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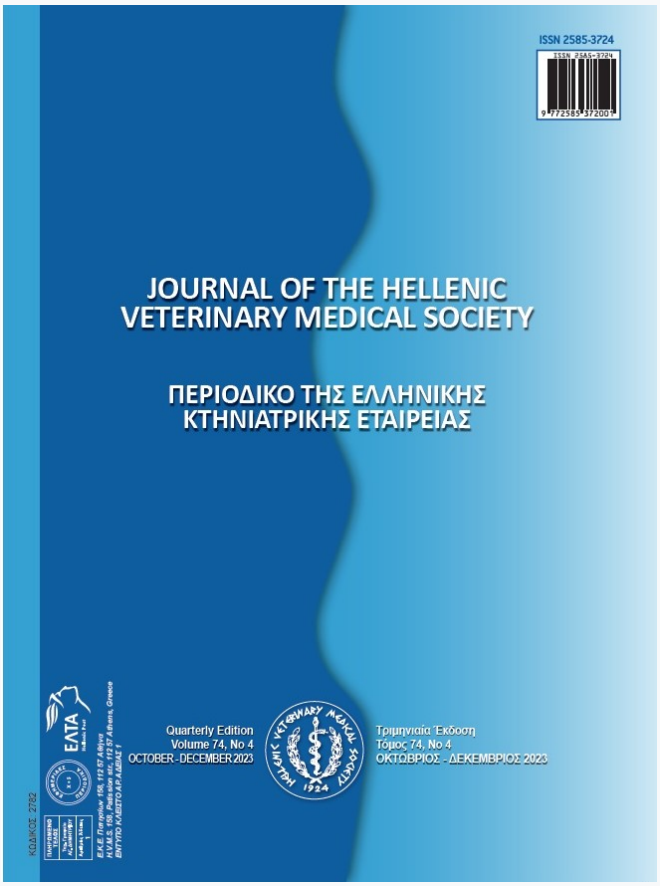
Journal of the Hellenic Veterinary Medical Society

Vol 74, No 4 (2023)



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doi: 10.12681/jhvms.30558

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To cite this article:

Jouybari, M., Sadeghi, A., Jouzani, G., Chamani, M., & Aminafshar, M. (2024). Effects of Bacillus subtilis on the immune parameters, intestinal morphology and mucin gene expression in broilers exposed to Salmonella enterica challenge . *Journal of the Hellenic Veterinary Medical Society*, *74*(4), 6399–6410. https://doi.org/10.12681/jhvms.30558

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J HELLENIC VET MED SOC 2023, 74 (4): 6399-6410
ΠΕΚΕ 2023, 74 (4): 6399-6410

Research article
Ερευνητικό άρθρο

Effects of *Bacillus subtilis* on the immune parameters, intestinal morphology and mucin gene expression in broilers exposed to *Salmonella enterica* challenge

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ABSTRACT: The objective of this study was to evaluate the effect of the probiotic *Bacillus subtilis* supplementation on bacterial population, morphometry and mucin gene expression of intestine, and on immune response in *Salmonella enterica* challenged broilers. Treatments were: 1) the negative control (no probiotic-no challenging); 2) probiotic treated (no challenging); 3) the positive control (no probiotic-salmonella challenging), and 4) Salmonella-challenged chicks receiving probiotic supplement. Salmonella infection resulted in significant decrease (P<0.05) in the relative weight of thymus. In challenged birds, dietary addition of probiotic increased (P<0.05) the relative weights of bursa and thymus. Population of lactic acid bacteria was higher (P<0.05) in probiotic supplemented groups as compared to the negative control or challenged chickens. Challenged chickens had the lowest (P<0.05) count of goblet cells and those receiving probiotic had the highest goblet cells count. The expression of mucin 2 gene was higher (P<0.05) in group receiving probiotic supplement as compared with other treatments. Difference for the relative expression of mucin gene was not observed in challenged chickens and the negative control (P>0.05). In conclusion, the dietary inclusion of *Bacillus subtilis* could alleviate the negative effects of *Salmonella enterica* infection on intestinal cells and bacterial population, and could improve the growth and development of immune organs and function in infected broilers.

***Keywords*:** antibody titers; bacterial population; Immune response; Intestinal morphology; Probiotics

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Date of initial submission: 14-06-2022
Date of acceptance: 05-07-2023

INTRODUCTION

The first exposure of chicks with microbes in the hatchery and farm may include pathogens in the digestive tract, which attach to the gut wall and multiply by fermentable carbohydrates. Absorption sites are blocked by colonization of pathogen bacteria and finally nutrient absorption decreases (Sikandar et al., 2020). *Salmonella* spp. a pathogen which can infect animal and humans, is prevalent throughout the commercial hatchery, poultry farms and products (Abudabos et al., 2017; Hayashi et al., 2018). *Salmonella* spp. has been isolated from chicken meat and eggs, and can cause dangerous consequences in human health (Knap et al., 2011; Hayashi et al., 2018; Sikandar et al., 2022; Zhou et al., 2022). In the United States, Centers for Disease Control (CDC, 2022) reported that 142,000 people are infected with *Salmonella enteritidis* and 30 people died during the year of 2022, specifically from chicken eggs. Approximately 16 million cases of inflammatory fever and 1.3 million cases of gastroenteritis occurred annually due to infection with *Salmonella* spp. and three million deaths are reported from salmonellosis worldwide (Wibisono et al., 2020). Hence, it is crucial to monitor and control the infection of *Salmonella* spp. in the farm level and also during chicken meat processing chain.

One Health is an integrated approach to create a sustainable balance in the health of humans, animals and ecosystems, focusing on disease control by establishing links between these fields. In recent years, One Health has become very important as human population increased, severe changes in climate, and animal transportation increased. These factors cause the spread of endemic or emerging diseases that transmitted between human and animals. Salmonellosis is a disease caused by *Salmonella* spp. in human and animals and is considered a zoonotic disease.

In the past, antibiotics were used in the diet of animals as the solving method for preventing pathogenic diseases, but todays its application is prohibited, because of their residues in animal products, microbial resistance and other negative effects (Reid and Friendship, 2002; Stein and Kil 2006). Due to the extensive use of antimicrobial agents in the chicken supply chain, *Salmonella* spp. has become resistant to various antimicrobial compounds such as streptomycin, chloramphenicol, ampicillin, sulfonamides, and tetracyclines (Ray, 2004). *Salmonella* spp. attaches to intestinal villi by binding to proteins called adhesins and forms a colony (Beachey, 1981). Therefore, the best and most effective way to prevent infection is to prevent *Salmonella enteritidis* from binding to intestinal epithelial cell receptors (Gaggia et al., 2010).As a consequence, antibiotic alternatives such as beneficial microorganisms termed probiotics has been considered to include in water or diet of animals (Reid and Friendship, 2002; Abudabos et al., 2019; Lipiński et al., 2021).

The use of probiotics which are classified in term of "Generally Regarded as Safe" has been shown to increase the beneficial bacteria population and reduce the growth of pathogenic bacteria in the gastrointestinal tract (Hayashi et al., 2018; Sikandar et al., 2022), also enhance the humoral antibody response to antigens in young animals, and can result in the production of cytokines and immunity modulation (Schierack et al., 2007; Gao et al., 2017; Ma et al., 2018; Ciurescu et al., 2020; Kim et al., 2022). The action modes of probiotics, which affect gut function and health in poultry, include of inhibiting colony forming of pathogens, maintaining a normal micro-flora and beneficial microbial population by competitive exclusion and antagonism (Yurong et al., 2007; Oh et al., 2017; Sikandar et al., 2020), and improving gut mucin composition and amount (Tsirtsikos et al., 2012). *Bacillus subtilis* is widely used as a probiotic supplement in industrial poultry feeding, because this strain of bacillus is safe and highly resistant to feed processing conditions, and also has a long shelf life and positive effects on the beneficial microflora population of the digestive tract. (Zhenhua et al., 2017; Sikandar et al., 2020). Overall, the use of *Bacillus subtilis* in the diet of broilers improves the survival rate, daily weight gain, and feed conversion ratio. Meanwhile, the immune parameters and antioxidant activities of chickens were significantly improved (Nair et al., 2021).

Although there are many studies on the immune enhancement by oral administration of probiotics (Majidi-Mosleh et al., 2016; Abd El-Hack et al., 2021), there is limited information on the effects of probiotics or infection, alone or in combination, on the antibody titers, gut morphology and the relative expression of mucin gene in broilers infected with *Salmonella* spp. Therefore, this study was conducted to determine the effect of *Bacillus subtilis* on the bacterial population, gut morphometry, the relative expression of mucin gene, and on the antibody titers against Newcastle virus and cell-mediated immune response in broilers, and the protection it can provide to the broilers against a *Salmonella enterica* challenge.

MATERIAL AND METHODS

This experiment was conducted in late April and early June 2021 in the research poultry farm of Nuclear Agriculture Research School (Karaj, Iran). The guidelines of Iranian Council of Animal Care (1995) were considered for executing all of the procedures. To comply with ethical principles, the chickens were sacrificed by cervical dislocation (Close et al., 1997). The approval of this research was obtained from the doctoral thesis committee of Islamic Azad University.

Salmonella culturing and challenging

Salmonella enterica serovar Enteritidis (ATCC® 9150™) was prepared as freeze-dried form from Iranian Biological Resource Centre (IBRC, Tehran, Iran). Briefly, Salmonella inoculum was transferred to tryptic soy broth (Acumedia Manufacturers Inc., Baltimore, MD, USA) and cultured at 37 °C for 8 h. Then, media was passed to fresh tryptic soy broth for three incubation periods, and after counting it was diluted to 5×10^7cfu/ml (Vila et al., 2019). The numbers of colony-forming units (cfu) were determined through decimal dilution series which performed in sterile buffered 0.9% peptone water (pH 7.2). For determination of cfu, diluted medium (0.1 mL) was transferred in Petri dishes containing Shigella-Salmonella agar and incubated at 37 °C for 24 h, then cfu per mL were calculated. Days 7,12 and 24 of age, 0.2 mL of suspension containing 5×10^4cfu of *Salmonella enterica* was orally gavaged to chicks in the challenged groups. Non-challenged chickens were received 0.2 mL sterile buffered peptone water by oral gavage. Pens of challenge chicks had two meters distance from each other, and these pens were also isolated from non-challenged pens. Cloacae samples (three swabs from each pen) were collected from challenged and non-challenged chickens on day 39 of age and were cultured to monitor the Salmonella infection. Cultures were assessed for the presence or absence of Salmonella, which grow as red colonies (Jose et al., 2008).

Experimental birds and treatments

Four hundreds broiler chicks (Ross 308) of the same breeder stock were purchased from a commercial hatchery. Forty chicks were removed on arrival for Salmonella monitoring, and also because of the weight below or over the range. Three hundred and sixty chicks (body weight= 40 ±2 g) were randomly divided into four treatment groups and placed in the isolated pens with identical size (1.8 × 1.5 m) covered with wood shaving, which sterilized by electron beam irradiation (beam energy of 10 MeV) at dose of 30 kGy (AEOI, Yazd center, Iran). Each treatment group had six replicates with 15 chicks per each. The treatments were considered as: 1) negative control (basal diet without probiotic inclusion and Salmonella challenging); 2) chicks fed diet containing probiotic without Salmonella challenge; 3) positive control or chicks fed basal diet and Salmonella challenged at days 7, 12 and 24 of age (5×104 cfu/bird), and 4) chicks fed diet containing probiotic and Salmonella challenged at days 7, 12 and 24 of age (5×10^4cfu/bird). Probiotic sample, Gallipro®, which contains *Bacillus subtilis* (8×10^7cfu/g) was purchased from Biochem Company (Zusatzstoffe GmbH, Germany) and used in the diets at 0.2 g/kg ration based on producer recommendation. During the experiment, all chicks had free access to fresh water and feed *ad libitum*. The diets were formulated for starter phase (1-10 days), grower phase (11-28 days) and finisher phase (29-42 days) based on corn grain-soybean meal without addition of antibiotics or other additives (Table 1). In the first days, house temperature was set at 33 °C and decreased weekly to 20 °C until the end of the experiment. Light program was 23 hours during the first week, and thereafter reduced to 19 h based on the Ross 308 management recommendations.

Vaccination and serology

On days 9 and 14 of age, the vaccination against Newcastle virus product of MERIAL, 17 rue Bourgelat 69002 (Lyon, France) (Batch no.: L395281) was done based on region veterinary recommendation. On days 28 and 42 of age, blood samples were collected from wing vein using sterile syringes. Samples (3.0 mL) were collected from 12 birds per treatment (two birds per pen). After collecting, 880 µL of blood was transferred to sterile plastic tube with 120 µL sodium citrate solution, and immediately content mixed. The tubes were placed in ice and sent to laboratory for counting white blood cells (WBC) based on the method of Wright's stain. The rest of blood sample was transferred in sterile glass tube and kept at 4 °C, and after 5 h tubes were centrifuged at 2000 × *g* for 15 min. Serum was separated and then inactivated at 56 °C for 30 min, and then stored at -20 °C until measurement of antibody titer against Newcastle virus. Hemagglutination-inhibition test was used for measurement of antibody titer against Newcastle virus. The widal test was performed to quantitatively evaluate the amount of antibody produced in the serum

Table 1. Ingredients and chemical composition of experimental rations [a]

Ingredients (%)	Starter Days 1-10	Grower Days 11-28	Finisher Days 29-42
Yellow corn	57.21	59.28	63.75
Soy bean meal (48% CP)	32.98	30.00	25.20
Fish meal	3.00	2.36	2.50
Pure starch	--	--	--
Soy bean oil	2.00	4.00	4.00
DCP	1.54	1.39	1.13
CaCO3	1.10	1.02	0.92
Salt	0.36	0.41	0.41
Mineral Premix[b]	0.20	0.20	0.20
Vitamin premix[c]	0.25	0.25	0.25
Methionine	--	0.08	0.03
Lysine	0.17	0.09	0.30
Celite	1.19	0.92	1.31
Chemical composition			
ME (kcal/kg)	2890	3050	3100
Protein (%)	21.0	19.5	18.00
Ether extract (%)	4.79	6.78	6.91
Linoleic acid (%)	2.41	3.47	3.55
Lysine (%)	1.25	1.21	1.24
Methionine (%)	0.55	0.43	0.36
Calcium (%)	1.01	0.90	0.80
Phosphorus (%)	0.50	0.45	0.40

[a]On an as-fed basis

[b] The mineral mix composition was as follows (amount in 10 g): 0.5 g Mg, 0.3 g S, 1.0 g Na, 1.6 g Cl, 6.0 mg Cu, 0.2 mg I, 45.0 mg Fe, 59 mg Mn, 0.2 mg Se and 29 mg Zn.

[c] The vitamin mix composition was as follows (amount in 10 g): 4000 IU vitamin A palmitate, 1000 IU cholecalciferol, 50 IU vitamin E acetate, 0.5 mg menadione sodium bisulfite, 0.2 mg biotin, 10 μg cyanocobalamin, 2 mg folic acid, 30 mg nicotinic acid, 16 mg calcium pantothenate, 7 mg pyridoxine-HCl, 6 mg riboflavin, 6 mg thiamin HCl.

against Salmonella at day 17 of age, 10 days after first infection with Salmonella. Levels of Immunoglobulin G (IgG) were assessed by using chicken specific commercial ELISA kits (CUSABIO Biotech Co., China) according to company's instruction.

On day 28 of age, 2 chicks per each replicate were randomly injected 0.1 mL of 5% SRBC (sheep red blood cell) solution in breast muscle. On day 28 of age, blood samples were collected from the wing vein, after clothing centrifuged and sera samples were poured in sterile glass tubes. The hemagglutination test was used to measure the antibody titer response against SRBC (Witkamp and Olson, 1963).

On day 28 of age, the test of cell-mediated immune response was done in two chickens per pen based on Corrier and DeLoach (1990) using cutaneous basophil hypersensivity test (CBH). Phytohemagglutinin P was prepared from Gibco Co. (Gibco, Invitrogen Corporation, Scotland, UK).

Bacterial populations in ileal contents

On days 28 and 42 of age, two chickens per each replicate were slaughtered after cervical dislocation. Ileal samples were collected to assay lactic acid bacteria (LAB) and *Escherichia coli* populations. The populations of *Escherichia coli* and LAB were estimated based on log 10 of colony forming units (cfu) per gram of ileal content. *Escherichia coli* was cultured at 37 °C for 24 h on MacConkey agar (Merck, Germany) and LAB was enumerated on MRS agar (Merck, Germany) after incubation at 37 °C for 72 h under anaerobic condition (Witkamp and Olso, 1963).

Morphological analyses

On day 28 of age, jejunal samples (3 cm) of two chickens per each replicate were taken and floated in buffered 10% formalin solution for morphometrical analyses and the count of goblet cells. Tissue preparation was performed based on the method of Iji et al. (2001). The counts of goblet cells were performed in the scale of 300 μm of epithelium length.

RNA isolation and Real-time PCR assessment

On day 28 of age and the end of the experiment, a 3-cm segment from the midpoint of the ileum of sacrificed birds (six birds per treatment) was removed, rinsed in phosphate buffered saline, kept in dry ice and then in freezer -70 °C. Total RNA was extracted using a commercial kit (RNX-PLUS, Sina-Clon Co., Tehran, Iran). The cDNA was synthesized according to the kit guideline manufactured by Vivantis Company (Selangor Darul Ehsan, Malaysia).

The relative gene expression of mucin 2 (muc2) in the ileal of chickens was quantified by relative real time PCR method. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control or housekeeping gene. The primers MUCIN2 F (5'-CAGCGTTAACACAGGGCTTA-3') and MUCIN2 R (5'-GCAGCAGACGTTGATCTCAT-3') were used for amplification of the target muc2 gene, and the primers GAPDH-F (5'-TCTCTGGCAAAGTCCAAGTG-3') and GAPDH-R (5'-TGCCCATTGATCACAAGTTT-3') were used for amplification of the GAPDH gene (Valizade et al., 2014). Real time PCR was performed using SYBR Green PCR Master Mix Kit (Takara SYBR ® Premix Ex TaqTM II, Kyoto, Japan). Real Time PCR thermo cycler (Applied Biosystems, USA) was used, and the temperature condition of reaction were as: the initial denaturation (95 °C for 30 s), annealing (60 °C for 34 s), followed by extension (72°C for 1 min). Real-time PCR data were normalized and statistically analyzed using LinRegPCR software (Ramakers et al., 2003). Results are expressed as fold change relative to the negative control chickens (Majidi-Mosleh et al., 2014).

Statistical analysis

Statistical analyses were done using the GLM procedure of SAS for Windows version 9.4 (SAS, 2010). The test of Kolmogorov-Smirnov was applied to evaluate the data normality before the analysis of variance. The mean comparison was done using Tukey test. Statistical differences were declared at $P < 0.05$.

RESULTS

As presented in Table 2, at day 28 of age, the relative weights of two tissue (spleen and bursa) were not affected, but thymus relative weight affected by treatments. Chickens in Salmonella challenged group had lower the relative weight of thymus than the negative control. Challenged chickens receiving probiotic *Bacillus subtilis* had no differ ($P>0.05$) for the relative weight of thymus compared to the negative control. In another time period (day 42 of age), differences ($P<0.05$) were observed among treatments for the relative weights of immune tissues. Chickens in the Salmonella challenged group had lower ($P<0.05$) the relative weights of the thymus by 17% as compared to the negative control. In challenged birds, dietary addition of probiotic increased ($P<0.05$) the relative weights of spleen, bursa and thymus. Challenged chickens receiving probiotic in the diet had higher ($P<0.05$) the relative weight of immune tissues as compared to the positive control.

As shown in Table 3, there was no differ ($P>0.05$) for white blood cells counts at day 28 of age, but a difference ($P<0.05$) observed at day 42 of age. In the latter period, chickens in the positive control and those receiving dietary probiotic had higher ($P<0.05$) white blood cells count as compared to chickens in the negative control ($P<0.05$). In the two periods, chickens in the positive control had the lowest ($P<0.05$) lymphocyte percentage as compared with the other treatments. At day 28 of age, a significant difference ($P<0.05$) exist for lymphocyte percentage between the negative control and chickens receiving probiotic with or without challenging. This finding was replicated at day 42 of age, but a significant decrease ($P<0.05$) in lymphocyte percentage was found in challenged chickens fed probiotic as compared with non-challenged chickens fed probiotic or the negative

Table 2. The relative weights* of immune tissues of chickens at days 28 and 42 of age
* As g per kg body weight

Treatments	day 28 of age			day 42 of age		
	Spleen	Bursa	Thymus	Spleen	Bursa	Thymus
The negative control	1.35	1.69	0.318^{a}	1.08^{ab}	1.35^{b}	0.231^{b}
Probiotic treated	1.38	1.59	0.321^{a}	1.17^{a}	1. 51^{a}	0.275^{a}
The positive control	1.24	1.55	0.258^{b}	0.94^{b}	1.33^{b}	0.193^{c}
Challenged and probiotic treated	1.28	1.57	0.263^{b}	1.05^{ab}	1.49^{a}	0.228^{b}
p-value	0.27	0.55	0.05	0.05	0.02	0.04
SEM	0.109	0.108	0.045	0.069	0.049	0.038

^{a,b,c} Within the same column, means with different superscripts are significantly differ ($p < 0.05$).

Table 3. Effects of experimental treatments on total and differential counts of white blood cells* at 28 and 42 d of age.

Treatments	WBC × 10^3	L (%)	H (%)	H/L
day 28 of age				
The negative control	26.3	72.5^{b}	27.5^{b}	0.37^{b}
Probiotic treated	27.1	74.9^{a}	25.1^{c}	0.33^{c}
The positive control	26.7	69.2^{c}	30.8^{a}	0.44^{a}
Challenged and prebiotic treated	26.6	75.7^{a}	24.3^{c}	0.32^{c}
p-value	0.35	0.03	0.04	0.03
SEM	1.78	2.51	1.92	0.032
42 d of age				
Negative control	24.5^{b}	79.5^{a}	18.5^{b}	0.23^{c}
Probiotic treated	26.2^{ab}	79.1^{a}	18.0^{b}	0.22^{c}
Positive control	28.9^{a}	68.4^{b}	24.2^{a}	0.35^{a}
Challenged and probiotic treated	26.3^{ab}	71.3^{b}	21.3^{ab}	0.29^{b}
p-value	0.02	0.05	0.001	0.001
SEM	0.81	1.85	1.52	0.026

^{a,b,c} Within the same column, means with different superscripts are significantly differ ($p < 0.05$).
* WBC: white blood cells count; L: Lymphocyte; H: Heterophil; H/L: Heterophil to Lymphocyte ratio; E: Eosinophils; M: Monocytes

control. At two time periods, chickens in the positive control had the highest (P<0.05) heterophil percentage and heterophil/lymphocyte ratio as compared to the other treatments. Challenged chickens receiving probiotic had lower (P<0.05) heterophil percentage and also the heterophil/lymphocyte ratio compared to the chickens in the positive control.

The mean of antibody titers (log 2) against Newcastle virus, CBH and SRBC titers at days 28 and 42 of age are presented in Table 4. At day 28 of age, there were no differences (P>0.05) for antibody titers against Newcastle virus, but at day 42 of age, a difference (P<0.05) was observed between the negative control and probiotic treated chickens. Chicken in the positive control had no significant differ (P>0.05) for antibody titers against Newcastle virus compared to chickens in the negative control. Challenged chickens fed probiotic had higher (P<0.05) CBH tests as compared to chicks in the negative control. The highest (P<0.05) means were observed in probiotic treated chickens at the both periods. Salmonella challenging and probiotic supplementation had no significant effect (P>0.05) on SRBC titers, however these treatments had numerically higher means as compared to the negative control.

Effect of treatments on the populations of lactic acid bacteria and *Escherichia coli* is presented in Table 5. At day 28 of age, the population of lactic acid bacteria was significantly higher (P<0.05) in chickens receiving probiotic supplement as compared to

Table 4. Cell-mediated immune response to PHA-P, and antibody titer response against NDV and SRBC

Treatments	Antibody titer (log_2) against NDV[2]		CBH[1] test (mm)		Antibody titer (log_2) against SRBC[3]	
	Day 28	Day 42	Day 28	Day 42	Day 28	Day 42
The negative control	3.3	3.3^{b}	0.483^{c}	0.532^{b}	2.3	2.5
Probiotic treated	2.6	5.7^{a}	0.783^{a}	0.892^{a}	2.8	3.3
The positive control	3.8	4.4^{ab}	0.645^{b}	0.585^{b}	2.6	3.5
Challenged and probiotic treated	3.3	5.3^{a}	0.697^{b}	0.507^{b}	2.7	3.3
p-value	0.09	0.05	0.002	0.05	0.12	0.15
SEM	0.41	0.48	0.053	0.043	0.33	0.59

1. Cutaneous basophil hypersensitivity reaction to phytohemagglutinin P injection into the toe web skin, and measured as skin swelling before and after injection
2. Antibody response against Newcastle disease virus.
3. Antibody response against Sheep Red Blood Cell.
^{a,b,c} Within the same column, means with different superscripts are significantly differ ($p < 0.05$).

the negative control or the positive control, but there was no difference among treatments at day 42 of age (P>0.05). At the two time periods, *Escherichia coli* population was affected (P<0.05) by treatments. Chickens in the positive control had significantly the highest (P<0.05) *Escherichia coli* population in the ileum, and the lowest (P<0.05) population was for groups receiving probiotic supplement.

The means of goblet cells counts, villus height of jejunum, crypt depth, and ratio of villus height/crypt depth are presented in Table 6. Chickens in the positive control had the lowest (P<0.05) goblet cells count and those receiving probiotic supplement had the highest (P<0.05) count. Differences (P<0.05) were observed among treatments for villus height, crypt depth, and the ratio of villus height/crypt depth. Chicken receiving *Bacillus subtilis* had greater villus height and smaller crypt depth and higher (P<0.05) villus height/crypt depth ratio compared to the negative control group (P<0.05). In contrast, chickens in the positive control had smaller (P<0.05) villus height and greater (P<0.05) crypt depth and lower (P<0.05) villus height/crypt depth ratio compared with the negative control. Probiotic supplementation in diet of challenged chickens had no significant effect on goblet cells count and villus height, but resulted in a decrease of crypt depth and an increase of height/crypt depth ratio.

The effects of treatments on the relative gene expression of mucin 2 in the ileum of chickens at days 28 and 42 of age are presented in Table 7. At day 28 of age, the relative expression of mucin 2 gene was higher (P<0.05) in group receiving probiotic supplement as compared with other treatments. Difference was not observed for the relative gene expression in the positive control and the negative control (P>0.05). Up-regulation of mucin 2 gene was observed (P<0.05) at day 42 of age in chickens receiving probiotic supplement, then in challenged chickens fed dietary probiotic supplement as compared with the negative control and the positive control. There was no differ for gene expression in challenged chickens and the negative control (P>0.05).

Table 5. Effect of treatments on intestinal lactic acid bacteria and E. *coli* population of broiler chickens (log 10 cfu/g of content)

Treatments	*lactic acid bacteria*		*E. coli* population	
	Day 28	Day 42	Day 28	Day 42
The negative control	2.30 [b]	3.37	5.98 [b]	6.06 [c]
Probiotic treated	2.61 [a]	3.54	5.64 [c]	5.81 [c]
The positive control	2.26 [b]	3.35	6.31 [a]	7.77 [a]
Challenged and probiotic treated	2.57 [a]	3.43	5.71 [c]	6.65 [b]
p-value	0.04	0.10	0.05	0.05
SEM	0.35	0.41	0.32	0.39

[a,b,c]Means with different superscripts within the same column differ significantly (*P*<0.05).

Table 6. Goblet cells counts (n per 300 μm of epithelium length), villus height, crypt depth and their ratio in the jejunum of chickens

	Goblet cell counts	Villus height (μm)	Crypt depth(μm)	Villus height/Crypt depth
The negative control	17.1 [ab]	880 [b]	175 [b]	5.02[a]
Probiotic treated	19.0 [a]	985[a]	164 [b]	6.01[a]
The positive control	15.9 [b]	867 [b]	210 [a]	4.12[b]
Challenged and probiotic treated	17.8 [ab]	938[ab]	169 [b]	5.55[a]
p-value	0.033	0.045	0.004	0.002
SEM	0.62	34.7	6.5	0.266

[a,b] Means with different superscripts within the same column differ significantly (p<0.05).

Table 7. Effects of treatments on the relative expression of mucin 2 gene

Treatments	day 28	day 42
The negative control	1.00 [b]	1.00 [c]
Probiotic treated	1.86 [a]	2.26 [a]
The positive control	0.98 [b]	1.08 [c]
Challenged and probiotic treated	1.09 [b]	1.81 [b]
p-value	0.04	0.05
SEM	0.138	0.141

[a,b] Means with different superscripts within the same column differ significantly (p<0.05).

DISCUSSION

Salmonellosis is an important problem in the poultry production and human health in the world (Saleem et al., 2016). The application of antibiotic inhibited and probiotics play as an important replacer to combat the Salmonella invasion. Probiotics improve performance and protect the epithelium cells from pathogen invasion (Hayashi et al., 2018; Knap et al., 2011; Sikandar et al., 2022). *Bacillus subtilis*, which is included in diet as feed additive, has become popular in the world due to the enhancement of the immune response and the positive effects it has on gut health (Dersjant-Li et al., 2014). Earlier studies (Schierack et al., 2007; Knap et al., 2011; Lourenco et al., 2012; Abudabos et al., 2019; Vimon et al., 2020; Sikandar et al., 2022), the positive effects of this probiotic in healthy chickens was reported. However, information about the preventive effects of *B. subtilis* in Salmonella-challenged broilers is scare. Therefore, the present study was done to evaluate the influence of *Bacillus subtilis* on broiler immune response, intestinal morphology and the relative gene expression of mucin in Salmonella-challenged broilers.

The immune system functions are closely related to the immune tissue health and development. At day 28 of age, the relative weight of thymus, and at day 42 of age, the relative weights of bursa and thymus were changed by Salmonella-challenging as compared with the negative control. Salmonella-challenging had no significant effect on spleen weight at days 28 and 42 of age. A study showed that during challenging corticosteroid production increase, which could induced immune tissue atrophy (Hu et al. 2009). Low bursa and thymus weights in challenged chickens is an indicator of low immune cell proliferation, low immune activity and antibody production, because these organs are major lymphoid organs in poultry. Differences in the relative weight of spleen were not found among treatments, as reported elsewhere (Saleem et al., 2016). Hu et al. (2009) showed that cortisol causes rapid degeneration of lymphatic tissues, including the bursa of Fabricius, due to apoptosis. An interesting study showed that microbial challenging could increase the level of interlukin-1 production, which results in an increase in serum corticosterone concentration (Post et al., 2003), and finally, it causes retardation of growth and weight loss of immune organs. In the present study, the dietary supplementation of *Bacillus subtilis* increased the relative weights of the immune organs in non-challenged and challenged chickens. The result of present study is in line with the report of Sikandar et al. (2020) who reported that the relative weights of thymus and bursa decreased in the salmonella infected group, but the weights increased with *Bacillus subtilis* supplementation. The mechanism of probiotic effect on the weight of immune organs is related to the reduction effects on cortisol concentration. It has been reported that probiotic supplementation eliminates the adverse effects of cortisol injection and improves lymphocyte density in the bursa of Fabricius (Hu et al., 2009). Moreover, increasing in the immune organs weight by probiotic supplementation may also be related to induce of immune organ to produce the antibodies, and an increase in the numbers of antibodies which produced to combat the disease invasion and condition (Shabani et al., 2019). This probiotic effect is a direct effect that the probiotic has on the lymphatic tissue, and its indirect effects are the changes that the probiotic causes on the microbial population of the digestive tract.

In the present study, significant differences observed in the antibody titers against Newcastle virus and SRBC, and also CBH test between non-challenged probiotic treated chickens and the negative control. Our results are in consistence with studies (Khaksefidi and Ghoorchi, 2006; Seifertet al., 2011), who reported that dietary inclusion of probiotic in non-challenged chickens had significant influence on the antibody titers against SRBC. In a study (Sikandar et al., 2020) demonstrated that *Bacillus subtilis* treated group exhibited a higher antibody titer against the Newcastle disease virus compared to the control group. In contrast, a number of other studies (Haghighi et al., 2005; Kim et al., 2006) reported probiotics treatment had no significant effect on immunity in broilers. These discrepancies among different studies concerning probiotic effects on immunity can be related to class of the antigen used, age of broilers, the probiotics included, and the probiotics doses used. As seen in Table 4, antibody titers against Newcastle was not significant at day 17 of age and was differ at day 27 of age and also a comparison between two antigens used (Newcastle virus and SRBC) for day 27 of age.

Based on the results of the present study, Salmonella infection had a negative effect on the antibody titer and sensitivity of broilers through a retardation of immune organs weights and white blood cell differentiation. Dietary addition of *Bacillus subtilis* had significant effect on the immune organs weights and antibody titers of chickens in non-challenged chickens, but it had no significant effect in challenged broilers.

Bacillus subtilis can be effective on immune parameters by stimulation of animal innate immune responses and production of immunomodulation materials, reducing pro-inflammatory cytokines and increasing anti-inflammatory cytokine (Gaggia et al., 2010). A proposed mechanism for the effects of probiotics is the stimulation of cytokine secretion from cells of the immune system, hence it has been suggested that some of the effects of probiotics are exerted through cytokines (Alkhalf et al. 2010).

In this study, white blood cells and the heterophil/lymphocyte ratio was increased in challenged chickens compared with the negative control and dietary inclusion of probiotic had no effect on WBC, but decreased the heterophil/lymphocyte ratio. Increases in the count of WBC is an indicator of inflammation process or infection. Our results are inconsistence with Rahimi and Khaksefidi (2010), who reported dietary supplementation of probiotic significantly increased WBC count of broilers in comparison to the control group.

A study (Swaggerty et al., 2006) reported that interleukin 1β is an important factor in the inflammatory response and its synthesis is increased by infections. Inflammatory factors could influence on hypothalamic which increase the production of corticotrophin releasing factor (Post et al., 2003) and finally increase the level of serum corticosterone. Corticosterone could decrease the functions of lymphocyte, and also the count of white blood cells. It has been found that corticosterone is an immunosuppressive hormone, and inhibit the antibodies production and actions (Post et al., 2003). Also it was reported that corticosterone could increase the heterophil/lymphocyte ratio and has negative effect on immune organ growth (Müller et al., 2011).

Subcutaneous injection of PHA-P stimulates T lymphocytes and creates thymus-dependent response, and is used in cellular immunity studies as a measure to determine the activity of T cells. After injection, PHA-P adheres to the surface of T cells through glycoproteins. These cells produces lymphokine which increase the vascular permeability and also invade of leukocytes in injection site (Grasman 2010), therefore skin became swell because of leukocytes presence and fluid filtration. In the present work, the thickness of injection site in challenged broilers was lower than control group which indicated lower cell-mediated immunity. The cell-mediated immune response after PHA-P injection in broilers receiving *Bacillus subtilis* in non-challenged broilers was the highest, which could stimulate an immune response to acute infections. In healthy chickens, studies (Dersjant-Li et al., 2014; Tarradas et al., 2020) showed that *B. subtilis* could modulate normal microflora, regulate the functions of systemic immuno-modulatory, and in enhance the resistance of broilers infected with coccidiosis.

Dietary inclusion of *Bacillus subtilis* reduced the Salmonella and *Escherichia coli* loads of cecum contents, which is in line with the finding of Knap et al. (2011). An interesting study (Lourenco et al. 2012) reported that oral feeding of *Bacillus subtilis* could decrease significantly Salmonella counts in chicken gut. A study (La Ragione and Woodward, 2003) showed that *Bacillus subtilis* changes the microbiota population through competitive elimination and ultimately reduces the recovery of Salmonella in the cecum of chickens. An interesting study (Baltzley et al. 2010) showed that dietary inclusion of *Bacillus subtilis* caused a 13-35% reduction in the presence of Salmonella in the swab compared to the control group (Knarreborg et al., 2008).

Studies (La Ragione and Woodward, 2003) have shown that *Bacillus subtilis* is an agent with successful competitive exclusion. It was reported that *Bacillus subtilis* supplementation resulted in an increase of the population of lactic acid bacteria, which has beneficial effects on gut health and may lower the intestinal pH (Knarreborg et al., 2008). By acidifying the intestinal contents, the growth of pathogenic and detrimental bacteria is prevented (e.g., Salmonella; Van Immerseel et al., 2006). *Bacillus subtilis* might protect the intestine epithelium of broilers against pathogen invasion through increase in bacterial diversity and functionality of epithelium metabolism and cells. Based on the report of (Gaggia et al., 2010) reduce in the functional diversity may suppress in the commensal-microbiota-mediated homeostasis.

Challenged chickens had lower jejunal villus height and goblet cells compared with the control. This reduction in the villi size could be attributed to feed mal-digestion and nutrient mal-absorption, because of inflammation of the intestinal villi caused by Salmonella (Sikandar et al., 2017).

Chickens receiving *Bacillus subtilis* supplement had greater villus height and counts of goblet cells than the negative control. In an interesting study (Abudabos et al., 2019), the inclusion of *Bacillus subtilis* in the diet increased energy supply mechanisms

such as carbohydrate digestion, transport and metabolism in intestinal cells, leading to a stable microbiota population as well as protection of intestinal integrity.

One of the mechanism of action of *Bacillus subtilis* is to improve the intestinal digestive capacity by releasing a wide range of digestive enzymes such as amylase, protease and lipase activity. In addition, with modulation of intestinal microbiota and intestinal microstructures can improve the conditions of digestion and absorption of nutrients and improve the growth of intestinal villi (Zhang et al. 2016; Gao et al., 2017).

In the study of Dersjant-Li et al. (2014), Salmonella-challenged group has the lowest intestinal villi height. An increase in the size of villi in group receiving probiotic supplement may be related to high nutrient absorption and cellular functionality and it is likely that the number of microbiomes increases in sympathetic association with the mucosa (Awad et al., 2016). Another mechanism of action of probiotics on the health of the intestine and its morphological parameters is to improve the conditions for microbial fermentation and the production of short-chain fatty acids in the small intestine, which in turn provides the energy needed for the growth of enterocytes. The increase in the production of volatile fatty acids due to the use of probiotics may also cause more proliferation of epithelial cells in the intestine of chickens, and as a result, the size of the villi and the relative weight of the intestine increase (Gao et al. 2017). Probiotic supplementation may be reduced the epithelium inflammation and it provides a thinner area as well as a bigger area for the absorption of available nutrients.

In the present study, Salmonella-challenged birds had deeper crypts, which is in agreement with finding of Yang and Liao, (2019) who demonstrated that rapid and higher tissue turnover occurs following mucosal tissue renewal to maintain maximal absorptive surface. According to Sikandar et al. (2017), the epithelium covering the villi is destroyed in challenged birds, thus the villi are reduced in size and appear ulcerated. This events ultimately reduces the length of the villi and reduces the available surface area for nutrient absorption. A study (Sharma and Schumacher, 1995) reported that microbiota could affect the number of intestinal goblet cells. In an interesting study (Rahimi et al., 2011), turkey poults receiving probiotic supplement had higher goblet cell counts in the small intestine compared with the control group. Goblet cells can protect the epithelial cells against the invasion of harmful and pathogenic bacteria by secreting mucin (Rahimi et al., 2011). The level of mucin secretion is positively correlated with the counts of goblet cells. During pathogen invasion and water or food contamination, goblet cells produce a thick mucus layer that can prevent the penetration of the pathogen into the intestinal cells. Broilers receiving probiotic supplement had higher the relative gene expression of mucin as compared with the negative control. Previously, increases in the mucin synthesis and secretion have been reported to occur with probiotic supplements consisting mainly of Lactobacillus and Bifidobacterium (Mack et al., 2003). A study (Tsirtsikos et al. 2012) showed that increase in the level of probiotic in diet can increased linearly the mucus layer thickness in the duodenum of broilers.

CONCLUSION

The dietary inclusion of *Bacillus subtilis* could alleviate the negative effects of Salmonella infection on intestinal cells, as well as on the beneficial bacterial population. *Bacillus subtilis* supplementation could improve the growth and development of immune organs and function in infected broilers.

CONFLICT OF INTEREST

None declared.

ACKNOWLEDGMENTS

The authors thank the Iranian Institute of Agricultural Biotechnology (Dr. Meisam Tabatabaei), and the Nuclear Institute of Science and Technology (Nuclear Agriculture Research School) and especially Dr. Parvin Shawrang for their cooperation and generous assistance in carrying out this research project. This paper was elicited from PhD student thesis (Mehdi Ghaderi Joybari) of Islamic Azad University, Science and Research Branch.

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