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P Kanagaraju
M.V. Sc. Scholar, Department of Poultry Science, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, TANUVAS, Chennai, Tamil Nadu, India

S Ravichandran
Assistant Professor, Department of Poultry Science, Madras Veterinary College, TANUVAS, Chennai, Tamil Nadu, India

K Kumanan
Professor and Head, Dept of Bioinformatics and ARIS Cell, MVC, Chennai, Tamil Nadu, India

P Muthusamy
Assistant Professor, PGRIAS, TANUVAS, Kattupakkam, Tamil Nadu, India

S Rathnapraba
Assistant Professor, Department of Animal Biotechnology, Madras Veterinary College, TANUVAS, Chennai, Tamil Nadu, India

G Srinivasan
Professor and Head, Department of Poultry Science, Madras Veterinary College, TANUVAS, Chennai, Tamil Nadu, India

Correspondence
P Kanagaraju
M.V. Sc. Scholar, Department of Poultry Science, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, TANUVAS, Chennai, Tamil Nadu, India

Effect of *in ovo* injection of probiotics bacteria *Lactobacillus acidophilus* on the gut microbial colonization in commercial broilers

P Kanagaraju, S Ravichandran, K Kumanan, P Muthusamy, S Rathnapraba and G Srinivasan

Abstract
The respective research trial was conducted to investigate the effect of *in ovo* injection of *Lactobacillus acidophilus* to 18 days old broiler chicken embryo on gut microflora of commercial broilers. On 18th day of incubation *in ovo* injection was carried out in which out of total 720 broiler hatching eggs, 144 eggs served as non injected control (T_1), 144 eggs served as sham control and the remaining 432 eggs (144 for each treatment group) were injected with 0.2 ml of 1x10^6 *Lactobacillus acidophilus* (T3), 0.2 ml of 1x10^9 *Lactobacillus acidophilus* (T4) and 0.2ml of 1x10^{12} *Lactobacillus acidophilus* (T5). The positive control group was injected with 0.2 ml of 0.9% normal saline solution. At hatch, 480 chicks were randomly selected (96 birds in each treatment) with six replicates of 16 birds each as per treatment wise. On 42nd day of experiment, data related to gut microflora count of intestinal contents were recorded and statistically analysed. *In ovo* injection of L. *acidophilus* how ed significant (P<0.01) improvement in colonization of beneficial *Lactobacillus spp.* and significant (P<0.01) reduction in harmful bacteria among treatment and control groups. Among the treatment group L. *acidophilus* of 1x10^{6}shows better values of 14.24 compared to other treatment groups with log value of 15.00 in LA 1x10^9 and 14.94 kg value in LA 1x10^{12} injected group. The results of the present trial indicated that the *in ovo* injection of *Lactobacillus acidophilus* has incresed beneficial bacteria counts and markedly reduced harmful pathogens in the intestinal contents of commercial broilers thereby improved gut efficiency and gut health of broiler chicken.

Keywords: *In ovo*, *Lactobacillus acidophilus*, gut microflora, intestine, broilers

Introduction
Traditionally, probiotics have been administered in the feed or water supply to 1-day-oldchicks. However, as soon as the chick hatches and is exposed to the external environment, it quickly begins to establish the microbial community in the intestine (Pedroso *et al.*, 2005) [1]. This resident microflora may affect the establishment of the probiotic microorganisms, and in order to promote early establishment of probiotic strains, employing *in ovo* technology may be the answer. *In ovo* technology represents a means to take advantage of this crucial time and promote early colonization of beneficial bacteria in order to stimulate intestinal and immune system development. Around embryonic day 18, the chick will have its first meal when it consumes the amniotic fluid before internal pipping (Ferket, 2006) [2].

In broiler nutrition, a variety of microbial species have been used as probiotics, including species of *Bacillus*, *Bifidobacterium*, *Enterococcus*, *E. coli*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, a variety of yeast species such as *Saccharomyces cerevisiae*, and undefined mixed cultures (Mountzouris *et al.*, 2007) [3]. However, there has been a recent increase in research on feeding *Lactobacillus* to livestock (Tellez *et al*., 2001) [4]. Since, they are able to release different substances like antimicrobials in to gastro intestinal tract of host system that interact with the Gut-Associated Lymphoid Tissue (GALT) that can stimulate cytokine production by lymphocytes, which leads to regulation of the innate and adaptive immune responses.

Though research in the areas of probiotic supplementation and *in ovo* feeding is quickly growing in popularity, little has been done to explore the effect of *in ovo* administration of probiotics on gut health and gut efficiency of broiler chicken. Though, there is a plethora of research in the areas of dietary probiotic supplementation and *in ovo* feeding of various nutrients, very few researchers have entertained the idea of extending the concept of *in ovo*

administration to probiotics. Work done by Cox *et al.* (1992) [5] is considered to be the first attempt at connecting the concepts of competitive exclusion and *in ovo* administration. Hence, a biological experiment has been planned to investigate the effect of *in ovo* injection of *Lactobacillus acidophilus* on gut microflora in commercial broilers

Materials and Methods

Seven hundred and twenty fertile eggs with uniform weight were randomly selected from 38 weeks old commercial broiler breeder flock (Cobb 400). *In ovo* injection of nutrient solutions was done as per the modified Noor *et al.* (1995) [6] method. On 18th day of incubation, out of total 720 eggs, 144 eggs served as non injected control (T_1), 144 eggs served as injected control (Positive control) and the remaining 432 eggs (144 for each treatment group with six replicates of 24 eggs each) were injected with 0.2 ml of $1x10^6$ *Lactobacillus acidophilus* (T3), 0.2 ml of $1x10^9$ *Lactobacillus acidophilus* (T4) and 0.2ml of $1x10^{12}$ *Lactobacillus acidophilus* (T5). The positive control group was injected with 0.2 ml of Sterile water (Sham). The *Lactobacillus acidophilus* (MTCC NO.10307) culture was obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India-160 030. *In ovo* injection was carried out in an empty incubation cabinet where the temperature and humidity was maintained at 37.5°C and 60 per cent, respectively. Each egg was candled and earmarked to identify the site of the injection (Aminion). After disinfection of egg shell surface with 99.90 % ethyl alcohol, a pin head size hole was made using a sharp egg puncture and 0.2 ml of respected treatment solution was injected into the amnion using a insulin syringe with 31g needle (0.25 mm x 8 mm) to a depth of about 8 mm without disturbing the air cell. The hole was sealed and incubated up to 21 days.

After completion of *in ovo* injection, all eggs were transferred to hatcher trays and incubation was continued till hatching of the chicks. Number of chicks hatched in each replicate within each treatment was noted and recorded. The hatch was taken on day 21 and the chicks were wing banded. The hatch weight of each chick was individually recorded on a balance of 0.01 g accuracy. The hatched out chicks were allotted in to five treatments with six replicates of 16 chicks each. Experimental birds were provided with standard broiler ration (BIS, 2007) [7]. Birds were provided with *ad libitum* feed and water. Standard management practices were followed throughout the experiment. At the end of the experiment (day 42), one bird was randomly selected from each replicate (6 birds per each treatment group) and jejunum contents were collected to determine the gut microbial populations of *Lactobacillus, Salmonella, E. coli* and *Staphylococcus* from six randomly selected birds from each treatment. Immediately after collecting, the jejunum contents were diluted to 10^{-1} dilution initially, followed by 10-fold serial dilutions were subsequently made in sterile condition. For each dilution, 0.1 ml was inoculated in an agar plate. The media used were DeMan, Rogosa, Sharpe (MRS) broth (HimediaM369) for *Lactobacillus*, Mac Conkey agar (Himedia M 083) for *E.coli*, Xylose Lysine De-oxy Cholate (XLD) agar (Himedia M 031)for *Salmonella* and Mannitol salt broth (M 383)for *Staphylococcus sp*

All the inoculated plates were incubated at 39°C. MRS agar plates were incubated anaerobically for 2 days in a Gas-Pak container and Mac Conkey agar plates, Xylose Lysine De-oxy Cholate (XLD) agar plate and Mannitol salt broth were incubated aerobically for 1 day. Total numbers of bacterial colonies were counted at the end of each incubation period by using colony counter. The data were analyzed by one way ANOVA using V.17 SPSS (1999) [8] software. Differences between treatment means were detected by the Tukey test.

Results and Discussion

Mean gut microbial count ($\log_{10}$cfu/ml) of jejunal contents of broiler chicken at sixth week of age as influenced by *in ovo* injection of *Lactobacillus acidophilus* are furnished in Table.1.

The results revealed that the birds injected with $1x10^6$L. *acidophilus* through intra amnion on 18th day of incubation had the best ($P< 0.01$) colonization of *Lactobacillus spp*. (log 10.93 to 14.21) and the least of harmful bacteria like *Salmonella, E. coli* and *Staphylococcus* in the jejuna content of broiler chicken at 6th week of age. *In ovo* injection of *Lactobacillus acidophilus* significantly ($P<0.01$) reduced *Salmonella* count in jejuna contents of broiler chicken at 42 days of age compared to control and sham. Among the treatment group L. *acidophilus* of $1x10^6$cfu received group had better log values of 14.24 compared to other treated groups with the log value of 15.00 in LA $1x10^9$ and 14.94 log value in LA 1x10*12* injected groups.. The same trend was followed in *E coli* and *Staphylococcus* count and the log values were significantly ($P<0.01$) reduced in treatment group compared to control and sham.

In ovo supplementation of higher concentration (LA $1x10^9$ and $1x10^{12}$) of L. *acidophilus* had no extra beneficial effect; but resulted in lesser concentration of *Lactobacillus spp*and higher concentration of harmful bacteria compared to LA $1x10^6$ injected birds but lesser than that of sham and negative control which cannot be explained or needs further experimentation. Sham control also resulted in higher *Lactobacillus spp* colonization as well as harmful bacteria than that of negative control but the values were lesser than that of *in ovo* groups. Based on these results, *in ovo* injection of *Lactobacillus acidophilus* at $1x10^6$ cfu in broilers had higher concentration of probiotic bacteria *Lactobacillus spp*. and lower concentration of harmful microbes in the jejunal contents of broilers compared to other *in ovo* treated groups. *In ovo* injection of L. *acidophilus* to broiler embryos on 18th day of incubation through intra amnion significantly increased colonization of beneficial bacteria *Lactobacillus spp*. and significantly suppressed harmful bacterial colonization of *Salmonella*, *E.coli* and *Staphylococcus* in the jejunum compared to sham and non injected control broilers.

The results of the present *in ovo* study are agreed with the earlier finding of Watkins *et al.* (1982) [9], who observed decreased level of coliforms in Turkeys by the dietary addition of *Lactobacillus.* Similarly, Watkins and Kratzer (1983) [10], Jin *et al.* (1998) [11] and Kabir *et al.* (2004) [12] also witnessed significantly reduced coliforms counts in cecal macerates of chicks by dietary supplementation of *Lactobacillus* strains in the diet. Dimcho Djouvinov *et al.* (2005) [13] also recorded decreased total counts and *E. coli* and significantly increased level of *Lactobacilli* concentrations in the caecal digesta by dietary supplementation of pure cultures of *Streptococcus thermophilus, Enterococcus faecium* and 4 strains of *Lactobacillus* in broiler feed.

The findings of Mountzouris *et al.* (2010) [14] are in agreement with the present results who reported significantly lowered coliform counts and increased concentrations of *Bifido bacterium* and *Lactobacillus* in the ceacal contents of multi species probiotic fed broilers at 42nd day of age. The findings of increased level of *Lactobacilli* concentrations and reduced

E. coli pathogens in the cecal samples of probiotics supplemented broilers by Altaher *et al.* (2015) [15] concurred with the present study results. In line with present findings, Majidi-Mosleh *et al.* (2017)[16] also recorded increased lactic acid bacteria and decreased E. coli count in the jejunum of newly hatched broiler chicken *in ovo* injected with various probiotics on 18th day of incubation. Similar reports was also published by Lourenco *et al.* (2012) [17] who found that feeding of *B. subtilis* significantly decreased the Salmonella population in the intestinal contents of broiler.

In contrary to our study, Ozcan Cengiz *et al.* (2015) [18] who reported that dietary probiotic supplementation on the gut microflora count in commercial broiler chicken did not show any significant effect on gut total aerobs, *Salmonella*, and *Lactobacilli* count. Similar contrary result was also registered by Yamawaki *et al.*(2013) [19] who found that *in ovo* injection of *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus salivarius* through air-cell route in an embryonated broiler eggs on 18th day of incubation did not significantly (P<0.05) affect protection against *Salmonella.* Dietary probiotic supplementation did not show any significant effect on gut total aerobs, *Salmonella*, and *Lactobacilli* count, the finding of Ozcan Cengiz *et al.*, (2015) [18] did not concurred with the present findings.

The beneficial effect of higher concentration of probiotics bacteria *Lactobacillus* and lower concentration of harmful microbes colonization in the jejunum *obtained in this study* by *in ovo* injection of *Lactobacillus acidophilus* on 18th d of incubation may be due to early intestinal colonization of beneficial bacteria i.e. before hatching not only prevents pathogenic bacteria-related intestinal disorders, but also improves intestinal maturation and integrity (Lan *et al.*,2013) [20]. Advances in intra amniotic administration techniques have made it possible to incorporate several nutrients and active compounds including in late-term embryos, and these substances are subsequently swallowed, by the embryos before hatching (De Oliveira *et al.*, 2014)[21] whereas, beneficial microorganisms in this study. However, successful colonization of probiotics depends on the survival and stability of the microbial strain used, their relationship with the host, dose and usage frequency, and host health, nutritional status, age, stress and genetics of birds (Mason *et al.*, 2005) [22].

Table 1: Mean (±SE) gut microbial count (log $_{10}$ cfu/g) of intestinal contents of broilers chicken as influenced by *in ovo* injection of *Lactobacillus acidophilus*

Treatments		Gut microbial count (log $_{10}$ cfu/g) of intestinal contents			
		Lactobacillus spp	***Salmonella spp***	***Escherichia coli***	***Staphylococcus spp***
Non injected control		$6.64^{c} \pm 0.21$	$16.37^{a} \pm 0.59$	$15.42^{a} \pm 0.18$	$19.21^{a} \pm 0.04$
In ovo injection of 0.2 ml of	Sterile water (Sham)	$10.32^{b} \pm 1.23$	$15.81^{a} \pm 0.35$	$15.59^{a} \pm 0.23$	$19.20^{a} \pm 0.05$
	L. acidophilus 1x10^{6}cfu	$14.21^{a} \pm 0.04$	$14.24^{b} \pm 0.24$	$11.90^{c} \pm 0.19$	$15.57^{c} \pm 0.19$
	L. acidophilus 1x10^{9}cfu	$10.93^{b} \pm 0.44$	$15.00^{ab} \pm .31$	$12.20^{b} \pm 0.05$	$16.05^{bc} \pm 0.21$
	L. acidophilus 1x10^{12}cfu	$11.57^{b} \pm 0.39$	$14.94^{ab} \pm 0.19$	$12.18^{b} \pm 0.06$	$16.22^{b} \pm 0.05$
F- value		22.40	5.19	134.42	185.46
Significance		**	**	**	**

No. of observations (N) = 6

Means within column bearing different superscripts differ significantly

** – Highly significant (P<0.01

Conclusion

In ovo injection of *Lactobacillus acidophilus* at the dose of 1x10^{6} significantly increased the concentration of probiotic bacteria *Lactobacillus spp.* and lowered the concentration of harmful microbes in the jejunal contents of broilers compared to other *in ovo* treated groups. Based on the results obtained from this experiment it can be concluded that the *in ovo* delivery of *Lactobacillus acidophilus* improved beneficial gut microflora of commercial broilers.

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