Acta Scientiarum
http://periodicos.uem.br/ojs
ISSN on-line: 1807-8672
Doi: 10.4025/actascianimsci.v47i1.71260



# Evaluating interventions on broiler chicken health using blood indices and graphical representation

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**ABSTRACT.** Heat stress significantly affects the physiological and performance aspects of broiler chickens, leading to reduced growth, feed intake, and feed efficiency. This research investigates the impact of various dietary additives and supplements on blood serum components in heat-stressed Ross 308 broiler chickens. A completely randomized design with eight treatment groups, each with four replications and 12 birds per replication, was used. Significant differences were found in blood parameters such as glucose, total protein, albumin, uric acid, and AST, as well as in heterophil percentage, lymphocyte count, heterophil to lymphocyte ratio, and bronchitis antibody titers ($p < 0.05$). Dietary interventions also significantly altered the *E. coli* population in the cecum ($p < 0.05$). Diets enriched with coenzyme $Q_{10}$ and vitamin C notably reduced *E. coli* populations under heat-stress. However, no significant differences were observed in tibia characteristics ($p > 0.05$). These results demonstrate the effectiveness of feed additives in mitigating heat stress effects in broiler chickens. We recommend incorporating vitamin C, coenzyme $Q_{10}$, and Eubiotic supplements to enhance the well-being and performance of broiler chickens under heat stress conditions.

**Keywords:** immunity; heat stress; feed additive; coenzyme $Q_{10}$; eubiotic.

Received on February 12, 2024.
Accepted on June 25, 2024.

## Introduction

Birds, including broiler chickens, are highly susceptible to a plethora of stressors, with environmental heat emerging as a pivotal and formidable factor. This is especially pertinent in tropical regions where the deleterious effects of heat stress on poultry performance are accentuated (Nawab et al., 2018). Heat stress, stemming from elevated environmental temperatures, gives rise to a cascade of adverse consequences, including reduced feed intake, growth inhibition, compromised amino acid digestibility, alterations in carcass composition, and an overall decline in performance, culminating in significant economic losses within the poultry industry (Khan et al., 2012; Talebi, Dolatkhah, & Joyani, 2022). While heat stress is commonly seasonal in temperate regions during the summer, it assumes a chronic and persistent character in tropical countries (Kingsolver, Diamond, & Buckley, 2013). The repercussions of high ambient temperatures extend beyond growth inhibition, affecting breast muscle volume in commercial broiler chickens, thereby inducing acid-base imbalances and heightened panting, potentially precipitating respiratory alkalosis (Borges, Silva, Majorka, Hooge, & Cummings, 2004).

The physiological repercussions of heat stress are multifaceted and encompass a spectrum of effects such as electrolyte imbalances, hormonal disruptions, diminished metabolic rates, lipid peroxidation, immune system suppression, and perturbations in gut microbial equilibrium (He et al., 2018). During heat stress, elevated catecholamine secretion from the adrenal gland's central region incites increased heart rate, subsequently stimulating the brain's brachial plexus nuclei, leading to escalated respiratory rates (Johnson, 2019). Furthermore, heat stress engenders a decline in packed cell volume (PCV) and hemoglobin (Hb) concentrations in the blood of broiler chickens, instigating conditions of hypoxia (Ayo & Ogbuagu, 2021).

Studies have underscored the adverse implications of heat stress on intestinal development, the balance of microbial populations within the digestive tract, and the avian immune system (Ahmad et al., 2022). Damage to the intestinal mucus compromises its functionality, potentially facilitating the proliferation of pathogenic microbes within the gut. Notably, heat stress disrupts the equilibrium of intestinal microorganisms, with detrimental consequences such as increased populations of pathogenic microorganisms and a decline in beneficial bacterial species. For instance, researchers demonstrated that heat

stress reduces the abundance of beneficial intestinal bacteria, including *Lactobacillus* and *Bifidobacterium*, while promoting the proliferation of coliform and clostridium bacteria (Song et al., 2014).

The notion of well-being encompasses a holistic evaluation of the health, quality of life, and overall welfare of living organisms. In the context of welfare assessment and validation, an array of hematological parameters and markers assumes significance as vital indicators of physiological health and welfare. These parameters encompass hemoglobin and hematocrit levels, a comprehensive blood count (CBC), blood glucose concentrations, lipid profiles, liver function assessments, kidney function evaluations, markers of inflammation, and the comprehensive metabolic panel (CMP). Additionally, thyroid function tests and blood coagulation profiles constitute integral components of this multifaceted evaluation (Righi et al., 2019).

In light of the considerable impact of heat stress on broiler production, various nutritional strategies have been proposed to mitigate its adverse effects. These strategies encompass the use of anionic-cationic salts, dietary protein reduction while maintaining amino acid balance, early heat shock treatments, dietary restrictions, and supplementation with antioxidant vitamins and minerals such as selenium and zinc (Vandana et al., 2021). Recognizing the pivotal role of heat stress in broiler production, nutritional interventions emerge as a compelling avenue to alleviate its ramifications. This study aims to investigate the effects of specific feed additives and supplements on blood factors and the immune response of broilers exposed to heat stress. The objective is to provide valuable insights into addressing the challenges posed by elevated temperatures in poultry production, intending to improve overall well-being and performance.

## Material and methods

### Birds and experimental conditions

A total of 384 one-day-old male broiler chickens of the Ross 308 strain were utilized in this experiment. The broilers were randomly allocated to 8 experimental groups, employing a completely randomized design (CRD). Each experimental group consisted of 4 replicates, with 12 chicks per replication. Throughout the experiment, the birds were subjected to a light regime comprising 23 hours of light exposure and 1 hour of darkness per day. Rearing management procedures adhered to the recommendations specific to the Ross strain, encompassing measures related to light exposure, humidity, ventilation, and vaccination. Continuous monitoring of feed was conducted multiple times throughout the day, ensuring that the birds had unrestricted access to drinking water. Heat stress was administered daily for 8 hours, commencing at 10 am and concluding at 6 pm. During the heat stress period, the ambient temperature within the rearing hall was elevated to 37ºC, with relative humidity levels fluctuating between 50 and 60%. These conditions were implemented to simulate and assess the effects of heat stress on the broiler chickens and their responses to various dietary interventions.

### Basal diet and experimental treatments

The experimental treatments encompassed eight distinct dietary regimens, each designed to investigate the effects of various nutritional interventions under heat stress conditions. The experimental groups and their corresponding treatments were as follows:

$T_1$: Basal diet under normal temperature conditions.
$T_2$: Basal diet under heat stress conditions.
$T_3$: Basal diet under heat stress conditions with supplementation of vitamin E and organic selenium.
$T_4$: Basal diet under heat stress conditions with supplementation of vitamin C and $Q_{10}$.
$T_5$: Basal diet under heat stress conditions with eubiotic supplementation.
$T_6$: Basal diet under heat stress conditions with a 10% increase in methionine levels.
$T_7$: Basal diet under heat stress conditions with a 20% increase in methionine levels.
$T_8$: Basal diet under heat stress conditions using a traditional method.

### Basal diet formulation

The basal diet employed in this research was meticulously formulated to meet the nutritional requirements of broiler chickens at different growth stages, including starter, grower, and finisher phases, as prescribed by the National Research Council (NRC) guidelines for poultry nutrition (National Research Council [NRC], 2011). Formulation of the diets was executed utilizing the UFFDA software. The key components of the basal diet, including the proximate composition, are presented in Table 1.

**Table 1.** Ingredients and nutrients composition of the basal diet (%).

| Ingredients | 1-10 days (Starter) | 11-24 days (Grower) | 25-42 days (Finisher) |
|---|---|---|---|
| Corn grain | 54.32 | 58.52 | 62.64 |
| Soybean meal | 39.55 | 35.43 | 31.27 |
| L-Lysine | 0.15 | 0.10 | 0.11 |
| DL-Methionine | 0.31 | 0.26 | 0.13 |
| L-threonine | 0.09 | 0.05 | 0.04 |
| Dicalcium phosphate | 1.94 | 1.72 | 1.59 |
| Oyster shell | 1.02 | 0.94 | 0.90 |
| Salt | 0.33 | 0.28 | 0.23 |
| Sodium bicarbonate | 0.20 | 0.20 | 0.20 |
| Mineral premix | 0.25 | 0.25 | 0.25 |
| Vitamin premix | 0.25 | 0.25 | 0.25 |
| Soybean oil | 1.60 | 2.00 | 2.40 |
| Total | 100 | 100 | 100 |
| Calculated composition | | | |
| Metabolizable energy (kcal $kg^{-1}$) | 2835 | 2911 | 2985 |
| Crude protein (%) | 21.73 | 20.19 | 18.66 |
| Energy/Protein | 130.40 | 144.10 | 160.00 |
| Crude fiber (%) | 3.96 | 3.76 | 3.53 |
| Ether extract (%) | 4.02 | 4.53 | 5.04 |
| Calcium (%) | 0.92 | 0.87 | 0.81 |
| Available phosphorus (%) | 0.48 | 0.43 | 0.40 |
| Calcium/Phosphorus | 2.00 | 2.00 | 2.00 |
| Lysine (%) | 1.21 | 1.08 | 0.98 |
| Methionine (%) | 0.60 | 0.53 | 0.39 |
| Methionine + Cysteine (%) | 0.89 | 0.81 | 0.66 |
| Threonine (%) | 0.81 | 0.72 | 0.66 |
| Crude protein/Lysine | 17.96 | 18.69 | 18.86 |
| Methionine+Cysteine/Lysine | 0.74 | 0.75 | 0.78 |
| Threonine/Lysine | 0.67 | 0.66 | 0.66 |
| Sodium (%) | 0.20 | 0.18 | 0.16 |

[a]The vitamin-mineral premix was incorporated into the diet at the following concentrations per kilogram: vitamin A (all-trans-retinal) - 10,000 IU, cholecalciferol - 2,000 IU, vitamin $K_3$ - 3.0 mg, thiamin - 1.1 mg, riboflavin - 18.0 mg, niacin - 50 mg, D-calcium pantothenic acid - 24 mg, vitamin $B_6$ - 2.94 mg, biotin - 0.5 mg, choline chloride - 450 mg, vitamin $B_{12}$ - 0.02 mg, folic acid - 3.0 mg, manganese (as $MnSO_4 \cdot H_2O$) - 110 mg, iron (as $FeSO_4 \cdot 7H_2O$) - 60 mg, zinc (as ZnO) - 90 mg, copper (as $CuSO_4$) - 10 mg, iodine (as $Ca(IO_3)_2$) - 0.46 mg, and selenium (as $_{Na2SeO3}$) - 0.2 mg. [b]Various nutritional components, such as dry matter (analyzed using method 934.01), crude protein (analyzed using method 954.01), ether extract (analyzed using method 920.39), ash (analyzed using method 942.05), calcium (analyzed using method 968.08), and phosphorus (analyzed using method 965.17), were quantified following the guidelines outlined in the AOAC (Feldsine, Abeyta, & Andrews, 2002). Additionally, gross energy content was assessed using an Adiabatic Bomb Calorimeter (Gallenkamp Autobomb, Leicestershire, UK). Neutral detergent fiber and acid detergent fiber levels were determined following the procedures detailed by Van Soest et al. with the utilization of sodium sulfite in the analysis (Van Soest, Robertson, & Lewis, 1991).

## Dietary additives

The experimental diets were enriched with specific dietary additives to investigate their influence on the response of broiler chickens to heat stress. The dietary supplements included:

Vitamin E (alpha-tocopherol acetate) at a concentration of 50 mg $kg^{-1}$ of the diet.

Organic selenium (Sel-Plex®) at a concentration of 0.3 ppm.

Vitamin C at a concentration of 300 mg $kg^{-1}$ of the diet.

Coenzyme $Q_{10}$ ($Q_{10}$) at a concentration of 40 mg $kg^{-1}$ of the ration.

An eubiotic product provided by Behinparvar Company, containing probiotics, prebiotics, and phytobiotics.

The incorporation of ice and non-alcoholic beer in the drinking water, administered at a rate of 2 kg per 1000 L of water.

These dietary additives were administered as part of the experimental treatments to assess their impact on blood factors and the immune responses of broiler chickens subjected to heat stress.

## Blood parameters

After the 42-day experimental period, two randomly selected broilers from each replicate pen were chosen for blood sample collection. These blood samples were subsequently transported to the laboratory for the quantification of various blood parameters. The assessed parameters included glucose, cholesterol, triglyceride, high-density lipoprotein (HDL), total protein, albumin, uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP).

The evaluation of blood serum parameters was executed utilizing Pars Azmoun kits and an autoanalyzer model (RA1000). This analytical approach provided accurate and consistent results for the quantification of the aforementioned blood parameters.

To assess changes in the ratio of heterophils to lymphocytes, a blood sample from the experimental birds was processed and stained according to the May-Giemsa-Grunwald staining method. Subsequently, the ratio of heterophils to lymphocytes was determined by manually counting 100 white blood cells, in line with the methodology described by (Li et al., 2021). This allowed for a detailed examination of the heterophil-to-lymphocyte ratio as an indicator of the birds' immune response to the experimental treatments.

## Serological analysis

During the rearing period, specifically at the ages of 21 and 42 days, blood samples were collected from two randomly selected birds in each pen. These blood samples were utilized for the determination of antibody titers against infectious bronchitis and Gumboro disease, employing a specific symbiotic Enzyme-Linked Immunosorbent Assay (Elisa) kit designed for this purpose.

To assess the agglutination response to Sheep Red Blood Cells (SRBC), a coordinated hemagglutination assay was conducted. Serum samples were initially incubated at 56°C for 30 min. to inactivate the complement system. A round-bottomed 96-well microplate was prepared, with each well containing 50 microliters of Phosphate-Buffered Saline (PBS) supplemented with 0.05% Bovine Serum Albumin (BSA). Subsequently, 50 µl of the serum samples were added to the wells, and a serial two-fold dilution was performed within the wells from columns 2 to 12. The first column, which contained only PBS, served as the blank control. Following this, 50 µl of a 1% SRBC suspension in PBS was added to all wells, resulting in a final volume of 100 µl in each well. The microplates were agitated for 1 min. and then incubated for 24 hours at 37°C to determine the agglutination titers. A positive result was recorded when at least 50% agglutination of SRBC was observed.

To quantify anti-SRBC $Ig_G$ and $Ig_M$ antibodies, serum samples underwent treatment with 0.2 M 2-mercaptoethanol (2-ME) for 30 min. at 37°C. This treatment effectively inactivated $Ig_M$, and any hemagglutination observed after the 2-ME treatment was primarily attributed to the presence of IgG antibodies. The distinction between the total antibody titers and $Ig_G$ titers was used to calculate the $Ig_M$ titer, following the approach described by (Alizadeh et al., 2021; Vilela et al., 2021). This serological analysis allowed for the assessment of the birds' immune response to infectious bronchitis and Gumboro disease, as well as their specific $Ig_G$ and $Ig_M$ antibody levels in response to SRBC.

## Intestinal microbial population

To assess the composition of the intestinal microbial population within the cecum, a subset of three birds was randomly selected from each pen for this analysis. The digestive system of each bird was carefully extracted, and approximately one gram of cecal content was collected for further examination.

Cultivation media consisting of Brain Heart Infusion (BHI), Man Rogosa Sharpe (MRS), and MacConkey Agar (MAC) were employed for the quantification of various bacterial populations within the cecal samples. These media allowed for the determination of the total aerobic bacterial count, lactobacillus count, and coliform count, respectively.

The collected cecal samples underwent dilution in the experiment, with dilution factors of $10^{-6}$, $10^{-7}$, and $10^{-8}$ utilized. Each dilution was then applied to the respective cultivation media.

The prepared culture media, seeded with the diluted cecal samples, were incubated for 24 hours. Following incubation, colonies that fell within the range of 30 to 300 were enumerated for each dilution.

To determine the total number of bacteria present in one gram of the cecal sample, the number of colonies observed was multiplied by the corresponding dilution factor. This calculation method was consistent with the approach outlined by (Li, Wang, Liu, & Guo, 2018). The findings from this analysis provided valuable insights into the microbial composition within the cecal region of the birds, contributing to a comprehensive understanding of the effects of the experimental treatments on the avian intestinal microbiota.

## Characteristics of the tibia

The characteristics of the tibia bone were assessed on the $42^{nd}$ day of the experiment, following the slaughter of the broiler chickens. For this analysis, two chickens from each experimental unit were selected, focusing on birds whose weight closely approximated the average weight of their respective pens. The left tibia bone was carefully isolated from each selected chicken for subsequent evaluation.

The length of the tibia was measured using a caliper with a precision of 0.01, measuring from one end to the other end of the bone. To calculate the volume of the tibia, each bone was placed into a graduated cylinder containing a known volume of water. The volume of the bone was determined based on the assumption that the specific gravity of water at room temperature is one gram per cubic centimeter. The relative weight of the tibia was expressed as a

percentage of the total body weight. The density of the tibia was calculated based on its volume and relative weight. To quantify the amount of ash in the tibia, the bones were first dried in an oven at 100°C for 24 hours. Subsequently, the dried bones were ground into a fine powder. The ash content was determined by heating the bone samples in porcelain crucibles at 600°C, and the percentage of ash was calculated. The methodology for assessing these tibia characteristics was following the procedures outlined by (Kim et al., 2004). These analyses provided valuable information regarding the structural and compositional attributes of the tibia bone in broiler chickens, contributing to a comprehensive understanding of the impact of the experimental treatments on bone health and development.

### Broiler health index (BHI)

The measurement of this index serves as a vital means to evaluate the influence of various treatments on the overall health status of broiler chickens, with a specific focus on individual blood parameters. To provide a comprehensive assessment of treatment outcomes, average indices were meticulously computed for each treatment group, and these values were subsequently utilized to create informative graphical representations. These graphical depictions play a critical role in visually portraying and elucidating the diverse impacts of the treatments on the health and physiological profiles of broiler chickens.

$BHI_1 = (\frac{(X-C)}{SD} + 100)$ and $BHI_2 = (\frac{(X-A)}{SD} + 100)$

$BHI_1$: Health index for the trait, which is the highest value of the control group.
$BHI_2$: Health index for the character that is not the highest value of the control group.
X: Each trait;
C: Control;
SD: Standard deviation.

This approach enables a nuanced comprehension of treatment effects, facilitating the identification of patterns, variations, and potential correlations that may affect the birds' health and overall well-being. The utilization of these graphical representations enhances the interpretability and communication of research findings, thereby promoting informed decision-making and the development of optimized strategies for the management and welfare of broiler chickens.

### Statistical model

Statistical analyses and comparisons were conducted utilizing the Generalized Linear Model (GLM) procedure available in SAS software, version 9.1. The means of the various treatment groups were compared using Duncan's test at a significance level of 0.05.

The statistical model employed for data analysis is represented as follows:

$Y_{ij} = \mu + T_i + e_{ij}$

where:
$Y_{ij}$ denotes the value of each observation.
$\mu$ represents the overall average or mean across all treatments.
$T_i$ signifies the effect of the $i^{th}$ treatment.
eij accounts for the error term, encompassing any residual variability not explained by the other components of the model.

This statistical model allowed for the systematic assessment of the impact of different treatments on the measured parameters while accounting for variability and error within the dataset. Duncan's test was then applied to compare treatment means and assess significant differences at the 0.05 significance level, facilitating the accurate evaluation of treatment effects and their implications on the experimental outcomes.

## Results

### Blood parameters

The analysis of blood parameters in broiler chickens, as presented in Table 2, revealed noteworthy variations among the different experimental treatments ($p < 0.05$). Among these parameters, blood glucose concentration exhibited significant differences between treatments, with the highest level observed in $T_2$ (294.00 mg $dL^{-1}$) and the lowest in $T_3$ (204.33 mg $dL^{-1}$). Similarly, chickens' total blood protein level displayed significant differences among the treatment groups, with T1 (5.32 mg $dL^{-1}$) and T5 (4.23 mg $dL^{-1}$) recording

the highest and lowest total protein concentrations, respectively. These findings underscore the potential impact of the applied treatments on the birds' metabolic and physiological profiles.

Furthermore, the data of blood serum analysis revealed significant variations in the concentration of albumin across the different treatment groups. Notably, T1 exhibited the highest albumin concentration, recording a value of 1524.3 mg dL-1, while T8 displayed the lowest albumin concentration, with a value of 1379 mg dL-1. These distinctions in blood serum components may signify diverse physiological responses to the treatments and could have implications for the health and well-being of the broiler chickens.

Additionally, the concentrations of uric acid and aspartate aminotransferase (AST) also exhibited significant differences among the treatment groups, suggesting potential implications for the metabolic processes and liver function in the birds. These variations underscore the need for further investigation into the specific effects of the applied treatments on broiler chicken physiology and overall health.

## Blood immune parameters and antibody titers

The findings, as delineated in Table 3, reveal statistically significant reductions in the percentage of heterophils, lymphocytes, and the heterophil-to-lymphocyte ratio, with a significance level of $p < 0.01$. Among the experimental treatments, $T_3$ exhibited the highest percentage of heterophils, registering at 20%, while $T_1$ displayed the lowest proportion, accounting for 16%. In terms of lymphocytes, $T_1$ and $T_2$ was recorded the highest and lowest percentages, standing at 44 and 37%, respectively. The highest heterophil-to-lymphocyte ratio was observed in the $T_2$ group, with a value of 0.62.

## The microbial population of the cecum

The study's results unveiled a substantial reduction in the population of *E. coli* within the cecal environment, primarily attributed to the dietary supplement enriched with coenzyme $Q_{10}$ and vitamin C, particularly under conditions of heat stress (Table 4). Notably, the $T_4$ group exhibited the lowest *E. coli* count, registering at 7.64 colony-forming units (CFU), while the $T_5$ group demonstrated the highest CFU count, reaching 8.00. These findings strongly suggest a favorable impact of the dietary supplement in diminishing the prevalence of deleterious bacteria within the digestive tract of the experimental avian subjects.

Under heat-stress conditions, the study observed a notable decrease in the antibody titer against bronchitis, which registered at 2456.0.

**Table 2.** The effect of experimental treatments on some blood parameters of broilers (mg $dL^{-1}$).

| Treatment | Glu | Tri | Cho | HDL | TP | Alb | UA | ALP | AST | ALT |
|---|---|---|---|---|---|---|---|---|---|---|
| $T_1$ | 206.67$^{c}$ | 113.00 | 158.33 | 82.66 | 5.32$^{a}$ | 1.70$^{a}$ | 5.65$^{ab}$ | 1524.3 | 237.33$^{abc}$ | 6.33 |
| $T_2$ | 294.00$^{a}$ | 127.67 | 151.33 | 77.00 | 4.33$^{c}$ | 1.13$^{b}$ | 5.70$^{ab}$ | 1491.0 | 246.00$^{abc}$ | 6.00 |
| $T_3$ | 204.33$^{c}$ | 123.33 | 159.00 | 77.66 | 4.61$^{bc}$ | 1.30$^{b}$ | 5.31$^{b}$ | 1500.7 | 211.67$^{c}$ | 4.66 |
| $T_4$ | 232.00$^{bc}$ | 131.00 | 155.67 | 73.66 | 4.86$^{b}$ | 1.26$^{b}$ | 6.63$^{a}$ | 1494.7 | 251.00$^{ab}$ | 7.33 |
| $T_5$ | 221.67$^{bc}$ | 128.33 | 147.33 | 75.00 | 4.23$^{c}$ | 1.33$^{b}$ | 5.39$^{b}$ | 1439.0 | 218.67$^{bc}$ | 5.00 |
| $T_6$ | 248.00$^{b}$ | 134.00 | 133.33 | 82.66 | 4.37$^{c}$ | 1.33$^{b}$ | 5.93$^{ab}$ | 1562.3 | 265.33$^{a}$ | 6.00 |
| $T_7$ | 243.67$^{b}$ | 126.33 | 167.67 | 85.33 | 4.42$^{bc}$ | 1.20$^{b}$ | 5.11$^{b}$ | 1327.3 | 221.67$^{bc}$ | 6.66 |
| $T_8$ | 238.00$^{bc}$ | 146.33 | 151.00 | 79.33 | 4.42$^{bc}$ | 1.10$^{b}$ | 5.34$^{b}$ | 1379.0 | 223.33$^{bc}$ | 5.66 |
| SEM | 3.68 | 5.22 | 3.95 | 2.02 | 0.05 | 0.03 | 0.12 | 28.56 | 3.94 | 0.35 |
| P-value | 0.001 | 0.882 | 0.589 | 0.813 | 0.001 | 0.009 | 0.039 | 0.505 | 0.042 | 0.654 |

Glu.: Glucose, Tri: Triglyceride, Cho: Cholesterol, HDL: High-density lipoprotein, TP: Total protein, Alb: Albumin, UA: Uric acid, ALP: Alkaline phosphatase, AST: Aspartate transaminase, ALT: Alanine transaminase. The means of each column with different letters have a significant difference at the 0.05 level.

**Table 3.** The effect of experimental treatments on blood immune parameters and antibody titers.

| Treatments | Immune parameters | | | Antibody titer | |
|---|---|---|---|---|---|
| | Heterophile (%) | Lymphocyte (%) | H/L | Bronchitis | Gumboro |
| $T_1$ | 16.00$^{c}$ | 44.00$^{a}$ | 0.36$^{c}$ | 4330.0$^{a}$ | 3274.3 |
| $T_2$ | 23.00$^{a}$ | 37.00$^{c}$ | 0.62$^{a}$ | 2456.0$^{d}$ | 3838.0 |
| $T_3$ | 20.00$^{b}$ | 40.00$^{b}$ | 0.50$^{b}$ | 3599.7$^{bc}$ | 3616.3 |
| $T_4$ | 19.00$^{b}$ | 41.00$^{b}$ | 0.46$^{b}$ | 3403.7$^{bc}$ | 3722.3 |
| $T_5$ | 18.33$^{b}$ | 41.66$^{b}$ | 0.44$^{b}$ | 3527.7$^{bc}$ | 3240.7 |
| $T_6$ | 19.33$^{b}$ | 40.66$^{b}$ | 0.47$^{b}$ | 3091.3$^{cd}$ | 4040.3 |
| $T_7$ | 18.66$^{b}$ | 41.33$^{b}$ | 0.45$^{bc}$ | 3820.0$^{ab}$ | 3919.3 |
| $T_8$ | 18.66$^{b}$ | 41.33$^{b}$ | 0.45$^{bc}$ | 3564.3$^{bc}$ | 3323.0 |
| SEM | 0.27 | 0.27 | 0.008 | 76.60 | 84.80 |
| P-value | 0.001 | 0.001 | 0.0007 | 0.001 | 0.185 |

H/L: Heterophil/Lymphocyte The means of each column with different letters have a significant difference at the 0.05 level.

**Table 4.** The effect of experimental treatments on the microbial population of broiler caecum (log CFU $g^{-1}$).

| Treatments | *Lactobacillus* | *E. coli* | Total microbial population |
|---|---|---|---|
| $T_1$ | 6.88 | $7.73^{ab}$ | 9.14 |
| $T_2$ | 6.97 | $7.92^{ab}$ | 9.23 |
| $T_3$ | 6.92 | $7.79^{ab}$ | 9.19 |
| $T_4$ | 6.98 | $7.64^{b}$ | 9.07 |
| $T_5$ | 6.91 | $8.00^{a}$ | 9.27 |
| $T_6$ | 7.17 | $7.77^{ab}$ | 9.24 |
| $T_7$ | 7.00 | $7.70^{ab}$ | 9.29 |
| $T_8$ | 6.90 | $7.87^{ab}$ | 9.18 |
| SEM | 0.03 | 0.03 | 0.02 |
| P-value | 0.641 | 0.011 | 0.367 |

The means of each column with different letters have a significant difference at the 0.05 level.

## Characteristics of the tibia

The assessment of bone characteristics represents a conventional and fundamental criterion for evaluating the quality of poultry diets, particularly concerning the mineral content, specifically calcium and phosphorus. Various methods exist for measuring bone mineralization, encompassing bone ash content, fracture point, bone weight, bone volume, and photon absorption (bone density) (Rao et al., 1993). However, in the context of this study, the results presented in Table 5 indicate that the incorporation of different dietary treatments did not exert a significant impact on the tibia characteristics.

**Table 5.** The effect of experimental treatments on the tibia characteristics of broiler.

| Treatments | Ash (%) | Relative length (mm) | Diameter (mm) | Volumetric mass |
|---|---|---|---|---|
| $T_1$ | 50.25 | 90.99 | 7.96 | 7.90 |
| $T_2$ | 52.28 | 92.73 | 8.49 | 8.23 |
| $T_3$ | 50.48 | 92.12 | 8.31 | 8.33 |
| $T_4$ | 49.54 | 89.36 | 8.81 | 8.13 |
| $T_5$ | 50.96 | 91.12 | 8.72 | 8.86 |
| $T_6$ | 51.04 | 92.12 | 7.91 | 8.53 |
| $T_7$ | 51.18 | 90.39 | 8.12 | 7.66 |
| $T_8$ | 50.96 | 89.81 | 8.03 | 8.36 |
| SEM | 0.28 | 0.46 | 0.15 | 0.20 |
| P-value | 0.501 | 0.594 | 0.725 | 0.882 |

## Broiler health index (BHI)

In the context of a specific experiment involving heat stress, the alterations in primary blood parameters, indicative of the health status of broiler chickens, were utilized to derive a quantitative health index (Table 6). The calculation of average health indices for each treatment modality was undertaken, followed by comparisons with the average index of the control group and chickens subjected to heat stress but not exposed to any specific treatment. Health indicators that exceeded the established average threshold were accorded priority status as suitable markers of treatment efficacy. Subsequently, the treatments responsible for inducing these favorable alterations were identified based on the graphical representation, as depicted in Figure 1.

**Table 6.** Broiler health index for different investigated traits.

| Treatment | Glu | Tri | Cho | HDL | TP | Alb | UA | ALP | AST | ALT | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $T_4$ | 99.11 | 98.10 | 100.26 | 97.80 | 98.72 | 97.63 | 97.96 | 99.62 | 99.27 | 98.84 | 98.73 |
| $T_6$ | 98.55 | 97.78 | 102.48 | 100.00 | 97.36 | 98.01 | 99.42 | 100.49 | 98.50 | 100.38 | 99.30 |
| $T_2$ | 96.93 | 98.45 | 100.69 | 98.62 | 97.25 | 96.93 | 99.90 | 99.57 | 99.53 | 100.38 | 98.83 |
| $T_1$ | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| $T_5$ | 99.47 | 98.38 | 101.09 | 98.13 | 96.97 | 98.01 | 100.54 | 98.91 | 101.00 | 101.54 | 99.40 |
| $T_8$ | 98.90 | 96.48 | 100.73 | 99.19 | 97.50 | 96.77 | 100.65 | 98.14 | 100.75 | 100.78 | 98.99 |
| $T_3$ | 100.08 | 98.91 | 99.93 | 98.78 | 98.03 | 97.85 | 100.71 | 99.70 | 101.38 | 101.94 | 99.73 |
| $T_7$ | 98.70 | 98.59 | 99.07 | 100.65 | 97.50 | 97.31 | 101.13 | 97.48 | 100.84 | 99.62 | 99.09 |

Glu.: Glucose, Tri: Triglyceride, Cho: Cholesterol, HDL: High-density lipoprotein, TP: Total protein, Alb: Albumin, UA: Uric acid, ALP: Alkaline phosphatase, AST: Aspartate transaminase, ALT: Alanine transaminase.

Analysis of the graphical data revealed that a subset of treatments, specifically $T_3$, $T_5$, $T_6$, and $T_7$, surpassed the established average threshold and thus were assigned to the category of treatments yielding favorable

health indices. Consequently, based on the presented findings, it is discerned that $T_3$ exhibits the most pronounced positive influence on animal health when compared to the remaining treatment modalities.

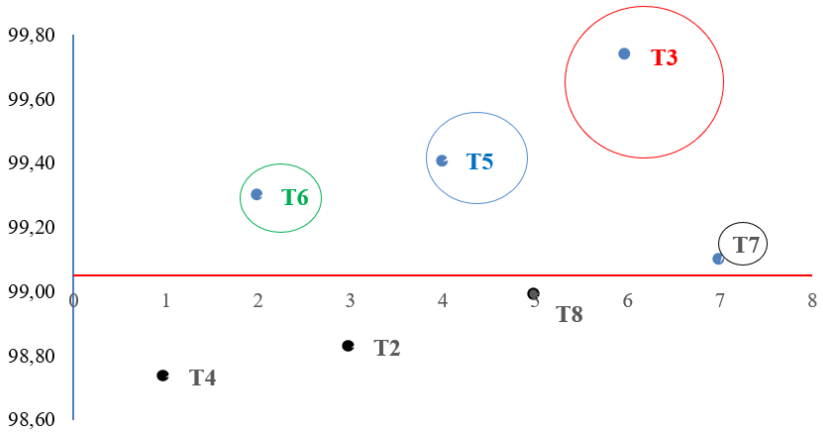


**Figure 1.** Position of different treatments based on broiler health index.

## Discussion

The observed increase in blood glucose concentration under heat stress conditions, specifically in treatment $T_2$, aligns with prior research that has documented elevated serum glucose levels in quails exposed to high temperatures (Sahin, Kucuk, Sahin, & Sari, 2002). Stress, characterized as the biological response to threats and disruptions in homeostasis, can initiate autonomic nervous system responses that affect various physiological systems, including cardiovascular, digestive, and endocrine functions (Kario, Bruce, & Thomas, 2003).

The effects of thermal stress on the body's physiological mechanisms are intricate and can manifest as both increases and decreases in various parameters. Of particular note, the observed reduction in thyroid size and subsequent reduction in thyroid hormone secretion in response to heat stress may contribute to the elevated serum glucose levels (Lumeij, 1997; Keshavarz & Fuller, 1980).

The decrease in serum protein levels among broilers subjected to heat stress is consistent with findings from previous studies. These studies have established a link between exposure to high temperatures and reduced protein synthesis, lower plasma amino acid levels, and increased catabolism (Feizi, Dadian, & Asadzadehmajdi, 2012). Intriguingly, the administration of vitamin C, as seen in treatment $T_4$, resulted in an improvement in total serum protein levels. Vitamin C plays a crucial role in corticosterone biosynthesis and functions as a significant glucocorticoid hormone that supports gluconeogenesis, aiding in the augmentation of energy reserves under heat-stress conditions (Talebi & Khademi, 2011; Karimi et al., 2015).

Additionally, the incorporation of organic selenium supplements appeared to reduce the levels of aspartate aminotransferase (AST) and alkaline phosphatase (ALP) enzymes in the serum, which corresponds with previous research findings (Shabani, Fakhraei, Yarahmadi, & Seidavi, 2020; Talebi & Ghazanfarpoor, 2021). Changes in these enzymes may reflect liver health, as the liver serves as a primary organ responsible for protecting against toxic substances. Elevated liver enzymes often signify liver damage (Pratt & Kaplan, 2000). Results from similarly indicated that dietary organic selenium sources can help reduce liver enzymes, suggesting a potential protective effect on liver function (Cai et al., 2012).

The observed response, characterized by a significant decrease in the percentage of heterophils, lymphocytes, and the ratio of heterophils to lymphocytes under heat stress conditions, signifies the profound impact of elevated temperatures on the poultry's immune system. This response underscores the intricate relationship between the intensity and duration of heat stress exposure. Heat stress is recognized for its detrimental effects, including a reduction in the weights of lymphoid organs such as the bursa, thymus, and spleen, a decrease in the total count of white blood cells, a decline in antibody titers, and an alteration in the heterophil-to-lymphocyte ratio (Ratriyanto & Mosenthin, 2018). Thus, the ratio of heterophils to lymphocytes emerges as a valuable indicator for assessing avian stress levels, with heightened stress levels often corresponding to an elevated ratio.

Broiler chickens exposed to heat stress tend to exhibit compromised immune systems, rendering them susceptible to diseases like Newcastle disease and secondary infections (Hirakawa et al., 2020). The

heightened production of corticosterone in response to heat stress inhibits antibody production, ultimately leading to reduced blood antibody titers (Saelao et al., 2018). Recent investigations have revealed that the administration of specific nutrients and compounds, including vitamins C and E, zinc, selenium, and plant extracts, can help mitigate the adverse effects of heat stress on the immune system (Cai et al., 2012).

The findings underscore the critical importance of managing and supporting the immune function of broiler chickens under heat-stress conditions. Implementing strategies aimed at addressing immune system suppression and striving to maintain or enhance antibody titers can significantly influence the overall health and disease resistance of poultry facing challenging environmental conditions.

The incorporation of nutritional antioxidants, such as vitamins C and E, has been previously associated with improvements in intestinal microbiology and immune characteristics. These antioxidants have also shown promise in reducing the ratio of *Escherichia coli* to beneficial bacteria like lactobacillus (Babinszky, Szabó, & Horváth, 2021). The microbial composition of the digestive tract is a critical determinant of avian physiology, influencing nutrition, detoxification of specific compounds, growth, and protection against pathogenic bacteria. In the absence of a natural microflora in chickens, the risk of bacterial infections is heightened. Conversely, a balanced and healthy microbial flora in the digestive system contributes to overall health and supports the host's immune system (Nosrati et al., 2017).

The observed reduction in the population of *E. coli* highlights the potential of dietary interventions to modulate the microbial composition within the cecal region. This modulation can have profound implications for the health and disease resistance of broiler chickens, especially under the stress conditions associated with heat stress. These findings underscore the importance of strategies aimed at enhancing the intestinal microbiota and mitigating the proliferation of harmful bacteria, particularly in the context of heat stress.

These results align with previous research by (Sørum & Sunde, 2001), which reported that various parameters related to tibia characteristics, such as epiphysis diameter, external and internal diameter, weight, and thickness, remained unaffected by varying levels of phytobiotic supplements in the diets of laying hens. The absence of significant changes in tibia characteristics in response to dietary treatments suggests that these interventions may not exert a pronounced influence on bone mineralization in this specific experimental context.

It is essential to consider that elevated ambient temperatures can adversely affect mineral metabolism, leading to increased mineral excretion. In this regard, Metwally documented that the addition of phytobiotics did not significantly affect specific tibial bone characteristics, including volume, length, density, and ash content, in broiler chickens (Jamali, Ghorbani, Tatar, Salari, & Chaji, 2017; Metwally, 2023). These results underscore the need to account for environmental factors, such as temperature, which can interact with dietary components and impact bone health.

## Conclusion

This study provides valuable insights into the physiological responses of broiler chickens to heat stress and the potential of dietary interventions to mitigate adverse effects. It highlights the importance of understanding the interplay between environmental stressors, dietary components, and avian metabolism to improve poultry health. The findings emphasize the need for further research into the mechanisms of heat stress and dietary strategies, particularly nutritional antioxidants and supplements, to enhance immune function and microbial composition in the cecal region. Overall, this study advances our knowledge of strategies to improve broiler chicken resilience and well-being under challenging conditions.

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