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Effects of two plant extracts and native *Lactobacillus* culture on immune response, lymphoid organs and antioxidant properties of broiler chickens

Amin DIBAMEHR[1], Mohsen DANESHYAR[1] (✉), Amir TUKMECHI[2], Seyyed Meysam ABTAHI FROUSHANI[2]

[1] Department of Animal Science, Faculty of Agricultural Science, University of Urmia, Postal Code 5715944931, Urmia, Iran

[2] Faculty of Veterinary Medicine, Urmia University, Postal Code 5715944931, Urmia, Iran

✉ Corresponding author: m.daneshyar@urmia.ac.ir

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ABSTRACT

Probiotics and phytogenics have been evaluated as potential alternatives to antibiotic growth promoters (AGP) in poultry feeds in terms of their ability to improve growth performance in commercial poultry production through improving growth performance, feed conversion ratio and immune response efficiency. This study investigated the benefits of *Lactobacillus* culture (LC), green tea extracts (GTE) and *Berberis vulgaris* extracts (BVRE) have been investigated on the immune response, lymphoid organs, and antioxidant properties of broiler chickens. A total of 320 one-day-old Ross 308 chicks were randomly allotted to 8 treatment groups, each including 4 replicates of 10 chicks. A 2×2×2 factorial arrangement of 8 dietary treatments was used to appraise the effects of the mixture of five LC (none vs. 1-5 × 10^8 cfu/g), GTE (none vs. 2500 ppm) and BVRE (none vs. 2500 ppm). The relative weight of lymphoid organs (spleen, thymus and bursa of Fabricius), antioxidant parameters of malondialdehyde (MDA) content and total antioxidant capacity (T-AOC) and immune response indices (white blood cells, antibody response to sheep red blood cell, respiratory burst and splenocytes proliferation) were assessed. According to the results of the current experiment, the relative weights of the spleen and bursa were significantly higher than the control group in broilers fed the LC diet ($P < 0.01$). The combination of LC and GTE significantly decreased MDA as compared to broilers fed the control diet ($P < 0.05$). Moreover, the GTE diet markedly increased the T-AOC compared to the control ($P < 0.01$). The LC and plant extract treatments significantly improved the humoral and cellular immune systems ($P < 0.01$). Based on obtained results, plant extracts in combination with *Lactobacillus* strains can improve the immune responses of broiler chickens.

Keywords: antioxidant activity, broiler, immune parameters, *Lactobacillus* strains, plant extracts

INTRODUCTION

Antibiotic growth promoters (AGP) are the feed additives to improve growth, feed conversion ratio and prevent disease in the poultry nutrition industry (Thomke and Elwinger, 1998). AGPs may alter the diversity and structure of the microbial population of the intestine and lead to the creation of optimal microbiota to increase energy usage and better growth performance in livestock. Numerous kinds of research have shown that the consumption of AGPs in livestock production can arise microbial resistance resulting in a potential threat to human health (O'Brien, 2002). Concerns regarding adverse side effects of AGPs have prompted researchers to consider alternative to antibiotics (Diarra and Malouin, 2014). Probiotics and phytogenics have been evaluated as potential alternatives to AGPs in poultry feeds in terms of their ability to improve growth performance in commercial poultry production. Probiotic properties are different from antibiotics in birds.

However, both can improve growth performance and feed conversion ratio. Therefore, probiotics can be considered as potential alternatives to growth-promoting antibiotics, but other attributes of probiotics should be evaluated. One of the mechanisms of probiotics is to change the microbial population which increases the production of short-chain fatty acids (SCFA), reduces the pH of the intestinal environment, and modulates the immune system (Rhayat et al., 2017; Pender et al., 2017). A few studies have shown that *Lactobacillus* strains as probiotics decreased the performance of the broilers through changing energy metabolism by bile salt hydrolyze enzyme (Begley et al., 2006; Sharifi et al., 2012; Dibamehr et al., 2021). But the advantages of probiotics include modifying the intestinal environment and strengthening the function of the intestinal barrier by useful microorganisms, competitive elimination of pathogens and stimulating the immune system and improving performance. Modification of the intestinal environment is considered an important probiotic impact and is evaluated as the basis of other probiotic benefits (Jha et al., 2020).

There are some reports regarding the improved response of broiler chickens by dietary inclusion of probiotics. In an experiment, Bai et al. (2017) evaluated the effect of *Bacillus subtilis* on intestinal immune characteristics and observed the positive effects of *Bacillus subtilis* on the intestinal T cell immune system. The combined effects of probiotics (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus fascium* and *bacillus subtilis*) on poultry's immune system were studied by Yitbarek et al. (2015) that observed modified immune system by fed the above-mentioned probiotics along with methylene methyl bacitracin and yeast carbohydrates to 300-day-old Lohmann chicken. In another study, dietary inclusion of different strains of *Lactobacillus* were used as probiotics in broilers infected with reduced the number of macrophages. The reduction in the number of macrophages in infected birds can be attributed to the reduction in bacterial load resulting from competitive elimination through the addition of probiotics (Higgins et al., 2007). Neveling et al. (2020) showed that feeding different probiotic strains in broilers improved weight gain, intestinal morphology and immune response. Another group of compounds that are considered alternatives to AGPs are phytogenic feed additives, also known as phytobiotics, which are natural active compounds derived from plants and used in animal and poultry feeds to increase production (Windisch et al., 2008). The beneficial effects of phytogenics are mostly related to their antibacterial and antioxidant properties. The consumption of phytogenics in the diets alters and stabilizes intestinal microbiota and reduces toxic metabolites in the gastrointestinal tract and also is directly related to the antibacterial properties on pathogenic bacteria, which reduces intestinal problems, and immune stress caused to improves performance (Zhang et al., 2013; Zhao et al., 2013). Another beneficial impact of using phytogenics is to reduce oxidative stress and increase antioxidant activity in various tissues, which improves health (Cao et al., 2012; Mueller et al., 2012). Phytogenics also can play an important role as immunomodulators such as increasing immune cell proliferation, cytokine expression and antibody titers (Kim et al., 2010; Pourhossein et al., 2015).

Catechins are a group of polyphenols present in green tea leaves composed of 4 compounds: epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate (Du et al., 2012). These compounds promote health by preventing oxidation. Whereas tea catechins have been reported to reduce peroxide formation in chicken fat even better than alpha-tocopherol and butyl hydroxy anisole (Chen et al., 2019). Studies on the properties and composition of *Berberis vulgaris* showed that the main activity of this plant is due to the presence of isoquinolic nuclei such as berberine, oxyacanthine, berbamine and palmitin (Iauk et al., 2007). Alkaloids are found in the skin, roots, stems and fruits of this plant, which is more in the skin and roots of *Berberis vulgaris* than other parts (Ivanovska and Philipov, 1996). *Berberis vulgaris* is known as a medicinal plant and in traditional medicine has favorable effects such as lowering blood pressure and antioxidant properties (Alimirzaee et al., 2009). Researchers have shown that supplementation of

the quail diet with *Berberis vulgaris* root (100 or 200 mg *Berberis vulgaris* root extract per kg for 12 weeks) reduces the adverse effects of heat stress by strengthening the immune system (Sahin et al., 2013).

Also, despite the issue of antimicrobial resistance in livestock, the livestock industry still relies heavily on AGPs due to the lack of practical and compatible approaches to finding suitable alternatives. Therefore, the aim of this article was to investigate the effect of *Berberis vulgaris* root and green tea extracts along with *Lactobacillus* strains isolated from chicken gastrointestinal tract on immune parameters, lymphoid organs and antioxidant properties of broilers.

MATERIAL AND METHODS

Preparation of Lactobacillus cultures (LC)

Five LC including *L. animalis*, *L. acidophillus*, *L. gallinarum*, *L. lactis*, and *L. returi* obtained from the microbiology laboratory (Urmia University, Iran) and were previously isolated from the gastrointestinal tract of the native chickens. The LC were separately cultured in de Man, Rogosa and Sharpe (MRS) broth (Scharlau, Spain) and stored in 10% glycerol at -20°C before use (Dibamehr et al., 2021).

The LC were grown in MRS broth (Scharlau, Spain) for 18 to 24 h at 37°C and shaking at 120 rpm. Then, strains were harvested using a refrigerated centrifuge (Hettich, Germany) at 4°C and 5000 × g for 10 min in order to use in the broiler chicken diet as dry powder of the strains culture. Supernatants were discarded and cell pellets were washed twice with sterile saline solution [NaCl; Sigma-Aldrich; 8.5 g/l (w/v)] and adjusted to 1 × 10^8 cfu/mL based on optical density (OD 600) next adding cryoprotective medium containing skim milk 10% (wt/vol) (Sigma-Aldrich) and then the cell pellet each LC were frozen at −40°C and freeze-dried (Vac05 ZirBus, Germany) separately, and then blend together in the equal ratio of 1:1:1:1:1 (w:w at 1×10^8 cfu/g). Before use, the freeze-dried culture was considered for total viable cell counts. The viable cell count was used by the standard dilution method on MRS agar after incubation at 37°C for 48 h. The combination of LC was stored at -20°C and used in diet as a dietary supplement for broiler chickens (Vandeplas et al., 2009; Shokryazdan et al., 2017).

Preparation of extracts

The *Berberis vulgaris* root and green tea were purchased, air-dried at room temperature, and ground to a mesh size of 1 mm. Then, each sample of the fine powder was dissolved in 70% ethanol (1:10) for 96 h, followed by filtering and concentrating to a small volume in order to remove the entire ethanol using a rotary evaporator. The plant extracts were kept at 4°C.

Birds and experimental diets

This research was conducted in the facilities of the Animal Science Department (Urmia University, Iran). In this study, a total of 320 one-day-old Ross 308 broiler chicks were acquired from a local hatchery and randomly assigned to 32-floor pens (100 × 100 cm) covered with pine shavings. The temperature was set at 32°C within 1 to 3 days of age and then was gradually reduced to 3°C per week until reached 22°C. This temperature was kept at 22°C until the end of the experiment. Moreover, the lighting program was performed with 23 L/1D under a white light (20 lux). Relative humidity was maintained between 50-60% during the experiment. A 2×2×2 factorial arrangement of 8 dietary treatments was used to appraise the effects of a mixture of five LC (none vs. 1-5 × 10^8 cfu/g), GTE (none vs. 2500 ppm) and BVRE (none vs. 2500 ppm). Therefore, chicks were randomly placed in 1 of 8 dietary treatments (4 pen replicates; 10 chicks per pen) and diets were formulated based on maize and soybean meal in the mash form to meet the nutrient requirements of broiler chickens (based on Ross 308 nutrient specifications) for starter (1 to 10 d), grower (11 to 25 d) and finisher (27 to 42 d) periods (Table 1). All experimental procedures were approved by the University of Urmia Animal Ethic Committee (692/RD, December 12, 2018).

Table 1. Diet formulation (%)

Ingredients	Starter stage (0 -10 days)	Grower stage (11-25 days)	Finisher stage (26-42 days)
Corn	54.30	58.58	62.76
Soybean meal	38.93	34.40	29.66
Vegetable oils	2.11	2.86	3.70
Dicalcium phosphate	2.15	1.84	1.71
Carbonate Calcium	1.00	0.89	0.83
Mineral and Vitamin premix*	0.50	0.50	0.50
NaCl	0.37	0.38	0.38
DL-Met	0.33	0.29	0.26
L-lys HCL	0.19	0.17	0.14
Threonine	0.12	0.09	0.06
Calculated composition			
ME, Mcal/kg	2.91	3.00	3.10
Protein, %	22.31	20.85	18.91
Arginine, %	1.37	1.32	1.18
Isoleucine, %	0.85	0.85	0.76
Leucine, %	1.65	1.67	1.54
Valine, %	0.93	0.93	0.85
Methionine, %	0.65	0.59	0.53
Lysine, %	1.34	1.21	1.07
Met+Cys, %	0.99	0.91	0.83
Calcium, %	0.931	0.817	0.765
Available Phosphorus, %	0.465	0.408	0.378
DCAB (mEq/kg)	230.55	211.69	191.52

Supplied by Faraz Daneh Avand Co., Tehran, Iran, and provided per kilogram of premix: vitamin A, 8800000.IU; vitamin D3, 2500000 IU; vitamin E, 22000 IU; vitamin K, 2500 mg; vitamin B12, 10 mg; thiamine, 1500 mg; riboflavin, 4000 mg; calcium pantothenic acid, 8000 mg; niacin, 35000 mg; pyridoxine, 2500 mg; folic acid, 600 mg; choline, 200 mg; manganese, 75000 mg; zinc, 65000 mg; iron, 75000 mg; copper, 6000 mg; iodine, 900 mg; and selenium, 200 mg.

Lymphoid organs

At the end of the experiment, 2 birds (including one from each sex) per replicate (8 birds/treatment) were randomly selected, individually weighed and killed by cervical dislocation. The abdominal cavity was opened and the total gastrointestinal tract was immediately exposed. The weight of the spleen, thymus and bursa of fabricius were expressed as percentages of respective live body weight.

Serum biochemistry

At day 41 of age, blood samples (1.5 ml) were taken from the brachial vein of 3 birds per replicate (12 birds/ treatment) to analyze the total Antioxidant Capacity (T-AOC) and Malondialdehyde (MDA) content using commercial diagnostic kits (Pars Azmoon, Tehran, Iran).

Immune System Parameters

Hematological analysis

Hematological analysis was carried out using the blood collected from the experimental chickens at the end of the experiment. The blood samples were collected from the wing vein of 3 selected birds per replicate. The blood samples were collected from each chicken and transferred immediately into test tubes with EDTA (Ethylenediamine tetraacetic acid) as anticoagulant for the direct measurements of cells (WBC) and blood smears were also performed to determine the H: L (heterophils to lymphocytes) ratio. For each collected blood sample, 3 smears were prepared and evaluated by two people in a double-blind manner.

Antibody response to Sheep Red Blood Cell (SRBC)

To measure the humoral immune response against SRBC, whole sheep blood collected in the heparinized tube was washed three times in sterile saline solution [NaCl; Sigma-Aldrich; 8.5 g/L (w/v)]. The blood plasma was then separated by centrifugation (2500 to 3000 rpm for 15 minutes) and the erythrocyte section was repeated with sterile saline solution and centrifuged. After centrifugation, the isolated erythrocytes were diluted with a sterile saline solution. This was repeated three times until, after centrifugation, the upper part containing the blood plasma in the tube was completely clear. After this step, the erythrocytes were isolated again and diluted and ready to be injected with 5% erythrocytes by physiological serum. The chicks were immunized with 1 ml of 5% diluted red blood cell solution in breast muscle on day 29. A booster dose of SRBC antigen was given at day 35. Blood samples were collected from the injected chickens at days 35 and 41 of age. The antibody titer produced against SRBC was measured by the hemagglutination (HA) method (Haghighi et al., 2005).

Respiratory burst potential of peripheral blood phagocytic cells

After taking a blood sample from broiler chickens at 41 days of age, each sample was collected in a heparinized tube. Intracellular generation of reactive oxygen species (ROS) was measured by NBT reduction as previously described (Nabi et al., 2005; Froushani and Galeh, 2014). In brief, the cells were incubated for 30 min at 37°C and then, an aliquot of NBT solution was added to the cells and incubated for 1 hour at 37°C. The unused NBT was removed through washing and the reduced dye was extracted in dioxin and quantitated at 520 nm.

Peripheral blood lymphocytes proliferation

The proliferation potential of peripheral blood lymphocytes population was evaluated by MTT assay. In brief, the heparinized blood sample was diluted with the same amount of Hanks balanced salt and the suspensions were overlaid onto Histopaque®1077 density gradient medium. The cells were centrifuged at 1800 rpm for 20 min. Lymphocytes were collected at the interface and rinsed three times in Hanks balanced salt. The lymphocytes were plated in 96-well flat-bottomed plates in RPMI 1640 medium supplemented with 10% fetal calf serum (1×10^5 cells/100 µl/well) and stimulated with 50 µl PHA solution (1 mg/ml) or medium alone. After 72-hour incubation, cultures were pulsed with 20 µl of the MTT solution (5 mg/ml) for 4 hours at 37°C. Then 150 ml DMSO was added and shaken vigorously to dissolve the formazan crystal. The optical density (OD) at 550 nm was measured using a microplate reader (Dynatech, Denkendorf, Germany). The experiments were done in triplicate sets. The results were expressed as the proliferation index according to the ratio of OD550 of stimulated cells with MOG35-55 to OD550 of non-stimulated cells (Miyamoto et al., 2002).

Statistical analysis

All the data were analyzed in a 2×2×2 factorial arrangement using the one-way ANOVA procedure of the SAS program to determine the main effects of dietary additives and the interaction effects among them by using the GLM procedure of SAS (version 8.0; SAS Institute, Cary, USA), followed by comparison among means us Duncan's multiple-range test and differences were considered at $P < 0.05$.

RESULTS AND DISCUSSION

The lymphoid organs' weights (spleen, bursa and thymus) and antioxidant parameters (MDA and T-AOC) are shown in Table 2. The relative weight of the spleen and bursa was significantly (P < 0.01) higher in LC-fed birds as compared to the control group. There was no interaction between the treatments for the relative weight of the thymus. Karimi Torshizi et al. (2010) reported that the relative weight of the spleen and bursa in broilers increased in response to probiotic supplements (0.5 and 1 g/kg proteins containing 9 bacterial species) consumed through water and feed. The high weight of the lymphoid organs (bursa of Fabricius and spleen) can be due to an improved immune system (Gore and Qureshi, 1997). Seidavi et al. (2017) represented that supplementation of broiler diets with 0.5 and 1% green tea powder increased the relative weight of the bursa and spleen compared with other experimental treatments.

Table 2. Effect of *Lactobacillus* culture (cfu/g), green tea extract (ppm) and *Berberis vulgaris* root extracts (ppm) on relative weights of lymphoid organs (% of live body weight) and antioxidant properties (µmol/mg)

Variable	Spleen	Thymus	Bourse	MDA	T-AOC
GTE × BVRE × LC					
None + none + none	0.09^{d}	0.22	0.10^{c}	2.77^{a}	1.22^{c}
None + BVRE + none	0.10^{cd}	0.26	0.13^{bc}	2.12^{b}	1.64^{ab}
GTE + none + none	0.11^{bc}	0.25	0.12^{bc}	1.75^{bc}	1.83^{a}
None + none + LC	0.14^{a}	0.26	0.19^{a}	1.85^{bc}	1.61^{ab}
GTE + BVRE + none	0.10^{cd}	0.26	0.13^{bc}	2.00^{bc}	1.65^{ab}
None + BVRE + LC	0.12^{bc}	0.27	0.15^{b}	1.87^{bc}	1.58^{b}
GTE + none + LC	0.13^{ab}	0.31	0.14^{b}	1.57^{c}	1.76^{ab}
GTE + BVRE + LC	0.11^{bc}	0.23	0.12^{bc}	1.82^{bc}	1.68^{ab}
Main Effects					
GTE					
None	0.11	0.25	0.14	2.15^{a}	1.51^{b}
GTE	0.11	0.26	0.12	1.78^{b}	1.73^{a}
LC					
None	0.10^{b}	0.25	0.12^{b}	2.16^{a}	1.59
LC	0.13^{a}	0.27	0.15^{a}	1.78^{b}	1.66
BVRE					
None	0.12	0.26	0.14	1.98	1.61
BVRE	0.11	0.26	0.13	1.95	1.64
P-Value					
GTE	0.64	0.55	0.07	0.003	0.001
LC	0.001	0.29	0.01	0.002	0.23
BVRE	0.09	0.90	0.36	0.78	0.60
GTE + BVRE + LC	0.03	0.08	0.006	0.02	0.006
SEM	0.01	0.04	0.02	0.32	0.16

Note: $^{a, b, c, d}$ means with different letters in the column represent significant differences at P < 0.05.

In relation to antioxidant parameters, broilers fed diets containing LC with GTE had lower MDA compared with the control group ($P < 0.05$) and the total antioxidant capacity in diets containing GTE without other additives was significantly higher than the control group ($P < 0.01$). Researchers have shown that supplementing the diet of broilers and quails with green tea extract reduces malondialdehyde levels in the liver, meat and serum (Biswas and Wakita, 2001; Sahin et al., 2010; Farahat et al., 2016). Yang et al. (2003) reported a rise of about 75% in the antioxidant capacity (reduction of malondialdehyde levels) of meat in broilers due to the use of diets containing green tea by-products at various levels of 2000-20000 mg/kg. It was concluded that tea catechins can be transmitted to meat tissues through food intake and thus can be protected against oxidative damage (Biswas and Wakita, 2001).

In studies on total antioxidant capacity, Chi et al. (2020) showed that contamination of broilers with cyclophosphamide (oxidative stress agent) increased the total antioxidant capacity of broilers fed tea extract. Bai et al. (2017) also presented different levels of probiotics (*Bacillus subtilis*) in the diet of broilers resulting in a significant reduction in malondialdehyde content in the liver and serum compared to controls. The liver is one of the vital organs of the body and by using probiotic supplements in diets, oxidation in liver tissue can be prevented (Rajput et al., 2013). Dietary probiotics are useful in ameliorating the damaging effects of oxidative stress and enhancing the activity of antioxidant enzymes (Sanders, 1993). It prevents an increase in reactive oxygen species (ROS) damage cells resulting in enhancing host health (Li et al., 2015).

Scientists found that some probiotics may be useful against oxidation caused to the inhibition of ROS activity and enhancement of antioxidant capacity (Wen et al., 2011). Due to the antioxidant capacity of probiotics as a natural source of the antioxidant defense system in animals, it prevents oxidative stress caused by ROS and strengthens the body's antioxidant defense system (Rajput et al., 2013).

The results of the immune system parameters of broiler chickens are shown in Table 3. The ratio of heterophils to lymphocytes in broilers fed supplementation with LC without plant extracts was significantly lower than in the control group ($P < 0.01$). The significance of heterophils to lymphocyte ratio was frequently studied because of its easy-to-measure characteristics of immunity. As the lower H: L ratio may be an indicator higher level of immunity and likelihood of resistance to pathogens (Sturkie, 1986). Research had shown that the H: L ratio in chickens fed probiotic supplementation decreased may be indexed by a reduction in stress and an increase in immune function (Al-Kassie et al., 2008).

In an experiment, dietary supplementation of probiotics (*Aspergillus niger*) and prebiotics (*Taraxacum officinale*) decreased the heterophile-to-lymphocyte ratio in broiler chickens (Al-Kassie et al., 2008). Therefore, in this study, supplementation of *Lactobacillus* strains leads to higher heterophils to lymphocytes ratio to more lymphocytes, which indicates a decrease in stress and improved immunity in the bird.

All three additives and their interaction effect had significantly result on proliferation of lymphocytes and respiratory burst of macrophages ($P < 0.01$). Also, GTE along with BVRE had significantly lower respiratory burst than the control group. Respiratory burst test showed that heterophil's burst was higher in chickens fed LC additive. Newly hatched chicks are immature in terms of the immune system and are most susceptible to pathogens during this period (Beal et al., 2004; Lowry et al., 2005). Adaptive immune response (immunity that develops when exposed to antigen or after vaccination) may take 1 to 2 weeks to clear the infection (Berndt and Methner, 2004). Heterophils, the first responders to the innate immune system of birds, can respond rapidly to a bacterial infection within 30 minutes (Kogut et al., 1995; He et al., 2003). Therefore, the main action of heterophiles in host animals is to trap and kill foreign particles by phagocytosis and increasing their efficiency is an important indicator to determine the presence of bacterial and pathogenic agents in the body (Nobakht and Aghdam Shariar, 2010).

Table 3. Effect of *Lactobacillus* culture (cfu/g), green tea extract (ppm) and *Berberis vulgaris* root extracts (ppm) on immune system parameters

Variable	Antibody titer	MTT	NBT	WBC (µl)	H: L
GTE × BVRE × LC					
None + none + none	384.00^{c}	2.40^{b}	1.69^{abc}	22289.25^{c}	0.82^{a}
None + BVRE + none	1638.40^{b}	1.61^{d}	1.45^{bcd}	22420.00^{c}	0.80^{ab}
GTE + none + none	1536.00^{b}	1.63^{d}	1.35^{de}	22427.00^{c}	0.74^{bcd}
None + none + LC	1609.14^{b}	2.65^{a}	1.97^{a}	27920.75^{a}	0.60^{e}
GTE + BVRE + none	2633.10^{a}	1.56^{d}	1.10^{e}	22423.25^{c}	0.77^{abc}
None + BVRE + LC	1755.42^{b}	2.04^{c}	1.44^{bcd}	25209.50^{b}	0.70^{cd}
GTE + none + LC	1609.00^{b}	1.95^{c}	1.37^{cde}	25214.75^{b}	0.67^{de}
GTE + BVRE + LC	1877.25^{ab}	2.38^{b}	1.71^{ab}	25618.00^{b}	0.75^{bc}
Main Effects					
GTE					
None	1346.56^{b}	2.17^{a}	1.64^{a}	24459.87	0.73
GTE	1914.00^{a}	1.88^{b}	1.38^{b}	23920.75	0.73
LC					
None	1548.00	1.80^{b}	1.40^{b}	22389.87^{b}	0.78^{a}
LC	1712.46	2.25^{a}	1.62^{a}	25990.75^{a}	0.68^{b}
BVRE					
None	1284.62^{b}	2.15^{a}	1.59^{a}	24462.93	0.71^{b}
BVRE	1975.93^{a}	1.90^{b}	1.42^{b}	23917.98	0.76^{a}
P-Value					
GTE	0.008	0.001	0.004	0.056	0.9
LC	0.41	0.0001	0.009	0.0001	0.0001
BVRE	0.001	0.004	0.04	0.054	0.01
GTE + BVRE + LC	0.03	0.001	0.009	0.0009	0.005
SEM**	558.29	0.17	0.22	761	0.047

Note: $^{a, b, c, d}$ means with different letters in the column represent significant differences at $P<0.05$

Naturally, enhancing the function of these cells will enhance the innate immunity of broilers. Research proves the importance of probiotics as a stimulus to the adaptive immune response in chickens and may also play an important role in enhancing the innate immune response (Koenen et al., 2004). Probiotics are non-pathogenic bacteria that can enhance the health of birds by reducing the establishment of pathogens in the gut (Mead, 2002). On the other hand, a very rapid reaction is observed in phagocytes stimulated with similar microbial agents and thus increases phagocytosis, killing pathogenic microbial agents by respiratory burst (Farnell et al., 2003; Lowry et al., 2005). In addition, diets supplemented with green tea and *Berberis vulgaris* root extracts significantly reduced respiratory bursts, which may be due to the antioxidant properties of the relevant plant extracts which eliminate

free radicals (Sahin et al., 2010). Peroxidation is caused by oxidative stress, which ultimately reduces the production of ROS and reduces the respiratory burst.

The results showed that chickens fed diets containing LC without plant extracts had the highest MTT, NBT and WBC compared to the control group and in contrast, MTT, NBT and WBC had the lowest amount in broilers fed diets supplementing GTE and BVRE. The lymphocyte proliferation test (MTT) was used to evaluate the cellular immune system. Kirjavainen et al. (1999) showed that *Lactobacillus* strains isolated from milk increased lymphocyte proliferation in laboratory mice. Stringfellow et al. (2011) also stated that the addition of probiotics to the water of vaccinated broilers resulted in a significant increase in a respiratory burst in monocytes and heterophiles, which in turn led to an increase in lymphocyte proliferation compared to other groups. The exact mechanism of enhancement in improving the immune response through probiotics is unclear because probiotics affect a wide range of host immune functions (Koenen et al., 2004; Donoghue et al., 2006). Previous studies have demonstrated the positive effects of *Lactobacillus salivarius* on growth performance and the non-specific immune level of broilers (Shokryazdan et al., 2017). Research has shown that adding *Lactobacillus plantrum* to broiler diets can improve intestinal health and reduce mortality in *Escherichia coli*-challenged chickens (Ding et al., 2019). Other research has also represented that probiotic bacteria activate the mucosal immune system by stimulating intestinal antigen-presenting cells (APCs) to provide protection (Clancy, 2003). Findings showed that after administration of lactic acid bacteria to humans and mice orally or by injection, T cells and macrophages can be stimulated (Cunningham-Rundles et al., 2000).

There are conflicting results about the effect of plant extracts on the humoral and cellular immune systems. In general, the effect of green tea extract on the stimulation of the humoral immune system is mainly attributed to its antioxidant components (polyphenolic catechins and their derivatives). The presence of sufficient amounts of antioxidants in the body maintains immune cells and protects them against adverse environments and oxidative stress, which in turn leads to the proliferation and differentiation of B lymphocytes within antibody-producing cells in plasma (Khan et al., 2016). On the other hand, Mahmoudi et al. (2016) examined the effect of berberine on the immune system of mice and concluded that mice treated with berberine had a toxic effect on the production and differentiation of T and B cells. The researchers also showed that berberine consumption in mice caused toxic effects in response to delayed hypersensitivity and lymphocyte proliferation. They suggested that the adverse effect of berberine on the immunity of mice could be due to a direct effect on lymphocyte activation and differentiation.

The results of experiments showed that experimental diets had a significant effect on the antibody titer of broilers against SRBC. Chickens fed diets containing GTE and BVRE had the highest antibody titers against SRBC ($P < 0.05$). GTE and BVRE interacted positively to markedly raise antibody titer compared with other treatments. In inconsistency with the results obtained in this experiment, Mahmoudi et al. (2016) showed that the antibody titer against SRBC was significantly lower in rats treated with high-dose berberine (10 mg/kg per day). Consumption of 5 mg/kg per day berberine had no significant effect on antibody titer against SRBC. Researchers conducted that berberine may have some moderating effects on the immune system.

In a study of the anti-inflammatory impacts of *Berberis vulgaris* root extract and some of its alkaloids in mice, it was shown that the whole alcoholic extract of the plant has the highest reduction effects on acute inflammation (Ivanovska and Philipov, 1996). It was also stated that supplementation of *Berberis* in chicken diets increased the immune response against diseases such as intestinal necrotic, Newcastle and Gamburo (Chand, 2008). Another study showed that the addition of green tea powder (0.25, 0.50 and 0.75%) to the growing diets of Japanese quail increased the antibody titer (Abdel-Azeem, 2005).

To sum up, *Lactobacillus* strains are considered immune stimulants on macrophages. Macrophages are recognized as the main cells influencing the innate immune system and are the first cellular line of defense against invasive pathogens (Smith, 2005). Macrophages can kill pathogens directly through phagocytosis and the production of nitric oxide or indirectly through antigen and secretion of cytokines and other mediators (Kaufmann and Dorhoi, 2016). This, in turn, is the beginning of a hierarchy that activates other cells in the immune system. In this context, evidence suggests that intestinal macrophages can be activated by intestinal microbiota and their metabolites (Bain and Mowat, 2014). Taha-Abdelaziz et al. (2017) in laboratory studies of *Lactobacillus* strains as immune modulators and anti-Campylobacter have concluded that *Lactobacillus* strains induce both pro-cytokines and anti-inflammatory drugs at the same time and cause unique immune function modulation by probiotic strains maintain the homeostasis of the immune system in the host.

CONCLUSION

Due to the positive effect of GTE and BVRE on humoral immunity, the additives, the object of the experiment, can be used as a modulator of the immune system. Compared with *Lactobacillus* strains have positive effects on the cellular immune system and can be used as stimulants. The consumption of plant extracts along with *Lactobacillus* strains may be beneficial to the humoral and cellular immune systems.

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