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Evaluation of Probiotic, Prebiotic, and Synbiotic on Performance, Immune Responses, and Gastrointestinal Health of Broiler Chickens

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Abstract

To investigate the effects of newly introduced probiotic, prebiotic, and synbiotic, 1080 d-old broiler chickens were used in a completely randomized design with six treatments and six replicates of 30 birds each. Dietary treatments were: basal diet (control), basal diet plus 500 g of probiotic/ton (Probiotic-500), basal diet plus 500 g and 300 g of probiotic/ton during days 0-24 and 25-42 of age, respectively (Probiotic-500-300), basal diet plus 300 g of probiotic/ton (Probiotic-300), basal diet plus 1 kg of prebiotic/ton (Prebiotic), basal diet plus 1 kg of synbiotic/ton (Synbiotic). Average daily weight gain and feed conversion ratio were only affected by dietary treatments during the starter period; the lowest average daily weight gain and the highest feed conversion ratio were recorded for the control treatments compared to the others. The antibodies titers (total, IgG, and IgA) against sheep red blood cells, and Newcastle and Influenza viruses were significantly *(P < 0.05)* increased by feeding diets containing all experimental additives compared with the control diet. The pH of the ileum decreased *(P < 0.05)* by using probiotic, prebiotic, and synbiotic in the diets. The highest number of lactobacillus and *E. coli* populations was observed in birds fed synbiotic and control diet, respectively. The height of villus in the jejunum and its ratio to crypt depth was higher in birds fed a symbiotic diet compared to birds fed probiotic or control diets *(P < 0.05)*. Furthermore, the cecum concentration of short-chain fatty acids was greater in chickens fed diets containing probiotic, prebiotic, or synbiotic than the control chickens *(P < 0.05)*. In conclusion, although dietary supplementation with probiotic, prebiotic, and synbiotic had no positive effect on growth performance parameters of broiler chickens, the use of these products could exert promising effects on poultry health.

Introduction

Nowadays, the main objective of poultry production is to achieve better performance and quality of products. Using fast-growing strains is one of the most important strategies to have a high level of economic efficiency in poultry production. Improving feed efficiency along with reducing the feed costs are the final goals of poultry nutritionists. Among the others, using dietary growth promoters in newly hatched chickens is one of the feeding strategies to enhance their beneficial intestinal microflora and immune system (Fuller, 1989). Antibiotics are growth promoters that can control poultry disease if they are used at safe doses (Ghahri *et al.*, 2013), but their extensive use can be dangerous to human health due to the emergence of antibiotic-resistant bacteria (Smith *et al.*, 2003). Currently, researchers have been focused on the production of antibiotic-free products without compromising the efficiency of production in poultry, to address some public health concerns (Luckstadt *et al.*, 2004; Baurhoo *et al.*, 2007). Due to the continuous research on safe growth-promoting products in the poultry industry, there is a greater tendency to use probiotics and prebiotics. Probiotics are live cultures including beneficial microflora, metabolites of microorganisms or living cells, and composition of gut microflora in both animals and humans (Fuller, 2012), which can be added to the diet to improve intestinal microbial balance and

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production performance of poultry (Mazhari *et al*., 2016). Prebiotics are indigestible feed ingredients, which can be utilized by anaerobic and aerobic bacteria (Gibson *et al*., 2004) and consequently stimulate the proliferation of beneficial bacteria in the gastrointestinal tract (Gibson and Roberfroid, 1995). The combination of probiotic and prebiotic which is named synbiotic having synergistic effects and stimulates the growth of useful bacteria in the gut (Ghahri *et al*., 2013) and improves the immune system of broiler chicks (Pandey *et al*., 2015).

In some studies, dietary supplementation with probiotic, prebiotic, and synbiotic supplementation had positive effects on broiler's performance and feed efficiency (Kim *et al*., 2011; Yang *et al*., 2012; Salim *et al*., 2013; Bozkurt *et al*., 2014; Ravangard *et al*., 2017), while in some others they did not affect the performance of broiler chicks (Sarangi *et al*., 2016). Although several reports exist on the administration of probiotics, prebiotics, and synbiotics in farm animals, it seems that newly introduced products should also be evaluated due to 1) variation in poultry responses to these products in different rearing conditions and 2) evaluation of quality control of these products. Therefore, the current study was conducted to evaluate the effects of recently introduced probiotic, prebiotic, and synbiotic to the poultry production market.

Materials and Methods

Birds and dietary treatments

Comprehensive protocols for poultry welfare and experimental procedures adhered to FASS (FASS, 2010) guidelines. In this experiment, 1080 day-old Ross 308 broiler chickens were reared for 42 days. The newly hatched chicks were individually weighed and randomly divided into six experimental treatments with six replicate pens of 30 birds each. The average weight of chickens was 45.89 g. The chickens had free access to feed and water during the study. The experimental diets were basal diet (control), basal diet plus 500 g of probiotic/ton (Probiotic-500), basal diet plus 500 g and 300 g of probiotic/ton during days 0-24 and 25-42 of age, respectively (Probiotic-500-300), basal diet plus 300 g of probiotic/ton (Probiotic-300), basal diet plus 1 kg of prebiotic/ton (Prebiotic), basal diet plus 1 kg of synbiotic/ton (Synbiotic). The diets were provided for starter (1-10 days), growing (11-24 days), and finishing (25-42 days) periods. All diets (Table 1) were formulated to meet or marginally exceed nutrient requirements provided by the Ross manual (Aviagen, 2014).

Table 1. Ingredients and nutrient composition of the basal diet.

	Starter (0-10 days)	Grower (11-24 days)	Finisher (25-42 days)
Ingredients (g/kg)			
Maize	494.2	575.5	610.2
Soybean meal (CP=42%)	379.3	342.7	307.7
Soybean Oil	29.5	37.7	42.9
Maize gluten meal (CP=60%)	50	-	-
Dicalcium phosphate	20.6	18.3	15.5
Limestone	11.4	10.0	8.8
Sodium bicarbonate	1.7	1.7	2.0
Salt	2.3	2.5	2.3
Vitamins premix[1]	3.0	3.0	3.0
Minerals premix[2]	3.0	3.0	3.0
DL-Methionine	2.5	3.2	2.8
L-Lysine HCl	2.2	1.9	1.5
L-Threonine	0.3	0.5	0.3
Nutrient Composition (g/kg)			
Metabolizable energy (kcal/kg)	3000	3100	3200
Crude protein	230	215	195
Digestible Lysine	12.8	11.5	10.2
Digestible Methionine	5.1	4.7	4.3
Digestible Methionine + cysteine	9.5	8.7	8.0
Digestible Threonine	8.6	7.7	6.8
Calcium	9.6	8.7	7.8
Phosphorus, available	4.8	4.4	3.9

[1]The vitamin premix supplied the following per kilogram of diet: 12000 IU vitamin A, 5000 IU vitamin D3, 80 IU vitamin E, 3.2 mg vitamin K, 3.2 mg vitamin B_1, 8.6 mg vitamin B_2, 65 mg Niacin, 20 mg Pantothenic acid, 1700 mg Choline, 0.22 mg Biotin, 2.2 mg Folic acid, 4.3 mg Pyridoxine and 0.017 mg vitamin B_{12}.

[2] The mineral premix supplied the following per kilogram of diet: 20 mg Fe, 110 mg Zn, 120 mg Mn, 1.25 mg I, 16 mg Cu, 0.30 mg Se.

Probiotic, prebiotic, and synbiotic product
The probiotic and prebiotic used in this experiment provided by a commercial company. The probiotic consisted of four bacterial species including *Enterococcus faecium*, *Pediococcus acidilactici*, *Bacillus subtilis*, and *Bacillus licheniformis*. The number of each strain per kg of this product was at least 10^{10} and the total number of probiotic strains per kg was at least 10^{11}. The prebiotic product was made from hydrolyzed yeast and yeast cell wall, which was mainly consisted of mannan oligosaccharides (70%). The synbiotic product was a 50:50 (weight: weight) combination of the probiotic and prebiotic.

Growth performance
The live weight of broilers and feed were recorded on days 0, 10, 24, and 42, and average daily weight gain (ADWG), as well as average daily feed intake (ADFI), were calculated accordingly. Feed conversion ratio (FCR) was calculated as the ratio of ADFI to ADWG during each feeding period of the experiment.

Characteristics of carcass and relative organs weight
At the end of the experiment, two birds per pen were humanely euthanized. The inner organs such as the heart, liver, gizzard, abdominal fat, and lymphoid organs (bursa of Fabricius, thymus, and spleen) were separated and weighed individually. The gastrointestinal parts were then removed to determine the eviscerated carcass weight. The relative organ weight was calculated as a percentage of live body weight.

Antibody production assessment of anti-SRBC
To evaluate the antibody production potential, 12 birds from each treatment were injected with 0.5 mL of 5% suspension of SRBC (sheep red blood cell) on days 28 and 35 of age, and blood samples were collected at 7 and 14 days after the first injection. The serum of each sample was inactivated at 56°C for 30 minutes and then total, mercaptoethanol-sensitive (IgA), and mercaptoethanol-resistant (IgG) anti SRBC antibodies were measured as previously described by Yamamoto and Glick (1982) and Cheema *et al*. (2003).

Antibody response against NDV and Influenza viruses
The birds were vaccinated with live virus vaccines against NDV (Newcastle disease virus; 13 and 21 days of age) and inactivated vaccine against avian influenza (AI) (13 days of age) to evaluate the antibody response against ND and AI. Serum samples of 12 birds from each treatment were collected on days 20 and 28 of age and stored at -20°C for antibody analysis. A hemagglutination inhibition was performed based on methods described by Akhlaghi *et al*. (2013) and Ma *et al.* (2010).

Collection of Samples and Enumeration of Bacteria
For enumeration of *Lactobacillus* and *Escherichia coli* in the intestinal contents, at 42 days of age, 12 birds from each treatment were randomly selected, killed and 1 g of their ileal contents (from the Meckel's diverticulum to the ileocecal junction) were collected and separately mixed with 5 mL of glycerol and rapidly frozen in liquid nitrogen (Ghazanfari *et al*., 2015). One mL of solution was then homogenized in 9 mL phosphate-buffered saline (PBS), and serially (1:10) diluted. The population of *Lactobacillus* bacteria was counted on the *lactobacilli* MRS agar (DeMan, Rogosa, and Sharpe) that were incubated in an anaerobic incubator at 37°C for 48 hours (h) (Baurhoo *et al*., 2007). *E. coli* was cultured on the Eosin-methylene blue agar medium. Plates were incubated at 37°C for 24 h (Dziuk and Duck, 1972). *Lactobacilli* bacteria as white colonies and *E. coli* bacteria as green sheen colonies were seen and identified on the culture surface. Formed colonies were expressed as log10 colony-forming units (CFU) per gram of ileal digesta (Hashemi *et al*., 2012).

pH measurement of ileum content
On day 42, fresh ileal digesta (from Meckel's diverticulum to the ileocecal junction) was collected from two birds per pen. One g of digesta from each bird was mixed with 9 mL of deionized water and its pH was determined using a digital pH meter (2211 pH/ ORP meters HI) as described by Al-Natour and Alshawabkeh (2005).

Morphology of small intestine
At the end of the experiment, a 1-cm segment from the midpoint of jejunum was separated and fixed in 10% formalin (Mahdavi *et al*., 2010). Crypt depth, villi height, and width, villi height: Crypt depth (VH: CD) and epithelial and muscular layers thickness were measured after preparation and stained with hematoxylin-eosin (HE). A total of 180 measurements per treatment were measured with an optical microscope (Olympus CX31, Tokyo, Japan).

The formula for calculating the villus surface area was $2\pi \times (\text{villus width}/2) \times \text{villus height}$. The average values for each cross-section were used for data analysis (Sakamoto *et al*., 2000). The apparent absorptive surface area was calculated based on the formula as described by Iji *et al.* (2001).

Analysis of Cecal Short Chain Fatty Acids (SCFAs)
Frozen cecal digesta (12 birds from each treatment) were thawed at 4°C and suspended in 4 mL of distilled water in a sterile tube. Samples were centrifuged at 4,000 × *g* for 15 min at 4°C. Then one mL of supernatant mixed with 0.2 mL metaphosphoric acid solution and were placed in an ice bath for 30 min. after that samples again were centrifuged (10 min at 11,000 × *g*) at 4°C and the

supernatant was analyzed by gas chromatography (GC) for concentrations of acetate, propionate, and butyrate (Roberfroid, 1998; Zhang *et al*., 2003).

Statistical analysis

Data were evaluated using the GLM procedures of SAS statistical software (SAS Institute, 1999) as a complete randomized design (CRD). Duncan's multiple range tests were used to determine the difference between the treatments and the control group. The significance level was considered at $P < 0.05$.

Results and Discussion

Performance

ADFI was not affected by experimental treatments during starter, grower, and finisher phases (Tables 2 and 3). ADWG and FCR were significantly *($P < 0.05$)* improved in birds fed diets supplemented with prebiotic, probiotic, and synbiotic compared with the control birds during the starter period (days 0-10). While dietary treatments had no significant effect on ADWG and FCR in growing, finishing, and overall periods. It has been reported that growth promoter additives are more effective in birds under stress conditions such as heat stress, crowding, poor management, and diseases (Hooge, 2004). In the current study, the birds were reared under good sanitary conditions (good bio-security, good ventilation, clean litter, and low stocking density) which may justify why the growth promoters used had little effects on ADFI, ADWG, and consequently on FCR of birds during the growing, finishing, and overall periods. Moreover, due to good sanitation conditions throughout the experiment birds were faced with a minimum bacterial challenge. Hence the eating behavior of broilers is more controlled by physical satiety mechanisms (Bokkers and Koene, 2003); therefore, due to the limited capacity of the gastrointestinal tract of broilers, ADFI was similar in all groups. The lack of increase in ADFI while adding specific additives to the diets, may lead to the same ADWG and FCR in all groups.

In agreement with our result, Ravangard *et al.* (2017), Fernandes *et al.* (2014), and Khodambashi Emami *et al.* (2012) also found that feeding broiler chickens with diets supplemented with prebiotics, probiotics, and essential oils of peppermint had no beneficial effect on their production performance. In contrast, several reports are showing that dietary supplementation with probiotic, prebiotic, and synbiotic improved the production performance of broiler chickens (Falaki *et al*., 2011; Yakhkeshi *et al*., 2012; Landy and Kavyani, 2013; Ghahri *et al*., 2013). These contradictory results could be attributed to the number of live organisms in the products, the strain of microorganisms, survivability of microorganisms, prebiotic ingredients, and dietary nutrient levels (Ghasemi and Taherpour, 2013).

The significant improvement of ADWG in treated broiler chicks during the starter period was reflected as a lower FCR in them compared with the control chickens. This can be explained by the unstable microbial population of the gut in the early ages of birds and selective stimulation of growth of beneficial bacteria due to dietary supplementation with prebiotics, probiotics, or a combination of them, which may lead to improvement of bird performance (Murshed and Abudabos, 2015).

Table 2. Effect of dietary supplementation with growth promoters on average daily feed intake (ADFI, g), average daily weight gain (ADWG, g), and feed conversion ratio (FCR) in broiler chickens

Treatments[1]	Starter (0-10 days)			Grower (11-24 days)		
	ADFI	ADWG	FCR	ADFI	ADWG	FCR
control	31.534	22.575[b]	1.437[a]	84.298	55.62	1.516
Probiotic-500[†]	31.856	24.409[a]	1.305[b]	86.330	55.96	1.543
Probiotic-500-300[†]	31.856	24.409[a]	1.305[b]	86.330	55.96	1.543
Probiotic-300	32.500	24.689[a]	1.316[b]	86.705	54.73	1.586
Prebiotic	32.436	23.974[a]	1.315[b]	86.486	56.18	1.540
Synbiotic	31.844	24.376[a]	1.306[b]	86.157	54.46	1.585
SEM	0.427	0.347	0.0058	0.0124	0.0131	0.0249
P-value	0.428	0.001	0.0001	0.404	0.523	0.295

[1]Basal diet (control), basal diet plus 500 g of probiotic/ton (Probiotic-500), basal diet plus 500 g and 300 g of probiotic/ton during days 0-24 and 25-42 of age, respectively (Probiotic-500-300), basal diet plus 300 g of probiotic/ton (Probiotic-300), basal diet plus 1 kg of prebiotic/ton (Prebiotic), basal diet plus 1 kg of synbiotic/ton (Synbiotic).

[†]Due to the identical level of probiotic in the starter period, they were considered to be one treatment with 12 replicates in the starting and growing periods (unbalanced random design).

[a,b] Means with no common superscripts within each column for any effect is significantly different *($P < 0.05$)*.

Table 3. Effect of dietary supplementation with growth promoters on average daily feed intake (ADFI, g), average daily weight gain (ADWG, g), and feed conversion ratio (FCR) in broiler chickens

Treatments[1]	Finisher (25-42)			Total period (0-42 days)		
	ADFI	ADWG	FCR	ADFI	ADWG	FCR
Control	156.184	90.69	1.727	102.634	62.82	1.635
Probiotic-500[†]	162.798	94.25	1.729	105.726	64.46	1.640
Probiotic-500-300[†]	157.192	93.66	1.691	104.069	65.03	1.604
Probiotic-300	158.582	90.17	1.762	104.603	62.76	1.668
Prebiotic	163.525	98.33	1.664	106.419	66.56	1.599
Synbiotic	155.010	90.77	1.710	102.734	62.73	1.637
SEM	0.045	0.051	0.035	0.054	0.049	0.019
P-value	0.118	0.322	0.494	0.248	0.148	0.147

[1]Basal diet (control), basal diet plus 500 g of probiotic/ton (Probiotic-500), basal diet plus 500 g and 300 g of probiotic/ton during days 0-24 and 25-42 of age, respectively (Probiotic-500-300), basal diet plus 300 g of probiotic/ton (Probiotic-300), basal diet plus 1 kg of prebiotic/ton (Prebiotic), basal diet plus 1 kg of synbiotic/ton (Synbiotic).
[†] Due to the identical level of probiotic in the starter period, they were considered to be one treatment with 12 replicates in the starting and growing periods (unbalanced random design).
[a,b] Means with no common superscripts within each column for any effect is significantly different *(P< 0.05)*.

Carcass and organs relative weight

The relative weights of carcass and internal organs such as heart, liver, abdominal fat, and gizzard were not affected by the experimental treatments (data not shown). Similar results were reported by Awad *et al.* (2009), Fallah and Rezaei (2013), and Ghahri *et al.* (2013). In contrast, significant enhancement in the relative weights of carcass and internal organs were reported by Khan *et al.* (1992) and Ozturk and Yildirim (2005) in broilers fed diets containing probiotic and prebiotic.

There was no significant difference among the experimental treatments for spleen weight (Table 4), which was in line with the findings of Teo and Tan (2007) who reported that dietary supplementation with growth promoters did not increase the relative weight of the spleen. In contrast, some studies showed an improvement in the spleen relative weight in broiler chicks fed diets containing probiotic compared with the control group (Willis *et al*., 2007; Alkhalf *et al*., 2010). The lack of significant effect of dietary treatments on spleen relative weight may be attributed to delay in the response of the spleen as a secondary lymphoid organ since its proper functions develop as age progresses in birds (Alkhalf *et al*., 2010).

The thymus and bursa of Fabricius' relative weights were significantly *(P < 0.05)* affected by dietary treatments (Table 4). The greatest relative weight of thymus was observed in birds fed diets supplemented with synbiotic and probiotic (treatment D) which was only differed from that of in control birds *(P < 0.05)*. Also, Alkhalf *et al*. (2010) observed an increased thymus relative weight in chickens fed diets containing probiotic. The heavier thymus in the treated broiler with growth promoters was probably due to the effect of probiotic bacteria on the functional activity of the immune response, which would lead to increased T lymphocytes (Alkhalf *et al*., 2010).

Furthermore, the relative weight of bursa of Fabricius was greater in synbiotic and probiotic supplemented birds than control *(P < 0.05)*. Shoeib *et al*. (1997) and Teo and Tan (2007) found that feeding broiler chickens with probiotic supplemented diet increased bursa of Fabricius weight, which were inconsistent with our findings. The increase in the relative weight of bursa can be related to the enhancement of lymphocyte B production level (Gibson and Roberfroid, 1995). Also, Shoeib *et al.* (1997) reported that the bursa of Fabricius in broilers chicks fed the probiotic products were characterized by an increased number of follicles with high plasma cell reaction in the medulla.

Table 4. Effect of dietary supplementation with growth promoters on carcass and organs relative weight (% of live body weight) in broiler chickens at 42 days of age

Treatments[1]	Carcass	Spleen	Thymus	Bursa of Fabricius
Control	65.29	0.113	0.237[b]	0.054[b]
Probiotic-500	64.56	0.125	0.283[ab]	0.076[a]
Probiotic-500-300	64.44	0.099	0.269[ab]	0.072[a]
Probiotic-300	63.97	0.135	0.333[a]	0.066[ab]
Prebiotic	64.96	0.098	0.272[ab]	0.062[ab]
Synbiotic	64.77	0.113	0.334[a]	0.074[a]
SEM	0.79	0.011	0.025	0.005
P-value	0.333	0.194	0.047	0.038

[1]Basal diet (control), basal diet plus 500 g of probiotic/ton (Probiotic-500), basal diet plus 500 g and 300 g of probiotic/ton during days 0-24 and 25-42 of age, respectively (Probiotic-500-300), basal diet plus 300 g of probiotic/ton (Probiotic-300), basal diet plus 1 kg of prebiotic/ton (Prebiotic), basal diet plus 1 kg of synbiotic/ton (Synbiotic).
[a,b] Means with no common superscripts within each column for any effect is significantly different *(P < 0.05)*.

Immunological Measurements

Antibody titers against SRBC

The highest levels of total antibody titer in the primary (35 d) and secondary (42 d) periods were observed in birds fed diets containing synbiotic (Tables 5 and 6). Also, total antibody concentration was higher in the second period than in the primary period in all treatments. The serum IgG concentration was affected by treatments in the second period, and the highest antibody titer was related to synbiotic treatment, which significantly differed from the other treatments *(P < 0.05)*. The IgG concentration of broiler chicks fed a synbiotic diet was higher in the second period compared with the primary period. The primary response of IgA production against SRBC was significantly affected by the experimental treatments *(P < 0.05)* with the highest antibody titer for birds in probiotic (Probiotic-500-300) and symbiotic treatments. In contrast, the secondary antibody response was not affected by treatments. Similarly, Kaufhold *et al*. (2000); Cetin *et al*. (2005); and Lillehoj *et al*. (2010) reported that antibody titer (total, IgG, and IgA antibody) against SRBC were affected by prebiotic and probiotic, while other researchers found no effect of using probiotic (Yakhkeshi *et al*., 2012; Salehimanesh *et al*., 2016), prebiotic and synbiotic (Midilli *et al*., 2008) on serum antibody concentration in broiler chickens. The higher humoral immune response obtained in the second period as compared with the first period can be due to the development of the immune system in the older broiler. It may lead to the production of more antibodies, which can help to protect the intestinal villus from damage (Ghahri *et al*., 2010; Toloei *et al*., 2010). Also using growth promoters may provide a better condition for digestion and absorption of feed, consequently may provide more amino acids for the synthesis of immunoglobulin (Roberfroid, 1998; Guo *et al*., 2003).

Table 5. Effect of growth promoters supplementation on total antibody titers against SRBC (log2) at primary (35 d) and secondary (42 d) response in broiler chickens

Treatments[1]	Total antibody	
	primary response (35 d)	secondary response (42d)
Control	1.374^{c}	1.437^{b}
Probiotic-500	1.524^{bc}	1.714^{ab}
Probiotic-500-300	1.841^{ab}	1.736^{ab}
Probiotic-300	1.581^{abc}	1.741^{ab}
Prebiotic	1.417^{c}	1.665^{b}
Synbiotic	1.899^{a}	2.004^{a}
SEM	0.123	0.113
P-value	0.019	0.044

[1]Basal diet (control), basal diet plus 500 g of probiotic/ton (Probiotic-500), basal diet plus 500 g and 300 g of probiotic/ton during days 0-24 and 25-42 of age, respectively (Probiotic-500-300), basal diet plus 300 g of probiotic/ton (Probiotic-300), basal diet plus 1 kg of prebiotic/ton (Prebiotic), basal diet plus 1 kg of synbiotic/ton (Synbiotic).

[a,b,c] Means with no common superscripts within each column for any effect is significantly different *(P < 0.05)*.

Table 6. Effect of growth promoters supplementation on IgG and IgA antibody titers against SRBC (log2) at primary (35 d) and secondary (42 d) response in broiler chickens

Treatments[1]	IgG[2]		IgA[3]	
	primary response (35 d)	secondary response (42 d)	primary response (35 d)	secondary response (42 d)
Control	0.431	0.646^{b}	0.333^{c}	0.167
Probiotic-500	0.514	0.531^{b}	0.563^{bc}	0.583
Probiotic-500-300	0.667	0.799^{b}	0.980^{a}	0.417
Probiotic-300	0.730	0.813^{b}	0.612^{bc}	0.382
Prebiotic	0.431	0.681^{b}	0.396^{bc}	0.583
Synbiotic	0.896	1.244^{a}	0.750^{ab}	0.583
SEM	0.20	0.14	0.12	0.14
P-value	0.544	0.011	0.012	0.257

[1]Basal diet (control), basal diet plus 500 g of probiotic/ton (Probiotic-500), basal diet plus 500 g and 300 g of probiotic/ton during days 0-24 and 25-42 of age, respectively (Probiotic-500-300), basal diet plus 300 g of probiotic/ton (Probiotic-300), basal diet plus 1 kg of prebiotic/ton (Prebiotic), basal diet plus 1 kg of synbiotic/ton (Synbiotic).

[2] Immunoglobulin G (mercaptoethanol-resistant anti-SRBC antibodies)

[3] Immunoglobulin A (mercaptoethanol-sensitive anti-SRBC antibodies)

[a,b,c] Means with no common superscripts within each column for any effect is significantly different *(P < 0.05)*.

Newcastle and Avian influenza antibody titer

Antibody production titers against NDV and AI in broiler chicks are indicated in Tables 7 and 8. Treatments had significant positive effects on

antibody titers against NDV at 7 and 14 days after vaccination *(P < 0.05)*. The lowest antibody levels in both periods were related to the birds of control. The antibody titer against NDV in the second period was higher than the first period in all birds.

Antibody titer against AI was not affected by the experimental treatments in the first period (20 d). In the second period (28 d), the antibody titer was affected by the experimental treatments *(P < 0.05)*, and the highest antibody titer was related to the probiotic-treated birds (Probiotic-500-300, Probiotic-300), while the lowest titer was recorded for the control. Our findings are in agreement with those reported by Sadeghi *et al*. (2013) where dietary inclusion of prebiotic enhanced antibody production. Some reports are showing probiotic supplemented diets increased systemic immune response against NDV in broiler chicks (Haghighi *et al*., 2006; Talebi *et al*., 2008). Also, Awad *et al*. (2009) and Naseri Alavi *et al.* (2012) reported that synbiotic supplementation had a positive effect on the immune response to ND. Talebi *et al*. (2015) shown that probiotics and synbiotic supplementation increased immune responses against the AI virus.

Overall, there was a systemic response to NDV and AI vaccination so that the antibody production was increased after the injection of viruses. The significant increase in antibody titer against the ND virus may be due to the immunostimulatory and immunomodulatory effects of probiotics as reported by Hatab *et al*. (2016). Some probiotics may stimulate the immune response and consequently enhance resistance against microbial pathogens (Noverr and Huffnagle, 2004). The same findings confirmed that adding prebiotic to the diets might improve the immune system by stimulation of phagocytes or macrophages and increased cytokines and antibody production (Kabir *et al*., 2004). Optimal nutrition can improve the immune system to produce more antibodies (Talebi *et al*., 2015). However, it has been reported that one of the most important reasons for the positive response of the immune system to antibody production can be attributed to the degree of stress in animals and/or unbalanced microbial populations in the digestive tract (Midilli *et al*., 2008).

Table 7. Effects of growth promoters supplementation on antibody titers against NDV (log_2) at primary (20 d) and secondary (28 d) response in broiler chickens

Treatments[1]	NDV[3]	
	primary response (20 d)	Secondary response (28 d)
Control	1.985[b]	3.057[b]
Probiotic-500[†]	2.334[a]	3.326[a]
Probiotic-500-300[†]	2.334[a]	3.322[a]
Probiotic-300[†]	2.405[a]	3.283[a]
Prebiotic	2.315[a]	3.327[a]
Synbiotic	2.265[a]	3.342[a]
SEM	0.092	0.028
P-value	0.042	0.0001

[1]Basal diet (control), basal diet plus 500 g of probiotic/ton (Probiotic-500), basal diet plus 500 g and 300 g of probiotic/ton during days 0-24 and 25-42 of age, respectively (Probiotic-500-300), basal diet plus 300 g of probiotic/ton (Probiotic-300), basal diet plus 1 kg of prebiotic/ton (Prebiotic), basal diet plus 1 kg of synbiotic/ton (Synbiotic).
[†] Due to the identical level of probiotic in the starter period, they were considered to be one treatment with 12 replicates in the starting and growing periods (unbalanced random design).
[3] Newcastle disease virus
[a,b,c] Means with no common superscripts within each column for any effect is significantly different *(P < 0.05)*.

Table 8. Effects of growth promoters supplementation on antibody titers against Avian influenza (log2) at primary (20 d) and secondary (28 d) response in broiler chickens

Treatments[1]	Avian influenza	
	primary response (20 d)	Secondary response (28 d)
Control	2.733	2.619[c]
Probiotic-500[†]	2.741	2.889[a]
Probiotic-500-300[†]	2.741	2.883[a]
Probiotic-300	2.752	2.729[b]
Prebiotic	2.678	2.715[bc]
Symbiotic	2.696	2.733[b]
SEM	0.024	0.033
P-value	0.225	0.0001

[1]Basal diet (control), basal diet plus 500 g of probiotic/ton (Probiotic-500), basal diet plus 500 g and 300 g of probiotic/ton during days 0-24 and 25-42 of age, respectively (Probiotic-500-300), basal diet plus 300 g of probiotic/ton (Probiotic-300), basal diet plus 1 kg of prebiotic/ton (Prebiotic), basal diet plus 1 kg of synbiotic/ton (Synbiotic).
[†] Due to the identical level of probiotic in the starter period, they were considered to be one treatment with 12 replicates in the starting and growing periods (unbalanced random design).
[a,b,c] Means with no common superscripts within each column for any effect is significantly different *(P < 0.05)*.

Enumeration of bacterial population of the ileum

The number of *Lactobacilli* colonies in chickens fed a diet containing synbiotic and prebiotic were significantly *(P < 0.05)* increased compared with the control treatment (Table 9). Also, the number of *E. coli* colony was affected by experimental treatments *(P < 0.05)* so that the lowest value related to synbiotic treatment while the highest value was for the control. In general, as age progresses, the density and diversity of the microbial population in different parts of the gastrointestinal tract will change in broiler chickens (Barnes *et al*., 1972). However, gut microflora can be affected by some factors, such as breed, health status, maintenance conditions, and diet composition (Van der Wielen *et al*., 2002; Lu *et al*., 2003). Additives such as prebiotics, probiotics, and synbiotic can inhibit the growth of harmful bacteria by decreasing the pH of the gastrointestinal tract to the levels that pathogens are not able to compete effectively (Gibson, 1999). The present results are consistent with the previous ones in which dietary supplementation with prebiotic and synbiotic increased the *lactobacillus* population and reduced the *Coliforms* population especially *E.coli* (Alloui *et al*., 2013; Dibaji *et al*., 2014; Bogusławska-Tryk, 2015; Mazhari *et al*., 2016). In contrast, Salehimanesh *et al*. (2016) found that growth promoters did not affect the ileal microbial population.

Ileum pH measurement

The highest ileal pH was observed in birds of control treatment as compared with other treatments (Table 9). This result is in agreement with findings of Gibson (1999) and Angel *et al*. (2005) who reported that probiotics and prebiotics play an important role in the production of beneficial bacteria and ultimately lead to reducing the pH of the gastrointestinal tract, while other authors found no effect of probiotic as well as prebiotic on broiler gut's pH (Hernandez *et al*., 2006; Jiang *et al*., 2015). The positive effects of increases in the number of lactic acid-producing bacteria are mainly related to their fermentation end-products. These bacteria produce a large number of fatty acids, such as acetic acid and lactic acid, which can reduce intestinal pH (Gibson, 1999). Also, the concentration of lactic and acetic acid may increase during the fermentation of prebiotics by *bifidobacteria* resulted in lower pH of the digestive tract (Apajalahti and Vienola, 2016).

Table 9. Effects of growth promoters supplementation on bacterial populations (log CFU/g) and pH value of ileum in broiler chickens at 42 days of age

Treatments[1]	Lactobacilli	E. coli	pH
Control	3.05^{c}	6.80^{a}	6.57^{a}
Probiotic-500	3.81^{c}	3.38^{ab}	6.02^{b}
Probiotic-500-300	4.70^{bc}	5.80^{ab}	6.06^{b}
Probiotic-300	7.88^{abc}	4.83^{ab}	5.98^{b}
Prebiotic	8.69^{ab}	8.69^{ab}	5.94^{b}
Synbiotic	9.89^{a}	3.05^{b}	5.92^{b}
SEM	1.32	1.96	0.053
P-value	0.025	0.043	0.0001

[1]Basal diet (control), basal diet plus 500 g of probiotic/ton (Probiotic-500), basal diet plus 500 g and 300 g of probiotic/ton during days 0-24 and 25-42 of age, respectively (Probiotic-500-300), basal diet plus 300 g of probiotic/ton (Probiotic-300), basal diet plus 1 kg of prebiotic/ton (Prebiotic), basal diet plus 1 kg of synbiotic/ton (Synbiotic).
[a,b,c] Means with no common superscripts within each column for any effect is significantly different (*P < 0.05*).

Morphology of jejunum

The results obtained from the effects of growth promoters on the development of morphological parameters of jejunum have been reported in Table 10. Villus height and villus height: crypt depth ratio (VH: CD) was significantly increased by dietary supplementation with synbiotic treatment *(P < 0.05)* as reported by Ghasemi and Taherpour (2013) as well as Ghahri *et al*. (2013). Feeding broiler chickens with a diet containing synbiotic numerically decreased villi width and crypt depth, while increased villi surface area and apparent absorptive surface area. Van Leeuwen *et al*. (2004) reported that the higher number of beneficial bacteria in the gastrointestinal tract may lead to a higher supply of nutrients that stimulate the development of the villus. Furthermore, the intestinal villus is the first tissue which is in contact with nutrients (Gartner and Hiatt, 2001); therefore, the taller villi increase the absorptive surface area and improve absorption of available nutrients (Caspary, 1992). Increasing villus height and villus height to crypt depth ratio is associated with an increment in epithelial cell turnover (Fan *et al*., 1997; Xu *et al*., 2003), and these changes can be a suitable indicator of digestive tract health (Pluske *et al*., 1996). In our study, the thickness of the muscular layer in the jejunum was not affected by dietary treatments as reported by Baurhoo *et al*. (2007). In contrast, in some studies morphological features of the small intestine were not affected by dietary inclusion of probiotic, prebiotic, and synbiotic (Houshmand *et al*., 2011; Salehimanesh *et al*., 2016).

Table 10. Effects of growth promoters supplementation on the morphology of jejunal in broiler chickens at 42 days of age

Item	Treatments[1]			*SEM*	*P*-value
	Control	Probiotic-500	Synbiotic		
Villi height (μm)	1195.5[b]	1220.8[b]	1471.8[a]	71.507	0.040
Villi width (μm)	136.00	128.00	111.00	7.361	0.144
Crypt depth (μm)	198.17	191.83	160.40	13.486	0.214
VH: CD	6.69[b]	6.33[b]	9.06[a]	0.543	0.013
Epithelium layer (μm)	43.00	38.33	37.83	2.155	0.207
Muscularis layer (μm)	264.00	232.33	241.00	23.198	0.617
Villi surface area (μm^2)	517921	505923	547109	38758.16	0.698
Apparent villi Absorptive area (μm^2)	164943	161122	174238	12343.36	0.698

[1]control diet (control); control diet plus 500 g of probiotic/ton (Probiotic-500); basal diet plus 1 kg of synbiotic/ton (Synbiotic).
[a,b] Means with no common superscripts within each row for any effect is significantly different ($P < 0.05$).

Cecal concentrations of SCFAs

The effect of growth promoters on cecal concentrations of short-chain fatty acids (*μ*mol/g) in broiler chicks at 42 days of age is shown in Table 11. The cecal concentration of acetic acid was significantly higher in synbiotic treated birds as compared with the control *(P < 0.05)*. Adding probiotics, prebiotic, and synbiotic resulted in a significantly higher cecal concentration of propionic acid compared with the control group *(P < 0.05)*. Also, chickens fed a diet supplemented with probiotic (D) and synbiotic had a higher cecal concentration of butyric acid compared with the control group *(P < 0.05)*.

Belenguer *et al*. (2007) reported that *Faecalibacterium prausnitzii* is one of the dominant bacteria in the cecum of broiler chickens. *F. prausnitzii* and some other bacteria can use produced lactate by *lactobacillus* and produce butyrate and propionate from it. Therefore, there is a positive correlation between populations of *lactobacilli* and cecal concentration of SCFAs. Also, increasing probiotic bacteria may increase the decomposition of indigestible carbohydrates and consequently production of SCFAs (Sakata *et al*., 2003). These results were in accordance with those derived from studies carried out to investigate the influence of growth promoter especially synbiotic on the cecal concentration of SCFAs in broiler chickens (Mookiah *et al*., 2014; Calik and Ergum, 2015). However, Rebole *et al*. (2010) reported that dietary supplementation with inulin as a prebiotic did not affect the cecal concentration of SCFAs in broiler chickens.

Table 11. Effects of growth promoters supplementation on cecal concentrations of SCFAs (*μ*mol/g) in broiler chickens at 42 days of age

Treatments[1]	Short Chain Fatty Acids (SCFAs)		
	Acetic acid	Propionic acid	Butyric acid
control	55.272[b]	9.752[b]	20.480[b]
Probiotic-500	57.500[b]	14.660[a]	25.813[ab]
Probiotic-500-300	59.468[b]	14.227[a]	25.693[ab]
Probiotic-300	69.334[ab]	14.198[a]	33.187[a]
Prebiotic	66.264[ab]	14.169[a]	21.153[b]
Synbiotic	81.624[a]	15.502[a]	32.727[a]
SEM	5.509	0.99	3.23
P-value	0.0241	0.0151	0.0199

[1]Basal diet (control), basal diet plus 500 g of probiotic/ton (Probiotic-500), basal diet plus 500 g and 300 g of probiotic/ton during days 0-24 and 25-42 of age, respectively (Probiotic-500-300), basal diet plus 300 g of probiotic/ton (Probiotic-300), basal diet plus 1 kg of prebiotic/ton (Prebiotic), basal diet plus 1 kg of synbiotic/ton (Synbiotic).
[a,b] Means with no common superscripts within each column for any effect is significantly different ($P < 0.05$).

Conclusion

Results of the current study show that dietary supplementation with probiotic, prebiotic, and synbiotic would be more effective when birds are reared under stressful conditions such as high ambient temperature, diseases, presence of unfavorable microorganisms, high flocking density, and poor management, as dietary supplementation they did not improve production performance of broiler chickens reared under the normal conditions of the present study. However, dietary supplementation of broiler chicken with synbiotic and probiotic improved relative weights of lymphoid organs, immune responses against SRBC, NDV, AI, microbial population, ileum acidity, villus height, VH: CD, and cecal concentrations of SCFAs in them.

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