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RESEARCH ARTICLE

Effects of *Hibiscus sabdariffa* Calyx Supplementation on Productive and Blood Parameters in Broiler Chickens Exposed to Intermittent Heat Stress

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Abstract

Objectives: To evaluate the effects of dietary supplementation with *Hibiscus sabdariffa* (Hs) calyces on productive performance, hematological parameters, plasma corticosterone and serum concentrations of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) in broiler chickens exposed to intermittent heat stress.

Materials and Methods: A total of 208 Cobb 500 broiler chickens were randomly allocated to four experimental groups: Control (thermoneutral conditions, no Hs), CHs (thermoneutral conditions, 2% Hs), Stre (heat stress for 5 consecutive hours per day, no Hs) and Stre/Hs (heat stress, 2% Hs). Two ambient temperature conditions (25 and 35°C) and dietary treatments were applied from day 28 to day 49 of age. Feed intake, water intake, body weight and feed conversion ratio were recorded throughout the experimental period. On day 49, blood samples were collected for leukocyte differential counts and determination of corticosterone, IL-6 and TNF-α concentrations.

Results: Broilers exposed to heat stress (Stre and Stre/Hs) exhibited reduced body weight and feed intake and increased feed conversion ratios, reflecting diminished productive efficiency compared with thermoneutral groups. Heat stress was associated with elevated circulating corticosterone and decreased proportions of lymphocytes and monocytes. Although, Hs supplementation did not improve productive performance, it significantly reduced serum IL-6 and TNF-α concentrations in both thermoneutral and heat-stressed birds, indicating a modulatory effect on the inflammatory response.

Conclusion: Dietary supplementation with 2% *H. sabdariffa* calyces mitigated the pro-inflammatory response induced by intermittent heat stress, as evidenced by reduced IL-6 and TNF-α concentrations, without significantly affecting productive performance in broiler chickens.

INTRODUCTION

Currently, poultry production particularly broiler production is increasingly constrained and disruptive climatic conditions, which are among the main limiting factors for production efficiency. In this context, elevated ambient temperatures represent the primary stressor, as they directly induce heat stress. This issue is especially critical in arid and tropical regions, where its impact on broiler production has been extensively documented over recent decades[1-3]. Numerous studies have demonstrated that broiler chickens acutely or chronically exposed to temperatures above 31°C exhibit reduced feed intake and body weight gain during the finishing phase, resulting in impaired feed conversion and increased mortality rates[4,5]. Moreover, heat stress disrupts hematological and serum by decreasing monocyte, lymphocyte and eosinophil counts, thereby indirectly increasing the heterophil to lymphocyte ratio and elevating corticosterone and heat shock protein concentrations[6,7]. At the immunological level, exposure to temperatures exceeding 31°C is associated with increased concentrations of pro-inflammatory cytokines, including interleukins 1-β, 2 and 6, tumor necrosis factor alpha (TNF-α) and interferon alpha (IFN-α), compared with birds maintained under thermoneutral conditions[8-10]. To mitigate the adverse effects of stress on poultry performance and health, several strategies have been implemented, including improved facility design, environmental temperature control systems (e.g., extractors and air coolers), reduced stocking density, dietary additives, electrolyte supplementation in drinking water and genetic selection. However, these approaches are costly and often fail to achieve the expected outcomes[11,13].

In recent years, calyces of *Hibiscus sabdariffa* (Hs) have been incorporated into poultry diets as a feed additive, primarily due to their antioxidant properties[14,15]. The use of Hs in broiler production has yielded promising results, as phenolic extracts from Hs have been evaluated in various heat stress models and shown to improve productive parameters such as final body weight, total feed intake and feed conversion ratio. Additionally, *Hibiscus sabdariffa* supplementation has been associated with reduced blood triglyceride concentrations, maintenance or improvement of hematological indices and modulation of humoral immune responses and antioxidant enzyme activity[16,18]. Therefore, the objective of the present study was to evaluate the effects of dietary supplementation with *Hibiscus sabdariffa* calyces on hematological parameters and pro-inflammatory cytokine concentrations in broiler chickens subjected to moderate intermittent heat stress during the final phase of the production cycle.

MATERIALS AND METHODS

Housing conditions: The study was conducted at the facilities of the University Center for Biological and Agricultural Sciences (CUCBA) of the University of Guadalajara (UDG), located in Zapopan, Jalisco, Mexico (1,567 m above sea level). The region has a temperate semi-dry climate, with a mean annual temperature of 23.5°C and average annual precipitation of 906.1 mm, with rainy season from June to October. The experiment was carried out during the temperate semi-humid season (November-January 2024-2025). The experimental broiler house was naturally ventilated, oriented east-west and measured 12×12 m. The poultry population consisted of 208 male broiler chickens of the Cobb 500 genetic line.

Experimental management: One-day with an average initial body weight of 41.5 g, were vaccinated at the hatchery against Marek's disease and a vaccination program against Gumboro diseases was implemented. On day 21, birds were randomly allocated to four experimental groups (n = 52 per group). Each groups was subdivided into four replicates of 13 birds and housed in pens measuring 80×165 cm at a stocking density of 10 birds/m^2. Each pen was equipped with a hopper feeder (10 kg capacity) and a drinker (5 L capacity). The experimental groups as follows: Control, maintained under thermoneutral conditions and fed a diet without Hs; CHs, maintained under thermoneutral conditions and fed a diet supplemented with 2% Hs; Stre, exposed to heat stress for 5 consecutive hours per day and fed a diet without Hs and Stre/Hs, exposed to heat stress and fed a diet supplemented with Hs. Four gas heaters were evenly distributed across the pens assigned to the heat stress condition. A double layer of plastic was installed between heated and thermoneutral areas to provide thermal insulation. The temperature in heat-stressed pens was maintained at 35°C for 5 consecutive hours daily (11:30-16:30 hrs) from day 28 to 49 of age. Temperature and Relative Humidity (RH) we using six thermohygrometers (Model RC-51H, Elitech) randomly positioned at floor level within the pens, with measurements recorded at 1 min intervals (Fig. 1) water temperature was continuously monitored using a mercury thermometer. Birds were weighed using a digital scale (Model EQB-100/200, Torrey). Animal management adhered to the guidelines of the Cobb Broiler Management Guide[19], the Manual of Good Livestock Practices in Broiler Chickens issued by the Secretaría de Agricultura y Desarrollo Rural[20] and the Terrestrial Animal Health Code of the World Organization for Animal Health[21] and was approved by the Ethics Committee of the University Center. The diets met the National Research Council nutritional requirements[20].

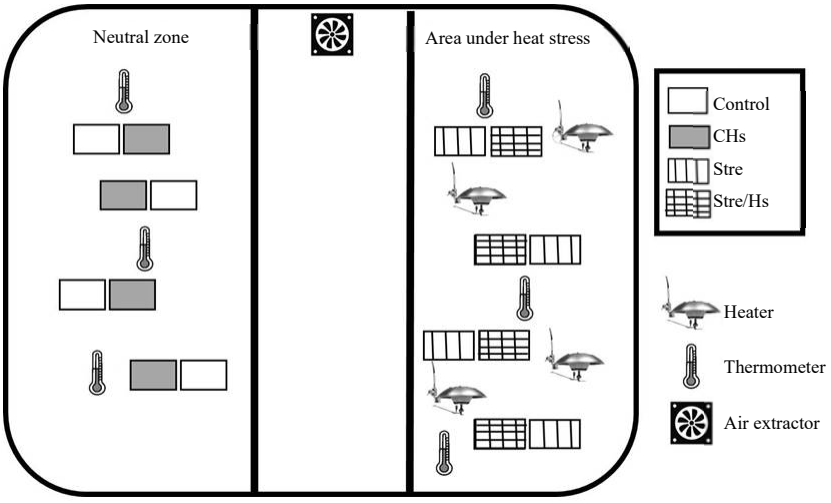

Fig. 1: Distribution of treatments and their repetitions in different pens of the experimental broiler house.

Table 1: Diet composition and ingredients by production stage

Ingredient	Initiation (%)	Finalization (%)
Maize	48.1800	59.28
Soybean paste	35.9200	26.34
Soybean oil	6.0000	6.00
mix*	8.0000	7.00
Calcium carbonate	1.2100	1.10
Dicalcium phosphate	0.6800	0.28
Crude protein	20.7450	18.02
Ether extract	4.3086	4.20
Raw fiber	2.1882	3.50
Ashes	4.6900	4.89
Nitrogen-free elements	60.2467	58.89
Metabolizable energy (Mcal/kg)	3157.2200	3395.00
Dry matter	88.1785	89.50
Humidity	9.8215	10.50

*The premix provides the following (per kg of complete diet). Vitamins A: 367500 IU, D3: 133500 IU, E: 1920 mg, K3: 84.42, B: 150 mg, B2: 150 mg, B3: 500 mg, B6: 177.5 mg, B12: 0.8 mg, PP: 600 mg, Folic acid: 24.5 mg, Biotin: 27 mg, Choline: 5767.5 mg, Fe: 2667 mg, Cu: 333.75 mg, Mn: 3334.06, Co: 203 mg, Zn: 2334.38 mg, Ca: 100.75 mg, Se: 10 mg, P: 65446.46 mg, DL Methionine: 36667.5 mg, Ethoxyquin: 200.02 mg, Flavophospholipol: 50 mg, Fish meal: 30 g, Wheat bran: 1800 g.

The feeding program consisted of a starter diet (days 1-21) and a finisher diet (days 22-49), provided by a commercial feed manufacturer (Nutrimentos Ramírez S.A. de C.V.) (Table 1).

Polyphenol quantification and diet supplementation: To determine the polyphenol content of the experimental diet, the total polyphenol concentration of the Hs calyces was first quantified. Briefly, 20 g of calyces were analyzed using the Folin-Ciocalteu method[23]. Samples were mixed with 200 mL of ethanol and filtered to obtain the liquid fraction, which was subsequently concentrated by rotary evaporation (Büchi, model B-100). Total polyphenol content was quantified in triplicate by UV-Vis spectrophotometry at 760 nm. Based on these measurements, the Hs treatment consisted of supplementing the basal diet with 2% ground Hs calyces (*Hibiscus sabdariffa* var. *nigeriana*), milled to a particle size of 1 mm to ensure homogeneous incorporation.

Data collection: Throughout the experimental period, feed and water intake were recorded daily and individual body weight was measured weekly. Feed conversion ratio was calculated as the ratio of total feed intake to total body weight gain. Mortality was expressed as the number of birds that died per treatment group during the experimental period. On day 49, twelve birds were randomly selected from each group for blood sampling (3 mL per bird) via the occipital sinus. Samples were collected into sterile tubes and centrifuged at 3,500 rpm for 4 min to obtain plasma and serum. Plasma was stored at -70°C until analysis. A blood smear was prepared from each sample, fixed in methanol for 30 sec and stained using the Romanowsky technique for differential leukocyte counting[24]. Serum concentrations of corticosterone, IL-6 and TNF-α were measured using commercial ELISA kits: Corticosterone ELISA Kit (Enzo Life Sciences®), Chicken IL-6 ELISA Kit (Invitrogen) and Chicken TNF-α ELISA Kit (MyBiosource®), according to the manufacturers' instructions. All assays were performed in triplicate and absorbance was read using a microplate reader (Multiskan Go, Thermo Fisher Scientific®) at 405 nm for corticosterone and 450 nm for both cytokines. Concentrations were calculated using Ascent Software, version 2.6 (Thermo LabSystems®).

Statistical analysis: Data were analyzed using two-way analysis of variance (ANOVA), followed by Tukey's post hoc test, to detect differences among groups for normally distributed variables at a significance level of $p \leq 0.05$. Variables that did not meet normality assumptions were analyzed using the Kruskal–Wallis H test. All analyses were conducted using SPSS software (version 21.0).

RESULTS

The mean absorbance obtained using the Folin-Ciocalteu assay was 1.073, corresponding to a gallic acid concentration of 4.989 µg/mL. Accordingly, the total polyphenol content of *H. sabdariffa* var. *nigeriana* calyces was 1,746.22 mg Gallic Acid Equivalents (GAE) per 100 g of sample.

Throughout the heat stress exposure period, birds exhibited normal growth and development and no clinical signs of disease were observed. Moreover, no rejection of the Hs-supplemented diets was detected. The thermoneutral environment had a mean temperature of 23.33°C with a relative humidity of 53.33%, whereas the heat stress environment averaged 35°C during the scheduled exposure period. Mortality across treatments was minimal (0.25-0.5%) and therefore was not subjected to statistical analysis.

Mean feed consumption for each group during the experimental weeks (day 28, week 1, day 42, week 2 and day 49, week 3) is presented in Table 2. Statistical analysis revealed significant differences among groups ($F_{[2]} = 22.083$, $p<0.001$), with lower feed intake in the Stre and Stre/Hs groups compared with the Control and CHs groups at the end of the first and second weeks ($p<0.05$). During the third week, feed consumption in the Stre/Hs group was lower than in all other groups and intake in the Stre group was also significantly lower than in the thermoneutral groups ($p<0.05$). Analysis of cumulative feed intake over the entire experimental period likewise showed significant differences among groups ($F_{[3]} = 297.52$, $p<0.001$), with lower intake in the Stre and Stre/Hs groups compared with the Control and CHs groups ($p<0.05$) (Fig. 2). Analysis of water intake indicated that at the end of the first week, the Stre group had the lowest mean consumption. In the second week, the Stre, CHs and Stre/Hs groups also exhibited lower water intake than the Control group. By the third week, only the Stre group maintained significantly lower water consumption ($p<0.05$, Table 3). No significant differences were observed in total water intake among groups over the experimental period.

Statistical analysis of body weight revealed significant differences among groups ($F_{[3]} = 42.841$, $p<0.001$). Weekly comparisons showed no differences during the first week of treatment ($p>0.05$). In the second week, the CHs group

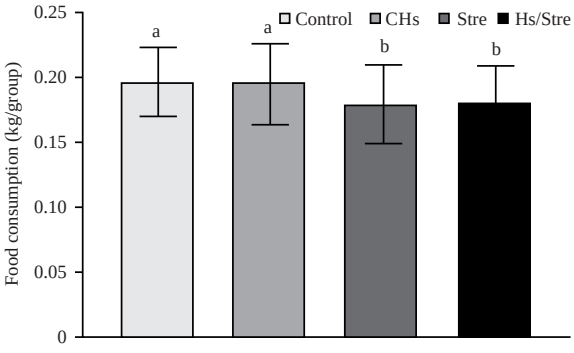

Fig. 2: Average feed intake throughout the experimental period.
Lower feed intake was observed in the Stre and Stre/Hs groups compared with the Control and CHs groups. Bars represent the mean±standard deviation; different letters indicate $p<0.05$.

Table 2: Feed consumption (kg) of the chickens during the experimental period

	Experimental group			
	Control	CHs	Stre	Stre/Hs
Week 1	0.1696±0.0099[a]	0.1653±0.0122[a]	0.1543±0.0114[b]	0.1559±0.0153[b]
Week 2	0.1964±0.0117[a]	0.1962±0.0166[a]	0.1821±0.0254[b]	0.1845±0.0211[b]
Week 3	0.2252±0.0119[a]	0.2319±0.0112[a]	0.2120±0.0166[c]	0.2043±0.0242[d]

Values are expressed as mean±standard deviation. Different letters represent statistical differences ($p<0.05$), the comparison is established within the same week of treatment.

Table 3: Water consumption (L) of the chickens during the experimental period

	Experimental group			
	Control	CHs	Stre	Stre/Hs
Week 1	0.4437±0.0328[a]	0.4305±0.0348[a]	0.4129±0.0489[b]	0.4364±0.0626[c]
Week 2	0.4876±0.0867[a]	0.4560±0.0815[b]	0.4326±0.0906[c]	0.4726±0.0816[d]
Week 3	0.4835±0.0808[a]	0.4903±0.0679[a]	0.4585±0.0804[b]	0.4777±0.0996[a]

Values are expressed as mean±standard deviation. Different letters represent statistical differences ($p<0.05$), the comparison is established within the same week of treatment.

Table 4: Body weight (kg) of chickens across experimental groups during the study period

	Experimental group			
	Control	CHs	Stre	Stre/Hs
Week 1	1.87±0.23[a]	1.88±0.23[a]	1.85±0.23[a]	1.89±0.18[a]
Week 2	2.54±0.34[ab]	2.56±0.29[a]	2.48±0.31[b]	2.52±0.23[ab]
Week 3	3.37±0.32[a]	3.28±0.35[b]	3.08±0.41[c]	3.01±0.22[c]

Values are expressed as mean±standard deviation. Different letters represent statistical differences (p<0.05), the comparison is established within the same week of treatment.

Table 5: Feed conversion ratio of chickens throughout the experimental period

	Experimental group			
	Control	CHs	Stre	Stre/Hs
Week 1	1.94±0.21[a]	2.24±0.55[b]	2.25±0.76[c]	2.18±0.51[c]
Week 2	1.84±0.21[a]	1.93±0.43[b]	2.01±0.73[c]	1.97±0.27[c]
Week 3	1.80±0.48[a]	2.04±0.32[b]	2.36±0.79[c]	2.42±0.49[c]

Values are expressed as mean±standard deviation. Different letters represent statistical differences (p < 0.05), the comparison is established within the same week of treatment.

Table 6: Effect of Hibiscus sabdariffa calyx supplementation on hematological parameters in broiler chickens

	Experimental group			
	Control	CHs	Stre	Stre/Hs
Heterophils (%)	50.75±7.97[a]	59.75±4.33[a]	51.82±4.21[a]	49.11±3.94
Lymphocytes (%)	8.90±5.45[a]	24.83±5.82[ab]	37.25±4.48[b]	31.67±6.15[ab]
Monocytes (%)	0.42±0.23[a]	0.91±0.50[a]	4.73±1.35[b]	4.92±1.03[b]
Eosinophils (%)	0.08±0.083[a]	0.33±0.23[a]	0.75±0.22[a]	0.60±0.30[a]
Basophils (%)	N/A	N/A	1.00±1.0[a]	1.00±1.0[a]
H/L ratio	1.33±0.55[a]	1.61±1.27[a]	1.44±0.23[a]	1.33±0.24[a]

Values are expressed as mean±standard deviation. Different letters represent statistical differences (p<0.05), the comparison is established within the same week of treatment. N/A: not applicable.

exhibited higher body weight than the Stre group. By the third week, the CHs, Stre and Stre/Hs groups all had lower body weights than the Control group. Pairwise comparisons indicated no differences between the Control and CHs groups, whereas the Stre group had the lowest body weight compared with the Control, CHs and Stre/Hs groups ($p<0.05$). The Stre/Hs group had a significantly lower body weight than the Control and CHs groups but a higher body weight than the Stre group ($p<0.05$, Table 4).

Analysis of feed conversion ratio (FCR) demonstrated that the heat-stressed groups (Stre and Stre/Hs) had significantly higher FCR values than the Control and CHs groups, indicating reduced productive efficiency ($p<0.05$). Additionally, the CHs group exhibited a higher FCR than the Control group ($p<0.05$) (Table 5).

Differential leukocyte counts were analyzed using the Kruskal-Wallis H test. A significant difference in monocyte percentages was observed among experimental groups [$\chi^2_{(3)} = 18.609$, $p<0.01$]. *Post hoc* comparisons revealed differences between the Control group and the Stre and Stre/Hs groups, as well as between the CHs group and the Stre and Stre/Hs groups; no differences were observed between the Control and CHs groups. Lymphocyte percentages also differed among groups [$\chi^2_{(3)} = 10.193$, $p<0.05$], with *post hoc* analysis indicating differences between the Stre and Control groups, whereas the Stre group did not differ from the other groups. In contrast, no significant differences were observed for heterophils [$\chi^2_{(3)} = 3.477$, $p>0.05$], eosinophils [$\chi^2_{(3)} = 7.563$; $p>0.05$], basophils, or the heterophil-to-lymphocyte ratio [$\chi^2_{(3)} = 1.455$, $p>0.05$] among experimental groups (Table 6).

Analysis of corticosterone concentrations showed that the Stre and Stre/Hs groups had significantly higher levels than the non-stressed groups (Control and CHs) ($p<0.001$). Conversely, analysis of IL-6 and TNF-α concentrations demonstrated that these cytokines were significantly lower in the Hs-supplemented groups (CHs and Stre/Hs) than in the Stre and Control groups ($p<0.001$) (Table 7).

DISCUSSION

Analysis of productive parameters demonstrated that exposure to heat stress resulted in reduced feed intake and diminished weight gain throughout the experimental period, leading to an increased feed conversion ratio and, consequently, reduced feed efficiency. Although water intake exhibited temporal variation among groups, no consistent

Table 7: Serum corticosterone, TNF-α and IL-6 concentrations (pg/mL) per treatment at day 49 of age

	Experimental group			
	Control	CHs	Stre	Stre/Hs
Corticosterone	106.220±51.98[b]	98.070±24.87[b]	154.080±34.56[a]	167.620±39.83[a]
TNF-α	39.991±0.08[b]	39.601±0.17[a]	40.017±0.20[b]	39.619±0.10[a]
IL-6	73.666±1.86[b]	64.833±4.07[a]	73.416±5.52[b]	65.250±2.46[a]

Values are expressed as mean±standard deviation. Different letters represent statistical differences ($p<0.05$), the comparison is established within the same week of treatment.

differences in cumulative water consumption were observed. Dietary supplementation with Hs did not adversely affect productive performance. Regarding physiological responses, heat stress induced a marked increase in circulating corticosterone concentrations regardless of dietary treatment. In contrast, birds receiving Hs supplementation exhibited significantly lower concentrations of the pro-inflammatory cytokines IL-6 and TNF-α, as well as greater stability in leukocyte profiles in response to the stressor. Consistent with these findings, previous studies in heat-stressed chickens have reported reduced feed intake, altered water consumption patterns, decreased weight gain and impaired feed efficiency as adaptive responses aimed at limiting endogenous heat production associated with nutrient metabolism[25,26].

It is also important to note that reduced feed consumption is directly associated with other performance parameters in broiler chickens[26]. This relationship was evident in the present study, in which birds consumed less feed and, consequently, less water. Alternatively, this pattern may have been influenced by environmental factors such as relative humidity, given that heat loss is dependent on the thermal gradient between the animal and its environment[27]. Relative humidity constrains evaporative heat loss primarily through respiratory surfaces and to a lesser extent, through non-feathered body regions, potentially impairing thermoregulation in heat-stressed birds[28,29].

Therefore, the observed production responses can be interpreted in light of previous studies demonstrating that heat stress induces oxidative stress, which compromises digestion and nutrient absorption and alters metabolism, ultimately reducing productive performance in birds exposed to this stressor. Additionally, other investigations have shown that administration of Hs calyx extracts can reduce intestinal lipid digestion and absorption, leading to lower circulating concentrations of cholesterol and triglycerides[14]. This effect may be reflected during the finishing stage, resulting in lighter and leaner birds compared with those fed diets without Hs[30]. However, these findings contrast with those reported by Al-Nasrawi[16], who observed increased feed intake and weight gain during the early growth period (first six weeks) of broiler chickens supplemented with Hs calyces under standard production conditions.

Previous studies have documented that exposure to heat stress decreases circulating lymphocyte and monocyte counts while increasing heterophil numbers, thereby elevating the heterophil-to-lymphocyte (H/L) ratio[31,32]. This pattern is widely regarded as indicative of a low-grade chronic inflammatory state[33]. In the present study, partially similar responses were observed: lymphocyte and monocyte percentages decreased in the heat-stressed group, whereas heterophil percentages and the H/L ratio did not change significantly. In contrast, leukocyte profiles in the Hs-supplemented groups tended to remain more stable. The reduction in lymphocyte counts under heat stress may be attributed to elevated circulating corticosterone concentrations, which exert lympholytic effects during chronic stress and inflammation. Accordingly, these findings suggest that Hs supplementation may attenuate, rather than completely prevent, the leukocyte response to heat stress. This interpretation is supported by the observation that lymphocyte and monocyte reductions were less pronounced in the Hs-supplemented groups.

Corticosterone is a well-established indicator of chronic stress intensity and its circulating concentration increases in response to various stressors, including elevated ambient temperatures, as reported by Ramiah *et al.*[8]. Beckford *et al.*[6] reported a sixfold increase over baseline corticosterone concentrations in broiler chickens exposed to heat stress at comparable temperatures but for longer durations and under caged housing conditions. Another study reported values ranging from 160-170 pg/mL, which are comparable to those observed in the heat-stressed groups in the present study[34].

Regarding pro-inflammatory cytokines, tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) are key mediators of the inflammatory response, produced primarily by mononuclear phagocytes. These cells initiate immune signaling, phagocytic activity and the upregulation of major histocompatibility complex receptors in other blood cells, such as heterophils and monocytes[35]. Several cytokines, including IL-1β, IL-6, TNF-α and IFN-α, have been reported to increase significantly in serum in response to heat stress[36,37]. In the present study, serum concentrations of both TNF-α and IL-6 were significantly lower in the Hs-supplemented groups, indicating that Hs supplementation modulates the inflammatory response associated with chronic heat stress.

CONCLUSION

Based on our estimations, supplementation with 2% calyces of the Nigerian variety of *Hibiscus sabdariffa* provided approximately 100 mg of polyphenols during the finishing stage. The present findings demonstrate that intermittent heat stress during this phase induces pronounced physiological and immunological alterations in broiler chickens, including elevated corticosterone concentrations, changes in leukocyte profiles and increased levels of pro-inflammatory cytokines. These responses are indicative of activation of a chronic stress response and a low-grade inflammatory state, which compromise physiological homeostasis.

Although, dietary supplementation with *H. sabdariffa* calyces did not counteract the negative effects of heat stress on productive performance, it exerted a clear modulatory effect on the inflammatory response, as evidenced by significantly reduced circulating concentrations of IL-6 and TNF-α in supplemented birds under both thermoneutral and heat stress conditions. These results suggest that *H. sabdariffa* acts primarily at the immunophysiological level by attenuating stress-associated inflammation rather than by directly enhancing productive parameters.

Collectively, these findings support the potential use of *H. sabdariffa* calyces as a functional dietary supplement to mitigate the inflammatory consequences of chronic heat stress in broiler chickens, thereby improving physiological resilience under adverse environmental conditions. Further research is warranted to elucidate the underlying molecular mechanisms and to determine whether longer supplementation periods or alternative inclusion levels may also influence productive performance.

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