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Oxidative status, immune response and growth performance of broiler chickens treated with different multi-strain probiotics

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ABSTRACT

This study was conducted to evaluate the effects of different sources of probiotics (based on Lactic acid bacteria or *Bacillus* strains) on growth performance, antioxidant status and immunity. Therefore, two hundred eighty-eight 1-d-old male Ross broiler chicks were allocated to three experimental groups for 35 days. The dietary treatments included: basal diet-unsupplemented (C), supplemented with lactic acid bacteria (LABP), and/or *Bacillus* strains-based probiotics (BP). LABP led to reduced feed intake, while both LABP and BP improved the feed conversion rate as compared to the control group on day 7 ($P < 0.05$). BP increased serum total protein level compared to the control group at the end of the experiment ($P < 0.05$). However, supplementation with probiotics did not affect the relative weight of carcass components, immune organs, malondialdehyde, reactive oxygen species levels and the heterophil/lymphocyte ratio. These findings suggest similar efficacy and potency between probiotics based on *Bacillus* strains-based and lactic acid bacteria-based probiotics in enhancing early-life growth performance and increasing blood total protein. However, there is insufficient evidence to support an improvement in antioxidant status or modulation of the immune system through the addition of LABP or BP.

Keywords: broiler, heterophil, malondialdehyde, probiotic, reactive oxygen

INTRODUCTION

Probiotics are feed additives that help maintain the balance of the host's intestinal microbiota by providing live beneficial microorganisms (El Jeni et al., 2021), which leads to improved performance (Zou et al., 2022). The concept of probiotics was first introduced in 1974 and approved by WHO and FAO (Santacroce et al., 2021). Since then, various probiotics, in terms of the type and number of microorganisms, have been introduced to the poultry industry (Rahmani Alizadeh et al., 2023; Naghibi et al., 2023).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are usually generated during normal metabolic processes. Oxidative stress, a common physiological phenomenon (Obianwuna 2023), is characterized as an imbalance or a transient or chronic increase in the levels of free oxygen/nitrogen radicals. This imbalance can result from either an excessive increase in their production or a decrease in their elimination by antioxidant systems (Dobrică 2022). Oxidative stress leads to elevated ROS levels, which can damage DNA, lipids, and proteins (Zhao et al., 2020). Malondialdehyde (MDA), a toxic production of lipid peroxidation by ROS, serves as a biological indicator of oxidative damage and reflects the extent of cell damage (Zou et al., 2022). Some researchers have reported probiotics can adjust oxidative stress (Deraz et al., 2019).

There are limited reports about the effectiveness of different sources of probiotics on broiler chickens. Therefore, the present study aimed to investigate the effects of lactic acid bacteria or *Bacillus* strains-based probiotics on the performance, serum biochemical parameters, and immune parameters in broiler chickens.

MATERIALS AND METHODS

Bird, diet, and experimental design

A total of 288 one-day-old Ross male broiler chickens were randomly allocated into three treatment groups in 8 replicates (12 chickens/replicate). The birds were fed with a corn-soybean meal-based diet (Control, C), the control diet plus a lactic acid bacteria-based probiotic (ALBP), and *Bacillus* probiotic strains (BP). The Lactic acid bacteria-based probiotic is commercially available as Lacto-Feed (TakgeneZist Co, Tehran, Iran), contains 1.7×10^8 CFU of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Enterococcus faecium* (the minimum amount of each of the bacteria was 2.5×10^7 CFU/g) as certified by the manufacturer. The *Bacillus* probiotic strains are commercially available as Parsilact (Biological Products Company, Roshd Mehrgan Campus, Shiraz, Iran), include *Bacillus* subtilis and *Bacillus* coagulans with 4×10^9 CFU/g respectively 2×10^{11} CFU/g, according to the manufacturer's certification. Probiotics were added to the control diet at 0.2 g/kg from day 1 to day 21 and at 0.1 g/kg from day 22 to day 35, following the manufacturer's instructions.

The lighting schedule, house temperature and basal diet (Table 1) were prepared according to the Ross Broiler Management Guide. All treatment groups received water and feed ad libitum. The broilers were reared on paper litter.

Broiler performance

To determine the body weight gain (BWG), all chicks in each pen were weighed at 0, 7, 21, and 35 days of age. Feed intake (FI) based on each replication was recorded. The feed conversion ratio (FCR) was calculated by dividing the FI by BWG (Salehizadeh et al., 2019).

Table 1. Composition and chemical analysis of the experimental diet

Ingredients (%)	Starter (0-7 d)	Grower (8-21 d)	Finisher (22-35 d)
Corn	55.3	59.9	63.4
Soybean meal	37.52	33.30	30.25
Soybean oil	2.5	2.6	2.79
DCP	1.9	1.75	1.2
Limestone	1.05	0.9	1.02
Methionine	0.30	0.25	0.24
Hcl- Lysine	0.35	0.3	0.14
Threonine	0.18	0.1	-
NaCl	0.22	0.22	0.2
Soduim bicarbonate	0.18	0.18	0.2
Vitamins [1]	0.25	0.25	0.25
Minerals [2]	0.25	0.25	0.25
Calculated analysis			
ME (kcal/kg)	2900	2950	3000
Protein (%)	20.5	19	17.18
Lysine (%)	1.39	1.25	1.05
Methionine+Cysteine (%)	0.92	0.85	0.80
Ca (%)	0.95	0.90	0.75
AvP (%)	0.40	0.38	0.35

[1] The vitamin supplement provided the following amounts per kilogram of feed: vitamin A, 9000 IU; vitamin B1, 1.8 mg; vitamin B2, 0.015 mg; biotin, 0.1 mg.; vitamin D3, 2000 IU; vitamin E, 18 IU; K3, 2 mg; choline chloride, 500 mg.

[2] Mineral supplements provided the following amounts per kilogram of feed: manganese (manganese oxide), 100 mg; iron (iron sulfate $7H_2O$), 50 mg; zinc (zinc oxide), 100 mg; copper (copper sulfate $5H_2O$), 10 mg; iodine (calcium iodate), 1 mg; selenium (sodium selenite), 0.2 mg.

Blood chemical analysis

At the end of the experiment, one bird per replication was randomly selected and weighed. Then, blood samples were taken from the wing vein. Serum samples were prepared according to Rahmani-Alizadeh et al., (2023). The blood biochemical parameters including total protein, and albumin were measured using the RA-XT. Autoanalyzer (Technicon Co., USA) and Pars Azmoon special kits.

Immune system parameters

Oxidative damage biomarkers and heterophil/lymphocyte ratio

The serum ROS was measured by using a Dichlorofluorescin-diacetate indicator and fluorescence spectrophotometer (JasCo, -Fp-6200- Japan) at the wavelength of 485 nm (Excitation) to 520 nm (Emission) and expressed as U/mg protein (Shokrzadeh et al., 2017). The MDA levels were quantified in micromols per milligram of protein after reacting with a Thiobarbituric acid reagent and using a Microplate Reader (Biotek Elx800- USA) at a wavelength of 532 nm (Dong et al., 2020).

To assess white blood cell differentiation count, blood samples were collected in tubes containing EDTA. Blood smears were prepared and stained with Wright-Giemsa solution. One hundred white blood cells were counted under a 100x magnification optical microscope (Olympus-Japan) based on the morphological criteria, the percentage of the heterophil (H) and lymphocyte (L) was measured and H/L ration was calculated (Amoozmehr et al., 2023).

Antibody titer

The broilers were vaccinated against Infectious Bronchitis Virus (IBV) and Bursal Disease (IBD). On the 30th day of the experiment, blood samples were taken from one chicken per replication for antibody titer analysis. An ELISA reader instrument (MINDRAY– China) and special kits (IDvet Elisa kit- France) were used to determine sera antibody titer against IBV and IBD.

Immune organs

To determine the relative weight of the immune organs at the end of the experiment, a randomly chosen bird from each replication was euthanized via cervical dislocation following weighing. The weight of the spleen and bursa of fabricius were weighed to the nearest 0.01 g using a digital scale (Centaurus scale-china). All data were calculated as a percentage of live weight.

Statistical analysis

The data were analyzed as a completely randomized design. Before analysis, normal distribution was examined using the Univariate procedure of SAS statistical software (SAS Institute Inc. 2003). Means were compared using Duncan's multiple range test, with significance set at $P < 0.05$.

RESULTS

Broiler performance

As seen in Table 2, at the starter phase, there was a difference in FI between the LABP and control group ($P < 0.05$). Additionally, FCR in the LABP and BP groups were better than in the control group ($P < 0.05$). However, no significant differences were noted in FCR, FI and BWG among the birds throughout the grower and finisher phases or during the overall periods ($P < 0.05$).

Immune response

As indicated in Table 3, there were no significant differences ($P > 0.05$) between experimental treatments for ROS, MDA as well as L or H count and H/L ratio.

Antibody titer, blood biochemical parameters and immune organ weight

According to the presented results (Table 4), the antibody titer against IBV and IBD was not affected ($P > 0.05$) by either LABP or BP (Table 4). Total protein value significantly increased ($P < 0.05$) in BP compared to total protein in the control group. There were no significant differences ($P > 0.05$) noted in albumin levels among the experimental treatments. Similarly, no significant differences ($P > 0.05$) were observed in the relative weights of immune system organs between the probiotic-treated groups and the control group.

Table 2. Feed intake, body weight gain, and feed conversion ratio of experimental groups at different ages

Variable	Age (day)	Experimental treatments*			*P* - value
		Control	LABP	BP	
Feed intake (g)	Starter	87.96 ± 6.50 [a]	78.24 ± 5.55 [b]	82.28 ± 4.96 [ab]	0.0095
	Grower	1012.70 ± 57.77	1032.28 ± 47.67	1019.73 ± 25.34	0.6900
	Finisher	1781.27 ± 96.11	1776.95 ± 131.95	1767.15 ± 88.15	0.9642
	1-35 (Total)	2881.93 ± 100.70	2887.48 ± 159.16	2869.16 ± 99.45	0.9545
Body weight gain (g)	Starter	91.48 ± 6.71	90.21 ± 7.61	97.56 ± 8.37	0.1425
	Grower	623.54 ± 31.96	617.14 ± 32.48	630.04 ± 15.85	0.6567
	Finisher	1079.55 ± 58.84	1029.93 ± 56.71	1077.97 ± 67.72	0.2079
	1-35 (Total)	1794.56 ± 50.69	1737.28 ± 67.46	1805.57 ± 77.67	0.1103
FCR (g:g)	Starter	0.97 ± 0.09 [a]	0.87 ± 0.08 [b]	0.85 ± 0.07 [b]	0.0155
	Grower	1.63 ± 0.11	1.67 ± 0.06	1.62 ± 0.06	0.3739
	Finisher	1.66 ± 0.15	1.73 ± 0.16	1.65 ± 0.18	0.5520
	1-35 (Total)	1.61 ± 0.09	1.66 ± 0.09	1.60 ± 0.12	0.3591

Means with the same superscripts in each row have no significant difference ($P < 0.05$)

*Control broilers that were fed a basal diet, LABP - basal diet supplemented with a lactic acid bacteria-based probiotic, BP - basal diet supplemented with *Bacillus* probiotic strains

Table 3. Oxidative damage biomarkers and heterophil/lymphocyte ratio

Variable	Experimental treatments*			*P* - value
	Control	LABP	BP	
ROS (U/mg protein)	19.50 ± 6.22	20.96 ± 6.22	25.51 ± 8.22	0.3422
MDA (µM/gr protein)	4.29 ± 1.56	4.51 ± 1.63	6.17 ± 2.37	0.1332
Heterophile (%)	21.58 ± 1.58	21.88 ± 2.23	19.75 ± 2.12	0.1162
(%) Lymphocyte	38.75 ± 1.57	38.13 ± 2.23	40.25 ± 2.12	0.1162
Heterophile/lymphocyte ratio	0.55 ± 0.06	0.58 ± 0.09	0.50 ± 0.08	0.1198
Heterophile (%)	21.58 ± 1.58	21.88 ± 2.23	19.75 ± 2.12	0.1162

Means with the same superscripts in each row have no significant difference ($P < 0.05$)

* Control - broilers that were fed a basal diet, LABP - basal diet supplemented with a lactic acid bacteria-based probiotic, BP - basal diet supplemented with *Bacillus* probiotic strains.

Table 4. Antibody titer, blood biochemical parameter and immune organ weight

Variable	Experimental treatments*			*P* - value
	Control	LABP	BP	
Albumin (g/dL)	1.00 ± 0.33	1.24 ± 0.26	1.05 ± 0.26	0.2590
Total protein(g/dL)	2.88 ± 0.99 ^{b}	3.81 ± 1.25 ^{ab}	4.47 ± 0.46 ^{a}	0.0203
Antibody titer against IBV (log10)	3.36 ± 0.18	3.52 ± 0.10	3.37 ± 0.16	0.1215
Antibody titer against IBD (log10)	3.72 ± 0.14	3.64 ± 0.10	3.69 ± 0.17	0.4929
Spleen (% of live weight)	0.18 ± 0.06	0.17 ± 0.05	0.17 ± 0.05	0.9703
Bursa of Fabricius (% of live weight)	0.14 ± 0.07	0.13 ± 0.09	0.17 ± 0.07	0.4662

Means with the same superscripts in each row have no significant difference ($P < 0.05$)

* Control - broilers that were fed a basal diet, LABP - basal diet supplemented with a lactic acid bacteria-based probiotic, BP - basal diet supplemented with *Bacillus* probiotic strains.

DISCUSSION

Performance traits

Some researchers have noted that the addition of probiotics to the diet can improve both growth performance and health by regulating enteric microbiota balance (Rahmani-Alizadeh et al., 2023). In the present study, supplementation of the diet with probiotics containing lactic acid bacteria or *Bacillus* strains led to a reduction in FI and improvement in FCR during the initial 1 to 7 days. The result of this study is in agreement with Hassan et al. (2022) and shows that probiotics have an important effect on growth performance in the early stages of broiler life. One-day-old chickens are affected by different environmental stress factors such as different hatching times, delays in access to water and feed after hatching, transferring of chickens from hatchery to farms, entry of chickens into new conditions, and high stocking density in broiler houses. These factors often result in decreased broiler performance (Alizadeh et al., 2022). The results of this experiment suggest that both lactic acid bacteria and *Bacillus* strain-based probiotics have similar positive effects on the improvement of the growth performance of broilers during the initial days after hatch.

Immune response

Appropriate ROS levels play a crucial role in regulating intercellular communication as well as the immune response against pathogens (Kim 2022). Authors indicated that some probiotics can activate the antioxidative enzymes such as Superoxide Dismutase, Catalase, and Glutathione Peroxidase involved in scavenging ROS and decreasing MDA (Bai et al,.2018; Dibamehr et al, 2023). However, in this experiment, the ROS level remained unchanged following supplementation with Lactic acid bacteria or *Bacillus* strains probiotics. This result is in agreement with Seifert et al. (2011) that had reported probiotic intervention under normal farming condition had no effect on ROS production. According to the results of this study neither the lactic acid bacteria-based probiotic nor *Bacillus* probiotic strain, altered MDA level, in line with ROS level. This result agrees with Aalaei et al. (2019) who indicated that dietary probiotics had no significant effect on MDA level significantly. Despite ROS being typically generated during normal metabolic processes (Obianwuna, 2023), these results emphasize the inability of probiotics under investigation to strengthen the antioxidant status under the normal situations of this experiment (Bai et al., 2018; Rahmani-Alizadeh et al., 2023)

Birds with a lower H/L ratio exhibit higher survival rates against infection (Minias, 2019). Previous research indicates that probiotics can modulate the host's immune system by activating lymphocytes and reducing the H/L ratio (Mazhari et al., 2016; Aalaei et al., 2019). However, in this study, the H/L was not affected by supplementation by lactic acid bacteria or *bacillus*-based probiotics. These results align with Aalaei et al. (2019) who found no change in the H/L ratio when single or multi-strain probiotics were added to the diet.

Contradictions between the results of various studies regarding the effect of probiotics on immune response indicators could be related to the genetic variations among birds, as these differences influence the bird's physiological and immunological response (Hangalapura et al., 2005).

Blood biochemical parameters, antibody titer and immune organ weight

Total protein comprises plasma albumin and globulins (Khabirov et al., 2021). Similar to the studies conducted by Wu et al. (2019), this study revealed that probiotics had no impact on serum albumin levels. However, a supplemented diet with *Bacillus* probiotic strains increased serum total protein levels. Beneficial bacteria employ competitive exclusion to prevent protein degradation and nitrogen utilization by pathogens, thereby enhancing intestinal protein absorption efficiency (Khaliq et al., 2016; Yazhini et al., 2018).

Several studies have highlighted probiotics' role in immune system regulation through cytokines secreted by stimulated immune cells (Rehman et al., 2020) which contribute to elevated antibody titers in vaccinated birds (Salehizadehet al., 2019). In this study, the addition of probiotics did not impact the antibody titers against IBV and IBD. These results are in harmony with Rehman et al. (2020) who indicated that multi-strain probiotics did not affect antibodies against Newcastle disease.

The weight of lymphoid organs reflects their functionality, with higher spleen and bursa of Fabricius weights indicating a potentially enhanced immune system (Dibamehr et al., 2023). Ahfeethah et al. (2023) didn't observe any significant change in the bursa relative weight due to the usage of probiotics. Similarly, this study has found that lactic acid bacteria or *Bacillus* strain-based probiotics do not affect the weight of the spleen and bursa. Generally, the effectiveness of probiotic consumption on the antioxidant capacity and immune system response in poultry can be influenced by various factors including the dosage of the probiotic administered, the strains of beneficial microbial delivered by the probiotic, the genetic structure and the age of the bird (Aliakbarpour et al., 2013; Behnamifar et al., 2019; El Jeni et al., 2021; Biswas et al., 2022; Deng et al., 2022).

CONCLUSION

In conclusion, the findings suggested that the probiotics based on lactic acid bacteria or *Bacillus* strains can improve the performance of broiler chicks during their early days and increase the blood's total protein level. However, the results of this study do not support an improvement in the humoral immune system, oxidative status indicators, and heterophil/lymphocyte ratio.

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