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Mitigating the negative effects of heat stress in broiler chickens using *Saccharomyces cerevisiae* and ascorbic acid

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DECLARATION

I, Victory Osirimade Sumanu declare that the thesis which I hereby submit for the degree of Doctor of Philosophy (PhD) in the Department of Anatomy and Physiology at the University of Pretoria is my own work. I have not submitted the content of this thesis for a degree at this or any other tertiary institution.

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CERTIFICATION

I, Prof Joseph Panashe Chamunorwa do hereby certify that this project was carried out by Dr Victory Osirimade Sumanu in the Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, Pretoria, under my supervision.

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But by the grace of God I am what I am: and His grace which *was bestowed* upon me was not in vain; but I laboured more abundantly than they all: yet not I, but the grace of God which was with me. 1 Corn. 15:10 (KJV)

DEDICATION

I dedicate this work to the Glory of God, my Help, Provider, Protector, Guarantor and the Lifter of my head

And
To my adorable and precious husband Dr Kennedy Ejebhiare Osuidia and my children.

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ABSTRACT

The production of broiler chickens is often affected by fluctuating environmental conditions leading to loses economically during the hot summer season globally. This has resulted in calls to evaluate the non-ventilating measures available for alleviating excessive heat stress in broiler chickens. Antistress and/or antioxidant agents are readily available and potent agents that are considered in mitigating the negative effect of heat stress in broiler chickens' production. The research focused on mitigating the negative effects of heat stress using *Saccharomyces cerevisiae* and ascorbic acid in broiler chickens. Fifty-six broiler chicks were divided into 4 groups of 14 each, control, probiotic-administered, ascorbic acid-administered and probiotic + ascorbic acid-administered. Broiler chickens were fed diet fortified with probiotic at a dose of 1 g/kg of feed and ascorbic acid at a dose of 200 mg/kg of feed from day (D) 1 to D35 of the study period.

Cloacal temperature (CT), temperature-humidity index, dry-bulb temperature and relative humidity and in the pen were obtained bi-hourly, from 07h00 – 19h00, while body surface temperature (BST) was measured thrice on D21, D28 and D35 of the study period. Feed intake, water intake and body weight were measured on D7, D14, D21, D28 and D35 of the study. Behavioural parameters were measured on D21, D28 and D35 of the study period. Interleukin 10 (IL-10) gene expression; 8-hydroxyl-2-dioxyguanosine (8-OHdG), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and malondialdehyde (MDA) activities and concentration, respectively, as well as erythrocyte osmotic fragility (EOF), haematology and small intestinal morphology, were evaluated after sacrificing the chickens at the end of the experiment.

Ambient temperature parameters were outside the thermoneutral zone which was indicative of thermal stress during this study. Cloacal temperature and body surface temperature (BST) obtained in the treatment groups were significantly higher ($P < 0.0001$) than those of the control. Water intake and body weight were significantly higher ($P < 0.01$) in the treatment groups when compared to the control. Improved tonic immobility and vigilance parameters were obtained in the treatment groups when compared with the control group. The administered antioxidants were efficacious in reducing the expression of oxidative gene damage and enhancing that of

interleukin-10. Superoxide dismutase, CAT, GPx, EOF and some haematological parameters were significantly lower ($P < 0.0001$) in the treatment groups when compared with the control. Small intestinal morphometry and goblet cells count were significantly higher ($P < 0.0001$) in the treatment groups in comparison with the control group. Interestingly, chickens on probiotic and/or ascorbic acid did not display the detrimental effects of heat stress compared to the control group and this was evident in their performance indices. We therefore conclude that both probiotic and ascorbic acid, anti-stress and antioxidant agents show potential to be effective in mitigating the negative effects of heat stress in broiler chickens, while the best performance was obtained in the *Saccharomyces cerevisiae* group of broiler chickens. Based on this result, production scale studies are recommended.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
A_s	Average surface area of the chickens
AT	Ambient temperature
BST	Body surface temperature
BRW	Washing buffer
CAT	Catalase
CRH	Corticotropic releasing hormone
CT	Cloacal temperature
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EL	Erythrocyte lysis
ELISA	Enzyme-linked immunosorbent assay
EOF	Erythrocyte osmotic fragility
GPx	Glutathione peroxidase
h	Hour of the day
hc	Heat transfer coefficient
HRP	Horseradish peroxidase

HSP Heat shock proteins

IL-10 Interleukin-10

MDA Malondialdehyde

MC Body mass

OVARU Onderstepoort Veterinary Animal Research Unit

OD Optical density

OFT Open-field test

PNMT Phenylethanolamine N-methyltransferase

Q_c Convective and conductive heat loss

RH Relative humidity

RLT Lysis buffer

RNA Ribonucleic acid

ROM Reactive oxygen metabolites

ROS Reactive oxygen species

RPE Mild washing buffer

RT-PCR Reverse transcription polymerase chain reaction

RW Washing buffer

SOD Superoxide dismutase

T_{at} Ambient temperature

THI Temperature-humidity index

TI Tonic immobility

TNZ Thermal neutral zone

$V_{ar,}$ Air velocity

V Vigilance

8-OHdG 8-hydroxy-2′-deoxyguanosine

CHAPTER ONE

1.0 INTRODUCTION

Stress can be described as the results of adverse effect of a management system/or an environment that causes changes in the behaviour or physiology of an animal (Sinkalu and Ayo, 2018; Aksoy et al., 2021). The body responds to stressors in a very non-specific way that has been termed the general adaptation syndrome (Gogoi et al., 2021). Anything that interrupts psychological and/or physiological stability is termed a stressor and the reaction to a stressor is what is called stress. Behavioural changes are mostly the primary responses and therefore first signs of stress. Stress is detrimental in livestock production because it decreases or impairs the animals' ability to fight against diseases and gain ample live weight (Aluwong et al., 2017). Fluctuating ambient temperature that occurs naturally during the hot season (summer) leads to temperatures above the thermoneutral zone in the tropics and sub-tropics, thereby inducing heat stress (Kim et al., 2021). This is often evident as high cloacal and body surface temperatures, poor performance, high morbidity and mortality rates of the chickens.

High relative humidity (RH) and ambient temperature (AT) are meteorological factors that have adverse effects on poultry production globally, including South Africa (Mutibvu et al., 2017). Heat stress is one of the dangers poultry production faces in the sub-tropical and tropical countries due to the increased climatic changes as a consequence of global warming (Egbuniwe et al., 2018; Lu et al., 2024). Broiler chickens, when heat stressed, reduce feed and water consumption in order to reduce the production of metabolic heat in the body (Gogoi et al., 2021). Heat stress also results in decreased growth performance of the birds due to decreased feed intake (Kim et al., 2021), and stimulates increased release of corticosterone, decreases

percentage of phagocytising macrophages and increases mortality (Archer, 2023). Reactive oxygen species (ROS) are chemically reactive chemicals that are produced as a by-product of oxygen metabolism. Reactive oxygen species have been shown to be responsible for the induction of lipoperoxidation of the cytomembranes resulting in cell destruction (Makeri et al., 2017).

Antioxidants are efficacious in mitigating peroxidation as they transfer their electrons to free radicals (Lee et al., 2019). Free radicals that gain electrons from an antioxidant do not have the efficacy to attack the cell, thus electron donation by antioxidant breaks the chain reaction of oxidation process. Hence, antioxidants are used widely as dietary supplements which are beneficial against tissue damage induced by oxidative stress (Sumanu et al., 2021). The enrichment of diets with antioxidant substances like probiotics, ascorbic acid, zinc gluconate, fisetin and other phytonutrients, may be of benefit to ameliorate environmental stress effect in chickens (Aluwong et al., 2017; Egbuniwe et al., 2018). Ascorbic acid protects the body against ROS deleterious effects. It achieves this by preventing the excessive production of ROS (Egbuniwe et al., 2021).

There are different strains of probiotics which are either the yeast probiotics or bacterial probiotics. Yeast probiotics are very rich in vitamin B, protein, fat and enzymes such as phytase and cellulose (Aluwong et al., 2017). Their cell walls contain 31% mannan, 1-2% chitin, 29-64% betaglucan and mannan oligosaccharide (MOS) which are known to be immune stimuli (Aluwong et al., 2013). Yeast probiotic cell walls contain antioxidant enzymes which prevents the excessive production of ROS (Aluwong et al., 2017). Therefore, yeast probiotics (such as *Saccharomyces cerevisiae*) may serve as an anti-stress, antioxidant and growth promoting agent.

Anti-inflammatory cytokines are produced in large quantity to inhibit inflammatory processes that occurs during heat stress. The IL-10 is a potent anti-inflammatory cytokine that mitigates the damage caused by the pro-inflammatory cytokines. It functions via the activation of mitogen-activated protein kinases (Arendt et al., 2019) during heat stress. Heat shock proteins (HSP) are also produced in the phase of stress. Heat shock protein inhibits apoptosis and provides cells with thermal stability in the phase of stress (Siddiqui et al., 2020). They are found in the liver, thymus and testis.

Broiler chickens exposed to heat stress also experience DNA damage. The concentration of 8-hydroxy-2′-deoxyguanosine (8-OHdG) serves as an oxidative gene damage biomarker for analysing the level of DNA damage in broiler chickens experiencing heat stress, it (Sumanu et al., 2023). The concentration of 8-OHdG is assayed via urine and/or serum samples (Zhu et al., 2019).

Behavioural responses to heat stress include various processes employed by broiler chickens to enhance heat loss via thermoregulation (Calefi et al., 2019). Fear is one of the components of stress in poultry. Increase in fear responses impairs their feed intake which subsequently impairs their live weight gain and might lead to disease conditions or death if not mitigated (Egbuniwe et al., 2018).

1.1 STATEMENT OF THE RESEARCH PROBLEM

The production of broiler chickens is a great source of income to farmers since broiler chicken producers can have about 8 to 10 production cycles per annum (Mohammed et al., 2018). Broiler chickens are easy to handle, and this has prompted a great deal of individuals to venture into

broiler chicken production, both subsistence and commercially. In Africa, especially South Africa broiler chicken production is affected adversely by thermal environmental conditions, inflicting heavy economic losses on the farmers (Mutibvu et al., 2017). High RH and AT, and the fluctuations occurring during the hot season (late September to late March) in South Africa, induces heat stress in poultry. Mutibvu et al. (2017), reported that broiler chickens reared during summer experienced heat stress. The study focused on the ambient temperature effects on different strains of chickens reared in an intensive and free-range management system. Heat stress inflicts significant losses to the farmers, hence the need for a mitigating strategy through evaluation of the antioxidants of choice that might be effective in improving their welfare and minimizing economic losses during heat stress.

1.2 JUSTIFICATION OF THE STUDY

Global warming is increasingly becoming a real threat to our planet. Higher ambient temperatures have become a reality to contend with, not only in poultry production, but across all sectors of the economy including human health. Thus, if relatively cheaper feed additives are found to be effective against the adverse effects of heat stress then an original contribution to solving this real threat to many livelihoods would have been made.

Yeast probiotic is a living microorganism that has been shown to serve as an anti-stress, antioxidant and growth promoting agent. Its constituents like mannan oligosaccharides serve as immune stimuli in the body (Aluwong et al., 2017). It has also been shown to be effective in the gut in protecting the intestinal epithelium against harmful microbes (Sugiharto et al., 2017). Aluwong et al. (2017) reported yeast probiotic to be a potent anti-stress agent that reduced the core body temperature of broiler chickens.

Ascorbic acid administration may improve the health status of broiler chickens, leading to an increase in productivity and profitability. Gan et al. (2018) noted that broiler chickens synthesise about 0.5 - 0.7% of ascorbic acid in their liver, but this quantity is insufficient to ameliorate oxidative stress, hence the need for its exogenous administration.

Since yeast probiotic and ascorbic acid have both been reported to be antioxidant, anti-stress and a growth promoting agents (Aluwong et al., 2013; Egbuniwe et al., 2018), the administration of a combination of these antioxidants might be better in enhancing protection against oxidation than when singly administered. This study sought to provide a basis for ameliorating the detrimental effects of heat stress through incorporation of probiotic and/or ascorbic acid in the feed. Performance indicator of growth rate and measures of stress were the markers of effectiveness or lack thereof and data obtained from the study is envisaged to create a basis for production for field application.

1.3 AIM OF THE STUDY

To mitigate the negative effects of heat stress in broiler chickens using *Saccharomyces cerevisiae* and ascorbic acid.

1.4 OBJECTIVES

i. To determine performance indices of broiler chickens fed diet fortified with probiotic and/or ascorbic acid and exposed to heat stress.

ii. To evaluate cloacal temperature, and body surface temperatures including wing, head, leg, breast muscle, back and comb surface temperatures of broiler chickens exposed to heat stress.

iii. To measure the haematological parameters: PCV, total leukocyte count estimation from a blood smear and differential leukocyte count of chickens fed diet fortified with probiotic and/or ascorbic acid and exposed to heat stress.

iv. To evaluate the activities and concentration of oxidative stress biomarkers (such as SOD, CAT, GPx and MDA) of broiler chickens exposed to heat stress.

v. To evaluate the level of expression of IL-10, HSP70 and 8-OHdG of broiler chickens fed diet fortified with probiotic and/or ascorbic acid and exposed to heat stress.

vi. To evaluate the behavioural parameters: tonic immobility, vigilance and open-field test of broiler chickens exposed to heat stress.

vii. To evaluate small intestinal morphometry and histochemistry of goblet cells of chickens fed diet fortified with probiotic and/or ascorbic acid and exposed to heat stress.

1.5 STATEMENT OF THE RESEARCH HYPOTHESES

i. *Saccharomyces cerevisiae* and/or ascorbic acid does not significantly affect the performance indices of broiler chickens exposed to heat stress

ii. *Saccharomyces cerevisiae* and/or ascorbic acid does not significantly affect the cloacal temperature and body surface temperatures of broiler chickens exposed to heat stress

iii. *Saccharomyces cerevisiae* and/or ascorbic acid does not affect the levels of oxidative biomarkers of broiler chickens exposed to heat stress

iv. *Saccharomyces cerevisiae* and/or ascorbic acid does not significantly affect the levels of cytokines, heat shock protein and oxidative gene damage of broiler chickens exposed to heat stress

v. *Saccharomyces cerevisiae* and/or ascorbic acid does not significantly affect the behavioural parameters of broiler chickens exposed to heat stress

vi. *Saccharomyces cerevisiae* and/or ascorbic acid does not affect the small intestinal morphology of broiler chickens exposed to heat stress

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CHAPTER TWO

LITERATURE REVIEW

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ADVERSE EFFECTS OF HEAT STRESS DURING SUMMER ON BROILER CHICKENS PRODUCTION AND ANTIOXIDANT MITIGATING EFFECTS

ABSTRACT

Poultry farming is a means of food production that comprises both meat (broilers) and egg (layers) production, and is considered a good source of protein. Furthermore, poultry litter containing faecal material has been reported to be highly nutritional and can be used in feed enrichment in ruminant production and serves as a source of organic manure for gardening. In the Southern part of Africa, most poultry farmers are faced with the challenges of heat stress during the summer (hot season) period which occurs during September to March (with little or no variation). Ambient temperature values that exceed the thermoneutral zone of broiler chickens expose them to heat stress and subsequently oxidative stress, which is evident during summer globally. Hence it is important to ameliorate heat stress's negative effects to ensure optimum productivity and profitability to the farmers. The aim of this chapter was to review scientific publications on the adverse effect of heat stress and the role of antioxidants in mitigating this effect in broiler chicken production. Heat stress induces oxidative stress which is the excessive production of reactive oxygen species above the antioxidant capacity of the body. Oxidative stress impairs physiological, behavioural and performance parameters of broiler chickens with evident decrease in feed and water intake, body weight gain and subsequently high morbidity

and mortality rates. Poor feed conversion ratio is a sequela to oxidative stress, due to the slow rate of absorption by the intestinal villi. Hence, antioxidants are explored to mitigate the negative effects of heat stress. Antioxidants are molecules that prevent the oxidation of other molecules. Probiotics, prebiotics, phytonutrients and some amino acids are potent antioxidants that can serve as an anti-stress or growth promoting agents thereby improving the welfare of the broiler chickens.

Key-words: Oxidative stress, heat stress, broiler chickens, antioxidants

2.0 INTRODUCTION

Stressors refer to any factor that threatens the health of the body or has an adverse effect on its functioning, such as cellular injury, disease or anxiety. Stress induces several changes in the physiology or behaviour of an animal, which leads to pathophysiology and malfunctioning (Aluwong et al., 2017; Gogoi et al., 2021). The general adaptation syndrome is a non-specific one-way physiological response of the body to all types of endogenous and exogenous stressors (Sumanu et al., 2019; Kim et al., 2021).

This general adaptation syndrome has three phases, namely, alarm, resistance and exhaustion (Gaidica and Dantzer, 2020).

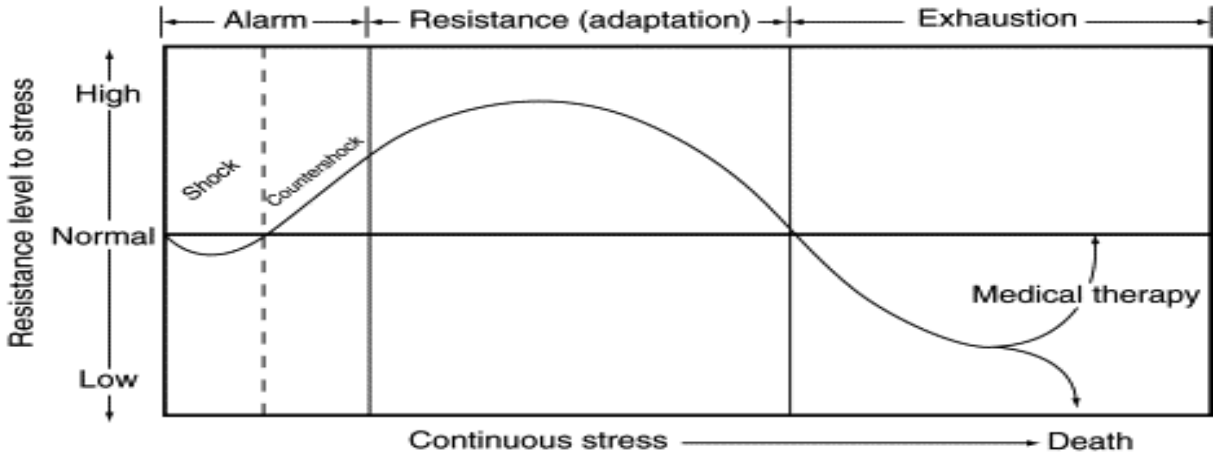

Fig. 1 General adaptation syndrome of animals to stress showing the alarm phase which is the first response, the resistance phase which is an adaptive response and the exhaustion phase which may result in death due to continuous exposure to the stressor (Source: Baffy 2020).

- Alarm phase (primary response): This phase involves the neuroendocrine system activation and stress hormone production (corticosterone). Corticotropic releasing hormone (CRH) is produced by the hypothalamus, which stimulates the pituitary gland to

release adrenocorticotropic hormone (ACTH). This further stimulates the rapid release of the stress hormone corticosterone from the adrenal gland (Getabalew et al. 2020), and corticosterone in turn increases the conversion of noradrenaline to adrenaline by stimulating the enzyme phenylethanolamine N-methyltransferase (PNMT) in the adrenal medulla (Baffy, 2020). During this phase, an increase in the level of corticosterone occurs in the blood stream. This further influence the diversion of broiler chickens from productive processes to adaptive mechanisms (Sejian et al., 2018).

- Resistance phase (secondary response): This phase involves the effects of corticosterone on the body/organ systems. It encompasses the physiological and biochemical effects induced via the stress hormones, as they induce alterations in haematology and blood chemistry (Turk et al., 2017). During this process, glycogenolysis and gluconeogenesis occur in the liver to increase the level of glucose for the muscle and brain uptake, due to an increase in energy demand (Gaidica and Dantzer, 2020). While both, corticosterone and adrenaline can increase the production of glucose, the effect induced by corticosterone is longer lasting due to it having a longer half-life than adrenalin. During this phase (for instance, thermal stress), the broiler chickens exhibit some adaptive behaviours like panting/gular flutter (evaporative cooling mechanism), spreading of the wings, decrease feed intake, increase water intake, resting on the litter or cages (Sanchez et al., 2022; Sejian et al., 2018).
- Exhaustion phase (tertiary response): This phase involves the consequences of the various physiological changes (Haque et al., 2019). That is, the observable changes associated with stress. Failure of the broiler chickens to cope with the stressor in this phase as a response to exhaustion subsequently lead to poor performance such as;

decrease body weight, high morbidity and finally death (Sanchez et al., 2022). To avoid the adverse effects of stress on the normal physiology of the chickens, early intervention is needed.

Thus, if stress is left to run its course in poultry production, it will reduce their ability to combat diseases and to gain live weight (Minka and Ayo, 2013). Stress evokes harmful responses that interfere with the immunity and general health resulting in immunosuppression (Makeri et al., 2017). Of the different stressors to poultry production, environmental stress due to high ambient temperatures has been reported to be a major problem especially during the hot summer season (Bhadauria et al., 2017). The sequela of climate change due to global warming can only be expected to make the situation worse, prompting an aggressive approach in adoption of stress mitigation strategies to sustain productivity and safeguard food security (Oke et al., 2021).

2.1 BROILER CHICKENS PRODUCTION DURING THE HOT SUMMER SEASON

Broiler chickens are Gallinaceous fowls that are domesticated and bred specially for the production of meat (Al-Zghoul et al., 2019; Darmani et al., 2019). They are subspecies of the red jungle fowl (*Gallus gallus*) and are hybrid of layers (Naidoo et al., 2007; Alemneh and Getabalew, 2019; Dong et al., 2019). They have yellowish skin with white feathers (which are used for ornamentation or further processed into fertilizers or animal food). Under wild conditions, the birds would take twenty months to reach their adult weight. However, through very efficient selective breeding programmes, current meat strains of broiler chickens usually attain slaughter weight at about five weeks of age (Hassan et al., 2019; Arrazola and Torrey, 2021) and grow much faster than the layers or dual-purpose breeds (Awad et al., 2020).

Today, these new breeds of chickens can be farmed in different ways, namely:

- Extensive system in which broiler chickens are not confined; that is, they are free to feed on their own. Here the broilers are exposed to various disease conditions, effective monitoring and control is required (Mutibvu et al., 2017).
- Semi-intensive system in which broiler chickens are allowed to move out in search of feed within a confined environment. The broilers are also exposed to various disease conditions hence, effective monitoring and control is advocated (Davoodi and Ehsani, 2020).
- Intensive system in which broiler chickens are confined in deep litter and/or battery cages within an environment that is efficiently controlled and spacious, feed and water are provided *ad libitum* (Amao et al., 2019). This system is recommended for large-scale broiler production.

Nowadays, the production of chicken meat in the tropics, sub-tropics and temperate regions is reliant on keeping heavy chicken breeds, which are sold as live birds, or as fresh or frozen meat (Mohammed et al., 2018; Sumanu et al., 2019, 2021a). The advantages of broiler chickens' production globally, are high turnover, ease of management, it wide acceptance for consumption and fast returns on investment. Broiler chicken meat is considered a high-quality protein with low level of fat (Bai et al., 2018). People also prefer chicken because being white meat, it is deemed healthier in comparison to red meat (Moataz et al., 2018). Broiler chicken production was reported to have risen to 92.7 million tonnes globally in 2018 and is forecast to reach 100 million tonnes by the end of 2022 (Olmez et al., 2021).

2.2 THERMONEUTRAL ZONE OF BROILER CHICKENS

The thermoneutral zone (TNZ) is the ambient temperature range at which regulatory changes in the production of metabolic heat or evaporative heat loss is not induced in broiler chickens (Egbuniwe et al., 2018). The diurnal ambient temperature often fluctuates in the tropics thereby exceeding the TNZ which results to heat stress in broiler chickens (Aluwong et al., 2017). Ambient temperatures that exceed TNZ of broiler chickens regardless of the age may elicit adverse effects on fitness and energy balance (Sinkalu and Ayo, 2018).

Increased temperature affects reproductive potentials, production, health status and immune responses broiler chicken. The TNZ for poultry in the tropics is reported to be between 18 – 24 °C and between 12 – 26 °C in temperate regions (Aluwong et al., 2017). Favourable temperature range for poultry production is 12 – 26 °C (Ravikumar et al., 2016). Thermoneutral zone for poultry production is between 18 – 24 °C in KwaZulu-Natal area of Southern Africa due to the fluctuation in ambient temperature (Mutibvu et al., 2017), but none have been reported in Pretoria North, Gauteng province of Southern Africa to the best of the author's knowledge. To understand homeotherm's thermodynamic responses to its environments, evaluation of the energy used for biological processes is advocated (dietary induced thermogenesis).

2.3 MAINTAINING TEMPERATURE IN THE POULTRY HOUSE

Adequate housing is paramount for broiler chicken production. The house must be well equipped for factors like temperature, light, air and moisture to be properly controlled. For efficient environmental control, heaters, exhaust fans, thermostats, air inlet and evaporative cooling system are required to maintain the correct temperature specific to the age of the chick

(Schauberger et al., 2020). These appropriate temperatures should be maintained all through the production cycle in the pen; temperate regions require more insulation while the tropical and sub-tropical regions require efficient air speed (Oke et al., 2021; Sumanu et al., 2021b). It is expedient that broiler chicks are maintained in brooding temperature of about 31 - 32 °C for the first 14 days of life which requires exogenous heating, but as they grow older, they require cooler temperature within the range of 12 - 26 °C for optimum growth which is dependent on cooling systems, air-flow and exhaust control (Egbuniwe et al., 2015). Adult broiler chickens often express changes in their behaviour like congregating, consumption of more feed and drinking less water when exposed to cooler temperatures and the reverse occurs when exposed to hot temperatures to keep warm when the temperature is not too favourable (Mack et al., 2013).

2.4 POULTRY HOUSE VENTILATION

In broiler chickens' production, large scale farmers generally use deep litter for housing birds intensively using an enclosed housing system. With modern houses being enclosed structures, harmful gases and odours could be trapped if no proper ventilating system is put in place. Poultry house ventilation not only provides fresh air and oxygen that is important in sustaining life, but it is also essential in reducing high temperature, air contamination and humidity to the bearable minimum\optimum level. Unfortunately, while the ventilated air expels excessive heat, odour, dust and moisture from the poultry house, it can also disperse disease organisms that are airborne which could result in other risks (Chirarattananon et al., 2012). Generally small-scale farmers do not need ventilation support to bring in fresh air and remove harmful gases, as they mostly use the backyard system of production (Kakati and Nath, 2022). This does come with the pitfall of being able to adequately maintain the temperature. In the third system of housing, farmers are

reliant on open sided naturally ventilated houses with the air change being dependent on natural airflow through the building. As evident with all three housing system, proper ventilation can be a great challenge due to the variations in temperature, season, humidity, time of the day, age of the chickens and wind (Samuel et al., 2013).

2.13.1 VENTILATION SYSTEM

Based on the housing systems mentioned above, ventilation systems can be divided into two major types: natural ventilation system and mechanical ventilation system.

Natural ventilation system: The essential requirements for this system of ventilation is that, there must be adequate fresh air supply and distribution in the poultry house brought about, by wind direction and speeds, the geographical features of the site and orientation of poultry house in respect of the more predominant wind patterns. This ventilation can be maintained by placing air vents close to the eaves of the poultry building. Curtains can also be used to maintain optimum ventilation because an equal opening often runs the length of the poultry building when curtains are lowered. The ventilation system requires some modifications between the winter and summer periods in order to keep the chickens in a comfortable state (Chirarattananon et al., 2012):

- Winter ventilation: This system is a bit complex than that for the summer months as the poultry house needs to be tightly closed to obtain maximum energy conservation and comfort, which results in humidity, odours, gases, etc. being trapped in tight enclosures which must be continually dealt with for optimum health status of the chickens (Bhadauria et al., 2017). This type of ventilation requires the removal of excessive moisture that is build up in the poultry house as compared to the summer ventilation

wherein the removal of heat is required. During winter there is less carbon dioxide and ammonia build up in the poultry house when compared with the summer season.

- Summer ventilation: During summer, natural ventilation system is required especially in poultry houses where side curtains are used, as the curtains are often raised up for optimum flow of natural air into the building whereas in winter, the curtains are mostly dropped (Xuan et al., 2012). Also, air vents are left opened to allow the influx of natural air into the building whereas it is left covered during the winter season. Here the removal of excessive heat is paramount due to the hot season as compared to winter ventilation where moisture is mostly at the extreme. In contemporary poultry production, environmental changes impair this system's effectiveness due to recurrent rise in temperature resulting from global warming (Bhadauria et al., 2017).

Mechanical ventilation system: In extreme climatic conditions, this system of ventilation is required to ensure proper ventilation of the house (Yahav and Hurwitz, 1996). Electric fans are used as principal components in this system for the movement of air in the poultry house. The components of this system are, fans required for air movement through the poultry house; air inlet and outlet vents and lastly; a control unit for the regulation of the fans (timers and thermostats).

- Fans: For this system of ventilation, one must consider the wide range of climatic conditions for appropriate selection of the desired fan that can cover a large area of the poultry house. Proper wiring should be done to avoid fire outbreaks or shocks from the fan which might pose great danger to the chickens and the farmers (Bhadauria et al., 2017).

- Inlets and outlets: The fan is the determinant of the rate of air exchange in the poultry house, but the adjustment, location and design of the air inlets are primary determinants of air uniformity. The recommended air inlet velocities in a poultry house are 600 – 1000 feet per minute for large size chickens and 300 – 500 feet per minute for average size chickens (Bhadauria et al., 2017).
- Controls: Thermostats alone or in combination with timers are used to control fans. Single stage thermostats through the activation and stoppage of fan control one or more single speed fans when temperature rises and drops, respectively (Schauberger et al., 2020). A double-throw switch thermostat also controls two-speed fans; it changes automatically from a high to low speed as the temperature increase or decrease, respectively. Therefore, when maximum ventilation is required to expel excessive heat in the poultry house, timers interconnected with thermostats are needed.

2.14 HEAT STRESS AND ITS ADVERSE EFFECTS ON BROILER CHICKEN PRODUCTION

Heat stress is caused by either high ambient temperatures (AT), lack of ventilation and high relative humidity (RH) or a combination of these factors. In the tropics, heat stress is caused by a combination of high RH and AT (Minka and Ayo, 2013). In the sub-tropical and arid regions, as expected, heat stress is more than the temperate regions, unless the broiler chickens are reared intensively with automated regulation of microclimatic conditions (Aluwong et al., 2017). In most rural areas in South Africa, broiler chickens are subjected to the adverse effects of heat stress by being raised under an extensive or free-range management system, which exposes them to significantly high AT during the hot season of the year (Mutibvu et al., 2017). The effects of high environmental parameters on broiler chickens do not only depend on the microclimate but

also on the intensity of their stocking density (Bhadauria et al., 2017). The physiological adjustments made by broiler chickens to altered environmental conditions via various mechanisms; including cardiovascular, neuro-endocrine, behavioural and respiratory thermoregulatory responses are overwhelmed in heat stress (Kikusato and Toyomizu, 2019).

High AT is detrimental to broiler chicken production through altered normal physiological functions such as: reduced feed intake which leads to reduced body weight (due to poor feed conversion ratio) and subsequently impaired health status, which may lead to morbidity and mortality (Amiri et al., 2019). Other effect includes lower reproductive performance of broiler chickens resulting in reduced egg fertility in breeder flocks (Ramiah et al., 2019); as well as interference with the rate at which hormones are secreted and their clearance from the body (Nyoni et al., 2019). Hence, the need to consider heat stress mitigation when designing poultry farms in the tropics.

2.15 THERMOREGULATION IN CHICKENS

Thermoregulation is the process by which the body maintains homeostasis of body temperature (Minka and Ayo, 2013). The various processes involved in thermoregulation in birds under conditions of high temperatures are described below:

2.15.1 CHEMICAL RESPONSE TO HEAT STRESS

The hypothalamus is responsible for the reception and integration of signals from thermal receptors to stimulate regulation in both humans and animals (Kumari and Nath, 2018). During heat stress in broiler chickens, signals are sent to the hypothalamus to release CRH, which further stimulates the pituitary gland to release ACTH that stimulates the adrenal gland to release

corticosterone into the blood stream (Gaidica and Dantzer, 2020). When this hormone is released in the body, the hypothalamus is furthermore stimulated to initiate the process of thermoregulation as a response to the increased level of corticosterone in the blood (a negative feedback mechanism) (Sugiharto et al., 2017; Xu et al., 2019). This further improves the general performance of the broiler chickens, decreasing the rate of morbidity and mortality (Wasti et al., 2020).

2.15.2 EVAPORATIVE COOLING

With chickens not having sweat glands, they are reliant on evaporative cooling from their lungs surface and air sacs to cool themselves (Xuan et al., 2012). As a process, the conversion of water to water vapour, expends energy in the form of heat. Therefore, as the water vaporises from the lungs surface of the broiler chickens heat is concurrently lost from the body (Ranjan et al., 2019).

2.15.3 BEHAVIOURAL RESPONSE TO PROMOTE HEAT LOSS

- Water intake: To enhance cooling further, behavioural responses include drinking of more water and eating less to enhance evaporative cooling (Shao et al., 2019).
- Food intake: In addition to the broiler chickens drinking more water, they also eat less to lower metabolic heat production from digestive processes. Also, the decrease in broiler feed intake is related to the need for more frequent respiration in high temperature environments to dissipate heat without being able to feed. The gastrointestinal tract and liver contribute to heat production as the suprahepatic rete which supplies them blood functions as heat exchanger that retains metabolic heat produced by the muscle. Thermal conduction from the vessels in the liver and warm blood supply are both responsible for transferring heat to the stomach (Law et al., 2019).

- Panting/gular flutter: This behavioural response heightens the smooth passage of air through the various systems of the body, thereby increasing evaporation cooling through the lungs, which subsequently lowers the temperature of the body (Al wakeel et al., 2019).
- Enhancing surface area: Broiler chickens exposed to heat stress usually spread out their wings to initiate effective radiation of heat from the body to the environment in order to keep the body cool. There is usually shunting of blood from the gut to the comb to reduce the rate of metabolic heat production/pick-up and increase the rate of heat loss from the body system, respectively through surface radiative cooling (Tickle and Codd, 2019).

2.16 EFFECTS OF HEAT STRESS ON THE PERFORMANCE OF BROILER CHICKENS

In the Southern and Northern hemispheres, heat stress season falls within the period of September to March and March to June, respectively, which is thermally stressful for broiler chicken production (Mutibvu et al., 2017; Aluwong et al., 2017). The ATs during this season are usually at the upper limit, which is an indicator that the season is thermally stressful to the broiler chickens (Slawinska et al., 2019). Broiler chickens are homeotherms, meaning they strive to keep their body temperature relatively constant in the face of either rising or decreasing AT; but when the rate of heat dissipation to the environment is lesser than the rate of metabolic heat production and heat gained from the environment, the body temperature rises concurrently (Yousaf et al., 2019).

The following changes have been associated with heat stress:

- Changes in performance (Production Efficiency): Performance refers to the general welfare of the broiler chickens, such as feeding and body weight gain (Sumanu et al.,

2021a). Heat stress greatly impact the feeding pattern of broiler chickens and this subsequently results into 33% decrease in body weight gain (Lara and Rostagno, 2013) which further impairs the production efficiency of the chickens (Sumanu et al., 2021b).

- Immunocompetence: The immune responses of broiler chickens are impaired during heat stress due to the presence of excessive reactive oxygen species (ROS) that cause cellular damage. The body's defence mechanisms are weakened over a period of time due to the increased level of pro-inflammatory cytokines in circulation.
- Haematology: The blood parameters of broiler chickens are negatively affected during heat stress (Karadagoglu et al., 2020). About 10 to 20% decrease in concentration of white blood cells have been reported and this affects the immunity of the chickens to a greater extent (Ogbuagu et al., 2018).
- Deoxyribonucleic acid (DNA) damage: Oxidative stress, due to the excessive production of ROS causes mutation in genetic makeup which leads to genetic disorders (Sofinska et al., 2020).
- Behavioural parameters: The effect of heat stress on broiler chickens generally affects the nervous system that controls all other systems of the body. Several behavioural changes in response to heat stress occurs in broiler chickens like panting, drooping feathers, drinking more water and feeding less. Also, fear responses are often heightened in broiler chickens exposed to heat stress (Sumanu et al., 2019).
- Sleep disorders: Heat stress creates an unfavourable condition for sleeping due to the high metabolic heat production and the lack of sweat glands to dissipate the heat by the broiler chickens (Sinkalu et al., 2016). Also, during heat stress there is decreased

secretion of melatonin, a hormone from the pineal gland responsible for the regulation of sleep.

- Bone abnormalities: Bone formation is impaired due to bone marrow degradation as corticosterone is proteolytic. It degrades organic bone matrix affecting strength, it also affects calcium and phosphate absorption, which contributes to the abnormalities (Sugiharto et al., 2017).

2.17 OXIDATIVE STRESS AND ANTIOXIDANT SYSTEM OF THE BODY

Despite the numerous pathological changes induced by heat stress, at the physiological level, much of the effects seen are due to oxidative stress. The disproportion between antioxidants and free radicals in the body system is known as oxidative stress. Hence, antioxidants are molecules that prevent the oxidation of other molecules (Aluwong et al., 2017), while free radicals are molecules that contain an unpaired electron. Some free radicals are produced naturally in the presence of inflammation or exercise in the body and they contribute in keeping the body healthy. The environment also contains pollutants and radiation that might also aggravate the level of exposure to oxidative stress (Sumanu et al., 2021b). An important feature of free radicals is their instability that allows them to react easily with other molecules. Their ability to react easily creates large chain reactions in the body, which are known as oxidation reactions.

Oxidative stress induced by heat stress is responsible for the impairment of several body functions like the digestion of feed, reproduction as seen in broiler breeders, etc. which eventually result in poor meat quality, increased morbidity and mortality rate, thereby decreasing their productivity (Awad et al., 2020; Sumanu et al., 2021b). Oxidative stress could also heighten fear responses in broiler chickens due to the increased level of epinephrine in the body which is

evident as long duration of tonic immobility and prolonged vigilance period in most behavioural studies (Egbuniwe et al., 2018; Sumanu et al., 2019). Reduced expression of messenger ribonucleic acid (mRNA) occurs as a result of oxidative stress due to impairment in the process of gene replication and transcription. This further leads to a decreased level of intestinal immunity locally (such as immunoglobulins) and increased apoptosis (Lee et al., 2019). Oxidative stress resulting from excessive free radical is virtually impossible to avoid especially due to the presence of heat stress resulting from the effects of global warming. Nevertheless, increased level of exogenous antioxidants could be helpful in preventing oxidative stress based on the equilibrium state these can provide.

Several organs and tissues of the body possess a distinct antioxidant system that respond to oxidative stress (Aluwong et al., 2013; Ogbuagu et al., 2018). This system defends cells against oxidative damage to proteins, lipids and DNA (Azeez et al., 2019). The body protects itself against the adverse effects of ROS via two important mechanisms, which are: regulation of membrane permeability and potential antioxidant system. Antioxidant metals such as copper, zinc and manganese are components of superoxide dismutase (SOD) enzyme that scavenge superoxide radical, which is vital in eliciting a defensive mechanism against ROS (Lee et al., 2019). Superoxide dismutase is responsible for the dismutation of free radicals to hydrogen peroxide, thereafter, glutathione peroxidase and catalase split the hydrogen peroxide into molecules of water and oxygen, thereby rendering the radicals inactive (Ogbuagu et al., 2018; Sumanu et al., 2019). During heat stress, the mitochondria of skeletal muscle in broiler chickens are responsible for the production of superoxide anions as a response to oxidative stress in order to neutralise the harmful effect of the increased levels of free radicals (Egbuniwe et al., 2018). Erythrocytes also show some level of antioxidant defence, but the erythrocyte life-span decreases

by 50% in the presence of oxidative stress based on the fact that the stability of the erythrocyte is reduced drastically leading to haemolysis (Ogbuagu et al., 2018). Cytokines, especially the anti-inflammatory cytokines are crucial in inhibiting inflammatory processes that occur during heat stress.

2.18 CYTOKINES AND THEIR ROLES IN THE BODY SYSTEM DURING HEAT STRESS

Cytokines are small proteins that are important in cell signalling and are unable to cross the lipid bilayer to penetrate the cytoplasm of a given cell (Wang et al., 2019). Examples of cytokines are the interleukins (IL), transforming growth factor (TGF), tumour necrosis factor (TNF), interferon (IF), lymphokines and chemokines. A broad range of cells is responsible for the production of cytokines, including T lymphocytes, B lymphocytes, macrophages, mast cells, stromal cells, fibroblasts and endothelial cells (Seremelis et al., 2019). The balance between cell-based and humoral immune responses is effectively modulated by these cytokines (Saleh and Al-Zghoul, 2019).

Cytokines generally play a role in the pathways regulating inflammatory process:

- Pro-inflammatory cytokines: They are molecules that try to act to resolve an infection and contain inflammatory foci via the activation of systemic or local inflammatory responses. They play a vital role in inflammatory disease of non-infectious and infectious origin (Zhang et al., 2019). The excessive release of these cytokines can lead to cytokine storm in the systemic circulation which may trigger several disease conditions like multiple organ failure and subsequently death. The activities of pro-inflammatory

cytokines are often inhibited by the synthesis of anti-inflammatory cytokines (He et al., 2019).

- Anti-inflammatory cytokines: These cytokines are molecules responsible for immune regulation, as they control the response of pro-inflammatory cytokines, they achieve this immune response regulation by acting in concert with cytokine inhibitors and soluble cytokine receptors that are specific (Dai et al., 2019). The release of anti-inflammatory cytokines often terminates the activities of pro-inflammatory cytokine (He et al., 2019).

Also, of importance is that oxidative stress is induced by several inflammatory cytokines from the oxidative burst reaction (Humam et al., 2019). During the oxidative burst reaction, the immune system rapidly releases macrophages and neutrophils as a means of defence. During heat stress, the circulatory level of pro-inflammatory cytokines like IL-6 and TNF-a is heightened in broiler chickens (Ma et al., 2019). When broiler chickens are exposed to heat stress, the microglia (cells responsible for immune defence in the central nervous system) can move from a resting state to an active state during which pro-inflammatory cytokines are not released actively (Cheng et al., 2019). However, it could be speculated that continuous exposure to this stressor (heat), may further weaken the microglia activities, and this could trigger the release of pro-inflammatory cytokines and HSP into the systemic circulation.

2.19 HEAT STRESS INDUCES HEAT SHOCK PROTEINS IN BROILER CHICKENS

Heat shock proteins (HSP) are a family of proteins which are produced by the body cells in response to an exposure to any form of stress such as heat, cold, tissue remodelling, disease conditions and wound healing (Erfani et al., 2021). Members that belong to this group are responsible for the stabilisation of newly formed

proteins to ensure an accurate folding and/or refolding of damaged proteins in the cell due to a stressor (Li et al., 2021). The upregulation of HSP dramatically is a vital process that occurs as a response to heat shock which is primarily induced by heat shock factor (HSF) (Kang and Shim, 2021). Heat shock proteins are present in both bacteria and humans, their nomenclature is based on their molecular weight, for instance; HSP90, HSP70 and HSP60 in kilodaltons (size), respectively. The identification of stress proteins also called HSP came to existence due to this discovery and they exhibit some important roles in the body system which includes;

Upregulation in stress: Increase levels of HSP are triggered via exposure to various types of environmental stress, such as starvation, inflammation, exercise, hypoxia, water deprivation, or infection. The mechanism by which these environmental stressors or heat shock activates the heat shock factor has been studied in bacteria (Tarkhan et al., 2020). It was reported that during heat stress, the outer membrane proteins do not fold and it fails to correctly insert into the outer membrane, therefore, they tend to accumulate in the periplasmic space. Nevertheless, some school of thought has it that, HSPs are triggered into action when there is an increase in abnormal or damaged proteins.

Serve as a chaperone and as monitors: Various HSP serves as vital intra-cellular chaperones for other proteins, via the protein-protein interactions that is, folding and establishment of proper protein shape or conformation, and the prevention of the aggregation of unwanted protein (Zheng et al., 2020). As these HSPs stabilises partially unfolded proteins, they also help in the transport of proteins within the cell. In all the various organisms, members of the HSP family are often expressed in a low to moderate levels due to their vital role in the maintenance of protein. Also, HSP often do occur in non-stressful conditions, and they are simply responsible for monitoring

the proteins of the cell in this stage (Humam et al., 2019). For instance, they carry old proteins to the cell's proteasome (the recycling bin of the cell) and thereby assist in the proper folding of the proteins that are newly synthesised. These activities are part of the repair system of the cell called the 'heat-shock response' or 'cellular stress response'. Due to slow proteolytic action, HSP are therefore more subject to self-degradation than other proteins.

Play a vital role in immunity: The function of HSP in immunity is attributed to their ability to bind both whole proteins and also peptides and the specificity and affinity of this interaction is very low (Miao et al., 2020). This ability is possessed by HSP70, HSP90, calreticulin and their peptide-binding sites. Also, these HSPs are involved in the stimulation of immune receptors that are vital in the accurate folding of proteins that partakes in pro-inflammatory signalling pathways (Baxter et al., 2020).

2.19.1 Heat Shock Protein 70

Heat shock protein 70 are known for their housekeeping functions in the cells of all living organism which includes broiler chickens, as they are mainly responsible for cellular protection against heat shock. They have been reported to modulate the immune system of broiler chickens via the appropriate folding of proteins and apoptosis regulation. The vital characteristics of the HSP70 are the non-disintegration of heat and their stability. The high conservation of its genetic structures is thought to be responsible for the stability of its role and the performance of its functions. There is consistency in the genetic expression of the catalysts responsible to produce these proteins that are highly effective under unsuitable conditions for the work of the proteins (Baxter et al., 2020). In mammals, HSP70 is specific for a key function (of protection) in the central nervous system, such that they remove or repair damaged proteins caused by a stressor

which might lead to harmful disease conditions like Parkinson's disease and various neurological diseases. Nevertheless, an increase in HSP70 genetic expression could lead to an increase in the cells' susceptibility to oxidation. Hence, the use of antioxidant agents that could limit their production in broiler chickens exposed to heat stress.

2.20 EFFECT OF HEAT STRESS ON OXIDATIVE DNA DAMAGE

One of the impacts of heat stress has been DNA damage and protein degradation which causes alterations in genes and cell death (Elnesr et al., 2019). Oxidative stress, which is an imbalance between the amount of ROS and endogenous antioxidants in the body, is the major factor that induces DNA damage (Sofinska et al., 2020). Reactive oxygen species induce several lesions to the DNA molecules such as inter-strand and intra-strand cross-links, DNA-protein cross-links, sugar and/or base alterations and sugar-base cyclisation, all of which further result into breaks of DNA strands (Cramer et al., 2019). Ultraviolet light and ionising radiation are other exogenous agents that induce DNA damage through oxidative stress hence, alleviation of the adverse effect of heat stress is of importance.

2.21 MITIGATION STRATEGY AGAINST THE ADVERSE EFFECTS OF HEAT STRESS ON BROILER CHICKEN PRODUCTION

With the detrimental effects of heat stress on poultry production, mitigation is necessary. In technologically advanced poultry farming, this may be achieved by the implementation of mechanical systems, as explained above. (Bhadauria et al., 2017). The disadvantages of this system are for instance; high cost of equipment, high maintenance cost, constant electricity supply and potential inability to recoup investment from sales. Due to these disadvantages, it

may not be feasible to implement mechanical systems in less developed economies. Hence, the existence of an alternative system or strategy that is cheaper and possibly more profitable needs to be investigated. Reactive oxygen species production and oxidative stress is a major factor in heat stress, mitigating these changes could result in alternate methods of managing the dangers of heat stress which could be cheaper (Sumanu et al., 2019, 2021b).

Antioxidants' in-built capability of preventing or slowing down other molecules' oxidation (Yang et al., 2019), could prove to be successful protection against heat stress. Antioxidants prevent the peroxidation of lipids by ROS (Aluwong et al., 2017; Ogbuagu et al., 2018; Sumanu et al., 2019). They are very effective because they easily donate their own electron to free radicals. When a given antioxidant donates an electron to a free radical the free radical is unable to cause further harm to cells as the oxidation chain reaction is broken. Oxidation is a chemical reaction in which an electron is transferred from one substance (reducing agent) to another (oxidising agent). Antioxidants are also currently used in dietary supplements as ingredients, and their supplementation is highly beneficial against stress-induced tissue damage (Ogbuagu et al., 2018; Sumanu et al., 2019). Antioxidants could be classified as enzymatic and nonenzymatic based on their site of action, mode of action and their effect in the body. Examples of the enzymatic antioxidants are catalase, superoxide dismutase, glutathione reductase and glutathione peroxidase, their activities or level in the body give a clue of the effectiveness of antioxidant defence in broiler chickens and they are endogenous (Marchi et al., 2022). The nonenzymatic antioxidants are low in molecular weight and some examples are vitamins (A, C, E), probiotics, prebiotics, flavonoids, carotenoids, phenolic acid, minerals, etc. They are often exogenous as most of them are obtained from plants and living organisms. The need for exogenous antioxidants is advocated when endogenous antioxidants is inadequate in exhibiting a defence

against ROS in the body (Bensid et al., 2022). Contemporarily, many natural antioxidants from plants and living organisms have been isolated and are readily available as therapeutic and prophylactic agents to mitigate the adverse effect of oxidative stress in both humans and animals (Sumanu et al., 2021b). Antioxidants exhibit various mechanisms of action such as: sequestering of transition metal ions, quenching of active prooxidants, scavenging free radicals, repairing cellular damages, enhancement of endogenous antioxidant defence and decomposing hydroperoxides or hydrogen peroxides. Hence, they are occasionally classified as chain-breaking or primary antioxidants and as preventive or secondary antioxidants.

Antioxidants for example, probiotic, phytonutrients and amino acids are widely used in farms and especially in poultry production as anti-stress agents via their function in the hypothalamus-pituitary-adrenal axis, which, therefore, reduce the level of corticosterone secreted in the blood (Sumanu et al., 2019; Elghandour et al., 2020). They also induce peripheral vasodilation with resultant increase in heat loss. They serve as growth promoters via the suppression of the satiety centres in the brain to induce feed intake which subsequently leads to an increased body weight gain (Hag and Poondla, 2021). They improve the immune system of the broiler chickens via the activation of the body defence mechanisms for instance, the endogenous antioxidant systems such as superoxide dismutase, catalase, glutathione peroxidase, which subsequently prevent outbreaks of several disease conditions (Adamu et al., 2014; Sumanu et al., 2021b). These antioxidants, when adequately used could also allow return to normal behaviour via thermoregulatory measures. Previous studies have reported several antioxidant substances such as probiotics of fungi and bacterium strains, and other prebiotics like curcumin, onion, avocado, etc. and phytonutrients such as fisetin, zinc gluconate, vitamins C, A and E, which have been speculated to be highly beneficial in attenuating the adverse effects of heat stress in broiler

chickens (Egbuniwe et al., 2018; Sumanu et al., 2021b). For this review, the results obtained with the use of some antioxidants in poultry production by certain researchers would be summarised as stated on Table 2.1.

In a study by Aluwong et al. (2017), the administration of *Saccharomyces cerevisiae* probiotic and zinc gluconate at the dose of 4.125 × 106 cfu/100 mL and 50 mg/kg, respectively to broiler chickens exposed to heat stress mitigated the adverse effect of heat stress which was evident as a decrease in cloacal temperature values during the study period. They concluded that the groups of broiler chickens treated with probiotic had better thermoregulation when compared with the zinc gluconate-administered and control groups, respectively. Wang et al. (2016) stated that the administration of *Bacillus subtilis* probiotic at a dose of 1×10^6 cfu/g to broiler chickens exposed to heat stress reduced inflammatory responses and heat stress-related behaviours. It was concluded that this strain of probiotic was potent in modifying gut microbiome with resultant decreased heat production in the broiler chickens. Sinkalu et al. (2016) reported that the administration of melatonin to broiler chickens at the dose of 0.5 mg/kg during the hot-dry season reduced fear responses in broiler chickens subjected to tonic immobility test when compared with the group that was not administered with melatonin. They concluded that the administration of melatonin to broiler chickens mitigated the deleterious effect of heat stress which was evident as an increment in boldness of the chickens. It could be speculated that as broiler chickens advance in age, there is a resultant increase in metabolic heat production, which increases their exposure to heat stress and subsequently increase fear responses. Therefore, the administration of antioxidants or anti-stress agents might be effective in reducing heat stress and subsequently fear responses which are seen as boldness and alertness in the broiler chickens based on the similarities in the path-way of fear and stress responses.

Habibian et al. (2014) stated that the administration of vitamin E at a dose of 200 mg/kg to broiler chickens exposed to thermal stress reduces the heterophil/lymphocyte ratio which was a prove that the chickens were able to strive better during heat stress condition as compared to the control group. Vitamin E, a potent fat-soluble vitamin, exhibits it antioxidant effect by preventing damages caused by free radicals during the process of oxidation in the body. It was further concluded that Vitamin E administration was able to improve blood parameters of the broiler chickens.

Egbuniwe et al. (2021) reported that the administration of betaine and ascorbic acid at a dose of 2 g/kg and 200 mg/kg, respectively to quails exposed to heat stress improved erythrocytic parameters and activities of serum sex and stress hormones. Betaine alone, and its combination with ascorbic acid was speculated to be more effective in enhancing erythrocytic and endocrine responses in quails. They attributed their findings to be a resultant of the thermotolerant effect of the antioxidant utilised for the study.

Sumanu et al. (2021b) stated that the supplementation of fisetin a flavonoid at a dose of 5 mg/kg was not effective in mitigating the detrimental effect of thermal stress in broiler chickens during the hot dry season. They hypothesised that fisetin was not effective in inducing thermoregulation because cloacal temperature responses obtained in the fisetin administered group was higher than other treatment groups. It was therefore concluded that fisetin was not beneficial as an antistress agent in poultry production.

Shimao et al. (2019) reported that the supplementation of oleuropein at a dose of 0.5 ppm in feed of broiler chickens was effective in enhancing hormonal effects and oxidative status. This finding was linked to the up-regulatory effect of oleuropein in the levels of mRNA expression of

reactive oxygen species reducing factors, manganese superoxide dismutase and avian uncoupling protein. They concluded that the antioxidant could be potent when administered through feed in small doses to broiler chickens. The table below summarises the effect of the antioxidants in poultry species exposed to thermal and transportation stress.

As evident from the above, the control of heat stress through the utilisation of antioxidants could be more economical and cost-effective. For instance, the use of *Saccharomyces cerevisiae* probiotic in poultry production could help in mitigating heat stress, improve immune status and also serve as a growth promoter. This agent could be cost-effective based on the fact that the broiler chickens' health status would be improved and there would be a reduced need for the use of antibiotics and anticoccidial agents, assuming efficient biosecurity measures are also in place. With an improved performance in immune status, one should also see a reduction in morbidity and mortality and possible better market weights at slaughter age. Most agents such as: ascorbic acid, betaine, melatonin, flavonoids (fisetin, quercetin), oleuropein, etc. could serve as antioxidant and anti-stress agents if appropriately administered to the host, they are also readily available and cost effective.

Table 2.1. Effects of supplementing poultry species diet with some antioxidants during exposure to heat and transportation stress

Antioxidants	Poultry species	Effects	References
Saccharomyces cerevisiae probiotic	Broiler chickens	Improved performance indices, carcass characteristics and small intestinal morphology.	Sumanu et al. (2021a)
		Decreased erythrocyte osmotic fragility, malondialdehyde concentration and Improved superoxide dismutase enzyme activity	Ogbuagu et al. (2018)
		Decreased fear responses, muscle tissue malondialdehyde concentrations and improved muscle tissue superoxide dismutase enzyme activity	Sumanu et al. (2019)
Betaine	Quails	Enhanced endocrine secretions and erythrocyte parameters	Egbuniwe et al. (2021)
Ascorbic acid	Broiler chickens	Increased alertness and haematological parameter	Egbuniwe et al. (2018)
Melatonin	Quails	Improved colonic temperature and erythrocyte osmotic fragility	Minka and Ayo (2013)
Fisetin	Broiler chickens	Little or no effect on cloacal temperature parameter	Sumanu et al. (2021b)
Oleuropein	Broiler chickens	Improved oxidative status and hormonal concentration	Shimao et al. (2019)

2.22 CONCLUSION

The purpose of this review was to address the adverse effects of heat stress during summer on broiler chicken production and antioxidant mitigating effects. Heat stress impairs the general performance of broiler chickens; it induces oxidative stress which elicits inflammatory responses

leading to the release of inflammatory cytokines in the body. It also induces single and double stranded DNA breaks thereby resulting in oxidative DNA damage. Therefore, the use of potent antioxidants is advocated to ameliorate the adverse effects of heat stress in broiler chicken production during the hot summer season of the year for optimum productivity.

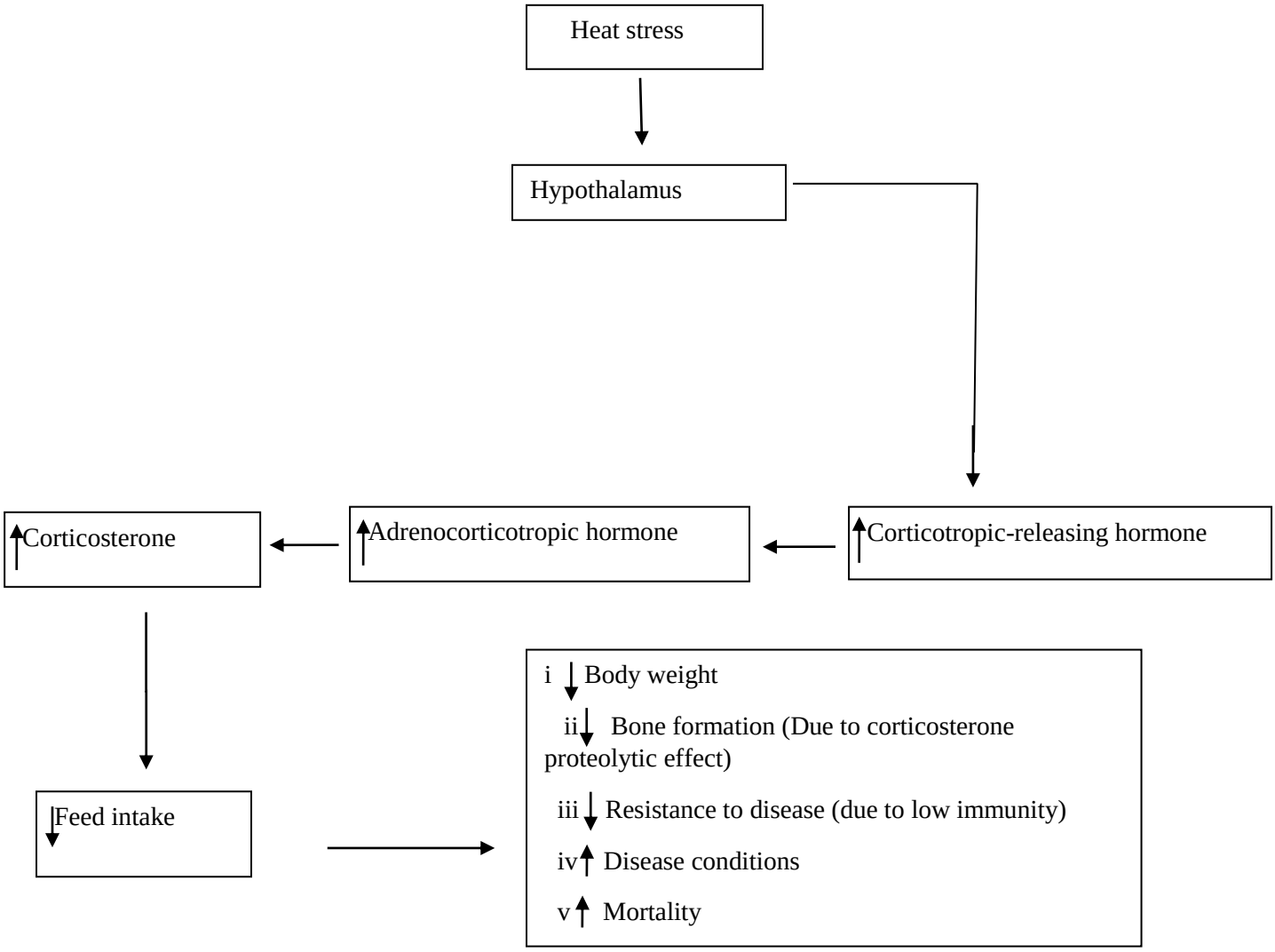

Fig 2.2. Schematic diagram summarising the adverse effect of heat stress on broiler chickens. The neuro-endocrine system responds to heat stress by stimulating the secretion of specific hormones that affect the performance parameters and welfare of the chickens.

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CHAPTER THREE

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EFFECTS OF PROBIOTIC (*Saccharomyces Cerevisiae*) AND ASCORBIC ACID ON OXIDATIVE GENE DAMAGE BIOMARKER, HEAT SHOCK PROTEIN 70 AND INTERLEUKIN 10 IN BROILER CHICKENS EXPOSED TO HEAT STRESS

ABSTRACT

Heat stress is a prominent factor responsible for losses economically in poultry meat industry due to adverse effects on the general performance of broiler chickens. In this study, we evaluated the effects of probiotic (*Saccharomyces cerevisiae*) and ascorbic acid on oxidative gene damage biomarker, heat shock protein 70 (*HSP70*) and interleukin 10 (*IL-10*) in broiler chickens exposed to heat stress under natural conditions. Fifty-six broiler chickens served as the subjects, they were divided into four groups of 14 as follows: group I (control), group II (probiotic *S. cerevisiae* at 1 g/kg of feed), group III (ascorbic acid at 200 mg/kg of feed) and group IV (probiotic + ascorbic acid at 1 g/kg and 200 mg/kg of feed, respectively). The treatments were administered via feed for 35 days (D1 to D35). Enzyme-linked immunosorbent assay (ELISA) and one step Real-time reverse transcription polymerase chain reaction (RT-PCR) was utilised to study the effects of heat stress on the expression levels of 8-hydroxy-2′-deoxyguanosine (*8-OHdG*), *HSP70* and *IL-10* respectively, in broiler chickens raised during the hot summer season. The level of *8-OHdG* gene was significantly lower in the probiotic administered group. The expression level of *HSP70* was lowest in the ascorbic acid group while, *IL-10* level of expression was highest in the probiotic + ascorbic acid group. The administered antioxidants were efficient in exhibiting anti-stress effects at the level of gene expression. We conclude that probiotic, ascorbic acid and probiotic + ascorbic acid reduced oxidative gene damage, affected the

expression of *HSP70* and increased the level of *IL-10* gene respectively, in broiler chickens exposed to heat stress.

Key-words: Antioxidants, broiler chickens, heat stress, oxidative gene damage,

3.0 INTRODUCTION

The world has been faced with the detrimental effects of heat stress arising from global warming (Moyo and Nsahlai, 2021). Global warming, especially in the sub-tropical and tropical regions of the world, has negatively affected the surface of the earth due to the emission of trapped solar radiation by several greenhouse gases. With the impact of global warming being widespread, negative impacts are now extending to agricultural systems (Oke et al., 2021). Of the various species farmed, intensively farmed poultry are highly susceptible species, due to the absence of sweat glands in the breed, making them highly dependent on other mechanisms for heat dissipation (Ouchi et al., 2022). In this species, increased thermal exposure leads to heat stress and subsequent oxidative stress in broiler chickens and other animals (Chen et al., 2018; Domenici and Seebacher, 2020; Shakeri and Le, 2022). Oxidative stress occurs when the production of reactive oxygen species (ROS) such as superoxide anion radical, hydroxyl radical, nitric oxide radical, hydrogen peroxide, peroxynitrite radical and oxygen singlet exceeds the body's capability to reduce them with resultant cellular damage (Amevor et al., 2022; Lin et al., 2022).

More specifically, increased levels of ROS have a negative impact on the nervous and endocrine systems which subsequently affect both cell-mediated and antibody immune responses (Alaqil, et al., 2022; Rattanasrisomporn et al., 2022). Alterations in the hypothalamic-pituitary-adrenal axis leads to an increase level of corticosterone as a response to the stressor (Ogbuagu et al., 2018). During chronic inflammation, the response of the body to inflammatory processes could eventually damage cells, tissues and organs that are apparently healthy (Fu et al., 2022). This can result to deoxyribonucleic acid (DNA) damage, other subcellular structures (plasma

membrane, nucleus, mitochondria) damage, death of tissue and scarring over time which are sequel to several diseases such as cancer (Hu et al., 2019). Reactive oxygen species effects on cells could also trigger the induction of autoimmune response during heat stress exposure. Heat shock proteins (HSP) are often triggered in broiler chickens when heat stress is intensed.

Heat shock proteins are molecular chaperones termed as stress protein; they are made when the cell is briefly exposed to temperatures above normal. They are also produced in response to oxidative stress (Roushdy et al., 2018). Their up-regulation is as a result of an individual's response to stressful conditions (Siddiqui et al., 2020). These *HSP* are responsible for the folding of proteins that are newly synthesized and the refolding of misfolded or damaged proteins (Siddiqui et al., 2020). In addition to upregulating ROS production, hydroxyl radicals can react with mitochondrial or nuclear DNA resulting to the formation of 8-hydroxy-2′-deoxyguanosine (*8-OHdG*), which is widely considered a biomarker of oxidative gene damage (Attia et al., 2019; Dai et al., 2019). Reactive oxygen species induce the modification of DNA bases by attacking guanine and leaving it unpaired (Zhu et al., 2019; Qing et al., 2019).

Following the release of inflammatory cytokines, their release triggers the release of anti-inflammatory cytokines (interleukin-10 (*IL-10*)) that inhibits further secretions of the previous cytokines and repair the damages caused by inflammation (Jiang et al., 2022). The balance between cell-based and humoral immune responses is effectively modulated by the cytokines (Saleh and Al-Zghoul, 2019). Reactive oxygen species exposure is known to interfere with this process with resultant increases in pro-inflammatory cytokine gene which could inhibit the release of anti-inflammatory cytokine like *IL-10* gene if not mitigated (Jiang et al., 2022).

The net impacts of oxidative stress in poultry production system are decreases in productivity and potentially death of birds (Aluwong et al., 2017). To understand the latter impact, one needs to consider the importance of poultry meat as a food source in the world. Poultry production, a form of animal husbandry encompasses the raising of domesticated birds like chickens, turkeys, etc., to produce meat or egg (Deng et al., 2022). Broiler chickens are bred for rapid growth to attain maximum meat production. Broiler meat is universally accepted because it is less expensive, has a quick turn-over and is thought to be more healthy than red meat, hence its value in countries with poorer economy (Sumanu et al., 2022).

To allow for optimal production, birds need to be either managed under controlled conditions which are expensive or through therapeutic means. For the latter the antioxidants, like ascorbic acid, are attractive therapeutic agents as they are known to inhibit the process of oxidation (Fu et al., 2022). Yeast probiotic, a living organism, may offer an alternate treatment as they possess both antioxidant and anti-stress potencies (Aluwong et al., 2017; Kim et al., 2022). The current study was therefore carried out to evaluate the effect of probiotic (*Saccharomyces cerevisiae*) and ascorbic acid in mitigating expression of *8-OHdG*, *HSP70* and *IL-10* genes in broiler chickens exposed to heat stress under natural conditions.

3.1 MATERIALS AND METHODS

3.1.1 EXPERIMENTAL SITES AND THERMAL ENVIRONMENTAL CONDITIONS

The study was undertaken at the Onderstepoort Veterinary Animal Research Unit (OVARU), Faculty of Veterinary Science, University of Pretoria, located in latitude 25° 39' 5"S and longitude 28° 10' 41.8"E, South Africa. The broiler chickens were raised in a natural environment of fluctuating ambient temperature during the hot period of the South African

summer. The study was approved by the Animal Ethics Committee of the University of Pretoria (REC050-20).

3.1.2 EXPERIMENTAL ANIMALS AND MANAGEMENT

Fifty-six (one-day-old) male and female broiler chicks (Ross), sourced from Alpha chicks, Gauteng, were used as the subjects. They were housed in an intensive management system (same building with four separate pens); brooding was done for 14 days with the aid of infra-red bulbs with an upper end of 32 °C. Feed (Epol feeds, South Africa) and water were made available to the chicks *ad libitum*, while wood shavings were used as litter. The poultry pen was constructed with concrete floor, cement blocks and aluminium roof. Efficient biosecurity measures were ensured during the study.

3.2 EXPERIMENTAL DESIGN

The individually marked chickens (n = 56) were allotted into four groups of fourteen each: Group I (control); Group II (probiotic, *Saccharomyces cerevisiae*; Sigma-Aldrich, South Africa), Group III (ascorbic acid; Medico Herbs, South Africa), Group IV (probiotic and ascorbic acid). Probiotic and ascorbic acid were administered at a dose of 1 g/kg (Parlat et al., 2001) and 200 mg/kg (Egbuniwe et al., 2021), respectively for 35 days.

3.2.1 DETERMINATION OF 8-OHDG, HSP70 AND IL-10 GENE EXPRESSION

Four (4) mL of blood sample was collected from the wing vein of 28 broiler chickens (7 per group) into 2 mL plain tubes and tubes containing ethylenediamine tetraacetic acid (EDTA) at D35 of the study before euthanising the entire (56) broiler chickens. The samples were transferred to the Department of Veterinary Tropical Diseases Laboratory for analysis. Plasma

samples were utilised for the *HSP70* and *IL-10* analyses while serum samples were used for *8-OHdG.*

3.2.1.1 8-HYDROXY-2′-DEOXYGUANOSINE QAUNTITATIVE ANALYSIS

The *8-OHdG* gene was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit specific for the gene (BIOCOM Africa, South Africa). The manufacturer's instructions were adhered to accordingly. Briefly, 50 µl of the sample was added to each well and 40 $ng \cdot \mu L^{-1}$of biotinylated detection antibodies was added immediately to the wells. It was incubated at 37 °C for 45 min and washed thrice. Forty nanograms per microlitre (40 $ng \cdot \mu L^{-1}$) of HRP conjugate was added to the wells and incubated for 30 min at 37 °C and washed, hence, 90 µl of substrate reagent was added and incubated at 37 °C for 15 min. Finally, 50 µl of stop solution was added and the optical density (OD) was determined immediately at 450 nm.

3.2.1.2 *HSP70* and *IL-10* gene expression analyses

I. procedure for ribonucleic acid (RNA) extraction from whole blood

The QIAamp RNA kit (BIOCOM Africa, South Africa) was used for the extraction. Two microgram per millilitre (2 µg/ml) of whole blood was mixed with 800 µl of buffer EL. The mixture was placed on ice for 15 minutes. The mixture was centrifuged at 400 × g for 10 minutes at 4 °C. The supernatant was completely removed and discarded. Fifty millimolar (50 mM) of buffer EL was added to the cell pellet and it was vortexed briefly. It was then centrifuged at 400 × g for 10 minutes at 4 °C. The supernatant was completely removed. Three hundred and fifty (350 µl) of buffer RLT was added to the pellet. Vortexing or pipetting was done to mix in order to remove any clumps. The lysate was pipetted directly into a QIA shredder spin column in a 2 mL collection tube, and centrifuged for 2 minutes at maximum speed to homogenise. The spin column was then discarded, and the homogenised lysate was saved. Three hundred and fifty (350

µL) of 70% ethanol was added to the homogenised lysate and mixed by pipetting. The sample was carefully pipetted (lysate + 70% ethanol), including the precipitate that was formed into a new spin column. It was centrifuged for 15 seconds at 10000 rpm. The spin column was transferred into a new collection tube. About 700 µl of buffer RW1 was added to the collection tube and centrifuged for 15 seconds at 10000 rpm. The spin column was placed into another new collection tube, 500 µl of buffer RPE was added into the collection tube and centrifuged for the third time for 15 seconds at 10000 rpm. The supernatant was discarded. The collection tube (spin column) was carefully opened and 500 µl of buffer RPE was added. It was closed and centrifuged for 3 minutes at maximum speed. The spin column was placed in a new collection tube. The old collection tube was then discarded with the lysate and buffer. It was centrifuged for 1 minute at full speed (without buffer). Finally, the spin column was transferred into a new microcentrifuge tube (final collection tube). About 50 µl of RNase-free water was pipetted into the membrane (tube), we then centrifuged for 1 minute at 10000 rpm to elute. Afterward, the RNA was stored in -80 °C.

II. Target genes

Expression of target genes for *IL-10* and *HSP70* were measured using RT-PCR Target genes and corresponding primers used for RT-PCR analysis are shown on Table 3.1.

Table 3.1. Primer sequences (F, forward; R, reverse) used for RT-PCR

Gene	Symbol	Nucleotide sequence (5′-3′)
Heat shock protein 70	*HSP70*	F: 5′-CCAAGAACCAAGTGGCAATGAA-3′
		R: 5′-CATACTTGCGGCCGATGAGA-3′
Interleukin 10	*IL-10*	F: 5′-AAGGCAGTGGAGCAGGTGAA-3′
		R: 5′-CCAGCAGACTCAATACACAC-3′

III. Standard curves and RT-PCR optimisation

Quantitative reverse transcription polymerase chain reaction was used to determine the differential gene expression *of IL-10* and *HSP70.* The RT-PCR for each gene was optimised first to determine the most appropriate annealing temperature for efficiency and specificity of amplification. Stock samples with relatively high RNA concentration (PP4, 55.50 ng/µl and CP2, 103.45 ng/µl) was used for the *HSP70* and *IL-10* gene, respectively. The number of copies of single stranded RNA was calculated using the formula:

Number of copies = [6.022E+23 (copies/mol) x Concentration (g/µl)]/[DNA/RNA length (bp) x 340 (g/mol)]

where, 6.022 x 10^{23} molecules/mole is the Avogadro's constant number, concentration as 5.5 x 10^{-8} g/µl for *HSP70* and 1.0345 x 10^{-7} g/µl for *IL-10,* and 340 Daltons is the average weight of a base in RNA. The length of RNA for the *HSP70* and *IL-10* are taken to be 2692 bases and 2300 bases, respectively.

Standard curved were generated using four 10-fold serial dilutions, with the aim of optimising and determining the efficiency of the RT-PCR assays. The diluent was nuclease-free water. Each dilution was analysed in triplicate in each RT-PCR run. To optimize the assays, annealing temperatures 53, 57, 60, 63°C and primer concentrations 0.2 and 0.4 μM were used. The mean of the C_q values from the dilutions in each RT-PCR test run were plotted against the logarithm of copy number of each gene to generate a standard curve. The PCR efficiency [E] (Livak and Schmittgen, 2001;Pfaffl, 2001; Vandesompele et al., 2002), expressed as a percentage, was determined using the following formula:

$$\%E = (10^{(-1/Slope)}-1) \times 100$$

where slope = slope of the derivative (tangent line) of the calibration curve. Dissociation curves were also evaluated for specificity of the PCR assays. After optimisation, the most appropriate annealing temperature and primer concentrations were used in the PCR reactions.

IV. RT-PCR for experimental samples

Twenty eight experimental chickens were randomly selected (7 per group) from the 56 broiler chickens and were analysed for the two genes in separate arrays. Each RT-PCR reaction comprised x1 (final concentration) Luna® Universal One-Step RT-qPCR Mix, x1 (final concentration) Luna WarmStart RT Enzyme Mix (New England BioLabs Inc., Ipswich, Massachusetts, USA), 0.4 μM of each primer and 1 μl of template RNA in a total reaction volume of 10 μl. The RT-qPCR conditions were set with an initial reverse transcription step at 55 °C for 10 min, followed by initial denaturation at 95 °C for 1 min, 45 cycles of denaturation at 95 °C for 10 s and annealing at optimised temperature for each primer for 10 s (60 °C for *HSP70* and 57 °C for *IL-10* and an extension step at 60 °C for 60 s). We included an additional step of 1

cycle for the melting curve analysis, with temperatures of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. A no-template control (without RNA template) was included in each PCR run. The RT-PCR was performed using a StepOnePlus™ Real-Time PCR System and the amplification data was collected using the StepOne™ Software 2.0 (Applied Biosystems, Life Technologies, South Africa).

Efficiency testing of the *HSP70* and *IL-10* primers was done prior to performing gene expression analysis.

V. Gene expression analysis

The RT-PCR quantification cycle (Cq) values of all experimental samples as well as 10-fold RNA dilutions were exported to Microsoft Excel to compute the Cq means and standard errors (SE) for each treatment group in each gene. Cq values of wells with undetectable values were excluded from further analyses. For quality control, Cq values above 35 were excluded from the analyses. The standard curves generated were used to estimate *HSP70* and *IL-10* copy numbers (copies per mL of blood) in the experimental chicken samples, using the corresponding linear regressions.

3.2.1.3 MALONDIALDEHYDE CONCENTRATION

The homogenates of breast muscle tissue were utilised to determine the Malondialdehyde (MDA) concentration using an ELISA kit (BIOCOM Africa, South Africa). The manufacturer's instructions were adhered to accordingly.

3.3 STATISTICAL ANALYSIS

Data (*HSP70* and *IL-10* genes) were log transformed to obtain a normal distribution. Comparison of gene expression levels across treatment groups in each target gene were performed using Generalised Linear Models with Gaussian distribution for *HSP70* and *IL-10*

genes. While one-way analysis of variance (ANOVA) was used to compare the means between the groups for *8-OHdG* gene. This was followed by the Tukey's Honest Significant Difference (Tukey's HSD) tests to compare differences between treatment pairs. Statistical analyses were performed using R software version 4.1.2 (R Core Team, 2021) at a significance level of 0.05.

3.4 RESULTS

3.4.1 8-HYDROXY-2′-DEOXYGUANOSINE GENE QUANTIFICATION

Results are presented as mean ± standard error of mean (SEM) for all the treatment groups. There was a highly significant difference between *8-OHdG* gene concentration obtained in the control and probiotic groups ($P < 0.0001$), but no significant difference ($P > 0.05$) between the control and ascorbic acid groups. Also, the difference in gene concentration between probiotic and ascorbic acid groups was significant ($P < 0.05$), however, there was no significant difference ($P > 0.05$) between probiotic and probiotic + ascorbic acid groups, and between ascorbic acid and probiotic + ascorbic acid groups, respectively (Fig. 3.1).

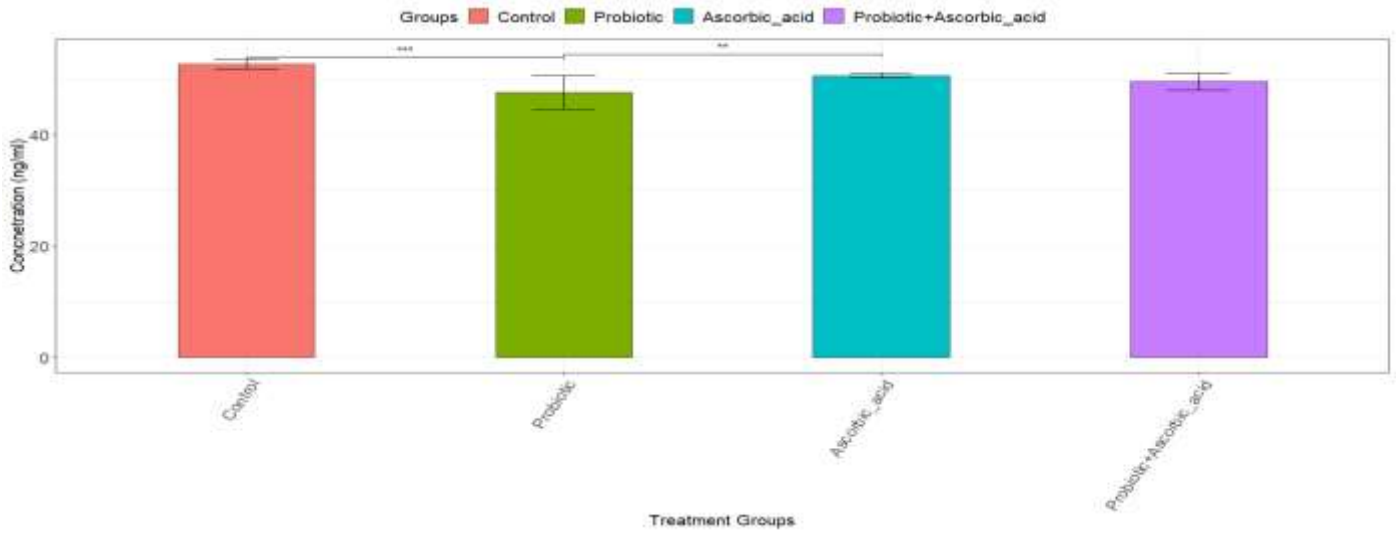


Fig. 3.1. 8-hydroxy-2′-deoxyguanosine (*8-OHdG*) gene concentration in blood from broiler chickens due to the adverse effects of heat stress. Four treatment groups: control, probiotic, ascorbic acid and probiotic + ascorbic acid. Data were expressed as mean fold change in gene concentration; the vertical bars represent standard error of mean. (n=7).

3.4.2 HEAT SHOCK PROTEIN 70 AND INTERLEUKIN 10 GENE EXPRESSION

Analysis of Cq values showed that *IL-10* (mean 27.14 and median 27.19) had higher mRNA expression than *HSP70* (mean 20.35 and median 19.88). The RT-PCR mean copy number expressions of the two different gene markers (*HSP70 and IL-10*) in the probiotic, ascorbic acid and co-administered groups compared to the control are shown in Table 3.2. The copy number is reported as mean ± standard error of mean for all experimental groups.

The Generalised Linear model showed that the expression of *HSP70* was 1.66 and 1.60 in the probiotic and probiotic + ascorbic groups, respectively compared with the control group. In contrast, *HSP70* had a lower expression (0.85 times) in the ascorbic group than the control group (Table 3.2). The *IL-10* gene had higher expression in probiotic + ascorbic group (1.16 times) compared with the control group, but similar expression in probiotic group and lower expression in ascorbic group (Table 3.2). The differences in both genes were not statistically significant ($P > 0.05$). Pairwise analysis using the Tukey's HSD showed no significant differences ($P > 0.05$) in *HSP70* and *IL-10* gene expression between groups.

Table 3.2. Gene expression of heat shock protein and interleukin 10 in four groups of chickens. This was in an experiment to determine the effect of ascorbic acid and probiotic treatments on diverse effects of heat stress.

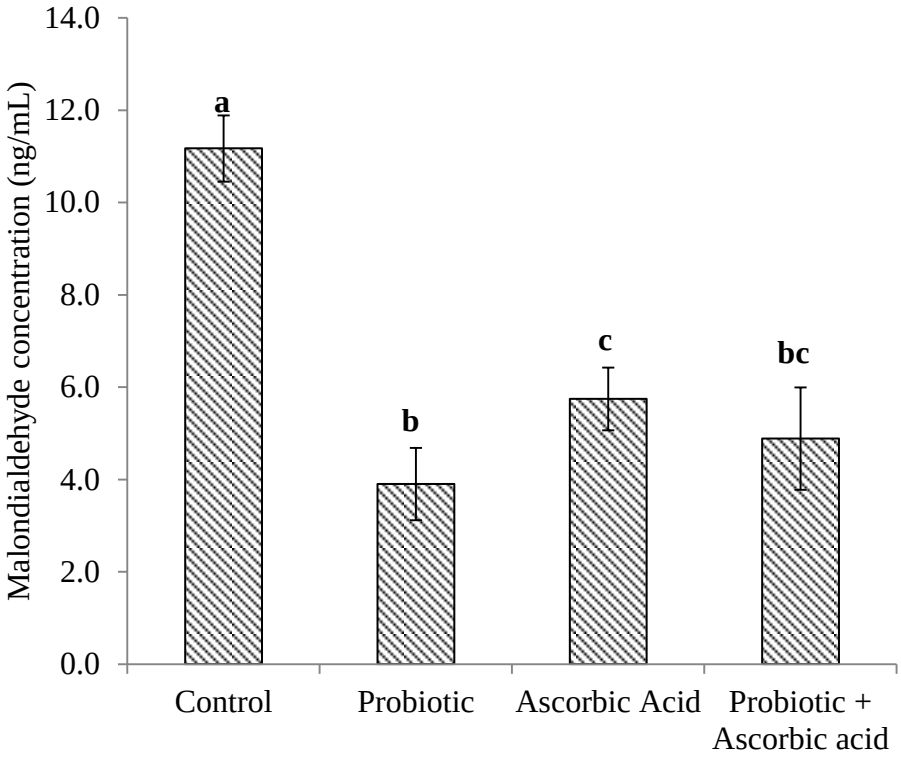
Parameter	Groups	Mean copy numbers ± SE ($x10^{12}$)	Coefficient	P-value
Heat shock protein 70	Control	1.633 ± 0.479		
	Probiotic	3.140 ± 0.684	1.662 [a]	0.149
	Ascorbic acid	1.155 ± 0.310	0.849 [b]	0.635
	Probiotic + ascorbic acid	3.110 ± 0.837	1.603 [a]	0.178

		$x10^8$		
Interleukin 10	Control	12.488 ± 4.725		
	Probiotic	12.623 ± 3.144	1.050[a]	0.896
	Ascorbic acid	5.044 ± 0.282	0.951[a]	0.897
	Probiotic + ascorbic acid	16.666 ± 5.689	1.156 [b]	0.699

Statistical analysis conducted using logarithm-transformed copy number data. SE, standard error of mean. n = 7. Values with superscripts [a,b] represent the level of significance.

3.4.3 MALONDIALDEHYDE CONCENTRATION

The concentration of MDA was significantly lower ($P < 0.02$) in the probiotic, ascorbic acid and probiotic + ascorbic acid groups of broiler chickens when compared to the control group during the study period (Fig. 3.2).



MDA concentration

Fig. 3.2. Malondialdehyde concentration in broiler chickens administered probiotic and ascorbic acid and exposed to heat stress. Data are expressed as Mean ± SEM. Vertical bars represent standard error of mean. Bars with superscripts [a,b,c] represent the level of significance.

3.5 DISCUSSION

Heat stress induces oxidative stress which promotes damages to the cell membranes; this process further stimulates various cellular activities as a response to the initial damages (Yan et al., 2022). The experimental period was thermally stressful to the broiler chickens and this fact was supported by the environmental temperature (20 – 30 °C) which outranged the thermoneutral zone (18 – 24 °C) for broiler chickens. To our knowledge, the use of yeast probiotic and ascorbic acid in alleviating oxidative stress using *HSP70, IL-10* gene expression and especially *8-OHdG* as biomarkers, have not been evaluated in broiler chickens. During this study, performance indices were negatively affected by heat stress in the group of broiler chickens void of antioxidants administration (control), while the treatment groups had better performance evident by an increase in body weight gain (See chapter four).

The serum concentration of *8-OHdG* was higher in the control group of broiler chickens, which could indicate that the broiler chickens were subject to heat stress during this study. This finding conforms to that of Soria-Meneses et al. (2022) who reported an increase in sperm concentration of *8-OHdG* in ram exposed to oxidative stress. The group of broiler chickens treated with probiotic had the least concentration of serum *8-OHdG*. With *8-OHdG* reported to be a genotoxicological effect via the cellular uptake of nanoparticles that are responsible for oxidative stress (Dai et al., 2019), it could be speculated that *Saccharomyces cerevisiae* probiotic was efficacious in modulating the production of ROS which triggers oxidative gene damage.

Saccharomyces cerevisiae probiotic functions as both anti-stress and antioxidant agent hence, its efficacy in inhibiting oxidative processes may be attributed to the decrease concentration of serum *8-OHdG* observed in this group of broiler chickens. This singular process may further confer immunity and a sense of wellness to the broiler chickens during the thermally stressful season. Ascorbic acid alone was not effective in down regulating the gene in broiler chickens; this could also explain the insignificant values obtained in the combination group during the study.

There was generally no significant difference in the expression of *HSP70* during the study, except in the ascorbic acid treatment group where there was down regulation of *HSP70* gene, in comparison to the control group. Heat stress is responsible for the induction of reactive oxygen metabolites (ROM) and *HSP70* (Gorman et al., 1999). Fatty acid oxidation increases to meet the requirement for energy in animals during heat stress, hence it is suggestive that the administration of ascorbic acid was effective in decreasing the accumulation as expected due to the potent antioxidant effect of the compound. The availability of ascorbic acid via diet could heighten the quantities of ascorbic acid oxidised to dehydroascorbic acid which would enhance the reduction of α-tocopheroxyl to α-tocopherol and functions to scavenge ROM produced during heat stress. Antioxidants generally slow down or decrease the process of oxidation, hence minimising the expression level of *HSP70* in birds (Sur et al., 2023). Hence, it could be speculated that animals treated with antioxidant and/or antistress agents during heat stress may inhibit the level of expression of *HSP70* in cells. That is, absence of oxidative stress could decrease the level of *HSP70* expression and vice versa. Gu et al. (2012) reported that the treatment of H_2O_2-stressed fish with melatonin decreased the level of *HSP70* due to its protective

effects against oxidative stress. Mahmoud et al. (2004) has previously reported a decrease in expression of *HSP70* in broiler chickens administered ascorbic acid and exposed to chronic stress. This was attributed to the ability of the supplement to act on the cellular antioxidant cascade to reduce the accumulation of reactive oxygen species. Our findings were the same as in the literature. The administered probiotic was not effective on the expression level of *HSP70* during the study. It could be speculated from our study that yeast probiotic had an inverse proportion between the expression levels of *HSP70* and *8-OHdG* genes in broiler chickens exposed to heat stress, which requires further investigation to ascertain the cause.

The combined administration of the probiotic + ascorbic acid was effective in increasing the expression level of *IL-10* gene (though was not significant) which indicated that systemic inflammation induced by heat stress in broiler chickens was being mitigated. Stress induces inflammatory responses, hence the up regulation of *IL-10* an anti-inflammatory cytokine serves as a protective measure against oxidative stress effect in the cells. This also revealed that ascorbic acid functioned best when combined with probiotic in enhancing the production of *IL-10* gene. Broiler chickens treated with the probiotic or ascorbic acid singly showed a decrease level of expression of *IL-10* gene in comparison with the co-administered group. This result shows that the effect of probiotic and especially ascorbic acid administration on a host could be affected by several factors like the duration and type of stressors, animal species and environment in enhancing immune response (Wang et al., 2018). Hence, the broiler chickens could have also been susceptible to the ascorbic acid administered in stimulating anti-inflammatory cytokine release and this could be responsible for the lowest value obtained in this group. The control group had a decrease expression of *IL-10* in comparison with the co-administered group. This may be attributed to the intensity of heat stress the broiler chickens

were exposed to during the study. Arendt et al. (2019) reported a decrease in expression of *IL-10* in the jejunum and cecum of broiler chickens infected with *Eimeria* which is a form of stressors to the chickens.

Antioxidants are responsible for converting free radicals to more stable products. Therefore, they play a vital role in enhancing the stability of lipids in the cell by preventing the oxidation of lipid during oxidative stress in broiler chickens (Ogbuagu et al., 2018). This could be attributed to the decrease in concentration of MDA in the treatment groups in comparison with those of the control. This finding supports that of Deng et al. (2022) who reported the antioxidant effect of yeast probiotic in broiler chickens.

The mechanisms responsible for the modulation of *8-OHdG* and *HSP70* gene expression inversely in broiler chickens administered probiotic and exposed to heat stress are unknown and should be threads for future research. Though the sample size may be seen as a limitation of this study, the biostatistician's calculation found 56 birds to be enough for the objectives of the study and the research ethics and animal ethics committees are strict on using the minimum number of animals when approving ethics application with respect to the animal welfare policy of the University of Pretoria.

3.6 CONCLUSION

Based on the above findings, we concluded that probiotic (*Saccharomyces cerevisiae*) reduced oxidative gene damage by mitigating the excessive production of ROS which could be responsible for genetic mutation in broiler chickens. Ascorbic acid was effective in reducing the production of *HSP70*, while probiotic + ascorbic acid increased the expression level of *IL-10* genes in broiler chickens exposed to heat stress. Hence, it could be deduced that the

administration of probiotic and ascorbic acid both singly and in combination were effective in alleviating the adverse effect of heat stress in broiler chickens.

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CHAPTER FOUR

Published in Animals

MITIGATION OF THE NEGATIVE EFFECTS OF HEAT STRESS IN BROILER CHICKENS FED DIET FORTIFIED WITH PROBIOTIC AND ASCORBIC ACID

ABSTRACT

Thermal stress negatively affects the welfare of broiler chickens leading to poor productivity and potentially death. This study was focused on mitigating the detrimental effects of thermal stress in chickens through the administration of probiotic and/or ascorbic acid. Performance indices, indicators of heat stress and intestinal morphology were evaluated under semi-natural conditions during the hot summer months at Onderstepoort, Pretoria, South Africa. The study made use of 56 chicks and were allotted into 4 groups (one control and three treatment groups) of 14 each and the administrations were given via feed for five weeks (D1 to D35). The effect of the antioxidants activities were highly significant ($P < 0.0001$) in the treatment groups in comparison with the control. Performance indicators were significantly higher ($P < 0.05$) in the probiotic and co-administered group. The height of duodenal, jejunal and ileal villi, and goblet cell counts in the treatment groups were significantly different ($P < 0.001$). The control group showed that heat stress negatively affects the performance, levels of endogenous antioxidants and the morphology of small intestinal epithelium. In conclusion, the administered antioxidants were efficacious in mitigating the negative effects of thermal stress in chickens.

Key words: ascorbic acid; probiotic; antioxidant enzymes; performance; small intestinal morphology

4.0 INTRODUCTION

Heat stress is a major problem experienced globally, especially during the summer in the sub-tropics and tropics (Elbaz et al., 2022; Madkour et al., 2022). It severely affects the profitability in both small- and large-scale farmers due to lower productivity and growth in the stressed birds (Goo et al., 2019). Heat stress induces oxidative stress, which is the impairment in balance between antioxidant enzymes and free radicals with resultant negative effects on the health status of broiler chickens. Of the various injuries induced during oxidative damage, one is lipid peroxidation where the free radicals tend to 'grab' electrons from lipids in the cell membrane which leads to damage in all cell types (Alagawany et al., 2022).

Endogenous antioxidants are adequate for preserving normal cellular functionality under normal physiology and increased ROS production such as during heat stress; they are often exhausted necessitating the need for these enzymes or precursors to be administered (Ebeid et al., 2022). Heat stress impairs the morphology of broiler chickens' intestine necessitating the administration of anti-stress agents like probiotic and ascorbic acid to protect the integrity of the intestine (Jahejo et al., 2016).

Yeast probiotic is rich in vitamin B, protein, fat, phytase and cellulase (Parlat et al., 2001; Aluwong et al., 2013). Ascorbic acid, a water-soluble vitamin, serves as both antioxidant and anti-stress agent. It protects the body against ROS's deleterious effects via the prevention of formation of excessive free radicals above what the body can deal with (Gouda et al., 2020). Therefore, these feed additives which are less expensive compared to some phytonutrient and are readily available, could serve as alternative measures in alleviating the negative effects of heat stress in broiler chickens, improving their growth performance.

Our aim was to alleviate the adverse effects of heat stress in broiler chickens during summer under semi-controlled conditions by fortifying their feeds with *Saccharomyces cerevisiae* and/or ascorbic acid as feed supplements.

4.1 MATERIALS AND METHODS

4.1.1 MANAGEMENT, AND ENVIRONMENTAL CONDITIONS

Fifty six day-old Ross 308 broiler chicks that were apparently healthy according to chick classification methods, were purchased from Gauteng poultry farmers. The sample size was calculated in G-power for the biomedical conditions under which the birds were housed, with an effect size f value of 0.59, and critical F of 2.78.

The broiler chicks were kept under an intensive management system under prevailing natural environmental conditions. Wood shavings were used as litter and the chicks were allowed access to feed (Epol feed, South Africa) and water. The broiler chicks were allotted into four groups of 14 each, they were given broiler starter (D1 - D14), broiler grower (D15 - D25) and broiler finisher (D26 - D35). The feed analysis is presented on Table 4.1. Biosecurity measures were ensured by the provision of foot baths (F10SC, Health and Hygiene (Pty) Ltd, South Africa), and of protective clothing and footwear for the assistants.

4.2 EXPERIMENTAL DESIGN

The experimental design is as described in chapter three, section 3.2.

Table 4.1. Composition of feed utilized for the study

Feed composition	**Starter**	**Grower**	**Finisher**
Ingredients (g/kg)			
Protein	180	170	160
Total lysine	11	10	9
Total methionine	4.2	3.8	3.4
Moisture	120	120	120
Fat	25	25	25
Fibre	50	60	70
Calcium	10	10	10
Phosphorus	5	4.5	5
Proximate Analysis (%)			
Protein	18.22	18.23	18.24
Total lysine	1.67	1.68	1.69
Total methionine	0.63	0.64	0.65
Fat	3.45	3.46	3.47
Fibre	5.54	5.55	5.56
Calcium	0.58	0.59	0.60
Phosphorus	0.52	0.53	0.54

4.2.1 MEASUREMENT OF PERFORMANCE PARAMETERS

4.2.1.1 Measurement of feed consumption

Feed consumption of broiler chickens was measured daily at 07:00 h using a weighing balance (8606 Greifensee, Switzerland). Total feed intake was calculated as the difference between the amount of feed offered and the amount of refusal at the end of each day (Aluwong et al., 2013).

4.2.1.2 Measurement of water intake

Daily water intake was measured by the difference between the quantity of water offered and the left-over using a graduated cylinder (Rutland Industries, South Africa).

4.2.1.3 Measurement of body weight gain

Upon arrival at the poultry pen, each broiler chick was weighed using a Mettler Toledo® weighing balance at D1 and these values served as the initial body weight. Their weights were measured once every week during the study period. The final body weight gain was determined on D35 of the study period.

4.2.1.4 Terminal Procedures

Broiler chickens were sacrificed via euthanasia with the use of gas mixtures (35% CO_2, 35% N_2 and 30% O_2). The breast muscle tissues were severed from 28 chickens (seven per group), and 5 g was placed in tubes and homogenised. Samples were collected at OVARU, transferred to the Physiology Laboratory of the University of Pretoria, South Africa, were it was processed and stored.

4.2.2 MEASUREMENT OF OXIDATIVE STRESS BIOMARKERS

4.2.2.1 Superoxide dismutase activity (SOD)

At D35, breast muscle tissue was randomly collected from 28 broiler chickens. Superoxide dismutase activity was determined using an ELISA kit (BIOCOM Africa, South Africa), adhering to the manufacturer's instructions. Optical density (OD) was determined at 550 nm using a spectrophotometer (UV-VIS, Perlong Medical Equipment Co., Ltd., China).

4.2.2.2 Catalase activity (CAT)

Catalase activity was determined using the homogenates of the breast muscle tissue with the aid of CAT ELISA kit (BIOCOM Africa, South Africa). The OD was measured at 405 nm with a spectrophotometer.

4.2.2.3 Glutathione peroxidase activity (GPx)

Glutathione peroxidase activity was determined using the homogenates of the breast muscle tissue with the aid of GPx ELISA kit (BIOCOM Africa, South Africa) and the manufacturer's instructions were adhered to accordingly. The OD was determined at 450 nm immediately after, with the aid of a spectrophotometer.

4.2.3 MEASUREMENT OF SMALL INTESTINAL MORPHOLOGY

Seven broiler chickens from each group (28 broiler chickens) were fasted for 12 h on D35 of the experiment (as stated in section 4.2.1.4). About 3 cm length of the small intestine for each broiler chicken were taken and stored in a tube containing 10% solution of buffered neutral formaldehyde (pH 7.2 - 7.4). After dehydration, the samples were placed in paraffin blocks, they were sectioned and stained with Periodic acid-Schiff reagent according to the method of Luna (1968). The crypt height and width, villus height and width, villus and crypt surface areas and goblet cells of the small intestinal epithelium were measured at 100 × magnification (Sigma Scan, USA).

Transmission electron microscopy was used to evaluate the morphology of the goblet cells. About 0.5 × 0.5 × 0.5 cm tissue blocks of various segments of the small intestine were taken and inserted into a tube containing 2.5% glutaraldehyde and processed according to the standard

method described by Cheville and Stasko (2014). Stained sections were visualised using a JEOL 1400 electron microscope operated at 80 kV.

4.3 STATISTICAL ANALYSIS

Values from all the analysis were subjected to one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison *post-hoc* test to compare differences between the treatment and control groups' means with significance at 0.05. Version 27 (Armonk, NY: IBM Corp) SPSS Statistics for Windows software was used for the analysis.

4.4 RESULTS

4.4.1 PERFORMANCE INDICES

Values of feed intake recorded in the treatment groups were not significantly different ($P > 0.05$) when compared with that of the control. Water intake recorded in the treatment groups was significantly higher ($P < 0.05$) during the study period. Body weight gain obtained in the probiotic group was significantly higher ($P < 0.05$) when compared with the control group during the study period (Table 4.2).

Table 4.2. Performance indicators in broiler chickens exposed to thermal stress and ameliorated using *Saccharomyces cerevisiae* and ascorbic acid.

	Group	Day 7	Day 14	Day 21	Day 28	Day 35
Feed intake (g)	Control	675.14 ± 157.20[a]	1177.57 ± 58.95[a]	1836.71 ± 80.19[a]	2505.43 ± 70.01[a]	2796.86 ± 45.88[a]
	Probiotic	674.43 ± 137.40[a]	981.57 ± 64.21[a]	1923.00 ± 134.98[a]	2821.86 ± 41.10[a]	3126.71 ± 56.54[a]
	Ascorbic acid	708.71 ± 153.98[a]	1115.00 ± 64.21[a]	2327.71 ± 175.52[a]	2646.00 ± 31.61[a]	2792.29 ± 70.02[a]
	Prob + AA	672.29 ± 139.07[a]	1152.29 ± 71.42[a]	2066.57 ± 87.43[a]	2771.43 ± 16.53[a]	2920.71 ± 62.78[a]
Water intake (ml)	Control	1400.00 ± 293.58[a]	2542.86 ± 184.98[a]	4585.71 ± 280.67[a]	5928.57 ± 184.80[a]	7500.00 ± 267.26[a]
	Probiotic	2000.00 ± 243.98[b]	2971.43 ± 176.90[a]	4900.00 ± 325.87[a]	6828.57 ± 164.34[b]	8628.57 ± 316.77[a]
	Ascorbic acid	1942.86 ± 220.23[a]	3200.00 ± 211.57[b]	5142.86 ± 348.37[b]	7057.14 ± 165.99[b]	8671.43 ± 222.23[b]
	Prob + AA	1928.57 ± 229.61[a]	2971.43 ± 178.24[a]	5085.71 ± 317.30[b]	6871.43 ± 171.43[b]	8471.43 ± 153.86[b]
Body weight gain (g)	Control	181.50 ± 2.24[a]	505.86 ± 15.14[a]	1031.21 ± 29.89[a]	1756.50 ± 48.23[a]	2138.50 ± 68.02[a]
	Probiotic	183.93 ± 1.16[a]	515.57 ± 19.33[a]	1289.07 ± 90.23[a]	1835.71 ± 43.00[b]	2730.79 ± 55.26[b]
	Ascorbic acid	190.07 ± 1.95[a]	521.14 ± 16.33[a]	1063.14 ± 30.99[a]	1768.86 ± 45.56[a]	2321.71 ± 58.36[a]
	Prob + AA	185.07 ± 2.93[a]	522.21 ± 15.89[a]	1077.64 ± 34.02[a]	1729.14 ± 61.73[a]	2432.64 ± 68.82[b]

Mean values with different superscript letters within the same column are significantly different at $P < 0.05$. Prob + AA = Probiotic + Ascorbic acid, n = 14. Values with superscripts [a,b,c] represent the level of significance.

4.4.2 SUPEROXIDE DISMUTASE ENZYME ACTIVITY

The activity of SOD was significantly higher ($P < 0.0001$) in the treatment groups when compared to the control. There were no significant differences ($P > 0.05$) in SOD activity obtained between all the treatment groups (Fig. 4.1).

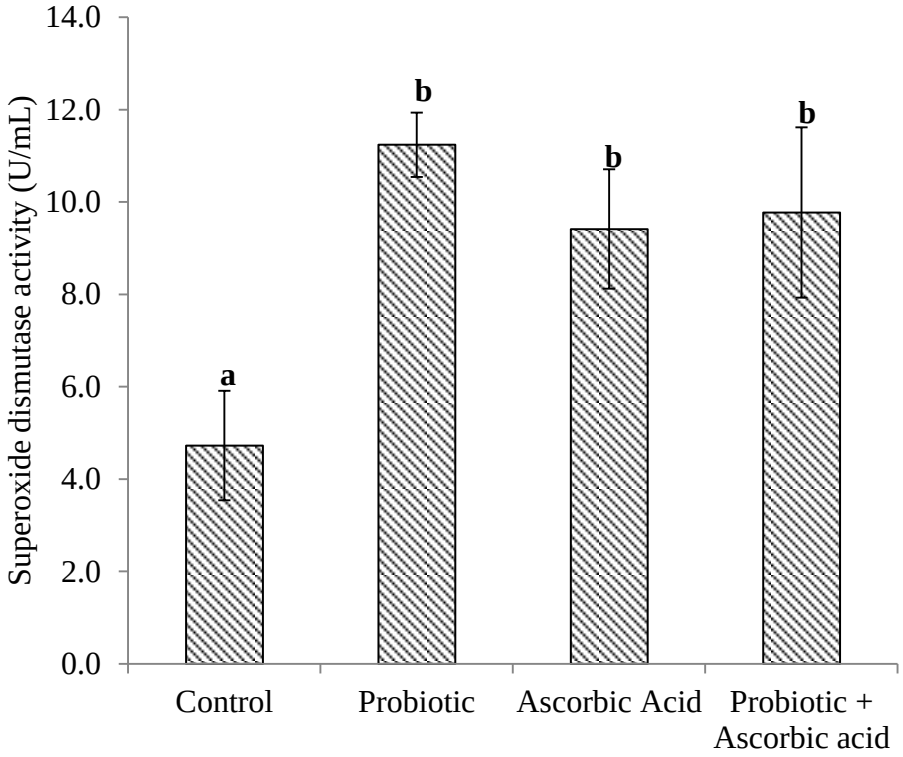

Fig.4.1. Superoxide dismutase activity in muscle tissue homogenates of chickens exposed to thermal stress and administered *Saccharomyces cerevisiae* and ascorbic acid. Bars with superscripts [a,b] represent the level of significance.

4.4.3 CATALASE ENZYME ACTIVITY

The activity of CAT was significantly higher ($P < 0.0001$) in treatment groups when compared to the control (Fig.4.2).

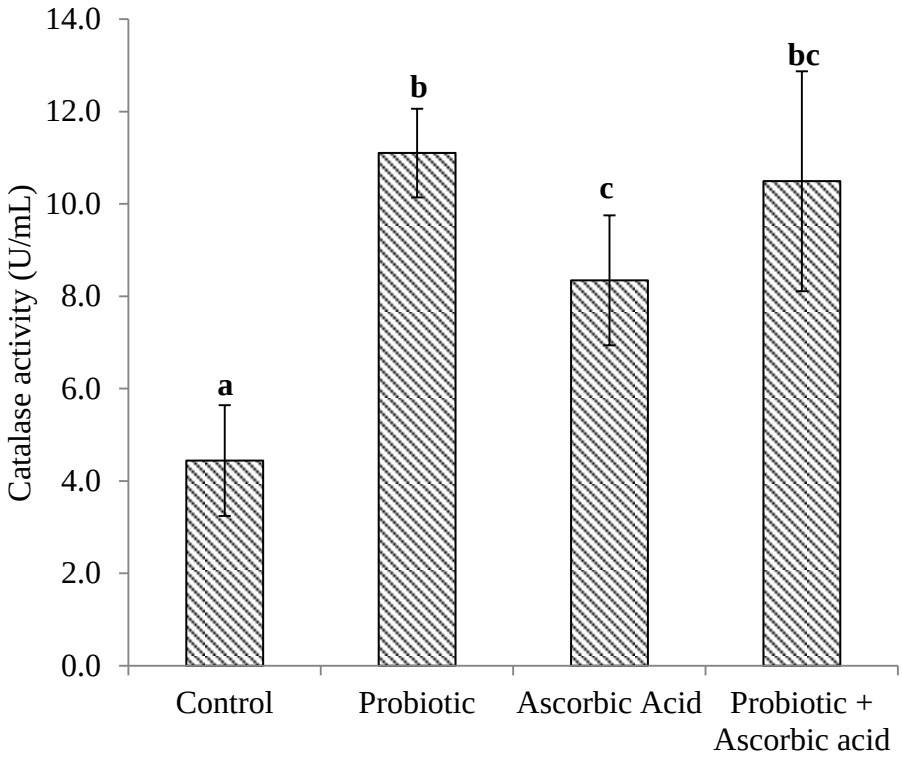

Fig 4.2. Catalase activity (U/mL) in broiler chickens muscle tissue exposed to thermal stress and administered *Saccharomyces cerevisiae* and ascorbic acid. Bars with superscripts [a,b,c] represent the level of significance.

4.4.4 GLUTATHIONE PEROXIDASE ENZYME ACTIVITY

The activity of GPx was significantly higher ($P < 0.0001$) in the treatment groups of broiler chickens (Fig. 4.3).

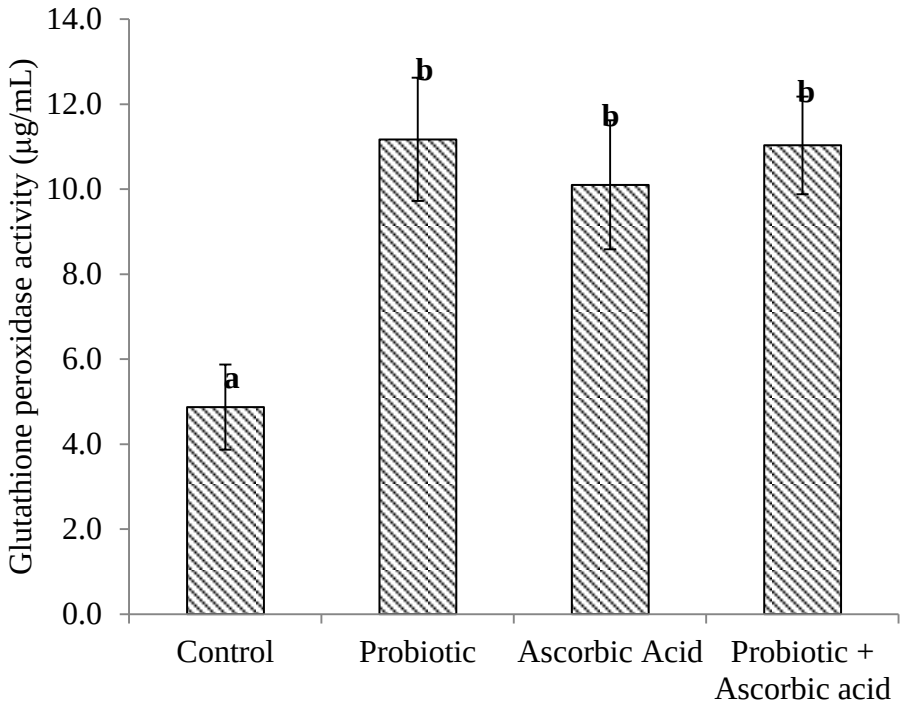

Fig.4.3. Glutathione peroxidase activity in muscle tissue homogenates of broiler chickens exposed to thermal stress and administered *Saccharomyces cerevisiae* and ascorbic acid. Bars with superscripts ^{a,b,c} represent the level of significance.

4.4.5 MORPHOLOGICAL ANALYSIS

Morphometric analysis of the small intestine was significantly different ($P < 0.001$) in all treatment groups in comparison with the control group. The crypt depth and width, were significantly greater ($P < 0.0001$) in the treatment groups when compared to the control group. The treatment groups had significantly different surface areas ($P < 0.05$) of villi and crypts compared to the control group. The goblet cells of the duodenum, ileum and jejunum were significantly greater ($P < 0.001$) in the treatment groups during this study (Table 4.3).

Table 4.3. Histomorphometry of the small intestine of broiler chickens administered probiotic and/or ascorbic acid and exposed to heat stress.

	Parameters	Control	Probiotic	Ascorbic acid	Probiotic + AA
Duodenum	Villus height (μm)	652.18 ± 39.94^{a}	1706.92 ± 129.15^{c}	1427.69 ± 66.69^{b}	1230.55 ± 81.85^{b}
	Villus width (μm)	117.33 ± 6.98^{a}	288.21 ± 53.41^{b}	173.55 ± 8.23^{a}	278.15 ± 44.77^{b}
	Crypt depth (μm)	95.61 ± 12.29^{a}	329.45 ± 37.26^{c}	267.20 ± 53.10^{b}	324.23 ± 43.89^{b}
	Crypt width (μm)	48.16 ± 4.86^{a}	159.57 ± 16.87^{b}	125.07 ± 20.47^{b}	140.95 ± 25.55^{b}
	Villus SA (mm^2)	5.40 ± 1.29^{a}	16.00 ± 2.98^{c}	7.50 ± 3.21^{b}	10.80 ± 1.32^{b}
	Crypt SA (mm^2)	2.80 ± 0.66^{a}	12.80 ± 2.13^{c}	3.80 ± 1.24^{a}	8.40 ± 1.03^{b}
	Goblet cells	58.00 ± 13.99^{a}	101.00 ± 4.92^{b}	108.20 ± 9.32^{b}	102.80 ± 12.14^{b}
Ileum	Villus height (μm)	284.58 ± 56.72^{a}	1025.07 ± 66.80^{d}	678.08 ± 58.14^{b}	865.22 ± 68.37^{c}
	Villus width (μm)	84.70 ± 19.19^{a}	236.10 ± 41.28^{c}	107.93 ± 8.03^{b}	230.98 ± 19.38^{c}
	Crypt depth (μm)	98.95 ± 5.09^{a}	159.73 ± 25.54^{b}	135.13 ± 21.12^{b}	231.73 ± 35.60^{c}
	Crypt width (μm)	40.80 ± 5.94^{a}	88.70 ± 10.96^{c}	65.38 ± 5.05^{b}	92.28 ± 7.71^{c}
	Villus SA (mm^2)	3.60 ± 0.93^{a}	10.40 ± 1.44^{b}	7.60 ± 1.60^{b}	9.00 ± 1.22^{b}
	Crypt SA (mm^2)	3.60 ± 0.68^{a}	7.40 ± 1.12^{b}	3.60 ± 1.21^{a}	7.40 ± 0.93^{b}
	Goblet cells	52.40 ± 9.69^{a}	183.20 ± 20.61^{c}	125.40 ± 22.65^{b}	122.20 ± 13.40^{b}
Jejunum	Villus height (μm)	362.95 ± 68.90^{a}	1205.70 ± 105.67^{b}	1010.33 ± 62.26^{b}	1066.50 ± 51.31^{b}
	Villus width (μm)	102.81 ± 11.09^{a}	204.58 ± 20.54^{b}	202.58 ± 37.60^{b}	220.48 ± 35.19^{b}
	Crypt depth (μm)	123.46 ± 11.16^{a}	303.43 ± 36.83^{b}	240.39 ± 39.34^{b}	279.19 ± 26.99^{b}
	Crypt width (μm)	48.47 ± 4.33^{a}	98.71 ± 14.12^{b}	84.21 ± 5.62^{b}	88.41 ± 9.57^{b}
	Villus SA (mm^2)	2.70 ± 0.49^{a}	10.20 ± 1.39^{b}	11.40 ± 3.06^{b}	9.80 ± 0.86^{b}
	Crypt SA (mm^2)	2.80 ± 0.66^{a}	8.20 ± 1.28^{b}	6.40 ± 1.57^{b}	7.80 ± 1.02^{b}
	Goblet cells	56.00 ± 8.15^{a}	150.60 ± 20.92^{c}	93.00 ± 9.95^{b}	102.60 ± 6.37^{b}

Mean values with different superscript letters along the same row are significantly different at $P < 0.05$. n = 7. AA = Ascorbic acid, SA = surface area.

The histological representations of the effect of stress on jejunal epithelium in the treatment group were significantly different ($P < 0.05$) when compared to the control groups of broiler (Figs 4.4 and 4.5).

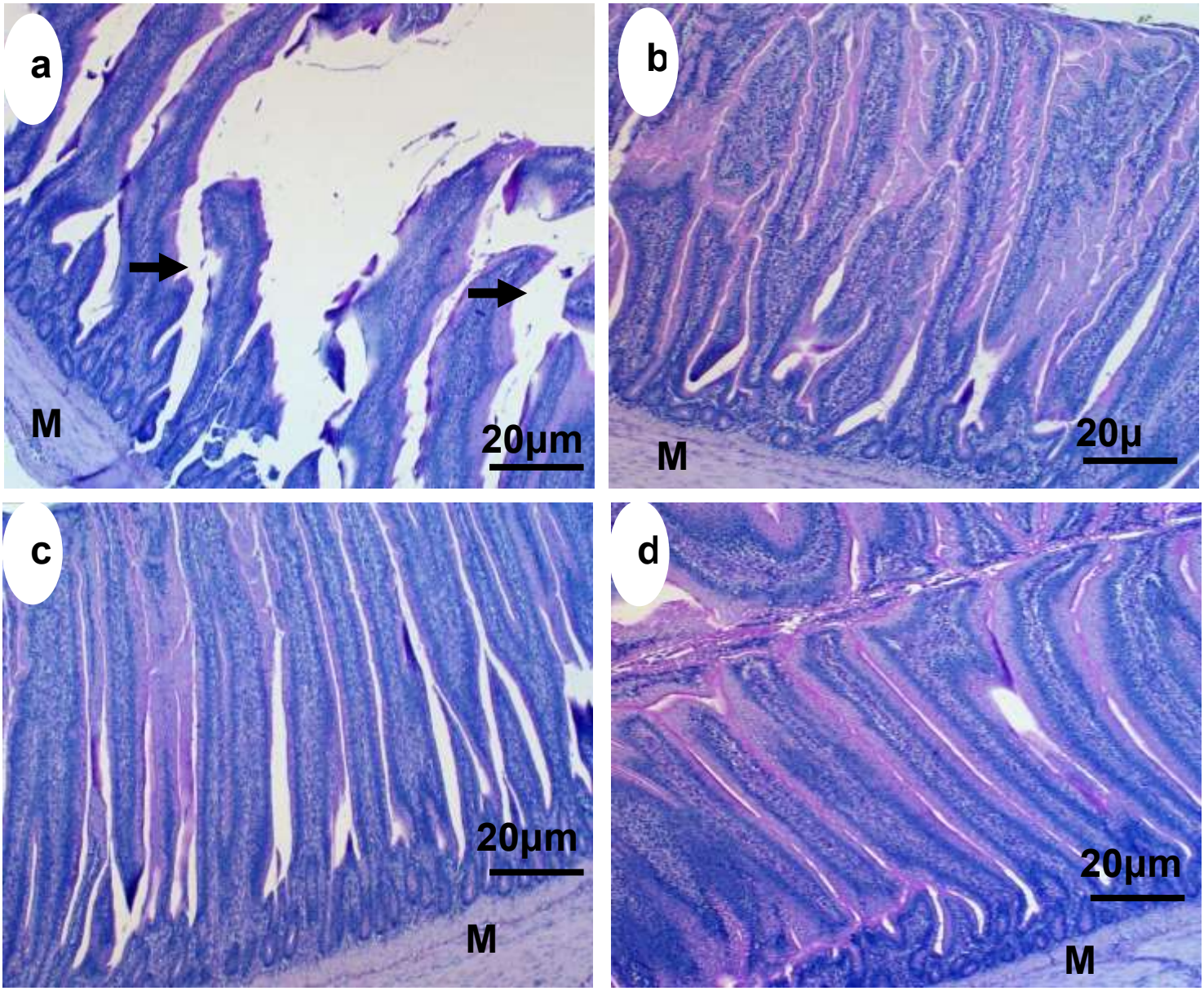

Fig. 4.4. Light microscopy showing the jejunal epithelia of broiler chickens during thermal stress (a) without treatment as a control group, (b) treated with probiotic, (c) treated with ascorbic acid, and (d) treated with probiotic and ascorbic acid. Arrows = widening of capillary lacteal due to distortion of epithelia, M = muscularis mucosa.

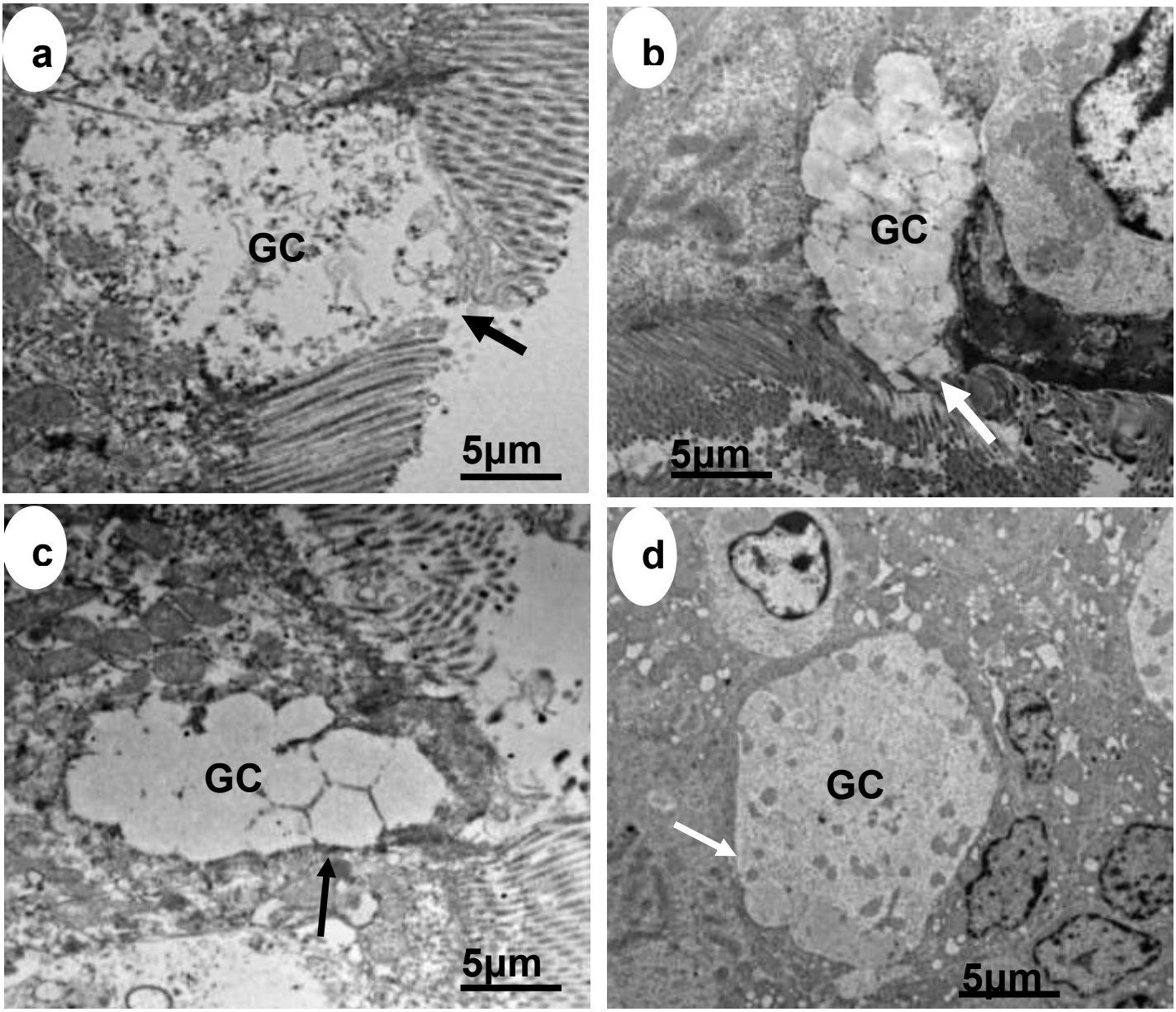

Fig. 4.5. Electron micrographs of jejunum of broiler chickens during thermal stress (a) without treatment, a distorted goblet cell without a defined contour and absence of mucin (thick black arrow), (b) treated with probiotic, showing the goblet cell and presence of mucin (thick white arrow), (c) treated with ascorbic acid, and there was no presence of mucin but presence of dilations within the lobules of the goblet cell (thin black arrow) and (d) treated with probiotic and ascorbic acid, in which the goblet cell has a well-defined contour (thin white arrow) GC = goblet cell.

4.5 DISCUSSION

The decreased water intake observed in the control may be based on their low feed intake, as the quantity of feed intake may be directly proportional to water intake to further enhance digestibility. Ascorbic acid-administered group had an increase in water intake, which could

be supported by the fact that feed intake stimulates an increase in water intake, especially during heat stress. Generally, increased water intake obtained in the treatment groups could also be assumed as a thermoregulatory measure to rid the body of excessive heat during the period of study. The increase in water intake recorded in the treatment groups agrees with Egbuniwe et al. (2018) who observed an increase in water intake in chickens administered ascorbic acid and/or betaine than the control group. They also attributed the increase to be a means of thermoregulation.

The lowest body weight gain was observed in the control. This may be in line with the lower feed (though not significantly different) and water intake compared with the treatment groups. It is important to note that Ross breed of broiler chickens utilized for this study was reported to attain the highest weight of 2.5 kg at 6 weeks of age under a thermoneutral condition according to the Ross broiler pocket guide (2020), but body weight gain was highest (2.8 kg) in the probiotic-supplemented group at D35 (5 weeks) of this study. This increase may be linked to the fact that probiotics are gut effective and increased water intake may further enhance the rate at which ingested feed is utilized for optimum body weight gain Aluwong et al. (2013). Additionally, probiotic could be vital in the process of nutrient digestion due to optimization of the gut microflora, although, its effect in the transportation of nutrients within the body system remains a grey area for further examination. To the best of the authors' knowledge, most researchers conducted their studies using probiotic to optimize the rate of performance in broiler chickens within 42 days (6 weeks), but this study achieved better outcomes within 35 days with an average weight of 2.7 kg being recorded in the probiotic-administered group. Aluwong et al. (2017) speculated that the administration of *Saccharomyces cerevisiae* via gavage is the fastest route that promotes broiler chickens' performance. Nevertheless, we administered the yeast probiotic via feed (oral route) and from the results, improved performance indices were observed in this group of broiler chickens

during our study. Wang et al. (2018) reported that probiotic (*Bacillus subtilis*) increased the body weight gain of broiler chickens to 2.3 kg at D43 of their study and this was attributed to the gut enhancement effect of the additive. Also, the thermoregulatory effect of probiotic *Saccharomyces cerevisiae* during heat stress could have been beneficial as feed intake is often improved when core body temperatures of broiler chickens are within the normal range (Bilal et al., 2023). Increased weight gain attained in the ascorbic acid group might be due to the improved feed and water consumption recorded in this group of broiler chickens, based on its antioxidant effects in improving wellness which could further stimulate an improvement in performance. This finding corresponds with Egbuniwe et al. (2018) who observed an increase in body weight gain in group of chickens treated with ascorbic acid, they presumed the increase to be a resultant of the anti-stress effect of the agent.

Increased production of ROS which occurs during heat stress generally impacts the activities of endogenous enzyme if no exogenous source is used for augmentation in broiler chickens (Wang et al., 2022). Heat stress stimulates the process of lipid peroxidation which subsequently leads to an exhaustion of their antioxidant defence system in broiler chickens. Xue et al. (2021) also noted a drastic depletion in antioxidant enzyme activities of broiler chickens during stress. They attributed this to the fact that exhaustion of antioxidant defence system occurs during heat stress due to cellular damages resulting from rapid oxidation processes. Following treatment, the probiotic group had the highest SOD, CAT and GPx activities followed by the combination and the ascorbic acid groups, respectively during this study period. Antioxidants directly react with free radicals converting them to non-radical products that are more stable; they are also vital in preventing lipid oxidation in the cell (Decker et al., 2002).

According to Winiarska-Mieczan et al. (2021) exogenous antioxidants induce an increase in the activities of endogenous antioxidants in the muscle tissue of broiler chickens by protecting the cellular membrane from peroxidation. Kumar et al. (2021) stated in their findings that the use of *Moringa oleifera* and ascorbic acid in chickens bred in the tropics augmented the activities of CAT, SOD and GSH, and lipid peroxidation was reduced significantly. The ability of the above antioxidants to attach to the cytoplasmic membrane and inhibit oxidases could be responsible for the observed changes. Deng et al. (2022) reported that the supplementation of broiler chicken's diet with yeast probiotic improved their antioxidant status which is suggestive of their strong antioxidant potential as compared to selenium which had no effect.

Generally, it is assumed that the non-significant value in feed intake observed between the control and treatment groups might be due to the degree of heat stress as water intake is prioritized over feed intake during prolonged exposure to thermal stress. This agrees with Moataz et al. (2018) who reported decreased feed intake in layers exposed to heat stress, when compared with the groups that were administered *Bacillus subtilis* probiotic. The authors associated the decrease to be a resultant of the degree of thermal stress.

In addition to the changes in the specific markers, the small intestinal epithelium was shortened, while the goblet cell count in the control group decreased. Intestinal distortion is basically a primary response to heat stress, and this could further compromise the integrity and barrier function of the intestine in broiler chickens as observed in this group.

Also, the jejunal epithelia lacteal was distorted which could be linked to the negative effects of stress in the control group. The epithelia lacteal absorbs fatty acids and cholesterol in the small intestine, therefore, any form of alteration in their morphology could affect the process of absorption. The gastrointestinal tract has been reported to be more susceptible to any form

of stressors, especially heat stress because it impairs the integrity of the epithelium via decreasing crypt depth and villus height (Song et al., 2013). Zampiga et al. (2021) stated that, heat stress induces damages in the mucosa of the small intestine by increasing the permeability of the intestine to endotoxins which subsequently leads to a reduction in their growth performance.

The treatment groups, especially the probiotic-administered broiler chickens had an improved epithelial lining and goblet cell count during the study. Enhanced epithelial turnover directly influences the height of the epithelium (in the small intestine) via the stem cells of the crypts of Lieberkuhn as yeast probiotic generally improve the intestinal epithelial cell integrity in chickens (Mohsin et al., 2022). The stimulation of mixing movements expose digesta to the enzymes and improve the process of digestion and absorption of nutrients, which could further enhance growth. The presence of adequate mucins in the goblet cells of broilers administered probiotic could have influenced their growth rate as they are responsible for protecting the epithelium and regulating the concentration and passage of some immune mediators (antimicrobial peptides), ions and water. Sen et al. (2012) suggested that increased intestinal morphometry enhances nutrient digestion and absorption in the small intestine of broiler chickens supplemented with *Bacillus subtilis*.

Even though changes to morphometry can account for part of *Saccharomyces cerevisiae* mechanisms of action, the specific mechanisms by which it influences transport across the epithelium remains an area for further research. Also, it would be important to determine if the effect evident under controlled conditions would be translatable to open ventilated broiler houses where birds would be exposed to both production conditions and higher thermal conditions.

4.6 CONCLUSION

Heat stress negatively affect endogenous antioxidant enzymes, performance and morphology of the small intestine of the broiler chickens, but probiotic and/or ascorbic acid administrations were efficacious in mitigating the negative effects of heat stress during this study. This was evident by an increase in endogenous antioxidant enzymes level. The morphology of the small intestinal epithelium was enhanced by these treatments in the phase of heat stress exposure. This could therefore be replicated in larger poultry production.

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CHAPTER FIVE

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HEAT STRESS EFFECTS ON DIURNAL RHYTHMS OF CLOACAL AND BODY SURFACE TEMPERATURES IN BROILER CHICKENS FED DIETS SUPPLMENTED WITH ASCORBIC ACID AND *SACCHAROMYCES CEREVISIAE* DURING THE HOT SUMMER SEASON

ABSTRACT

Thermal stress greatly affects poultry production across the globe, hence the need for its mitigation. The effects of thermal stress on cloacal and body surface temperatures (CT and BST) in broiler chickens fed diet supplemented with probiotic and ascorbic acid was evaluated. Fifty six broiler chicks were used for the study and divided into control; probiotic; ascorbic acid (1 g/kg of feed); and combination of probiotic and ascorbic acid (1 g/kg of feed and 200 mg/kg of feed, respectively) for 35 days (D1 – D35). Pen ambient temperature (AT), CT and BST were measured during the study. All administration were given via feed. Values of AT obtained during the study surpassed the thermoneutral zone (TNZ) specified for broiler chickens. Cloacal temperature and BST were significantly different ($P < 0.05$) in the treatment groups. In conclusion, the antioxidants mitigated the detrimental effects of thermal stress by enhancing thermoregulation in broiler chickens.

Key-words: Heat stress, cloacal temperature, body surface temperature, probiotic, ascorbic acid

5.0 INTRODUCTION

The ambient temperature that prevails during the hot summer season induces heat stress in broiler chickens. Heat stress has been a major problem in poultry production due to geographical location and now increasingly due to global warming (Aluwong et al., 2017). The thermoneutral (TNZ) for broilers is the range of environmental temperatures wherein evaporative heat loss and metabolic heat production are in equilibrium. Environmental temperatures that exceed the TNZ stipulated for broiler chickens adversely affect their welfare. In the tropics and subtropics; high relative humidity (RH) and ambient temperature (AT) are meteorological factors that are of significance especially in broiler chicken production (Aluwong et al., 2017). A surge in the parameter induces heat stress which adversely affects broiler chickens performance efficiency (Qaid et al., 2021). While it may be argued that chickens reared in the temperate regions are predominantly in an intensive system wherein the microclimatic conditions are regulated this is not the case in poorer farming areas where natural ventilation and open side housing is practiced in broiler rearing (Schauberger et al., 2020).

Cloacal temperature (CT) is a potent physiological biomarker used for the measurement of stress (Gonzalez-Rivas et al., 2020). It depicts the core body temperature. Body surface temperature (BST) gives an idea of the ability of the animal to cope with dissipation of the heat broiler chickens are generating (Iyasere et al., 2021) as vasodilation occurs to ensure optimum heat loss through the body surface to the environment via convection.

Therefore, the detrimental effects of thermal stress on broiler chickens may be alleviated by supplementing their diet with agents that possess anti-stress and antioxidant activities for optimum productivity (Lee et al., 2021).

For this study we focused at heat stress effects on CT and BST in broiler chickens and the benefit of combining two products with different mechanisms of action, for their beneficial effect during the natural hot summer season.

5.1 MATERIALS AND METHODS

5.1.1 ENVIRONMENTAL CONDITIONS IN THE EXPERIMENTAL SITES

The chickens after brooding were under the thermal environmental conditions of fluctuating high RH and high AT that prevailed, which are characteristic of the hot summer season in Pretoria, South Africa, the study site.

5.1.2 EXPERIMENTAL ANIMALS AND MANAGEMENT

Ceramic heaters (34 °C) were used as a source of heat in the brooding of broiler chicks for 14 days. Biosecurity measures were ensured by the provision of footbaths using F10SC (Health and Hygiene (Pty) Ltd, Roodepoort, South Africa) at a concentration of 1:500, footwear and clothing were provided for all the assistants. The antioxidants were added to the chickens' feed from days 1 to 35 (D1 to D35). Each broiler chicken was identified using colour markings and wing tags for accurate records.

5.2. EXPERIMENTAL MEASUREMENTS

5.2.1 THERMAL ENVIRONMENTAL PARAMETERS

An electronic sensor (Hobo) in the poultry pen was used to measure the AT and RH. On each day of the diurnal rhythm determination, AT and RH were recorded twice on days 21, 28 and 35 of the experiment inside the poultry pen. The formula below was used to determine the temperature-humidity index (THI):

THI = $(1.8 \times AT + 32) - (0.55 - 0.55 \times RH) \times [(1.8 \times AT + 32) - 58]$ (Aluwong et al., 2017):

where THI = temperature-humidity index, AT = Ambient temperature (°C) and RH = Relative humidity (%).

5.2.2 CLOACAL AND BODY SURFACE TEMPERATURE MEASUREMENTS

Digital clinical thermometer was used to record the CT (Zhengzhou AiQURA Intelligent Technology Co., Ltd, China). The CT recordings were taken on D21, D28 and D35 of the study. The AT and RH were recorded concurrently with the CT.

For the BST, seven broiler chickens (from each group) were selected at random on D21, D28 and D35, respectively of the study period, measurements were taken with the aid of an infrared thermometer (Rutland Industries, South Africa).

5.2.3 CALCULATION OF CONVECTIVE AND CONDUCTIVE HEAT LOSS

Sensible heat loss by convection and conduction to the environment in broiler chickens was calculated using a modified formula (Ruvio et al., 2018):

$Q_c = A_s \times h\ (T_s - T_{at})$

Where:

Q_c is conductive and convective heat loss;

A_s is the surface area of the bird (m^2) ($A_s = 3.86 \times MC^{0.74}$);

MC is the body mass of the broiler chicken (kg);

hc is the heat transfer coefficient ($hc = 0.336 \times 4.184 \times (1.46 + \sqrt{V_{AR}} \times 100)$);

V_{AR} is air velocity ($V_{AR} = 0$);

T_s is the average surface temperature of birds (°C) and

T_{at} is the ambient temperature (°C).

5.3 STATISTICAL ANALYSIS

Data were transformed statistically to assume a normal distribution and then subjected to repeated measure analysis of variance (ANOVA). Tukey's HSD test was used to compare the differences between the means obtained from the control and treatment groups with significance placed at 0.05. SPSS Statistics for Windows, Version 27 software (Armonk, NY: IBM Corp) was used for the analysis.

5.4 RESULTS

5.4.1 AMBIENT TEMPERATURE AND CLOACAL TEMPERATURE RESPONSES

On D21, D28 and D35 of the study period, the AT values obtained exceeded the thermoneutral zone specified for chickens (Table 5.1.). On D21, D28 and D35, the CT values in the probiotic group were significantly lower ($P < 0.05$) when compared with the values obtained in the control. At 9h00 the CT value recorded in the treatment groups were significantly lower ($P < 0.05$) than that of the control (Table 5.2.).

Table 5.1. Temperature and humidity Indices on days 21, 28 and 35 of the study.

Time (h)	DBT (°C)	RH (%)	THI
7:00	27.67 ± 0.33 (27 - 28)	83.33 ± 2.19 (79 - 86)	27.33 ± 0.32 (26.7 - 27.7)
9:00	28.33 ± 0.33 (28 - 29)	74.67 ± 2.19 (72 - 73)	27.80 ± 0.31 (27.4 - 28.4)
11:00	28.67 ± 0.33 (28 - 29)	79.00 ± 0.00 (79)	28.27 ± 0.33 (27.6 - 28.6)
13:00	33.33 ± 1.67 (30 -35)	81.67 ± 4.33 (73 - 86)	32.80 ± 1.70 (29.4 - 34.6)
15:00	34.00 ± 1.00 (33 - 36)	84.00 ± 2.00 (80 - 86)	33.40 ± 1.00 (32.4 - 35.4)
17:00	31.33 ± 0.67 (30 - 32)	80.00 ± 0.00 (80)	30.87 ± 0.64 (29.6 - 31.6)
19:00	28.33 ± 0.33 (28 - 29)	77.00 ± 2.00 (73 - 79)	27.87 ± 0.27 (27.6 - 28.4)
Overall mean±SEM	30.24 ± 0.60 (27 - 36)	79.95 ± 1.00 (72 - 86)	29.76 ± 0.59 (26.7 - 35.4)

RH = Relative humidity; DBT = Dry-bulb temperature; THI = Temperature-humidity index.

Table 5.2. Changes in cloacal temperature of broiler chickens given probiotic and ascorbic acid

Day	Time (h)	Group			
		Control	Probiotic	Ascorbic acid	Probiotic + AA
21	07:00	41.21 ± 0.27^{a}	39.98 ± 0.13^{b}	40.78 ± 0.11^{a}	41.04 ± 0.15^{a}
	09:00	40.35 ± 0.30^{a}	40.07 ± 0.20^{a}	40.46 ± 0.11^{a}	40.96 ± 0.08^{b}
	11:00	40.99 ± 0.25^{a}	40.72 ± 0.62^{a}	40.85 ± 0.09^{a}	41.06 ± 0.09^{a}
	13:00	41.00 ± 0.25^{a}	40.45 ± 0.08^{a}	40.80 ± 0.11^{a}	40.83 ± 0.12^{a}
	15:00	41.14 ± 0.20^{a}	40.84 ± 0.06^{a}	40.97 ± 0.04^{a}	40.98 ± 0.09^{a}
	17:00	41.53 ± 0.15^{a}	40.36 ± 0.10^{b}	41.11 ± 0.12^{a}	41.02 ± 0.08^{a}
	19:00	41.69 ± 0.18^{a}	40.84 ± 0.05^{b}	41.36 ± 0.14^{a}	41.20 ± 0.08^{a}
28	07:00	41.63 ± 0.15^{a}	39.80 ± 0.18^{c}	40.63 ± 0.14^{b}	40.52 ± 0.16^{b}
	09:00	41.39 ± 0.32^{a}	40.07 ± 0.20^{b}	40.46 ± 0.12^{b}	40.96 ± 0.08^{a}
	11:00	41.79 ± 0.22^{a}	40.78 ± 0.22^{b}	41.03 ± 0.22^{b}	41.01 ± 0.13^{b}
	13:00	41.69 ± 0.11^{a}	40.68 ± 0.13^{b}	40.78 ± 0.13^{b}	40.75 ± 0.12^{b}
	15:00	41.14 ± 0.20^{a}	40.84 ± 0.06^{a}	40.97 ± 0.06^{a}	40.98 ± 0.09^{a}
	17:00	41.71 ± 0.14^{a}	40.87 ± 0.16^{b}	41.03 ± 0.16^{b}	40.86 ± 0.14^{b}
	19:00	41.69 ± 0.17^{a}	41.10 ± 0.14^{a}	41.23 ± 0.14^{a}	41.07 ± 0.09^{a}
35	07:00	41.53 ± 0.20^{a}	40.36 ± 0.18^{b}	40.97 ± 0.15^{a}	41.01 ± 0.16^{a}
	09:00	41.15 ± 0.27^{a}	40.16 ± 0.18^{b}	40.49 ± 0.12^{b}	40.90 ± 0.14^{a}
	11:00	41.24 ± 0.24^{a}	40.86 ± 0.12^{a}	40.81 ± 0.11^{a}	41.02 ± 0.13^{a}
	13:00	41.47 ± 0.22^{a}	40.59 ± 0.13^{b}	40.89 ± 0.14^{b}	40.73 ± 0.12^{b}
	15:00	41.24 ± 0.20^{a}	40.81 ± 0.12^{a}	40.98 ± 0.11^{a}	40.86 ± 0.11^{a}
	17:00	41.49 ± 0.16^{a}	40.34 ± 0.12^{b}	41.09 ± 0.12^{a}	40.94 ± 0.11^{a}
	19:00	41.66 ± 0.17^{a}	40.94 ± 0.12^{b}	41.27 ± 0.13^{a}	40.98 ± 0.12^{b}

Mean values with different superscript letters along the same row are significantly different at $P < 0.05$. n =14.

5.4.2 BODY SURFACE TEMPERATURE (BST)

On D21, D28 and D35, broiler chickens in the treatment groups had a significant difference ($P < 0.05$) in comb, head, back, wing and foot temperatures when compared with the values obtained in the control group during the study (Table 5.3.).

Table 5.3.Variations in head, comb, wing, back and foot temperature of broiler chickens given probiotic and ascorbic acid.

Area	Day	Time (h)	Treatment groups			
			Control	Probiotic	Ascorbic acid	Probiotic + Ascorbic acid
Head	21	07:00	37.01 ± 0.53^{a}	36.79 ± 0.17^{a}	35.87 ± 0.15^{a}	33.00 ± 1.38^{b}
		13:00	36.03 ± 0.23^{a}	37.06 ± 0.23^{a}	34.00 ± 0.85^{b}	34.57 ± 0.91^{b}
		19:00	36.34 ± 0.18^{a}	37.16 ± 0.28^{a}	35.69 ± 0.48^{b}	32.34 ± 0.68^{c}
	28	07:00	37.10 ± 0.43^{a}	36.26 ± 0.19^{a}	35.83 ± 0.21^{a}	32.59 ± 1.49^{b}
		13:00	35.91 ± 0.25^{a}	36.89 ± 0.25^{a}	35.09 ± 0.23^{a}	35.07 ± 0.73^{a}
		19:00	37.89 ± 0.62^{a}	37.00 ± 0.21^{a}	38.20 ± 0.60^{a}	36.06 ± 0.41^{b}
	35	07:00	37.17 ± 0.42^{a}	36.67 ± 0.17^{a}	35.93 ± 0.22^{a}	36.16 ± 0.86^{a}
		13:00	36.79 ± 0.49^{a}	36.91 ± 0.28^{a}	36.17 ± 0.32^{a}	35.97 ± 0.51^{a}
		19:00	37.49 ± 0.41^{a}	35.84 ± 0.50^{b}	35.69 ± 0.65^{b}	35.20 ± 0.29^{b}
Comb	21	07:00	36.63 ± 0.42^{a}	35.97 ± 0.20^{a}	35.51 ± 0.11^{a}	35.21 ± 1.51^{a}
		13:00	35.86 ± 0.16^{a}	36.21 ± 0.07^{a}	33.19 ± 0.58^{b}	33.50 ± 0.81^{b}
		19:00	36.96 ± 0.25^{a}	36.53 ± 0.10^{a}	37.73 ± 0.44^{a}	36.00 ± 0.69^{a}
	28	07:00	36.37 ± 0.36^{a}	36.76 ± 0.48^{a}	35.59 ± 0.18^{a}	36.30 ± 0.17^{a}
		13:00	35.74 ± 0.18^{a}	36.24 ± 0.21^{a}	35.19 ± 0.50^{a}	35.64 ± 0.38^{a}
		19:00	35.60 ± 0.47^{a}	36.41 ± 0.11^{a}	37.73 ± 0.48^{b}	36.14 ± 0.48^{a}
	35	07:00	35.27 ± 0.42^{a}	37.59 ± 0.58^{b}	36.23 ± 0.26^{a}	37.14 ± 0.41^{b}
		13:00	34.53 ± 0.49^{a}	36.50 ± 0.33^{b}	35.90 ± 0.23^{a}	36.43 ± 0.41^{b}
		19:00	35.93 ± 0.42^{a}	37.96 ± 0.67^{b}	37.53 ± 0.41^{b}	36.57 ± 0.54^{a}
Wing	21	07:00	36.23 ± 0.34^{a}	39.16 ± 0.27^{b}	40.47 ± 0.32^{c}	37.23 ± 0.50^{a}
		13:00	39.83 ± 0.20^{a}	39.23 ± 0.46^{a}	39.01 ± 0.50^{a}	38.44 ± 0.33^{a}
		19:00	39.83 ± 0.20^{a}	40.07 ± 0.13^{b}	39.90 ± 0.35^{a}	38.30 ± 0.07^{a}
	28	07:00	35.76 ± 0.16^{a}	39.07 ± 0.29^{c}	40.09 ± 0.26^{c}	37.39 ± 0.48^{b}
		13:00	39.83 ± 0.48^{a}	40.54 ± 0.40^{a}	39.30 ± 0.72^{a}	40.44 ± 0.47^{a}
		19:00	37.87 ± 0.84^{a}	40.34 ± 0.26^{b}	40.59 ± 0.34^{b}	39.73 ± 0.68^{b}
	35	07:00	35.71 ± 0.36^{a}	39.30 ± 0.30^{c}	40.40 ± 0.28^{c}	37.53 ± 0.43^{b}
		13:00	36.81 ± 0.73^{a}	39.14 ± 0.45^{b}	38.90 ± 0.49^{b}	37.87 ± 0.65^{b}
		19:00	37.81 ± 1.09^{a}	40.10 ± 0.13^{b}	39.71 ± 0.31^{b}	38.86 ± 0.30^{b}
Back	21	07:00	37.43 ± 0.59^{a}	36.06 ± 0.23^{a}	36.96 ± 0.38^{a}	31.81 ± 0.84^{b}
		13:00	36.36 ± 0.11^{a}	36.61 ± 0.28^{a}	36.69 ± 0.91^{a}	34.89 ± 0.62^{a}
		19:00	37.36 ± 0.11^{a}	34.26 ± 0.33^{c}	36.66 ± 0.88^{b}	35.50 ± 0.69^{b}
	28	07:00	36.87 ± 0.78^{a}	35.83 ± 0.25^{a}	36.67 ± 0.45^{a}	35.61 ± 0.68^{a}

		13:00	36.37 ± 0.13^{a}	36.53 ± 0.18^{a}	37.27 ± 0.85^{a}	35.57 ± 0.36^{a}
		19:00	39.96 ± 0.97^{a}	37.16 ± 0.35^{b}	38.69 ± 0.95^{a}	36.07 ± 0.37^{c}
	35	07:00	37.36 ± 0.67^{a}	36.01 ± 0.26^{a}	36.89 ± 0.38^{a}	36.39 ± 0.88^{a}
		13:00	37.56 ± 0.62^{a}	36.04 ± 0.30^{b}	36.06 ± 0.81^{b}	35.60 ± 0.40^{b}
		19:00	38.31 ± 0.13^{a}	36.04 ± 0.36^{b}	37.46 ± 0.82^{b}	35.64 ± 0.21^{c}
Foot	21	07:00	36.74 ± 0.57^{a}	35.94 ± 0.18^{a}	36.34 ± 0.25^{a}	36.06 ± 0.19^{a}
		13:00	35.39 ± 0.17^{a}	35.50 ± 0.24^{a}	34.16 ± 1.04^{a}	35.34 ± 1.14^{a}
		19:00	38.30 ± 0.16^{a}	37.57 ± 0.36^{a}	37.33 ± 0.19^{a}	35.09 ± 1.60^{b}
	28	07:00	36.70 ± 0.51^{a}	35.89 ± 0.18^{a}	36.24 ± 0.24^{a}	35.94 ± 0.22^{a}
		13:00	35.50 ± 0.20^{a}	35.47 ± 0.21^{a}	34.01 ± 0.98^{b}	34.36 ± 0.95^{b}
		19:00	39.16 ± 1.02^{a}	37.57 ± 0.36^{b}	38.33 ± 0.19^{a}	35.66 ± 1.27^{c}
	35	07:00	36.67 ± 0.59^{a}	35.86 ± 0.19^{b}	36.27 ± 0.23^{a}	36.03 ± 0.25^{a}
		13:00	35.71 ± 0.22^{a}	35.26 ± 0.29^{a}	34.41 ± 0.83^{b}	36.06 ± 0.80^{a}
		19:00	37.50 ± 0.66^{a}	37.29 ± 0.36^{a}	37.59 ± 0.59^{a}	36.46 ± 1.08^{a}

Mean values with different superscript letters along the same row are significantly different at $P< 0.05$. n=14.

5.4.3 CONVECTIVE AND CONDUCTIVE HEAT LOSS

On D35 heat loss recorded in the treatment groups were significantly higher ($P < 0.05$) in comparison with the control, also the THI was within the TNZ during the morning period of the study (Fig 5.1.). At noon and evening time, although the THI exceeded the TNZ, there was a significant difference ($P < 0.05$) in heat loss obtained in the probiotic group when compared with the control group (Fig 5.2. and Fig 5.3.).

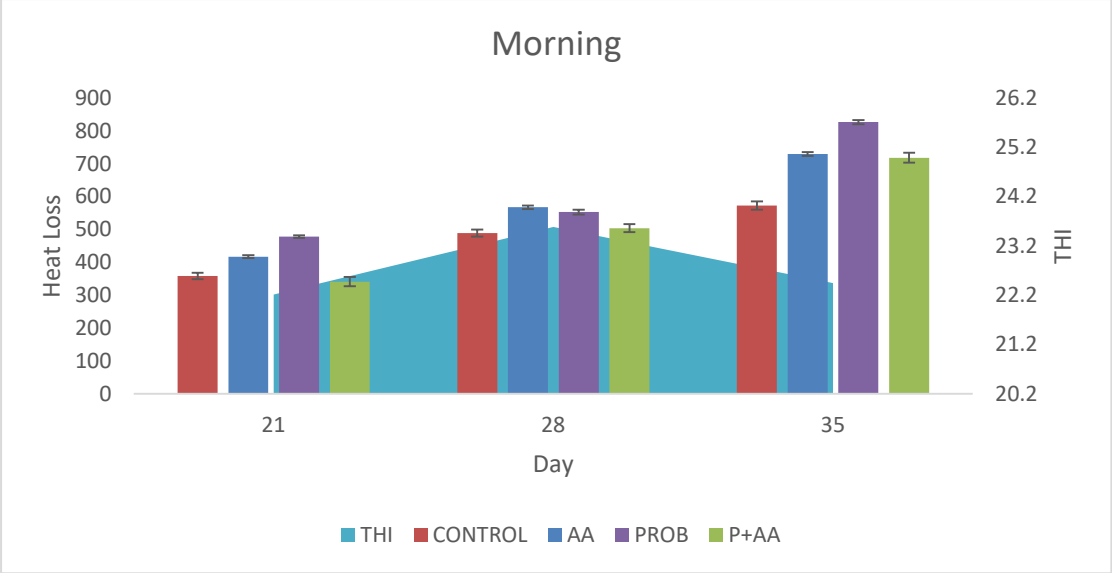

Fig 5.1. Convective and conductive heat loss obtained during the morning hours of the study period. THI was within the TNZ stipulated for broiler chickens which influenced the degree of heat loss positively during this period of the study (n = 7). THI; temperature-humidity index, TNZ; thermoneutral zone.

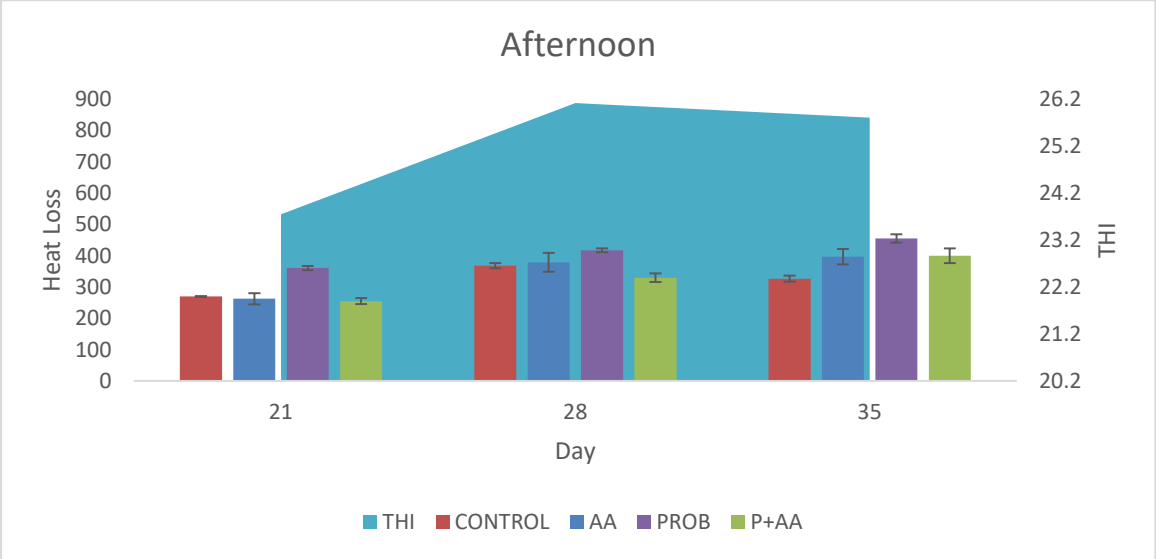

Fig 5.2. Convective and conductive heat loss obtained during the afternoon hours of the study period. THI exceeded the TNZ for broiler chickens which influenced the degree of heat loss negatively during this period of the study (n = 7). THI; temperature-humidity index, TNZ; thermoneutral zone.

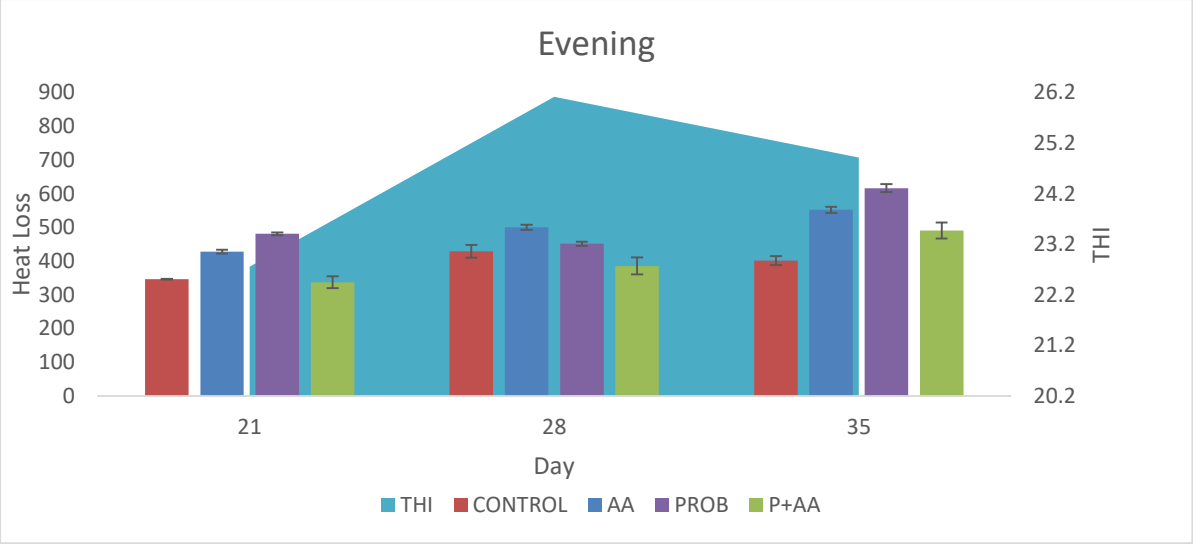

Fig 5.3. Convective and conductive heat loss obtained during the evening hours of the study period. The probiotic and ascorbic acid groups had a significantly higher ($P < 0.05$) value of heat loss when compared with the control group on D21 and D35. THI was outside the TNZ stipulated for broiler chickens which negatively influenced the degree of heat loss during this period of the study (n = 7). THI; temperature-humidity index, TNZ; thermoneutral zone.

Heat loss obtained on D21 was all within the TNZ (yellow zone) stipulated for broiler chickens, while those recorded on D28 and D35 of the study surpassed the TNZ (red zone) (Fig 5.4.).

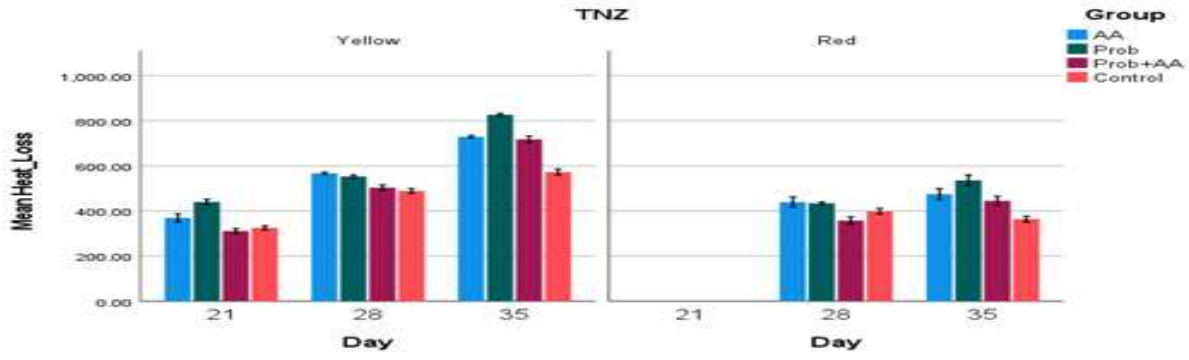

Fig 5.4. Convective and conductive heat loss within (yellow zone) and outside (red zone) the TNZ in broiler chickens treated with probiotic and ascorbic acid. At D21, heat loss values obtained were within the TNZ, while those recorded on D28 and D35 surpassed the TNZ stipulated for broiler chickens during the period of study (n = 7).

5.5 DISCUSSION

High CT values recorded in the control group may be an indication of the reduced capacity of birds to handle thermal stress as they advance in age. In addition, the fact that this group of chickens was not exposed to any form of treatment contributed to the rise in CT observed especially during the afternoon and evening periods. This corresponds with Egbuniwe et al. (2015), who observed an increase in CT of chickens devoid of betaine and ascorbic acid. The low CT values recorded in the probiotic-treated group may be attributed to the anti-stress effect of the antioxidant. This may be valid as yeast probiotic has been reported to be a potent anti-stress agent, promoting the performance of broiler chickens when administered in adequate amounts during the thermally stressful season (Aluwong et al., 2017). This agrees with Sugiharto et al. (2017), who demonstrated that increased metabolic processes that accompany an increase in body weight gain generate more heat in broiler chickens, and the administration of probiotic could modulate these adverse effects. Regarding the CT values of the co-administered group, it may be deduced that there was no additive effect between the two antioxidants despite the fact that ascorbic acid has been reported to be effective in reduction of corticosterone concentration in the systemic circulation via a negative feedback mechanism (Habibian et al., 2015). But during the period of this study, ascorbic acid was not as effective as the probiotic in ameliorating the negative effects of thermal stress in the broilers. This could be attributed to varying susceptibility of the broiler chickens to these agents, or the dose of the antioxidant administered.

The treatment groups had a lower head, wing, back and foot temperatures which could be associated to the antioxidant effect of the agents. During heat stress it could be speculated that the oxygen intake of the broiler chickens increases to meet demand for thermoregulatory mechanisms e.g., evaporative cooling (panting). Excessive buildup of reactive oxygen species, a byproduct of oxygen metabolism, can occur depending on the concentration of

endogenous antioxidants, leading to oxidative stress. Therefore, the use of exogenous antioxidants like *Saccharomyces cerevisiae* and ascorbic acid could inhibit the generation of oxygen radicals at the cellular level. While the increase BST observed in the control group may be supported by the fact that the chickens were not able to elicit thermoregulation to control the level of heat stress they are being exposed to as evident by their CT values. This agrees with Kim et al. (2021) who reported that BST is an effective indicator of the level of exposure of poultry species to heat stress, as high environmental temperature directly influences the BST, although laying hens were used as their research subjects.

The THI during the morning hours of the study was within the TNZ specified for broiler chickens' optimum production. This further influenced thermoregulatory measures which was evident via convective and conductive heat loss in the treatment groups. During the study period, heat was gained from the environment and through metabolic heat production by the broiler chickens, but our focus was basically on the quantity of sensible heat loss obtained in the treatment and control group. Nevertheless, it could be speculated that heat loss was not only influenced by the administered antioxidants, but it was also dependent on the degree of THI throughout the study. This finding agrees with that of Tao and Xin (2003), who reported that THI optimum for broiler chickens' production was 21. During the afternoon and evening hours of the study, THI exceeded the TNZ stipulated for broiler chickens' optimum production. This would be expected to lead to a low degree of heat loss by conduction and convection in the broiler chickens. The probiotic group had a higher degree of heat loss when compared with the control on D21 and D35. This could therefore be attributed to the high THI values which were predominant during this study. This agrees with Sinkalu et al. (2015) and Aluwong et al. (2017), who both reported that THI above 21 induces heat stress in broiler chickens using CT as a biomarker of heat stress.

The TNZ is also known as the comfort zone for broiler chickens. AT values obtained within the TNZ for broiler chicken favours optimum performance indices, this is known as the zone of comfort (yellow zone), while values obtained that surpass the TNZ induce discomfort or danger, and it is called the zone of discomfort (red zone) (Tao and Xin, 2003). During the study period, THI recorded on D21 and the morning hours of D28 and D35 were within the TNZ for broiler chickens. This enhanced the process of thermoregulation in the broiler chickens, which was evident as optimum heat loss through convection and conduction. But THI values obtained during the afternoon and evening hours of D28 and D35 were outside the TNZ which negatively affected the process of thermoregulation. It could be deduced from our study that the higher the AT, the poorer the process of thermoregulation in the absence of treatment with anti-stress agents and vice versa.

5.6 CONCLUSIONS

In conclusion, *Saccharomyces cerevisiae* probiotic alone decreased CT and BST biomarkers, meaning that it was best in the induction of convective and conductive heat loss. Therefore, the antioxidants may be of benefit in mitigating the detrimental effects of thermal stress, enhancing the welfare of broiler chickens during the hot summer season.

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CHAPTER SIX

Proposed to be submitted to Behavioural Processes

HEAT STRESS INDUCED BEHAVIOURAL AND HAEMATOLOGICAL CHANGES IN BROILER CHICKENS FED DIETS FORTIFIED WITH ASCORBIC ACID AND YEAST PROBIOTIC

ABSTRACT

Heat stress affecting poultry industry adversely affects bird productivity and in the process compromises enterprise profitability. The study evaluated the behavioural and selected haematological changes stimulated by heat stress in chickens treated with yeast probiotic and/or ascorbic acid under natural conditions. Regarding this, 56 purpose raised broiler chickens were divided into four groups comprising 14 chicks each, control; probiotic; ascorbic acid and combination of probiotic and ascorbic. The antioxidants were given from day (D) 1 to D35 of the study. Open-field test (OFT), tonic immobility (TI) and vigilance (V) were evaluated at D21, D28 and D35 of the study and some haematological indicators were evaluated at D35 of the study. The treatment groups had a significant difference ($P = 0.001$) in OFT, TI and V in comparison with the control group during this study. Erythrocyte osmotic fragility, lymphocyte, monocyte, eosinophil counts, and heterophil/lymphocyte ratio were significantly lower ($P < 0.0001$) in the treatment groups. The administered antioxidants were potent in reducing fear responses, increasing the resistance of erythrocytes to haemolysis and improving leucocyte counts of broiler chickens, hence their use in broiler chickens' production could be advocated.

Key-words: Open-field test, tonic immobility, vigilance, leucocyte counts, antioxidants, heat stress

6.0 INTRODUCTION

Thermal stress is one of the dangers poultry productions face due to increase climatic changes (Abaza et al., 2021; Abidin et al., 2022). During stress the hypothalamic-pituitary-adrenal axis are involved in responding either negatively or positively, as in the case during fear. Fear which is a form of stress is the reaction to any form of danger (Ghareeb et al., 2014). Previous experience can also elicit fear in animals which is termed conditional fear (Mohammed et al., 2021). The behavioural patterns linked with fear differ greatly based on the peculiarity of the threat (Oso et al., 2022). Immobility, escape, flight, hiding and vigilance are both passive and active strategies of fear expression (Franco et al., 2022). Latency, preening, defaecation, facial expressions, are all specific indication for fear responses in broiler chickens (Egbuniwe et al., 2018). Other parameters like open-field test could be evaluated to ascertain the level of fear responses in birds.

Open-field test, tonic immobility and vigilance are all behavioural markers which have been used in evaluating fear responses in broiler chickens (Wang et al., 2014; Carli and Farabollini, 2022). Since these parameters evaluate stress responses, they can be used to estimate the level of stress the broiler chickens face, especially at maturity when body organs are fully developed, irrespective of the insult (Anderson et al., 2021). Not surprisingly these responses are heightened by heat stress, it adversely affect the chickens' performance, resulting in poor productivity and profitability (Makinde and Adewole, 2022). Heat stress also induces oxidative stress which negatively impact the physiological, haematological and biochemical parameters of animals (Bastami et al., 2022). The knowledge of broiler chickens' haematology is a vital tool for diagnosis as it serves as an indicator to detect the level of stress they are posed to (Iyaode et al., 2020). The measurement of erythrocyte osmotic fragility (EOF) in broiler chickens is a good indication of cell membrane fragility as

the erythrocytes become more susceptible to osmotic lysis following membrane peroxidation, it is a potent biomarker of oxidative stress induced by thermal stress.

Other haematological components like heterophils/lymphocytes ratio are vital parameters used in monitoring the health status of the birds (Ogbuagu et al., 2022). Antioxidants which prevent oxidation reactions can be of advantage in inhibiting the production of free radicals responsible for cellular damages (Uyanga et al., 2021). Yeast probiotics are anti-stress agents that may mitigate the negative effects of the stress the hormones adrenalin and corticosterone produced in presence of a stressor (Bilal et al., 2021). Therefore, the study aimed at evaluating the behavioural and haematological changes in broiler chickens induced by heat stress and fed diets fortified with ascorbic acid and probiotic.

6.1 MATERIALS AND METHODS

6.1.1 EXPERIMENTAL ANIMALS AND MANAGEMENT

A total of 56 chicks (Ross 308) served as the research subjects and were kept under an intensive management system. Electric sensors (Hobo, Onset Computer Corporation MA) were used to monitor the ambient temperature of the pen with efficient biosecurity measures in place.

6.2 EXPERIMENTAL DESIGN

The experimental design is as described in chapter three, section 3.2.

6.2.1 MEASUREMENT OF BEHAVIOURAL PARAMETERS

6.2.1.1 Open-field test

Twenty-eight broiler chickens (7 per group) were selected at random for this study, each broiler chicken was subjected to an open-field test for 5 minutes at 08h00, 13h00 and 18h00

of D21, D28 and D35 of the study period. Four open-field boxes, each measuring 45 cm × 35 cm × 18 cm were used. Each broiler chicken was tested independently in different boxes, which were 1 m apart in an enclosed environment to prevent the broiler chickens from seeing one another and to prevent escape. The floor of each box was divided into 5 squares by markings. Each broiler chicken was placed in the middle of the box, carefully observed and assessed by camera and visual observation for the following behavioural events: time taken by the broiler chicken to move immediately after placement in the box (Latency); amount of time taken by the broiler chicken to rest after movement in the box (Rest); number of subdivisions or boundaries crossed by the broiler chicken (lines crossed); number of times attempts were made to jump outside the box (attempts to jump); sounds made by the broiler chicken (vocalization); number of boluses excreted by the broiler chicken (defaecation) and number of times the broiler chicken use its beak to rearrange its feathers (preening frequency).

Latency and rest were measured using a stopwatch, while the preening frequency, defaecation, vocalisation, attempts to jump and number of lines crossed were measured by counting each event. Each box was cleaned after usage to ensure proper hygiene. The individuals that assisted in this study were trained prior to the commencement of the study in order to ensure accuracy (Egbuniwe et al., 2018).

6.2.1.2 Tonic immobility and ranking of vigilance behaviour

Induction of tonic immobility and vigilance was done on D21, D28 and D35, using a modified method described by Sinkalu et al. (2016) and Wang et al. (2014) in 28 randomly selected broiler chickens (7 in each group).

6.2.2 MEASUREMENT OF HAEMATOLOGICAL INDICATORS

Four (4) mL of blood sample was collected from the wing vein of 56 broiler chickens on D35 into tubes containing an anticoagulant, EDTA. After collection, the blood samples (2 mL each) were transferred to the Physiology Laboratory and Clinical Pathology Laboratory, University of Pretoria, for EOF and haemogram analysis, respectively.

The EOF test was performed using a modified method of Ogbuagu (2022). Haemolysis in each tube was expressed as a percentage, taking haemolysis in distilled water (0% NaCl) as 100%:

$$\text{Percentage (\%) haemolysis} = \frac{\text{Optical density of test}}{\text{Optical density of standard}} \times 100$$

For the haemogram, blood samples were analysed using manual methods as described by Campbell (2007). Briefly, sample was prepared for a microhaematocrit and the PCV was read according to the microhaematocrit standard operating procedure (SOP). Blood smear was prepared according to the SOP for haematology specimen preparation, the Wright-Giemsa staining method was used. Blood smear evaluation and manual leucocyte differential count was performed as described in the SOP for blood smear evaluation: the smear was scanned at low magnification (10 x objective) to locate the optimal area and a higher magnification (50-100 x objective) was used to perform a differential leucocyte count (Campbell, 2007).

6.3 STATISTICAL ANALYSIS

The behavioural parameters (OFT, TI and vigilance) were log transformed to assume a normal distribution, they were then subjected to repeated measure analysis of variance (ANOVA), Tukey's HSD test was used to compare the differences between the means obtained from the control and treatment groups with significance placed at 0.05. SPSS

Statistics for Windows, Version 27 software (Armonk, NY: IBM Corp) was used for the analysis.

RESULTS

6.4.1 OPEN-FIELD TEST

There was a significant difference ($P < 0.001$) in the period of latency and rest, number of defaecation, attempts to jump, number of lines crossed and the number of preening and vocalisation obtained in the treatment groups in comparison with the controls (Fig 6.1., 6.2., 6.3., 6.4., 6.5., 6.6. and 6.7.).

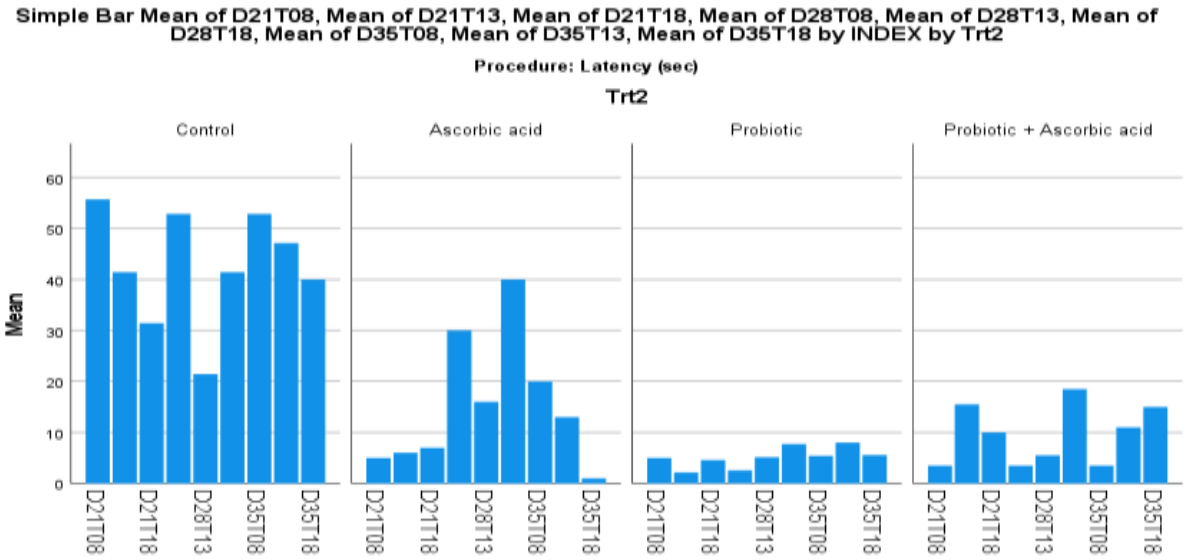

Fig 6.1. Latency (sec) of broiler chickens given probiotic and ascorbic acid during thermal stress. There was a significant difference in period of latency obtained in the treatment groups. n = 7.

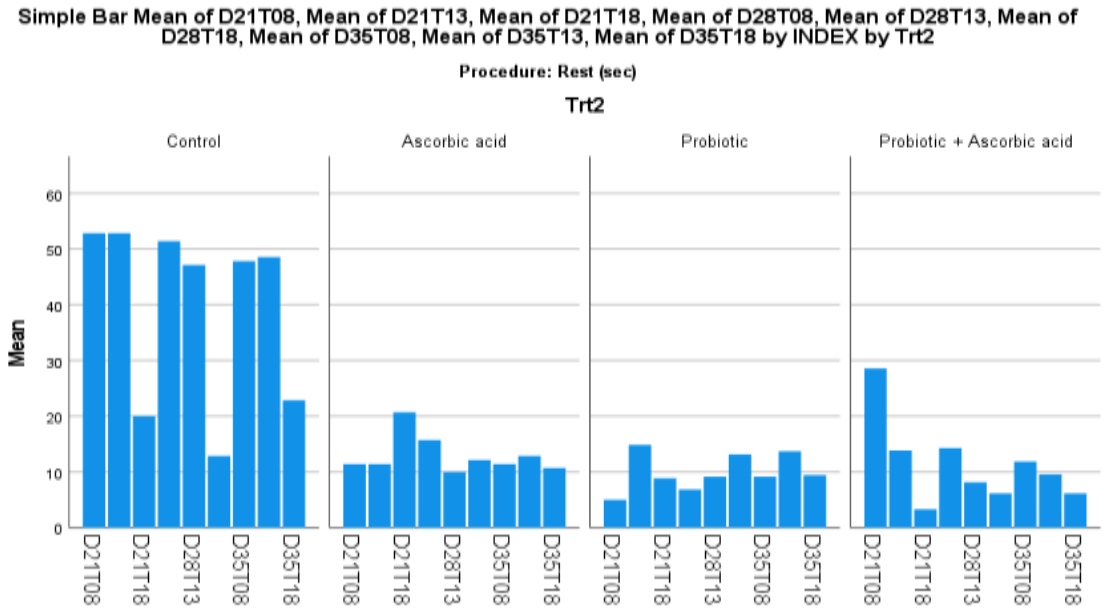

Fig 6.2. Period of rest (sec) of broiler chickens given probiotic and ascorbic acid during thermal stress. There was a significant difference in period of latency obtained in the treatment groups. n = 7.

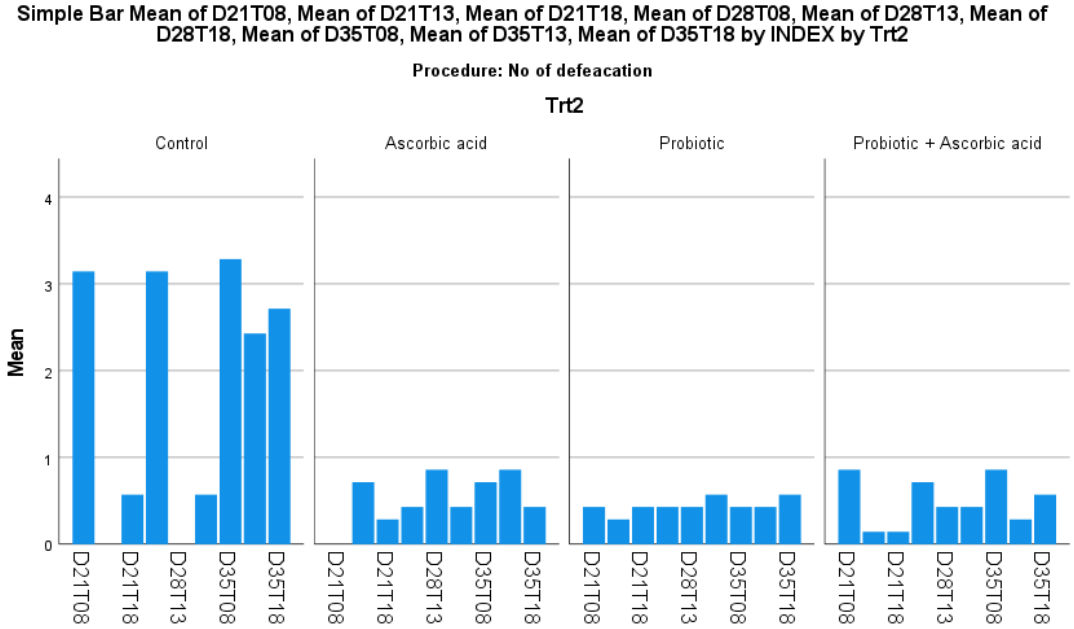

Fig 6.3. Number of defaecation by broiler chickens given probiotic and ascorbic acid during thermal stress. There was a significant difference in period of latency obtained in the treatment groups. n = 7.

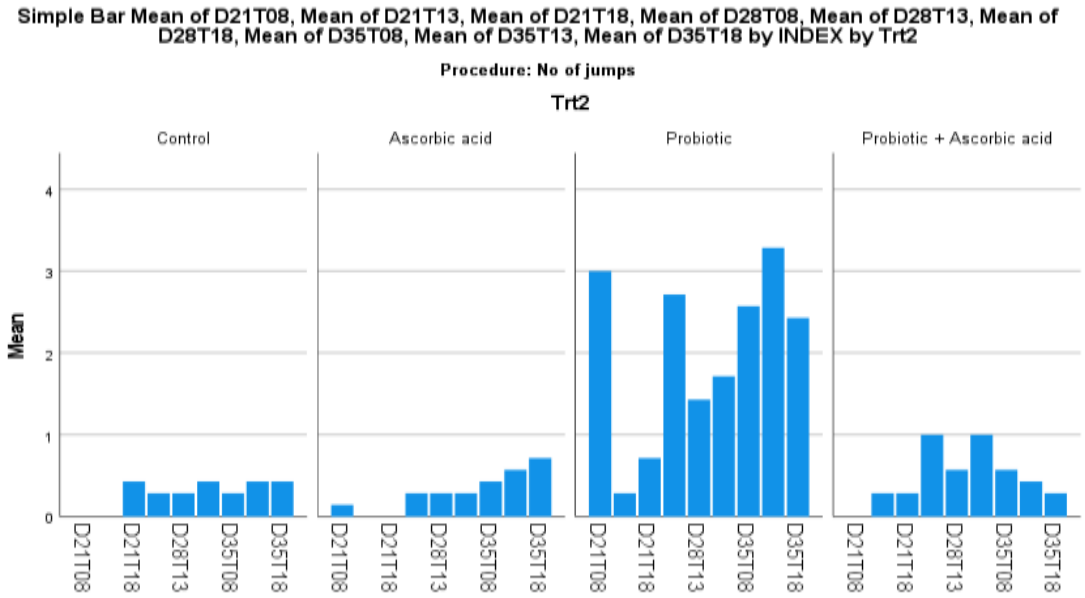

Fig 6.4. Number of attempts to jump by broiler chickens given probiotic and ascorbic acid during thermal stress. There was a significant difference in period of latency obtained in the treatment groups. n = 7.

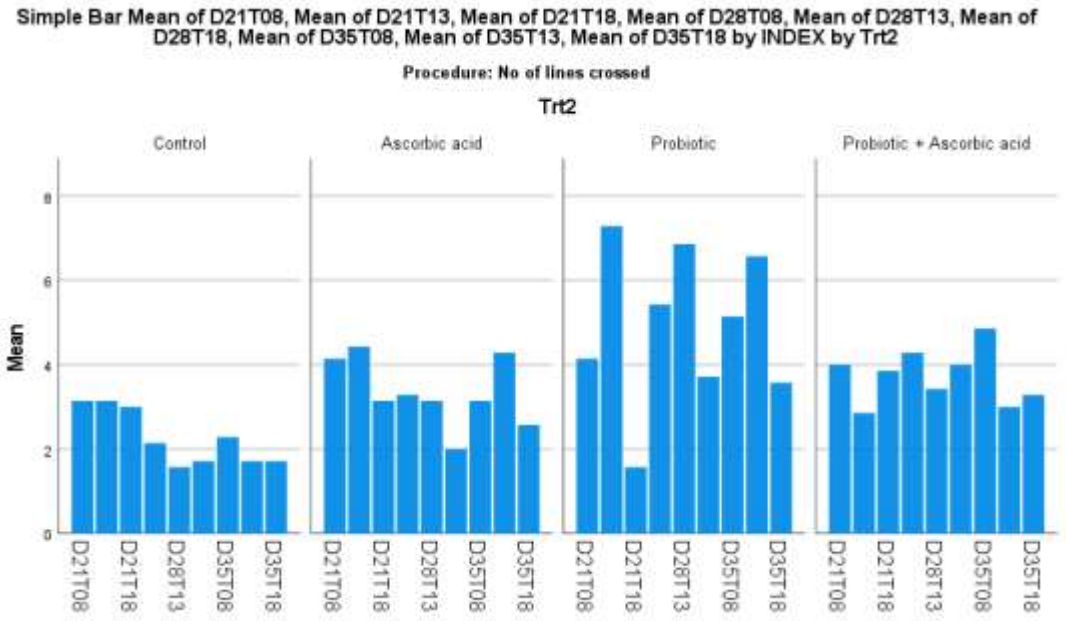

Fig 6.5. Number of lines crossed by broiler chickens given probiotic and ascorbic acid during thermal stress. There was a significant difference in period of latency obtained in the treatment groups. n = 7.

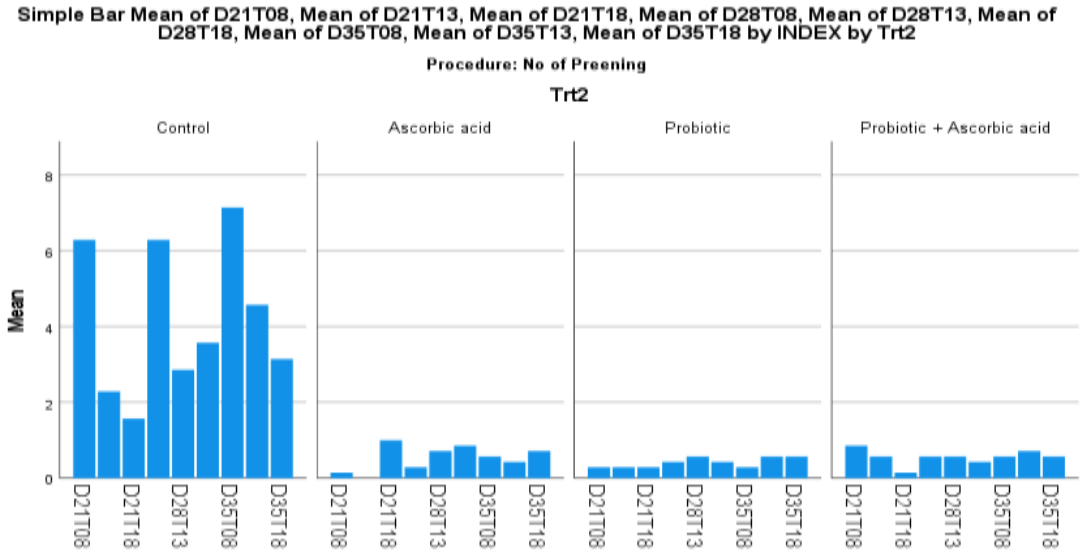

Fig 6.6. Number of preening by broiler chickens given probiotic and ascorbic acid during thermal stress. There was a significant difference in period of latency obtained in the treatment groups. n = 7.

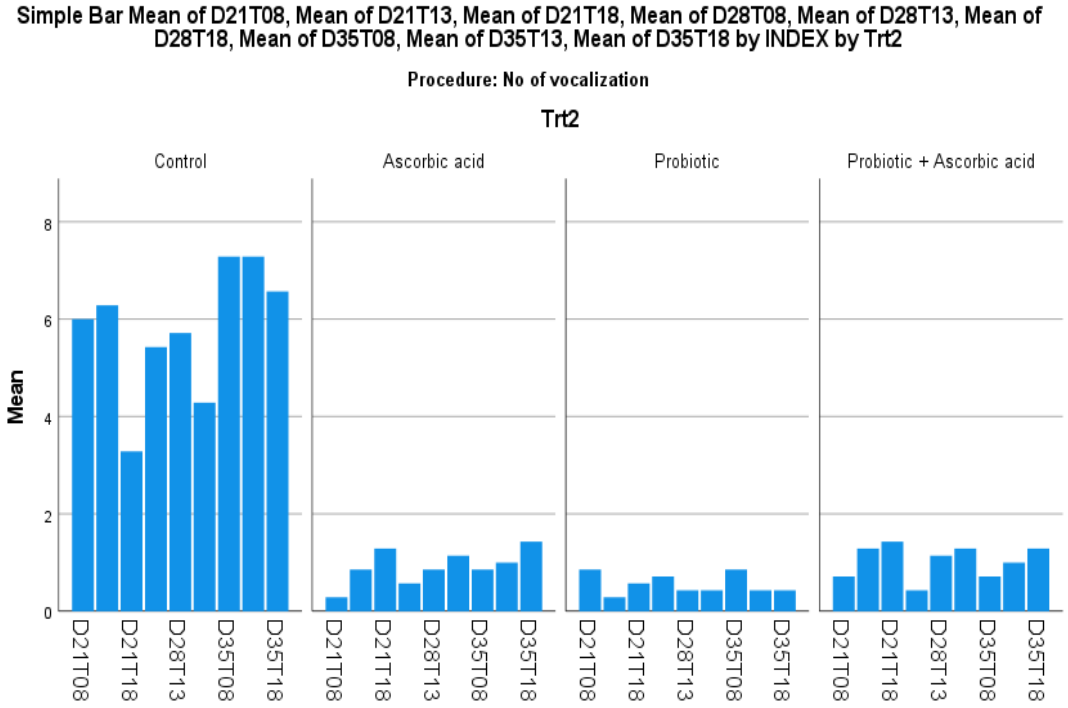

Fig 6.7. Number of vocalisation of broiler chickens given probiotic and ascorbic acid during thermal stress. There was a significant difference in period of latency obtained in the treatment groups. n = 7.

6.4.2 TONIC IMMOBILITY AND VIGILANCE TEST

There was a significant difference ($P < 0.0001$) in duration of TI in broiler chickens in the treatment groups compared with those of the control. Broiler chickens in the treatment groups were significantly more vigilant ($P < 0.001$) when compared to the control group (Fig 6.9.).

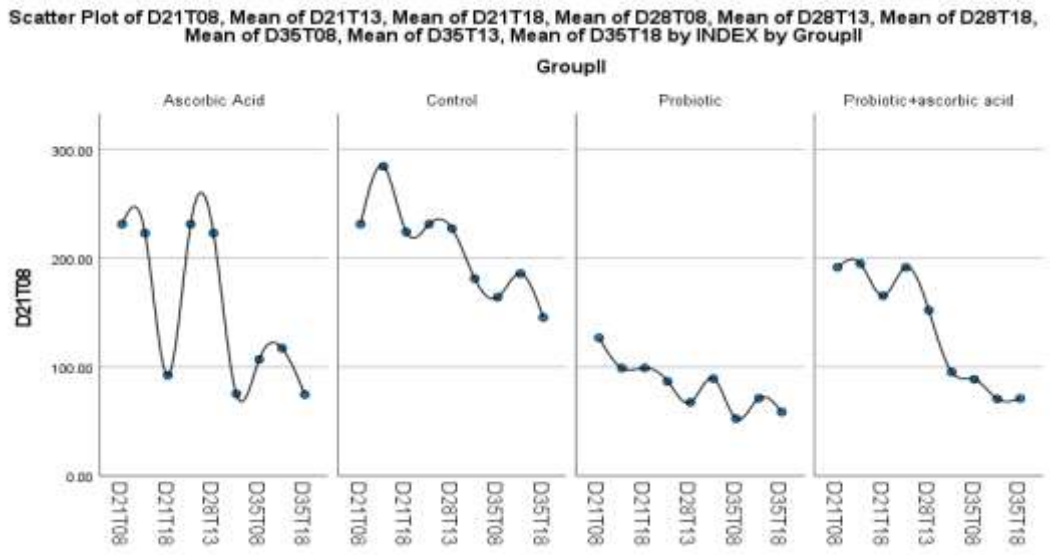

Fig 6.8. Tonic immobility (sec) in broiler chickens treated with probiotic and ascorbic acid during thermal stress. n = 7.

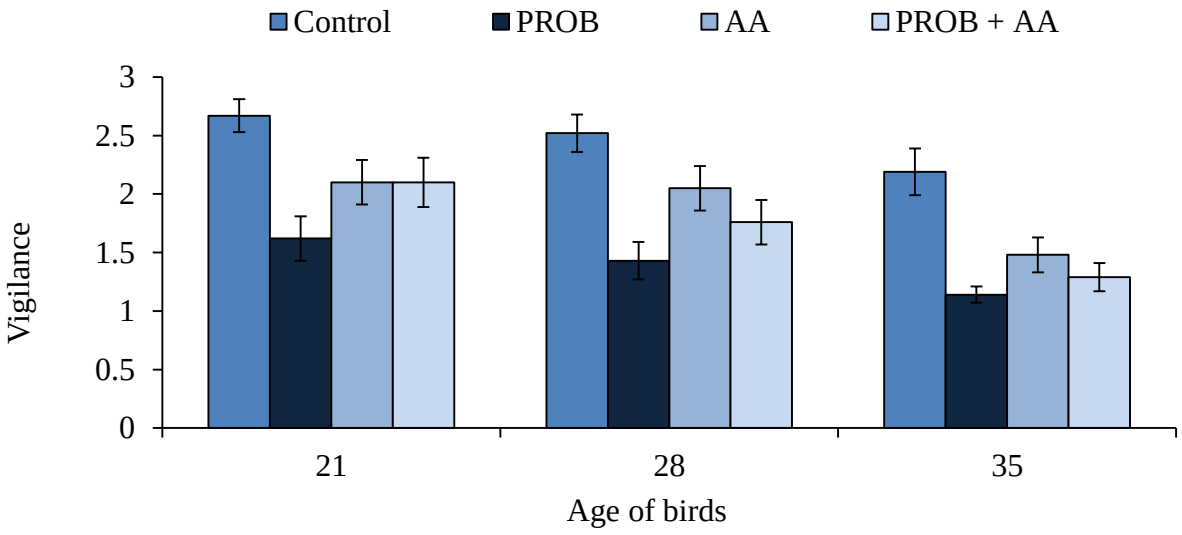

Fig 6.9. Vigilance in broiler chickens treated with probiotic and ascorbic acid during thermal stress. n = 7. PROB= Probiotic; AA= Ascorbic acid.

6.4.3 HAEMATOLOGICAL INDICATORS

At 0.1%, 0.3%, 0.5%, 0.7% and 0.9% concentrations of NaCl the EOF of broiler chickens was significantly lower ($P < 0.0001$) in the treatment groups when compared to the control (Fig. 6.10.). The PCV, band cells, basophils, eosinophils, lymphocytes, monocytes and heterophils/lymphocytes ratio were significantly different in some of the treatment groups when compared with the untreated group (Table 6.1).

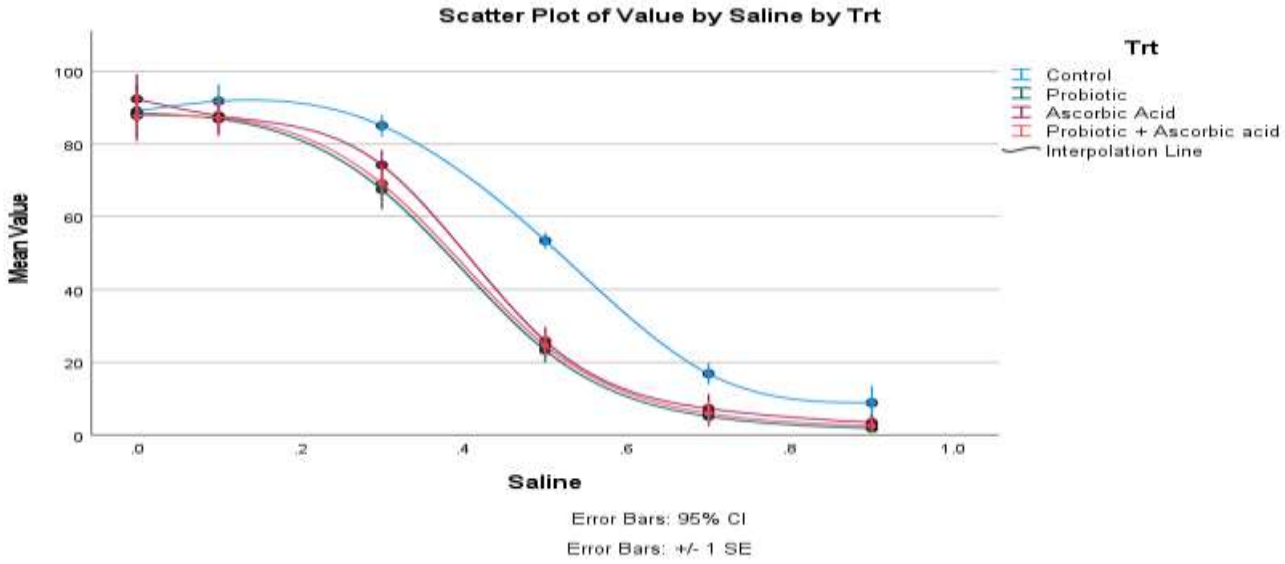

Fig. 6.10. Erythrocyte osmotic fragility in broiler chickens administered probiotic and ascorbic acid during heat stress.

Table 6.1. Some haematological parameters of broiler chickens treated with probiotic and ascorbic acid and exposed to thermal stress.

	Control	Probiotic	Ascorbic acid	Probiotic + AA
PCV (%)	32.25 ± 0.73^{a}	31.87 ± 0.68^{a}	31.25 ± 1.35^{a}	29.21 ± 0.50^{a}
WBC (x 10^{9}/l)	10.82 ± 0.72^{a}	11.92 ± 0.87^{b}	13.18 ± 0.75^{b}	13.53 ± 0.81^{b}
Heterophil	6.21 ± 0.61^{a}	5.82 ± 0.53^{a}	6.10 ± 0.94^{a}	6.30 ± 0.61^{a}
Band	0.07 ± 0.00^{a}	0.03 ± 0.01^{a}	0.10 ± 0.01^{b}	0.06 ± 0.01^{a}
Lymphocyte	3.13 ± 0.37^{a}	4.01 ± 0.48^{b}	5.59 ± 0.59^{c}	5.20 ± 0.39^{c}
Monocyte	0.59 ± 0.10^{a}	0.93 ± 0.21^{b}	0.74 ± 0.15^{b}	0.93 ± 0.13^{b}
Eosinophil	0.80 ± 0.10^{a}	1.10 ± 0.28^{b}	0.62 ± 0.15^{a}	0.91 ± 0.13^{a}
Basophil	0.00 ± 0.00^{a}	0.01 ± 0.01^{a}	0.00 ± 0.00^{a}	0.02 ± 0.02^{a}
H:L	2.80 ± 0.03^{a}	1.91 ± 0.03^{b}	2.21 ± 0.15^{a}	1.30 ± 0.02^{b}

Mean values with different superscript letters across the same row are significantly different at $P < 0.05$. AA = Ascorbic acid, H:L = Heterophil/Lymphocyte ratio. n = 7.

6.5 DISCUSSION

The poor responses to open-field test, tonic immobility and vigilance obtained in the control group throughout the study might be linked to the negative effects of heat stress observed. Heat stress directly and indirectly affects the biological functions of animals causing structural and functional damage to the brain (Egbuniwe et al., 2018). Increase in metabolic heat production occurs when the cell uses more oxygen to produce ATP, more heat is further dissipated and the body temperature rises, leading to an increase in fear. Branco et al. (2021) stated that the sequences of behavioural response in chickens are often retarded by heat stress due to the increase fear hormone in circulation which generally alters their behaviours.

During the cause of our study, the probiotic-administered group of broiler chickens had an optimum behavioural response. These improved markers of open-field test, TI and vigilance

could further validate the fact that yeast probiotic is potent in ameliorating thermal stress based on the decrease in fear responses observed. Physiologically, increase in age increases basal metabolic heat production in broilers; this could be mitigated by the administration of probiotic which could further improve their behaviours. Yeast probiotics are vital in the induction of thermoregulation which subsequently inhibit or regulate the secretion of both stress and fear hormones controlled via the hypothalamic-pituitary-adrenal (HPA) axis. This regulation further enhances the optimum behaviour of the broiler chickens, evident as decrease in fear responses.

The ascorbic acid group of broiler chickens had a short duration of TI and vigilance during the study. This may be due to the anti-stress effect of the agent which might have reduced the level of fear hormone in circulation evident by an improved behaviour. According to Zulkifli et al. (2000), broiler chickens administered ascorbic acid had a short duration of TI and this was associated with the production of catecholamines and the conversion of dopamine to norepinephrine which could be stimulated by ascorbic acid. Interesting as norepinephrine is an excitatory neurotransmitter in the brain, more neurotransmitters, could mean more wakefulness and this could explain the better response time observed in this group.

In comparison to the controls all the treatments had a protective effect against osmotic fragility of the erythrocytes. It could be speculated that probiotic and ascorbic acid were effective as an anti-stress agent in enhancing the resistance of the erythrocyte to haemolysis. Based on their antioxidant effect, they decrease damages to the erythrocyte membrane via the inhibition or reduction of lipid peroxidation. This is responsible for increasing the integrity of the membrane and protecting the erythrocytes from heat-stress effects (Ogbuagu et al., 2018; Egbuniwe et al., 2021).

Nevertheless, higher PCV was obtained in the control group. Heat stress induces haemoconcentration due to dehydration resulting into higher PCV. Shandya et al. (2015)

reported that increase in PCV is often stimulated by heat stress in animals due to dehydration. In the treatment group, the lower PCV obtained could be attributed to the absence of dehydration which enhances the general wellbeing of the chickens. Iyaode et al. (2020) also stated that broiler chickens supplemented with agents that function as an antioxidant could be effective in improving their haematological profile. Although, they used an agent (ginger) which possesses antioxidant effect and reported a decrease PCV in broiler chickens supplemented with ginger in comparison with the unfed group, they attributed the supplement to be effective on the bone marrow of the chickens.

There was an increase in lymphocyte, monocyte and eosinophil counts in the treatment groups which serves as our reference values for this study while the combination group had a decrease H:L ratio (a potent indicator of heat stress in broiler chickens). This could be linked to the absence of dehydration or the correction of dehydration that might have occurred due to heat stress exposure during the study. Also, ascorbic acid and especially yeast probiotic could improve the immune organs of broiler chickens leading to immunocompetence when administered adequately due to their anti-stress effect in the HPA axis and antioxidant effect in decreasing the production of ROS. Therefore, this could also be responsible for the changes obtained. Akhavan-Salamat and Ghasemi (2016) reported an improved lymphocyte, heterophil and monocyte counts in broiler chickens administered antioxidants such as betaine and turmeric and exposed to heat stress. They associated this with the reversal effect of the antioxidants on the HPA axis in reducing the synthesis of corticosterone in broiler chickens during heat stress. The control group had a decrease in lymphocyte, monocyte and eosinophil counts in relation to the treatment groups this could be a resultant of the heat stress this group was exposed to, as high ambient temperature has been reported to negatively affect the components of circulating leucocytes in broiler chickens (Altan et al., 2000).

6.6 CONCLUSION

Probiotic and/or ascorbic acid were efficacious in improving the behavioural and some haematological indicators in broiler chickens during the phase of thermal stress. Their erythrocytes were also resistant to haemolysis resulting from heat stress due to the administered antioxidants. Their effect on the HPA axis played a vital role in regulating fear responses and improving their leucocyte profile.

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CHAPTER SEVEN

7.0 GENERAL DISCUSSION, CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

7.1 GENERAL DISCUSSION

This study was undertaken to ascertain the potential value of specific exogenous compounds in mitigating the impact of thermal stress in broiler chickens reared under semi-intensive and intensive conditions in the absence of mechanical ventilation and artificial cooling. Under said conditions, birds tend to perform poorly due to mismatch between environmental conditions and the birds thermoneutral zone. This therefore has spurred the use of alternate strategies or systems that are less expensive, readily available and profitable in poultry production, the use of anti-stress and antioxidant and/or growth promoting agents such as yeast probiotic and ascorbic acid. Birds were placed under thermal stress by allowing rearing under a typical Pretoria summer.

The study period was thermally stressful (DBT 27- 36 °C; RH 72-86% and THI 26.7-35.4) to the broilers with high AT, RH and THI values exceeding the range specific for raising broiler chickens. This agrees with Mutibvu et al. (2017), who observed ambient temperature values above the thermoneutral zone specific for broilers production during the hot summer.

High CT values (40.35 – 41.79 °C) obtained in the control group may be an indication of the reduced capacity of broiler chickens to handle heat stress as they advance in age and this manifests as the high indexes of discomfort (THI) obtained during the study period. Increased rate of metabolism, which subsequently leads to an increase in metabolic heat production – hence heat-gain, occurs in adult broiler chickens (Aluwong et al., 2017). The low CT values obtained in the treated groups may be linked with the anti-stress effect of the antioxidant. This may be valid as yeast probiotic has been reported to be a potent anti-stress agent, promoting the performance of broiler chickens when administered in adequate amounts

during the thermally stressful season (Zhang et al., 2014; Mohammed et al., 2018). The treatment groups had a lower comb, head, wing, back and foot temperatures; the increased value obtained in the control may be supported by the fact that the chickens were not able to elicit thermoregulation to control the level of heat stress the broiler chickens are being exposed to as evident by their CT values. During heat stress, it could be speculated that the oxygen intake of broiler chickens increases to meet demand for thermoregulatory mechanisms e.g., evaporative cooling, which further leads to the excessive build ups of ROS. Therefore, the administration of exogenous antioxidants like probiotic and/or ascorbic acid could enhance thermoregulatory mechanisms thereby reducing the adverse effect of thermal stress. This finding agrees with Kim et al. (2021) who reported that BST is an effective indicator of the level of exposure of poultry species to heat stress, as high environmental temperature directly influences their BST.

The performance of broiler chickens was impaired by heat stress as observed in the control group with reduced feed and water intake which subsequently decrease their body weight gain. Probiotic group had the best performance which could be attributed to the growth promoting and anti-stress effects of the antioxidant as mentioned earlier in the above chapter. According to Al-Ali et al. (2023), probiotic stimulates the growth of broiler chickens due to its effect in the gut which enhances competitive exclusion of the harmful bacteria. The distortion in the epithelia lacteal and absence of mucin in some of the goblet cells which was a resultant of heat stress effect in the small intestine could be a contributing factor to the poor weight gain obtained in the control group. Because epithelia lacteal is responsible for the digestion and absorption of nutrient while mucin is responsible for protecting the epithelium and regulating the concentration and passage of some immune mediators (antimicrobial peptides), ions and water, therefore, heat stress could impact these processes in broiler chicken production if not mitigated. Probiotic and ascorbic acid were effective in mitigating

the negative effect of heat stress on the expression level of 8-OHdG, HSP70 and IL-10 gene either singly or in combination, respectively and in improving the antioxidant status of the treated broiler chickens due to their effect in reducing or inhibiting cellular damages which is sequel to ROS production. Mahmoud et al. (2004) and El-Senousey (2017) reported a decline in the expression levels of IL-10 and HSP70 genes, respectively in chickens supplemented with antioxidants and exposed to thermal stress. They attributed this to the ability of the agent to inhibit lipid peroxidation.

Lipid peroxidation, the free radicals' tendency to extract electrons in the cell membrane leads to cell damage (Alagawany et al., 2022). Endogenous antioxidants are important enzymes that improve the immune status of the individuals especially during oxidative stress (Abeyrathne et al., 2021; Lee et al., 2022). While the concentrations of these enzymes are adequate for preserving normal cellular functionality under normal physiology, under increased ROS production such as during heat stress, they are often exhausted necessitating the need for exogenous antioxidants (Jiang et al., 2021; Attia et al., 2022) or for precursors to be administered (Gopi et al., 2018; Ebeid and Al-Homidan, 2022).

The haematological indicators were enhanced in broiler chickens supplemented with ascorbic acid and especially yeast probiotic. It could be speculated that the agents improved the immune organs of the broiler chickens leading to immunocompetence when administered adequately due to their anti-stress effect in the HPA axis and their antioxidant effect in decreasing the production of ROS. Heterophil:lymphocyte ratio increase could portray the degree of thermal stress in broilers. The treatment groups had decreased H/L ratio which connoted the protection conferred against the negative effect of heat stress on these chickens by the administered antioxidants. This was similar to the report of Akhavan-Salamat and Ghasemi (2016), who stated that the incorporation of ascorbic acid in broiler chickens feed,

had a positive impact on the H/L ratio based on its ability to function as an immunomodulator.

Broiler chickens administered probiotic and/or ascorbic acid had a short duration of tonic immobility, were more vigilant and performed better in the open-field test during the study. This may therefore be attributed to the anti-stress effects of the administered antioxidants, as decrease in heat stress further decreases the fear responses of the birds. Ahmed et al. (2021) reported an improved behaviour in broiler chickens supplemented with antioxidants during thermal stress; this was linked to the anti-stress nature of the agents.

7.2 CONCLUSION

This study sought to provide a basis for alleviating the detrimental effects of heat stress through the use of probiotic and/or ascorbic acid as cheaper feed additives that exhibit either anti-stress or antioxidant effects or also functions as growth promoters. In doing so, we confirmed the effect of thermal stress in the untreated (control) group as their CT and BST were heightened all through the study. This was also responsible for the poor behavioural responses obtained in this group. The performance indicators, intestinal epithelium, IL-10, HSP70, oxidative gene damage biomarker, MDA, SOD, CAT, GPx and haematological indicators were generally impaired by thermal stress in the untreated group. Based on the obtained results, probiotic and/or ascorbic acid was efficacious as a means of intervention in alleviating the detrimental effects of thermal stress in chickens due to their anti-stress, antioxidant and/or growth promoting effects that were observed during the study. Although, the present study encountered few limitations as indicated below, however, it was able to achieve it set objectives.

7.3 LIMITATIONS

The major limitation encountered in this present study was the unavailability of funds to purchase extra ELISA kits and qPCR mastermix to carry out a pilot study before the commencement of the main research analysis. But in any case, this does not negate the validity of our findings as we were able to conduct a pre-analysis of the various parameters via random selection of samples from each research group to examine the adverse effects of heat stress on antioxidant enzymes activities, HSP70, 8-OHdG, and IL-10 genes, respectively before embarking on the main analysis. This was done meticulously to avoid wastage.

Also, there was no reference value for the antioxidants enzymes activities of broiler chickens raised within the thermoneutral zone of a given locality which was expedient.

7.4 RECOMMENDATIONS

Since this study was interventionist in design, a production study should be carried out to translate the results into a more commercial environment.

In addition to CT and BST, other indicators such as oxidative gene damage and behavioural responses may be useful diagnostic tools for evaluating the negative effects of stress in broiler chickens. More studies are required to explain the molecular mechanism by which probiotic (*Saccharomyces cerevisiae*) administration, both singly and in combination with ascorbic acid enhance nutrient transportation in the gut.

It is also expedient for research to be carried out in evaluating the antioxidants enzyme activities of broiler chickens raised within the thermoneutral zone of a given locality, for reference values to be made available for future studies.

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Appendix I

Letter For Sample Size

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

2 Ju

Approval Certificate
New Application

AEC Reference No.:	REC050-20
Title:	EVALUATION OF THE EFFECTS OF PROBIOTIC (Saccharomyces cerevisiae) AND ASCORBIC ACID ON THE ADVERSE EFFECTS OF HEAT STRESS IN BROILER CHICKENS
Researcher:	Dr VO Sumanu
Student's Supervisor:	Prof JP Chamunorwa

Dear Dr VO Sumanu,

The **New Application** as supported by documents received between 2021-02-24 and 2021-07-02 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2021-07-02.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number**
Poultry - cobb500	64
Samples Blood	64
muscles, intestinal segments, reproductive organ, brain	28

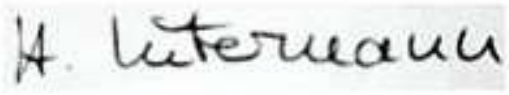
2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2022-07-02.
3. Please remember to use your protocol number (REC050-20) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modificatior monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be require if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely



Dr Heike Lutermann
DEPUTY CHAIRMAN: UP-Animal Ethics Committee

Appendix II

Published Literature Review

International Journal of Biometeorology
https://doi.org/10.1007/s00484-022-02372-5

REVIEW PAPER

Adverse effects of heat stress during summer on broiler chickens production and antioxidant mitigating effects

V. O. Sumanu[1] · V. Naidoo[2] · M. C. Oosthuizen[3] · J. P. Chamunorwa[1]

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Abstract
Broiler chicken meat is a good source of protein consumed universally, and is one of the most commonly farmed species in world. In addition to providing food, poultry non-edible byproducts also have value. A major advantage of broiler chicken production is their short production cycle, which results in a greater rate of production in comparison to other species. However, as with any production system, there are constraints in broiler production with one of the most pressing being energy requirements to keep the birds warm as chicks and cool later in the growth cycle, as a result of the cost needing mechanical heating and cooling. While this is feasible in more advanced economies, this is not readily affordable in developing economies. As a result, farmers rely on natural ventilation to cool the rearing houses, which generally becoming excessively warm with the resultant heat stress on the birds. Since little can be done without resorting to mechanical ventilation and cooling, exploring the use of other means to reduce heat stress is needed. For this review, we cover the various factors that induce heat stress, the physiological and behavioral responses of broiler chickens to heat stress. We also look at mitigating the adverse effect of heat stress through the use of antioxidants which possess either an anti-stress and/or antioxidant effects.

Keywords Heat stress · Oxidative stress · Global warming · Broiler chickens · Welfare

Introduction

Stressors refer to any factor that threatens the health of the body or has an adverse effect on its functioning, such as cellular injury, disease, or anxiety. Stress is a consequence of the physiological and adverse effects of the environment or management system, which induces several changes in the physiology or behavior of an animal, which in the latter case; if unchecked, leads to pathophysiology and malfunctioning (Altuwong et al. 2017; Gogoi et al. 2021). Thus, stress assists the animal to cope with its environment, i.e., physiological stress. The general adaptation syndrome is a non-specific one-way physiological response of the body to all types of endogenous and exogenous stressors (Sumanu et al. 2019; Kim et al. 2021).

The general adaptation syndrome has three phases, namely alarm, resistance, and exhaustion (Gaidica and Dantzer 2020). The exhaustion phase signals that the animal has given all it had and is no longer able to fight; not only the stressor, but also opportunistic infections, and it will succumb in death (Fig. 1).

- Alarm phase (primary response): This phase involves the neuroendocrine system activation and stress hormone production (corticosterone). Corticotropic releasing hormone (CRH) is produced by the hypothalamus, which stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH). This further stimulates the rapid release of the stress hormone corticosterone from the adrenal gland (Getabalew et al. 2020), and corticosterone in turn increases the conversion of noradrenaline to adrenaline by stimulating the enzyme Phenylethanolamine *N*-methyltransferase (PNMT) in the adrenal medulla (Bailly 2020).

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Appendix III

Published Article

Animal Gene 28 (2023) 200150

Contents lists available at ScienceDirect

Animal Gene

journal homepage: www.sciencedirect.com/journal/animal-gene

Effects of probiotic (*Saccharomyces cerevisiae*) and ascorbic acid on oxidative gene damage biomarker, heat shock protein 70 and interleukin 10 in broiler chickens exposed to heat stress

Victory Osirimade Sumanu [a,*], Charles Byaruhanga [b], Anna-Mari Bosman [b], Sunday Ochonu Ochai [b], Vinny Naidoo [c], Marinda Catharina Oosthuizen [b], Joseph Panashe Chamunorwa [a]

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Keywords:
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Oxidative gene damage

ABSTRACT

Heat stress is a prominent factor responsible for losses economically in poultry meat industry due to adverse effects on the general performance of broiler chickens. In this study, we evaluated the effects of probiotic (*Saccharomyces cerevisiae*) and ascorbic acid on oxidative gene damage biomarker, heat shock protein 70 (*HSP70*) and interleukin 10 (*IL-10*) in broiler chickens exposed to heat stress under natural conditions. Fifty-six broiler chickens served as the subjects, they were divided into 4 groups of 14 as follows: group I (control), group II (probiotic *S. cerevisiae* at 1 g/kg of feed), group III (ascorbic acid at 200 mg/kg of feed) and group IV (probiotic + ascorbic acid at 1 g/kg and 200 mg/kg of feed, respectively). The treatments were administered via feed for 35 days (D1 to D35). Enzyme-linked immunosorbent assay (ELISA) and one step real time reverse transcription polymerase chain reaction (RT-PCR) was utilised to study the effects of heat stress on the expression levels of 8-hydroxy-2′-deoxyguanosine (*8-OHdG*), *HSP70* and *IL-10* respectively, in broiler chickens raised during the hot summer season. The level of *8-OHdG* gene was significantly lower in the probiotic administered group. The expression level of *HSP70* was lowest in the ascorbic acid group while, *IL-10* level of expression was highest in the probiotic + ascorbic acid group. The administered antioxidants were efficient in exhibiting anti-stress effects at the level of gene expression. We conclude that probiotic, ascorbic acid and probiotic + ascorbic acid reduced oxidative gene damage, affected the expression of *HSP70* and increased the level of *IL-10* gene respectively, in broiler chickens exposed to heat stress.

Appendix IV

Published Article

animals

MDPI

Technical Note

A Technical Report on the Potential Effects of Heat Stress on Antioxidant Enzymes Activities, Performance and Small Intestinal Morphology in Broiler Chickens Administered Probiotic and Ascorbic Acid during the Hot Summer Season

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Simple Summary: Thermal stress is an environmental factor that negatively affects poultry production globally. It elicits behavioural and physiological changes in broiler chickens, hence the need to find ways of ameliorating its detrimental effects which are mainly expressed as oxidative stress. This study was designed as an intervention on the effect of heat stress during the hot summer season in broiler chickens' production using probiotic and ascorbic acid as anti-stress agents. From the results, probiotic and/or ascorbic acid were effective in enhancing the antioxidant enzyme activities and performance of the broiler chickens. This study stands as a basis for application in animal production trials with a larger sample size.

Abstract: Oxidative stress negatively affects the welfare of broiler chickens leading to poor productivity and even death. This study examined the negative effect of heat stress on antioxidant enzyme activities, small intestinal morphology and performance in broiler chickens administered probiotic and ascorbic acid during the hot summer season, under otherwise controlled conditions. The study made use of 56 broiler chickens; which were divided into control; probiotic (1 g/kg); ascorbic acid (200 mg/kg) and probiotic + ascorbic acid (1 g/kg and 200 mg/kg, respectively). All administrations were given via feed from D1 to D35 of this study. Superoxide dismutase, glutathione peroxidase and catalase activities were highly significant ($p < 0.0001$) in the treatment groups compared to the control. Performance indicators (water intake and body weight gain) were significantly higher ($p < 0.05$) in the probiotic and probiotic + ascorbic acid group. The height of duodenal, jejunal and ileal villi, and goblet cell counts of broiler chickens were significantly different in the treatment groups. In conclusion, the study showed that heat stress negatively affects the levels of endogenous antioxidant enzymes, performance and the morphology of small intestinal epithelium, while the antioxidants were efficacious in ameliorating these adverse effects.

Keywords: ascorbic acid; probiotic; antioxidant enzymes; performance; small intestinal morphology

check for updates

Citation: Sumanu, V.O.; Naidoo, V.; Oosthuizen, M.; Chamunorwa, J.P. A Technical Report on the Potential Effects of Heat Stress on Antioxidant Enzymes Activities, Performance and Small Intestinal Morphology in Broiler Chickens Administered Probiotic and Ascorbic Acid during the Hot Summer Season. *Animals* 2023, 13, 3407. https://doi.org/10.3390/ani13213407

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