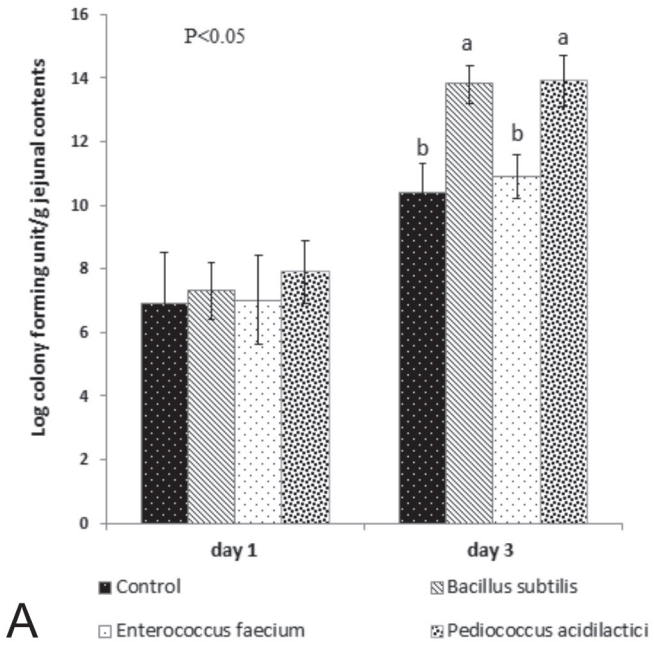
Treatments	Pre-hatch	Post-hatch
	day 21	day 3
Control	1.00 [b]	1.00 [b]
Bacillus subtilis	4.16 [a]	3.76 [a]
Enterococcus faecium	1.84 [b]	1.78 [b]
Pediococcus acidilactici	2.02 [ab]	2.61 [ab]
p value	0.002	0.001
SEM	0.343	0.308

[a,b] Means with different superscripts within the same column differ significantly (p<0.05).

Effect of *in ovo* infusion probiotics on lactic acid bacteria population in the jejunum of broiler chicken is presented in Figure 1A. The population of lactic acid bacteria in the jejunum of one-day old chicks was not affected (p>0.05) by *in ovo* infusion, but a difference appeared on day 3 of post-hatch (p<0.05). The highest lactic acid bacteria population of *in ovo* infused groups was for *Pediococcus acidilactici* and *Bacillus subtilis* on day 3 of age.

Figure 1B represents the effect of *in ovo* infusion of probiotics on *E. coli* population in the jejunum of broiler chicken. Infusion of probiotics had no effect (p>0.05) on *E. coli* population in one-day old chicks, but decreased its population on day 3 of age, compared with the control group. No differences (p>0.05) were found among different probiotics for *E. coli* population on day 3 of age.

Effects of *in ovo* infusion of probiotics strain on daily feed intake, average gain and feed conversion ratio are presented in Table 3. There were no significant differences (p>0.05) among treatments for performance parameters at different periods or in the whole period.



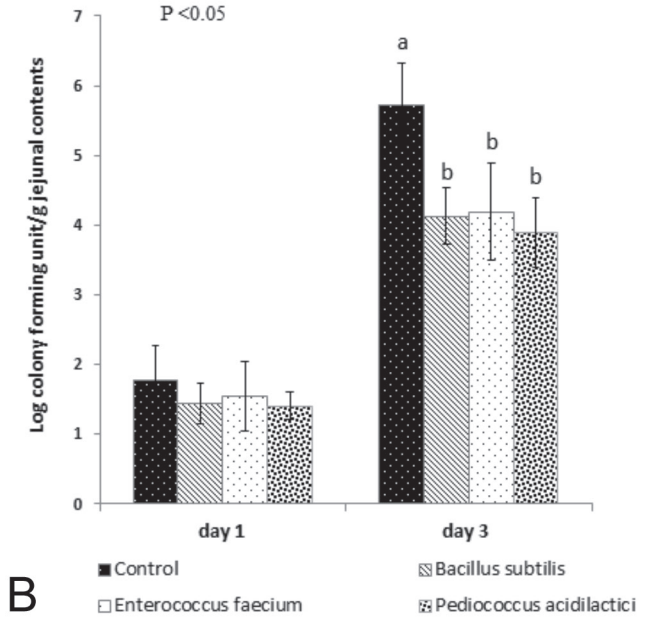


Figure 1 – Total lactic acid bacteria (A) and *E. coli* (B) population in the jejunum of chicks at days 1 and 3 of age

Table 3 – Average feed intake (g/day), average weight gain (g/day) and feed conversion ratio of broiler chickens infused probiotic strains

Periods*	d 1-10	d 1-42	d 1-10	d1-42	d 1-10	d 1-42
Control	31.0	109	22.0	54	1.41	2.02
Bacillus subtilis	30.5	110	21.4	55	1.43	2.01
Enterococcus faecium	31.7	106	21.5	53	1.48	1.99
Pediococcus acidilactici	31.2	111	21.8	55	1.44	2.02
p value	0.466	0.328	0.710	0.667	0.268	0.941
SEM	0.497	2.10	0.438	1.21	0.023	0.037

*d 1-10: starter; d1-42: whole period

Means without superscripts within the same column not differ significantly (p<0.05).

DISCUSSION

In this study, *in ovo* infusion of *Bacillus subtilis* into the amnionic fluid increased the expression of mucin gene on day 21 of incubation and day 3 of post-hatch. The gut mucin amount and composition are arranged based on bacterial glucodisase, activities responsible for removal of monosaccharide residues from mucin carbohydrate side chains (Hoskins *et al*., 1985), as well as via regulation of intestinal glycosylation (Bry *et al*., 1996; Bryk *et al*., 1999; Freitas *et al*., 2005; Sharma & Schumacher, 1995). An increased mucin synthesis and secretion have been shown to occur with probiotics consisting mainly of *Lactobacillus* and *Bifidobacterium* (Mack, 1999; Mack 2003). According to the study of Tsirtsikos *et al*. (2012), mucus layer thickness is increased linearly with probiotic inclusion level in the duodenum of chickens on day 14 and 42 of age.

Before hatching, digestive tract of chicks is free of microorganism; early replacement of beneficial bacteria in gut can prepare suitable conditions for cloning of normal microflora and improving quality and health of the gut. Our results indicated that infusion of the probiotic strains into amniotic fluid increased lactic acid bacteria and decreased *E. coli* populations on day 3 of post-hatch. Lourenco *et al*. (2012) indicated that oral feeding *Bacillus subtilis* decreased significantly Salmonella population in broiler gut. Probiotic improves performance and health of bird due to balance of microbial population (Awad *et al*., 2009). *Pediococcus acidilactici* prevents growth and development of intestinal small bacteria such as *Shigella*, *clostridium* and *E. coli*. Therefore, *Pediococcus acidilactici* increases the resistance of birds to pathogenic bacteria (Lee *et al*., 2007).

In this study, performance parameters were not affected by *in ovo* infusion. In the numerous studies, the probiotic effects appeared in a three weeks period after addition to the diet (Ghasemi *et al*., 2010). An infusion in the late fetus period could not affect on performance, and following it, feeding of probiotic is necessary to achieve better results.

As a conclusion, it seems that in ovo infusion of probiotic strains to the late-term fetus has no effect on performance parameters, but has a benefit effect on mucin gene expression and beneficiary microbial colonization in the jejunum of chicks. The best strain, considering mucin gene expression, among the strains used in this study was *Bacillus subtilis* and for beneficiary bacteria colonization was *Bacillus subtilis* and *Pediococcus acidilactici*.

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