THE INFLUENCE OF FUNGAL β-GLUCAN ON NONSPECIFIC IMMUNITY IN BROILER CHICKS

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The influence of β-1,3/1,6-D-glucan isolated from Pleurotus ostreatus added to the diet at a level of 20 g/t (group B_{20}) and 40 g/t (group B_{40}) for the whole trial and 40 g/t from the 1st to the 14th day and 20 g/t from the 15th to the 42nd day of the trial (group B_{40+20}) was examined on some immune markers in hybrid Ross 308 chicks. A significantly higher number of monocytes (p<0.01, p<0.05) and significantly higher phagocytic indiced of leukocytes (p<0.01) and heterophiles (p<0.001) after 35 days of the trial were observed in group B_{40} in comparison to the control group and group B_{20}. Significantly higher phagocytic indices of leukocytes (p<0.01) and heterophiles (p<0.001) in comparison to the control group and group B_{20} was found also in group B_{40+20}. The total number of leukocytes, heterophiles, eosinophiles, basophiles and lymphocytes were not significantly influenced, but heterophil/ lymphocyte ratio was significantly lower in group B_{40} (p<0.05) in comparison to the control group. These results show that β-glucan from Pleurotus ostreatus can stimulate nonspecific immunity and reduce the susceptibility of stress in broiler chicken, whereas higher concentrations have a stronger effect.

Key words: haematology, oyster mushroom, phagocytosis, poultry

INTRODUCTION

β-glucans are long chain polysaccharides with only one kind of structural unit – i.e. β-glucose. They are the main structural elements of cell walls of cereals, yeast, fungi, algae and some bacteria (Volman *et al.*, 2008). Differences in the macromolecular structure of β-glucans depend on their origin. Non-branched β-glucans composed of β-D-glycopyranosic subunits bound through (1→4) and (1→3) glycosidic bonds can be found in cereals. The richest source of β-glucan from all cereals is barley (*Hordeum vulgare* L, 3-11%) and oat (*Avena sativa* L, 3-7%). In rye (*Secale cereale* L), wheat (*Triticum*), corn (*Zea mays* L), rice (*Oryza sativa* L), sorghum (*Sorghum bicolor* L) and in millet (*Pennisetum americanum* L) β glucan is found at lower concentrations (Chovancová and Šturdík, 2005;

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Havrlentová and Kraic, 2006). Highly branched β-glucans are found in yeast and fungi. The main chain of these β-glucans is built of β-D-glycopyranosic units bound through (1→3) glycosidic bonds. Along the main chains are randomly branched side chains of β-D-glycopyranosic units bound through (1→6) bonds. Best-known yeast β-glucan is PGG-glucan (betafectin) isolated from *Saccharomyces cerevisiae*. To β-glucans of fungi belong lentinan from *Lentinus edodes*, grifolan (GRN) from *Grifola frondosa*, schizophyllan (SPG) from *Schizophyllum commune* and pleuran from *Pleurotus ostreatus* (Chovancová and Šturdík, 2005; Volman *et al.*, 2008).

According to many studies, β-glucans have significant immune-stimulating effects (Estrada *et al.*, 1997; Rogers *et al.*, 2005; Chen *et al.*, 2008; Rajapakse *et al.*, 2010; Tang *et al.*, 2011), whereby the stronger stimulating force have glucans with more side chains (Brown and Gordon, 2001). Immune-stimulating effects of β-glucans consist in the activation of white blood cells (WBC), mainly neutrophiles/heterophiles, monocytes, macrophages and dendritic cells. The activation mechanism is based on the binding of β-glucans to specific receptors of white blood cells (Novák, 2007). Some of the known receptors are toll-like receptor 2 (TLR-2), dectin-1, complement receptor 3 (CR3), lactosylceramide and others. Binding of β-glucans to these receptors increase the chemokinesis and chemotaxis of WBC, increase also intracellular processes like respiratory burst (stronger production of bactericidal substances and free radicals), higher activity of hydrolytic and metabolic enzymes, and production and release of primary and secondary cytokines (e.g. IL-1, IL-6, TNF-α) (Chovancová and Šturdík, 2005; Novák, 2007).

Besides this immune-stimulating effect, β-glucans can possess also antibacterial (Markova *et al.*, 2003; Lowry *et al.*, 2005; Revolledo *et al.*, 2009) and antivirotic effects (Liu and Li, 1999; Jung *et al.*, 2004; Xiao *et al.*, 2004), and may also defuse coccidial infection (Cox *et al.*, 2010a). β-glucans show also prebiotic properties, they can stimulate the growth of beneficial microorganisms in the gut (Snart *et al.*, 2006; Synytsya *et al.*, 2009).

The objective of the present study was to investigate the influence of β-glucan isolated from oyster mushroom on haematological parameters, on some parameters of nonspecific immunity and on the weight of immune organs in broiler chicks.

MATERIALS AND METHODS

Birds and Diets

Two hundred healthy 1-day-old mixed-sex Ross 308 broiler chicks obtained from a commercial supplier were randomly divided into four groups (one control and three treatment groups; 50 chicks per group) and housed on deep bedding in agreement with the technological instructions for Ross 308 chicks, with controlled room temperature, hygiene and feeding regime. The birdhouse was lit 24 hours a day. Birds in the control group were fed with antibiotic growth promoters- and anticoccidials-free corn-wheat-soybean meal-based basal diets (according to the

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stages of growth) in mash form. In the treatment groups purified β-1,3/1,6-D-glucan ($93 \pm 2\%$) isolated from oyster mushroom (*Pleurotus ostreatus*) was added to the basal diets: in the first treatment group (B_{20}) 20 g/t for the whole trial, in the second treatment group (B_{40}) 40 g/t for the whole trial and in the third treatment group (B_{40+20}) 40 g/t for the 1st - 14th day and 20 g/t for the 15th-42nd day of the trial. The respective amount of β-glucan was first mixed with small amounts of the basal diet and then with a larger amount of the basal diet until the total amount of the respective diets were homogeneously mixed. Diets and drinking water were offered to birds *ad libitum*.

The experiment was carried out in the barns of the Institute of Animal Nutrition and Dietetics at the University of Veterinary Medicine and Pharmacy in Košice in compliance with the ethical requirements.

Collection, Processing and Analysis of Blood Samples

Blood samples were collected from six birds in each group on the 14th and 35th day of the experiment from the jugular vein into a set of glass tubes with 1.5% EDTA for haematological (white blood cells (WBC) count and differential count of WBC) and immunological assays (phagocytic activity and index of phagocytic activity of leukocytes and heterophiles, index of metabolic activity).

Haematological assays were performed only on the 35th day of the experiment. The count of WBC was determined in Bürker's chamber after mixing the blood with Fried-Lukáčová solution (Lukačová and Fried, 1962). The number of individual species of leukocytes was determined in Papenhaim stained blood smears in a total count of 200 cells per slide. Heterophiles and lymphocytes were used to calculate heterophil/lymphocyte (H/L) ratio.

The phagocytic activity of leukocytes and heterophiles was evaluated using microspheric hydrophilic particles (MSHP, Artim, s.r.o., Czech Republic) according to Vetvička *et al.* (1982). Briefly, MSHP diluted with phosphate buffered saline (PBS) to an approximate concentration 5×10^8 particles/mL were mixed in a test tube with 0.1 mL of whole blood. After incubation for 75 min at 37^oC with occasional shaking, blood smears were prepared using Pappenheim's standard panoptic method. Phagocytic activity tests were performed by evaluating at least 200 leukocytes capable of phagocytosis. Cells containing 3 or more absorbed particles were considered as phagocytizing. The values of phagocytic activity were expressed as the percentage of cells with phagocytised particles compared to the total number of leukocytes regardless of the lymphocytes.

Iodo-nitro-tetrazolium (INT) reductase test was used for the determination of metabolic activity (MA) of polymorphonuclear cells during phagocytosis according to the method of Mareček and Procházková (1986). Leukocytes were separated by the osmotic shock method and resuspended in RPMI 1640 medium (Biotech, Germany). Pure RPMI medium in a volume of 50 μL was placed into eight wells in a row of a 96- well tissue culture plate (Sarstedt, USA), which were used as a blank sample. Another line of wells contained 25 μL of leukocyte suspension (15.10^6 per mL) and 25 μL of 0.1% INT (Sigma-Aldrich, USA). The first four wells were supplemented with a solution of Zymozan (Sigma-Aldrich, USA) in an amount of 10 μL. Zymozan served as a cell stimulator. Saline was added into

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the other four wells to present non-stimulated cells. Immediately after 45 min incubation at 37°C, the reaction was stopped by adding 100 μL of hydrochloric acid to each well and the plates were centrifugated 200-300x g for 10 min. The supernatant was removed and the plates were dried at 37-40°C during 30-45 min. Then 100 μL of DMSO (dimethylsulphoxide, Sigma-Aldrich, Germany) was added into each well and allowed to stand for 10 min at 20°C. The optical density (OD) was then measured spectrophotometrically at 450 nm. The results were described in the form of an index of metabolic activity (IMA) based on the ratio between OD of the suspension of spontaneous active cells and the OD of suspension of cells activated after stimulation by zymozan.

The immune organs

Spleen and bursa of Fabricius were collected from eight birds in each group after their weighting and slaughtering by cervical dislocation on the 14th and 35th day of the experiment, weighed and calculated as a percentage of body weight (relative weight).

Statistical Analysis

The results were analyzed by one-way ANOVA and significant differences between the groups were tested by Tukey's Multiple Comparison Test (level of significance set at $p < 0.05$, $p < 0.01$, $p < 0.001$).

RESULTS

The mean values of haematological parameters are shown in Table 1. The supplementation of β-glucan in used concentrations led to the increase of WBC count in all treatment groups, but this increase was not statistically influenced. The highest count of WBC was observed in the group with β-glucan supplementation at the higher concentration during the whole trial period (group B_{40}). The evaluation of differential count of WBC showed a significantly higher number of monocytes in the B_{40} group than in the control ($p < 0.01$) and B_{20} group ($p < 0.05$).

Table 1. Results of hematological examination (10^6/L) on day 35 of the experiment (n=6, x±SEM)

	Control	B20	B40	B40+20
WBC	31.67±5.78	35.00±4.47	48.20±7.11	32.83±4.20
Heterophiles	10.71±1.98	11.18±1.38	13.51±2.28	9.69±1.21
Eosinophiles	1.37±0.46	1.49±0.50	2.10±0.60	1.69±0.46
Basophiles	2.01±0.51	2.04±0.52	1.79±0.19	1.59±0.31
Monocytes	0.15±0.08[c]	0.23±0.06[b]	0.54±0.09[a]	0.26±0.07
Lymphocytes	17.42±3.47	20.06±3.10	30.24±4.58	19.60±2.66

[ab]<0.05, [ac]$p < 0.01$

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The count of heterophiles, eosinophiles, basophiles and lymphocytes was not statistically influenced. However, H/L ratio was significantly lower in the treatment group B_{40} ($p<0.05$) in comparison to the control group (Figure 1). Also in groups B_{20} and B_{40+20} a lower value of this marker was measured than in the control group, but not significantly.
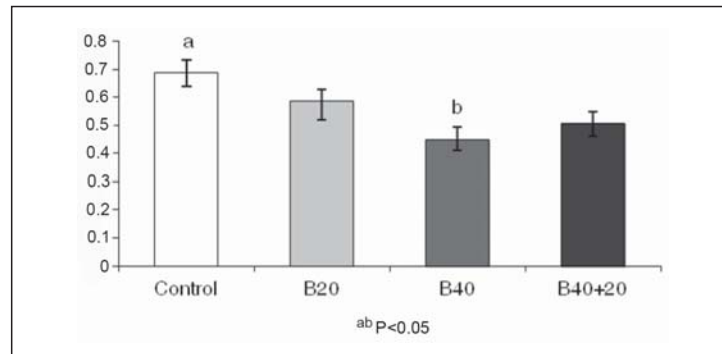

Figure 1. Heterophil/lymphocyte ratio

Table 2. Parameters of nonspecific immunity after 14 and 35 days of experiment ($n=6$; $x\pm SEM$)

	Control	B_{20}	B_{40}	B_{40+20}
	After 14 days			
PA_{Le} (%)	36.60±6.47	29.00±2.05	26.00±1.15	33.33±8.25
IPA_{Le}	6.40±0.66	5.17±0.25	4.80±0.19	4.75±0.60
PA_{He} (%)	91.42±5.34	87.60±2.44	93.27±2.88	81.37±4.48
IPA_{He}	7.17±0.59	5.70±0.11	5.34±0.17	5.81±0.54
IMA	1.24±0.02	1.23±0.03	1.35±0.05	1.28±0.02
	After 35 days			
PA_{Le} (%)	55.20±3.12	46.40±2.25	50.25±4.01	46.80±3.02
IPA_{Le}	6.84±0.34[a]	6.50±0.27[a]	12.54±0.99[c]	12.65±2.03[c]
PA_{He} (%)	98.48±0.76	94.38±2.04[a]	97.38±0.93	100.00±0.00[b]
IPA_{He}	7.02±0.45[a]	6.92±0.40[a]	15.82±0.63[d]	14.05±1.98[d]
IMA	2.19±0.07	2.30±0.14	3.16±0.25	2.28±0.46

PA_{Le} – phagocytic activity of leukocytes, IPA_{Le} – index of phagocytic activity of leukocytes, PA_{He} – phagocytic activity of heterophiles, IPA_{He} – index of phagocytic activity of heterophiles, IMA – index of metabolic activity; [ab]$p<0.05$, [ac]$p<0.01$, [ad]$p<0.001$

The values of the monitored indicators of nonspecific immunity, phagocytic activity and index of phagocytic activity of leukocytes and heterophiles and index of metabolic activity are shown in Table 2. No significant differences in monitored

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parameters of nonspecific immunity were observed after 14 days of the trial. A significantly higher index of phagocytic activity of leukocytes (p<0.01) and heterophiles (p<0.001) was found in groups B_{40} and B_{40+20} after 35 days of trial when compared to the control and B_{20} group. The phagocytic activity of heterophiles in the B_{40+20} group was significantly higher than in the B_{20} group (p<0.05), but not than in the control group. The phagocytic activity of leukocytes and index of metabolic activity were not significantly influenced after 35 days by the β-glucan supplementation.

Relative weights of the immune organs, spleen and bursa of Fabricius, were not significantly influenced by dietary supplementation of β-glucan (Table 3).

Table 3. Relative weight of immune organs after 14 and 35 days of experiment (n=8, x±SEM)

	Control	B_{20}	B_{40}	B_{40+20}
	After 14 days			
Spleen	0.091±0.012	0.077±0.007	0.068±0.006	0.091±0.010
B. of Fabricius	0.228±0.023	0.283±0.015	0.221±0.012	0.231±0.022
	After 35 days			
Spleen	0.087±0.003	0.103±0.006	0.096±0.007	0.110±0.008
B. of Fabricius	0.245±0.021	0.195±0.016	0.198±0.011	0.256±0.024

DISCUSSION

The influence of purified β-glucan examined in the present study showed that the supplementation of the diet with this substance may lead to improvement of non-specific immunity in broiler chicks. In the treated group, where the β-glucan supplementation was performed in a dose of 40 g/t (group B_{40}), a significantly higher count of monocytes (p<0.01, p<0.05) and significantly higher index of phagocytic activity of leucocytes (p<0.01) and heterophiles (p<0.001) was observed after 35 days of trial in comparison to the control and B_{20} group. A significantly higher index of phagocytic activity of leucocytes (p<0.01) and heterophiles (p<0.001) in comparison to the control and B_{20} group was found also in the group where the supplementation of β-glucan was done in combined concentrations (group B_{40+20}). In this group, significantly higher percentage of phagocytic heterophiles was noticed when compared to group B_{20} (p<0.05), but not to the control group. Similar results were observed by Lowry *et al.* (2005) in roosters fed with a diet supplemented with purified β-glucan of unknown origin. This supplementation led to a significantly higher percentage of phagocytic heterophiles and a significantly higher index of phagocytic activity and respiratory burst. In our experiment, the index of metabolic activity was not significantly influenced, but values of this parameter were numerically higher in all treatment groups than in the control group. Chen *et al.* (2008) observed higher phagocytic ability of abdominal macrophages in chicks fed a diet supplemented with β-

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glucan isolated from *Schizophyllum commune* in 0.1% concentration. These authors investigated also the direct influence of this β-glucan on abdominal macrophages *in vitro*. The phagocytic activity of macrophages from β-glucan-free chicks was significantly increased due to the effect of β-glucan. The increase of the macrophage phagocytic activity was observed also in the study by Guo *et al*. (2003) in broiler chicks, which were fed a diet containing 20 and 40 mg/kg yeast β-glucan in the starter and 20 and 20 mg/kg in the grower diet.

One of the haematological parameters, the H/L ratio, is changed during exposition to stress and might be used as a reliable indicator of stress in birds. Lymphocytes under the force of stress migrate from the blood to lymphatic nodes, spleen and skin. On the other side, heterophiles are released from the bone marrow into the blood and so the H/L ratio is pushed (Gross and Siegel, 1983). We observed a significantly lower value of H/L ratio in the group fed the diet supplemented with β-glucan in higher concentration for the whole trial (group B_{40}) than in the control group ($p<0.05$). These result show that addition of β-glucan from oyster mushroom into the diets might lead to decreased susceptibility of chicks to stress factors, and so reduce production losses caused by stress. Controversially, Cox *et al.* (2010b) did not observe any changes in H/L ratio in chicks fed the diets supplemented with β-glucan from *Saccharomyces cerevisiae*.

The spleen and bursa of Fabricius are important lymphoid organs. The spleen is the major site of immune response to blood borne antigens (Chen *et al.*, 2003) and bursa of Fabricius is the site of B-cell maturation and differentiation responsible for production of immunoglobulin and humoral immune response (Chen *et al.*, 2008). Chen *et al.* (2003) observed increased splenocytes proliferation in broiler chicks, supplemented with lentinan (β-glucan with shown immunostimulating and antitumor functions isolated from mushroom *Lentinus edodes*) in doses of 40, 80, and 160 μg/mL. Chen *et al.* (2008) noticed a significantly increased relative weight of the bursa of Fabricius in chicks after treatment with β-glucan from *Schizophyllum commune* in 0.1% concentration. Similar results were obtained by Guo *et al.* (2003) in broiler chicks, which ingested yeast β-glucan in a concentration of 20 or 40 mg/kg in the starter and 20 and 20 mg/kg in the grower diet. The relative weight of lymphoid organs observed in our study was not significantly influenced after β-glucan treatment. In the same manner, no change in the relative weight of spleen and bursa of Fabricius in broiler chicks were observed by Cox *et al.* (2010b), who investigated the effect of the yeast β-glucan (isolated from *Saccharomyces cerevisiae*) added to the diets in 0.02 and 0.1 % concentration.

These variable results due to β-glucan supplementation observed in the literature might result from differences in the source and preparation of β-glucan (Cox *et al*., 2010a).

In conclusion, the addition of β-glucan to the diet increased the count of monocytes, and also increased the phagocytic activity of leukocytes and heterophiles. Moreover, the supplementation of β-glucan to the diet led to a significant decrease in H/L ratio indicating higher resistance of chicks to stress. Although several sources showed the impact of β-glucan on the weight of

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lymphoid organs, in our study the weight of spleen and bursa of Fabricius were not influenced. We showed that an addition of β-glucan isolated from *Pleurotus ostreatus* to the diets can stimulate the nonspecific immunity and reduce the susceptibility of stress in broiler chicken, whereas higher concentrations have stronger effects.

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SADRŽAJ

Ispitivan je uticaj β-1,3/1,6-D-glukana izolovanog iz *Pleurotus ostreatus-a* na neke pokazatelje imunskog statusa pilića hibrida Ross 308. Ovaj preparat je dodavan u hranu za brojlere u količini od 20 g/t (grupa B_{20}) i 40 g/t (grupa B_{40}) tokom celog ogleda i u količini od 40 g/t od prvog do 14. dana i 20g/t od 15-42. dana ogleda (grupe B_{40+20}). Posle 35 dana, u grupi B_{40} je zapaženo značajno povećanje broja monocita (p<0,01, p<0,05) i značajno veći fagocitni indeks kod leukocita (p<0,01) i heterofilnih granulocita (p<0,001) u poređenju sa kontrolnom grupom i grupom B_{20}. U grupi B_{40+20} je takođe zabeležen značajno veći fagocitni indeks leukocita (p<0,01) i heterofilnih granulocita (p<0,001) u odnosu na kontrolnu grupu i grupu B_{20}. Ukupni broj leukocita, heterofila, oezinofila, bazofila i limfocita nije bio značajno promenjen, ali je odnos heterofili/limfociti bio značajno niži u grupi B_{40} u odnosu na kontrolnu grupu. Ovi rezultati uklazuju na to da β-glukan izolovan iz *Pleurotus ostreatus*-a može da stimuliše nespecifične imunske mehanizme i tako smanji osetljivost brojlera na stres. Više koncentracije β-glukana ispoljavaju snažniji efekat.