Effects of propolis and stocking density on growth performance, nutrient digestibility, and immune system of heat-stressed broilers

Shaban Chegini a, Ali Kiani a, Bahman Parizadian Kavan a and Hassan Rokni b

aDepartment of Animal Science, Lorestan University, Khorramabad, Iran; bThe Ministry of Agriculture Jihad, Deputy of Animal Products, Tehran, Iran

ABSTRACT
Two experiments were conducted to study the effects of propolis (bee glue; BG) supplementation and stocking density on growth performance, ileal nutrient digestibility, gut morphology, and immune response of broilers exposed to heat stress. In Experiment I, a total of 300 1-day-old male chicks (Ross 308) were assigned to six treatments, with five replications of 10 birds each. The birds were fed diet containing no additive (CON) or diet supplemented with different levels of BG (1, 2, 3, 4, and 5 g/kg of feed). Broilers-fed BG at 4 g/kg had higher average daily gain (ADG) (linear and quadratic, \( p < .05 \)) and average daily feed intake (ADFI) (linear, \( p < .01 \)) than those fed the CON diet, but BG supplementation (5 g/kg of feed) reduced (quadratic and linear, \( p < .01 \)) levels of triglycerides, total cholesterol and low-density lipoprotein (LDL). Inclusion of BG (4 and 5 g/kg of feed) increased villus height (VH) and villus height-to-crypt depth ratio (VH/CD) in the jejunum (linear and quadratic; \( p < .01 \)), and plasma IgM concentration (linear; \( p = .021 \)). In Experiment II, 1-day-old Ross 308 male broiler chickens (\( n = 240 \)) were allocated to four experimental groups for 42 days. The experimental treatments consisted of a 2 x 2 factorial arrangement with two levels of BG (0 or 4 g/kg of diet) and two levels of stocking density (low stocking density; LSD, 10 birds/m² or medium stocking density; MSD, 14 birds/m²). From Day 22 on the birds were either kept at thermoneutral zone (22 °C) or subjected to cyclic heat stress by exposing them daily to 33 °C for 10 h (from 08.00 to 18.00) and 22 °C from 18.00 to 08.00. The results showed that ADG, ADFI, and jejunum VH were decreased (\( p < .05 \)) more in the birds stocked at MSD than in those housed at LSD. In the birds stocked at MSD, dietary BG supplementation increased (\( p < .05 \)) the nitrogen digestibility, AME, blood IgG and heterophil:lymphocyte ratio (H:L). In conclusion, the optimum inclusion level of BG in broiler diets is 4 g/kg, and feeding BG had positive effects on immune system and stress indicators of heat-stressed broilers housed at a MSD.

Introduction
In the modern broiler chicken production systems, stress can arise from a variety of factors, such as elevated rearing densities and high ambient temperature. These unfavourable or stressful environmental conditions can negatively affect the broiler’s welfare, health, growth performance, and well-being (Gomes et al. 2014). In this regard, stocking density is a subject of serious concern in poultry industry. Different stocking densities are used, depending on the country and production system (Estevez 2007). A higher profitability per kilogram chicken can be obtained by increasing the stocking density if the performance of birds remains constant. However, a high stocking density (HSD) can be stressful and has deleterious effects on productive performance and the immune system (Houshmand et al. 2012). Increased heat production has been offered as an explanation for the decreased performance at HSD, especially in hot environments. Under these conditions, airflow at the level of the birds is often reduced, decreasing convective thermolysis. Consequently, a high environmental temperature is observed in the microclimate of the bird resulting in a reduced dissipation of body heat to the environment (Cengiz et al. 2015).

In the tropical and subtropical regions of the world, heat stress (HS) is one of the major challenges facing the poultry industry (Lara and Rostagno 2013). It has been well-documented that exposing broilers to high temperatures leads to chronic HS and resulted in an...
impairment of performance parameters (Lara and Rostagno 2013; Song et al. 2014). Concisely, HS is characterised by decreased feed intake, body weight gain and feed efficiency, endocrine disorders, reduced metabolic rate, lipid peroxidation, systemic immune dysregulation, electrolyte imbalance, damage to the mucosal epithelium, increased intestinal injury, decreased intestinal immune activity, and impairment of intestinal morphology following pathogenic bacteria invasion of the body through the intestinal epithelium (Bartlett and Smith 2003; Akbarian et al. 2014).

One of the possible approaches employed to maintain the performance, intestinal health, and humoral immunity, and to avoid physiological stress in broiler exposed to HS (Hosseini et al. 2016) and overcrowding stress (Chegini et al. 2018), is the use of propolis (bee glue; BG) as a natural antioxidant. Propolis has strong antioxidant, antifungal, antiviral, antibacterial, anti-inflammatory, and immunorestorative properties, and cytotoxic and hepatoprotective activities (Acikgoz et al. 2005). These properties are attributed to the chemical composition of BG, including flavonoids, aromatic acids, diterpene acids and phenolic compounds (Seven et al. 2012). We have shown that dietary supplementation of BG is effective on growth performance and immune status of broiler chickens subjected to heat stress and HSD (Chegini et al. 2018), which can be attributed to positive effects of BG on reduction in the deleterious effects of oxidative stress (Seven et al. 2012). Therefore, the use of BG in broiler diets was recommended as a way to alleviate the detrimental effects of HS (Hosseini et al. 2015) and overcrowding (Chegini et al. 2018) in broilers.

The primary objective of the current study was to determine the optimal dietary BG level for broilers housed under thermoneutral condition. The second objective of this research was to investigate the influence of dietary supplementation of BG on immune status, gut morphology, and nutrient digestibility of heat-stressed broilers reared at a medium stocking density.

**Material and methods**

All procedures carried out in this experiment were reviewed and approved by the Animal Care and Use Committee of Lorestan University, Khorramabad, Iran.

**Preparation of BG**

The BG was prepared and extracted in the same way as described in the previous research (Chegini et al. 2018).

**Birds, diets, and housing**

This study consisted of two experiments, and Experiment I was conducted before Experiment II.

In Experiment I, 300 1-day-old Ross 308 male chicks were randomly distributed into 30 floor pens to examine the effects of 0, 1, 2, 3, 4, or 5 g/kg BG in five replicates of 10 birds each. In Experiment II, 240 1-day-old chicks were distributed in a completely randomised design with four treatments, each of which was replicated five times. The experimental treatments consisted of a 2 × 2 factorial arrangement with two levels of BG (0 or 4 g/kg of diet; the optimum level in Experiment I) and two levels of stocking density. The Iranian Animal Welfare Commission recommends a maximum stocking density of 37 kg/m² (0.067 m²/bird) for broilers in mechanically ventilated sheds with water-based cooling. For non-mechanically ventilated sheds, the maximum is 28 kg/m² (0.084 m²/bird). In Experiment II, the birds were placed at a low stocking density (LSD) of 10 birds/square m or a medium stocking density (MSD) of 14 birds/m², according to practical management conditions. These recommendations follow the guidelines published by Barnett et al. (2008). For both experiments, the chickens were fed corn–soybean basal diets (CON) that were formulated for starter (Day 0–21) and finisher (Day 22–42) according to the National Research Council (NRC) (1994) recommendations (Table 1). A temperature of 33 ± 1°C was maintained for the

| Item | Starter (0–21 d) | Finisher (22–42 d) |
|------|-----------------|-------------------|
| Corn | 50.21           | 57.43             |
| Soybean meal (48% CP) | 36.25 | 28.20 |
| Wheat gluten | 5.00 | 5.00 |
| Soybean oil | 3.94 | 5.22 |
| Limestone | 1.20 | 1.32 |
| Dicalcium phosphate | 1.88 | 1.33 |
| Salt | 0.25 | 0.25 |
| Choline chloride | 0.08 | 0.08 |
| Sodium bicarbonate | 0.05 | 0.05 |
| L-Lysine | – | 0.08 |
| α-Methionine | 0.14 | 0.04 |
| Vitamin–mineral premix | 1.00 | 1.00 |
| Metabolisable energy (MJ/kg) | 12.77 | 13.40 |
| Crude protein (N × 6.25) | 23.00 | 20.00 |
| Calcium (%) | 1.00 | 0.90 |
| Available phosphorus (%) | 0.45 | 0.35 |
| Lysine (%) | 1.13 | 1.01 |
| Methionine + cystine (%) | 0.90 | 0.72 |
| Threonine (%) | 0.86 | 0.74 |
| Crude fibre | 3.45 | 3.41 |

*Permix provided the following per kg of feed: retinol, 2.7 mg; cholecalciferol, 0.05 mg; α-tocopherol, 20 mg; nicotinic acid, 30 mg; cyanocobalamin, 0.12 mg; calcium pantothenate, 10 mg; menadione, 2 mg; thiamine, 1 mg; riboflavin, 4.2 mg; pyridoxine hydrochloride, 1.7 mg; folic acid, 0.5 mg; biotin, 0.5 mg; Fe, 80 mg; Cu, 10 mg; Mn, 100 mg; Zn, 80 mg; Co, 0.2 mg; I, 1.0 mg; Se, 0.3 mg; monensin, 100 mg.*
first 3 d and was gradually decreased until a constant temperature of 22°C was achieved. In Experiment II, chickens were exposed to heat stress (33 ± 1°C for 10 h, from 08.00 to 18.00 h, and 22°C from 18.00 to 08.00 h) from 22 to 42 d of age. In both experiments, the birds had free access to feed and water and were maintained on a 23-h lighting programme during 0–7 d, which was stepped down to 20L:4D by Day 8, this being maintained until the end of the study. Each pen was equipped with a tube feeder, automatic waterer, and rice hulls for bedding. The pens had dimensions of 1.2 m length × 0.85 m width × 0.7 m height.

Performance parameters
Feed intake and body weight for each replicate were recorded. Average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were further calculated.

Nutrient digestibility
For each experiment, the metabolic trial was conducted from 30 to 34 d of age. At Day 30, three birds per replicate with BW similar to the average of the corresponding pen were selected and transferred to battery cages (three birds in each) with a wire mesh bottom and excreta collection trays. The dimensions of cages were 60 × 30 × 30 cm, length × width × height. Experimental diets for the adjustment period were mixed with titanium oxide (3 g/kg) as an indigestible marker to determine the effect of treatments on apparent metabolisable energy (AME) and the ileal apparent nutrient digestibility coefficients (CIAD) for nitrogen (N), calcium (Ca), and phosphorus (P). On Day 35, all of the birds were killed by intravenous injection (1 mL per 2 kg live weight) of sodium pentobarbitone and then ileal digesta were collected from the ileum, according to the procedures described by Ravindran et al. (2005). The ileum samples were placed into plastic containers and freeze-dried. The samples were ground to pass through a 0.5 mm sieve before chemical analysis.

Chemical analysis
Gross energy was determined by adiabatic bomb calorimetry (Gallenkamp Autobomb, London, UK) standardised with benzoic acid. Samples were assayed for Ti on a UV spectrophotometer following the method of Short et al. (1996). Nitrogen content of the diets and ileal digesta was determined by combustion [method 968.06; Association of Official Analytical Chemists (AOAC) 2005] using a CNS-200 carbon, N and sulphur auto-analyser (LECOR Corporation, St. Joseph, MI). Dry matter content was determined using standard procedures (method 930.15; AOAC 2005). Samples of the diets and ileal digesta were analysed for Ca and P concentrations by colorimetric methods after ashing the samples at 550°C and acid digestion in 6.0 M HCl using standard procedures (method 968.08D; AOAC 2005).

Calculations
The AME values of the diets were calculated using the following formula:

\[
AME (MJ/kg) = \frac{\text{Feed intake} \times \text{GE}_{\text{diet}} - \text{Excreta output} \times \text{GE}_{\text{excreta}}}{\text{Feed intake}}
\]

Ileal apparent nutrient digestibility coefficients (CIAD) were calculated using the following formula:

\[
\text{CIAD of diet component} = \frac{(\text{Diet component/Ti})_d - (\text{Diet component/Ti})_i}{(\text{Diet component/Ti})_d}
\]

where \((\text{Diet component/Ti})_d\) is the ratio of diet component to Ti in the diet, and \((\text{Diet component/Ti})_i\) is the ratio of diet component to Ti in the ileal digesta.

Gut morphology
In both experiments, feed was removed from all pens 12 h before slaughter. On Day 42, 10 broilers (for each experiment) per treatment were randomly selected and euthanised with carbon dioxide and killed by cervical dislocation. Broiler organs including the crop, gizzard, liver, and small intestine were cleaned with physiological saline solution, dried with filter paper and were weighed and expressed as a percentage of body weight.

Samples (5 cm in length) were excised from the middle of the jejunum (from the distal portion of the duodenal loop to Meckel’s diverticulum). These samples were flushed with cold saline and immediately placed in 70% formalin. Samples were transferred into 70% ethanol after 72 h. The variables measured were villus height (VH), crypt depth (CD), and then VH/CD ratio was calculated.

Blood collection and analyses
In each trial, a 4 mL blood sample was obtained aseptically from the wing vein of two broilers in each replicate (10 samples per treatment) after a 2-h feeding withdrawal. In Experiment I, the blood sample was collected into two tubes (2 mL in each tube). The first
tube containing heparin as the anticoagulant, and the tube was centrifuged at 5000g for 10 min at 4°C. The collected plasma was stored at −20°C until further analysis. The plasma IgG and IgM concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit from Bethyl Laboratories (Montgomery, TX). The ELISA procedure was carried out according to the protocol of the manufacturer and absorbance was measured at 450 nm. The blood samples in the second tube were placed at room temperature for 2 h for serum separation. Samples for serum analysis were then centrifuged at 3000g for 10 min. Total cholesterol (Tch), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides (TG) concentrations in the serum samples were analysed with an autoanalyzer (Autolab, BT 3500, Autoanalyzer Medical System, Rome, Italy) and were measured using reagent kits (Wako Pure Chemical Industries, Osaka, Japan).

In Experiment II, haematological analysis was conducted to measure the levels of heterophils (H) and lymphocytes (L) by using an automatic haematological analyser (Sysmex XE-2100\textsuperscript{TM} Automated Hematology System; Sysmex America, Inc., Lincolnshire, IL). These concentrations were also used to calculate the H:L ratio index. The plasma IgG and IgM concentrations were also measured as described in Experiment 1.

**Statistical analysis**

Data were analysed using the GLM procedures of SAS (SAS Inst. Inc., Cary, NC) in a completely randomised design. All of the treatment means were compared using Tukey–Kramer’s test. For the different statistical tests, significance was declared at \( p < .05 \). In Experiment I, orthogonal polynomial contrasts were used to determine linear and quadratic effects of dietary BG supplementation on performance, gut morphology, nutrient digestibility and blood parameters. In Experiment II, data were analysed with a \( 2 \times 2 \) factorial treatment arrangements, including the main effects of BG and stocking density.

**Results**

**Experiment I**

The performance data (Table 2) showed that dietary treatments did not affect FCR, but inclusion of 3 and 4 g/kg of BG linearly increased (\( p = .005 \)) ADFI compared with birds fed the CON diet. The inclusion of 4 g/kg of BG increased (linear and quadratic, \( p < .05 \)) ADG compared with the CON group.

Nutrient digestibility for N, Ca and P, and AME of feed are summarised in Table 2. Dietary treatments had no significant effect on nutrient digestibility. However, the BG had a high tendency to improve CIAD of Ca (\( p = .081 \)) and P (\( p = .064 \)).

The effect of dietary BG supplementation on gut morphology of broilers is given in Table 3. Inclusion of BG did not affect the relative weight of crop, gizzard, liver, and small intestine, but there was a tendency (\( p = .068 \)) toward smaller crop weight with increasing BG levels. Dietary BG increased VH and VH/CD in the jejunum (linear and quadratic, \( p < .001 \)). However, the jejunum CD was not affected by increasing BG level.

The supplementation of BG significantly decreased the concentration of TG (quadratic, \( p = .048 \)), Tch (linear, \( p = .002 \); quadratic, \( p = .006 \)) and LDL (linear, \( p = .003 \); quadratic, \( p = .009 \)), but did not affect the HDL level in serum (Table 4). Inclusion of BG (4 and 5 g/kg) resulted in an increase (linear, \( p = .012 \)) the plasma IgM level compared with the CON diet, but did not affect the IgG concentration.

**Experiment II**

The effects of BG supplementation and stocking density on the growth performance and nutrient digestibility of broilers exposed to heat stress are shown in Table 2. As expected, MSD depressed ADG (\( p = .014 \)) and ADFI (\( p = .037 \)). Compared with the basal diet, supplementation of BG increased ADG and ADFI (\( p < .05 \)). In heat-stressed chickens stocked at MSD, BG supplementation increased (\( p < .001 \)) CAID of N and AME of feed compared to those fed basal diet and housed at LSD. The CIAD of Ca and P were unaffected by the treatments. No interaction between BG and stocking density was observed on performance and nutrient digestibility.

The effects of dietary BG supplementation and stocking density on the gut morphology of broilers subjected to heat stress are given in Table 3. Inclusion of BG and stocking density had no significant effect on relative weight of digestive organs (crop, gizzard, liver, and small intestine). Stocking density had a negative effect on jejunum morphometry. The MSD decreased (\( p < .001 \)) significantly the VH in comparison to the LSD. No interaction between BG supplementation and stocking density was observed on gut morphology in this study.

The effects of the BG and stocking density on the gut morphology of broilers exposed to heat stress are shown in Table 5. There was a significant BG × stocking density interaction related to H/L ratio (\( p < .001 \)); supplemental BG increased this stress.
indicator to a greater extent in broilers exposed to LSD. Inclusion of BG supplementation and MSD significantly increased the plasma IgG ($p < .001$) level compared with the LSD and CON diet.

**Discussion**

Some studies have shown impaired performance under acutely stressful conditions (Abudabos et al. 2013; Cengiz et al. 2015; Chegini et al. 2018). Estevez (2007) suggested that the reduction in the airflow around the bird may account for the lowered feed intake and weight gain leading to poor FCR. A reduction in access to feed and water, increase in ammonia, and poor air quality, because of insufficient air exchange at the level of bird are other factors that may negatively affect the bird’s performance. Another possible reason for poor performance in broilers housed at MSD may be a change in the behavioural patterns of birds, and because of these new behaviours, their energy efficiency will decrease (Cengiz et al. 2015). In addition, more energy is expanded to adapt to the stress conditions; therefore, less energy was used for growth, leading to the decreased growth performance in birds exposed to heat stress (Houshmand et al. 2012). In the present study, dietary BG supplementation improved broiler performance in normal density and overcrowded conditions. It has been well demonstrated that BG supplementation can be used in poultry feeds as a natural growth promoter and improved growth performance in broilers reared under high environmental temperatures (Hosseini et al. 2016) and overcrowded stress (Chegini et al. 2018). Acikgoz et al. (2005), Ichi et al. (2009) and Attia et al. (2014) revealed that the beneficial effect of BG may be due to the bioactive substances such as total flavonoids and benzoic acid that might enhance the animal metabolic process. Therefore, increase in feed consumption and body weight could be attributed to the flavonoid content and palatable properties of the BG diet.

The results of the current research are in agreement with Acikgoz et al. (2005) who reported that no enhancement in the nutrient digestion was observed in birds reared under thermoneutral conditions. Acikgoz et al. (2005) suggested that BG supplementation could not be recommended as a growth

| Experiment | ADFI, g | ADG, g | FCR | CIAD of N | CIAD of Ca | CIAD of P | AME (MJ/kg) |
|------------|--------|--------|-----|----------|-----------|----------|-------------|
| Exp. I     |        |        |     |          |           |          |             |
| Basal diet (CON) | 85.6b | 51.3b  | 1.72| 0.651    | 0.428     | 0.493    | 12.20       |
| CON + 1 BG g/kg | 87.0bc| 51.5bc | 1.68| 0.641    | 0.431     | 0.490    | 12.17       |
| CON + 2 BG g/kg | 86.2bc| 51.7bc | 1.66| 0.657    | 0.426     | 0.511    | 12.16       |
| CON + 3 BG g/kg | 88.4bc| 52.4bc | 1.69| 0.647    | 0.427     | 0.512    | 12.16       |
| CON + 4 BG g/kg | 90.2bc| 53.8bc | 1.68| 0.641    | 0.412     | 0.516    | 12.08       |
| CON + 5 BG g/kg | 85.8b | 51.8b  | 1.65| 0.655    | 0.428     | 0.505    | 12.12       |
| Pooled SEM  | 0.043  | <.001  | 0.687| 0.206    | 0.081     | 0.064    | 0.613       |
| p value     | 0.043  | <.001  |    |          |           |          |             |

| Exp. II    |        |        |     |          |           |          |             |
| Stocking density |        |        |     |          |           |          |             |
| LSD 0     | 77.7   | 44.3   | 1.75| 0.522    | 0.437     | 0.488    | 11.03       |
| LSD 4     | 80.4   | 46.5   | 1.73| 0.617    | 0.426     | 0.503    | 12.29       |
| MSD 0     | 77.2   | 43.2   | 1.79| 0.519    | 0.442     | 0.482    | 11.17       |
| MSD 4     | 79.3   | 44.5   | 1.76| 0.624    | 0.429     | 0.489    | 12.27       |
| Pooled SEM| 0.6    | 0.1    | 0.01| 0.006    | 0.009     | 0.008    | 0.07        |

| Stocking density |        |        |     |          |           |          |             |
| LSD 79.0bc | 45.4bc | 1.74  | 0.570| 0.432    | 0.496     | 11.66    |
| MSD 77.7b  | 43.9b  | 1.78  | 0.572| 0.436    | 0.486     | 11.72    |
| BG 77.5b   | 43.8b  | 1.77  | 0.521b| 0.440    | 0.485     | 11.10b   |
| BG 79.3c   | 45.5c  | 1.75  | 0.620c| 0.428    | 0.496     | 12.28c   |

| p value Stocking density |        |        |     |          |           |          |             |
| BG 0.014  | 0.037  | 0.185 | 0.347| 0.172    | 0.118     | 0.616    |
| BG 0.011  | 0.029  | 0.125 | <.001| 0.263    | 0.264     | <.001    |

| Stocking density × BG |        |        |     |          |           |          |             |
| 0.170     | 0.342  | 0.354 | 0.284| 0.429    | 0.312     | 0.395    |

| a,bMeans in the same column without the same superscript differ significantly ($p < .05$).
| cFrom Day 22 to 42, birds were either raised in a thermoneutral zone (22°C, Exp. I) or subjected to cyclic heat stress by exposing them to 33 ± 1°C for 10 h (from 08.00 to 18.00) and 22°C from 18.00 to 08.00 (Exp. II).

ADFI: average daily feed intake; ADG: average daily gain; FCR: feed conversion ratio; LSD: low stocking density (10 birds/m²), MSD: medium stocking density (14 birds/m²).
promoter in broilers exposed to thermoneutral conditions. In the present study, BG supplementation increased the N digestibility and AME values of the feed in the birds exposed to heat stress. This improvement may be due to the stimulation of saccharase, amylase and phosphatase activities (Seven et al. 2012).

In this way, the effects of the dietary BG supplementation on nutrient digestibility may appear more powerful under stress. Consequently, the BG efficiency may be related not only to the dose used but also to the study conditions (heat stress, overcrowding stress, or not).

Table 3. Effects of propolis (BG) supplementation and stocking density on gut morphology of broilers, Experiments I and II.

| Experiment | Relative weight (% of BW) | Jejunum morphology (μm) |
|------------|--------------------------|-------------------------|
|            | Crop | Gizzard | Liver | Small intestine | VH | CD | VH/CD |
| Exp. I Basal diet (CON) | 0.325 | 1.399 | 1.908 | 1.546 | 817<sup>b,c</sup> | 214 | 3.80<sup>b</sup> |
| CON + 1 BG g/kg | 0.317 | 1.396 | 1.906 | 1.624 | 821<sup>b,c</sup> | 215 | 3.83<sup>b</sup> |
| CON + 2 BG g/kg | 0.315 | 1.411 | 1.904 | 1.580 | 814<sup>c</sup> | 213 | 3.84<sup>b</sup> |
| CON + 3 BG g/kg | 0.322 | 1.384 | 1.893 | 1.537 | 828<sup>b</sup> | 215 | 3.85<sup>b</sup> |
| CON + 4 BG g/kg | 0.311 | 1.362 | 1.862 | 1.513 | 861<sup>d</sup> | 209 | 4.11<sup>d</sup> |
| CON + 5 BG g/kg | 0.324 | 1.417 | 1.905 | 1.582 | 859<sup>d</sup> | 211 | 4.06<sup>d</sup> |
| Pooled SEM | 0.068 | 0.132 | 0.366 | 0.667 | <.001 | 0.059 | <.001 |
| Orthogonal polynomial | Linear | 0.069 | 0.077 | 0.052 | 0.172 | <.001 | 0.073 | <.001 |
| Quadratic | 0.791 | 0.153 | 0.299 | 0.246 | <.001 | 0.119 | <.001 |

Exp. II Stocking densityBG (g/kg) | Lipid blood parameters (mg/dL) | Plasma immunoglobulin (log.)
| LSD 0 | 0.373 | 1.414 | 1.879 | 1.592 | 745 | 228 | 3.27 |
| LSD 4 | 0.350 | 1.385 | 1.904 | 1.557 | 739 | 226 | 3.27 |
| MSD 0 | 0.348 | 1.444 | 1.893 | 1.589 | 728 | 227 | 3.20 |
| MSD 4 | 0.366 | 1.415 | 1.887 | 1.581 | 730 | 229 | 3.19 |
| Pooled SEM | 0.057 | 0.018 | 0.012 | 0.016 | 3.2 | 3.8 | 0.06 |
| Orthogonal polynomial | Linear | 0.361 | 1.430 | 1.886 | 1.590 | 736 | 227 | 3.24 |
| Quadratic | 0.358 | 1.400 | 1.896 | 1.569 | 734 | 228 | 3.23 |

<sup>a,b,c</sup>Means in the same column without the same superscript differ significantly (p < .05).
<sup>d</sup>From Day 22–42, birds were either raised in a thermoneutral zone (22 °C, Exp. I) or subjected to cyclic heat stress by exposing them to 33 ± 1 °C for 10 h (from 08.00 to 18.00) and 22 °C from 18.00 to 08.00 (Exp. II).

VH: villus height; CD: crypt depth; VH/CD: villus height to crypt depth ratio; LSD: low stocking density (10 birds/m²); MSD: medium stocking density (14 birds/m²).

Table 4. Effects of propolis (BG) supplementation on blood parameters and plasma immunoglobulin, Experiment I.

| Treatments | TG | Tch | LDL | HDL | IgG | IgM |
|------------|----|-----|-----|-----|-----|-----|
| Basal diet (CON) | 98.3<sup>b</sup> | 184<sup>a</sup> | 74.5<sup>d</sup> | 92.9 | 4.03 | 0.99<sup>a</sup> |
| CON + 1 BG g/kg | 99.8<sup>b</sup> | 186<sup>b</sup> | 75.2<sup>a</sup> | 93.3 | 4.07 | 1.00<sup>b</sup> |
| CON + 2 BG g/kg | 97.0<sup>b</sup> | 183<sup>b</sup> | 72.7<sup>a</sup> | 91.7 | 4.17 | 1.01<sup>b</sup> |
| CON + 3 BG g/kg | 101.3<sup>c</sup> | 186<sup>b</sup> | 76.4<sup>a</sup> | 92.7 | 4.08 | 1.04<sup>b</sup> |
| CON + 4 BG g/kg | 95.6<sup>b,c</sup> | 174<sup>b,c</sup> | 67.3<sup>b</sup> | 88.9 | 4.09 | 1.16<sup>a</sup> |
| CON + 5 BG g/kg | 91.1<sup>c</sup> | 170<sup>c</sup> | 62.0<sup>b</sup> | 89.3 | 4.13 | 1.15<sup>a</sup> |
| Pooled SEM | 1.2 | 1.8 | 1.3 | 1.1 | 0.069 | 0.083 | 0.021 |
| Orthogonal polynomial | Linear | <.001 | <.001 | <.001 | 0.069 | 0.083 | 0.021 |
| Quadratic | 0.154 | 0.002 | 0.003 | 0.057 | 0.263 | 0.012 |

<sup>a</sup>Means in the same column without the same superscript differ significantly (p < .05).

Tg: triglycerides; Tch: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein.
Regarding the gut morphology, dietary BG supplementation did not have a significant effect on relative weights of the crop, gizzard, liver, and intestine, but increased villus height and villus height-to-crypt depth ratio of broilers reared under a thermoneutral condition. These findings were considered to improve growth performance, nutrient utilisation, and anti-inflammatory. It has been well documented that BG supplementation may stimulate the digestive and absorptive functions of broilers (Hosseini et al. 2016) and may be helpful in explaining the improvement in the performance observed in this study. Attia et al. (2014) suggested that higher villus height-to-crypt depth ratio increases digestive and absorptive capacities in the jejunum. Thus, the increased ratios obtained here can be attributed to the beneficial effect of BG supplementation in controlling proliferation of pathogenic bacteria and avoiding possible damage to the intestinal mucosa, which could result in reduced dimensions of the villus.

The results of the present study indicated that serum IgG concentrations in the birds fed BG supplementation reared under both normal and high temperatures were higher than those in control birds, suggesting that BG could modulate humoral immunity. Dietary BG supplementation activates the immune system in birds, increasing activity of macrophage and natural killer cells, and raising levels of cytokine (Sforcin 2007). The cytokines enhance B-lymphocytes activity, which would be able to produce immunoglobulins. Therefore, the increased levels of IgM and IgG in broilers-fed dietary BG are probably associated with the B-lymphocyte stimulation by cytokines (Hosseini et al. 2016). The levels of IgG in plasma were increased in the broilers stocked at MSD. This result may be related to reduce in immunological memory, thus leading to an increase in pathogen susceptibility, and might be a mechanism used by the immune system to respond to increasing density (Abudabos et al. 2013).

Table 5. Effects of propolis (BG) supplementation and stocking density on blood heterophil (H) and lymphocyte (L) concentrations, H:L ratio and immunity status, Experiment II.a

| Treatments             | Heterophils (× 10⁹/L) | Lymphocytes (× 10⁹/L) | H:L  | IgG  | IgM  |
|------------------------|-----------------------|-----------------------|------|------|------|
| Stocking density       |                       |                       |      |      |      |
| LSD                    | 41.2                  | 99.6                  | 0.41b| 936.84| 24.14|
| LSD                    | 44.8                  | 100.7                 | 0.45c| 968.54| 25.37|
| MSD                    | 39.2                  | 101.4                 | 0.38d| 944.09| 25.79|
| MSD                    | 42.1                  | 101.3                 | 0.41b| 978.74| 25.88|
| Pooled SEM             | 1.8                   | 1.9                   | 0.02 | 4.31 | 0.56 |
| Stocking density       |                       |                       |      |      |      |
| LSD                    | 43.0                  | 100.2                 | 0.43 | 952.69b| 24.76|
| MSD                    | 40.7                  | 101.4                 | 0.40 | 961.47c| 25.83|
| BG                     | 40.2                  | 100.5                 | 0.40 | 940.46b| 24.97|
| +                      | 43.5                  | 101.0                 | 0.43 | 973.64c| 25.63|
| p value                |                       |                       |      |      |      |
| Stocking density       | 0.092                 | 0.463                 | <0.001| <0.001| 0.135|
| BG                     | 0.106                 | 0.311                 | <0.001| <0.001| 0.285|
| Stocking density × BG  | 0.247                 | 0.752                 | <0.001| 0.064| 0.344|

a From Day 22 to 42, birds were subjected to cyclic heat stress by exposing them to 33 ± 1 °C for 10 h (from 08.00 to 18.00) and 22 °C from 18.00 to 08.00.

b-d Means in the same column without the same superscript differ significantly (p < 0.05).

LSD: low stocking density (10 birds/m²); MSD: medium stocking density (14 birds/m²).

874 S. CHEGINI ET AL.
change in H:L ratio and gradual decline with continuity of stress have been reported that reflect the involvement of highly dynamic immunological response with quick and long lasting effects (Attia et al. 2014). The higher H:L ratio observed in heat-stressed broilers-fed diets containing BG implies the positive influence of flavonoids on reducing stress in broilers (Seven et al. 2012).

Conclusions

The results indicated that addition of 4 g/kg of BG to the diet improved almost all zootechnical indices in broiler chickens. The dietary supplementation with BG (4 g/kg of feed) partially growth performance and nutrient digestibility in broilers reared under heat stress and overcrowding stress. According to these results, dietary use of BG as a beneficial additive may offer a nutritional strategy in broiler farming to overcome the deleterious effects of thermal or social stress.

Ethical approval

All procedures carried out in this experiment were reviewed and approved by the Animal Care and Use Committee of Lorestan University, Khorramabad, Iran.

Acknowledgements

The authors gratefully thank Dr. Morteza Rezaei (Animal Science Research Institute of Iran) for his helpful discussions and technical assistance.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was financially supported by the Vice Chancellor in Research and Technology of the Lorestan University.

ORCID

Ali Kiani http://orcid.org/0000-0003-4731-1546

References

Abudabos AM, Samara EM, Hussein EOS, Al-Ghadi MQ, Al-Atiyat RM. 2013. Impacts of stocking density on the performance and welfare of broiler chickens. Ital J Anim Sci. 12:e11–e71.

Acikgoz Z, Yucel B, Altan O. 2005. The effects of probopis supplementation on broiler performance and feed digestibility. Archiv Geflügelk. 69:117–122.

Akbarian A, Michiels J, Golian A, Buyse J, Wang Y, De Smet S. 2014. Gene expression of heat shock protein 70 and anti-oxidant enzymes, oxidative status, and meat oxidative stability of cyclically heat-challenged finishing broilers fed Origanum compactum and Curcuma xanthorrhiza essential oils. Poult Sci. 93:1930–1941.

Association of Official Analytical Chemists (AOAC) International. 2005. Official methods of analysis. 18th edn. Washington (DC): AOAC International.

Attia YA, Abd Al-Hamid AE, Ibrahim MS, Al-Harthi MA, Bovera F, El-Naggar A. 2014. Productive performance, biochemical and hematological traits of broiler chickens supplemented with propolis, bee pollen, and mannan oligosaccharides continuously or intermittently. Livest Sci. 164:87–95.

Barnett JL, Edge ME, Thomson L, Mackenzie M, Sansom G, Kite V. 2008. National animal welfare standards for the chicken meat industry, the standards. Australia: Australian Poultry CRC Pty Ltd.

Bartlett JR, Smith MO. 2003. Effects of different levels of zinc on the performance and immunocompetence of broilers under heat stress. Poult Sci. 82:1580–1588.

Cengiz Ö, Köksal BH, Tatlı O, Sevim Ö, Ahsan U, Üner AG, Ulutaş PA, Beyaz D, Büyüköyrik S, Yakan A, et al. 2015. Effect of dietary probiotic and high stocking density on the performance, carcass yield, gut microflora, and stress indicators of broilers. Poult Sci. 94:2395–2403.

Chegini S, Kiani A, Rokni H. 2018. Alleviation of thermal and overcrowding stress in finishing broilers by dietary propolis supplementation. Ital J Anim Sci. 17:377–385.

Fuliang HU, Hepburn HR, Xuan H, Chen M, Daya S, Radloff SE. 2005. Effects of propolis on blood glucose, blood lipid and free radicals in rats with diabetes mellitus. Pharmacol. Res. 51:147–152.

Estevez I. 2007. Density Allowances for Broilers: Where to set the limits? Poult Sci. 86:1265–1272.

Gomes AV, Quinteiro-Filho WM, Ribeiro A, Ferraz-de-Paula V, Pinheiro ML, Baskeville E, Akamine AT, Astolfi-Ferreira CS, Ferreira AJ, Palermo-Neto J. 2014. Overcrowding stress decreases macrophage activity and increases Salmonella enteritidis invasion in broiler chickens. Avian Pathol. 43:82–90.

Hosseini SM, Afshar M, Ahani S, Vakili-Azghandi M. 2015. Heat shock protein 70 mRNA expression and immune response of heat-stressed finishing broilers fed propolis (bee glue) supplementation. Arch Anim Breed. 58:407–413.

Hosseini SM, Vakili Azghandi M, Ahani S, Nourmohammadi R. 2016. Effect of bee pollen and propolis (bee glue) on growth performance and biomarkers of heat stress in broiler chickens reared under high ambient temperature. J Anim Feed Sci. 25:45–50.

Houshmand M, Azhar K, Zulkifli I, Bejo MH, Kamyab A. 2012. Effects of prebiotic, protein level, and stocking density on performance, immunity, and stress indicators of broilers. Poult Sci. 91:393–401.

Ichichi I, Hori H, Takashima Y, Adachi N, Kataoka R, Okihara K, Hashimoto K, Kojo S. 2009. The beneficial effect of...
propolis on fat accumulation and lipid metabolism in rats fed a high-fat diet. J Food Sci. 74:H127–H131.
Kolankaya D, Selmanoghlu G, Sorkun K, Salih B. 2002. Protective effects of Turkish propolis on alcohol induced serum lipid changes and liver injury in male rats. Food Chem. 78:213–217.
Lara LJ, Rostagno MH. 2013. Impact of heat stress on poultry production. Animals. 3:356–369.
National Research Council (NRC). 1994. Nutrient requirements of poultry. 9th rev. ed. Washington, DC: National Academy Press.
Ravindran V, Hew Li, Ravindran G, Bryden WL. 2005. Apparent ileal digestibility of amino acids in dietary ingredients for broiler chickens. Anim Sci. 81:85–97.
Seven I, Aksu T, Tatli Seven P. 2012. The effects of propolis and vitamin C supplemented feed on performance, nutrient utilization and carcass characteristics in broilers exposed to lead. Livest Sci. 148:10–15.
Sforcin JM. 2007. Propolis and the immune system: a review. J Ethnopharmacol. 113:1–14.
Short FJP, Gorton J, Wiseman J, Boorman KN. 1996. Determination of titanium oxide added as an inert marker in chicken digestibility studies. Anim Feed Sci Technol. 59:215–221.
Song J, Xiao K, Ke YL, Jiao LF, Hu CH, Diao QY, Shi B, Zou XT. 2014. Effect of a probiotic mixture on intestinal microflora, morphology, and barrier integrity of broilers subjected to heat stress. Poult Sci. 93:581–588.