Evaluation of marama bean as an alternative to soybeans in broiler nutrition

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DECLARATION

I declare that this thesis submitted to the North-West University for the degree of *Doctor of Philosophy in Agriculture in Animal Science* has not been previously submitted to any other university or institution and that it is my own original work. Material and information from other sources have been fully recognized and acknowledged.

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GENERAL ABSTRACT

The incorporation of non-conventional feed ingredients in poultry diets could potentially promote sustainable poultry production. Marama bean (MB) (*Tylosema esculentum*) is one such non-conventional feed resource with potential applications in poultry nutrition. However, its utility as a feed ingredient for poultry may be limited by the presence of high levels of trypsin inhibitors and other antinutritional factors. Therefore, this study was designed to evaluate the soyabean replacement efficacy of MB in broiler chicken diets. The first experiment investigated the maximum tolerance level of raw full-fat marama bean meal (MBM) as a partial alternative protein source in Ross 308 broiler chicken diets based on nutrient digestibility, haematological and biochemical indices, and growth performance. The second experiment assessed the effect of graded levels of raw full-fat MBM on carcass attributes, meat quality measurements, and meat stability. Three-hundred and eighty-five, two-week old Ross 308 broiler chickens (359.7 ± 25.48 g live-weight) were reared for four weeks (14 – 42 days). The broilers were fed five isonitrogenous and isoenergetic experimental diets containing graded levels of raw full-fat MBM in place of soyabean products as follows: 1) MBM0 = a standard grower diet with no MBM, 2) MBM4 = a standard grower diet in which soyabean products were replaced with MBM at 40 g/kg, 3) MBM9 = a standard grower diet in which soyabean products were replaced with MBM at 90 g/kg, 4) MBM14 = a standard grower diet in which soyabean products were replaced with MBM at 140 g/kg, and 5) MBM19 = a standard grower diet in which soyabean products were replaced with MBM at 190 g/kg. The five experimental diets (in mash form) were randomly allocated to 35 pens (experimental units) resulting in seven replicates per dietary treatment. The level of MBM inclusion induced neither quadratic nor linear responses (P >0.05) for methionine and lysine digestibility, and feed intake, however, negative quadratic responses (P <0.05) were recorded for all other nutrient digestibility values. Positive and negative quadratic effects were recorded for final body weight (R^2 = 0.724, P =

0.011) and feed conversion ratio (R^2 = 0.806, P = 0.0004), respectively, in response to increasing MBM levels. Hot and cold carcass weights were quadratically reduced with increased levels of MBM. Meat pH, redness, and shear force showed a negative linear response (P <0.05) to MBM levels. For these experiments, it was concluded that while replacing up to 190 g/kg of soyabean products with raw full-fat MBM in broiler diets did not affect mortality and feed intake, it resulted in poor calcium, and sodium utilization, growth performance, and carcass yield and heavier visceral organs (P <0.05). It was established that the protein value of MB in broiler diets is limited by antinutritional components such as trypsin inhibitors and possibly phytate and tannins. The trypsin inhibitor activity (TIA) in MB may negatively affect digestion and absorption of nutrients, which results in growth inhibition, hypertrophy, and hyperplasia of visceral organs such as the pancreas.

The outcomes of these two experiments afore-mentioned, instigated the need to evaluate strategies that would improve the nutritive value of MB by ameliorating the TIA. This was investigated in the third experiment, by applying physical treatments including soaking, cooking, and autoclaving to MB and soyabean samples. Soyabean was used as the control sample due to it being the 'golden standard' protein source in animal nutrition. The effects of these treatments were assessed on crude protein (CP), amino acids composition and TIA of MB and soyabean. Triplicate samples of MB and soyabean were independently subjected to the physical treatments. Soaking, cooking, and autoclaving caused a significant (P <0.05) increase in CP concentration of the beans compared to raw samples. However, ash content was lower (P <0.05) in treated samples than the raw, especially the samples that were soaked or steamed before autoclaving. All the treatments (except soaking MB for 24 hr) decreased the concentrations of TIA (P < 0.0001) and ash content (P <0.05) when compared with the raw samples. Soaking for 24 hr alone was less effective than any of the thermal methods in reducing the TIA in soyabean. More importantly, soaking prior to autoclaving yielded (P <0.05) beans

with lowest TI concentrations. While most of these methods may reduce the TI contents of the beans, they also resulted in lower ash content and amino acids concentration. In conclusion, this study showed that feeding raw MB to Ross 308 broiler chickens is detrimental to the growth performance, haematological and biochemical features, as well as meat quality properties of the chicken. Hence, thermal pre-treatment of MB would be necessary to reduce the anti-nutritional activities of TI in MB and improve its nutritional value for animal nutrition. Therefore, *in vivo* trial involving the incorporation of pre-treated MB in the diets of broiler chickens requires further research to enhance the utility of MB as an alternative protein source.

Keywords: autoclaving, broilers, cooking, marama bean, trypsin inhibitor, soaking

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DEDICATION

I dedicate this thesis to my beautiful daughters, Charis and Shalom Alabi, whom I deprived many fun times together during this study. I hope you grow up to be proud of this great achievement and beat this record.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PEER-REVIEW ARTICLES FROM THIS THESIS

Alabi, F., Kiarie, E.G., Mnisi, C.M. & Mlambo, V., 2022. Physical treatment reduces trypsin inhibitor activity and modifies chemical composition of marama bean (*Tylosema esculentum*). Molecules, 27:4451. **[Published]**.

Alabi, F., Mnisi, C.M. & Mlambo, V., 2023. Soybean replacement efficacy of marama bean (*Tylosema esculentum*) in diets of growing broilers as measured by feed utilization, physiological parameters, and meat quality. Anim. Feed Sci. Technol. **[Under Review]**.

LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AWFI	Average weekly feed intake
AWG	Average weekly gain
BWG	Body weight gain
CCW	Cold carcass weight
FBW	Final body weight
FCR	Feed conversion ratio
FI	Feed intake
GIT	Gastrointestinal tract
GGT	Gamma glutamyl transferase
Hb	Haemoglobin
HCW	Hot carcass weight
MB	Marama bean
MBM	Marama bean meal
MCH	Mean corpuscular haemoglobin
MCV	Mean corpuscular volume
PCV	Packed-cell volume
PUFA	Polyunsaturated fatty acid

RBC	Red blood cell
TI	Trypsin inhibitor
TIA	Trypsin inhibitor activity
TIU	Trypsin inhibitor units
SB	Soyabean
SBM	Soyabean meal
SDMA	Symmetric dimethylarginine
WBC	White blood cell
WHC	Water holding capacity

1 CHAPTER ONE - GENERAL INTRODUCTION

1.1 Background

Soyabean meal (SBM) is globally used as a major source of dietary protein in compound animal feeds. This is attributed to its high protein content and excellent amino acid profile, minimal variation in nutrient composition, and relatively free of intractable anti-nutritive factors if properly processed (Amonsou *et al*., 2011; Wickramasuriya *et al*., 2015). However, the high cost of soyabeans driven by the rise in its demand on the world market negates efforts to attain food and nutrition security in resource-poor communities. South Africa is traditionally a net importer of soyabean, partly because of high demand, mostly for animal feed against low local production (Dlamini *et al*., 2014). Furthermore, subsistence farmers rarely grow soyabean because of the perception that it is a commercial crop (Dlamini *et al*., 2014). Therefore, South Africa largely relies on imported soyabean for animal feed, especially to formulate commercial poultry diets (USDA, 2019).

Consequently, the identification and evaluation of non-conventional, locally available, and low-cost protein alternatives is crucial for the formulation of sustainable diets (Wickramasuriya *et al*., 2015). A number of these alternatives including canola meal, cowpea, and chickpea, have been investigated for their usefulness in animal nutrition (Talebali & Farzinpour, 2005; Akanji *et al*., 2016; Danek & Majewska *et al*., 2021). In addition to these afore-mentioned alternatives, marama bean (MB; *Tylosema esculentum*), an orphan legume that is currently undergoing domestication in southern Africa deserves special research attention.

Marama bean is an under-utilized perennial legume, native to the Kalahari desert and neighbouring sandy regions of southern Africa (Monaghan & Halloran, 1996), with exceptionally high nutritional value (Hartley *et al*., 2002). Morphologically, MB has anatomical characteristics, including seed coat, embryo and two cotyledons like legumes,

similar to soyabeans (Maruatona *et al*., 2010). Marama bean is considered a good source of protein (Amarteifio & Moholo, 1998) like soyabeans (Garcia *et al*., 1998) and peanuts (Venkatachalam & Sathe, 2006). The use of MB in animal nutrition could promote the production and availability of animal products that meet consumer expectations from locally available and indigenous feed resources.

1.2 Problem statement

Despite MB being a good source of protein (Amarteifio & Moholo, 1998) like other oilseed legumes such as soyabeans (Garcia *et al*., 1997) and peanuts (Venkatachalam & Sathe, 2006), its utility in animal nutrition is not common while its effect on performance of broiler chicks is largely unknown. Furthermore, MB contains anti-nutritional factors, including, but not limited to, polyphenols (tannins; Tibe *et al*., 2016), phytates (Jackson *et al*., 2010), and anti-elastase and trypsin inhibitors (Nadaraja *et al*., 2009). The primary anti-nutritional factor is the trypsin inhibitor (TI) (Araba & Dale, 1990), which is a globulin-type protein having a molecular weight of 24,000 and isoelectric point of 4.5 (Kunitz, 1947). It inhibits the conversion of zymogens to active proteases of trypsin and chymotrypsin. The mechanism of action is not the same for trypsin and chymotrypsin (Kunitz, 1947). The TI binds with trypsinogen resulting in an irreversible compound preventing the formation of an active protease (Dozier & Hess, 2011). Conversely, the action of TI in chymotrypsin is, to a lesser extent, forming a reversible dissociated compound (Dozier & Hess, 2011).

The concentration of TI in MB is significantly higher than that found in many other legumes. Indeed, TI in MB constitute about 20% of the total marama protein (Bower *et al*., 1988), which is 2 - 4 times higher than the range of 5 - 10% of the total protein found in many other legumes (Nadaraja *et al*., 2009). This could limit the inclusion level of MB as an alternative protein source in commercial broiler diets. Trypsin inhibitors in raw soyabeans have been linked to

growth inhibition, pancreatic hypertrophy, and hyperplasia in experimental animals (Liener & Kakade, 1980; Liener, 1981; Rackis & Gumbman, 1981). Hence, pre-treatment of MB prior to incorporation into animal diet could be necessary to improve its nutritive value.

1.3 Justification

Investigations on the use of MB as an alternative protein source in animal nutrition is crucial to ascertain its optimum inclusion level and assess the need for improvement of its nutritive quality. Further, adoption of physical treatments to reduce TI activity in full-fat MB may be required to improve its nutrient bioavailability as a possible replacement to soyabean products in commercial broiler diets. Heat treatment has been demonstrated to enhance SBM quality by denaturing the native protein structure and destroying protease inhibitors (Purushotham *et al*., 2007). The heat-lability of TI involves swift destruction of disulphide bonds, which are essential for the heat stability of the inhibitor and thus facilitating denaturation of the inhibitor (Friedman *et al*., 1984). Thermal treatment using a conventional oven set at 200°C for 20 minutes was shown to diminish the activity of TI in whole soyabean flour (Andrade *et al*., 2016). Similarly, hot air drying at 100°C for 2 hr reduced the trypsin inhibitor activity (TIA) of soy flour by 80%, without affecting its nitrogen solubility and colour (Agrahar-Murugkar & Jha, 2010). In the same study, steaming of soyabean flour for 10 minutes followed by hot air drying at 60°C reduced the inhibitor activity levels to 80% after 3 hours (Agrahar-Murugkar & Jha, 2010). Heat-inactivation of protease inhibitors in legume seeds has been stated to be a function of several variables including particle size, temperature, moisture content and duration of heat treatment (Rackis, 1965; Osman *et al*., 2002).

An alternative to the thermal treatment of legume seeds may be soaking. Soaking is the simplest and more economical processing method for inactivation of TI. In addition, it hydrates legume seeds and enhances the removal of a wide range of water-soluble compounds, and

antinutritional factors, in the discarded soaking solution (Avilés-Gaxiola *et al*., 2018). The soaking process followed by cooking, enhances TI inactivation compared with cooking only, which is likely due to the leaching of TI into the steep water. It also reduces the level of other antinutritional factors, such as lectins (Pedrosa *et al*., 2015; Shi *et al*., 2017).

Pre-soaking followed by microwave treatment of legumes significantly reduced the activity of TI compared to microwaving dry substrates (Petres *et al*., 1990). This is because of the higher moisture content in the soaked soyabeans, which could have resulted in better dielectric properties (Vagadia *et al*., 2017). Seeds of fava beans (*Vicia faba*), chickpeas (*Cicer arietinum*), soyabeans, lentils (*Lens culinaris*), and common beans (*Phaseolus vulgaris*) showed similar extent of TI inactivation by microwave heating method at 2450 MHz and 0.54 kW power compared to conventional heating treatment (boiling followed by hot air treatment at 60°C for 20 h) (Hernandez-Infante *et al*., 1998).

It has been established in literature that MB is comparable to SB in terms of crude protein and some other nutrients (Bower *et al*., 1988; Amarteifio & Moholo, 1998; Nkama & Filli, 2006; Holse *et al*., 2010). In addition, the need for exploration of locally sourced feed ingredients in animal nutrition is essential (Wickramasuriya, 2015) to meet the growing demand for animal protein at affordable prices (Industrial Development Corporation, 2016). However, the candidacy or suitability of MB as a protein source or its potential to be an alternative replacement to soy products in animal nutrition, particularly poultry, and the need to improve its nutritive quality have not been investigated. Therefore, this study was designed to assess the effect of incorporating different levels of raw full-fat MB into diets on nutrient utilization, blood and sera attributes, growth performance, carcass characteristics and meat quality of Ross 308 broiler chickens; determine the optimum inclusion level of marama bean meal (MBM) in broiler chickens' diets; and evaluate the physical treatment methods as strategies to improve the nutritive value of full-fat marama bean as a protein source for broilers' diets. The

application of physical treatment methods, especially soaking and heating, has been used to reduce the contents of antinutritional factors such as TI, oligosaccharides, phytates and tannins in legume seeds (Oliveira *et al*., 2001; Ramı´rez-Ca´rdenas *et al*., 2008; Mohamed *et al*., 2011). Thus, this study represents the first-ever attempt to improve the nutritional value of MB as an alternative protein source using physical treatment methods.

1.4 Objectives

The study was designed to assess the response of broiler chicken to diets containing raw full-fat MBM and determine the effectiveness of physical treatment strategies to enhance the feed value of MB.

The following specific objectives guided the study:

i. To determine the feed utilisation, growth performance, haemo-biochemical parameters of Ross 308 broiler chickens fed with diets containing partially replaced soy products with incremental inclusion levels of raw full-fat MBM.
ii. To determine the carcass characteristics, meat quality traits and bone breaking strength of Ross 308 broiler chickens reared on diets containing partially replaced soy products with incremental inclusion levels of raw full-fat MBM.
iii. To identify an optimal inclusion level of raw full-fat MBM in Ross 308 broiler chicken diets based on feed utilization and growth performance.
iv. To investigate the effect of physical treatments, including soaking, cooking, and autoclaving, on trypsin inhibitor activity, dry matter, ash, crude protein, and amino acids profile of MB and soyabean.

1.5 Hypotheses

The study tested the following alternative hypotheses:

i. Higher inclusion levels of raw full-fat MBM would reduce feed utilization, growth performance and haemo-biochemical parameters of Ross 308 broiler chickens.

ii. Higher inclusion levels of raw full-fat MBM would suppress carcass characteristics, meat quality traits and bone breaking strength of Ross 308 broiler chickens.

iii. The physiological parameters and meat quality traits in Ross 308 broiler chickens respond to incremental levels of MBM in a quadratic fashion.

iv. Physical treatments, including soaking, cooking, and autoclaving, would reduce the trypsin inhibition activity and improve the nutritive value of MBM.

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2 CHAPTER TWO - LITERATURE REVIEW

2.1 Introduction

In Africa, agricultural growth plays a pivotal role in steering economic development. Within agriculture, the poultry industry has shown rapid growth in many southern African countries in recent years. This promotes industrialisation and trade-in of poultry and animal feed products across the region (Samboko *et al*., 2018). Poultry production continuously indicates a growing trend, outperforming other animal protein sources, including beef and pork. This may partly be attributed to their short generation intervals, faster growth rates, early maturity, and relatively lower grain-based diet to meat yield compared to ruminants (Bounds & Zinyemba, 2018). According to the Department of Trade, Industry and Competition (DTIC, 2019), the poultry subsector plays an important role in South Africa by being an inexpensive source of protein for numerous families, and it is important for food processing, value addition and job creation across the country.

Despite the previously mentioned benefits, the poultry industry has since been impacted by feeding costs, given that animal feed accounts for 65 to 70% of broiler production costs (Bagopi *et al*., 2014; Zengeni, 2014). South Africa is the largest market for animal feed and poultry products in southern Africa, with strong dependency on Latin America and Europe for the supply of these commodities (Samboko *et al*., 2018). This dependency is due to limited availability of the locally produced soybean (Samboko *et al*., 2018). This shows that there is potential for regional growth if these imports can be replaced with regionally produced feed resources. Furthermore, there is a growing decline in global supply of plant-based protein sources used for broiler chickens, especially soybean meal. This calls for the assessment of underutilised plant protein sources (El-Deek *et al*., 2020). Alternative protein sources such as MB, is a legume that is locally available and adapted to dry or low moisture conditions in

Kalahari part of Southern Africa (Omotayo & Aremu, 2021). Presently, MB is underutilized in animal nutrition (Cullis *et al*., 2019). Hence, exploring its use in animal nutrition could reduce the over-reliance on soybean and, ultimately, contribute to sustainable broiler production.

2.2 Broiler industry in South Africa

In South Africa, the broiler industry is the biggest within the agricultural sector in terms of production value (Davids & Meyer, 2016). The industry generated R46.2 billion of gross value in 2018 and 2019, accounting for 16.2% of total gross value of agricultural products. In addition, 34% of total animal products is accounted for by broiler chicken, and its meat contributes approximately 90% to the total poultry-meat production (DALRRD, 2020). In addition, South Africa produced a total of 1.76 million tons of broiler meat, while its consumption was at 2.3 million tons during the year 2018/2019. The gap between consumption and production remains broad causing South Africa to become the growing net importer of broiler meat (DALRRD, 2020).

Despite the tremendous contribution of broiler production to the South African agricultural sector and rising demand for broiler meat, the industry has struggled to generate profits as a result of the rising feed costs. One of the key determinants of higher production costs for broiler production in South Africa, compared to countries such as Brazil and the USA, is that South Africa imports approximately 90% of its soybean meal (SBM) requirements (Davids, 2013). Consequently, feed costs contribute significantly to increasing broiler prices (Louw *et al*., 2013). Furthermore, the agricultural sector in South Africa can be characterised as a two-fold industry comprising of commercial farmers and small-scale farmers (Louw *et al*., 2017). The South African broiler industry is controlled by a few large, integrated firms, which make up the largest part of the supply chain. Broiler producers raise birds on contractual terms for these firms (Davids, 2013). A contractual off-take agreement enables market security and reduces

risk. According to the report by Industrial Development Corporation (IDC) in the year 2016, in South Africa, small scale farmers contribute only 5% of total broiler chicken production, of which 65% is from contract growers for large corporate companies. Whereas 33% supply about 1.3 million live birds per year to the informal market, and the remaining 2% sell to formal outlets such as supermarkets, restaurants, and butcheries (IDC, 2016). Furthermore, the scale requirement in broiler production, in South Africa, is dependent on price determination process (Louw *et al*., 2017). Domestic price determination is affected by large import portions from countries in the Northern Hemisphere and Brazil. Although, South Africa's performance is comparable to these countries where technical efficiency is concerned (Louw *et al*., 2017) but, once input costs, especially that of feed, are considered, local producers lose ground (Davids & Meyer, 2016). Hence, it is imperative to find alternative, locally sourced feed ingredients for broiler chicken production to meet the demand of consumers and to ensure higher profits for the farmers.

2.3 Ross 308 broiler chicken

The Ross 308 is known globally as a broiler that consistently and efficiently performs in the broiler house. Customers value the growth rate, feed efficiency and robust performance of the Ross 308 (Aviagen, 2014). This breed is characterized by high proportion of chest and calf of the legs from the carcass weight. Ross 308 is a tetra-linear hybrid, which was originated by Ross Breeders company, in Ingliston, Edinburgh, United Kingdom (Pascalau *et al*., 2017). Ross lines were introduced to the then National Poultry Breeders (now Ross Poultry Breeders) of South Africa in 1971. Ross broiler breeders are imported into the country as ancestors (Goga & Bosiu, 2019).

Although Ross 308, Cobb 500, and Arbor Acres are the main international breeds of broiler chickens that are used in the South African poultry industry (Goga & Bosiu, 2019). However,

Ross and Cobb are the most extensively produced broiler strains for meat production across the globe (Khalid *et al*., 2021). The comparative studies of the Ross 308 and cobb 500 have shown that the two strains are different in terms of growth performance and yields of carcass and carcass cuts (Lobago *et al*., 2003; Nogueira *et al*., 2019; Khalid *et al*., 2021). For instance, greater breast yield, lower thigh yield, were recorded in Cobb strain when compared to Ross 308 (Nogueira *et al*., 2019).

2.4 Marama bean

Marama bean (family *Fabaceae*) is an indigenous perennial legume in Southern Africa commonly called Morama, Braaiboontjie, Marumama, Gemsbokboontjies, Muraki, Gembok beans, Tamani and berry (Omotayo & Aremu, 2021). In South Africa, MB grows naturally in the arid and semiarid areas of the Northern Cape, Limpopo, Gauteng, and North West provinces (DAFF, 2014). Marama bean seeds are dark brown in colour (Figure 2.1), and each weigh between 2 and 3 g (Tibe *et al*., 2016). The outer shell of MB is hard and inedible, while the inner portion of the seed has an edible two-lobes (National Research Council, 2006).

Traditionally, immature MB seeds, together with the seed coat, are used as vegetables (Mosele, 2011a). The matured seeds are roasted as snack, or ground and boiled to prepare porridge, and for producing fat or oil (Omotayo & Aremu, 2021) in some parts of Botswana, Namibia, and South Africa. Marama bean flour can also be used to prepare coffee or cocoa-like beverages (Mosele, 2012). The use of MB as an alternative protein source to SB can be limited by the high concentration of antinutritional factors, including, but not limited to trypsin inhibitors (TI) (Maruatona *et al*., 2010). However, MB could be a potential alternative to soybean in poultry diets if its inclusion level is investigated to ascertain safe and beneficial utilization without compromising growth and health performance of chickens.

Figure 2.1. *Tylosema esculentum* (Source: Jackson *et al*., 2010).

2.4.1 Nutritional composition of marama bean in comparison to soybean

Marama bean appear to compare favourably with soyabean in terms of moisture, crude protein, and crude fibre. However, soyabean is richer in total carbohydrates and ash, and lower in crude fat content than MB (Table 2.1).

2.4.1.1 Moisture and dry matter in Marama bean

The moisture content is about 67% (Mosele *et al*., 2011a) for immature seeds (cotyledon), which is higher than 1.3 – 7.1% moisture in mature seeds (Bower *et al*., 1988; Holse *et al*., 2010; Mosele *et al*., 2011a; Amonsou *et al*., 2011). The dry matter content of the matured MB ranges from 93.4 to 98.7%. Therefore, the moisture content is quite low and differences in moisture level in MB has been associated with factors such as soil composition, weather conditions, stage of maturity and harvest time (Holse *et al*., 2010).

2.4.1.2 Crude protein in Marama bean

Marama bean has a significant amount of crude protein, which ranges between 29 and 40% in mature seeds (Holse *et al*., 2010; Amonsou *et al*., 2011; Mosele *et al*., 2011a). The MB protein content is like that of soyabeans (Nkama & Filli, 2006) and is higher than that of peanuts (*Arachis hypogaea*) as stated by Amonsou *et al*. (2011). Marama bean proteins are found to comprise of globulins (53%), followed by albumins (23.3%), prolamins (15.5%), alkali-soluble glutelins (7.7%) and acid-soluble glutelins (0.5%) (Bower *et al*., 1988). This differs greatly from soyabean proteins, which is predominantly made up of 90% globulins and the remaining 10% being albumins (Gueguen, 1983).

The major amino acids in marama seed are found to be glutamic acid, arginine, aspartic acid, and tyrosine (Bower *et al*., 1988; Maruatona *et al*., 2010; Kayitesi *et al*., 2012). Bower *et al*. (1988) reported that glutamic acid is the highest at about 15% followed by arginine at approximately 11% (Bower *et al*., 1988). The tyrosine content is substantially higher than that of most other legumes such as soyabeans and peanuts (USDA, 2011). Like soyabean protein, the MB protein is also relatively high in lysine but limiting in the sulphur-containing amino acids (cysteine and methionine). The content of leucine, phenylalanine, threonine, and valine of the MB protein, match or exceed the level for a protein to be classified as a quality protein (Maruatona *et al*., 2010).

2.4.1.3 Carbohydrates in Marama bean

The total carbohydrate content, calculated by difference, in mature MB has been reported to be in the range of 18.3 - 24.1% (Bower *et al*., 1988; Amarteifio & Moholo, 1998; Amounsou *et al*., 2011; Mosele *et al*., 2011a). Carbohydrate content in soyabean could be up to 27.4% which is slightly higher than that of MB. The carbohydrates in MB are predominantly insoluble polysaccharides such as pectin and cellulose and negligible quantities of starch and soluble

sugars with both far less than 1% (Mosele *et al*., 2011a). Glucose and fructose are monosaccharides in immature seeds, and mainly arabinose in mature MB (Mosele *et al*., 2011b). The disaccharides in MB are sucrose and maltose (Mosele *et al*., 2011b), while the main oligosaccharide is raffinose (Holse *et al*., 2011). Oligosaccharides include prebiotic carbohydrates that selectively stimulate the growth of either *bifidobacteria* or *lactobacilli* in the human colon (Mosele, 2012).

2.4.1.4 Dietary fibre in Marama bean

Total dietary fibre makes up to 27% of the dry matter in mature seeds (Holse *et al*., 2010), constituting the major portion of carbohydrates in MB. On the other hand, SB contain more total dietary fibre at 54.8% (with 2.7% soluble dietary fibre and 52.1% insoluble dietary fibre), on dry matter basis (Martín-Cabrejas *et al*., 2008). Legumes generally contain a high amount of insoluble dietary fibre (Mosele, 2012), which was also found in 14 samples of MB (*Tylosema esculentum*) collected from Botswana, Namibia and South Africa as reported by Holse *et al*. (2010). The insoluble dietary fibre was 23.6% DM and soluble dietary fibre was 0.9% in MB (Holse *et al*., 2010).

2.4.1.5 Lipids in Marama bean

The total lipid content of MB ranges from 32.0 to 41.9% DM, which exceeds by far the lipid content of other pulses and compares with that of sunflower seeds (22 – 36%) and rapeseeds (22 – 49%; Holse *et al*., 2010; Holse, 2012). In terms of the fatty acid composition, the fat in MB comprises of mainly unsaturated fatty acids with approximately 43 to 48% oleic acid (Ketshajwang *et al*., 1998; Holse *et al*., 2011), being the highest fatty acid by composition, followed by linoleic acid (26.4%) (Ketshajwang *et al*., 1998). In contrast, the oleic acid and linoleic acid contents of SB oil were reported to be 23.4% and 53.4%, respectively (Gunstone,

2002). This shows that MB oil and/or MBM may be less prone to oxidation than soyabean oil and/or SBM due to less 1,4 penta-diene structure in linoleates of MB (Maruatona, 2008).

2.4.1.6 Minerals and vitamins in Marama bean

The ash content of MB varies between 3.0 and 3.7% (Mosele *et al*., 2011a; Omotayo & Aremu, 2021). This is lower than 4.8% observed in SB (Amonsou *et al*., 2011). The macro elements in MB include phosphorus, calcium, magnesium, sulphur, and potassium (Holse *et al*., 2010). The micro elements include zinc, manganese, iron, and copper. Marama bean is a good source of vitamins E (Holse *et al*., 2010) and B-complex (Müseler-Schönfeldt, 2006).

Table 2.1. Comparison of the chemical composition (g/100g) of the marama bean and soybean.

Nutrient	**Marama bean**	**Soyabean**
Moisture	5.1 – 7.1	7.0
Crude protein	30.6 – 37.3	45*
Crude fat	29.2 – 36.2	14.9
Crude fibre	7.9 – 8.4	8.6
Ash	3.2 – 3.4	4.8
Total carbohydrates	14.9 – 18.3	31.3

Sources: Amonsou *et al.* (2011); *Lokuruka (2010).

2.4.2 Antinutritional factors in Marama bean

It has been reported that MB has a high content of dietary fibre, which ranges between 19% and 27%, and a high content of lignans but there is no content of cyanogenic glycosides, and the potent allergens that have been identified in peanut and lupin (Holse *et al*., 2010). The main antinutritional factor that has been reported in MB is trypsin inhibitor (TI), which on average

account for 20% of the total protein. However, trypsin inhibitor activity can be eliminated significantly by dry heating, and boiling (Bower *et al*., 1988). In addition to TI, marama bean contains condensed tannins which is 0.38% leucocyandin equivalent (Tibe *et al*., 2016).

2.4.2.1 Condensed tannins

Tannins are classified into two mains groups, namely hydrolysable and condensed (Khanbabaee & van Ree, 2001). During hydrolysis, tannins release ellagic acid and gallic acid, which exhibit excellent anti-oxidative properties, and have been tested to be beneficial to overall chicken health (Badhani *et al*., 2015; Samuel *et al*., 2017). On the other hand, condensed tannins are polyphenolic substances that are widely found in plants including particularly legumes. They interact with protein and starch to form complexes, which inhibit enzymatic hydrolyses (Agugo *et al*., 2013; Aberoumand, 2012), hence reducing the bioavailability of nutrients including but not limited to protein and carbohydrates.

2.4.2.2 Protease inhibitors

2.4.2.2.1 Trypsin inhibitor

Kunitz trypsin inhibitor (molecular weight; 20 KDa) and Bowman Birk inhibitor (molecular weight; 8KDa) are the two trypsin inhibitors that have been reported to negate protein utilisation (Clemente *et al*., 2013). They inhibit the activity of the enzymes trypsin and chymotrypsin in the gut, thus preventing protein digestion (Dhaliwal *et al*., 2022). Kunitz trypsin inhibitor was the first protease inhibitor to be isolated by Kunitz in 1946 (Kunitz, 1946) and it is referred to as serine protease inhibitor as it forms stable complexes with digestive enzymes, decreasing their activity to very low levels (Savage & Morrison, 2003). The Kunitz-type trypsin inhibitor family comprises mainly of protease inhibitors from legume seeds. Present in these inhibitors are 170 to 200 amino acid residues and one or two intra-chain disulphide bonds.

Kunitz trypsin inhibitor binds to the site directly involved in inhibitor interaction with trypsin (a core protein for proper digestion of food), in a way as it does to the substrate protein. This results in hydrolysis of the peptide bonds between reactive site residues 63 (arginine) and 64 (isoleucine) of the inhibitor or substrates as shown in Figure 2.1 (Savage & Morrison, 2003). This molecule binds with trypsin in a stoichiometric fashion, whereby, one molecule of the inhibitor inactivates one molecule of trypsin (Mohan *et al*., 2016). Inhibitors differ from substrate in that, the reactive site residues are clenched between disulphide bonds. Post hydrolysis, the altered inhibitor is held together with the same conformation, because of the disulphide bond. Hence, a stable enzyme-inhibitor complex is formed (Savage & Morrison, 2003). However, the activity of Kunitz trypsin inhibitor is preserved only in native state. Increase in temperature results in its denaturation, and consequent loss of its inhibiting potential (Kunitz, 1945). The Kunitz-type inhibitors show activity against trypsin, thiol proteases, subtilisin, aspartic proteases, and elastase (Nadaraja *et al*., 2010). Among the antinutritional factors, Kunitz trypsin inhibitor is a major antinutrient that reduces the availability of protein and suppresses the activity of digestive proteases (Dhaliwal *et al*., 2022).

The Bowman–Birk inhibitor is a smaller peptide molecule (molecular weight is about 8 000 Da) with 71 amino acids, and it is broadly distributed in various legume seeds (Mohan *et al*., 2016). Bowman–Birk inhibitor prevents both trypsin and chymotrypsin at two different binding sites; a trypsin-reactive site (lysine 16 and serine 17) and a chymotrypsin-reactive site (leucine 43 and serine 44). In contrast to the Kunitz inhibitors, the Bowman–Birk inhibitors are very rich in disulphide bonds having seven disulphide bridges (Figure 2.3). This feature is attributed to its very tight, compact, three-dimensional structure and resistance to gastric juices and proteolytic enzymes (Mohan *et al*., 2016).

Trypsin inhibitors exist in a variety of plants, and they have been studied extensively in grain legumes including mung beans, chickpeas, and soyabeans (Savage & Morrison, 2003). While

TI are present in most legumes, their concentration varies considerably. Most legume species including chickpea, common bean, pea, lentil, navy beans, fava bean and a couple of varieties of kidney beans, have less than half of the trypsin inhibitor activity (TIA) of soyabeans. Lima bean contains higher TIA than the previously mentioned legumes, but not up to that of soyabean as indicated in Table 2.2 (Savage & Morrison, 2003). While MB may not be as common as the afore-mentioned legume species, it contains a higher level of TI than soyabean (250.8 vs 57.6 TIU/mg sample; Maruatona, 2008). Trypsin inhibitor plays an important role in agronomy by providing defence against insect pests in plants (Dhaliwal *et al*., 2022). Nevertheless, TI have been linked with adverse effects such as poor nutrient digestibility, growth inhibition, pancreatic hypertrophy, and hyperplasia in mammals (Liener, 1995).

The nutritional status of MB (Table 2.2) makes it suitable for animal diets owing to the presence of protein, oil, and crude fibre. Despite all these advantages, the presence of antinutritional factors especially, TIA in MB seeds may be a constraint limiting their use as an animal feed ingredient. Processing such as moist heating has been identified to reduce or eliminate antinutrients including protease inhibitors (Aberoumand, 2012).

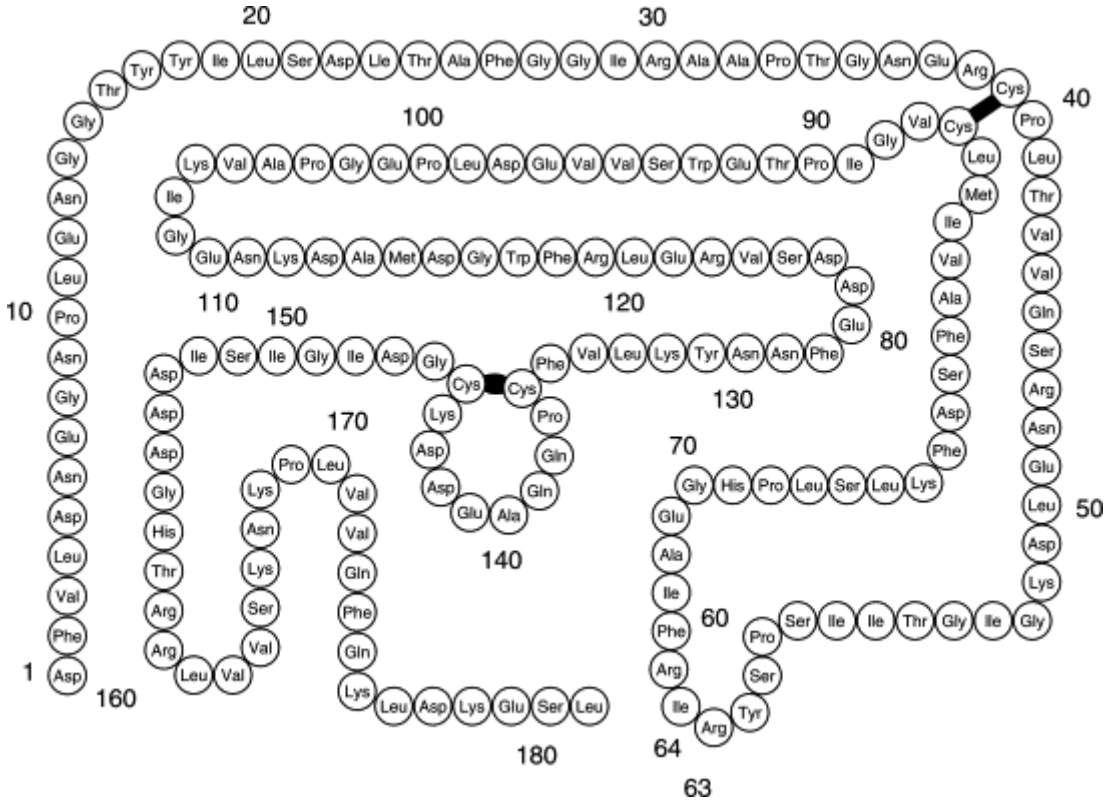

Figure 2.2. Structure of the Kunitz soyabean trypsin inhibitor (Source: Koide & Ikenaka, 1973).

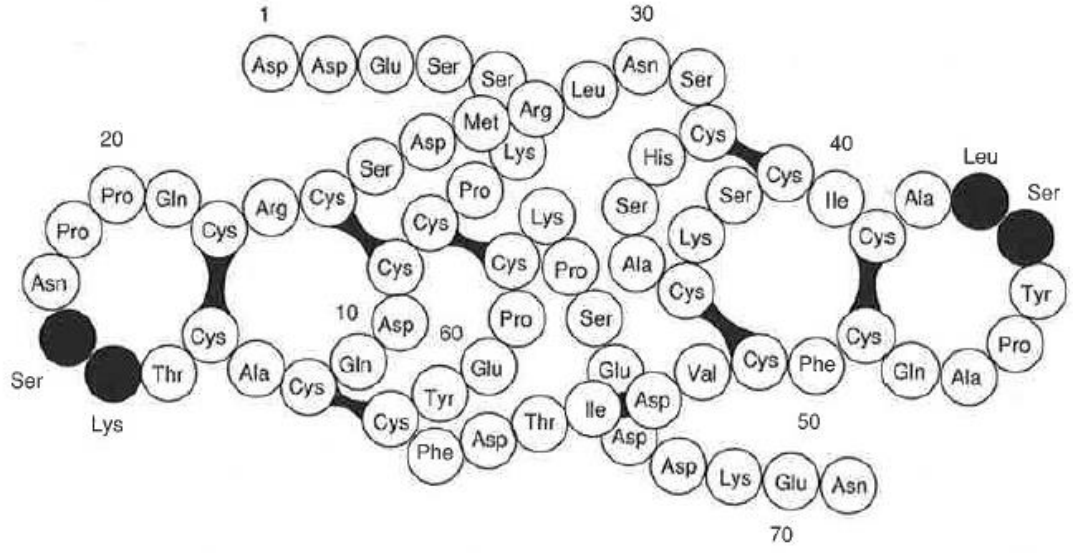

Figure 2.3. Structure of the Bowman Birk soyabean trypsin inhibitor (Source: Odani & Ikenaka, 1973).

2.4.2.2.2 Elastin inhibitor

Protease inhibitors in soyabean have extensively been studied more than other plant inhibitors, understandably due to their common availability and contribution to the human diet. It is quite interesting that apart from TI, elastase inhibitors have been isolated from MB (Elfant *et al*., 1985). Elastase inhibitors are Kunitz-type serine protease inhibitors that are specific for pancreatic and neutrophil elastase. With these inhibitors, virtually no inhibition of chymotrypsin, however, strong inhibition of elastase was observed (Nadaraja *et al*., 2010). The study by Nadaraja *et al*. (2010), showed that elastase inhibitors amount to approximately 14% of the total acid-extractable protein from MB. They were detected as two separate 17.8 kDa and 20 kDa molecular weight proteins (due to carbohydrate differences), with apparently identical amino acid sequences.

2.5 Determination of trypsin inhibitor activity

Quantifying TI is of importance to soyabean processors who are concerned with producing a high-quality product for animal feed (Stauffer, 1990). The standard method of measuring TI in soyabean has been that of American Association of Cereal Chemistry (method-22-40.01) developed in 1991 based on the findings by Kakade *et al*. (1974). This method involves the use of benzoyl-DL-arginine-*p*-nitroanilide (BAPA) as a substrate for porcine or bovine trypsin. The ability of aliquots of meal extract to inhibit the activity of trypsin towards this substrate is used to calculate the amount of TI in a soyabean (meal) sample. The demerit of this assay is that it can only be used to estimate protein-type inhibitors activity with trypsin without differentiating between the two main protease inhibitors, Kunitz and bowman birk (Savage & Morrison, 2003). Similar method has been adopted to assess the trypsin activity in MB (Maruatona, 2008).

Trypsin inhibitor activity is frequently expressed as trypsin inhibitor units (TIU) in a milligram of sample. One TIU is defined as a decrease of 0.01 absorbance units at 410 nm per 10 ml assay solution under specified assay conditions (Kakade *et al*., 1974).

Table 2.2. Trypsin inhibitor activity (TIU/mg sample) in selected legume seeds in comparison to soyabean.

Legume	**Trypsin inhibitor activity**
Soyabean (*Glycine max*)	43 – 84*
Lima beans (*Phaseolus lunatus L*)	38.4**
Common bean (*Phaseolus vulgaris*)	21 – 25*
Chickpea (*Cicer arietinum)*	15 – 19*
Pea (*Pisum sativum*)	6 – 15*
Lentil (*Lens culinaris*)	3 – 8*
Fava bean (*Vicia faba*)	5 – 10*
Marama bean (*Tylosema esculentum)*	251***

Sources: *Guillamón *et al.* (2008); **Hove & King (1979); ***Maruatona (2008).

2.6 Physical inactivation of trypsin inhibitors

Inactivation of trypsin inhibitors is commonly achieved during the processing of legumes for human consumption and/or animal feeds (Savage & Morrison, 2003). The processing includes, but not limited to, oil-extracting, heating, and drying (Stanojević *et al*., 2004). Prior to processing, the hulls of the legume seeds are separated from the cotyledons by dehulling. For instance, in soyabean processing, beans are cracked to coarse particles by cracking machines,

which are made of counter-rotating, grooved rolls (Maruatona, 2008). This activity detaches the hulls and allows their separation by aspiration (Maruatona, 2008).

Removal of antinutritional factors such as TI can improve the nutritional quality of legumes and, therefore, help to effectively incorporate them in animal diets. Several food processing methods such as autoclaving (Stanojević *et al*., 2004), soaking, dehulling, and cooking, are known to lower anti-nutritional factors effectively and increase the nutritional quality of legumes (Mohamed *et al*., 2011).

2.6.1 Soaking

Soaking is one of the inexpensive means of inactivating antinutritional factors in legume seeds (Ibrahim *et al*., 2002). It enables hydration of legume seeds and enables the removal of a variety of water-soluble compounds including antinutritional factors in the discarded soaking solution (Avil´es-Gaxiola *et al*., 2018). While some of these antinutritional factors, such as lectins and trypsin inhibitors are thermolabile, reducing when properly cooked, others are heat stable. However, their concentrations are reduced by dissolution in water (Haro, 1983; Silva & Silva, 1999, 2000). Soaking is usually applied prior to subjecting the legume seeds to heating. It has been proven that soaking can greatly lower the amounts of antinutritional factors such as oligosaccharides, tannins and phytates (Oliveira *et al*., 2001; Ramı´rez-Ca´rdenas *et al*., 2008). Temperature, pH, the duration of soaking (El-Adawy *et al*., 2000), and the variety of legume seed (Fernandes *et al*., 2010) are some of the factors that determine the effectiveness of soaking. The use of tap or distilled water at ambient temperature has been used for the removal of TI, and oftentimes, the longer the duration (comparing 8, 12 and 16 hr) of soaking, the more TI are removed from the seeds into the soaking water (Ibrahim *et al*., 2002). The major drawback with soaking is that nutrients, for instance, soluble proteins may also be lost with the discarded

water, resulting in the protein content of the legume seed being reduced. The intensity of nutrient loss may be dependent on the duration of soaking (Avil´es-Gaxiola *et al*., 2018).

2.6.2 Thermal treatment

Heat processing is widely accepted as an effective method of inactivating thermolabile antinutritional factors of legume grains. Some of the methods of thermal applications are boiling, cooking, autoclaving, roasting and microwaving (Avil´es-Gaxiola *et al*., 2018). Thermal treatments enable a rapid way to inactivate TI in legumes like soyabean, peanut, lentil, and mung bean, with the added benefit that all these treatments are fully standardized and can be carried out at industrial or farm or household level (Avil´es-Gaxiola *et al*., 2018). Heat treatment is usually applied prior to including legumes in the human diet. This enhances the protein quality by inactivating anti-physiological factors, particularly trypsin inhibitors and haemagglutins and by releasing the protein structure, therefore making them more prone to attack by digestive enzymes (Sathe *et al*., 1984).

Steam heating (cooking) tends to be more effective than dry heating (Carlini & Udedibie, 1997). However, the choice to discard water from cooking or soaking of legume seeds has been a subject of debate (Fernandes *et al*., 2010). It is noteworthy that nutrients such as minerals and protein are leached into the cooking water and they may be lost if the water is got rid of (Huma *et al*., 2008). Hence, bean preparations consumed with the cooking water keep those nutrients (Fernandes *et al*., 2010). On the other hand, the antinutritional factors such as tannins, phytic acids and TIA lixiviate into the cooking water (Ibrahim *et al*., 2002) and can be eliminated by discarding the cooking solution.

2.6.2.1 Cooking

Cooking is widely used to inactivate heat sensitive anti-nutritive factors such as trypsin and chymotrypsin inhibitors and volatile compounds (Akande & Fabiyi, 2010). Domestically,

cooking may be applied with or without prior soaking of beans,although soaking before cooking has been recommended for legume seeds such as common beans (Fernandes *et al*., 2010).

The degree to which TIA may be destroyed by cooking is dependent on temperature, duration of cooking, particle size of the legume seed and moisture content (Savage & Morrison, 2003; Akande & Fabiyi, 2010). These variables are closely controlled in the industrial processing of SBM to derive a product's maximum nutritional value (Savage & Morrison, 2003). Cooking for 45 minutes at 100°C was adequate to inactivate 87% TIA in cowpea (Ibrahim *et al*., 2002). Bressani & Elias (1979) showed that about 30 – 40% of polyphenols can be extracted from common beans (*Phaseous vulgaris*) by cooking and discarding the cooking water solution. Mohamed *et al*. (2011) subjected soy, mung, and kidney beans to three different cooking periods of 30, 60 and 90 minutes, taking the period of cooking as starting from boiling. It took 90 minutes to eliminate TIA in soyabean and mung bean and 99% TIA in kidney bean.

2.6.2.2 Autoclaving

Autoclaving is the process of cooking under high pressure. The application of pressure results in lower time of cooking when compared with ordinary cooking (Akande *et al*., 2010). Pressure cooking is more effective than ordinary cooking in decreasing trypsin inhibitor (Ibrahim *et al*., 2002). In addition to inactivation of TIA by autoclaving, other anti-nutritional components, such as hemagglutinins, tannins and phytic acid can be reduced or inactivated as well (Ibrahim *et al*., 2002; Avil´es-Gaxiola *et al*., 2018). The level of residual TIA of soyabean flour after physical or chemical treatment, is subjected to certain factors such as treatment method, temperature level, pressure, heating time (Rackis, 1974), moisture content (Fulmer, 1989), pH conditions and the presence of reducing agents (Sessa & Ghantous, 1987; Friedman *et al*., 1989). For instance, dry heat treatment based on autoclaving at a temperature of 96°C for 5 to

15 minutes showed no satisfactory effect on the reduction of TIA in soyabean flour with average residual 43.4 - 84.3% TIA in treated samples (Stanojević *et al*., 2014). Commercial heated meals may contain up to 20% of the residual TIA with no adverse effect in nutrition (Rackis *et al*., 1974; Brandon & Friedman, 2002). In addition, dry heat has very low reducing effect on TIA, even at 121°C. Treatments with steam cooking (Veličković *et al*., 2000) and steam jet cooking (Johnson *et al*., 1980; Wang & Johnson, 2001) were proven to be more effective in reducing TIA in soyabean products than autoclaving at pressure of 0.5 to 2.0 bar (Veličković *et al*., 2000). Similarly, autoclaving pre-soaked samples of soyabean, mung and kidney bean seeds at 15 atmospheric pressure and 121°C for 10 minutes in distilled water (1:10 w/v) completely inactivate TIA. Therefore, it is clear that steam autoclaving could potentially reduce the TIA in MBM.

2.6.2.3 Effects of thermal treatment

Thermal treatments have become the most effective methods used for TI inactivation because of their efficiency to diminish TIA and ease to apply in households and industrial setups (Avilés-Gaxiola *et al*., 2018). The TIA can be destroyed by dry-heating that eliminates 70%, and by boiling (moist heat) which removes 80% of TIA (Bower *et al*., 1988). To inactivate anti-nutritional factors in MB, Maruatona (2008) found that dry-heating at 150ºC for 20 min was effective in reducing the levels of TI from 251 trypsin units inhibited (TIU/mg flour) to 3 TIU/mg flour. Similarly, a reduction in TIU from 44.2 to 2.6 TIU/mg when soyabean flakes were autoclaved at 121°C for 36 min was reported by Batal *et al*. (2000). Trypsin inhibitors can be reduced by 80 - 95% of the activity originally present by heat processing (Rackls *et al*., 1986). Continued heat treatment to destroy the remaining residual activity would impair the proteins, thereby lowering the nutritive value and nitrogen solubility, and limiting functional properties (Sessa *et al*., 1988). Hence, heating may also negatively affect the functional properties of the MB product.

In relation to soyabeans, thermal treatments involving boiling at 100°C for approximately 9 minutes, cooking for 7 to 30 minutes, and autoclaving at 121°C for 15 minutes can inactivate most of the TIA (Avilés-Gaxiola *et al*., 2018). However, despite the effectiveness of these processes, it has been observed that temperatures more than 80°C can impair some important nutrients, such as lysine, sulphur-amino acids, and heat-labile vitamins (Egounlety & Aworth, 2003; Bara´c-Stanojevi´c, 2005; Machado *et al*., 2008; Chen *et al*., 2014; Yang *et al*., 2014).

2.6.2.4 Constraints of heat processing

Heat processing of beans is by far the most efficient method to reduce antinutrients, but also nutrient contents (Fernandes *et al*., 2010). Over processing by heat treatment may result in amino acid unavailability because of early Maillard reaction. Lysine is particularly susceptible because of its exposed ε-amino group, which readily reacts with reducing sugars (Heger *et al*., 2016). As Maillard reaction progresses, cross-linking may happen between most amino acids and polypeptide chains, hence lowering the efficiency of intestinal proteases (Heger *et al*., 2016). For instance, excessive heat treatment was stated by Sibbald (1980) to reduce the availability of some amino acids, with lysine being the most sensitive. Similarly, the effects of over-processing dehulled, solvent-extracted SBM by autoclaving at 121ºC and 105 kPa for 0, 20, 40, and 60 minutes were assessed by Anderson-Hafermann *et al*. (1992). It was found that increasing the time of heating reduced total amount of lysine, arginine, and cysteine, but other amino acids were not influenced by over-processing. True amino acid digestibility of lysine, cysteine, histidine, and aspartic acid was greatly reduced, whereas digestibility of threonine, serine, alanine, and leucine was decreased to a lesser extent (Anderson-Hafermann *et al*., 1992). Hence, determination of the effects of physical treatments of MB is important to indicate the optimum temperature and duration required to improve the nutritive value of the beans.

2.7 Influence of trypsin inhibitor on gastrointestinal tract, nutrient utilization, and growth performance in broiler chicken

Legumes represent a major source of nutrients, including valuable protein. However, the nutritive value of legumes depends upon the processing methods, presence, or absence of nutrients with other feed components (Ghadge *et al*., 2008; Akande & Fabiyi, 2010). Utilization of nutrients may vary based on the components of ingredient present in the supplied feed as well as the function of the gut. The identification and amelioration of factors that inhibit nutrient utilisation are necessary for successful poultry production (Jha & Mishra, 2021). Among potential factors reducing nutrient bioavailability are TI (Liener, 1995; Dhaliwal *et al*., 2022) in ingredients such as soyabeans and marama beans (Maruatona, 2008).

Despite being a monogastric animal, the gastrointestinal tract (GIT) of poultry is significantly different from human and pig in terms of being shorter and lighter. The GIT system performs many functions comprising of digestion, absorption, and protection. The structure of the gut is well adapted to perform these functions (Jha & Mishra, 2021). To elucidate, the poultry GIT maximizes nutrient utilization to lower substrates for bacteria and supports epithelial cell growth and differentiation. Similarly, the GIT aids gut tissue integrity, prevents attachment of pathogenic bacteria, balances microbial populations with low amounts of potentially pathogenic strains, promotes appropriate immune response, and controls inflammation (Shang *et al*., 2018). Therefore, the effective functioning of the GIT and its health are important metrics in determining animal growth performance, meat, and egg quality (Jha *et al*., 2019).

Broilers are notably efficient in converting feed to high quality meat; however, this is not always the case because much of the nutrients ingested are not adequately digested and utilized but are lost through excretion (Chang'a *et al*., 2019). Broiler chickens' void a significant amount of nutrients in droppings, including 30% dry matter, 25% gross energy, 50% nitrogen and 55% phosphorus (Ravindran, 2013), which could have been absorbed and used by the birds

for growth. This is because performance of broiler chickens is strongly influenced by anti-nutrients in the feed, the physical form of feed, nutrient density, among other factors (Olukosi *et al*., 2008). For instance, TI form complexes with pancreatic proteases, thus impeding their activity in the upper small intestine. To ameliorate the lack of enzymes, hypersecretion by the exocrine pancreas is prompted followed by pancreatic hypertrophy and hyperplasia (Grant *et al*., 1995). The combined effect of endogenous loss of essential amino acids (particularly sulphur-containing amino acids) and reduced intestinal proteolysis may result in reduced growth performance of chickens.

Pacheco *et al*. (2014) reported a decrease in performance of male broiler chicks when TI in SBM containing diets were greater than 18.9 TIU/mg. Additionally, TI have been shown to adversely affect protein digestibility and amino acid availability (de Coca-Sinova *et al*., 2008), by forming indigestible complexes with pancreatic-produced trypsin (Liener, 1994; Pacheco *et al*., 2014). Further, a study involving feeding young growing rats with either raw or heated MBM as a source of 10% of dietary protein in the diet (Ripperger-Suhler, 1983; Nadaraja *et al*., 2009), showed reductions in feed intake and growth rates. This was attributed to high TI level in MB (Nadaraja *et al*., 2009).

Globally, the broiler industry has adopted different nutritional strategies to improve nutrient utilization and bird productivity. Physical processing of feed and the use of additives, particularly exogenous enzymes, have been used to improve quality of feeds and nutrient utilization by poultry (Chang'a *et al*., 2019). Therefore, the inactivation of TI antinutritional activities in MB through thermal treatment is crucial for optimal broiler performance.

2.8 Serum biochemical constituents of broiler chickens

There are many substances that make up serum, including proteins, enzymes, lipids, hormones, minerals, and glucose. The evaluation of these various substances yields information about

the tissues, organs, and metabolic state of the animal. Biochemical parameters are essential in maintaining osmotic pressure between the circulating fluid and the fluid in the tissue spaces so that transportation of materials between the blood and cells could be expedited (Ilo *et al*., 2019). They also promote the viscosity and maintenance of normal blood pressure and pH (Ladokun *et al*., 2008).

The sera biochemical parameters give an assessment of protein quality of feed, animal health and nutritional status (Obun *et al*., 2017). Although there are no studies that have reported the feed value of MB in poultry, several studies have investigated the effect of replacing soyabean with alternative feed ingredients such as rapeseed, kenaf seed, and cowpea on sera biochemical components of broiler chickens (Xu *et al*., 2012; Odetola *et al*., 2014; Balaiel, 2014; Obun *et al*., 2017). Some of these studies on serum biochemical indices have shown prospect for replacement options for conventional feed ingredients. For instance, partial substitution (100 or 200 g/kg) of SBM by soaked-toasted tallow seed meal in broiler diets had no adverse effect on broiler chickens' biochemical indices (serum total protein, albumin, globulin, urea, and glucose) of broiler chicks (Obun *et al*., 2017). On the other hand, despite the good nutritive value (approximately 22 - 25% crude protein) of fava bean, its incorporation into poultry diets as an alternative to conventional feed ingredients is limited due to the presence of many anti-nutritional factors, such as protease inhibitors, and tannins (Omar *et al*., 2021).

2.8.1 Serum proteins

Serum proteins comprise of albumin and globulin, and they are all almost synthesized and secreted by hepatocytes or liver cells (Tóthová *et al*., 2016). The major exceptions are the immunoglobulins that are formed by the immune system comprising of the reticuloendothelial tissues, lymphoid and plasma cells (Eckersall, 2008). Non-hepatic tissues, including the intestine, lung, adipose tissue, and mammary gland, may also produce some serum proteins for

specific functions (Friedrichs *et al*., 1995; Vreugdenhil *et al*., 1999). Concentrations of proteins in serum are tightly regulated to balance their physiological functions of immunity, transportation of small molecules, coagulation, and inflammation (Tóthová *et al*., 2016). Any malfunction or loss of balance in the concentrations of proteins can initiate or result from disease processes (Pieper *et al*., 2003).

A decrease in the concentration of albumin (hypoalbuminaemia) can be caused by a decreased hepatic synthesis due to liver diseases such as chronic hepatitis, cirrhosis (Lee, 2012). Further, hypoalbuminaemia may be induced by renal diseases and nephrotic syndrome, in which there is a rapid loss of albumin in urine caused by glomerular impairment (Grauer, 2005; Kodner, 2009). Furthermore, low albumin concentrations may be related to chronic malnutrition, insufficient dietary protein, gastrointestinal diseases, and protein-losing enteropathy (Don & Kaysen, 2004; Diogenes *et al*., 2010). On the other hand, an increase in albumin concentration indicates dehydration (Tóthová *et al*., 2016).

The concentrations of globulins may rise due to imbalance in homeostasis or tissue injury (Gabay & Kushner, 1999). For instance, the replacement of SBM with graded levels of fermented rapeseed meal was shown to increase the immunoglobulin G content in the serum of birds as the replacement levels increase from 5, 10 and 15%. This was attributed to increase of rapeseed bioactive peptides in fermented rapeseed meal (Xu *et al*., 2012). Similarly, an increase in sera immunoglobulin M concentration was observed as the levels of fermented fava bean incorporated into broiler chickens' diets increased (Omar *et al*., 2021). Globulins specifically function in the regulation of inflammatory processes and/or antibody production, mostly at the site of inflammatory lesions, however, they may also function systemically (Gabay & Kushner, 1999). In other words, the main function of globulins is to defend the host against pathological damage, promote the restoration of homeostasis and assist in the regulation of different stages of inflammation (Petersen *et al*., 2004). Increased concentrations of

globulins are often associated with infectious diseases and immune-mediated disease. Therefore, assessment of serum albumin level in broilers chicken can, therefore, provide information about the function of the liver, kidneys, and digestive system.

2.8.2 Kidney and pancreatic function

Poultry renal function can be assessed by serum urea and uric acid measurements. The rise of these parameters in the serum happens when 30% or less of the kidneys are functional (Lumeiji, 1997; Campbell, 2007). In poultry, an increase in serum urea concentration occurs after glomerular filtration rate is reduced. This may indicate a renal disease or a physiological response to fluid restriction. In addition, the blood concentration of symmetric dimethylarginine (SDMA) was reportedly increased in patients with chronic renal diseases and has a strong positive correlation with serum urea and creatinine in renal failure patients (Fleck *et al*., 2003). Serum parameters can also be increased after high protein intake since they are required in nitrogen metabolism (Campbell, 2007; Schmidt *et al*., 2007). The amount of amylase and lipase in broilers serum can be measured for pancreatic function evaluation (Traesel *et al*., 2011). An increase in these serum parameters might be an indication of a pancreatic (Lumeiji, 1997) or kidney injury (González & Silva, 2006).

2.8.3 Liver function and its enzymes

Hepatic function of poultry can be assessed by serum concentration of hepatic enzymes; alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT). The ALP concentration could increase due to a bone disorder or reduction in bile flow into the liver, while ALT usually rises when the cells of the liver are damaged or stressed. An elevation in AST serum levels may be initiated by hepatocellular disease (Campbell, 2007), whereas a rise in GGT level is usually related to hepatobiliary disease (Tennant, 1997) or blockage in bile ducts. Therefore, ALT is a more

specific indicator of liver injury than AST, ALP and GGT. For instance, AST may also be affected by muscle injuries, as shown by a simultaneous increase in creatine kinase (CK) concentrations (Tennant, 1997; Campbell, 2007; Schmidt *et al*., 2007). In addition, cholesterol may be used to determine the state of the liver, since it is synthesized in the liver (González & Silva, 2006; Traesel *et al*., 2011).

2.9 Haematological parameters of broiler chicken

Haematological examination is one of the methods that may contribute to the detection of some changes in health and physiological status, which may not be obvious during physical examination or growth performance, but which influence the health or well-being of the animal (Bamishaiye *et al*., 2009). Haematological analysis entails the evaluation of various blood parameters including, but not limited to packed cell volume (PCV), white blood cells (WBC; eosinophils, lymphocytes, monocytes, and neutrophils), red blood cell (RBC) count, haemoglobin (Hb), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and platelets.

A readily available and rapid way of assessing clinical and nutritional health condition of animals on feeding assays may be the use of blood analysis because ingestion of dietary feed ingredients have quantifiable effects on blood composition (Church *et al*., 1984; Maxwell *et al*., 1990) and may be taken as indicator of long-term nutritional status (Olabanji *et al*., 2007). This is because blood distributes or transports nutrients to various parts of the body. Therefore, effects of factors including medication, pathogens or nutrition on blood parameters impact the entire body in terms of health, growth, maintenance, and reproduction (Oke *et al*., 2007).

Haematological parameters have been assessed in many different species of animals, including poultry, and these parameters are known to vary with many factors, one of which is dietary composition (Amakiri *et al*., 2009). For instance, PCV, RBC, and Hb concentrations of broiler

chickens reduced as the graded level of moist heat-treated *Gmelina arborea* seed meal increased from 25 to 30%, and this was linked to rise in crude fibre content of the diets (Fatokun *et al*., 2013). Haemoglobin performs cellular respiration which is vital in metabolic reactions (McDonald *et al*., 1995), thus a fall in haemoglobin is an important indicator of anaemia, which may probably result in a decrease in the oxygen carrying capacity of blood (Ilo *et al*., 2019). Furthermore, the blood concentrations of WBC, lymphocytes, and neutrophils may be used to determine immune status of an animal. For example, lymphocytes are known to contribute immensely to immune defence system of animals (Ameen *et al*., 2007). A rise in neutrophil:lymphocyte ratio is a good index of stress (Minka *et al*., 2007; Adekonla *et al*., 2008) which could be nutritional stress (Etim, 2014).

2.10 Effect of nutrition on broiler meat quality

The recent surge in consumer preference for chicken meat over beef, mutton or lamb and pork, may be attributed to socio-cultural and economic factors, and increasingly adherence to healthier dietary options. In addition, the poultry subsector has shifted its focus, in recent decades, from whole bird product to a diverse components industry producing deboned meat, cut-up and processed products (Henchion *et al*., 2014; Metro *et al*., 2017; FAO, 2021; Baéza *et al*., 2022). This switch has obviously resulted in the rise in consumers' expectations and the need for improving standards in chicken meat (Baéza *et al*., 2022). Subsequently, broad attempts have been made in the broiler production system to improve the quality of meat in terms of reducing muscle malformations (Petracci *et al*., 2015; Petracci *et al*., 2017; Petracci *et al*., 2019), and enhancing technological (Bianchi *et al*., 2007), sensory (Parrot *et al*., 2017), microbial (Pasquali *et al*., 2017; Rouger *et al*., 2017) features and nutritional characteristics.

Considering the nutritional properties, chicken meat has proven to be an excellent model for making useful food, primarily due to the huge versatility that enables producers to derive many

attractive, suitable, and easy-to-use products containing significant quality proteins, some important fat-soluble vitamins, and minerals (Biesalski, 2005; Decker & Park, 2010; Albergamo *et al*., 2022). Enhancing the consumer preference for poultry meat may be achieved either by dietary adjustment during the rearing stage or by enrichment with certain ingredients with functional and/or therapeutic attributes during meat processing (Albergamo *et al*., 2014; Pogorzelska-Nowicka *et al*., 2018; Aly *et al*., 2019; Youssef *et al*., 2021). The nutritional adjustment has broadly shown how incorporation and intake of desirable feed ingredients tend to yield animal muscles with better biochemical, nutritional, and functional attributes (Fazio *et al*., 2021; Juárez *et al*., 2021; Morshedy *et al*., 2021). Enhancement of most consumed broiler chicken meat through dietary modification, apart from giving health advantages to consumers, has an added advantage of improving bird health (Kalakuntla *et al*., 2017).

In this regard, concerted efforts have been geared towards improving the fatty acid profile of chicken meat, especially in terms of n-3 polyunsaturated fatty acids (PUFA), by provision of diets rich in PUFA such as flaxseed (Albergamo *et al*., 2022), grape and pomegranate seed oil (Banaszkiewicz *et al*., 2018), soyabean oil, mustard oil, linseed oil, fish oil (Kalakuntla *et al*., 2017), and olive oil (Buccioni *et al*., 2009). Marama bean is uniquely rich in PUFAs with a range of 48 – 49% oleic and 19 – 26% linoleic acids (Omotayo & Aremu, 2021), but also contains anti-nutrients such as TI that may adversely affect the health and well-being of broilers, if not properly extracted. Hence, prior heat treatment on MB seed may reduce the TI contents and enable the chicken to safely derive the nutrients including the essential oleic and linoleic acids when fed heat-treated MB containing diets.

Furthermore, linoleic acid can be metabolized into eicosapentaenoic acid and docosahexaenoic acid by desaturating and elongating enzymes (Anjum *et al*., 2013; Kumar *et al*., 2020). The high unsaturation degree is highly beneficial to consumers' health in terms of initiation of anti-inflammatory cascades, slowing down onset of aging associated neurological degeneration, and

lowering the risk of certain cancers and heart diseases (Seo *et al*., 2005). However, the increased unsaturation may affect the oxidative stability of the meat, hence inevitably resulting in the deterioration of its nutritional and functional value (Cortinas *et al*., 2005; Betti *et al*., 2009; Juskiewicz *et al*., 2017). Lipid oxidation and discolouration are the major initiators of meat quality deterioration during storage (Ryu *et al*., 2005, 2006). Oxidation reactions reduce the nutritional value of meats due to the loss of essential fatty acids and vitamins (Domínguez *et al*., 2019). The onset of lipid peroxidation results in a gradual reduction of sensory quality factors. These include occurrence of rancid odour and flavour, and changes in colour, and texture, which affects consumer acceptance (Purriños *et al*., 2011).

After slaughtering of an animal, a decline in muscle pH is expected for conversion of muscle to meat; however, a sharp fall in pH of the muscle yields a pale, soft and exudative (PSE) meat accompanied by a pale appearance with reduced water holding capacity (Jung *et al*., 2015). On the other hand, an increase in muscle pH results in dark, firm, and dry (DFD) meat with inferior storage stability (Allen *et al*., 1997). Therefore, pH is an important metric that can be used to assess meat quality as it affects physical meat quality parameters including colour and water holding capacity. These two factors are known to impact perceptions of the acceptability of the meat product by the consumers and they change with the species, muscle function, age of the animal and storage conditions (Berri, 2000). Hence, evaluating the extent of change in muscle pH and colour after slaughter is crucial to the production of chicken meat. Water holding capacity has been defined as the ability to retain naturally occurring or included water during application of external forces such as pressing, cutting, heating, or grinding (Aberle *et al*., 2001). Muscle tissue is made up of about 75% water and 20% protein, with the remaining component shared by fat, carbohydrates, vitamins, and minerals (Huff-Lonergan & Lonergan, 2005). The ability of meat to retain water is affected by several factors including lactic acid synthesis (low pH), energy loss, onset of rigor mortis, and alterations of cell structure in relation

to proteolytic enzyme activity (Aberle *et al*., 2001; Huff-Lonergan & Lonergan, 2005). In addition, quality attributes of meat such as colour, texture, and juiciness are influenced by water-holding capacity (Aberle *et al*., 2001). Meat tenderness (shear force) is strongly related to eating quality and variations in tenderness informs the consumer decision to re-purchase.

Moreover, it has been established that correlation exists between meat colour and pH (Boulianne & King, 1995, 1998; Allen *et al*., 1997; Fletcher, 1999; Qiao *et al*., 2001). These two factors can also affect the moisture and water holding capacity of the meat (Qiao *et al*., 2001). Therefore, the evaluation of broiler's meat, fed MBM containing diet, for meat colour, tenderness, pH and water holding capacity can be used to determine the oxidative stability of the meat.

2.11 Summary

The nutritional composition of MB in comparison to soyabean suggests it could be a potential replacement for soyabean products in poultry diet formulation. In addition, the increase in demand for affordable and high-quality broiler meat, creates a need for research into the incorporation of locally sourced feed ingredients to promote sustainable meat production. The use of MB in commercial broiler production has not been investigated and documented. Apart from other anti-nutritional factors such as phytates (Jackson *et al*., 2010) and tannins (Tibe *et al*., 2016), trypsin inhibitors are found in higher concentrations in MB than in legumes that are commonly used in broiler diets (Bower *et al*., 1988). The TI have the capacity to impede nutrient digestion, adversely alter gastrointestinal tract morphology and functions and subsequently undermine growth performance. The inclusion of MBM in rat diets has shown to negatively affect feed intake and growth (Ripperger-Suhler, 1983). Thus, the incorporation of MB at high levels in chicken diets might impair nutrient digestion and growth performance. Hence, it is important that the optimum dietary inclusion level of MB be determined so as not

to compromise growth performance of the broiler. Where higher dietary inclusion levels of MBM are desired, it is imperative to investigate means by which the main antinutritional components of MB, trypsin inhibitor, can be ameliorated. The objective of this thesis is to plug the information gap that exist in this respect.

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3 CHAPTER THREE: SOYABEAN PRODUCT-REPLACEMENT EFFICACY OF MARAMA (*TYLOSEMA ESCULENTUM*) BEAN IN BROILER GROWER DIETS AS MEASURED BY NUTRIENT DIGESTIBILITY, GROWTH PERFORMANCE, AND BLOOD PARAMETERS

3.1 Abstract

Orphan legumes native to semi-arid areas of southern Africa, such as marama bean (MB) (*Tylosema esculentum*), could be used as sustainable soyabean alternatives in broiler diets. However, because MB has not benefitted from crop improvement, its utility as a dietary protein source for broilers could be compromised by the presence of antinutritional factors that are normally found in low quantities in soyabeans. Therefore, this study evaluated the effect of partial substitution of soyabean ingredients with raw full-fat marama bean meal (MBM) on nutrient digestibility, physiological, and haematological and serum biochemical characteristics of growing Ross 308 broiler chickens. A total of 385, two-week-old chicks (359.7 ± 25.48 g live-weight) were randomly assigned to five diets formulated to replace soyabean products in a standard broiler grower diet with MBM at 0, 40, 90, 140, and 190 g/kg. Each diet was allocated to 7 replicate pens (experimental units). Except for methionine and lysine, digestibility of all nutrients showed negative quadratic responses to increasing MBM levels. Diets had no effect on overall feed intake, but final body weight and feed conversion ratio showed negative and positive quadratic responses (P <0.05), respectively, to increasing levels of MBM. Sera urea showed positive while alanine transaminase showed negative quadratic responses, respectively. Haemoglobin and calcium linearly declined, while symmetric dimethylarginine, alkaline phosphatase, and γ-glutamyl transferase linearly increased with MBM levels. Calculated optima inclusion levels of MBM in broiler chicken diet using the quadratic responses for crude protein digestibility, feed conversion ratio, and final body weight was 20 g/kg, 20 g/kg, and 4 g/kg respectively, suggesting that very small quantities of MBM

could be used to partially replace soyabean products in grower diets of Ross 308 broiler chickens without compromising these parameters. It was concluded that the inclusion of higher levels of MB caused poor growth performance possibly due to higher levels of trypsin inhibitors.

Keywords: broilers, growth performance, marama bean meal, nutrient digestibility, trypsin inhibitor

3.2 Introduction

Soyabean is recognised as a standard dietary plant protein source for animal nutrition due to its high protein and balanced amino acid profile and minimal variation in nutrient content, and low levels of anti-nutrients. However, the low local production of soyabean with concurrent rise in demand as feed and food in South Africa negates efforts to improve food and nutrition security in resource-poor communities. South Africa's climatic conditions are suboptimal for soyabean growth, thus producing this crop requires high levels of inputs that include irrigation water, fertilizers, and pesticides.

To ensure sustainability of animal diets in these regions, native legumes could be valuable alternatives to soyabean meal (SBM) (Wickramasuriya *et al*., 2015). Indeed, the utility of some orphan legumes, such as cowpea (Akanji *et al*., 2016) and chickpea (Danek-Majewska *et al*., 2021), have been evaluated as dietary protein sources in animal diets. Ideally, these SBM alternatives must be sufficiently adapted to local growing conditions such that they require minimal levels of inputs like irrigation water, fertilizers, and pesticides. One such example is the marama beans (MB), an indigenous legume in southern Africa that is undergoing domestication at various sites in southern Africa (Chimwamurombe, 2011). Marama seed oil ranges between 24 and 48% and it is majorly made up of mono- and di-unsaturated fatty acids and without cholesterol (Jackson *et al*., 2010). It is also a good source of minerals including

potassium, phosphorus, calcium, magnesium, sulphur (Amonsou *et al*., 2011), iron, zinc, and B vitamins, including folate (Jackson *et al*., 2010).

The full-fat MB have a crude protein content of 30 - 37% dry basis (Amarteifio & Moholo, 1998; Amonsou *et al*., 2011) and a crude fat content of about 40% (Omotayo & Aremu, 2021), among other nutrients. Regarding essential amino acids in percent of the bean, MB and soyabeans have comparable arginine (5.96 – 9.58 vs 7.93), cystine (0.78 – 2.01 vs 1.78), histidine (2.25 – 3.57 vs 2.65), isoleucine (3.75 – 5.66 vs 5.90), lysine (5.22 – 6.51 vs 6.93), methionine (0.76 – 1.32 vs 1.34), phenylalanine (4.58 – 5.81 vs 5.42), threonine (2.89 – 4.97 vs 4.32), tryptophan (1.29 - 1.55 vs 1.51) and valine (4.18 – 6.12 vs 5.75) levels (Bower *et al*., 1988). While leucine is lower (5.58 – 7.70 vs 8.46) in MB, tyrosine is four times higher (11.13 – 14.54 vs 3.49) than in soyabean (Bower *et al*., 1988). The use of MB in animal diets could reduce the need for imported soyabeans. However, MB is also known to contain trypsin inhibitors (TI) (Bower *et al*., 1988; Nadaraja *et al*., 2009), which could impede nutrient absorption and compromise animal health and growth (Liener, 1994). These inhibitors constitute about 20% of the total marama protein (Bower *et al*., 1988), a concentration that is about four times higher than in many other legumes (Nadaraja *et al*., 2009).

Apart from TI, druse, and globoids (crystalline) are present within the protein bodies of MB flour (Amonsou *et al*., 2011), this implies that phytic acid may predominantly be in an insoluble protein-phytate complex form. Consequently, this may drastically reduce protein digestibility in MB compared to soyabean where the phytate occurs as soluble protein-phytate (Amonsou *et al*., 2011). Indeed, lower *in vitro* protein digestibility in MB compared to soyabean has been reported (Maruatona, 2008). In addition, while common legume plant protein sources, such as chickpea, cowpea, and canola meal, have been evaluated for their worthiness as SB alternatives

in animal nutrition (Akanji *et al*., 2016; Danek-Majewska *et al*., 2021), the use of MB for this purpose remains unknown.

The appreciation of MB's dietary value is limited to indigenous people of Botswana, Namibia, and South Africa (Jackson *et al*., 2010). Thus, the MB seeds production is presently low. However, harnessing its use in animal nutrition could potentially increase the demand and subsequently raise the cultivation of the MB. Therefore, there is a need to evaluate how replacing soyabean with MB in poultry diets might influence nutrient digestibility, growth, serum biochemistry and haematology, given these potential shortcomings. This will inform current MB improvement programs as well as short-term strategies to reduce the level and bioactivity of antinutritional factors. To the best of our knowledge, the information on the utility of MBM as a soyabean substitute in broiler chicken diets is very limited. In anticipation of growth in the commercial production of MB, this study was designed to examine the effect of partially replacing soyabean ingredients in standard commercial broiler grower diets with raw full-fat MBM on nutrient utilisation, growth performance, and blood parameters in Ross 308 broiler chickens. This study tested the hypothesis that replacing soyabean-based ingredients in a standard commercial broiler diet does not compromise nutrient utilisation, growth performance, and blood parameters in growing Ross 308 broiler chickens.

3.3 Materials and methods

3.3.1 Ethics statement

Rearing and slaughtering procedures used for this study underwent a review by the Animal Production Research Ethics Committee of the North-West University and approval number NWU-02007-20-A5 was issued by the Committee.

3.3.2 Description of the study site

The feeding trial was done in Rooigrond private farm (25°55′0″ S; 25°48′0″ E), North West province of South Africa. The feeding trial was conducted in winter (July - August) and temperatures during this time ranged from -3°C to 25°C. However, the broiler house temperature was maintained at 35°C using infrared electric bulbs for the first 14 days.

3.3.3 Source of feed ingredients

Raw MB were harvested in 2019 from Malwelwe village, Botswana (23.94282°S; 25.1999° E), where Kalahari sands are the common soil type. The village has annual rainfall of 350 - 450 mm, and with temperatures ranging from 34 to 36°C during summer, and 0 to 5°C during winter. The remaining dietary components were sourced from a commercial feed manufacturing company, Nutroteq SA, while the broiler chickens were supplied by Superior Chicks (Pty) Ltd, a reputable chicken hatchery in Pretoria, South Africa.

3.3.4 Preliminary chemical analysis

Triplicate samples of milled (Hamilton Beach® grinder 80393C, China) MB were used for the determination of dry matter (DM), ash, crude protein, gross energy, and crude fat. The DM was determined by weighing approximately 4 g of sample into pre-weighed crucibles and placed in an oven set at 105°C for 2 hr by AOAC method 930.15 (AOAC, 2005). The percent moisture content was measured as proportion of weight loss on drying to weight test portion (g/g) and multiplied by 100. The DM was then calculated as the difference between initial weight and moisture content. The determination of ash content was achieved by placing the samples used for moisture content analysis in the furnace for ashing at 600°C for 12 hr (AOAC method 942.05). Total nitrogen content was determined by Dumas' combustion method (AOAC method 968.06) using Leco Nitrogen Analyzer (Leco Corporation, St. Joseph, MI, USA). The nitrogen (N) was converted to crude protein by multiplying the percentage N content by a factor

of 6.25 and expressed as percent of DM. Gross energy was assayed for MB and soyabean seed samples by complete combustion in a bomb calorimeter (IKA Calorimeter System C 6000; IKA Works, Wilmington, NC, USA). Crude fibre was evaluated by AOAC method 978.10. Crude fat analysis was done using petroleum ether extraction in an ANKOM XT 20 Extractor (ANKOM Technology, Fairport, NY, US). Minerals including calcium, phosphorus, chloride, and sodium were evaluated by digestion using acid followed by spectrophotometry assay; AOAC method no. 991.25.

3.3.5 Formulation of experimental diets

The acquired nutritional components were used to formulate five isonitrogenous and isoenergetic experimental grower diets, using Format® nutritional software, to meet the daily nutritional requirements of growing chickens according to NRC (1994) guidelines. The diets were formulated to include graded levels of MBM as follows: 1) a standard grower diet with no MBM (MBM0); 2) a standard grower diet in which soyabean products were replaced with MBM at 40 g/kg (MBM4); 3) a standard grower diet in which soyabean products were replaced with MBM at 90 g/kg (MBM9); 4) a standard grower diet in which soyabean products were replaced with MBM at 140 g/kg (MBM14); and 5) a standard grower diet in which soyabean products were replaced with MBM at 190 g/kg (MBM19). The ingredient composition of the five diets is presented in Table 3.1. The diets were further analysed as described in Section 3.3.4 and the chemical components are presented in Table 3.2.

Table 3.1. Gross ingredient composition (g/kg, *as fed*) of experimental broiler grower diets.

Ingredients	Diets				
	MBM0	MBM4	MBM9	MBM14	MBM19
Yellow maize	552.2	558.3	564.0	573.6	584.5
Full-fat soya	120.0	120.0	120.0	84.4	28.8
Soya oilcake	231.9	217.9	203.9	215.8	244.7
Crude soya oil	21.0	12.1	3.2	0.0	0.0
Marama bean meal (MBM)	0.0	16.2	32.5	48.7	65.0
Sunflower oilcake	35.0	35.0	35.0	35.0	35.0
Limestone	12.4	12.4	12.3	12.1	12.0
Monocalcium phosphate	9.0	9.2	9.4	9.6	9.9
Salt	2.7	2.7	2.5	2.5	2.5
Sodium bicarbonate	1.3	1.4	1.5	1.6	1.6
DL Methionine	4.1	4.3	4.3	4.4	4.4
L-Threonine	1.1	1.2	1.2	1.3	1.3
L-Tryptophan	0.1	0.1	0.1	0.1	0.1
Lysine HCl	2.9	3.2	3.4	3.6	3.8
Lignobond	2.0	2.0	2.0	2.0	2.0
Premix	3.0	3.0	3.0	3.0	3.0
Axtra phytase	0.1	0.1	0.1	0.1	0.1
Antioxidant	0.3	0.3	0.3	0.3	0.3
Salinomycin 12%	0.5	0.5	0.5	0.5	0.5
Zinc bacitracin	0.5	0.5	0.5	0.5	0.5

MBM0 = a standard grower diet with no MBM, MBM4 = a standard grower diet in which soyabean products were replaced with MBM at 40 g/kg, MBM9 = a standard grower diet in which soyabean products were replaced with MBM at 90 g/kg, MBM14 = a standard grower diet in which soyabean products were replaced with MBM at 140 g/kg, and MBM19 = a standard grower diet in which soyabean products were replaced with MBM at 190 g/kg.

Table 3.2. Chemical composition (g/kg DM) of marama bean meal-containing grower diets.

	[1]Diets				
	MBM0	MBM4	MBM9	MBM14	MBM19
Dry matter	929.6	925.3	930.7	930.2	934.4
Metabolizable energy	14.8	14.8	14.8	14.7	14.8
Crude protein	225.0	225.1	225.8	224.9	225.1
Lysine	6.1	5.7	6.1	5.9	6.1
Methionine	3.8	3.8	3.0	3.5	3.2
Crude fat	71.6	69.7	68.6	66.3	67.6
Crude fibre	40.0	40.3	43.1	42.0	43.9
Ash	68.5	67.9	60.6	66.0	60.6
Phosphorus	4.6	4.3	4.3	4.4	4.7
Calcium	8.6	8.6	9.0	8.7	8.7
Chloride	2.5	2.5	2.5	2.5	2.5
Sodium	2.4	2.4	2.4	2.3	2.5

[1]Diets = MBM0 = a standard grower diet with no MBM, MBM4 = a standard grower diet in which soyabean products were replaced with MBM at 40 g/kg, MBM9 = a standard grower diet in which soyabean products were replaced with MBM at 90 g/kg, MBM14 = a standard grower diet in which soyabean products were replaced with MBM at 140 g/kg, and MBM19 = a standard grower diet in which soyabean products were replaced with MBM at 190 g/kg.

3.3.6 Experimental design

Four-hundred, day old Ross 308 broiler chicks were obtained from Superior Chicks (PTY) Ltd (Gauteng, South Africa). The chicks were administered vitamins and electrolytes (stress pack) in drinking water for the first three days. They were also fed with a standard commercial starter diet until day 10 post hatch, when the acclimatization period ended. For the first 14 days, temperature was kept at 35°C using infrared electric bulbs. The design was a completely

randomized design composed of the following treatments; (i) a standard broiler grower diet without MBM (MBM0), (ii) a standard grower diet in which soyabean products were replaced with MBM at 40 g/kg (MBM4), (iii) a standard grower diet in which soyabean products were replaced with MBM at 90 g/kg (MBM9), (iv) a standard grower diet in which soyabean products were replaced with MBM at 140 g/kg (MBM14), and a standard grower diet in which soyabean products were replaced with MBM at 190 g/kg (MBM19). At day 11, 385 chicks were randomly selected, weighed, and distributed to 35 replicate pens, each measuring 3.5 m × 1.0 m × 1.85 m (length × width × height), and with sunflower husk-covered floor. Each pen was also equipped with a feeder and two drinkers that were cleaned daily and filled with fresh feed and water, were offered *ad libitum* respectively. The dietary treatments were replicated 7 times with each pen carrying 11 chickens. Chicks were gradually introduced to treatment diets over a period of three days. Measurements including mortality were taken from day 14 to 42.

3.3.7 Feeding and measurements

Dietary treatments and fresh water were provided *ad libitum*. Initial body weight was measured for all birds in the 35 pens at the end of starter phase (day 14) and subsequently weighed weekly until day 42. Average daily feed intake was measured from week 2 to week 6. Feed intake was taken daily, and live weight was measured weekly. Prior to feeding, feed was weighed, and refusals were collected each morning before feeding. The measurements of feed, refusals and birds were taken using ADAM® scale, readability of 0.5 g to 2 g, Adam Equipment S.A. PTY, Johannesburg, South Africa. Average weekly feed intake (AWFI) and average weekly gain (AWG) were used to calculate feed conversion ratio (FCR).

3.3.8 Nutrient digestibility trial

At 35 days of age, two chickens per pen were randomly selected and housed individually in metabolic cages (1.75 m × 0.5 m × 0.925 m, L × W × H) for the determination of apparent

digestibility of nutrients. These birds were allowed a three-day acclimatization period followed by a four-day collection period (Rafiu *et al*., 2022). Each pen was equipped with a feeder and a drinker that were cleaned daily and filled with fresh feed and water, respectively. Each of the five experimental diets described earlier in section 3.3.4, was allocated to 7 replicate pens and feed intake was quantified as already described above. Samples of excreta were collected and stored at -20ºC daily (Manangi *et al*., 2018). The excreta samples were oven-dried at 55°C (Morris *et al*., 2019) for 48 hr, milled, and stored in airtight plastic bags until analysed as described in Section 3.3.4. The proximate contents of feed and excreta including ash, crude protein, crude fibre, crude fat, lysine, methionine, sodium, chlorine, and calcium were used to calculate the digestibility percent for each nutrient as follows:

$$Digestibility\ (\%) = \left(\frac{nutrient\ in\ feed - nutrient\ in\ excreta}{nutrient\ in\ feed}\right) \times 100$$

3.3.9 Blood collection and analysis

Collection of blood samples was done at 40 days of age from the brachial vein of two birds, randomly selected from each pen, using 23-gauge needles and 5 ml syringes. The samples were immediately transferred into serum and whole blood tubes. An automated IDEXX® LaserCyte Haematology Analyzer (model no. 93−30001−01 IDEXX Laboratories (Pty) Ltd., Gauteng, South Africa) was used to measure the concentrations of red blood cells (RBC), haematocrit (HCT), haemoglobin, mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), red blood cells distribution width (RDW), reticulocyte, white blood cells (WBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, platelets, mean platelet volume (MPV), and platelet distribution width (PDW). Sera biochemical attributes including symmetric dimethylarginine (SDMA), glucose, urea, phosphorus, calcium, total protein, albumin, globulin, albumin:globulin ratio (ALB/GLOB), alkaline phosphatase (ALP), alanine transaminase (ALT), gamma-glutamyl transferase (GGT), cholesterol, amylase, and lipase

were determined with the use of automated IDEXX® Vet Test Chemistry Analyzer (model no. 89−92525−00, IDEXX Laboratories (Pty) Ltd., IDEXX® Laboratories (Pty) Ltd., Gauteng, South Africa).

3.3.10 Statistical analysis

Overall feed intake, growth performance, and blood characteristics were analysed in a completely randomized design using one-way analysis of variance (ANOVA) by means of general linear model (GLM) procedure of SAS, with diet being the only factor. The means among variables were separated using Duncan's Multiple Range Test. Statistical significance was considered at P <0.05. The linear statistical model used was as follows:

$$Y_{ik} = \mu + D_i + E_{ik}$$

Where, Y_{ik} = dependant variable, μ = population mean, D_i = effect of diets and E_{ik} = random error associated with observation *ik*, assumed to be normally and independently distributed.

All tested variables were evaluated for linear and quadratic effects using polynomial functions. Response surface regression analysis (Proc RSREG; SAS 2010) was used to describe responses of parameters to graded levels of MBM in diets fed to Ross 308 broiler chickens, using the following quadratic model:

$\boldsymbol{y = ax^2 + bx + c}$, where $\boldsymbol{y}$ = response variables, $\boldsymbol{a}$ and $\boldsymbol{b}$ are the coefficients of the quadratic equation; $\boldsymbol{c}$ is intercept; $\boldsymbol{x}$ is dietary MBM level (%). The optimal values were calculated as:

$$Optimal\ value = \frac{b}{-2a}$$

Data from repeatedly measured variables, to include average weekly feed intake, body weight gain and feed conversion ratio were subjected to repeated measures analysis in GLM procedure of SAS (SAS, 2010) to evaluate the interaction effect between dietary treatment and time (in weeks). The following statistical linear model was employed:

$Y_{ijk} = \mu + D_i + W_j + (D \times W)_{ij} + E_{ijk,}$ where, Y_{ijk} = dependant variable, μ = population mean, D_i = effect of diets, W_j = effect of week, $(D \times W)_{ij}$ = effect of interaction between diets and week, E_{ijk} = random error related to observation *ijk*, assumed to be normally and independently distributed.

3.4 Results

3.4.1 Nutrient digestibility

There were significant effects of diets on digestibility of all nutrients, excluding lysine and methionine (Table 3.3). Similarly, negative quadratic responses (P <0.05) were observed in the digestibility values of all dietary parameters as MBM levels increased, except for lysine, methionine, and ash. Hence, the optimum MBM inclusion levels were determined and presented in Table 3.4 for these parameters.

Table 3.3. Nutrient digestibility (%) in diets containing graded marama bean meal fed to Ross 308 broiler chickens.

	[1]Diets						
Parameters	MBM0	MBM4	MBM9	MBM14	MBM19	[2]SEM	*P* value
Dry matter	82.86[a]	82.89[a]	82.80[a]	77.16[b]	70.16[c]	0.42	< 0.0001
Ash	72.60[a]	71.77[a]	72.79[a]	59.22[b]	58.86[b]	1.49	< 0.0001
Crude protein	77.68[a]	78.65[a]	74.83[a]	68.02[b]	57.12[c]	1.70	0.0002
Crude fibre	75.26[a]	77.14[a]	76.56[a]	63.88[b]	56.23[c]	1.19	< 0.0001
Crude fat	91.77[a]	91.12[a]	91.51[a]	88.77[b]	82.21[c]	0.31	< 0.0001
Lysine	88.46	90.45	85.64	89.21	86.23	2.06	0.861
Methionine	91.72	93.25	91.25	93.29	90.64	1.18	0.329
Sodium	65.53[b]	68.32[a]	65.93[ab]	56.78c	45.01[d]	0.90	< 0.0001
Chlorine	71.52[a]	72.54[a]	71.73[a]	61.45[b]	54.70[c]	0.67	< 0.0001
Calcium	51.09[a]	53.89[a]	53.89[a]	42.75[b]	19.49[c]	1.57	< 0.0001

[1]Diets: MBM0 = a standard grower diet with no MBM, MBM4 = a standard grower diet in which soyabean products were replaced with MBM at 40 g/kg, MBM9 = a standard grower diet in which soyabean products were replaced with MBM at 90 g/kg, MBM14 = a standard grower diet in which soyabean products were replaced with MBM at 140 g/kg, and MBM19 = a standard grower diet in which soyabean products were replaced with MBM at 190 g/kg. [2]SEM: standard error of mean. [a-d] Within a row, means without common letter(s) are significantly different (P <0.05).

Table 3.4. The regression equations for nutrient digestibility in Ross 308 broiler chickens fed diets containing marama bean meal.

	Regression equations	*P* value	R^2	[1]COIL
Dry matter	$y = 82.6(\pm0.39) + 0.046(\pm0.01)\ x - 0.001\ (\pm0.12)\ x^2$	< 0.0001	0.185	23
Ash	$y = 73.1(\pm1.59) - 0.007(\pm0.04)\ x$	< 0.0001	0.603	Nil
Crude protein	$y = 77.9(\pm1.40) + 0.04(\pm0.03)\ x - 0.001(\pm0.0002)\ x^2$	0.0002	0.816	20
Crude fibre	$y = 75.72(\pm1.17) + 0.07(\pm0.03)\ x - 0.001(\pm0.0002)\ x^2$	< 0.0001	0.870	35
Crude fat	$y = 91.5(\pm0.30) + 0.02(\pm0.01)\ x - 0.0003(\pm0.00004)\ x^2$	< 0.0001	0.896	33
[2]ME (Kcal/Kg)	$y = 84.3(\pm0.35) + 0.05(\pm0.01)\ x - 0.001(\pm0.00005)\ x^2$	< 0.0001	0.955	25
Lysine	Nil	0.861	0.030	Nil
Methionine	Nil	0.329	0.044	Nil
Sodium	$y = 65.6(\pm0.77) + 0.09(\pm0.02)\ x - 0.001(\pm0.0001)\ x^2$	< 0.0001	0.945	45
Chlorine	$y = 71.8(\pm0.74) + 0.05(\pm0.02)\ x - 0.001(\pm0.0001)\ x^2$	< 0.0001	0.925	25
Calcium	$y = 50.2(\pm1.35) + 0.21(\pm0.04)\ x - 0.02(\pm0.0002)\ x^2$	< 0.0001	0.923	20

[1]COIL: calculated optimum MBM inclusion in g/kg of gross ingredient composition. [2]ME: Metabolizable energy.

3.4.2 Mortality

The mortality rate recorded during the study was 3% (a total of 12 birds died). Six birds died during the day 14 – 35 of feeding the experimental diets; two each in the MBM0 (control) and MBM4, and one each in MBM9 and MBM19 diets. In the last week (day 36 – 42) of the trial, two birds, each in MBM0 and MBM9, and one each in MBM14 and MBM19 diets, died.

3.4.3 Feed intake and growth performance

Repeated measures analysis revealed no (P >0.05) diet × week (chicken age) interaction effect on average weekly feed intake, average body weight gain, and average feed conversion ratio. There were dietary effects (P <0.05) on final body weight (FBW), body weight gain, and feed

conversion ratio (FCR) as presented in Table 3.5. There were no linear and quadratic trends (P >0.05) for overall feed intake (FI) in response to graded levels of MBM. However, overall body weight gain (BWG) linearly declined with increasing inclusion levels of dietary MBM [y = 2093.2 (±57.54) – 1.410(±1.49) x; R^2 = 0.575, P < 0.0001]. Final body weight and FCR showed negative and positive quadratic responses, respectively, to graded levels of MBM levels [y = 2444.9 (±44.08) – 0.120 (±1.14) x – 0.014 (±0.006) x^2; R^2 = 0.724; P = 0.021; y = 1.610 (±0.031) – 0.0004 (±0.0008) x + 0.00001(±0.000004) x^2; R^2 = 0.807; P = 0.001]. The MBM optimum inclusion level of 4 g/kg and 20 g/kg of gross ingredient composition were calculated for FBW and FCR, respectively, using the quadratic models.

Table 3.5. The effect of MBM inclusion levels on overall feed intake and growth performance (g/bird, unless otherwise indicated) of Ross 308 broiler chickens.

[1]Diets	[2]FBW	Overall Feed intake	Overall [3]BWG	Overall [4]FCR (g:g)
MBM0	2453.6[a]	3330.8	2091.1[a]	1.60[d]
MBM4	2405.6[ab]	3329.6	2044.6[ab]	1.63[cd]
MBM9	2310.3[bc]	3163.6	1862.5[bc]	1.70[c]
MBM14	2175.6[c]	3275.5	1800.7[c]	1.82[b]
MBM19	1904.7[d]	3240.2	1556.0[d]	2.08[a]
		Statistics		
[5]SEM	49.30	78.40	63.50	0.034
[6]GLM	<0.0001	0.540	<0.0001	< 0.0001
Linear	<0.001	0.0279	<0.001	< 0.001
Quadratic	0.0099	0.0156	0.2482	0.0004
R^2	0.723	0.043	0.568	0.805

[1]Diets: MBM0 = a standard grower diet with no MBM, MBM4 = a standard grower diet in which soyabean products were replaced with MBM at 40 g/kg, MBM9 = a standard grower diet in which soyabean products were replaced with MBM at 90 g/kg, MBM14 = a standard grower diet in which soyabean products were replaced with MBM at 140 g/kg, and MBM19 = a standard grower diet in which soyabean products were replaced with MBM at 190 g/kg. [2]FBW: Final body weight. [3]BWG: Body weight gain. [4]FCR: Feed conversion ratio. [5]SEM: standard error of mean.[6]GLM = general linear model. [a-d] Within a row, means without common letter(s) are significantly different (P <0.05).

3.4.4 Haematological attributes

Except for eosinophils, there were no quadratic effects (P >0.05) in response to incremental levels of dietary MBM on all haematological parameters (Table 3.6). Eosinophil concentration quadratically declined as the inclusion level of MBM increased [y = 5.66(±0.78) + 0.090(±0.020) x – 0.001(±0.0001) x^2; R^2 = 0.471, P < 0.0001]. The optimum MBM inclusion level for eosinophil concentration was calculated to be 45 g/kg diet. Haemoglobin [y = 9.37(±0.213) – 0.002(±0.006) x; R^2 = 0.190, P = 0.011], neutrophils, [y = 3.71(±0.281) – 0.007(±0.007) x; R^2 = 0.260, P = 0.002] and monocytes [y = y = 4.85(±0.456) – 0.027(±0.012) x; R^2 = 0.272, P = 0.001] linearly decreased with increasing levels of MBM in the diets.

Table 3.6. Effects of feeding marama bean meal containing diets on haematological characteristics in Ross broiler chickens.

[4]Parameters	[1]Diets					[2]SEM	Significance		
	MBM0	MBM4	MBM9	MBM14	MBM19		[3]GLM	Linear	Quadratic
Red blood cell									
RBC (10^{12}/L)	1.29^{b}	1.76^{ab}	2.08^{a}	1.81^{ab}	1.97^{ab}	0.26	0.196	0.077	0.161
Haematocrit (%)	9.65	14.30	13.71	16.11	13.21	2.46	0.409	0.255	0.162
Haemoglobin (g/dl)	9.45^{a}	8.96^{ab}	9.45^{a}	8.52^{b}	8.66^{b}	0.23	0.008	0.011	0.760
MCV (fL)	50.73	63.84	58.56	61.30	61.24	5.13	0.364	0.260	0.343
MCH (pg)	50.09	50.33	47.20	39.37	50.84	6.35	0.669	0.660	0.399
RDW (%)	34.69	26.78	27.02	29.71	29.77	3.11	0.322	0.500	0.105
White Blood Cell									
Neutrophil (10^{9}/L)	3.89^{a}	3.15^{ab}	2.95^{bc}	3.26^{ab}	2.25^{c}	0.32	0.010	0.002	0.972
Monocyte (10^9/L)	4.71^{a}	4.29^{ab}	2.67^{c}	2.89^{bc}	2.63^{c}	0.54	0.012	0.001	0.186
Lymphocyte (10^{9}/L)	42.02^{b}	122.31^{a}	132.94^{a}	36.51^{b}	100.65^{a}	13.67	<.0001	0.607	0.068
Eosinophil (10^{9}/L)	5.76^{bc}	7.80^{ab}	10.20^{a}	6.88^{b}	3.97^{c}	0.91	0.0002	0.073	<0.0001
Reticulocyte (K/µL)	334.47^{a}	304.40^{ab}	288.84^{ab}	342.99^{a}	218.84^{b}	35.71	0.099	0.072	0.382
Platelet (K/µL)	2500^{a}	2500^{a}	2219^{b}	2500^{a}	2500^{a}	93.47	0.108	0.990	0.089
MPV (fL)	2.59	2.29	2.37	2.45	2.19	0.16	0.424	0.196	0.903
PDW (%)	14.64	14.56	15.02	14.38	14.42	0.31	0.569	0.515	0.415

[1]Diets: MBM0 = a standard grower diet with no MBM, MBM4 = a standard grower diet in which soyabean products were replaced with MBM at 40 g/kg, MBM9 = a standard grower diet in which soyabean products were replaced with MBM at 90 g/kg, MBM14 = a standard grower diet in which soyabean products were replaced with MBM at 140 g/kg, and MBM19 = a standard grower diet in which soyabean products were replaced with MBM at 190 g/kg. [2]SEM: standard error of mean. [3]GLM: general linear model. [4]Parameters: MCV = mean corpuscular volume, MCH = mean corpuscular haemoglobin, RDW = red blood cells distribution width, MPV = mean platelet volume, PDW = platelet distribution width. [a-c]Within a row, means without common letter(s) are significantly different (P <0.05).

3.4.5 Sera biochemical attributes

As presented in Table 3.7, symmetric dimethylarginine, alanine transaminase, alkaline phosphatase, cholesterol, and lipase were the only serum biochemical parameters of the Ross 308 broiler chickens that were affected by the experimental diets. A positive quadratic response was recorded for urea [$y = 0.68(\pm0.013) - 0.001(\pm0.0004)\ x + 0.00001(\pm0.000002)\ x^2$; $R^2 = 0.182$] while alanine transaminase [$y = 32.55(\pm2.713) + 0.16(\pm0.071)\ x - 0.001(\pm0.0004)\ x^2$; $R^2 = 0.278$] showed a negative quadratic response to graded levels of MBM. The optimum inclusion levels of MBM were estimated to be 50 and 80 g/kg diet for urea and alanine transaminase, respectively.

Symmetric dimethylarginine [$y = 29.31(\pm2.673) + 0.09(\pm0.070)\ x$; $R^2 = 0.363$], alkaline phosphatase [$y = 160.25(\pm14.595) + 1.017(\pm0.381)\ x$; $R^2 = 0.208$], γ-glutamyl transferase [$y = 15.16(\pm1.305) + 0.07(\pm0.034)\ x$; $R^2 = 0.117$], cholesterol [$y = 2.44(\pm0.133) + 0.004(\pm0.003)\ x$; $R^2 = 0.235$] and lipase [$y = 191.27(\pm29.010) + 0.67(\pm0.785)\ x$; $R^2 = 0.010$] levels increased linearly in response to increasing inclusion levels of MBM. On the other hand, serum calcium levels linearly reduced [$y = 1.42(\pm0.041) - 0.003(\pm0.001)\ x$; $R^2 = 0.018$], with a rise in inclusion level of MBM. Graded levels of MBM had neither linear nor quadratic effects ($P > 0.05$) on serum amylase concentration of the chickens.

Table 3.7. Serum characteristics of Ross broiler chickens fed diets containing graded levels of marama bean meal.

	[1]Diets						*P* value	
[4]Parameters	MBM0	MBM4	MBM9	MBM1	MBM19	[2]SEM	[3]GLM	Linear	Quadratic
Glucose (mmol/L)	7.55	7.87	8.01	7.81	8.57	0.47	0.560	0.143	0.758
SDMA (µg/dL)	27.36^{c}	36.57^{b}	35.86^{b}	38.67^{ab}	45.79^{a}	3.09	0.002	0.0002	0.919
Urea (mmol/L)	0.68^{a}	0.64^{ab}	0.64^{ab}	0.63^{b}	0.67^{a}	0.02	0.115	0.614	0.015
Phosphorus (mmol/L)	2.26	2.29	2.26	2.30	2.30	0.10	0.996	0.781	0.958
Calcium (mmol/L)	1.40^{a}	1.36^{a}	1.28^{ab}	1.21^{b}	1.29^{ab}	0.05	0.057	0.018	0.093
Total protein (g/L)	40.36	43.43	41.71	42.08	43.36	2.26	0.827	0.493	0.879
Albumin (g/L)	12.57	12.00	11.36	11.67	11.71	0.55	0.527	0.214	0.223
Globulin (g/L)	27.79	31.43	30.36	30.42	31.64	1.99	0.601	0.268	0.614
Albumin: Globulin	0.46^{a}	0.39^{ab}	0.39^{ab}	0.40^{ab}	0.37^{b}	0.03	0.183	0.068	0.354
ALT (U/L)	33.36^{ab}	35.79^{a}	39.36^{a}	35.6^{7a}	25.43^{b}	3.27	0.037	0.076	0.006
ALP (U/L)	157.83^{b}	195.79^{ab}	232.64^{a}	214.00^{a}	226.29^{a}	17.27	0.019	0.005	0.065
GGT (U/L)	15.42^{b}	16.57^{ab}	20.43^{a}	18.70^{ab}	19.14^{ab}	1.54	0.120	0.044	0.156
Cholesterol (mmol/L)	2.37^{c}	2.79^{ab}	2.54^{bc}	3.03^{a}	2.98^{a}	0.15	0.008	0.004	0.742
Amylase (U/L)	466.8^{ab}	391.4^{b}	464.9^{ab}	601.5^{a}	542.1^{ab}	55.77	0.184	0.083	0.619
Lipase (U/L)	189.6^{b}	223.0^{b}	244.9^{b}	388.1^{a}	415.6^{a}	44.12	0.0003	0.010	0.981

[1]Diets: MBM0 = a standard grower diet with no MBM, MBM4 = a standard grower diet in which soyabean products were replaced with MBM at 40 g/kg, MBM9 = a standard grower diet in which soyabean products were replaced with MBM at 90 g/kg, MBM14 = a standard grower diet in which soyabean products were replaced with MBM at 140 g/kg, and MBM19 = a standard grower diet in which soyabean products were replaced with MBM at 190 g/kg. [2]SEM: standard error of mean. [3]GLM: general linear model. [4]Parameters: SDMA= symmetric dimethylarginine, ALT = alanine transaminase, ALP = alkaline phosphatase, GGT = gamma-glutamyl transferase. [a-d] Within a row, means without common letter(s) are significantly different (P <0.05).

3.5 Discussion

The use of marama bean in animal nutrition has not been widely practiced and there are no documented studies on its application as a partial replacement for soyabean products in broiler chicken diets. In addition, the effects of marama bean meal (MBM) on feed utilisation, growth performance and blood indices of broiler chickens have not been reported in literature.

3.5.1 Nutrient digestibility

Digestibility trial results indicate that increasing the level of MBM in diets reduced the digestibility of all nutritional components, except for lysine and methionine. This result agrees with the findings by Erdaw *et al*. (2017), where a decline in apparent ileal digestibility of crude protein and most dispensable and indispensable amino acids was recorded with increasing levels of raw full-fat soyabean in broiler diets. This was linked with an increase in trypsin inhibitor activity (TIA) as inclusion levels of raw full-fat soyabean increased from 0, 45 or 75 g/kg of diet. Reduction in digestibility of dietary components observed in the current study is consistent with expected biological effects of anti-nutritional factors that are known to be present in marama bean. Trypsin inhibitors (Nadaraja *et al*., 2009) and phytic acid (Jackson *et al*., 2010) have been reported to be part of antinutritional factors present in MB. Trypsin inhibitors disrupt the biological activity of endogenous trypsin, hence, adversely affect protein digestibility and amino acid availability by making indigestible complexes with pancreatic-produced trypsin (Liener, 1994). It was, however, surprising that the digestibility of lysine and methionine was not affected by increasing levels of MBM.

The digestibility of calcium was the most adversely affected when dietary levels of MBM increased. The poor digestibility of mineral may suggest the presence of phytate in MB. Phytic acid in marama bean has been stated to be predominantly found in an insoluble nutrient-phytate complex form, which becomes detrimental to nutrient digestibility (Amonsou *et al*., 2011).

Contradictorily, phytic acid is mostly present in the form of soluble protein-phytate in soyabeans (Amonsou *et al*., 2011). Phytate forms complexes with other dietary nutrients such as minerals, proteins, free amino acids, and starch (Casartelli *et al*., 2005) resulting in reduced digestibility. Therefore, it is not surprising that in the current study, the digestibility of calcium was reduced as the levels of MBM increased in broiler diets.

3.5.2 Feed intake and growth metrics

The nutritional components of MBM indicate its potential as a substitute for soyabean products in broiler diets, however pre-treatment of MB may be required prior to its inclusion in broiler chickens' diets. Bird mortality was low and evenly distributed across all five experimental diets. Thus, there was no evidence that MBM inclusion in broiler diets induced bird mortality. Diet-induced modifications in growth performance parameters were independent of the age of the birds, as shown by the lack of a diets × week (bird age) interaction effects on average weekly FI, body weight gain, and feed conversion ratio. Results from this study suggest that the inclusion of MBM in chicken diets up to 190 g/kg had no effect on overall feed intake (FI). This may be attributed to absence of anti-palatability factors such as cyanogenic glycosides (Holse *et al*., 2010) and glucosinolates in MB. These factors have been reported to adversely alter palatability and subsequently reduce feed intake in animals (Woyengo *et al*., 2011; Abdulshaheed, 2018). This finding on FI reported in this presented study is consistent with previous findings when wringed beans were included in broiler diets at 0, 2.5, and 5% (Nurpaidah *et al*., 2021). However, this contradicts the decline in FI as the levels of raw full-fat soyabean replacing soyabean meal increased as reported by Erdaw *et al*. (2017). Despite similar feed intake levels, increasing MBM levels reduced final body weight and overall body weight gain while reducing feed utilization efficiency. Further, the optimal inclusion levels of 20, 4 and 20 g/kg diet calculated for protein digestibility, FBW and FCR, respectively, using the regression models suggest that the lowest MBM inclusion level of 40 g/kg (MBM4) in this

present study was too high and induced negative results on nutrient digestibility and growth performance in the chickens. This suggests that while birds consumed the same amount of feed across the experimental diets, conversion to meat was poor as the levels of dietary raw full-fat MBM increased. In a related study, growth depression in broiler chicken was attributed to the cumulative effect of loss of essential amino acids and reduced intestinal proteolysis caused by trypsin inhibitors in raw soyabeans (Chohan *et al*, 1993). Earlier findings by Maruatona (2008) showed that MB is lower (0.46 g/100 g) in methionine, the first limiting amino acid in practical broiler's diet, compared to soybean (0.71 g/100 g) and this could have resulted in poor feed utilization efficiency observed in the present study at higher MBM inclusion levels.

3.5.3 Haematological parameters

The dietary inclusion of MBM resulted in eosinophil (except for 65 g/kg MBM) values that were markedly higher than in the control birds. On another hand, monocytes and neutrophils were reduced in the blood of broiler chicks fed diets containing MBM. Neutrophils constitute about half of the WBC and are the major pathogen-fighting immune cells (Mayadas *et al*., 2014). The fatty acid profile and the expression of antimicrobial properties reported in MB (Chingwaru *et al*., 2011), may have provided effective antimicrobial protection, thereby reducing the need for high neutrophils in the blood of chickens. The haemoglobin concentration linearly declined due to incorporation of MBM, which could be a consequence of poor nutrient utilization. This is because proteins and minerals (such as iron, calcium, and phosphorus) are essential for the formation of haemoglobin (Nworgu *et al*., 2007). Given that crude protein digestibility declined as MBM inclusion levels increased, this suggests that poor protein utilization played a role in the low haemoglobin concentrations observed in chickens reared on MBM-containing diets.

3.5.4 Sera biochemical profile

The sera urea and alanine transaminase showed positive and negative quadratic responses, respectively, to levels of MBM in the diets, however calculated MBM optimum inclusion level for urea and ALT were 50 and 80 g/kg. This indicates that the urea was inferior when MBM was included at a rate below 50 g/kg. Linear increases were observed for symmetric dimethylarginine, alkaline phosphatase, and lipase concentrations as the level of MBM in the diets increased. Our results on ALP agreed with the previous findings on serum biochemical components when laying Japanese quail were reared on diets supplemented with canola seeds (Ibrahim *et al*., 2020). The rise in concentrations of these enzymes (ALP, SDMA, and lipase) in response to high levels of MBM in the diets, may be an indication of stress on the liver, kidney, and pancreas. For instance, higher lipase concentration may be attributed to pancreas functional changes due to pancreatic hypertrophy, and hyperplasia (Liener, 1994) because of the increase in level of TIA in the MB (Alabi *et al*., 2022). Similarly, SDMA has been shown to be an accurate biomarker for estimating renal dysfunction in animals (Hall *et al*., 2014). Although, the biochemical assessment of plasma uric acid is presently the primary method of diagnosing renal disease in birds, however, it is a waste product of protein metabolism synthesized by the liver and excreted from the kidneys (Lierz, 2003). On the other hand, SDMA is a by-product of cellular proteolysis solely excreted by the kidneys (van Zanten & Xie, 2023). Although the proportion of different enzymes related to metabolism and function of heart, liver and kidney are used to determine feed toxicity, liver enzymes such as ALT or GGT have low diagnostic value for nutritional merit due to their high variability in the blood (Enemor *et al*., 2005). In this study, serum calcium in the blood linearly decreased as dietary MBM increased. This reduction in calcium in response to increasing MBM inclusion levels may be attributed to the high level of antinutritional factors, especially phytic acid in MB (Jackson *et al*., 2010) and poor Ca digestibility as reported in Section 3.5.1. This corroborates the findings reported when

incremental levels (0, 5, 10, 15, 20, and 25%) of raw suckle pod seed meal were used to replace roasted soyabean in broiler chickens' diets (Augustine *et al*., 2017). A positive linear response of sera cholesterol to dietary MBM level was also recorded. Generally, high lipid levels in circulation indicate enhanced *de novo* lipogenesis, while low lipid profile in blood indicates an increased rate of amino acid transportation and improved lipid metabolism with consequent decreased fat deposition (Zhao *et al*., 2009). Hence, it can be deduced that the increase in cholesterol concentration in sera samples might have been caused by poor nutrient utilisation due to antinutritional factors in MB.

3.6 Conclusions

This study showed that incremental inclusion levels of MBM as a partial replacement of soyabean products in Ross 308 broiler chickens adversely affected nutrient utilization and growth performance. The optimum MBM inclusion level in a broiler grower diet that would promotes crude protein digestibility and feed conversion ratio is 20 g/kg. Lower optimum level inclusion of 4 g MBM per kg diet was calculated for final body weight. However, the optimum inclusion levels of MBM were estimated to be 45, 50 and 80 g/kg diet for sera eosinophil, and blood urea and alanine transaminase, respectively. Therefore, it can be concluded that the lowest level of 40 g/kg MBM in diet was detrimental to nutrient utilization and growth performance in Ross 308 broilers. Hence, a further investigation to assess the influence of MBM on carcass traits and meat quality of broiler chickens is highly recommended.

3.7 References

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4 CHAPTER FOUR: EFFECT OF PARTIALLY REPLACING SOYABEAN PRODUCTS IN BROILER GROWER DIETS WITH MARAMA BEAN ON INTERNAL ORGANS, CARCASS AND MEAT QUALITY TRAITS, AND BONE BREAKING STRENGTH

4.1 Abstract

Currently, chicken meat is commonly distributed and sold as parts or processed products, revealing the significance of meat quality in the poultry industry. As living standards have improved, the qualitative attributes of meat need to be ascertained to meet the consumer demand for high quality animal products. This part of the study evaluated the effect of partial substitution of soyabean product with marama bean meal (MBM) on carcass weights, carcass parts, and internal organs as well as breast meat colour attributes, cooking loss, shear force, water holding capacity and tibial bone breaking strength of Ross 308 broiler chickens. A total of three hundred and seventy-three, six-week-old broiler chickens previously assigned to five diets formulated with MBM in a standard grower broiler diet at 0, 40, 90, 140, and 190 g/kg of the diet, with each di*et al*located to 7 replicate pens, were slaughtered. Breast weight linearly decreased (P <0.05) while the weights of visceral organs (jejunum, caeca, gizzard, proventriculus, pancreas, and ileum) were enhanced as MBM inclusion level increased. Meat pH had negative linear response (P <0.05) while meat lightness and shear force linearly increased (P <0.05) with increasing levels of dietary MBM. Experimental diets had no effect (P >0.05) on water holding capacity, cooking loss and bone breaking strength. There were negative quadratic effects on thigh, hot carcass weight (HCW) and cold carcass weight (CCW). Hence, the calculated optimal inclusion levels of MBM as a component of diet that is required to maximize HCW, CCW, and thigh weights were 22, 26, and 50 g/kg, respectively. It was concluded that increasing levels of MBM from 40 g/kg in broilers diets is detrimental to carcass attributes, caused atrophy of some internal organs, and increased meat toughness.

Keywords: carcass attributes, meat quality, meat shelf life, marama bean meal, trypsin inhibitor

4.2 Introduction

The increase in global demand for meat has caused a direct rise in production of animal protein (FAO, 2015), with a major attention to meet consumers' preference for quality meat (Hocquette *et al*., 2005; Melo *et al*., 2016). Meat quality is evaluated by many ways, these include, but are not limited to sensory characteristics (include colour, juiciness, texture, tenderness, taste, and odour), technical attributes (water holding capacity, pH, and thawing loss) and nutritional composition (fatty acid profile, fat content, protein fraction, minerals, and vitamins), according to Hocquette *et al*. (2005) and dos Santos *et al*. (2019). Carcass characteristics and meat quality parameters, such as colour and tenderness, are important for consumer acceptance (dos Santos *et al*., 2019).

The effects of diets containing certain legumes such as soyabean and pea on poultry carcass traits and/or meat quality parameters have gained attention (Casaubon-Huguenin *et al*., 2004; Laudadio & Tufarelli, 2011; Erdaw *et al*., 2017; Maidala *et al*., 2017; Rada *et al*., 2017). However, the effect of MBM-containing diets on carcass attributes and meat quality parameters of broiler chickens have not been reported in literature. Moreover, the replacement of conventional protein sources in poultry diets with non-conventional ones may have the potential to alter carcass and meat quality. Furthermore, the previous study (Chapter 3) revealed the detrimental effect of MBM incorporation on broiler performance and these may be related to the antinutritional factors including trypsin inhibitors (Bower *et al*., 1988), elastase inhibitors (Nadaraja *et al*., 2010) and phytates (Mosele *et al*., 2011; Amonsou *et al*., 2011), which are present in MB. Some of these aforementioned antinutrients have been indicated to influence carcass and meat parameters of poultry. For instance, pancreatic hypertrophy has been linked

to TI (Liener, 1995; Rada *et al*., 2017). Similarly, skin yellowness increased linearly in broilers fed raw full-fat soyabean due to anti-components (Casaubon-Huguenin *et al*., 2004). Hence, the effects of MBM inclusion in broilers' diets need to be investigated to determine its mode of action on chicken carcass and meat quality traits. Therefore, this study attempted to evaluate the carcass yield, carcass parts, intestinal weight, meat quality attributes and bone breaking strength of Ross 308 broiler chickens.

4.3 Materials and methods

4.3.1 Ethics statement

The procedures used were reviewed and approved as described in Chapter 3, section 3.3.1.

4.3.2 Diets and experimental design

The diets were formulated, and birds reared in a completely randomized design as described in the Chapter 3, sections 3.3.5 and 3.3.6.

4.3.3 Slaughter procedures

At 42 days of age, all 373 broiler chickens were taken to Rooigrond poultry abattoir (North West province, South Africa) for slaughter. At the abattoir, the birds were stunned using an electric shock and hung onto a portable metal rack by their feet and exsanguinated by cutting the jugular vein with a sharp knife and bled out. Thereafter, chickens were defeathered and each carcass was packed in a labelled plastic bag. All carcases were taken to the NWU Meat Science Laboratory for measurements.

4.3.4 Carcass traits and internal organs

At the NWU Meat Science Laboratory, the following were removed and weighed for each carcass: breasts, thighs, drumsticks, wings, gizzard, liver, pancreas, proventriculus and spleen.

Length and weight of duodenum, ileum, jejunum, ceca, and colon were also measured and recorded. Immediately after slaughter, hot carcass weight (HCW) was measured while cold carcass weight (CCW) was measured after the carcasses had been refrigerated at 6°C for 24 hr. Carcass yield was calculated as the ratio of HCW to FBW. Also, the value of each carcass organ was estimated as the ratio of organ weight to HCW.

4.3.5 Meat quality measurements

Immediately after slaughter, meat pH (pH_0) was evaluated on the central part of the breast muscle with pH-temperature meter (Corning Model 4, Corning Glass Works, Medfield, MA) fitted with an Ingold spear-type electrode (Ingold Messtechnik AG, Udorf, Switzerland). Whole carcasses were stored under refrigeration to maintain a cool temperature of 6°C. Exactly 24 hr post-mortem, pH ($pH_{24)}$ and colour attributes of the meat were measured. Calibration of pH meter was done after every ten measurements using standard solutions (pH 4, 7, and 10). After each pH was determined, the breast meat colour attributes (lightness, *l*, redness, *a*, and yellowness, *b*) were measured with a Minolta colour-guide (BYK-Gardener GmbH, Geretsried, Germany), following the guidelines by the manufacturer for colour guide and calibration of the device.

Breast meat samples, free of external fat, were weighed (ADAM® scale, readability of 0.001 g to 0.01 g, Adam Equipment S.A. PTY, Johannesburg, South Africa), transferred into foil plates and cooked in an electric oven to reach an internal temperature of 75°C (Honikel, 1998). The samples were removed immediately when the 75°C temperature was attained and then allowed to cool for 30 minutes. The meat was then taken from the foil plate, gently blotted dry and weighed to determine the final weight. Cooking loss was calculated as the difference between the weight of raw meat sample and cooked meat and expressed as a proportion of the weight of the raw meat sample:

$$Cooking\ loss\ (\%) = \left(\frac{weight\ of\ raw\ meat - weight\ of\ cooked\ meat}{weight\ of\ raw\ meat}\right) \times\ 100$$

Water holding capacity (WHC) was evaluated in raw breast meat samples by expressing water from the meat held under pressure (60 kg pressure) using the filter-paper method as described by Grau & Hamm (1957). Percent WHC was calculated using the formula below:

$$WHC\ (\%) = \left(\frac{Initial\ weight\ of\ the\ meat - weight\ of\ the\ meat\ after\ press}{Initial\ weight\ of\ the\ meat}\right) \times\ 100$$

For the measurement of meat shear force (N), freshly cut breast meat was sheared with the use of a Meullenet-Owens Razor Shear Blade (A/MORS) placed on a Texture Analyzer (TA-XT plus, Stable Micro Systems, Surrey, UK).

4.3.6 Bone breaking strength

Bone breaking strength was determined in duplicate tibia bones. Freshly cut drumstick was placed in thin-walled plastic bags and immersed in water bath at 100°C for 30 minutes. The meat was then allowed to cool at room temperature for 15 minutes. Thereafter, bones were de-fleshed and cleaned of all tissues, followed by placing the bone on an adjustable three-point bend/snap fixture fitted on a heavy-duty TA-XT platform of the Texture Analyser (TA-XT plus, Stable Micro Systems, Surrey, UK) and then broken with a 6-cm flat head probe attached to a 50-kg load cell reporting the breaking force in Newtons. The breaking bone strength was measured as the peak load before bone breakage (Disetlhe *et al*., 2017).

4.3.7 Meat shelf life

A selected breast meat sample from each replicate pen was used to determine broiler meat shelf life at room temperature. The breast meat samples were placed in labelled foil trays and stored on top of the table at NWU Meat Science laboratory. Meat pH and colour (lightness, redness, and yellowness) were then recorded daily from 24 hr post-mortem for a period of six (6) days.

4.3.8 Statistical analysis

All tested variables were evaluated for linear and quadratic effects as previously mentioned in Chapter 3. Shelf-life data, including daily pH, the breast meat lightness, redness, and yellowness were subjected to repeated measures analysis in SAS software package (SAS, 2010) to evaluate the interaction effect between dietary treatment and time (in days). The statistical linear model was employed as described in section 3.3.10.

The pH and the breast meat colour attributes (lightness, *l*, redness, *a*, and yellowness, *b*) were analysed in a completely randomized design using one-way analysis of variance (ANOVA) by means of general linear model procedure of SAS, with diet being the only factor. The means among variables were separated using Duncan's Multiple Range Test. Statistical significance was considered at $P < 0.05$. The linear statistical model used was as stated in Chapter 3 (section 3.3.10)

4.4 Results

4.4.1 Carcass traits

There were dietary effects ($P < 0.05$) on HCW, CCW, and drumstick, thigh, breast, duodenum, jejunum, ileum, gizzard, proventriculus and pancreas weights (Table 4.1). There was no quadratic effect on all the parameters measured due to inclusion of increased levels of MBM, except for a negative quadratic effect on thigh weight [$y = 7.135(\pm0.20) - 0.010(\pm0.005)\ x - 0.0001(\pm0.00003)\ x^2$; $R^2 = 0.128$, $P = 0.041$], HCW [$y = 1899.2(\pm39.76) + 0.441(\pm1.037)\ x - 0.013(\pm0.005)\ x^2$; $R^2 = 0.071$, $P = 0.018$] and CCW [$y = 1887.1(\pm39.14) + 0.509(\pm1.021)\ x - 0.013(\pm0.005)\ x^2$; $R^2 = 0.074$, $P = 0.016$]. Therefore, the calculated optimal inclusion levels of MBM as a component of diet that maximized HCW, CCW, and thigh weights were calculated to be 22, 26, and 50 g/kg, respectively.

Breast weight linearly declined [$y = 23.60(\pm0.70) – 0.04(\pm0.018)\ x$; $R^2 = 0.512$, $P < 0.0001$] as the inclusion level of MBM increased. There were positive linear responses, as MBM inclusion level increased, in the weight of intestinal organs comprising of duodenum [$y = 0.585(\pm0.036) + 0.002(\pm0.0009)\ x$; $R^2 = 0.318$, $P = 0.0004$], jejunum [$y = 1.531(\pm0.06) + 0.003(\pm0.001)\ x$; $R^2 = 0.535$, $P < 0.0001$], caeca [$y = 0.628(\pm0.03) + 0.001(\pm0.0007)\ x$; $R^2 = 0.129$, $P = 0.035$], gizzard [$y = 1.960(\pm0.07) + 0.002(\pm0.001)\ x$; $R^2 = 0.356$, $P = 0.0002$], proventriculus [$y = 0.461(\pm0.02) + 0.001(\pm0.0005)\ x$; $R^2 = 0.553$, $P < 0.0001$], pancreas [$y = 0.278(\pm0.03) + 0.001(\pm0.0007)\ x$; $R^2 = 0.736$, $P < 0.0001$] and ileum [$y = 1.365(\pm0.05) + 0.0001(\pm0.001)\ x$; $R^2 = 0.432$, $P < 0.0001$].

Table 4.1. The effects of marama bean meal-containing diets on carcass traits and weights of internal organs in Ross 308 broiler chickens (%HCW, unless stated otherwise).

	[1]Diets						*P* value	
[4]Parameters	MBM0	MBM4	MBM9	MBM14	MBM19	[2]SEM	[3]GLM	Linear	Quadratic
Carcass yield (%)	77.82^{ab}	76.44^{b}	81.74^{a}	76.53^{b}	79.68^{ab}	1.77	0.145	0.461	0.727
[4]HCW (g)	1913.00^{a}	1852.08^{a}	1891.09^{a}	1660.87^{b}	1519.52^{c}	45.14	< 0.0001	< 0.0001	0.018
[5]CCW (g)	1904.68^{a}	1836.11^{a}	1884.63^{a}	1666.38^{b}	1518.23^{c}	44.28	< 0.0001	< .0001	0.016
Wing	4.99^{ab}	4.95^{ab}	4.67^{b}	5.19^{a}	5.20^{a}	0.15	0.096	0.174	0.110
Drumstick	5.92^{a}	5.97^{a}	5.36^{b}	6.13^{a}	6.15^{a}	0.17	0.011	0.274	0.050
Thigh	7.09^{a}	7.03^{a}	6.27^{b}	7.15^{a}	7.08^{a}	0.21	0.018	0.860	0.041
Breast	23.06^{a}	23.24^{a}	19.32^{b}	19.16^{b}	18.17^{b}	0.77	< 0.0001	< 0.0001	0.401
Duodenum	0.62^{b}	0.61^{b}	0.78^{a}	0.81^{a}	0.77^{a}	0.04	0.001	0.0004	0.100
Jejunum	1.59^{c}	1.54^{c}	1.82^{b}	2.01^{a}	2.01^{a}	0.07	< 0.0001	< 0.0001	0.799
Colon	0.13	0.16	0.12	0.13	0.13	0.02	0.449	0.640	0.893
Ceaca	0.64^{b}	0.65^{ab}	0.73^{a}	0.72^{ab}	0.71^{ab}	0.03	0.147	0.035	0.184
Ileum	1.36^{c}	1.41^{c}	1.42^{bc}	1.58^{ab}	1.71^{a}	0.06	0.001	< .0001	0.185
Liver	2.39^{b}	2.28^{b}	2.44^{ab}	2.67^{a}	2.45^{ab}	0.09	0.047	0.091	0.545
Gizzard	1.99^{b}	2.03^{b}	2.08^{b}	2.38^{a}	2.30^{a}	0.08	0.002	0.0002	0.904
Proventriculus	0.47^{c}	0.48^{c}	0.55^{b}	0.59^{ab}	0.62^{a}	0.02	< 0.0001	< 0.0001	0.774
Spleen	0.15	0.15	0.16	0.17	0.15	0.01	0.418	0.740	0.130
Pancreas	0.28^{c}	0.33^{c}	0.44^{b}	0.49^{b}	0.61^{a}	0.03	< 0.0001	< 0.0001	0.661

[1]Diets: MBM0 = a standard grower diet with no MBM, MBM4 = a standard grower diet in which soyabean products were replaced with MBM at 40 g/kg, MBM9 = a standard grower diet in which soyabean products were replaced with MBM at 90 g/kg, MBM14 = a standard grower diet in which soyabean products were replaced with MBM at 140 g/kg, and MBM19 = a standard grower diet in which soyabean products were replaced with MBM at 190 g/kg. [2]SEM: standard error of mean. [3]GLM: general linear model. [4]HCW: hot carcass weight. [5]CCW: cold carcass weight. [abc] Within a row, means without common letter(s) are significantly different (P <0.05).

4.4.2 Meat quality attributes

Experimental diets had no effect (P >0.05) on meat quality parameters measured immediately after slaughter, except for a negative linear response recorded for meat pH [y = 6.04(±0.043) – 0.001(±0.001) x; R^2 = 0.191, P = 0.011] as the levels of MBM increased in the diets (Table 4.2).

Table 4.2. The effects of graded marama bean meal containing diets on pH_0 and colour attributes of Ross broiler chickens.

	[1]Diets						Significance		
	MBM0	MBM4	MBM9	MBM14	MBM19	[2]SEM	[3]GLM	Linear	Quadratic
pH	6.04[a]	6.00[ab]	5.96[ab]	5.94[ab]	5.87[b]	1.009	0.165	0.011	0.994
Lightness (*l*)	58.65	59.60	60.51	59.65	61.03	1.031	0.465	0.115	0.751
Redness (*a*)	1.94[ab]	1.73[ab]	1.93[ab]	2.18[a]	1.54[b]	0.189	0.154	0.482	0.223
Yellowness (*b*)	10.12[b]	10.06[b]	10.92[ab]	11.49[a]	10.09[b]	0.474	0.128	0.403	0.073

[1]Diets: MBM0 = a standard grower diet with no MBM, MBM4 = a standard grower diet in which soyabean products were replaced with MBM at 40 g/kg, MBM9 = a standard grower diet in which soyabean products were replaced with MBM at 90 g/kg, MBM14 = a standard grower diet in which soyabean products were replaced with MBM at 140 g/kg, and MBM19 = a standard grower diet in which soyabean products were replaced with MBM at 190 g/kg. [2]SEM: standard error of mean. [3]GLM: general linear model. [ab]Within a row, means without common letter(s) are significantly different (P <0.05).

Similarly, meat pH measured 24 hr post-mortem showed a negative linear response [y = 6.10(±0.035) – 0.002(±0.001) x; R^2 = 0.149, P = 0.022], while meat lightness linearly increased [y = 58.90(±0.667) + 0.015(±0.017) x; R^2 = 0.161, P = 0.021] with increasing levels of dietary MBM. Experimental diets had no effect (P >0.05) on meat redness and yellowness measured 24 hr post-slaughter (Table 4.3).

Table 4.3. The effects of graded marama bean meal containing diets on pH_{24} and colour attributes of Ross broiler chickens.

	[1]Diets						Significance		
	MBM0	MBM4	MBM9	MBM14	MBM19	[2]SEM	[3]GLM	Linear	Quadratic
pH	6.11[a]	6.02[ab]	5.98[b]	5.98[b]	5.98[b]	0.042	0.128	0.022	0.138
Lightness (*l*)	58.77	59.74	60.04	60.51	61.21	0.807	0.238	0.021	0.848
Redness (*a*)	2.03[a]	1.47[ab]	2.06[a]	1.83[ab]	1.25[b]	0.264	0.106	0.145	0.338
Yellowness (*b*)	9.15[b]	9.13[b]	9.88[ab]	10.45[a]	9.20[b]	0.479	0.200	0.373	0.105

[1]Diets: MBM0 = a standard grower diet with no MBM, MBM4 = a standard grower diet in which soyabean products were replaced with MBM at 40 g/kg, MBM9 = a standard grower diet in which soyabean products were replaced with MBM at 90 g/kg, MBM14 = a standard grower diet in which soyabean products were replaced with MBM at 140 g/kg, and MBM19 = a standard grower diet in which soyabean products were replaced with MBM at 190 g/kg. [2]SEM: standard error of mean. [3]GLM: general linear model. [ab]Within a row, means without common letter(s) are significantly different (P <0.05).

4.4.3 Cooking loss, water holding capacity, shear force and bone breaking strength

Table 4.4 shows that experimental diets had no effect (P >0.05) on cooking loss, water holding capacity and bone breaking strength. Shear force linearly increased [y = 3.48(±0.326) + 0.096(±0.02) x; R^2 = 0.642, P < 0.0001] with increasing levels of dietary MBM.

Table 4.4. The effects of graded marama bean meal containing diets on meat quality characteristics and bone breaking strength of Ross broiler chickens.

	[1]Diets						*P* value		
	MBM0	MBM4	MBM9	MBM14	MBM19	[2]SEM	[3]GLM	Linear	Quadratic
Cooking loss	25.17	25.20	24.84	24.22	25.98	0.62	0.352	0.685	0.166
[4]WHC (%)	15.32	15.94	12.92	15.22	16.51	1.42	0.464	0.728	0.217
Shear force	3.62[b]	4.50[b]	6.27[a]	6.54[a]	6.94[a]	0.37	< 0.0001	< 0.0001	0.072
[5]BBS (N)	332.9	329.9	338.3	285.5	337.7	20.4	0.319	0.638	0.560

[1]Diets: MBM0 = a standard grower diet with no MBM, MBM4 = a standard grower diet in which soyabean products were replaced with MBM at 40 g/kg, MBM9 = a standard grower diet in which soyabean products were replaced with MBM at 90 g/kg, MBM14 = a standard grower diet in which soyabean products were replaced with MBM at 140 g/kg, and MBM19 = a standard grower diet in which soyabean products were replaced with MBM at 190 g/kg. [2]SEM: standard error of mean. [3]GLM: general linear model. [4]WHC: water holding capacity. [5]BBS (N): bone breaking strength in Newton. [ab]Within a row, means without common letter(s) are significantly different (P <0.05).

4.4.4 Meat shelf life measurements

Repeated measures analysis showed no diet × shelf life (day) interaction effect (P >0.05) on meat pH, and meat colour attributes (lightness, *l*; redness, *a*; and yellowness, *b*). The effect of experimental diets on overall meat quality is presented in Table 4.5. There was no significant effect due to experimental diets on meat lightness. Overall meat pH [y = 6.01(±0.02) – 0.0003(±0.0006) x; R^2 = 0.175, P = 0.015} and redness [y = 5.90(±0.306) – 0.026(±0.008) x; R^2 = 0.423, P < 0.0001] linearly declined with increasing levels of dietary MBM.

Table 4.5. The effects of graded marama bean meal containing diets on breast meat stability of Ross broiler chickens.

	[1]Diets						Significance		
	MBM0	MBM4	MBM9	MBM14	MBM19	[2]SEM	[3]GLM	Linear	Quadratic
pH	6.00[a]	6.00[a]	6.00[a]	5.91[b]	5.94[ab]	0.03	0.040	0.015	0.895
Lightness (*l*)	34.47	35.06	33.26	34.20	33.68	0.55	0.135	0.145	0.663
Redness (*a*)	6.10[a]	4.61[b]	4.22[bc]	4.09[bc]	3.64[c]	0.36	0.0002	< 0.001	0.059
Yellowness (*b*)	5.97[c]	8.21[a]	6.04b[c]	7.22[ab]	6.58[bc]	0.43	0.002	0.995	0.310

[1]Diets: MBM0 = a standard grower diet with no MBM, MBM4 = a standard grower diet in which soyabean products were replaced with MBM at 40 g/kg, MBM9 = a standard grower diet in which soyabean products were replaced with MBM at 90 g/kg, MBM14 = a standard grower diet in which soyabean products were replaced with MBM at 140 g/kg, and MBM19 = a standard grower diet in which soyabean products were replaced with MBM at 190 g/kg. [2]SEM: standard error of mean. [3]GLM: general linear model. [abc]Within a row, means without common letter(s) are significantly different (P <0.05).

4.5 Discussion

4.5.1 Carcass characteristics

Diet is one of the factors that influence the quantity and quality of poultry meat and the return on investment (Biesek *et al*., 2020). In other words, feed supplied to chickens may affect their growth rate, feed intake, feed conversion ratio, body weight gain and carcass yield. In this presented study, diet depressed the weights of hot and cold carcass, and breast. Similarly, Biesek *et al*. (2020) recorded a lower body weight gain and carcass parts (breast and legs) weight in Ross 308 broiler chickens fed with pea or fava bean in comparison to those offered genetically modified soyabean meal. However, no significant differences in carcass, breast, neck, and wing yields. On the contrary, feeding low-quality fava beans or high-quality seed at 15%, 30%, and 45% faba bean in partial or total replacement of soyabean meal had no effect

on cold carcass weight, dressing percentage or yield of breast and thigh of broiler chickens (Smit *et al*., 2021). The lower weights of carcass and breast may be caused by a lower level of essential amino acids or the content of tannins, phytic acid as well as the other antinutritional factors in MB.

The weight of the internal organs of the birds, comprising of jejunum, duodenum, gizzard, proventriculus, ileum and pancreas linearly increased as the level of MBM also increased in diets. This result is in line with those of other researchers (Erdaw *et al*., 2017; Maidala *et al*., 2017; Rada *et al*., 2017) who reported that birds fed diets containing raw soyabean had heavier pancreases, small intestines, and gizzards. The increased weights of these internal organs may be due to metabolic differences, caused by nature of the diets, and cellular hypertrophy or hyperplasia. Indeed, trypsin inhibitor ingestion has been reported to increase production and secretion of trypsin and chymotrypsin enzymes leading to pancreatic hypertrophy (Rada *et al*., 2017). Thus, the heavier visceral organs may be attributed to declining nutrient digestibility and compromised feed utilization efficiency recorded in this study.

4.5.2 Meat quality parameters

The evaluation of meat quality entails physical traits including immediate pH, muscle colour and shear force. The meat pH and redness within 24 hr post-mortem linearly declined while meat lightness and shear force linearly increased in response to dietary MBM inclusion levels. Contrary to our study, a report by Laudadio & Tufarelli (2011) indicated that a replacement of soyabean meal with dehulled-micronized fava beans (310 g/kg) in Hubbard broiler chicken diet did not affect the breast muscle colour but enhanced redness of thigh skin. In addition, Casaubon-Huguenin *et al*. (2004) reported that there was no effect on broiler skin redness, but skin yellowness was improved in a study in which increasing inclusion level (0, 20, 40, 60 and 100%) of raw full-fat soybean was used as a replacement for soyabean meal. The difference in

the current study and previous ones could be due to different protein sources, birds, and study environments feed ingredients. Apart from nutrition, the decrease in post-mortem meat pH naturally occurs due to lactic acid secretion in response to muscle contraction and energy production and degradation (Dransfield & Sosnicki, 1999). Nevertheless, the immediate and 24 hr post-mortem pH recorded in this study agrees with the optimum pH (ranges between 5.35 and 6.10) during 24 hr post-mortem that has been established (Janocha *et al*., 2022).

The increase in meat toughness (shear force) as MBM levels increased in this study, may have been caused by the decline in nutrient digestibility and utilization related to presence of antinutritional factors (especially TI). Furthermore, protein, fat, and water are the three main elements that determine the overall texture, flavour, and palatability of meat. Protein plays an important role in the contraction of muscles and, consequently, impact the tenderness of the meat and its ability to retain moisture (Samuel, 2009). Also, with reference to the decrease in haemoglobin, which was attributed to poor protein and mineral utilization in Chapter 3, it was not surprising that breast meat lightness increased, and meat redness reduced during shelf-life assay. This is because blood pigments influence meat colour (Froning, 1995).

4.6 Conclusions

This study shows that up to 90 g MBM of MBM can replace soya oilcake in a diet for Ross 308 broiler chickens, without compromising meat pH, colour attributes including, redness and yellowness during 24 hr post-mortem. However, meat stability (pH and redness) may be adversely impacted by inclusion level of MBM. In addition, the inclusion of increasing levels of raw full-fat MB in the diets resulted in poor carcass weight and heavier visceral organs. Therefore, further studies with different methods of reducing the trypsin inhibitor activity in MB are needed to promote its use in broiler diets.

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5 CHAPTER FIVE: EFFECT OF PHYSICAL TREATMENTS ON TRYPSIN INHIBITOR ACIVITY, NUTRIENT COMPOSITION, AND AMINO ACIDS IN MARAMA BEAN (*TYLOSEMA ESCULENTUM*) AND SOYABEAN (*GLYCINE MAX*).

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5.1 Abstract

The use of marama bean (MB) as an alternative protein source to soyabean (SB) can be limited by high level of trypsin inhibitors activity (TIA), as indicated by poor growth performance and carcass traits of broilers in Chapters 3 and 4 of this thesis. It is thus imperative that ways of reducing the TIA in MB be identified. Physical treatment of MB has the potential to ameliorate the antinutritional activities of trypsin inhibitors and alter other chemical components. Therefore, this study investigated the effects of physical treatments including soaking, cooking, and autoclaving on chemical components and TIA in MB and SB. For this study, 17 bean substrates were produced as follows: R = Raw (not treated); S = ambient temperature (20 – 22°C) soaking in ultra-pure water for 24 hr; C = electric stove cooking at 100°C for 10, 20 and 30 minutes (C10; C20 & C30); A1 = steam autoclaving at a temperature of 110°C and a pressure of 7 pounds per square inch (psi) for 5, 15 and 30 minutes (A1/5; A1/15; & A1/30); A2 = steam autoclaving at temperature of 121°C and 7 psi for 5, 15, and 30 minutes (A2/5; A2/15; & A2/30); P1 = pre-soaked autoclaving at 110°C (7 psi) for 5, 15 and 30 minutes (P1/5; P1/15; & P1/30); P2 = pre-soaked autoclaving at 121°C (17 psi) for 5, 15 and 30 minutes (P2/5; P2/15; & P2/30). Each treatment method was independently applied to three replicate bean substrates. The raw and treated MB and SB substrates were analysed for proximate composition (moisture, ash, and protein), gross energy, neutral detergent fibre (NDF), and acid detergent fibre (ADF), amino acids, and TIA. The ash, crude protein, NDF and ADF contents

were greater in raw SB than MB. However, MB contained more crude fat and gross energy than SB. Treated MB and SB had higher (P <0.05) crude protein concentration than raw samples. All the treatments methods (except 24 hr soaking of MB) reduced (P <0.05) the TIA and ash content in MB and SB substrates. Marama and SB are significantly different (P <0.05) in protein content and also their amino acids profile and TIA differed. Soaking for 24 hr was less effective in inactivating TIA in MB and SB, compared to the thermal methods, and it was detrimental to the ash and amino acids composition of the two beans. Although, soaking before steam autoclaving resulted in beans with the lowest TI concentrations, steam autoclaving with or without prior 24 hr of soaking and cooking effectively reduced the TIA in both beans. In conclusion, thermal treatments reduced the TI contents and altered the level of ash, crude protein, and amino acids profile of the marama bean and soyabean.

Keywords: autoclaving, cooking, marama bean, soaking, soyabean, trypsin inhibitor activity

5.2 Introduction

Results from chapter 3 indicate that the soyabean replacement efficacy of marama bean (*Tylosema esculentum*) in diets of growing broilers as measured by nutrient digestibility and growth performance is rather poor. This is despite the native legume containing 34 - 37% crude protein on a dry matter (DM) basis (Amarteifio & Moholo, 1998; Amonsou *et al*., 2011), which is comparable to that of soyabean (33 - 48% DM) (Zaworska-Zakrzewska *et al*., 2020). Marama bean contains antinutrients such as TI, anti-elastase, tannins, and phytic acid (Nadaraja *et al*., 2009; Jackson *et al*., 2010), similar to other legume grains. However, the TI in MB is higher than that found in soyabean (Maruatona, 2008). Trypsin inhibitor is the prime anti-nutritional factor in raw soyabeans (Araba & Dale, 1990). It is a globulin type protein with a molecular weight of 24,000 dalton and isoelectric point of 4.5 (Kunitz, 1947). The mode of action is different for trypsin and chymotrypsin (Kunitz, 1947). The TI attaches with trypsinogen to

form an irreversible compound disrupting the formation of an active protease. On the contrary, TI action on chymotrypsin is less evident, forming a reversible dissociated compound (Dozier, 2011). Trypsin inhibitors in raw soyabeans have been implicated in stagnant growth, pancreatic hypertrophy, and hyperplasia in farm animals (Liener, 1981). Marama bean contains significantly higher levels of TI than in many other legumes (Bower *et al*., 1988). The TI is usually 5 - 10% of total protein in legumes (Ryan 1981; Bower *et al*., 1988). On the other hand, TI is approximately 20% of the total marama protein (Bower *et al*., 1988), which is 2 – 4 times higher than in many other legumes (Nadaraja *et al*., 2009). This can impede the utilization of MB as a dietary protein source in animal nutrition, especially monogastric as it was recorded in Chapters 3 and 4. Therefore, strategies to reduce the activity of TI in MB should be investigated to improve its utilization, especially in diets of simple non-ruminants.

There are many treatments that are used to ameliorate TIA in legumes seeds. The common ones are physical, chemical, and biological processes (Avil´es-Gaxiola *et al*., 2018). Soaking is an easy, inexpensive physical treatment that can reduce the amount of soluble antinutrients, which are removed with the soaking solution (Ibrahim *et al*., 2002). Alternatively, thermal processing is widely considered as the most effective physical means of improving the overall nutritive value of legume seeds because it increases protein digestibility mainly through the inactivation of heat sensitive antinutritional factors, especially TI (Avil´es-Gaxiola *et al*., 2018). The various heat methods, including microwaving, cooking, extrusion, roasting, and toasting, have been reported to be useful in reducing the amount and activity of thermolabile TI (Ibrahim *et al*., 2002; Habiba, 2002; Ari *et al*., 2017). Furthermore, growth performance has been reportedly improved in terms of higher body weight gain and lower feed conversion ratio, in broiler chickens that were fed thermally processed SB-containing diets, when compared to those fed a raw soyabean-containing diet (Tousi-Mojarrad *et al*., 2014). This could be attributed to an enhanced nutrient availability following TI reduction. Autoclaving and cooking are

regarded as the main thermal treatments that lower the concentration of TI in protein legumes (Habiba, 2002).

Despite the effectiveness of these thermal processes, it has been documented that high temperatures (greater than 80°C) can negatively alter some important nutrients components, such as lysine, methionine, tryptophan, and heat-labile vitamins (Egounlety & Aworth, 2003; Bara´c-Stanojevi´c, 2005; Machado *et al*., 2008; Chen *et al*., 2014; Yang *et al*., 2014; Avil´es-Gaxiola *et al*., 2018). Therefore, the objective of this study was to evaluate the effect of physical treatments, including soaking, cooking, and autoclaving, on the proximate composition, amino acids and TIA of MB and SB. The study investigated the hypothesis that physical treatments would decrease TIA and improve the nutritive value of MB and SB. The choice of methods of physical treatment, including soaking, cooking, and autoclaving was premised on low cost (Ibrahim *et al*., 2002), and ease of application (Avilés-Gaxiola *et al*., 2018). in domestic and/or rural setting.

5.3 Materials and methods

5.3.1 Procurement and preparation of the bean samples

The marama beans were harvested in 2019 from Malwelwe village described in Chapter 3 (Section 3.3.3). Whole MB were cracked into 2 - 5 pieces and kernels separated from the hulls. Both soyabean seeds and MB kernels were ground using coffee grinding machine (Hamilton Beach, 80393C, China) and stored in polythene sample bags under refrigeration (2°C) pending physical treatments. Raw soyabeans seeds were procured from University of Guelph, Feed Mill Unit (Guelph, Canada). The physical treatments comprising of soaking, cooking and steam autoclaving, and subsequent chemical analyses were carried out at Monogastric Nutrition Laboratory, Department of Animal Biosciences, University of Guelph, Canada. Each physical treatment method was independently applied to triplicate samples of MB and SB.

5.3.2 Physical processing

5.3.2.1 Soaking

Triplicate samples (20 g each) of cracked MB and whole SB samples were weighed into 250 ml conical flask and soaked in 200 ml ultra-pure water (Milli-Q IQ 7000, Millipore SAS, 67120 Molsheim, France) for 24 hr at ambient temperature ranging between 22 – 25°C. The samples were then gently rinsed and drained for about 2 hr, followed by transferring drained samples into aluminium foil bowls and then oven dried for 48 hr at 45 - 50°C in.

5.3.2.2 Cooking

For each cooking time of 10 and 20 minutes, MB, and SB samples (20 g per sample) were measured in triplicate and each sample poured into an aluminium pot containing 200 ml (1 g:10 ml; Ibrahim *et al*., 2002) of ultra-pure water on electric coil stove and heated to boiling point (100°C) after which cooking time countdown began. The same procedure was repeated for beans samples cooked for 30 minutes, except for the use of 500 ml of water to boil 20 g of the bean substrates. After cooking for required timing, samples were rinsed, drained, and oven-dried for 48 hr at 45 – 50°C.

5.3.2.3 Autoclaving

Triplicate samples (20 g each) of MB and SB were soaked in ultra-pure water for 24 hr at room temperature ranging between 22 - 25°C, after which soaking water was discarded. Thereafter, soaked samples were rinsed twice with fresh water during each rinsing. Both soaked and non-soaked samples were steam autoclaved for 5, 15, and 30 minutes at either 110°C (7 psi) or 121°C (17 psi) in conical flasks covered with aluminium foil. The ratio of seed to water was 1:7.5 (g/ml) for autoclaving. The heating time countdown started automatically when the

internal temperature of the autoclave (3870E Heidolph, Tuttnauer®, US) reached 110℃ or 121℃.

All soaked, cooked, and autoclaved samples were spread in aluminium bowls in one layer and oven dried at 45 - 50°C (Ibrahim *et al*., 2002), for 48 hr before being ground to pass through 70-mesh sieve, stored in plastic (ziplock) bags, and thereafter refrigerated at 2°C pending chemical analyses.

Table 5.1. Description of marama bean and soyabean samples.

Sample (SB/MB) [1]	Description
R	Raw
S	Soaked for 24 hr
C10	Cooking for 10 min
C20	Cooking for 20 min
C30	Cooking for 30 min
A1/5	Steam autoclaving at 110 °C and 7 psi for 5 min
A1/15	Steam autoclaving at 110 °C and 7 psi for 15 min
A1/30	Steam autoclaving at 110 °C and 7 psi for 30 min
A2/5	Steam autoclaving at 121 °C and 17 psi for 5 min
A2/15	Steam autoclaving at 121 °C and 17 psi for 15 min
A2/30	Steam autoclaving at 121 °C and 17 psi for 30 min
S1/5	Presoaked for 24 hr and steam autoclaving at 110 °C and 7 psi for 5 min
S1/15	Presoaked for 24 hr and steam autoclaving at 110 °C and 7 psi for 15 min
S1/30	Presoaked for 24 hr and steam autoclaving at 110 °C and 7 psi for 30 min
S2/5	Presoaked for 24 hr and steam autoclaving at 121 °C and 17 psi for 5 min
S2/15	Presoaked for 24 hr and steam autoclaving at 121 °C and 17 psi for 15 min
S2/30	Presoaked for 24 hr and steam autoclaving at 121 °C and 17 psi for 30 min

[1]Sample: SB = soyabean; MB = marama bean.

5.3.3 Proximate analyses

The raw and treated MB and SB substrates (Table 5.1) were analyzed for proximate composition, fibre, and gross energy as described in Chapter 3 (3.3.4). Neutral detergent fibre

(NDF) and acid detergent fibre (ADF) of raw samples were evaluated according to Van Soest *et al*. (1991), using ANKOM^{2000} Fibre Analyzer (ANKOM Technology, Fairport, NY, USA). All determinations were done in triplicates, and the outcomes were expressed as the mean.

5.3.4 Determination of amino acids

Samples of SB and MB were subjected to fat extraction using hexane in the ratio of 1:3 (ground bean: hexane). The mixture was stirred for 1 hr with a magnetic stirrer at low setting. The mixture was allowed to settle for 30 min, and then the hexane removed (Maruatona, 2008). This step was repeated thrice, with fresh hexane each time. The defatted samples were placed in a fume hood overnight to evaporate the n-hexane. This was followed by accurately weighing 60 mg and 50 mg each of treated MB and SB respectively, into test tubes and to the sample was added 2.5 ml of concentrated HCl for 24 hr at 110°C to initiate digestion by acid hydrolysis. The digested samples were then neutralized with 6 N NaOH and cooled to room temperature. Amino acids in the raw and treated samples were assessed using Norvaline (AccQ-Tag Ultra Waters Corporation, Milford, MA, USA), as the amino acid internal standard for ultra-performance liquid chromatography (UPLC; Waters Corporation, Milford, MA, USA).

5.3.5 Trypsin inhibitor activity assessment

The TIA of raw and treated samples of defatted MB and SB was quantified using spectrophotometric method by American Association of Cereal Chemists (AACC method 22–40), as modified by (Maruatona, 2008). This method requires N-benzoyl-DL-arginine p-nitroanilide (BAPA) as a substrate for porcine trypsin, and the ability of aliquots of SB and MB extracts to inhibit the activity of trypsin towards this substrate was utilized to measure the amount of TI in the samples.

To prepare the substrate solution, 40 mg of BAPA hydrochloride was dissolved in 1 ml of dimethyl sulfoxide and diluted to 100 ml with tris-buffer (0.05 M, pH 8.2; containing 0.02 M CaCl2: 6.05 g hydroxymethylamino methane and 2.94 g calcium chloride dissolved in 900 ml of ultra-pure water. The pH was adjusted to 8.2 using HCl, and the volume brought to 1 litre with water. The BAPA solution was made fresh daily and kept at 37°C while in use. The trypsin solution was prepared by weighing 4 mg of porcine trypsin and dissolved in 200 ml of 0.001 M HCl.

For the TIA assessment, 1 g of finely ground defatted sample of each SB and MB, sieved through 100-mesh screen, were extracted with 50 ml 0.01 N NaOH/g sample by stirring for 3 hr. Thereafter, the mixture was filtered using Whatman filter paper No. 3. The sample extracts were diluted using Tris buffer to a point where 1 ml yields trypsin inhibition of 40 – 60%. Trial dilutions were done to achieve this inhibition value. Portions (0, 0.6, 1.0, 1.4, and 1.8 ml) of diluted extract were pipetted into test tubes and made to 2.0 ml with water. This was then followed by addition of 2 ml of trypsin solution added to each test tube and placed in a water bath at 37°C, followed by adding 5 ml prewarmed (37°C) BAPA hydrochloride substrate. After 10 min, the reaction was terminated by adding 1 ml of 30% acetic acid solution and mixed using a vortex. The absorbance at 410 nm was determined with a spectrophotometer (PowerWaveTM XS, BIO-TEK® Instruments, Inc., Santa Clara, CA, USA) at room temperature. One trypsin unit is equal to a rise of 0.01 absorbance unit at 410 nm per 10 ml of reaction mixture, in terms of trypsin inhibitor units (TIU; Coscueta *et al*., 2017).

5.3.6 Statistical analysis

Proximate composition, fibre, energy, amino acids, and TIA data of MB and SB were subjected to a two-way analysis of variance (ANOVA), by means of the general linear model procedure of the Statistical Analysis System (SAS, 2010). The bean substrate (2 levels) and physical

treatment methods (17 levels) were the main factors. Means were separated using the Duncan's Multiple Range Test in SAS software. Statistical significance was declared at $P < 0.05$.

5.4 Results

5.4.1 Chemical composition of soyabean and marama bean

The raw bean samples of the two legumes are different in their contents of dry matter, ash, crude protein, crude fat, NDF, ADF and gross energy (Table 5.2). The dry matter, crude fat and gross energy content of MB was higher than that of SB, whereas the ash, crude protein, and NDF and ADF contents were lower in MB than in SB.

Table 5.2. Comparison of proximate composition (% *as is*, except for gross energy) of raw soyabean and marama bean samples.

	Dry matter	Ash	Crude protein	Crude fat	NDF	ADF	Gross energy (J/g)
SB	90.2 (0.05)	4.6 (0.55)	34.8 (0.21)	17.2 (1.13)	14.7 (5.03)	8.3 (0.31)	22,800.3 (3.62)
MB	94.0 (0.11)	2.9 (1.63)	32.7 (0.37)	38.7 (1.57)	7.5 (4.02)	3.4(0.36)	27,982.0 (3.12)

Values in parenthesis indicate coefficient of variation (CV); NDF = neutral detergent fiber; ADF = acid detergent fiber; SB = soyabean; MB = marama bean.

There were significant substrates, treatments, and substrates × treatments interaction effects on DM, ash, and CP components of SB and MB, due to the physical treatments (Table 5.3). The moisture content after oven drying were different across all the treatments and substrates. The values of DM of SB varied across all the treatments ($P < 0.05$), with soaking, cooking, and autoclaving resulting in higher values than the raw SB sample. The DM values in SB was 90.2 (in raw) and reduced to the lowest value of 85.2 % (S2/30). There was no significant difference in DM between cooking for 20 min (C20) and 30 min (C30), and between steam autoclaving

at 110°C; 7 psi (A1/30) and 121°C; 17 psi (A2/30) for 30 min. Also, steam autoclaving at 121°C, 17 psi for 15 min (A2/15), was not significantly different (P >0.05) from presoaked and autoclaved at 121°C, 17 psi for 5 min (S2/5). Further, presoaked and autoclaved at 110°C, 7 psi for 30 min (S1/30), was not significantly different from presoaked and autoclaved at 121°C, 17 psi for 30 min (S2/30). The lowest values for cooking, steam autoclaving at 110°C, 7 psi steam autoclaving at 121 °C, 17 psi presoaked and autoclaved at 110°C, 7 psi and presoaked and autoclaved at 121°C, 17 psi were 88.2, 88.1, 86.1, 85.4, and 85.2%, respectively. This indicates that, presoaked, and autoclaved at 121°C, 17 psi yielded the highest loss of 6% in DM content in SB.

Dry matter value was 94.0% in raw and 87.3% for presoaked and autoclaved at 121°C, 17 psi for 30 min (S2/30) MB. Like SB, all the physical treatments led to a DM value lower than the raw MB, and the highest loss of 7% was also caused by presoaked and autoclaved at 121°C, 17 psi for 30 min (S2/30). The dry matter content in soaked MB was lesser than it was at 10 and 20 min of cooking, but greater than the presoaked and autoclaved values. There was no significant difference between 20 min cooking (C20) and steam autoclaving of MB at 110°C, 7 psi for 5 min (A1/5), 30 min cooking (C30), steam autoclaving of MB at 110°C, 7 psi for 15 min, presoaked and autoclaved treatments; S2/5 and S2/15. Additionally, steam autoclaving of MB; A1/30, A2/15, A2/15, and A2/15 were not significantly different.

The ash content in each of SB sample was higher when compared with MB (P <0.05). Soaking, cooking, and autoclaving adversely affected the amount of ash recorded in SB and MB samples. Among the physical treatments, cooking resulted in a lesser reduction in ash content in both SB and MB, compared to the autoclaving methods. There was no significant difference caused by 20 min and 30 min cooking times in ash contents of SB and MB. Autoclaving (S2/5) greatly decreased the ash content of SB by 39% (5.1% in raw sample to the lowest value of 3.1% in S2/5). There was no difference (P >0.05) in ash content in cooked (C10, C20 and C30)

and steam- and presoaked-autoclaved (A1/5, A1/15, A1/30, A2/5, A2/15, A2/30, S1/5, S1/15, S1/30, S2/15, and S2/30) MB. Furthermore, the ash content value reduced from 3.1% in raw MB to the lowest of 1.9% (S1/5), resulted in highest 38% loss in ash content.

Like ash composition, SB contained higher CP values than MB across all the treatments (P <0.05). Crude protein values for SB ranged between 38.6% in raw sample to 47.5% (S2/30; sample soaked and autoclaved at 121°C, 17 psi for 30 min), while that of MB ranged from 34.8 (raw) to 36.50% (S2/30; sample soaked and autoclaved at 121°C, 17 psi for 30 min). The CP values were recorded to be higher in the treated samples of both beans than in the raw samples, with soaked and autoclaved (S2/30; sample soaked and autoclaved at 121°C, 17 psi for 30 min) having the highest CP value. The crude protein content of MB samples treated by soaking (S), cooking for 20 min, steam autoclaving at 110°C, 7 psi for 5 min (A1/5) and 30 min (A1/30), and S1/15, S2/5, S2/15, and S2/30 treatments were not different, and these substrates had the highest CP value

Table 5.3. Chemical composition (% DM, except for moisture) of soyabean and marama bean samples.

		Treatments [1]																
Parameters	**Sub[3]**	**R**	**S**	**C10**	**C20**	**C30**	**A1/5**	**A1/15**	**A1/30**	**A2/5**	**A2/15**	**A2/30**	**S1/5**	**S1/15**	**S1/30**	**S2/5**	**S2/15**	**S2/30**	**SEM [2]**
Moisture	SB	9.78 ^{aA}	5.50 ^{klA}	7.16 ^{bcdA}	7.66 ^{bA}	7.68 ^{bA}	6.43 $^{e–hA}$	7.35 ^{bcA}	6.95 ^{cdeA}	6.03 $^{h–kA}$	6.28 $^{f–iA}$	4.54 ^{mnoA}	6.02 $^{h–kA}$	6.80 $^{c–fA}$	6.62 $^{d–gA}$	6.22 $^{f–jA}$	6.17 $^{g–jA}$	5.12 ^{mlA}	2.04
	MB	5.97 $^{h–kB}$	4.60 ^{mnB}	5.65 ^{jklB}	5.90 $^{h–kB}$	5.78 ^{ijkB}	4.54 ^{noB}	4.52 ^{noB}	3.70 ^{pB}	4.50 ^{noB}	4.49 ^{noB}	3.87 ^{pB}	4.47 ^{noB}	4.56 ^{mnoB}	3.60 ^{pB}	4.51 ^{noB}	3.99 ^{opB}	2.68 ^{qB}	
DM [4]	SB	90.22 ^{cB}	87.50 ^{hB}	89.51 ^{dB}	88.33 ^{fgB}	88.24 ^{fgB}	88.46 ^{fB}	87.03 ^{ijkB}	86.05 ^{lmB}	87.26 ^{hijB}	86.78 ^{kB}	86.10 ^{lmB}	86.98 ^{jkB}	86.20 ^{lB}	85.38 ^{nB}	86.78 ^{kB}	85.84 ^{mB}	85.24 ^{nB}	0.16
	MB	94.03 ^{aA}	89.40 ^{deA}	91.30 ^{bA}	90.13 ^{cA}	89.08 ^{eA}	90.36 ^{cA}	90.23 ^{eA}	89.48 ^{dA}	89.50 ^{dA}	89.51 ^{dA}	89.12 ^{eA}	89.10 ^{eA}	88.30 ^{fgA}	88.07 ^{gA}	87.49 ^{hA}	87.51 ^{hA}	87.30 ^{hiA}	
Ash	SB	5.07 ^{aA}	4.44 ^{bcA}	4.66 ^{bA}	4.40 ^{cA}	4.26 ^{cA}	3.68 ^{dA}	3.49 ^{deA}	3.51 ^{deA}	3.69 ^{dA}	3.58 ^{deA}	3.50 ^{deA}	3.16 ^{gA}	3.41 ^{efA}	3.61 ^{deA}	3.07 ^{gA}	3.47 ^{deA}	3.21 ^{fgA}	0.85
	MB	3.06 ^{gB}	2.53 ^{hB}	2.40 ^{hB}	2.32 ^{hB}	2.48 ^{hB}	2.07 ^{jB}	1.92 ^{jB}	2.09 ^{jB}	1.95 ^{jB}	2.09 ^{jB}	1.96 ^{jB}	1.89 ^{jB}	1.93 ^{jB}	1.98 ^{jB}	2.09 ^{ijB}	2.05 ^{jB}	2.06 ^{jB}	
CP [5]	SB	38.57 ^{jA}	41.70 ^{fgA}	39.40 ^{ijA}	40.35 ^{hiA}	41.12 ^{hgA}	42.17 ^{fA}	45.33 ^{cdeA}	44.62 ^{eA}	42.22 ^{fA}	44.41 ^{eA}	44.39 ^{eA}	44.80 ^{eA}	47.25 ^{abA}	46.31 ^{bcA}	45.11 ^{deA}	46.05 ^{cdA}	47.46 ^{aA}	3.63
	MB	34.80 ^{nB}	36.87 ^{klmB}	35.78 ^{mnB}	36.46 ^{klmB}	35.95 ^{lmB}	36.42 ^{klmB}	36.84 ^{klB}	36.62 ^{klmB}	36.89 ^{klB}	35.91 ^{lmB}	37.17 ^{kB}	37.02 ^{kB}	36.60 ^{klmB}	36.88 ^{klB}	36.36 ^{klmB}	36.60 ^{klmB}	36.50 ^{klmB}	

[1] Treatments: R = raw soyabean/marama bean; S = soyabean/marama bean sample soaked for 24 hr; C10 = soyabean/marama bean cooked for 10 min; C20 = soyabean/marama bean cooked for 20 min; C30 = soyabean/marama bean cooked for 30 min; A1/5 = steam soyabean/marama bean sample autoclaved at 110 °C, 7 psi for 5 min; A1/15 = steam soyabean/marama bean sample autoclaved at 110 °C, 7 psi for 15 min; A1/30 = steam soyabean/marama bean sample autoclaved at 110 °C, 7 psi for 30 min; A2/5 = steam soyabean/marama bean sample autoclaved at 121 °C, 17 psi for 5 min; A2/15 = steam soyabean/marama bean sample autoclaved at 121 °C, 17 psi for 15 min; A2/30 = steam soyabean/marama bean sample autoclaved at 121 °C, 17 psi for 30 min; S1/5 = soyabean/marama bean sample soaked and autoclaved at 110 °C, 7 psi for 5 min; S1/15 = soyabean/marama bean sample soaked and autoclaved at 110 °C, 7 psi for 15 min; S1/30 = soyabean/marama bean sample soaked and autoclaved at 110 °C, 7 psi for 30 min; S2/5 = soyabean/marama bean sample soaked and autoclaved at 121 °C, 17 psi for 5 min; S2/15 = soyabean/marama bean sample soaked and autoclaved at 121 °C, 17 psi for 15 min; S2/30 = soyabean/marama bean sample soaked and autoclaved at 121 °C, 17 psi for 30 min. [2] SEM = standard error of mean. [3] Sub = substrate (SB, soyabean; MB, marama bean). [4] DM = dry matter. [5] CP = crude protein. $^{a–q}$ Means within each row with no common superscripts are significantly different (P <0.05). ^{A,B} Means within each column with no common superscripts are significantly different (P <0.05).

5.4.2 Non-essential amino acids profile of soyabean and marama bean

The concentrations of non-essential amino acids in raw and treated MB and SB samples are presented in Table 5.4. There were significant (P <0.05) substrate type, treatments, and interaction effects for all non-essential AAs. For raw samples, SB had greater values (P <0.05) of glutamic acid, and alanine than MB, while arginine, aspartic acid, glycine, tyrosine, and proline were higher (P <0.05) in MB than SB. However, the isoleucine concentration was similar (P >0.05) for raw SB and MB. In comparison to raw substrates, soaking for 24 hr significantly decreased all the AAs (except for aspartic acid, glutamic acid, and serine in SB) concentrations in both SB and MB. In addition, soaking SB for 24 hr yielded the lowest values for alanine (1.4%), arginine (2.4%), glycine (1.5%), and tyrosine (1.2%). Soaking resulted in reduction in contents of arginine (6.0%), tyrosine (6.0%), and alanine (5.0%). Physical treatments resulted in aspartic acid, glutamic acid, serine, and proline values that were equal to or greater than those in raw SB. Soaking MB for 24 hr (S) resulted in the lowest values for alanine (1.2%), aspartic acid (3.8%), and serine (2.0%). Presoaked steam-autoclaving at 121°C and 17 psi for 30 min (S2/30) gave lowest values for arginine (2.4%), glutamic acid (4.0%), glycine (2.4%), and tyrosine (3.6%) in MB.

On the other hand, some physical treatments caused an increase in AAs values of MB. Steam-autoclaving at 110°C and 7 psi for 30 min (A1/30) gave the highest values for alanine (1.6%), glutamic acid (5.2%), proline (3.9%), serine (2.7%), threonine (1.5%), and valine (2.3%). The highest values for arginine (2.9%) and aspartic acid (4.6%) were found in MB samples cooked for 10 min (C10). Steam autoclaving at 121°C (A2/30) had the highest values for glycine (2.8%). Tyrosine value was decreased in the MB samples by all the physical treatments, with the highest value of 5.1% being recorded in the raw sample.

For SB, certain physical treatments resulted in a rise in AAs values. For instance, presoaked steam-autoclaving at 121°C and 17 psi for 15 min (S2/15) yielded highest values for glycine (2.5%), serine (2.7%), isoleucine (2.0%), and threonine (1.9%). Also, presoaked steam-autoclaving at 110°C and 7 psi for 15 min (S1/15) and presoaked steam-autoclaving at 121°C and 17 psi for 15 min (S2/15) gave the highest values for isoleucine and threonine in SB. Presoaked steam-autoclaving of SB at 110°C and 7 psi for 15 min (S1/15) yielded the highest values for aspartic acid (5.4%), glutamic acid (7.1%), and proline (2.6%). The highest concentrations for alanine (2.1%), arginine (3.6%), and tyrosine (2.1%), were recorded in the SB samples treated with steam-autoclaving at 110°C and 7 psi for 15 min (A1/15).

Table 5.4. Non-essential amino acids profile (%, dry matter basis) of raw and treated marama and soyabean samples.

Parameter	Sp [3]	R	S	C10	C20	C30	A1/5	A1/15	A1/30	A2/5	A2/15	A2/30	S1/5	S1/15	S1/30	S2/5	S2/15	S2/30	SEM [2]
Alanine	SB	1.49 $^{f–kA}$	1.43 $^{h–lA}$	1.69 ^{deA}	1.55 $^{e–jA}$	1.82 ^{bcdA}	1.59 $^{e–hA}$	2.05 ^{aA}	1.93 ^{abcA}	1.71 ^{deA}	1.93 ^{abcA}	1.66 ^{defA}	1.81 ^{cdA}	2.01 ^{abA}	1.64 $^{d–gA}$	1.91 ^{abcA}	1.95 ^{abcA}	1.81 ^{cdA}	0.07
	MB	1.31 $^{k–nB}$	1.20 ^{nB}	1.45 $^{g–lB}$	1.24 ^{mnB}	1.32 $^{k–nB}$	1.31 $^{k–nB}$	1.28 ^{lmnB}	1.56 $^{e–iB}$	1.35 $^{k–nB}$	1.27 ^{lmnB}	1.45 $^{g–lB}$	1.23 ^{nB}	1.25 ^{mnB}	1.39 $^{i–nB}$	1.28 ^{lmnB}	1.38 $^{i–nB}$	1.36 $^{j–nB}$	
Arginine	SB	2.48 ^{klmB}	2.34 ^{mB}	2.94 $^{d–gA}$	2.85 $^{e–kA}$	3.03 $^{c–fA}$	3.19 $^{a–eA}$	3.55 ^{aA}	3.19 $^{a–eA}$	2.86 $^{e–jA}$	3.16 $^{b–eA}$	3.31 ^{abcA}	3.14 $^{b–eA}$	3.35 ^{abcA}	2.66 $^{g–mA}$	3.48 ^{abA}	3.31 ^{abcA}	2.92 $^{e–hA}$	0.13
	MB	2.71 $^{f–mA}$	2.51 $^{j–mA}$	2.88 $^{e–iB}$	2.48 ^{klmB}	2.56 $^{h–mB}$	2.51 $^{j–mB}$	2.52 $^{i–mB}$	3.14 $^{b–eB}$	2.72 $^{f–lB}$	2.49 ^{klmB}	2.93 $^{e–hB}$	2.50 $^{j–mB}$	2.60 $^{g–mB}$	2.62 $^{g–mA}$	2.42 ^{lmB}	2.65 $^{g–mB}$	2.35 ^{mB}	
Aspartic acid	SB	4.00 $^{i–lB}$	4.11 $^{h–lA}$	4.45 $^{e–kB}$	4.25 $^{f–lA}$	4.79 $^{b–fA}$	4.78 $^{b–fA}$	5.08 ^{abcA}	5.11 ^{abA}	4.51 $^{c–jA}$	5.01 $^{a–dA}$	3.90 ^{klB}	4.76 $^{b–gA}$	5.41 ^{aA}	4.41 $^{e–kA}$	4.96 $^{a–eA}$	5.04 ^{abcA}	4.64 $^{b–hA}$	0.21
	MB	4.08 $^{h–lA}$	3.78 ^{lB}	4.63 $^{b–hA}$	3.81 ^{lB}	4.12 $^{h–lB}$	4.04 $^{i–lB}$	3.99 $^{i–lB}$	4.95 $^{a–eB}$	4.22 $^{f–lB}$	3.94 ^{jklB}	4.54 $^{b–iA}$	3.82 ^{lB}	3.88 ^{klB}	4.20 $^{g–lB}$	3.89 ^{klB}	4.17 $^{h–lB}$	4.19 $^{g–lB}$	
Glutamic acid	SB	5.24 $^{f–iA}$	5.24 $^{f–iA}$	6.00 $^{b–fA}$	5.75 $^{e–hA}$	6.30 $^{b–eA}$	5.30 $^{f–iA}$	6.72 ^{abA}	6.63 ^{abcA}	5.87 $^{c–gA}$	6.50 $^{a–eA}$	5.31 $^{f–iA}$	6.28 $^{b–eA}$	7.11 ^{aA}	5.76 $^{e–hA}$	6.56 $^{a–dA}$	6.47 $^{a–eA}$	5.83 $^{d–gA}$	0.28
	MB	4.98 ^{hijB}	4.57 ^{ijkB}	5.25 $^{f–iB}$	4.30 ^{jkB}	4.55 ^{ijkB}	4.18 ^{kB}	4.20 ^{kB}	5.20 ^{ghiB}	4.41 ^{jkB}	3.93 ^{kB}	5.04 ^{hijB}	3.94 ^{kB}	4.10 ^{kB}	4.19 ^{kB}	3.95 ^{kB}	4.10 ^{kB}	4.00 ^{kB}	
Glycine	SB	1.55 ^{opB}	1.45 ^{pB}	1.89 $^{k–nB}$	1.81 ^{mnB}	2.00 $^{j–lB}$	2.08 ^{jkB}	2.35 ^{ghiB}	2.06 ^{jkB}	1.83 ^{lmnB}	2.03 $^{j–lB}$	2.43 ^{fghB}	2.05 $^{j–klB}$	2.13 ^{ijB}	1.73 ^{noB}	2.19 ^{hijB}	2.45 ^{efgB}	1.88 $^{k–nB}$	0.08
	MB	2.70 ^{bcdA}	2.44 ^{efgA}	2.73 ^{bcA}	2.51 $^{c–gA}$	2.46 ^{efgA}	2.44 ^{efgA}	2.46 ^{efgA}	3.04 ^{aA}	2.67 $^{b–eA}$	2.47 $^{d–gA}$	2.81 ^{abA}	2.44 ^{efgA}	2.54 $^{c–gA}$	2.63 $^{b–fA}$	2.47 $^{d–gA}$	2.56 $^{c–gA}$	2.35 ^{ghiA}	
Proline	SB	1.90 ^{jB}	1.81 ^{jB}	2.18 ^{hiB}	1.97 ^{ijB}	2.40 ^{fghB}	2.58 ^{fB}	1.98 ^{ijB}	2.52 ^{fgB}	2.18 ^{hiB}	2.48 ^{fgB}	2.48 ^{fgB}	2.48 ^{fgB}	2.63 ^{fB}	2.18 ^{hiB}	2.52 ^{fgB}	3.14 ^{eB}	2.29 ^{ghB}	0.09
	MB	3.46 ^{bcdA}	3.16 ^{eA}	3.52 ^{bcA}	3.24 ^{deA}	3.17 ^{eA}	3.11 ^{eA}	3.26 ^{cdeA}	3.93 ^{aA}	3.46 ^{bcdA}	3.22 ^{deA}	3.53 ^{bA}	3.18 ^{eA}	3.23 ^{deA}	3.31 $^{b–eA}$	3.34 $^{b–eA}$	3.26 ^{cdeA}	3.20 ^{eA}	
Serine	SB	1.88 ^{m}	1.87 ^{m}	2.16 $^{i–l}$	2.04 ^{lm}	2.25 $^{e–k}$	2.37 $^{c-i}$	2.5 $^{a–d}$	2.44 $^{c–g}$	2.25 $^{f-l}$	2.40 $^{c-h}$	2.38 $^{c–i}$	2.32 $^{c–j}$	2.51 $^{a–d}$	2.10 $^{j–m}$	2.69 ^{ab}	2.74 ^{a}	2.23 $^{g–l}$	0.08
	MB	2.26 $^{e–l}$	2.04 ^{lm}	2.54 ^{abc}	2.06 ^{klm}	2.22 $^{g–l}$	2.18 $^{h–l}$	2.13 ^{jkl}	2.69 ^{b}	2.30 $^{d–j}$	2.22 $^{g–l}$	2.48 $^{b–f}$	2.09 $^{j–m}$	2.25 $^{f–l}$	2.49 $^{b–e}$	2.17 $^{h–l}$	2.39 $^{c–i}$	2.23 $^{f–l}$	
Tyrosine	SB	1.29 ^{lmB}	1.21 ^{mB}	1.57 $^{j–mB}$	1.45 ^{klmB}	1.65 $^{i–lB}$	1.69 $^{i–lB}$	2.06 ^{hiB}	1.74 ^{ijkB}	1.56 $^{j–mB}$	1.73 ^{ijkB}	2.44 ^{hB}	1.74 ^{ijkB}	1.78 ^{ijkB}	1.48 ^{klmB}	1.99 ^{iB}	1.95 ^{ijB}	1.66 $^{i–lB}$	0.15
	MB	5.10 ^{aA}	4.52 ^{bcA}	4.70 ^{abA}	4.25 ^{cdA}	4.07 ^{defA}	3.61 ^{gA}	3.80 ^{efgA}	4.73 ^{abA}	4.12 ^{cdeA}	3.65 ^{gA}	4.79 ^{abA}	3.64 ^{gA}	3.91 $^{d–gA}$	3.69 ^{fgA}	3.65 ^{gA}	3.59 ^{gA}	3.53 ^{gA}	

[1]Treatments: R = raw soyabean/marama bean; S = soyabean/marama bean sample soaked for 24 hr; C10 = soyabean/marama bean cooked for 10 min; C20 = soyabean/marama bean cooked for 20 min; C30 = soyabean/marama bean cooked for 30 min; A1/5 = steam soyabean/marama bean sample autoclaved at 110°C, 7 psi for 5 min; A1/15 = steam soyabean/marama bean sample autoclaved at 110°C, 7 psi for 15 min; A1/30 = steam soyabean/marama bean sample autoclaved at 110°C, 7 psi for 30 min; A2/5 = steam soyabean/marama bean sample autoclaved at 121°C, 17 psi for 5 min; A2/15 = steam soyabean/marama bean sample autoclaved at 121°C, 17 psi for 15 min; A2/30 = steam soyabean/marama bean sample autoclaved at 121°C, 17 psi for 30 min; S1/5 = soyabean/marama bean sample soaked and autoclaved at 110°C, 7 psi for 5 min; S1/15 = soyabean/marama bean sample soaked and autoclaved at 110°C, 7 psi for 15 min; S1/30 = soyabean/marama bean sample soaked and autoclaved at 110°C, 7 psi for 30 min; S2/5 = soyabean/marama bean sample soaked and autoclaved at 121°C, 17 psi for 5 min; S2/15 = soyabean/marama bean sample soaked and autoclaved at 121 °C, 17 psi for 15 min; S2/30 = soyabean/marama bean sample soaked and autoclaved at 121°C, 17 psi for 30 min. [2] SEM = standard error of mean. [3] Sp = substrates (SB, soyabean; MB, marama bean). $^{a–p}$ Means within each row with no common superscripts are significantly different (P <0.05). ^{A,B} Means within each column with no common superscripts are significantly different (P <0.05).

5.4.3 Essential amino acids profile of soyabean and marama bean

The contents of essential amino acids in raw and treated MB and SB samples is presented in Tables 5.5. There were significant (P <0.05) substrate type, treatments, and interaction effects for all AAs, except for serine, which had no significant substrate effect. For raw samples, SB had greater values (P <0.05) of lysine, threonine, and leucine than MB, while valine, phenylalanine, and histidine were higher (P <0.05) in MB than SB. However, the isoleucine concentration was similar (P >0.05) for raw SB and MB. In comparison to raw substrates, soaking for 24 hr significantly decreased all the essential AAs (except for isoleucine and valine in MB) concentrations in both SB and MB. In addition, soaking SB for 24 hr yielded the lowest values for histidine (0.9%), isoleucine (1.3%), leucine (2.5%), lysine (2.14%), phenylalanine (1.7%), threonine (1.3%), and valine (1.5%). The loss in AAs concentrations in SB ranged from 2 to 15%, and valine was most adversely affected by 24 hr soaking (1.75 vs. 1.49%). Soaking resulted in reduction in contents of isoleucine (13.0%), histidine (7.0%), leucine (6.0%), and phenylalanine (6.0%). Also, soaking MB for 24 hr (S) resulted in the lowest values for leucine (2.3%), lysine (1.9%), and threonine (1.1%). Presoaked steam-autoclaving at 121 °C and 17 psi for 30 min (S2/30) gave lowest value for isoleucine (0.98%) in MB. The detrimental effect of thermal treatment on AAs was higher in MB than SB. The loss of AAs concentrations of MB was as high as 36.0% (1.54% in raw reduced to 0.98%), as found with isoleucine in MB treated with presoaked steam autoclaving (S2/30), with the lowest being a 5.0% shortage with lysine and leucine. Other essential amino acids in MB, including phenylalanine, histidine, and threonine, were reduced up to 11.0% (due to steam autoclaving at 110°C and 7 psi for 5 min; A1/5), 8.0% (caused by presoaked and autoclaved at 121°C and 17 psi for 5 and 30 min, and also by steam autoclaving 110°C and 7 psi for 15 min), and 7.0% (due to soaking, cooking for 20 min and presoaked and autoclaved at 110°C and 7 psi for 5 min), respectively.

On the other hand, some physical treatments caused an increase in AAs values of MB. Steam-autoclaving at 110℃ and 7 psi for 30 min (A1/30) gave the highest values for histidine (1.3%), leucine (2.9%), lysine (2.6%), phenylalanine (2.3%), threonine (1.5%), and valine (2.3%). Steam autoclaving at 121°C (A2/30) had the highest value for isoleucine (1.9%).

For SB, certain physical treatments resulted in a rise in AAs values. For instance, presoaked steam-autoclaving at 121℃ and 17 psi for 15 min (S2/15) yielded highest values leucine (3.8%), phenylalanine (2.8%), isoleucine (2.0%), and threonine (1.9%). Also, presoaked steam-autoclaving at 110°C and 7 psi for 15 min (S1/15) and presoaked steam-autoclaving at 121℃ and 17 psi for 15 min (S2/15) gave the highest values for isoleucine and threonine in SB. Presoaked steam-autoclaving of SB at 110°C and 7 psi for 15 min (S1/15) yielded the highest value for lysine (2.9%). The highest concentration for valine (2.4%) was recorded in the SB samples treated with steam-autoclaving at 110°C and 7 psi for 15 min (A1/15). Histidine value was maximized (1.4%) by steam-autoclaving at 121℃ and 17 psi for 30 min.

Table 5.5. Essential amino acids profile (%, dry matter basis) of raw and treated marama and soyabean samples.

Parameter	Sp [3]	R	S	C10	C20	C30	A1/5	A1/15	A1/30	A2/5	A2/15	A2/30	S1/5	S1/15	S1/30	S2/5	S2/15	S2/30	SEM [2]
Histidine	SB	0.94 ^{lmB}	0.87 ^{mB}	1.10 $^{e–lB}$	1.05 $^{g–lA}$	1.15 $^{d–kA}$	1.32 $^{a–dA}$	1.26 $^{a–eA}$	1.18 $^{c–hA}$	1.05 $^{g–lA}$	1.16 $^{d–jA}$	1.37 ^{abA}	1.20 $^{b–gA}$	1.24 $^{a–fA}$	1.01 $^{h–mB}$	1.39 ^{aA}	1.35 ^{abcA}	1.08 $^{f–lA}$	0.06
	MB	1.07 $^{f–lA}$	1.00 $^{i–mA}$	1.12 $^{e–kA}$	1.01 $^{h–mB}$	1.01 $^{h–mB}$	1.00 $^{j–mB}$	0.98 ^{klmB}	1.26 $^{a–eB}$	1.10 $^{e–lB}$	1.00 $^{j–mB}$	1.18 $^{c–hB}$	1.00 $^{i–mB}$	1.04 $^{g–mB}$	1.06 $^{g–lA}$	0.98 ^{klmB}	1.07 $^{f–lB}$	0.98 ^{klmB}	
Isoleucine	SB	1.54 ^{ijkA}	1.34 ^{kB}	1.72 $^{e–iA}$	1.46 ^{jkB}	1.87 $^{b–eA}$	1.83 $^{b–fA}$	2.15 ^{aA}	1.89 $^{b–eB}$	1.60 $^{g–iB}$	1.96 $^{a–dA}$	1.77 $^{d–hB}$	1.84 $^{b–fA}$	2.03 ^{abA}	1.64 $^{e–iB}$	1.95 $^{a–dA}$	1.96 $^{a–dA}$	1.86 $^{b–eA}$	0.08
	MB	1.54 ^{ijkA}	1.55 ^{ijkA}	1.70 $^{e–iA}$	1.59 $^{g–iA}$	1.64 $^{f–iB}$	1.70 $^{e–iB}$	1.61 $^{g–iB}$	2.00 ^{abcA}	1.76 $^{d–hA}$	1.52 ^{ijkB}	1.87 $^{b–eA}$	1.60 $^{g–iB}$	1.58 ^{hijB}	1.71 $^{e–iA}$	1.58 ^{hijB}	1.80 $^{c–gB}$	0.98 ^{lB}	
Leucine	SB	2.71 ^{jklA}	2.54 $^{l–oA}$	3.05 ^{ghiA}	2.82 ^{ijkA}	3.27 ^{efgA}	3.31 ^{defA}	3.76 ^{abA}	3.50 ^{cdA}	3.11 ^{fghA}	3.53 ^{bcdA}	3.41 ^{deA}	3.41 ^{deA}	3.68 ^{abcA}	3.00 ^{hiA}	3.65 ^{abcA}	3.79 ^{aA}	3.37 ^{deA}	0.08
	MB	2.41 $^{m–pB}$	2.27 ^{pB}	2.63 ^{klmB}	2.31 ^{opB}	2.40 $^{m–pB}$	2.37 ^{nopB}	2.38 ^{nopB}	2.93 ^{hijB}	2.56 ^{lmnB}	2.38 ^{nopB}	2.71 ^{jklB}	2.33 ^{nopB}	2.39 ^{nopB}	2.48 $^{l–pB}$	2.39 $^{m–pB}$	2.55 ^{lmnB}	2.56 ^{lmnB}	
Lysine	SB	2.18 $^{g–oA}$	2.14 $^{i–oA}$	2.44 $^{c–gA}$	2.22 $^{f–nA}$	2.60 ^{bcdA}	2.56 $^{b–eA}$	2.60 ^{bcdA}	2.66 ^{abcA}	2.33 $^{e–jA}$	2.67 ^{abcA}	2.23 $^{f–nB}$	2.55 $^{b–eA}$	2.88 ^{aA}	2.32 $^{e–jA}$	2.48 $^{b–fA}$	2.72 ^{abA}	2.39 $^{d–i}$	0.09
	MB	2.04 $^{l–oB}$	1.94 ^{oB}	2.42 $^{c–gA}$	1.94 ^{oB}	2.19 $^{g–oB}$	2.20 $^{g–oB}$	2.11 $^{j–oB}$	2.57 $^{b–eB}$	2.17 $^{h–oB}$	1.99 ^{noB}	2.36 $^{d–jA}$	2.00 ^{mnoB}	2.04 $^{l–oB}$	2.21 $^{g–nB}$	2.09 $^{k–oB}$	2.26 $^{f–lB}$	2.25 $^{f–m}$	
Phenylalanine	SB	1.77 ^{jkB}	1.67 ^{kB}	2.15 $^{d–jA}$	2.63 ^{abcA}	2.27 $^{c–iA}$	2.59 ^{abcA}	2.82 ^{aA}	2.38 $^{b–eA}$	2.15 $^{d–jA}$	2.41 $^{a–eA}$	2.65 ^{abcA}	2.37 $^{b–fA}$	2.48 $^{a–dA}$	2.03 $^{e–kA}$	2.75 ^{abA}	2.81 ^{aA}	2.28 $^{c–hA}$	0.14
	MB	2.02 $^{e–kA}$	1.86 ^{ijkA}	2.02 $^{e–kB}$	1.89 $^{h–kB}$	1.86 ^{ijkB}	1.81 ^{jkB}	1.85 ^{jkB}	2.31 $^{c–gB}$	2.05 $^{e–kB}$	1.95 $^{g–kB}$	2.14 $^{d–jB}$	1.86 ^{ijkB}	1.93 $^{g–kB}$	1.92 $^{g–kB}$	1.86 ^{ijkB}	1.97 $^{f–kB}$	1.93 $^{g–kB}$	
Threonine	SB	1.32 $^{h–kA}$	1.30 $^{i–lA}$	1.58 ^{defA}	1.51 ^{efgA}	1.64 $^{b–fA}$	1.57 ^{defA}	1.79 ^{abcA}	1.72 $^{a–dA}$	1.58 ^{defA}	1.73 $^{a–dA}$	1.66 $^{b–eA}$	1.62 $^{c–fA}$	1.80 ^{abA}	1.48 ^{fghA}	1.87 ^{aA}	1.87 ^{a}	1.59 ^{defA}	0.06
	MB	1.22 $^{i–lB}$	1.14 ^{lB}	1.38 ^{ghiB}	1.14 ^{lB}	1.23 $^{i–lB}$	1.20 ^{klB}	1.19 ^{klB}	1.49 ^{fgB}	1.28 $^{i–lB}$	1.21 ^{jklB}	1.37 $^{g–jB}$	1.15 ^{lB}	1.21 ^{jklB}	1.26 $^{i–lB}$	1.17 ^{klB}	1.29 $^{i–lB}$	1.25 $^{i–lB}$	
Valine	SB	1.75 ^{jkA}	1.49 ^{lB}	1.91 $^{e–jB}$	1.65 ^{klB}	2.07 $^{b–fA}$	2.02 $^{c–iA}$	2.37 ^{aA}	2.07 $^{b–fB}$	1.76 ^{jkB}	2.17 $^{a–d}$	1.95 $^{d–jB}$	2.01 $^{c–iA}$	2.24 ^{abcA}	1.85 $^{f–jB}$	1.76 ^{jkB}	2.23 ^{abcA}	2.05 $^{c–hA}$	0.1
	MB	1.77 ^{jkA}	1.78 ^{jkA}	1.95 $^{d–jA}$	1.83 $^{g–kA}$	1.88 $^{f–iB}$	1.95 $^{d–jB}$	1.84 $^{g–kB}$	2.28 ^{abA}	2.01 $^{c–iA}$	1.74 ^{kB}	2.13 $^{b–eA}$	1.82 $^{h–kB}$	1.80 ^{ijkB}	1.97 $^{d–jA}$	1.80 ^{ijkA}	2.05 $^{b–gB}$	2.04 $^{c–hA}$	

[1] Treatments: R = raw soyabean/marama bean; S = soyabean/marama bean sample soaked for 24 hr; C10 = soyabean/marama bean cooked for 10 min; C20 = soyabean/marama bean cooked for 20 min; C30 = soyabean/marama bean cooked for 30 min; A1/5 = steam soyabean/marama bean sample autoclaved at 110°C, 7 psi for 5 min; A1/15 = steam soyabean/marama bean sample autoclaved at 110°C, 7 psi for 15 min; A1/30 = steam soyabean/marama bean sample autoclaved at 110°C, 7 psi for 30 min; A2/5 = steam soyabean/marama bean sample autoclaved at 121°C, 17 psi for 5 min; A2/15 = steam soyabean/marama bean sample autoclaved at 121°C, 17 psi for 15 min; A2/30 = steam soyabean/marama bean sample autoclaved at 121°C, 17 psi for 30 min; S1/5 = soyabean/marama bean sample soaked and autoclaved at 110°C, 7 psi for 5 min; S1/15 = soyabean/marama bean sample soaked and autoclaved at 110°C, 7 psi for 15 min; S1/30 = soyabean/marama bean sample soaked and autoclaved at 110°C, 7 psi for 30 min; S2/5 = soyabean/marama bean sample soaked and autoclaved at 121°C, 17 psi for 5 min; S2/15 = soyabean/marama bean sample soaked and autoclaved at 121 °C, 17 psi for 15 min; S2/30 = soyabean/marama bean sample soaked and autoclaved at 121°C, 17 psi for 30 min. [2] SEM = standard error of mean. [3] Sp = substrates (SB, soyabean; MB, marama bean). $^{a–p}$ Means within each row with no common superscripts are significantly different (P <0.05). ^{A,B} Means within each column with no common superscripts are significantly different (P <0.05).

5.4.4 Trypsin inhibitor activity

The result of assessment of TIA in raw and treated MB and SB samples is presented in Table 5.6. There were significant substrate, treatment, and substrate × treatment interaction effects on TIA. Cooking (C30), steam-autoclaving (A1/30, A2/5, A2/15, and A2/30), and pre-soaked autoclaving (S1/30, S2/5, S2/15, S2/30) gave a similar (P >0.05) trypsin inhibitor unit (TIU). Treated MB had (P <0.05) higher level of TIA, compared to treated SB. The highest reduction in TIA in SB (66.98 TIU/mg to 1.51 TIU/mg) was recorded with the substrate that was subjected to steam autoclaving at 121 C, 17 psi for 30 min (A2/30). In MB, the TIA was reduced from 245.25 TIU/mg (raw) to 2.66 TIU/mg (S2/30).

Table 5.6. Trypsin inhibitor activity ([1]TIU /mg) of soyabean and marama bean samples.

[2] Treatments	Substrates	
	SB	**MB**
R	66.98 ^{Cb}	245.25 ^{bA}
S	54.04 ^{dB}	271.98 ^{aA}
C10	11.52 ^{gB}	47.08 ^{eA}
C20	5.21 ^{hijB}	18.22 ^{fA}
C30	2.49 ^{jB}	9.12 ^{ghA}
A1/5	3.69 ^{hijB}	8.85 ^{ghiA}
AI/15	3.09 ^{hijB}	7.32 $^{g–jA}$
A1/30	2.49 ^{jB}	5.40 $^{g–jA}$
A2/5	1.89 ^{JB}	6.56 $^{g–iA}$
A2/15	1.89 ^{jB}	4.23 ^{hijA}
A2/30	1.51 ^{jB}	3.00 ^{hijA}
S1/5	3.38 ^{hijB}	7.73 $^{g–jA}$
S1/15	2.69 ^{ijB}	5.76 $^{g–jA}$
S1/30	2.19 ^{jB}	5.05 ^{hijA}
S2/5	1.91 ^{jB}	6.26 $^{g–jA}$
S2/15	2.12 ^{jB}	4.22 ^{hijA}
S2/30	1.60 ^{jB}	2.66 ^{ijA}
	[3]SEM = 2.22	

[1]TIU = trypsin inhibitor unit, [2] Treatments: R = raw soyabean/marama bean; S = soyabean/marama bean sample soaked for 24 hr; C10 = soyabean/marama bean cooked for 10 min; C20 = soyabean/marama bean cooked for 20 min; C30 = soyabean/marama bean cooked for 30 min; A1/5 = steam soyabean/marama bean sample autoclaved at 110 °C, 7 psi for 5 min; A1/15 = steam soyabean/marama bean sample autoclaved at 110 °C, 7 psi for 15 min; A1/30 = steam soyabean/marama bean sample autoclaved at 110 °C, 7 psi for 30 min; A2/5 = steam soyabean/marama bean sample autoclaved at 121 °C, 17 psi for 5 min; A2/15 = steam soyabean/marama bean sample autoclaved at 121 °C, 17 psi for 15 min; A2/30 = steam soyabean/marama bean sample autoclaved at 121 °C, 17 psi for 30 min; S1/5 = soyabean/marama bean sample soaked and autoclaved at 110 °C, 7 psi for 5 min; S1/15 = soyabean/marama bean sample soaked and autoclaved at 110 °C, 7 psi for 15 min; S1/30 = soyabean/marama bean sample soaked and autoclaved at 110 °C, 7 psi for 30 min; S2/5 = soyabean/marama bean sample soaked and autoclaved at 121 °C, 17 psi for 5 min; S2/15 = soyabean/marama bean sample soaked and autoclaved at 121 °C, 17 psi for 15 min; S2/30 = soyabean/marama bean sample soaked and autoclaved at 121 °C, 17 psi for 30 min. [3] SEM = standard error of mean. $^{a–j}$Means within each row with no common superscripts are significantly different (P <0.05). ^{A,B}Means within each column with no common superscripts are significantly different (P <0.05).

5.5 Discussion

The proximate composition of MB and SB reported in the present study aligns with what has already been reported in the literature (Mosele *et al*., 2011; Siulapwa & Mwambungu, 2014). The decline in DM observed in soaked and thermal-treated samples, compared to the raw samples, may be related to sample loss in soaking, cooking, or autoclaving discarded water (Sharma *et al*., 2014). The ash value for SB recorded in this present study was greater than that in MB, in agreement with previous findings (Amonsou *et al*., 2011). Similarly, the ash content of 2.88% (as is) recorded for MB was comparable to the 3% (as is) result in existing literature (Mosele *et al*., 2011). However, the ash content was reduced in both SB and MB by the application of various physical treatments. This loss was more pronounced with samples that were soaked before being autoclaved or steam autoclaved. This may point to leaching of nutrients in water during the physical treatments.

The crude fat in MB was approximately triple that in SB, and the value compares well with the 40% crude fat content of MB that was reported previously (Mosele *et al*., 2011). The NDF and ADF were found to be lower in MB than in soyabean. In raw samples, MB compares favourably with SB in terms of CP content (34.80 vs. 38.57% DM basis) in agreement with earlier findings (Amarteifio & Moholo, 1998; Amonsou *et al*., 2011). Crude protein was found to be greater in treated samples of both beans than in the raw samples. This result corroborates with previous findings (Emiola *et al*., 2002), where an increase in the CP concentration of raw kidney beans was observed upon being subjected to boiling. This may suggest a reduction in moisture content without protein or nitrogen loss. Also, phytic acid is naturally occurring in MB as a phytate-protein complex that can partially impede the availability of the MB protein (Mosele *et al*., 2011), and thermal treatment has been reported to lower the phytate in cowpea (Ibrahim *et al*., 2002).

The difference in AA levels of SB and MB, particularly with higher aromatic AAs (tyrosine and phenylalanine) and proline in MB, despite the similar crude protein contents of these two legumes as found in this study, have been previously reported (Bower *et al*., 1988; Maruatona, 2008). Furthermore, findings from this study showed that tyrosine, glutamic acid, aspartic acid, and proline are the dominant AAs in MB, which aligns with the earlier investigation reports on purified marama protein extract (Mmonatau, 2005) and MB flour (Maruatona, 2008).

The rise in the concentration of some AAs in substrates subjected to physical treatments may be related to the increase in CP values. However, the loss in concentrations of AA observed with certain treatments (soaking and pre-soaked steam-autoclaving at 121°C and 17 psi for 30 min) could have been caused by partial loss of essential and non-essential AAs, with other nitrogenous compounds formed as protein was chemically broken down into water soluble AAs, due to prolonged soaking, thermal treatment, and/or pressure (Huma *et al*., 2008). The recorded reduction in AAs content aligns with previous report of significant effects on the AAs profile of SB samples autoclaved for different durations; 0, 5, 10, 15, and 20 min (Jannathulla *et al*., 2017).

The TIA of raw MB was about four times greater than that found in SB. The value of 245.25 TIU/mg MB was close to the 250.8 TIU/mg previously reported (Maruatona, 2008). However, the TIA in raw SB of reported in this study was higher than previous findings (Maruatona, 2008). The differences observed in the TIA of the SB may be attributed to varietal and/or origin (growth environment) differences (Karr-Lilienthal *et al*., 2004). Further, the cooked (100°C) and steam autoclaved (110°C) soyabean TIA values recorded in this study agreed with those reported by Kaewtapee *et al*. (2017). Soaking has been shown to be less effective in lowering TIA in legumes. For instance, soaking cowpea for 16 hr in water only resulted in a 16% decrease in TIA (Ibrahim *et al*., 2002). The same was observed in the current study with 24 hr soaking of SB, which resulted in 19% (66.98 vs. 54.04 TIU/mg) decrease in TIA. The TIA was

found to continuously decrease by 96% (66.98 vs. 2.49 TIU/mg) and 96% (245.23 vs. 9.12 TIU/mg) as cooking time increased to 30 min (C30) for both SB and MB, respectively.

The lowest values for TIA in SB and MB were recorded with steam-autoclaving at 121°C and 17 psi for 30 min, with (S2/30) or without pre-soaking (A2/30). Higher losses in TIA with pressure cooking (1 kg/cm^2; 14.2 psi for 20 min) compared to cooking at 100°C for 45 min has been reported earlier (Ibrahim *et al*., 2002). This may imply that despite the TIA of raw MB being about four times that of raw SB, the use of thermal treatments was able to reduce it by 99% (245.23 vs. 2.66 TIU/mg). Therefore, the TI in both SB and MB are heat-labile, and heating SB and MB at the temperature of 121°C and 17 psi for 30 min was effective in altering the conformation of the inhibitors and permanently inactivating them. Thermal processing of soyabean for animal feed has been found to improve body weight gain and feed efficiency in animals (Tousi-Mojarrad *et al*., 2014). In addition, the inclusion of autoclaved (121 °C for 20 and 30 min) soyabean meal in the diets enhanced growth performance and feed efficiency in broiler chickens, when compared with those fed raw SBM (Tousi-Mojarrad *et al*., 2014). In this study, autoclaving at 121°C, 17 psi for 30 min yielded the lowest TIA values.

5.6 Conclusion

Marama bean compares well with soyabean in terms of crude protein; however, the amino acids profile and trypsin inhibitor activity differ between the two beans. Threonine, lysine, leucine, glutamic acid, and alanine values were higher in SB than in MB while MB protein was richer in arginine, proline, glycine, and tyrosine. Soyabean contains approximately one-fourth of trypsin inhibitor concentration compared to MB. Steam treatments (conventional cooking and autoclaving) are effective in reducing TIA in MB and should be considered to improve its SB replacement value in broiler diets. However, thermal methods of inactivating TIA in these legumes may cause a significant leaching of nutrients, especially amino acids, and minerals.

Steam-treated MB needs to be evaluated against SB in broiler diets to confirm that the method can enhance the SB replacement value of this native, orphan legume.

5.7 References

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6 CHAPTER SIX – GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 General discussion

The increase in demand for soyabean as a direct food for the growing human population and as the major protein source in broiler nutrition drives the price of this commodity in global market. This necessitates the need to find local alternative feed resources for animals to ensure sustainability. The poultry industry produces relatively inexpensive and healthy animal protein, when compared to beef industry, to feed the growing human population. This is because broilers have a short generation interval and high nutrient conversion efficiency compared to cattle (Bounds & Zinyemba, 2018). The broiler strain of interest in this study, Ross 308, has shown several attributes such as fast growth rates and tolerance in relation to feed quality conditions (Pascalau *et al*., 2017).

Several authors found marama bean (MB) comparable to soyabean in terms of crude protein content (Maruatona *et al*., 2010; Holse *et al*., 2010; Amonsou *et al*., 2011). This suggests that MB could be a possible substitute for soyabean in animal diet formulation. However, the higher trypsin inhibitor activity that is found in MB compared to other common legumes raises a concern (Bower *et al*., 1998; Nadaraja *et al*., 2009). The first two studies were, therefore, designed to evaluate the utility of MBM as a component of broiler diets.

The productivity of MB seeds is presently low, as it is largely obtained from the wild. According to Chimwamurombe (2011), the domestication of MB is ongoing in southern Africa with the goal of increasing the productivity of this crop. Research on the potential of MB as animal feed ingredient is necessary to promote awareness on the benefits of this seed and increase the desire and effort towards its domestication. Thus, this study was conducted to determine the effect of graded levels of dietary MB meal on nutrient digestibility, growth

performance, haematology, serum biochemistry, carcass characteristics and meat quality parameters of Ross 308 broiler chickens. Due to current shortage in supply of MB, the incorporation of MB in broiler chicken diets in this presented study was limited to the grower phase. To test the null hypothesis that inclusion of raw full-fat MBM at higher level would reduce feed utilization, growth performance and haemo-biochemical parameters of Ross 308 broiler chickens, the MBM was incorporated in commercial broiler grower diet at MBM0 = commercial chicken grower diet without MBM; MBM4 = commercial chicken grower diet containing 40 g of MBM per kg of soyabean products; MBM9 = commercial chicken grower diet containing 90 g of MBM per kg of soyabean products; MBM14 = commercial chicken grower diet containing 140 g of MBM per kg of soyabean products; and MBM19 = commercial chicken grower diet containing 190 g of MBM per kg of soyabean products, producing five isonitrogenous and isoenergetic diets, which were fed to three-hundred and eighty-five Ross 308 broiler chickens. Nutrient digestibility, feed intake, body weight gain, haematology, serum biochemistry, carcass characteristics and meat quality parameters were determined. There was no dietary effect on feed intake, however, nutrient digestibility, growth performance, haemoglobin and sera calcium contents were reduced. Thus, the null hypothesis was accepted for nutrient digestibility, body weight gain, feed conversion efficiency, haemoglobin and sera calcium and rejected for feed intake. Dietary inclusion of marama bean up to 20 g/kg diet, were estimated as optimum levels for crude protein digestibility and feed conversion ratio. These were lower than the lowest inclusion level of 40 g/kg fed to broiler chickens in this study. This suggests that higher dietary MBM levels require pre-processing to safely replace soyabean products in broilers' diets.

The observed reduction in nutrient digestibility, poor growth performance and low haemoglobin and sera calcium contents in the first study indicates that the amount of MBM that can be included in broiler diets may be limited by antinutritional factors such as trypsin

inhibitors and phytate. This outcome necessitated the need to ascertain the effect on the animal product (carcass and meat) in terms of attributes such as colour, pH, water holding capacity and shear force, as they are dependent on variables found to be negatively affected in the previous study. For instance, the haemoglobin concentration in the blood would affect haem pigment found in poultry meat the latter mostly influences meat colour (Froning, 1995). Hence, the appearance of poultry meat is largely influenced by the quantity of myoglobin, the chemical state of myoglobin and the chemical and physical state of other meat components (Lawrie, 1998). Colour is the most important attribute by which consumers determine the product acceptability or meat quality (Conforth, 1995). Therefore, the poor meat colour attributes (redness and yellowness) and tenderness (high shear force) observed in the second study as a result of increase in level of MBM in the broiler chickens' diets validates the hypothesis that higher inclusion levels of raw full-fat MBM in diets would impair carcass characteristics, meat quality traits of Ross 308 broiler chickens. These findings can be attributed to presence of antinutrients (such as trypsin inhibitors, phytates and tannins), particularly trypsin inhibitors. Indeed, trypsin inhibitors are known to alter biological activity of endogenous trypsin, protein digestibility and amino acid availability by forming indigestible complexes with pancreatic-produced trypsin (Liener, 1994). Similarly, Amonsou *et al*. (2011) mentioned that the phytate in MBM may bind to nutrients, thus reducing their availability. Hence, the hypothesis was accepted.

Subsequently, the third study involved laboratory assay to evaluate the nutritional, major antinutritional (trypsin inhibitor) concern and the effect of physical treatment methods on TIA, crude protein, and amino acids profile. The methods applied included: R = Raw; S = ambient temperature (20 – 22°C) soaking for 24 hr; C = electric stove cooking at 100°C for 10, 20 and 30 minutes (C10; C20 & C30); A1 = steam autoclaving at temperature of 110°C and pressure of 7 pounds per square inch (psi) for 5, 15 and 30 minutes (A1/5; A1/15; & A1/30); A2 =

steam autoclaving at temperature of 121℃ and 7 psi for 5, 15, and 30 minutes (A2/5; A2/15; & A2/30); P1 = pre-soaked autoclaving at 110℃ (7 psi) for 5, 15 and 30 minutes (P1/5; P1/15; & P1/30); P2 = pre-soaked autoclaving at 121℃ (17 psi) for 5, 15 and 30 minutes (P2/5; P2/15; & P2/30). Cooking and autoclaving effectively reduced the TIA in MB and soyabean but led to loss of amino acids and ash. Hence, the hypothesis that application of physical treatments to MB and soyabean reduce the trypsin inhibitor activity and improve the nutritive value of MB and soyabean was accepted.

Strategies such as heat treatment were shown to be effective in inactivating TIA in MBM. Hence thermal treatment and exogenous phytase enzyme pre-treatment of MB may be considered to reduce anti-nutritional factors and enhance nutrient utilization in broiler chickens. In addition, the improvement programs for MB currently underway in southern Africa should also include reduction of antinutrients in this orphan legume as a breeding objective. This will allow widespread utilization of this bean as a dietary protein source for both humans and animals.

6.2 Conclusions

While the feeding trial established that MBM could be incorporated at low level in broiler chicken diets to avoid detrimental effect due to higher trypsin inhibitor activity in MB compared to soyabean, the lack of significant effect on feed intake with incorporation of graded levels of MBM could implied a good palatability and acceptance by the Ross broiler chickens. Results from the subsequent study showed that physical treatment methods, especially steam heating, can effectively eradicate TI in MB. However, caution should be taken to avoid leaching of nutrients, especially amino acids, and minerals. Hence, MB has the potential to be included in formulating sustainable diets if properly processed to reduce the antinutritional

factors, especially trypsin inhibitors, prior to its inclusion into broiler chickens' diets and thus reduce the need for importation of soyabean.

6.3 Recommendations

To improve the nutritional quality and provide effective utilization of MB for poultry, it is essential that the anti-nutritional factors be removed or reduced prior to its inclusion in broiler chickens' diets. Therefore, the following recommendations are made:

- Assessment of other pre-treatment techniques such as chemical and biological, of marama bean with focus on minimising nutrient loss is suggested.
- Future research is required to evaluate the effect of processed marama bean on broiler chicken performance, fatty acid and amino acid profiles, gut histomorphology, and meat quality and stability to establish processing method(s) that may allow higher inclusion levels of MB.

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7 APPENDICES

APPENDIX 7.1. Ethics Certificate

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Web: http://www.nwu.ac.za

Research Ethics Regulatory Committee
Tel: 018 299-4849
Email: nkosinathi.machine@nwu.ac.za

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the **North-West University Animal Production Sciences Research Ethics Committee (NWU-AnimProdREC)** on 17/09/2020, the NWU Animal Production Sciences Research Ethics Committee hereby **approves** your study as indicated below. This implies that the North-West University Senate Committee for Research Ethics (NWU-SCRE) grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Towards the amelioration of trypsin inhibitor activity in marama bean (Tylosema esculentum) for broiler nutrition

Study Leader/Supervisor (Principal Investigator)/Researcher: Prof K Mnisi

Student: ALABI FA

Ethics number: N W U - 0 2 0 0 7 - 2 0 - A 5
Institution Study Number Year Status

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single Study
Commencement date: 2022/09/25 **Risk Category:** 2
Expiry date: 2023/09/24
Approval of the study is initially provided for a year, after which continuation of the study is dependent on receipt and review of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation.

Special in process conditions of the research for approval (if applicable):

- Any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the NWU-AnimProdREC. Ethics approval is required BEFORE approval can be obtained from these authorities.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The study leader/supervisor (principle investigator)/researcher must report in the prescribed format to the NWU-AnimProdREC:*
  - *annually (or as otherwise requested) on the monitoring of the study, whereby a letter of continuation will be provided, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the study leader/researcher must apply for approval of these amendments at the NWU-AnimProdREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *Annually a number of studies may be randomly selected for an external audit.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-SCRE and NWU-AnimProdREC reserves the right to:*

APPENDIX 7.2. Language Editing Certificate

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Date: 20th February 2023

To Whom It May Concern,

REF: Language Editing and Proof-reading of Dissertations/Theses

Dear Sir or Madam,

This serves to confirm that I have proof-read and edited the **Doctoral thesis** for **FA Alabi** (Student number: **30552613**; orcid.org/**0000-0001-5361-7790**) entitled: **Evaluation of marama bean as an alternative to soybeans in broiler nutrition.** The candidate then later corrected all the identified language and technical errors to the satisfaction of the supervisor. Thus, the document presented here is of sufficient and acceptable academic standards.

Editor

Prof. O Ruzvidzo

Supervisor

Prof. CM Mnisi

APPENDIX 7.3. Thesis Turnitin

Evaluation of marama bean as an alternative to soybeans in broiler nutrition

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APPENDIX 7.4. Publication in Molecules

molecules

MDPI

Article

Physical Treatment Reduces Trypsin Inhibitor Activity and Modifies Chemical Composition of Marama Bean (*Tylosema esculentum*)

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Abstract: The utility of the marama bean (MB) as an alternative protein source to soybean (SB) can be limited by the high concentration of trypsin inhibitors (TI). The physical treatment of MB has the potential to ameliorate the antinutritional activities of TI and modify other chemical components. Thus, this study investigated the effects of physical treatments on the chemical components and trypsin inhibitor activity (TIA) of raw MB and SB. The bean substrates were subjected to each of the following treatment methods: (1) room temperature (20–22 °C) soaking for 24 h; (2) electric stove cooking at 100 °C for 10, 20, and 30 min; (3) steam autoclaving at a temperature of 110 °C and pressure of 7 pounds per square inch (psi), as well as a temperature of 121 °C and 7 psi for 5, 15, and 30 min; (4) pre-soaked autoclaving at 110 °C (7 psi) and 121 °C (17 psi) for 5, 15, and 30 min. Treated MB and SB had greater ($p < 0.05$) crude protein content than untreated samples. All the treatments (except 24 h soaking of MB) reduced ($p < 0.05$) the TIA and ash content. Marama and SB are similar in protein content, but their amino acids profile and TIA are quite different. Soaking for 24 h was less effective in reducing TIA in MB and SB, compared to the thermal methods, and it was detrimental to the ash and amino acids profile of the two beans. Soaking prior to autoclaving yielded beans with the lowest TI concentrations. In conclusion, thermal methods reduced the TI contents and modified the level of proximate components and amino acids profile of the beans.

Keywords: autoclaving; cooking; marama bean; soaking; soybean; trypsin inhibitor activity

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1. Introduction

The marama bean (MB; *Tylosema esculentum*) is an indigenous legume in southern Africa that is directly consumed by indigenous people of Botswana, Namibia, and South Africa [1]. The matured seeds are used for producing porridge, oil, and butter [2]. Marama nutritionally contains oil ranges between 24–48%, predominantly mono- and di-unsaturated fatty acids and without cholesterol [1]. It is also a good source of potassium, phosphorus,