EVALUATION OF HAEMATOLOGICAL, BIOCHEMICAL AND SMALL INTESTINAL MORPHOMETRIC CHANGES IN BROILER CHICKENS ADMINISTERED WITH FISETIN AND PROBIOTIC DURING THE EARLY RAINY SEASON

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN VETERINARY PHYSIOLOGY

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OCTOBER, 2018

DECLARATION

I declare that the work reported in this dissertation, titled **'Evaluation of Haematological, Biochemical and Small Intestinal Morphometric Changes in Broiler Chickens Administered with Fisetin and Probiotic During the Early Rainy Season'** was carried out by me in the Department of Veterinary Physiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria. The information derived from literature has been duly acknowledged in the text and in the list of references provided. No part of this dissertation has been presented for another degree or diploma at any university.

Ngozi Ejum OGBUAGU

…….. ....

(Name of Student) Signature Date

CERTIFICATION

This dissertation, entitled **'EVALUATION OF HAEMATOLOGICAL, BIOCHEMICAL AND SMALL INTESTINAL MORPHOMETRIC CHANGES IN BROILER CHICKENS ADMINISTERED WITH FISETIN AND PROBIOTIC DURING THE EARLY RAINY SEASON',** carried out by Ngozi Ejum OGBUAGU meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

I dedicate this project to Almighty God, mummy and my husband.

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ABSTRACT

The aim of the study was to evaluate the effects of fisetin and probiotic administration on haematological, biochemical and small intestinal morphometric changes in broiler chickens during the early rainy season. Sixty day-old broilers of both sexes used for the study were divided into four groups: Group I (control) was given distilled water; Group II, fisetin; Group III, probiotic; and Group IV, fisetin + probiotic, each orally for 7 days. Blood samples were collected from each bird in the four groups on day 42 for determination of erythrocyte osmotic fragility, serum concentration of malondialdehyde (MDA) and activity of superoxide dismutase (SOD), packed cell volume, red blood cell count, haemoglobin concentration, total and differential leucocyte counts and total plasma protein concentration. Three birds per group were sacrificed to evaluate small intestinal morphology. The dry-bulb temperature, relative humidity and temperature-humidity index ranges during this experiment were 25.00 - 36.00 °C, 47.00 - 100.00% and 25.00 - 35.55, respectively, indicating the birds were subjected to heat stress. At 0.5% and 0.3% NaCl, fisetin and/or probiotic significantly ($P < 0.001$) reduced erythrocyte osmotic fragility, compared to control broiler chickens. The MDA concentrations in probiotic (5.66 ± 1.06 nmol/L), fisetin (14.37 ± 1.15 nmol/L) and fisetin + probiotic (4.14 ± 0.58 nmol/L) administered groups were significantly ($P < 0.05$) lower than that of the control group (22.64 ± 2.95 nmol/L). A significant increase in serum SOD activity in the treated groups: fisetin (6.34 ± 0.24 IU/L), probiotic (5.67 ± 0.09 IU/L), fisetin + probiotic (5.93 ± 0.13 IU/L), were obtained when compared to the control group (5.37 ± 0.09 IU/L). The values of packed cell volume, red blood cell, haemoglobin and total protein concentration in the treated groups were higher ($P < 0.05$) than those of the control group. Total leucocyte, eosinophil, basophil and lymphocyte counts in the treated groups did not differ significantly ($P > 0.05$) from the corresponding values in the control group. Heterophil/lymphocyte ratio was lower in the treated groups (fisetin, 0.10 ± 0.04; probiotic, 0.09 ± 0.04; fisetin + probiotic, 0.11 ± 0.03),

than that of the control group (0.54 ± 0.12). Values obtained for the small intestine showed that villus height, villus width, crypt height, crypt width, villus height/crypt height ratio, surface area of villus and crypt were significantly higher ($P < 0.05$) in the treated groups, when compared to those of the control group. It was concluded that the administration of fisetin and probiotic decreased MDA concentration and heterophil/lymphocyte ratio, and increased SOD activity as well as surface area of villus in broiler chickens exposed to heat stress. The administration of fisetin and probiotic alleviated the detrimental effects of heat stress and may enhance broiler chicken production during the early rainy season in the zone.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BAS	Basophil
°C	Degree celsius
CAT	Catalase
DBT	Dry-bulb Temperature
DNA	Deoxyribonucleic acid
EOF	Erythrocyte Osmotic Fragility
EOS	Eosinophil
FAO	Food and Agricultural Organisation
Fl	Femtolitre
g/dL	Grams per litre
GP_X	Glutathione Peroxidase
H_o	Null hypothesis
H_A	Alternate hypothesis
HB	Haemoglobin
HET	Heterophil
IgA	Immunoglobin A
IgG	Immunoglobin G
IgM	Immunoglobin M
IU/L	International unit per litre
kCal/kg	Kilocalorie per kilogram
LYM	Lymphocyte
M	Metre
MCH	Mean Corpuscular Haemoglobin

MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MDA	Malondialdehyde
mg/Dl	Millligram per decilitre
mg/kg	Milligram per Kilogram
mL	Millilitre
mm	Millimetre
MON	Monocyte
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
Nm	Nanometre
nmol/L	Nanomole per litre
OD	Optical Density
PCV	Packed Cell Volume
RBC	Red Blood Cell
RH	Relative Humidity
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
SOD	Superoxide Dismutase
THI	Temperature-humidity Index
Th1	Type 1 T helper
Th2	Type 2 T helper
µL	Microlitre
%	Percentage

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

The yearly increase in population of the world including Nigeria has resulted in an increased demand for protein intake, and this is readily met with poultry meat supplies (Henchion *et al*., 2017; Onuk *et al.*, 2017). Broiler chicken was reported to be the third most consumed meat in the world, after beef and pork (Atilgan and Hayati, 2006; Marangoni *et al.,* 2015). Broiler chicken production is profitable because it has a good net return in investment (Amid *et al.*, 2015), and is managed both in small and large scales in Nigeria. Broilers chickens are subjected to oxidative stress under certain unsuitable physiological and environmental conditions (Zheng *et al.,* 2016). Meteorological factors such as high ambient temperature and high humidity exert adverse effects on poultry production in many tropical countries, including Nigeria (Chand *et al.,* 2017). These impose heat stress on broiler chicken, affecting feed intake, structural integrity of the intestine, feed conversion and weight gain (Gupta *et al*., 2017).

Antibiotics have been used in broiler chicken production to suppress the population of bacteria in intestine and protect the birds from the harmful effects of pathogenic enteric microorganisms (Broom, 2018). Due to the emergence of antibiotic-resistant bacteria, drug residues in poultry meat and the irrational use of antibiotics in poultry meat, it has become necessary to search for alternative additives such as probiotics and plant extracts (Wang *et al.,* 2016), beneficial in broiler chicken production.

The word probiotic in Greek means ‘for life’ (Gibson and Fuller, 2000), is defined as live microbial feed additives that maintain microbial balance in digesta in the gastrointestinal tract of the host animal (Fuller, 1989). Probiotics stimulate growth, health and also modify the ecology of the intestine in a beneficial manner to the host (Aluwong *et al.,* 2013; Gao *et al.,*

2017). They modify the intestinal environment by reducing the pH, supplying digestive enzymes and increasing enzyme activity in the gastrointestinal tract (Sarangi *et al.,* 2016). Probiotics increases the population of beneficial bacteria, decreases harmful bacteria, reduces harmful caecal bacterial enzymes and improves intestinal morphology of broilers (Shokryazdan *et al.,* 2017). Probiotics changing microflora could be due to competitive exclusion by competing for nutrients and attachment sites on the intestinal epithelial wall, or production of antimicrobial substances by probiotic strains or a synergy of both actions (Hayek *et al.,* 2013). Several studies have reviewed the potential benefits of probiotics in improving the intestinal morphology and integrity of birds subjected to heat stress (Zhang *et al.,* 2017a). Probiotic increase villus height and crypt depth (Bai *et al.,* 2013; Bai *et al.,* 2018) and it also reverses impaired villus-crypt structure of stressed birds by controlling the corticosterone level (Lei *et al.,* 2013). Furthermore, it increases intestinal length which is indicative of larger digestive and absorptive area (Wang *et al.,* 2016). Probiotic administration has been found to enhance antibody responses (Manafi *et al*., 2018), elevating the concentration of IgG and IgM levels, which has been proposed to contribute to more positive growth performance and resistance of animals against diseases (Cetin *et al.,* 2015). Probiotic lowers heterophil to lymphocyte (H/L) ratio and improves immune responses (Beski and AlSardary, 2015).

Fisetin is a plant polyphenol from the flavonoid group emerging as potent antioxidant effective against a wide range of free radical mediated diseases. Flavonoid is present in fruits and vegetables, such as strawberries, apples, persimmons, onions and cucumbers (Sahu *et al*., 2014). Numerous studies have demonstrated the beneficial effects of flavonoid-rich foods, including anticancer, anti-inflammatory and cardiovascular protective effects, as well as a protective role in degenerative diseases (Subramanian *et al.,* 2015; Kashyap *et al.,* 2017). They are known to enhance the gut morphology and functionality, mucosal and cellular immunity and amelioration of heat stress in farm animals, suggesting that they promote health and exert

beneficial effects in animal production (Kamboh *et al.*, 2015). When an imbalance occurs between antioxidants and reactive oxygen species (ROS), it results in oxidative stress and is a consequence of a mismatch between the production of the ROS and the ability to defend against them (Slimen *et al.*, 2014). Flavonoids prevent ROS-induced cell injury (Prochazkova *et al.*, 2011; Treml and Simjkal, 2016) by direct scavenging of ROS, activation of antioxidant enzymes, metal chelating activity, reduction of α-tocopheryl radicals, inhibition of oxidases and mitigation of oxidative stress (Surai, 2014).

Stress induces increased ROS generation, which leads to lipid peroxidation (Puttachary *et al.*, 2015). Lipid peroxidation is the oxidative degradation of polyunsaturated fatty acids. It occurs in biological membranes, inactivates several membrane bound enzymes, and impairs membrane fluidity and integrity (Azeez *et al.*, 2013). Flavonoid binding to the erythrocyte membranes significantly inhibits lipid peroxidation, and at the same time enhances their integrity against hypotonic lysis (Kamboh and Zhu, 2013). Malondialdehyde (MDA) is a product of peroxidised polyunsaturated fatty acids, and increased MDA concentration is an important indicator of lipid peroxidation (Egbuniwe *et al*., 2018a). Activities of antioxidant enzymes are used to assess tolerance in broiler chickens (Surai *et al.*, 2015). Erythrocyte osmotic fragility is a potential biomarker of oxidative membrane damage to cells, including xenobiotic-induced oxidative membrane damage to erythrocytes (Sharma *et al.*, 2010). Poultry health status, especially in broiler chickens, is linked to changes in haematological parameters (Etim *et al*., 2014; Jaiswal *et al.*, 2017). Northern Nigeria is characterised by fluctuating ambient temperature and relative humidity during the early rainy season, which has been shown to induce thermal stress in poultry (Dunning *et al*., 2016).

1.2 Statement of Research Problems

Broiler meat is a good source of protein, the intake of which increases yearly due to the rapid growth of the population in Nigeria (Heise *et al*., 2015). Challenges facing broiler chicken production in the developing world, including Nigeria, apart from heat stress is the increasing need to improve production efficiency, influenced by management factors, including the use of antibiotics as additive. Farmers in an attempt to enhance growth of birds have resorted to the use of additives, right from day one and all through to the point of sale. Although antibiotics have been used as feed additives in sub-therapeutic dosage to promote growth and protect broiler chickens (Goodarzi *et al.*, 2014; Yazdi *et al.,* 2014). They exert adverse health effects on broiler chickens and consumers of broiler meat raised with irrational use of antibiotics, such effects include poor antimicrobial resistance to disease conditions (Zaheer *et al*., 2017). Non-observance of withdrawal period poses a health risk of antimicrobial resistance in consumer. More alarming is the increased potency of products of antibiotics in broiler meat, even after thermal processing (Nyuyen *et al*., 2015).

1.3 Justification

The use of antibiotics in broiler production is associated with negative effects, including antimicrobial resistance in human diseases, resulting in treatment failure (Capital and Alonso-Calleja, 2013; Xiong *et al*., 2018); therefore, administration of probiotics and fisetin in broiler production may be beneficial in reducing or even abolishing the negative effects associated with the use of antibiotics. In addition, probiotics and fisetin may ameliorate the negative effect of heat stress associated with broiler production in the region of the study. Probiotics exhibit antioxidant activity and, thus, increase the body antioxidant levels, which in turn improve the health status of broiler chickens (Tabidi *et al*., 2013). Therefore, the administration of probiotics to increase broiler production, yet with high yield, safe and high-quality meat (Park

et al., 2016), may reduce or abolish the high antibiotic resistance, often associated with the irrational use of antibiotics in broiler chicken production. Fisetin as an antioxidant scavenges ROS, which damage lipids, amino acids, carbohydrates and nucleic acids (Sinha *et al*., 2014). Therefore, its administration may prevent the adverse effects of ROS; enhancing immunity, prolonging shelf-life and preserving quality and taste of meat (Zhang *et al.,* 2015). There is, therefore, the need to search for new alternatives to the use of antibiotics as feed additives in poultry production in order to produce wholesome broiler meat, fit for human consumption (Bai *et al.,* 2016).

1.4 Aim of the Study

The aim of the study was to evaluate the effects of administration of fisetin and probiotic on haematological, biochemical and small intestinal morphometric changes during the early rainy season in broiler chickens.

1.5 Objectives of the Study

The objectives of the study were to determine the effects of fisetin and probiotic administration on changes in:

i. Haematological profiles during the early rainy season
ii. Biochemical profiles during the early rainy season
iii. Small intestinal morphometric profiles during the early rainy season

1.6 Statement of Research Hypotheses

H_o: Fisetin and probiotics do not exert significant effect on haematological, biochemical and small intestinal morphometric parameters in broiler chickens during the early rainy season.

H_A: Fisetin and probiotics exert significant effect on haematological, biochemical and small intestinal morphometric parameters in broiler chickens during the early rainy season

CHAPTER TWO

LITERATURE REVIEW

2.1 Broiler Chicken Production

Broiler chicken production is an important integral part of poultry for economic benefits (Scanes, 2007). Production of chickens for consumption started as far as 4000 years ago, since then selective breeding of parent stock has been going on to achieve specific desired trait (Kalmar *et al.,* 2013). In Nigeria over decades importation of poultry meat into the market have affected poultry industry and economy of the nation. President Olusegun Obasanjo during the National Economic Empowerment Development Strategy (NEEDS) placed a ban on imported poultry products which has improved the poultry industry in Nigeria (Heise *et al.,* 2015). Broiler production was not regarded as an important occupation in the past, but recently it has become developed and occupied an important position among the livestock enterprise due to its fast turn-over (Marangoni *et al*., 2015). Thus, in a developing country like Nigeria, where the utmost priority is to achieve self-sufficiency in food production with special emphasis on animal products, it becomes imperative to encourage farmers to venture into broiler chicken production (Majid and Hassan, 2014). Broiler chickens are also considered important biological model for researches in biomedical field (Neves *et al.,* 2014).

Poultry enterprise is becoming complex by the day, given the rapid strides in technology, changing market dynamics and growing scale of production (Afzal and Khan, 2017). In broiler chicken production, entrepreneurs are faced with numerous constraints starting from the procurement of chicks to their final disposal, which must be handled properly to achieve maximum output (Amid *et al*., 2015). Factors such as heat stress in broiler chickens raised under unfavourable conditions result in economic losses. Considerations should therefore be given to the microclimate within the broiler houses as they experience heat stress (Akyuz and

Boyaci, 2010). Some of the challenges faced in broiler production, especially in developing countries, include high cost of feed and veterinary drugs, poor quality of commercial feeds due to wrong formulation by the manufacturers, inadequate capital investment, inadequate knowledge of nutrients and energy requirements, and especially the abuse of antibiotic use (Nwandu *et al.,* 2016). Even with these challenges, it has been projected that a 20% annual growth in the poultry industry may occur between 2010-2020, driven by increased large population (Mottet and Tempio, 2017).

2.2 Importance of Broiler Chicken Production

Broiler chickens are kept in most parts of the world and provide generally acceptable form of animal protein to most people throughout the world (Wolk, 2017). Intensively kept broiler chickens are ways of rapidly increasing animal protein supplies for the rapidly increasing urban populations. Broiler chickens are relatively low priced, reproduce rapidly, and have a high rate of productivity (Tallentire *et al*., 2016). Their production is characterised by high economic return due to short production cycle of 6-7 weeks, while the production cycle of local breed of chickens takes 3 to 12 months. Therefore, the capital cycle is very rapid in broiler production as compared with that of other types of livestock production (Horne, 2013; Onuk *et al*., 2017). The business of broiler chicken production is beneficial and profitable with prospects of increasing the accessibility to high-quality protein for the consumption of humans in a short period of time (Afzal and Khan, 2017). Poultry farming besides its nutritional value, is an important source of livelihood and employment for millions of people worldwide; and its allied business of feed production, hotel and restaurant industry, retail and wholesale businesses, carriage and transport industry (Ohajianya *et al.*, 2013). Broiler chicken industry has significantly developed in the world and is now an important source of foreign exchange earnings for many countries. It is expected that poultry meat will increase seven-fold in the first two decades of 21st century (Scanes, 2007).

2.3 Adverse Effects of Meteorological Factors on Broiler Chicken Production during Seasons

The town of Zaria (11°, 12′; 7° E, 38′) is located in the Northern Guinea Savannah of Nigeria. It has annual ambient temperature, ranging between 18.0 ± 3.7 and 31.8 ± 3.2°C, and has harmattan (the cold-dry, November-February), hot-dry (March-May) and rainy (June-October) seasons (Dzenda *et al.,* 2013; Ayo *et al*., 2014). Climatic conditions affect the health, welfare and productivity of broiler chickens. With the change in climatic conditions in the world, and especially with global warming, broiler chicken production has been negatively affected (Part *et al*., 2016). Despite technological advancements such as improved varieties and genetically modified livestock organism, climate is still a key factor in poultry productivity (Okere, 2013).

Meteorological factors such as ambient temperature and relative humidity, directly influence production of broiler chickens (Nayak *et al*., 2015). During the hot-dry season, weather conditions are characterised by high ambient temperature, low relative humidity and high radiant energy, which decrease the ability of broiler chickens to eliminate heat from the body resulting in mortality due to hyperthermia (Sinkalu *et al*., 2014; Aluwong *et al.,* 2017). The harmattan (cold-dry) season is characterised by low ambient temperature, low relative humidity and low radiant energy (Nguyen *et al*., 2016). Hot and cold-dry as well as rainy seasons generate stress. Therefore, it is very important to ascertain, modify and control the environmental parameters of rearing broiler chickens that may adversely affect their performance (Minka and Ayo, 2016). Heat stress, occurring during the hot-dry season is generated from high ambient temperature and relative humidity, and it negatively affect poultry performance (Mohammed *et al*., 2018). The temperature-humidity index (THI), an index of thermal comfort integrating the effects of ambient temperature and relative humidity, is used to evaluate the degree of thermal stress in animals (Dash *et al*., 2016). Thermal stress in animals during hot weather, particularly when the heat is prolonged or at extreme ambient temperatures,

results in reduced feed intake, growth, production, reproduction, welfare and health (Cottrell, 2015).

The thermoneutral zone is the range of ambient temperature, which does not affect regulatory changes in metabolic heat production or evaporative heat loss in birds (McWhorter *et al*., 2018). The thermoneutral zone for poultry in the tropics is between 18 and 24 °C and between 12 and 26 °C in temperate regions (Holik, 2009; Vinoth *et al*., 2015). In the tropics, the fluctuations in ambient temperature at twilight usually exceed the thermoneutral zone of chickens, resulting in heat stress (Nayak *et al*., 2015). Ambient temperatures outside the thermoneutral zone of birds, irrespective of age, may negatively affect their energy balance and fitness (Egbuniwe *et al*., 2018b). Metabolic disorders such as ascites and sudden death syndromes may occur in broilers reared above the temperature of thermal comfort in the first week of life of the broiler chickens (Ayo *et al.*, 2014). Broiler chickens subjected to heat stress show elevated corticosterone levels and lower levels of thyroid hormones (Mahmoud *et al*., 2014). Elevated ambient temperature inducing heat stress negatively affects production, reproductive potential, immune responses and health status of broiler chickens (Fathi *et al.*, 2017)

2.4 Heat Stress and Its Adverse Effects on Broiler Chicken Production

In broiler chicken production, the main source of stress is heat. High ambient and high humidity exert heat stress on broiler chickens in many tropical countries, including Nigeria (Rhoads *et al*., 2013). In broiler chickens, the maximum heat load exceeds the bird's carrying capacity for heat dissipation. Heat stress results from a negative imbalance between the net amount of energy flowing from the broiler's body to its surrounding environment and the amount of heat energy produced by the bird. This imbalance may be caused by variations of a combination of environmental factors (Akbarian *et al*., 2016). Maurya *et al.,* (2015) reported that heat stress is

a critical problem in broiler production systems and has a negative effect on health status, reproduction, metabolism and productivity. In tropical, subtropical and arid regions, high ambient temperature is the main factor causing heat stress, having adverse effects, particularly on high productivity of broiler chickens (Sinkalu *et al.*, 2015). This is because heat stress along with high relative humidity causes reduced heat dissipation by evapotranspiration (Vasantha *et al*., 2016), suppressing the immune response and increasing the susceptibility of broiler chickens to diseases. This is manifested in decrease in body weight, feed conversion efficiency and growth rate in broiler chickens (Belhadj *et al*., 2015).

In addition, heat stress damages the intestinal barrier in broiler chickens due to oxidative stress, and negatively influences the welfare of the birds (Hu *et al.,* 2017). Heat stress also disrupts intestinal morphology decreasing the rate of absorption of nutrients, which reduces the body weight of broiler chickens. Heat stress also stimulates a rise in serum corticosterone concentration, mortality and decreases the percentage of phagocytising macrophages (Ayo *et al.,* 2014; Santos *et al*., 2015).

2.5 Biomarkers of Heat Stress in Broiler Chicken Production

The health status of birds may be evaluated based on some important haematological parameters (Hrabrakova *et al*., 2014). Corticosteroid concentration in the blood has been used as a measure of environmental stress in birds (Fallahsharoudi *et al*., 2015). It increases in heat stress condition, adversely and significantly affecting proportions of leucocyte components. Leucocytic responses have been used as indicator of heat stress, demonstrated to disrupt leucocyte counts (Osti *et al*., 2017).

Exposure of broilers to high temperature has been shown to cause decrease in haematocrit values (Egbuinwe *et al*., 2018b). The erythrocytes and thrombocytes of heat-stressed broiler chickens are longer and thinner and these structural changes identified in the cells affect the

functional ability in raising an immune response (Maxwell *et al.,* 1992). Heterophil/lymphocyte ratio is usually utilised as a panacea for evaluating heat stress. When it is high above 0.20, it indicates the birds are undergoing heat stress, which adversely affect their immunity (Lentfer *et al*., 2015).

Measurement of cloacal temperature by a thermometer reflects the body temperature, and changes in this parameter is used as an index of welfare for broiler chickens when exposed to high ambient temperature (Selvan *et al*., 2018). Body temperature of broiler chickens reared under heat conditions are usually high; which tends to disrupt homeostasis, exerting deleterious effects on production and efficiency of the birds (Aluwong *et al*., 2017).

The erythrocyte osmotic fragility (EOF) is an important haematological parameter recently adopted for the evaluation of lipid peroxidation in broiler chichkens (Minka and Ayo, 2008; Egbuinwe *et al*., 2018a). The degree of lipid peroxidation of the cytomembranes, manifested in the extent of EOF is often used as an indicator of ROS-mediated damage (El-Bahr, 2013; Niki, 2014). The erythrocyte membrane damaged by lipid peroxidation, resulting from increased generation of ROS is indirectly measured by the MDA concentration. Thus, the concentration of MDA in blood and tissues is generally used as biomarkers of lipid peroxidation (Rodríguez-Ortega *et al.,* 2017; Nasiroleslami *et al*., 2018).

Behavioural responses also serve as a pointer for evaluating the welfare of broiler chickens in heat stress conditions (Mahmoud *et al*., 2015). High levels of cortisosterone and heat-shock protein 70 may indicate different stages of heat stress in broiler chickens (Zulkifli *et al.,* 2014). This could result in high intensity levels of fear responses, shown by protracted immobility in heat-stressed broiler chickens (Pusch *et al*., 2018). Heat stress predisposes broiler chickens to various behaviours such as panting, depression-like behaviour, preening behaviour, wing drooping behaviour, postration, shunking of chest and abdomen, staggering of legs, stretching

of body, restlessness, weakness, and squatting close to the ground as a means of thermoregulation (Li *et al*., 2015; Egbuniwe *et al*., 2016).

Analyses of the activities of enzymes are also important in determining oxidative stress reactions, induced in cells and ROS-induced cell or organs damage (Ognik and Krauze, 2016). Activities of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase have been quantified in plasma as measures of antioxidant capabilities. Decrease in antioxidant enzymes is an indicator of stress in broiler chickens. Measurement of the MDA concentration in the tissues and the activities of antioxidant enzymes, including the liver and serum have been investigated and demonstrated to serve as biochemical markers of oxidative stress in broiler chickens exposed to heat stress (Lui *et al*., 2014). The findings show that MDA concentration increases, while the activities of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) decrease in the birds (Akbarian *et al*., 2016; Ayo *et al*., 2018).

2.6 Antioxidants and their Roles in Heat Stress Alleviation in Broiler Chicken Production

Supplementation with antioxidant agents has been shown to boost the capacity of the biological antioxidant barrier in combating excess ROS generated in broiler chicken production (Pisoschi and Pop, 2015). Dietary antioxidants exhibit *in vivo* and post-mortem effects by preventing oxidative stress and oxidative rancidity in broiler chickens and their products, respectively (Salami *et al.*, 2015). The *in vivo* effect of antioxidants enhance the health, welfare of broiler chickens and improves the performance of the birds. It also decreases the deterioration of meat quality due to lipid peroxidation and stabilises meat oxidation after slaughter (Jiang and Xiong, 2016). Maintaining optimal performance of broiler chickens in conditions of oxidative stress is imperative for preventing economic loss in the broiler chicken industry (Zulkifli *et al*., 2014). The antioxidants also improve erythrocytic indices of broiler chickens subjected to heat stress

(Kamboh *et al.,* 2018). Vitamin E has been shown to improve antioxidant capacity in broiler chickens (Niu *et al*., 2018). It is also essential for the integrity of reproductive, muscular, circulatory, nervous and immune systems of broiler chickens (Habiban *et al.,* 2014). Vitamin C has been shown to improve the tolerance of broiler chickens during heat stress by increasing the immunity of the birds (Attia *et al.,* 2017). Selenium and vitamin E supplementation has also been shown to significantly improve total carotenoid concentrations in the plasma, which enhances skin and meat colour (Karados *et al.,* 2016).

2.7 Fisetin as Antioxidant and its Potential Role in Stress

Fisetin (3,3′,4′,7-tetrahydroxyflavone) is a naturally occurring flavonoid (Fig. 2.1), which is found in fruits and vegetables such as strawberries, apples, persimmons, grapes and onions (Lall *et al*., 2016).

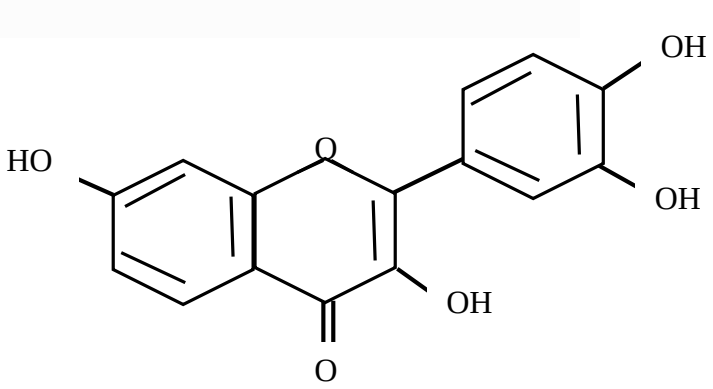


Figure 2.1: Molecular structure of fisetin

The ability of flavonoids to scavenge free radicals contributes to their marked antioxidant activity and significant biological effects. The oxygen radicals damage lipids, amino acids, carbohydrates, and nucleic acids. When an imbalance occurs between antioxidants and reactive oxygen species, it results in oxidative stress and is a consequence of a mismatch between the production of the reactive oxygen species and the ability to defend against them (Khan *et al*., 2013). It has been implicated in the development of many diseases, including diabetes mellitus, retinal degeneration, neurodegenerative diseases, mutagenesis, carcinogenesis, and ageing

(Naeimi and Alizadeh, 2017). It has high antioxidant activity and interacts with common target sites of lipid peroxidation in cell membranes to scavenge the ROS. It easily penetrates through cellular membranes and effectively protects cellular components such as DNA and proteins from oxidative disruption (Prasath *et al.,* 2013; Naeimi and Alizadeh, 2017). Fisetin inhibits the adverse effects of ROS on cellular activities and intracellular oxidative processes. Fisetin as a flavonoid, scavenges for ROS, which is an important role in the antioxidant capacity of the compound (Panche *et al.,* 2016). Generally, flavonoids, including fisetin, transfer an electron from the hydroxyl radical on their nucleus and produce fewer reactive radicals in the free-radical scavenging process. It has been shown that fisetin has scavenging activities even higher than ascorbic acid, making it a good and potent antioxidant agent which aids in therapeutic or preventive effect of oxidative stress-associated diseases (Shon *et al.,* 2016).

Fisetin also modulates ROS production, thereby affecting the activities of oxidant enzymes, such as xanthine oxidase and NADPH oxidase (Piao *et al*., 2013). Fisetin has potential effects on the levels of antioxidants, reversing the depleted levels of different enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic antioxidants, including vitamins C and E to near normal levels (Naeimi and Alizadeh, 2017). Fisetin has the capability to chelate transition metal cations, thereby protecting cells against the potential toxicity of ROS, generated via the catalytic action of metal ions (Kasprzak, *et al*., 2015). Fisetin has the ability of protecting erythrocytes from extracellular oxidative agents by donating electron to plasma membrane oxidoreductase, maintaining the state of plasma (Tedesco *et al*., 2015). The therapeutic benefits of fisetin are demonstrated through its interactions with diverse redox signaling pathways, including the regulation of many cytoprotective, antioxidant enzymes and multi-drug resistance-associated proteins (Zhang *et al.,* 2017b).

2.8 Probiotic as Antioxidant and its Role in Stress

The term "probiotic" is defined as live microorganisms which, when consumed in appropriate amounts, confers a health benefit on the host through improvements of the intestinal microbial balance (FAO, 2014). A variety of microbial species have been used as probiotics, including species of *Bacillus*, *Bifidobacterium*, *Enterococcus*, *E. coli*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, a variety of yeast species, and undefined mixed cultures. *Lactobacillus* and *Bifidobacterium* species have been used most extensively in humans, whereas species of *Bacillus*, *Enterococcus*, and *Saccharomyces* yeast have been the most common organisms used in livestock (Wang *et al*., 2017)**.** Broiler chickens are exposed to natural or induced stressors, which induce ROS generation and cause morphological and/or physiological malfunctions (Sugiharto *et al.*, 2016). Probiotics have been reported to improve the antioxidant status in broiler chickens by decreasing the production of ROS (Wang *et al*., 2017). Probiotic supplementation modulates the dynamics of oxidants and antioxidants in the body, inhibiting lipid peroxidation (Abudabos *et al*., 2016). Probiotic supplementation has been confirmed in numerous scientific investigations to improve physiological conditions, intestinal morphology and structure, immune system and, thus, performance and well-being of heat-stressed poultry (Al-Fataftah and Abdelqader, 2014; Jahromi *et al*., 2015). It modulates the composition of the gut microflora by eliminating the pathogenic bacteria (Pandey *et al*., 2014) and reducing intestinal oxidative injuries (Porto *et al*., 2015).

Probiotic supplementation enhances resistance to diseases and improve the immune responses by reducing circulating level of corticosterone, leading to lowered heterophil to lymphocyte (H/L) ratio (Beski and AlSardary, 2015). Yang *et al.* (2015) suggested that corticosterone possesses immunosuppressive effects in poultry; therefore, the reduced corticosterone level may be beneficial for restoring the normal function and development of immune system of broiler chickens. Probiotic supplementation has been shown to increase performance, body

weight and enhance serum antioxidant enzyme activities of broiler chickens (Aluwong *et al.*, 2013; Wang *et al*., 2018).

2.9 Effects of Probiotic on Gastrointestinal Tract

The primary function of the gastrointestinal tract is to digest, absorb nutrients and also serves as a vital barrier preventing the entry of potentially harmful pathogens. A well-balanced gastrointestinal microbiota is crucial for optimal broiler chicken health and performance (Gao *et al*., 2017). Probiotics help maintain a healthy balance of microorganisms within the intestine, which is accomplished through multiple modes of action (Aluwong *et al*., 2013). Although the exact manner in which they exert their effects is still not fully elucidated. These ranges from bacteriocin and short chain fatty acid production, lowering of gut pH, and nutrient competition to stimulation of mucosal barrier function and immunomodulation (Sarangi *et al*., 2016). Probiotics influence several aspects of the acquired and innate immune response by inducing phagocytosis and IgA secretion, modifying T-cell responses, enhancing Th1 responses, and attenuating Th2 responses (Manafi *et al*., 2018). The presence of probiotics reduces colonization of the gastrointestinal tract by pathogenic bacteria and attenuates enteric diseases, leading to improved intestinal integrity and morphology which ultimately result in enhanced performance of broiler chickens (Zhang *et al*., 2017a).

2.10 Pathophysiological Mechanism of Heat Stress in Blood of Broiler Chickens

The first step in the pathophysiology of heat stress appears to be an increase in cellular energy demand (Lu *et al*., 2017). The enhanced energy demand of cells leads to increase in production of ROS which are important triggers and modulators of cell signalling and, consequently, cell behaviour (Akbarian *et al*., 2016). The blood system is particularly sensitive to changes in temperature and an important indicator of physiological responses of broilers to stressors (Tawfeek *et al*., 2014). Stress induces changes in blood cells, causing decrease in percentages

of leucocytes and leucocyte differentiation (Ravikumar *et al*., 2016) erythrocyte count and haemoglobin in the erythrocytes (Beski and Al-Sardary, 2015). Under stressful situations with release of adrenocorticotropic hormone, there is a reduction in the number of circulating lymphocytes, contributing to the increase of the heterophil/lymphocyte ratio (Habibian *et al*., 2014). It has been well documented that changes in haematological values of the heterophil/lymphocyte ratio (H/L) is a good indicator of stress in broilers (Cengiz *et al*., 2015; Scanes, 2016).

Blood pictures vary based on the causative agent of stress; severe food restriction heteropenia, lymphocytosis and significant basophilia; climatic and environmental stressors significantly raise heterophil/lymphocyte ratio and induce basophilia, respectively; Psychological stressors such as fasting and noise show different leucocyte responses (Maxwell, 1993; Tamzil *et al*., 2014). The release of corticosterone in stressful conditions may cause involution of lymphoid tissue (thymus, spleen and cloacal bursa) and the suppression of humoral and the cell-mediated immunity (Sugiharto *et al.*, 2016). Consequently, broiler chickens become more susceptible to acquiring secondary illnesses, a consequence of heat stress. Heat stress also promotes a decrease in phagocytic activity of macrophages, which negatively affects immune response of broiler chickens (Gomes *et al*., 2014; Youseff *et al.*, 2016).

2.11 Pathophysiological Mechanism of Stress in Serum of Broiler Chickens

Stress causes an increase in the serum concentrations of glucose, triglycerides, total cholesterol, and low-density lipoprotein-cholesterol but a decrease in serum high density lipoprotein-cholesterol (Habiban *et al*., 2014). It also causes increase in activities of aspartate aminotransferase, alanine aminotransferase and creatine phosphokinase in birds exposed to stress (Hosseini-Vashan *et al.,* 2016). The increase in the activities of these enzymes in the plasma or serum has been suggested to result from damage of various tissues or cause disease

conditions (Bejaei and Cheng, 2014). It has been reported that heat stress decreases plasma protein, vitamins and minerals present in the serum (Sahin *et al.,* 2001; Mosleh *et al*., 2018). In response to stress, the level of various hormones changes. Stress enhances secretion of a number of hormones, including glucocorticoids, growth hormone and prolactin; the effect of which is to increase mobilisation of energy sources and adaptation by the individual to its new circumstance (Kataria *et al.,* 2008; Scanes *et al*., 2016). Stress modulates the concentrations of biochemical parameters. It causes increase in MDA concentration, which shows increase in lipid peroxidation, and decrease in oxidative stress biomarkers (catalase, superoxide dismutase and glutathione peroxidase) in serum (Zheng *et al.,* 2016; Wan *et al*., 2017).

2.12 Pathophysiological Mechanism of Heat Stress in Intestine of Broiler Chickens

Broiler chicken production under stressful conditions undergoes neuroendocrine, physiological and behavioural responses that enable them to acclimatize to the conditions to maintain homeostasis (Marchini *et al*., 2016). The strain generated by heat stress may trigger changes in intestinal development by reducing the intestine weight, the number of villi, and the proliferation rate of enterocytes (Akbarian *et al.,* 2016). One of the main features that make broiler chickens so productive is the great capacity of its intestinal epithelium to absorb nutrients (Pan and Yu, 2014). Damage to intestinal morphology is one of the first consequences of stress, and it depends on the stressor intensity and duration. Acute stress reduces enterocyte proliferation in chicks and reduces ileum crypt depth, without changing villus height or the villus/crypt ratio (Tang *et al*., 2013; Souza *et al*., 2018). It directly affects the bird's capacity to digest and absorb the nutrients, required for maintenance and production (Zhang *et al.*, 2017a). The strain caused by heat stress may reduce growth rate, food intake and weight gain, as well as feed conversion ratio (Osti *et al*., 2017). Thus, performance of birds subjected to heat stress is often compromised by deficient intestinal development and diminished food intake.

The resultant reduction of feed intake as a consequence of heat stress which is one of the factors that may change intestinal morphology; for instance, by reducing villus height and crypt depth; and by reducing enterocyte proliferation in the small intestine. The factors would directly affect the capacity to digest and absorb nutrients, necessary for maintenance and production (Abdelqader and Al-fataftah, 2016). Crypt depth reduction along with increased mucus secretion may reduce absorption capacity of the intestine (Marchini *et al*., 2016). Impaired digestion and absorption may be cause by stress, which leads to poor growth and development in broiler chickens (Hu *et al*., 2017).

2.13 Probiotics and Fisetin as Alternatives to Antibiotics

In poultry industry, antibiotics have been widely used to improve growth, performance, production and to protect birds from the harmful effects of pathogenic enteric microorganism (Mehdi *et al*., 2018). Antibiotic administration in broiler chicken production has resulted to problem of resistance of pathogens to antibiotics, drug residues in poultry products, imbalance of normal microflora, and reduction in beneficial intestinal microflora (Li *et al*., 2017; Muaz *et al*., 2018). Due to problems associated with the use of antibiotics, new alternative additives are now being encouraged to avoid such problems. Probiotic as an additive improves the body antioxidant levels (Bai *et al.,* 2016), stabilises microbial communities, and promotes health, which offer a better solution on broiler health and broiler meat safety (Song *et al.,* 2014). Shokryazdan *et al*. (2017) showed that probiotics improve body weight, reduce total cholesterol, increase population of beneficial bacterial, decrease harmful bacteria and improve intestinal morphology of broilers.

Fisetin is a flavonoid, commonly found in most plants. It is used as an additive, and has been known to improve the health status of animals (Khan *et al.,* 2013). Fisetin acts in various biological activities as antioxidant, preventing damaging effects of ROS and products of their

metabolism (Surai *et al*., 2014), anticarcinogenic, anti-inflammatory (Kunchari Kalaimathi and Sudhandiran, 2016), antibacterial and antiviral agent (Mansuri, *et al*., 2014). Flavonoids have been shown to improve body weight, decrease the total cholesterol and low-density lipoprotein cholesterol level in broiler chickens, which tend to provide healthy meat for human consumption (Ouyang *et al.,* 2016).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental Site and Thermal Environmental Conditions

The experiment was conducted at the Department of Veterinary Physiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria (11° 10/ N, 07° 38/ E), located in the Northern Guinea Savannah zone of Nigeria. It was carried out from June to July, 2017 during the early rainy season (Dzenda *et al.*, 2013).

3.2 Ethical Approval

Ethical approval for this work was sought and obtained from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC), with Reference Number: ABUCAUC/2018/020.

3.3 Experimental Birds and Management

A total of 60 day-old, apparently, healthy broilers (Arbor Acres), comprising both sexes, served as the experimental subjects. They were kept under a deep litter system. The poultry pen was made of concrete floor that was littered with wood shavings, and zinc roof, having cardboard ceiling. The dimension of the pen was 8.4 m x 5.6 m x 1.91 m, and the broiler chickens were stocked at 15 birds/m^2 (Muniz *et al*., 2006). The pen was partitioned into four parts to house each group.

The broiler chickens were given access to water and feed *ad libitum*. They were fed with broiler starter (day 0-28) and broiler finisher (day 29-42). The proximate analysis of the feed was carried out as shown in Table 4.1. The birds were randomly divided into the following four groups of 15 birds each: Control (I), fisetin (II), probiotic (*Saccharomyces cerevisiae*) (III),

and combination of probiotic (*Saccharomyces cerevisiae*) and fisetin groups (IV). Probiotic (*Saccharomyces cerevisiae*) (Montajat Pharmaceuticals, Biosciences Division, Dammam 31491, Saudi Arabia) at 4.125 x 10^6 cfu/100 mL and fisetin (Sigma-Aldrich, St. Louis, MO, USA) at 5 mg/kg were administered for 7 days, consecutively, and respectively by the oral route, from one-day old. Each bird was tagged using a masking tape on the leg for identification and proper recordings. Biosecurity measures were ensured by providing foot bath, foot wears and clothing for everyone who assisted in the experiment. The pen was not accessible to non-essential persons, animals, other birds or rodents.

3.4 Meteorological Data from the Study Area

The dry-bulb temperature (DBT) and wet-bulb temperature (WBT) were recorded thrice per day at 07:00 h, 13:00 h and 18:00 h, at the experimental site inside the poultry pen using a wet- and dry-bulb thermometer (Brannan®, Cumbria, England) which was kept 1 m above the ground. The RH was calculated using Osmon's hygrometric table (Narinda Scientific Industries®, Haryana, India). The THI was determined using the following formula (Tao and Xin, 2003):

THI = 0.85 (Tdb) + 0.15Twb (for broilers)

Where THI = temperature-humidity index for broilers, Tdb = dry-bulb temperature and Twb = wet-bulb temperature.

The parameters were recorded inside the poultry house on each day of the experiment.

3.5 Collection of Blood Samples

Blood sample (3 mL) was collected at 8:00 h from the wing vein of each broiler chicken at six weeks of age, 2 mL was dispensed into plain tubes while 1 mL was dispensed into a tube with anticoagulant, sodium ethylenediaminetetraacetate. The blood samples without anticoagulant

were allowed to clot and then centrifuged at 3000 *g* for 10 minutes and serum was harvested and stored at 4°C until assayed (Ramnath *et al.,* 2008). The blood was transferred to the Physiology Research Laboratory, Department of Veterinary Physiology and Clinical Pathology Research Laboratory, Department of Veterinary Pathology, Ahmadu Bello University, Zaria for analyses. The serum was used for determination of biochemical parameters while the anticoagulated blood was used for determination of haematological parameters

3.6 Determination of Haematological Parameters

3.6.1 Packed cell volume determination

The packed cell volume (PCV) was determined using standard technique as described by Rehman *et al.* (2003). Briefly, a non-heparinised capillary tube was filled up to 3/4 of its length from one end and the second end was heat-sealed using Bunsen burner. The blood in the sealed capillary tube was centrifuged for 5 minutes at 4,383 x g using the Saitexiangyi TG12MX® Micro-haematocrit centrifuge machine (Shanghai, China). Thereafter, the proportion of cells in the total volume of blood was measured and recorded as a percentage using the Hawksley® Micro-haematocrit Reader (Sussex, UK).

3.6.2 Red blood cell and total leucocyte counts

Red blood cell (RBC) and total white blood cell (TWBC) counts were determined using Natt-Herrick solution (1:200 dilution) and the improved Neubauer haemocytometer. Both counts were prepared directly from the same sample placed in the haemocytometer (Campbell and Ellis, 2007).

The blood samples with sodium ethylenediaminetetraacetate were slightly agitated and the RBC diluting pipette was used to pipette the blood to the 0.5 marking. The tip of the pipette was cleaned properly using tissue paper, same pipette was also used to pipette the diluting

solution (Natt-Herrick) to the 101 mark (1:200) without entirely immersing the pipette tip into the diluting fluid. The mixture was shaken for a minute to obtain equal distribution then empty into a clean sample bottle. The mixture was agitated a little and a capillary tube was used to withdraw a small aliquot. Both sides of the haemocytometer were filled up and left for 5 minutes for cells to settle down.

The light microscope (Olympus-XSZ-107BN), at low power magnification (x 40), was used to view the cells, and counting was done using tally counter. For TWBC count, the four outer large squares of the haemocytometer were counted and calculated using the formula:

$N/20 = WBC \times 10^9/L$, where N = number of WBC counted in the four large squares (or in 64 small squares)

For RBC count, the cells in the four corner and central squares in the mid-section of the haemocytometer were counted. The RBC count was calculated using the formula:

$N/100 = RBC \times 10^{12}/L$, where N = number of RBC counted in the 5 square in the mid-section of the haemocytometer (or in 160 square).

Both charged sides of the haemocytometer were counted for both the RBC and TWBC and the average was calculated.

3.6.3 Differential leucocyte count

The count was determined using the method of Campbell and Ellis (2007). Briefly, a pair of smear was made from each blood samples. A small drop (2 µL) of blood was immediately used for the preparation of blood smear each using the standard slide-to-slide technique. The air-dried smears were properly labelled using a pencil on the frosted end of the slide and then fixed in a fixing jar containing methanol for 3 minutes and air-dried. Staining was done by flooding the smear with Wright-Giemsa stain for 3 minutes. An equal amount of Sorensen's buffer (pH

6.8) was added then mixed gently by blowing using a pipette until green metallic sheen forms on the surface. It was allowed to stand for further 6 minutes. The smears were rinsed with the Sorensen's buffer and the back of the smear was wiped with a tissue paper to remove the excess stain and allowed to air dry. It was neatly packed into a box until viewing.

Examination of the blood smears was done using a light microscope (Olympus-XSZ-107BN) under high-power magnification with oil immersion (x 1000). A total of 100 TWBC was counted and classified based on their morphologic features (Campbell and Ellis, 2007). The counting was carried out using the Marble® Blood Cell Calculator. The differential WBC count was expressed as a percentage of the individual cell group. The percentage of each cell was converted into absolute numbers by reference to the TWBC using the formula below:

$$\text{Percentage of WBC counted} \times \text{TWBC}/100 = \text{Absolute Number} \times 10^9/\text{L}$$

3.6.4 Haemoglobin concentration

Blood haemoglobin concentration was assayed colorimetrically as cyanomethaemoglobin using the Drabkin method, described by Kwiecien *et al*. (2015). Briefly, 5 mL of Drabkin solution (consisting of potassium cyanide and potassium ferricyanide) was measured using a 5 mL syringe into plastic test tubes. Twenty microlitres (20 µL) of blood was measured using a micropipette and added to the Drabkin solution in the test tube and properly mixed by gently shaking the test tube. It was centrifuged at 1,509 x g for 15 minutes to separate the empty RBC from interfering with the reading. The supernatant was separated into a sample bottle. The mixture was absorbed into the haemoglobin meter (XF-1C, Perlong Medical Equipment Co., Ltd., Nanjing, China). After the wiggling pump had stopped, the value displayed on the screen was recorded in g/dL as the haemoglobin concentration.

3.6.5 Erythrocytic osmotic fragility test

The erythrocyte osmotic fragility (EOF) test was performed as described by Oyewale (1992). Briefly, 0.02 mL of blood was added to six tubes, containing increasing concentrations (0%, 0.1%, 0.3%, 0.5%, 0.7% and 0.9%) of phosphate-buffered sodium chloride (NaCl) solution at pH 7.4. The tubes were gently mixed and incubated at room temperature (26 ± 2 °C) for 30 minutes. The content of each tube was centrifuged at 150 x *g* for 10 minutes and the supernatant decanted. Optical density (OD) of the supernatant was determined using spectrophotometer (23A, Perlong Medical Equipment Co., Ltd., Nanjing, China) at wavelength of 540 nm. Haemolysis in each tube was expressed as a percentage, taking haemolysis in distilled water (0% NaCl) as 100%:

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

3.6.6 Erythrocytic indices

The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) were calculated using the following standard formulae (Campbell and Ellis, 2007):

MCV = (PCV x 10)/RBC = MCV (fl)

MCH = (Hb x 10)/RBC = MCH (pg)

MCHC = (Hb x 100)/PCV = MCHC (g/L).

3.7 Determination of Biomarkers of Oxidative Stress

3.7.1 Evaluation of malondialdehyde concentration

Serum lipid peroxidation was evaluated as described by Janero (1990). Briefly, serum sample (100 µL) was mixed with sodium dodecyl sulphate-acetate buffer (pH 3.5) and aqueous

solution of thiobarbituric acid. It was heated at 95 °C for 60 minutes, the red pigment produced was extracted with n-butanol-pyridine mixture and estimated by measuring spectrophotmetrically the absorbance at 532 nm. Tetramethoxy-propane was used as an external standard, and lipid peroxidation level was expressed in nmol/L.

3.7.2 Superoxide dismutase assay

The activity of SOD was measured using the Northwest Life Science Specialties SOD kit (NWLSS™ NWK-SOD02) based on the method of monitoring the auto-oxidation rate of haematoxylin originally, described by Martin (Jr) *et al*. (1987), and modified to enhance reliability. In the presence of SOD enzyme at specific assay pH, the rate of auto-oxidation was inhibited and the percentage of inhibition was linearly proportional to the amount of SOD present within a specific range. Sample SOD activity was determined by measuring ratios of auto-oxidation rates in the presence and absence of the sample and expressed as McCord-Fridovich "cytochrome c" units.

3.8 Evaluation of Small Intestinal Morphometry

Three broiler chickens were randomly selected from each group at age of six weeks and sacrificed by exsanguination. Small intestine tissues were dissected at mid-point, flushed with cold sterile saline solution and opened longitudinally. They were harvested and fixed in 10% formol saline. Fixed tissues were histologically processed according to the method of Bancroft and Stevens (2008). Tissues were dehydrated using ascending grades of alcohol (70%, 90%, and 100%) for 2 hours each. The tissues were then cleared in xylene for 2 hours, later impregnated and embedded in paraffin wax. In addition, tissues were sectioned at 5 µm thickness using Rotary Microtome Machine (Leica RT 25, Newcastle, UK). Furthermore, sectioned tissues were mounted on slides, dried and stained using haematoxylin and eosin stain. Tissues stained slides were photomicrographed using Amscope Digital Camera (Tokyo, Japan)

for microscope version 2.0. Histomorphometric analysis was carried out using Digimizer image analysis software, version 4.5 (Medcalc Software, Oostende, Belgium) for measurement of villus height and width, crypt height and width. Villus height/crypt ratio was calculated. Villus height was measured from the tip of the villus to the villus crypt junction, and the crypt depth was measured from its base up to the region of transition between the crypt and villus. Surface area of villus and crypt were calculated using the following formula (Solis de los Santos *et al*., 2005):

$(2\pi) \times (VW/2) \times (VH)$, Where VW = villus width and VH = villus height

$(2\pi) \times (CW/2) \times (CH)$, Where CW = crypt width and CW = crypt height

3.9 Data Analyses

Data obtained were expressed as mean ± standard error of the mean (mean ± SEM) and analysed using one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test. The analyses were done using GraphPad 4.0 for Windows (San Diego, California, USA). Values of $P < 0.05$ were considered significant (Snedecor and Cochran, 1994).

CHAPTER FOUR

RESULTS

Table 4.1: Composition and Proximate Analysis of Broiler Chicken Diet

Feed Composition	Starter	Finisher
Ingredients (%)		
Crude protein	22.00	19.50
Fat	5.10	3.80
Crude fibre	4.30	3.00
Calcium	1.20	1.20
Available phosphorus	0.45	0.44
Methionine	0.56	0.50
Lysine	1.30	1.20
Metabolisable energy (kCal/kg)	3000.00	3100.00
Proximate analysis		
Crude protein (%)	22.00	21.00
Fat (%)	7.90	6.80
Crude fibre (%)	4.30	3.00
Calcium (%)	2.00	2.00
Available phosphorus (%)	0.80	0.70
Methionine (%)	0.56	0.50
Lysine (%)	1.20	1.20
Metabolisable energy (kCal/kg)	2900.00	2980.00

4.1 Thermal Environmental Parameters in the Poultry Pen

During the period of study, the DBT in the pen ranged from 25.00 - 36.00 °C, while RH fluctuated from 47.00 - 100.00%. The THI ranged from 25.00 - 35.55. The overall mean DBT value recorded during the study period was 31.11 ± 0.28 °C, while those of the RH and THI were 79.32 ± 0.97% and 30.65 ± 0.26, respectively. The lowest mean value of DBT (27.68 ± 0.20 °C) was recorded at 07:00 h, and rose to 33.15 ± 0.34 °C with the hour of day to attain its peak at 13:00 h; thereafter, it decreased to 32.50 ± 0.32 °C at 18:00 h. The lowest mean value of RH (72.74 ± 1.18%) was obtained at 13:00 h, while that of the THI (27.45 ± 0.19) was recorded at 07:00 h. The highest mean values of RH (87.98 ± 1.66%) and THI (32.54 ± 0.34) were recorded at 07:00 h and 13:00 h, respectively (Table 4.2).

4.2 Erythrocyte Osmotic Fragility in Broiler Chickens during the Study Period

During the study period, at 0.5% NaCl, the mean EOF was significantly ($P < 0.05$) lower in the fisetin, probiotic and fisetin + probiotic group (34.26 ± 0.98%, 35.65 ± 0.81% and 34.25 ± 1.98%, respectively), than that of the control group (48.42 ± 0.40%). At 0.3% NaCl, the mean EOF in the groups treated with fisetin (57.94 ± 2.72%), probiotic (59.29 ± 1.55%) and fisetin + probiotic (58.91 ± 2.82%) was significantly ($P < 0.001$) lower than that of the control group (78.33 ± 2.29%) (Figure 4.1).

Table 4.2: Mean (± SEM) Thermal Environmental Parameters during the Study Period

Hour (h)	DBT (°C)	RH (%)	THI
7:00	27.68 ± 0.20[a] (25.00 - 30.00)	87.98 ± 1.66[a] (65.00 - 100.00)	27.45 ± 0.19[a] (25.00 - 29.70)
13:00	33.15 ± 0.34[b] (28.00 - 36.00)	72.74 ± 1.18[b] (47.00 - 93.00)	32.54 ± 0.34[b] (26.80 - 35.55)
18:00	32.50 ± 0.32[c] (28.00 - 36.00)	77.24 ± 1.20[c] (59.00 - 93.00)	31.96 ± 0.30[c] (27.55 - 35.10)
Overall mean ± SEM	31.11 ± 0.28 (25.00 - 36.00)	79.32 ± 0.97 (47.00 - 100.00)	30.65 ± 0.26 (25.00 - 35.55)

Values in parenthesis are minimum and maximum

[a, b, c] = Values in the same row with different superscript letters are significantly ($P < 0.05$) different. DBT, dry-bulb temperature; RH, relative humidity; THI, temperature-humidity index

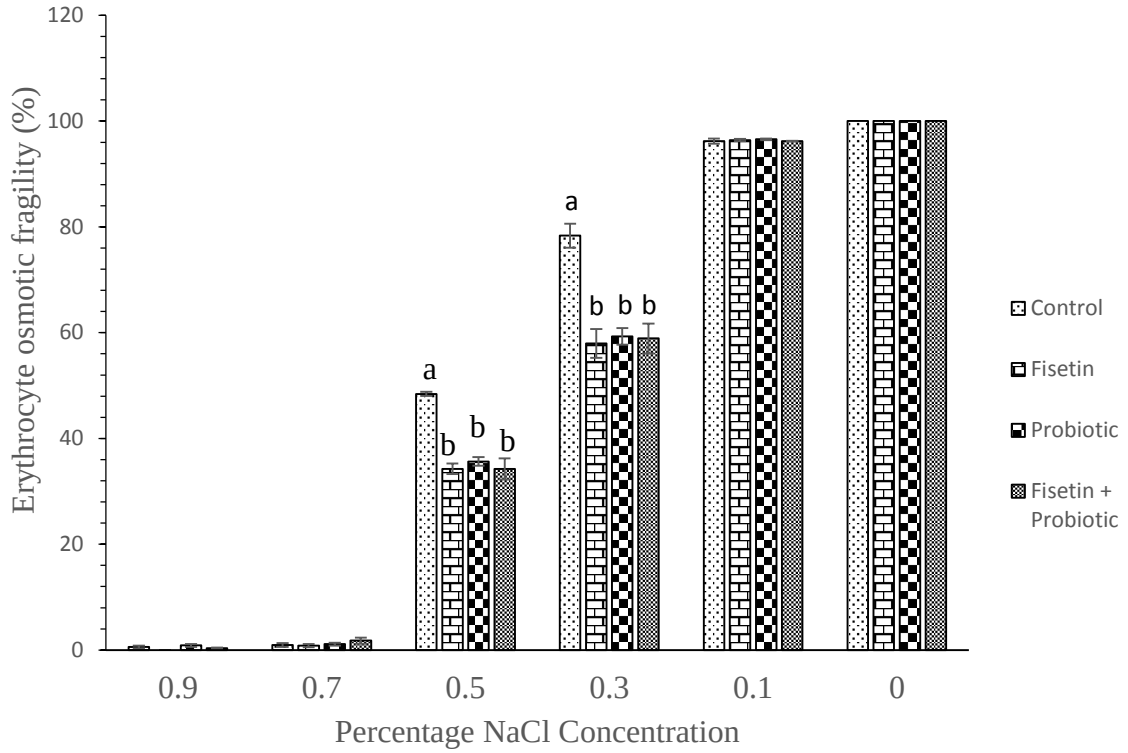


Figure 4.1: Mean (± SEM) Erythrocyte Osmotic Fragility Profiles of Fisetin-, Probiotic-, Fisetin + Probiotic-treated and Control Broiler Chickens during the Early Rainy Season

^{a,b} = Values with different superscript letters are significantly ($P < 0.05$) different, n = 7

4.3 Malondialdehyde Concentration in Broiler Chickens during the Study Period

The mean MDA concentration in broiler chickens administered with fisetin, probiotic and fisetin + probiotic (14.37 ± 1.15 nmol/L, 5.66 ± 1.06 nmol/L, 4.136 ± 0.58 nmol/L, respectively) were significantly ($P < 0.05$) lower than the concentration in the control group (22.64 ± 2.95 nmol/L). The mean MDA concentration in the group administered with fisetin + probiotic was significantly ($P < 0.05$) lower than that of the control or fisetin-treated group. The mean concentration of MDA in probiotic and fisetin + probiotic-treated groups did not differ significantly (5.66 ± 1.06 nmol/L and 4.136 ± 0.58 nmol/L, respectively) (Figure 4.2).

4.4 Superoxide Dismutase Activity in Broiler Chickens during the Study Period

The mean activities of SOD in the fisetin, probiotic and fisetin + probiotic groups (6.34 ± 0.24 IU/L, 5.67 ± 0.09 IU/L and 5.93 ± 0.13 IU/L, respectively) were significantly ($P < 0.05$) higher than that of the control group (5.37 ± 0.09 IU/L). The mean activity of SOD in fisetin treated group (6.34 ± 0.24 IU/L) was significantly ($P < 0.05$) higher than that of controls (5.37 ± 0.09 IU/L). The mean SOD activities in probiotic and fisetin + probiotic-treated groups (5.67 ± 0.09 IU/L and 5.93 ± 0.13 IU/L, respectively) did not differ significantly ($P > 0.05$) (Figure 4.3).

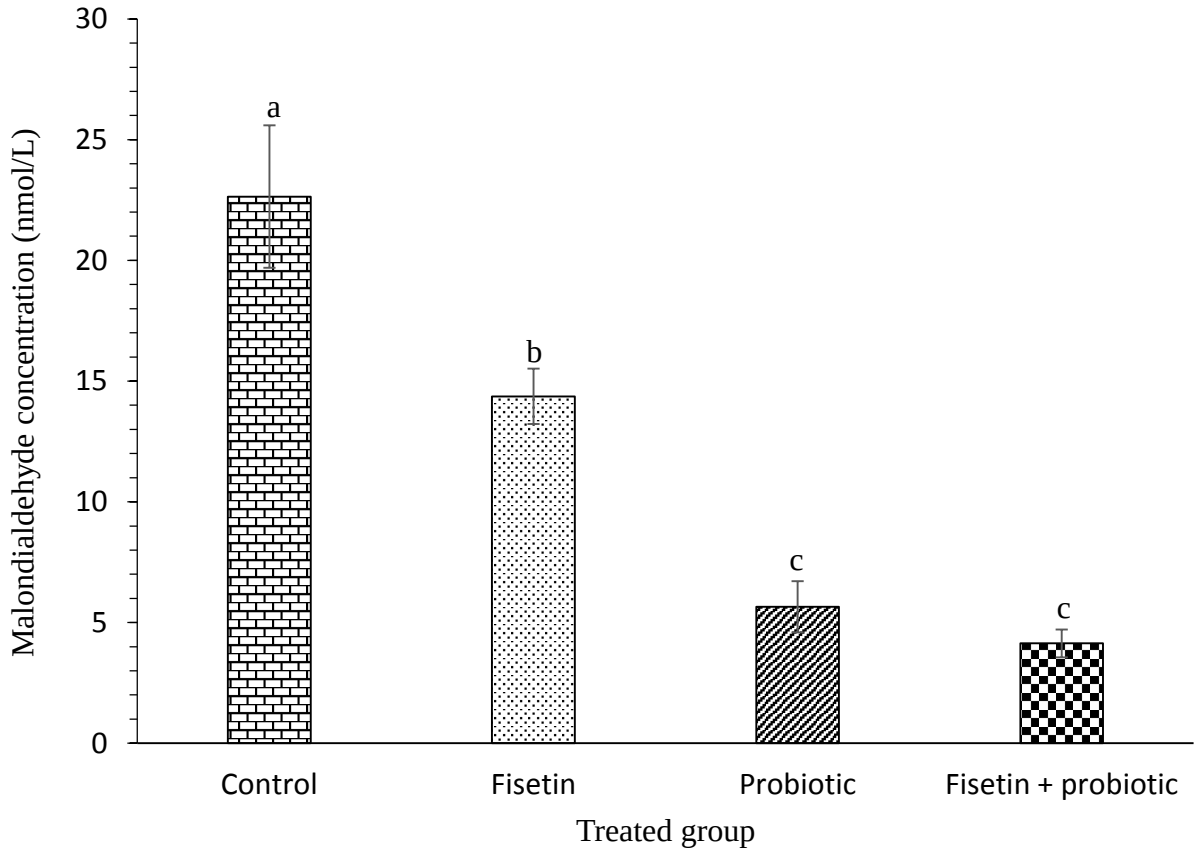


Figure 4.2: Mean (± SEM) Malondialdehyde Concentration in Fisetin-, Probiotic-Fisetin + Probiotic-treated and Control Broiler Chickens during the Early Rainy Season

^{a,b,c} = Values with different superscript letters are significantly ($P < 0.05$) different, n = 7

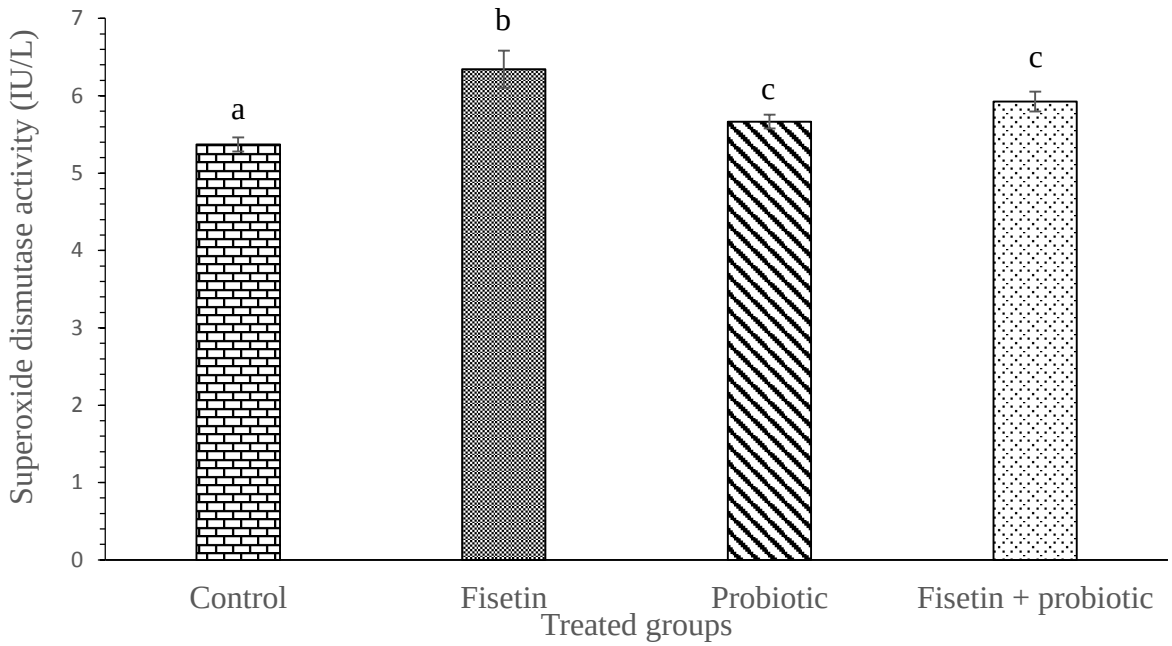


Figure 4.3: Mean (± SEM) Superoxide Dismutase Activity in Fisetin-, Probiotic-, Fisetin + Probiotic-treated and Control Broiler Chickens during the Early Rainy Season

^{a,b,c} = Values with different superscript letters are significantly ($P < 0.05$) different, n =7

4.5 Haematological Parameters of Broiler Chickens during the Study Period

4.5.1 Erythrocytic parameters

The mean PCV values in the groups administered with fisetin, probiotic and fisetin + probiotic (32.00 ± 2.33%, 27.14 ± 1.49%, 29.00 ± 1.45%, respectively) were significantly ($P < 0.05$) higher than in the control group (24.86 ± 1.30%). The mean PCV value in fisetin group was significantly ($P < 0.05$) higher, when compared with other treated groups. The mean RBC counts in control (1.60 ± 0.04 x 10^6/µL), fisetin (1.62 ± 0.08 x 10^6/µL), probiotic (2.00 ± 0.06 x 10^6/µL) and fisetin + probiotic (2.00 ± 0.10 x 10^6/µL) groups did not differ significantly ($P > 0.05$). The mean Hb concentrations in fisetin (10.66 ± 0.78 g/dL), probiotic (9.05 ± 0.49 g/dL) and fisetin + probiotic (9.67 ± 0.48 g/dL) groups were higher ($P < 0.05$) than that of the control group (8.29 ± 0.43 g/dL). There was no significant difference among values in the treated groups.

The mean TP concentrations in the groups treated with fisetin (286.60 ± 18.10), probiotic (294.60 ± 10.32 mg/dL) and fisetin + probiotic (296.40 ± 7.86 mg/dL) were significantly ($P < 0.05$) higher than that in the control group (271.20 ± 20.57 mg/dL) (Table 4.3).

The mean MCV values in probiotic (136.7 ± 9.58 fl) and fisetin + probiotic (145.9 ± 7.62 fl) groups were lower ($P < 0.05$) than that of the control group (155.50 ± 8.48 fl). The mean MCV was higher ($P < 0.05$) in fisetin group (197.54 ± 12.58 fl) than in the control group (155.50 ± 8.48 fl). The mean MCH in probiotic (45.58 ± 3.19 ρg) and fisetin + probiotic (48.65 ± 2.54 ρg) groups were lower ($P < 0.05$) than that of the control group (51.85 ± 2.82 ρg). The mean MCH in fisetin group (67.73 ± 7.52 ρg) was higher ($P < 0.05$) than that of the control group. The mean MCHC in control and treated groups did not differ significantly ($P > 0.05$) (Table 4.3).

Table 4.3: Mean (± SEM) Erythrocyte Parameters in Fisetin-, Probiotic-, Fisetin + Probiotic-treated and Control Broiler Chickens during the Early Rainy Season

Parameters	Control	Fisetin	Probiotic	Fisetin + Probiotic
PCV (%)	24.86 ± 1.30[a] (20.00 - 30.00)	32.00 ± 2.33[b] (24.00 - 43.00)	27.14 ± 1.49[c] (23.00 - 34.00)	29.00 ± 1.45[d] (22.00 - 33.00)
RBC (x 10^{12}/L)	1.60 ± 0.04 (1.52 - 1.83)	1.62 ± 0.08 (1.30 - 1.94)	2.00 ± 0.06 (1.85 - 2.27)	2.00 ± 0.10 (1.62 - 2.34)
Hb (g/dL)	8.29 ± 0.43[a] (6.67 - 10.00)	10.66 ± 0.78[b] (8.00 - 14.33)	9.05 ± 0.49[b] (7.67 - 11.33)	9.67 ± 0.48[b] (7.33 - 11.00)
TP (mg/dL)	271.20 ± 18.57[a] (202.8 - 327.30)	286.60 ± 20.10[b] (233.00 - 353.50)	294.60 ± 10.32[c] (253.70 - 326.10)	296.40 ± 7.86[c] (268.60 - 325.80)
MCV (fl)	155.50 ± 8.48[a] (125.70 - 178.60)	197.54 ± 12.58[b] (149.50 - 292.50)	136.7 ± 9.58[c] (101.30 - 174.40)	145.9 ± 7.62[c] (117.10 - 178.80)
MCH (ρg)	51.85 ± 2.82[a] (41.91 - 59.52)	67.73 ± 7.52[b] (49.79 - 97.48)	45.58 ± 3.19[c] (33.79 - 58.10)	48.65 ± 2.54[c] (39.05 - 59.61)
MCHC (g/dl)	33.33 ± 0.01 (33.32 - 33.35)	33.33 ± 0.003 (33.31 - 33.33)	33.34 ± 0.01 (33.32 - 33.35)	33.33 ± 0.004 (33.32 - 33.35)

Values in parenthesis are minimum and maximum.
[a, b, c] = Values in the same row with different superscript letters are significantly ($P < 0.05$) different. PCV, packed cell volume; RBC, red blood cell count; Hb, haemoglobin concentration; TP, total protein; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration, n = 7

4.5.2 Total and differential leucocytic counts

The mean total leucocyte count in control (1.90 ± 0.35 x 10^3/µL), fisetin (1.84 ± 0.24 x 10^3/µL), probiotic (2.02 ± 0.08 x 10^3/µL) and fisetin + probiotic (1.64 ± 0.12 x 10^3/µL) groups did not differ significantly (P > 0.05). The mean eosinophil and basophils counts in control and treated groups did not significantly differ (P > 0.05). The mean monocyte count in control (0.25 ± 0.01 x 10^3/µL) was significantly (P < 0.05) higher than that in the treated groups fisetin (0.14 ± 0.03 x 10^3/µL), probiotic (0.16 ± 0.04 x 10^3/µL) and fisetin + probiotic (0.17 ± 0.03 x 10^3/µL). The mean lymphocyte counts in fisetin (1.81 ± 0.17 x 10^3/µL), probiotic (1.72 ± 0.14 x 10^3/µL) and fisetin + probiotic (1.72 ± 0.18 x 10^3/µL) groups were significantly (P < 0.05) higher than that of the control group (1.52 ± 0.19 x 10^3/µL). The mean heterophil counts in fisetin (0.17 ± 0.05 x 10^3/µL), probiotic (0.13 ± 0.05 x 10^3/µL) and fisetin + probiotic (0.18 ± 0.04 x 10^3/µL) groups were significantly (P < 0.05) lower than that of the control group (0.72 ± 0.10 x 10^3/µL). The mean heterophil counts in the treated groups, fisetin did not differ significantly (P > 0.05) from one another (Table 4.4).

The mean heterophil/lymphocyte ratio in fisetin (0.10 ± 0.04), probiotic (0.09 ± 0.04) and fisetin + probiotic (0.11 ± 0.03) groups were significantly (P < 0.05) lower, when compared to control group (0.54 ± 0.12). The mean heterophil/lymphocyte ratio in groups, fisetin (0.10 ± 0.04), probiotic (0.09 ± 0.04) and fisetin + probiotic (0.11 ± 0.03) groups, did not differ significantly (P > 0.05) (Table 4.4).

Table 4.4: Mean (± SEM) Total and Differential Leucocyte Counts in Fisetin-, Probiotic-Fisetin + Probiotic-treated and Control Broiler Chickens during the Early Rainy Season

Parameters	Control	Fisetin	Probiotic	Fisetin + Probiotic
WBC (x 10^9/L)	1.90 ± 0.35 (0.80 - 3.50)	1.84 ± 0.24 (0.65 - 2.35)	2.02 ± 0.08 (1.70 - 2.40)	1.64 ± 0.12 (1.10 - 2.10)
EOS (x 10^9/L)	0.00 ± 0.00 (0.00 - 0.03)	0.04 ± 0.02 (0.00 - 0.14)	0.02 ± 0.01 (0.00 - 0.07)	0.03 ± 0.01 (0.00 - 0.09)
MON (x 10^9/L)	0.25 ± 0.01[a] (0.03 - 0.74)	0.14 ± 0.03[b] (0.05 - 0.28)	0.16 ± 0.04[b] (0.04 - 0.29)	0.17 ± 0.03[b] (0.04 - 0.23)
BAS (x 10^9/L)	0.00 ± 0.00 0.00 - 0.00	0.00 ± 0.00 0.00 - 0.00	0.00 ± 0.00 0.00 - 0.00	0.00 ± 0.00 0.00 - 0.00
LYM (x 10^9/L)	1.52 ± 0.19[a] (1.66 - 2.34)	1.81 ± 0.17[b] (1.16 - 2.34)	1.72 ± 0.14[b] (1.21 - 2.23)	1.72 ± 0.18[b] (1.52 - 2.46)
HET (x 10^9/L)	0.72 ± 0.10[a] (0.01 - 0.70)	0.17 ± 0.05[b] (0.07 - 0.47)	0.13 ± 0.05[b] (0.00 - 0.42)	0.18 ± 0.04[b] (0.06 - 0.32)
H/L	0.54 ± 0.12[a] (0.15 - 0.86)	0.10 ± 0.04[b] (0.03 - 0.32)	0.09 ± 0.04[b] (0.00 - 0.30)	0.11 ± 0.03[b] (0.03 - 0.28)

Values in parenthesis are minimum and maximum.
[a, b, c] = Values in the same row with different superscript letters are significantly ($P < 0.05$) different. WBC, total leucocyte count; EOS, eosinophil; MON, monocyte; BAS, basophil; LYM, lymphocyte; HET, heterophil; H/L, heterophil/lymphocyte ratio, n = 7

4.6 Small Intestinal Morphometric Parameters of Broiler Chickens during the Study Period

Mean villus heights in fisetin (1.02 ± 0.05 mm), probiotic (1.13 ± 0.07 mm) and fisetin + probiotic (1.24 ± 0.05 mm) groups were higher ($P < 0.05$) than in the control group (0.80 ± 0.03 mm). Mean villus widths in fisetin (0.10 ± 0.01 mm), probiotic (0.11 ± 0.01 mm) and fisetin + probiotic (0.13 ± 0.00 mm) groups were higher ($P < 0.05$), when compared to that of the control group (0.06 ± 0.01 mm). Mean crypt heights in fisetin (0.35 ± 0.01 µm), probiotic (0.35 ± 0.01 mm) and fisetin + probiotic (0.36 ± 0.02 mm) groups increased ($P < 0.05$), when compared to that of the control group (0.29 ± 0.01 mm). Mean crypt width in fisetin (0.04 ± 0.00 mm), probiotic (0.06 ± 0.00 mm) and fisetin + probiotic (0.06 ± 0.00 mm) groups were higher ($P < 0.05$) than in control group (0.02 ± 0.00 mm).

Mean villus height/crypt height ratio in fisetin (3.01 ± 0.22), probiotic (3.33 ± 0.25) and fisetin + probiotic (3.61 ± 0.27) groups were higher ($P < 0.05$) than in control group (2.86 ± 0.19). Mean villus surface areas in fisetin (0.31 ± 0.03 mm^2), probiotic (0.41 ± 0.04 mm^2) and fisetin + probiotic (0.52 ± 0.03 mm^2) groups were higher ($P < 0.05$), when compared to that of the control group (0.15 ± 0.02 mm^2). Mean crypt surface area in fisetin (0.04 ± 0.00 mm^2), probiotic (0.07 ± 0.00 mm^2) and fisetin + probiotic (0.07 ± 0.00 mm^2) groups were higher ($P < 0.05$) than in control group (0.02 ± 0.00 mm^2) (Table 4.5).

Table 4.5: Mean (± SEM) Small Intestinal Parameters in Fisetin-, Probiotic-, Fisetin + Probiotic-treated and Control Broiler Chickens during the Early Rainy Season

Parameters	Control	Fisetin	Probiotic	Fisetin + Probiotic
Villus height (mm)	0.80 ± 0.03^{a}	1.02 ± 0.05^{b}	1.13 ± 0.07^{b}	1.24 ± 0.05^{b}
Villus width (mm)	0.06 ± 0.01^{a}	0.10 ± 0.01^{b}	0.11 ± 0.01^{b}	0.13 ± 0.00^{b}
Crypt height (mm)	0.29 ± 0.01^{a}	0.35 ± 0.01^{b}	0.35 ± 0.01^{b}	0.36 ± 0.02^{b}
Crypt width (mm)	0.02 ± 0.00^{a}	0.04 ± 0.00^{b}	0.06 ± 0.00^{c}	0.06 ± 0.00^{c}
Villus height/Crypt height	2.86 ± 0.19^{a}	3.01 ± 0.22^{b}	3.33 ± 0.25^{b}	3.61 ± 0.27^{b}
Villus surface area (mm^2)	0.15 ± 0.02^{a}	0.31 ± 0.03^{b}	0.41 ± 0.04^{b}	0.52 ± 0.03^{b}
Crypt surface area (mm^2)	0.02 ± 0.00^{a}	0.04 ± 0.00^{b}	0.07 ± 0.00^{c}	0.07 ± 0.00^{c}

$^{a, b, c}$ = Values in the same row with different superscript letters are significantly ($P < 0.05$) different, $n = 3$

CHAPTER FIVE

DISCUSSION

The results of the present study showed that high thermal environmental conditions of DBT and RH prevailed during the early raining season, and that the conditions were unfavourable for the rearing of broiler chickens in the zone as they induced heat stress. This is because the values were outside the thermoneutral zone of 18 - 24 °C (Olarewaju *et al*., 2016) and the optimum range of RH (65 – 70%) for broilers between the ages of 3-7 weeks (Akyuz and Boyaci, 2010; Egbuniwe *et al*., 2018b). The findings agree with that of Aluwong *et al*. (2017), who showed that high DBT and RH in broiler chickens raised in the zone induced heat stress. The THI above 20.8 has been shown to induce heat stress in broiler chickens (Tao and Xin, 2003; Sinkalu *et al.,* 2015). The overall mean value of THI recorded in the present study (30.65 ± 0.26) showed that the broiler chickens were subjected to heat stress, which may result in poor performance, hyperthermia, immune suppression and high mortality (Kamel *et al*., 2017). Thus, the administration of fisetin and probiotics, which are potent antioxidants (Abudabos *et al*., 2017; Abu Hafsa and Ibrahim, 2018) may ameliorate the adverse effects of heat stress on the performance and health of broiler chickens, reared during the early raining season.

The study showed that administration of fisetin and probiotic to broiler chickens maintained the integrity of erythrocyte membrane during the early raining season. The finding shows that fisetin exerted antioxidant effect on the cell membrane (Belscak-Cvitanovic *et al*., 2018), thus protecting the cells from the deleterious effects of heat stress-induced lipid peroxidation. The result agrees with that of Belkhiri *et al.* (2017), who demonstrated that the binding of flavonoids to erythrocyte membranes significantly inhibits lipid peroxidation, and consolidates their integrity against hypotonic lysis. The result also supports the previous finding of Aluwong *et al*. (2013) who reported that probiotic has an antioxidant effect by preventing cells against

adverse effects of heat stress, thus protecting the cell integrity and inhibiting lipid peroxidation. The study showed that administration of both fisetin and probiotic has no additive or synergistic effect on the maintenance of the integrity of the cell membrane. However, Egbuniwe *et al*. (2018a) demonstrated that combined administration of antioxidants, betaine and ascorbic acid, reduced EOF in broiler chickens under stressful hot-dry conditions.

Oxidative stress increases the production of ROS, which cause damage to the RBC membrane via lipid peroxidation (Duranti *et al*., 2018). It decreases erythrocyte diapole potential (a biophysical determinant of membrane function) and results in the formation of haemoglobin-spectrin complexes, which stiffen the membrane (Jewell *et al*., 2013; Welbourn *et al*., 2017), and impairs erythrocyte functions. Heat stress decreases of pH in the erythrocyte environment due to loss of some ions, which increases EOF (Oyewale *et al*., 2011; Chauhan *et al*., 2015). The erythrocytes of stress-induced broiler chickens are mis-shaped, impairing the functions of the cells (Maxwell *et al*., 1992; Farag and Alagawany, 2017). The result of the present study showed that impairments induced by oxidative stress were ameliorated by the administration of antioxidants, fisetin and/or probiotic, to broiler chickens exposed to heat stress during the early raining season. Thus the antioxidants protect the erythrocytes from the adverse effects of heat stress, by consolidating the membrane integrity.

The findings of the study that fisetin, a flavonoid decreased serum MDA concentration and significantly increased SOD activity agrees with that of Iskender *et al.* (2016), who reported a decrease in MDA in laying hens administered with a flavonoid, quercetin. The findings of the study that fisetin increased serum SOD agrees with that of Lui *et al*. (2014), who reported an increase in SOD in laying hens administered with a flavonoid, quercetin. Thus, fisetin, a flavonoid, conferred some protection on the broiler chickens against the negative effects of oxidative stress, resulting from heat stress. Fisetin has potential effects on the levels of

antioxidants, reversing the depleted levels of different enzymatic and non-enzymatic antioxidants (Naeimi and Alizadeh, 2017). Similarly, findings from the present study that probiotic decreased serum MDA concentration agrees with that of Bai *et al.* (2016), who reported that probiotic, as an antioxidant, decreases serum MDA concentration in broiler chicken reared under thermal stress. The finding of the study that probiotic increased SOD agrees with that of Aluwong *et al*. (2013), who reported an increase in SOD activity in broiler chickens. Probiotic has been shown to improve the antioxidant status in broiler chickens by decreasing the production of ROS (Kim *et al.,* 2017). Probiotic modulates the dynamics of oxidants and antioxidants in the body, inhibiting lipid peroxidation (Mishra *et al*., 2015; Abudabos *et al*., 2016). The result shows that administration of antioxidants, such as fisetin and/or probiotic, to broiler chickens exposed to heat stress during the early raining season was protective against the adverse effects of excess ROS. The result also demonstrated that the two antioxidants did not exert additive effects in reducing MDA concentration.

The findings of the study showed that there was significant increase in PCV, Hb, MCV, MCH, in the group treated with fisetin. This result agrees with the findings of Toumi *et al.* (2016), who reported increased Hb concentration, MCV in stressed pregnant rats, administered with quercetin, a flavonoid. Similarly, the present finding also showed significant increase in PCV, Hb in the group treated with probiotic. The result agrees with the finding of Aguihe *et al*. (2015) that probiotic supplementation significantly increases erythrocyte count, Hb concentration and PCV values in broiler chickens. In contrast to the findings Tang *et al.* (2017) demonstrated that probiotic supplementation significantly decreased, PCV, Hb, MCV and MCH in laying hens. The type and dose of probiotic microorganisms used may explain the above discrepancy. The present finding showed significant increase in total protein concentration in the group treated with probiotic. The result agrees with the finding of Attia *et al*. (2017) that probiotic significantly increased total protein concentration in broiler chickens subjected to heat stress.

The result of the study showed that there was no significant increase in WBC, EOS, and BAS counts in the groups treated with fisetin or probiotic, which agrees with the findings of Park and Kim (2015) that total leucocyte and differential leucocyte counts are not significantly altered by probiotic supplementation. The result of the study showed decreased lymphocyte and increased monocyte count in the control group when compared to those of the treated groups. The result agrees with the findings of Bai *et al*. (2013), who reported a decrease lymphocyte and increase monocyte count in broiler chickens administered with probiotic supplement. There was a decrease in heterophil count in groups treated with fisetin and/or probiotic, which is similar to the finding of Owosibo *et al.* (2013), who reported a decrease in lymphocyte and heterophil counts in broiler chickens administered with probiotic-base diet. The increased heterophil/lymphocyte ratio in the control group was an evidence of the physiological stress imposed on the broiler chickens. Heterophil/lymphocyte ratio has been used as an index of stress in broilers subjected to heat stress (Lentfer *et al*., 2015; Scanes, 2016 The reference values for the heterophil/lymphocyte ratios are about 0.20, 0.50 and 0.80 for low, optimal and high degrees of stress, respectively (Maxwell and Robertson, 1998; Lentfer *et al*., 2015). The findings from the recent study showed that the broiler chickens were exposed to heat stress during the early rainy season stressed and that fisetin and probiotic alleviated the stress.

Heat stress has been shown to impair the intestinal morphology and barrier integrity of chickens (Song *et al.,* 2017), inducing disorders, and also increased digestive disease conditions. The findings from the current study showed significant increase in the small intestine villus height, villus width, crypt height, crypt width, villus height/crypt height ratio, surface area of the villus and crypt in the group treated with fisetin agrees with the finding of Mehaisen *et al*. (2017), who reported that propolis rich in flavonoid preserves the integrity of intestinal villi and increases the crypt depth in heat-stressed quails. The findings of the present study also showed

increases in villus height, villus width, crypt height, crypt width, surface area of the villus and crypt in probiotic-treated group. Shokryazdan *et al*. (2017) reported an increased villus height, which agrees with the findings of the present study and non-significant increase in villus crypt in broiler chickens fed with probiotic. Song *et al.* (2014) reported an increased villus height/crypt height ratio in broiler chickens subjected to heat stress and fed with probiotic, which also agrees with the findings of the present study. Wang *et al.* (2016) observed insignificant changes in small intestinal morphology in broiler chickens, administered with probiotic only without exposure to environmental stress factors.

Bai *et al*. (2016) reported the beneficial effect of probiotic villi height and crypt in broiler chickens. An increase in villus height equates to an increase in surface area, resulting in more effective absorption of available nutrients (Al-Sultan *et al*., 2016). Crypts are origin area for production of new epithelial and villus cells. Villus height/crypt height ratio is an indicator of the capacity of the intestinal mucosa to absorb nutrients. The probiotics may reverse the impaired villus-crypt structure in heat-stressed birds by controlling the corticosterone level (Lei *et al*., 2013; Al-Fataftah and Abdelqader, 2014) and excessive release of pro-inflammatory agents that may cause intestinal tissue injuries and increase the permeability of the intestine (Deng *et al*., 2012; Gupta *et al*., 2017). Beneficial effects in the systemic immune response against heat stress by probiotics ingestion may be related to alterations in the intestinal barrier. Since *Saccharomyces cerevisiae* acts in the intestine forming a layer close to the epithelium, which establishes eubiosis and promotes its thickening (Gibson and Roberfoid, 1995), it can be suggested that the deeper crypt occurred as a result of this layer being formed by the ingestion of the probiotic, which may act as a barrier against pathogenic microorganisms. It has also been suggested that alteration in villi height and crypt depth may lead to poor nutrient absorption, digestive enzyme secretion in the gastro-intestinal tract and eventually lower growth and performance in broilers chickens (Xu *et al.*, 2003; Awad *et al*., 2018).

The findings of the present study showed, for the first time, that the administration of antioxidants, fisetin and probiotic to broiler chickens, exposed to heat stress during the early raining season protected the intestinal morphology from the adverse effects of heat stress. Therefore, probiotic and fisetin may be beneficial in increasing broiler chicken productivity during the heat-stressed early rainy season.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i. The DBT (25.00 - 36.00 °C), RH (47.00 - 100%) and THI (25.00 – 35.55) prevailing during the early rainy season were outside the thermoneutral zone, indicating that the broiler chickens were exposed to heat stress during the early rainy season

ii. The administration of fisetin + probiotic to broiler chickens alleviated the impact of heat stress by decreasing MDA concentration (4.136 ± 0.58 nmol/L), when compared to that of the control group (22.64 ± 2.95 nmol/L)

iii. Activity of SOD increased ($P < 0.05$) in broiler chickens administered with fisetin (6.34 ± 0.24 IU/L), when compared to that of the control group (5.37 ± 0.09 IU/L).

iv. The EOF decreased ($P < 0.05$) in broiler chickens administered with fisetin (34 ± 0.98%), probiotic (35.65 ± 0.81%) and fisetin and probiotic (34.25 ± 1.98%), when compared to that of the control group (48.42 ± 0.40%) during the early rainy season

v. Heterophil/lymphocyte ratio in broiler chickens administered with fisetin (0.10 ± 0.04), probiotic (0.09 ± 0.04) and fisetin and probiotic (0.11 ± 0.03) were lower ($P < 0.05$) than that of the control group (0.54 ± 0.12) during the early rainy season

vi. Surface area of the villus of the small intestine increased ($P < 0.05$) in the treated broiler chickens (fisetin, 0.31 ± 0.03 mm^2; probiotic, 0.41 ± 0.04 mm^2; probiotic + fisetin, 0.52 ± 0.03 mm^2), when compared to the value obtained in the controls (0.14 ± 0.02 mm^2)

6.2 Recommendations

i. Fisetin and/or probiotic should be administered to broiler chickens, exposed to heat to improve their health and production during the early rainy season

ii. Farmers should be encouraged to use fisetin and/or probiotic additive as alternative to antibiotics in the feed for broiler chickens.

iii. Further studies are required to ascertain the optimal doses for combined administration of fisetin and probiotic to broiler chickens during the early raining season.

iv. Further studies are required to elucidate the mechanism of action of fisetin and probiotic on haemato-biochemical and small intestinal morphometric parameters in broiler chickens subjected to heat stress.

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LIST OF APPENDICES

Appendix I: Mean, Minimum, Maximum and Range Dry-bulb Temperature (DBT) of the Study Area during the Early Rainy Season

Hour (h)	Mean DBT (°C)	Minimum DBT (°C)	Maximum DBT (°C)	Range DBT (°C)
7:00	27.68 ± 0.20	25.00	30.00	5.00
13:00	33.15 ± 0.34	28.00	36.00	8.00
18:00	32.50 ± 0.32	28.00	36.00	8.00
Overall mean ± SEM	31.11 ± 0.28	27.00 ± 0.24	34.00 ± 0.30	7.00 ± 0.14

Appendix II: Mean, Minimum, Maximum and Range Relative Humidity (RH) of the Study Area during the Early Rainy Season

Hour (h)	Mean RH (%)	Minimum RH (%)	Maximum RH (%)	Range RH (%)
7:00	87.98 ± 1.66	65.00	100.00	35.00
13:00	72.74 ± 1.18	47.00	93.00	46.00
18:00	77.24 ± 1.20	59.00	93.00	34.00
Overall mean ± SEM	79.32 ± 0.97	57.00 ± 0.52	95.33 ± 1.30	38.33 ± 0.44

Appendix III: Mean, Minimum, Maximum and Range Temperature-humidity Index (THI) of the Study Area during the Early Rainy Season

Hour (h)	Mean THI	Minimum THI	Maximum THI	Range THI
7:00	27.45 ± 0.19	25.00	29.70	4.70
13:00	32.54 ± 0.34	26.80	35.55	8.75
18:00	31.96 ± 0.30	27.55	35.10	7.55
Overall mean ± SEM	30.65 ± 0.26	26.45 ± 0.24	33.45 ± 0.28	7.00 ± 0.18

Appendix IV: Mean (± SEM) Erythrocyte Osmotic Fragility of Broiler Chickens during the Early Rainy Season (n=7)

NaCl Concentration	Control	Fisetin	Probiotic	Fisetin + Probiotic
0.9	0.57 ± 0.26	0.00 ± 0.00	0.86 ± 0.25	0.33 ± 0.17
0.7	0.93 ± 0.36	0.83 ± 0.32	1.12 ± 0.27	1.78 ± 0.58
0.5	48.42 ± 0.40	34.26 ± 0.98	35.65 ± 0.81	34.25 ± 1.98
0.3	78.33 ± 2.29	57.94 ± 2.72	59.29 ± 1.55	58.91 ± 2.81
0.1	96.22 ± 0.49	96.41 ± 0.20	96.56 ± 0.14	96.23 ± 0.05
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Appendix V: Mean (± SEM) Malondialdehyde Concentration and Superoxide Dismutase Activity in Broiler Chickens during the Early Rainy Season (n = 7)

Parameters	Control	Fisetin	Probiotic	Fisetin + Probiotic
MDA (nmol/L)	22.64 ± 2.95	14.37 ± 1.15	5.66 ±1.06	4.136 ± 0.58
SOD (IU/L)	5.37 ± 0.09	6.34 ± 0.24	5.67± 0.09	5.93 ± 0.13

Appendix VI: Erythrocytic Parameters in Broiler Chickens during the Early Rainy Season (n = 7)

Groups	RBC ($x10^6$/µL)	PCV (%)	Hb (g/dL)	MCV (fl)	MCH (pg)	MCHC (g/dl)
Control						
A1	1.53	30.00	10.00	196.72	65.57	33.33
A2	1.60	27.00	9.00	168.75	56.25	33.33
A3	1.79	23.00	7.67	128.67	42.89	33.33
A4	1.48	20.00	6.67	135.59	45.20	33.33
A5	1.58	22.00	7.33	139.68	46.56	33.33
A6	1.44	27.00	9.00	187.83	62.61	33.33
A7	1.66	25.00	8.33	150.38	50.13	33.33
Fisetin						
B1	1.94	30.00	10.00	154.84	51.61	33.33
B2	1.54	30.00	10.00	195.12	65.04	33.33
B3	1.56	43.00	14.33	275.20	91.73	33.33
B4	1.51	31.00	10.33	204.96	68.32	33.33
B5	1.59	24.00	8.00	151.18	50.39	33.33
B6	1.35	37.00	12.33	274.07	91.36	33.33
B7	2.03	29.00	9.67	143.21	47.74	33.33
Probiotic						
C1	2.00	26.00	8.67	130.00	43.33	33.33
C2	1.88	30.00	10.00	160.00	53.33	33.33
C3	1.78	26.00	8.67	146.48	48.83	33.33
C4	2.16	23.00	7.67	106.36	35.45	33.33
C5	2.06	23.00	7.67	111.52	37.17	33.33
C6	2.03	34.00	11.33	167.90	55.97	33.33
C7	2.23	28.00	9.33	125.84	41.95	33.33
Fisetin + Probiotic						
D1	1.72	32.00	10.67	186.86	62.29	33.33
D2	2.33	26.00	8.67	111.83	37.28	33.33
D3	2.55	31.00	10.33	121.57	40.52	33.33
D4	2.20	30.00	10.00	136.36	45.45	33.33
D5	2.20	30.00	10.00	136.36	45.45	33.33
D6	1.45	22.00	7.33	151.72	50.57	33.33
D7	2.20	33.00	11.00	150.00	50.00	33.33

PCV, packed cell volume; RBC, red blood cell count; Hb, haemoglobin concentration; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration

Appendix VII: Leucocytic Parameters in Broiler Chickens during the Early Rainy Season (n = 7)

Groups	WBC ($x10^3/\mu L$)	BAS ($x10^3/\mu L$)	EOS ($x10^3/\mu L$)	MON ($x10^3/\mu L$)	LYM ($x10^3/\mu L$)	HET ($x10^3/\mu L$)
Control						
A1	1.47	0.00	0.00	0.06	1.20	0.21
A2	2.00	0.00	0.00	0.10	1.82	0.28
A3	1.80	0.00	0.00	0.36	1.26	0.18
A4	3.00	0.00	0.00	0.63	1.77	0.60
A5	0.67	0.00	0.00	0.07	0.55	0.05
A6	2.60	0.00	0.00	0.42	1.77	0.42
A7	1.20	0.00	0.024	0.02	1.14	0.01
Fisetin						
B1	1.40	0.00	0.01	0.06	1.25	0.08
B2	1.93	0.00	0.04	0.14	1.55	0.21
B3	2.13	0.00	0.13	0.24	1.34	0.43
B4	2.10	0.00	0.06	0.25	1.67	0.08
B5	2.00	0.00	0.02	0.08	1.76	0.14
B6	0.47	0.00	0.01	0.04	0.37	0.05
B7	1.67	0.00	0.00	0.13	1.64	0.09
Probiotic						
C1	2.20	0.00	0.00	0.07	2.05	0.09
C2	2.10	0.00	0.00	0.29	1.36	0.41
C3	1.73	0.00	0.02	0.03	1.63	0.05
C4	1.93	0.00	0.00	0.12	0.87	0.95
C5	2.00	0.00	0.00	0.06	1.86	0.08
C6	1.47	0.00	0.06	0.22	1.04	0.15
C7	1.73	0.00	0.02	0.26	1.40	0.05
Fisetin + Probiotic						
D1	1.80	0.00	0.00	0.27	1.30	0.23
D2	1.87	0.00	0.00	0.13	1.38	0.35
D3	1.93	0.00	0.04	0.21	1.53	0.15
D4	1.00	0.00	0.02	0.04	0.86	0.08
D5	0.00	0.00	0.00	0.00	0.00	0.00
D6	1.67	0.00	0.03	0.12	1.47	0.05
D7	1.67	0.00	0.08	0.22	1.07	0.30

WBC, total leucocyte count; EOS, eosinophil; MON, monocyte; BAS, basophil; LYM, lymphocyte; HET, heterophil

Appendix VIII: Mean (± SEM) Duodenal Morphometric Parameters in Broiler Chickens during the Early Rainy Season (n = 3)

Parameters	Control	Fisetin	Probiotic	Fisetin + Probiotic
Villus height (mm)	0.86 ± 0.03	1.21 ± 0.06	1.48 ± 0.08	1.45 ± 0.03
Villus width (mm)	0.08 ± 0.00	0.10 ± 0.00	0.13 ± 0.00	0.15 ± 0.00
Crypt height (mm)	0.27 ± 0.01	0.37 ± 0.02	0.38 ± 0.01	0.36 ± 0.03
Crypt width (mm)	0.14 ± 0.04	0.20 ± 0.05	0.22 ± 0.05	0.21 ± 0.05
Surface area of villus (mm^2)	0.10 ± 0.01	0.24 ± 0.04	0.28 ± 0.03	0.40 ± 0.02
Surface area of crypt (mm^2)	0.01 ± 0.00	0.04 ± 0.01	0.08 ± 0.01	0.07 ± 0.00

Appendix IX: Mean (± SEM) Jejunal Morphometric Parameters in Broiler Chickens during the Early Rainy Season (n = 3)

Parameters	Control	Fisetin	Probiotic	Fisetin + Probiotic
Villus height (mm)	0.74 ± 0.06	0.97 ± 0.03	1.01 ± 0.05	1.27 ± 0.07
Villus width (mm)	0.04 ± 0.00	0.10 ± 0.02	0.11 ± 0.01	0.13 ± 0.01
Crypt height (mm)	0.22 ± 0.02	0.34 ± 0.02	0.34 ± 0.02	0.34 ± 0.03
Crypt width (mm)	0.03 ± 0.00	0.04 ± 0.00	0.06 ± 0.00	0.06 ± 0.00
Surface area of villus (mm^2)	0.11 ± 0.03	0.31 ± 0.05	0.34 ± 0.03	0.49 ± 0.03
Surface area of crypt (mm^2)	0.02 ± 0.00	0.05 ± 0.00	0.10 ± 0.00	0.10 ± 0.01

Appendix X: Mean (± SEM) Ileal Morphometric Parameters in Broiler Chickens during the Early Rainy Season (n= 3)

Parameters	Control	Fisetin	Probiotic	Fisetin + Probiotic
Villus height (mm)	0.61 ± 0.03	0.87 ± 0.07	0.88 ± 0.05	1.01 ± 0.02
Villus width (mm)	0.05 ± 0.00	0.08 ± 0.01	0.10 ± 0.00	0.13 ± 0.00
Crypt height (mm)	0.16 ± 0.01	0.39 ± 0.04	0.32 ± 0.03	0.39 ± 0.04
Crypt width (mm)	0.02 ± 0.00	0.04 ± 0.00	0.06 ± 0.01	0.05 ±0.00
Surface area of villus (mm^2)	0.10 ± 0.01	0.24 ± 0.04	0.28 ± 0.03	0.39 ± 0.02
Surface area of crypt (mm^2)	0.01 ± 0.00	0.04 ± 0.00	0.06 ± 0.01	0.06 ± 0.01