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RESEARCH ARTICLE

The Impacts of Prebiotic Supplementation on Humoral and Cell-Mediated Immune Responses of Broilers under Combined Stresses Caused by Feed Restriction and *Salmonella enteritidis* Challenge

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Summary

This experiment was conducted to investigate the impacts of prebiotic on humoral and cell-mediated immune responses of broilers under combined stresses caused by feed restriction and *Salmonella enteritidis* challenge. 320 male broilers (Ross 308) at a 23 factorial arrangement in randomized complete block design were divided into 8 treatment groups with 4 replicates per each. The Impact of these factors and their interaction on cell-mediated immune response to cutaneous basophil hypersensivity (CBH) test, antibody titer response against Newcastle disease virus (NDV) and sheep red blood cell (SRBC), and the relative weight of lymphoid organs were measured. Prebiotic caused an increase and feed restriction caused a decrease in cell-mediated immune responses on days 15 and 30 respectively, and interaction among the factors was differ (P<0.05). Also, factors' interaction had a significant effect on antibody titer response against NDV (P<0.05) at day 27. *Salmonella* and feed restriction significantly reduced the relative weight of thymus at day 21 and bursa of Fabricius at day 42, respectively (p < 0.05). Differences among other measured parameters were not significant (P>0.05).

Keywords: *Prebiotic, Feed restriction, Salmonella, Humoral immune response, Cell-mediated immune response*

Prebiyotik İlavesinin Yem Kısıtlaması ve *Salmonella enteritidis* İnokulasyonu İle Kombine Stres Altındaki Broylerlerde Sıvısal ve Hücresel İmmun Yanıt Üzerine Etkileri

Özet

Bu çalışma, prebiyotik ilavesinin yem kısıtlaması *Salmonella enteritidis* inokulasyonu ile kombine stres altındaki broylerlerde sıvısal ve hücresel immun yanıt üzerine etkileri araştırmak üzere gerçekleştirildi. Rastgele tam blok tasarım içinde 23 faktoriyel düzenlemede 320 erkek boyler (Ross 308) 4 tekrar olmak üzere 8 tedavi grubuna bölündü. Bu faktörlerin ve onların etkileşiminin hücresel immun cevap üzerin etkilerini belirlemek üzere kutanöz bazofil aşırı duyarlılık testi (CBH), Newcastle hastalığı (NDV) virüsü ve koyun eritrositlerine (SRBC) karşı gelişen antikor titreleri ve lenfoid organların göreceli ağırlıkları belirlendi. Hücresel immun yanıtı prebiyotik 15. günde arttırırken yem kısıtlamasını 30.günde azalttığı ve faktörler arası etkileşimin faklı (P<0.05) olduğu gözlemlendi. Ayrıca, faktörlerin etkileşiminin 27. gündeki NDV'ye karşı gelişen antikor titresinin gelişimi üzerine önemli etkisinin (P<0.05) olduğu belirlendi. *Salmonella* ve yem kısıtlamasının, timus ve bursa fabricius'un göreceli ağırlığını, sırasıyla 42. ve 21. günde azalttığı belirlendi (P<0.05). Diğer ölçülen parametreler arasında farklılıklar anlamlı bulunmadı (P>0.05).

Anahtar sözcükler: *Prebiyotik, Yem kısıtlaması, Salmonella, Sıvısal bağışıklık cevabı, Hücresel bağışıklık cevabı*

INTRODUCTION

In recent years, feed restriction has been used for improving broilers' performance and their carcass quality. One of the common methods is restriction in the starter feeding period on the base of compensatory growth. It has been reported that decrease in broilers' primary growth reduces the rate of metabolic reactions; therefore there

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is less need to oxygen, and the metabolic disorders such as ascites and SDS (sudden death syndrome) resulted from fast growth are prevented [1]. Beside the advantages, feed restriction has some disadvantages too. Limitations in access to food make stress for the chickens, and this stress changes the microbial population of their gut. It has been shown that hunger in birds reduces the amount of *Lactobacilli* spp. in the crop [2]. Also there are reports on the suppressing effects of feed restriction on some immune system parameters in chickens [3,4].

Furthermore, broilers commonly meet several internal and external stressors like stocking density, temperature, transportation, feed contamination and microbial infections [5]. Among the microbial infections, *Salmonella* spp. is one of the main, and it is the leading cause of human food-borne infections associated with consumption of poultry products in the world [6].

The reduced immunocompetence caused by feed restriction beside the effects of bacterial enteric pathogens like *Salmonella* spp. make severe stresses in broilers. In such a condition, using additives such as prebiotics for improving the immune function and gut microflora is helpful. Prebiotics beneficially affect the host by making changes in the population of gut microflora in favor of useful bacteria [7] as well as increasing its humoral and cell-mediated immune responses [8]. In addition, prebiotics help the host resist against the bacterial infections [9].

Several studies have been done on the useful effects of prebiotics on infections [10,11] and the negative effects of feed restriction on immune system [3,4]. Gursoy et al.[12] reported that exposure to combined stresses may have synergistic, additive, or adaptive effects on the immune system parameters. In the literature, there was no report concerning the effect of dietary prebiotic on immune responses of broilers under combined stresses caused by infection and feed restriction. Therefore, our purpose in this study was to investigate the effect of prebiotic supplementation on immune system parameters of broiler chickens under feed restriction and *Salmonella* challenge.

MATERIAL and METHODS

Birds, Diets, and Management

Three hundred twenty 1-d-old male broilers (Ross 308) were obtained from a commercial hatchery. At a 2^3 factorial arrangement in randomized complete block design, they were divided into 8 treatment groups with 4 replicates and 10 broilers in each replicate, and were then put in special cages. Environmental temperature in the first week of life was 32°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. Chickens had access to *ad libitum* water and a diet based on corn and soybean meal which provided 3.000 kcal/kg ME and 21.7% CP up to day 21, and 3075 kcal/kg ME and 19.25% CP from day 21 to day 42. This basal diet was unmedicated.

Prebiotic Supplementation

The prebiotic was gained from TechnoMOS (Biochem, Lohne, Germany). It was used at the amount of 0.1% of the diet. This product consists of mannanoligosaccharide (MOS) and β-1,3-Glucan and has been obtained from *Saccharomyces cerevisiae*.

Feed Restriction Procedure

From day 7 to day 14, the daily feed intake of the groups getting ad libitum consumption was measured, and 80% of this feed was given to feed restricted treatment groups the next day.

Salmonella Culturing, Counting and Challenge

Salmonella enteritidis (PTCC 1709) was provided freeze-dried from the Persian Type Culture Collection (IROST, Tehran, Iran) isolated from the liver of chickens. Freeze-dried inoculum was grown in nutrient broth media at 37°C for 8 h and passed to fresh nutrient broth for 3 incubation periods. Counting colony-forming units (cfu) was done with Neobar lam at day 10. After counting, chickens in the challenged groups received 2.6×10^5 cfu/chick of passaged medium through oral gavage with utilizing micropipette on the same day.

Treatment Groups

The treatment groups included 1. a control group with no supplementation and challenge, 2. a group under feed restriction (Control + R), 3. a *Salmonella* challenged group (Control + S), 4. a group under feed restrictin and Salmonella challenge (Connrol + R + S), 5. a prebiotic treated group, 6. a prebiotic treated and feed restricted group (Prebiotic + R), 7. a prebiotic treated under *Salmonella* challenge (Prebiotic + S), 8. a prebiotic treated under feed restriction and *Salmonella* challenge (Prebiotic + R + S).

Immunological Tests

Cell-mediated immune response was assessed according to the method of Corrier and DeLoach [13] by a cutaneous basophil hypersensivity (CBH) test using phytohem-agglutinin P (PHA-P) produced by Gibco company (Gibco, Invitrogen Corporation, Scotland, UK). At days 15 and 30, the toe web of the right foot (2 chickens per pen) was measured with a Caliper with an accuracy of 0.01 mm. Immediately after measurement, 100 µg of PHA-P (suspended in 0.1 mL of PBS) was injected into the toe web. The toe web swelling was measured 24 h after injection. The response was measured by subtracting the skin thickness of the first measurement from the skin thickness 24 h after dermal injection. At day 7, vaccination against Newcastle disease virus (NDV) was done by instilling

B1 strain of NDV (Intervet International BV, Boxmeer, Netherlands) in the chickens' eyes, and 2 birds per each pen were injected in breast muscle by 0.1 ml of 5% sheep red blood cell (SRBC) diluted in PBS. The chickens were bled from the wing vein at days 17 and 27, and sera were collected individually in separate sterile vials. The hemagglutination inhibition and hemagglutination tests were done to determine the antibody titer response against NDV [14] and SRBC [15], respectively.

At days 21 and 42, a chicken from each replicate pen was weighed and then killed by cervical cutting. Carcasses were dissected immediately after euthanasia. Thymus, bursa of Fabricius, and spleen were then precisely removed and weighed separately on a sensitive digital scale.

Statistical Analysis

All data were subjected to GLM procedures of SPSS version 18 software as a 2^3 factorial arrangement of treatments in randomized complete block design that included prebiotic supplementation, feed restriction and *Salmonella* challenge as the main factors and their respective interactions. The treatment means were compared by Duncan's multiple range tests. Probability values of less than 0.05 ($P<0.05$) were considered significant.

RESULTS

As it is shown in *Table 1*, there were significant differences among treatments regarding the cell-mediated immune response to CBH test ($P<0.05$). Prebiotic caused an increase at day 15, and feed restriction caused a decrease at day 30 in toe web thickness, and interaction among the factors was significant ($P<0.05$). There were no significant differences among treatments and factors' interaction regarding antibody titer responses against SRBC at days 17 and 27, and to NDV at day 17 ($P>0.05$); but there were significant differences among factors' interaction regarding antibody titer response against NDV at day 27 ($P<0.05$).

Table 2 shows the relative weight of lymphoid organs at days 21 and 42. *Salmonella* challenge had a significant effect on thymus relative weight at day 21 ($P<0.05$). Feed restriction significantly reduced the bursa of Fabricius at day 42 ($P<0.05$). There were no significant differences among treatments and factors' interaction regarding relative weight of spleen at days 21 and 42 ($P>0.05$).

DISCUSSION

In this study, prebiotic had advantageous and feed restriction had disadvantageous effects on cell-mediated immune response to PHA-P injection. Savino [3] expressed that sever feed restriction can cause significant decrease of mitogenic lymphocyte responses in vitro. Also in the study done by Hangalapura et al.[4], feed restriction reduced cell-mediated immune response which is likely because the cellular components of immune system are energy demanding. Savino and Dardenne [16] reported that infections as well as malnutrition can cause thymus atrophy leading

Table 1. *Cell-mediated immune response to PHA-P, and antibody titer response against NDV and SRBC*
Tablo 1. *PHA-'ye karşı hücresel immun yanıt ve NDV ve SRBC'ye karşı antikor yanıtı*

Treatment	CBH[1] Test (mm)		Antibody Titer (log_2) Against NDV[2]		Antibody Titer (log_2) Against SRBC[3]	
	Day 15	Day 30	Day 17	Day 27	Day 17	Day 27
Control	0.643^{bc}	0.580^{cd}	3.25	4.625^{ab}	2	2.5
Control + R[4]	0.695^{abc}	0.528^{d}	2.5	5.250^{a}	2	3
Control + S[5]	0.783^{ab}	0.888^{a}	3.75	5.625^{a}	1.25	3.5
Control + R + S	0.480^{c}	0.503^{d}	3.25	3.250^{b}	2.25	3.375
Prebiotic	0.905^{a}	0.843^{ab}	3.25	5.000^{a}	2	2.625
Prebiotic + R	0.693^{abc}	0.530^{d}	3.25	4.500^{ab}	2.5	3.5
Prebiotic + S	0.840^{ab}	0.728^{abc}	3	4.750^{a}	2	3.5
Prebiotic + R + S	0.890^{a}	0.663^{bcd}	2.75	4.875^{a}	2.5	3.625
SEM	0.052	0.043	0.412	0.458	0.330	0.592
P-value						
Prebiotic	0.002	0.135	0.672	0.775	0.122	0.607
Restriction	0.061	0.001	0.212	0.116	0.043	0.421
Salmonella	0.786	0.093	0.672	0.506	0.597	0.171
P×R×S[6]	0.007	0.003	0.672	0.011	0.296	0.941

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 virüs,* ***3.*** *Antibody response against Sheep Red Blood Cell,* ***4.*** *Restriction in feed,* ***5.*** *Salmonella challenge,* ***6.*** *Interaction among prebiotic, feed restriction and Salmonella challenge,* *[a,b,c,d] Within the same column, means with different superscripts are significantly differ (P<0.05)*

***Table 2.** The relative weights (% of live BW) of lymphoid organs at days 21 and 42*
***Tablo 2.** 21. ve 42. günlerde lenfoid organların göreceli (% canlı ağırlığa göre) ağırlığı*

Treatment	Day 21			Day 42		
	Bursa (%)	Thymus (%)	Spleen (%)	Bursa (%)	Thymus (%)	Spleen (%)
Control	0.213	0.373[a]	0.078	0.220[a]	0.218	0.125
Control + R[1]	0.250	0.263[ab]	0.075	0.188[ab]	0.193	0.110
Control + S[2]	0.268	0.288[ab]	0.075	0.228[a]	0.275	0.120
Control + R + S	0.208	0.258[ab]	0.085	0.140[bc]	0.140	0.133
Prebiotic	0.243	0.363[ab]	0.080	0.223[a]	0.165	0.118
Prebiotic + R	0.218	0.323[ab]	0.075	0.180[abc]	0.188	0.110
Prebiotic + S	0.260	0.228[b]	0.083	0.218[a]	0.210	0.105
Prebiotic + R + S	0.253	0.310[ab]	0.083	0.125[c]	0.200	0.105
SEM	0.023	0.042	0.011	0.018	0.043	0.013
P-value						
Prebiotic	0.596	0.726	0.081	0.566	0.610	0.198
Restriction	0.407	0.424	0.935	0.001	0.235	0.793
Salmonella	0.329	0.04	0.570	0.065	0.610	1.00
P×R×S[3]	0.91	0.726	0.807	0.923	0.527	0.601

***1.** Restriction in feed, **2.** Salmonella challenge, **3.** Interaction among prebiotic, feed restriction and Salmonella challenge, [a,b,c] Within the same column, means with different superscripts are significantly differ (P<0.05)*

to decrease in cellular immune response mediated by T-cells. As Hooge [17] expressed, MOS advantages are more noticeable in stressor conditions which is in agreement with our findings showing that MOS increased cell-mediated immune response against the stresses caused by feed restriction and *Salmonella* challenge. The mechanism of prebiotic's function in immunity regulation is not yet exactly known. But some studies show that *Salmonella* infections stimulate inflammatory cytokine, interleukin-1 production [10,11]. Furthermore, MOS can competitively adsorbs pathogens from mannose and prevent their attack to gut epithelium [18]; this can cause increase in energy intake which is helpful in cell-mediated immunity.

Antibody titer responses against NDV had significant differences among treatment groups at day 27. The group under feed restriction and *Salmonella* challenge had the least antibody titer response against NDV and its difference with the group which was supplemented with prebiotic under the same condition, was significant. This indicates the advantageous effect of prebiotic on immunity in agreement with Alavi et al.[19], but in contrast with Silva et al.[20] findings. Also, Fanoosi and Torki [21] did not observe any decrease in antibody titer response against NDV under 10% feed restriction, but Jahanpour et al.[22]) reported that feed restriction as amount as 75% standard catalogue during days 8 to 14 decreased antibody titer response against NDV. There was no significant difference among treatments regarding antibody titer response against SRBC. Khalaji et al.[23] and Hangalapura et al.[4] found similar results using prebiotic in broilers and feed restriction in line chickens respectively; but Khajavi et al.[24] reported that antibody titer response increased against SRBC under moderate feed restriction in broilers which is in contrast with Jahanpour et al.[22] who reported that feed restriction as amount as 75% standard catalogue during days 8 to 21 decreased antibody titer response against SRBC. Riberio et al.[25] expressed that prebiotic effect on IgG against *Salmonella enteritidis* is not significant in broilers; whereas Cetin et al.[26] and Woo et al.[27] indicated that IgG level increases by MOS supplementation in turkeys and layers respectively. These contradictions might be because of differences in strain and age of the birds, the prebiotic type, feed restriction severity and intensity of exposure to *Salmonella*. However it seems that stresses and nutrient intake decrease have less suppressive effect on humoral immunity than on cell-mediated immunity.

There was a significant difference regarding the relative weight of thymus at day 21 among treatments. The treatment group with prebiotic under *Salmonella* challenge had the least relative weight of thymus. The significant difference between this treatment and the control treatment indicates that despite using prebiotic, *Salmonella* caused a decrease in the weight of thymus. Savino and Dardenne [16] expressed that infections cause thymus gland Atrophy. At day 42, the relative weight of bursa of Fabricius had a significant difference among treatments. The treatment group under prebiotic supplementation, restriction and *Salmonella* challenge had the least relative weight of bursa of Fabricius. Feed restriction and *Salmonella* infection stresses might be the main reason of decrease in the relative weight of this lymphoid organ. The plasma corticosterone level can increase because of feed restriction [4] and microbial challenge [28] stresses. The high level of plasma corticosterone can cause a decrease in the immunpcompetance and the weight of lymphoid organs.

The results of this study show that using prebiotics during combined stresses can have some advantages in broilers. Although feed restriction has beneficial effects on broilers, its suppressive impact on immunity system especially when used severely must be considered. These suppressive impacts are more noticeable in cell-mediated immunity than humoral immunity, because the cellular immunity components are more sensitive to energy demand. Therefore, prebiotics are helpful in such a condition as well as in *Salmonella* infections.

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