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Effect of *Agaricus blazei* in the diet of broiler chickens on immunity, serum parameters and antioxidant activity

Efeito do *Agaricus blazei* na dieta sobre a imunidade, parâmetros séricos e ação antioxidante na carne de frangos de corte

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Abstract

The effect of adding *Agaricus blazei* to the diet of broilers on immunity, serum parameters, and antioxidant activity was evaluated. A total of 840 1-day-old chicks were used, distributed among five levels of a completely randomized design (0.0, 0.05, 0.10, 0.15, and 0.20% *A. blazei*), with six replications and 28 birds per experimental unit. The weights of the thymus, spleen and cloacal bursa were not influenced ($P > 0.05$). Leukocytes, macrophages and nitric oxide were unaffected ($P > 0.05$), but at each supplementation level compared with the control, differences appeared in the percentages of eosinophils and macrophages ($P < 0.05$) at inclusion levels of 0.10, 0.15 and 0.20%. At 28 days, the antibody titer against Newcastle disease showed a quadratic response ($P < 0.05$) with supplementation, and from the estimated level of 0.08% the production of antibodies was stimulated; however, the same behavior was not observed ($P > 0.05$) at 42 days. Hypocholesterolemic effect was demonstrated ($P < 0.05$), but the serum triglyceride concentration was not affected ($P > 0.05$). The antioxidant activity of mushroom showed a positive linear effect ($P < 0.05$) on DPPH capture on day zero of meat cooling. The inclusion of *A. blazei* in the diet of broilers provided an immunostimulatory activity and hypocholesterolemic effect. Residual compounds with antioxidant activity were present in the meat, which may promote tissue protection of the animal *in vivo*, making possible the use of *A. blazei* as a natural additive.
Key words: Cholesterol. Free radical. Immunoglobulins. Leukocytes. Mushroom.

Resumo

Avaliou-se o uso do *Agaricus blazei* na alimentação de frangos de corte sobre a imunidade, parâmetros séricos e a atividade antioxidante. Utilizou-se 840 pintos distribuídos em delineamento experimental inteiramente casualizado, cinco níveis (0,0; 0,05; 0,10; 0,15 e 0,20%) de *Agaricus blazei*, seis repetições e 28 aves por unidade experimental. O peso do timo, baço e bolsa cloacal não foi influenciado pelos tratamentos experimentais (P>0,05). Os leucócitos, macrófagos e o óxido nítrico não foram afetados pelos níveis de *Agaricus blazei* (P>0,05), no entanto a porcentagem de eosinófilo e de macrófagos diferenciaram-se (P<0,05) nos níveis de 0,10, 0,15 e 0,20% em relação ao controle. Aos 28 dias, os níveis de anticorpos contra a doença de Newcastle apresentaram resposta quadrática (P<0,05) à suplementação, e a partir do nível estimado de 0,08% estimulou-se a sua produção, contudo o mesmo

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não ocorreu (P>0,05) aos 42 dias. A ação hipocolesterolêmica foi comprovada (P<0,05) mas a suplementação não interferiu (P>0,05) na concentração sérica de triglicerídeo. A atividade antioxidante do cogumelo apresentou efeito linear positivo (P<0,05) sobre o DPPH no dia 0 de refrigeração da carne. A inclusão do cogumelo proporcionou atividade imunoestimulante e hipocolesterolêmica. Observou-se a presença de compostos residuais antioxidantes, proteção tecidual do animal "in vivo" e possibilidade de uso do *Agaricus blazei* como um aditivo natural em rações de frangos.
Palavras-chave: Cogumelo. Colesterol. Imunoglobulinas. Leucócitos. Radical livre.

Introduction

The immune response is the body's first line of defense against potentially harmful organisms (ABBAS et al., 2012). To activate the immune system, a number of cellular components and substances are needed that perform various defense functions in the bird's body (GERTNER et al., 2008). During the first week of life, the bird experiences a great increase in the number of leukocytes and the development of lymphoid organs. These events will modulate all of the bird's immunity throughout its life, which makes the early stage of development a nutritionally critical period, as deficiencies or excesses can impact the immune system (KLASING, 1998). The productive potential of farms and the pressure exerted by pathogenic and non-pathogenic agents on immunity and animal metabolism has intensified, and supplemental additives have become an essential tool in the search for improved performance and economic results.

In the search for new types of additives, *Agaricus blazei*, also known as the sun mushroom and native to Brazil, and other species of medicinal mushrooms have been studied in recent decades for their medicinal effects *in vitro* and *in vivo* (RESHETNIKOV et al., 2001). *A. blazei* is considered a nutraceutical food because, in addition to its nutritional value, its secondary metabolites are chemically diverse and have a wide spectrum of biological activity (KHATUN et al., 2012). Among the bioactive compounds are polysaccharides, glucan peptides, glycoproteins, triterpenoids, polyphenols, vitamins, minerals, and polyunsaturated fatty acids (SINGH et al., 2011). It is believed that the mushroom can be classified as a new type of prebiotic due to the presence of several types of carbohydrates, many of them resistant to hydrolysis by gastric enzymes (AIDA et al., 2009). Among the polysaccharides of interest, the betaglucans have drawn attention for their ability to enhance the immune system. The bioactive substances of mushrooms enhance the proliferation of B and T lymphocytes and the synthesis of immunoglobulins (FANG; LIN, 2003), reduce cholesterol and diabetes (FIRENZUOLI et al., 2008), and possess antioxidant (MAU et al., 2004) and bacteriocidal activities (RANA et al., 2008). Foods containing antioxidants can help to reduce the production of free radicals, which compromise tissue integrity (KOZARSKI et al., 2011). The replacement of synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylhydroxyanisole (BHA), and etoxiquim, among others, with natural substances has shown positive results (BOTSOGLOU et al., 2003; FASSEAS et al., 2007). Mushrooms have, as part of their constitution, phenolic compounds whose antioxidant activity is characterized by the ability to stabilize free radicals (MORAES; COLLA, 2006). Thus, the objective was to study the effect of *A. blazei* mushroom supplementation in the diet of broiler chickens on the immune system, serum parameters, and antioxidant activity in the meat.

Materials and Methods

The experiment was conducted at the Iguatemi Experimental Farm Poultry sector belonging to the State University of Maringa, under the approval of Experimentation in Animal Ethics Committee – CEAE/EMU (Registration N°. 028/2013). Male broiler chicks (n = 840) of the commercial line

"Cobb" were distributed in an air-conditioned shed in a completely randomized design with five *A. blazei* mushroom powder levels in the diet (0.0, 0.05, 0.10, 0.15 and 0.20%) and six replicates of 28 birds each. The feeding program was divided into three stages: initial (1 to 7 days of age), growth (8 to 21 days of age) and final (22 to 42 days). The feed formulations (Table 1) were based on corn and soybean meal, using the chemical composition values of foods and nutritional requirements for male broilers at each stage, according to Rostagno et al. (2011).

Table 1. Percentage and calculated composition of experimental diets for 1 to 7 days, 8 to 21 days, and 22 to 42 days of age.

Ingredients (%)	**1-7 days**	**8-21 days**	**22-42 days**
Corn	54,60	58,70	63,59
Soybean meal	38,36	34,88	29,59
Dicalcium phosphate	1,97	1,56	1,22
Calcareous	0,78	0,80	0,71
Soy oil	2,36	2,34	3,27
Salt	0,5	0,48	0,45
DL-methionine, 99%	0,33	0,26	0,22
L-lysine, 78%	0,29	0,22	0,21
L-threonine, 99%	0,11	0,06	0,04
Suppl. Min and Vitam.[1,2]	0,40	0,40	0,40
Inert[3]	0,30	0,30	0,30
TOTAL	100	100	100
Calculated Composition			
Energy Met. (kcal kg^{-1})	2.950	3.000	3.125
Crude protein (%)	22,20	20,80	18,75
Digestible lysine (%)	1,310	1,174	1,044
Digestible Met + Cis (%)	0,944	0,846	0,762
Digestible tryptophan (%)	0,226	0,206	0,175
Digestible threonine (%)	0,852	0,763	0,678
Digestible valine (%)	0,918	0,866	0,783
Digestible arginine (%)	1,418	1,323	1,173
Calcium (%)	0,920	0,819	0,685
Available phosphorus (%)	0,470	0,391	0,320
Sodium (%)	0,217	0,210	0,198
Potassium (%)	0,860	0,809	0,726
Chlorine (%)	0,350	0,340	0,322

- Electrolyte balance: 215,76 mEq kg^{-1} (1 to 7 days); 202,26 mEq kg^{-1} (8 to 21 days); 180,88 mEq kg^{-1} (22 to 42 days).

[1](1-21 days old) Vitamin supplement (content kg^{-1} of premix): Vit. A 2.916.670 UI kg^{-1}; Vit.D3 583.330 UI kg^{-1}; Vit. E 8.750 UI kg^{-1}; Vit. K3 433.33 mg kg^{-1}; Vit.B1 408.33 mg kg^{-1}; Vit.B2 1.333,33 mg kg^{-1}; Vit. B12 4.166,67 mcg kg^{-1}; Niacin 8.983,33 mg kg^{-1}; Calcium Pantothenate 3.166,67 mg kg^{-1}; Folic Acid 200 mg kg^{-1}; Biotin 25 mg kg^{-1}. Mineral Supplement (content kg^{-1} of premix): Iron 12.6 g kg^{-1}; Covers 3.072 mg kg^{-1}; Iodine 248 mg kg^{-1}; Zinc 12.6 g kg^{-1}; Manganese 15 g kg^{-1}; Selenium 61.20 mg kg^{-1}; Cobalt 50.40 mg kg^{-1}.

[2](22-42 days old) Vitamin supplement (content kg^{-1} of premix): Vit. A 2.250.000 UI kg^{-1};
Vit.D3 450.000 UI kg^{-1}; Vit. E 7.000 UI kg^{-1}; Vit. K3 418 mg kg^{-1}; Vit.B1 300 mg kg^{-1}; Vit.B2 1000 mg kg^{-1}, Vit.B12 3000 mcg kg^{-1}; Niacin 7000 mg kg^{-1}; Calcium Pantothenate 2500 mg kg^{-1}; Folic Acid 140 mg kg^{-1}; Biotin 14 mg kg^{-}. Mineral Supplement (kg-content of premix): Iron 12.5 g kg^{-1}; Covers 3000 mg kg^{-1}; Iodine 250 mg kg^{-1}; Zinc 12.5 g kg^{-1}; Manganese 15 g kg^{-1}; Selenium 75 mg kg^{-1}; Cobalt 50 mg kg^{-1}.

[3]Caulim: the *A. blazei* levels were included to replace the inert during the three stages of growth.

After seven days of age, the entire batch was vaccinated against Newcastle disease (Fort Dodge®) and after 28 and 42 days of age, blood was collected for antibody titration using an enzyme immunoassay according to the manufacturer's instructions (IDEXX®).

After 28 days, the leukocyte profile was determined by collecting blood samples in the presence of an anticoagulant (EDTA) from two animals per experimental unit. For the evaluation, according to Charles Noriega (2000), differential counts were performed on blood smears on glass slides, stained using the Giemsa – May Grunwald method and observed under an optical microscope with an immersion objective.

The evaluation of phagocytic activity (%) and peritoneal macrophage nitric oxide production was performed according to the method of Quershi et al. (1986) with some modifications. The production of macrophages was stimulated in six birds per level of supplementation, on the 35th day of life, by intra-abdominal injection of Sephadex G-50® (Sigma) 3% (1 mL 100 g^{-1} live weight). After 42 h, the birds were sacrificed by cervical dislocation and carried by inoculation of 20 mL of heparinized sterile PBS solution (0.5U mL^{-1} Liquemine® – Roche), after which 15 mL of abdominal fluid were collected. The material was centrifuged at 1500 rpm for 10 min and the pellet resuspended in 2 mL RPMI 1640® (Sigma). One hundred fifty microliters of this suspension was added to each well of a 24-well macrophage culture plate containing 13-mm diameter coverslips. After incubation for 1 hr at 37 °C with 5% CO_2 and washing of the plate with RPMI 1640 solution, 200 sheep erythrocytes were added and incubated for 1 h. Then, the plate was washed with sterile PBS and the coverslips stained using a commercial kit (Fast Panotic LB® – Laborclin). Macrophages were counted (200 per coverslip), checking the number of cells with engulfed red blood cells. The phagocytic activity (FA) was calculated as the ratio of the number of macrophages containing phagocytosed erythrocytes to the total number of macrophages counted. The nitric oxide concentration (μmol mL^{-1}) produced by adhering macrophages was determined in another plate; after the second washing, the wells were maintained for 24 h in an oven with 200 mL of RPMI 1640. The concentration of nitric oxide was performed using the Griess reaction and subsequent reading in a spectrophotometer.

At 21 days old, the lymphoid organs (thymus, spleen and cloacal bursa) were collected from one bird each. The relative weight was obtained after dissection and removal of exogenous tissue, depending on body weight. At 28 and 42 days of age, serum levels of total cholesterol (mg dL^{-1}) and triglycerides (mg dL^{-1}) were determined by means of an enzymatic-colorimetric procedure using commercial kits (Diagnostic Gold analyzes Ltda.) and spectrophotometric reading at 500 nm in a Bio-2000 (Bioplus Laboratory Products Ltd.) according to the manufacturer's instructions.

At 42 days of age, lipid oxidation of the meat and the antioxidant action of the mushroom were verified by two analyses on day 0 and after five days of refrigeration at 4 °C: 1) analytical determination of thiobarbituric acid (TBA) reactive substances (TBARS) according to Sorensen and Jorgensen (1996) consisted of a 5 g sample of ground beef homogenized with 15 ml of trichloroacetic acid solution (7.5%), gallic acid (0.1%) and EDTA (0.1%). The solution was filtered and 1.5 mL were taken and mixed with 1.5 mL of TBA and taken to a water bath for 40 min. After centrifugation at 3000 RPM for 10 min, the absorbance of the final products of lipid peroxidation, which reacted with thiobarbituric acid, was read at a wavelength of 532 nm in a spectrophotometer. For the calculations, the values obtained for the samples were plotted on a malonaldehyde standard curve and the data were expressed as μM of malonaldehyde (MDA) g^{-1} of sample. 2) the test for antioxidant activity for the capture of organic free radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH) consisted of mixing 100 μL of the extracted solution with 1.9 mL of the

DPPH solution. Subsequent readings took place in the spectrophotometer at 515 nm for 1 h to allow the absorbance to stabilize (LI et al., 2009).

The amount of beta-glucan mushroom polysaccharide was determined by the Spectroscopy Laboratory, Department of Physics, of Maringa State University, through Raman spectroscopy analysis and the concentration of beta-glucan is given in percent per 100 g of sample (NOVAK et al., 2012). The concentration of polyphenols was determined using the Folin-Ciocalteu method (SINGLETON; ROSSI JUNIOR, 1965). The extraction was carried out using 0.1 g mushroom in 10 mL of methanol. The solution was homogenized and allowed to rest for 10 min. This procedure was performed three times and centrifuged for 10 min at 3000 rpm. A 125 µL volume was recovered from the supernatant and added to 125 µL of Folin-Ciocalteu reagent and 2250 µL sodium carbonate. The solution was incubated for 30 min, the reading took place in a spectrophotometer at 725 nm and the value was expressed in milligrams of gallic acid equivalents per gram of mushroom.

The results were subjected to ANOVA and polynomial regression according to the statistical program SAEG: Analysis Statistics and Genetic Systems (UFV, 2005). A comparison of data between the control and the other levels of inclusion of *A. blazei*, was carried out using Dunnett's test at a 5% probability. The following mathematical model was used: $\mathbf{Y_{ij} = \mu + b_1A + b_2A^2_i + \varepsilon_{ij}}$, where Y_{ij} = dependent variable; µ = general average; b_1 = linear regression coefficient depending on the level of *A. blazei* (0.5%, 0.10%, 0.15% and 0.20%); A = *A. blazei* level for each phase; b_2 = quadratic regression coefficient depending on the level of *A. blazei*; and e_{ij} = random error.

Results and Discussion

The concentrations of beta-glucans and polyphenols found in *A. blazei* were 18% and 5.07 mg gallic acid equivalents, respectively. At 28 days of age, it was revealed that supplementation promoted a quadratic response ($P < 0.05$) in antibody titre against Newcastle disease broilers vaccinated for seven days (Table 2). From the estimated level of addition, there was a 0.08% stimulation of antibody production, confirming that the fractions of polysaccharides present may enhance the humoral immune response (GUO et al., 2004). The level of 0.20% was different ($P < 0.05$) from that of the controls, indicating a greater stimulation of stimulating immunoglobulins by the chicken defense system, however this increase in production of antibodies was not associated with an increase in the number of lymphocytes. Similar behavior was observed by Chan et al. (2007), wherein the intragastric administration of *A. blazei* elevated serum levels of immunoglobulin G in rats, but did not increase the lymphocyte proliferation rate, indicating an increase in immunoglobulin secretion efficiency by B lymphocytes elicited by polysaccharides of *A. blazei*.

At 42 days of age, antibody titration was greater than at 28 days, but was not influenced ($P > 0.05$) by the addition of mushroom. Vaccination stimulates B lymphocytes through interaction of their antigen receptor with the antigen of the vaccine itself, and betaglucans have the ability to leverage this response, resulting in augmentation of the adaptive immune response (DARPOSSOLO et al., 2010). The adjuvant activity of the polysaccharide against coccidiosis vaccine was demonstrated by Guo et al. (2005) to work with the fungus species *Lentinus edodes* and *Tremella fuciformis* and the herb *Astragalus membranaceus*.

Table 2. Titre of antibodies (log_{10}) (± standard error) against the virus of Newcastle disease in broilers supplemented with *Agaricus blazei* at various levels.

A. blazei (%)	28 days	42 days
0	2,715 ± 0,053	2,939 ± 0,073
0,05	2,747 ± 0,041	3,262 ± 0,075
0,10	2,642 ± 0,010	3,161 ± 0,053
0,15	2,923 ± 0,031	3,034 ± 0,095
0,20	3,302 ± 0,164*	3,148 ± 0,131
CV (%)	6,27	6,43
Regression	Q^1= 0,08	NS

* Significant 5% by Dunnett's test ($P < 0.05$); NS = not significant; Q = Quadratic
[1]$Q = 3,022 – 8,208x + 48,4x^2$ ($R^2 = 0.98$; $P < 0.0001$).

The index of macrophage phagocytosis and nitric oxide concentration (Table 3) were not affected ($P > 0.05$). However, when comparing each level with the control, macrophages were increased ($P < 0.05$) at the levels of 0.10, 0.15 and 0.20% inclusion of *A. blazei*. It is likely that the betaglucans present in the mushroom activated immune functions by increasing the concentration of macrophages, and this is possible only in the presence of the polysaccharide receptors that have been identified on the surfaces of macrophages (BATTLE et al., 1998). Sorimachi et al. (2001) concluded that *A. blazei* is capable of stimulating macrophages *in vitro*, resulting in the induction of cytokines and the production of nitric oxide. Many prophylactic techniques are presented to the poultry industry to prevent diseases, and the immunostimulatory action of the active ingredients of mushrooms can make them a great candidate to promote animal health (GUO et al., 2003).

Table 3. Phagocytosis Index (%) and nitric oxide concentration (μm mL^{-1}) (± standard error) in broilers fed diets supplemented with *Agaricus blazei* at various levels.

A. blazei (%)	Phagocytosis Index	Nitric Oxide
0	17,63 ± 1,34	0,35 ± 0,16
0,05	22,63 ± 1,57	0,51 ± 0,10
0,10	25,00 ± 2,27*	0,54 ± 0,16
0,15	24,56 ± 1,79*	0,59 ± 0,11
0,20	24,31 ± 1,39*	0,41± 0,16
CV (%)	21,13	72,22
Regression	NS	NS

* Significant 5% by Dunnett's test ($P < 0.05$); NS = not significant.

With respect to leucocyte differential count, there was no effect of the addition of mushroom ($P > 0.05$) on cells of the immune system or the heterophil:lymphocyte ratio (Table 4).

The supplementation of *A. blazei* in the diet of broilers did not affect ($P > 0.05$) the weight of organs related to the immune system, such as the thymus, spleen, and cloacal bursa, at 21 days (Table 5), although it has been shown that the mushroom immunoactvators in broilers include a variety of compounds, such as polysaccharides, alkaloids and organic acids, which stimulate the immune system (GUO et al., 2004). Similar results were found using *Saccharomyces cerevisiae* and *P. ostreatus* (DARPOSSOLO et al., 2010; DANESHMAND et al., 2011).

Table 4. Leukocyte values (%) and the heterophil:lymphocyte ratio (H:L) (± standard error) in broilers at 28 days of age supplemented with *Agaricus blazei* at various levels.

A. blazei (%)	Lymphocyte	Heterophils	Eosinophil	Basophil	Monocyte	H:L
0	68,55±1,82	9,49±1,09	2,83±0,60	5,99±0,68	7,16 ±1,70	0,14±0,02
0,05	67,67±2,59	10,0±1,46	4,33±0,71	5,00±1,15	7,33 ± 1,20	0,15±0,03
0,10	71,67±2,03	8,17±0,79	5,67±0,71*	4,17±0,75	6,50 ± 1,57	0,11±0,01
0,15	65,00±1,98	8,70±1,58	5,83±0,87*	4,67±0,61	10,33 ±1,26	0,14±0,03
0,20	69,83±1,33	8,00±1,51	6,00±0,52*	5,67±0,67	7,50 ±1,36	0,12±0,02
CV (%)	7,11	36,52	34,48	38,28	45,10	42,46
Regression	NS	NS	NS	NS	NS	NS

* Significant 5% by Dunnett's test ($P < 0.05$); NS = not significant.

Table 5. Relative weight (%) (± standard error) of the spleen, thymus and cloacal bursa of broiler chickens at 21 days of age fed diets supplemented with *Agaricus blazei* at various levels.

A. blazei (%)	Spleen	Cloacal Bursa	Thymus
0	0,123 ± 0,020	0,212 ± 0,014	0,484 ± 0,045
0,05	0,093 ± 0,019	0,214 ± 0,020	0,500 ± 0,031
0,10	0,095 ± 0,011	0,243 ± 0,017	0,494 ± 0,055
0,15	0,106 ± 0,010	0,279 ± 0,024	0,526 ± 0,046
0,20	0,104 ± 0,005	0,275 ± 0,031	0,642 ± 0,067
CV (%)	28,04	21,84	23,24
Regression	NS	NS	NS

* Significant 5% by Dunnett's test ($P < 0.05$); NS = not significant.

Supplementation did not stimulate the production of lymphocytes ($P > 0.05$), the percentage of which was within the standard 60% to 65% in broilers (MACARI; LUQUETTI, 2008) and lymphocyte counts were similar to the values found by Cardoso and Tessari (2003) and Roll et al. (2010). The percentage of heterophiles was even below the expected 25% to 30% (MACARI; LUQUETTI, 2008); however, they still constituted the most abundant granulocyte in the blood of the birds. The percentage of monocytes was found to be below the stipulated 10% (SWENSON; REECE, 1996; MACARI; LUQUETI, 2008) and the percentage of basophils was close to the reported value of 4% (SWENSON; REECE, 1996). The local blood puncture and laboratory technique used, the age of the birds, the environmental conditions, and the form of accommodation that they are in at the time of the experiments are all factors that can interfere with hematological parameters (CARDOSO; TESSARI, 2003; BORSA, 2009). The study birds were kept under good environmental and sanitary conditions throughout the production cycle as, according to Daneshmand et al. (2011) the presence or absence of health challenge can also influence the outcome of the mushroom operation.

Eosinophils showed differences ($P < 0.05$) at *A. blazei* supplementation levels of 0.10, 0.15 and 0.20% compared with the control, and their percentage was found to be above the 2% stipulated for leukocytes in broilers (MACARI; LUQUETTI, 2008), but within the stipulated 3-8% proposed by Swenson and Reece (1996). In humans, the ability of betaglucans to modulate the immune system has been reported, both at the humoral and cellular levels, through the stimulation of macrophages, neutrophils, eosinophils, and lymphocytes. Much of the gastrointestinal betaglucans are phagocytosed by macrophages, being driven into the bone marrow. Here, fragments of polysaccharides bind to

CR3 receptors on granulocytes, such as neutrophils and eosinophils. These activated granulocytes, when recruited by inflammatory stimuli, act on tumors (FIRENZUOLI et al., 2008). Therefore, it is possible to suggest that the slight increase observed in the number of eosinophils, associated with an increase in the number of macrophages, is related to the activation of the same against the presence of betaglucans.

There was no effect of *A. blazei* ($P > 0.05$) on serum cholesterol and triglyceride parameters at 28 days of age (Table 6). However, after 42 days, the hypocholesterolemic effect was apparent and serum cholesterol showed a negative linear response ($P < 0.05$); supplementation levels of 0.15 and 0.20% showed the lowest values ($P < 0.05$) when compared with the control. This behavior was not observed ($P > 0.05$) for the blood concentration of triglycerides.

The reduction in cholesterol levels is complex because it possibly involves the combination of bioactive compounds in mushrooms, such as short chain fatty acids, and fiber fermentation by the intestinal flora (JEONG et al., 2010). The decline may also be due to the reduction in cholesterol absorption by the nondigestible polysaccharides such as β-(1→ 3) glucans and the interaction of cholesterol with bile acids, which reduces their reabsorption into the enterohepatic circulation and causes the liver to divert cholesterol to bile acid production, thus reducing the cholesterol concentration in the blood (CHEUNG, 1996). The hypocholesterolemic and hypoglycemic effects of betaglucans, which are present in fungi of the Agaricus genus, attested to in rats, suggests a protective action on the biochemical and metabolic parameters of the animals and consequently, in maintaining the body's homeostasis (KIM et al., 2005; MASCARO et al., 2014).

Table 6. Total serum cholesterol concentration (mg dL^{-1}) and triglycerides (mg dL^{-1}) (± standard error), at 28 and 42 days in broilers fed diets supplemented with *Agaricus blazei* at various levels.

A.blazei (%)	Cholesterol 28d	Triglycerides 28d	Cholesterol 42d	Triglycerides 42d
0	92,47 ± 10,62	48,24 ± 8,65	112,62 ± 3,92	131,73 ± 43,54
0,05	89,18 ± 3,46	82,04 ± 33,87	103,22 ± 0,54	163,98 ± 7,69
0,1	90,69 ± 3,11	96,83 ± 19,96	101,89 ± 5,08	69,64 ± 7,69
0,15	105,16 ± 10,02	92,12 ± 6,24	96,78 ± 5,69*	81,58 ± 34,35
0,2	108,40 ± 2,93	36,67 ± 12,48	93,45 ± 3,78*	127,43 ± 12,22
CV (%)	14,36	53,55	7,01	45,03
Regression	NS	NS	L[1]	NS

* Significant 5% by Dunnett's test ($P < 0.05$); NS = not significant; L = Linear.
[1]L= 107.44 – 68.856X ($R^2 = 0,96$; $P = 0.0321$).

The lipid oxidation indicator in meat, TBARS was not affected ($P > 0.05$) by the inclusion of *A. blazei* on days 0 and after five days of cooling, as shown in Table 7. The meat from both control and supplemented birds showed differences ($P < 0.05$) in the final production of TBARS over time. As lipid oxidation occurred, the concentration of these compounds increased between day 0 and day 5 of cooling, regardless of the presence of mushroom. Despite the high concentration of polyphenols presented by *A. blazei*, of which the value was 5.07 mg of gallic acid equivalents per gram of mushroom, the levels added to the diet were not sufficient to inhibit degradation of the lipid compounds of the flesh or to promote tissue protection. Oxidative-reduction by the proven antioxidant action of the medicinal mushrooms was expected (CHEUNG et al., 2003; TSAI et al., 2007), due to the presence

of phenolic compounds, which have the ability to inactivate free radicals (MORAES; COLLA, 2006).

Using *Agaricus bisporus* in the diet of broilers and turkeys, Giannenas et al. (2010, 2011) observed, after five days of refrigeration, the presence of a higher content of malondialdehyde as a result of lipid oxidation in the meat of birds without supplementation compared with birds supplemented with the mushroom. It has been suggested that antioxidant activity is dose dependent and that the protective effect of *A. bisporus* is a result of the ability of fractions of polysaccharides and polyphenols to stabilize free radicals by electron transfer. Even with negative results on lipid protection, the presence of antioxidant compounds in chickens arising from *A. blazei* is observed. The capture of the free radical DPPH showed a positive linear effect ($P < 0.05$) only on day zero of meat cooling and levels of 0.15 and 0.20% mushroom showed ($P < 0.05$) higher percentages (75.04 and 75.36%) when compared with controls (Table 7). This difference indicates that both supplementation levels were associated with the highest free-radical sequestration capacity due to the higher concentration of antioxidant compounds present in the meat, resulting in tissue preservation of the animal *in vivo*. Phenolic compounds of low molecular weight, despite their low liquid absorption in the intestine, reach the bloodstream and promote antioxidant activity in animals (GIANNENAS et al., 2010).

Table 7. Evaluation of lipid oxidation by TBARS values expressed in µM of malondialdehyde g^{-1} of sample and the percentage (%) of the free radical DPPH (± standard error) in the meat of broiler chickens fed diets supplemented with levels of *Agaricus blazei*.

***A. blazei* (%)**	**TBARS**		**P-value**	**CV (%)**
	DAY 0	**DAY 5**		
0	0,59 ± 0,10 B	3,19 ± 0,38 A	<0,0001	29,97
0,05	0,73 ± 0,10 B	2,93 ± 0,38 A	0,0025	65,12
0,1	0,65 ± 0,10 B	2,52 ± 0,38 A	<0,0001	34,26
0,15	0,80 ± 0,10 B	3,21 ± 0,38 A	<0,0001	32,43
0,2	0,65 ± 0,11B	2,89 ± 0,43 A	0,003	39,86
CV (%)	37,88	36,01		
Regression	NS	NS		
***A. blazei* (%)**	**DPPH[1]**		**P-value**	**CV (%)**
	DAY 0	**DAY 5**		
0	74,15 ± 0,18A	67,70 ± 0,37 B	<0,0001	2,3
0,05	73,73 ± 0,18A	65,45 ± 0,37*B	<0,0001	4,64
0,1	74,34 ± 0,18A	64,94 ±0,37*B	<0,0001	5,79
0,15	75,04 ± 0,18*A	67,18 ± 0,37 B	<0,0001	2,39
0,2	75,36 ± 0,18*A	65,76 ± 0,37*B	<0,0001	2,43
CV (%)	2,25	5,06		
Regression	L[2]	NS		

[*] Significant 5% by Dunnett's test ($P < 0.05$); NS = not significant; L = Linear.
[1] DPPH: 2,2-diphenyl-1-picryl-hydrazyl; [2] L = 73.22 + 11.18x ($R^2 = 0.98$; $P < 0.0001$)
[a,b] Means with different letters in the same row differ by Tukey's test.

After five days of cooling, the residual antioxidant capacity in meat to sequester the free radical DPPH was reduced ($P < 0.05$) compared with day 0. When compared with the control, the levels of 0.5, 0.10 and 0.20% mushroom inclusion had lower ($P < 0.05$) protective activity, revealing that the antioxidant effect observed at day 0 decreased over time and with increased oxidative stress. Bao et al. (2009),

ascertained by DPPH analysis a protective effect of *Flammulina velutipes* mushroom on chilled fish meat, indicating no loss of scavenging activity of electrons and that foods containing antioxidants can help to reduce the production of free radicals, which compromise the integrity of the final meat product.

The results of the use of medicinal mushrooms as additives still have varying responses according to Guo et al. (2003), due to physico-chemical properties, type of sugars, and the structure and molecular weight of the compounds present. The quantity and quality of the chemical composition of fungi are influenced by their genetic and environmental growing conditions, the nature of the substrate, and storage conditions (MIZUNO, 2002; CHANG; MILES, 2004; SINGH et al., 2011). The effective use of mushrooms as natural additives in poultry nutrition depends on the correct patterning of their dosages, since researchers are working with different concentrations and species, and experimental conditions. In addition, we need to better understand how compounds with biological activity act on the animal organism, whether or not they are employed in dealing with health and/or environmental challenges, enabling better decision making with regard to the application of mushroom in animal production.

Conclusion

The use of *A. blazei* as a natural additive provided a better immune response in birds by stimulating immunoglobulins and macrophages. A hypocholesterolemic effect was seen after 42 days of age, and although the lipid protection of the flesh was not favored "in natura" or upon cooling, the presence of residual mushroom compounds with antioxidant activity allowed the protection of animal tissue *in vivo*.

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References

ABBAS, A. K.; LICHTMAN, A. H.; PILLAI, S. *Imunologia celular e molecular*. 7. ed. Rio de Janeiro: Elsevier, 2012. 564 p.

AIDA, F. M. N. A.; SHUHAIMI, M.; YAZID, M.; MAARUF, A. G. Mushroom as a potential source of prebiotics: a review. *Trends in Food Science & Technology*, Wageningen, v. 20, n. 11-12, p. 567-75, 2009.

BAO, H. N. D.; SHINOMIYA, Y.; IKEDA, H.; OHSHIMA, T. Preventing discoloration and lipid oxidation in dark muscle of yellowtail by feeding an extract prepared from mushroom (*Flammulina velutipes*) cultured médium. *Aquaculture*, Amsterdam, v. 295, n. 3-4, p. 243-249, 2009.

BATTLE, J.; HA, T.; LI, C.; BEFFA, V. D.; RICE, P.; KALBFLEISCH, J.; BROWDER, W.; WILLIAMS, D. Ligand binding to the (1→3)-β-d-glucan receptor stimulates NFkB activation, but not apoptosis in U937 cells. *Biochemical and Biophysical Research Communications*, Amsterdam, v. 249, n. 2, p. 499-504, 1998.

BORSA, A. Valores hematológicos em frangos de corte de criação industrial. *Colloquium Agrariae*, Presidente Prudente, v. 5, n. 1, p. 25-31, 2009.

BOTSOGLOU, N. A.; FLETOURIS, D. J.; FLOROU-PANERI, P.; CHRISTAKI, E.; SPAIS, A. B. Inhibition of lipid oxidation in long-term frozen stored chicken meat by dietary oregano essential oil and a-tocopheryl acetate supplementation. *Food Research International*, Toronto, v. 36, n. 3, p. 207-213, 2003.

CARDOSO, A. L. S. P.; TESSARI, E. N. C. Estudo dos parâmetros hematológicos em frangos de corte. *Arquivos do Instituto Biológico*, São Paulo, v. 70, n. 4, p. 419-24, 2003.

CHAN, Y.; CHANG, T.; CHAN, C.; YEH, Y.; CHEN, C.; SHIEH, B.; LI, C. Immunomodulatory effects of Agaricus blazei Murril in Balb/cByJ mice. *Journal of Microbiology, Immunology and Infection*, Amsterdam, v. 40, n. 3, p. 201-8, 2007.

CHANG, S. T.; MILES, P. G. *Mushrooms*: cultivation, nutritional value, medicinal effect, and environmental impact. 2th ed. Boca Raton: CRC Press, 2004. 451 p.

CHARLES NORIEGA, M. L. V. C. *Apuntes de hematología aviar*: material didático para curso de hematologia aviária. Cidade do México: Universidad Nacional Autónoma de México, Departamento de Produccíon Animal, 2000. 70 p.

CHEUNG, L. M.; CHEUNG, P. C. K.; OOI, V. E. C. Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chemistry*, Amsterdam, v. 81, n. 2, p. 249-255, 2003.

CHEUNG, P. C. K. The hypocholesterolemic effect of two edible mushrooms: Auricularia auricula (tree-ear) and Tremella fuciformis (white jelly-leaf) in hypercholesterolemic rats. *Nutrition Research*, Amsterdam, v. 16, n. 10, p. 1721-1725, 1996.

DANESHMAND, A.; SADEGHI, G. H.; KARIMI, A.; VAZIRY, A. Effect of oyster mushroom (Pleurotus ostreatus) with and without probiotic on growth performance and some blood parameters of male broilers. *Animal Feed Science and Technology*, Amsterdam, v. 170, n. 1-2, p. 91-96, 2011.

DARPOSSOLO, F. P. B.; QUINTANA, L. R.; MAGNANI, M.; OBA, A.; VENÂNCIO, E. J.; CASTRO-GOMÉZ, R. J. H. Avaliação do potencial imunoestimulante da Carboximetil-glucana de *Saccharomyces cerevisiae* em frangos de corte (*Gallus domesticus*). *Semina*: *Ciências Agrárias,* Londrina, v. 31, n. 1, p. 231-240, 2010.

FANG, C.; LIN, Z. B. Effect of Agaricus brasiliensis S. Wasser et al. (Agaricomycetideae) extract on the functions of lymphocytes in mice in vitro. *International Journal of Medicinal Mushrooms*, Danbury, v. 5, n. 3, p. 287-291, 2003.

FASSEAS, M. K.; MOUNTZOURIS, K. C.; TARANTILIS, P. A.; POLISSIOU, M.; ZERVAS, G. Antioxidant activity in meat treated with oregano and sage essential oils. *Food Chemistry*, Amsterdam, v. 106, n. 3, p. 1188-1194, 2007.

FIRENZUOLI, F.; GORI, L.; LOMBARDO, G. The medicinal mushroom Agaricus blazei murrill: review of literature and pharmaco-toxicological problems. *Evidence-Based Complementary and Alternative Medicine*, New York, v. 5, n. 1, p. 3-15, 2008.

GERTNER, L. R. S.; SANTIN, E.; SAAD, M. B. Influência da fumonisina sobre a resposta imunológica de aves: revisão bibliográfica. *Revista Acadêmica: Ciências Agrárias e Ambientais,* São José dos Pinhais, v. 6, n. 3, p. 401-11, 2008.

GIANNENAS, I.; PAPPAS, I. S.; MAVRIDIS, S.; KONTOPIDIS, G.; SKOUFOS, J.; KYRIAZAKIS, I. Performance and antioxidant status of broiler chickens supplemented with dried mushrooms (Agaricus bisporus) in their diet. *Poultry Science*, Champaign, v. 89, n. 2, p. 303-311, 2010.

GIANNENAS, I.; TSALIE, E.; CHRONIS, E. F.; MAVRIDIS, S.; TONTIS, D.; KYRIAZAKIS, I. Consumption of *Agaricus bisporus* mushroom affects the performance, intestinal microbiota composition and morphology, and antioxidante status of turkey poults. *Animal Feed Science and Technology*, Amsterdam, v. 165, n. 3-4, p. 218-219, 2011.

GUO, F. C.; KWAKKEL, R. P.; WILLIAMS, B. A.; SUO, X.; LI, W. K.; VERSTEGEN, M. W. A. Coccidiosis immunization: effects of mushroom and herb polysaccharides on immune responses of chickens infected with Eimeria tenella. *Avian Diseases*, Ithaca, v. 49, n. 1, p. 70-73. 2005.

GUO, F. C.; KWAKKEL, R. P.; WILLIANS, B. A.; PARMENTIER, H. K.; LI, W. K.; YANG, Z. Q.; VERSTEGEN, M. W. A. Effects of mushroom and herb polysaccharides on cellular and humoral immune responses of Eimeria tenella infected chickens. *Poultry Science*, Champaign, v. 83, n. 7, p. 1124-1132, 2004.

GUO, F. C.; SAVELKOUV, H. F. J.; KWAKKEV, R. P.; WILLIAMS, B. A.; VERSTEGEN, M. W. A. Immunoactive, medicinal properties of mushroom and herb polysaccharides and their potential use in chicken diets. *World's Poultry Science Journal*, Cambridge, v. 59, n. 4, p. 427-440, 2003.

JEONG, S. C.; JEONG, Y. T.; YANG, B. K.; ISLAM, R.; KOYYALAMUDI, S. R.; PANG, G.; CHO, K. Y.; SONG, C. H. White button mushroom (*Agaricus bisporus*) lowers blood glucose and cholesterol levels in diabetic and hypercholesterolemic rats. *Nutrition Research*, Amsterdam, v. 30, v. 1, p. 49-56, 2010.

KHATUN, S.; ISLAM, A.; CAKILCIOGLU, U.; CHATTERJEE, N. C. Research on mushroom as a potential source of nutraceuticals: a review on indian perspective. *American Journal of Experimental Agriculture*, Tarakeswar, v. 2, n. 1, p. 47-73, 2012.

KIM, Y. W.; KIM, K. H.; CHOI, H. J.; LEE, D. S. Anti-diabetic activity of b-glucans and their enzymatically hydrolyzed oligosaccharides from *Agaricus blazei*. *Biotechnology Letters*, Dordrecht, v. 27, n. 7, p. 483-487, 2005.

KLASING, K. C. Nutritional modulation of resistance to infectious diseases. *Poultry Science*, Champaign, v. 77, n. 8, p. 1119-1125, 1998.

KOZARSKI, M.; KLAUS, A.; NIKSIC, M.; JAKOVLJEVIC, D.; HELSPER, J. P. F. G.; VAN GRIENSVEN, L. J. L. D. Antioxidative and immunomodulating activities of polysaccharide extracts

of the medicinal mushrooms *Agaricus bisporus, Agaricus brasiliensis*, *Ganoderma lucidum* and *Phellinus linteus*. *Food Chemistry*, Amsterdan, v. 129, n. 4, p. 1667-1675, 2011.

LI, W.; HYDAMAKA, A. W.; LOWRY, L.; BETA, T. Comparison of antioxidant capacity and phenolic compounds of berries, chokecherry and seabuckthorn. *Central European Journal of Biology*, Warsaw, v. 4, n. 4, p. 499-506, 2009.

MACARI, M.; LUQUETTI, B. C. Fisiologia cardiovascular. In: MACARI, M.; FURLAN, R. L.; GONZALES, E. (Ed.). *Fisiologia aviária aplicada a frangos de corte*. 2. ed. Jaboticabal: Funep/Unesp, 2008. 375 p.

MASCARO, M. B.; FRANÇA, C. M.; ESQUERDO, K. F.; LARA, M. A. N.; WADT, N. S. Y.; BACH, E. E. Effects of dietary supplementation with *Agaricus sylvaticus* Schaeffer on glycemia and cholesterol after streptozotocin-induced diabetes in rats. *Evidence-Based Complementary and Alternative Medicine*, New York, v. 2014, Article ID 107629, p. 1-10, 2014.

MAU, J. L.; CHANG, C. N.; HUANG, S. J.; CHEN, C. C. Antioxidant properties of methanolic extracts from Grifola frondosa, Morchella esculenta and Termitomyces albuminosus mycelia. *Food Chemistry*, Amsterdan, v. 87, n. 1, p. 111-118, 2004.

MIZUNO, T. Medicinal properties and clinical effects of culinary-medicinal mushroom *Agaricus blazei* Murrill (Agaricomycetideae) (Review). *International Journal of Medicinal Mushrooms*, Danbury, v. 4, n. 4, p. 299-312, 2002.

MORAES, F. P.; COLLA, L. M. Alimentos funcionais e nutracêuticos: definições, legislação e benefícios à saúde. *Revista Eletrônica de Farmácia*, Goiânia, v. 3, n. 2, p. 109-122, 2006.

NOVAK, M.; SYNYTSYA, A.; GEDEON, O.; SLEPICKA, P.; PROCHÁZKA, V.; SYNYTSYA, A.; BLAHOVEC, J.; HEJLOVÁ, A.; COPÍKOVÁ, J. Yeast (1-3),(1-6)-d-glucan films: preparation and characterization of some structural and physical properties. *Carbohydrate Polymers*, Amsterdam, v. 87, n. 4, p. 2496-2504, 2012.

QUERSHI, M. A.; DIETERI, R. R.; BACON, L. D. Genetic variation in the recruitment and activation of chicken peritoneal macrophages. *Proceedings of the Society for Experimental Biology and Medicine*, New York, v. 181, n. 4, p. 560-568, 1986.

RANA, I. S.; KANOJIYA, A.; SANDHU, S. S. Evaluation of antibacterial potential of two species of genus Agaricus L.: Fr. (Agaricomycetideae) from India. *International Journal of Medicinal Mushrooms*, Danbury, v. 10, n. 2, p. 163-169, 2008.

RESHETNIKOV, S. P.; WASSER, S. P.; TAN, K. K. Higher basidiomycota as a source of antitumor and immunostimulating polyssaccharides (review). *International Journal of Medicinal Mushrooms*, Danbury, v. 3, n. 4, p. 361-394, 2001.

ROLL, V. F. B.; LOPES, L. L.; ROSSI, P.; ANCIUTI, M. A.; RUTZ, F.; XAVIER, E. G.; SILVA, S. S. Hematologia de frangos alimentados com dietas contendo aflatoxinas e adsorvente de toxinas. *Archivos de Zootecnia*, Córdoba, v. 59, n. 225, p. 93-101, 2010.

ROSTAGNO, H. S.; ALBINO, L. F. T.; DONZELE, J. L.; GOMES, P. C.; OLIVEIRA, R. F.; LOPES, D. C.; FERREIRA, A. S.; BARRETO, S. L. T.; EUCLIDES, R. F. *Tabelas brasileiras para aves e suínos. Composição de alimentos e exigências nutricionais.* 3. ed. Viçosa, MG: Universidade Federal de Viçosa, 2011. 252 p.

SINGH, M.; VIJAY, B.; KAMAL, S.; WALKCHAURE, G. C. *Mushrooms cultivation, marketing and consumption*. *Directorate of mushroom research*. Solan: Directorate of Mushroom Research (ICAR), 2011. 266 p.

SINGLETON, V. L.; ROSSI JUNIOR, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, Davis, v. 16, n. 3, p. 144-158, 1965.

SORENSEN, G.; JORGENSEN, S. S. A critical examination of some experimental variables in the 2-thiobarbituric acid (TBA) test for lipid oxidation in meat products. *Zeitschrift Lebensmittel Untersuchung Forschung*, Dresden, v. 202, n. 3, p. 205-210, 1996.

SORIMACHI, K.; AKIMOTO, K.; IKEHARA, Y.; INAFUKU, K.; OKUBO, A.; YAMAZAKI, S. Secretion of TNF- α, II-8 and nitric oxide by macrophages activated with *Agaricus blazei* Murrill fractions in vitro. *Cell Structure and Function*, Kyoto, v. 26, n. 2, p. 103-108, 2001.

SWENSON, M. J.; REECE, W. O. *Dukes*: fisiologia dos animais domésticos. 11. ed. Rio de Janeiro: Guanabara Koogan, 1996. 856 p.

TSAI, S. Y.; TSAI, H. L.; MAU, J. L. Antioxidant properties of *Agaricus blazei*, Agrocybe cylindracea, and Boletus edulis. *LWT – Food Science and Technology*, Campinas, v. 40, n. 8, p. 1392-1402, 2007.

UNIVERSIDADE FEDERAL DE VIÇOSA – UFV. SAEG – Sistema de análises estatísticas e genéticas: Versão 5. 0. Viçosa, MG: UFV, 2005.