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Probiotics and ascorbic acid improved radiographic bone density and mitigated oxidative stress and multiple organ dysfunction induced by heat stress in rats

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ABSTRACT

Heat stress is a substantial environmental challenge that adversely affect health and performance. This study aimed to investigate the effect of advanced short-term dietary supplementation of probiotics or vitamin C on serum biochemical parameters, antioxidant status, and radiographic bone density in heat-stressed rats. A 48 male albino rat were randomly allocated into six groups: Control, Heat stress, Probiotics, Probiotics+Heat stress, Vitamin C, Vitamin C+Heat stress group. Rats in supplementation groups received probiotics or ascorbic acid orally from week 1 to 8. Heat stress groups were subjected to elevated temperature (42±1 ˚C and relative humidity 65±2%) 60 minutes daily for 4 weeks starting from week 5 to 8, while other groups were maintained under standard laboratory conditions. Heat stress resulted in increased serum ALT, AST, urea and creatinine ($P<0.001$), decreased calcium, increased phosphorus and osteocalcin ($P<0.001$), increased serum MDA, decreased TAC ($P<0.001$), decreased radiographic bone density ($P<0.001$) compared to other groups. Histopathological examination of liver, kidney and adrenal glands reflected the ongoing heat-stress damage. Administration of probiotics and ascorbic acid demonstrated substantial protection, suggesting their potential efficacy against heat stress. The obtained findings hold promise for the development of novel strategies to enhance heat stress resilience in animals.

Introduction

Heat stress (HS) is a substantial environmental challenge that can adversely affect health and performance of human and animals (Díaz *et al*., 2006; Dou *et al*., 2020). It is a non-specific body response occurs when the amount of heat produced by the body surpasses the body's capacity to dissipate heat to the surrounding environment. Without effective first aid measures, increased body temperature may progress to a cascade of events including systemic inflammatory response, multiple organ dysfunction, disseminated intravascular coagulation, or even death (Li *et al*., 2021). Heat stress has a detrimental effect on metabolism by creating imbalance in glucocorticoids, adrenocorticotropic hormone, norepinephrine and growth hormone (Wang *et al*., 2015; Pragna *et al*., 2018). Additionally, it affects the proteins involved in the antioxidant-stress response and inflammatory response, including catalase, glutathione peroxidase, and C-reactive Protein (Dou *et al*., 2020; Song *et al*., 2020). Recent studies have indicated that glucocorticoids and reactive oxygen species induced by heat stress may have an influence in bone quality and mass resulting in skeletal damage (D'Amelio and Sassi, 2018; Zhang *et al*., 2021). Due to the lack of specific-heat stress medicinal therapy, prevention is essential rather than conservative treatment following exposure to high environmental temperature. Improved housing conditions and formulating nutritional regimens may have a positive influence in controlling the adverse effects of heat stress (Eskander *et al*., 2024). Intestinal barrier and gut microbiota seem to be more sensitive to heat stress compared to other organs. Heat stress alters the functional intestinal barrier which has a key role in the pathogenesis and pathophysiology resulting in gut-derived endotoxemia that triggers the systemic inflammatory responses and multiple organ injury (Lian *et al*., 2020; Ogden *et al*., 2020). As a result, the intestines can be thought to be the primary pathway for the progression of heat stress and maintaining the intestinal barrier may might help to prevent heat stress adverse effect on multiple organs (Li *et al*., 2021).

Probiotics are viable microorganisms that, when given in appropriate quantity may benefit the host. Dietary supplementation with probiotics was proven to inhibit the colonization of pathogenic bacteria, improve intestinal barrier and intestinal microflora (Hill *et al*., 2014). It has been suggested that probiotics have the potential to reduce the negative impacts caused by heat stress in poultry and laboratory settings (Deng *et al*., 2012; Lei *et al*., 2013; Song *et al*., 2017).

Ascorbic acid (vitamin C) is a water-soluble vitamin with potent antioxidant activity. Although vitamin C could be synthetized in many animal species (Zhou *et al*., 2020; Akinmoladun 2021; Du *et al*., 2022), human bodies cannot synthetize it due to the lack of L-gulonolactone lactone oxidase, an enzyme that convert glucose to vitamin C in the last biosynthetic pathway (Combs and McClung 2016). Subclinical vitamin C deficiency was diagnosed in mine workers during a 12-month period of employment with rapid decline in serum ascorbic acid during the first 3 months of employment in spite of adequate vitamin C intake (Visagie *et al*., 1973). It has been suggested that mine workers undergoing heat acclimatization may need to be supplemented with ascorbic acid (Strydom *et al*., 1976; Schneider, 2016). Even though vitamin C is naturally synthesized in most animal species, it is rapidly depleted in stress and diseased conditions resulting in decreased plasma concentration of ascorbic acid (Kim *et al*., 2012). In stress conditions, the animal's body consume vitamin C faster than its ability to synthetize it causing oxidative damage. It has been proposed that dietary supplementation of vitamin C during heat stress may mitigate the physiological and metabolic damage caused by heat stress through promoting the immune and antioxidant systems.

The direct effect of dietary probiotic and ascorbic acid supplementation for modifying bone health associated with heat stress, especially bone density and remodelling are largely lacking. We hypothesize that incorporating probiotic or ascorbic acid into the diet could potentially mitigate the adverse effects of heat stress, particularly when administered for advanced short duration.

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The aim of the present study was to investigate the ameliorative effect of the advanced short-term dietary supplementation of probiotics and vitamin C on serum biochemical parameters, antioxidant status, and bone density in rats subjected to heat-stress.

Materials and methods

Animals

The present study was conducted on 48 male Sprague Dawley albino rats weighing 170-200 g that were raised in specific and parasite free animal centre (Egyptian Holding Company for Biological Products and Vaccines-VACSERA, Giza, Egypt). Rats were grouped (4/cage) and housed in standard plastic cages (30.80×59.37×22.86 cm) with galvanized iron filter tops. Rats were maintained in ambient temperature of 22-25˚C and relative humidity 60-65% with a 12 h light/dark cycle. A standard rat diet with calculated nutritional parameters was provided, the compositions of the experimental diets are detailed in Table 1. Food and water were provided ad libitum. Rats were kept for 2 weeks for acclimatization before the start of the experiment.

All study procedures were done at the Department of Physiology-Faculty of Veterinary Medicine- Cairo University, Egypt. Experimental procedures were approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine- Cairo University (approval # VET/CU/20092022495) and done in according to the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments.

Table 1. Composition of the standard experimental diet fed to rats.

Ingredient	g/kg
Casein	200
L-cystine	3
Corn starch	150
Sucrose	500
Cellulose	50
Corn oil	50
Mineral mix S10001	35
Vitamin mix V10001	10
Choline bitartrate	2

Study Design

Rats were randomly allocated into 6 groups (n=8). The required number of rats was determined using a power analysis with an alpha level of 0.05 and a power of 0.80.

Group I: Control group (Control): rats were fed standard diet without heat stress.

Group II: Heat stress group (HS): rats were fed standard diet and subjected to heat stress.

Group III: Probiotic group: (Probiotic): rats were fed standard diet, supplied with probiotics without heat stress.

Group IV: Probiotic-heat stress group (Probiotic+HS): rats were fed standard diet, supplied with probiotics and subjected to heat stress.

Group V: Vitamin C group (Vit C): rats were fed standard diet, supplied with vitamin C without heat stress.

Group VI: Vitamin C- heat stress group (Vit C+HS): rats were fed standard diet, supplied with vitamin C and subjected to heat stress.

All rats were fed according to animal grouping starting from the beginning of the experiment (week 1-week 8), and heat stress was induced according to animal grouping (group II, IV, VI) starting from the 5th to the 8th week of the experiment. Rats were monitored daily and weighed weekly till the end of the study at 8 weeks.

Probiotics and vitamin C administration

A multi-strain commercially available probiotic powder (Bacterio-cell®, LANCOM Bio Pharm Co., USA) containing 5 strains (Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacilus brevis, Pediococcus pentosaceus, Enterococcus faecium) of bacteria was dissolved in water and administered once daily for 8 weeks at dose (3.3×10^8 CFU/ml/day) (Collins *et al*., 2017).

Ascorbic acid (Vitamin C® 500 mg, Vitabiotics Ltd., London, United Kingdom) powder was dissolved in water and was administered once daily for 8 weeks at a dose of 120 mg/kg/day) (Abu Zeid *et al*., 2018).

Heat stress protocol

An artificial heating chamber with controlled environmental temperature adjusted at 43.0±1.0˚C (Halder *et al*., 2020) and relative humidity of 65.0±2.0% was used to induced heat stress. Rats included in group II, IV and IV were placed in the heating chamber for 60 minutes daily for 4 weeks starting from the 5th week till the 8th week of the experiment. Rats were placed in the hot and humid conditions without food or water with continuous monitoring of the temperature. After 60 minutes of heat stress, rats were transferred to regular housing and allowed free access to food and water.

Blood and tissue sampling

Whole blood samples were collected from all rats at the end of the experiment through retrograde puncturing of the orbital venous plexus using capillary tubes. Plasma samples were taken on EDTA (1.5 mg/1 ml blood) for hematological examination. Sera were separated by centrifugation at 4000 rpm for 15 min and stored at -20˚C for biochemical analyses and at -80˚C for oxidative stress parameters.

Just following blood sampling, rats were euthanized using cervical dislocation under the effect of isoflurane inhalation anesthesia. Tissue samples were harvested from liver, kidney, and adrenal glands for histopathologic examination.

The left hind limb was collected from all rats and sent for radiographic bone density measurements.

Hematological examination

Complete blood count including red blood cells (RBCs) count, hemoglobin concentration (Hb), white blood cells (WBCs) count, and platelet count were measured using Coulter counter (ProCyte One Haematology Analyser, IDEXX, USA) (Kakel, 2013).

Serum biochemical parameters of liver and kidney function

Serum biochemical markers of internal organ injury were determined including Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), blood urea nitrogen, and creatinine were determined using commercial kits (Egyptian Company for Biotechnology-Spectrum diagnostics designs, Cairo, Egypt) and analysed using UV spectrophotometer (Jasco, V-730, Japan).

Serum calcium, phosphorous, and osteocalcin

Serum calcium concentration was measured spectrophotometrically at 565nm, using QCA calcium kits (Calcium Colorimetric Kit MAK002, Sigma-Aldrich, USA). At alkaline pH, calcium forms a coloured complex with O-cresolpthalein, 8-hydroxyquinolein is added to the reagent as a chelating agent of magnesium ions which can interfere with the reaction (Baginski *et al*., 1973). Serum inorganic phosphorus was measured spectrophotometrically at 625nm (Phosphate Colorimetric Kit MAK030,

Sigma-Aldrich, USA).

Serum osteocalcin, a late marker of osteoblastic activity and bone formation marker, was determined using rat-specific osteocalcin using Enzyme-Linked Immunosorbent Assay (ELISA) kit obtained from Sigma Chemical Co. (Cairo, Egypt) catalogue no. E-EL-R0243.

Serum corticosterone level

Serum corticosterone level was determined using rat-specific cortisone ELISA kit obtained from (Sigma Chemical Co., Cairo, Egypt) catalogue no. E-EL-0160.

Oxidative stress biomarkers

Total antioxidant capacity (TAC) and Malondialdehyde (MDA) concentrations were determined using commercial kits provided by Bio-diagnostic Company, Cairo, Egypt (Catalogue no. TA 25 13 and MD 25 29).

Histopathological examination

Tissue samples were harvested from the liver, kidney and adrenal gland and fixed in neutral buffered formalin for 48 hours. Samples were routinely processed, embedded in paraffin wax, sectioned at 5 µm, stained with H&E for histopathological examination (Bancroft and Cook, 1994).

Radiographic bone density measurement

Digital radiographs were taken for the left femur of all rats using Poskom machine (PCMAX-60H(LED), Poskom Co., Gyeonggi-do, Korea) using the same automated setting and read by digital radiography system. Standardization of radiographic density was made through an aluminium step wedge (1mm/10 step) of uniform density that was positioned next to bone samples during the same radiographic exposure.

The obtained radiographic images were analysed using Sante DICOM Software (Sante DICOM viewer, SanteSoft LTD, Nicosia, Cyprus). To create a calibration curve, the radiographic density was measured on a circular region of interest on each step of the wedge. The bone density of all left femurs was then measured in triplicates from lateral radiographs, using a circular region of interest selected at the proximal third of the femur. After calibrating the images to optical density, the automatic selection tool was employed to delineate the bone area. The mean grey intensity of each pixel within the outlined region was then recorded (Kinds *et al*., 2011; Castro *et al*., 2019).

Statistical analysis

Data were tabulated and presented as mean±standard error. Normality of the data was tested using Kolmogorov-Smirnov test. A one-way analysis of variance (ANOVA) was used to determine statistically significant differences between groups. When statistically significant differences were detected, LSD test was used for pairwise comparison. Statistical significance was set at $P < 0.05$. Data were analysed using the Statistical Package for Social Sciences (SPSS Version 28, SPSS Inc., Chicago, IL, USA).

Results

Body weight

The initial and final body weight of the rats included in the study did not change significantly at the start and end of the experiment in all groups ($P > 0.05$). A slight decrease in body weight of rats in heat stress group, however this decrease was not statistically significant.

Hematological examination

RBCs count, platelet count and Hb concentration was significantly increased in heat-stress group compared to all groups ($P< 0.001$). The WBCs count was significantly increased in all groups compared to control group ($P< 0.001$) (Fig. 1).

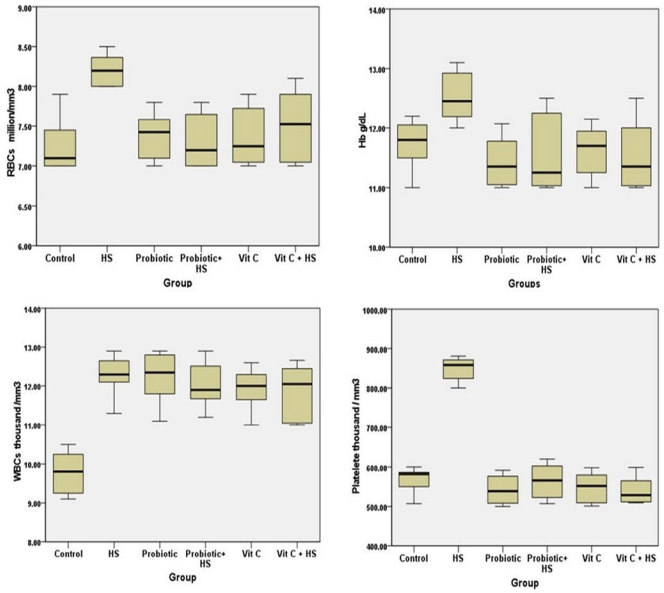

Fig. 1. Box plot representation of hematological examination of control, heat stress (HS), Probiotic, Probiotic+ heat stress, Vitamin C, Vitamin C+ heat stress evaluated at 8 weeks. A statistically significant increase in red blood cells (RBCs), platelet and hemoglobin (Hb) was recorded in HS group compared to other groups ($P < 0.001$). The white blood cells (WBCs) count was significantly increased in control group compared to other groups ($P < 0.001$).

Serum biochemical parameters of liver and kidney function

Serum activity of ALT and AST levels demonstrated a statistically significant increase liver function tests in heat-stressed rats compared to all other groups ($P<0.001$).

Dietary supplementation with vitamin C did not change serum ALT and AST without (Vit C group) or with heat stress (Vit C+HS group) compared to control group ($P=1.000$, 0.929 and 0.936, 0.440 respectively).

Probiotic supplementation resulted in statistically significant decrease in serum ALT and AST levels both without heat stress (Probiotic group) and with heat stress (Probiotic+HS group) compared to control rats ($P< 0.001$). No statistically significant differences were recorded in ALT and AST levels both in probiotic and probiotic+HS groups ($P=1.000$) (Table 2).

Table 2. Liver and kidney function parameters of control, heat stress, Probiotic, Probiotic+ heat stress, Vitamin C, Vitamin C+ heat stressed rats evaluated at 8 weeks.

Parameter	Control	HS	Probiotic	Probiotic-HS	Vitamin C	Vitamin C-HS
ALT (IU/L)	54.82±0.73[a]	58.61±0.64[b]	51.38±0.26[c]	51.49±0.41[c]	54.69±0.43[a]	55.35±0.28[a]
AST (IU/L)	90.50±1.72[a]	105.00-1.56[b]	79.62±0.92[c]	80.62±1.75[c]	88.62±1.45[a]	84.88±0.88[a]
Urea (mg/dl)	47.15±0.92[a]	59.38±1.49[b]	43.96±0.52[a]	42.44±0.52[c]	45.21±0.70[a]	42.10±0.58[c]
Creatinine (mg/dl)	0.78±0.00[a]	0.88±0.01b	0.71-0.01[b]	0.72±0.01[b]	0.76±0.01[a]	0.78±0.01[a]

* Data are presented as mean±SE. Different superscript letters in the same row indicate statistically significant differences. (ALT: alanine aminotransferase, AST: aspartate aminotransferase, HS: heat stress)

Blood urea nitrogen was significantly increased in heat stress group compared to all groups (P<0.001). Rats in probiotic+HS and Vit C+HS groups had significantly lower blood urea level compared to control rats (P=0.005 and 0.002 respectively).

Serum creatinine level of heat stress group was significantly increased compared to all groups (P < 0.001). Probiotic and probiotic+HS groups had significantly lower creatinine level compared to control rats (P<0.001). While creatinine level did not change significantly between Vit C, Vit C+HS groups compared to control group (P=0.240 and 1.000 respectively) (Table 2).

Serum calcium, phosphorous, and osteocalcin

Serum calcium level was significantly decreased in heat stress group compared to all groups (P<0.001). Rats supplied with vitamin C without heat stress had significantly higher serum calcium level compared to all groups (P<0.001). While Vit C+HS group had significantly increased calcium level compared to control and heat stress groups (P<0.001), while this increase was not statistically significant compared to probiotic (P=0.972) and probiotic +HS (P=0.911) groups.

A statistically significant decrease in serum phosphorus concentration was detected in heat stress group compared to all groups (P< 0.001). No statistically significant differences were detected in phosphorus level in all other groups (P>0.05).

Serum osteocalcin was significantly decreased in heat stress group compared to all groups (P<0.001). Control group had significantly lower osteocalcin level compared to all groups with dietary supplementation (P<0.001). No statistically significant differences were recorded in osteocalcin level of probiotic and Vit C group (P=1.000) or probiotic+HS and Vit C+HS group (P=0.983) (Table 3).

Serum corticosterone level

Serum corticosterone level was significantly increased in heat stress group compared to all groups (P<0.001). No significant differences were recorded in corticosterone level of probiotic and Vit c groups compared to control group (P=0.115 and 1.000 respectively). No significant differences were recorded in between probiotic +HS and Vit C+HS groups (P=0.990) (Table 4).

Oxidative stress biomarkers

A statistically significant sharp increase in serum MDA activity was detected in rats exposed to heat stress compared to all groups (P< 0.001). No statistically significant difference between MDA level in Vit C group compared to control rats (P=1.000) and probiotic group (P=1.000). While a statistically significant difference in MDA activity was recorded between Vit C group and Vit C+HS (P<0.001) and probiotic+HS group (P<0.001).

Rats exposed to heat stress had significantly lower TAC compared to all groups (P<0.001). TAC did not change significantly in Probiotic and Vit C groups compared to control group (P=0.974 and 0.983 respectively). No significant differences were recorded in TAC between probiotic and vit C groups (P=1.000) or between probiotic+HS and Vit C+HS groups (P=0.854) (Table 4).

Histopathological examination

Histopathological sections obtained from the liver of control rats demonstrated typical hepatic architecture with hexagonal lobules and acini. The hepatocytes were arranged in cords radiating from the central veins with the presence of portal triad containing branches of the portal vein, hepatic artery, and bile duct.

Rats exposed to heat stress demonstrated moderate histopathological changes in hepatic sections. Moderate disarrangement of the hepatic cords was visualized with moderate congestion of the hepatic artery, sinusoids, and portal veins, along with dilation of the portal veins. Focal inflammatory cell infiltration, hemorrhage within blood sinusoids and pyknotic nuclei were also detected (Fig. 2).

No remarkable histopathological changes were recorded in liver sections obtained from both probiotic and vitamin C-treated rats.

Rats supplemented with probiotics and exposed to heat stress demonstrated normal hepatic architecture with mild mononuclear cell infiltration while rats supplemented with vit C and exposed to heat stress had normal hepatic architecture with mild congestion of blood sinusoids and presence of pyknotic nuclei (Fig. 2).

Histopathological sections obtained from the kidney of control rats demonstrated normal architecture of the glomeruli, Bowman's spaces in between and normal tubular structure.

Heat-stressed rats demonstrated moderate shrunken glomeruli with dilatation of the urinary spaces and focal lymphocytic infiltration and interstitial hemorrhage. No remarkable changes were recorded in kidney sections obtained from probiotic and vit C groups with or without heat stress (Fig. 3).

Control rats demonstrated normal adrenal cortex and medulla with well distinct three cortical zones (zona glomerulosa, zona fasciculata, zona reticularis). The cells had oval to rounded nuclei and basophilic cytoplasm. Heat stressed rats demonstrated moderate degenerative changes, inflammatory cell aggregates, hemorrhage, vacuolated cells with pyknotic nuclei. No remarkable changes could be detected in adrenal gland microstructure obtained from probiotic and vit C groups with or without heat stress (Fig. 4).

Table 3. Serum calcium, phosphorus and osteocalcin levels of control, heat stress (HS), Probiotic, Probiotic+ heat stress, Vitamin C, Vitamin C+ heat stressed rats evaluated at 8 weeks.

Parameter	Control	HS	Probiotic	Probiotic-HS	Vitamin C	Vitamin C-HS
Calcium (mg/dl)	8.69±0.98^{a}	7.40±0.20^{b}	10.88±0.23^{c}	10.81±0.21^{c}	12.09±0.22^{d}	11.09±0.18^{c}
Phosphorus mg/dl)	4.50±0.07^{a}	3.53±0.10^{b}	4.37±0.11^{a}	4.43±0.09^{a}	4.37±0.10^{a}	4.29±0.09^{a}
Osteocalcin (g/mL)	4.99±0.31^{a}	2.96±0.29^{b}	10.84±0.22^{c}	8.70±0.41^{d}	10.95±0.18^{c}	8.97±0.24^{d}

* Data are presented as mean±SE. Different superscript letters in the same row indicate statistically significant differences. (HS: heat stress)

Table 4. Oxidative stress parameters of control, heat stress, Probiotic, Probiotic+ heat stress, Vitamin C, Vitamin C+ heat stressed rats evaluated at 8 weeks.

Parameter	Control	HS	Probiotic	Probiotic-HS	Vitamin C	Vitamin C-HS
MDA (mmol/L)	5.65±0.13^{a}	22.67±0.23^{b}	5.77±0.07^{a}	7.85±0.20^{c}	5.70±0.24^{a}	7.66±0.13^{c}
TAC (mmol/L)	0.77±0.01^{a}	0.45±0.01^{b}	0.76±0.01^{a}	0.65±0.01^{c}	0.76±0.02^{a}	0.63±0.01^{c}
Corticosterone (µg/L)	17.38±0.19^{a}	21.71±0.14^{b}	16.70±0.21^{a}	18.96±0.21^{c}	17.42±0.09^{a}	18.80±0.22^{c}

* Data are presented as mean±SE. Different superscript letters in the same row indicate statistically significant differences. (HS: heat stress; MDA: malondialdehyde, TAC: total antioxidant capacity.

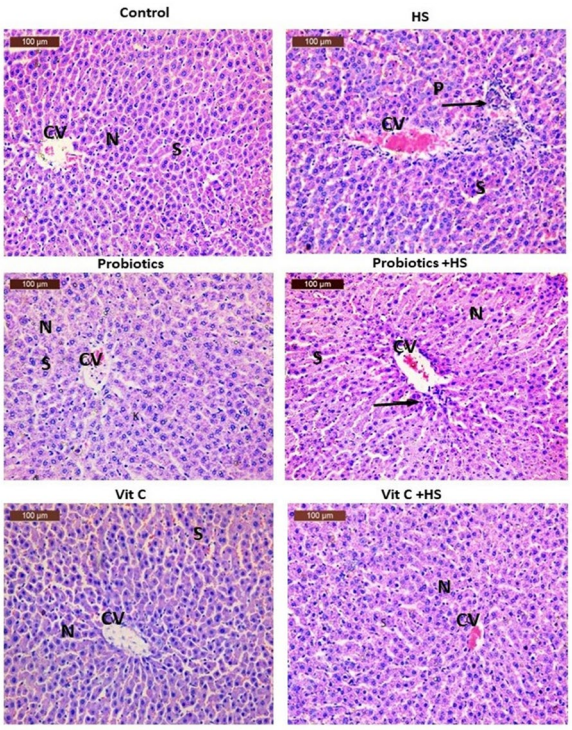

Fig. 2. Photomicrograph of liver sections stained with H&E of control, heat stress (HS), Probiotic, Probiotic+ heat stress, Vitamin C, Vitamin C+ heat stress groups evaluated at 8 weeks. Rats exposed to heat stress demonstrated moderate disarrangement of the hepatic cords with moderate congestion of the hepatic artery, sinusoids (S), and central veins (CV), along with dilation of the portal veins (P). Focal inflammatory cell infiltration, hemorrhage within blood sinusoids and pyknotic nuclei were also detected. No remarkable histopathological changes were recorded in liver sections obtained from both probiotic and vitamin C-treated rats (scale bar= 100µm).

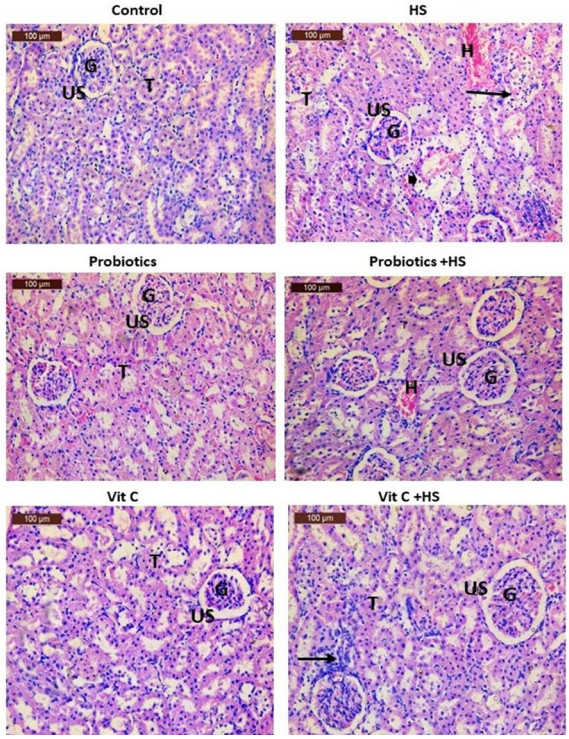

Fig. 3. Photomicrograph of kidney sections stained with H&E of control, heat stress (HS), Probiotic, Probiotic+ heat stress, Vitamin C, Vitamin C+ heat stress groups evaluated at 8 weeks. Heat-stressed rats demonstrated moderate shrunken glomeruli (G) with dilatation of the urinary spaces (US) and focal lymphocytic infiltration and interstitial hemorrhage (H). No remarkable changes were recorded in kidney sections obtained from probiotic and vit C groups with or without heat stress (scale bar= 100µm).

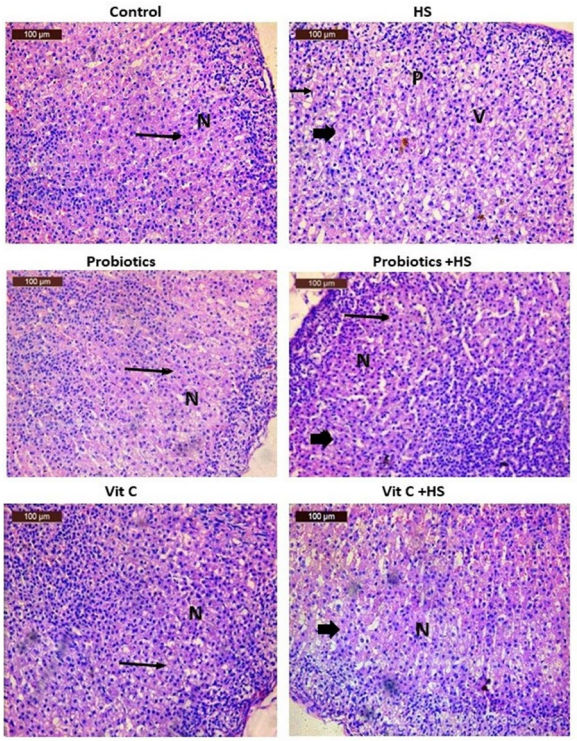

Fig. 4. Photomicrograph of adrenal gland sections stained with H&E of control, heat stress (HS), Probiotic, Probiotic+ heat stress, Vitamin C, Vitamin C+ heat stress groups evaluated at 8 weeks. Heat stressed rats demonstrated moderate degenerative changes, inflammatory cell aggregates, hemorrhage, vacuolated cells (V) with pyknotic nuclei (P). No remarkable changes could be detected in adrenal gland microstructure obtained from probiotic and vit C groups with or without heat stress (scale bar= 100µm).

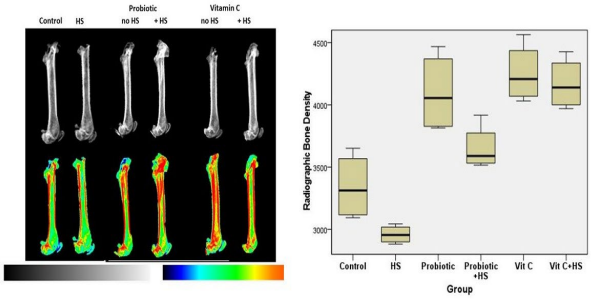

Fig. 5. Representative digital radiographs of the left femur of rats in different groups. Radiographic bone density is represented in color scale correlated to bone radiodensity and radiolucency radiographic femoral bone density was changed significantly among different groups. Rats subjected to heat stress demonstrated decreased bone density compared to control (P=0.03) and all other groups (P<0.001). Boxplot visualization of radiographic bone density demonstrated a statistically significant increase in bone density was recorded in rats supplemented with probiotics (P<0.001) and ascorbic acid (P<0.001) compared to control and heat stressed rats. Heat-stressed rats supplemented with probiotics and ascorbic acid also demonstrated statistically significant increase in bone density compared to control and heat stressed rats (P=0.020 and 0.005 respectively).

Radiographic bone density

Radiographic femoral bone density was changed significantly among different groups (P< 0.001). Rats subjected to heat stress demonstrated decreased bone density compared to control (P=0.03) and all other groups (P<0.001) (Fig. 5).

A statistically significant increase in bone density was recorded in rats supplemented with probiotics (P<0.001) and ascorbic acid (P<0.001) compared to control and heat stressed rats.

Heat-stressed rats supplemented with probiotics and ascorbic acid also demonstrated statistically significant increase in bone density compared to control and heat stressed rats (P=0.020 and 0.005 respectively).

Discussion

The present study documented the protective role of probiotics and ascorbic acid in mitigating the negative impact of heat stress on physiological parameters and bone health in rats, therefore our hypothesis was confirmed. Excessive exposure to heat has triggered a series of physiological responses that was manifested by the reduction body weight,

hemoconcentration, multiple organ dysfunction, oxidative stress damage and altered bone health that could be ameliorated by dietary supplementation of probiotics or vitamin C.

The initial negative impact of heat stress is "dehydration" that occurs due to evaporative heat loss, excessive sweating, and fluid loss to maintain core body temperature along with decreased water intake. Dehydration usually results in electrolyte imbalance, increased serum sodium levels (hypernatremia), hemoconcentration as indicated by the elevated RBCs and WBCs count (Odo *et al*., 2019). Dehydration also results in increased in blood urea nitrogen and creatinine levels due to decreased fluid intake, reduced blood flow to the kidney with subsequent decreased glomerular filtration rate and decreased renal function as documented in the current study by the elevated urea and creatinine levels in blood and confirmed by the ongoing histopathological changes in the kidney following exposure to heat stress. Additionally, heat stress had negative impact on liver function (Odo *et al*., 2019). The elevated liver enzymes have indicated liver injury that was confirmed by histopathological changes detected in heat-stressed rats.

Histopathologic changes detected in the adrenal gland in heat-stressed rat and the associated increased oxidative stress parameters could be attributed to the release of adrenocortical hormones triggered by heat stress. It has been reported that increased adrenocortical hormones decreases ascorbic acid level within the adrenal gland (Strydom *et al*., 1976). Normally, ascorbic acid inhibits the synthesis of adrenocortical hormones, but with insufficient vitamin C stores, the production of these hormones continues even after the stress has ceased. This can potentially result in adrenal fatigue and a lack of production of anti-stress hormones (Strydom *et al*., 1976). In the present study, heat stress has resulted in elevated serum TAC, MDA and corticosterone levels indicating the presence of heat-induced cellular damage, reflecting the ongoing systemic oxidative damage and release of free radicals.

Quantitative evaluation of radiographic bone density revealed that heat-stressed rats had reduced bone density compared to other groups. There is a lack of direct evidence regarding how high temperatures modify bone health, particularly bone remodelling. Heat stress may be associated with decreased feed consumption and deficient calcium intake, decreased nutrient absorption. The impaired gut integrity and the increased systemic inflammation and the activation of innate immune system was found to provoke osteoclastic activation and induce bone resorption (D'Amelio and Sassi, 2018). Osteoclasts are the main cells responsible for bone resorption, osteoclastic activation results in accelerated bone breakdown contributed to decreased bone density. Another proposed mechanism is the impact of heat stress on vitamin D metabolism responsible for calcium absorption and bone health. Heat can alter vitamin D synthesis in the skin and reduce activation of vitamin D by the kidney that subsequently affect bone health (Zhang *et al*., 2021). Additionally, dehydration may negatively affect bone health by impairing the delivery of nutrients to bone cells and hinder the clearance of waste products compromising bone integrity. Moreover, excess glucocorticoids and reactive oxygen substances that are triggered by heat stress, have been found to interfere with both bone formation and resorption, ultimately contributing to skeletal damage (Wauquier *et al*., 2009).

Ascorbic acid, supplemented as vitamin C, is a potent antioxidant acting as a scavenger of free radicals which are highly reactive molecules causing cellular damage (Alhassan *et al*., 2016). Results obtained in the current study demonstrated that vitamin C neutralized the oxidative damage induced by heat stress and help to protect tissue against damage.

It has been reported that vitamin C supplementation enhances heat exchange between the body and the surroundings, while also regulating oxygen consumption by controlling physiological and metabolic changes (Minka and Ayo, 2012). Dietary supplementation of vitamin C has been shown to improve growth performance, alleviate stress-related metabolic symptoms, enhance immune function, and reduce mortality (Abidin and Khatoon, 2013; Shakeri *et al*., 2019). However, the exact mechanisms by which vitamin C promotes the function of the antioxidant and immune systems are not fully understood (Watson *et al*., 2010; Sorice *et al*., 2014). Previous studies have indicated that vitamin C influences the production of cell adhesion factors in phagocytes, inflammatory cytokines, lymphocytes, and monocytes (Watson *et al*., 2010; Sorice *et al*., 2014). In comparison to other drugs used for treating heat stress, vitamin C has demonstrated a non-pressurizing, safer, and more practical effectiveness in alleviating stress. Furthermore, vitamin C is affordable, easy to administer and absorb, non-toxic, does not require a withdrawal period, and does not have any after-effects of overdose (Seifi *et al*., 2010).

In the present study, vitamin C has resulted in statistically significant increase in radiographic bone density in rats compared to control and probiotic groups which could be attributed to the additional role of vitamin C in maintaining bone health. Vitamin C is antioxidant protecting bone cells from oxidative stress and maintain normal bone remodelling. It prevents or reduces the inflammatory reaction and bone loss by inhibiting osteocyte apoptosis and mitigating osteoclastic activity which ultimately increase osteoblast activity and induce osteogeneisis (Domazetovic *et al*., 2017). Besides being antioxidant, vitamin C is essential for collagen production, a crucial component of bone tissue providing the structural support of bone to maintain strength and integrity. Moreover, vitamin C has been reported to promote osteoblastic differentiation and activity which are the main cells responsible for new bone formation. Vitamin C also enhances the absorption of calcium from diet contributing to optimum bone mineralization and density.

Results obtained from the present study revealed that probiotics had a beneficial effect to countermeasure the effect of heat stress which could be attributed to their role in enhancing intestinal barrier function and modulating gut flora. Pre-administration of Bacillus licheniformis for 7 days had a preventive effect against heat stroke in rats by sustaining the intestinal barrier, attenuating multiple organ injury and decreasing serum inflammatory cytokines (Li *et al*., 2021). During exposure to heat stress, excessive heat cannot be dissipated from the body and body temperature homeostasis cannot be maintained producing a direct thermal injury and heat cytotoxicity as an acute phase response with subsequent vascular endothelial damage (Li *et al*., 2021). Furthermore, the cutaneous blood flow increases to aid in heat loss and diminish heat production, while the visceral blood flow is reduced as a means of compensation. If the reduced visceral blood flow persists for a long time, visceral ischemia can lead to oxidative stress, damage to the functional gut barrier, disruption of the microbiota, and ultimately intestinal injury (Lian *et al*., 2020; Li *et al*., 2021). Hence maintaining the intestinal barrier by probiotic administration plays a key role in prevention of heat stress onset and supress the subsequent pathological cycle.

The interaction between gut microbiota and the immune system likely plays a significant role in bone metabolism. In mice, oral supplementation of probiotics has been reported to alter gut microbiota and was associated with bone development and remodelling and change in mechanical strength of bone through its effect on the immune system, endocrine system, and calcium absorption. On the other hand, altering gut microbiota has been linked to decreased bone density and mass both in trabecular and cortical bone (Chen *et al*., 2017; D'Amelio and Sassi, 2018). Mice that are lacking gut microbiota exhibit a reduced number of osteoclasts and lower levels of interleukin-6 (IL-6), receptor activator of nuclear factor-κ B ligand (RANKL), tumor necrosis factor alpha (TNF-α), and CD4+ T cells in their bones (Sjögren *et al*., 2012; Ohlsson *et al*., 2017). In addition, probiotics enhance the absorption of minerals especially calcium by increasing the production of short-chain fatty acids that promote calcium absorption and influence bone metabolism. The evidence linking the direct effect of probiotics and bone metabolism is still limited. Further studies are required to understand the exact mechanism linking probiotics, and bone health, the optimum probiotic strain and optimum dosage which should be considered in future studies.

Limitations of the present study may include the lack of a parallel bone histologic study documenting the ongoing changes in bone morphometry. Further investigations are warranted to elucidate the underlying molecular mechanisms and optimize the dosage and duration of probiotic and ascorbic acid supplementation.

Conclusion

The present study highlighted the potential efficacy of probiotics and ascorbic acid in mitigating the adverse effects of heat stress in rats. These findings hold promise for the development of novel strategies to enhance heat stress resilience that could be applied in human and animals.

Conflict of interest

The authors have no conflict of interest to declare.

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