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Immune tissue development in pathogen challenged broiler chicks fed diet supplemented with probiotic (*Bacillus subtilis*)

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Abstract

In the present study, an experiment was conducted to evaluate the effects of a commercial probiotic (*Bacillus subtilis*) on immune tissue development in *Salmonella* challenged broiler chicks. One hundred and sixty 1-d-old broiler chicks were randomly assigned to four treatments at a completely randomized design. The treatments were control group, probiotic (200 g per ton of diet) treated group, challenged group (1.0×10^5 cfu/chick at day 7 of age) and challenged probiotic treated group. There were differences among the weights of spleen or bursa at days 21 and 42 of age. *Salmonella* challenging resulted in tissue degeneration and depletion of spleen and bursa from lymphoid. Inclusion of probiotic to diet of challenged chicks ameliorated the negative effects of *Salmonella* challenging on tissue structure. *Salmonella* challenging decreased ($P < 0.05$) the weights of spleen and bursa by 6 and 27% compared to those of control group, respectively. Inclusion of probiotic to diet of these chicks increased ($P < 0.05$) the weight of spleen and bursa by 4 and 20%, respectively. The results showed that *Salmonella* challenging had negative effects on immune organ weight and tissue structure and probiotic supplementation could improve immune organ development of infected chicks.

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Introduction

Salmonella enteritidis is a genus of rod-shaped, Gram-negative, non-spore-forming and predominantly motile enterobacteria. *Salmonella* can cause infection of chickens in the absence of a clinical disease (Gast and Beard, 1990). Several measures to control *Salmonella* have been used, among them the use of antibiotics. The use of antibiotics in broiler feeds has become undesirable, because of the drug residues in meat (Burgat, 1999), development of drug-resistant bacteria, and imbalance of normal microflora (Sorum and Sunde, 2001). Therefore, it has become necessary to develop alternatives such as beneficial microorganisms termed probiotic. Probiotics have previously been utilized for reduction of *Salmonella* in chicks with success (Waters *et al.*, 2005; Okamura *et al.*, 2005; Menconi *et al.*, 2011).

It was reported that *Salmonella* infection and probiotic supplementation could modulate the systemic antibody response to antigens in chickens (Koenen *et al.*, 2004; Haghighi *et al.*, 2005; Menconi *et al.*, 2011; Elangovan, *et al.*, 2011; Seifert *et al.*, 2011). Despite the fact that several studies have shown positive effects of oral administration of probiotics on antibody production in unchallenged chicks, there is a dearth of information regarding the effects of probiotic on the immune organ development of broiler chicks challenged with *Salmonella enteritidis*. Therefore, the present study was carried out to determine the effects of probiotic, *Bacillus subtilis*, on the spleen and bursa development in broiler chicks.

Materials and methods

Salmonella culturing

Salmonella Enteritidis (PTCC 1709) was obtained as freeze-dried from the Persian Type Culture Collection (IROST, Tehran, Iran) isolated from the liver of chickens. Briefly, freeze-dried inoculum was added and grown in tryptic soy broth (Acumedia Manufacturers Inc., Baltimore, MD) at 37 °C for 8 h and passed to fresh tryptic soy broth for 3 incubation periods. Determination of the number of colony-forming units (cfu) through decimal dilution series

was performed in sterile buffered peptone water with pH 7.2. For this, 0.1 ml of diluted medium was inoculated in Petri dishes containing *Shigella–Salmonella* agar (SS agar) and cultivated for 24 h at 37 °C, then cfu counted.

Experimental birds and treatments

At a completely randomized design, 160 one-d-old broiler chicks (Ross 308) were randomly divided into four groups and housed in pens of identical size (1.3 × 1.2 m) in a litter system with wood shavings. Each group had four replicates with 10 birds per each replicate. The treatments were negative control (basal diet without probiotic supplementation and *Salmonella* challenge), probiotic treated group (basal diet supplemented with 200 g probiotic per ton of diets, without pathogen challenge), challenged group (basal diet, with *Salmonella* challenge) and challenged probiotic treated group (basal diet supplemented with probiotic and *Salmonella* challenge). Basal diet was formulated based on corn-soybean meal without enzyme and antibiotic supplementations (Table 1). At day 7 of age, chicks in challenged groups received 1.0×10^5 cfu/chick utilizing micropipette. Unchallenged birds received the same amount of sterile buffered peptone water by micropipette.

Probiotic sample, Gallipro®, was prepared from Iranian Baltec Co. (subsidiary of Biochem Co. Zusatzstoffe GmbH, Germany), included at the amount of 200 g powder per ton of diet and fed during the total period of rearing. The birds had free access to water and feed. Environmental temperature in the first week of life was 33 °C and decreased to 20 °C until the end of the experiment. During the first week, 22 h of light was provided with a reduction to 20 h afterwards.

Weighting of immune organs

On days 21 and 42, a total of 32 birds (8 per treatment; two per pen) with nearly the same weight was selected, individually weighed, stunned, killed by cervical dislocation and plucked in slaughterhouse. The carcasses were opened, then spleen and bursa

removed and weighed.

Histology

The spleen and bursa of Fabricius were excised and fixed in 10% neutral buffered formalin for histology. Samples were dehydrated, cleared, and paraffin embedded. Tissue samples were sectioned at 6 µm thickness, placed on glass slides, and processed by hematoxylin and eosin stain for examination by light microscopy.

Statistical analysis

Statistical analyses were conducted with the general linear model procedure of SAS for Windows version 9.1 (SAS Institute Inc., Cary, NC) to determine if variables differed between groups. The Kolmogorov-

Smirnov test was used to test the normal distribution of the data before statistical analysis was performed. The data were compared between groups by Tukey test. Probability values of less than 0.05 ($P < 0.05$) were considered significant.

Results

Immune organ weights

As shown in Figure 1, the weights of spleen and bursa at 21 d of age were not affected by treatments. At this period, *Salmonella* challenging resulted in numerically decrease of the weights of spleen and bursa, compared with those of control group. Probiotic supplementation to diet of challenged chicks increased numerically the weights of immune organs.

Table 1. Ingredients and chemical composition of experimental rations.^a

Ingredients (%)	Starter	Grower	Finisher
Yellow corn	59.12	62.31	63.84
Soy bean meal	32.62	30.09	29.13
Fish meal	3.00	2.00	--
Soy bean oil	1.71	2.42	3.87
DCP	1.53	1.43	1.48
CaCO ₃	1.10	1.05	1.03
Salt	0.36	0.24	0.18
Mineral Premix ^b	0.10	0.10	0.10
Vitamin premix ^c	0.25	0.25	0.25
Methionine	0.17	0.03	0.05
Lysine	--	0.05	0.05
Probiotic	0.02	0.02	0.02
Chemical composition			
ME (kcal/kg)	2920	3000	3100
Protein (%)	21.0	19.5	18.00
Calcium (%)	1.00	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.

The weights of spleen and bursa at d 42 of age are shown in Figure 2. Differences among the weights of spleen or bursa of chicks were appeared at this period compared with day 21 of age. Inclusion of probiotic to

diet of unchallenged chicks increased ($P < 0.05$) the weights of spleen and bursa. *Salmonella* challenging decreased ($P < 0.05$) the weights of spleen and bursa by 6 and 27% compared to those of control group,

respectively. Inclusion of probiotic to diet of these chicks increased ($P < 0.05$) the weight of spleen and bursa by 4 and 20%, respectively.

Immune tissue structure

Figures 3 and 4 show histological status of spleen and bursa related to challenged chicks and challenged probiotic treated chicks at days 21 and 42 of age, respectively. *Salmonella* challenging resulted in depletion of spleen and bursa from lymphoid and tissue degeneration. Inclusion of probiotic to diet of challenged chicks ameliorated the negative effects of *Salmonella* challenging.

Discussion

Litter moisture contents and mortality in challenged groups were higher than unchallenged ones (data not shown), which indicate a developing of infection through challenging program. Chicks before dead were weak, lethargic with distinct green diarrhea. Dietary inclusion of probiotic decreased mortality of challenged chicks, significantly, which indicate its reducing effect on *Salmonella* colonization in intestine.

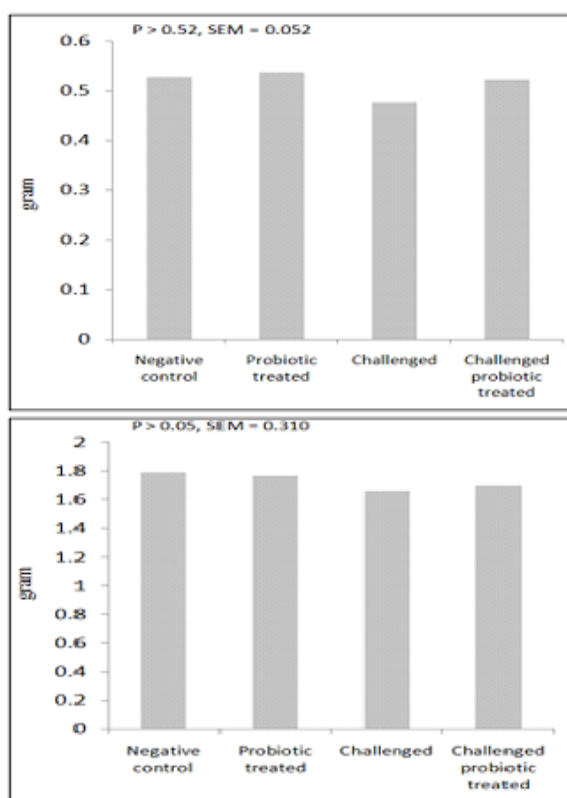


Fig. 1. The spleen (up) and bursa (down) weights of chicks at day 21 of age.

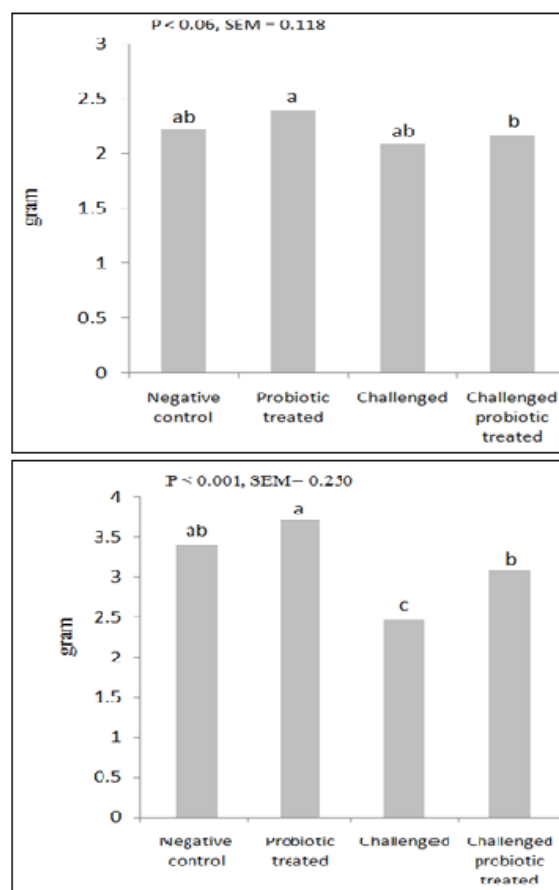


Fig. 2. The spleen (up) and bursa (down) weights of chicks at day 42 of age.

Immune tissue development is the basis of immune system functionality. At day 42 of age, the weights of bursa and spleen were influenced negatively by *Salmonella* challenging. It has been shown that *Salmonella* stimulate different subsets of immune system cells to produce cytokines, especially interleukin-1 β (Okamura *et al.*, 2005), which in turn play a role in the induction and regulation of the immune response (Maassen *et al.*, 2000). The lower humoral immune response of challenged broilers can be explained by an association between the production of interleukin-1 and the stress on the hypothalamic-pituitary-adrenal axis. Interleukin-1 stimulates the hypothalamus, leukocytes, or both to produce the corticotropin-releasing factor, which stimulates the production of adrenocorticotrophic hormone (ACTH) by the anterior pituitary, leukocytes, or both. Adrenocorticotrophic hormone then stimulates corticosterone production from the adrenal gland (Maassen *et al.*, 2000). Corticosterone has been found to be immunosuppressive (Post *et al.*,

2003), inhibiting the production and actions of antibodies (Gross *et al.*, 1992), increasing in heterophil to lymphocyte ratio (Vleck *et al.*, 2000) and depressing immune organ growth (Khansari *et al.*, 1990). Stress-induced bursal atrophy has been suggested to be caused by an increased corticosteroid production (Riddell, 1987). Low bursa weight could be interpreted as an indicator of low immune activity because it is a major lymphoid organ in poultry. The decrease of immune tissue weight produces an effect on immune cell phenotypes, immune cell proliferation, and antibody production. As mentioned previously, challenging could induce production of interleukin-1, which finally resulted in increase of serum corticosterone concentration (Okamura *et al.*, 2005). It was approved (Khansari *et al.*, 1990) that under this condition, retardation of immune organs occurs.

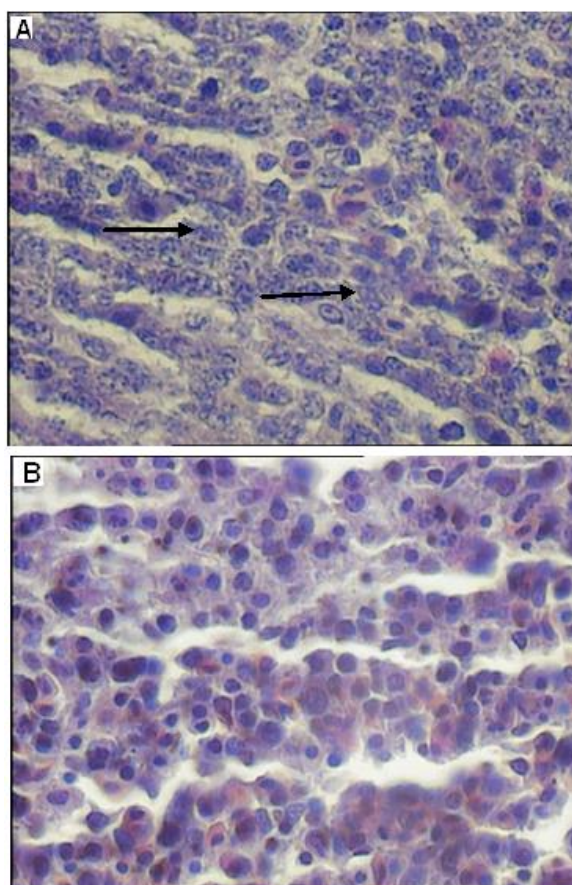


Fig. 3. Image A indicate the hematoxylin and eosin staining of spleen related to challenged chick and B related to challenged probiotic treated chicks. Arrows show degenerations and lymphoid depletion which occurred in the spleen of challenged chicks (image A), but these events were not observed in image B.

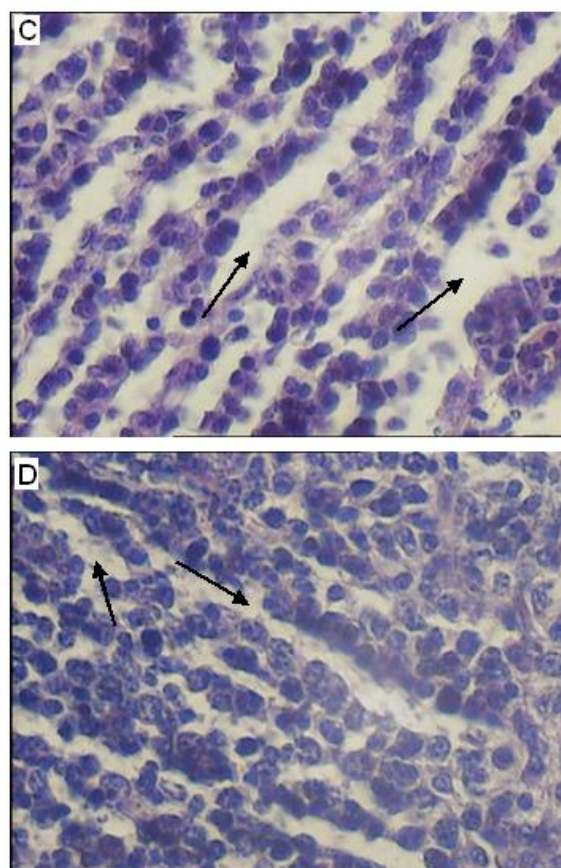


Fig. 4. Image C indicate the hematoxylin and eosin staining of bursa related to challenged chick and D related to challenged probiotic treated chicks. Lymphoid depletion with severe fat around in tissue (in image C) and lymphoid in the follicles with mild fat deposited around them (in image D) was observed.

The addition of probiotic to diet of unchallenged and challenged chicks had significant effect on the weights of spleen and bursa. The two most likely mechanisms by which probiotic reduce the negative effects of *Salmonella* on antibody titers involve competitive exclusion through competition for receptor sites, production of volatile fatty acids that are inhibitory of certain enteric pathogens, production of bacteriocins, or competition with pathogens and native flora for limiting nutrients or stimulation of a host innate immune response (Gaggia *et al.*, 2010).

The results of this study indicated that *Salmonella* challenging had negative effect on immune organ and tissue development of broiler chicks. Dietary inclusion of probiotic has significant effect on immune organ weight and ameliorates the negative

effects of challenging on immune tissue structure.

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