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Original article

EFFECTS OF *IN OVO* SYNBIOTIC INJECTION ON GROWTH PERFORMANCE, INTESTINAL BACTERIAL LOAD AND ANTIBODY TITRES IN BROILER CHICKENS VACCINATED AGAINST INFECTIOUS BURSAL DISEASE

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Summary

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The present study investigated the efficacy of a synbiotic (*Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus salivarius* subsp. *thermophilus,* and mannan oligosaccharide) along with an infectious bursal disease (IBD) vaccine in Cobb 500 broilers. A total of 1200 embryonated chicken eggs were randomly allocated in 10 groups with eight replicates. The first group did not receive any treatment. The second group was vaccinated post-hatch (PV), the third group was vaccinated *in ovo* (IV), the fourth group received dietary synbiotic and *in ovo* vaccination (DS+IV), the fifth group was treated *in ovo* with synbiotic (IS), the sixth group received *in ovo* and dietary synbiotic (IS+DS), the seventh group received *in ovo* synbiotic plus post-hatch vaccination (IS+PV) and the eighth group – *in ovo* and dietary synbiotic and post-hatch vaccination (IS+DS+PV). In the ninth group, the synbiotic and the vaccine were administered *in ovo* (IS+IV) while the tenth group received *in ovo* and dietary synbiotic, plus *in ovo* vaccine (IS+DS+IV). The *in ovo* treatment with the synbiotic in combination with DS, IV or PV had a positive effect on weekly weight gain. The sixth group provided a better feed conversion ratio at the end of fourth week. The synbiotic application, individually in sixth group or along with PV or IV treatment in eighth and tenth groups, elevated feed intake in fifth and sixth weeks respectively.The antibody titre of IBD was higher for groups which received IV along with IS. It is concluded that the application of synbiotic along to IBD vaccine improved growth performance and had positive effects on IBD antibody titres.

Key words: broiler, *in ovo* injection, infectious bursal disease vaccine, synbiotic

INTRODUCTION

Due to the increase in the world population that is associated with an increased demand for protein, there is a rising trend in poultry production, especially in developing countries (Alexandratos & Bruinsma, 2012; Seto & Ramankutty, 2016). Hence, the need for new approaches and methods for the prevention of poultry di-

seases, the improvement of food safety and nutritional management is increasing (Ricke, 2018).

In ovo technology, a route to inject various substrates into eggs, was primarily applied in 1982 for a vaccine against Marek's disease (Sharma & Burmester, 1982). Currently, in addition to the Marek's vaccine, vaccines against Newcastle disease, fowl pox, coccidiosis and infectious bursal disease (IBD) are approved for *in ovo* administration (Peebles, 2018). *In ovo* delivery of vaccines provides earlier stimulation of immune responses against pathogens compared to post-hatch vaccination. The stress related to the handling of chicks is eliminated by using this method. Additionally, the vaccination is performed rapidly and also diminishes labour costs (Peebles, 2018). It is reported that *in ovo* vaccination has no negative effects on hatchability and production (Negash *et al.,* 2004). Due to the presence of maternally derived antibodies, the immune complex vaccines which are insensitive to maternal immunity and less virulent are developed. Therefore, these vaccines can be administered in the incubation period or early days after hatching (Jeurissen *et al.,* 1998). Immune complex vaccines bind the virus to the virus-neutralising factor and prevent it from neutralisation by maternally derived antibodies. When maternally derived antibodies declined, the virus replicates and acts as an immune stimulator (Haddad *et al.,* 1997).

The concern about antibiotic resistance in humans, led to use of alternatives such as probiotics and prebiotics which could reduce the use of antibiotics in poultry industry (Alloui *et al.,* 2013; Van Boeckel *et al.,* 2017). Probiotics play a role in preventing the colonisation of pathogens via improving the gut microbial balance and immune response regulation (Park *et al.,* 2016). The administration of probiotics to turkey and broiler chicks could reduce the risk of diarrhoea, salmonellosis and also, lead to an increase in body weight gain (Tellez *et al.,* 2013). Prebiotics are non-digestible carbohydrate compounds that stimulate growth and activity of beneficial bacteria and result in modulating gut microbiome (Babazadeh *et al.,* 2011; Pandey *et al.,* 2015). The synbiotic is a fusion product of probiotic and prebiotic compounds that promote the survival and implantation of live microorganisms of food supplement in the digestive system (Babazadeh *et al.,* 2011; Pandey *et al.,* 2015). The *in ovo* injection of synbiotics have been applied in some researches, however, these supplements are usually administered in-diet or in-water during the post-hatching period. Since the chickens are more susceptible to infection in the first day after hatching, it is proposed that *in ovo* injection of synbiotics can confer early protection against pathogens. It is declared that *in ovo* delivery of useful bacteria by establishing the gut microbiome, similar to *in ovo* vaccination promote the immune system (Roto *et al.,* 2016). Therefore, the goal of the present study was to investigate the effects of *in ovo* injection of a synbiotic along with *in ovo* administration of IBD vaccine on growth performance, IBD antibody titre, and gut microbiome in broiler chickens.

MATERIALS AND METHODS

A total of 1200 embryonated chicken eggs (Cobb 500) were purchased from a commercial hatchery (Pasargad Co, Iran) and incubated under standard conditions for 18 days. Then, the infertile eggs and dead embryos were discarded when the eggs were transferred from the setter to the hatcher. The fertilised eggs were ran-

domly divided into 10 groups. A total of 960 live hatched chickens were transferred from the hatcher to the rearing farm at Agricultural Research Center of Jahad, Mashhad, Iran after finishing incubation period (21 days).

All of the one-day old chickens were divided into the pens based on their groups on hatcher (10 groups with 8 replicates; 12 birds/1 m^2). Sanitation principles and health measures of birds were applied and no medications were used during the rearing period. The chickens were raised on litter, feed and water were provided *ad libitum*. The actual ambient temperature, light, humidity, and air conditions were prepared based on the last recommendations of broiler management guide (Anonymous, 2018). A prepared commercial starter, grower, and finisher broiler diets were used (Gohar Daneh Shargh, Iran). The chemical analysis of diet is presented in Table 1.

Synbiotic preparation

The bacteria were obtained from Persian Type Culture Collection, Tehran, Iran. *L. delbrueckii* subsp. *bulgaricus* strain ATCC 11842 were cultured anaerobically on MRS agar that was prepared by adding glucose and yeast extract, at 37–43 °C in 5% CO_2 for 24 h. *S. salivarius* subsp. *thermophilus* strain ATCC 9649 were grown in Columbia blood agar under aerobic conditions at 37 °C for 24 h. The synbiotic prepared in laboratory of Bioran Co, Karaj, Iran contained *Lactobacillus delbrueckii* subsp. *bulgaricus* (10^9 cfu/ kg), *Streptococcus salivarius* subsp. *thermophilus* (10^9 cfu/kg) and 0.1% mannan oligosaccharide (MOS) extracted from the *Saccharomyces cerevisiae*, which was received daily with the diet. The synbiotic solution administered *in ovo* consisted of 0.2 mL 0.1% MOS, *L. delbrueckii* subsp. *bulgaricus (*10^3 cfu/ egg) and *S. salivarius* subsp. *thermophilus* (10^3 cfu/egg).

In ovo inoculation

In ovo injection of IBD vaccines and synbiotics was performed on the 18^{th} day of incubation using an automated egg injection machine (Wakenell *et al.,* 2002). A dose of 0.2 mL/egg synbiotic solution was administered in each injection inside the IBD vaccine (CEVAC® Transmune®, Ceva-Phylaxia, Budapest, Hungary) that was delivered at a 0.05 mL dose into the air cell. The eggs of control groups were injected with sterile phosphate buffered saline of 0.25 mL/egg. The injection site in the eggshell was immediately sealed with adhesive tape and eggs were returned to the hatcher until the end of the incubation period.

Table 1. Analysis of diet for the 6-week rearing period in broiler chickens

Analysis results	Starter (Day 1–14)	Grower (Day 15–28)	Finisher (Day 29–42)
ME (kcal/kg)	2.98	3.04	3.11
Crude protein (%)	21.3	18.8	17.42
Methionine (%)	0.53	0.47	0.44
Methionine +cysteine (%)	0.87	0.77	0.72
Lysine (%)	1.05	1.04	0.99
Threonine (%)	0.77	0.69	0.65
Calcium (%)	0.98	0.97	0.97
Available phosphorus (%)	0.51	0.50	0.49
Sodium (%)	0.15	0.15	0.15

Treatment groups

The first group was negative control 1 (NC) and it did not receive the IBD vaccine and synbiotics either *in ovo* or in diet. The second group was negative control 2 (PV) which received only the live IBD vaccine (IBDL, Ceva Animal Health, Budapest, Hungary) orally on day 20 (calculated based on the maternal antibody). In the third group, eggs were injected with IBD vaccine (IV). The fourth group received synbiotics in diet and also *in ovo* vaccine against IBD (IV+DS). In the fifth group, eggs were injected *in ovo* only with the synbiotic compound (IS). The sixth group received both synbiotic *in ovo* and in diet (IS+DS). The seventh group received synbiotics *in ovo* and also were delivered post-hatch vaccination orally during the rearing period (IS+PV). The eighth group received synbiotics both *in ovo* and in diet and in addition, was vaccinated orally in the rearing period (IS+DS+PV). In the ninth group, synbiotics and IBD vaccine were administered *in ovo* (IS+IV). In the tenth group, synbiotics and IBD vaccine were administered *in ovo*, this group received also dietary synbiotic during the rearing period (IS+ DS+IV).

Sampling and measurements

On the first day and at the end of the third and sixth weeks of age, blood samples were taken from the wing vein from 32 birds randomly in each group (4 birds per replicate). Serum samples were screened using a commercial ELISA kit (IDEXX, Maine, USA) to determine the antibody titres against IBD.

The faecal drops (one g per sample) were collected on the second and last days of rearing from one bird in each replicate that received the synbiotic on hatcher to trace the *in ovo* injected bacteria. The samples were prepared according to the method described by Sattar *et al.* (2018). The samples were cultured anaerobically on MRS agar which was prepared by adding glucose and yeast extract, at 37–43 °C in 5% CO_2 for 24 h for detecting *L. delbrueckii* and in Columbia blood agar under the aerobic condition at 37 °C for 24 h for detecting *S. salivarius*.

At 1, 14, 28 and 42 days of age, two birds per replicate were randomly euthanised by severing the jugular vein. The bursa of Fabricius weight (g) was recorded for calculation of bursal index (B/B): 100× bursa of Fabricius (g)/body weight (g).

The feed intake (FI) and mortality rate (MR) were recorded for each replicate of groups daily. Feed conversion ratio (FCR), body weight (BW) and body weight gain (BWG) were recorded weekly.

Statistical analysis

Data were analysed in SPSS version 21 software (SPSS Inc., USA) by one-way ANOVA followed by Duncan's *post hoc* test to determine the statistically significant differences in mean values of all data. $P<0.05$ was considered statistically significant.

Ethical approval

All experiments were conducted after institutional approval of the Animal Use Committee of Shiraz University, Shiraz, Iran. Also, slaughtered chickens were humanely handled.

RESULTS

Body weight gain and feed conversion rate

Table 2 presents the body weight gain (BWG) per groups during the study. In weeks 1 and 2, there were no significant

differences between groups in term of BWG. The BWG of NC group in the third week was significantly higher than that of PV, IV and IS+PV groups (P<0.05). The IS+DS+PV group had higher weight gain than the PV and IV+DS groups on the 28th day of the study and this difference was statistically significant (P<0.05). In the fifth week same as fourth week, the highest BWG was observed for the IS+DS+PV group which had a significant difference vs the PV group (P<0.05). In the last week, the BWG in IV, IS+DS+PV and IS+DS+IV groups were significantly higher than those of IS+IV and PV groups (P<0.05). Feed intake (FI) on the 7th day and in the IS+DS+PV group was significantly higher than other groups (P<0.05). In SI group, FI was significantly higher compared to PV group on day 14 (P<0.05). There was no significant difference between groups on 21th and 28th days of rearing period. FI on 35th day in the IS+DS and IS+DS+PV groups was significantly higher than the NC, PV and IS+IV groups (P<0.05). In addition, on the 42nd day, FI in IS+DS+IV group was significantly higher than NC, PV and IS+IV groups (P<0.05) (Table 3). The lowest value of FCR was observed in IS+DS group at fourth week, which had a significant difference with IV+DS group (P<0.05). No significant difference was found in term of FCR between groups in the first, second, third, fifth and sixth weeks of rearing (P>0.05) (Table 3).

Mortality

Total mortality in this rearing period was 28 (2.91%) (Table 4). The highest mortality (0.41%) was detected for the PV, IS and IS+PV groups.

Bursal index

Table 4 presents data of B/B ratio. On the first day, the B/B ratio in the IS+DS, IS+PV, and IS+IV groups was significantly higher than values in other groups, except the IS+DS+PV and IS+DS+IV groups (P<0.05). The B/B ratio on the 14th day of rearing was significantly higher in the IS+DS and IS+PV groups than the IS+DS+IV group (P<0.05). On the 28th day, B/B ratio was significantly

Table 2. Weekly body weight gain of experimental Cobb-500 broilers groups during the different rearing weeks. The values are expressed as mean±SD (n=8)

	Weekly body gain (g)					
	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
G1 (NC)	91^{a}±0.8	269^{a}±1.2	421^{a}±1.5	496^{ab}±1.4	581^{ab}±3.3	670^{ab}±26
G2 (PV)	91^{a}±4.5	267^{a}±11	375^{b}±30	468^{b}±46	542^{b}± 90	650^{b}±55
G3 (IV)	91^{a}±2.3	293^{a}±28	380^{b}±30	507^{ab}±50	574^{ab}±93	719^{a}±67
G4 (IV+DS)	96^{a}±2.8	277^{a}±15	395^{ab}±39	470^{b}±37	584^{ab}±20	668^{ab}±47
G5 (IS)	95^{a}±1	283^{a}±7.7	395^{ab}±26	518^{ab}±54	586^{ab}±43	673^{ab}±38
G6 (IS+DS)	91^{a}±4.9	272^{a}±11	389^{ab}±22	517^{ab}±10	624^{ab}±27	677^{ab}±28
G7 (IS+PV)	96^{a}±2.3	268^{a}±6	378^{b}±11	531^{ab}±25	584^{ab}±25	666^{ab}±11
G8 (IS+DS+PV)	97^{a}±6.2	280^{a}±16	386^{ab}±19	553^{a}±55	644^{a}±27	723^{a}±46
G9 (IS+IV)	94^{a}±7.4	269^{a}±9.8	399^{ab}±17	499^{ab}±24	595^{ab}±52	646^{b}±21
G10 (IS+DS+IV)	95^{a}±1.9	276^{a}±16	387^{ab}±9	492^{ab}±35	606^{ab}±63	719^{a}±43

G1–10: Groups 1–10, NC: negative control, untreated; PV: post-hatch vaccination; IV: *in ovo* vaccination; IS: *in ovo* synbiotic; DS: dietary synbiotic. Different superscript letters within the same column indicate significant differences (P<0.05).

Table 3. Feed conversion ratio and feed intake of experimental Cobb-500 broilers groups during the different rearing weeks. The values are expressed as mean± SD (n=8)

	Feed conversion ratio						Feed intake (g)					
	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
G1 (NC)	1.11^{a}±0.0	1.34^{a}±0.0	1.56^{a}±0.1	1.74^{ab}±0.1	1.88^{a}±0.1	2^{a}±0.0	100^{b}±2.7	368^{ab}±9	630^{a}±36	875^{a}±35	1094^{b}±52	1314^{b}±29
G2 (PV)	1.09^{a}±0.1	1.34^{a}±0.0	1.60^{a}±0.0	1.77^{ab}±0.1	1.95^{a}±0.1	2.03^{a}±0.1	100^{b}±5.8	358^{b}±11	601^{a}±32	843^{a}±44	1101^{b}±92	1309^{b}±99
G3 (IV)	1.06^{a}±0.0	1.28^{ab}±0.1	1.67^{a}±0.2	1.8^{ab}±0.1	1.87^{a}±0.7	1.98^{a}±0.1	100^{b}±0.0	373^{ab}±13	632^{a}±23	910^{a}±50	1174^{ab}±69	1391^{ab}±81
G4 (IV+DS)	1.08^{a}±0.0	1.37^{a}±0.1	1.59^{a}±0.1	1.86^{a}±0.1	1.98^{a}±0.1	2^{a}±0.0	102^{b}±3.6	379^{ab}±14	628^{a}±40	887^{a}±40	1137^{ab}±46	1337^{ab}±76
G5 (IS)	1.06^{a}±0.0	1.36^{a}±0.0	1.59^{a}±0.0	1.75^{ab}±0.1	2.01^{a}±0.1	2.03^{a}±0.0	100^{b}±6.2	383^{a}±8.1	627^{a}±32	903^{a}±45	1178^{ab}±38	1369^{ab}±60
G6 (IS+DS)	1.07^{a}±0.0	1.34^{a}±0.0	1.62^{a}±0.0	1.68^{b}±0.1	1.9^{a}±0.1	2.01^{a}±0.0	98^{b}±6.8	365^{ab}±12	632^{a}±30	887^{a}±50	1193^{a}±48	1371^{ab}±99
G7 (IS+PV)	1.06^{a}±0.0	1.35^{a}±0.0	1.59^{a}±0.0	1.74^{ab}±0.1	1.98^{a}±0.1	2^{a}±0.0	101^{b}±3.8	364^{ab}±6.5	603^{a}±18	893^{a}±13	1156^{ab}±35	1336^{ab}±15
G8 (IS+DS+PV)	1.1^{a}±0.0	1.34^{a}±0.0	1.60^{a}±0.0	1.74^{ab}±0.1	1.86^{a}±0.1	1.98^{a}±0.0	133^{a}±38	377^{ab}±20	635^{a}±40	866^{a}±89	1194^{a}±56	1438^{ab}±99
G9 (IS+IV)	1.08^{a}±0.0	1.36^{a}±0.0	1.56^{a}±0.1	1.76^{ab}±0.1	1.86^{a}±0.1	2.02^{a}±0.0	101^{b}±3.8	367^{ab}±18	624^{a}±10	883^{a}±26	1105^{b}±54	1305^{b}±50
G10 (IS+DS+IV)	1.04^{a}±0.0	1.36^{a}±0.0	1.62^{a}±0.0	1.76^{ab}±0.1	1.9^{a}±0.1	1.98^{a}±0.1	100^{b}±0.0	374^{ab}±16	629^{a}±24	853^{a}±40	1155^{ab}±63	1454^{a}±61

G1–10: Groups 1–10, NC: negative control, untreated; PV: post-hatch vaccination; IV: *in ovo* vaccination; IS: *in ovo* synbiotic; DS: dietary synbiotic. Different superscript letters within the same column indicate significant differences ($P<0.05$).

Table 4. B/B ratio of experimental Cobb-500 broilers groups during the different rearing weeks and mortality rate at the end of study. The values are expressed as mean±SD (n=16)

	B/B ratio				Mortality rate number (%)
	Day 1	Day 14	Day 28	Day 42	Day 42
G1 (NC)	0.09^{b}±0.01	0.19^{ab}±0.01	1.25^{abc}±0.13	1.96^{b}±0.04	3 (0.31%)
G2 (PV)	0.09^{b}±0.01	0.19^{ab}±0.01	1.12^{c}±0.14	0.84^{cd}±0.11	4 (0.41%)
G3 (IV)	0.07^{b}±0.01	0.17^{ab}±0.01	1.12^{cd}±0.09	0.86^{c}±0.04	2 (0.2%)
G4 (IV+DS)	0.09^{b}±0.01	0.17^{ab}±0.01	1.16^{bc}±0.03	0.9^{c}±0.07	2 (0.2%)
G5 (IS)	0.09^{b}±0.02	0.18^{ab}±0.02	1.29^{ab}±0.05	2.06^{ab}±0.12	4 (0.41%)
G6 (IS+DS)	0.12^{a}±0.01	0.20^{a}±0.01	1.38^{a}±0.03	2.14^{a}±0.12	2 (0.2%)
G7 (IS+PV)	0.12^{a}±0.01	0.20^{a}±0.03	1.11^{cd}±0.04	0.72^{d}±0.07	4 (0.41%)
G8 (IS+DS+PV)	0.1^{ab}±0.01	0.19^{ab}±0.01	0.99^{d}±0.10	0.73^{d}±0.04	2 (0.2%)
G9 (IS+IV)	0.12^{a}±0.01	0.18^{ab}±0.01	1.19^{bc}±0.06	0.87^{c}±0.06	3 (0.31%)
G10 (IS+DS+IV)	0.1^{ab}±0.01	0.16^{b}±0.01	1.18^{bc}±0.04	0.91^{c}±0.03	2 (0.2%)

B/B = 100 × bursa of Fabricius (g)/body weight (g); G1–10: Groups 1–10, NC: negative control, untreated; PV: post-hatch vaccination; IV: *in ovo* vaccination; IS: *in ovo* synbiotic; DS: dietary synbiotic. Different superscript letters within the same column indicate significant differences (P<0.05).

Table 5. Infectious bursal disease antibody titre experimental Cobb-500 broilers groups during the 42-day rearing period. The values are expressed as mean±SD (n=32)

	IBD antibody titre		
	Day 1	Day 21	Day 42
G1 (NC)	6581^{a}±289	162^{c}±66	22^{e}±20
G2 (PV)	6420^{a}±789	200^{bc}±33	4469^{cd}±458
G3 (IV)	6652^{a}±601	420^{a}±99	4582^{c}±314
G4 (IV+DS)	6530^{a}±410	465^{a}±84	4372^{d}±370
G5 (IS)	6486^{a}±916	104^{c}±13	6^{e}±5
G6 (IS+DS)	6504^{a}±674	194^{bc}±15	12^{e}± 7
G7 (IS+PV)	6397^{bcd}±351	269^{b}±61	4296^{d}±106
G8 (IS+DS+PV)	6534^{a}±863	434^{a}±71	5047^{b}±225
G9 (IS+IV)	6607^{a}±261	451^{a}±58	6018^{a}±192
G10 (IS+DS+IV)	6447^{a}±213	467^{a}±48	5951^{a}±156

G1–10: Groups 1–10, NC: negative control, untreated; PV: post-hatch vaccination; IV: *in ovo* vaccination; IS: *in ovo* synbiotic; DS: dietary synbiotic. Different superscript letters within the same column indicate significant differences (P<0.05).

higher in the IS+DS group than the other groups, except the IS and NC groups (P<0.05). The lowest value of B/B ratio on the 42nd day was detected in IS+DS+PV and IS+PV groups, which had a significant difference with other groups, except for PV group (P<0.05).

Infectious bursal disease antibody titre

On the first rearing day, the IBD antibody titres were not significantly different among groups. On the 21st day of rearing, the IBD antibody titres in the IV, IV+DS, IS+DS+IV, IS+IV and IS+DS+PV groups

Table 6. Detection of probiotic bacteria in faecal samples of Cobb-500 broilers at the beginning and end of rearing period.

	Lactobacillus delbrueckii		*Streptococcus salivarius*	
	Day 2	Day 42	Day 2	Day 42
G1 (NC)	–	4.71×10^{4b}	–	3.66×10^{3b}
G2 (PV)	–	4.98×10^{4b}	–	3.96×10^{3b}
G3 (IV)	–	5.14×10^{4ab}	–	4.02×10^{3b}
G4 (IV+DS)	–	5.28×10^{4ab}	–	4.13×10^{4a}
G5 (IS)	2.34×10^{2c}	–	1.97×10^{3b}	–
G6 (IS+DS)	4.28×10^{3b}	6.24×10^{4ab}	5.62×10^{2c}	4.56×10^{3b}
G7 (IS+PV)	6.36×10^{2c}	–	5.37×10^{2c}	–
G8 (IS+DS+PV)	3.83×10^{2c}	7.33×10^{4a}	4.88×10^{2c}	4.94×10^{3b}
G9 (IS+IV)	4.79×10^{3b}	–	5.29×10^{4a}	–
G10 (IS+DS+IV)	5.09×10^{4a}	7.21×10^{4a}	6.9×10^{4ca}	5.76×10^{4a}

G1–10: Groups 1–10, NC: negative control, untreated; PV: post-hatch vaccination; IV: *in ovo* vaccination; IS: *in ovo* synbiotic; DS: dietary synbiotic. Different superscript letters within the same column indicate significant differences ($P<0.05$).

were significantly higher vs the other groups ($P<0.05$). The IBD antibody titres on the 42nd day and in the IS+DS+IV and IS+IV groups exceeded significantly those of the other groups ($P<0.05$) (Table 5).

Faecal Lactobacillus and Streptococcus

Table 6 presents the count of probiotic microbes in faecal samples of birds at second and last day of rearing period. The highest microbial load was significantly observed in the tenth group which received continuously the synbiotic in rearing period and *in ovo* injection ($P<0.05$).

DISCUSSION

In nature, one day old chicks are in contact with mother and other adult birds, so an early exposure to the microorganisms and gut colonisation occur. However, because of the lack of contact with adult birds in artificial incubation, an opportunity for colonisation of pathogens is provided (Hashemzadeh *et al.,* 2010). In the poultry industry, the early administration of beneficial bacteria as probiotics and synbiotics can diminish pathogen colonisation in the gut through competitive exclusion (de Oliveira *et al.,* 2014). In the present study MOS from the cell wall of yeast *Saccharomyces cerevisiae* inside *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were used as a synbiotic component. There are different reports about application of the components of this synbiotic on immune reactions, hatchability and performance of poultry (Burton *et al.,* 2017; Saeed *et al.,* 2017).

It is shown that balance in gut microbial population helps to improve the general health of the poultry and ultimately growth performance (Saeed *et al.,* 2017). The obtained results in the present study revealed that the IS+DS+PV treatment group in last three weeks of rearing period had better weekly weight gain than the other treated broilers. Similar to this finding, Nikpiran *et al.* (2013) reported positive effects of dietary additive of *Saccharomyces cerevisiae* and its cell wall on BW, FI and FCR in comparison with con-

trol group. It is noted that dietary supplementation of MOS in avian species indicated higher rates of hatchability, fertility, BW gain and FCR (Saeed *et al.,* 2017). Nikpiran *et al.* (2014) reported that administration of MOS in the poultry diet increased growth hormone and insulin secretion and lead to a better FI and growth performance. In agreement with the mentioned studies, the eighth treatment group which contained symbiotic *in ovo* and in diet along with post-hatch vaccination obtained a better FI and FCR in last weeks of rearing period. Regardless of the present findings, Natsir *et al.* (2018) evaluated the effects of a phytobiotic (Black Cincau leaves) and a probiotic (*Lactobacillus* and *Bacillus*) in an encapsulated form included in diet of laying hens, and reported no significant effects on FI and FCR improvement.

The development of immunologic functions of birds occurs before hatching. The goal of *in ovo* vaccination in this study at the late incubation period was to stimulate early immune response which is reported in previous studies (Sharma, 1985; Johnston *et al.,* 1997; Kelemen *et al.,* 2000). Furthermore, one of the aims of using the synbiotic inside the vaccine was the stimulation of immune system. Synbiotics have a role in elevating secretion of pro-inflammatory cytokines such as interleukin-4 and interleukin-6 and increasing effects on immunoglobulin production by plasmacytes (Saeed *et al.,* 2019). It is reported that MOS via activation of mannose-binding protein secretion and ultimately complement system could stimulate the immune system (Chacher *et al.,* 2017). The beneficial effects of MOS in producing antibodies against avian influenza virus was reported which resulted in reduction of viral shedding (Saeed *et al.,* 2017). A similar study reported that application of dietary synbiotic (Biomin Imbo) in broiler chickens led to elevation of antibody titres against IBD (Talebi *et al.,* 2015).

In the current study, *in ovo* vaccination along with synbiotic (*in ovo* or in diet) conferred significant antibody response compared to the other groups and also the birds that received the IBD live vaccine in rearing period. According to the results, it is reported the immune complex vaccines provided an elevated rate in antibody production compared to other IBD vaccines (Negash *et al.,* 2004). This finding can be related to the superiority of *in ovo* vaccination to conventional IBD vaccination in term of immune stimulation. Moreover, it can be noted that MOS could have favourable impacts on humoral immunity (Burton *et al.,* 2017; Chacher *et al.,* 2017).

It is stated that the IBD vaccination from 1 to 14 days of age provided poor antibody response due to the presence of maternally derived antibodies (Ahmed & Akhter, 2003). The antibody titres in the first three weeks of this study was reduced which attributed to decline in maternally derived antibodies (Hassanzadeh *et al.*, 2006). On day 42, the antibody titres of groups which received the vaccine and synbiotic *in ovo* were higher than the other groups. Hassanzadeh *et al.* (2006) reported a progressive increase of antibody titres from three weeks of age in broilers that received immune complex vaccine *in ovo*. Moreover, the obtained results in the present study were in agreement with the findings of Cazaban *et al.* (2018) that reported elevated antibody titres measured by IDEXX kit in broiler chickens that were vaccinated *in ovo*, from 22 days until the end of rearing. Similarly, Iván *et al.* (2005) administered immune complex vaccine subcutaneously in one-day-old broilers and declared that

the subcutaneous application leads to delayed virus replication in the bursa of Fabricius compared to *in ovo* delivery. After *in ovo* vaccination, an expansion in macrophage population in the bursa of Fabricius has been observed and thus it can be dedicated that *in ovo* inoculation activates the innate immunity (Negash *et al.,* 2004).

The antibody titres of present study in birds vaccinated through *in ovo* route were higher than the titres reported by Kundu *et al.* (2017) that vaccinated broiler chickens with two types of live vaccines belonging to intermediate plus strain of IBDV by intraocular route. In the mentioned study, vaccination was performed on day 17, a booster vaccine on day 24 and antibody titres were measured by ELISA method and IDEXX kit. This finding can demonstrate that the administration of vaccine along with synbiotic through *in ovo* delivery did not influence the efficacy of vaccine and conferred high protection. A study conducted by Camilotti *et al.* (2016) investigated the efficacy of recombinant, immune-complex and intermediate vaccines, administered in one-day-old specific pathogen free (SPF) white Leghorn chickens. The results revealed that immune-complex vaccine induced a protection level against IBD challenge similar to other two vaccines (Camilotti *et al.,* 2016).

The virus of IBD replicates in bursa, destroys B cells and ultimately impairs antibody production and humoral immunity. Moreover, lesions and atrophy are observed followed by IBD incidence (Yamazaki *et al.,* 2017). The B/B ratio is used to determine the pathogenicity of IBD virus and also to assess the level of protection provided by IBD vaccine (Mazariegos *et al.,* 1990; Bolis *et al.,* 2003). In 2015, a study was conducted to determine the B/B ratio in male Cobb 500 chickens that were unvaccinated and uninfected in term of IBD. The results indicated the standard B/B ratio from 7 to 42 days of age was 0.11 or above which was consistent to the results of present study (Cazaban *et al.,* 2015). A similar study reported B/B ratio in a range of 0.20 to 0.24 from 7 to 21 days in Ross 308 chickens vaccinated with Transmune (Cazaban *et al.,* 2018). The differences in B/B ratio can be attributed to the differences in genotype of broilers and various factors such as stress, hygiene, route of vaccine administration and pathogenicity of environmental viruses (Alloui *et al.,* 2005; Fellah *et al*., 2014; Cazaban *et al.,* 2015; 2018; Camilotti *et al*., 2016).

CONCLUSION

In conclusion, *in ovo* application of synbiotic and IBD vaccine resulted in an improvement of growth performance values of broiler chickens. *In ovo* or in diet supplementation of synbiotic significantly affected the microbial population balance. *In ovo* vaccination against IBD accompanied by synbiotic treatments is a possible method that exerted positive effects on titres of IBD antibody. The consistency of the results in B/B ratio, titres of antibody against IBD and the rate of mortality in vaccinated birds revealed that *in ovo* supplementation of immune-complex vaccine and synbiotic product have established needful protection against IBD. According to the necessity of replacing antibiotics with safer substances, further studies are needed to compare different injection sites and determine the most effective day of incubation for *in ovo* injection of synbiotic. It is essential to identify the other appropriate compositions and doses of synbiotic for *in ovo* injection inside other vaccines.

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