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The Effects of Various Feed Forms and Dietary Supplements (Probiotic and Antibiotic) on Performance, Immune System, Cecal Microbiota, and Intestinal Morphology in Broiler Chickens

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Abstract

This study was conducted to evaluate the effects of feed form (FF) and dietary supplements on performance, immune system, cecal microflora, and intestinal morphology in broiler chickens. A total of 960 one-day-old Ross 308 mixed-sex chickens were distributed to 8 treatments consisting of 6 replicates (20 birds/pen). The experimental design was a 2×4 factorial arrangement of treatments evaluating two feed forms (mash or pellet) and dietary supplements [without a supplement, *Lactobacillus acidophilus* (*L. acidophilus*), *Bacillus subtilus* (*B. subtilus*), and Avilamycin (an antibiotic)]. Considering the main effects, dietary supplements and pellet diets significantly improved growth performance parameters (FI, BWG, and FCR) compared to the other treatments. Birds fed with a pellet diet had a reduced relative weight of the gizzard and pancreas, increased villus height, and gained the relative weight of the liver and small intestinal. Regardless of the FF, *B. subtilis* supplementation tended to greater villus height, lower crypt depth, and higher villus height to crypt depth ratio compared to other groups. Birds fed with mash diets supplemented with *L. acidophilus* and *B. subtilis* and a pelleted diet supplemented *B. subtilis* had higher villus height, goblet cell, and *Lactobacillus* population in the gut compared to the other treatments. Probiotics supplementation reduced the percentage of heterophils compared to other diets. The significant interaction between FF and dietary supplements showed that *L. acidophilus* in the mash diet tended to enhance the percentage of lymphocytes and reduce the heterophil to lymphocyte ratio compared to the pelleted diet. The main factors had no significant effect on anti-SRBC antibody titer. The results from this study indicated that the probiotic *L. acidophilus and B. subtilis* used in the mash diet may serve as alternatives to an antibiotic.

Introduction

Feed additive is used in the poultry industry to enhance the growth rate. Antibiotic growth promoters have been used to achieve better production, stimulate growth and feed conversion of animal and poultry feed and reduce the mortality rate in livestock and poultry production. However, since 2006, the European Union has banned the use of antibiotics in animal and poultry diets due to the development of resistant bacteria, the storage of antibiotics in livestock products such as meat and eggs, environmental problems, and encouragement of alternative additives (Castanon, 2007; Dibner and Richards, 2005; Mohebodini *et al*. 2020). Numerous additives have been studied to find alternatives to dietary antibiotics, and some results suggest probiotics, prebiotics, organic acids, and herbs

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(Abudabos *et al.* 2017; Darabighane and Nahashon, 2014; Zhang *et al.* 2015).

Probiotics are live microorganisms that participate in the health and maintenance of the host gastrointestinal tract and are considered feasible substitute for antibiotics as growth promoters (Fuller, 1989). The mechanism of action of probiotics in poultry has been expressed in several ways: 1) Preservation of intestinal microflora through competition with pathogens; 2) Change in metabolism by increasing the activity of digestive enzymes; 3) Strengthening the immune system by producing specific polysaccharides; and 4) Improving feed intake and digestion (Abudabos *et al.* 2019; Jha *et al.* 2020; Sen *et al.* 2012). Some probiotic strains most commonly used in poultry feed include *Lactobacillus bulgaricus, Lactobacillus plantarum, Lactobacillus acidophilus, Streptococcus therthermophilescillus subtilis, Bifidobacterium bifidum,* and *Aspergillus oryzae* (Khaksefidi and Rahimi, 2005).

Lactobacillus acidophilus (*L. acidophilus*) is one of the probiotics used for broilers. Including probiotics containing L. *acidophilus* in the diet of broilers improves the body weight and feed conversion ratio by increasing the crypt depth and the villus height (Li *et al.* 2018). *Bacillus subtilis* (*B. subtilis*) is a gram-positive bacterium used as a relatively new probiotic in poultry diets (Rhayat *et al.* 2017). In addition, not only is *B. subtilis* used as a growth promoter but it also enhances animal health through the development of intestinal microflora (Sen *et al.* 2012; Sohail *et al.* 2012). Furthermore, supplementing the diet with *Bacillus subtilis* helps maintain intestinal health against pathogenic bacteria (Abudabos *et al.* 2019). Nevertheless, poultry nutritionists are interested in applying probiotics to induce the immune system and have protective effects against many diseases (Jha *et al.* 2020; Seifi *et al.* 2018; Teo and Tan, 2007).

Commercial feed mills produce forms of feed for broilers of different ages. Feed form (FF) and particle size significantly affect the broilers' growth and feed intake (Dozier *et al.* 2010). While the pelleted feed increases feed cost, it can be balanced out by improved performance (Jahan *et al.* 2006). Many studies indicated that feeding broilers with pellets reduces feed wastage, decreases selective feed, and improves the taste and digestibility of food (Amerah *et al.* 2007; Amerah *et al.* 2008; Chewning *et al.* 2012; McKinney and Teeter, 2004). Nowadays, most feed is produced in the form of pellets, and poultry feed manufacturers use it to improve poultry performance (Abdollahi *et al.* 2010; Dozier *et al.* 2010; Lemme *et al.* 2006).

Probiotics based on *Bacillus* species are attractive for the poultry industry due to the spore-forming ability of the bacteria that made them resistant to heat, and give them a longer shelf-life at room temperature. So, a high percentage of the ingested bacteria can reach the small intestine intact (Cutting, 2011). Although some species of *Bacillus* and *Lactobacillus* used for probiotics are resistant to high temperatures in vitro (Mbye *et al.* 2020), the viability of these bacteria under normal pelletizing conditions is unclear. Amerah *et al.* (2013) reported that some probiotics based on some *Bacillus* can tolerate the high temperatures of normal pelletizing conditions and affect the immune system of chickens.

This study was conducted to investigate the effect of various feed forms (mash and pellet) and supplementations (*Lactobacillus acidophilus*, *Bacillus subtilus*, and Avilamycin) on performance, immune system, cecal microflora, and intestinal morphology in broilers

Materials and Methods

Birds and experimental treatments

The Animal Ethics Committee of Saravan Higher Education Complex approved all the animal protocols used in the current experiment. A total of 960 one-day-old mixed-sex Ross chickens were randomly distributed in a 2×4 factorial design, composed of two feed forms (mash or pellet) and four types of supplementation (without a supplement, with probiotic *L. acidophilus*, probiotic *B. subtilis*, or antibiotic avilamycin (AA)), totaling eight treatments with six replications with 20 birds per pen. The basal diets, starter (1-10 days), grower (11-24 days), and finisher (25-42 days) were formulated to meet the Ross 308 strain recommendations (Ross, 2014) for broilers (Table 1). The pelleting process was performed at a temperature of 75 °C, and the pellet diameter was 2.5 mm in the starter and 3.0 mm in the grower and finisher feeds. Diet supplemented with the probiotic based on *L. acidophilus* and *B. subtilis* and at a rate of 1.5×10^5 cfu/g diets, and Avilamyat 150 g/ton feed. A bacteria strain of *B. subtilis* (ATCC 6051a) and *L. acidophilus* (CECT 4529) were purchased from the Iranian Research Organization for Science and Technology (IROST). Each dietary treatment was fed *ad libitum* to six replicate pens. Birds of mash and pellet treatments were fed from d 1-42. Broilers were housed in 60×200×130 cm floor pens with four nipples per pen. The environmental temperature was maintained at 33 °C at d 1 and was reduced gradually to 24 °C until the end of the experiment.

Table 1. Composition and nutrient levels of the basal diet (as-fed basis)

Items	Starter 1-10 days	Grower 11-24 days	Finisher 25-42 days
Corn	57.90	60.20	61.20
Soybean meal (44% CP)	33.20	31.00	31.00
Corn gluten (60% CP)	3.9	3	1.5
Supplement[1]	-	-	-
Limestone (36% Ca)	1.21	1.08	1.05
Di-calcium phosphate	1.87	1.6	1.45
Salt	0.36	0.33	0.32
Na-bicarbonate (soda)	0.11	0.11	0.11
L-threonine	0.06	0	0
L-lysine	0.3	0.18	0.06
DL-methionine	0.19	0.2	0.18
Sunflower oil	0.4	1.8	2.63
Premix[2]	0.5	0.5	0.5
Calculated nutritive values			
Metabolizable energy (Kcal kg^{-1})	2880	2993	3039
Crude protein (%)	22.35	20.9	19.96
Calcium (%)	0.99	0.86	0.81
Available Phosphorus (%)	0.48	0.43	0.4
Methionine (%)	0.48	0.43	0.39
Methionine + Cystine	1.02	0.9	0.82
Lysine (%)	1.36	1.18	1.04
Sodium	0.19	0.18	0.17
Dietary cation-anion balance (mEq kg^{-1})	287	280	285

[1]Diet supplemented with the probiotic based on *L. acidophilus* and *B. subtilis* and at a rate of 1.5×10^5 cfu/g diets, and Avilamyat 150 g/ton feed.

[2]Vitamin premix provided per kilogram of diet: vitamin A (transretinyl acetate), 11,000 IU; vitamin D_3 (cholecalciferol), 2300 IU; vitamin E (dl-α-tocopherol acetate), 121 IU; vitamin K_3 (bisulphate menadione complex), 3 mg; thiamine (thiamine mononitrate), 3 mg; riboflavin, 9 mg; nicotinic acid, 50 mg; pantothenic acid (D-calcium pantothenate), 15 mg; vitamin B6, 4 mg; d-biotin, 0.1 mg; folic acid, 2 mg; vitamin B_{12} (cyanocobalamin), 0.02 mg and choline (choline chloride), 1000 mg. Mineral premix provided per kilogram of diet: iron ($FeSO_4 \cdot 7H_2O$), 55 mg; iodine (Ca (IO3)2), 1.3 mg; manganese ($MnSO_4 \cdot H_2O$), 120 mg; zinc (ZnO), 100 mg; copper ($CuSO_4 \cdot 5H_2O$), 16 mg; selenium (Na_2SeO_3), 0.2 mg.

Measurement of growth performances

At the end of the experiment, chickens were weighed by pen, and feed consumption was recorded. Body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR), including mortality weight, were calculated for each phase.

Digestive tract and lymphoid organs' weight

On day 42, two broiler chickens were randomly selected and weighed from a total of 20 birds per replicate. They were then slaughtered by exsanguinations after CO_2 stunning. The feathers, legs, and head were removed, the internal organs were carefully removed, and intestinal specimens were finally collected. The lymphoid organs (thymus, spleen, and bursa of Fabricius), gizzard, pancreas, liver, and small intestine were carefully dissected and weighed. Meanwhile, the duodenum, jejunum, and ileum were removed and the empty digestive tracts were weighed. Their relative weights were determined as percentages of the live weight.

Immune response

At the age of 28 days, two birds from each replicate (selected and identified by wing staining) were injected intravenously (brachial vein) with 0.1 mL of sheep red blood cells **(**SRBCs) suspension (5% v/v PBS). Antibody production against SRBCs was evaluated after seven days of inoculation. Titers were expressed as log 2 of the reciprocal of the highest dilution in which there was agglutination (Wegmann and Smithies, 1966). At the end of the trial, two broilers per pen (8 birds per treatment) were randomly selected and blood samples were drawn from the brachial vein. For hematological parameters, approximately 10 mL of blood samples were taken into vacuum tubes containing EDTA as an anticoagulant. The blood was centrifuged at 2500 × g for 10 min at 4 °C, then stored at −20 °C until used to measure blood parameters. Blood smears were also made from blood samples to calculate the ratio of heterophils to lymphocytes, and they were then stained with Wright's stain (Lucas, 1961). Blood smears were scanned with a microscope (1000× magnification) and the first 150 white blood cells were differentiated (heterophils and lymphocytes)

Intestinal morphological measurements

On 42 d one cm segment of the middle region of the jejunum from the killed birds was removed, washed with PBS, and then fixed in 10% phosphate-buffered formalin for 42 h. Samples were dehydrated through graded alcohol levels (absolute alcohol, 70% an,d 95%) and embedded in wax. Two samples of each section (5 µm thickness) were cut with a microtome (MicroTec, Walldorf, Germany) and stained using

hematoxylin-eosin and alcian blue (Kumar and Kiernan, 2010). Crypt depth, Villus height, and height-to-depth ratio were measured on the stained sections using a light microscope (Carl Zeiss, Promenade, Germany) equipped with a digital camera (U-TV1X; Olympus). Measurements were made from photomicrographs taken at 100× magnification. The density of goblet cells was calculated as the number per 100 μm of villus length.

Cecal microflora population

At the end of the experiment, the contents of the cecum from slaughtered birds were collected and stored in the refrigerator, and immediately transferred to a microbiological laboratory for analysis. Colonies of cecal microflora were measured as described by Weese (2002). In brief, one gram of ceca content was transferred to 9 mL of sterile saline solution and thoroughly mixed. Subsequently, 10-fold serial dilutions of each sample were made to obtain up to 8-10dilutions. The populations of *Escherichia coli*, lactic acid bacteria, and *bifidobacterium* in cecum contents were estimated as CFU per gram (Weese, 2002; Pattananandecha *et al*. 2016).

Statistical Analysis

All data of each pen were considered for statistical analysis. The experimental unit was statistically analyzed under a completely randomized design in 2×4 factorial arrangements using the GLM procedure of the Statistical Analysis System (Mukhopadhyay *et al.* 2008). Duncan's multiple range comparison tests were carried out to examine significant differences between dietary treatments. The following model was used: $Y_{ij}=\mu+\alpha_i+\beta_j+\alpha\beta_{ij}+ e_{ijk}$

Where Y_{ij} =an observation, μ =the overall mean, α_i =fixed effect of feed form, β_j =fixed effect of the supplement, $\alpha\beta_{ij}$= fixed effect of interaction between feed form and supplement, and e_{ijk} =random error associated with each observation.

Result

Growth performance

The effect of dietary treatments on performance characteristics (FI, BWG, and FCR) of broiler chickens are presented in Table 2. In this study, the benefits of pelleting on weight gain, feed intake, and feed conversion ratio in broilers well demonstrated. The diet containing *B. subtilis* and antibiotic improved BWG in birds compared with the diet without supplements ($P < 0.05$). None of the treatments had a sigficant effect on FI and FCR. Also, no interactions ($P > 0.05$) were observed on performance parameters.

Table 2. Influence of feed form and dietary supplements on productive performance of broiler chickens

Feed form	Supplement	Performance parameters[1]		
		FI	BWG	FCR
Mash	WS	91.97	51.82	1.77
Mash	*LA*	90.49	52.64	1.72
Mash	*BS*	90.38	52.72	1.71
Mash	AA	91.57	52.85	1.73
Pellet	WS	99.02	57.85	1.71
Pellet	*LA*	99.02	57.88	1.71
Pellet	*BS*	98.11	59.21	1.66
Pellet	AA	99.02	59.40	1.67
SEM		1.12	0.34	0.02
Main Effects				
Feed form				
Mash		91.10[b]	52.51[b]	1.74[a]
Pellet		98.79[a]	58.59[a]	1.69[b]
SEM		1.02	0.31	0.01
Supplement				
WS		95.50	54.84[b]	1.74
LS		94.76	55.26[ab]	1.71
BS		94.25	55.97[a]	1.69
AA		95.30	56.13[a]	1.70
SEM		1.00	0.32	0.02
***P*-value**				
Feed form		0.01	0.01	0.01
Supplement		0.08	0.04	0.07
Feed form ×Supplement		0.12	0.12	0.20

WS: Without Supplements; LS: *Lactobacillus acidophilus*; BS: *Bacillus subtilis*; AA:Avilamycin Antibiotic; *SEM*: Standard error of the means

[a–b]Means within the same column with different superscripts are significantly different ($P < 0.05$).

[1] FI= Feed intake; BWG= Body weight gain; FCR= Feed conversion ratio.

Digestive tract and lymphoid organs' weight

Table 3 shows that feeding the pellet diet reduced the relative weight of the pancreas, gizzard, and small intestine and increased the relative weight of the liver. None of the treatments significantly affected the relative weight of the bursa of Fabricius, spleen, and thymus.

Table 3. The effect of feed form and dietary supplement on the relative weight of digestive tract and lymphoid organs

Feed form	Supplement	Bursa (%)	Spleen (%)	Thymus (%)	Gizzard (%)	Pancreas (%)	Liver (%)	Small intestine(%)
Mash	WS	0.132	0.159	0.606	1.460	0.290	2.700	6.240
Mash	*LA*	0.141	0.162	0.615	1.430	0.270	2.710	6.420
Mash	*BS*	0.139	0.164	0.620	1.450	0.260	2.780	6.280
Mash	AA	0.137	0.160	0.604	1.470	0.270	2.790	6.350
Pellet	WS	0.135	0.157	0.605	1.190	0.210	2.950	5.740
Pellet	*LA*	0.140	0.165	0.618	1.220	0.240	2.990	5.820
Pellet	*BS*	0.139	0.164	0.616	1.190	0.220	3.030	5.920
Pellet	AA	0.141	0.159	0.614	1.200	0.230	3.010	5.720
SEM		0.006	0.08	0.009	0.04	0.01	0.10	0.25
Main Effects								
Feed form								
Mash		0.137	0.161	0.611	1.453[a]	0.273[a]	2.745[b]	6.323[a]
Pellet		0.139	0.161	0.613	1.200[b]	0.225[b]	2.995[a]	5.800[b]
SEM		0.005	0.007	0.007	0.03	0.01	0.09	0.22
Supplement								
WS		0.134	0.158	0.606	1.325	0.250	2.825	5.990
LA		0.141	0.164	0.617	1.325	0.255	2.850	6.120
BS		0.139	0.164	0.618	1.320	0.240	2.905	6.100
AA		0.139	0.160	0.609	1.335	0.250	2.900	6.035
SEM		0.006	0.007	0.008	0.03	0.01	0.09	0.23
P-value								
Feed Form		0.75	0.91	0.79	0.004	0.04	0.02	0.031
Supplement		0.12	0.09	0.24	0.49	0.71	0.42	0.72
Feed form × Supplement		0.45	0.58	0.39	0.31	0.28	0.42	0.33

WS: Without Supplements; LS: *Lactobacillus acidophilus*; BS: *Bacillus subtilis*; AA:Avilamycin Antibiotic; *SEM*: Standard error of the means.

[a–b]Means within the same column with different superscripts are significantly different ($P < 0.05$).

Morphology of jejunum

The effect of dietary treatments on the morphological parameters of the jejunum is shown in Table 4. Diets containing the supplements, as the main factor, had a shallower crypt, and greater villus height to crypt depth ratio in the jejunum compared to the supplement-free mash diet. Moreover, The use of AA and *L. acidophilus* improved the villus height to crypt depth ratio than supplementing of *B. subtilis*. A significant feed form×supplement interaction was observed for villus height and the number of goblet cells in the jejunum. Birds fed pelleted diets containing AA and *B. subtilis* had higher villus compared to other treatments. Also, supplementing pellet diets with *B. subtilis* increased the villus height more than the AA treatment. It was shown that feed form and dietary supplements alone had no significant effect on the goblet cell of the small intestinal, but a significant feed form×supplement interaction was observed for the goblet cell ($P = 0.03$) in the jejunum. Birds who received *L. acidophilus and B. subtilis* in the mash diet or *B. subtilis* in the pellet diet had a higher number of goblet cells in jejunum compared to the other treatments. The number of goblet cells was greater in birds fed with the mash diets supplemented with *L. acidophilus* or *B. subtilis* than in *B. subtilis*- supplemented pellet diet.

Intestinal microflora

The influence of treatments on the bacteria population in the ceca of broilers on d 42 is summarized in Table 5. An interaction was observed between the feed form and dietary supplements on the lactic acid bacteria population in the supplementing of mash diet with *L. acidophilus* and *B. sutilis* increased the count of lactic acid bacteria compared to the other treatments. Furthermore, supplementing of pellet diet with L. acidophilus increased the count of lactic acid bacteria in the jejunum than those of both forms of diets alone or with AA. All dietary supplements reduced the population of *E-Coli*. The lactic acid bacteria population in the cecum of broilers was significantly increased in the probiotic *L. acidophilus and B. subtilis* relative to the other treatments. Two-way interaction was observed for the

cecal lactic acid bacteria population. According to the main effects of dietary supplements, the *E. coli* population was the highest in the jejunum of birds that received the supplement-free mash diet.

Table 4. The effect of feed form and dietary supplement on the morphology of jejunum of broilers

Feed form	Supplement	Villus height (µm)	Crypt depth (µm)	Villus height / Crypt depth	Gobbet cell (N/100 μm)
Mash	WS	1134.00^{c}	168.00	6.67	7.20^{c}
Mash	*LA*	1175.30^{c}	157.12	7.48	8.20^{ab}
Mash	*BS*	1189.50^{c}	154.50	7.70	8.35^{a}
Mash	AA	1181.35^{c}	161.40	7.32	7.21^{c}
Pellet	WS	1195.90^{c}	160.12	7.47	7.11^{c}
Pellet	*LA*	1201.80^{c}	153.62	7.82	7.31c
Pellet	*BS*	1304.20^{a}	148.15	8.80	8.02^{b}
Pellet	AA	1251.10^{b}	151.40	8.26	7.00^{c}
SEM		22.46	4.26	0.53	0.31
Main Effects					
Feed form					
Mash		1170.54^{b}	160.26	7.31	7.74
Pellet		1238.25^{a}	153.32	8.09	7.36
SEM		1.02	0.31	0.01	0.29
Supplement					
WS		1164.95^{b}	164.06^{a}	7.11^{c}	7.16
LA		1188.55^{b}	155.37^{b}	7.65^{b}	7.76
BS		1246.85^{a}	151.33^{b}	8.25^{a}	8.19
AA		1216.23^{ab}	156.40^{b}	7.79^{b}	7.11
SEM		1.00	0.27	0.01	0.25
P-value					
Feed form		0.001	0.14	0.21	0.48
Supplement		0.01	0.02	0.01	0.11
Feed form × Supplement		0.04	0.12	0.2	0.03

WS: Without Supplements; LS: *Lactobacillus acidophilus*; BS: *Bacillus subtilis*; AA: Avilamycin Antibiotic; *SEM*: Standard error of the means

$^{a–b}$Means within the same column with different superscripts are significantly different ($P < 0.05$).

Table 5. The effect of feed form and dietary supplement on the microflora population (log_{10} cfu/g) in the jejunum of broilers

Feed form	Supplement	*E-Coli*	LAB	*Bifidobacteriom*
Mash	WS	8.4	3.42^{c}	4.51
Mash	*LA*	6.4	7.34^{a}	5.12
Mash	*BS*	6.22	8.41^{a}	5.19
Mash	AA	6.3	3.66^{c}	5.68
Pellet	WS	8.1	3.41^{c}	5.24
Pellet	*LA*	6.81	5.30^{b}	5.09
Pellet	*BS*	6.38	8.54^{a}	5.51
Pellet	AA	6.26	3.42^{c}	5.71
SEM		82.46	4.26	0.53
Main Effects				
Feed form				
Mash		6.83	5.71	5.13
Pellet		6.89	5.17	5.39
SEM		1.02	0.31	0.01
Supplement				
WS		8.25^{a}	3.42^{c}	4.88
LA		6.61^{b}	6.32^{b}	5.11
BS		6.30^{b}	8.48^{a}	5.35
AA		6.28^{b}	3.54^{c}	5.70
SEM		1.00	0.27	0.01
P-value				
Feed form		0.42	0.35	0.29
Supplement		0.01	0.001	0.31
Feed Form × Supplement		0.34	0.002	0.21

WS: Without Supplements; LS: *Lactobacillus acidophilus*; BS: *Bacillus subtilis*; AA:Avilamycin Antibiotic;LAB: Lactic acid bacteria; *SEM*: Standard error of the means

$^{a–b}$Means within the same column with different superscripts are significantly different ($P < 0.05$).

Immune response

The immune response in the tested broiler used in the study is presented in Table 6. Probiotics supplementation reduced the percentage of heterophils compared to other diets. Interaction of feed form and supplement were observed in lymphocyte percentage and heterophil to lymphocyte ratio. Birds fed *B. subtilis* had the highest percentage of lymphocytes and a lower heterophil to lymphocyte ratio. A significant interaction was observed for feed form×supplemented diets in the percentage of lymphocyte and heterophil to lymphocyte ratio. All dietary treatments (except for AA in the pellet diet reduced the heterophil to lymphocyte ratio compared to the supplement-free mash diet. Moreover, the use of *B. subtilis* significantly decreased the heterophil to lymphocyte ratio than those of other treatments. Anti-SRBC antibody titers were not affected by the effects of feed form and supplements used in the diet. However, a slight increase in anti-SRBC antibody titers (Ig M, Ig Y, and Ig T) was observed in birds fed the supplemental diet.

Table 6. The effect of feed form and supplements on immune system response parameters in broilers

Feed form	Supplementation	Heterophil (%)	Lymphocyte (%)	H:L	Anti-SRBC titer(Log_2)		
					IgM	IgY	IgT
Mash	WS	46	54^{c}	0.85^{a}	0.85^{b}	3.82	4.67
Mash	LA	*37*	62^{ab}	0.60^{cb}	1.04^{ab}	4.39	5.43
Mash	BS	*36*	65^{a}	0.55^{c}	1.13^{a}	4.26	5.39
Mash	AA	41	55^{c}	0.75^{b}	1.02^{b}	4.07	5.09
Pellet	WS	42	53^{c}	0.79^{b}	0.80^{b}	3.72	4.52
Pellet	LA	*39*	56^{c}	0.70^{b}	1.00^{b}	4.15	5.15
Pellet	BS	*37*	66^{a}	0.56^{c}	1.07^{ab}	4.11	5.18
Pellet	AA	41	54^{c}	0.76^{ab}	1.00^{b}	4.00	5.00
SEM	WS	0.342	0.401	0.020	4.26	0.53	82.46
Main Effects							
Feed form							
Mash		59.00	0.69	59.00	1.01	4.14	5.15
Pellet		57.25	0.70	57.25	0.97	4.00	4.96
SEM		0.241	0.382	0.019	0.31	0.01	1.02
Supplementation							
WS		44.00^{a}	53.50^{b}	0.82^{a}	0.83	3.77	4.60
LA		38.00^{b}	59.00^{ab}	0.65^{b}	1.02	4.27	5.29
BS		36.50^{b}	65.50^{a}	0.56^{b}	1.10	4.19	5.29
AA		41.00^{a}	54.50^{b}	0.75^{ab}	1.01	4.04	5.05
SEM		0.230	0.390	0.021	0.27	0.09	1.00
P-value							
Feed form		0.42	0.24	0.38	0.35	0.43	0.42
Supplements		0.04	0.01	0.02	0.31	0.28	0.59
Feed form × Supplements		0.08	0.001	0.001	0.002	0.2	0.34

WS: Without Supplements; LS: *Lactobacillus acidophilus*; BS: *Bacillus subtilis*; AA:Avilamycin Antibiotic; *SEM*: Standard error of the means; H:L: Heterophil to Lymphocyte ratio

[a–b]Means within the same column with different superscripts are significantly different ($P < 0.05$).

Discussion

The importance of physical feed form in the growth performance of broilers is well known (Amerah *et al.* 2007). Currently, various feed forms are typically produced for birds by commercial feed mills, and feed processing by pelleting has become commonplace and is widely used by feed manufacturers to improve livestock performance. (Lv *et al.* 2015). Previous studies have shown that the weight gain of chickens fed pellet diets is higher than those fed mash (Abadi *et al.* 2019; Lv *et al.* 2015). Broilers fed pellet diet supplemented with probiotics and enzymes showed the best performance in broilers due to synergistic effects (Hosseini *et al.* 2017) Another study showed that increasing nutrient concentrations as well as feed intake in chickens fed pellet diets improved starch gelatinization and reduced feed waste (Amerah *et al.* 2007), which is supported by the finding of Lv *et al.* (2015), who found that pelleting affected weight gain and feed intake. In the current study, pellet feeds enhanced performance by approximately 9% at the end of the period compared with mash diets. The findings of improved feed conversion ratio for pellet diets were found to contradict those of Chewning *et al.* (2012).

Some studies have reported that the inclusion of probiotics in poultry diets improves growth performance by increasing the population of symbiotic microbiota in the intestines of broilers (Latorre *et al.* 2017; Mountzouris *et al.* 2007; Rhayat *et al.* 2017). Shah *et al.* (2019), showed that supplementation of broiler diets with *Lactobacillus* as a probiotic enhances intestinal villi height and absorption capacity in broilers, which leads to more

body weight at the end of the rearing period. The current results are in line with the findings of Cavazzoni *et al*. (1998), who found that feeding broiler chickens with *B. coagulans* as a probiotic significantly improved broiler body weight, an effect comparable to virginiamycin. In contrast to these findings, Yousefi and Karkoodi (2007), reported that weight gain was not affected by the supplementation of probiotics in a broiler diet. Also, according to Sen *et al.* (2012) probiotics based on *B. subtilis* improved weight gain, feed intake, and feed conversion. Similar results in feed intake with the findings of some other scientists have shown that probiotic supplements had no effect on feed consumption (Sohail *et al.* 2012).

Although some reports indicate that the inclusion of probiotics in the diet of broilers improves the conversion ratio (Awad *et al.* 2009; Zamanizadeh *et al.* 2021), our results did not show a statistically significant difference with the supplementation of *L. acidophilus* and *B. subtilis*. However, controversial results of probiotic supplements on broiler performance might be due to the method of preparation, various kinds of probiotics, dose, feed form of diet, and health status of birds.

No significant interactions between supplement type and feed form on chick performance were shown. Nevertheless, *L. acidophilus* supplementation in the mash diet seems to be more effective than in the pellet diet comparison of *B. subtilis* supplemented in mash and pellet diets showed that it performed better than the control in both feed forms. It may be explained that *B. subtilis* is resistant to heat and poor storage conditions, and it is considered safe to be used as probiotic (Fuller, 1989).

However, high temperatures during processes such as pelleting are likely to increase cell membrane fluidity and may therefore lead to cellular dysfunction (Mbye *et al.* 2020). It has been reported that only a few strains of *lactobacilli* can survive at temperatures between 45 and 80 °C (Grujović *et al.* 2019). The mitigated performance of pellet-fed birds supplemented with *LA* may be due to high temperatures in pellet processing.

A comparison of the relative weight of the internal organs of birds fed with pellet and mash diets showed that not only the relative weight of the pancreas and gizzard is lower in the birds fed with pellets, but also the relative weight of the liver and small intestine is relatively heavier than those fed mash diets. These results are in line with the studies by Abadi *et al.* (2019). The weight loss of birds fed pellet diets can be attributed to insufficient mechanical stimulation by pellet feed (Lv *et al.* 2015). Pelletizing the feed reduces the particle size and shortens the shelf life of small particles compared to large particles in the gizzard (Coarse particles might decrease the passage time of fine particles in the diet), thereby reducing mechanical stimulation (Mateos *et al*. 2012) and reducing organ size. Similar results have been reported by Chewning *et al.* (2012) and Lv *et al.* (2015) for the relative increase in intestinal weight in pellet-fed chickens.

Results of carcass evaluation in this experiment showed that supplementations alone produced no significant difference in the carcass traits. Data on the digestive tract parameters of broilers (relative weight of the pancreas, gizzard, and small intestine and increased relative weight of the liver) are consistent with the findings of Khan *et al.* (2011).

According to Teo and Tan (2007), birds supplemented with *B. subtilis* had a significantly heavier bursa weight than the control groups. The increase in the relative weight of a lymphoid organ was in agreement with Khan *et al.* (2011), who found that the supplemented diet broilers on the probiotic increased the relative weights of the bursa, spleen, and thymus of the treatment group.

Few studies have focused on the simultaneous effect of feed form and probiotics on intestinal morphological parameters in broilers. Conversely, considerable studies have been conducted on the effects of feed form and probiotics separately (Teo and Tan, 2007; Khan *et al*. 2011; Chewning *et al*. 2012; Lv *et al*. 2015; Abadi *et al*. 2019). Our finding in the jejunum agrees with those of a previous study by Zang *et al.* (2009), which indicated that villus height increased in broiler-fed pelleted diets. Further, Abadi *et al.* (2019) stated that probably in birds fed pelleted diets, the extent of villus height could be positively regulated proportionally to the greater load of nutrients which reach the proximal section of the intestine.

The form of feed had no significant effect on the goblet cells of the small intestine. However, the number of goblet cells was numerically lower in chickens fed pellet diets. The association between intestinal bacterial load and goblet cell or mucin production has been reported by Fasina *et al.* (2010). Some heat-sensitive bacteria may be reduced during the pelleting process, leading to a decrease in goblet cells at the surface of the villi. This condition causes less demand for mucin production to maintain the health of the host intestine against harmful bacteria (Abadi *et al*. 2019).

Several studies have found the effects of probiotic supplementation on the gut microbiota and in the intestinal histomorphometry of broiler chickens. Experimental findings suggest that supplementation with a diet containing probiotics can affect villus height and crypt depth in the small intestine (Olnood *et al.* 2015; Abd El-Moneim and Sabic, 2019; El-Moneim *et al.* 2020). Inoculation of some bifidobacterial strains into the yolk sac in the last days of incubation did not affect crypt depth, but the height of the ileal villus showed a significant increase (El-Moneim *et al*. 2020). The prolongation of villi

may be based on the hypothesis that probiotic supplements activate mitotic cell division and proliferate intestinal epithelial cells (Bai *et al.* 2013; Samanya and Yamauchi, 2002). Zamanizadeh *et al*. (2021) stated that dietary supplements from probiotics (*S. cerevisiae*) significantly increase the villus height of the ileum in laying Japanese quail. As the villi height increases, absorption surface area and therefore the uptake of many nutrients will increase the inefficiency. Similar results have been reported by Jin *et al*. (1996) when they examined the effects of L. *acidophilus* inclusion in broiler diets. In general, increasing crypt depth alone may reduce the secretion of certain digestive enzymes, reduce nutrient uptake, and ultimately impair the growth performance of broilers (Singh *et al*. 2011). It can be concluded that in addition to villi length, shape and size of villi are also very important effects for *L. acidophilus* and *B. subtilis* on microflora, morphology, and morphometry of the intestine did not show significant differences between the groups (Forte *et al.* 2016), which is not consistent with the results of the present experiment.

In the current study, a significant interaction between feed form and supplementation was observed for villus height and goblet cells in the jejunum.

Probiotic *B. subtilis* showed these effects in both pellet and mash diets compared to the control, while the effect of *L. acidophilus* in mash diets seems to be greater. It can be suggested that *L. acidophilus* is more susceptible than *B. subtilis* during the pelleting process. The lower resistance of lactobacillus to high temperatures has been previously reported by Grujović *et al.* (2019).

The important role of various intestinal microbiota on host metabolism, growth performance, nutrient digestion, and the health of birds has been documented (Jha *et al.* 2020). Occasionally, the composition of the gut microbiota can be drastically altered by several factors especially early in life, genotype, and dietary/food additives (Jha *et al.* 2020). The results of this research showed that broilers fed with mash and pellet diets had no bacteria population in the ceca. Similar results have been reported by Abadi *et al*. (2019).

Similar and insignificant effects were observed on the total number of aerobics and *Salmonella* in the intestine with the addition of probiotics (multistrain) in mash diets (Sohail *et al.* 2015). Nevertheless, it contradicts the findings of Singh *et al.* (2011), who reported an increase in *Lactobacilli* spp cecum from mash diets. Engberg *et al*. (2002) reported that the feed form had no significant effect on cecal *Coliform* spp. population. Also, feed form or supplements had no significant effect on the cecal *Bifidobacterium* population. It has been reported that the use of *B. subtilis* probiotic supplement in broiler diets has no significant effect on cecum microflora (Sohail *et al.* 2015). The present study showed a decrease in the populations of E. coli in the intestine of in broilers fed diet added with *B. Subtilis*, *L. acidophilus*, and antibiotic which agree with the findings of Gao *et al.* (2017) who indicated that the addition of B. subtilis and antibiotics to broiler diets can significantly reduce the amount of E. coli in the cecum.

It is stated that the antibacterial effect occurs due to the "microbial consumption of oxygen". Because *B. subtilis* is an aerobic bacterium, oxygen is required in large quantities for its growth and reproduction. By consuming free oxygen in the animal's gut, the growth of anaerobic probiotics is enhanced and the balance is maintained to stop anaerobic substances such as *E. coli*. There are conflicting reports in this regard, a study found that the addition of *B. subtilis* to the diet did not show a significant change in the number of intestinal *E-coli* in broilers, but in another study, that chickens supplemented with *lactobacilli*, the population of *E-coli* had been significantly reduced (Jin *et al.* 1996).

It has been reported that in diets containing probiotics, the pelleting temperature affects the secreted IgA concentration. For instance, temperatures above 90°C reduce the concentration of secreted IgA more than temperatures of 75 or 85 °C. In addition, a decrease in serum IgM has been reported at a pelleting temperature of 90 ℃ compared to 85 ℃ in diets containing probiotics (Amerah *et al.* 2013). Through these observations, we observed a decrease in the humoral immune responses in the pellet diet only numerically. These results may be explained by chemical reactions caused by pellet temperature (McCracken, 2002) that reduce stimulation in the immune response.

Sheep red blood cell injection is used as an antigen in most experiments to assess the humoral immune response of birds. The inclusion of probiotics in the diets of birds increased the serum concentrations of IgM and IgA compared to the control group, while the amount of IgY was not affected by supplements (Zhang and Kim, 2014). Teo and Tan (2007) found that a probiotic containing *B. subtilis* improved intestinal morphology and enhanced the immune response through a higher degree of phagocytosis and a heavier relative bursa weight. In the present experiment, a slight improvement in the plasma immunoglobulin of broilers fed probiotics was observed. Strengthening the immune system may be attributed to the immunomodulatory activities of bacteria in probiotics (Paturi *et al.* 2008). Most factors that stimulate the bird's immune system affect the H: L ratio. Previous research has shown that dietary supplementation with the probiotic PrimaLac reduces the H:L ratio and increases the antibody titer against the Gambro vaccine (Nayebpor *et al.* 2007). These results were also observed in the present experiment. Interactions

of probiotics and feed form on anti-SRBC antibody titer do not seem to have a definite trend.

Conclusion

The results from this study indicated that pellet feeds improved performance by approximately 9% at the end of the rearing period compared with mash diets. However, the probiotic *L. acidophilus and B. subtilis* used in the mash diet may serve as an alternative to the antibiotic. Overall, pellet and mash diets containi *B. subtilis* improved the performance of broiler chickens through positive changes in intestinal morphology, while chickens fed *with L. acidophilus* showed positive effects only in mash diets.

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