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The Effect of Silymarin on Antioxidant, Performance, Immunoglobulin Protein Levels, Cecal Microbiota, and Hemobiochemical Indicators in Heat Stressd Broilers

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Abstract

This study examined the effects of dietary inclusion of silymarin on performance, and physiological responses of broilers exposed to heat stress (HS) conditions. A flavonoid complex known as silymarin is extracted from the *Silybum marianum* plant, which is renowned for its medicinal properties. A total of 500 one-day-old male broiler chickens (Ross 308) were divided into five dietary treatment groups with five replicates each. Thermoneutral (TN), heat stress (HS), and HS with a diet supplemented with four different silymarin at rates of zero, 150, 300, and 450 mg/kg, respectively (S0, S1, S2, and S3 groups). Broiler chickens were reared under normal conditions until d 25 and after that the heat stress (34 ± 2◦C) was applied for eight hours (0900 to 1700 h) from d 25 to 42. Exposure to HS significantly reduced feed intake, weight gain, antioxidant capacity, hematocrit (HCT), hemoglobin (HGB), and Lymphocytes (LYM) in and elevated feed conversion ratio (FCR), mortality, serum malondialdehyde, and heterophils (HET) compared to the TN group. Moreover, HS decreased the immunoglobulin G (IgG), immunoglobulin M (IgM), and caecal lactic acid bacteria population (CLBP**),** but increased caecal coliform population (CCFP), and total cholesterol (TC), triglyceride (TG), aspartate transaminase (AST), alanine transaminase (ALT) levels in serum ($P < 0.01$). Among the HS groups, supplementary silymarin improved growth performance indices and reduced mortality ($P < 0.01$). The HET and HET to LYM ratio was reduced by silymarin supplementation. The HS-induced effects on of TG, TC, ALT, and AST concentration in serum were alleviated by dietary silymarin supplementation ($P < 0.01$). Moreover, the HCT, HGB, glutathione peroxidase, superoxide dismutase, IgM, and IgG level were increased. Silymarin inclusion reduced malondialdehyde in serum ($P < 0.01$). In addition, CLBP increased, and CCFP decreased by silymarin inclusion. In conclusion, silymarin inclusion may be used to alleviate the physiological responses of broilers exposed to heat stress.

Introduction

Due to the environmental changes, high temperatures affect the poultry industry, specifically commercial broilers, and have been extensively researched due to the significant economic losses they can cause (Pawar *et al*., 2016). Heat stress (HS) has a negative impact on the global poultry industry, particularly in tropical regions such as the Middle East and Iran (Attia and Hassan, 2017; Israr *et al*., 2021). Heat stress leads to physiological variation accompanied by hormonal changes and reduced feed intake that leads to a loss in production and physiological performance. More recently, the involvement of heat stress in inducing oxidative stress has received much interest (Akbarian *et al*., 2016; Ghanima *et al*., 2020). In addition, HS can have a detrimental effect on the gastrointestinal microflora and disrupt the balance of the gastrointestinal ecosystem, weakening beneficial microorganisms and increasing the chances of pathogens colonizing (Lin *et al*., 2011).

Several strategies have been developed to reduce the adverse effects of HS on poultry production. The

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main emphasis of current strategies primarily centers on dietary adjustments as a result of the significant expenses linked to poultry building cooling (Syafwan *et al.*, 2011). Over the past several years, there has been a growing focus on the significance of antioxidants in reducing oxidative stress and the resulting physiological disorders and impairments.

Silymarin is an extract containing unique flavonoid compounds from the fruits and seeds of milk thistle (Silybum marianum [L.] Gaertn.), such as silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin and flavonoid (a family of flavonolignans) (Federico *et al*., 2017). Silybin (or silibinin) is the main biologically active component (70-80%) present in silymarin extract, which is a mixture of silybin A and silybin B. In addition, silymarin extract contains about 20-35% of polyphenolic and fatty acid compounds (Esmaeila *et al*., 2017). Previous studies show that silymarin has antimicrobial properties against all types of harmful bacteria such as *Staphylococcus aureus*, *Escherichia coli, Campylobacter jejuni***,** *Escherichia coli*, and *Staphylococcus aureus* (Evren and Yurtcu, 2015; Radhika *et al*., 2017; Kareem *et al*., 2020; Jahanian *et al*., 2021).

In addition, silymarin has the properties of inhibiting free radicals and has a high ability to enhance endogenous antioxidant systems (Vargas-Mendoza *et al*., 2014). Silymarin can inhibit the growth of human breast, cervical, and prostate cancer cells in test tubes. Furthermore, silymarin, due to its antioxidant effects, can protect the brain, heart, liver and other vital internal organs (Karimi *et al*., 2011).

Studies show that silymarin acts as a strong scavenger of active free radicals by increasing the activity of endogenous non-enzymatic antioxidants (vitamins E and C) in serum, liver and muscle tissues; as a result, they inhibit lipid and protein peroxidation (El-Gazayerly *et al*. 2014; Alhidary *et al.* 2017). Another mechanism of action that they express for the antioxidant effects of silymarin, the high ability of silymarin to increase cellular glutathione content, inducing superoxide dismutase, and inhibiting lipid and protein peroxidation or metal ions (copper, iron) chelation ability of this compound (Esmaeila *et al*., 2017).

The anti-arthritic and anti-inflammatory effects of silymarin are due to scavenging free radicals and excellent antioxidant properties, which act as pro-inflammatory agents (Abdelazim, 2017***)***. Research results showed that silymarin inclusion in the diet of The study consisted of two controlled environments, each with its temperature control system. Each room was equipped with portable electric heaters that were controlled by thermostats. The rooms were equipped with a humidifier to regulate the relative humidity (RH) within a range of 55% ± 5%. The temperature laying hens in summer improved the antioxidant status, liver function and lipid profile (Abou-Shehema *et al*., 2016) and improved egg laying rate and nutrient content of eggs (Quarantelli *et al*., 2009). In another report, they showed that silymarin inclusion in broiler chickens improved the chemical composition and oxidative stability of thigh and breast meat (Schiavone *et al*., 2007).

Despite the above-reported information about silymarin supplementation, so far, there is no study on the effects of silymarin in broiler chickens under heat stress and its effect on growth performance and physiological changes. Therefore, this research study aimed to investigate the effect of silymarin supplementation on growth performance and some biochemical parameters and intestinal bacterial population in broilers under heat stress.

Materials and Methods

The study was conducted and approved by the Institutional Review Board of Payam Noor University (No. 11.1402).

Animal husbandry

This study was performed on a total of 500 unsexed one-day-old Ross male 308 broiler chicks with an average body weight of 44±3 g, purchased from a traditional company in Gorgan, Iran. The birds were randomly distributed among five groups. Each group consisted of five replications, with each replicate pen housing 20 birds. In this study, broilers were assigned to one of five diets beginning on day 25. All the feeding and managemental protocols in accordance with brooding standards were followed as recommended by Aviagen (2014). The treatment groups were as follows: (1) thermoneutral (TN) group**,** which received the basal diet (Table 1) reared under thermoneutral conditions; (2) S0 group, which received the basal diet and reared under HS conditions; (3) S1 group**,** (4) S2 group, (5) S3 group, which received the basal diet supplemented with 150, 300, and 450 mg/kg of silymarin respectively and reared under HS conditions. Supplements were included in the diets from d 25 to 42 of the experiment. Silymarin, with a molecular weight of 482.44 g/mol and purity (UV 80%) used in this search, was manufactured by Zardband LTD., Tehran, Iran. The chickens were given unrestricted access to mash feed and water.

Environmental conditions

of the chickens was carefully regulated throughout the experiment. Initially, all chickens were kept at a temperature range of 33 to 34 °C for the first 3 days. Two environmental conditions were utilized from day 25 to the 42-day study period: The TN group had a gradual decrease in temperature by 3 °C per week

until reaching 22-23 °C. The HS groups initially remained at a constant temperature of 34 ± 2◦C for 8 hours (9 am to 5 pm). Then, they decreased to match the TN group's temperature for the remaining 16 hours daily under summer conditions from June 23 to August 2, 2023 (Mohammed *et al*., 2019; Bahrampour *e*t *al.*, 2021). A continuous-flow, pressure-controlled ventilator was used for air movement, and the lighting program included continuous light for the first 3 days, followed by a cycle of 23 hours of light and 1 hour of darkness until the end of the trial. All experimental groups were reared under identical managerial and hygienic procedures. The chicks were housed in deep litter partitions measuring 2 × 1 m. The vaccinations against infectious Bronchitis virus (IBV) were administered through drinking water on the first day, while vaccinations against Newcastle disease were given on the 8th and 21st day. Additionally, the chicks were vaccinated against flu (first day), and infectious bursal disease (14 and 23 days) during the period of study.

Table 1. Composition (as-fed) and calculated analyses of the basal diet fed from 25 to 42 d of age

Ingredients	(%)
Corn	57.57
Soybean meal, 44%CP	32.35
Soybean oil	6.29
Limestone	1.05
Di-calcium phosphate	1.34
Vitamin premix[a]	0.25
Mineral premix[b]	0.25
NaCl	0.40
DL-Methionine, 99%	0.28
L-Lysine, 78%	0.22
Calculated values	
Metabolizable energy, kcal/kg	3218
Crude protein, %	19.3
Calcium, %	0.79
Available phosphorus, %	0.361
Sodium, %	0.16
Met, %	0.58
Met + Cys, %	0.89
Lys, %	1.17
Arg, %	1.30
Thr, %	0.78
Try, %	0.29

[a] Vitamin concentrations per kilogram of diet: retinol, 13.50 mg; cholecalciferol, 4.15 mg; tocopherol acetate, 32.00 mg; vitamin K3, 2 mg; thiamin, 2 mg; riboflavin, 6.00 mg; biotin, 0.1 mg; cobalamin, 0.015 mg; pyroxidine, 3 mg; niacin, 11.00 mg; d-pantothenic acid, 25.0; menadione sodium bisulphate, 1.10; folic acid, 1.02; choline chloride, 250 mg; nicotinamide, 5 mg.

[b] Mineral concentrations per kilogram of diet: calcium pantothenate, 25 mg; Fe (from ferrous sulphate), 35 mg; Cu (from copper sulphate), 12.5 mg; Mn (from manganese sulphate), 8 mg; Zn (from zinc sulphate), 75 mg; I (from calcium iodate), 0.6 mg; Se (from sodium selenite), 0.3 mg.

Growth performance analysis

Chicks were weighed individually, and their initial body weight (IBW) and final body weight (FBW) at six weeks of age were recorded to the nearest gram using a digital scale. Body weight gain (BWG) was calculated as the difference between initial and final body weight. Feed intake was determined daily by subtracting the remaining feed from the previous day from the feed offered to each replicate within each group. Feed conversion ratio (FCR) was also computed as the proportion of total feed intake to total weight gain. Mortality percentages were recorded on a replicate basis every day and totaled for the entire experimental period (Hu *et al.*, 2021).

Sample collection

On day 42 of the experiment, 2 birds were selected from each replicate based on their average BW. Blood samples were obtained from the wing veins of each bird and then divided into two parts. The first portion was collected in tubes containing 10% EDTA, an anticoagulant, to measure hematological parameters analysis. The second part was collected in coagulation tubes for serum extraction to determine the serum biochemical parameters of the birds. Birds were euthanized by cervical dislocation after blood sampling and caecal samples were collected.

Blood Biochemical parameters

Hematological parameters were recorded using the HBVET-1 automated hematology analyzer,

SINNOWA Medical Science and Technology CO., LTD., depending on the principle of electro-impedance and photometric analysis. These parameters included hemoglobin (HGB) and hematocrit (HCT), concentration, lymphocyte (LYM) percentage, and heterophil (HET) percentage.

Serum biochemical *indices* analysis

The second portion of the blood sample was collected in tubes without anticoagulant and centrifuged at 3500 × g for 12-15 minutes to separate the serum. The serum was collected and stored at -20 °C until it was used to determine biochemical parameters. The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), along with the concentrations of triglyceride (TG), and total cholesterol (TC), were evaluated using biochemical kits (Pars Azmoun Co., Tehran, Iran). The measurements were carried out using an automatic spectrophotometer (Chem 200, Gesan Production Srl, Campobello, Italy).

Determination of immunoglobulin G (IgG), and immunoglobulin M (IgM) against non-pathogenic were measured using commercial diagnostic kits according to the protocol provided by the manufacturer (DIASORIN S.P.A. Italia) by using an autoanalyzer (Technicon RA1000, Bayer Diagnostics, Puteaux, France) as described by Piquer *et al*. (1991).

Serum antioxidant analysis

The levels of superoxide dismutase (SOD), Glutathione peroxidase (GPx), and malonaldehyde (MDA) were measured using commercial kits (RANDOX/RANSEL Laboratories) following the manufacturer's instructions and the methodologies of Fathi *et al*., (2022).

The bacterial population of cecum

Analysis of the microbial population in the cecum of broiler chickens was estimated as follows: In a screw-top bottle, gut samples of broiler chickens (ten birds per group) were collected and quickly transported to the laboratory. Samples of broiler cecum (Amount of 10 g) were collected by gentle pressure in a tube containing 4.5 ml of sterile saline. Then 1 gram of each sample was homogenized in 9 ml of sterile water for 30 seconds by a vortex shaker and stored at at−20°C to count the microbial population. Enumeration of target bacterial groups was done using selective agar media (Merck, Darmstadt, Germany) for coliforms (McConkey agar medium); and Lactobacillus spp. (MRS Agar) (Abolfathi *et al.*, 2019). Microbiological count units were expressed as log10 CFU/g of fresh sample.

Statistical design and analysis

The collected data in the current study were analyzed using SPSS 18.0 software (SPSS Inc., IL). To compare the TN control and S0 groups, the *t* test was performed. To determine the significance of these differences, Tukey's test was employed, with adjustments made at a significance level $P < 0.05$. Orthogonal polynomial contrasts were employed to estimate the optimal amount of dietary silymarin and detect linear and quadratic effects of silymarin levels, respectively. The results are presented as means accompanied by the pooled standard error of the mean (*SEM*).

Results

Growth performance and mortality

Table 2 shows the performance results for broiler chickens grown under HS conditions until 42 days of age. The results indicated that heat stress significantly decreased the FBW, BWG and FI, but it increased the FCR and mortality in broilers ($P < 0.05$). Broilers in the S3 and S4 group (heat stress + 300 and 450 mg/kg silymarin treatment) exhibited higher FBW and BWG, lower FCR and mortality compared with those in the S0 group ($P < 0.01$). Under heat conditions, performance growth and mortality were linearly affected by dietary silymarin inclusion (Table 2) ($P < 0.01$).

Table 2. Effect of dietary supplementation of silymarin on performance in broiler chickens subjected to heat stress (HS) (1- 42 d)

Indices	Treatments					*SEM*	P-Value	
	TN	HS					Linear	Quadratic
		Level of silymarin [mg/kg diet]						
		S0	S1	S2	S3			
IBW (g)	843	845	841	842	842	11.05	0.590	0.621
FBW (g)	2680^{a}	2150^{c}	2180^{c}	2410^{b}	2430^{b}	37.20	0.002	0.490
BWG (kg)	1845^{a}	1302^{c}	1341^{c}	1572^{b}	1593^{b}	19.20	0.001	0.601
Feed Intake (g)	4475^{a}	4257^{b}	4272^{b}	4386^{a}	4422^{a}	85.10	0.001	0.492
Feed conversion ratio	1.67^{c}	1.98^{a}	1.96^{a}	1.82^{b}	1.85^{b}	0.11	0.001	0.721
Mortality (%)	4.00^{b}	12.00^{a}	10.00^{a}	4.00^{b}	4.00^{b}	0.35	0.001	0.391

$^{a, b, c}$ Mean values in the same row with different superscript letters were significantly ($P < 0.05$). (N=10)

IBW, initial body weight; FBW, final body weight; BWG, body weight gain; TN; thermoneutral, HS; Heat Stress, S0-, S1, S2 and S3 indicate the supplementation of silymarin at the rate of 0, 150, 300, and 450 mg/kg respectively.

Hematology parameters

As shown in Table 3, the HS induction increased values for heterophils (HET), and reduced the hematocrit (HCT), hemoglobin (HGB), and lymphocyte (LYM) compared with those in the control group ($P < 0.01$). Silymarin-treated groups (S1-S3) afforded a significant increase in HGB, HCT and LYM and a decrease in HET when compared with those of the HS control group (S0) ($P < 0.01$). Under high-temperature conditions, hematological indices were linearly affected by dietary silymarin inclusion.

Table 3. Effect of dietary supplementation of silymarin on hematological parameters of broiler chickens subjected to heat stress (HS) at day 42 of age

Indices	Treatments					*SEM*	P-Value	
	TN	HS						
		Level of silymarin [mg/kg diet]						
		S0	S1	S2	S3		Linear	Quadratic
HGB (g/dl)	15.40^{a}	12.90^{b}	13.60^{b}	13.20^{b}	16.70^{a}	0.260	0.021	0.291
HCT (%)	36.30^{b}	31.00^{c}	30.80^{c}	36.31^{b}	39.65^{a}	1.01	0.001	0.721
HET (%)	18.02^{c}	27.03^{a}	25.20^{b}	19.31^{c}	19.03^{c}	1.02	0.001	0.629
LYM (%)	59.10^{a}	48.41^{c}	52.50^{b}	58.51^{a}	57.46^{a}	1.52	0.001	0.483
HET / LYM Ratio	0.30^{c}	0.56^{a}	0.48^{b}	0.33^{c}	0.33^{c}	0.02	0.001	0.390

$^{a, b, c}$ Mean values in the same row with different superscript letters were significantly ($P < 0.05$) different. (N=10)

TN; thermoneutral, HS; Heat Stress, S0, S1, S2 and S3 indicate the supplementation of silymarin at the rate of 0, 150, 300, and 450 mg/kg, respectively. HGB, hemoglobin HCT, Hematocrit; HET, Heterophil; LYM, Lymphocyte,

Antioxidant status

Table 5 presents effect of heat stress (HS) on antioxidant status and showed that HS increased serum MDA level, and decreased ($P < 0.05$) SOD and GPx levels in serum of broilers than the TN control group (Table 5). Moreover, broilers in the S1-S3 groups (heat stress + 150, 300 and 450 mg/kg silymarin treatment) exhibited higher serum SOD and GPx and lower serum MDA than HS treatment ($P < 0.05$). Under HS condition, antioxidant status was linearly affected by dietary inclusion of silymarin (Table 5).

Biochemical indices

As shown in Table 4, heat induction dramatically decreased the TG, TC, AST, ALT, IgG, and IgM levels in serum of broilers compared with those in thermoneutral group ($P < 0.01$). Broilers in the S1-S3 groups (heat stress + 150, 300 and 450 mg/kg silymarin treatment) exhibited lower serum TG, TC, AST, ALT, IgG, and IgM levels than those in the S0 group (heat stress group). Under high temperature condition, serum TP, TG, and T4 were linearly affected by dietary inclusion of silymarin (Table 4).

Table 4. Effect of dietary supplementation of silymarin on some biochemical parameters of broiler chickens subjected to heat stress (HS) at day 42 of age

Indices	Treatments					*SEM*	P-Value	
	TN	HS						
		Level of silymarin [mg/kg diet]						
		S0	S1	S2	S3		Linear	Quadratic
TG (mg/dL)	79.58^{c}	128.73^{a}	111.58^{b}	107.51^{b}	82.75^{c}	2.60	0.000	0.720
TC (mg/dL)	97.05^{c}	124.15^{a}	113.05^{b}	108.04^{b}	107.14^{bc}	3.01	0.001	0.592
ALT (U/L)	8.27^{d}	30.11^{a}	18.48^{b}	13.57^{c}	12.91^{cd}	2.50	0.001	0.391
AST (U/L)	122.85^{d}	195.22^{a}	145.38^{b}	139.30^{b}	133.24^{bc}	8.10	0.002	0.482
IgM (U/ml)	5.27^{b}	2.86^{c}	2.91^{c}	3.48^{b}	3.51^{b}	0.23	0.000	0.621
IgG (U/ml)	6.77^{a}	5.00^{b}	5.10^{b}	5.23^{b}	5.78^{ab}	0.29	0.021	0.310

$^{a, b, c}$ Mean values in the same row with different superscript letters were significantly ($P < 0.05$). (N=10)

TN; thermoneutral, HS; Heat Stress, S0, S1, S2 and S3 indicate the supplementation of silymarin at the rate of 0, 150, 300, and 450 mg/kg respectively. TG, triglyceride; TC, total cholesterol; ALT, alanine transaminase; AST, aspartate aminotransferase; IgG: Immunoglobulin IgM: Immunoglobulin M

Caecal microbiota

As shown in Table 6, Heat stress (S0 group) decreased ($P < 0.05$) the caecal *Lactobacillus* population and increased the caecal *coliform* population in broilers compared with that in the TN control group (Table 6). As shown in Table 6, broilers in the S1-S3 groups (heat stress + 150, 300 and 450 mg/kg silymarin treatment) exhibited a lower caecal *coliform* population and higher caecal *Lactobacillus* population than those in the S0 group (heat stress group). ($P < 0.05$). Under heat induction conditions, the caecal microbiota was linearly affected by the dietary inclusion of silymarin (Table 6).

Table 5. Effect of dietary supplementation of silymarin on antioxidant status in broiler chickens subjected to heat stress (HS) at day 42 of age

Indices	Treatments					*SEM*	P-Value	
	TN	HS						
		Level of silymarin [mg/kg diet]						
		S0	S1	S2	S3		Linear	Quadratic
GPx (U/g hemoglobin)	194.50[a]	85.25[c]	126.52[b]	128.95[b]	149.01[b]	10.15	0.001	0.810
SOD (U/g hemoglobin)	518.66[a]	359.86[c]	373.56[c]	441.50[b]	484.66[a]	18.60	0.001	0.783
MDA (nm/mL)	7.61[d]	10.39[a]	9.36[b]	9.27[b]	8.68[c]	0.30	0.000	0.692

[a, b, c] Mean values in the same row with different superscript letters were significantly ($P < 0.05$) different. (N=10 what?)
TN; thermoneutral, HS; Heat Stress, S0, S1, S2 and S3 indicate the supplementation of silymarin at the rate of 0, 150, 300, and 450 mg/kg, respectively. GPx, glutathione peroxidase; SOD, *superoxide dismutase; MDA,* malondialdehyde.

Table 6. Effect of dietary supplementation of silymarin on caeca bacterial population of broiler chickens subjected to heat stress (HS) at day 42 of age

Microbiological Count (Log 10 CFU/g fresh digesta)	Treatments					*SEM*	P-Value	
	TN	HS						
		Level of silymarin [mg/kg diet]						
		S0	S1	S2	S3		Linear	Quadratic
Coliform	5.41[b]	6.95[a]	6.89[a]	5.95[b]	5.38b[b]	0.21	0.001	0.623
Lactic acid bacteria	7.55[a]	2.02[c]	2.60[c]	5.90[b]	6.10[b]	0.11	0.001	0.810

[a, b, c] Mean values in the same row with different superscript letters were significantly ($P < 0.05$) different. (N=10)
TN; thermoneutral, HS; Heat Stress, S0, S1, S2 and S3 indicate the supplementation of silymarin at the rate of 0, 150, 300, and 450 mg/kg, respectively. CFU, Colony Forming Unit.

Discussion

Heat stress conditions, can disrupt thermoregulation and homeostasis, reduce growth performance, welfare and health, and lead to a drop in economic indicators of growth and production in broiler chicken farming. The decrease in growth efficiency and the drop in economic indicators of growth in broiler chickens during heat stress are attributed to the decrease in the tendency of these birds to feed intake (Peng *et al*., 2023). Similar to the findings of the current study, previous investigations have indicated that broiler chickens exhibit a decrease in feed consumption when exposed to hot environmental conditions (Amiri *et al*., 2019; Gouda *et al*., 2020; Peng *et al.*, 2023). A decrease in nutrient intake is associated with a subsequent decrease in body heat production. Furthermore, it has been reported that HS can decrease feed intake in broiler chickens by influencing the presence and distribution of fenteroendocrine cells, which play a crucial role in regulating appetite (Kim *et al*., 2021; Mazzoni *et al*., 2022). In addition, it is shown that heat stress, both by reducing feed consumption and by reducing the efficiency of digestion and absorption ultimately leads to weight loss and growth performance in broiler chickens (Liu *et al*., 2019).

According to previous research (Ghasemi and Nari, 2023), broilers subjected to heat stress showed elevated serum corticosterone concentrations. The observed elevation in corticosterone levels was observed to impede the growth and development of broiler chickens through the inhibition of thyroid hormone synthesis and the restriction of feed intake.

According to the findings of Zhang *et al*. **(**2015 a,b), Mazur-Kusnirek *et al*. (2019), Xue *et al*. (2017), Bahrampour *et al*. (2021), and Hu *et al*. (2021), including antioxidants such as phloretin, epigallocatechin gallate, vitamin E, curcumin, and plant antioxidant in the diet can alleviate adverse effects of heat stress and maintain broiler growth.

The antioxidant effects of silymarin have been reported in several studies (Dixit *et al*., 2009; Shaker *et al*., 2010; Ghosh *et al*., 2010**;** Abdelazim, 2017). It has been reported that the activity of pancreatic trypsin and α-amylase, as well as intestinal maltase, increased due to the consumption of mixtures of plant extracts containing capsaicin, cinnamaldehyde, and carvacrol (Jang *et al*., 2007). Previous research has shown that including plant extract in the diet can result in a decrease in intestinal pathogenic bacteria (Park and Kim, 2020), an improvement in gut morphology and an increase in intestinal barrier function (Srinivasan, 2005). Therefore, it appears that by enhancing gut health (Table 6) through changing intestinal microflora due to silymarin, there is potential for improvements in nutrient digestion and absorption, which could subsequently lead to enhancements in growth performance. In this study, the productive performance of chickens and mortality had linearly improved in heat-stressed broilers fed silymarin-supplemented diets.

Hematological variables are currently used as health indexes in animals and humans, indicating potential infections, intoxication, dehydration, or blood clotting issues (Talebi *et al*., 2005). This study revealed that heat stress challenge resulted in a decrease in the hematocrit and hemoglobin and an

alteration in the leukocyte profile. It suggests that birds subjected to heat stress conditions experienced adverse effects on hematopoiesis and the immune response. The cardiovascular system in broiler chickens is a reliable indicator of physiological responses to stressors due to its susceptibility to temperature variations.

According to previous studies (Ayo and Ogbuagu, 2021; Kolluri *et al.,* 2022), heat stress has been found to cause a decrease in RBC count and hemoglobin concentration. The detrimental impact of heat stress on the hematological profile is emphasized in our findings, indicating potential implications for overall health and well-being. The investigation of leukocyte counts, specifically the H/L ratio, is crucial for identifying chronic stress conditions in avian species (Gross and Siegel, 1983). According to previous studies (Amiri *et al*., 2019; Ghasemi *et al*., 2021), exposing broiler chickens to heat stress challenge results in an increase in the H/L ratio. The study findings showed that adding silymarin supplements to the diet of the S2 and S3 groups improved the chickens' resistance to chronic heat stress conditions. This improvement in heat stress resistance was demonstrated by a decrease in the H/L ratio, which was comparable to that in the S0 group. Apparently, the hemodilution occurring during heat stress causes a decrease in hematocrit and hemoglobin, an adaptive response that allows for evaporative water loss, with most evaporative water loss coming from the extracellular compartment without compromising plasma volume (Borges *et al*., 2004).

Furthermore, according to previous studies (Borges, 1997), exposing broiler chickens to heat stress challenges results in a reduction in the number of lymphocytes and an increase in the number of circulating heterophils in chickens. In line with our research, Mohammad *et al*. (2015) stated that supplementing mice diet with silymarin increased HCT and HGB. Probably, silymarin has an antioxidant effect on red blood cell membranes by reducing oxidative damage, and in this way, it has improved the total number of red blood cells and subsequently improved blood hematocrit and hemoglobin radical (Dixit *et al*., 2009; Shaker *et al*., 2010; Ghosh *et al.*, 2010**;** Abdelazim, 2017).

Assessing blood biochemical characteristics, including serum metabolic profiles, enzyme activities, and hormone profiles, is widely used to evaluate animal health. This study revealed that heat stress had negative effects on broilers, as evidenced by an increase in triglyceride, total cholesterol, AST and ALT activity, and immune parameters, including IgM. Liver damage followed by an increase in serum levels of liver enzymes, including ALT, and AST levels (Huang *et al*., 2018; Cheng *et al*. 2019a; Lan *et al*., 2020) and an increase in serum cholesterol and triglycerides have been reported during heat stress in broiler chickens (Huang *et al*., 2018). In addition, heat stress adversely affects immune response (Attia *et al*., 2011; Abou-Shehema, 2013). Concerning serum lipid profile, broilers fed diets supplemented with various levels of silymarin showed a significant dose-dependent decrease in cholesterol and triglycerides. These results indicate a hypolipidemic effect of silymarin supplementation. The current findings align with those obtained by Metwally *et al*. (2009), who reported that rats fed silymarin had lower serum total lipid, triglyceride, and total cholesterol levels compared to the control group. It has been shown to boost the expression of LDL cholesterol receptors on hepatocytes, resulting in a higher LDL uptake by the liver hepatocytes and ultimately lowering blood LDL concentrations. Therefore, therapeutic plants such as silymarin have been shown to possess cholesterol-lowering effects (Ghasemi *et al*., 2014).

The current findings align with those obtained by Krecman *et al.* (1998,) who reported that broiler chicks fed silymarin had lower serum LDL-C, total lipid, triglyceride, and total cholesterol levels compared to the control group. Nassuato *et al*. (1991), in laboratory studies using silybin, noted that the possible effect of reducing serum lipids will be through the inhibition of the 3 hydroxy 3-methylglutaryl coenzyme A reductase, which is an important enzyme in cholesterol synthesis.

Regarding liver function parameters, ALT and AST levels exhibited a significant dose-dependent decrease in broilers fed silymarin compared to the control group. These findings indicate improved liver function parameters due to adding silymarin to broiler diets. The present study's findings of reduced liver enzyme activity support the safety of the studied herbal plant at the recommended dosage. These results are consistent with those obtained by Fathi *et al.* (2023, 2024) who reported decreased liver enzymatic activities following herbal supplementation. Our findings align with a previous investigation, which has demonstrated the positive effects of silymarin on hepatocytes. The protective effects on serum ALT and AST activity observed in our study when silymarin was used in the diet suggested that silymarin increased antioxidant defense in hepatocytes. Previous studies have demonstrated the antioxidant effects of silymarin supplementation in eliminating excessive oxidative substances and preserving the body's antioxidant system homeostasis in Japanese quails (Tahir *et al*., 2017; Khaleghipour *et al*., 2020), Bandarah chickens (Abou-Shehema *et al*., 2016), and broiler chickens (Shaker *et al.* (2010).

In this study, IgG and IgM levels were significantly increased by silymarin inclusion, suggesting an improvement in humoral immunity. The observed improvements in IgG and IgM could be

correlated with the birds' immune and health status due to herbal supplementation (Fathi *et al*., 2023, 2024). Several studies have demonstrated that natural products can exert various actions on the immune system, such as enhancing macrophage activity, promoting IL1(Oršolić and Jazvinšćak Jembrek, 2022), IL2, and IL4 production (Park *et al*., 2004). This finding indicates stimulation of the immune system's B lymphocytes by these cytokines, which subsequently transform into plasma cells to generate antibodies (Gharagozloo *et al.,* 2013).

Our results showed that, heat stress increased MDA levels in the serum, liver and spleen of heat-stressed broilers and oxidative stress developed. Our findings align with a previous investigation, which has demonstrated that heat stress induces high production of free radicals, including reactive oxygen species (ROS); so that the body's antioxidant system is not able to neutralize this high volume of free radicals, the oxidant-antioxidant balance of the body is disturbed and eventually contributed to oxidative stress (Lan *et al*., 2020; Fatima *et al*., 2022; Uyanga *et al*., 2022).

Increased levels of MDA production are a reliable marker of lipid peroxidation during oxidative stress that occurs during heat stress in broilers (Cheng *et al*., 2019 a,b). Also, Hu *et al.* (2021) reported that heat stress reduced the expression of serum antioxidant factors in broilers. Our findings also suggest that within the HS groups, the birds that were fed diets containing silymarin had the highest SOD (superoxide dismutase), and GSH-Px (glutathione peroxidase) levels. Consistent with our results, the antioxidant effects of silymarin have been reported in several studies (Dixit *et al*., 2009; Shaker *et al*., 2010; Ghosh *et al*., 2010; Abdelazim, 2017).

Our findings align with a previous investigation, which has demonstrated the antioxidant effects of incorporating a supplement containing silymarin on broilers during the summer season (Abdalla *et al*., 2018). Moreover, including silymarin in broiler chicken diets has been found to improve hepatic glutathione synthesis (Yu *et al.*, 2018). The activity of intestinal microflora and bacterial populations can be affected by various types of stress, especially heat stress. Heat stress can indirectly affect the feeding of broiler chickens through the effect on the hypothalamus's feeding center and leading to the decline of broiler growth performance (Wang, 2013). Moreover, stresses, especially heat stress, can directly affect the expression of bacterial genes in the intestine of a broiler and thus change the composition of the intestinal bacterial community in favor of harmful bacteria (Zampiga *et al*., 2018).

Our findings align with a previous investigation, which has demonstrated the positive effect of silymarin in increasing the population of beneficial bacteria (*Lactobacillus*) and reducing harmful bacteria (*E. coli* and Salmonella bacteria) in the cecum has been reported in previous research (Abdelazim, 2017; Shanmugam *et al*., 2022**)**. The dietary silymarin supplementation resulted in reduced bacterial counts in broiler chicks (Jahanian *et al.,* 2017***)***. As we all know, a decreased E. coli and ***an*** increase in Lactobacillus count in broilers results in better gastrointestinal health (Jin *et al.,* 2000). In this study, lactic acid bacteria (*Lactobacillus*) were linearly increased, and coliform bacteria (*E. coli*) showed a tendency to reduce by increasing the level of silymarin concentration in the diet.

Conclusion

In conclusion, supplementing broiler chickens with silymarin improved their growth performance and health status under HS conditions. The positive effects included reduced stress markers in blood and harmful bacteria in the cecum, as well as better antioxidant status. These findings indicate that the administration of silymarin may protect broiler chickens from HS and provide opportunities for further research on their mechanism of action. It is possible to conclude that adding silymarin to broiler feed at concentrations of up to 450 mg/kg of diet positively impacts liver function, immunoglobulin proteins, antioxidant status, lipid profiling, and cecum microbiota population.

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