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Crude propolis as an immunostimulating agent in broiler feed during the starter phase[1]

Própolis bruta como agente imunoestimulante na alimentação de frangos de corte na fase inicial

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Abstract

The experiment herein was conducted to evaluate the efficacy of supplementing a broiler diet with crude propolis on the animals' immune response (humoral and cellular), lymphoid organ weight and hematological profile. One hundred sixty-eight male broilers raised in metabolic cages until 21 days of age were used in the experiments. The birds were randomly distributed in an experimental design with six treatments that consisted of different crude propolis doses (0, 100, 200, 300, 400 and 500 ppm); the experimental unit was repeated seven times with four birds each. Including crude propolis in the food did not affect ($P>0.05$) the relative weights of the thymus, spleen and cloacal bursa or serum antibody production against Newcastle disease. The macrophage phagocytic activities were quadratic ($P<0.05$) in accordance with the level of crude propolis; the lowest activity observed corresponded to a 350.72 ppm dose of crude propolis. Moreover, when each crude propolis dose was compared with the control group, the birds fed 100 ppm and 500 ppm of crude propolis had higher levels of phagocytic activity ($P<0.05$) and a greater number of red blood cells phagocytized per macrophage ($P<0.05$), respectively. No change in nitric oxide production was observed ($P>0.05$). The interdigital reaction to the phytohemagglutinin displayed quadratic behavior as a function of time and crude propolis dose. Using the adjusted equation, the 275.45 ppm crude propolis dose and 39.35 hours produced the lowest and highest reaction values. Inclusion of 100 ppm crude propolis in the broiler feed was an effective immunostimulatory agent for cell-mediated responses.

Key words: Antibody, cellular immune response, humoral immune response, immune system, macrophages

Resumo

Este experimento foi realizado para avaliar a eficácia da suplementação de própolis bruta nas dietas de frangos de corte sobre as respostas imunes (humoral e celular), peso dos órgãos linfóides e perfil hematológico. Foram utilizados 168 pintos de corte, machos, criados em gaiolas de metabolismo até os 21 dias de idade. As aves foram distribuídas em um delineamento experimental inteiramente casualizado, com seis tratamentos, que consistiram em diferentes níveis de inclusão da própolis (0, 100, 200, 300, 400 e 500 ppm), com sete repetições e quatro aves por unidade experimental. A inclusão de própolis bruta nas rações não afetou ($P>0,05$) o peso relativo do timo, baço e bolsa cloacal e a produção dos

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anticorpos séricos contra a doença de Newcastle. Observou-se comportamento quadrático (P<0,05) para a atividade fagocítica dos macrófagos em função dos níveis de própolis bruta, com menor atividade ao nível de 350,72 ppm. Além disso, quando comparado cada nível de inclusão com o controle observou-se que as aves que receberam rações contendo 100 ppm e 500 ppm de própolis bruta apresentaram maior atividade fagocítica (P<0,05) e maior número de hemácias fagocitadas por macrófago (P<0,05), respectivamente. Para a produção de óxido nítrico não foi observada alteração (P>0,05). A reação interdigital a fitohemaglutinina apresentou comportamento quadrático em função do tempo e dos níveis de inclusão. De acordo com a equação ajustada, o nível de 275,45 ppm de própolis bruta e o tempo de 39,35 horas resultou no menor e maior valor da reação, respectivamente. A inclusão de 100 ppm de própolis bruta nas rações de frangos de corte se mostrou eficaz como agente imunoestimulante nas respostas mediadas por células.
Palavras-chave: Anticorpo, macrófagos, resposta imune humoral, resposta imune celular, sistema imune

Introduction

Expansion of the poultry industry was associated with routine use of chemotherapeutic agents in broiler feed to fight pathogenic agents in the animals' bodies, which decreased the performance loss caused by clinical and subclinical diseases (DIBNER; RICHARDS, 2005). However, public and scientific pressures regarding the possible harm to human health upon indiscriminate use of such additives led to an ongoing search for natural ingredients to substitute for the harmful agents (HAN et al., 2010).

One such alternative is propolis, which is produced by honeybees that collect resins and balsams from various plant parts and reprocess this material while adding wax and salivary secretions (DAUGSCH et al., 2008). The chemical composition of propolis contributes to its antioxidant, antimicrobial, anti-inflammatory, antiviral, immunomodulatory, and other biological properties (LOFTY, 2006).

Thus, these substances, including polyphenols, flavonoids, phenolic acids, aromatic aldehydes, esters, amino acids, minerals and vitamins (BANKOVA, 2005; TRUSHEVA et al., 2006), can affect various phases of immune system activation, such as the increased expression of cytokines, which are involved in initiating the immune response (FREITAS et al., 2011); antibody production (ZIARAN; RAHMANI; POURREZA, 2005; GALAL et al., 2008a); and macrophage phagocytic activity (DIMOV et al., 1991, SFORCIN, 2007).

Microflora and the intestinal epithelium interact with the immune system. The bioactive elements of propolis act on the microbial composition of the gastrointestinal tract (TAHERI; RAHMANI; POURREZA, 2005), resulting in an increase in and diversification of the immune response (HAGHIGHI et al., 2005). In certain studies, animals without germs produced better developed lymphoid tissue associated with mucous. This tissue is responsible for providing immune cells and products to fight pathogenic agents (JANARDHANA et al., 2009).

Little is known about the effect of natural propolis on the immune response in chickens because studies are typically conducted using an ethanol or oily propolis extract (TAHERI; RAHMANI; POURREZA, 2005; ZIARAN; RAHMANI; POURREZA, 2005; SHAHRYAR et al., 2011). Therefore, the objective of the study herein was to evaluate the efficacy of supplementing a broiler's diet with crude propolis on the immune response (humoral and cellular), lymphoid organ weight and hematological profile.

Material and Methods

Animals and experimental design

One hundred and sixty-eight one-day-old male Cobb-Vantress broilers were raised in metabolic cages until they reached 21 days of ages. The experimental design included broilers randomly assigned to six treatments. The treatments comprised

different crude propolis doses (0, 100, 200, 300, 400 and 500 ppm) in the feed; the experimental units were repeated seven times with four birds each. The control treatment (0 ppm) did not include an additive. The experimental diets were formulated to meet the nutritional requirements proposed by Rostagno et al. (2005) for the 1- to 7-day-old and the 8- to 21-day-old age groups (Table 1).

Table 1. Percent and calculate composition of experimental diets.

Ingredients (%)	1 to 7 days	8 to 21 days
Corn	56.38	59.26
Soybean meal, 45%	36.92	34.27
Soybean oil	1.99	2.30
Limestone	0.92	0.88
Dicalcium phosphate	1.94	1.80
NaCl	0.40	0.40
Inert*	0.20	0.20
DL- Met, 98%	0.35	0.24
L- Lys HCl, 78%	0.35	0.19
L- Thr, 98%	0.15	0.05
Supplement mineral and vitaminic[1]	0.40	0.40
Total	100	100
Calculated composition		
CP (%)	22.04	20.79
AME (kcal/kg)	2.950	3.000
Ca (%)	0.939	0.884
Avaiable P (%)	0.470	0.442
Digestible Met + Cis (%)	0.944	0.814
Digestible Lys (%)	1.330	1.146
Digestible Thr (%)	0.865	0.745
Digestible Trp (%)	0.213	0.183

[1]Vitamin Supplement (content/kg of premix): Vit. A 2.916.670 UI/kg; Vit. D3 583.330 UI/kg; Vit. E 8.750 UI/kg; Vit. K3 433.33 mg/kg; Vit. B1 408.33 mg/kg; Vit. B2 1.333,33 mg/kg, Vit. B12 4.166,67 mcg/kg; Niacin 8.983,33 mg/kg; Calcium pantothenate 3.166,67 mg/kg; Folic acid 200 mg/kg; Biotin 25 mg/kg. Mineral Supplement (content/kg of premix): Iron 12.6 g/kg; Cooper 3.072 mg/kg; Iodine 248 mg/kg; Zinc 12.6 g/kg; Manganese 15 g/kg; Selenium 61.20 mg/kg; Cobalt 50.40 mg/kg.
*The crude propolis was added to the diets as an inert replacement.
Source: Elaboration of the authors.

Crude propolis

The crude propolis used in this experiment was acquired from Maringá-State of Paraná-Brazil in its solid form and stored at 2-8°C until use. The crude propolis was then ground to supplement the experimental diets. The total polyphenols was determined according to Singleton and Rossi (1965) and Pierpoint (2004) and the flavonoids content was evaluated using the aluminum chloride colorimetric methods.

Antibody Titer

The broilers were vaccinated via an intraocular route for Newcastle disease at 14 and 21 days of age, and blood samples were taken from six birds per treatment. The blood was separated, and the serum was stored to measure antibody production against Newcastle disease, which was tested through indirect ELISA (IDEXX©) according to the manufacturer's instructions. The titers evaluated refer to the vaccine response.

Hematological profile and lymphoid organ collection

At 21 days of age, six broilers in each treatment group with a representative weight (average±5%) were selected and, after insensibilization by electric shock, the animals were sacrificed by cervical dislocation to determine the hematological profile and relative weight (% of live weight) of the lymphoid organs (cloacal bursa, spleen and thymus).

Blood-smear stains using the May Grunwald – Giemsa method were prepared to determine the hematological profile. Differential cell counts performed with an optical microscope and an immersion objective classified the cells into lymphocytes, heterophils, eosinophils, monocytes and basophils. The proportion of each cell type counted/bird was calculated (LUCAS; JAMROZ, 1961).

Cell-mediated in vivo immune response

Five broilers from each treatment group were also selected at 21 days of age to evaluate the *in vivo* cell-mediated immune response according to the protocol described by Corrier and DeLoach (1990). Phytohemagglutinin PHA-M® (Invitrogen) at 0.1 mL was intradermally injected between the third and fourth interdigital folds of each animal's right foot. The same volume of saline solution was applied to the left foot as a negative control. The skin thickness was measured for both feet using a digital caliper before inoculation, as well as 12, 24, 48 and 72 hours after inoculation. The results are reported as the difference between the phytohemagglutinin-injected and control animals at each different time point.

Peritoneal macrophage phagocytic activity and nitric oxide production

Five birds per treatment were used to evaluate peritoneal macrophage phagocytic activity according to the protocol by Qureshi, Dieteri and Bacon (1986). At 21 days, a 3% Sephadex G-50® (Sigma) solution (0.9% saline solution) was injected at 1 mL/100 g of body weight into each animal's peritoneal cavity.

After 42 hours, the birds were sacrificed by cervical dislocation; each bird's abdomen was cleaned (neutral detergent), sanitized (70% alcohol) and inoculated with 20 mL of sterile heparinized PBS (0.5 U/mL Liquemine® - Roche); approximately 15 mL of the abdominal liquid was collected and immediately conditioned in plastic tubes on ice. The collected material was centrifuged at 1,500 rpm/10 minutes, and the pellet was resuspended in 1.5 mL of RPMI 1640® (Sigma). A total of 150 µL of this suspension was added to each well of the culture plate with a 13-mm diameter glass coverslip. After an hour in the incubator at 37°C with 5% CO_2, each well was washed with RPMI 1640 solution to remove the non-adhered cells. Next, 200 µL of sheep erythrocytes was added (suspension of 3% red blood cells in RPMI 1640), and the mixture was incubated again for one hour. After incubation, each well was washed with RPMI 1640 and stained using a commercial kit (Panótico Rápido LB® - Laborclin).

After the slides were dried and cover slips fixed, 200 macrophages were counted in duplicate for each bird to verify the number of macrophages with phagocytized erythrocytes and the number of erythrocytes in each macrophage. Phagocytic activity was calculated by dividing the number of macrophages with phagocytized erythrocytes by the total number of macrophages counted.

Simultaneously, the same process was conducted with a second plate; however, during the second wash, 200 µL of RPMI 1640 was stored per well. The plates were then placed in an incubator for an additional 24 hours to measure nitric oxide production in the macrophages. Each sample contained a positive control (MØ + RBC) and negative control (MØ), which differed based on the presence or absence of red blood cells; the negative control represented spontaneous production of nitric oxide in the macrophages. After 24 hours, the supernatant was collected and nitrite levels were measured using the Griess reaction (QURESHI; DIETERI; BACON, 1986).

Statistical analysis

The data on the relative weight of the lymphoid organs, macrophage activity and antibody titer were analyzed using an analysis of variance (ANOVA) and polynomial regression with the Systems of Statistical and Genetic Analyses software (SSGA; Sistemas de Análises Estatísticas e Genéticas, SAEG) (UFV, 1999).

The hematological data were first analyzed using the Shapiro-Wilk test to verify whether the data had a normal distribution. The variables with a normal distribution were analyzed using an ANOVA and simple linear regression; the remaining variables were analyzed using generalized linear

models with the gamma distribution as an inverse function. The Shapiro-Wilk test was also used for the interdigital reaction to phytohemagglutinin, and the variables with a normal distribution were analyzed using an ANOVA and multiple linear regression, which considered the timing and doses for crude propolis. The results are shown in the surface response graph. Both analyses were performed using the R software (*R Development Core Team, 2009*).

A Dunnett test with a 5% probability was used to compare results between the control and crude propolis doses, and a Tukey test with a 5% probability was performed to discern the interdigital reaction to phytohemagglutinin as a function of the time elapsed between measurements.

Results

The crude propolis used in this experiment had 78.75 mg/kg polyphenols and 11.95 mg/kg flavonoids.

Including crude propolis in the feed did not influence (P>0.05) the relative weights of the thymus, spleen and cloacal bursa in 21-day-old chickens (Table 2).

Table 2. Relative weights ± standard error (%) of thyme, spleen and cloacal bursa of broiler chickens at 21 days of age fed diets with different doses of crude propolis.

Doses of crude propolis	Thyme	Spleen	Cloacal brusa
Control	0.682±0.042	0.114±0.008	0.248±0.025
100 ppm	0.605±0.078	0.131±0.013	0.271±0.030
200 ppm	0.638±0.059	0.105±0.007	0.229±0.027
300 ppm	0.597±0.079	0.131±0.0016	0.239±0.012
400 ppm	0.568±0.084	0.119±0.014	0.215±0.020
500 ppm	0.624±0.081	0.120±0.010	0.226±0.028
CV (%)	28.47	24.31	25.27
Regression	ns	ns	ns

Not significant by Dunnett test (P>0.05)
ns = not significant
Source: Elaboration of the authors.

Including crude propolis in the diet did affect (P<0.05) the percentage of eosinophils with a negative linear response (Y=6.480897–0.006555x). However, when each dose of crude propolis was compared with the control treatment, no significant difference was observed (P>0.05). In addition, no differences were detected (P>0.05) in the number of lymphocytes, heterophils, basophils and monocytes or in the heterophil:lymphocyte ratio (Table 3).

Table 3. Hematological values (%) and heterophil:lymphocyte ratio (H:L) ± standard error of broiler chickens at 21 days of age fed diets with different doses of crude propolis.

Doses of crude propolis	Lymphocyte	Heterophil	Bashophil	Monocyte	Eosinophil	H:L
Control	62.71±3.88	22.66±3.45	8.19±0.71	1.17±0.48	5.27±0.72	0.38±0.07
100 ppm	60.57±3.35	20.92±2.82	10.58±2.43	2.41±0.85	5.52±0.29	0.35±0.06
200 ppm	68.16±1.17	17.63±1.48	5.79±0.43	3.14±0.26	5.28±0.88	0.26±0.03
300 ppm	66.30±1.11	20.13±1.60	6.45±1.30	2.38±0.61	4.74±0.64	0.30±0.03
400 ppm	65.19±2.51	18.86±2.09	9.97±1.76	1.68±0.60	4.29±0.59	0.29±0.04
500 ppm	63.95±3.50	23.79±2.70	7.93±1.61	1.49±0.47	2.83±0.52	0.38±0.06
CV (%)	9.50	25.50	39.85	62.58	33.29	33.39
Regression	ns	ns	ns	ns	Linear[1]	ns

Not significant by Dunnett test (P>0,05)
ns = not significant
[1]Y = 6,480897 – 0,006555x (R^2=0,41); (P<0,002)
Source: Elaboration of the authors.

Quadratic behavior (Y=34.0325–0.0710378x+ 0.000101273x^2) (P<0.05) was observed for the macrophage phagocytic activity in response to different crude propolis doses, and the lowest activity was observed when the diet was supplemented with 350.72 ppm of crude propolis. When each dose of crude propolis inclusion was compared with the control group, the birds that received feed supplemented with 100 ppm and 500 ppm propolis displayed higher phagocytic activity and a greater number of red blood cells phagocytized per macrophage (P<0.05), respectively. Changes in nitric oxide production were not observed (P>0.05) (Table 4).

Table 4. Phagocytic activity (%), phagocytosed erythrocytes and nitric oxide concentration (µM/mL) ± standard error of broiler chickens at 21 days of age fed diets with different doses of crude propolis.

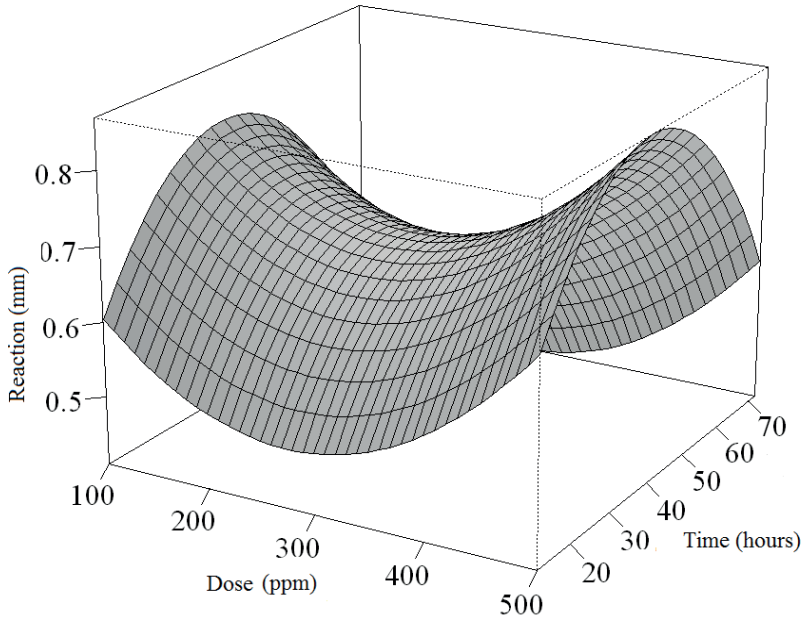
Doses of crude propolis	Phagocytic activity	Phagocytosed erythrocytes	Nitric oxide	
			MØ + RBC[1]	MØ[2]
Control	19.788±2.235	5.158±0.298	11.460±2.724	6.532±1.382
100 ppm	27.894±2.114*	5.383±0.173	16.743±1.348	5.306±0.638
200 ppm	24.237±1.098	5.761±0.149	12.543±0.987	7.971±1.356
300 ppm	20.981±1.160	5.541±0.203	12.398±3.473	8.902±2.503
400 ppm	23.118±2.576	4.480±0.392	11.730±2.607	7.374±2.329
500 ppm	23.585±1.213	6.039±0.242*	12.662±0.722	8.087±1.685
CV (%)	15.63	9.37	39.63	51.56
Regression	Quadratic[3]	ns	ns	ns

[1] Positive control = macrophages + red blood cells
[2] Negative control = macrophages
*Significant by Dunnett test (P<0.05)
ns = not significant
[3] Y = 34.0325 – 0.0710378x + 0.000101273x^2 (R^2 = 0.91); (P<0.02); Minimum point: 350.72ppm.
Source: Elaboration of the authors.

Moreover, an interaction (P>0.05) was not observed between the treatment factors and time for the interdigital reaction to phytohemagglutinin; the analysis of variance indicated that this variable was only affected by the time elapsed (P<0.05) between measurements (Table 5). However, the reaction

showed quadratic behavior (P<0.05) for the treatment timing and doses. According to the adjusted equation (Y=0.5771+0.01967*hour–0.0002499*hour^2–0.002105*dose+0.000003821*dose), 275.45 ppm of crude propolis and 39.35 hours produced the least and greatest reaction values (Figure 1).

Figure 1. Response surface of interdigital reaction to phytohemagglutinin (mm) as a function of dose of inclusion of crude propolis and analysis time of broiler chickens at 21 days of age fed diets with different doses of crude propolis.



Source: Elaboration of the authors.

Table 5. Interdigital reaction to phytohemagglutinin (mm) ± standard error of broiler chickens at 21 days of age fed diets with different doses of crude propolis.

Doses of crude propolis	12h	24h	48h	72h	Treatment average
Control	0.530±0.046	0.564±0.065	0.759±0.058	0.424±0.033	0.569
100 ppm	0.555±0.067	0.673±0.093	0.901±0.060	0.600±0.088	0.682
200 ppm	0.453±0.089	0.573±0.107	0.683±0.158	0.474±0.081	0.546
300 ppm	0.531±0.127	0.551±0.105	0.634±0.130	0.304±0.094	0.505
400 ppm	0.740±0.136	0.759±0.168	0.828±0.109	0.493±0.089	0.705
500 ppm	0.641±0.075	0.834±0.141	0.753±0.116	0.573±0.087	0.700
Time average	0.566bc	0.652ab	0.745a	0.469c	
CV (%)	39.15	40.02	35.59	42.43	
Anova					
Treatment			ns		
Time			*		
Regression					
Treatment x Time			ns		
Time effect			Quadratic[1] (P<0.0002)		
Treatment effect			Quadratic[1] (P<0.01)		

ns = not significant
*Means followed by different letters, in thesamerow, differ by Tukey test (P<0.05)
[1]Y = 0.5771 + 0.01967*hour – 0.0002499*hour^2 – 0.002105*dose + 0.000003821*dose^2 (R^2 = 0.72)
Source: Elaboration of the authors.

Diet supplementation with crude propolis did not influence (P>0.05) the concentration of antibodies against Newcastle disease at 21 days of age (Table 6).

Table 6. Antibody titer (Log_{10}) ± standard error of broiler chickens at 21 days of age fed diets with different doses of crude propolis.

Doses of crude propolis	Titer
Control	2.450±0.068
100 ppm	2.444±0.085
200 ppm	2.435±0.083
300 ppm	2.565±0.083
400 ppm	2.476±0.079
500 ppm	2.365±0.092
CV (%)	7.47
Regression	ns

Not significant by Dunnett test (P>0.05)
ns = not significant.
Source: Elaboration of the authors.

Discussion

The levels of polyphenols and flavonoids in the crude propolis used herein were of adequate quality and were consistent with values reported in the literature (FUNARI; FERRO, 2006) as well as with the minimum requirements of the Ministry of Agriculture, Livestock and Supply of the Brazilian government, 5% and 0.5% for polyphenols and flavonois, respectively (Brasil – Ministério da Agricultura - Normativa n°3 – Anexo VI, 2001).

The relative weight of the lymphoid organs is often used to evaluate the immunological system because changes are related to animal health (CHICHLOWSKI et al., 2007). Although crude propolis does not impair such organ growth, studies have observed positive effects on immune organ development from diet supplementation using natural additives (ASHAYERIZADEH et al., 2009; LI; ZHAO; WANG, 2009).

Propolis is comprised of compounds with biological activities that can stimulate immunological factors (ZIARAN; RAHMANI; POURREZA, 2005). Thus, a positive effect on the relative organ weights due to increased immune system cell production was expected because these organs are the primary tissues responsible for humoral and cellular immunity (WARNER, 1964). However, despite its importance, weight should not be the sole consideration; other immune factors must also be correlated (KABIR et al., 2004).

Hematological assessments were used to diagnose quantitative physiological alterations (AL-MANSOUR et al., 2011). As changes were only observed in the number of eosinophils, there was insufficient evidence to infer that crude propolis compromised the cell-mediated immune response.

Inconsistencies for hematological factors have been reported in the literature. They range from no influence by propolis supplementation in leukocyte counts (ORSOLIC; BASIC, 2003; ÇETIN et al., 2010; SHAHRYAR et al., 2011) to an increase in lymphocytes and a decrease in heterophils (ZIARAN; RAHMANI; POURREZA, 2005; GALAL et al., 2008a).

It has been suggested that the bioactive propolis components can increase the immune response because macrophage phagocytic activity increases and more cytokines are produced (DIMOV et al., 1991), which can stimulate the proliferation of other immune cells (TAHERI; RAHMANI; POURREZA, 2005). Macrophages play an important role in animals' physiological defenses through phagocytosis, chemotaxis, mediating inflammatory processes and the secretion of substances involved in immune processes (QURESHI, 1998; ORSI et al., 2000).

Propolis at 100 ppm and 350.72 ppm produced the highest and lowest phagocytic activities, respectively, which suggests a dose-dependent response according to Ziaran, Rahmani and Pourreza (2005). In those studies, propolis extract doses at 40 and 70 ppm produced the best immune responses. The difference observed between the studies herein

and the studies discussed above may be due to the form of the feed supplementation because the propolis used was in its raw form without previous processing, which could have produced lower quantities of the bioactive compost. In addition to the different supplementation form, the propolis composition could vary with the region, season and available plants (CHANG et al., 2008), which could also have generated the observed differences.

Although the phagocytic activity was not significant for the 500 ppm crude propolis treatment, the macrophages were more active in animals fed a diet with this dose of crude propolis. However, this effect would be valid if the phagocytic activity was also increased because a higher number of cells with phagocytic potential is more interesting than an increase in approximately one phagocytized blood cell per macrophage.

Immunomodulatory activity can also be evaluated by measuring nitric oxide production in macrophages, which is a microbicide compost associated with macrophage activity (BOGDAN, 2001; SFORCIN, 2007). Nitric oxide production in animals given a 100 ppm propolis supplement was higher compared with other propolis doses, which may be associated with the higher phagocytic activity observed; however, the increase observed was not significant.

The interdigital reaction to phytohemagglutinin is an evaluation of cell-mediated immunity, and the response is lymphocyte-T-dependent (GALAL et al., 2008b). Phytohemagglutinin can cause an increase in the lymphocyte population, which results in the accumulation of these cells and the infiltration of macrophages 24 hours after the initiation of the cutaneous response (McCORKLE; OLAH; GLICK, 1980). This test is also referred to as the cutaneous basophil hypersensitivity reaction because basophils are associated with these reactions.

Although the 275.45 ppm crude propolis dose produced the lowest reaction value, no changes ($P>0.05$) in the thymus weight or the number of T blood cells were observed considering that 80 to 90% of the circulating leukocytes are T lymphocytes (ZIARAN; RAHMANI; POURREZA, 2005). Therefore, if we evaluate both of these responses, we may infer that the cell-mediated immunological response of the animals fed this level of crude propolis were not impaired.

For the time variable, the greatest reaction was observed at 39.35 hours, regardless of the crude propolis dose, which suggests that the animals did not have a rapid immunological response compared with other studies that have also evaluated cellular immunity in birds given propolis (GALAL et al., 2008a; ABD EL - MOTAAL et al., 2008). The reaction decrease over time was expected given the regulation of physiological events underlying recruitment of cells that fight foreign agents.

The addition of propolis to the feed in its crude form might explain the quadratic behavior observed in both the macrophage activity and interdigital reaction to phytohemagglutinin. Natural propolis is composed of approximately 30% wax; 50% resin and vegetal balsams; 10% essential aromatic oils; and 5% pollen and other substances (BURDOCK, 1998). Thus, the different compounds complexed with these components are typically released through an extraction process with solvents such as ethanol, methanol and water (CUNHA et al., 2004). Therefore, the crude propolis might have been resistant to the digestive enzymes in the gastrointestinal tract, and these functional elements may not have been fully available to the birds.

Studies have shown that the substances in propolis are beneficial to humoral immunity (ZIARAN; RAHMANI; POURREZA, 2005; ÇETIN et al., 2010; FREITAS et al., 2011). The mechanism of action has not been completely elucidated because the propolis chemical composition is complex (SALATINO et al., 2005); however, the humoral and cellular immune responses may be synergistic. The propolis compounds may interfere with macrophage

activation and cytokine production (DIMOV et al., 1991; ORSI et al., 2005; RAMOS; MIRANDA, 2007); further, these substances are involved in stimulating the B lymphocytes that can produce antibodies (ANSORGE; REINHOLD; LENDECKEL, 2003; TAHERI; RAHMANI; POURREZA, 2005).

Therefore, an adjuvant action by propolis was expected because this supplement could influence macrophage phagocytic activity, and, thus, stimulate antibody production. However, the maximum level of antibodies may have been produced later, which was documented by Kong et al. (2006), who observed that animals produced greater antibody titers 21 days after the Newcastle vaccine was administered. If the antibody titers were determined at later time points for the study described herein, better results may had been observed.

Conclusion

Crude propolis at 100 ppm in the broilers' feed was an effective immunostimulating agent for cell-mediated responses during the initial phase.

Ethics and biosecurity committee

The animal experimental protocol used was previously approved by the Committee of Ethical Conduct in Animal Experimentation of State University of Maringá (Universidade Estadual de Maringá) (number 048/2010).

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