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Effect of *in ovo* feeding of probiotic, prebiotic and synbiotic to Broiler embryos on growth performance, *Mucin-2* gene expression and gut colonization of microbiota

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ABSTRACT

Supplementation of antibiotics in poultry diet was banned in several countries due to development of antibiotic resistance. *In ovo* feeding of prebiotics, probiotics and synbiotics have gained more attention recently. The present study was carried out in 2018 to investigate the effect of *in-ovo* feeding of probiotic, prebiotic and symbiotic on growth performance and gut microbiome of broiler chicken. On 18th day of incubation, 600 eggs were randomly divided into five treatments each with four replicates of 30 eggs each and were injected with different bio-active compounds, viz. 0.2 ml of *Lactobacillus acidophilus* 3×10^7 cfu, 0.5% Mannan-oligosaccharide (MOS), synbiotic (0.1 ml each of *Lactobacillus acidophilus* 3×10^7 cfu and 0.5% MOS**)** along with injected and non-injected controls. After hatch, 400 chicks were sorted out as per treatment with four replicates of 20 chicks each. Birds were reared under deep litter system and fed with experimental diet *ad lib. In ovo* feeding of *Lactobacillus acidophilus,* MOS either separately or in combination significantly improved hatch weight, fifth week body weight and gain. However, hatchability, cumulative feed intake, cumulative FCR and cumulative livability were not affected. Improved colonization of *Lactobacillus acidophilus* and suppressed colonization of *Salmonella, Escherichia coli* and *Staphylococcus* in all intra-amniotic groups was noticed. Ileal *Mucin-2* gene was significantly up-regulated in the order of MOS, *L. acidophilus* and synbiotic injected broilers. The results concluded that the *in ovo* delivery of *Lactobacillus acidophilus* and MOS either separately or in combination had beneficial effect on growth and gut health of broiler chicken.

Keywords: Broilers, Growth performance, Gut health, *In ovo* feeding, *Lactobacillus acidophilus*, Mannan-oligosaccharide

Most of the countries banned the use of antibiotics in poultry feed due to development of antibiotic resistance in bacteria as well as in human beings and alteration of natural gut microbiota (Bostoglou *et al*. 2002). Hence, use of alternatives such as prebiotics, probiotics, synbiotics, enzymes, phytogenic, organic acids and other feed additives have been advocated to improve the production and health performance of broilers (Borazjanizadeh *et al*. 2011). Recently, *in ovo* feeding of prebiotics, probiotics and synbiotics is the alternative to the traditional system of dietary feeding and has gained more attention. Uni and Ferket (2003) developed a modern tool of *in ovo* injection of different nutrients was found to be an effective option to achieve the task of early delivery of probiotics. Pre-hatch birds naturally consume the amniotic fluids on 19th day of incubation. Therefore, deposition of essential nutrients in the amniotic fluid would deliver the same into the embryo's gut before pipping and in turn improve chick quality, hatch weight, better absorption, faster growth rate and increased marketing weight.

Lactobacillus acidophilus is a probiotics bacterial strain exerts its beneficial effects on the host, viz. by competitive exclusion of pathogenic bacteria, by producing the anti-bactericidal substance, by stimulation of immune system, by improving digestion and absorption of nutrients, and also responsible for neutralization of enterotoxins (Menten 2002). Mannan-Oligosaccharide is the one of the prebiotics that exerts its beneficial effect in the host by selectively stimulate the colonization of beneficial microbes in the colon (Gibson and Roberfroid 1995). Synbiotic is the combination of prebiotics and probiotics and act synergistically in the host's gut (Ashraf *et al*. 2013). More literatures are available on traditional dietary supplementation. However, studies on the intra-amniotic supplementation of prebiotics, probiotics and synbiotics in broilers are very much limited in India. Hence, the present study was undertaken to investigate the effects of intra-amniotic delivery of prebiotic, probiotic and synbiotic

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supplement on production performance and gut colonization microbiota in commercial broilers.

MATERIALS AND METHODS

This study was carried out in accordance with the Institutional Animal Ethics Committee (IAEC, No.2140/SA/DFBS/IAEC/2017 dated 30.10.2017) of TANUVAS, Chennai, India.

Preparation of Lactobacillus acidophilus*, Mannan-oligosaccharide and their combination: Lactobacillus acidophilus* (MTCC 10307) was purchased from MTCC, Chandigarh. Culture was grown in specific MRS broth and incubated at 37°C for 48 hrs in an anaerobic chamber. Colony forming unit was found to be 3×10^7 cfu/ml. Mannan-oligosaccharide (Lot I3I00Ib) was imported (Israel) and supplied by M/s Exotic Biosolution Private Limited, Mumbai. Mannan-oligosaccharide was dissolved at 0.5% in normal saline. Combination was prepared by mixing of *Lactobacillus acidophilus* (3×10^7 cfu) with Mannan-oligosaccharide (0.5%).

Intra-amniotic administration: Six hundred fertile eggs were collected from 35th weeks old broiler breeder flock (Cobb 400) and incubated under optimal conditions at a commercial hatchery. On 18th day of incubation eggs were divided into five groups with four replicates of 30 eggs each. On same day, intra-amniotic injection of 0.2 ml of *Lactobacillus acidophilus* 3×10^7cfu (T_3); 0.2 ml of 0.5% Mannan Oligosaccharide (T_4); 0.2 ml of *Lactobacillus acidophilus* 3×10^7 cfu + 0.5% Mannan-oligosaccharide (T_5) and 0.2 ml of Normal saline (T_2) was carried out by adopting Uni and Ferket (2003) method and T_1 was as a non-injected control.

Biological trial: After hatch, a total of 400 day-old broiler chicks were sorted out based on different *in ovo* treatment for conducting biological trial. Eighty chicks for each treatment were selected with four replicates of 20 chicks each. The biological study was conducted at Environmentally Controlled Broiler House facility established at Madras Veterinary College, Chennai funded by Indian Council of Agricultural Research, New Delhi. Birds were reared on a deep litter system and standard management practices were followed. Birds were provided with clean, potable drinking water and fed *ad libitum* with pre-starter, starter and finisher diet as per BIS (2007) from 0–7 d, 8–21 d and 22–35 d of age, respectively. The lighting programme was 23 h of light and 1 h of darkness with the intensity of 1 watt per chick from 1 to 7 days and 18 h of light and 6 h of darkness from 8 to 35 days. The birds were immunized as per recommended vaccination schedule.

Parameters recorded: Per cent hatchability and hatch weight were recorded. Birds were individually weighed by using electronic weighing balance of 0.1 g accuracy and the weights were recorded. The body weight gain was calculated. Cumulative feed consumption of each group was measured at the same time periods as body weights with cumulative averages calculated. Cumulative feed conversion ratio was calculated by dividing average cumulative feed consumption by average body weight gain. Mortality was recorded on a weekly basis throughout the trial and livability was calculated by using the following formula.

$$\text{Livability (\%)} = \frac{\text{Total number of birds alive}}{\text{Total number of birds started with}} \times 100$$

Sample collected: For microscopic studies, three days old chick's jejunum samples (four samples of male + four samples of female from each treatment) were collected and transferred to vials and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (*p*H 7.2) for 24 h at 4°C. The ileums were collected for expression of *Mucin-2* gene on the day old, 7th day and 14th day of age. A section of the ileum (approximately 3.5 cm) were sampled, rinsed in cold phosphate buffer saline (PBS), and placed in RNA later (Sigma) for subsequent gene expression analysis.

Gut colonization of microbiota under TEM: The fixed jejunum sample was washed with PBS for 45 min two times, post fixed in 1% aqueous osmium tetroxide for 2 h, later washed with deionized distilled water for 4 times each 45 min. The samples were dehydrated in series of graded alcohols, infiltrated and embedded in araldite 6005 resin or spur resin (Spurr 1969) and incubated at 80°C for 48 h for complete polymerization. Ultrathin (60 nm) sections were made on ultra-microtome (Leica ultracut UCT-GA-D/E-1/100), mounted on copper grids and stained with saturated aqueous uranyl acetate and counter stained with Reynolds lead citrate and were observed under the transmission electron microscope (Make: Hitachi, H-7500, JAPAN) at College of Veterinary Sciences, PV Narsimha Rao Telangana Veterinary University, Hyderabad, India.

Gene expression of Mucin-2 *by using quantitative RT-PCR*: Eight samples of ileum from each treatment (one sample each for either sex in each replicate) were collected for *Mucin-2* gene expression study. The RNA was extracted from cells of ileum using Trizol® reagent (Cat # 15596018) as per the manufacture's instruction. The concentration of RNA was estimated in a spectrophotometer (Biophotometer Plus, Eppendorf). The synthesis of cDNA was carried out using PrimeScript™ synthesis kit (Cat. #RR037A) as per manufacturer's instruction.

The sequences of the published primers used for expression analysis were *Mucin*-2 F- CCTGTGCAGACC-AAGCAGAAA, R-TCTGAGTTTTT-CAGCAAAGA-ACAC and β-*actin* F-TGCTGTGTTCCC-ATCTATCG, R-TTGGTGACAATACCGTGTTCA. Quantitative PCR (q-PCR) was carried out using SYBR® Green following the manufacturer's instruction. The Real Time PCR was carried out in CFX96 Touch Real Time PCR detection system, Bio-Rad.

Statistics analysis: Data recorded in this biological experiment were subjected to one-way analysis of variance (ANOVA). Means were compared by Duncan multiple range comparison test (Steel and Torrie, 1981) with level of significance ($P<0.05$). Average gene expression of *Mucin*-2 was calculated relative to the β-actin endogenous control by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen

2001) for ANOVA analysis. Gene expression fold change, standard error and statistical significance were calculated based on the formula developed by Pfaffl's (2001). Treatment means were compared by Duncan multiple range comparison test (Steel and Torrie 1981) with level of significance ($P<0.05$).

RESULTS AND DISCUSSION

Effect of Intra-amniotic administration of *Lactobacillus acidophilus*, Mannan-oligosaccharide and their combination on per cent hatchability and hatch weight, fifth week body weight and gain, Feed consumption, Feed

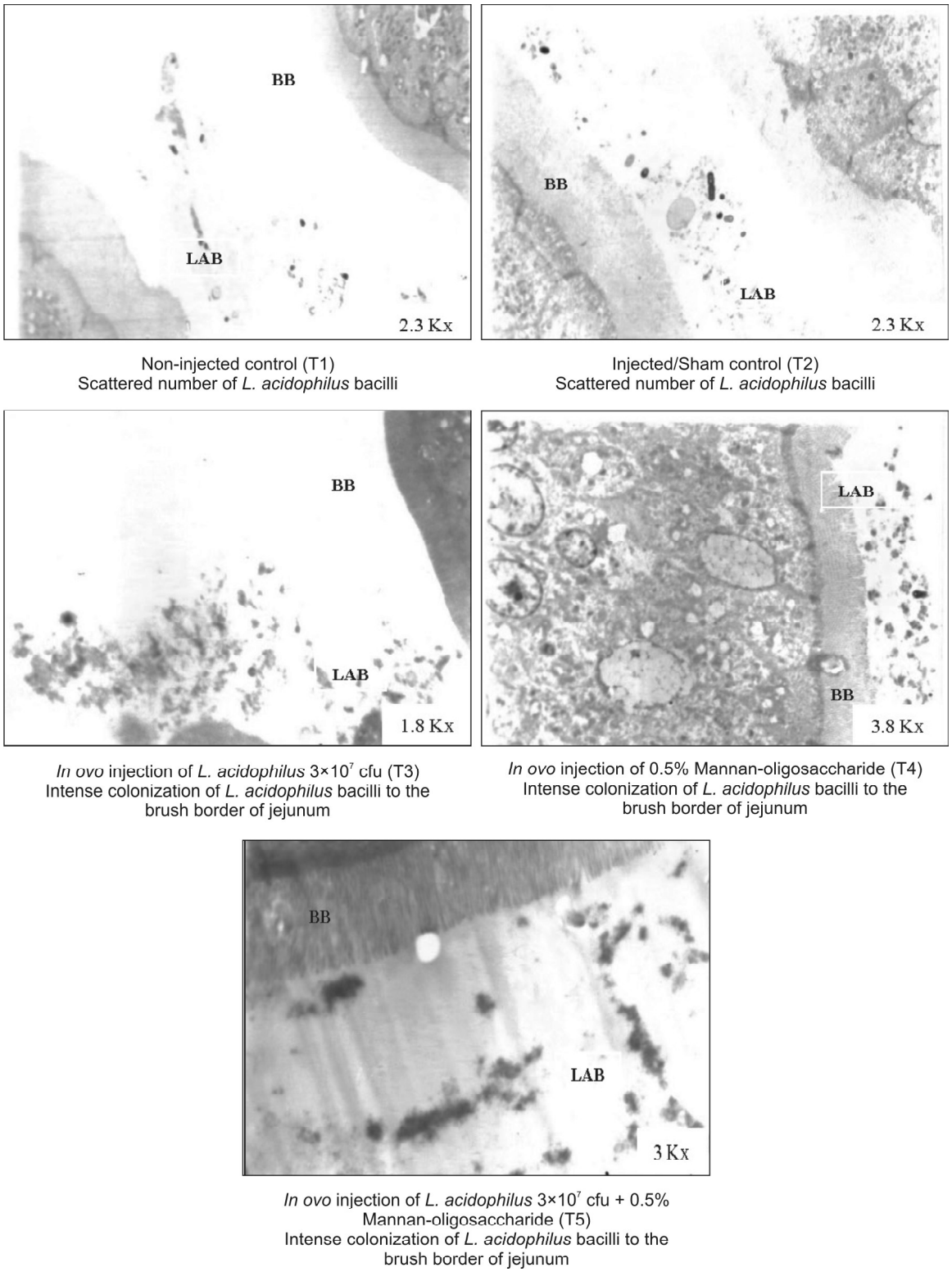

Fig.1. Gut colonization of *Lactobacillus acidophilus* in three days old broiler chicks under Transmission Electron Microscope.

conversion ratio, livability are presented in Table 1.

Hatchability: The Intra-amniotic injection of *Lactobacillus acidophilus*, Mannan-oligosaccharide and their combination were not significantly (P<0.05) affected the per cent hatchability and ranged from 88.19 (T4) to 91.32 (T3). Whereas the per cent hatchability of embryonated eggs belongs to T1, T2 and T5 were observed as 89.53, 89.30 and 89.78 respectively. This beneficial effect could be due to establishment of healthy gut microbiota without any adverse effect on hatching performance due to *in ovo* deposition of either *Lactobacillus acidophilus*, Mannan-oligosaccharide separately or in combination. The present finding is in agreement with the report of Calick *et al*. (2017) and Majidi-Mosleh *et al*. (2017) who found that the hatchability was not affected by *in ovo* delivery of probiotics in broilers. However, Bednarczyk *et al*. (2016) observed better hatchability with different prebiotics than control due to optimized conditions of *in ovo* delivery of prebiotics for broiler chicken. Whereas, De Oliveira *et al.* (2014) observed a reduction in hatchability by 10% with *in ovo* infusion of commercial probiotics when compared with non-injected control and saline injected group in broilers.

Growth performance: The Intra-amniotic injection of *L. acidophilus*, MOS and *L. acidophilus* + Mannan-oligosaccharide (MOS) significantly (P<0.01) increased (39.54 to 40.25 g) the day old body weight of chicks than non-injected control (38.39 g) and was comparable with injected control chicks (39.92 g). The present findings are in agreement with the finding of Aleksandra *et al*. (2017) who reported that day old body weight was significantly (P<0.05) higher in chicks treated with *L. plantarum* + Raffinose family oligosaccharides (RFO) by *in ovo* when compared with other group of chicks. Similar findings were also reported by Ravichandran (2017) who observed that *in ovo* injection of *L. acidophilus* at the level of 1×10^{12} cfu significantly (P<0.01) increased the day-old chick weight compared with non-injected control chicks.

All the Intra-amniotic treatments recorded significantly higher marketing body weight at 35 d of age and ranged from 2100.23 g (T3) to 2135.25 g (T5) when compared to (T1) 2022.99 g with no significant difference among Intra-amniotic treated groups. Whereas the T2 group recorded intermediary body weight of 2080.13 g. Similar finding was observed by Pruszynska-Oszmalek *et al*. (2015) and Mista *et al*. (2016) who observed significant increase in the final body weight of broilers received prebiotic when compared with control. The beneficial effect of *in ovo* feeding of probiotics on marketing weight was confirmed by Pender *et al*. (2017b) and De Oliveira (2014) who reported that *in ovo* supplementation of probiotic bacteria significantly increased the body weight of broilers when compared to the birds of negative control and sham control (P=0.01). whereas Maiorano (2012), Sobolewska *et al*. (2017) and Majidi-Mosleh *et al*. (2017) reported contrary findings of non-significant effect on body weight. Similar trend was observed in body weight gain also with significantly (P<0.05) higher marketing body weight gain in Intra-

Table 1. Mean (±SE) hatchability and growth performance of broiler chicken as influenced by *in ovo* delivery of probiotic, prebiotic and synbiotic

Treatment	Hatchability (%) (n=120)	Hatch weight (g) (n=80)	5th Week body weight (g)	5th week body weight gain (g)	Cumulative feed consumption (g) (n=4)	Cumulative feed conversion ratio (FCR) (n=4)	Livability (%) (n=4)
Non injected control (T1)	89.53±0.71	38.39^{b}±0.24	2022.99^{b}±21.44 (n=58)	1984.58^{b}±21.21 (n=58)	3313.37±53.61	1.67±0.02	96.25±3.75
In ovo delivery of 0.2 ml of:							
Normal saline (Sham) (T2)	89.30±0.91	39.92^{a}±0.17	2080.13^{ab}±16.59 (n=60)	2040.31^{ab}±16.51 (n=60)	3385.65±45.99	1.66±0.02	98.75±1.25
Probiotic (*L. acidophilus* 3×10^{7} cfu) (T3)	91.32±1.30	40.26^{a}±0.19	2100.23^{a}±33.70 (n=62)	2059.85^{a}±33.60 (n=62)	3407.17±128.39	1.65±0.07	96.25±1.25
Prebiotic (0.5% Mannan-oligosaccharide) (T4)	88.19±1.03	39.71^{a}±0.21	2121.84^{a}±15.26 (n=60)	2082.08^{a}±15.42 (n=60)	3346.13±103.38	1.60±0.05	98.75±1.25
Synbiotic (*L. acidophilus* 3×10^{7} cfu + 0.5% Mannan-oligosaccharide) (T5)	89.78±1.17	39.54^{a}±0.13	2135.25^{a}±26.83 (n=61)	2095.79^{a}±26.94 (n=61)	3471.66±97.61	1.65±0.05	95.00±2.04
F-value	1.41	12.89	3.42	3.58	0.43	0.22	0.6
Significant	NS	**	*	*	NS	NS	NS

n, number of observations; NS, Not significant; *, Significant (P<0.05); **, Significant (P<0.01). Mean values within each column having the same superscript do not differ significantly.

amniotic treated broilers T3 (2059.85 g), T4 (2082.08 g) and T5 (2095.79) compared to T2 (2040.31 g) and T1 (1984.58 g) birds. The results of present study concurs with the earlier reports of Bednarczyk *et al*. (2016) who stated that during the 6th week age body weight gain was significantly (P<0.05) higher in broilers injected *in ovo* with raffinose oligosaccharide in comparison with the control. Contrarily, Maiorano (2012) reported non-significant improvement in the body weight gain of birds received *in ovo* prebiotic and synbiotic treatment compared with the control group. These discrepancies could be due to a variety of factors including, but not limited to, strain(s) of bacteria utilized, composition and viability of the probiotic, preparation method, dosage, application method, and frequency of application, overall diet, drug interactions, and condition of the animal (Mountzouris *et al*. 2007).

In ovo delivery of *Lactobacillus acidophilus*, MOS and *Lactobacillus acidophilus* +MOS did not have significant (P<0.05) influence on the fifth week cumulative feed consumption and cumulative FCR in broilers. The fifth week cumulative feed consumption was numerically higher in all Intra-amniotic treatments compared to non-injected control. The results of the present study on feed consumption are in line of agreement with those of Maiorano (2012) and Pender *et al*. (2017b) who found that the *in ovo* treatment with prebiotics did not significantly affect the feed intake in broilers. However, Pruszynska-Oszmale *et al*. (2015) found that *in ovo* administration of prebiotic and synbiotic marginally increased the mean daily feed intake per bird when compared to control group. The fifth week age cumulative FCR value ranged from 1.60 (T4) to 1.67 (T1). The present findings are in agreement with Pender *et al*. (2017b) who reported that the *in ovo* injection of Primalac® through amniotic route did not significantly affect the FCR when compared to sham and negative control birds.

There was numerical improvement in the survivability per cent of broilers from group T3 (96.25), T4 (98.75) and T5 (95.00) compared with T1 (96.25) by Intra-amniotic injection of *L. acidophilus*, MOS and their combination at fifth week of age. Similar results were observed by Adrianna *et al*. (2017) who found that the injection of prebiotic did not have any significant impact on livability up to 42 d of rearing. Similarly, Pender *et al*. (2017b) also observed no significant effect on mortality of broilers injected with commercial probiotic on 18th day of incubation. Bednarczyk *et al*. (2016) also reported similar finding that the mortality of broiler chicken was not influenced by the type of prebiotics and route of delivery.

Gut colonization: The Transmission Electron Micrographs of microbial colonization adhering to the brush border of jejunum in 3 day-old chicks are depicted in Fig. 1. The aggregation of the rod-shaped colonized microbes in the injected chicks was also seen more clearly in the magnified TEM micrograph (T3, T4, and T5 at 1.8, 3.8 and 1 Kx). Conversely, scattered colonized microbes occurred only in small numbers on the lumen surface of the sham control and non-injected chicks (T1 and T2 at 2.3 and 2.3 Kx) and significantly suppressed colonization of harmful bacteria like *Salmonella, E. coli* and *Staphylococcus* when compared to sham and non-injected control broilers. The early colonization of beneficial bacteria could be due to engulfing of *L. acidophilus,* Mannan-oligosaccharide that are deposited in to amniotic fluid on 18th day of incubation. The developing embryo engulfs the amniotic fluid on 19th day of incubation. In this study, *L. acidophilus* gets colonized on third day after hatching as witnessed by electron microscopic study. Moreover, the prebiotic Mannan-oligosaccharide may be selectively increased the growth of beneficial bacteria in the respective groups.

The results of present study were confirmed by Villaluenga *et al*. (2004), who observed that significant increase of *Bifidobacteria* in the colon of two-day old chicken *in ovo* injected with prebiotic than the injected control group. Similarly, Aleksandra *et al*. (2017) observed that microbial populations of *Lactobacillus* spp. and *Enterococcus* spp. in the ileum were significantly higher in broilers injected with *in ovo* treatment with different symbiotic combination group 1 and group 2 than that of control in broilers. Mookiah *et al*. (2014) also reported the similar findings significantly (P<0.05) higher populations of *Lactobacilli* in caecal contents of broiler chickens fed on probiotic, prebiotic, and synbiotic supplemented diet than that of the control birds at 21 days of age.

In contrary to present study, Yamawaki *et al*. (2013) 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 higher level of *Salmonella enteritidis* oral administration.

Mucin-2 gene expression: The effect of intra-amniotic infusion of *Lactobacillus acidophilus*, MOS and their combination on *Mucin-2* gene expression on day old, first week and second week of age is presented in Table 2. The result indicated that *L. acidophilus*, MOS and their combination when administered before hatch had greater influence on the mRNA abundance of *Mucin*-2 in the ileum of broiler chicken.

The *Mucin*-2 gene responsible for gut health was up-regulated on day old (72 h after administration) in broiler chicken subjected to *in ovo* treatments of *L. acidophilus*, MOS, *L. acidophilus* + MOS and sham control injected treatment (1.17, 2.83, 1.40 and 1.01 respectively). However, the level of *Mucin*-2 expression on day-old chicks was significantly (P<0.05) higher in MOS injected birds followed by probiotic and synbiotic infused groups. The present finding is agreed with Majidi-Mosleh *et al*. (2017) who conducted research by *in ovo* infusion of different probiotics strains on 18th day of its embryonic life and concluded that *Bacillus subtilis* upregulated *Mucin*-2 gene expression 4 and 3.7 folds higher than the control group in day old. Contrary to the present findings, Cheled-Shoval *et al*. 2011 found that *in ovo* feeding of prebiotic (MOS) on

Table 2. Mean relative expression (fold change) of *Mucin*-2 gene in the ileum of broiler chicken as influenced by *in-ovo feeding* of probiotic (*L. acidophilus*), prebiotic (Mannan-oligosaccharide) and synbiotic (*L. acidophilus* + Mannan-oligosaccharide) on 18th day of incubation (n=8)

Treatment	Day old	1st week	2nd week
Non injected control (T1)	$1.00^{c}\pm 0.00$	$1.00^{c}\pm 0.00$	$1.00^{d}\pm 0.00$
In ovo delivery of 0.2 ml of:			
Normal saline (Sham) (T2)	$1.01^{c}\pm 0.06$	$1.99^{b}\pm 0.02$	$3.76^{c}\pm 0.11$
Probiotic (*L. acidophilus* 3×10^{7} cfu) (T3)	$1.17^{b}\pm 0.05$	$1.75^{b}\pm 0.04$	$5.87^{b}\pm 0.05$
Prebiotic (0.5% Mannan-oligosaccharide) (T4)	$2.83^{a}\pm 0.02$	$2.38^{a}\pm 0.02$	$9.19^{a}\pm 0.01$
Synbiotic (*L. acidophilus* 3×10^{7} cfu + 0.5% Mannan-oligosaccharide) (T5)	$1.40^{b}\pm 0.01$	$2.28^{a}\pm 0.01$	$3.60^{c}\pm 0.02$
F-value	18.75	51.69	41.82
Significant	*	*	**

*, Significant (P<0.05); **, Significant (P<0.01). Mean values within each column bearing common superscript do not differ significantly.

18th day of embryonic life, did not affect the *Mucin*-2 expression on the day of hatch. On first week of age, the *Mucin*-2 gene expression level was significantly (P<0.05) more in MOS and MOS+*L. acidophilus* injected birds when compared to other groups. However, the expression level was comparable between probiotic and sham control birds and least recorded in non-injected birds. The highest level (P<0.01) of *Mucin*-2 gene expression was noticed in MOS infused broilers, followed by probiotic, sham control, symbiotic birds and least with non-injected broilers on 2nd week of age. However, the *Mucin*-2 gene expression level was not significantly altered between symbiotic and sham control birds. At all three periods (0-day, 1st and 2nd week), among *in ovo* treatments, MOS injected chicks shown higher folds compare to *Lactobacillus acidophilus* and synbiotic groups. The present finding is in agreement with the results of Smirnov *et al*. (2004) who found that the birds fed with commercial probiotic containing *Bifidobacterium bifidum*, *L. acidophilus, L. casei,* and *Enterococcus faecium* had increased the expression of *Mucin*-2 mRNA by 160% in the jejunum over that of control groups. Whereas, *Mucin*-2 gene expression was not affected in other parts of small intestinal. Pender *et al*. (2017a), who found that *in ovo* inoculation of commercial probiotic at different doses (1×10^{5}, 1×10^{6} and 1×10^{7} cfu) did not affect the expression level of *Mucin*-2 gene in the ileum of broilers on 4th, 6th, 8th, 15th and 22nd day age was contrary to the present finding.

The result of the present study indicated that the intra-amniotic administration of *Lactobacillus acidophilus* and MOS either separately or in combination had beneficial effect on growth and survivability of commercial broilers. These may be due to these *in ovo* delivered bio-active compounds by selective increase in the population of beneficial bacteria (*Bifidobacterium* and *Lactobacillus*) by depressing the colonization of potentially pathogenic bacterial populations. In addition, the gut health and gut efficiency was improved by the pre hatch supplementation of *Lactobacillus acidophilus* and MOS and their combination on day old, 1st and 2nd week of growing period which is confirmed by the up regulation of gut health related *Mucin*-2 genes.

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