EFFECTS OF DIETARY SUPPLEMENTATION WITH β-SITOSTEROL ON COBB 500 BROILER CHICKEN PRODUCTIVITY, HEALTH AND PRODUCT QUALITY

MALEBOGO AUDREY BOPAPE

A thesis submitted to the Faculty of Health Sciences, University of Witwatersrand, School of Physiology, in fulfilment of the requirements for the degree of Doctor of Philosophy.

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DECLARATION

I, **Malebogo Audrey Bopape**, student number **2076139**, declare that this thesis, hereby submitted for the degree Doctor of Philosophy at the University of the Witwatersrand, is my own work and that it has not been submitted to any other institution for an award. All other sources of information cited in this thesis have been adequately and clearly acknowledged. The experiments were performed with approval by the Animal Ethics Screening Committee of the University of Witwatersrand.

_____
(Signature of candidate)

16th day of October 2024 in Kimberley, Northern Cape

DEDICATION

To the loving memory of my dear father

Shima Moses Bopape

1st January 1953 – 10th June 2005

In memory of my dear friend and sister

Nare Abrina Mpebe

10th October 1983 –2nd February 2023

PRESENTATIONS ARISING FROM THIS RESEARCH

Oral presentations

i. **Malebogo Audrey Bopape,** Kiahrah Reese Rajah, Busisani Wiseman Lembede, Kennedy Erlwanger and Eliton Chivandi. Effect of dietary β-sitosterol on broiler chicken growth performance, feed utilisation efficiency and carcass yield. *Sol Plaatje University Academic Symposium*, 7-8th December 2020. Sol Plaatje University, Kimberley.

ii. **M.A. Bopape.,** K.H. Erlwanger., B.W. Lembede & E. Chivandi. β-sitosterol as an alternative to oxytetracycline: effect on growth performance, feed intake and utilization efficiency, and meat quality of broiler chickens. *53rd South African Society of Animal Science (SASAS) Congress,* 26-28th September 2022, University of Kwazulu Natal, Pietermaritzburg.

iii. **Malebogo Audrey Bopape**, Kennedy Erlwanger, Busisani Wiseman Lembede and Eliton Chivandi. Effect of dietary β-sitosterol on growth performance, feed intake and utilization efficiency, and meat quality of broiler chickens. Sol Plaatje University Annual Teaching, Learning and Research Symposium, 21-22 November 2022. Hybrid MS Team and Central Campus, Kimberley.

Poster presentation

i. **M.A. Bopape,** K.H. Erlwanger, B.W. Lembede & E. Chivandi. Effect of dietary β-sitosterol supplementation on the carcass yield and meat quality of Cobb 500 broiler chickens. ***39th Scientific Symposium World's Poultry Science Association***, 7-8th March 2023. Pretoria, Gauteng, South Africa.

PUBLICATION ARISING FROM THIS RESEARCH

i. **Malebogo A. Bopape,** Kennedy H. Erlwanger, Busisani W. Lembede and Eliton Chivandi. 2023. β-sitosterol as an alternative to oxytetracycline: Effect on growth performance, feed intake and utilization efficiency, and viscera macromorphometry of Cobb 500 broiler chickens. ***Veterinary and Animal Science***, 19: 1-6. DOI: https://doi.org/10.1016/j.vas.2022.100283

ABSTRACT

Antibiotic use as growth promoters in chicken feeds results in antibiotic resistance and environmental pollution. To mitigate these challenges alternatives natural growth promoters are required. Beta-sitosterol is one of several phytosterols with chemical structures similar to that of cholesterol. β-sitosterol has antimicrobial, antioxidant, anti-inflammatory and hypocholesterolaemic activities, thus might replace synthetic antibiotics as a feed supplement in chicken feeds. The current study evaluated β-sitosterol's potential to replace oxytetracycline in Cobb 500 broiler chicken feeds by determining its effects on growth performance, meat yield and quality and bird health. β-sitosterol replaced oxytetracycline at 0 (control: 50 mg/kg oxytetracycline), 500, 1000 and 1500 mg/kg feed for diet 1 to 4, respectively with doses similar in starter, grower, and finisher diets. Chickens were fed from day 1 to 42 days of age. Body mass and feed intake were measured. Body mass gain, average daily gain and feed conversion ratio were computed. Terminally, broiler chickens were fasted, weighed, humanely slaughtered and dressed. The carcass yield, viscera morphometry and plasma surrogate markers of health were also determined. Meat pH, colour, thawing and cooking loss (TL; CL), water holding capacity (WHC), tenderness and myofibrillar fragmentation length (MFL) and nutrient content were determined. Femora and tibiae mass, length, breaking strength and liver fat content and histology were determined. Dietary β-sitosterol had similar ($P > 0.05$) effects as oxytetracycline on the chickens' growth performance and feed intake utilization efficiency, gastrointestinal tract (GIT) and GIT accessory viscera macromorphometry, meat yield, meat colour, pH, TL, CL, WHC, tenderness and MFL. However, breast meat crude protein content of chicken fed diet 4 was higher ($P < 0.0001$) compared to that of counterparts fed diets 2 and 3. Breast meat fat content of chicken fed diet 2 and diet 4 was higher ($P < 0.0001$) compared to that of counterparts fed diets 1 and 3. Dietary β-sitosterol had similar ($P > 0.05$) broiler chickens' breast meat total saturated fatty acids (TSFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), palmitic, oleic and linoleic content as oxytetracycline. Dietary treatments had no effect on chickens' tibiae masses and breaking strength ($P > 0.05$) albeit tibiae from chickens fed diet 4 were shorter ($P < 0.01$) than those of counterparts fed diet 2. Dietary β-sitosterol at 1000 and 1500 mg/kg feed increased ($P < 0.05$) liver lipid content but had no effect on hepatic microarchitecture. However, at 1500 mg/kg feed it caused micro- and macro hepatic steatosis and lobular inflammation and higher ($P < 0.05$) non-alcoholic fatty liver disease activity scores (NAS). Compared to control, dietary β-

sitosterol decreased ($P < 0.0001$) the chickens' malondialdehyde (MDA) concentration but increased superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) activities and glutathione S-transferase (GST) and glutathione (GSH) concentration ($P < 0.05$). Diet 4 increased ($P < 0.01$) plasma AST and GGT activities compared to diet 1 (control). At 1000 and 1500 mg/kg feed it increased plasma cholesterol concentration compared to control and β-sitosterol at 500 mg/kg feed. β-sitosterol can replace oxytetracycline as growth promoter in Cobb 500 broiler chicken diets without negatively affecting growth performance, meat yield and quality and potentially mitigates oxidative stress by upregulating systemic antioxidant enzymes activities. However, at 1500 mg/kg feed, it can increase the risk of fatty liver disease development and hypercholesterolaemia.

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NOMENCLATURE

ADG: Average daily gain

AGP: Antibiotic growth promoter

ALP: Alkaline phosphatase

ALT: Alanine transaminase

AMYL: Amylase

ANOVA: Analysis of variance

ARC: Agricultural Research Council

AREC: Animal Research Ethics Committee

AST: Aspartate Aminotransferase

BUN: Blood urea nitrogen

BWG: Body weight gain

CAT: Catalase

CP: Crude protein

CREA: Creatinine

DAFF: Department of Agriculture, Forestry and Fisheries

DM: Dry matter

EE: Ether extract

EU: European Union

EFSA: European Food Safety Authority

FI: Feed intake

FCR: Feed conversion ratio

FLHS: Fatty liver hemorrhagic syndrome

GDP: Gross domestic product

GGT: Gamma-glutamyl transferase

GIT: Gastrointestinal tract

GSH: Glutathione

GSH-Px: Glutathione peroxidase

GST: Glutathione S-transferase

LDL: Low-density lipoprotein

MDA: Malondialdehyde

NAMC: National Agricultural Marketing Council

NAS: Non-alcoholic fatty liver disease activity score

NGP: Natural growth promoter

NRC: National Research Council

OECD-FAO: Organisation for Economic Co-operation Development, Food and Agriculture Organisation

pHi: Initial pH

pHu: Ultimate pH

PSD: Plain starter diet

PUFA: Polyunsaturated fatty acids

SA: South Africa

SAPA: South African Poultry Association

SOD: Superoxide dismutase

SSA: Sub-Saharan Africa

TBIL: Total Bilirubin

TRIG: Triglyceride

USDA: United States Department of Agriculture

WHC: Water holding capacity

WHO: World Health Organisation

WRAF: Wits Research Animal Facility

CHAPTER ONE – INTRODUCTION AND JUSTIFICATION

1.0 Thesis overview

Animal derived foods, including meat, are a key source of nutrients in human diets. Global meat production was estimated at 360 million tonnes in 2022, an increase of 1.2% compared to 2021 (Food and Agriculture Organization in Africa, 2023). Broiler poultry production is a key contributor to meat for human consumption. Human population growth, expansion of urban settlements and increases in incomes are major drivers of increased demand of meat. To meet this increased demand, feed technologies, for example, use of synthetic antibiotics to fortify broiler chicken feeds to boost productivity, are employed (Krysiak et al., 2021). However, routine use of antibiotics as growth promoters causes antibiotic resistance, contamination of meat with antibiotic residues and also pollution of the environment (Basit et al., 2020). Health and environmental challenges associated with the routine use of synthetic antibiotics in broiler chicken production necessitate that acceptable natural alternatives that mimic the growth promoting properties of synthetic antibiotics, without adverse effects, be explored (Ribeiro et al., 2023). In light of this, study evaluated the potential of β-sitosterol; a phytosterol with antimicrobial, antioxidant, anti-inflammatory and gastrointestinal tract and immune modulating properties, to replace oxytetracycline in broiler chicken feed. The thesis is presented in a divided block format. Chapter 1, in addition to presenting a general background to the Sub-Saharan Africa and South African poultry industries, it gives a narrative on routine use of synthetic antibiotics to fortify poultry feeds productive efficiency. The narrative takes the reader to an understanding of the health and environmental challenges associated with such routine use of antibiotics and proposes and justifies the need to seek alternatives. Phytochemicals, and specifically β-sitosterol, are identified as a possible alternative. The study aim, associated objectives and hypotheses are presented. Chapter 2 provides a detailed account of literature pertinent to this study. Major highlights include discourse on the routine use of synthetic antibiotics as growth promoting feed supplements in poultry production with effects on productive performance, meat and eggs quality and consumer health. A discourse on potential interventions to mitigate the synthetic antibiotics induced challenges is given with a specific focus on β-sitosterol. Chapter 3 is the first experimental chapter. A description of the evaluation of β-sitosterol's potential to replace oxytetracycline on the growth performance of Cobb 500 broiler chicken. The chapter presents study results and concludes with a discussion of the findings. Chapter 4 is narrative of the evaluation of the effects of dietary fortification of Cobb 500 broiler chicken feeds with β-sitosterol on meat yield and quality. Results of this evaluation and a discussion are presented.

Chapter 5 focusses on evaluation of the effects of dietary β-sitosterol in Cobb 500 broiler chickens' femora and tibiae mass, length and breaking strength as measures of growth and bone health. This chapter focused on effect on long/antigravity bones because a functional skeletal system is critical to broiler chicken production and welfare. Chapter 6 reviews the importance of ensuring flock welfare and health in the process of producing broiler chicken meat for human consumption. The chapter narrates the effect of dietary β-sitosterol on Cobb 500 broiler chickens' kidney and liver health, key metabolic organs. Chapter 7 gives a summary of the key findings and conclusions from the current study, identified limitation and recommendations for future studies. Chapter 8 presents a list of the references cited in the thesis. A list of appendices including a copy of the ethical clearance certificate for the study.

1.1 Introduction

In Sub-Saharan Africa (SSA), there is an increase in the demand of meat, milk and eggs for human consumption (Thornton, 2010). This increased demand is driven by human population growth, expansion of urban settlements and an improvement in incomes (Thornton, 2010). Broiler chicken and pullet production are major contributors to animal-derived protein for human consumption (Olobatoke and Mulugeta, 2011; Bozkurt et al., 2012). Compared to other animal-derived dietary protein sources, chicken meat and eggs are relatively more affordable (King, Osmond-McLeod and Duffy, 2018; Saleh et al., 2018). Chicken meat, due to its low fat, saturated fatty acid and cholesterol content is considered as a healthier product when compared to red meats (Kralik et al., 2018).

The South African poultry industry makes a significant contribution to agriculture's input to the national gross domestic product in 2021, it contributed 16.6% to the national gross domestic GDP [(South African Poultry Association (SAPA), 2021)]. Additionally, the industry provides employment to over 110 000 people (SAPA, 2021). The South African poultry industry's enormous contribution to the economy and job creation emanates from its relative affordability to establish, ease of management and a fair return on investment (SAPA, 2021). Despite the poultry industry's huge footprint nationally, local production of poultry meat fails to meet demand and is currently exacerbated by the outbreak of bird flu (United State Department of Agriculture, 2021). Thus South Africa imports poultry products from Brazil and European Union countries (Jörnling, 2017), thereby draining the country of much needed foreign currency. This reliance to meet poultry meat short falls from imports

not only threatens local poultry producers when poultry products are dumped in the country at prices that undercut local producers, but also negatively impacts job creation.

Several strategies and technologies have been and continue to be utilized in broiler and pullet chicken productivity. The utilization of synthetic antibiotics as poultry feed supplements has been and continues to be one such intervention (Huyghebaert et al., 2011). While some antibiotics have been and continue to be used as therapeutic agents to improve bird health and well-being, routine antibiotic use in the poultry and livestock industry is often for sub-therapeutic purpose, whose aim is to improve growth performance and feed utilisation efficiency (Huyghebaert et al., 2011). In situations where antibiotics use targets to improve livestock productivity, they are viewed as antimicrobial growth promoters (AGPs).
As AGPs, antibiotics mediate improved animal productive performance by suppressing undesirable gastrointestinal tract resident microorganisms that would otherwise drain nutrients meant for the host and cause ill health (Dudek-Wicher et al., 2018). Furthermore, AGPs contribute to improve animal productive performance by mitigating the incidence and severity of subclinical infections and enhancing intestinal nutrient absorption (Brennan et al., 2003). Interestingly, AGPs also reduce the amount of growth-depressing metabolites produced by undesirable gut-resident bacteria (Knarreborg et al., 2004). Antibiotic growth promoters have been shown to inhibit feed- and/or disease-induced inflammation of the small intestines of poultry (Buret, 2010). The observation that AGPs are not effective in germ-free livestock gives credence to the possible mechanisms through which they mediate growth-promoting effects (Adedokun and Olojede, 2019).

Commercial poultry farmers have used and in some instances continue to use AGPs as feed supplements to improve flock gut health (Paul et al., 2022) and enhance productive performance and meat quality (Olobatoke and Mulugeta, 2011; Gadde et al., 2017). Although the use of AGPs yielded tremendous gains with regards to animal productive performance, their introduction in the food chain has caused perceived and serious consumer health challenges and concerns (Sethiya, 2016). This use of AGPs has been observed to be a major cause of the emergence of microbes resistant to antibiotics currently used to cure animal and human microbial infections (Sethiya, 2016; Landers et al., 2012). Due to increased consumer awareness and health concerns arising from the routine use of antibiotics in the food production chain, consumers are objecting to the routine use of these synthetic antibiotics, for

example, penicillins, sulphonamides, tetracyclines and tylosin in livestock production (Valenzuela-Grijalva et al., 2017).

Oxytetracycline has been one of the most commonly used AGPs in poultry production (Odore et al., 2001). It remediates intestinal inflammation (Niewold, 2007) resulting from either feed or disease (Khadem et al., 2014). Intestinal inflammation be it of food or microbial origin, has been shown to compromise growth performance and flock health (Khadem et al., 2014). Literature points to varied effects when oxytetracycline is used as a dietary supplement to fortify broiler chicken diets. At 50 mg oxytetracycline/kg feed it has been reported to elicit an immunosuppressant effect (Al-Ankari and Homeida, 1996) while at 200 mg oxytetracycline/kg feed as it was shown to improve body weight gain and feed intake (Khadem et al., 2014). However, long-term use of AGPs has been shown to cause imbalances in gut flora typified by excessive reproduction of bacteria and concomitant development of endogenous infection (Francino, 2016). Previous studies have demonstrated that routine use of streptomycins, penicillins, sulphonamides and tetracycline elicit allergies and cause cancer, hepatic and renal injury in consumers of the animal products in which the antibiotic residues are found (Rafiq et al., 2022). Furthermore, this routine use of AGPs causes antimicrobial resistance both in livestock and humans. The resistant bacteria populations enter the body through handling of poultry meat and eggs and consumption of antibiotic residue in livestock products (Selaledi et al., 2020).

Globally, antimicrobial resistance is currently responsible for 700,000 deaths per year (Kariuki et al., 2022) and forecasts suggest that it could cause 10 million deaths per year by 2050 (World Health Organization, 2019). This high loss of life due to antimicrobial resistance has led consumers to demand the elimination, from the poultry (livestock) production chain, the routine use of AGPs (Valenzuela-Grijalva et al., 2017; Mak et al., 2022). On the backdrop of the health challenges posed by the use of AGPs, the European Union Commission, instituted and invoked a special regulation (EC Regulation No. 1831/20031) that saw a phased withdrawal and eventual ban in United States of America and European countries (Sweden, Denmark and Canada) because of their use as dietary supplements in livestock feeds (Huyghebaert et al., 2011). The global concern of the negative impacts of AGPs which has seen the phasing out and, in some instances a total ban of antibiotics has not been without

cost to poultry and livestock production. This is in line with the one health approach (Pokharel et al., 2020).

Research has provided evidence demonstrating that removal of AGPs negatively impacts livestock productive performance through increased feed conversion ratios and increased, albeit, subclinical necrotic enteritis (Haque et al., 2020; Abd El-Hack et al., 2022). Abreu et al. (2023) report that since the ban of the use of AGPs in 2006 by the European Union Commission, the poultry industry has seen the emergence of a disease syndrome commonly referred to as dysbacteriosis which is characterised by bacterial overgrowth in the small intestines, maldigestion and malabsorption (Abd El-Hack et al., 2022). The health and environmental challenges that ensue from the use of AGPs and the associated decrease in poultry and livestock productive performance on their withdrawal requires that alternatives be developed. It is crucial that the proposed alternatives resonate with consumer demands, expectations and that they mitigate the public health challenge of antibiotic resistance. Consumers consider phytochemicals as more natural and deem them less toxic than synthetic chemicals (Gadde et al., 2017).

Phytochemicals are plant-derived secondary compounds that protect plants against herbivory and attack by insect pests (Sethiya, Thakore and Mishra, 2009) and microorganisms. They also exhibit biological activities that can be harnessed for use in human health management and livestock production (Park et al., 2023). These phytochemicals, in low doses are considered less toxic, less likely to result in residues in animal products and are therefore seen as potential feed additives in animal food production with potential to replace AGPs (Al-Mnaser et al., 2022; Rafiq et al., 2022).

A multiplicity of phytochemicals has been and are considered for use as natural growth promoters (NGPs) for enhanced poultry and livestock productive performance (Krauze, 2021; (Hotea et al., 2023). It is argued that phytochemicals as NGPs exert their positive effects on poultry and livestock productive performance via several mechanisms improve the flavour and palatability of feed which translates into improved feed intake and productive performance (Valenzuela-Grijalva et al., 2017). However, caution must be exercised as higher dietary phytochemical inclusion could cause reduced feed intake due to odour and flavour tainting (Yan et al., 2011). Importantly, a significant number of the phytochemicals

exhibit antimicrobial activity and are thus hypothesised to reduce the negative effect of undesirable gut-resident microbes on nutrient digestion and absorption (Lillehoj et al., 2018; Shehata et al., 2022). It is argued that through health beneficial activities, inclusive of antimicrobial, and immunomodulatory antioxidant activity, phytochemicals exert health promoting effects on the gastrointestinal tract and the gut-brain axis (Yang et al., 2009). Phytosterols make up one of the dominant groups of phytochemicals.

The phytosterols are unsaponifiable components of edible vegetable oil products and have an hypocholesterolaemic effect (Moreau et al., 2018). β-sitosterol, campesterol and stigmasterol are some of the most common phytosterols which occur either as free isoforms or as esters of free fatty acids, sugar moieties or phenolic acids (Li et al., 2022). Known to be found in high concentration in *Moringa oleifera* LAM, β-sitosterol can potentially be used as a NGP in poultry production. It has been reported to possess antioxidant, antimicrobial, antibacterial, antifungal and immunomodulatory activities (Rajanandh and Kavitha, 2010; Saeidnia, Manayi, and Gohari, 2014) that can potentially improve bird gut morphology and health (Valenzuela-Grijalva et al., 2017). The health beneficial biological activities of β-sitosterol are known to modulate the gut microflora and control the adhesion of antimicrobial pathogens which maintain intestinal epithelium integrity and health status by reducing toxins and increasing the availability of nutrients for absorption (Dhama et al., 2014). Reactive oxygen species produced from lipid peroxidation attack intestinal mucosa compromising nutrient absorption (Valenzuela-Grijalva et al., 2017).

Through its antioxidant activity β-sitosterol eliminates the reactive oxygen species thus helping to maintain a healthier intestinal mucosa and desirable gut microflora population and balance (Valenzuela-Grijalva et al., 2017; Ding et al., 2021). By virtue of its antimicrobial, and antioxidant properties, if used as a dietary supplement, β-sitosterol can potentially reduce the gastrointestinal tract (GIT) microbial load and quench reactive oxygen species thus mitigate feed and microbial induced intestinal oxidative stress. These activities can tremendously benefit chicken production. Use of sterols to fortify poultry diets has been shown to promote growth performance, enhance carcass traits, meat and egg quality and mitigate oxidative stress (Goliomytis et al., 2014; Saeed et al., 2017). Several studies have shown that phytosterols when used as dietary supplements mediated reduced cholesterol absorption by absorptive intestinal enterocytes (Sachiko et al., 2000; Trautwein et al., 2003;

Poli et al., 2021). Importantly and interestingly, β-sitosterol has been reported to inhibit the small intestine cholesterol absorption (Ding et al., 2021). The risk of hypercholesterolaemia that is normally associated with cardiovascular can be reduced with inclusion of β-sitosterol in broiler chicken diets. Reduced cholesterol absoprtion reduces the risk of hypercholesterolaemia and which may prevent cardiovascular diseases (Choudhary and Tran, 2011; Ding et al., 2021). Furthermore, the higher cholesterol present in chicken body may be deposited into the poultry meat and eggs. In older chickens which are prone to increased plasma cholesterol concentrations, β-sitosterol when used as a dietary supplement was shown to reduce cholesterol concentrations in both their serum and liver (Dam et al., 1978). A sharp decrease in the egg yolk cholesterol content was observed when quail were fed diets fortified with 2% β-sitosterol (Dam et al., 1978). The supplemental β-sitosterol-mediated reduction in serum and liver cholesterol concentration in chicken (Kudchodkar et al., 1976) and in quail eggs (Dam et al., 1978) benefits the birds and consumers of products from such supplemented birds. Egg laying hens can be supplemented with high phytosterols doses up to 800 mg/kg feed without key organ or systemic toxicity (Shi et al., 2014; Shen et al., 2015).

Despite the positive effects on bird health, productive performance and product quality, some research has flagged that supplementing poultry diets with β-sitosterol may lead to poor intestinal nutrient absorption in pullets (Kudchodkar et al., 1976) hence the need to closely monitor growth performance in such supplemented birds. Further to reducing nutrient absorption, the β-sitosterol in fortified chicken diets might also be poorly absorbed which would limit its positive effects on gut health and growth promoting effects (Kudchodkar et al., 1976). However, it can be argued that the lower absorption reported by other researchers could be due lower dietary concentration of β-sitosterol used as other researchers have reported positive effects when β-sitosterol is used as a dietary supplement (Mattson, Volpenhein and Erickson, 1977; Dam et al., 1978; Dhama et al., 2014). The health beneficial biological activities of β-sitosterol can potentially be exploited in poultry production by using it as a NGP in place of AGPs to fortify broiler chicken and pullet chicken diets. Past research has demonstrated that when used as a dietary supplement in broiler chicken and fish feeds β-sitosterol improved the growth performance (Naji et al., 2014; Chakraborty et al., 2014). Despite the availability of some data on the positive effects of dietary β-sitosterol on broiler chicken productive performance, there is need for more scientifically validated information regarding its effects in broiler chicken with respect to flock oxidant and antioxidant status, bone, kidney and liver health and meat quality.

1.2 Study aim

This study evaluated the potential effect of β-sitosterol to replace oxytetracycline, a synthetic antibiotic, as a NGP feed supplement, in Cobb 500 broiler diets by assessing its effects on bird performance, meat yield and quality and bird health.

1.3 Study objectives

Specifically, the current study determined the effects of dietary supplementation with β-sitosterol on:

(a) growth (body mass gain, average daily gain, terminal body mass) and meat (slaughter mass, carcass mass and dressing percentage) yield and GIT organ and GIT accessory organs macromorphometry.

(b) physical meat properties (meat pH, colour, water-holding capacity, drip loss, tenderness and myofibrillar fragmentation length and nutrient (proximate, mineral, and fatty acid) content.

(c) long bone health by assessing effects on femora and tibiae lengths, masses, mass to length ratio, breaking strength and mineral (calcium, magnesium, phosphorus and potassium) content.

(d) circulating (blood glucose, cholesterol and triglyceride) and stored (liver lipid) metabolic substrate content, plasma surrogate markers [alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT) activities and total bilirubin concentration] of liver health, plasma surrogate markers (blood urea nitrogen and creatinine concentration) of kidney health and plasma surrogate markers (amylase activity and calcium concentration) of general health.

(e) marker of oxidative stress (plasma malondialdehyde concentration) and markers of natural antioxidant system response [plasma glutathione (GSH), glutathione-S-transferase (GST), glutathione-peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT) activities].

1.4 Hypotheses of the study

a) H_0: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline does not affect chickens' growth performance, and GIT visceral macromorphometry.

H_1: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline affects chickens' growth performance, and GIT visceral macromorphometry.

b) H_0: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline does not affect the birds' meat yield and meat physico-chemical properties.

H_1: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline affects the birds' meat yield and meat physico-chemical properties.

c) H_0: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline does not affect birds' femora and tibia growth, strength and mineral content.

H_1: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline affects the birds' femora and tibiae growth, strength and mineral content.

d) H_0: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline does not affect birds' metabolic substrate content, oxidant and antioxidant status, kidney, liver and general health.

H_1: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline affects the birds' metabolic substrate content, oxidant and antioxidant status, kidney, liver and general health.

e) H_0: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline does not affect birds' oxidant and antioxidant status.

H_1: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline affects the birds' oxidant and antioxidant status.

Having given the background, problem statement and study justification, aim, objectives and hypotheses, the next chapter gives a discourse on the literature pertinent to this study.

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CHAPTER TWO – LITERATURE REVIEW

2.0 Introduction

Not only are poultry meat and eggs the mostly widely consumed animal protein worldwide (Paul et al., 2022), there is an increase globally in the demand of the poultry meat as a source of animal protein for human consumption (Barbut and Leishman, 2022). In order to meet the demand of food for an estimated 10.9 billion of human population by 2050, there is a need for food production (De Wrachien et al., 2021). The increase in the demand of poultry meat is driven by an increase in the human population, expansion of urban settlements and improvement in incomes (Nkukwana, 2018; Hafez and Attia, 2020). In Sub-Saharan Africa (SSA), consumption of poultry meat have been projected to increase by 214% by 2050 (Erdaw, 2023).

In line with global patterns, poultry meat is one of the most common and widely consumed meat in SSA due to its relative affordability compared to other meats (King, Osmond-McLeod and Duffy, 2018). Due to its high protein, low fat, low saturated fatty acid and cholesterol content, poultry meat, specifically broiler chicken meat, is considered to be a healthy product (Kim et al., 2020). However, the poultry industry is facing multiple infectious threats caused by pathogenic microbes. Therefore, healthy chicken production needs proper microbial control to maintain bird health, ensure efficient feed utilisation (Huyghebaert et al., 2011) and for the production of quality meat (Abdulla et al., 2017). Synthetic antibiotics are routinely used to control and treat microbial infections in broiler chicken and other livestock (Mehdi et al., 2018). Van den Honert et al. (2018) contend that misuse, overuse or inappropriate use of antibiotics as growth promoters in poultry and livestock feed creates enormous pressure, which increases and accelerates the genesis of bacteria adapted to these antibiotics that then multiply to produce more resistant populations.

The emergence of antibiotic resistant bacteria due to routine use of antibiotics in poultry production has led to serious public health concerns (Castanon, 2007; Aarestrup et al., 2010) due to the development of pathogenic bacterial resistance. Toghyani et al. (2011) confirmed the challenge of routine antibiotic use as feed supplements in the emergence of antibiotic-resistant bacterial strains, for example, *Salmonella typhymurium*, that could be transferable to consumers. In addition to mediating the emergence of hazardous antibiotic resistance in both poultry/livestock and consumers, use of antibiotics as growth promoters results in the deposition of their residues in meat and eggs as well as in waste from poultry causing

environmental pollution (Abdulla et al., 2017; Ahmad et al., 2017; Tian et al., 2021). The antibiotic residues cause toxic effects including, among others, mediating allergies, triggering immunopathological reactions, carcinogenesis and nephropathy as well the transfer of antibiotic resistant bacteria to humans (Nisha, 2008; Manyi-Loh et al., 2018). It is because of these public health and environmental concerns and the dire for cleaner poultry and livestock production that this "irrational" and routine use of antibiotics faces resistance and, in some instances, has been banned (Dibner and Richards, 2005). Several potential alternatives, among them, phytobiotics, botanical powders and extracts, essential oils and phytochemicals, are touted as alternatives to antibiotics in poultry and livestock production (Sethiya, 2016; Valenzuela-Grijalva et al., 2017).

2.1 Overview of the poultry production industry

The United States of America, Brazil and China are the major producers of poultry products globally (United States Department of Agriculture, 2020). The world poultry meat production is estimated at approximately 140 million metric tons per year (Statista, 2023). In South Africa the total production of poultry meat from duck, geese, guinea fowl and turkey were estimated at 1.915 million tonnes in 2021 (SAPA, 2021). Poultry production has been increasing rapidly worldwide. Between the early 1990s to 2005 the industry recorded a 158% increase in production (Nkukwana, 2018). According to the Organisation for Economic Co-operation and Development and the Food and Agriculture Organization (OECD-FAO, 2023) poultry meat is expected to account for 41% of the protein consumed from all meat sources in 2032. The relatively lower poultry production costs compared to other livestock enterprises coupled with the lower cost per kilogram of poultry meat versus other meats have been and continue to be the main drivers of an increment in poultry production (OECD-FAO, 2019). On a global scale, the current per capita poultry meat is 34.1 kg and this is expected to increase by 15% by 2032 (OECD-FAO, 2021). In the SSA region, the per capita consumption of chicken meat is 3.9 kg and is projected to increase to 9.9 kg per year by 2032 (Statistica, 2023).

In Africa, South Africa is the largest commercial poultry producer followed by Egypt, Morocco and, Nigeria (Nkukwana, 2018). In 2018, South Africa produced 1.9 million tonnes chicken meat higher than the 1.3 million tonnes and 821.5 thousand metric tons of chicken meat for Egypt and Morocco, respectively (Statistica, 2023). In South Africa, poultry meat is

one of the highest consumed animal proteins [(South African Poultry Association (SAPA, 2020)]. Broiler chicken per capita consumption in 2021 in South Africa was at 39.27 kg. and 2.8 million tonnes of broiler chicken meat were produced (SAPA, 2021). Currently, the consumption of poultry meat in South Africa is 37.3 kg per capita (Unted States Department of Agriculture, 2023). The South African poultry industry, as elsewhere globally, plays a significant role in the provision of household food security especially animal derived protein security (Queenan et al., 2021; Mnisi et al., 2021). Chicken meat exports from South Africa made up 96.8% of total poultry meat exports and turkey meat contributed 1.1% while the combination of duck, goose and guinea fowl made the remaining 2.1% (Agriseta, 2023). In 2021, a total of 50 611 tonnes of poultry products (chicken, turkey, ducks, geese and guinea fowl) at the value of R4.712 billion were exported by South Africa (SAPA, 2021). Turkey, geese, duck, guineafowl, and mixed duck-geese-guinea fowl meat exports totalled 553, 242, 57, 6, and 756 tonnes, respectively (SAPA, 2021). Poultry production in South Africa contributed approximately 18.4 % in 2020 to the local agricultural economy and about 16.6% to the country's gross domestic product (SAPA, 2021). In 2021, the South African poultry industry contributed 39.9% of the total agricultural gross value of animal products (SAPA, 2021).

In 2020, the production volume of slaughtered chicken in South Africa amounted to nearly 968 million heads which was an increase from the 966.1 million heads slaughtered in 2019 (Mdletshe and Obi, 2023). In 2016 South Africa slaughtered more than 935 million broiler chicken which was 3.1% less than the number of broilers slaughtered in 2015 (Pilusa, Belete and Baloi, 2020). Despite the seemingly high productivity, the South African poultry industry does not meet local demand for broiler chicken meat and the shortfall is filled by imports from Brazil and EU countries (Jörnling, 2017). The National Agricultural Marketing Council, (2023) reports that first quarter of 2023 South Africa imported 122 455 tons of chicken meat compared to 73 590 tons imported in the last quarter of 2022. This high import bill could be reduced by investing in improvements in local broiler poultry production. Improved chicken breeds have been selected for high growth rate, high breast-meat yield, and a high feed utilisation efficiency (Dawkins and Layton, 2012). Feed costs are critical to the competitiveness of poultry production as they account for up to 70% of the total production costs (Bagopi et al., 2014). It is therefore important that the local poultry industry, particularly broiler chicken producers adopt strategies that enhance and boost the efficiency

with which feed is utilised to get a reasonable return on investment. Such strategies must aim at having antibiotic residue-free poultry products.

2.2 Growth promoters in poultry production

Growth promoters are chemical and biological substances which are added to poultry feed with the aim of improving growth performance and feed utilisation efficiency (Fallah et al., 2013). There are several types of growth promoters including but not restricted to antibiotics, probiotics, prebiotics, exogenous enzymes, antioxidants, and coccidiostats (Dhama et al., 2015). These growth promoters, each exerting a unique mechanism of action to enhance growth performance, are usually administered in relatively low concentrations, ranging from 2.5 mg/kg to 125 mg/kg in feed (Brown et al., 2017). The utilization of growth promoters as a strategy to improve productivity has resulted in great benefits through the meat production chain (Valenzuela-Grijalva et al., 2017). In addition to improving growth performance and enhancing feed utilisation efficiency, these growth promoting substances have also led to an improvement in the meat quality characteristics (Hao et al., 2014). Among the growth promoters employed by the poultry and livestock feed industry, antibiotics have been mostly and continue to be used.

2.3 Antibiotics as growth promoters

Antibiotics are organic substances synthesized naturally by microorganisms, fungi and plants or artificially and they can kill (bacteriocidal) or inhibit the growth (bacteriostatic) of microbes (Abreu et al., 2023). These antibiotics have been incorporated in poultry and livestock feed for decades. In addition to use as growth promoters and enhancers of feed utilisation efficiency, they have also been used as prophylaxis to reduce mortality by regulating intestinal microbiota (Salim et al., 2018). Additionally, the antibiotics used as growth promoters enhance broiler chicken health and well-being by modulating immune system function (Dhama et al., 2015) resulting in enhanced mitigation of gastrointestinal infections such as necrotic enteritis (Mund et al., 2017). They also modify intestinal microbiota populations by promoting the growth of desirable microbial species (Yadav and Jha, 2019; Singh et al., 2013). Mechanistically, antibiotics used as growth promoters have been shown to exert positive effects on performance parameters by reducing the incidence and severity of sub-clinical infections (Rahman et al., 2022) and mitigating nutrient drain in the gastrointestinal tract to gut-resident microbiota, especially in the small intestines (Yadav and Jha, 2019). These antibiotics have also been shown to mediate improved nutrient

absorption by altering the functional morphology of the intestinal wall and reducing the amount of growth-depressing metabolites produced by gram-positive bacteria (Knarreborg et al., 2004).

Oxytetracycline, bacitracin, sulfonamide, penicillin, virginiamycin, neomycin, gentamycin, tylosine, erythromycin, amoxicillin, avilamycin, enrofloxacin, monensin, lincomycin, tiamulin and furazolidone are the antibiotics that are commonly used as growth promoters (Dhama et al., 2015; Mund et al., 2017). These antibiotics are incorporated either in the feed and/ or drinking water (Pokrant et al., 2021; Abreu et al., 2023). The use of antibiotics as dietary supplements has been reported to improve poultry weight by 9 to 14% and feed utilisation efficiency by 3.8 to 6.2% (Plata et al., 2022). Oxytetracycline is one of the first antibiotics to be introduced as a growth promoter (Pokrant et al., 2021) and is one of the most commonly used antibiotics in poultry production because of its low cost, efficacy, and relative low risk of side effects (Odore et al., 2015). Antibiotics are likely to act by remodelling microbial diversity and relative abundance in the intestine to provide an optimal microbiota for growth (Mehdi et al., 2018). Dietary supplementation with virginiamycin has been associated with an increased abundance of *Lactobacillus* species in broiler duodenal loop and proximal ileum (Mehdi et al., 2018) demonstrating that it alters the composition of chicken gut microbiota (Mehdi et al., 2018). Some antibiotics control and limit the growth of *Clostridium perfringens* which is known to be harmful to the host (Lillehoj et al., 2018). Furthermore, dietary virginiamycin has been reported to decrease Salmonella in the duodenal loop (Dumonceaux et al., 2006). Dietary supplementation of broiler chickens with tylosin, a bacteriostat, has been shown to significantly reduce *Lactobacillus* population in the ilea of chicken compared to non-supplemented counterparts (Lin, 2014).

The antibiotic-induced decrease in the *Lactobacillus* reduces the intestinal activity of the bile salts hydrolases increasing relative abundance of conjugated bile salts (Dong and Lee, 2018; Mehdi et al., 2018), resulting in enhanced lipid metabolism, energy harvesting and increased animal weight gain (Lin, 2014). A change in the microbiota of chickens can affect the bird's health. Chicken caeca contain the highest density of microbial biomass (Lv et al., 2021) and changes in the caecal environment impacts fermentation and production of short chain fatty acids which are used as an energy source (Costa et al., 2017). Antibiotics used as AGPs can be exploited to manipulate caecal microbiota populations of chickens and improve growth

efficacy in the chicken through improved nutrient absorption and mitigating pathogenic invasions (Stanley et al., 2014; Huang et al., 2017).

2.3.1 Effect of antibiotic growth promoters on poultry health

The gastrointestinal tract plays a significant role in the digestion and absorption of nutrients required for growth and maintenance of the animal body. The proliferation of pathogens in the intestines often results in inflammatory responses that cause productivity losses and an increase in mortality and poultry meat and eggs (Baurhoo, Ferket and Zhao, 2009). Research has amply demonstrated the impact of AGPs on the chicken gut microflora (Singh et al., 2013). Sub-therapeutic antibiotic doses have been used in broiler diets to control intestinal pathogens and enhance growth performance (Baurhoo, Ferket and Zhao, 2009). Antibiotic growth promoters such as oxytetracycline, salinomycin, bacitracin and virginiamycin are widely used in the starter, grower and finisher phases of broiler chicken production. Their biological effects on birds' growth are most likely derived from effects on the intestinal microbiota by reducing opportunistic pathogens and sub-clinical infections and decreasing microbial competition for host nutrients (Dibner and Richards, 2005). Supplementation of broiler chicken diets with penicillin has been demonstrated to mediate an increase in the proportion of firmicutes type of bacteria from 58.1 to 91.5% but decreased the proportion of bacteriodetes type of bacteria to between 2.9% and 31.1% in the gut of broiler chicken (Singh et al., 2013). These penicillin-mediated changes in the proportion of broiler chicken gut microbiota have been shown to promote bird intestinal health and growth performance (Singh et al., 2013).

2.3.2 Effect of antibiotic growth promoters on broiler chicken meat quality

Meat quality is defined as the sum of the physico-chemical, nutritional, sensory, health and food safety characteristics that could yield greater acceptance and a higher price in the market (Kralik et al., 2018; Nusairat, Tellez-Isaias and Qudsieh, 2022; Choi et al., 2023). Meat colour, which is a function of its lightness (L*), redness (a*) and yellowness (b*), influences the consumers' perception its freshness, quality and acceptability (Popova, 2017). While the meat's fat content is a key nutritional factor to the consumer (Wood et al., 2008), its fatty acid profile and cholesterol content are critical to consumer health (Valenzuela-Grijalva et al., 2017). It is for these reasons that synthetic growth promoters are deployed as both a production strategy and a technology to decrease fat deposition in the meat in order to satisfy consumer preference for lean meat (Walker et al., 2005). Poultry meat has numerous

desirable nutritional characteristics: low lipid content and relatively high concentrations of PUFAs. Fatty acid composition is an important component of meat quality, associated with its dietetic value. However, lipid oxidation is a major cause of meat quality deterioration, resulting in rancidity and the formation of undesirable odours and flavours, which lowers the functional, sensory and nutritive values of meat products; and therefore, consumer acceptability (Huang and Ahn, 2019).

Antibiotics as dietary supplements reportedly improve meat quality by decreasing the meat's fat content (Abdulla et al., 2017). However, routine therapeutic and or prophylactic use of antibiotics in poultry production has been shown to result in the deposition of significant quantities of antibiotic residues in chicken meat (Kabir et al., 2004; Abreu et al., 2023). Deposited antibiotic residues in meat compromise its colour and decrease tenderness (Mehdi et al., 2018; Thema et al., 2019). Use of antibiotics as growth promoters has been shown to increase the concentration of polyunsaturated fatty acids (PUFAs) in the meat (Valenzuela-Grijalva et al., 2017). Nutritionally higher dietary PUFAs enhance consumer health but an increased degree of poultry muscle (meat) membrane unsaturation increases its susceptibility to peroxidation which leads to deterioration of meat colour, odour, texture, flavour and nutritional value (Goliomytis et al., 2015).

2.3.3 Challenges associated with antibiotic growth promoters in poultry production

As highlighted earlier, the use of antibiotics in poultry and livestock production for growth promotion and disease prevention has been demonstrated to cause to the development of antibiotic resistant bacteria in poultry, livestock and humans (Marshall and Levy, 2011). This emergence of antibiotic-resistant bacteria has become a global public health concern. The challenge of antibiotic resistance has been exacerbated by the "misuse" of antibiotics as growth promoters in poultry and livestock feeds (Mehdi et al., 2018; Van den Honert et al., 2018). Consumption of poultry meat and or eggs with high tetracycline residues has been shown to cause subnormal foetal development, staining teeth in young children and gastrointestinal and autoimmune disorders (Lawal et al., 2015; Mund et al., 2017). Moreover, it can also result in allergic reactions, an imbalance in the intestinal microbiota population and development of antibiotic-resistant bacteria that are transmitted to the environment and to consumers (Mund et al., 2017; Muaz et al., 2018; Ma et al., 2021). Several studies have reported the transfer of genes responsible for resistance against antibiotics from animals to human microbiota (Muaz et al., 2018; De Mesquita Souza Saraiva et al., 2022).

Irrefutable evidence shows that antibiotic resistant genes can be transmitted from animal microbiota to human microbiota (Ma et al., 2021) setting up a chain of zoonoses. Additionally, it has been demonstrated that antibiotic resistant genes present in the gut microbiome can be exchanged between the resident species and can be horizontally transferred from pathogenic species (Francino, 2016). *Escherichia coli* (*E. coli*), a member of the enterococci, is an important indicator of antibiotic resistance and is frequently found on food surfaces (European Food Safety Authority, 2015). In addition to the risk of direct infection by antibiotic resistant bacteria, there is also the added challenge of toxicity caused to consumers by drug residues in poultry and livestock products. The intake of nitrofuran metabolites via poultry products has been shown to cause toxicosis and to have carcinogenic side effects (Meskarpour Amiri et al., 2014). Other drug residues such as nitroimidazole and 3-nitrofuran have also been shown to cause different cancers in humans (Steven and Sundlof, 1994; Teuber, 2001; Anderson et al., 2003). Furthermore, consumption of drug residues through chicken products creates a vicious circle of challenges as it leads to the production and proliferation of drug-resistant bacteria in human beings that cause therapeutic failures (Nisha, 2008; Karmi, 2014), which increases the cost of health delivery. Furthermore, they have been shown to elicit gastrointestinal disorders and to mediate pro-inflammatory, cytotoxic, and immuno-pathological effects (Idowu et al., 2010; Lawal et al., 2015). Penicillin residues in poultry products are associated with increased risk of severe anaphylactic reactions such as skin allergies in consumers (Idowu et al., 2010).

The strain on the public health delivery imposed by the challenge of antibiotic resistance is huge and this leads to serious economic loss. The European Union reports that annually antibiotic resistance mediated increase in medical costs due to decreased efficacy of antibiotics equates to €1.5 billion in hospital costs (European Commission Public Health, 2022). In addition to increased hospital costs, the EU reports that 35 000 patients die annually due to infections caused by antibiotic resistant bacterial strains (European Centre for Disease Prevention and Control, 2022). Loss of human life due to poultry and livestock mediated genesis of antibiotic-resistant bacteria is not limited to the EU region. In the United States of America, over 2.8 million people become infected with “antibiotic resistant bacteria” annually and at least 48 700 of them die as a direct result of these infections (European Centres for Disease Control and Prevention, 2019). The World Health Organization (2019) has reported increased incidence of antibiotic-resistance in the continent of Asia accounting for over 50 000 deaths annually. In the developing countries, there is poor monitoring of the

effects of use of antibiotics as feed supplements on human and livestock health due to resource constraints, but this does not wish away the problem.

In South Africa, the use of antibiotics in the poultry and livestock production has been in place since the 1990s and a whopping 29% of the antibiotics are delivered in the form of feed premixes and intensively used in chicken production (Henton et al., 2011; Selaledi et al., 2020). Henton et al. (2011) have reported high levels of resistance to tetracyclines, fluoroquinolones and sulphonamides in the South African poultry industry. Antibiotic resistance in C*ampylobacter jejuni* isolated from chicken abattoirs in KwaZulu-Natal showed high resistance (>95%) to tetracyclines and ceftriaxone (Henton et al., 2011). The threat to human, poultry and livestock health from use of antibiotics, especially at sub-therapeutic doses in the promotion of poultry and livestock productivity is real. Withdrawal of the use of antibiotics as grow promoters in poultry production has been shown to compromise growth performance, feed utilisation efficiency and product quality (Cowieson and Kluenter, 2019). However, their continued use has a huge cost on human health and on the cost of public health delivery. Due to serious concerns around the safety of the use of antibiotics in poultry and livestock feeds, some countries, Sweden for example, have banned their use in the food production chain (Teuchert et al., 2014). The World Health Organisation (WHO) has called for the reduction and ultimate removal of the use of antibiotics as growth promoters in poultry and livestock feed (World Health Organization, 2019). In order to address consumers concerns around the negative effects of antibiotic use, there is need to search for and develop alternatives. The envisaged alternatives should mimic the beneficial effects of synthetic antibiotics but without eliciting microbial resistance and the problem of toxicity associated with antibiotic residues in poultry and livestock products. This is more so considering that poultry meat and eggs still represent a major part of animal protein consumed by humans (Nkukwana, 2018). It is necessary to consider and evaluate the efficacy of potential natural growth promoters (NGPs) to replace synthetic antibiotic growth promoters in poultry and livestock production.

2.4 Natural growth promoters

Broadly, phytobiotics are natural growth promoters (NGPs). Several natural compounds, among others, essential oils, plant-derived extracts and phytochemicals, have potential to replace AGPs in poultry diets (Gadde et al., 2017) due to their antibacterial, antifungal, antioxidant, GIT microbiome and immune modulating activities (Lillehoj et al., 2018). Thus,

the biological activities of these natural compounds can potentially improve poultry health and protect against various infectious diseases by enhancing gut health and bird productivity (Sethiya, 2016). These natural compounds, whose biological activities mimic synthetic antibiotics, can potentially mitigate nutrient by gut resident microbiota (Mehdi et al., 2018). Research has also demonstrated that some of these potential natural growth promoters are immunomodulatory and so inhibit the production and excretion of cytokines by macrophages and also suppress the proliferation of gut microbiota that elicit inflammatory responses (Huyghebaert et al., 2011; Sethiya, 2016). Plant-derived feed additives have a high content of natural antioxidants that can be tapped into to enhance poultry growth performance and feed utilisation efficiency without compromising product quality (Pashtetsky et al., 2019). These plant-derived organics have been shown to disrupt microbial cell membranes and to reduce microbial virulence (Jamroz et al., 2003). Importantly, these natural compounds, in addition to stimulating the proliferation of beneficial gut microbiota (Sethiya, 2016), have also been shown to stimulate the growth and proliferation of GIT ileal enterocyte absorptive cells and to elicit immune-stimulation which results in improved GIT health (Yadav and Jha, 2019). Some of the natural compounds with potent health beneficial biological activities are phytochemicals. Figure 2.1 below is an illustration of various classes of natural growth promoters and antibiotic alternatives.

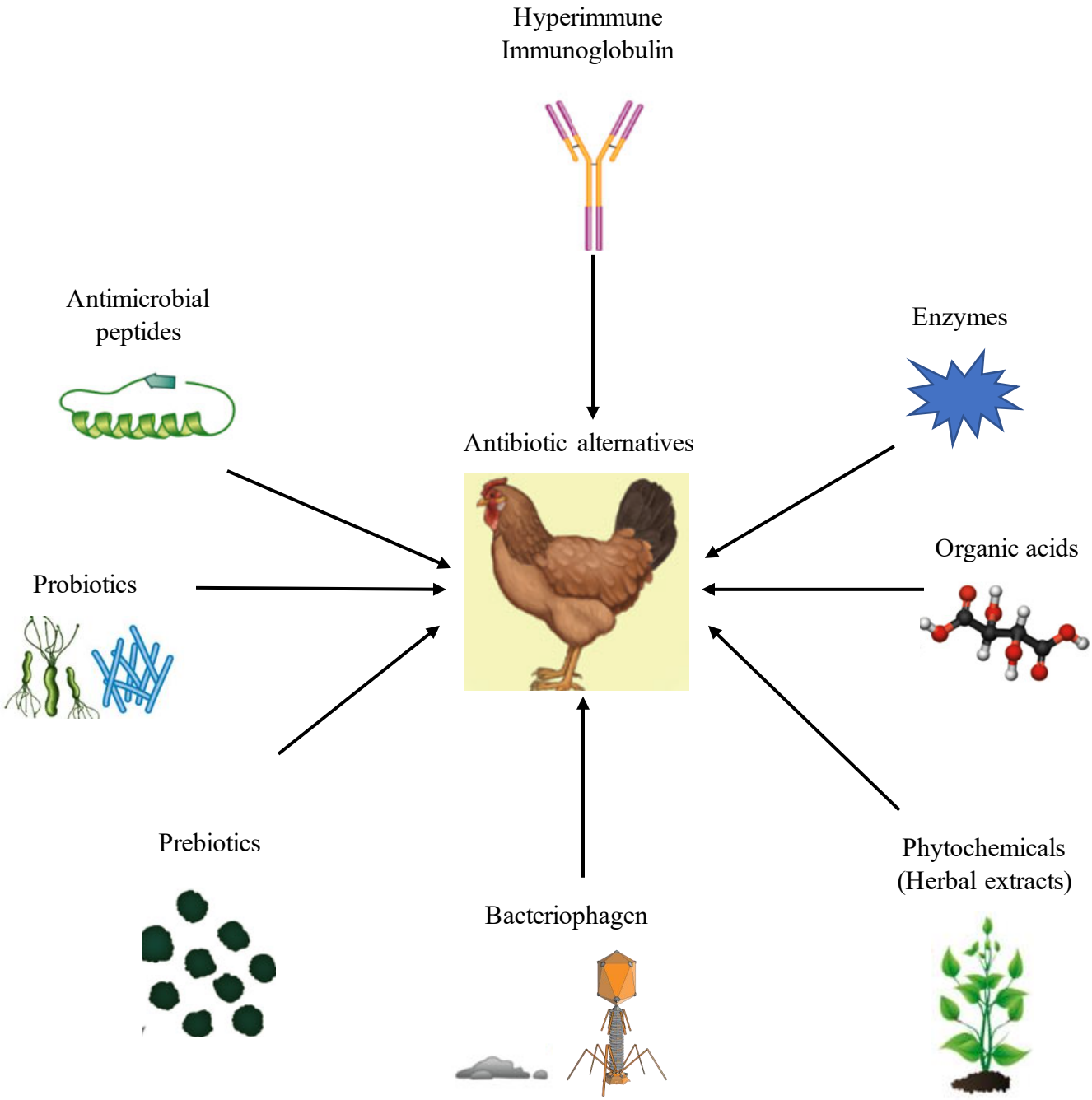

Figure 2.1: Antibiotics alternatives used in poultry production (Modified from Gadde et al., 2017).

2.4.1 Phytochemicals: effects on poultry productivity and health

Phytochemicals are secondary plant metabolites that exhibit health beneficial biological activities (Sethiya, Thakore and Mishra, 2009) that essentially protect plants against herbivory and insect pest attack (Sethiya, 2016). These compounds inhibit the growth and proliferation of harmful GIT-resident bacteria (Sethiya, 2016). They also stimulate the production and secretion of digestive enzymes by activating small intestinal and exocrine pancreatic cells secretory activity resulting in improvement in digestion, nutrient absorption

and growth performance (Sethiya, 2016). Dietary supplementation with phytochemicals positively impact broiler chicken growth performance and feed utilisation efficiency (Nkukwana et al., 2014; Dhama et al., 2015). The beneficial effects of phytochemicals accrue when dietary supplementation with the target phytochemical is made at all growth stages (Valenzuela-Grijalva et al., 2017). In some instances, dietary supplementation with a single purified phytochemical, for example, quercetin, genistein, and or isoflavone individually, has produced adverse effects (Jiang et al., 2007; Kamboh and Zhu, 2014). Dietary supplementation with quercetin and/ or Camellia leaf extract compromised growth performance, and feed utilisation of poultry and livestock (Marcinčáková et al., 2011). The difference in the outcomes of dietary supplementation with phytochemicals suggests a dire need to carefully evaluate the efficacy and potential benefits of each of these compounds.

Herbal medicines have been used extensively in ethnomedicine and ethnoveterinary medicine for the maintenance of human and poultry/livestock health (Dhama et al., 2015). In the poultry industry, herbal plants such as *Azadirachta indica, Allium sativum, Oreganum compactum, Thymus vulgaris, Piper nigrum, Zingiber officinale, Rosmarinus officinalis* and *Cinnamomum zeylanicum,* have been utilised as ethnoveterinary medicines due to the presence of phytochemical constituents with health beneficial biological activities inclusive of antioxidant, antifungal, antibacterial, antiviral, anti-inflammatory, anticoccidial and immunomodulatory activities (Sethiya, 2016; Valenzuela-Grijalva et al., 2017). In poultry, supplementation with dietary mannanoligosaccharides elicits colonization of the ileum with beneficial bacteria thus conferring intestinal health (Baurhoo, Ferket and Zhao, 2009). The phytochemical additives have been reported to have immunostimulatory and antioxidant effects, which result in favourable conditions for poultry/livestock health (Valenzuela-Grijalva et al., 2017).

Positive effects of the phytochemical supplementation have been reported on intestinal morphology of broilers chicken (Valenzuela-Grijalva et al., 2017). Supplementing poultry feeds with phytochemicals has been reported to improve gut health by reducing intestinal inflammation (Karadas et al., 2014; Pirgozliev et al., 2018). Their mode of action is associated with improved availability of dietary energy, enhanced immune status and hepatic antioxidant content of the birds (Pirgozliev et al., 2018). Additionally, Rubio, (2019) propose that phytochemical feed additives may act by over-expressing antioxidant enzymes, which might down-regulate the inflammatory process. Several studies have also reported the

significant improvement in the intestinal villus/crypt ratio of broiler chicken reared diets fortified with phytochemicals (Khalaji et al., 2011; Amad, Wendler and Zentek, 2013). Furthermore, broiler chickens fed diets supplemented with thymol, a phytochemical, had oxidative stress typified by lower malondialdehyde (MDA) content in their duodenal mucosa and better intestinal integrity (Placha et al., 2014).

2.4.2 Phytochemicals: effect on chicken meat quality

The shelf life of meat is enhanced when lipid peroxidation is kept in check and or is slowed (Mutwakil, 2011; Velasco and Williams, 2011). Lipid peroxidation by free radicals is a key mechanism in the deterioration of meat quality (Kanner, 1994). This oxidative deterioration is initiated in polyunsaturated fatty acid fraction of the meat leading to generation of hydroperoxides (Valenzuela-Grijalva et al., 2017). These hydroperoxides decompose further to other compounds such as aldehydes, ketones and other oxygen-carrying freed radicals leading to further deterioration of the meat (Maraschiello et al., 1998). The free-radical mediated deterioration of the meat manifest with development of poor colour, off flavours and a loss of nutritional value culminating in reduced shelf life (Ponnampalam et al., 2022).

While increasing the concentration of PUFAs in meat through dietary manipulation has health benefits (Simopoulos, 2000), this increase in PUFAs increases the risk of lipid peroxidation through oxidative deterioration (Engberg et al., 1996). Dietary supplementation of broiler chicken feed with isoflavone, a soyabean-derived phytochemical, increase the WHC and pH of the meat (Jiang et al., 2007). An increase in the meat's WHC is associated with the improvement of tenderness and juiciness (Mir et al., 2017). An increase in meat pH results in higher WHC and causes meat to have a more compact structure that prevents oxygen diffusion in the muscles. Oxygen diffusion in muscle leads to dark meat colour (Petracci et al., 2019). Thus, dietary supplementation with soy isoflavone increased the meat's lightness improved the WHC and pH of broiler chicken breast meat (Jiang et al., 2007). Due to the negative effects of synthetic antioxidants on consumer health, considerable interest on the use of natural antioxidants, for example, tocopherols, carotenoids as supplements to preserve and improve meat quality, has increased (Yanishlieva and Marinova, 2001). In addition to mitigating the challenge of antibiotic and synthetic antioxidant residues in the meat, natural alternatives lead to a cleaner environment (Yanishlieva and Marinova, 2001). Oregano essential oil contains high amounts of thymol and carvacrol (Contreras-

Castillo et al., 2007). These compounds inhibit free radicals and delay lipid oxidation by giving off hydrogen atoms (Dragoev, 2024).

The use of natural antioxidants extend shelf life and increase the acceptability of meat during retail display (Fellenberg and Speisky, 2006; Simitzis et al., 2011). Dietary supplementation with lipo-soluble vitamin E as an antioxidant promote the uniform incorporation of tocopherol into the subcellular membranes where it effectively inhibits oxidative reactions at local sites resulting in improved meat quality due to delayed lipid oxidation and muscle discoloration (Simitzis et al., 2011). Ri et al. (2017) reported that use of Oregano extract as a dietary supplement improved the systemic antioxidant capacity of broiler chicken leading to effective inhibition of lipid peroxidation resulting in better quality meat. It has also been observed that dietary supplementation with rosemary, sage and oregano improved the oxidative stability and reduced lipid peroxidation of raw and pre-cooked broiler chicken meat in refrigeration (Botsoglou et al., 2002). It is important to note that these herbs contain phytochemicals with significant antioxidant activity.

2.5 Phytosterols and poultry production

Phytosterols are bioactive compounds found in foods such as fruits and vegetables and have a similar structure to cholesterol (Bo et al., 2015; Cabral and Klein, 2017). They are widely used in human and livestock feeds because of their cholesterol-lowering properties. The most predominant physterols that positively impact human health and nutrition are β-sitosterol, stigmasterol, campesterol (Saeidnia et al., 2014). β-sitosterol is the most abundant phytosterol and is present in almost all plant foods (Cheng et al., 2020). In livestock production, phytosterols have been approved as functional feed additives by the Chinese Ministry of Agriculture since 2008 (Zhao et al., 2019). Several studies reported that, when used as feed/food supplements, phytosterols, for example β-sitosterol, stigmasterol, campesterol and brassicasterol significantly lower serum total cholesterol and low-density lipoprotein cholesterol (Cheng et al., 2020; Feng et al., 2020; Poli et al., 2021).

The physiological effects of phytosterols have been considered extensively but public health recommendations regarding their intake remain disputed (Li et al., 2022). It has been stated that phytosterols improve antioxidant capacity, immune function, and intestinal morphology of pigs and chicken (Hu et al., 2017; Cheng et al., 2020). In broiler chickens, it has been demonstrated that phytosterols improve growth performance and meat quality (Naji et al.,

2013; Zhao et al., 2019). Meat with a high proportion of PUFAs is favourable due to its positive health impacts on consumers (Cartoni Mancinelli et al., 2022). However, high PUFAs content make meat more susceptible to lipid peroxidation which reduces both quality and shelf life hence intervening with phytosterols could come in handy. While fortifying poultry diets with phytosterols has been proven to enhance gut health, promote growth and boost productive performance, (Bo et al., 2015; Saeed et al., 2017; Zhao et al., 2019) the phytosterols' mode of action is not as yet fully known. β-sitosterol, one of the many phytosterols with health beneficial biological activities can potentially be used as a natural growth promoter in poultry production.

2.5.1 Effect of phytosterols on broiler chicken health

Phytosterols play an important role in immune regulation and exert positive effects on disease resistance and anti-inflammatory activity (Nashed et al., 2005; Zhao et al., 2019), which may have a benefit to the health status of poultry and livestock. In addition, consumption of phytosterols improved the growth performance of broiler chickens as shown by improved feed intake and final body weight (Naji et al., 2014). Hamady et al. (2015) reported the increase in body weight and weight gain in broiler chickens fed diets supplemented with 10 g/kg of phytosterol-rich pomegranate peel extract. Several studies reported that phytosterols could act as modest radical scavengers, enhance the activities of peroxide dismutase (SOD) and glutathione peroxidase (GSH-Px); the natural antioxidant enzymes (Yoshida and Niki, 2003; Vivancos and Moreno, 2005; Panda et al., 2009) synergistically mitigate oxidative stress and hence reduce MDA concentration; the end product of lipid peroxidation (Zhao et al., 2019).

Dietary phytosterols increase the relative abundance of probiotics and inhibit the growth of harmful bacteria in the intestine of broilers (Feng et al., 2020). *Lactobacillus,* a widely known probiotic, has been shown to help maintenance of paracellular permeability which enhances the mucous layer and stimulates the immune system mediating improved animal health (Liu et al., 2015; Mehdi et al., 2018; Wickramasuriya et al., 2022). Dietary phytosterols have been shown to decrease the relative abundance of undesirable gut microbiota (Feng et al., 2020) and also to positively impact intestinal morphology of broiler chicken (Ding et al., 2021).

2.5.2 Effect of phytosterols on chicken meat quality

Poultry meat from broiler chicken fed diets supplemented with phytosterols has been shown to have an increased PUFA: SFA ratio which improved meat quality (Naji et al., 2014; Ding et al., 2021). Several studies shows that dietary supplementation with phytosterols significantly decreased meat lightness (L^*) 24 h post-slaughter and reduced drip loss of chicken breast meat at 24 and 48 h post-slaughter (Cheng et al., 2019; Zhao et al., 2019). Duck and chicken meat from bird fed diets supplemented with phytosterols exhibited decreased meat drip loss at 24 and 48 h post-slaughter (Wu et al., 2012; Zhao et al., 2019). Decreased meat drip loss helps prevent loss of valuable micronutrients, flavour compounds and water (Ding et al., 2021).

There is an increase in the demand for functional poultry products enriched with PUFAs. Meat with a high proportion of PUFAs is favourable due to its positive health impacts on consumers (Yasin et al., 2012). However, high PUFAs content can make meat more susceptible to lipid peroxidation which reduces both quality and shelf life despite the PUFAs' positive impact on consumer health, hence interventions with phytosterols could be of benefit. While fortifying poultry diets with phytosterols has been proven to enhance gut health, promote growth and boost productive performance (Bo et al., 2015; Saeed et al., 2017; Zhao et al., 2019) the phytosterols' mode of action is not as yet fully known. β-sitosterol, one of among the many potential phytosterols with health beneficial biological activities, can potentially be used as a natural growth promoter in poultry production.

2.6 β-sitosterol and its biological activities

β-sitosterol's structure is similar to that of cholesterol which is found in abundance in plant oils, seeds and nuts, cereals and legumes (Baskar et al., 2012). It exhibits antioxidant, antimicrobial, antibacterial, antifungal, anti-inflammatory, immunomodulatory and hypocholesterolaemia activities (Saeidnia, 2014). β-sitosterol competitively inhibits the absorption of cholesterol from the intestine thus increases hepatic excretion of cholesterol into bile (Cheng et al., 2020). Additionally, β-sitosterol improves immune function by increasing weight of thymus, spleen and/ or bursa fabricius and immunoglobins content in the intestine (Cheng et al., 2020). β-sitosterol improves cellular antioxidant capacity by scavenging free radicals and activating antioxidant signalling pathway, which may also contribute to the improved redox status (Cheng et al., 2020; Feng et al., 2020; Xie et al., 2022). It also mitigates inflammation by inhibiting the survival of pathogenic bacteria and

maintaining intestinal barrier function and integrity (Ding et al., 2019; Xie et al., 2022). Thus, through the aforementioned biological activities β-sitosterol potentially could enhance poultry and livestock health and welfare. In addition to its other stated biological activities, β-sitosterol lowers plasma LDL cholesterol levels (Jones and AbuMweis, 2009). Broiler chicken breast meat from birds fed diets supplemented with β-sitosterol exhibited improved antioxidant status (Cheng et al., 2019; Zhao et al., 2019) which translates to improved shelf life. The observed improvement in the meat's antioxidant status is attributed to β-sitosterol's ability to directly neutralise free radicals and/ or its ability to donate hydrogen atoms (Cheng et al., 2019; Zhao et al., 2019; Ding et al., 2021).

2.6.1 β-sitosterol: potential to enhance poultry production and meat quality

Fortifying broiler poultry diets with β-sitosterol improve growth performance, feed utilization efficiency, and enhance meat quality of Ross 308 and Arbor Acres broiler chickens (Naji et al., 2014; Cheng et al., 2019). Meat from poultry fed diets supplemented with β-sitosterol reduced lightness and cooking loss, increased pH and decreased breast meat drip loss at 24 h post-slaughter (Cheng et al., 2019; Zhao et al., 2019). While some improvement in poultry growth performance and feed utilisation efficiency has been reported with dietary supplementation with β-sitosterol, a more focused interrogation of the potential beneficial effects of dietary β-sitosterol in broiler chicken productive performance, health and meat quality needs to be done.

The next chapter gives a narrative on the first experiment with content focusing on the effects of supplemental β-sitosterol on the growth performance, and viscera macromorphometry of Cobb 500 broiler chicken.

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CHAPTER 3 – EFFECT OF REPLACING OXYTETRACYCLINE WITH β-SITOSTEROL ON GROWTH PERFORMANCE, FEED INTAKE AND UTILIZATION EFFICIENCY AND MACROMORPHOMETRY OF COBB 500 BROILER CHICKENS

Preamble

3.1 Aim of the study

The study aimed to evaluate the effect of dietary β-sitosterol on growth performance, feed intake and utilization efficiency, feed conversion ratio and viscera macromorphometry of Cobb 500 broiler chicken.

3.2 Objectives of the study

The objectives of this study were to evaluate the effect of dietary β-sitosterol on:

a) growth performance (body mass gain, average daily gain and terminal body mass)

b) feed utilisation efficiency (daily feed intake and feed conversion ratio) during the starter, grower and finisher phase

c) viscera gastrointestinal tract and accessory organs of broiler chicken (masses and lengths where appropriate)

3.3 Hypotheses of the study

a) H_0: Dietary supplementation with β-sitosterol does not affect the growth performance (body mass, average daily gain and terminal body mass) of broiler chicken

   H_1: Dietary supplementation with β-sitosterol affects the growth performance (body mass, average daily gain and terminal body mass) of broiler chicken

b) H_0: Dietary supplementation with β-sitosterol does not affect feed utilization efficiency (feed intake and feed conversion ratio) of broiler chicken

   H_1: Dietary β-sitosterol improves feed utilization efficiency (feed intake and feed conversion ratio) of broiler chickens

c) H_0: Dietary supplementation with β-sitosterol had no effect on viscera gastrointestinal tract and accessory organs of broiler chicken

   H_1: Dietary supplantation with β-sitosterol affects viscera gastrointestinal tract and accessory organs of broiler chickens.

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3.4 Abstract

Antibiotics are used to fortify broiler chicken feeds as growth promoters. Chronic antibiotic use pollutes the environment and causes the development of antibiotic resistance. Natural alternatives that mimic the properties of antibiotics, without causing health and environmental challenges are required. β-sitosterol has antimicrobial, antioxidant, digestive and immune system modulating and growth stimulating activities. We evaluated its potential to replace oxytetracycline as a growth-promoter in broiler chicken feeds. Two hundred and forty, one-day-old Cobb 500 broiler chicks were randomly allocated to four diets where β-sitosterol replaced oxytetracycline at 0 mg/kg (control; fortified with 50 mg/kg oxytetracycline), 500 mg/kg, 1000 mg/kg and 1500 mg/kg (w/w) feed and fed for 6 weeks: 2 weeks for each growth phase. Each diet was replicated thrice with 20 chicks per replicate. Initial, weekly and terminal body mass (TBM) and daily feed intake (FI) were measured. Body mass gain (BMG), average daily gain (ADG) and feed conversion ratio were computed. Terminally, the chickens were fasted for 4 h then slaughtered and dressed. Gastrointestinal tract (GIT) and GIT accessory viscera masses and small and large intestine lengths were measured. Dietary fortification with β-sitosterol had similar effects ($P > 0.05$) to oxytetracycline (50 mg/kg of feed) on the chickens' TBM, BMG, ADG, FI and utilisation efficiency and GIT organ macromorphometry. In conclusion, β-sitosterol can replace oxytetracycline in Cobb 500 broiler chicken feeds without compromising growth performance, feed intake and utilisation efficiency and GIT organ growth and development.

3.5 Introduction

The global human population is predicted to be 10.9 billion by 2050 and the demand for food is expected to increase by 70% (Wrachien et al., 2021). According to Lynch et al. (2018) meat production should increase by 200 million tons to reach 525 million tons annually to meet the demand of the growing global population by 2050. In sub-Saharan Africa the increase in the demand of chicken meat and eggs (Nkukwana, 2018) is driven by an increase

in population, expansion of urban settlements and improved economic status of the younger populace (Wickramasuriya et al., 2022). Chicken meat is one of the most common and widely consumed meats (Wahyono and Utami, 2018) and together with chicken eggs make a significant source of animal-derived protein for human consumption. Compared to beef, pork, lamb, mutton and chevon, broiler chicken meat and eggs are relatively more affordable (King, Osmond-Mcleod, et al., 2018). Importantly, chicken meat compared to red meats has lower fat, saturated fatty acid and cholesterol content which from a consumer health perspective, is nutritionally a better product (Zhao et al., 2019). Despite chicken meat being relatively more affordable and nutritionally superior compared to other meats, the South African poultry industry fails to meet local demand of broiler chicken meat such that the country resorts to imports to make up for the shortfall (Jörnling, 2017).

In order to enhance broiler chicken productivity and improve profitability, antibiotics are often used at sub-therapeutic doses as growth promoters in broiler chicken feeds (Mehdi et al., 2018). Antibiotics boost broiler chicken productivity by inhibiting the growth of harmful intestinal microbes and promoting growth of favourable intestinal gut microbiota (Lillehoj et al., 2018). Antibiotic-induced reduction in unfavourable gut microbiota results in improved gut health and reduced inflammation of the GIT mucosa translating into increased functional efficiency (Mehdi et al., 2018) characterised by increased digestion, absorption and assimilation of nutrients (Kallam and Sejian, 2021). However, use of antibiotics as growth promoters in the production of broiler chicken and other livestock is a key driver of the development of antibiotic resistant bacteria, in addition to polluting the environment with antibiotic residues in chicken waste (Selaledi et al., 2020). In addition to causing the development of antibiotic resistant bacteria, the accumulation of antibiotic residues in chicken meat and eggs negatively impacts human health (Mund et al., 2017). The threat of antibiotic-resistant pathogens and residual environmental pollution has led to a growing interest in the exploration and development of alternative growth promoters for use in poultry production (Mehdi et al., 2018).

Phytochemicals are secondary plant metabolites produced to mitigate herbivory and infection by microorganisms (Sethiya, 2016). They are potential targets to replace antibiotics as feed-based growth promoters in chicken production due to their health beneficial biological activities that mirror those of antibiotics (Valenzuela-Grijalva et al., 2017). In addition to

mirroring the biological activities of antibiotics, phytochemicals have been shown to boost feed flavour thereby increasing feed intake (Valenzuela-Grijalva et al., 2017). Phytochemicals also stimulate secretion of digestive enzymes; resulting in enhanced broiler chicken production efficiency (Alagawany et al., 2021). However, supplementing broiler chicken diets with some phytochemical feed additives at dietary inclusion levels greater than 1500 mg/kg feed (w/w) has been reported to compromise feed intake due to the phytochemical-induced strong odour and flavour (Valenzuela-Grijalva et al., 2017).

One of the well characterised phytochemicals is β-sitosterol, known as (3S,8S,9S,10R,13R,14S,17R)-17-[(2R,5R)-5-ethyl-6-methylheptan-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol), a phytosterol which possesses antioxidant, antimicrobial, antibacterial, antifungal and immunomodulatory activities (Saeidnia, Manayi and Gohari, 2014). β-sitosterol has been shown to modulate and reduce adhesion of pathogenic gut bacteria and to increase nutrient availability to the host (Dhama et al., 2014). We hypothesised that β-sitosterol can potentially replace oxytetracycline as a growth-promoting supplement in broiler chicken feeds hence we evaluated its effects on broiler chicken growth performance, feed intake and feed utilisation efficiency. Previous studies have shown that dietary β-sitosterol has been successfully utilized as a growth promoter in Arbor Acres (Xie et al., 2022), White feather broiler (Ding et al., 2021) Yellow-feather (Feng et al., 2020), Partridge Shank (Zhao et al., 2019), Arbor Acres (Cheng et al., 2019) and Ross 308 broiler chicken strains (Naji et al., 2014). In this study, the Cobb 500 broiler chicken breed was selected as it has a good growth rate and good performance on low cost feed rations (Awad et al., 2020). Furthermore, the breed is internationally recognised as one of the world's most production efficient breed for meat production (Singh et al., 2021). It is also widely used in Africa (Cobb, 2022). Brazil is one of the main contributors of broiler chicken meat in the world, they produce about 60% of the Cobb 500 broiler chicken with a production of 14.3 million tonnes per year (Miller et al., 2022). Despite the reported successful utilization of the β-sitosterol as a growth promoter in different broiler chicken breeds, its potential has not been evaluated on the productive performance of Cobb 500 broiler chicken breed.

3.6 Materials and methods

3.6.1 Ethical clearance and study site

Ethical clearance for the study was granted (AREC number: 2018/50/10/B) by University of the Witwatersrand's Animal Research Ethics Committee. The feeding trial was conducted at the Wits Research Animal Facility in Johannesburg, South Africa.

3.6.2 β-sitosterol, oxytetracycline and feed ingredients

β-sitosterol (purity: ≥70%) was obtained from Sigma-Aldrich Private Limited, Germany. Groenvoer product cc (Olifantfontein, Pretoria, South Africa) supplied the oxytetracycline. Yellow maize grain, soyabean meal, canola oil, wheat bran, feed grade limestone, dicalcium phosphate, choline chloride, and sodium chloride were purchased from Obaro Animal Feed Manufacturers (Pty Ltd), Pretoria, South Africa. Corn gluten meal (60%) was bought from Tongaat Hullet Starch (Germiston, South Africa). The vitamin-mineral premix, synthetic lysine and methionine were purchased from Trouw Nutrition (Isando, Johannesburg, South Africa).

3.6.3 Diet formulation

The diets were formulated to meet the nutritional requirements of broiler chickens at starter, grower, and finisher growth stages as per National Research Council (NRC, 1994) recommendations. Table 3.1 shows the ingredient and chemical composition of the starter, grower, and finisher diets.

Table 3.1: The ingredient and chemical nutrient composition of the starter, grower and finisher diets

Ingredients	**Starter**				**Grower**				**Finisher**			
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**
Yellow maize meal (g/kg)	447.94	447.94	447.94	447.94	494.09	494.09	494.09	494.09	520.65	520.65	520.65	520.65
Soyabean meal (g/kg)	383.95	383.95	383.95	383.95	264.69	264.69	264.69	264.69	225.62	225.62	225.62	225.62
Wheat bran (g/kg)	18.28	18.28	18.28	18.28	105.88	105.88	105.88	105.88	121.49	121.49	121.49	121.49
Corn gluten meal (g/kg)	118.84	118.84	118.84	118.84	88.23	88.23	88.23	88.23	82.44	82.44	82.44	82.44
Canola oil (g/kg)	-	-	-	-	22.94	22.94	22.94	22.94	26.03	26.03	26.03	26.03
Limestone (g/kg)	20.11	20.11	20.11	20.11	14.12	14.12	14.12	14.12	13.88	13.88	13.88	13.88
DL-Methionine, 99% (g/kg)	1.28	1.28	1.28	1.28	1.24	1.24	1.24	1.24	1.21	1.21	1.21	1.21
Dicalcium phosphate (g/kg)	2.74	2.74	2.74	2.74	2.21	2.21	2.21	2.21	2.17	2.17	2.17	2.17
Salt (g/kg)	2.29	2.29	2.29	2.29	2.21	2.21	2.21	2.21	2.17	2.17	2.17	2.17
*Vitamin and min premix (g/kg)	4.57	4.57	4.57	4.57	4.41	4.41	4.41	4.41	4.34	4.34	4.34	4.34
Oxytetracycline (mg/kg)	50	-	-	-	50	-	-	-	50	-	-	-
β-sitosterol (mg/kg)	-	500	1000	1500	-	500	1000	1500	-	500	1000	1500
Analysed chemical composition												
Dry matter (%)	91.94	92.00	91.98	91.89	92.00	92.16	92.08	91.89	92.00	92.10	92.04	92.07
Ash (%DM)	28.13	28.49	28.29	28.64	28.04	26.91	28.20	27.07	28.34	28.27	28.50	28.43
Ether extract (% DM)	2.35	2.35	2.35	2.34	6.10	6.15	6.15	6.12	5.75	5.75	5.80	5.76

Neutral detergent fibre (%DM)	10.40	10.43	10.42	10.44	13.49	13.44	13.45	13.44	15.90	15.88	15.89	15.90
Acid detergent fibre (%DM)	4.66	4.56	4.45	4.55	5.11	5.07	5.09	5.08	5.97	6.19	6.06	6.04
Calcium (%)	0.90	0.91	0.90	0.90	0.81	0.81	0.80	0.81	0.90	0.89	0.90	0.90
Phosphorus (%)	0.50	0.51	0.50	0.50	0.46	0.45	0.46	0.46	0.46	0.44	0.47	0.47
Magnesium (%)	0.16	0.15	0.16	0.15	0.12	0.11	0.12	0.12	0.12	0.13	0.12	0.10
Potassium (%)	1.26	1.25	1.26	1.26	1.20	0.19	1.20	1.20	1.15	1.16	0.15	1.13
Gross energy (MJ/kg DM)	17.66	17.62	17.80	17.66	18.51	18.72	18.14	18.48	18.07	17.93	17.81	18.12

* Vit A: 2000000.000, Vit B_1 (Thiamine): 003.000g, Vit D_3 (500 000): 3000000.000 IU, Vit E (500 IU): 40000.000 IU, Vit K_3 (43%): 003.000g, Vit B_2 (80%) : 010.000g, Vit B6 98% (pyrod): 005.000g, Vit B_{12} 1g/kg (m): 100.000mg, Niacine 99.5%: 060.000g, Choline (Chloride 60): 606.060g, Biotine 2%: 200.000 mg, Manganese ($MnSO_4$-31%): 160.000g, Copper ($CuSO_4$-25.2%): 005.000g, Cobalt ($CoSO_4$- 20%): 100.000mg, Selenium (Na_2SeO_3 4.5%): 400:000mg, Calcium pantothenate: 020.000g, Folic acid (96 pure): 001.000g, Anty-ox Vit Dry: 100.000g, Zinc ($ZnSO_4$-35%): 090.000g, Iodide (KI 76.45%): 001.000g, Ferrous (FeSO4-30%): 035.000g, Limestone: 2647.133g; DL-Methionine with purity of 99%.

3.6.4 Chicken management: housing and feeding

One-day-old Cobb 500 broiler chicks vaccinated against Marek's, Newcastle and infectious bursal disease were purchased from Alfa Kuikensplaas chicks (Onderstepoort, Pretoria, South Africa). During the two-day habituation period, before the feeding trial, the chicks were fed a starter diet and dewormed with piperazine (Kyron Laboratories Pvt Ltd, Johannesburg, South Africa) in drinking water at 90 mg/L. The chicks were housed in a deep litter system with each group of 20 chicks housed in a pen measuring 1.7 m × 1.1 m × 1.3 m length, width, and height, respectively. Clean dry wood shavings served as bedding. Feed and cleaning drinking water were provided *ad libitum*. The controlled housing temperature was set to meet the requirements of broiler chicken at starter, grower and finisher growth stages as according to Zhao et al. (2019). Infra-red lights provided supplemental heat throughout the experiment. A 12-hour lighting programme was followed: with lights on from 6:00 hours to 18:00 hours as recommended by the South African Poultry Association (SAPA, 2012).

3.6.5 Experimental design and measurements

Two hundred and forty-one-day-old Cobb 500 broiler chicks (n = 240) were, in a completely randomised design, allocated to four diets where β-sitosterol replaced oxytetracycline at 0 mg/kg (control; fortified with 50 mg/kg oxytetracycline), 500 mg/kg, 1000 mg/kg and 1500 mg/kg (w/w) for diets 1 to 4, respectively. The chicks were fed for 6 weeks: 2 weeks for each of the starter, grower, and finisher phases with similar β-sitosterol inclusion in the starter, grower, and finisher diets. Each diet was replicated thrice with 20 chicks per replicate (Singer et al., 2007). Initial, weekly and terminal body mass and daily feed intake were measured. Initial, weekly and terminal body mass of each bird were measured using electronic balance (SnowrexEQ-1200, Snowrex International Company, Taipei, Taiwan).

3.6.6 Computations

The body mass gain, average daily gain and feed conversion ratio were computed from data on body mass and feed intake. The body mass gain (BMG) and average daily gain (ADG) were computed using the equations:

$$Body\ mass\ gain = \text{Final body mass} - \text{initial body mass}$$

$$Average\ daily\ gain\ (\text{g}) = \frac{Body\ mass\ gain}{Duration\ (days) of the\ feeding\ trial}$$

Daily feed intake (FI) by growth phase and trial were computed using the equation:

$$Feed\ intake = \text{Offered feed} - \text{residual feed}$$

The FI and BMG were used to compute the feed conversion ratio (FCR) by growth phase and for the trial period using the equation:

$$Feed\ conversion\ ratio = \frac{Feed\ intake}{Body\ mass\ gain\ (g)}$$

3.6.7 Terminal procedures, measurements and sample collection

Three days after the end of the 6-weeks feeding trial, thirty broiler chickens from each dietary treatment group were randomly selected and then subjected to a 4-hour fast with access to clean drinking water. Due to constraints with availability of the slaughter facilities all the birds were killed three days after ending of the performance measurements of the feeding trial. However, the birds were kept on their respective diets, groups and pens until slaughter. Each fasted chicken was weighed and then humanely slaughtered by decapitation using a guillotine (Harvard Apparatus, Massachusetts, United States). Feathers were hand-plucked and GIT (proventriculus, ventriculus, caeca, small and large intestine) and accessory GIT (liver and pancreas) viscera were dissected out. Viscera masses were measured using an electronic balance (SnowrexEQ-1200, Snowrex International Company, Taipei, Taiwan). Prior to weighing each GIT segment, digesta was gently squeezed out. The small and large intestines were gently stretched on a dissection board and their lengths measured using a ruler.

3.6.8 Statistical analyses

Data are presented as mean ± standard deviation. GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, California, USA) was used to analyse the data. All multiple-group parametric data were analysed using one-way ANOVA. The differences between the treatment means were determined using Tukey *post hoc* test. Significance was set at $P < 0.05$.

3.7 Results

3.7.1 Growth performance of broiler chicken

The effect of dietary β-sitosterol on the growth performance, feed intake and feed utilisation efficiency of broiler chicken during starter (day 3-16), grower (day 17-30) and finisher (day 31-44) growth phases is shown on Table 3.2. The initial body weight, BWG, ADG, FI and FCR had similar ($P > 0.05$) results in all dietary treatments. There were no significant differences ($P > 0.05$) observed on the birds' initial body weight, BWG, ADG, FI and FCR.

Table 3.2: Effect of dietary β-sitosterol on the growth performance, feed intake and feed utilisation efficiency of broiler chicken

Parameter	Growth phase	Dietary treatments				P-value
		Diet 1	Diet 2	Diet 3	Diet 4	
Initial body mass (g)		63.00 ± 1.23	63.00 ± 2.10	63.43 ± 0.65	62.30 ± 0.35	0.7513
Terminal body mass (g)		2204.00 ± 181.60	1938.00 ± 105.2	1956.00 ± 161.00	2016.00 ± 119.4	0.6167
BMG (g)	Starter	257.10 ± 52.71	259.00 ± 24.06	271.60 ± 52.91	272.40 ± 36.26	0.9549
	Grower	641.20 ± 91.74	676.60 ± 50.49	668.50 ± 48.57	695.70 ± 119.00	0.8793
	Finisher	968.70 ± 77.72	939.20 ± 81.55	914.90 ± 84.20	985.70 ± 85.88	0.8333
Total BMG (g)		1867.00 ± 124.00	1875.00 ± 104.10	1893.00 ± 161.50	1953.00 ± 119.40	0.8416
ADG (g)	Starter	18.33 ± 3.76	18.53 ± 1.71	19.43 ± 3.79	19.47 ± 2.63[a]	0.9518
	Grower	45.83 ± 6.57	48.33 ± 3.59	46.93 ± 4.17	49.67 ± 8.52[a]	0.8739
	Finisher	69.20 ± 1.25	67.10 ± 5.81	65.33 ± 3.15	70.40 ± 1.13[a]	0.8326
ADG trial (g)		44.47 ± 2.91	44.63 ± 2.52	44.13 ± 5.29	45.80 ± 4.04	0.9538
FI (g)	Starter	891.60 ± 80.30	947.10 ± 28.45	905.60 ± 74.44	908.50 ± 43.40	0.8913
	Grower	1162.00 ± 103.30	1203.00 ± 34.51	1243.00 ± 68.04	1225.00 ± 52.02	0.5464
	Finisher	2654.00 ± 113.10	2899.00 ± 208.00	2826.00 ± 174.80	2791.00 ± 83.06	0.3266
FI trial (g)		4708.00 ± 135.71	5065.00 ± 226.30	5009.00 ± 274.80	4925.00 ± 163.90	0.2411
FCR	Starter	3.60 ± 0.96	3.68 ± 0.34	3.45 ± 0.85	2.83 ± 0.08	0.9629
	Grower	1.83 ± 0.14	1.78 ± 0.11	1.80 ± 0.21	1.80 ± 0.33	0.9954
	Finisher	2.74 ± 0.17	3.09 ± 0.23	3.20 ± 0.85	2.83 ± 0.08	0.5794
FCR trial		2.52 ± 0.10	2.70 ± 0.05	2.67 ± 0.37	2.53 ± 0.24	0.6906

No significant differences in growth performance, FI and FCR across dietary treatments ($P > 0.05$). BMG – body mass gain, ADG – average daily gain, FI – feed intake, FCR – feed conversion ratio. Diet 1 – oxytetracycline (50 mg/kg of feed); diet 2 – 500 mg/kg of feed of β-sitosterol; diet 3 – 1000 mg/kg of feed of β-sitosterol; diet 4 – 1500 mg/kg of feed of β-sitosterol. Data is presented as mean ± standard deviation; for growth performance n = 60 broiler chickens per dietary treatment group

3.7.2 Viscera macromorphometry

Table 3.3 shows the effect of β-sitosterol on the macromorphometry of GIT and GIT accessory organs. β-sitosterol similarly ($P > 0.05$) affected the proventriculi, ventriculi, small and large intestines and caeca, pancreata and liver masses and small and large intestine lengths of the chickens as oxytetracycline.

Table 3.3: Effect of dietary β-sitosterol on viscera gastrointestinal organ and accessory organ masses and lengths of broiler chicken

	Dietary treatments				P-value
Parameter	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	
Heart (g)	12.01 ± 2.17	11.82 ± 2.69	11.87 ± 3.05	12.24 ± 2.80	0.9320
Relative to body mass (%)	0.51 ± 0.09	0.51 ± 0.10	0.49 ± 0.07	0.51 ± 0.08	0.9078
Liver (g)	43.13 ± 9.72	41.12 ± 10.04	41.88 ± 10.52	40.90 ± 9.98	0.8262
Relative to body mass (%)	1.83 ± 0.35	1.76 ± 0.32	1.75 ± 0.22	1.70 ± 0.28	0.3988
Pancreas (g)	4.18 ± 0.84	4.00 ± 0.75	4.20 ± 1.09	4.07 ± 0.78	0.7830
Relative to body mass (%)	0.18 ± 0.04	0.17 ± 0.03	0.18 ± 0.03	0.17 ± 0.03	0.7548
Spleen (g)	2.05 ± 0.61	1.94 ± 0.49	1.97 ± 0.58	2.02 ± 0.64	0.8905
Relative to body mass (%)	0.09 ± 0.02	0.08 ± 0.02	0.08 ± 0.02	0.08 ± 0.02	0.9007
Proventriculus (g)	8.24 ± 1.44	7.90 ± 1.95	7.61 ± 1.67	7.76 ± 1.55	0.5089
Relative to body mass (%)	0.35 ± 0.06	0.34 ± 0.07	0.32 ± 0.04	0.33 ± 0.06	0.1757
Ventriculus (g)	40.00 ± 5.29	39.37 ± 7.45	40.33 ± 9.68	40.87 ± 8.06	0.8991
Relative to body mass (%)	1.71 ± 0.19	1.70 ± 0.25	1.69 ± 0.23	1.73 ± 0.29	0.9642
Small intestines (g)	42.99 ± 6.10	40.78 ± 8.52	42.74 ± 9.28	43.04 ± 8.20	0.6618
Relative to body mass (%)	1.84 ± 0.24	1.76 ± 0.30	1.80 ± 0.21	1.82 ± 0.30	0.6798
Small intestines length (mm)	1684.00 ± 148.90	1690.00 ± 247.50	1681.00 ± 206.00	1698.00 ± 216.30	0.9886
Relative to body mass (mm/g)	0.73 ± 0.10	0.74 ± 0.12	0.73 ± 0.13	0.73 ± 0.14	0.9761
Large intestine (g)	3.21 ± 0.51	3.44 ± 0.96	3.20 ± 1.02	3.05 ± 0.99	0.4082
Relative to body mass (%)	0.14 ± 0.03	0.15 ± 0.05	0.14 ± 0.03	0.13 ± 0.03	0.1025

Large intestines length (mm)	101.80 ± 18.84	101.20 ± 19.10	99.63 ± 21.09	100.30 ± 24.49	0.9801
Relative to body mass (mm/g)	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.02	0.7228
Caecum (g)	7.15 ± 1.44	7.06 ± 1.36	6.70 ± 1.50	6.56 ± 1.27	0.3041
Relative to body mass (%)	0.31 ± 0.06	0.31 ± 0.07	0.28 ± 0.05	0.28 ± 0.05	0.0777
Visceral fat (g)	39.12 ± 16.51	41.77 ± 14.81	42.73 ± 13.65	45.15 ± 14.88	0.4752
Relative to body mass (%)	1.64 ± 0.58	1.92 ± 0.62	1.82 ± 0.62	1.98 ± 0.67	0.2189

No significant differences in viscera gastrointestinal organ and accessory organ masses and lengths across dietary treatments ($P > 0.05$); Diet 1 – oxytetracycline (50 mg/kg of feed); diet 2 – 500 mg/kg of feed of β-sitosterol; diet 3 – 1000 mg/kg of feed of β-sitosterol; diet 4 – 1500 mg/kg of feed of β-sitosterol, data is presented as mean ± standard deviation; n = 28-32 birds per dietary treatment group.

3.8 Discussion

3.8.1 Growth performance, feed intake and utilisation efficiency

We evaluated the potential of β-sitosterol, supplemented at 500 mg/kg, 1000 mg/kg and 1500 mg/kg of feed, respectively, to replace oxytetracycline as a growth promoter in broiler chicken starter, grower, and finisher diets, respectively. Our findings show that β-sitosterol-supplemented chickens had similar growth performance, feed intake and utilisation efficiency as their oxytetracycline-supplemented (control) counterparts. These findings show that supplemental β-sitosterol at the three dietary inclusion levels used in the current study did not compromise the growth performance, feed intake, and feed utilization efficiency of the broiler chickens. The three dietary inclusion levels of β-sitosterol were equally effective in promoting the growth performance of the broiler chickens, suggesting that one can save on production costs by making use of the lowest dietary β-sitosterol inclusion level without compromising performance, feed intake and feed utilization efficiency of the broiler chickens. The three dietary inclusion levels of β-sitosterol were equally effective in promoting the growth performance of the broiler chickens, suggesting that one can save on production costs by making use of the lowest dietary β-sitosterol inclusion level without compromising performance. It must be noted that in our study we used general poultry guidelines for diet formulation according to the National Research Council (NRC, 1994). The diet was formulated to mimic common practice in general broiler chicken rearing where farmers typically purchase feeds that are commercially available without targeting specific breeds. Additionally, the study did not target breeder Cobb 500 stock where there is a greater emphasis on precision diet formulation.

Ding et al. (2021) reported that β-sitosterol, at 40 and 80 (mg/kg) dietary inclusion as a growth promoting dietary supplement in broiler chicken diets resulted in improved average daily gain (ADG) and average daily feed intake (ADFI). Although findings from the current study did not show β-sitosterol-induced improvement in FCR, it is important to note that the β-sitosterol did not compromise feed utilization efficiency. Thus, the broiler chicken grew significantly. Our results are consistent with the findings of other authors who observed similar effects of β-sitosterol on DFI, ADG and FCR of broiler chickens (Feng et al., 2020; Zhao et al., 2019) using lower dietary concentrations (20, 40, 80 mg/kg) of β-sitosterol as well as the findings of Naji et al. (2014) who made use of higher (25 000, 50 000 and 75 000 mg/kg) dietary concentrations of the phytosterol compared to ours. In the current study, we

supplemented β-sitosterol at 500, 1000 and 1500 mg/kg dietary inclusion levels. However, we observed similar growth performance, feed intake and feed utilisation with oxytetracycline supplement control counterparts. The 42-day terminal body mass of the broiler chicken fed β-sitosterol fortified diets in the current study ranged from 1938 – 2016 g which was similar to that of the oxytetracycline control counterparts.

Our findings differ from those of Naji et al. (2014) who reported an increased final body mass (3104 – 3286 g) in broiler chicken whose diets were supplemented with different doses of β- sitosterol (25 000, 50 000 and 75 000 mg/kg of feed) compared to the control birds final body mass after 6 weeks (2881 g). However, there was no difference reported in FI and FCR in their study across the dietary treatments. It is important to note that Naji et al. (2014) made use of substantially higher dietary β-sitosterol inclusion levels and Ross308 broiler chicken in their study. Ross strains 308 and 708 have been reported to be rapidly growing broiler chicken reaching 2.2 – 2.9 kg by 35 – 40 days (Bedford et al., 2017). Ding et al. (2021) reported an increased final body mass (2670 – 2731 g) in White Feathered broiler chicken with lower doses of β-sitosterol (10 – 80 mg/kg of feed) than in the current study. Possible reasons for the differences in the terminal body mass of broiler chickens in the current study compared to other studies include breed differences, diet formulations, β-sitosterol doses, feeding environment and management. Importantly, our findings show similarities in terminal 42-day mass, BMG, ADG, DFI and FCR of chicken fed β-sitosterol supplemented diets and those fed the oxytetracycline supplemented diet which suggests that β-sitosterol was equally effective as oxytetracycline in sustaining the growth performance and efficient feed utilization by the Cobb 500 broiler chicken. At 30-45-days of age, Cobb 500 broiler chickens are expected to have a body mass range of approximately 2000 to 2500 g (Singh et al., 2021) which was observed in our study. Some phytochemicals when used as natural growth promoters enhance the flavour (palatability) and aroma of feeds following stimulation of the olfactory nerve and taste buds which increases appetite and hence feed intake (Valenzuela-Grijalva et al., 2017; Jiang et al., 2007). Our findings show similar DFI across dietary treatments which (similarities in DFI) seem to suggest that β-sitosterol, though a phytochemical, may not have had any stimulatory effect on appetite and hence feed intake.

3.8.2 Viscera macromorphometry

The gastrointestinal tract is the first point of contact for ingested substances, thus it interfaces directly with the exogenous environment (Mukonowenzou et al., 2021). Constituents of this

external environment, for example, feed ingredient type and form, feed additives and diet nutrient composition impact the development and functional efficiency of the GIT (Kogut, 2019). Phytochemicals through their effects on gut microflora can mediate precocious maturation of the GIT that enhances nutrient digestion and absorption (Mukonowenzou et al., 2021). Therefore, we evaluated β-sitosterol's effects on GIT organ and GIT accessory organ macromorphometry of Cobb 500 broiler chicken. We observed similarities in the masses of the proventriculi, ventriculi, small and large intestine, pancreata and livers and the lengths of the small and large intestines of broiler chickens supplement with β-sitosterol and control counterparts whose diets were fortified with oxytetracycline. The similarities in the GIT and GIT accessory organ macromorphometry suggest that dietary β-sitosterol neither stimulated nor compromised the growth and development of the GIT. However, our findings show significantly lower absolute and relative liver masses compared to the results observed by Feng et al. (2020) despite them having supplemented the broiler chicken diets with β-sitosterol at 25 mg/kg of feed which was lower than the inclusion level used in our study.

On the contrary, Naji et al. (2014) observed that supplemental β-sitosterol caused significant increases in the liver and proventriculi masses of the chicken. We speculate that the high dietary inclusion level of β-sitosterol by Naji et al. (2014) might have mediated "hypertrophy" of the chickens' proventriculi and livers. Apart from its cholesterol-lowering effect, β-sitosterol has been reported to have liver-protective properties (Feng et al., 2020). Contradicting results have been reported on the effect of phytosterols on the liver mass and index in broiler chickens with higher or lower dietary phytosterol doses. In a study with layer chicken Shi et al. (2014) observed that supplementing the diets with β-sitosterol at 400 and 800 mg/kg of feed resulted in similar liver masses with those fed the control diet; a finding that is similar to observations from the current study albeit differences in dietary inclusion levels of the phytosterol. The liver, which plays an important role in detoxification, is an important organ for nutrient metabolism and a sensitive indicator of toxicity (Dong et al., 2020). Higher dietary inclusion levels of phytosterols have been shown to affect liver lipid metabolism largely mediating increased hepatic fat accumulation a sign of fatty liver disease in the liver (Sissener et al., 2017). This did not seem to be the case in our study.

3.9 Conclusion

β-sitosterol supplemented diets had similar effects on the broiler chickens' terminal body mass, body mass gain, feed intake, feed conversion ratio and GIT and accessory GIT viscera macromorphometry as with oxytetracycline-fortified control diet. We therefore conclude that β-sitosterol can replace oxytetracycline as growth promoter in broiler chicken diets with no risk of compromising growth performance, feed intake and utilisation efficiency and the growth and development of GIT and accessory GIT organs.

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CHAPTER FOUR - EFFECT OF DIETARY SUPPLEMENTATION WITH β-SITOSTEROL ON MEAT YIELD AND QUALITY OF COBB 500 BROILER CHICKENS

Preamble

Having considered the effect of dietary supplementation with β-sitosterol on the growth performance and the gastrointestinal tract viscera macromorphometry of Cobb 500 broiler chicken in the previous chapter, this chapter focuses on the meat yield and quality.

4.0 Introduction

Chicken meat is a preferred meat by consumers for its high protein and unsaturated fatty acid content and low lipid content (Li et al., 2021; Baéza, Guillier and Petracci, 2022). Furthermore, broiler chicken meat when compared to red meat is lower in calories per unit mass thus is associated with reduced risk of causing metabolic diseases and cancer (Giromini and Givens, 2022). In addition to nutritive value, meat colour and texture are key quality attributes that influence consumers' decisions to purchase meat since they impact the perception of meat's freshness and hence attractiveness to the consumer (Barbut, 2015; Mir et al., 2017). Species and bird age at slaughter, muscle type and myoglobin concentration and state affect meat colour (Moyo et al., 2020). Post-slaughter temperature, degree of protein denaturation and pH influence the amount of light reflected by meat's surfaces and has a bearing on the physical appearance of the meat (Lawrie and Ledward, 2006). Stress in lairage and/or at slaughter has been reported to result in pale soft exudative (PSE) due to an increased rate of post-mortem metabolism, accelerated glycolysis and pre-mature on set of rigor mortis (Mir et al., 2017).

Synthetic antibiotics used to fortify broiler chicken feeds as growth promoters have been shown to reduce muscle glycogen content through anaerobic glycolysis (Sabow et al., 2015). This antibiotic-induced anaerobic glycolysis causes the accumulation of lactic acid and a concomitant acid mediated decline in meat colour (Abdulla et al., 2017). Furthermore, supplementing broiler chicken diets with oxytetracycline and/or neomycin has been shown to negatively impact tenderness of broiler chickens' breast meat (Abdulla et al., 2017). The meat's water holding capacity and propensity to lose moisture, which are strongly correlated to pH (Baéza, Guillier and Petracci, 2022), also impact meat colour. As pH declines, the meat becomes paler, softer, and experience a higher degree of moisture loss (Choi et al., 2023).

Despite their known benefits at improving broiler chicken productive performance and bird gastrointestinal tract health (Mehdi et al., 2018), antibiotics used to fortify broiler chicken

feeds result in their metabolites being deposited as residues in the meat (Abreu et al., 2023). These residues can elicit, in consumers of such contaminated meat, allergies that lead to diarrhoea and other formal of ill-health (Mund et al., 2017; Jammoul and El Darra, 2019). In addition, by contaminating meat, the antibiotics and their residues also cause the development of antibiotic-resistant bacteria further increasing risk to consumer health.

These multiple challenges associated with use of synthetic antibiotics as growth promoters in broiler chicken feeds have led to the search of alternatives. Phytochemicals, based on their widely reported antimicrobial, antioxidant, immune modulating and gastro-protective activities (Valenzuela-Grijalva et al., 2017), are potential alternatives to antibiotics in poultry production. β-sitosterol which possesses antibiotic, antioxidant, and hypolipidaemic activities when used as a dietary supplement can potentially be exploited as a dietary supplement in broiler chicken feeds. While previous research has demonstrated that dietary β-sitosterol improved broiler chicken meat yield (Cheng et al., 2019; Zhao et al., 2019; Ding et al., 2021) and decreased fat and saturated fatty acid content of broiler chicken breast meat (Naji et al., 2014), none of the studies evaluated its effects in Cobb 500 broiler chickens. The Cobb 500 broiler chicken breed is a highly competitive chicken meat breed with superior and higher productive performance compared to other broiler chicken breeds (Hassan, Atallah and Reda, 2021). Thus, this study sought to determine, in this unique broiler chicken breed, the effects of substituting the antibiotic, oxytetracycline with β-sitosterol, on meat yield and quality.

4.1 Aim of the study

This study evaluated the effects of fortifying Cobb 500 broiler chicken's diets with β-sitosterol as a growth promoter on the broiler chicken's meat yield and quality.

4.2 Objectives of the study

The specific objectives of the study were to determine, in Cobb 500 broiler chicken, the effect of dietary supplementation with β-sitosterol on:

a) slaughter and carcass mass and dressing percentage
b) meat colour, pH, thawing and cooking loss, tenderness and myofibrillar fragmentation length
c) meat proximate, calcium, phosphorus, magnesium, potassium, and fatty acid content.

4.3 Hypotheses of the study

a) H_0: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline does not affect chickens' slaughter and carcass mass and dressing percentage.

   H_1: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline affects the chickens' slaughter and carcass mass and dressing percentage.

b) H_0: Dietary supplementation with β-sitosterol as a growth promoter in place of oxytetracycline does not affect meat colour, pH, thawing and cooking loss, tenderness and myofibrillar fragmentation length.

   H_1: Dietary supplementation with β-sitosterol as a growth promoter in place of oxytetracycline affects meat colour, pH, thawing, and cooking loss, tenderness and myofibrillar fragmentation length.

c) H_0: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline does not affect the meat's proximate, mineral and fatty acid content.

   H_1: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline affects meat chemical nutrient, mineral composition, and fatty acid content.

4.4 Materials and methods

4.4.1 Study site and ethical clearance

The study site and ethical clearance for the study for this experiment are as described in Chapter 3; subheading 3.3.1.

4.4.2 Diet formulation

Diet formulation and experimental design of the study are described in Chapter 3; subheading 3.3.3.

4.4.3 Animals and management

Bird management was as described in Chapter 3; subheading 3.3.4.

4.4.4 Experimental design

The experimental design is as described in Chapter 3 subheading 3.6.5.

4.4.5 Terminal procedures and measurements

At the end of the 6-week feeding trial, 30 chickens per dietary treatment (total of 120 birds) were randomly selected, fasted for 4 hours with access to drinking water to prevent dehydration. The fasted broiler chickens were weighed with an electronic balance (SnowrexEQ-1200, Snowrex International Company, Taipei, Taiwan) before slaughter. Thereafter, broiler chickens were slaughtered using a guillotine (Harvard Apparatus, Massachusetts, United States). Feathers were hand-plucked. Gastrointestinal tract and accessory GIT viscera were dissected out. The mass of each dressed carcass was then weighed on an electronic balance. Carcass dressing percentage was calculated using the formula:

$$Dressed\ carcass\ mass\ \% = \frac{carcass\ mass\ of\ broiler\ chicken}{live\ mass\ of\ broiler\ chicken} \times\ 100$$

Immediately thereafter, the left side of each dressed broiler chicken breast was carefully dissected out and transferred into a vacuumed Ziplock plastic bag and stored in a freezer until analysis for meat quality.

4.4.6 Determination of meat quality

The meat's physical and chemical quality parameters were determined.

4.4.6.1 Determination of meat pH and colour

A portable calibrated pH meter with a piercing electrode [HANNA Instruments, (Pty) Ltd, Bedfordview, South Africa] was used to determine the chickens' breast meat pH. The pH was determined following a two-point (pH 4.0 and pH 7.0) calibration of the pH meter. The breast meat's initial pH was determined at 45 minutes post-slaughter (initial pH = pH_i) and at 24 hours post-slaughter (ultimate pH = pH_u) following storage at 4ºC. The chickens' breast and thigh meat colour values [Lightness (L*), Redness (a*), Yellowness (b*), Chroma (C*) and Hue (H*)] were determined using a Lovibond colour meter which was calibrated on a red plate (LC 100 Spectrophotometer, Lasec, China) 30 minutes and 24 hours post-slaughter. The

colour and pH measurements from the breast and thigh meat were determined on three points and the mean value recorded.

4.4.6.2 Determination of thawing loss

The breast meat thawing and cooking loss was assessed as described by Jama et al. (2008). Briefly, individual frozen breast meat sample in Ziplock plastic bag were weighed to determine the initial mass. Immediately after determination of the initial mass, the sub-sample was thawed in a cold room at 4ºC for overnight. The thawed samples were blotted dry with paper towel and re-weighed using a balance (Radwag PS 750/C/2; Radwag Balances and Scales, Radom, Poland). Thaw loss was calculated as the difference between the weights of the frozen and thawed sub-sample and expressed as a percentage using the equation:

$$Thawing\ loss\ (\%) = \frac{Initial\ mass - Final\ mass}{Initial\ mass} \times 100$$

4.4.6.3 Determination of cooking loss

The meat's cooking loss was determined as described by the American Meat Science Association (AMSA, 1995). In summary, each sample from the determination of thawing loss was weighed and broiled in an oven (Mielé, model H217, Mielé and Cie, Gütersloh, Germany) set at 200ºC. Prior to placement into the oven, each sub-sample was wrapped in an aluminium foil. Each sub-sample was broiled till its core temperature recorded by a hand-held digital probe (Model Kane-May 1012, Kane International Ltd, Hertfordshire, United Kingdom) reached 80-85ºC. Immediately after broiling the final mass of each broiled sub-sample was measured using an electronic balance. Sample cooking loss (CL) was computed using the equation:

$$\text{CL} = \frac{Weight\ of\ thawed\ meat\ sample\ (g) - Weight\ of\ cooked\ meat\ sample\ (g)}{Weight\ of\ thawed\ meat\ sample\ (g)} \times 100$$

4.4.6.4 Determination of tenderness

Immediately after determination of cooking loss, the broiled breast meat samples were cooled down at room temperature for 2 hours following which meat tenderness was determined using a Warner Braztler shear device mounted on an Instron Universal Testing Machine (Model 4301, Instron Ltd, Buckinghamshire, England) as described by Raharjo et al. (1992). Briefly, 4 × 10 × 10 mm thickness strips were cut from randomly chosen breast meat samples

per dietary treatment. Each meat strip was sheared once through the centre at a crosshead speed of 200 mm/minutes with a 1 kN load cell perpendicular to the muscle fibre. The average shear force of three cuttings per sub-sample were recorded on a computer screen connected to the Universal Testing Machine and the data was downloaded from the computer.

4.4.6.5 Determination of myofibrillar fragmentation length

The myofibrillar fragmentation length (MFL) of each breast meat sub-sample was determined as described by Culler et al. (1978) but with modifications described by Heinze and Bruggemann (1994). Briefly, each 50 g of frozen (-20˚C) breast meat sample was thawed at room temperature. Each sub-sample of 3 g pieces were cut into thin slices with a knife and the fat was trimmed. Each of the 3 g sub-sample pieces was then placed into 50 mL Bühler round bottom glass which contained 30 mL of 0.02M potassium phosphate extraction buffer. The sub-sample was homogenised for 30 seconds using a Bühler HO_4 homogeniser (Type H0-4 NR 3012, Labortechnik Admund Bühler, Wehingen, Germany). Immediately after homogenising, each mixture was centrifuged at 4ºC for 15 minutes at 1000 × *g* using a Hermle centrifuge (Hermle Labortechnik GmbH Z 36 HK, Wehingen, Germany). The supernatant was discarded, and the pellet re-suspended in 50 mL extraction buffer and centrifuged for the second time at 4ºC for 15 minutes at 1000 × g. The supernatant was discarded, and the pellet re-suspended in 10 mL extraction buffer and centrifuged for the third time for 10 minutes at 1000 × g at 4ºC. Immediately after centrifuging, the suspension was then filtered under vacuum using 1000 µm polyethylene terephthalate mesh (Dacron® velour fabric, USCI, Massachusetts, USA). Thereafter, an additional of 5 mL MFL buffer was used to facilitate the passage of myofibrils through the strainer. The resultant filtrate was then filtered under vacuum using 250 µm polyethylene terephthalate mesh. To measure the MFL of the meat, a drop (50 µL) of the filtrate was placed on a microscope slide covered with a coverslip and viewed at a 400X magnification using an Olympus system microscope model BX41 (Olympus Corporation, Tokyo, Japan). The microscope was connected with a XC30 Colour Camera (Olympus Soft Imaging Systems Gmbh, Münster, Germany) and a cellSens Dimenstion software package (Soft Imaging Systems Gmbh, Münster, Germany). The MFL was determined as the average length of 100 single myofibril fragments per sub-sample.

4.4.6.6 Determination of meat proximate content

Twelve breast meat samples per dietary treatment randomly selected were pooled to make a composite sample. Each composite sample was then lyophilised in a freeze drier (Custon, SSE Engineering, North America) and milled through a 2 mm screen. The DM, CP, EE and ash content of the breast meat samples were determined as described by the Association of Official Analytical Chemists procedures (AOAC, 2005; method numbers: 930.15, 942.15, 984.01 and 920.39, respectively). Each assay was done in triplicate.

4.4.6.7 Determination of the meat mineral content

The chickens' breast meat calcium, magnesium, phosphorus, and potassium mineral content were determined as described by Huang and Schulte (1985). Briefly, 0.5 g of each breast meat sample was digested in 25 mL of 65% nitric acid and 5 mL of perchloric acid at 200˚C. The digest solution was then used to spectrophotometrically determine the mineral content of the meat using inductively coupled plasma-optical emission spectrometry (ICP-OES) on a Varian Liberty 200 spectrometer (Varian, Perth, Australia). Each determination was done in triplicate.

4.4.6.8 Determination of the fatty acid profile

The Soxhlet method was used to extract the oil from the breast muscles as described by Association of Analytical Chemists procedures (AOAC, 2005, method number: 920.39). The fatty acid profile was determined using gas chromatography as described by Christopherson and Glass (1969). Briefly, the oil extracts were methylated with 2M methanol sodium hydroxide. The resultant fatty acid methyl esters were extracted in heptane, filtered and dried under the nitrogen. Thereafter they were separated by a temperature gradient over 45 minutes on a gas chromatography with nitrogen as carrier gas on a DB-23 capillary column (90 cm × 250 μm × 0.25μm) (Supelco, Sigma-Aldrich). The chromatograph consisted of an HP6890 gas chromatography (Hewlett Packard, Bristol, United Kingdom) with flame ionisation detector (FID). The detector and injector temperatures were set at 300ºC. A personal computer equipped with Chemstation software (Agilent Technologies Inc., Santa Clara, CA, USA) was used for quantification.

4.5 Data analyses

Data for slaughter mass, dressed carcass weight, dressing percentage physical and chemical meat properties was analysed using Graph Pad Prism Version 8.0.2 (Graph-pad Software Inc, San Diego, USA) statistical package. A one-way ANOVA was used to analyse data. The Tukey *post hoc* test was used to compare treatment means. The level of significance was set at $P < 0.05$. The statistical model used was:

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

where Y_{ij} = dependent variable of interest

μ = is the overall mean common to all observations

T_i = is the fixed effect of the i^{th} dietary treatment ($i = 1, 2, 3, 4$)

e_{ij} = is the random residual error.

4.6 Results

4.6.1 Slaughter mass and carcass yield

The effect of dietary supplementation with β-sitosterol on the slaughter mass, empty carcass mass and dressing percentage of Cobb 500 broiler chickens is presented on Table 4.1. Supplementing Cobb 500 broiler chicken diets with β-sitosterol had similar effects ($P > 0.05$) as when supplemented with oxytetracycline (control) on the slaughter and empty carcass masses and dressing percentage of the chickens.

Table 4.1: Effect of dietary supplementation with β-sitosterol on the slaughter mass, empty carcass mass and dressing percentage of Cobb 500 broiler chickens

Parameters	Dietary treatments				P-value
	Diet 1	Diet 2	Diet 3	Diet 4	
Slaughter mass (g)	2392.00 ± 187.30	2263.00 ± 125.40	2333.00 ± 296.90	2343.00± 157.90	0.8684
Empty carcass mass (g)	1954.00 ± 157.50	1827.00± 187.11	1864.00 ± 223.40	1901.00 ± 140.86	0.7438
Dressing percentage (%)	81.72 ± 0.72	81.53 ± 1.77	79.93 ± 0.82	80.88 ± 1.49	0.3103

No significant differences in slaughter mass, empty carcass mass and dressing percentage across dietary treatments ($P > 0.05$); Diet 1 – fortified with oxytetracycline at 50 mg/kg of feed; diet 2 – fortified with β-sitosterol at 500 mg/kg of feed; diet 3 – fortified with β-sitosterol at 1000 mg/kg of feed; diet 4 – fortified with β-sitosterol at 1500 mg/kg of feed; data is expressed as mean ± SD; n = 30 broiler chickens.

4.6.2 Physical meat properties

The effect of dietary supplementation with β-sitosterol on the colour and pH of breast meat from carcasses of Cobb 500 broiler chicken is presented on Table 4.2. Supplementing Cobb 500 broiler chicken diets with β-sitosterol had similar effects ($P > 0.05$) as when supplemented with oxytetracycline on Cobb 500 broiler chickens' breast meat colour and pH at 45 minutes and 24 hours post-slaughter.

Table 4.2: Effect of dietary supplementation with β-sitosterol on the colour and pH of breast meat from Cobb 500 broiler chickens

	Parameter	**Dietary treatment**				**P-value**
45-min post slaughter		**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	
	pH_i	6.03 ± 0.33	5.96 ± 0.28	6.00 ± 0.28	6.00 ± 0.34	0.8231
	L^*	46.26 ± 3.15	45.24 ± 2.07	46.62 ± 3.23	45.68 ± 2.48	0.2273
	a^*	-1.20 ± 1.16	-1.21 ± 0.85	-1.14 ± 0.86	-0.92 ± 1.98	0.8048
	b^*	12.46 ± 1.78	11.92 ± 2.02	12.27 ± 2.33	11.54 ± 2.10	0.3231
	C^*	12.41 ± 1.73	12.13 ± 1.92	12.39 ± 2.20	12.01 ± 2.41	0.4110
	H*	96.02 ± 5.40	95.03 ± 5.22	95.36 ± 8.62	95.95 ± 8.46	0.9392
	pH_u	5.69 ± 0.15	5.70 ± 0.20	5.73 ± 0.16	5.69 ± 0.13	0.6580
24-hour post slaughter	L^*	60.52 ± 17.56	58.96 ± 16.52	67.16 ± 20.60	66.57 ± 20.42	0.2274
	a^*	-0.65 ± 0.84	-0.64 ± 1.14	-0.67 ± 1.45	-0.69 ± 1.46	0.6280
	b^*	13.44 ± 5.75	13.44 ± 5.60	12.58 ± 7.32	11.59 ± 6.86	0.6403
	C^*	14.45 ± 5.87	13.48 ± 5.58	12.95 ± 7.34	11.68 ± 6.79	0.1329
	H*	100.20 ± 14.84	95.91 ± 11.68	98.83 ± 12.23	99.42 ± 11.66	0.5579

No significant differences in broiler chicken's breast meat colour pH across dietary treatments ($P > 0.05$); Diet 1 – fortified with oxytetracycline at 50 mg/kg of feed; diet 2 – fortified with β-sitosterol at 500 mg/kg of feed; diet 3 – fortified with β-sitosterol at 1000 mg/kg of feed; diet 4 – fortified with β-sitosterol at 1500 mg/kg of feed; chickens L* - lightness, a* - redness, b* - yellowness, C* - Chroma, H* - Hue angle, pHi – initial pH, pH_u – Ultimate pH data is expressed as mean ± SD; n = 30 broiler chickens.

Table 4.3 shows the effect of dietary supplementation with β-sitosterol on the colour and pH of thigh meat from the carcasses of broiler chicken. Supplementing Cobb 500 broiler chicken diets with β-sitosterol had similar effects ($P > 0.05$) as when supplemented with oxytetracycline on Cobb 500 broiler chicken thigh meat colour and pH at 45 minutes and 24 hours post-slaughter.

Table 4.3: Effect of dietary supplementation with β-sitosterol on the colour and pH of thigh meat from Cobb 500 broiler chicken

	Parameter	**Dietary treatment**				**P-value**
45-min post slaughter		**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	
	pH_i	5.99 ± 0.26	6.02 ± 0.28	6.04 ± 0.31	5.97 ± 0.28	0.8237
	L^*	52.10 ± 2.67	54.94 ± 12.37	51.79 ± 2.95	51.49 ± 2.53	0.1626
	a^*	1.89 ± 1.89	1.99 ± 1.84	1.66 ± 2.36	2.21 ± 1.67	0.7508
	b^*	15.38 ± 3.72	14.15 ± 4.96	15.64 ± 4.24	16.69 ± 4.40	0.1655
	C^*	15.41 ± 3.43	14.20 ± 5.00	15.86 ± 4.26	16.75 ± 3.98	0.1329
	H*	85.05 ± 5.87	86.78 ± 10.24	85.49 ± 7.89	83.60 ± 6.30	0.4658
24-hour post slaughter	pH_u	5.95 ± 0.26[a]	5.90 ± 0.25[a]	5.97 ± 0.27	5.94 ± 0.26	0.7881
	L^*	62.54 ± 20.26	64.20 ± 19.19	70.74 ± 21.68	65.92 ± 20.31	0.4418
	a^*	1.53 ± 1.96	1.17 ± 2.62	1.43 ± 2.36	1.11 ± 2.54	0.8909
	b^*	11.79 ± 6.78	11.68 ± 5.81	10.78 ± 7.89	11.07 ± 7.55	0.9353
	C^*	12.13 ± 6.76	12.45 ± 5.60	11.07 ± 8.07	11.41 ± 7.65	0.8659
	H*	90.55 ± 18.19	90.70 ± 17.13	95.33 ± 17.85	94.12 ± 20.19	0.6697

No significant differences in broiler chicken's thigh meat colour pH across dietary treatments ($P > 0.05$); Diet 1 – fortified with oxytetracycline at 50 mg/kg of feed; diet 2 – fortified with β-sitosterol at 500 mg/kg of feed; diet 3 – fortified with β-sitosterol at 1000 mg/kg of feed; diet 4 – fortified with β-sitosterol at 1500 mg/kg of feed; L* - lightness, a* - redness, b* - yellowness, C* - Chroma, H* - Hue angle, pHi – initial pH, pH_u – Ultimate pH; data is expressed as mean ± SD. n = 30 broiler chickens.

The effect of dietary supplementation with β-sitosterol on the physical properties of broiler chicken's breast meat is presented on Table 4.4. Supplementing Cobb 500 broiler chicken diets with β-sitosterol had similar effects ($P > 0.05$) as when supplementing with oxytetracycline on the thawing loss, cooking loss, tenderness and myofibrillar fragmentation length from the breast meat of broiler chicken.

Table 4.4: Effect of dietary supplementation with β-sitosterol on the physical properties of broiler chicken's breast meat

Parameter	Dietary treatments				P-value
	Diet 1	Diet 2	Diet 3	Diet 4	
Thawing loss (%)	9.22 ± 2.58	9.55 ± 3.19	8.82 ± 3.53	8.43 ± 2.76	0.5250
Cooking loss (%)	27.78 ± 3.31	25.89 ± 2.89	26.08 ± 3.74	24.85 ± 2.55	0.1651
Tenderness (N)	2.05 ± 0.55	1.78 ± 0.40	1.69 ± 0.43	1.65 ± 0.36	0.0953
Myofibrillar fragmentation length (μM)	23.16 ± 2.11	22.64 ± 3.01	23.35 ± 1.86	22.85 ± 2.01	0.8653

No significant differences in the meat's thawing loss, cooking loss, tenderness and myofibrillar fragmentation length across dietary treatments ($P > 0.05$); Diet 1 – fortified with oxytetracycline at 50 mg/kg of feed; diet 2 – fortified with β-sitosterol at 500 mg/kg of feed; diet 3 – fortified with β-sitosterol at 1000 mg/kg of feed; diet 4 – fortified with β-sitosterol at 1500 mg/kg of feed; data is expressed as mean ± SD; n = data is expressed as mean ± SD; n = 12.

4.6.3 Meat chemical nutrient content

The effect of dietary supplementation with β-sitosterol on the proximate content from the breast meat of Cobb 500 broiler chicken is presented on Table 4.5. Supplementing Cobb broiler chicken diets with β-sitosterol had similar effects (P = 0.6062) as oxytetracycline (control) on the dry matter of broiler chicken's breast meat. The crude protein content of the breast meat from the carcasses of Cobb 500 broiler chicken fed diet 4 increased (P = 0.0205) with an increase in β-sitosterol dietary supplementation. Supplementing the chickens' diets with higher β-sitosterol levels (1000 and 1500 mg/kg) increased (P = 0.0031) ash content of chickens' breast meat. The breast meat from broiler chicken fed diets 2 and 4 had a significantly (P = 0.0006) higher ether extract compared to that from counterparts fed diets 1 and 3, respectively.

Table 4.5: Effect of dietary supplementation with β-sitosterol on the proximate content of breast meat of Cobb 500 broiler chicken

Proximate (%DM)	Dietary treatments				P-value
	Diet 1	Diet 2	Diet 3	Diet 4	
Dry matter	93.50 ± 0.61[a]	93.16 ± 0.58[a]	94.22 ± 0.03[a]	94.12 ± 0.03[a]	0.6062
Crude protein	74.76 ± 0.05[b]	73.72 ± 0.04[c]	74.62 ± 0.05[b]	75.39 ± 0.37[a]	0.0205
Ash	7.00 ± 0.05[b]	7.00 ± 0.05[b]	8.67 ± 0.13[a]	8.67 ± 0.03[a]	0.0031
Ether extract	7.61 ± 0.18[b]	8.61 ± 0.08[a]	7.79 ± 0.15[b]	8.49 ± 0.21[a]	0.0006

[a,b.c] Within row means with different superscripts differ significantly at $P < 0.05$. Dietary supplementation with β-sitosterol significantly increased crude protein, ash and ether extract mineral content of breast meat of broiler chicken at $P < 0.05$. No significant differences in broiler chicken's breast meat proximate content across dietary treatments ($P > 0.05$). Supplemental β-sitosterol at 1500 mg/kg significantly increased crude protein. Diet 1 – fortified with oxytetracycline at 50 mg/kg of feed; diet 2 – fortified with β-sitosterol at 500 mg/kg of feed; diet 3 – fortified with β-sitosterol at 1000 mg/kg of feed; diet 4 – fortified with β-sitosterol at 1500 mg/kg of feed. Data is expressed as mean ± SD; n = 12.

Table 4.6 shows the effect of dietary supplementation with β-sitosterol on the calcium, magnesium, phosphorus and potassium mineral content of Cobb 500 broiler chicken breast meat. Supplementing Cobb 500 broiler chicken diets with β-sitosterol at 1500 mg/kg dietary inclusion significantly increased ($P = 0.0017$) the chickens' breast meat phosphorus content than those fed diets fortified with β-sitosterol at 1000 mg/kg and 500 mg/kg as well counterparts fed the oxytetracycline fortified control diet. Dietary β-sitosterol had no effect ($P > 0.05$) on the calcium content from breast meat from the carcasses of Cobb 500 broiler chicken. However, fortifying the broiler chickens' diets with β-sitosterol significantly increased ($P = 0.0162$) the breast meat's calcium content compared to that of control counterparts fed the oxytetracycline fortified control diet. The magnesium and potassium content of the Cobb 500 broiler chickens breast meat significantly increased ($P < 0.01$) with an increase in dietary β-sitosterol.

Table 4.6: Effect of dietary supplementation with β-sitosterol on the calcium, magnesium, phosphorus and potassium mineral content of breast meat of Cobb 500 broiler chicken

Mineral content	**Dietary treatments**				**P-value**
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	
Calcium (mg/kg)	22.01 ± 5.10^{b}	26.33 ± 7.83^{ab}	34.41 ± 8.73^{ab}	41.59 ± 12.45^{a}	0.0162
Magnesium (mg/kg)	220.40 ± 14.46^{b}	225.40 ± 15.43^{b}	289.10 ± 85.76^{ab}	328.90 ± 126.50^{a}	0.0034
Phosphorus (mg/kg)	1987.00 ± 108.70^{c}	2046.00 ± 130.5^{bc}	2143.00 ± 134.90^{b}	2794.00 ± 180.70^{a}	0.0017
Potassium (mg/kg)	3082.00 ± 134.50^{b}	3217.00 ± 177.90^{b}	3910.00 ± 292.87^{ab}	4414.00 ± 387.74^{a}	0.0113

^{a,b,c} Within row means with different superscripts differ significantly at $P < 0.05$. Dietary supplementation with β-sitosterol significantly increased calcium, magnesium, phosphorus and potassium mineral content of breast meat of broiler chicken ($P < 0.05$). Diet 1 – fortified with oxytetracycline at 50 mg/kg of feed; diet 2 – fortified with β-sitosterol at 500 mg/kg of feed; diet 3 – fortified with β-sitosterol at 1000 mg/kg of feed; Data is expressed as mean ± SD; n = 12.

The effect of dietary supplementation with β-sitosterol on the fatty acid profile of the Cobb 500 broiler chickens breast meat is presented on Table 4.7. Dietary supplementation with β-sitosterol had similar effect ($P > 0.05$) as dietary oxytetracycline (control) on TSFAs, TMUFAs and TPUFAs content of the broiler chickens breast meat. Dietary supplementation with β-sitosterol had no effect ($P > 0.05$) on the palmitic acid content of broiler chicken's breast meat. However, dietary fortification with β-sitosterol at 1500 mg/kg feed significantly increased ($P < 0.01$) the stearic acid content of broiler chicken's breast meat. Supplementing the broiler chickens' diets with β-sitosterol had no effects ($P > 0.05$) on the oleic acid content of the broiler chicken's breast meat. However, dietary supplementation with β-sitosterol at 500 and 1000 mg/kg feed increased ($P < 0.01$) palmitoleic acid content of Cobb 500 broiler chicken breast meat compared to supplementing with oxytetracycline. Dietary supplementation with β-sitosterol had no effect ($P > 0.05$) on the linoleic acid content of the Cobb 500 broiler chickens breast meat. However, supplementing Cobb 500 broiler chickens' diets with β-sitosterol at 1000 mg/kg significantly ($P < 0.05$) increased their breast meat's α-linolenic acid compared to that from counterpart fed diets fortified with β-sitosterol at 500 mg/kg. The Cobb 500 broiler chickens fed diets with β-sitosterol at 1500 and 500 mg/kg had significantly ($P < 0.05$) increased arachidonic acid and docosahexaenoic acid content in their breast meat compared to that from counterpart fed diets fortified with β-sitosterol at 1000 mg/kg.

Table 4.7: Effect of dietary supplementation with β-sitosterol on the fatty acid profile of breast meat of Cobb 500 broiler chicken

Fatty acid (%)	**Dietary treatment**				**P-value**
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	
Saturated					
Myristic acid (C14:0)	0.45 ± 0.03^{a}	0.47 ± 0.01^{a}	0.46 ± 0.01^{a}	0.49 ± 0.00^{a}	0.0747
Palmitic acid (C16:0)	25.28 ± 0.79^{a}	25.88 ± 0.26^{a}	24.79 ± 0.47^{a}	25.72 ± 0.46^{a}	0.1300
Margaric acid (C17:0)	0.11 ± 0.01^{a}	0.09 ± 0.00^{b}	0.09 ± 0.01^{b}	0.11 ± 0.01^{a}	< 0.01
Stearic acid (C18:0)	8.44 ± 0.08^{a}	8.13 ± 0.10^{ab}	7.45 ± 0.50^{b}	8.20 ± 0.06^{a}	< 0.01
Arachidic acid (C20:0)	0.10 ± 0.01^{a}	0.10 ± 0.01^{a}	0.08 ± 0.00^{a}	0.09 ± 0.01^{a}	0.0589
Behenic acid (C22:0)	0.03 ± 0.01^{a}	0.03 ± 0.00^{a}	0.03 ± 0.01^{a}	0.03 ± 0.00^{a}	0.2058
Lignoceric acid (C24:0)	0.19 ± 0.01^{a}	0.20 ± 0.00^{a}	0.17 ± 0.03^{a}	0.19 ± 0.01^{a}	0.5957
TSFA	34.59 ± 0.94^{a}	34.90 ± 0.38^{a}	33.07 ± 1.03^{a}	34.83 ± 0.56^{a}	0.3497
Monounsaturated					
Myristoleic acid (C14:1)	0.10 ± 0.01^{a}	0.11 ± 0.00^{a}	0.11 ± 0.01^{a}	0.11 ± 0.00^{a}	0.0519
Palmitoleic acid (C16:1)	4.13 ± 0.23^{b}	4.65 ± 0.05^{a}	4.61 ± 0.20^{a}	4.40 ± 0.09^{ab}	< 0.05
Oleic acid (C18:1n9c)	39.52 ± 1.27^{a}	39.43 ± 0.30^{a}	40.16 ± 0.42^{a}	39.45 ± 0.54^{a}	0.5938
Elaidic acid (C18:1n9t)	0.16 ± 0.00^{a}	0.16 ± 0.00^{a}	0.14 ± 0.01^{b}	0.15 ± 0.01^{ab}	< 0.01
Eicosenoic acid (C20:1)	0.36 ± 0.02^{a}	0.33 ± 0.01^{b}	0.35 ± 0.01^{a}	0.33 ± 0.01^{b}	< 0.01
Nervonic acid (C24:1)	0.12 ± 0.01^{a}	0.11 ± 0.01^{a}	0.09 ± 0.02^{a}	0.11 ± 0.01^{a}	0.0839
TMUFA	44.39 ± 1.53^{a}	44.79 ± 0.35^{a}	45.46 ± 0.65^{a}	44.55 ± 0.65^{a}	0.5629

Polyunsaturated					
Linoleic acid (C18:2n6c)	14.48 ± 1.10[a]	13.48 ± 0.49[a]	14.69 ± 0.63[a]	13.77 ± 0.69[a]	0.3073
α-linolenic acid (C18:3n3)	0.99 ± 0.05[a]	0.77 ± 0.11[b]	1.19 ± 0.22[a]	0.91 ± 0.20[ab]	0.0064
γ-linolenic acid (C18:3n6)	0.09 ± 0.01[a]	0.08 ± 0.01[a]	0.09 ± 0.02[a]	0.08 ± 0.01[a]	0.5279
Eicosadienoic acid (C20:2)	0.37 ± 0.02[a]	0.29 ± 0.03[b]	0.34 ± 0.01[ab]	0.34 ± 0.01[ab]	< 0.01
Eicosatrienoic acid (C20:3n3)	0.04 ± 0.01[a]	0.03 ± 0.01[ab]	0.03 ± 0.01[b]	0.03 ± 0.00[b]	< 0.05
Dihomo-gamma-linolenic acid (C20:3n6)	0.45 ± 0.05[a]	0.44 ± 0.02[a]	0.35 ± 0.06[a]	0.41 ± 0.03[a]	0.0743
Arachidonic acid (C20:4n6)	1.80 ± 0.18[a]	1.54 ± 0.05[a]	1.32 ± 0.23[b]	1.61 ± 0.11[a]	< 0.05
Eicosapentaenoic acid (C20:5n3)	0.06 ± 0.01[a]	0.06 ± 0.01[a]	0.05 ± 0.01[a]	0.05 ± 0.01[a]	0.2869
Docosahexaenoic acid (C22:6n3)	0.24 ± 0.03[a]	0.20 ± 0.01[ab]	0.15 ± 0.03[b]	0.20 ± 0.01[ab]	< 0.01
TPUFA	18.52 ± 1.46[a]	16.89 ± 0.73[a]	18.21 ± 1.22[a]	17.40 ± 1.07[a]	0.3172
PUFA:SFA ratio	0.54 ± 0.105[a]	0.48 ± 0.11[a]	0.55 ± 0.14[a]	0.50 ± 0.116[a]	0.2164

[a,b] Within row means with different superscripts are significantly different at $P < 0.05$. Dietary supplementation with β-sitosterol did not affect TSFAs, TMUFAs and TPUFAs of Cobb 500 broiler chickens breast meat ($P > 0.05$); Diet 1 – fortified with oxytetracycline at 50 mg/kg of feed; diet 2 – fortified with β-sitosterol at 500 mg/kg of feed; diet 3 – fortified with β-sitosterol at 1000 mg/kg of feed; Diet 1 – oxytetracycline 50 mg/kg of feed; diet 2 – 500 mg/kg of feed of β-sitosterol; diet 3 – 1000 mg/kg of feed of β-sitosterol; diet 4 – 1500 mg/kg of feed of β-sitosterol; TSFA = Total Saturated Fatty Acid, TMUFA = Total monounsaturated fatty acid, TPUFA = Total polyunsaturated fatty acid; Data is expressed as mean ± SD; n =10 samples per dietary treatment were pooled together.

4.7 Discussion

4.7.1 Carcass characteristics

This study evaluated the effect of supplemental β-sitosterol (500 mg/kg, 1000 mg/kg and 1500 mg/kg of feed) as a growth promoter in place of oxytetracycline on Cobb 500 broiler chickens' meat yield and quality. Current study findings show similarities in slaughter and dressed mass and dressing percentage of Cobb 500 broiler chickens fed the oxytetracycline (control) and β-sitosterol (test) fortified diets. Similarities in these parameters across dietary treatments demonstrate that β-sitosterol can be used, in place of oxytetracycline, to fortify Cobb 500 broiler chicken diets with no risk to meat yield. The Cobb 500 broiler chicken at 42 days of age have an expected slaughter mass ranging from 2500 to 3000 g (Kokoszyński et al., 2017). Findings from the current study showed slaughter masses that ranged from 2263 – 2392 g at 45 days of age which shows that dietary fortification of Cobb 500 broiler chicken diets with β-sitosterol 500, 1000 and 1500 mg/kg of feed and were comparable with other studies. Assuming that the 2500 g slaughter weight at 42 days of age, this suggests that despite the extra 3 days of feeding, on average the chickens were 108 to 237 g below the expected 42-day Cobb 500 broiler chicken slaughter weight. The slightly lower slaughter mass albeit after a 3-day extra feeding duration could have been due to breed effect and diets. Findings from the current study are in agreement with those obtained by Nkukwana et al. (2014) and Mpofu et al. (2016). In their study, Nkukwana et al. (2014) who fortified Cobb 500 broiler chicken diets with *Moringa oleifera* leaf meal reported 42-day slaughter weights ranging from 2147 – 2236 g. Similarly, Mpofu et al. (2016) in their study where they fortified Cobb 500 broiler chicken diets with 5 and 12 g of *Lippia javanica* per kg of feed reported 42-day slaughter weights that ranged from 2035 – 2213 g. The slaughter mass at 42 days is within the range reported by other studies (Banaszak et al., 2021; Kokoszyński et al., 2017).

Dressed carcass weight and dressing percentage, indicators of slaughter value of broiler chickens (Nematbakhsh et al., 2021), are important economic traits in broiler chicken production as key determinants of the marketable portion. In addition to diet type, these economic traits also depend on genotype (breed) and strain, sex and age at slaughter (Mir et al., 2017; Franco et al., 2020). Broiler chickens' dressing percentage is high averaging 74.81% (Hussein et al., 2019). At 42-days of age Cobb 500 broiler chicken have an expected

dressed carcass weight ranging 1934 g to 2414 g and their dressing percentage ranges from 70.52 to 77.80 g (Nikolic et al., 2019; Cobb Vantress, 2022). Findings from the current study showed that at 45 days of age the dressed carcass weight and dressing percentage ranged from 1827 to 1901 g and 73.93 to 80.88%, respectively. Importantly, there were no differences in the dressed carcass weight (empty carcass weight) and dressing percentage of chickens fed the oxytetracycline-fortified control diet and counterparts reared on β-sitosterol-fortified diets. These findings show that β-sitosterol could be used to replace oxytetracycline as a growth promoter in chicken diets as it did not compromise carcass characteristics of Cobb 500 broiler chicken. In their study using Cobb 500 broiler chickens fed *Moringa oleifera* leaf meal fortified diets, Mpofu et al. (2016) reported dressed carcass weights and dressing percentage of 1470 and 1480 g and 76.8 to 78.5 g, respectively. In another study with Cobb 500 broiler chickens fed *Lippia javanica* fortified broiler chicken diets. Mpofu et al. (2016) recorded dressed carcass weights that ranged from 1470 g to 1480 g. The higher dressed carcass weight and dressing percentage of Cobb 500 broiler chickens fed β-sitosterol fortified diets compared to findings by Nkukwana et al. (2014) and by Mpofu et al. (2016) could have been due to shanks being retained as part of the dressed carcass.

4.7.2 Physical meat quality characteristics

Meat quality, an indicator of-product wholesomeness and freshness (Mir et al., 2017; Nusairat, Tellez-Isaias and Qudsieh, 2022) is largely dependent on the appearance and texture of meat which inform the consumer's decision to purchase. Findings from the current study showed that broiler chicken's breast meat from birds fed β-sitosterol fortified diets had similar pHi (5.96-6.00) and pHu (5.69-5.73). Importantly, the breast meat's pH range was within the expected broiler chicken breast meat pH range of 5.7 – 6.2 (Mir et al., 2017). These findings suggest that the breast meat pH obtained in our study is within the normal range. Lower than expected meat pH is associated with it is low pH which decreases the ability to bind to water which causes shrinkage of the myofibrils and loss of water (Huff-Lonergan and Lonergan, 2005). Furthermore, low pH poultry meat due to its negative effect on WHC results in increased cook and drip losses and decreased tenderness and shelf life (Baéza, Guillier and Petracci, 2022). Following slaughter hypoxia sets in resulting in anaerobic metabolism in the muscle/meat which produces lactic acid (Maynard et al., 2023). The lactic acid induced decline in the meat's pH mediates denaturation loss of

solubility of the meat's protein in addition to causing decreased water binding by muscle proteins (Mir et al., 2017; Sun et al., 2019). Petracci et al. (2004) contend that broiler chicken breast meat with a pH value under 5.7 at 45 minutes post-slaughter is most likely to be pale soft exudative (PSE). The development of PSE in broiler chickens meat is attributed to a rapid post-mortem decline in pH due to pre-slaughter stress and temperature (Petracci et al., 2015; Mir et al., 2017). Dry, firm and dark meat results from higher ultimate meat pH values greater 6.2 that result from rapid depletion of muscle glycogen during post rigour (Qiao et al., 2001; Petracci et al., 2004) which results from higher than normal myofibril protein (Moyo et al., 2020). Current study findings show that dietary β-sitosterol did not compromise Cobb 500 broiler chickens breast meat pHi and pHu at 45 minutes and 24 hours post-slaughter, respectively. This suggests that β-sitosterol did not alter the metabolism of glycogen and protein in the muscle post-slaughter during the process of conversion of muscle to meat. Thus, β-sitosterol can potentially be used as a dietary supplement in Cobb 500 broiler chicken diets with not risk of causing either PSE or dry, firm and dark meat.

Consumers select the meat based on its colour, freshness, texture, storage time and juiciness (Mir et al., 2017). Chicken breast meat generally have a pink colour, which is a desirable characteristic by consumers (Choo et al., 2014; Nduku et al., 2021). In the current study, the lightness (L*) of the Cobb 500 broiler chicken breast meat at 45 minutes post-slaughter which was similar across β-sitosterol dietary treatments and ranged from 45.24 to 46.62 and was within the normal range (43 - 53) according to Zhang and Barbut (2005). This indicates that supplementation of Cobb 500 broiler chicken diets with β-sitosterol did not affect or compromise the breast meat lightness of broiler chicken at 45 minutes post-slaughter. Our results are consistent with previous studies which reported the range of 40 to 66 of the lightness values of broiler chicken breast meat (Zhao et al., 2019; Ding et al., 2021). However, the current study observed an increased lightness values which ranged from 58 to 66 at 24-hour post-slaughter.

There are inconsistencies in the literature on the lightness (L*) and pH values for broiler chicken meat. According to Petracci et al. (2004), the lightness of broiler breast meat ranges between 50 and 56, pale meat is >56, and darker meat is <56. However, Qiao et al. (2001) reported that lightness (L*) values of broiler chicken breast meat range between 45.1–55.1. It has been reported that breast colour lightness (L*) values above 49.0 are suggestive of a poor

WHC and increased shear force and a low pH (Mir et al., 2017). In the current study, we obtained a normal range of 45.24 to 46.2 of breast meat colour 45 minutes post-slaughter and is consistent with other studies (Zhao et al., 2019; Cheng et al., 2019). On the other hand, the lighter colour is indicative of increased reflectance caused by the increased water leakage that accumulates on the surface of the breast and reflects the light rays during the measurement of the colour (Bromfield et al., 2021). Fletcher (1995) reported a direct correlation between the colour of the breast fillets and the meat pH. It is believed that a low pH causes the protein in the muscles to spread out, causing the light to reflect directly from the surface, resulting in the light colour (Mir et al., 2017). The variations in breast meat colour, mainly due to pH effects, were shown to affect shelf life, odour development, moisture pick up in marination, drip loss, water-holding and cooking loss (Qiao et al., 2001). Meat pH and colour are highly correlated.

The yellowness (b*) meat colour depends on the total content of lipids (Mir et al., 2017). The higher the lipid content, the lighter the meat and higher yellowness meat colour. The yellowness colour of the breast meat from the current study ranged from 11.54 – 13.44 and was within the normal range (-3 – 12) as described by Petracci et al. (2004). Our results are consistent with findings reported by other studies which supplemented broiler chicken diet with β-sitosterol (Zhao et al., 2019; Ding et al., 2021). Supplementing the broiler chicken with dietary β-sitosterol did not compromise the yellowness colour of breast meat of Cobb 500 broiler chicken. Dietary supplementation of broiler chicken with β-sitosterol had similar effect as oxytetracycline on chroma (C*) and hue (H*) of breast and thigh meat of broiler at 45 minutes and 24 hours post-slaughter. Dietary supplementation with β-sitosterol had similar effect as oxytetracycline on initial and ultimate thigh meat pH of broiler chicken. The pH of thigh meat reported in the current study was within the normal range at both 45 minutes and 24 hours post-slaughter. The current study obtained similar ultimate pH meat as the findings of Ding et al. (2021).

In the current study, the lightness (L*) value of thigh meat colour ranged from 51.94 – 54.94 at 45 minutes post-slaughter and was within the normal range. However, our study observed increased lightness value ranged from 64.20 – 70.74 after 24 hours post-slaughter. The observed lightness (L*) value of thigh meat is not with the lightness value obtained by Ding et al. (2021). The redness (a*) and yellowness (b*) value of thigh meat obtained in the current study was within the normal range as described in the literature (Qiao et al., 2001; Petracci et

al., 2004). However, Ding et al. (2021) showed an increased redness (a*) and yellowness (b*) values of thigh meat colours than the current study. The differences may be due to the age of birds at slaughter, bird strain and dietary treatments. There are factors such as stress, pre-slaughter handling, genetic strain and temperature and pH that may affect the meat quality. During the conversion of muscle to meat, lactic acid builds up in the tissue leading to a reduction in pH of the meat. From the point at which a bird is slaughtered during the meat production process, it is inevitable that water will be lost from the carcass affecting the moisture characteristics (Mir et al., 2017).

Water represents between 70% and 80% of the weight of raw poultry meat (Huff-Lonergan and Lonergan, 2005; Mir et al., 2017). The loss of water is a key concern for meat producers as this water content is reported to contribute to the organoleptic (juiciness, tenderness, texture, smell and colour) of the meat products. Increased water and fat content at the time of consumption are generally associated with increased juiciness. However, cooking induced a decrease in water binding capacity and loss of moisture which can increase juiciness (Baéza Guillier and Petracci, 2022).

Dietary supplementation with β-sitosterol had similar thawing loss of broiler chicken breast meat as the oxytetracycline (control). The results shows that supplementing broiler chickens with β-sitosterol did not compromise the thawing loss of broiler chicken breast meat. The thawing loss of broiler chicken breast meat observed in the current study ranged from 8.43 to 9.55%. Our results are supported by the finding of Akuru et al. (2020) who observed similar thawing loss of 7.87% in the breast meat of broiler chickens fed 8 g/kg pomegranate peel powder (phytochemical). However, the current results were not in agreement with the results of Akuru et al. (2020) who reported thawing loss values of 2.85 to 5.76% with supplementation of 2 to 6 g/kg of pomegranate peel powder to broiler chicken diets. The reason of higher thawing loss obtained in the study could be due to factors such as handling and storage of meat, freezing and thawing conditions which negatively affect the meat quality.

Cooking loss is defined as the loss of sample weight in percentage of water that is lost during cooking (Cao et al., 2012; Strydom et al., 2016). Cooking loss depends on shape and size of the sample, temperature during cooking, final cooking temperature and environment during cooking (Barbut and Leishman, 2022).

The higher the final temperature and the slower the speed of heating, the higher the cooking loss (Kralik et al., 2018) According to Petracci et al. (2004) the normal cooking loss of breast meat is 21.13. The percentages of cooking loss for breast meat observed in this study were higher (24.85 – 26.08) than those reported by Cheng et al. (2019) but comparable to other reports (Matshogo et al., 2020). In addition, high cooking loss percentage shows the reduced ability of the meat to hold water during processing and storage (Petracci and Cavani, 2011).

Meat tenderness is an important meat quality characteristic. The shear force is used to measure the textural integrity of cooked meat. The lower the shear force, the greater the meat tenderness. The decrease in shear force observed in this study for dietary treatment could have been influenced by rigour resolution due to enzymatic breakdown of collagen holding meat fibres together. The shear force results observed in this study are consistent with the results obtained by Cruz et al. (2020) but inconsistent with other studies (Cheng et al., 2019; Ding et al., 2021). The differences might be due to several factors such as chicken breed strain and β-sitosterol inclusion levels in poultry diet used in the studies (Cheng et al., 2019; Ding et al., 2021). Other factors such as gender, age at slaughter, handling of bird prior to slaughter, handling of meat during processing, and cooking method may negatively affect the meat tenderness (Mir et al., 2017). The wide diameter of the muscle fibres and a low score of fat tissue produce tough meat, while the small diameter of muscle fibres and high score of fat tissue produce tender meat (Chartrin et al., 2006). Meat tenderness depends on the degree of contraction of the sarcomere length length, the extent of degradation of the structural myofibrillar proteins, and the connective tissue content (Koohmaraie et al., 2002).

Myofibrillar fragmentation length (MFL) is based on the amount of extracted myofibrillar proteins, the background of post-mortem meat tenderization (Santos et al., 2008). Myofibrillar fragmentation length is strongly associated with indices of meat tenderness such as Warner Bratzler shear force (WBSF) and a useful indicator of meat tenderness (Santos et al., 2008). It correlates with both WBSF values and objective masses of tenderness of cooked meat (Santos et al., 2008). The degree of toughening and of subsequent tenderization in poultry muscle is dependent on the rate and extent of post-mortem glycolysis (Cruz et al., 2020). The smaller MFL is correlated with a reduction in the shear force of meat (Cruz et al., 2020). The higher MFL values indicate a significant change in the structure of meat myofibrils (Barido and Lee, 2021).

In the current study, dietary β-sitosterol had similar effect of MFL as oxytetracycline and that shows that our treatment did not compromise the MFL of broiler chicken breast meat. Factors such as sex can influence the MFL and tenderness, female gives a higher MFL with more tender muscle than males. This could relate to the differences in metabolism and activity between males and females. However, the current study determined the effect of dietary β-sitosterol on the MFL of unsexed Cobb 500 broiler chicken and did not study the gender effect.

4.7.3 Nutrient composition of meat

We determined the effect of dietary supplementation with β-sitosterol on the chemical meat properties (proximate, mineral composition and fatty acid profile) of broiler chicken breast meat.

In the current study, dietary β-sitosterol had similar effect as the oxytetracycline on dry matter (DM) of broiler chicken breast meat. However, the higher inclusion levels of dietary β-sitosterol (1000 and 1500 mg/kg) had increased crude protein than breast meat of broiler chicken supplemented with β-sitosterol at 500 mg/kg and oxytetracycline (control). In the current study, dietary β-sitosterol had similar DM content as the control diet. However, our results disagree with other studies who observed significantly lower or decreased DM (63.77 – 74.40%), CP (22.35 – 23.68%) and ash (0.20 – 2.45%) with dietary supplementation of different phytochemical supplementation to broiler chicken diets (Nkukwana et al., 2016; Ahmad et al., 2017; Al-Baadani et al., 2023). The nutritional and chemical composition showed a large variation between the current and previous studies for crude protein, ash and ether extract when broiler chickens were supplemented with phytosterol or phytochemicals. It was also noted that different breeds, dietary treatments and ages of birds and bird strain may contributed to the differences in the studies.

Mineral composition is one of the determinants of meat quality which affect the colour, odour, tenderness and oxidation (Mir et al., 2017). The mineral content (mainly calcium, phosphorus and potassium) of poultry meat is 1.1% (Rabot, 1998). This parameter is little affected by feeding and other rearing factors as long as the dietary intakes cover the animal requirements (Baéza, Guillier and Petracci, 2022). Moreover, meat quality in chickens is affected by various potential intrinsic and extrinsic factors. Among factors, phosphorus is a critical and expensive mineral in poultry nutrition. Dietary β-sitosterol had similar calcium

content but higher to the control diet. However, high calcium level is usually accompanied by a reduced fat content in meat (Zhang et al., 2022). Higher inclusion level of β-sitosterol (1500 mg/kg) resulted in significantly increased phosphorus than other treatments and the control diet. There are limited studies on the effect of dietary phytochemicals (phytosterol) on mineral composition of broiler chicken's breast meat. The ether extract obtained in the current study ranged from 7.79 – 8.61 and is not in agreement with the findings of Nkukwana et al. (2016) (2.03 – 2.21), Ahmad et al. (2017) (1.69 – 2.06) and Al-Baadani et al. (2023) (2.23 – 2.46), respectively. The differences of ether extract obtained in the studies is due to bird strain, age of bird, and dietary treatments.

Poultry meat is considered by consumers as healthier protein source due to its relatively lower fat content compared with red meat (Barbut and Leishman, 2022). Fatty acid composition plays a critical role in meat quality as it contributes to the sensory attributes, nutritional value and oxidative stability of the meat (Mir et al., 2017). Fatty acid composition varied among animal species and breed (Klensporf-Pawlik et al., 2012), type of muscle and cut of meat (Gruffat et al., 2021), animal diet (Waheed et al., 2018) and fat content (Wood et al., 2008). The fatty acid composition of meat is influenced by factors such as diet, genotype and age of the animal (Milićević et al., 2014). Nevertheless, genetic factor affects the fatty acid composition of meat to a lower extent than the diet and other factors (De Smet, Raes and Demeyer, 2004). The results of the current study showed similar effect on fatty acid composition of broiler chicken meat and that shows that dietary β-sitosterol did not had any influence on total meat SFA, MUFA and PUFA. In the current study, dietary supplementation with β-sitosterol had similar effect of palmitic acid (C16:0) as other treatments and the control diet. As one of the most dominant SFA, it shows that dietary β-sitosterol did not affect the palmitic acid (C16:0). Although it was slightly higher than the normal range of SFA content in broiler chicken. Nowadays, consumers are health conscious and prefer meat with low SFA content which is healthier. Palmitic acid has often been found to increase blood cholesterol level, oleic has been found to have an opposite effect on the blood cholesterol levels (Mpofu et al., 2016). However, the lack of differences of palmitic acid in this study is similar with other studies which did not observe any significant differences on palmitic acid (C16:0) with supplementation of phytochemicals or phytosterol to broiler chickens (Nkukwana et al., 2016; Ding et al., 2021). Dietary supplementation with β-sitosterol at 500 and 1500 mg/kg had significantly higher (8.12 – 8.20) stearic acid (C18:0) than other treatment group (7.44).

Our results are consistent with Mpofu et al. (2016) and Mthana et al. (2022) who observed similar results of stearic acid (8.46 – 9.47) when broiler chickens were supplemented with phytochemicals (*Lippia janica* and *Mucuna pruriens* seed meal). There were no significance differences of Palmitoleic acid (C16:1) among β-sitosterol dietary in the current study and our results are consistent with other studies (Marcinčáková et al., 2011; Mpofu et al., 2016; Mthana et al., 2022). The current study had similar oleic acid (C18:1n9c) across dietary β-sitosterol (39.43 – 40.16) and the control diet (39.45). The result in this study is in agreement with findings of Marcinčáková et al. (2011) who obtained increased (37.03 – 39.43) oleic acid (C18:1n9c). Our results are in contrast with other studies (22.53 – 34.07) who supplemented broiler chickens with either phytosterol or phytochemicals (Nkukwana et al., 2014; Ding et al., 2021). Supplementation of the dietary β-sitosterol in broiler chicken diet increased the levels of elaidic acid (C18:1n9t), α-linolenic acid (C18:3n3) and arachidonic acid (C20:4n6) compared to other β-sitosterol treatment and the control diet. Our results are in line with other studies (Nkukwana et al., 2014; Mpofu et al., 2016; Mthana et al., 2022) but different to the results of Marcinčáková et al. (2011) and Ding et al. (2021) who also supplemented broiler chickens with dietary β-sitosterol or phytochemical. The differences observed in the studies might be due to breed effect and dietary treatment. However, dietary β-sitosterol had similar effect of linoleic acid (C18:2n6c) as the control group. In this study, dietary supplementation with β-sitosterol had significantly higher docosahexaenoic acid (C22:6n3) in diet 4 than diet 3 (1000 mg/kg) but similar with other treatment group and the control diet. Higher PUFA content could make the meat more susceptible to oxidation and reduce the shelf life of produced meat (Marcinčáková et al., 2011; Ding et al., 2021). The n-3 PUFA have preventive and therapeutic effects on cardiovascular diseases, while DHA and EPA are currently the most important n-3 PUFAs (Colussi et al., 2017). It is reported that DHA influences normal blood lipids and has a significant effect on lowering blood lipids in people with hyperlipidemia (Egert et al. 2009).

The positive is mainly increase in the proportion of EPA and DHA, which are essential for the human organism and their lack is in the diet. Meat fatty acid composition is considered as a valuable indicator of meat quality from the human health perspective. However, concern over excessive SFA or PUFA intakes by humans has been reported to have a negative impact to their health. According to World Health Organization (WHO, 2023) only 30% daily intake of the total energy and saturated fats limit to 10% of the calorie has been recommended with cholesterol intake of less than 300 mg per day. Dietary intake of unsaturated fatty acids has

been shown to reduce the risk of cardiovascular disease and possibly the incidence of some cancers (Milićević et al., 2014). Dietary supplementation with phytosterol can change the fatty acid profile of broiler muscle and reduce its SFA content (Ding et al., 2021).

Previous studies have shown that dietary SFA intake is closely related to vascular heart disease (Ramasamy et al., 2006). Therefore, from a health perspective, meat with relatively low SFA content can help reduce the risk of cardiovascular heart disease (Cheng et al., 2019). The recommended ratio of polyunsaturated fatty acids (PUFAs) to SFAs should be above 0.4, with the normal ratio of meat at around 0.1. The ratio of n-6/n-3 PUFAs is considered to be a risk factor in cancers and coronary heart disease, and it is recommended that this ratio be less than 4.0 (Wood et al., 2008). In the current study, the PUFA:SFA ratio of the lower inclusion level (500 mg/kg) of dietary β-sitosterol is within the acceptable range of fatty acid composition. However, other β-sitosterol treatment had slightly higher PUFA:SFA ratio than the normal range. Achieving a better balance of fatty acids in the diet, by decreasing intakes of cholesterol and saturated fats, is therefore seen as an important and effective strategy by which to reduce the incidence of these diseases. Overall, we observed that supplementation of dietary β-sitosterol improved the profile of fatty acids composition of broiler chicken's breast meat. In addition, phytosterol can increase the ratio of PUFA: SFA by changing the fatty acid composition of broiler chicken breast meat (Ding et al., 2021). The antioxidative effects of polyphenols would account for their beneficial effects on improving meat quality and decreasing lipid peroxidation in chicken meat (Choi et al., 2023).

4.8 Conclusion

β-sitosterol can be used to fortify Cobb 500 broiler chicken diets as a growth promoter without compromising meat yield and quality. It can be used to increase the long chain fatty acid content of the meat hence improve nutritional quality. However, caution needs to be exercised in its use as the increased fatty acids it mediates could reduce the shelf life of broiler chicken meat.

Having presented the effect of dietary β-sitosterol on meat yield and quality of Cobb 500 broiler chicken in the current study, the next chapter is the experimental chapter whose content focuses on the effects of supplemental β-sitosterol on the long bone health of Cobb 500 broiler chicken.

Many studies focus on the poultry products (meat) but tend to neglect the health and welfare of the birds hence the next chapter focuses on bone health of Cobb 500 broiler chicken.

4.9 References

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CHAPTER FIVE - EFFECT OF DIETARY β-SITOSTEROL ON FEMORA AND TIBIAE INDICES AND MINERAL CONTENT OF COBB 500 BROILER CHICKENS

5.0 Introduction

Broiler chickens are fast-growing and accrete muscle in a relatively short period hence the need for a functional skeletal support system (Damaziak et al., 2019). The selection of broiler chicken to optimise growth rate and increase meat yield results in disorders of locomotor system function (Zhao et al., 2014). This occurs due the rapid growth and increased muscle mass imposes strain and stress on the chickens' skeletal system especially the femora and tibiae (Talaty, Katanbaf and Hester, 2010) resulting in weakened legs. The strain and stress on these long bones manifest with leg bone problems that compromise movement and in extreme cases cause lameness (Yan et al., 2019; Choppa and Kim, 2023) and fractures (Yamada et al., 2021). Further to causing pain and discomfort, leg lameness limits access to feed and water (Talaty Katanbaf and Hester, 2010). This compromises access to drinking water causing dehydration and to feed intake negatively affecting growth performance (Talaty, Katanbaf and Hester, 2010; Wilcox et al., 2024). Further to reducing growth performance, strained and stressed leg bones increase mortality from osteoporotic fractures (Shim et al., 2012; Hartcher and Lum, 2020; Biesek et al., 2022). In commercial broiler chicken production spontaneous fractures associated with leg skeletal pathology compromise bird welfare (Dinev et al., 2019) and decrease enterprise profitability through increased osteoporotic-induced mortality and downgrading of carcasses in slaughterhouses (Huang et al., 2019; Damaziak et al., 2019).

The skeletal system helps with locomotion, support, and protection of key organs (Alkhtib et al., 2021). Bone is formed by osteoblasts that lay down new bone during skeletal development (Ottewell, 2016). Osteoblasts also indirectly communicate with bone-resorbing osteoclasts. Through direct and indirect mechanisms osteoblasts and osteoclasts regulate and help in the maintenance of the homeostatic balance between bone formation and bone resorption in a process called remodelling (Ottewell, 2016). Bone mineral matrix is largely made up of hydroxyapatite; a composite predominantly made up of calcium and phosphorus (Rath et al., 2000). Calcitonin, parathyroid hormone (PTH) and vitamin D_3 regulate plasma and bone calcium homeostasis (Dacke et al., 2015). In avian species PTH stimulates the calcium release from bones by activating osteocytes; a process that can lead to bone resorption (Brown, 1991; Silva and Bilezikian, 2015). In response to hypercalcaemia, calcitonin stimulates osteoblasts to deposit calcium in bones and inhibits both renal calcium reabsorption and intestinal calcium absorption (Gay, Gilman and Sugiyama, 2000; Dacke et al., 2015; Tonon et al., 2022).

In broiler chicken production, further to selection-induced bone pathologies and diseases, nutritional deficiencies are a key cause of the development of leg problems that affect bird growth (Bessei, 2006). Adequate bone mineralization is vital to skeletal integrity and allows for withstanding gravity and additional loading (Shim et al., 2012; Nakhon et al., 2019). Poor bone mineralisation increases the incidence of leg and bone weakness (Onyango et al., 2003; Sanchez-Rodriguez et al., 2019). The diet is the major source of calcium and phosphorus required for bone formation, growth and development (Imari et al., 2020; Li et al., 2020). Inadequate dietary supply of these key minerals increases susceptibility of broiler chickens to bone fracturing.

While genetic defects that cause bone weakness in poultry are not well known, tibial dyschondroplasia has been shown to be heritable and could indirectly contribute to bone weakness during early growth stages (Wong-Valle et al., 1993; Huang et al., 2019). A consequence for breeding poultry for increased growth rate and superior meat production is its negative on bone integrity and its ability to withstand the increased load (Dixon, 2020).

The use of phytochemicals, β-sitosterol included, to fortify broiler chicken feeds, in place of synthetic antibiotic growth promoters, has given positive results with respect to feed efficiency, growth performance and meat and egg quality (Shini, Shini, and Bryden, 2019; Cheng et al., 2019; Ding et al., 2021). No specific study has evaluated the effects of supplemental β-sitosterol on bone integrity, in broiler chickens. β-sitosterol has been shown to inhibit the activity of bone resorption cells and to promote bone formation in layer chickens (Shini, Shini, and Bryden, 2019). Importantly, in a rat model of senile postmenopausal osteoporosis, β-sitosterol was shown to mediate increased bone mineral density through upregulating the expression of bone mineral density and osteogenesis-related factors via the phosphoinositide- 3-kinase–protein kinase B signal pathway (Liu and Xue, 2023). Potentially, therefore, fortifying of broiler chicken diets with β-sitosterol might positively impact femora and tibiae indices of the chickens.

5.1 Aims of the study

This study evaluated the effects of fortifying Cobb 500 broiler chicken's diets with β-sitosterol as a growth promoter on the chickens' femora and tibiae mass, length, mass to length ratio, breaking strength and mineral content.

5.2 Objectives of the study

The specific objectives of the study were to determine the effect of dietary β-sitosterol on Cobb 500 broiler chickens:

a) femora and tibiae bone lengths, mass and mass to length ratio.
b) tibiae breaking strength and tibiae calcium, magnesium, phosphorus and potassium content.

5.3 Hypotheses of the study

a) H_0: Dietary supplementation with β-sitosterol does not affect femora and tibiae mass, length, mass to length ratio and tibiae breaking strength and calcium, magnesium, phosphorus and potassium content of Cobb 500 broiler chicken.

b) H_1: Dietary supplementation with β-sitosterol affect femora and tibiae mass, length, mass to length ratio, tibiae breaking strength and calcium, magnesium, phosphorus and potassium content of Cobb 500 broiler chicken.

5.4 Materials and methods

5.4.1 Housing, feeding and watering of chickens

The management (housing, lighting, feeding and watering) of the chickens was as described in Chapter 3; subheading 3.6.4.

5.4.2 Dietary ingredients and diet formulation

The types and sources of dietary ingredients and formulation of the experimental diets are as described in Chapter 3; subheadings 3.6.2 and 3.6.3.

5.4.3 Experimental design, samples collection and preparation

The experimental design is as described in Chapter 3 subheading 3.6.5. Immediately following slaughter and dressing, thirty Cobb 500 broiler chicken carcasses from each dietary treatment were randomly selected and femora and tibiae dissected from the left side of each carcass, defleshed and all soft tissues removed. The defleshed bones were dried in an oven (Salvis®, Salvis Lab, Switzerland) at 50ºC for 5 days to constant mass.

5.4.4 Determination of mass, length and mass to length ratio

On removal from the oven the femora and tibiae were allowed to cool to room temperature and then each bone was weighed using electronic scale (Snowrex EQ-1200, Snowrex International Company, Taipei, Taiwan). Femur and tibia lengths were measured with a digital Vernier calliper (SDP-S-ETP-1001, Major Tech, Johannesburg, South Africa). Femur and tibia length were determined by measuring the distance from the centre of the distal articular surface to the distance from the centre of the lateral condylar surface. The mass to length ratio (Seedor ratio) of each femur and tibia was computed as described by Seedor et al. (1991) using the equation:

$$Seedor = \frac{bone\ mass\ (g)}{bone\ length\ (mm)}$$

5.4.5 Determination of tibiae calcium, magnesium, phosphorus and potassium content

The dried tibiae were ashed in a furnace as described by the Association of Analytical Chemists (AOAC 2005; method number: 932.16). Tibiae calcium, magnesium, phosphorus and potassium content was determined using inductively coupled optical emission spectroscopy (ICO- ES) as described by Huang and Schulte (1985). Briefly, the tibia from each bird was ashed in a muffle furnace (BDI 73 muffle furnace, Labotec, Sheffield, England) at 600ºC for 6 hours. The ash was cooled to room temperature before being milled using a barrel mill (250 cc single pot barrel mill, Eriez Lab Equipment, Delta, Canada). The milled ash was digested in concentrated nitric acid and perchloric acid at 200ºC (Zasoski and Burau, 1977). Thereafter, an aliquot from the digest solution was used for determination of calcium, magnesium, phosphorus and potassium content on a Varian Liberty 200 spectrometer (Varian, Perth, Australia).

5.4.6 Determination of tibiae breaking strength

Tibiae from the right side of ten randomly selected Cobb 500 broiler chicken carcasses were used to determine bone breaking strength. The tibiae were dissected out, defleshed and cleaned of all soft tissues and each then wrapped in a mutton cloth soaked in 0.9% saline and stored in a freezer at -20ºC pending the breaking strength measurement. Following overnight thawing at 5ºC, the breaking strength of each tibia was determined by three-point bending test using an Instron 4502 material testing machine (Instron Ltd., High Wycombe, UK) modified method by Incharoen et al. (2016). Default specimen dimensions were setup as circular

geometry of 10 mm diameter, a 30 mm anvil height and a crosshead speed of 50 mm/minutes. Vertical hydraulic force was applied at the midpoint of the bone shaft to minimize splintering (Incharoen et al., 2016). The load defined as force (Newton, N) of cross-sectional area represented the bone breaking strength.

5.5 Data analyses

Group data on the long bone weights, lengths, weight to length ratio, breaking strength and mineral content was analysed using Graph Pad Prism Version 8.0.2 (Graph Pad Prism Version 8.0.2 (Graph-pad Software Inc, San Diego, USA) statistical package. A one-way ANOVA was used to analyse data. The Tukey *post hoc* test was used to compare treatment means. The level of significance was set at $P < 0.05$. The statistical model used was:

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

where Y_{ij} = dependent variable of interest

μ = is the overall mean common to all observations

T_i = is the fixed effect of the i^{th} dietary treatment (i = 1, 2, 3, 4)

e_{ij} = is the random residual error.

5.6 Results

5.6.1 Femora and tibiae indices and breaking strength

The effect of dietary β-sitosterol on femora and tibiae length, mass, mass to length ratio and tibia breaking strength of Cobb 500 broiler chicken is shown on Table 5.1. Feeding Cobb 500 broiler chicken with β-sitosterol fortified diets had similar effects as feeding them the control oxytetracycline fortified diet on femora length, mass, mass to length ratio of the chickens ($P = 0.6001$). However, while the β-sitosterol had a similar effect as oxytetracycline on tibiae masses, tibiae from chickens fed the diet fortified with β-sitosterol at 1500 mg/kg of feed (diet 4) had significantly shorter tibiae compared to that of counterparts fed the control oxytetracycline fortified diet (diet 1) and diet 2 fortified with β-sitosterol at 500 mg/kg feed ($P = 0.0018$). The tibia mass to length ratio of chickens fed diet 2 was significantly lower ($P = 0.0142$) than that of tibiae from chickens fed dietary β-sitosterol at 1500 mg/kg of feed (diet 4). Cobb 500 broiler chicken fed diets fortified with β-sitosterol (diets 2 to 4) had tibiae with significantly greater breaking strength compared to that of counterparts from chicken fed the oxytetracycline fortified control diet ($P < 0.0001$).

Table 5.1: Effect of dietary supplementation with β-sitosterol on femora and tibiae length, mass, mass to length ratio and tibia breaking strength of Cobb 500 broiler chicken

Parameter	Dietary treatments				P-value
	Diet 1	Diet 2	Diet 3	Diet 4	
Femur					
Dry mass (g)	5.63 ± 0.62[a]	5.38 ± 0.53[a]	5.63 ± 1.56[a]	5.94 ± 1.53[a]	0.6001
Length (mm)	72.58 ± 3.14[a]	71.33 ± 2.73[a]	70.38 ± 4.67[a]	73.49 ± 2.62[a]	0.0597
Seedor ratio (g/mm)	0.08 ± 0.01[a]	0.08 ± 0.01[a]	0.08 ± 0.02[a]	0.08 ± 0.02[a]	0.8918
Tibia					
Dry mass (g)	7.78 ± 1.34[a]	7.09 ± 0.95[a]	7.50 ± 1.55[a]	7.59 ± 1.46[a]	0.5301
Length (mm)	99.81 ± 3.49[a]	97.85 ± 3.87[a]	91.57 ± 3.54[ab]	86.65 ± 4.04[b]	0.0018
Seedor ratio (g/mm)	0.07 ± 0.01[ab]	0.08 ± 0.01[b]	0.09 ± 0.03[ab]	0.10 ± 0.03[a]	0.0142
Breaking strength (N)	159.58 ± 58.29[b]	263.70 ± 46.42[a]	245.20 ± 43.01[a]	285.60 ± 59.84[a]	< 0.0001

[a, b] Within row means with different superscripts differ significantly at ($P < 0.05$). No significant differences in broiler chicken's femora dry mass, length and tibiae mass to length ratio across dietary treatments ($P > 0.05$). Broiler chicken fed diet supplemented with β-sitosterol at 1500 mg/kg had significantly shorter ($P = 0.0018$) tibia than counterparts fed diets supplemented with β-sitosterol at 500 mg/kg and or oxytetracycline. Chikens fed diet fortified with β-sitosterol at 500 mg/kg had significantly lower ($P = 0.0142$) tibia mass to length ratio compared to the rest. Tibiae breaking strength was lowest in chickens fed the oxytetracycline fortified control fiet. Diet 1- fortified with oxytetracycline at 50 mg/kg of feed; diet 2 – fortified with β-sitosterol at 500 mg/kg of feed; diet 3 – fortified with β-sitosterol at 1000 mg/kg of feed; diet 4 - fortified with β-sitosterol at 1500 mg/kg of feed; data is expressed as mean ± SD; n = 10-12 per dietary treatment.

5.6.2 Tibiae calcium, magnesium, phosphorus and potassium content

Table 5.2 shows the effect of dietary β-sitosterol on the mineral content of Cobb 500 broiler chicken's tibiae. The chicknes' tibiae calcium, magnesium, phosphorus and potassium content were similar ($P > 0.05$) across dietary treatments.

Table 5.2: Effect of dietary supplementation with β-sitosterol on the calcium, magnesium phosphorus and potassium bone mineral content of the Cobb 500 broiler chicken

Parameter	Dietary treatments				P-value
	Diet 1	Diet 2	Diet 3	Diet 4	
Calcium (%)	14.08 ± 0.98	13.17 ± 0.84	14.01 ± 1.02	13.02 ± 2.48	0.1824
Magnesium (%)	0.29 ± 0.03	0.28 ± 0.03	0.29 ± 0.03	0.42 ± 0.03	0.0865
Phosphorus (%)	6.86 ± 0.48	6.42 ± 0.42	6.79 ± 0.47	6.34 ± 1.22	0.2080
Potassium (%)	0.36 ± 0.03	0.35 ± 0.03	0.36 ± 0.04	0.72 ± 0.08	0.1003

No significant differences in broiler chicken's mineral content across dietary treatments ($P > 0.05$); Diet 1 – fortified with oxytetracycline at 50 mg/kg of feed, diet 2 – fortified with β-sitosterol at 500 mg/kg of feed, diet 3 – fortified with β-sitosterol at 1000 mg/kg; diet 4 – fortified with β-sitosterol at 1500 mg/kg of feed; data is expressed as mean ± SD; n = 10 – 12 per dietary treatment.

5.7 Discussion

5.7.1 Effect of dietary β-sitosterol on femora and tibiae mass, length, tibiae breaking strength and mineral content

This study evaluated the effect of fortifying Cobb 500 broiler chickens' diets with β-sitosterol on the chickens' femora and tibiae length, mass, mass to length ratio, tibiae breaking strength and tibiae calcium, magnesium, potassium and phosphorus content. Dietary β-sitosterol at 1500 mg/kg feed decreased tibiae lengths of Cobb 500 broiler chicken compared to those of counterparts fed the control oxytetracycline fortified and the 500 mg/kg feed β-sitosterol fed counterparts. An increase in tibiae length would be expected to correlate with the bone mass and strength (Kolakshyapati et al., 2019) which was the case in the current study. If the bone length continues to grow and increase without the increase the strength that could lead to skeletal problems.

Bone is a dynamic tissue whose properties are a function of physiological, nutritional, genetic and mechanical and physical activities (Rath et al., 2000). Avian long bones are made up of the outer compact cortical bone which surrounds the cancellous or trabecular bone and marrow space (Rath et al., 2000). Bone quality, length, and calcium and phosphorus content are important indicators of bone metabolism and strength (Ma et al., 2020). The mass of material per unit volume of bone, which consists of inorganic and organic components, constitutes bone density (Muszyński et al., 2019). The inorganic matrix makes up the major component of the extracellular matrix, hence bone mineral density is considered to reflect the status of bone health (Muszyński et al., 2019).

Low bone density is a risk factor for fracture (Kolakshyapati et al., 2019). Bone responds to placed mechanical forces by increasing its mass and mineral content per unit bone length (McDevitt et al., 2006; Talaty, Katanbaf and Hester, 2010) and adapts to changes in physical loading and activities by modelling and remodelling (Rath et al., 2000). The ability of bone to endure stress that is related to ultimate load or stress at which it will break constitutes its strength (Rath et al., 2000). The load at break is the sum of all forces and moments applied to bone (Muszyński et al., 2019). Bone mass and strength increases with use (Kolakshyapati et al., 2019).

Growth rate, sex, age, genetics, physical loading, nutrition, disease and hormones affect bone strength (Biesek et al., 2022). Growth hormone facilitates cortical bone growth, and steroid hormones increase cancellous bone formation (Rath et al., 2000). Oestrogen deficiency is a known risk factor for osteoporosis and bone fragility (Rath et al., 2000). Growth is an important determinant of bone strength: bone mass increases with growth and its strength is proportional to its mass (Kolakshyapati et al., 2019). Yamada et al. (2021) reported an age-dependent increase in bone strength in osteoporotic chicken. Adequate dietary calcium and phosphorus are key determinants of poultry bone strength. Calcium and phosphorus, the primary inorganic constituents of bone make up 95% of the bone mineral matrices and are important for bone health and strength (Hart et al., 2020).

The similarities in Cobb 500 broiler chickens' femora mass, length, mass to length ratio across dietary treatments suggest that dietary fortification with β-sitosterol in place of

oxytetracycline neither compromised nor improved the growth and development of the femora. Importantly, the similarities in the tibiae calcium, magnesium, potassium and phosphorus content of the Cobb 500 broiler chickens across dietary treatments suggest that as a dietary supplement β-sitosterol in place of oxytetracycline did not alter the metabolism and deposition of these key bone minerals.

The observed increased tibiae strength in Cobb 500 broiler chickens fed diets fortified with β-sitosterol compared to control oxytetracycline counterparts. This suggest that the use of this natural growth promoter could mitigate skeletal and locomotion challenges associated with breeding and selection of broiler chicken for higher growth rates and increased meat yield that (increased growth rates and meat yield) are associated increased incidences of lameness and fractures. The tibiae strengths reported by the current study in Cobb 500 broiler chickens are comparable to those reported by Nkukwana et al. (2014) in Cobb 500 broiler chickens whose diets were fortified with *Moringa oleifera* leaf meal. *Moringa oleifera* is a rich source of β-sitosterol (Anwar et al., 2007) thus the similarities in the tibiae strength in Cobb 500 broiler chickens in the current study and that by Nkukwana et al. (2014) maybe ascribed to the phytosterol. Tibiae strengths reported by Nkukwana et al. (2014) in Cobb 500 broiler chickens whose diets were fortified by *Moringa oleifera* leaf meal are higher compared to those reported by the current study. While this difference may not be easy to explain, it is important to note that *Moringa oleifera* leaf meal further to being a rich source of β-sitosterol also contains flavonoids, alkaloids, tocopherol, and phenolics (Anwar et al., 2007) which have been shown to increase bone strength (Nkukwana et al., 2014). Furthermore, *Moringa oleifera* leaf meal is a rich source of calcium (Gopalakrishnan, Doriya and Kumar, 2016). It could, therefore, be speculated that synergistic availability of cocktail of phytochemicals and increased calcium availability from the *Moringa oleifera* leaf meal resulted in bone with a more optimal matrix and mechanical strength. The high flavonoids content and antioxidant activity of *Moringa oleifera* leaf meal (Anwar et al., 2007; Pakade et al., 2013) have been shown to deposits calcium and phosphorus for bone development in broilers (Zhang et al., 2020) and also regulate calcium and phosphorus metabolism in bone (Wang et al., 2022) thus the higher tibiae/femora strength observed in Cobb 500 broiler chicken reported by Nkukwana et al. (2014) could have been due to the antioxidant activity and flavonoid effect of the leaf meal. Current study findings show that the three dietary β-

sitosterol inclusion levels (low, medium and high) resulted in tibiae with greater strength compared to counterparts reared on the oxytetracycline fortified control diet. It can therefore be recommended that Cobb 500 broiler chicken diets can be supplemented with β-sitosterol at 500 mg/kg feed, the lowest inclusion level used in the current study, which helps save production costs without risking skeletal and locomotion challenges.

5.8 Conclusion

β-sitosterol can be used to fortify Cobb 500 broiler chicken diets without compromising femora and tibiae growth and development. Importantly, it can potentially reduce fracturing of tibiae compared to oxytetracycline since it resulted in greater strength as demonstrated by the higher breaking strengths of tibiae from Cobb 500 broiler chicken reared on β-sitosterol fortified diets.

Having evaluated the effect of dietary β-sitosterol on Cobb 500 broiler chicken's femora and tibiae growth and development, mineral content and breaking strength, it became pertinent to evaluate its effect on bird health. The next chapter therefore is a narrative on the evaluation of dietary β-sitosterol on Cobb 500 broiler chicken health and welfare by specifically interrogating its effects on oxidative stress and systemic antioxidant status and on plasma markers of general, liver and kidney health.

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CHAPTER SIX - EFFECT OF DIETARY β-SITOSTEROL ON THE HEALTH OF COBB 500 BROILER CHICKENS

6.0 Introduction

Commercial production of broiler chickens exposes the birds to environmental and physiological stresses. These stressors have severe consequences on productive performance, immune response and health status of the exposed birds (Alagawany et al., 2018; El-Hack et al., 2018). Poor biosecurity practices, housing and feeding strategies are other challenges experienced in broiler chicken farming which can contribute to stress and compromise the bird's health resulting in loss of profitability. Stocking density is an important management factor that may create a compromised microenvironment characterised with inadequate space and high ammonia concentration that may affect bird welfare and performance. Ammonia is a primary contaminant in poorly ventilated poultry houses which can decrease growth rate and body mass and alter feed efficiency of broiler chicken (Han et al., 2021). Long term exposure to ammonia can cause several health problems and compromises the bird's welfare (Mishra and Jha, 2019). Poor air and litter quality, high temperature and intense competition for feed and drinking water cause stress and that can also compromise chicken welfare and can increase the mortality rate (Sugiharto, 2022).

Environmental and physiological stressors associated with commercial broiler chicken production lead to oxidative stress by overwhelming the birds' systemic antioxidant system (Mishra and Jha, 2019; Talebi, Dolatkhah and Joyani, 2022). Oxidative stress is a major concern in the poultry industry which can negatively impact poultry health, production and quality of the products (Mishra and Jha, 2019). It results from redox imbalance between the pro-and antioxidants with increased or higher levels of pro-oxidants in the body (Desbruslais and Wealleans, 2022). Oxidative stress leads to an increase in the production of reactive oxygen species that damage tissues, cells and organelles in addition to causing lipid peroxidation and apoptosis (Chen et al., 2022).

Failure to overcome stress can cause a progression to dysregulation and derangement of the homeostatic mechanisms which usually leads to death (Puron et al., 1995). In commercial poultry production, synthetic antibiotics have and, in some regions, continue to be used to fortify feeds at sub-therapeutic doses to mitigate environment-induced stress (Abreu et al., 2023). Such use of antibiotics protects the birds from pathogenic gut-resident microbes

(Kogut, 2019) and mitigates microbe-induced irritation and inflammation of the gastrointestinal tract mucosa (Mehdi et al., 2018). Furthermore, in addition to reducing the incidence and severity of subclinical infections, fortification of poultry diets with subtherapeutic doses of synthetic antibiotics also reduces the toxic bacterial products produced by gut resident bacteria that damage gut mucosae (Mehdi et al., 2018; Yadav and Jha, 2019). Thus the use of synthetic antibiotics as growth promoters has had and continues to have positive effects on broiler chicken health, especially gut health, via suppression of undesirable gut microbes and mitigating inflammation of the gut mucosae (Abd El-Hack et al., 2022; Paul et al., 2022). However, it has been shown to compromise the environment and cause antibiotic resistance and cause the deposition of antibiotic residues in meat which can have negative implications to consumer health (Alagawany et al., 2021).

There is a dire need to search and develop acceptable natural alternatives, for example, phytochemicals that mimic synthetic antibiotics on broiler poultry health and welfare, without negative residual effects. β-sitosterol possesses antimicrobial, antioxidant, gut and immune system modulating, hepato- and reno-protective activities (Ding et al., 2021; Xie et al., 2022). β-sitosterol, therefore, can potentially be used to replace synthetic antibiotics in broiler chicken diets based on its health-beneficial biological activities both *in vitro* and *in vivo*. The data on the effects of dietary fortification of broiler chicken diets with β-sitosterol on bird health and welfare, especially with regards to metabolic substrate content and on general, liver and kidney health is limited.

6.1 Study aim

This study evaluated the effect of fortification of Cobb 500 broiler chicken diets with β-sitosterol in place of oxytetracycline on metabolic substrate content, oxidant and antioxidant status and liver and kidney health of the birds.

6.2 Study objectives

Specifically, the study determined in Cobb 500 chicken, the effects of dietary β-sitosterol on:

a) blood glucose, cholesterol and triglyceride concentration and liver lipid content.
b) oxidative stress by assaying for MDA and antioxidant status by assaying for CAT, GSH, GST, GSH-Px and SOD
c) liver function by assaying for plasma ALP, ALT, AST, GGT activities and total bilirubin and globulin concentrations, kidney function by examining plasma blood urea nitrogen and creatinine concentration and pancreas by assessing amylase.

6.3 Hypotheses of the study

a) H_0: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline does not affect chicken's blood glucose, cholesterol and triglyceride concentration and liver lipid content.

   H_1: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline affects chicken's blood glucose, cholesterol and triglyceride and liver lipid content.

b) H_0: Dietary supplementation with β-sitosterol as a growth promoter and as replacement of oxytetracycline does not affect oxidative stress (MDA) and antioxidant status (CAT, GSH, GST, GHS-Px and SOD activities)

   H_1: Dietary supplementation with β-sitosterol as a growth promoter and as replacement of oxytetracycline affects oxidative stress (MDA) and antioxidant status (CAT, GSH, GST, GSH-Px and SOD) activities.

c) H_0: Fortifying Cobb 500 broiler chicken diets with β-sitosterol as a growth promoter in place of oxytetracycline does not affect plasma surrogate markers of liver and kidney function and other general health markers.

6.4 Materials and methods

6.4.1 Ethical clearance and study location

Study location and ethical clearance for the study are described in Chapter 3; sub-heading 3.6.1.

6.4.2 Diet formula

The diet formulation of the study is described in Chapter 3; sub-heading 3.6.3.

6.4.3 Chicken management: housing, feeding and watering of chickens

The management of chickens was as described in Chapter 3; sub-heading 3.6.4.

6.4.4 Experimental design

The experimental design is as described in Chapter 3; subheading 3.6.5.

6.4.5 Termination, sample collection and procedures

At the end of the 6-week feeding trial, the chickens were fasted for 4 hours but with access to clean drinking water to prevent potential dehydration. Immediately thereafter, thirty birds randomly selected from each treatment group, were humanely slaughtered by decapitation with a guillotine (Harvard Apparatus, Holliston, Massachusetts, United States). The free-flowing blood from the jugular veins was collected into heparinised blood collection tubes (Cheng et al., 2019). Blood collected into heparinised blood collection tubes was centrifuged in a Thermo Sorvall® MC 12V centrifuge (Du Pont Instruments, Connecticut, USA) at the room temperature for 15 minutes at a force of 5000 × *g*. Plasma was then decanted into labelled 1.5 mL microtubes tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) and then frozen stored at -20ºC till analyses for oxidant and antioxidant status and surrogate markers of liver, general health and kidney. The liver from each dressed chicken carcass was weighed, and then a sample was frozen stored at -20ºC pending determination of liver lipid content, and the other sample was stored in 10% phosphate buffered formalin for histological assays.

6.4.6 Determination of blood glucose

A drop of blood was drawn from blood in the heparinized collection tubes and used to determine fasting blood glucose concentration. A calibrated Contour-plus glucometer (Contour Plus®, Bayer Corporation, and Mishawaka, United State) was used to measure each chicken's blood glucose concentration as per the manufacturer's guidelines.

6.4.7 Determination of hepatic lipid content

Each frozen liver sample was thawed at room temperature for 30 minutes. Then the thawed liver sample was lyophilized in a freeze-dryer (VirTis BenchTop BK-FD12, SP 90 Scientific, NewYork, USA) connected to a vacuum pump (Sogevac SV28, Leybold GmbH, Cologne, Germany) over 24-hour period at conditioner temperature of -50ºC and shelf temperature of 26ºC. The freeze-drying was done according to the manufacturer's instructions. Each freeze-dried liver sample was grounded using a grinder (Retsch ZM200, Retsch-Allee 1-5 42781, Haan, Germany) to pass through a 1 mm sieve. For each ground liver sample, liver lipid content was then determined using the Soxhlet apparatus with petroleum ether as a solvent as described by the Association of Analytical Chemists (AOAC, 2005; method number 920.39). In summary, 5 g of freeze-dried ground liver sample was weighed, put into an extraction thimble, and covered with absorbent cotton. Two hundred (200) mL of petroleum ether was added to a pre-weighed flask. The thimble was then inserted into the flask and placed onto the Soxhlet heating mantle. The samples were extracted for 2 hours at 55ºC, the boiling point of petroleum ether. The oil extracted into the flask was then dried using a rota-evaporator (Labex®, Krugersdorp, South Africa) connected to a vacuum at 37ºC. The flask and the oil were then dried in an oven (Salvis®, Salvis Lab, Switzerland), at 50ºC for 30 mins and cooled in a desiccator for 30 minutes before being re-weighed to determine the lipid content. The percentage of fat extracted from each liver sample was calculated using the equation:

$$\%\ \text{Liver lipids (oil)} = \frac{mass\ of\ flask\ with\ oil\ (g) - mass\ of\ empty\ flask(g)}{mass\ of\ liver\ sample\ (g)} \times 100$$

6.4.8 Determination of liver histology

The liver tissues preserved in 10% buffered phosphate formalin were processed using an automatic tissue processor (Micron STP 120, Thermo Fischer Scientific, USA) and then

embedded in paraffin wax. Each processed sample was then sectioned at 5µm and stained with haematoxylin and eosin (H and E) on a glass slide and covered with a glass coverslip as described by Reyes-Gordillo et al. (2007). To assess the hepatocellular changes, at least two random fields per slide were viewed under a light microscope (Leica Biosystems, USA) at 40X magnification. A camera which uses ZEISS ZEN microscope software mounted onto the microscope was used to capture images. The liver sections were examined for steatosis and assigned a score relative to the level of fat deposits in the sample as per the classification by Kleiner et al. (2005). The liver histology sections were assessed and scored for ballooning, hepatic steatosis, and lobular inflammation using the semi-qualitative non-alcoholic fatty liver disease activity score system (NAS): steatosis scoring 0: <5%; 1: 5–33%; 2: 34–66%; 3: >66%; foci of lobular inflammation scoring 0: none; 1: <2; 2: 2–4; 3: >4; hepatocellular ballooning scoring 0: none; 1: few ballooned cells; 2: many ballooned cells; total NAS (sum of values recorded for each criteria) < 2: not steatohepatitis; 3 and 4: uncertain; >5: probable or definite steatohepatitis (Kleiner et al., 2005).

6.4.9 Determination of surrogate markers of liver and kidney function

The plasma surrogate markers of liver function [alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyl transferase (GGT) activities, globulin and total bilirubin (TBIL) concentrations] and markers of kidney function [blood urea nitrogen (BUN) and creatinine concentrations] and as well as amylase activity and other markers of health (calcium, cholesterol, triglyceride concentrations) of the chicken were determined using the IDEXX colorimetric clinical chemistry analyzer (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc., Westbrook, Maine, USA) was done at as per the manufacturer's instructions. Briefly, the stored plasma samples were allowed to thaw at room temperature and gently inverted to mix the contents. Three hundred microlitres of plasma from each sample were drawn using a pipette and transferred into a catalyst sample cup which was then inserted in a colorimetric-based clinical chemistry analyser along with pre-loaded disks for analyses of the various health profile components. The machine automatically analysed the samples, and a printout of results was provided.

6.4.10 Determination of oxidative stress and antioxidant status

The plasma catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione-peroxidase (GSH-Px), activities and glutathione (GSH) and malondialdehyde (MDA) concentration were measured using the enzyme-linked immunosorbent assay (ELISA) kits (Bioassay Technology Laboratory, Shanghai, China) as described by Gungor, Altop and Erener (2021) following the manufacturer's instructions.

6.5 Data analyses

Data are presented as mean ± standard deviation. GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, California, USA) was used to analyse the data. The steatosis grades and inflammation data are expressed as median and range and were analysed by a Kruskal-Wallis followed by multiple-comparisons Dunn's *post hoc* test. The rest of the data were analysed by a one-way analysis of variance followed by a multiple-comparisons Turkey *post hoc* test. Significance was set at $P < 0.05$.

The statistical model used was:

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

where Y_{ij} = dependent variable of interest

μ = is the overall mean common to all observations

T_i = is the fixed effect of the i^{th} dietary treatment ($i = 1, 2, 3, 4$)

e_{ij} = is the random residual error.

6.6 Results

6.6.1 Circulating metabolic substrate concentrations

The effect of dietary supplementation with β-sitosterol on the circulating metabolic substrates content of Cobb 500 broiler chicken is presented in Table 6.1. The fasting blood glucose concentration, triglyceride concentration and calcium concentration of Cobb 500 broiler chickens fed diets fortified with β-sitosterol was similar ($P > 0.05$) to that of counterparts whose diet was fortified with oxytetracycline. However, the plasma cholesterol concentration of broiler chickens fed diet 4 fortified with 1500 mg/kg of β-sitosterol was significantly higher ($P < 0.0001$) than that of chicken fed diets fortified with 500 mg/kg of β-sitosterol and 50 mg/kg of oxytetracycline. The plasma cholesterol concentration of broiler chickens fed diet 4 fortified with 1500 mg/kg feed β-sitosterol was similar ($P > 0.05$) to that of counterparts fed diet 3 with β-sitosterol at 1000 mg/kg feed.

Table 6.1: Effect of dietary supplementation with β-sitosterol on the circulating metabolic substrates content of Cobb 500 broiler chickens

Parameter	**Dietary treatments**				**P-value**
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	
Blood glucose (mmol/L)	10.42 ± 1.24^{a}	10.28 ± 0.78^{a}	10.24 ± 0.98^{a}	10.17 ± 1.29^{a}	0.8327
Triglycerides (mg/dL)	114.00 ± 18.49^{a}	101.20 ± 11.27^{a}	113.30 ± 13.76^{a}	103.30 ± 15.81^{a}	0.3458
Cholesterol (mg/dL)	127.50 ± 15.83^{c}	156.20 ± 27.90^{b}	182.20 ± 8.95^{ab}	191.20 ± 8.47^{a}	< 0.0001
Calcium (mg/dL)	8.75 ± 1.18^{a}	8.22 ± 0.66^{a}	8.35 ± 0.25^{a}	8.67 ± 0.77^{a}	0.6084

[a, b, c] Within row means with different superscripts differ significantly at $P < 0.05$. No significance differences ($P > 0.05$) were observed on the blood glucose, triglycerides and calcium concentrations of broiler chickens across treatments. Chickens supplemented with β-sitosterol at 1500 mg/kg had increased ($P < 0.0001$) cholesterol concentration than those supplemented with 500 mg/kg and oxytetracycline (control). Diet 1 – fortified with oxytetracycline at 50 mg/kg of feed; diet 2 – fortified with β-sitosterol at 500 mg/kg of feed; diet 3 – fortified with β-sitosterol at 1000 mg/kg of feed; diet 4 – fortified with β-sitosterol at 1500 mg/kg of feed; n = 30 per dietary treatment.

The effect of dietary supplementation with β-sitosterol on the liver lipid content of Cobb 500 broiler chicken is presented on Figure 6.1. The liver lipid content of the chickens fed diets 3 and 4 was significantly higher ($P < 0.0001$) than that fed diet 1 ($P < 0.0001$). However, there was no significant difference ($P > 0.05$) in the liver lipid content of the chickens fed β-sitosterol fortified diets.

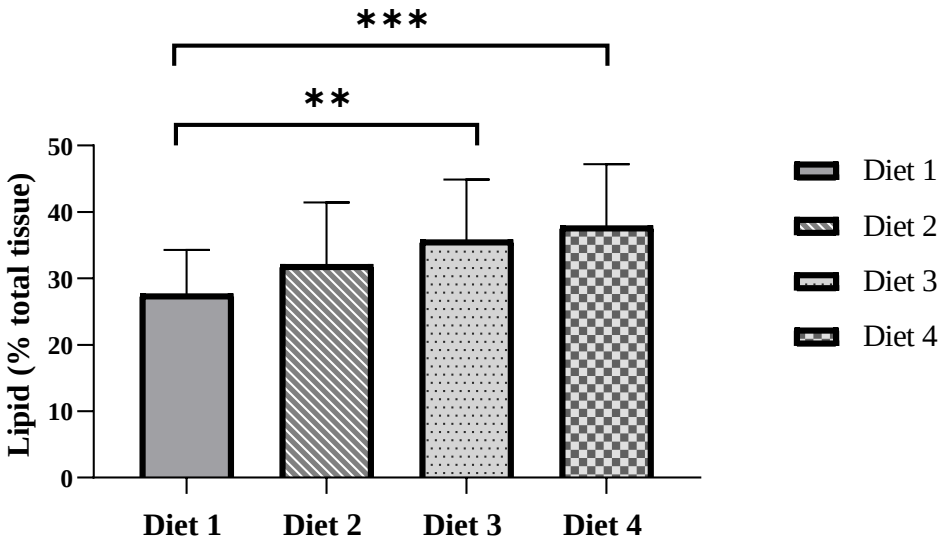

Figure 6.1: Effect of dietary supplementation with β-sitosterol on the liver lipid content of Cobb 500 broiler chicken. Chickens fed diets 3 and 4 had significantly higher liver lipid content ($P < 0.0001$) compared to those fed the control oxytetracycline fortified diet 1. Diet 1- fortified with oxytetracycline at 50 mg/kg of feed; diet 2 – fortified with β-sitosterol at 500 mg/kg of feed; diet 3 – fortified with β-sitosterol at 1000 mg/kg of feed; diet 4 - fortified with β-sitosterol at 1500 mg/kg of feed; data is expressed as mean ± SD; n = 24 per dietary treatment.

6.6.2 Liver histology: microarchitecture

Figure 6.2 shows the effect of dietary supplementation with β-sitosterol on liver histology of Cobb 500 broiler chicken. Hepatocytes from chickens fed the control (oxytetracycline fortified) and low dose (500 mg/kg feed) β-sitosterol had normal centrally located nuclei with no steatosis. However, photomicrographs of liver sections from chickens fed diets 3 and 4 (fortified with 100 and 1500 mg/kg feed β-sitosterol showed presence of hepatic steatosis.

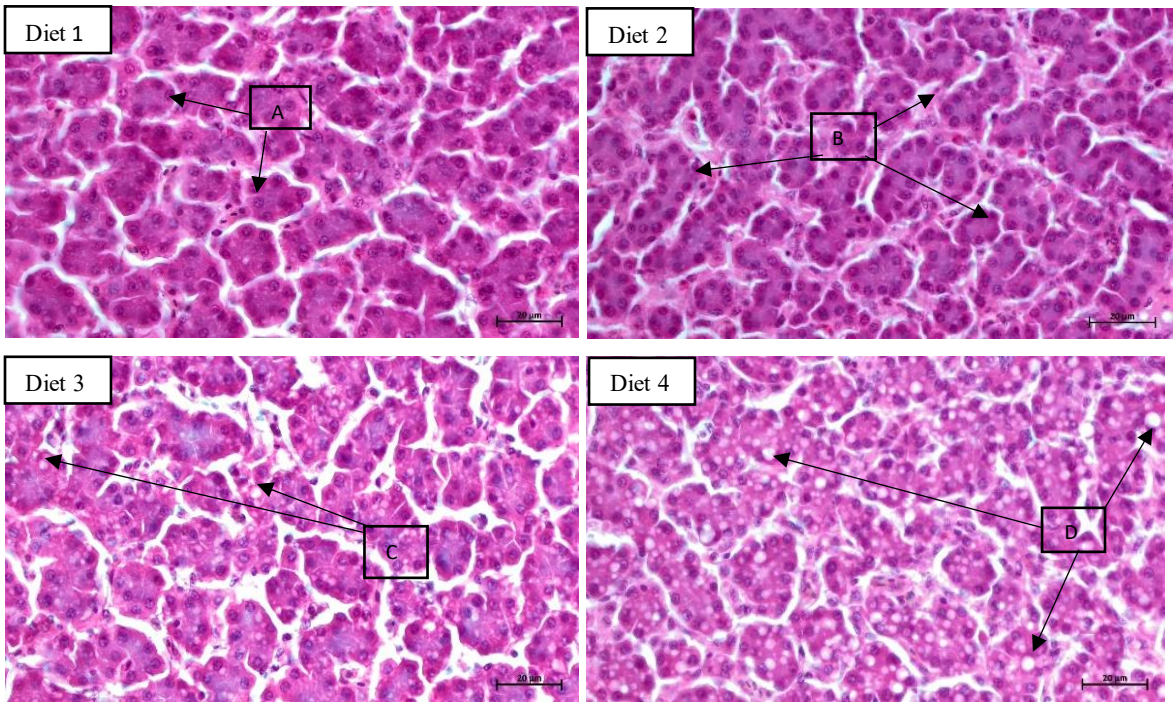

Figure 6.2: Representative photomicrographs (H and E staining, 40X magnification) of liver sections from Cobb 500 broiler chickens fed diets fortified with β-sitosterol. Liver section from chicken fed diet 1 (control, 50 mg/kg of oxytetracycline) and diet 2 (500 mg/kg of β-sitosterol had normal hepatocytes with centrally located nuclei (Arrows A and B). Liver section from chickens fed diet 3 showed hepatic micro-steatosis (Arrow C) and counterparts fed diet 4 showed macro-steatosis (Arrow D). Diet 1- fortified with oxytetracycline at 50 mg/kg of feed; diet 2 – fortified with β-sitosterol at 500 mg/kg of feed; diet 3 – fortified with β-sitosterol at 1000 mg/kg of feed; diet 4 - fortified with β-sitosterol at 1500 mg/kg of feed.

6.6.3 Liver histology: steatosis, ballooning, inflammation and disease activity score

The effect of dietary supplementation with β-sitosterol on hepatic steatosis, ballooning, inflammation and disease activity score of Cobb 500 broiler chicken is shown in Table 6.2. There was no significant difference ($P > 0.05$) in the hepatic ballooning scores of the chickens across dietary treatments. However, chickens fed diets 3 and 4 had significantly higher hepatic steatosis scores compared to counterparts fed diet 1(control) and diet 2 (low dose β-sitosterol ($P < 0.05$). Furthermore, chicken fed β-sitosterol fortified diets had significantly higher hepatic lobular inflammation and total NAS scores compared to control counterparts.

Table 6.2: Effect of dietary supplementation with β-sitosterol on hepatic steatosis, ballooning and inflammation of Cobb 500 broiler chickens

Parameter	**Dietary treatments**				**P-value**
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	
Macro-steatosis	0 (0;0)^{c}	0 (0;0)^{c}	0 (1;1)^{b}	2 (1;2)^{a}	0.0352
Micro-steatosis	0 (0;0)^{c}	0 (0;0)^{c}	1 (1;0)^{b}	1 (1;2)^{a}	0.0008
Ballooning score	0 (0;0)^{a}	0 (0;0)^{a}	0 (0;0)^{a}	0 (0;0)^{a}	0.8951
Lobular inflammation	1 (0;1)^{c}	2 (1;1)^{ab}	2 (2;1)^{ab}	2 (3;1)^{a}	0.0319
Total NAS	**1 (1;0)^{c}**	**1 (2;1)^{b}**	**1 (3;0)^{ab}**	**2 (4;0)^{a}**	**0.0003**

$^{a, b, c}$ Within row means with different superscripts differ significantly at $P < 0.05$. Chickens fed diets 3 and 4 had significantly higher ($P < 0.05$) steatosis scores compared to counterpart fed diet 1 (control) diet 2 (500 mg.kg feed β-sitosterol). Hepatic ballooning scores were similar across dietary treatments ($P > 0.05$). Chickens fed β-sitosterol fortified diets had significantly higher hepatic lobular inflammation and total NAS score than control counterpart ($P < 0.05$). Diet 1 – fortified with oxytetracycline at 50 mg/kg of feed; diet 2 – fortified with β-sitosterol at 500 mg/kg of feed; diet 3 – fortified with β-sitosterol at 1000 mg/kg of feed; diet 4 – fortified with β-sitosterol at 1500 mg/kg of feed; data is expressed as median and range; n = 8 per dietary treatment.

6.6.4 Surrogate markers of liver function

Effect of dietary supplementation with β-sitosterol on the plasma surrogate markers of liver function of Cobb 500 broiler chicken is presented on Table 6.3. Plasma alkaline phosphatase, alanine aminotransferase activities and globulin and total protein concentrations of the chickens fed diets fortified with β-sitosterol was similar ($P > 0.05$) to that of oxytetracycline supplemented control counterparts. The plasma aspartate aminotransferase and gamma-glutamyl-amino transferase activities of the chickens fed diet 4 (1500 mg/kg feed β-sitosterol) were significantly higher ($P < 0.05$) compared to control diet but similar ($P > 0.05$) to the other β-sitosterol fortified diets.

Table 6.3: Effect of dietary supplementation with β-sitosterol on the plasma surrogate markers of liver function of Cobb 500 broiler chicken

Parameter	**Dietary treatment**				**P-value**
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	
Alkaline phosphatase (U/L)	201.20 ± 54.80[a]	195.50 ± 27.44[a]	198.00 ± 18.35[a]	207.70 ± 16.35[a]	0.9270
Alanine aminotransferase (U/L)	11.17 ± 2.40[a]	10.65 ± 1.66[a]	10.22 ± 0.40[a]	10.53 ± 0.49[a]	0.7405
Aspartate aminotransferase (U/L)	248.30 ± 97.90[b]	313.50 ± 30.57[ab]	330.70 ± 18.48[ab]	371.80 ± 57.21[a]	< 0.05
Gamma-glutamyl transferase (U/L)	16.55 ± 1.24[b]	18.33 ± 0.52[ab]	18.50 ± 0.84[ab]	19.67 ± 2.34[a]	< 0.01
Globulin (mg/dL)	223.50 ± 21.89[a]	192.20 ± 22.29[a]	210.80 ± 23.18[a]	190.20 ± 34.54[a]	0.1148
Total bilirubin (mg/dL)	0.15 ± 0.08[a]	0.13 ± 0.05[a]	0.12 ± 0.04[a]	0.17 ± 0.02[a]	0.7356

[a, b] Within row means with different superscripts differ significantly at ($P < 0.05$). No significant differences ($P > 0.05$) in plasma alkaline phosphatase and alanine aminotransferase activities and globulin and total bilibirubin concentration across dietary treatment ($P>0.05$). Aspartate aminotransferase and gamma-glutamyl transferase activities were higher ($P < 0.05$) in chicken fed diet 4 (1500 mg/kg of β-sitosterol) compared oxytetracycline supplement control counterparts. Diet 1 – fortified with oxytetracycline at 50 mg/kg of feed; diet 2 – fortified with β-sitosterol at 500 mg/kg of feed; diet 3 – fortified with β-sitosterol at 1000 mg/kg of feed; diet 4 – fortified with β-sitosterol at 1500 mg/kg of feed; data is expressed as mean ± SD; n = 6 per dietary treatment.

6.6.5 Surrogate markers of kidney function and pancreatic health

The effect of fortifying Cobb 500 broiler chicken diets with β-sitosterol on the chickens' blood urea nitrogen (BUN) and creatinine concentrations (markers of kidney function) and plasma amylase activity (marker of pancreatic health) is shown in Table 6.4. There were no significant differences ($P > 0.05$) in the BUN and creatinine concentrations and plasma amylase activity of the Cobb 500 broiler chickens across dietary treatments.

Table 6.4: Effect of dietary supplementation with β-sitosterol on the plasma surrogate markers of kidney function and pancreatic health of Cobb 500 broiler chicken

Parameter	**Dietary treatment**				**P-value**
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	
Kidney					
Blood urea nitrogen (mg/dL)	2.98 ± 0.07[a]	2.99 ± 0.17[a]	3.00 ± 0.11[a]	3.00 ± 0.12[a]	0.9340
Creatinine (mg/dL)	0.12 ± 0.04[a]	0.10 ± 0.01[a]	0.10 ± 0.01[a]	0.11 ± 0.02[a]	0.9418
Pancreas					
Amylase (U/L)	358.00 ± 73.67[a]	363.50 ± 27.96[a]	374.50 ± 80.28[a]	381.70 ± 63.46[a]	0.9196

[a] Within row means with the same superscripts are not significantly different ($P > 0.05$). Plasma BUN and creatinine concentrations and amylase activity were similar across dietary treatments ($P > 0.05$) Diet 1 – fortified with oxytetracycline at 50 mg/kg of feed; diet 2 – fortified with β-sitosterol at 500 mg/kg of feed; diet 3 – fortified with β-sitosterol at 1000 mg/kg of feed; diet 4 – fortified with β-sitosterol at 1500 mg/kg of feed; data is expressed as mean ± SD; n = 6 per dietary treatment.

6.6.6 Oxidative stress and antioxidant status

The effect of dietary supplementation with β-sitosterol on the plasma oxidative stress marker malondialdehyde (MDA) concentration and plasma system antioxidant enzymes activities of Cobb 500 broiler chicken is presented on Table 6.5. Chicken fed β-sitosterol fortified diets had significantly lower ($P < 0.05$) plasma MDA concentration compared to oxytetracycline fortified diet controls. The plasma SOD and CAT activities of the Cobb 500 broiler chickens increased ($P < 0.0001$) with increasing dietary β-sitosterol but was similar ($P > 0.05$) in chickens fed diets 3 and 4. Plasma GSH-Px and GST concentrations were similar ($P > 0.05$) in birds fed diets 1 through to 3 but highest ($P < 0.05$) in chickens fed diet 4. There was a general increase in antioxidant enzyme activities and decrease in MDA concentration with increasing dietary β-sitosterol.

Table 6.5: Effect of dietary supplementation with β-sitosterol on plasma malondialdehyde concertation and systemic antioxidant enzymes activities/concentrations of Cobb 500 broiler chicken

Parameters	**Dietary treatments**				**P-value**
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	
MDA (nmol/mL)	1.25 ± 0.08^{a}	1.12 ± 0.10^{b}	1.01 ± 0.11^{c}	1.04 ± 0.05^{c}	< 0.0001
SOD (U/mL	69.11 ± 8.43^{c}	85.56 ± 10.63^{c}	111.10 ± 13.34^{b}	136.40 ± 17.00^{a}	< 0.0001
CAT (ng/mL)	5.16 ± 1.19^{c}	5.83 ± 1.73^{bc}	7.25 ± 1.53^{b}	9.00 ± 1.70^{a}	< 0.0001
GST (ng/mL)	13.02 ± 4.72^{b}	13.56 ± 4.42^{b}	17.97 ± 5.85^{b}	26.79 ± 7.19^{a}	< 0.01
GSH-Px (ng/mL)	113.80 ± 14.30^{b}	126.00 ± 17.34^{b}	130.00 ± 18.33^{b}	165.30 ± 27.63^{a}	< 0.05
GSH (nmol/L)	2.07 ± 0.37^{c}	2.55 ± 0.50^{b}	3.01 ± 0.39^{a}	3.30 ± 0.47^{a}	< 0.0001

[a, b, c] Within row means with different superscripts differ significantly at $P < 0.05$. Plasma MDA concentration of chickens fed β-sitosterol fortified diets were significantly lower compared to controls. Plasma CAT and SOD concentrations were highest ($P < 0.05$) in chickens fed diet 4. Plasma GSH-Px, GST and GSH increased with increasing dietary β-sitosterol and was highest in chickens fed diet 4 ($P < 0.05$). CAT – Catalase, SOD – Superoxide dismutase, GST – Glutathione *S*-transferase, GSH-Px – Glutathione peroxidase, GSH – Glutathione and MDA – Malondialdehyde. Diet 1 – fortified with oxytetracycline at 50 mg/kg of feed; diet 2 – fortified withβ-sitosterol at 500 mg/kg of feed; diet 3 – fortified with β-sitosterol at 1000 mg/kg of feed; diet 4 – fortified with β-sitosterol at 1500 mg/kg of feed; data is expressed as mean ± SD; n = 16 per dietary treatment.

6.7 Discussion

This study evaluated the effect of dietary supplementation with β-sitosterol in place of oxytetracycline in Cobb 500 broiler chickens' circulating and stored metabolic substrates content, oxidative stress levels and antioxidant status, kidney and liver health.

6.7.1 Effect of dietary β-sitosterol on circulating metabolic substrates content

Dietary β-sitosterol had no effect on blood glucose and plasma triglycerides, and calcium concentration but at higher (1000 and 1500 mg/kg feed) inclusion levels it increased the chickens' plasma cholesterol concentration. These findings show that β-sitosterol can be used to replace oxytetracycline as a growth promoting feed supplement without negatively impacting blood glucose and calcium homeostasis and triglyceride metabolism.

The endocrine pancreas derived insulin and glucagon mediate blood glucose homeostasis and the use of glucose as an energy source (Kawasaki et al., 2020). Insulin stimulates lipoprotein lipase to mediate triglyceride uptake into cells including adipocytes and glucagon stimulates hormone-sensitive lipase to hydrolyse stored triglycerides to free fatty acids and glycerol (Buyse and Decuypere, 2015). In broiler chickens, the normal blood fasting blood glucose concentration ranges from 5.6 to 11 mmol/L (Makino et al., 2021). Findings from the current study show fasting blood glucose range of 10.17 to 10.42 mmol/L which is within the reported normal range (Makino et al., 2021). Xie et al. (2022) reported blood glucose concentrations ranging from 9.10 to 11.39 mmol/L in broiler chickens fed diets supplemented with β-sitosterol, a finding congruent with the current study findings despite the differences in the chickens' ages.

Triglycerides are the major storage form of lipids that are synthesized in the intestinal mucosa and liver (Bogusławska-Tryk et al., 2016; Zaefarian et al., 2019). An increase in the mobilisation of triglycerides may occur during starvation especially in obese birds (Hochleithner, 2013). In avian species the normal triglyceride ranges from 150 to 220 mg/dL (Gustavsen et al., 2016; Zaefarian et al., 2019). High plasma triglyceride levels are associated with increased risk of severe steatosis in birds (Hermier, 1997). However, the triglyceride concentration obtained in the current study ranged from 101.20 to 113.30 mg/dL and was within the normal range from literature. It can therefore be inferred that dietary β-sitosterol

can be used without negatively impacting the endocrine pancreatic function especially with regards to glucose and triglyceride metabolism.

In chickens, cholesterol metabolism is correlated with diet, age, sex, growth rate, physiological status, feeding environment, and management (Wood et al., 1961; Xie et al., 2022). Plasma cholesterol concentration in chickens have been shown to decrease with increasing cholesterol deposition in the tissues (Wood et al., 1961). In avian species normal cholesterol concentration ranges from 180 to 250 mg/dL (Jones, 2008; Zaefarian et al., 2019). In the current study, cholesterol concentration ranged from 156.20 to 191.20 mg/dL. An increase of cholesterol concentration can compromise the bird's health and is associated with lipaemia and liver disease such as fatty liver haemorrhagic syndrome (FLHS) (Stanford, 2005; Lin et al., 2021). In addition, higher plasma cholesterol concentration is associated with the risk of cardiovascular disease in birds (Gustavsen et al., 2016). While the plasma cholesterol reported in the current study falls within the range reported for avian species by Jones (2008), the use of β-sitosterol as a dietary supplement may predispose broiler chickens to hypercholesterolemia since at high dietary inclusion it caused significant increase in plasma cholesterol concentration.

Cholesterol and triglycerides as non-polar liquid substances are transported in the plasma bound by various lipoprotein particles (Stanford, 2005). The very low-density lipoprotein (VLDL), intermediate density lipoproteins (IDLs), low-density lipoprotein (LDL) and high-density lipoproteins (HDL) are among lipoprotein particles which are synthesized and secreted by the liver (Zaefarian et al., 2019). Increase LDL-cholesterol concentration is associated with increased risk of cardiometabolic diseases while increased HDL-cholesterol is associated with reduced risk (Yuan et al., 2019; Xie et al., 2022). While Ding et al. (2021) reported that supplementary β-sitosterol decreased broiler chicken plasma total cholesterol, LDL cholesterol and triglyceride levels, however, findings from the current study are at variance with other studies as they report increased plasma cholesterol. The differences in cholesterol values between the current study and the previous study could be due to the different doses of β-sitosterol and chicken breed. The current study supplemented Cobb 500 broiler chicken diets with high doses of β-sitosterol (500 -1500 mg/kg of feed) and Ding et al. (2020) supplemented White feather broiler chicken diets with lower doses of β-sitosterol (10 – 80 mg/kg of feed). Calcium is critical in the maintenance of bone health, nerve impulse conduction and muscle contraction and is also required for energy metabolism. Alteration in

calcium homeostasis manifest with poor bone health and fatigue. In birds, the normal plasma calcium concentration ranges from 8 to 13 mg/dL (Harr, 2002) and in birds hypocalcemia has been shown to cause seizures and renal complications (Hochleithner, 2013) while hypercalcemia has been shown to cause hemolysis (Jones, 2008). The Cobb 500 broiler chicken plasma calcium ranged from 8.22 to 8.67 mg/dL which is the normal range reported by Harr (2002). Importantly, the current study also showed similarities in bone calcium concentration across treatments. Collectively, these findings demonstrate that β-sitosterol can be used as a dietary supplement in Cobb 500 broiler chicken diets without altering calcium metabolism.

6.7.2 Effects of dietary β-sitosterol on liver lipid content and histology

The liver is a key metabolic organ which metabolises fats, carbohydrates, proteins, vitamins and minerals, detoxifies xenobiotics, regulates the half-life of signalling molecules including hormones and also excretes waste products of metabolism (Zaefarian et al., 2019). It is a major site for *de novo* lipogenesis and the fat it produces is metabolised in all tissues (Hermier, 1997). *De novo* hepatic lipogenesis generates triglycerides, phospholipids and cholesterol (Emami et al., 2020). In chickens, triglycerides produced from hepatic *de novo* lipogenesis are exported to peripheral tissues (Nematbakhsh et al., 2021) where lipoprotein lipase catalyses their storage in adipose tissue whence, they are mobilised via hydrolysis by hormone sensitive lipase for energy production via β-oxidation (Lange, 2004; Watt and Hoy, 2012; Xiao, 2020). The feeding of high energy diets has been shown to cause excessive hepatic fat deposition as well as synthesis and export of triglycerides to other tissues (Zaefarian et al., 2019; Emami et al., 2020). Findings from the current study showed that dietary fortification with β-sitosterol at 1000 and 1500 mg/kg feed significantly increased the chickens' liver lipid content by 35.8 and 37.9%, respectively and it also caused hepatic macro- and micro-steatosis when compared to control. Findings from the current study reported liver fat content ranging from 31to 38% which is within the 30 to 40% range reported from livers of normally fasted broiler chickens (Van Ryssen, Webb and Myburgh, 2023). While the reported liver fat content in the current study falls within the expected liver fat content of broiler chickens, dietary β-sitosterol mediated increase in liver fat content at 1000 and 1500 mg/kg feed shows significantly increased liver fat content which may increase the risk of developing fatty livers and associated diseases.

Measuring total fat does not show the distribution within the liver and whether despite similar quantities, the interventions caused pathology. Therefore, histological analyses were performed on the liver samples. Indeed, histological assessment confirmed the development of steatosis and lobular inflammation with dietary fortification with β-sitosterol, an indication of the risk to the birds' liver health with high level of dietary β-sitosterol.

Excessive lipid storage in the hepatocytes results in increased ROS production from cellular electron transfer reactions during nutrient metabolism (Chen et al., 2022). Increased lipid peroxidation in fatty livers results in the inflammation (Emami et al., 2020). Current study finding showed increased hepatic steatosis, hepatic lobular inflammation and total NAS scores in chickens fed diet with the highest (1500 mg/kg) β-sitosterol inclusion which suggests that its use at this level may alter hepatic microarchitecture in broiler chickens.

6.7.3 Effects of dietary β-sitosterol on plasma markers of health

Biochemical indicators in the blood can be used to display the health status of birds (Rehman et al., 2017). The liver is a key metabolic organ. Many metabolic processes that occur in the liver are catalysed by intracellular liver enzymes (Zaefarian et al., 2019). Damage to the liver increases permeability of liver cells resulting in the leakage of liver cell components including enzymes that are usually found in liver cells (Johnson and Sherding, 2009). When there is hepatocellular damage, the alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) are released into the circulatory system (Jonathan et al., 2021). The plasma activity of these enzymes can be used as surrogated markers of liver health (Toghyani et al., 2011; Zhu et al., 2014). High plasma activities of ALT and AST are indicative of liver damage (Frasinariu et al., 2022) and high plasma alkaline phosphatase (ALP) activity is associated with damage to cells lining bile canaliculi and bile ducts (Mahmoud, Haggag and El-Gebaly, 2012).While ALT activity is largely expressed in the liver, the enzyme is also distributed in the heart muscles and in the kidney (Mahmoud, Haggag and El-Gebaly, 2012; Yuan et al., 2021). However, AST is largely distributed in liver tissue and myocardial cells and contends that ALT is a more specific indicator of liver damage (Yuan et al., 2021) and have been found to increase in laying hens with FLHS (Hamid et al., 2019). Furthermore, many proteins in the blood, including those related to lipid metabolism, are synthesized in the liver, and the abundance and structure of these proteins also change with the development of liver disease, making them potential biomarkers for liver diseases (Zhu et al., 2021).

The findings from the current study showed that dietary supplementation with β-sitosterol had no effect on the Cobb 500 broiler chickens' plasma ALP and ALT activities but chickens whose diets fortified with β-sitosterol at 1500 mg/kg feed had significantly higher plasma AST and GGT activities compared to counterparts fed diet 1. However, the AST and GGT activities of chicken fed β-sitosterol fortified diets were similar. Taken together, it can be argued that the similarities in the chickens' plasma ALT activity, which is highly expressed in hepatocytes (Yuan et al., 2021), across dietary treatments, suggests that dietary β-sitosterol may not have caused hepatocellular damage in the chickens. Furthermore, similarities in the plasma ALP activity of the chickens across dietary treatments also suggests that β-sitosterol may be used as a dietary supplement in Cobb 500 broiler chicken diets without risk to causing damage to the biliary system. However, the high plasma GGT activity seen in chicken fed diets fortified with 1500 mg/kg β-sitosterol could be a pointer to its possible detrimental effects on liver health since Jonathan et al. (2021) contends high plasma aminotransferases activity, including GGT activity, is indicative of hepatocellular damage. This is confirmed by the high NAS scores seen in the current study.

Further to its metabolic and detoxification functions, the liver is also synthesises plasma proteins including albumins and globulins as well as clotting factors such as fibrinogens (Friedman and Martin, 2017). Compromised liver function manifests with decreased plasma liver-derived proteins concentration which in extreme cases can cause oedema (Rueda and Lipsett, 2016). Findings from the current study showed similarities in the chickens' plasma globulin concentration which suggest that although dietary β-sitosterol might have caused some degree of hepatocellular damage (increased plasma GGT activity), it did not compromise the liver's synthetic function.

In addition to the liver, the kidney is another key organ with multiple physiological functions (Echols, 2006; Zaefarian et al., 2019). In humans, livestock and poultry both acute and chronic kidney injury are associated with high mortality rates (Imtiaz et al., 2019). The primary causes of kidney injury (KI) include ischemia, hypoxia or nephrotoxicity (Rueda and Lipsett, 2016). A key manifestation of KI is a rapid decline in the glomerular filtration rate (GFR) whose decrease is usually associated with decreased renal blood flow (Echols, 2006). Among their many functions, kidneys also filter and excrete metabolic wastes including urea, creatinine and bilirubin (Echols, 2006; Jones, 2008) hence high plasma concentrations of these metabolic wastes could be indicative of compromised kidney metabolic waste excretory

function. The normal plasma creatinine concentration in birds ranges from 0.1 to 0.4 mg/L (Hochleithner, 2013). Findings from the current study showed plasma creatinine concentrations that ranged from 0.10 to 0.11 mg/dL which is congruent to the normal range reported by Hochleithner (2013). Furthermore, findings from the current study showed similarities in plasma blood urea nitrogen (BUN) and total bilirubin (TBIL) concentration across dietary treatments. The chickens' BUN and TBIL concentrations ranged from 2.99 to 3.00 mg/dL and 0.12 to 0.17 mg/dL, respectively. Literature shows that in broiler chickens the normal plasma BUN and TBIL ranges from 0.0 to 4.0 mg/dL and 0.1 to 1.2 mg/dL, respectively (Kolmstetter and Ramsay, 2000; Jahantigh et al., 2019). Although not the gold standard, plasma BUN and TBIL can be used as some of the surrogate markers of kidney health (Jones, 2008; Hartung, 2016). The similarities in the plasma BUN and TBIL concentrations in the chickens across dietary treatments suggest that β-sitosterol can potentially be used, at the three evaluated doses, as a dietary supplement in Cobb 500 broiler chicken diets with no negative effects on the kidneys' excretory role.

Amylase is a key digestive enzyme produced by salivary glands and exocrine pancreatic acini. Together with the exocrine pancreatic ductular cells, the acini secrete into the duodenum pancreatic juice (Guo et al., 2021) composed of a cocktail of digestive enzymes and a bicarbonate rich fluid. In avians, plasma amylase activity ranges from 100 to 600 IU/L (Sakas, 2013). An increase in plasma amylase activity is associated with acute pancreatitis (Jones, 2008) and inflammation of the pancreatic duct which results in obstruction (Harr, 2002) backflow and rapture of exocrine pancreatic cells releasing contents into systemic circulation. Rapture of exocrine pancreatic acini manifests with plasma increased activities of pancreatic digestive enzymes. Current study findings show similarities in the plasma amylase activity (range: 363.10 to 281.70 U/L) of the Cobb 500 broiler chickens across dietary treatments. The plasma amylase activity reported in by the current study falls within the normal plasma amylase activity as reported by Sakas (2013). It can therefore be inferred that at the dietary inclusion levels evaluated by the current study β-sitosterol can be used to fortify Cobb 500 broiler chicken diets without negative effects on exocrine pancreatic synthetic and secretory roles.

6.7.4 Effects of dietary β-sitosterol on oxidant and antioxidant status

Overproduction of free radicals lead to oxidative stress when the produced ROS overwhelms the natural systemic antioxidant system (Ponnampalam et al., 2022). *In vivo,* the first line of

defence against oxidative stress is provided by catalase, glutathione peroxidase (GPx) and superoxide dismutase (SOD); the systemic antioxidant enzymes (Surai et al., 2019). Superoxide dismutase has been shown as the main driver of cellular and overall bodily adaptation to stress in commercial poultry production (Surai et al., 2019). The additional synthesis of SOD in stress conditions is an adaptive mechanism to decrease ROS formation that seeks to protect against oxidative stress by quenching excess ROS and help establish homeostasis (Azadmanesh and Borgstahl, 2018). Fortifying poultry diets with phytosterols has been shown to upregulate the activity of antioxidant enzymes to markedly reduce oxidative stress (Munawar et al., 2023). β-sitosterol has been shown to improve antioxidant enzymes activities both *in vitro* (Vivancos and Moreno, 2005) and *in vivo* (Summanen et al., 2003). Findings from this study have shown that fortifying Cobb 500 broiler chicken diets with β-sitosterol at 1500 mg/kg of feed markedly decreased plasma MDA concentration but increased SOD, CAT and GSH-Px activities. Further to confirming the reported antioxidant activity of β-sitosterol (Summanen et al., 2003; Vivancos and Moreno, 2005), the current study findings indicate that β-sitosterol protects against oxidative damage by upregulating the systemic antioxidant enzymes activities. Upregulation of the activities of these enzymes eliminate excess ROS and resulting in the maintenance of homeostasis. Dong et al. (2020) observed that in broiler chickens, dietary fortification with quercetin, also a phytochemical, decreased plasma MDA concentration but increased the activities of the systemic antioxidant enzymes, a finding mirrored by the current study thus demonstrating the protective antioxidant activities of the phytochemicals. Zhao et al. (2019) also reported that dietary supplementation of broiler chicken diets with β-sitosterol increased plasma SOD, CAT and GSH, GSH-Px activities, a finding also observed in the current study. Surai (2016) reported that an increase in the natural antioxidant enzymes activity demonstrates strong antioxidant capacity, thus it can be inferred that in the current study, dietary β-sitosterol at 1500 mg/kg feed stimulated enhanced natural antioxidant response as demonstrated by the increased SOD, CAT and GSH-Px activities and decreased MDA. In their studies on fortification of broiler chicken diets with phytochemicals, Cheng et al. (2019) and Ding et al. (2021) wherein they used a cocktail of phytosterols (β-sitosterol, stigmasterol, campesterol and brassicasterol), reported positive outcomes on antioxidant enzyme activities. The current study made use of only β-sitosterol and observed upregulated natural antioxidant enzymes activities demonstrating that it can potentially be used on its own and still mediate enhance antioxidant enzyme activities thus potentially being protective against ROS induced damage.

While the exact mechanism by which β-sitosterol upregulates systemic antioxidant enzymes activities, the observed antioxidant status points to its effectiveness.

6.8 Conclusion

β-sitosterol can potentially be used as dietary supplement in broiler chicken diets with no risk of altering glucose and triglycerides metabolism and without compromising renal and pancreatic function as well as liver synthetic function. The decrease in plasma MDA concentration and increased SOD, CAT and GSH-Px activities in Cobb 500 broiler chickens fed diets fortified with β-sitosterol demonstrates its potential to mitigate oxidative stress. However, β-sitosterol should be used with caution since at high dietary (1500 mg/kg) inclusion it triggered increased plasma cholesterol concentration and caused hepatic steatosis and lobular inflammation, possibly putting the birds at risk to development of fatty liver disease.

6.9 References

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CHAPTER SEVEN – CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

7.1 Conclusions

Diet intervention studies in poultry tend to focus on the productivity traits without deliberately delving into the health implications to the birds. The current study sought to determine the potential of β-sitosterol, a phytochemical, to replace oxytetracycline, as a growth promoting feed supplement in Cobb 500 broiler chicken diets, by specifically evaluating its (β-sitosterol) effects on growth performance, GIT development, meat yield and quality and long bone, kidney liver and general health of Cobb 500 broiler chicken. Thus, both the productivity outcomes and health/welfare implications to the birds were considered in the study.

Dietary supplementation with β-sitosterol mediated similar growth performance and GIT viscera growth and development, meat yield and quality as with oxytetracycline supplementation demonstration its potential to replace the latter without compromising productive performance and product (meat) quality. Furthermore, it can be inferred that β-sitosterol can be used to fortify Cobb 500 broiler chicken diets in place of oxytetracycline with no risk to femora and tibiae growth and development. In fact, its use can be tapped into to mitigate tibiae fracturing since it mediated greater strength as demonstrated by the higher breaking strengths of tibiae from Cobb 500 broiler chicken reared on β-sitosterol fortified diets. Importantly, the use of β-sitosterol as dietary supplement in broiler chicken diets did not alter the metabolism of glucose and triglycerides, two key fuel biomolecules. This study demonstrated that it (β-sitosterol) can be used without compromising renal and pancreatic function as well as liver synthetic function and importantly, it decreased plama MDA concentration and upregulated SOD, CAT and GSH-Px activities in the chickens demonstrating its potential to mitigate oxidative stress. However, β-sitosterol should be used with caution since at high dietary inclusion it triggered increased plasma cholesterol concentration and caused hepatic steatosis and lobular inflammation.

7.2 Study limitations and recommendations

The current study did not sex the chicks and importantly the study mimicked commercial broiler poultry production wherein birds are not reared by sex but in future studies this advice will be implemented. In determining the effects of dietary β-sitosterol on femora and tibiae health, current study findings demonstrated increased tibiae mechanical strength with dietary β-sitosterol shown by the increased breaking strength. Further to the determined bone mass, length, mass to length ratio and breaking strength, this study could also have evaluated effects

of the intervention on plasma calcitonin, 1,25-dihydroxycholecaliferol and parathyroid; hormones that regulate bone homeostasis and health. Determination of the bone regulating hormones could have helped with some indication of the mechanism by which the β-sitosterol mediated increased tibiae strength. It is therefore recommended that future studies to consider effects on hormones regulating bone health.

The current study reports that at high dietary inclusion, β-sitosterol caused increased plasma (total) cholesterol concentration. Indeed, increased plasma cholesterol concentration raises the risk of developing cardiovascular diseases through deposits of cholesterol plagues in the endothelia of arteries and emboli that may develop when the plaques peel off from arterial endothelia. However, a better picture would have been observed had the cholesterol determination gone further to quantify HDL-cholesterol and LDL-cholesterol. The HDL-cholesterol eliminate cholesterol from the periphery to liver where it is oxidised and conjugated and then excreted through the GIT mediating decreased cholesterol-induced metabolic risk while LDL-cholesterol transports cholesterol from the liver to the peripheral tissues resulting in heightened metabolic disease risk. Thus, it is important that future studies evaluate the effects of interventions such as β-sitosterol on total cholesterol, it is critical to further dissect the effect on HDL- and LDL-cholesterol concentrations in order to be able to draw more informed inferences.

Although the current study evaluated the effects of β-sitosterol on the chickens' oxidant and antioxidant status, it could have gone further to evaluate in more detail bird welfare by determining effects of the intervention on faecal and feather corticosterone, a marker of stress. Moreover, although the current study determined the effects of dietary β-sitosterol on the Cobb 500 broiler chicken meat quality by assessing effects on physical traits, proximate and fatty acid composition, it could have gone further to evaluate effects on the meat's amino acid composition so as to have a better perspective on effects protein quality of the product. Furthermore, it is always important to determine effects on the meat's aroma, flavour, and taste of any intervention, β-sitosterol included, as these sensory attributes play a critical role in product (meat) acceptability. Hence further studies must consider evaluating the effects of β-sitosterol and/ or any other phytosterol on water holding capacity and these critical sensory attributes that impact consumers' perception of meat quality. The gut microbiome is an important determinant of health and therefore the impact of β-sitosterol on the gut microbiome should be further explored in future studies.

Although the current study evaluated the effects of dietary supplementation with β-sitosterol on the nine different plasma surrogate markers of liver, kidney and pancreatic health, the number of replicates per dietary treatment was less than optimal due to limited research funds. It is thus recommended that future studies seek to evaluate the effects of potential phytochemicals to replace synthetic antibiotics in broiler chicken's feeds as growth promoting supplements and to consider the designed studies with the optimal number of replicates.

APPENDICES

Appendix I: Animal ethics clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL RESEARCH ETHICS COMMITTEE (AREC)

CLEARANCE CERTIFICATE NO. 2018/10/50/B

APPLICANT: Ms M Bopape

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Effects of dietary supplementation with B-Sitosterol on Broiler and Pullet chicken productivity, Health and product quality

Number and Species

300X one-day old unsexed broiler chickens (Gallus gallus domesticus) and 75X 20-weeks old female Layer pullets (Gallus gallus domesticus)

Approval was given for the use of animals for the project described above at an AREC meeting held on 2018/10/30. This approval remains valid until 2021/06/13.
Unreported changes to the application may invalidate the clearance given by the AREC
An annual progress report must be provided

The use of these animals is subject to AREC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

Signed: _____ (Chairperson, AREC) Date: 20/6/2019

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 20 June 2019.

cc: Supervisor: Prof E Chivandi and Dr B Lembede
Director: CAS

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Appendix II: Published paper

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β-sitosterol as an alternative to oxytetracycline: Effect on growth performance, feed intake and utilization efficiency and viscera macromorphometry of Cobb 500 broiler chickens

Malebogo A. Bopape [a,b,*], Kennedy H. Erlwanger [b], Busisani W. Lembede [b], Eliton Chivandi [b]

[a] University of the Witwatersrand, School of Physiology, Faculty of Health Sciences, 7 York Road, Parktown, Johannesburg, 2193, South Africa
[b] Sol Plaatje University, School of Natural and Applied Science, Department of Biological and Agricultural Sciences, Private Bag X5008, Kimberley, 8300, South Africa

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ABSTRACT

Antibiotics are used to fortify broiler chicken feeds as growth promoters. Chronic antibiotic use pollutes the environment and causes the development of antibiotic resistance. Natural alternatives that mimic the properties of antibiotics, without causing health and environmental challenges are required. β-sitosterol has antimicrobial, antioxidant, digestive and immune system modulating and growth stimulating activities. We evaluated its potential to replace oxytetracycline as a growth-promoter in broiler chicken feeds. Two hundred and forty, one-day-old Cobb 500 broiler chicks were randomly allocated to four diets where β-sitosterol replaced oxytetracycline at 0 mg/kg (control; fortified with 50 mg/kg oxytetracycline), 500 mg/kg, 1000 mg/kg and 1500 mg/kg (w/w) feed and fed for 6 weeks: 2 weeks for each growth phase. Each diet was replicated thrice with 20 chicks per replicate. Initial, weekly and terminal body mass (TBM) and daily feed intake (FI) were measured. Body mass gain (BMG), average daily gain (ADG) and feed conversion ratio were computed. Terminally, the chickens were fasted for 4 h then slaughtered and dressed. Gastrointestinal tract (GIT) and GIT accessory viscera masses and small and large intestine lengths were measured. Dietary fortification with β-sitosterol had similar effects ($P > 0.05$) to oxytetracycline on the chickens' TBM, BMG, ADG, FI and utilisation efficiency and GIT organ macromorphometry. In conclusion, β-sitosterol can replace oxytetracycline in Cobb 500 broiler chicken feeds without compromising growth performance, feed intake and utilisation efficiency and GIT organ growth and development.

1. Introduction

The global human population is predicted to be 10.9 billion by 2050 and the demand for food is expected to increase by 70% (Wrachien et al., 2021). According to Lynch et al. (2018) meat production should increase by 200 million tons to reach 525 million tons annually to meet the demand of the growing global population by 2050. In sub-Saharan Africa the increase in the demand of chicken meat and eggs (Nkukwana, 2019) is driven by an increase in population, expansion of urban settlements and improved economic status of the younger populace (Wickramasuriya et al., 2022). Chicken meat is one of the most common and widely consumed meats (Wahyono & Utami, 2018) and together with chicken eggs make a significant source of animal-derived protein for human consumption. Compared to beef, pork, lamb, mutton and chevon, broiler chicken meat and eggs are relatively more affordable (King et al., 2018). Importantly, chicken meat compared to red meats has lower fat, saturated fatty acid and cholesterol content which from a consumer health perspective, is nutritionally a better product (Zhao et al., 2019). Despite chicken meat being relatively more affordable and nutritionally superior compared to other meats, the South African poultry industry fails to meet local demand of broiler chicken meat such that the country resorts to imports to make up for the shortfall (Jörnling, 2017).

In order to enhance broiler chicken productivity and improve profitability, antibiotics are often used at sub-therapeutic doses as growth promoters in broiler chicken feeds (Mehdi et al., 2018). Antibiotics boost broiler chicken productivity by inhibiting the growth of harmful intestinal microbes and promoting growth of favourable intestinal gut microbiota (Lillehoj et al., 2018). Antibiotic-induced reduction in unfavourable gut microbiota results in improved gut health and reduced inflammation of the GIT mucosa translating into increased functional

* Corresponding author.
E-mail address: malebogo.bopape@spu.ac.za (M.A. Bopape).

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efficiency (Mehdi et al., 2018) characterised by increased digestion, absorption and assimilation of nutrients (Kallam & Sejian, 2021). However, use of antibiotics as growth promoters in the production of broiler chicken and other livestock is a key driver of the development of antibiotic resistant bacteria, in addition to polluting the environment with antibiotic residues in chicken waste (Selaledi et al., 2020). In addition to causing the development of antibiotic resistant bacteria, the accumulation of antibiotic residues in chicken meat and eggs negatively impacts human health (Mund et al., 2017). The threat of antibiotic-resistant pathogens and residual environmental pollution has led to a growing interest in the exploration and development of alternative growth promoters for use in poultry production (Mehdi et al., 2018).

Phytochemicals are secondary plant metabolites produced to mitigate herbivory and infection by microorganisms (Sethiya, 2015). They are potential targets to replace antibiotics as feed-based growth promoters in chicken production due to their health beneficial biological activities that mirror those of antibiotics (Valenzuela-grijalva et al., 2017). In addition to mirroring the biological activities of antibiotics, phytochemicals have been shown to boost feed flavour thereby increasing feed intake (Valenzuela-grijalva et al., 2017). Phytochemicals also stimulate secretion of digestive enzymes; resulting in enhanced broiler chicken production efficiency (Alagawany et al., 2021). However, supplementing broiler chicken diets with some phytochemical feed additives at dietary inclusion levels greater than 1500 mg/kg feed (w/w) has been reported to compromise feed intake due to the phytochemical-induced strong odour and flavour (Valenzuela-grijalva et al., 2017).

One of the well characterised phytochemicals is β-sitosterol, known as (3S,8S,9S,10R,13R,14S,17R)-17-[(2R,5R)-5-ethyl-6-methylheptan-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol), a phytosterol which possesses antioxidant, antimicrobial, antibacterial, antifungal and immunomodulatory activities (Saeidnia et al., 2014). β-sitosterol has been shown to modulate and reduce adhesion of pathogenic gut bacteria and to increase nutrient availability to the host (Dhama et al., 2014). We hypothesised that β-sitosterol can potentially replace oxytetracycline as a growth-promoting supplement in broiler chicken feeds hence we evaluated its effects on broiler chicken growth performance, feed intake and feed utilisation efficiency. Previous studies have shown that dietary β-sitosterol has been successfully utilized as a growth promoter in Arbor Acres (Xie et al., 2022), White feather broiler (Ding et al., 2021) Yellow-feather (Feng et al., 2020), Partridge Shank (Zhao et al., 2019), Arbor Acres (Cheng et al., 2019), Ross 308 broiler chicken strains (Naji et al., 2014). In this study, the Cobb500 broiler chicken breed was selected as it has a good growth rate and good performance on low cost feed rations (Awad et al., 2020). Furthermore, the breed is internationally recognised as one of the world's most production efficient breed for meat production (Singh et al., 2021). It is also widely used in Africa (Cobb, 2022). Brazil is one of the main contributors of broiler chicken meat in the world, they produce about 60% of the Cobb 500 broiler chicken with a production of 14.3 million tonnes per year (Miller et al., 2022). Despite the reported successful utilization of the β-sitosterol as a growth promoter in different broiler chicken breeds, its potential has not been evaluated on the productive performance of Cobb 500 broiler chicken breed. Therefore, the present study evaluated the effect of dietary β-sitosterol on growth performance, feed intake and utilization efficiency, feed conversion ratio and viscera macromorphometry of Cobb 500 broiler chicken.

2. Materials and methods

2.1. Ethical clearance and study site

Ethical clearance for the study was granted (AREC number: 2018/50/10/B) by University of the Witwatersrand's Animal Research Ethics Committee. The feeding trial was conducted at the Wits Research Animal Facility in Johannesburg, South Africa.

2.2. β-sitosterol, oxytetracycline and feed ingredients

β-sitosterol (purity: ≥70%) was obtained from Sigma-Aldrich Private Limited, Germany. Groenvoer product cc (Olifantfontein, Pretoria, South Africa) supplied the oxytetracycline. Yellow maize grain, soyabean meal, canola oil, wheat bran, feed grade limestone, dicalcium phosphate, choline chloride, and sodium chloride were purchased from Obaro Animal Feed Manufacturers (Pty Ltd), Pretoria, South Africa. Corn gluten meal (60%) was bought from Tongaat Hullet Starch (Germiston, South Africa). The vitamin-mineral premix, synthetic lysine and methionine were purchased from Trouw Nutrition (Isando, Johannesburg, South Africa).

2.3. Diet formulation

The diets were formulated to meet the nutritional requirements of broiler chickens at starter, grower, and finisher growth stages as per National Research Council (NRC, 1994) recommendations. Table A.1 shows the ingredient and chemical composition of the starter, grower, and finisher diets.

2.4. Chicken management: housing and feeding

One-day-old Cobb 500 broiler chicks vaccinated against Marek's, Newcastle and infectious bursal disease were purchased from Alfa Kuikensplaas chicks (Onderstepoort, Pretoria, South Africa). During the two-day habituation period, before the feeding trial, the chicks were fed a starter diet and dewormed with piperazine (Kyron Laboratories Pvt Ltd, Johannesburg, South Africa) in drinking water at 90 mg/L. The chicks were housed in a deep litter system with each group of 20 chicks housed in a pen measuring 1.7 m × 1.1 m × 1.3 m length, width, and height, respectively. Clean dry wood shavings served as bedding. Feed and cleaning drinking water were provided *ad libitum*. The controlled housing temperature was set to meet the requirements of broiler chicken at starter, grower and finisher growth stages as according to Zhao et al. (2019). Infra-red lights provided supplemental heat throughout the experiment. A 12-hlighting programme was followed: with lights on from 6:00 h to 18:00 h as recommended by the South African Poultry Association (SAPA, 2012).

2.5. Experimental design and measurements

Two hundred and forty-one-day-old Cobb 500 broiler chicks (n=240) were, in a completely randomised design, allocated to four diets where β-sitosterol replaced oxytetracycline at 0 mg/kg (control; fortified with 50 mg/kg oxytetracycline), 500 mg/kg, 1000 mg/kg and 1500 mg/kg (w/w) for diets 1 to 4, respectively. The chicks were fed for 6 weeks: 2 for each of the starter, grower, and finisher phase with similar β-sitosterol inclusion in the starter, grower, and finisher diets. Each diet was replicated thrice with 20 chicks per replicate. Initial, weekly and terminal body mass and daily feed intake were measured.

2.6. Computations

The body mass gain, average daily gain and feed conversion ratio were computed from data on body mass and feed intake. The body mass gain (BMG) and average daily gain (ADG) were computed using the equations:

i BMG = final body mass – initial body mass and
ii ADG = body mass gain/duration (days) of the feeding trial, respectively.

Daily feed intake (FI) by growth phase and trial were computed using the equation:

FI = offered feed – residual feed.

The FI and BMG were used to compute the feed conversion ratio (FCR) by growth phase and for the trial period using the equation:

FCR = feed intake (g) /body mass gain (g).

2.7. Terminal procedures, measurements and sample collection

Three days after the end of the 6-weeks feeding trial, thirty broiler chickens from each dietary treatment group were randomly selected and then subjected to a 4-h fast with access to clean drinking water. Due to constraints with availability of the slaughter facilities all the birds were killed three days after ending of the performance measurements of the feeding trial. However, the birds were kept on their respective diets, groups and pens until slaughter. Each fasted chicken was weighed and then humanely slaughtered by decapitation using a guillotine (Harvard Apparatus, Massachusetts, United States). Feathers were hand-plucked and GIT (proventriculus, ventriculus, caecum, small and large intestine) and accessory GIT (liver and pancreas) viscera were dissected out. Viscera masses were measured using an electronic balance (SnowrexEQ-1200, Snowrex International Company, Taipei, Taiwan). Prior to weighing each GIT segment, digesta was gently squeezed out. The small and large intestines were gently stretched on a dissection board and their lengths measured using a ruler.

2.8. Statistical analyses

Data are presented as mean ± standard deviation. GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, California, USA) was used to analyse the data. All multiple-group parametric data were analysed using one-way ANOVA. The differences between the treatment means were determined using Tukey *post hoc* test. Significance was set at $P < 0.05$.

3. Results

3.1. Mortality

During the trial, an overall 3% mortality spread as 0.00%, 8.33%, 3.33% and 1.67% for diets 1 through to 4, respectively was recorded.

3.2. Growth performance

The effect of dietary β-sitosterol on the growth performance, feed intake and utilization efficiency by growth phase and trial are shown in Table A.2. β-sitosterol had similar ($P > 0.05$) effects as oxytetracycline on the chickens' terminal body mass (TBM) and total body mass gain (TBMG) as well as their BMG, ADG, FI and FCR by growth phase and overall.

3.3. Viscera macromorphometry

Table A.3 shows the effect of β-sitosterol on the macromorphometry of GIT and GIT accessory organs. β-sitosterol similarly ($P > 0.05$) affected the proventriculi, ventriculi, small and large intestines and caeca, pancreata and liver masses and small and large intestine lengths of the chickens as oxytetracycline.

4. Discussion

4.1. Growth performance, feed intake and utilisation efficiency

We evaluated the potential of β-sitosterol, supplemented at 500 mg/kg, 1000 mg/kg and 1500 mg/kg of feed, respectively, to replace oxytetracycline as a growth promoter in broiler chicken starter, grower, and finisher diets, respectively. Our findings show that β-sitosterol-supplemented chickens had similar growth performance, feed intake and utilisation efficiency as their oxytetracycline-supplemented (control) counterparts. These findings show that supplemental β-sitosterol at the three dietary inclusion levels used in the current study did not compromise the growth performance, feed intake, and feed utilization efficiency of the broiler chickens. The three dietary inclusion levels of β-sitosterol were equally effective in promoting the growth performance of the broiler chickens, suggesting that one can save on production costs by making use of the lowest dietary β-sitosterol inclusion level without compromising performance. It must be noted that in our study we used general poultry guidelines for diet formulation according to the National Research Council (NRC, 1994). The diet was formulated to mimic common practice in general broiler chicken rearing where farmers typically purchase feeds that are commercially available without targeting specific breeds. Additionally, the study did not target breeder Cobb 500 stock where there is a greater emphasis on precision diet formulation.

Ding et al. (2021) reported that β-sitosterol, at 40 and 80 (mg/kg) dietary inclusion as a growth promoting dietary supplement in broiler chicken diets resulted in improved average daily gain (ADG) and average daily feed intake (ADFI). Although findings from the current study did not show β-sitosterol-induced improvement in FCR, it is important to note that the β-sitosterol did not compromise feed utilization efficiency. Thus, the broiler chicken grew significantly. Our results are consistent with the findings of other authors who observed similar effects of β-sitosterol on DFI, ADG and FCR of broiler chickens (Feng et al., 2020; Zhao et al., 2019) using lower dietary concentrations (20, 40, 80 mg/kg) of β-sitosterol as well as the findings of Naji et al. (2014) who made use of higher (25 000, 50 000 and 75 000 mg/kg) dietary concentrations of the phytosterol compared to ours. In the current study, we supplemented β-sitosterol at 500, 1000 and 1500 mg/kg dietary inclusion levels. However, we observed similar growth performance, feed intake and feed utilisation with oxytetracycline supplement control counterparts. The 42-day terminal body mass of the broiler chicken fed β-sitosterol fortified diets in the current study ranged from 1938 – 2016 g which was similar to that of the oxytetracycline control counterparts.

Our findings differ from those of Naji et al. (2014) who reported an increased final body mass (3104 – 3286 g) in broiler chicken whose diets were supplemented with different doses of β- sitosterol (25 000, 50 000 and 75 000 mg/kg of feed) compared to the control birds final body mass after 6 weeks (2881 g). However, there was no difference reported in FI and FCR in their study across the dietary treatments. It is important to note that Naji et al. (2014) made use of substantially higher dietary β-sitosterol inclusion levels and Ross308 broiler chicken in their study. Ross strains 308 and 708 have been reported to be rapidly growing broiler chicken reaching 2.2-2.9 kg by 35-40 days (Bedford et al., 2017). Ding et al. (2021) reported an increased final body mass (2670 – 2731 g) in White Feathered broiler chicken with lower doses of β-sitosterol (10 – 80 mg/kg of feed) than in the current study. Possible reasons for the differences in the terminal body mass of broiler chickens in the current study compared to other studies include breed differences, diet formulations, β-sitosterol doses, feeding environment and management. Importantly, our findings show similarities in terminal 42-day mass, BMG, ADG, DFI and FCR of chicken fed β-sitosterol supplemented diets and those fed the oxytetracycline supplemented diet which suggests that β-sitosterol was equally effective as oxytetracycline in sustaining the growth performance and efficient feed utilization by the Cobb 500

Table A1
The ingredient and chemical nutrient composition of the starter, grower and finisher diets.

Ingredients	Starter				Grower				Finisher			
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 1	Diet 2	Diet 3	Diet 4	Diet 1	Diet 2	Diet 3	Diet 4
Yellow maize meal (g/kg)	447.94	447.94	447.94	447.94	494.09	494.09	494.09	494.09	520.65	520.65	520.65	520.65
Soyabean meal (g/kg)	383.95	383.95	383.95	383.95	264.69	264.69	264.69	264.69	225.62	225.62	225.62	225.62
Wheat bran (g/kg)	18.28	18.28	18.28	18.28	105.88	105.88	105.88	105.88	121.49	121.49	121.49	121.49
Corn gluten meal (g/kg)	118.84	118.84	118.84	118.84	88.23	88.23	88.23	88.23	82.44	82.44	82.44	82.44
Canola oil (g/kg)	-	-	-	-	22.94	22.94	22.94	22.94	26.03	26.03	26.03	26.03
Limestone (g/kg)	20.11	20.11	20.11	20.11	14.12	14.12	14.12	14.12	13.88	13.88	13.88	13.88
DL-Methionine, 99% (g/kg)	1.28	1.28	1.28	1.28	1.24	1.24	1.24	1.24	1.21	1.21	1.21	1.21
Dicalcium phosphate (g/kg)	2.74	2.74	2.74	2.74	2.21	2.21	2.21	2.21	2.17	2.17	2.17	2.17
Salt (g/kg)	2.29	2.29	2.29	2.29	2.21	2.21	2.21	2.21	2.17	2.17	2.17	2.17
*Vitamin and min premix (g/kg)	4.57	4.57	4.57	4.57	4.41	4.41	4.41	4.41	4.34	4.34	4.34	4.34
Oxytetracycline (mg/kg)	50	-	-	-	50	-	-	-	50	-	-	-
β-sitosterol (mg/kg)	-	500	1000	1500	-	500	1000	1500	-	500	1000	1500
Analysed chemical composition												
Dry matter (%)	91.94	92.00	91.98	91.89	92.00	92.16	92.08	91.89	92.00	92.10	92.04	92.07
Crude protein (% DM)	31.62	30.80	30.25	30.92	24.51	24.26	24.44	24.38	21.11	21.24	21.61	21.30
Crude fibre (% DM)	27.96	28.00	28.15	24.68	19.11	19.23	20.12	19.48	19.51	19.62	18.04	18.38
Ash (%DM)	28.13	28.49	28.29	28.64	28.04	26.91	28.20	27.07	28.34	28.27	28.50	28.43
Ether extract (% DM)	2.35	2.35	2.35	2.34	6.10	6.15	6.15	6.12	5.75	5.75	5.80	5.76
Neutral detergent fibre (%DM)	10.40	10.43	10.42	10.44	13.49	13.44	13.45	13.44	15.90	15.88	15.89	15.90
Acid detergent fibre (%DM)	4.66	4.56	4.45	4.55	5.11	5.07	5.09	5.08	5.97	6.19	6.06	6.04
Calcium (%)	0.90	0.91	0.90	0.90	0.81	0.81	0.80	0.81	0.90	0.89	0.90	0.90
Phosphorus (%)	0.50	0.51	0.50	0.50	0.46	0.45	0.46	0.46	0.46	0.44	0.47	0.47
Magnesium (%)	0.16	0.15	0.16	0.15	0.12	0.11	0.12	0.12	0.12	0.13	0.12	0.10
Potassium (%)	1.26	1.25	1.26	1.26	1.20	0.19	1.20	1.20	1.15	1.16	0.15	1.13
Gross energy (MJ/kg DM)	17.66	17.62	17.80	17.66	18.51	18.72	18.14	18.48	18.07	17.93	17.81	18.12

*Vit A: 2000000.000, Vit B_1 (Thiamine): 003.000g, Vit D_3 (500 000): 3000000.000 IU, Vit E (500 IU): 40000.000 IU, Vit K_3 (43%): 003.000g, Vit B_2 (80%): 010.000g, Vit B6 98% (pyrod): 005.000g, Vit B_{12} 1g/kg (m): 100.000mg, Niacine 99.5%: 060.000g, Choline (Chloride 60): 606.060g, Biotine 2%: 200.000 mg, Manganese ($MnSO_4$-31%): 160.000g, Copper ($CuSO_4$-25.2%): 005.000g, Cobalt ($CoSO_4$- 20%): 100.000mg, Selenium (Na_2SeO_3 4.5%): 400:000mg, Calcium pantothenate: 020.000g, Folic acid (96 pure): 001.000g, Anty-ox Vit Dry: 100.000g, Zinc ($ZnSO_4$-35%): 090.000g, Iodide (KI 76.45%): 001.000g, Ferrous (FeSO4-30%): 035.000g, Limestone: 2647.133g; DL-Methionine with purity of 99%.

broiler chicken. At 30-45-days of age, Cobb 500 broiler chickens are expected to have a body mass range of approximately 2000 to 2500 g (Singh et al., 2021) which was observed in our study. Some phytochemicals when used as natural growth promoters enhance the flavour (palatability) and aroma of feeds following stimulation of the olfactory nerve and taste buds which increases appetite and hence feed intake (Valenzuela-grijalva et al., 2017; Jiang et al., 2007). Our findings show similar DFI across dietary treatments which (similarities in DFI) seem to suggest that β-sitosterol, though a phytochemical, may not have had any stimulatory effect on appetite and hence feed intake.

4.2. Viscera macromorphometry

The gastrointestinal tract is the first point of contact for ingested substances, thus it interfaces directly with the exogenous environment (Mukonowenzou et al., 2021). Constituents of this external environment, for example, feed ingredient type and form, feed additives and diet nutrient composition impact the development and functional efficiency of the GIT (Kogut, 2019). Phytochemicals through their effects on gut microflora can mediate precocious maturation of the GIT that enhances nutrient digestion and absorption (Mukonowenzou et al., 2021). Therefore, we evaluate β-sitosterol's effects on broiler chicken growth performance and feed intake and utilisation efficiency we also determined its effects on GIT organ and GIT accessory organ macromorphometry. We observed similarities in the masses of the proventriculi, ventriculi, small and large intestine, pancreata and livers and the lengths of the small and large intestines of broiler chickens supplement with β-sitosterol and control counterparts whose diets were fortified with oxytetracycline. The similarities in the GIT and GIT accessory organ macromorphometry suggest that dietary β-sitosterol neither stimulated nor compromised the growth and development of the GIT. However, our findings show significantly lower absolute and relative liver masses compared to the results observed by Feng et al. (2020) despite them having supplemented the broiler chicken diets with β-sitosterol at 25 mg/kg of feed which lower than the including level used in our study.

On the contrary, Naji et al. (2014) observed that supplemental β-sitosterol caused significant increases in the liver and proventriculi masses of the chicken. We speculate that the high dietary inclusion level of β-sitosterol by Naji et al. (2014) might have mediated "hypertrophy" of the chickens' proventriculi and livers. Apart from its cholesterol-lowering effect, β-sitosterol has been reported to have liver-protective properties (Feng et al., 2020). Contradicting results have been reported on the effect of phytosterols on the liver mass and index in broiler chickens with higher or lower dietary phytosterol doses. In a study with layer chicken Shi et al. (2014) observed that supplementing the diets with β-sitosterol at 400 and 800 mg/kg of feed resulted in similar liver masses with those fed the control diet; a finding that is similar to observations from the current study albeit differences in dietary inclusion levels of the phytosterol. The liver, which plays an important role in detoxification, is an important organ for nutrient metabolism and a sensitive indicator of toxicity, (Dong et al., 2020). Higher dietary inclusion levels of phytosterols have been shown to affect liver lipid metabolism largely mediating increased hepatic fat accumulation a sign of fatty liver disease in broiler chicken liver (Sissener et al., 2017). This did not seem to be the case in our study.

5. Conclusion

β-sitosterol supplemented diets had similar effects on the broiler chickens' terminal body mass, body mass gain, feed intake, feed conversion ratio and GIT and accessory GIT viscera macromorphometry as with oxytetracycline-fortified control diet. We therefore conclude that β-sitosterol can replace oxytetracycline as growth promoter in broiler chicken diets with no risk of compromising growth performance, feed intake and utilisation efficiency and the growth and development of GIT and accessory GIT organs.

Table A.2
Effect of dietary β-sitosterol on the growth performance, feed intake and feed utilisation efficiency of broiler chicken.

Parameter	Growth phase	Dietary treatments				Significance level
		Diet 1	Diet 2	Diet 3	Diet 4	
Initial body mass (g)		63.00 ± 1.23	63.00 ± 2.10	63.43 ± 0.65	62.30 ± 0.35	0.7513
Terminal body mass (g)		2204.00 ± 181.60	1938.00 ± 105.2	1956.00 ± 161.00	2016.00 ± 119.4	0.6167
BMG (g)	Starter	257.10 ± 52.71	259.00 ± 24.06	271.60 ± 52.91	272.40 ± 36.26	0.9549
	Grower	641.20 ± 91.74	676.60 ± 50.49	668.50 ± 48.57	695.70 ± 119.00	0.8793
	Finisher	968.70 ± 77.72	939.20 ± 81.55	914.90 ± 84.20	985.70 ± 85.88	0.8333
Total BMG (g)		1867.00 ± 124.00	1875.00 ± 104.10	1893.00 ± 161.50	1953.00 ± 119.40	0.8416
ADG (g)	Starter	18.33 ± 3.76	18.53 ± 1.71	19.43 ± 3.79	19.47 ± 2.63[a]	0.9518
	Grower	45.83 ± 6.57	48.33 ± 3.59	46.93 ± 4.17	49.67 ± 8.52[a]	0.8739
	Finisher	69.20 ± 1.25	67.10 ± 5.81	65.33 ± 3.15	70.40 ± 1.13[a]	0.8326
ADG trial (g)		44.47 ± 2.91	44.63 ± 2.52	44.13 ± 5.29	45.80 ± 4.04	0.9538
FI (g)	Starter	891.60 ± 80.30	947.10 ± 28.45	905.60 ± 74.44	908.50 ± 43.40	0.8913
	Grower	1162.00 ± 103.30	1203.00 ± 34.51	1243.00 ± 68.04	1225.00 ± 52.02	0.5464
	Finisher	2654.00 ± 113.10	2899.00 ± 208.00	2826.00 ± 174.80	2791.00 ± 83.06	0.3266
FI trial (g)		4708.00 ± 135.71	5065.00 ± 226.30	5009.00 ± 274.80	4925.00 ± 163.90	0.2411
FCR	Starter	3.60 ± 0.96	3.68 ± 0.34	3.45 ± 0.85	2.83 ± 0.08	0.9629
	Grower	1.83 ± 0.14	1.78 ± 0.11	1.80 ± 0.21	1.80 ± 0.33	0.9954
	Finisher	2.74 ± 0.17	3.09 ± 0.23	3.20 ± 0.85	2.83 ± 0.08	0.5794
FCR trial		2.52 ± 0.10	2.70 ± 0.05	2.67 ± 0.37	2.53 ± 0.24	0.6906

ns = not significant $P > 0.05$. BMG – body mass gain, ADG – average daily gain, FI – feed intake, FCR – feed conversion ratio. Diet 1 – oxytetracycline (50 mg/kg of feed); diet 2 – 500 mg/kg of feed of β-sitosterol; diet 3 – 1000 mg/kg of feed of β-sitosterol; diet 4 – 1500 mg/kg of feed of β-sitosterol. Data is presented as mean ± standard deviation; for growth performance n = 60 broiler chickens per dietary treatment group.

Table A.3
Effect of dietary β-sitosterol on viscera gastrointestinal organ and accessory organ masses and lengths of broiler chicken.

Parameter	Dietary treatments				Significance Level
	Diet 1	Diet 2	Diet 3	Diet 4	
Heart (g)	12.01 ± 2.17	11.82 ± 2.69	11.87 ± 3.05	12.24 ± 2.80	0.9320
Relative to body mass (%)	0.51 ± 0.09	0.51 ± 0.10	0.49 ± 0.07	0.51 ± 0.08	0.9078
Liver (g)	43.13 ± 9.72	41.12 ± 10.04	41.88 ± 10.52	40.90 ± 9.98	0.8262
Relative to body mass (%)	1.83 ± 0.35	1.76 ± 0.32	1.75 ± 0.22	1.70 ± 0.28	0.3988
Pancreas (g)	4.18 ± 0.84	4.00 ± 0.75	4.20 ± 1.09	4.07 ± 0.78	0.7830
Relative to body mass (%)	0.18 ± 0.04	0.17 ± 0.03	0.18 ± 0.03	0.17 ± 0.03	0.7548
Spleen (g)	2.05 ± 0.61	1.94 ± 0.49	1.97 ± 0.58[a]	2.02 ± 0.64	0.8905
Relative to body mass (%)	0.09 ± 0.02	0.08 ± 0.02	0.08 ± 0.02[a]	0.08 ± 0.02	0.9007
Proventriculus (g)	8.24 ± 1.44	7.90 ± 1.95	7.61 ± 1.67[a]	7.76 ± 1.55	0.5089
Relative to body mass (%)	0.35 ± 0.06	0.34 ± 0.07	0.32 ± 0.04[a]	0.33 ± 0.06	0.1757
Ventriculus (g)	40.00 ± 5.29	39.37 ± 7.45	40.33 ± 9.68	40.87 ± 8.06	0.8991
Relative to body mass (%)	1.71 ± 0.19	1.70 ± 0.25	1.69 ± 0.23	1.73 ± 0.29	0.9642
Small intestines (g)	42.99 ± 6.10	40.78 ± 8.52	42.74 ± 9.28	43.04 ± 8.20	0.6618
Relative to body mass (%)	1.84 ± 0.24	1.76 ± 0.30	1.80 ± 0.21	1.82 ± 0.30	0.6798
Small intestines length (mm)	1684.00 ± 148.90	1690.00 ± 247.50	1681.00 ± 206.00	1698.00 ± 216.30	0.9886
Relative to body mass (mm/g)	0.73 ± 0.10	0.74 ± 0.12	0.73 ± 0.13	0.73 ± 0.14	0.9761
Large intestine (g)	3.21 ± 0.51	3.44 ± 0.96	3.20 ± 1.02	3.05 ± 0.99	0.4082
Relative to body mass (%)	0.14 ± 0.03	0.15 ± 0.05	0.14 ± 0.03	0.13 ± 0.03	0.1025
Large intestines length (mm)	101.80 ± 18.84	101.20 ± 19.10	99.63 ± 21.09	100.30 ± 24.49	0.9801
Relative to body mass (mm/g)	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.02	0.7228
Caecum (g)	7.15 ± 1.44	7.06 ± 1.36	6.70 ± 1.50	6.56 ± 1.27	0.3041
Relative to body mass (%)	0.31 ± 0.06	0.31 ± 0.07	0.28 ± 0.05	0.28 ± 0.05	0.0777
Visceral fat (g)	39.12 ± 16.51	41.77 ± 14.81	42.73 ± 13.65	45.15 ± 14.88	0.4752
Relative to body mass (%)	1.64 ± 0.58	1.92 ± 0.62	1.82 ± 0.62	1.98 ± 0.67	0.2189

ns = not significant $P > 0.05$. Diet 1 – oxytetracycline (50 mg/kg of feed); diet 2 – 500 mg/kg of feed of β-sitosterol; diet 3 – 1000 mg/kg of feed of β-sitosterol; diet 4 – 1500 mg/kg of feed of β-sitosterol, data is presented as mean ± standard deviation; n = 28-32 birds per dietary treatment group.

Ethical statement

Ethical clearance for the study was granted (AREC number: 2018/50/10/B) by University of the Witwatersrand's Animal Research Ethics Committee. The feeding trial was conducted at the Wits Research Animal Facility in Johannesburg, South Africa.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Malebogo Bopape reports financial support was provided by National Research Foundation. Malebogo Bopape reports a relationship with South Africa Department of Higher Education and Training that includes: funding grants.

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Appendices

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