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Immune responses to dietary inclusion of prebiotic-based mannan-oligosaccharide and β-glucan in broiler chicks challenged with *Salmonella enteritidis*

Ali Asghar SADEGHI[1,*], Amineh MOHAMMADI[1], Parvin SHAWRANG[2], Mehdi AMINAFSHAR[1]

[1]Department of Animal Science, Science and Research Branch, Islamic Azad University, Tehran, Iran

[2]Agricultural, Medical, and Industrial Research School, Nuclear Science and Technology Research Institute, Atomic Energy Organization of Iran, Karaj, Iran

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Abstract: This study was performed to evaluate the effect of dietary prebiotic-based mannan-oligosaccharide and β-glucan on immune organ weights, white blood cell profiles, and antibody titers against Newcastle and infectious bursal disease viruses in broiler chicks challenged with *Salmonella enteritidis*. The addition of prebiotics to the diet of unchallenged chicks had no effect on antibody titers and other measured parameters. Salmonella challenging decreased the relative weights of immune organs, increased white blood cells and the heterophil-to-lymphocyte ratio, and decreased the antibody titers against Newcastle and infectious bursal disease viruses. Inclusion of prebiotics to the diet of challenged chicks increased the relative weight of the spleen, decreased the heterophil-to-lymphocyte ratio, and increased antibody titers. The results showed that dietary inclusion of prebiotic-based mannan-oligosaccharide and β-glucan has no significant effect on immune parameters on chicks in the noninfected group, but it displays an efficacy on chicks in the group infected with pathogens and can improve the immune responses and health of infected chicks.

Key words: Mannan-oligosaccharide, β-glucan, antibody titers, *Salmonella* challenge, immune cell profile

1. Introduction

There is a worldwide attempt to reduce antibiotic usage as a growth promoter in poultry production, because of its residues in meat, development of resistant bacteria, and imbalance of normal microflora (1,2). Alternatives to antibiotics, such as prebiotic-based mannan-oligosaccharide (MOS) or β-glucans, have been developed to counter the growth- or immune-depressing effects that certain strains of bacteria elicit in poultry.

MOS, derived from mannans on yeast cell surfaces, acts as a high-affinity ligand, offering a competitive binding site for the bacteria (3). Unfavorable gram-negative bacteria such as *Escherichia coli* and *Salmonella* have mannose-specific fimbriae on their bacteria surface, which they use to attach to and then colonize the intestinal wall. These pathogens adsorb to the MOS instead of attaching to intestinal epithelial cells and, therefore, move through the intestine without colonization (4–7).

The β-glucans are polymers of glucose that can be derived from the cell walls of yeast, bacteria, fungi, and cereals. One of these molecules, the β-1,3-glucan from the cell wall of *Saccharomyces cerevisiae*, is recognized as foreign by the immune systems of mammals, fish, and birds and has been shown to be protective in a number of disease challenge studies (8–11).

Despite the fact that several studies have shown immune enhancement of unchallenged chicks resulting from oral administration of MOS or β-glucans alone, there is a dearth of information regarding the effects of prebiotics on the immune response of broiler chicks challenged with *Salmonella enteritidis*.

Therefore, the present study was carried out to determine the effects of a prebiotic-based MOS and β-glucans on immune organs weights, antibody titers against Newcastle and infectious bursal disease viruses in broilers, and the potential protection they might provide to the birds against a *Salmonella* challenge.

2. Materials and methods

2.1. Preparation of prebiotic sample

The prebiotics were obtained from TechnoMOS (Biochem, Lohne, Germany) and included in the diets at the amount of 1 g/kg (0.1% of dry matter). This product is rich in the same amounts of MOS and β-1,3-glucan and has been obtained from *Saccharomyces cerevisiae*.

* Correspondence: a.sadeghi@srbiau.ac.ir

2.2. Salmonella culturing and counting

Salmonella enteritidis (PTCC 1709) was obtained as freeze-dried samples from the Persian Type Culture Collection (IROST, Tehran, Iran) isolated from the liver of chickens. Freeze-dried inoculum was grown in tryptic soy broth (Acumedia Manufacturers Inc., Baltimore, MD, USA) 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 (cfus) 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 incubated for 24 h at 37 °C, and the cfus were then counted.

2.3. Birds and experimental design

A total of 160 broiler chicks (Ross 308), 1 day old, were obtained from a commercial hatchery. With a completely randomized design, the birds were divided into 4 groups and housed in isolated pens of identical size (1.3 × 1.2 m) in a litter of wood shavings. Each group had 4 replicates with 10 birds each. 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 afterward. Chicks were provided ad libitum access to water and an unmedicated diet based on corn grain and soybean meal (Table 1).

The treatments were a negative control (basal diet without prebiotic supplementation and challenging), a prebiotics-treated group (basal diet supplemented with 1 g prebiotics/kg diet, without pathogen challenge), a challenged group (basal diet with pathogen challenge), and a challenged prebiotics-treated group (basal diet supplemented with prebiotics and pathogen challenge). At 7 days of age, the chicks in the challenged groups received by oral gavage 1.0×10^5 cfu/chick of passaged medium utilizing a micropipette. The unchallenged birds received the same amount of sterile buffered peptone water by oral gavage.

At days 20 and 40 of age, litter moisture contents were determined in each pen by collecting 3 samples (500 g) in areas of the pen away from feeders and drinkers. The 3 samples were mixed together and 1 homogeneous subsample was weighed (50 g), oven-dried at 105 °C for 24 h, and reweighed to determine moisture content.

2.4. Vaccination and serology

The immunization program included vaccination against Newcastle disease virus (B1, day 10, eye drops; Lasota, days 19 and 32, in drinking water) and infectious bursal disease virus (Gumboro, D78, days 12 and 24, eye drops). Blood samples (3.0 mL) of 8 birds per treatment (2 birds per pen) were collected from the wing veins, using sterile syringes, on days 21 and 42 of age. Immediately after collection, 900

Table 1. Ingredients* and chemical composition of experimental rations.

Ingredients (%)	Starter	Grower	Finisher
Yellow corn	59.04	62.03	63.76
Soy bean meal	32.62	30.09	29.13
Fish meal	3.00	2.00	--
Soy bean oil	1.71	2.42	3.87
Di-calcium phosphate	1.53	1.43	1.48
$CaCO_3$	1.10	1.05	1.03
Salt	0.36	0.24	0.18
Mineral premix	0.10	0.10	0.10
Vitamin premix	0.25	0.25	0.25
Methionine	0.17	0.03	0.05
Lysine	--	0.05	0.05
Prebiotic**	0.10	0.10	0.10
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

*On an as-fed basis.

**The prebiotic (TechnoMOS) has the same amounts of MOS and β-1,3-glucan.

μL of blood was transferred to a microtube containing 100 μL of sodium citrate solution (3.85 mg/100 μL) and immediately mixed. The tubes were transferred to the Veterinary Laboratory of Mabna (Karaj, Iran) for counting total and differential white blood cells. The remainder of the blood samples (2.1 mL) was transferred to clear glass tubes, kept at room temperature for 2 h and then overnight at 4 °C in a refrigerator (to clot), and centrifuged at 1500 × *g* for 15 min. Serum was obtained, inactivated at 56 °C for 30 min, and stored at –20 °C until analyses of antibody titers. The titers of antibodies against Newcastle disease and infectious bursal disease viruses were determined by enzyme-linked immunosorbent assay (ELISA) using related Antibody Test Kits from IDEXX Laboratories Inc. (Westbrook, ME, USA).

2.5. Immune organ weights

On days 21 and 42, a total of 32 birds (8 per treatment; 2 per pen) were randomly selected, individually weighed, stunned, killed by cervical dislocation, and plucked in a slaughterhouse. The carcasses were opened, and then the spleen and bursa were removed and weighed. Relative weights of the spleen and bursa were calculated as [organ weight (g) / body weight (kg)].

2.6. Statistical analysis

The Kolmogorov–Smirnov test was used to test the normal distribution of the data before statistical analysis was performed. Statistical analyses were conducted with the general linear model procedure of SAS for Windows version 9.1 (SAS Institute Inc., Cary, NC, USA) to determine if variables differed between groups. The data were compared between groups with the Tukey test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Effect on litter moisture

The effects of *Salmonella* challenging and prebiotic treatment on the moisture content of the litter at days 20 and 40 of age are shown in Figure 1. At 20 days of age, the lowest litter moisture was found in the prebiotic-treated group and the highest in the challenged groups. At 2 periods the litter moisture decreased significantly in the challenged prebiotic-treated chicks as compared with that of the challenged group.

3.2. Effect on viability

The effect of challenging and prebiotic supplementation on viability of chicks is shown in Figure 2. Mortality was measured from days 3 to 10 of age. The highest survival percentage was recorded in the prebiotic-treated group and the lowest percentage in the challenged groups. Dietary inclusion of prebiotics in the diet of challenged chicks significantly increased their viability.

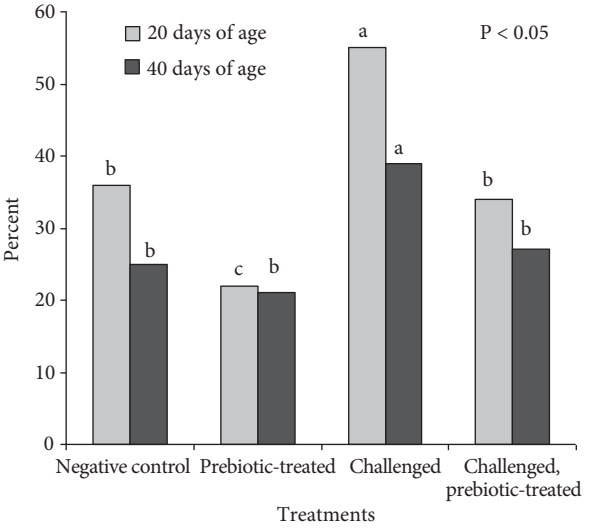

Figure 1. Litter moisture contents in different treatments. Different letters above bars reflect statistically significant values.

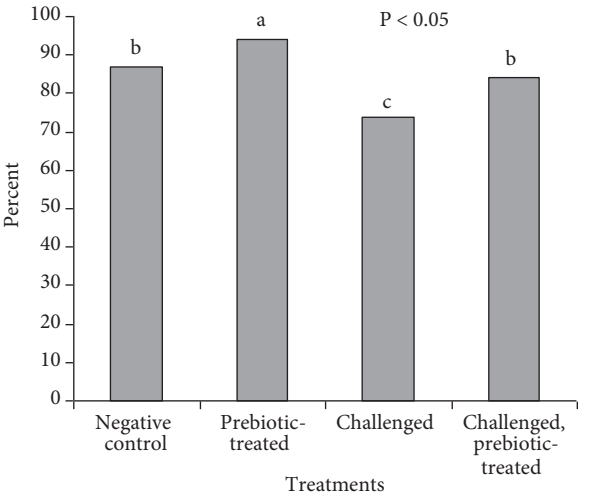

Figure 2. Viability of chicks in different treatments. Different letters above bars reflect statistically significant values.

3.3. Immune tissue weights

As shown in Table 2, the relative weights of the bursa of Fabricius and spleen at 21 days of age were not affected by prebiotic supplementation within either the unchallenged or the challenged treated group when compared with control birds. At 42 days of age, differences were found in the relative weights of the spleen or bursa of chicks in different treatment groups. Dietary inclusion of prebiotics increased the relative weights of the spleen but had no effect on the relative weight of the bursa. The relative weights of the bursa of Fabricius and spleen were decreased due to *Salmonella* challenge by 12% and 7% compared to the negative control, respectively. Addition of prebiotics to

Table 2. The relative weights* of spleen and bursa of chicks at 21 and 42 days of age.

Treatments	21 days of age		42 days of age	
	Spleen	Bursa	Spleen	Bursa
Negative control	1.24	1.62	1.08^{ab}	1.50^{a}
Prebiotic–treated	1.27	1.57	1.12^{a}	1.49^{a}
Challenged	1.13	1.53	0.92^{b}	1.38^{b}
Challenged and prebiotic–treated	1.19	1.54	1.03^{ab}	1.44^{ab}
SEM	0.101	0.103	0.061	0.047
P-value	0.25	0.62	0.04	0.01

*As g/kg body weight.
^{a,b}Within the same column, means with different superscripts are significantly different ($P < 0.05$).

the diet increased the relative weight of the spleen by 10% compared to chicks in the challenged group but had no effect on the relative weight of bursa.

3.4. Effects on immune cell profiles

The results of total and differential counts of white blood cells at days 21 and 42 of age are shown in Table 3. At 21 days of age, there was no significant difference for total white blood cells, monocytes, or eosinophils among treatments ($P > 0.05$). However, significant differences were observed for heterophil, lymphocyte, and their ratio. Challenged chicks had the lowest and prebiotic-treated chicks had the highest lymphocyte percentage. There were no significant differences in lymphocyte percentage among the negative control and challenged prebiotic-treated chicks. In contrast to lymphocytes, challenged chicks had the highest heterophil percentage and therefore the highest heterophil

Table 3. Effects of experimental treatments on total and differential counts of white blood cells* at 21 and 42 days of age.

Treatments	WBC × 10^3	L (%)	H (%)	H/L	E (%)	M (%)
21 days of age						
Negative control	24.8	74.7^{ab}	22.7^{b}	0.30^{b}	0.7	2
Prebiotic-treated	26.7	76.3^{a}	21.3^{b}	0.28^{b}	0.0	2.4
Challenged	27.1	69.7^{b}	26.3^{a}	0.38^{a}	2.0	2.0
Challenged and prebiotic-treated	27.6	72.7^{bc}	24.0^{ab}	0.34^{ab}	1.0	2.3
SEM	1.76	2.30	1.73	0.032	0.76	0.64
P-value	0.31	0.029	0.038	0.031	0.06	0.84
42 days of age						
Negative control	25.2^{c}	79.7^{a}	18.3^{b}	0.23^{b}	0.3^{b}	1.7^{b}
Prebiotic-treated	25.9^{bc}	77.7^{a}	18.6^{b}	0.24^{b}	1.0^{b}	2.7^{ab}
Challenged	27.7^{a}	69.3^{b}	25.4^{a}	0.36^{a}	3.0^{a}	2.3^{b}
Challenged and prebiotic-treated	26.8^{ab}	72.0^{b}	22.7^{ab}	0.31^{a}	1.3^{b}	3.7^{a}
SEM	0.75	1.73	1.52	0.026	0.64	0.57
P-value	0.015	0.003	0.001	0.001	0.01	0.01

^{a,b}Within the same column, means with different superscripts are significantly different ($P < 0.05$).
*WBC: white blood cell count; L: lymphocytes; H: heterophils; H/L: heterophil-to-lymphocyte ratio; E: eosinophils; M: monocytes.

to lymphocyte (H/L) ratio compared to the other treatment groups. The addition of prebiotics to the diet of challenged chicks reduced the heterophil percentage and therefore the H/L ratio, compared to chicks in the challenged group, by 9% and 10%, respectively.

At 42 days of age, the lowest and highest total white blood cell counts were observed in the negative control and challenged groups, respectively. *Salmonella* challenging increased the total white blood cells when compared to the negative control. The highest lymphocyte percentage and lowest heterophil percentage were found in the negative control and the prebiotic-treated groups. The opposite was found for the challenged chicks. *Salmonella* challenging resulted in increase in H/L ratio and dietary inclusion of prebiotic decreased it numerically. There were significant differences for eosinophils and monocytes among treatments.

3.5. Effects on antibody titers

The geometric means of antibody titers against Newcastle and infectious bursal viruses at 21 and 42 days of age are shown in Figures 3 and 4. At both periods and for both antigens, there were no differences in antibody titers between the negative control and prebiotic-treated chicks, although the addition of prebiotics to the diet increased the

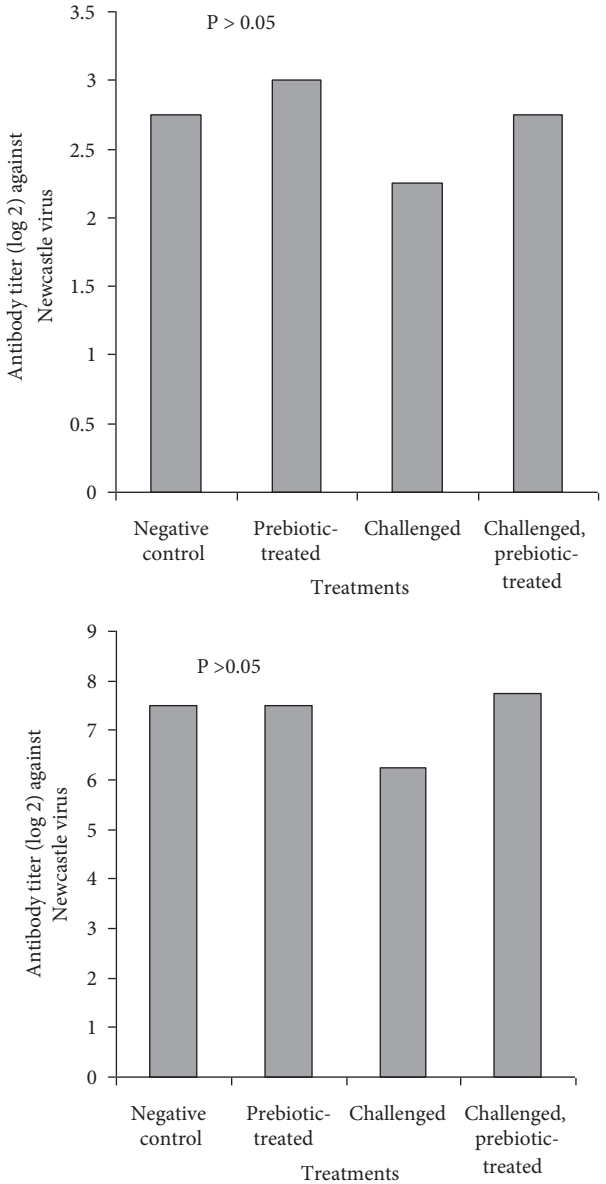

Figure 3. Antibody titers (log 2) against Newcastle disease virus of chicks at days 21 (upper image) and 42 (lower image) of age.

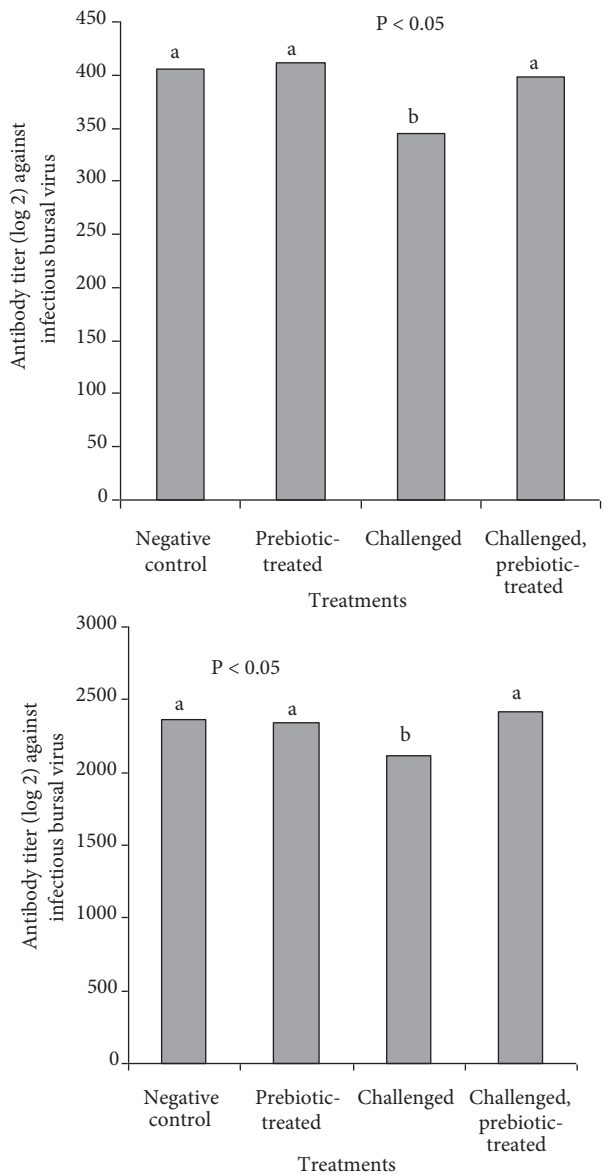

Figure 4. Antibody titers (log 2) against infectious bursal disease virus of chicks at days 21 (upper image) and 42 (lower image) of age.

antibody titers numerically. Challenging with *Salmonella* decreased ($P < 0.05$) antibody titers against both antigens, as the lowest means were observed in challenged chicks at both periods. At 42 days of age, antibody titers against infectious bursal disease virus in challenged prebiotic-treated chicks increased significantly by 12% as compared with that of challenged chicks.

4. Discussion

The present study was designed to evaluate the effects of the dietary inclusion of prebiotic-based MOS and β-glucan on immune responses in *Salmonella*-challenged broiler chicks that were vaccinated with Newcastle and infectious bursal disease viruses as antigens. The effects of these treatments on the white blood cell count and the relative weights of immune organs were also measured. We hypothesized that *Salmonella* challenging would decrease the antibody titers and that the addition of prebiotics to the diet of unchallenged and challenged chicks would enhance the antibody production.

Litter moisture of the negative control was 36%, near the normal range of 25% to 35% stated by Carr et al. (12). The litter moisture in the challenged groups was higher than in the unchallenged ones, which indicates a developing *Salmonella* infection through the experimental period. *Salmonella* challenging also increased the white blood cell counts (Table 3), which is an indication of an infection or inflammation process. At 20 days of age, dietary inclusion of prebiotics decreased litter moisture both in unchallenged and challenged chicks, and at 40 days of age in challenged chicks, significantly. The reason for this could be the removing of unfavorable gram-negative bacteria from the digestive tract by MOS (4,13), which may have results in the reduction of bacteria colonization and their toxic by-products.

Mortality in the challenged groups increased 3 days after the *Salmonella* infection. Dying chicks were weak and lethargic with distinctive green diarrhea. Dietary inclusion of prebiotics decreased the mortality of challenged chicks significantly, which indicates its reducing effect on *Salmonella* colonization in the intestine.

Salmonella challenging decreased the antibody titers against Newcastle and infectious bursal disease viruses (Figures 3 and 4), depressed the immune organ growth (Table 2), and decreased the lymphocyte percentage (Table 3). The exact mechanisms that mediate the immunomodulatory activities of probiotics are not clear. However, several in vitro and in vivo studies have shown that *Salmonella* infection stimulates different subsets of immune cells to produce the inflammatory cytokine interleukin-1 (14–16). The lower humoral immune response of challenged broilers can be explained by the lower lymphocyte count and lower immune organ weights, because of the inflammatory effects of interleukin-1. Inflammatory factors stimulate the hypothalamic production of corticotrophin-releasing factor (17). Interleukin-1 stimulates the hypothalamus, leukocytes, or both to produce the corticotropin-releasing factor, which stimulates the production of adrenocorticotropic hormone by the anterior pituitary, leukocytes, or both. Adrenocorticotropic hormone then stimulates corticosterone production from the adrenal gland (14). Corticosterone has been found to be immunosuppressive (17), inhibiting the production and actions of antibodies (18), increasing the H/L ratio, and depressing the immune organ growth (19). The results shown in Table 3 led us to conclude that the challenged chicks were in a physiological stress state. The H/L ratio has been accepted as a reliable index for determining stress in poultry (20). Heterophils are parts of natural immunity and cellular defense against microbial infections, and lymphocytes are cells that produce antibodies. The increases in the H/L ratio in challenged chicks may be attributed to increased corticosterone secretion (19), which finally resulted in the decrease of the antibody titers. Stress-induced bursal atrophy has been suggested to be caused by increased corticosteroid production (21). Low bursa weight could be interpreted as an indicator of low immune activity, because the bursa 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.

Although dietary inclusion of prebiotics had no effect on the immune response of unchallenged chicks, in *Salmonella*-challenged chicks significant positive effects were observed. In agreement with our results, previous studies reported that MOS (5–7) and β-glucan (8–10) are effective on humoral and cell immunity.

It is well documented that MOS competitively adsorbs to the mannose-specific type 1 fimbriae of pathogens, thereby limiting their colonization of the intestinal epithelium; they are ultimately excreted from the intestines (4,13). An increase in mucin secretion and an increase in growth of beneficial bacteria have been reported as an additional mechanism underlying MOS actions against intestinal pathogens (22). Furthermore, prebiotics with high mannose levels will bind to macrophage reception sites by recognizing specific sugars found in glucoproteins of the epithelial surface, triggering a cascading reaction that would eventually activate macrophages and release cytokines, thereby activating the acquired immune response (23). Moreover, MOS-based prebiotics can modulate the gut microflora and performance of broiler chickens (7). In a recent study, Yang et al. (6) demonstrated considerable decreases in the ileal and cecal populations of coliforms in broilers fed diets containing MOS.

The immunostimulatory properties of β-glucan (in various forms) have been reported in a variety of species (8,9). In agreement with our results, Chen et al. (24) and Cox et al. (11) reported that β-glucan had immune enhancing effect on *Eimeria*-challenged chickens. Results of another study (25) of mammalian species noted that proinflammatory cytokines were reduced in response to β-glucan treatment.

The results of this study indicated that *Salmonella* challenging had negative effects on the immune response of broiler chicks by reducing the immune organs' growth, changing immune cell profiles, and decreasing antibody production. Dietary inclusion of prebiotics had no significant effect on the immune parameters on chicks in the noninfected group, but it displayed an efficacy on chicks in the group infected with pathogens and can improve immune responses and health of infected chicks.

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