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Research Article
Brazilian Propolis Effects on Growth, Productivity Performance, Gut Characteristics and Physiological Changes in Broiler Chickens

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Abstract

Objective: This study aimed to examine the effect of dietary Brazilian propolis on the growth performance, physiological homeostasis and gut characteristics in broiler chickens reared under mild chronic heat stress. **Materials and Methods:** Five hundred and four 15 days old male broiler chicks were fed one of six diet (0.0, 100, 250, 500, 1000 and 3000 mg kg^{-1} propolis). Growth performance was evaluated in terms of Body Weight (BW), Body Weight Gain (BWG), Feed Intake (FI) and Feed Conversion Ratio (FCR) at 2 weeks intervals to 42 day of age. At 42 day of age 12 birds from each group were randomly selected and sacrificed for determination of the relative weight of internal organs and cecal contents were collected for microbial enumeration. Duodenal, jejunal and ileal tissue samples were collected for measuring villus height and width, crypt depth and villus crypt ratio. Also, blood was collected for subpopulations of leukocytes counts and serum chemical and hormonal analysis. In addition, brain samples were collected for determination of the heat stress-induced changes of the Heat Shock Protein 70 (HSP70) gene expression. The data were analyzed by one-way analysis of variance using the General Linear Models (GLM) procedure. **Results:** The results indicated that dietary propolis supplementation had no effect on growth performance and liver, heart, gizzard and spleen weights ($p>0.05$). While, compared to controls, the abdominal fat weight was increased with propolis supplementation ($p = 0.035$). Propolis did not affect cecal concentrations of *Escherichia coli,* total coliforms*, Enterococcus* spp. and total lactobacilli ($p>0.05$). However, compared to controls, the *Bifidobacterium* spp., population was lower in birds fed diet with propolis at 1000 mg kg^{-1} ($p = 0.005$). Propolis had no effect ($p>0.05$) on intestinal villus height and width, crypt depth and villus:crypt ratio. Compared to controls, propolis dietary supplementation did not affect the populations of eosinophils, monocytes and basophils; and serum concentrations of total proteins, globulins, phosphate, calcium, glucose and thyroid hormones as well as HSP70 mRNA expression in brain tissues ($p>0.05$, respectively*).* However, propolis regardless of dose reduced the number of heterophils, heterophil:lymphocyte ratio (H/L) and serum corticosterone and aminotransferase (AST) concentrations ($p<0.05$, respectively). In addition, all doses of propolis, except for 100 mg kg^{-1}, significantly increased circulating lymphocytes and reduced uric acid concentrations. In addition, there was an effect of propolis on serum albumin and tri-iodothyronine:thyroxin (T_3/T_4) ratio. Compared to the control group, birds fed 250 mg kg^{-1} propolis had a significantly higher T_3/T_4 ratio; while both 100 and 3000 mg kg^{-1} propolis groups had significantly increased the serum albumin concentrations. **Conclusion:** It is concluded that dietary supplementation of green Brazilian propolis at the tested doses, improves health status of birds by reducing initiation of heat stress responses, such as reduced concentrations of corticosterone, H/L ratio, AST and uric acid and increased T_3/T_4 ratio.

Key words: Broiler, cecal microbiota, intestinal morphology, heat shock protein, heat stress

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

High ambient temperature is one of the most serious problems faced by poultry producers, especially in the tropical and subtropical regions, such as Egypt[1,2]. Heat stress reduces Feed Intake (FI) of broiler chickens by 3.6% for every 1°C increase in environmental temperature[3], resulting in a worse Feed Conversion Ratio (FCR), 23-35% of less body weight (BW) gain[4,5] and 9-10% loss in their final BW[6,7]. Heat stress also changes the internal organs and fat contents, (i.e., increasing liver, heart and gizzard weight[8,9]), while decreasing abdominal fat weight[10]. Also, heat stress-suppressed immunity with high mortality of birds causes further economic loss to poultry producers[11]. Heat stress causes approximately $165 million loss to the U.S. poultry industry annually[12]. Moreover, heat stress disrupts the balance of the intestinal microbial ecology and stimulates proliferation of harmful pathogens including *Escherichia, Salmonella* and total aerobic bacteria[13]. Furthermore, heat stress causes a series of changes in physiological homeostasis, such as increased corticosterone concentrations, heterophil:lymphocyte (H/L) ratio and heat shock protein (HSP70) expression as well as metabolic changes including tri-iodothyronine (T_3) and thyroxin (T_4) concentrations and reduced total protein and globulin concentrations[14,15].

These changes lead to oxidative stress in broiler chickens by disturbing the balance between the production of Reactive Oxygen Species (ROS) and antioxidant systems[16]. One of the most important and effective strategies to prevent or reduce the negative effects of heat stress is to improve chickens internal antioxidative system through providing dietary supplementation of synthetic antioxidants such as vitamins A, C and E[17-19] or natural antioxidants such as plants extracts[20,21] to regulate ROS synthesis and inhibit its harmful effects. Propolis is one of the most fascinating bee products. Scientific research and commercial interests to propolis are growing continuously since 1960s, it has been used as a component of health additives due to its versatile biological activities in antioxidant, antibacterial, antiviral, antifungal, immunomodulatory, cytotoxic and anti-inflammatory effects[22]. Recently, propolis and its extracts have been used as nutritional substances in broiler chickens based on positive effects on health status and economic profiles; improving feed conversation ratio, productivity performance, intestinal microbial contents and nutritional status in chickens; and increasing meat quality and the production cost efficiency[23-26]. However, few studies have been conducted to investigate the effects of dietary supplementation of propolis, as a new strategy, to prevent the negative effects of heat stress on physiological and metabolic changes in broiler chickens[27,28]. The aim of this study was to determine if green Brazilian propolis can be used as a growth promoter to improve Feed Intake (FI) and Feed Conversion Ratio (FCR), to regulate intestinal microbial populations and to improve blood chemical biomarkers and brain HSP70 expression in broiler chickens reared under heat stress.

MATERIALS AND METHODS

All procedures and protocols were approved by the Purdue Animal Care and Use Committee, Purdue University (IN, USA); PACUC protocol No. 1111000262A003.

Propolis and its chemical analysis: Green propolis (No. 00900) was purchased from Apis Flora Co. (Ribeir˜ao Preto, São Paulo, Brazil). Its chemical compositions were analyzed at The Bindley Bioscience Center of Purdue University (IN, USA) for identification of soluble plant metabolites.

Birds and husbandry: Five hundred thirty 1 day-old male chicks of the Ross 708 strain were obtained from a local hatchery (Pine Manor/Miller Poultry, Goshen, Indiana, USA). At 15 days, 504 birds were weighed individually and randomly assigned to 24 floor pens (1.45×1.45 m per pen) in the same room at Purdue Poultry Research Farm during the summer of 2013. Wood shavings (5 cm depth) were used as litter. The brooding temperature was 34°C for the first 3 days then gradually reduced by 3°C per week up to 15 days of age, thereafter, all the chicks were exposed to 32°C for 9 h (08:00-17:00) daily up to d 42. Actual pen temperatures and humidity were measured every 30 min by using two data loggers/room (HOBO®, Onset Computer Corporation, Bourne, MA) which was fixed 30 cm above the litter surface (Table 1). All chicks were fed diets that meet or exceed the dietary recommendations for nutrients by the Ross management guide (Aviagen, 2014). A starter diet with 23.43% CP and 3,050 kcal ME kg^{-1} from day 1-14, grower diet with 22.81% CP and 3,150 kcal ME kg^{-1} from day 15-28 and then finisher diet with 19.17% CP and 3,200 kcal ME kg^{-1} from day 29-42 (Table 2). Each pen was equipped with one UV resistant plastic feeder and water troughs. Throughout the experiment, the chickens had *ad libitum* access to feed and water. The lighting regimen was constant at 30 lx for 23 L: 1D of light until 3 day, then 10 lx for 20 L: 4D up to 42 day.

Experimental design: At 15 day of age, 504 birds were weighed individually and assigned to 24 floor pens as that

Table 1: Temperature and humidity levels at different ages of birds

Birds age (week)	Temperature (°C) Night time (18:00-08:00)	Temperature (°C) Day time (08:00-18:00)	Humidity (RH %) Night time (18:00-08:00)	Humidity (RH %) Day time (08:00-18:00)
3rd week	29.96±0.18	32.34±0.26	48.13±1.33	44.82±1.42
4th week	28.86±0.27	32.02±0.13	58.92±0.85	58.18±1.09
5th week	28.31±0.19	31.16±0.19	63.97±0.19	63.21±1.60
6th week	27.91±0.20	31.42±0.17	60.56±0.69	57.59±1.21

Table 2: Dietary formulation and calculated nutrient and energy composition

Ingredient (%)	Starter	Grower	Finisher
Corn	52.0	52.3	62.8
Soybean meal, 48% CP	40.0	39.1	29.7
Soy oil	3.59	4.97	4.11
Sodium chloride	0.51	0.46	0.43
DL methionine	0.30	0.24	0.23
L-lysine HCL	0.13	-	0.07
Threonine	0.06	-	-
Limestone	1.29	1.15	1.12
Monocalcium phosphate	1.75	1.48	1.17
Vitamin/mineral premix[1]	0.35	0.35	0.35
Calculated analyses			
Crude protein (%)	23.4	22.8	19.2
Poultry (ME kcal kg^{-1})	3050	3151	3200
Calcium (%)	0.95	0.85	0.75
Available phosphorus (%)	0.50	0.44	0.36
Methionine (%)	0.66	0.59	0.53
Methionine+cystine (%)	1.04	0.97	0.86
Lysine (%)	1.42	1.29	1.09
Threonine (%)	0.97	0.89	0.74
Na (%)	0.22	0.20	0.19

[1]Provided per kilogram of diet: Vitamin A 13,233 IU, vitamin D_3 6,636 IU, vitamin E 44.1 IU, vitamin K 4.5 mg, thiamine 2.21 mg, riboflavin 6.6 mg, pantothenic acid 24.3 mg, niacin 88.2 mg, pyridoxine 3.31 mg, folic acid 1.10 mg, biotin 0.33 mg, vitamin B_{12} 24.8 µg, choline 669.8 mg, iron from ferrous sulfate 50.1 mg, copper from copper sulfate 7.7 mg, manganese from manganese oxide 125.1 mg, zinc from zinc oxide 125.1 mg, iodine from ethylene diamine dihydriodide 2.10 mg, selenium from sodium selenite 0.30 mg

each pen average body weight and weight distribution was not different. The experiment was carried out in a completely randomized design with 6 dietary treatments. In each treatment, there were 4 replicates of 21 birds for each. The experimental groups were as follows: Treatment 1 (control) was fed with a basal diet only and treatments 2-6 were fed with the basal diet supplemented with 100, 250, 500, 1,000 and 3,000 mg kg^{-1} propolis, respectively.

Data collection and sampling

Performance and internal organs weight: At the end of both the grower phase (day 15-28) and finisher phase (day 29-42), Feed Intake (FI), Body Weight (BW) and Body Weight Gain (BWG) were recorded on a pen basis and Feed Conversion Ratio (FCR) was calculated.

Blood biomarkers and gut characteristics: At 42 day of age, 3 birds were randomly taken from each pen and euthanized immediately (12 birds per treatment). The internal organs (the heart, liver, gizzard and spleen as well as abdominal fat pad), cecal content and brain samples were collected immediately following blood collection. To account for any circadian rhythmicity in hormones and neurotransmitters, the sampling time was standardized and followed the cycle one bird per treatment until the end.

Blood collection: At 42 day of age, a 5 mL blood sample was collected from each sampled bird (3 birds per pen×4 pens per treatment) within 2 min from taking the bird out from its cage via cardiac puncture following sedation with sodium pentobarbital (30 mg mL^{-1}) and then euthanized by cervical dislocation. Blood samples were collected into a serum separator tube without anticoagulant and held for 2-3 h at room temperature to clot. Following centrifuging at 3000×g for 15 min, serum was collected and stored at -80°C until the analysis.

Leukocyte populations and heterophil: lymphocyte ratio: Following blood sampling, duplicate blood smears per bird were prepared immediately from un-heparinized blood using previously published laboratory method[29,30]. After drying, within 3 h after preparation, blood smears were stained with Hema 3 Stain (Thermo Fisher Scientific Inc. Waltham, USA). One hundred white blood cells were counted from each stained slide (200 cells per bird) and examined at 2,000 times magnification. Heterophils, lymphocytes, monocytes, basophils and eosinophils were identified based on their characteristics described by Campbell[31], from which the heterophil:lymphocyte (H/L) ratios were determined[29].

Hypothalamus: The entire brain was removed from the skull of each sampled bird (12 birds per treatment) and the hypothalamus was dissected on ice based on the landmarks described by Kuenzel and Masson[32] and then immediately flash frozen on dry ice. Upon completion of the sample collection, all hypothalamic tissue samples were stored at -80°C for future analysis[33].

Internal organs weight: The heart, liver, gizzard, spleen and abdominal fat were harvested and weighed individually with data expressed as a percentage of body weight.

Cecal contents: The cecal contents (1 g) of each sampled chicken were collected aseptically for enumeration of *Bifidobacterium* spp., *Escherichia coli,* total coliforms, Enterococcus spp. and total lactobacilli. The samples were stored in cryovials at -80°C prior to further analyses.

Duodenum, jejunum and ileum tissue samples: The whole intestinal tract was removed with the duodenum, jejunum and ileum were identified based on the following anatomical makers: (1) The duodenum was from the gizzard to pancreatic and bile ducts, (2) The jejunum was from the bile duct entrance to Meckel's diverticulum and (3) The ileum was from Meckel's diverticulum to a point 40 mm proximal to the ileo-cecal junction[20]. Tissue samples (2 cm) were buffered at the midpoint of each intestinal section and fixed in 4% formalin solution until analysis.

Physiological assays and intestinal characteristics measurement

Avian health profile: The serum samples were used for biochemical analysis of the concentrations of albumin, aminotransferase (AST), calcium, globulin, glucose, total protein and uric acid using the Vet Test 8008 and Avian Health Profile kits (IDEXX Laboratories, Inc. USA).

Inorganic phosphate concentration: Serum inorganic phosphate concentrations were monitored using the QuantiChrom™ phosphate assay kits (Bioassay System, Hayward, CA, USA).

Thyroid hormones: Analysis of serum concentrations of total thyroxin (T_4) and total tri-iodothyronine (T_3) were performed by using the commercial chicken ELISA kits (MyBioSource, Inc., San Diego, CA, USA). The intra-assay and inter-assay CV of the T_4 assay was 5.0 and 8.5% and those of the T_3 assay were 4.8 and 8.2%, respectively.

Corticosterone radioimmunoassay: Serum concentrations of corticosterone were measured in duplicate using the commercial immuchem™ ^{125}/ radioimmunoassay kits (MP Biomedicals, Inc., Santa Ana, CA, USA) by using Cheng *et al.*[29] previously published protocol.

Heat shock protein 70 mRNA expression: The HSP70 mRNA expression in the brain tissues (the hypothalamus) was detected by real-time PCR using HSP70 (5-3) forward primer (CACCATCACTGGCCTTAACGT); reverse primer (TTATCCAAGCCATAGGCAATAGC) and Taqman probe (ATGCGTATTATCAATGAGCCCA) which was developed by (Applied Biosystems) using previously published protocol[33]. The β-actin was used as a housekeeping gene. The quantity of Hsp70 in each sample was normalized using method described by Yu and Bao[34].

Gastrointestinal microbial analysis: Miniaturized plating of microbes was carried out with modifications of the method described by Sieuwerts[35]. Briefly, intestinal contents were serially diluted (10-fold) in buffered peptone water (Neogen Corporation, Lansing, MI). Samples (10 µL) were plated on various types of agars for different intestinal microbial populations: MacConkey agar (Neogen Corporation, Lansing, MI) for enumeration of total coliforms, EMB agar (Fisher Scientific/Becton, Dickinson Co., Sparks, MD) for *Escherichia coli*, Rogosa agar (Fisher Scientific/Becton, Dickinson Co., Sparks, MD 38800) for total lactobacilli; m-*Enterococcus* agar (m-Ent)-(Neogen Corporation, Lansing, MI) for *Enterococcus* spp. and BSM agar (Sigma-Aldrich Co., 3050 Spruce Street, St. Louis MO) for *Bifidobacterium* spp. The first three seeded agars were incubated for 24 h at 37°C under aerobic conditions; whereas the other two were incubated for 48 h at 37°C under anaerobic conditions. After incubation, colonies were counted and recorded in a spreadsheet as colony units per gram of sample.

Intestinal morphology: A single 0.5 cm segment was dissected from each intestinal sample and then dehydrated in a graded series of absolute ethanol (50, 70, 80, 90 and 100%). Following dehydration, the tissue samples were cleared with xylene (Sub-X, Surgipath Medical Industries, Richmond, IL) and then embedded in paraffin wax (Polysciences, Warrington,PA). Sections of 7 µm thickness (4 cross-sections for each sample) were cut, mounted onto slides and stained with haematoxylin (Gill #2, Sigma, St. Louis, MO) and eosin (Sigma). The stained slides were examined using Olympus BX40 F-3 microscope (Olympus Cooperation, Tokyo, Japan) fitted with a digital video camera (Q-imaging, 01-MBF-200R-CLR-12, SN:Q32316, Canada). The villus height, villus width and crypt depth in the duodenum, jejunum and ileum were determined by the stereological image software, Stero-investigator (Version 10) (MBF Bioscience Inc, USA). The villus height/crypt depth ratio was determined. Villi were only measured from those having an intact lamina propria. The crypt-villus measures randomly taken from four points per cross section and four sections per

intestinal segment per birds to minimize sectioning variances (total 16 crypt villus measures/each intestinal segment). The data were averaged within the pen for villus height, villus width, crypt depth and villus height/crypt depth ratio of the duodenum, jejunum and ileum per bird, respectively.

Statistical analysis: In this study complete randomized block design was used. For the analysis, cage was considered as the experimental unit. The data were analyzed by one-way analysis of variance using the General Linear Models (GLM) procedure, significance was designated as $p<0.05$. Means were compared by Duncan's test when a significant difference was detected. For statistical analysis of enumeration of microbial colony forming units (CFUs), colony counts (CFU g^{-1}) were subjected to logarithm transformation (log_{10}) before statistical analysis. The Shapiro-Wilk test was used to analyze the normality of the data.

RESULTS

During this experiment, the average room temperature and relative humidity during the day time were 31.7±0.3°C and 56±4%; while at night were 28.8±0.4°C and 58±3%, respectively (Table 1).

Table 3 shows that propolis supplementation did not affect the BW, BWG, FI and FCR of birds, also the relative weights of the internal organs (the liver, heart, gizzard and spleen) were not affected by the propolis supplementation. However, the results clarified that propolis had a dose-associated effect on abdominal fat content, as the birds fed 100, 250 or 500 mg kg^{-1} propolis had significantly higher abdominal fat content than control birds.

In the current study (Table 4), compared to controls, propolis treated broiler chickens had a significantly higher lymphocyte percentage with a lower percentage of heterophils, resulting in a low H/L ratio ($p<0.05$). However, propolis had no effect on the populations of monocytes, eosinophils and basophils ($p>0.05$). Also, broiler chickens treated with 100 or 3000 mg kg^{-1} propolis had significantly higher serum albumin concentrations compared to control chickens ($p<0.05$). There were no differences in total protein and globulin concentrations as well as albumin:globulin ratio between propolis treated broiler chickens and controls ($p>0.05$).

Table 4 shows that propolis, regardless of dose, significantly ($p<0.05$) reduced serum AST and corticosterone concentrations in broiler chickens in comparison to control birds. Additionally, serum uric acid concentrations were significantly ($p<0.05$) reduced in 250, 500, 1000 and 3000 mg kg^{-1} propolis treated groups compared to the control group. In addition, the 250 mg kg^{-1} propolis fed birds had a significant increase in T_3/T_4 ratio in comparison to the control group and other propolis groups. However, results clarified no changes in serum calcium, phosphate and glucose in propolis fed broilers compared to the control group.

Dietary supplementation of propolis had no effects on the populations of lactic acid, *Enterococcus* spp., *E. coli* and total coliforms (Table 5). However, propolis caused a significant decline in *Bifidobacteria* spp., population in broiler chickens fed with propolis at 1000 mg kg^{-1} compared to control group. Also, the results indicted no differences in the villus height, villus width, crypt depth and villus:crypt ratio among propolis fed birds and the control group.

DISCUSSION

The results indicated that Brazilian propolis contained 420 chemical compounds. The major bioactive contents were: total flavonoids (quercetin) = 0.0 4%, total flavonoids (rutin) = 0.08%, artepillin C = 0.015%, caffeic acid = 0.03%, p-coumaric acid = 0.4%, benzoic acid = 0.6%. Similarly, Hori *et al.*[36] reported that the quantities of the main component of green Brazilian propolis from Apis Flora Co. were caffeic (0.024%), p-coumaric (0.148%) and trans-cinnamic (0.014%) acids, the flavonoid aromadendrin (0.0423%) and the prenylated compound artepillin C (0.369%).

Results of the present study showed that propolis supplementation did not affect the BW, BWG, FI and FCR of birds (Table 3). However, Seven and Seven[25] reported that BW, BWG and FCR were improved in heat stressed broiler chickens fed propolis at 5000 mg kg^{-1} diet. Contrary to this, Mahmoud *et al.*[37] recorded that dietary supplementation of propolis (100, 250, 500 or 750 mg kg^{-1}) to heat stressed broiler chickens significantly reduced BW but not FI and FCR. The different findings among the current and previous studies could be related to the differences in: (1) Type and chemical composition of propolis, (2) Bird strain and age and (3) Severity and length of stressors used in each study.

The current study also determined that the relative weights of the internal organs (the liver, heart, gizzard and spleen) were not affected by propolis supplementation. Similar results were obtained in broiler reared under normal temperature[37]. Conversely, Hassan and Abdulla[38] noted heavier liver weight from broilers fed a diet with 400 mg kg^{-1} propolis compared to control group.

The current study revealed that propolis had a dose-associated effect on abdominal fat content (Table 3). The birds fed diets supplemented with propolis at 100, 250 or

Table 3: Effect of different concentrations of propolis on performance characteristics and relative weight of internal organs of broiler chicks reared under heat stress

Propolis (mg kg^{-1} diet)	*BW (kg)	*BWG (kg)	*FI (kg)	*FCR	Liver (%)	**Heart (%)	**Spleen (%)	**Gizzard (%)	**Abdominal fat (%)
0	2.35±0.05	1.98±0.05	3.44±0.14	1.74±0.04	2.50±0.15	0.37±0.02	0.10±0.01	1.33±0.05	1.59±0.08^{b}
100	2.37±0.02	2.01±0.02	3.49±0.11	1.74±0.06	2.27±0.20	0.37±0.01	0.08±0.01	1.27±0.06	1.93±0.10^{a}
250	2.38±0.09	2.00±0.10	3.54±0.06	1.78±0.07	2.42±0.18	0.36±0.02	0.09±0.01	1.25±0.04	1.92±0.04^{a}
500	2.38±0.06	2.01±0.06	3.38±0.02	1.70±0.05	2.37±0.13	0.39±0.02	0.09±0.02	1.35±0.04	1.84±0.09^{a}
1000	2.40±0.12	2.02±0.11	3.65±0.14	1.81±0.04	2.32±0.08	0.35±0.03	0.08±0.01	1.37±0.04	1.71±0.08^{ab}
3000	2.41±0.03	2.04±0.04	3.53±0.08	1.73±0.04	2.36±0.28	0.42±0.02	0.08±0.01	1.30±0.05	1.79±0.04^{ab}
Probability of a diet effect	0.995	0.995	0.546	0.694	0.96	0.315	0.904	0.798	0.035

^{a,b}Means±SE with different superscripts in the same column differ significantly (p<0.05), *Means represented 4 pens per diet, 21 birds per pen, **Means represented 4 pens per diet, 3 birds per pen

Table 4: Dose effect of propolis on the differential leukocytes count, serum blood biochemical parameters and brain HSP70 mRNA expression in broiler chickens reared under heat stress from 15-42 day

				Serum blood biochemical indicators			Brain tissue		
Propolis concentration	Different leucocytes (%)			Albumin		Uric acid	Corticosterone		HSP70
(mg kg^{-1})	Heterophil	Lymphocyte	H/L	(g dL^{-1})	AST (U L^{-1})	(mg dL^{-1})	(ng mL^{-1})	T3/T4 ratio	mRNA
0.0	39.33±2.48^{a}	51.75±3.42^{b}	0.79±0.10^{a}	0.92±0.05^{b}	496.00±54.73^{a}	8.80±0.34^{a}	13.85±0.50^{a}	1.34±0.21^{b}	4.60±0.36
100	30.58±3.90^{b}	60.25±5.56^{ab}	0.55±0.10^{b}	1.13±0.08^{a}	391.50±33.77^{b}	8.15±0.14^{ab}	10.38±0.71^{bc}	0.92±0.08^{b}	4.66±0.51
250	22.67±2.68^{b}	65.17±2.35^{a}	0.36±0.05^{b}	1.08±0.06^{ab}	382.50±32.66^{b}	6.55±0.50^{bc}	9.09±0.40^{c}	2.66±0.91^{a}	4.19±0.81
500	24.17±1.70^{b}	67.37±2.91^{a}	0.37±0.04^{b}	0.95±0.03^{b}	353.75±33.42^{b}	6.48±0.89^{bc}	10.61±0.63^{bc}	0.92±0.07^{b}	5.08±0.88
1000	24.67±1.65^{b}	66.67±2.00^{a}	0.38±0.03^{b}	1.08±0.05^{ab}	350.25±22.87^{b}	6.03±0.87^{c}	9.16±0.69^{c}	0.84±0.09^{b}	5.71±0.49
3000	25.17±3.17^{b}	65.67±4.38^{a}	0.41±0.08^{b}	1.15±0.05^{a}	342.50±13.28^{b}	6.30±0.23^{c}	11.65±0.75^{b}	0.81±0.08^{b}	3.61±0.69
Probability of a diet effect	0.003	0.056	0.004	0.042	0.049	0.014	0.000	0.023	0.339^{ab}

$^{a-c}$Means±SE in the same column with different letters differ significantly (p<0.05), *Means represented 4 pens per diet, 3 birds per pen

Table 5: Effect of different concentrations of propolis on cecal bacterial count (log_{10} CFU g^{-1}) and overall intestinal-morphometry in heat stressed broiler chickens at 42 day

	Cecal bacterial count (log^{10} CFU g^{-1})				Intestinal-morphometry				
Propolis (mg kg^{-1} diet)	*Bifidobacterium* spp.	*E. coli*	Coliforms	*Enterococcus* spp.	Lactobacilli	Villus height (mm)	Villus width (mm)	Crypt depth (mm)	Villus: crypt ratio
0.0	7.69±0.16^{ab}	5.66±0.60	6.07±0.72	5.76±0.83	7.77±0.15	2.67±0.12	0.39±0.03	0.43±0.03	6.23±0.32
100	8.02±0.09^{a}	5.32±0.74	6.74±0.26	6.38±0.32	7.59±0.14	2.53±0.10	0.37±0.09	0.45±0.03	5.94±0.28
250	7.59±0.15^{abc}	4.15±0.89	6.72±0.26	6.07±0.26	7.58±0.21	2.43±0.10	0.35±0.02	0.41±0.01	5.90±0.22
500	7.44±0.16^{bc}	3.88±0.85	5.89±0.58	6.65±0.29	7.88±0.09	2.42±0.11	0.39±0.03	0.39±0.03	6.52±0.58
1000	7.08±0.18^{c}	5.33±0.60	6.48±0.33	6.19±0.61	7.82±0.11	2.45±0.03	0.38±0.03	0.43±0.02	5.93±0.16
3000	7.64±0.11^{ab}	4.48±0.97	6.50±0.46	6.35±0.41	7.46±0.21	2.51±0.09	0.38±0.02	0.45±0.03	5.81±0.41
Probability of a diet effect	0.002	0.513	0.790	0.886	0.401	0.482	0.812	0.475	0.72

^{a,b}Means±SE with different superscripts in the same column differ significantly (p<0.05), *Means represented 4 pens per diet, 3 birds per pen

500 mg kg^{-1} propolis had significantly higher abdominal fat content than control birds. The increased amounts of abdominal fat deposition in propolis fed birds may improve the birds ability to cope with heat stress. It is proposed that increased internal fat contents in pigs[39] and broiler chickens[40] improve their thermal insulation thus helping adapt to high ambient temperature, the more dietary energy stored as fat, the lower heat produced, resulting in less heat needing to be dispersed. Although the cellular mechanisms of increased fat contents in heat-stressed broiler chickens has not been examined in the current study, they could be similar to the ones reported by Lu *et al.*[40]. The increased amounts of abdominal fat deposition in propolis fed birds may be a result of propolis antioxidants and flavonoids contents acting as inhibitors of lipid peroxidation by scavenging polyunsaturated fatty acid peroxy radicals and interrupting the chain reactions[41].

The heterophil:lymphocyte ratio has been used as a stress indicator in chickens[29,42]. Chronic heat stress increases the number of heterophil cells but decreases the number of lymphocytes, leading to an increase in H/L ratio in broiler chickens[15,43]. In the current study (Table 4), propolis prevented the negative effects of heat stress on the populations of leukocytes. Compared to controls, propolis fed broilers had a significantly higher lymphocyte percentage with a lower percentage of heterophils, resulting in a low H/L ratio ($p<0.05$). However, propolis had no effect on the populations of monocytes, eosinophils and basophils ($p>0.05$). Similar results were reported in both propolis fed broiler chickens and laying hens reared under normal temperature conditions[44]. These results may reflect the antioxidant, antibacterial, immunomodulatory and or anti-inflammatory functions of propolis[22], improving birds immunity and health status by reducing the negative effects of heat stress.

Imik *et al.*[14] reported that heat stress reduces blood total protein, albumin and globulin concentrations and increases albumin: globulin ratio in broiler chickens. The current results indicated that only broiler chickens fed with 100 or 3000 mg kg^{-1} propolis had significantly higher serum albumin concentrations compared to control birds ($p<0.05$). Also, there were no differences in total protein, globulin concentrations and albumin:globulin ratio between propolis treated broiler chickens and controls ($p>0.05$). Similar results were previously obtained in heat stressed broilers[28].

Exposing broilers to high environmental temperatures significantly increases the concentrations of AST[45] as a biomarker of tissue damage[46]. The current results (Table 4) showed that propolis, regardless of dose, significantly ($p<0.05$) inhibited heat stress-induced increase of AST serum concentrations in broiler chickens. Propolis protected tissue damage, resulting in reducing AST concentrations and were also recorded in laying hens fed propolis[44] at 100 or 150 mg kg^{-1} and broiler chickens fed at 300 mg kg^{-1} propolis[47] under regular management conditions. Conversely, Seven *et al.*[24] reported that propolis had no effects on AST concentrations in broiler chickens exposed to heat stress or lead toxicity.

Serum uric acid concentration, as another biomarker of tissue damage[48], is significantly increased in broiler chickens reared under heat stress[49]. The current study clarified that serum uric acid concentrations were significantly ($p<0.05$) reduced in 250, 500, 1000 and 3000 mg kg^{-1} propolis treated groups compared to the control group. This effect may be attributed to the xanthine oxidase (XOD) inhibitory activity of propolis bioactive contents, such as chrysin, galangin, caffeic acid phenethyl ester, p-coumaric acid and artepillin C[50]. Similar protection effects have been also found in both rats fed propolis orally and guinea pigs injected propolis intraperitoneal[51]. Denli *et al.*[52], however, reported no differences in serum uric acid concentrations between propolis treated quail (0.5, 1 or 1.5 g kg^{-1}) and controls under thermo-neutral environmental conditions. Improvement in uric acid and AST concentrations may be attributable to the protective effects of propolis on the liver and the kidney from its phenolic components (including flavonoids) and their anti-oxidant effects inhibiting lipid oxidation in cell membranes[53].

Corticosterone has been used as a stress indicator in various animals including chickens[29]. Chronic heat stress induces increases in serum corticosterone concentrations have been found in broiler chickens[27]. In this study, the negative effects of heat stress were prevented or inhibited in propolis fed broiler chickens, regardless of dose. Although its mechanism has not been examined in this study, it may be similar to the one reported in mice exposed to a forced-swim stress test[54]. Lee *et al.*[54] reported that propolis reduces the stress response of the limbic hypothalamic-pituitary-adrenal axis. Propolis attenuated serum corticosterone concentrations, correlated with the changes of the numbers of the c-fos immunoreactive neurons in the hippocampal dentate gyrus; by which propolis decreased the neural activity to normalize HPA activity through the inhibitory feedback system[54]. C-fos has been used as a marker of activated neurons[55]. Conversely, Mahmoud *et al.*[27] reported that propolis at 250 mg kg^{-1} did not affect serum corticosterone concentrations in heat-stressed broilers. The difference between the current and the results reported by Mahmoud *et al.*[27] may be related to the differing responses of chicken strains (Ross 308 vs. Ross 708),

thermal-stress conditions (38 vs. 32°C) and types of propolis (Chinese propolis vs. Brazilian propolis), used in each study.

Thyroid hormones control metabolic heat production, which is necessary for the maintenance of constant body temperature in animals[56]. Lin *et al.*[16] reported that heat stress significantly reduced T_3/T_4 ratio in birds. The current results showed that 250 mg kg^{-1} propolis treated group had a significant increase in T_3/T_4 ratio in comparison to the control and other propolis treated groups. The change in 250 mg kg^{-1} propolis fed chickens may have a higher T_4 to T_3 conversion, resulting in a lower concentration of T_4 (2.11 vs. 2.48) but a higher concentration of T_3 (5.26 vs. 3.62) than controls.

The current results confirmed no changes in serum calcium, phosphate and glucose in propolis treated broiler chickens compared to the controls. Similar results have been obtained in broiler chickens reared under the recommended environmental conditions or exposed to various stressors such as heat stress or lead toxicity[24,57].

The current results showed that the dietary supplementation of propolis had no effects on the populations of lactic acid, *Enterococcus* spp., *E. coli* and total coliforms. Similar observations were reported in broiler chickens fed with propolis at doses among 400, 800 and 1000 mg kg^{-1} under normal temperature[58,59]. In the current study, propolis caused a significant decline in *Bifidobacteria* spp., population in broilers fed with propolis at 1000 mg kg^{-1} compared to controls. This finding is supported by Haddadin *et al.*[60] who reported that propolis had an adverse effect on the growth of the *Bifidobacteria* spp., of human intestinal origin, while Abdel-Mohsein *et al.*[23] reported that supplementation of propolis (100, 250, 500 or 750 mg kg^{-1} diet) had stimulated growth of both *Lactobacillus* spp. and *Bifidobacteria* spp., in broilers under both recommended normal temperature and chronic heat stress conditions.

Several studies have shown that heat stress negatively affects the lining epithelium of the intestine, inducing a reduction in villus height and crypt depth[61]. Results of the present study indicted no differences in the villus height, villus width, crypt depth and villus:crypt ratio among propolis groups and compared to the control group (Table 3). Similar to these results, Eyng *et al.*[62] reported that 1000 or 2000 mg kg^{-1}propolis had no effect on the crypt depth or the villus height:crypt ratio in the jejunum and ileum and the villus height in the duodenum, jejunum and ileum. These results may be due to the low anti-pathogenic biologic activity of plant extracts, which may not overcome the negative effects of heat stress on the intestinal microenvironment[20]. Contrary to this, Tekeli *et al.*[63] suggested that under normal temperatures, the intestinal villi length of broiler chickens was significantly improved in broilers supplemented with 1000 mg kg^{-1} propolis compared to controls. The differences between the findings of the present study and previously published results may be attributable to the type of propolis used, as the composition of the different propolis sources can differ greatly, depending on the location and season of the year they were collected[64]. In addition, the imposed temperature used in this study may not be severe enough to cause the microstructural changes in the intestine so that propolis would not elucidate a substantial effect.

CONCLUSION

It is concluded that dietary supplementation of green Brazilian propolis at the concentrations used in this study did not impact growth performance and intestinal morphology of broiler chickens reared under heat stress. However, there were significant dose-associated differences in abdominal fat weight and cecal *Bifidobacterium* spp., populations between the propolis fed groups and control group. Also, the results revealed that dietary supplementation of propolis reduced physiological stress responses, reductions in the concentrations of AST, uric acid, corticosterone and the H/L ratio but increased the T_3/T_4 ratio and albumen, in heat stressed broiler chickens.

SIGNIFICANCE STATEMENTS

This study discovered that green Brazilian propolis can be used as a growth promoter to improve production performance and health status in broiler chickens, particularly during hot seasons. The improvement in stress indicators may reflect improving the birds ability to cope with chronic high environmental temperature. The study will help research to further investigate the effects of propolis on chicken welfare and health; especially, with increasing demand for organic animal products to reduce or eliminate antibiotics used in agriculture. Thus new guidelines and management practices may be developed.

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