The impact of underutilised crops as feed and food ingredients

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Abstract

In the face of population growth and climate change, alternative feed sources are urgently needed to replace traditional sources such as soybean and wheat. The bambara groundnut and mezquite trees are two underutilised crops of current interest but are underutilised due to lack of widespread knowledge on cultivation or potential uses.

The aim of this work was to investigate the impact of bambara groundnut and mezquite pod flour as potential feed and food ingredients by studying their nutritional and antinutritional concentrations and testing mezquite pod flour as a potential feed ingredient for broiler chickens, both raw and following the application of post-harvest processing and exogenous enzymes.

For both bambara groundnut and mezquite pod flour there were significant ($p<0.05$) differences in crude protein between landraces or species. Protein content was the main focus of this analysis, due to this being the most expensive consideration in animal feed. The crude protein quantity was found to be lower than reported for soybean seeds. For bambara groundnut seeds, the crude protein content ranged from 15.82- 25.85 g/100g, while the mezquite pod flour ranged from 10.78-13.72 g/100g. However, the essential amino acid concentrations within the protein are comparable for bambara groundnut and within the mezquite dry weight, shows a similar profile to soybean. This is important as it indicates a high protein quality.

Both alternative crops had significant variation between the landraces or species in their antinutrient content. For the bambara groundnut seeds the total phenolic content ranged from 5.64-59.62 mg TAE/g and the phytic acid content from 0.210-1.162 g/100g. In the mezquite pod flour the total phenolic content ranged from 5.75-8.96 mg TAE/g and the phytic acid from 0.0260-0.3034 g/100g. The total phenolic content was used as an estimate of the tannin content, which reduced protein digestibility. This was therefore the antinutrient that could be expected to have the greatest impact within the following broiler chicken trials.

A 15% (w/w) inclusion of *Prosopis (P.) juliflora* pod flour within broiler chicken's feed, significantly reduced the final body weight ($p<0.001$) whilst not affecting feed intake, resulting in a significant negative increase in the feed conversion ratio ($p<0.001$). *P. juliflora* was chosen as the mezquite species due to previous literature research on this species, alongside availability. However, it also had a total phenolic content that fit in the middle of the range previously studied. Proportional breast muscle weights of birds fed the *P. juliflora* feed was significantly lower ($p<0.001$) whist liver weights were not affected. The apparent ileal digestibility coefficients for both crude protein and multiple essential amino acids were also reduced by the *P. juliflora* inclusion ($p<0.05$). When two alternative mezquite pod species (*P. velutina* and *P. articulata*) were tested, either raw or heat processed along with a phytase inclusion; there was a significant interaction between feed variety and processing for final body weight ($p=0.006$) and feed conversion ratio ($p=0.049$). Processing the *P.*

velutina resulted in this feed giving the same characteristics as the control feed, whilst *P. articulata* containing feeds, either processed or not, was significantly different from the control. In addition, there was a significant effect of feed variant on feed intake ($p<0.001$) as birds eating any diet containing mezquite had a significantly higher feed intake than control birds. Mezquite pod inclusion significantly reduced the apparent ileal crude protein digestibility coefficients ($p<0.001$), whilst there was a significant interaction between feed variant and processing ($p<0.05$) for all essential amino acids exceot phenylalanine. This indicated that processing *P. velutina* pod flour resulted in this feed giving similar characteristics as the control feed, whilst *P. artiulata* containing feeds were significantly different from the control, irrespective of whether or not they had been processed.

In conclusion, bambara groundnut seeds show potential as future feed and food ingredients due to their nutritional profiles. Though their use is limited by antinutritional factors, these can be reduced by seed dehulling or potentially by heat treatment. Mezquite pod flour has a variable nutritional and antinutritional profile. Its negative impact on broiler chickens feed digestibility is potentially due to its antinutritional profile. While this may be improved by heat treatment and the application of exogenous phytase; this is dependent on the species of mezquite pod flour used. It should therefore be recommended that the trees local to a community be profiled before use.

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1 Introduction

1.1 The need for alternative food sources

Providing the population with adequate diets to support a healthy lifestyle, while using sustainable food systems is a pressing challenge across the globe. With an ever-growing world population, increasingly long lifespans and the added pressure of climate change; it is becoming increasingly important to find new alternative sources of food, both for human and animal diets. Malnutrition, including undernutrition, micronutrient deficiencies and obesity is increasingly high, presenting economic impact across the world as well as an impact of related diseases and mortality, (Willett et al. 2019; The state of food and agriculture 2013).

This has been considered in the United Nations sustainable development goals. An estimated 8.9 percent of the world population are currently considered hungry, measured by the prevalence of undernourishment. The highest prevalence, calculated as percentage of people by region, in 2020 could be seen in Somalia, Central African Republic, Madagascar and Haiti (FAO, 2022) (Figure 1.1). This has led to the zero-hunger goal, aiming to end hunger and all forms of malnutrition by 2030. This goal includes targets to ensure sustainable food production systems and to strengthen their capacity for adaptation to climate change including extreme weather, droughts and flooding. It also aims to double the productivity and incomes of small-scale food producers including women and indigenous peoples (United Nations 2022).

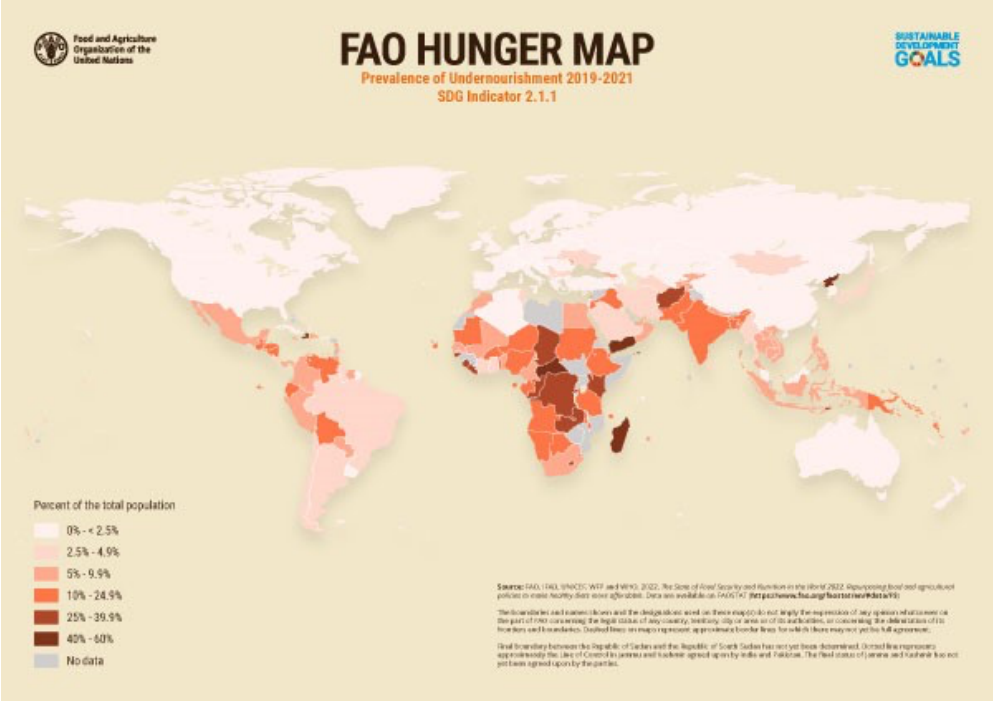


Figure 1.1 The prevalence of undernourishment across the world from 2019-2021 (FAO, 2022).

1.2 The importance of protein in human diets

It is essential to have enough protein, and of the right quality, in human diets. In the UK the reference nutrient intake for protein is 0.75g/kg/ body weight (BW), while internationally it is 0.8g/kg/BW (Lonnie et al. 2018). However, this figure indicates the minimum protein intake necessary to maintain nitrogen balance and does not consider age or activity levels (Wolfe et al. 2008). It is estimated that one billion people worldwide suffer from protein deficiency, a problem which is most pronounced in Central Africa and South Asia; and particularly in children. Protein deficiency can result in a weakened immune system; leading to greater infection risks, as well as slow wound healing and weakened bones (Gakpo, 2020).

Meeting the protein requirement of an individual is dependent on protein quality as well as quantity. To meet an individual's protein requirements, dietary proteins must contain adequate amounts of essential amino acids, higher quality proteins contain higher concentrations of these. Essential, or indispensable, amino acids are required to maintain health and support growth, and higher levels are required to support pregnancy and lactation and for growth in children. These are amino acids which are not naturally synthesised within the body and therefore must come from the diet. A selection of other amino acids are considered conditionally essential, meaning their natural synthesis can be limited in certain conditions. The remaining amino acids are considered non-essential, or dispensable, which are those which can be naturally synthesised within the body (United Nations 2022; World health organisation 2007; Reeds 2000). These categories of amino acids in humans are displayed in table 1.

Table 1.1. The essential, conditional and non-essential amino acids in humans.

Essential Amino Acids		Conditional Amino Acids	Non-essential Amino Acids
Amino Acid	**RDA[1] (mg/kg per day)**		
Leucine	14	Arginine	Alanine
Isoleucine	10	Cysteine	Aspartic Acid
Valine	10	Glutamine	Asparagine
Lysine	12	Glycine	Glutamic Acid
Threonine	7	Proline	Serine
Tryptophan	3.5	Tyrosine	
Methionine	13	Taurine	
Phenylalanine	14		
Histidine	8-12		

Adapted from (Reeds 2000; World health organisation 2007)

[1]Reccommended daily allowance for adults (National research council, 1989).

Alongside the amino acid profile, the second component that determines protein quality is digestibility. Digestibility is measured by the difference between intake and losses, to give an idea of the absorption of nutrients in the gastrointestinal tract. In the case of protein, both the amount of crude protein and the individual essential amino acids absorbed is important. If a protein source is not highly digestible, the essential amino acids contained will not be available for use when consumed (World health organisation 2007). Protein digestibility is often measured using either faecal matter or ileal digesta, followed by digestibility calculations to compare the levels of the nutrient within the faeces or digesta in comparison to the levels within the feed. The methods used is dependent on whether this is being calculated for human or animal studies; and whether a general crude protein digestibility estimation is needed, or a more in depth look at the digestibility of specific amino acids (Rutherfurd et al. 2015). Digestibility scores for protein are particularly important for the feeding of production animals. Here, a low protein digestibility will result a wastage of amino acids due to them being unavailable. This will not only result in poor health and slower growth due to amino acid deficiencies but will have a direct economic impact (Moughan 2003). There are also factors to consider which can affect the digestibility of a protein. These include changes to the proteins themselves through processes such as heating and fermentation, and the interaction of proteins with non-protein components within the protein source. These non-protein components may include the levels of dietary fibre, lipids, polyphenols and ant-nutritional factors

such as tannins and protease inhibitors, which have been shown to reduce protein digestibility (Pushparaj, Urooj 2011).

In western diets, with a rise in population there has also been a rise in the consumption of meat and dairy products. Similarly, with increasing wealth in developing countries there is also a trend of greater consumption of animal diets (Thornton n.d.; Speedy n.d.). It is predicted that by 2050 the production of meat from poultry, swine and beef, as well as dairy products, should double to keep up with demand; while fish production should triple (Binns et al. 2021; Parisi et al. 2020) (Figure 1.2).

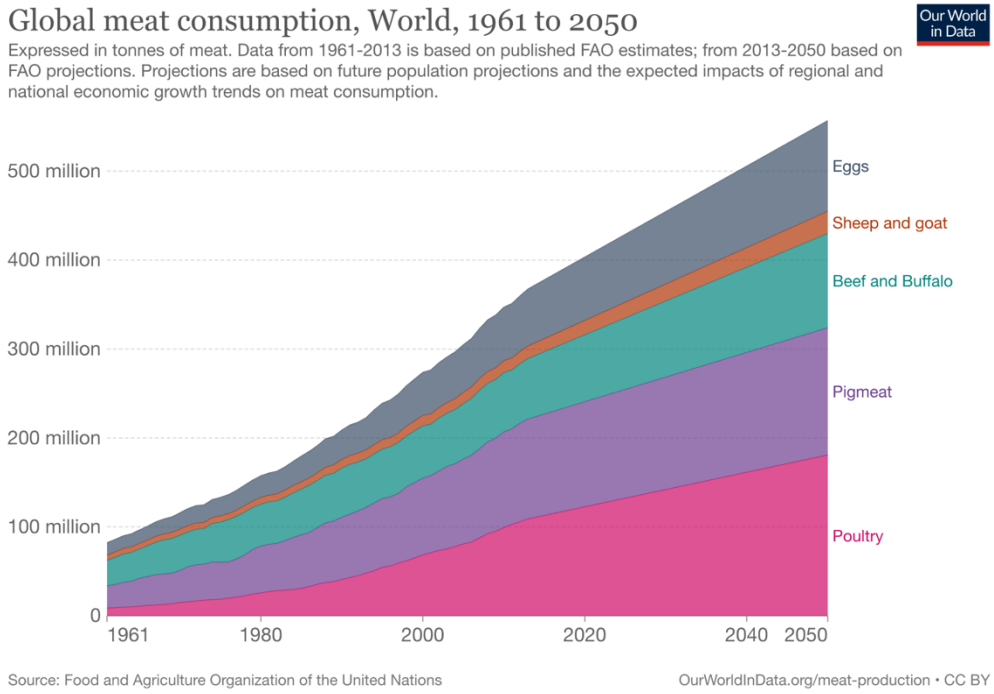


Figure 1.2. The global meat consumption from 1961 and the predicted consumption until 2050, taken from (Ritchie et al. 2019).

However, there are health risks associated with high meat consumption; a high consumption of red meat, particularly when processed, is associated with increased risks of colorectal cancer (Sasso, Latella 2019). Additionally, in western countries, diseases related to obesity such as diabetes and cardiovascular disease are on the rise. This is associated with greater calorie intakes and the consumption of diets rich in saturated fat (Micha, Mozaffarian 2010) and refined carbohydrates (Jebb 2015). There is also often a perceived benefit of high protein diets, particularly for athletic performance, which can lead to a higher excretion of nitrogen (Shibata et al. 2017). Considering the average global meat intake (Figure 1.2), it can be recommended on the whole that individuals in wealthier countries may benefit from a reduction in animal product intake. However, in developing countries, an increased consumption would likely be beneficial to many to ensure a high-quality protein intake (Speedy n.d.).

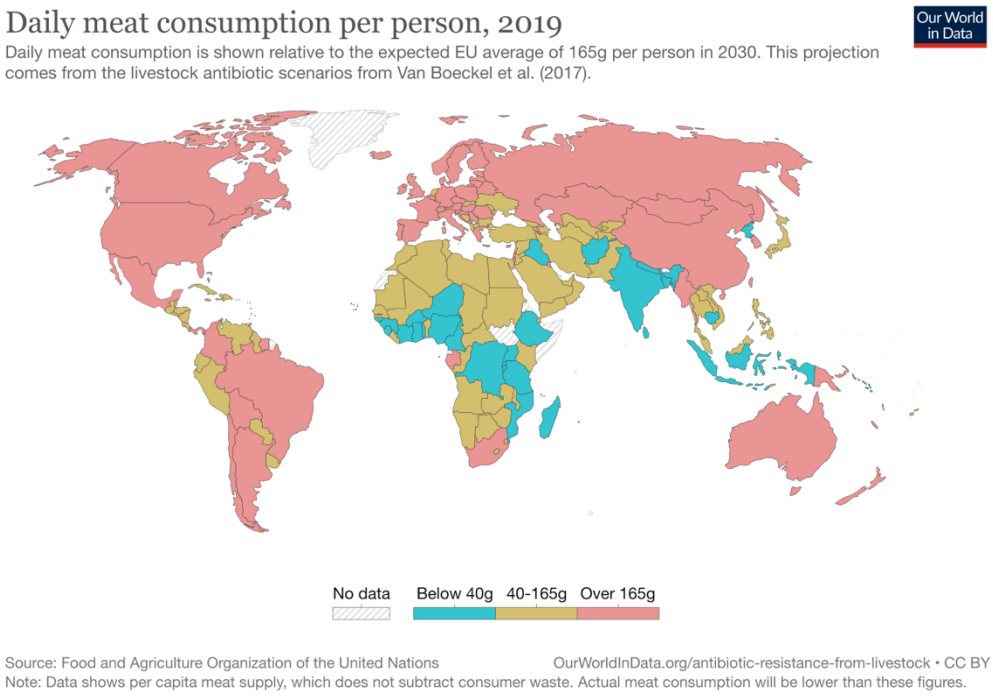


Figure 1.3. The global estimated average daily meat consumption in 2019, taken from (Ritchie et al. 2019).

Plant-based protein sources are often lower quality proteins compared to animal sources, due to lower protein contents as well as the concentrations of essential amino acids in their protein diverging from the requirements of animals. In addition, there is also the presence of antinutritional factors influencing digestibility and accessibility, therefore bioavailability is reduced. However, this can often be combatted by consuming plant-based proteins from multiple sources, increasing the range of essential amino acids consumed and compensating for low concentrations of particular essential amino acids in certain sources. This means that both omnivorous and vegetarian diets can provide a sufficient quantity of protein. This may not be the case in poorer populations though, those with access to a less varied diet

are more likely to obtain their protein from a single crop, making deficiencies more common (Sarwar Gilani et al. 2002). Globally, the primary sources of plant-based proteins come from the staple cereals- wheat, rice and maize; but reliance on these crops as the major protein source in the diet can lead to deficiencies in essential amino acids, particularly lysine (Pellett, Ghosh 2004). Nuts and legumes can also be a high-quality protein source, with the Planetary Diet proposed by the Eat Lancet Commission suggesting a major increase in protein rich plant-based foods including legumes, nuts and pulses, to replace a large portion of the protein from animal sources currently consumed (EAT-Lancet 2019). In general, these foods are recommended in a plant-based diet due to them having adequate levels of lysine, rather than for the amount of protein contained; as it is lysine that is most likely to be limiting in the staple cereal crops of a plant-based diet (Venter et al. 2013).

1.3 The livestock production industry

The livestock production industry provides food sources for human diets in the form of meat, eggs and dairy products. A rise has been observed in the consumption and production of all sectors of livestock production, but poultry meat has seen the highest increase (Speedy n.d.). However, this is not uniform globally, there is a noticeable divide between developed and developing countries (Thornton 2010).

While the impact of a higher consumption of animal products on human health has already been mentioned, there is also the environmental impact of production animals to consider. Livestock production alone accounts for

approximately one-fifth of total greenhouse gas emissions (Walsh et al. 2015) with food production as a whole being responsible for 20-30% of greenhouse gas emissions. Livestock systems also occupy approximately 30% of the worlds ice-free terrestrial surface area. This is only likely to increase with the expansion of livestock numbers in developing countries, particularly ruminants (Thornton 2010).

1.3.1 The broiler chicken industry

Population growth has led to the intensification of animal production amongst all species, but the most dramatic changes have been observed in the poultry sector (Bessei 2018). The majority of poultry meat comes from broiler chickens, these are specific breeds selected for meat production. The demand for poultry meat products has put pressure on the production of greater quantities of meat in shorter amounts of time and for lower costs. Genetic selection has led to the production of broiler chicken species with rapid growth rates, a younger slaughter age and a better feed conversion ratio (FCR) (Hartcher, Lum 2019). This was illustrated by (Zuidhof et al. 2014) who showed that from 1957 to 2005, the growth of broiler chickens increased by over 400% from unselected 1957 University of Alberta Meat Control strains to 2005 Ross 308 broilers (Figure 1.2), with a 50% reduction in feed conversion ratio.

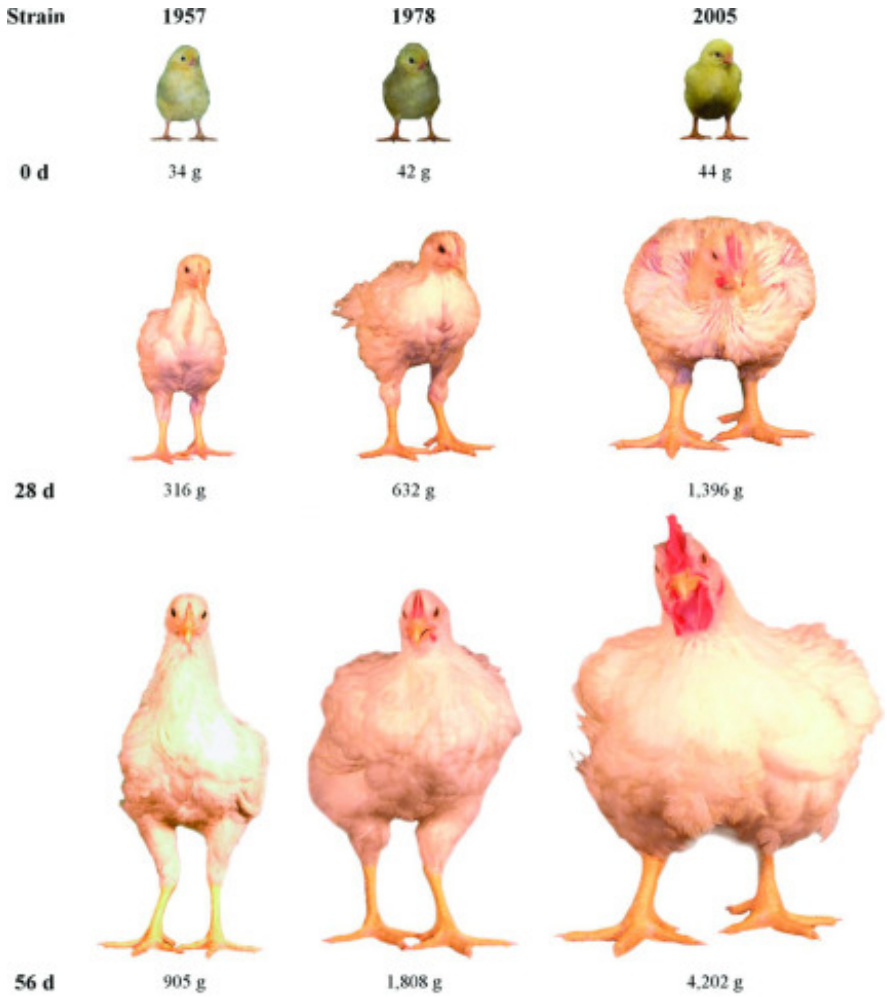


Figure 1.4. The age related size changes at day 0, day 28 and day 56 as a comparison of University of Alberta Meat Control strains unselected since 1957 and 1978, and Ross 308 broilers from 2005 (Zuidhof et al. 2014). Image sourced from (Zuidhof et al. 2014).

A massive upscale in production has been achieved in a short space of time, this is partly due to the high heredity of desired traits, but also an increased knowledge of nutrition in order to develop diets for maximum growth and a

manipulation of commercial production environments to give optimal growth conditions (Kalmar et al. 2013).

(Zuidhof et al. 2014) looked at two strains of two University of Alberta Meat Control broiler strains that were unselected since 1957 and 1978, compared to a 2005 strain of Ross 308 Broilers. They fed all birds on starter diets with 23% crude protein, a grower diet with 20.15% crude protein and a finisher diet with 19% crude protein; meeting the 2003 Ross 308 Broiler nutritional guidelines. They found that 42-day broiler growth increased 400% between 1957 and 2005, while FCR decreased by 50%; showing the effect of genetic selection. Supporting this, (Havenstein et al. 2003b) found that at 43 days of age, Athens-Canadian Randombred control birds, representative of those used in 1957, had an average carcass weight of 322g on a 1957 representative diet and 360g on a 2001 diet. Comparatively, Ross 308 broilers representative of those used commercially in 2001 has an average carcass weight of 1460g on the 1957 diet and 1926g on the 2001 diet. The 1957 diet here contained 23.7% crude protein in the starter phase and 19.2% crude protein in the grower-finisher stage. In the 2001 diet there was 24.8% protein in the starter phase, 22.2% in the grower 1 phase, 20.9% in the grower 2 phase and 19.8% in the finisher phase(Havenstein et al. 2003b; Havenstein et al. 2003a). This shows that more modern breeds, that have faced a more intensive selection pressure, already have a massive head-start for rapid growth, but that a focus on dietary requirements can further increase growth.

Ross 308 broilers remain one of the prominent fast growing broiler strains for use in both production and research. They are valued for their fast growth rate and optimised FCR and are supplied to over 100 countries by Aviagen (Aviagen 2018; Kokoszyński et al. 2022). Fast growing broilers such as these can grow to slaughter weight in 5-6 weeks, reducing the costs of production or research trials (Dixon 2020). For slower growing broilers, Hubbard strains are often used (Šťastník et al. 2022).

With the intensification of industrial broiler production, there comes concerns about bird welfare (Bessei 2018). In faster growing broiler strains, larger growth is associated with decreased activity. This can be linked to leg weakness, lameness and the occurrence of skin lesions due to an increased amount of time spent sitting. For example, (Dixon 2020) found that slower growing Hubbard broilers spent less time sitting and more time active than three faster growing broiler strains. This led to improved welfare measure scores. Cardiovascular problems are also a concern, with incidence of cardiac arrhythmia much higher in fast growing broilers than slow growing strains (Hartcher, Lum 2019).

1.3.2 Livestock feed

Animal feeds play an important role in the livestock production industry. Supplying feeds with the correct nutrition is essential for enabling intensive production in the most economically efficient way, while maintaining animal welfare(FAO, IFIF 2010). With demand increasing, there also must be a rapid increase in the feed supply needed to produce these animals, which requires a

significant amount of agricultural land and a significant proportion of global fresh-water use (Parisi et al. 2020). Ruminant species require a greater land usage, due to the need for grazing, but they do convert feed that cannot be utilised by many species, such as grass, into protein; making for more cost-effective feeding. However, an overexploitation of these production systems puts pressure on forage sources, land conversion and water usage (Michalk et al. 2019). Monogastric species, particularly poultry, are usually produced in more intensive systems, meaning land use is less of an issue. However, they are often fed a diet containing ingredients which could either be used directly for human food or are grown on land that may otherwise have been used for human food production. Only 34% of protein produced is consumed by humans, with 60% being consumed by production animals (Salter, Lopez-Viso 2021). This is particularly problematic as proteins are the most expensive feed ingredients in production animal feed formulation (Parisi et al. 2020) and feed currently accounts for approximately 60-70% of production costs (Salter, Lopez-Viso 2021).

Previously, processed animal proteins (PAPs) were a commonly used protein source in animal feeds. PAPs are a feed ingredient made from animal by-products and include meat meal and bone meal (Iwasaki 2017). Meat and bone meal were valued for feed due to a high protein concentration of around 50g/100g and high digestibility, as well as being a good source of phosphorus and calcium (Bozkurt et al. 2004). It is also a by-product of animal production and the rendering industry, meaning their use is sustainable and low cost

(Coutand et al. 2008; Cascarosa et al. 2012). However, the Bovine Spongiform Encephalopathy (BSE) epidemic in 1986 and the link between BSE and the emergence of neurodegenerative prion disease Creutzfeldt - Jakob disease (CJD) (Iwasaki 2017) resulted in a ban of the use of PAPs in animal diets (Woodgate, Wilkinson 2021). This resulted in increasing both the cost and reliance on plant protein sources such as soybean meal. However, the use of fishmeal in aquaculture has been maintained. Fishmeal has traditionally been used as the main source of protein in the aquafeed industry due to its high crude protein content of 60-70% (w/w) (Miles, Chapman 2021) and balanced essential amino acid profile. However, fishmeal production is unsustainable, and the increased demand coupled with the shortage of global production has meant that it has become too expensive for use in other animal feeds (El-Sayed 2020). In 2013, the ban on PAPs was partially lifted, allowing the use of non-ruminant PAPs in aquaculture systems (Moutinho et al. 2017). There is also current consideration for lifting the PAPs ban in poultry and pigs, though public perception is critical of this idea, which may play a role in the uptake (Parisi et al. 2020).

Currently, protein ingredients for animal feeds come from plant sources including forages, cereals and protein concentrates. The most commonly used protein source in monogastric diets is currently soybean (*Glycine max*) meal (Beski et al. 2015). Soybean is a species of leguminous crop native to Eastern Asia. They have a high economic value globally due to the seeds having a high crude protein content of around 36.5g/100g, a good amino acid profile

including high lysine, and a high oil content (Sakkas et al. 2019; Vagadia et al. 2017). 75% of soybean crop produced goes into animal feed, and it represents the most common source of protein used in feed for monogastric production animals (Vagadia et al. 2017). Soybean oil is extracted for use in cooking and within body care items such as soaps and moisturisers. The high protein substance left from this extraction process, soybean meal, is then what is used for animal feed (Parisi et al. 2020). However, there is also an increasing popularity of soy-based meat and dairy alternatives in human diets (Boukid 2020).

Despite its popularity, soybean production is largely considered to be unsustainable. Soya beans and soybean meal are largely imported into Europe (De Visser et al. 2014) from the United States and South America. Here, it has been linked to deforestation due to the land usage required for its production; driven by high exportation demand (Liu et al. 2021). Soybean production also uses 1,300 to 2,300 tonnes of water per tonne of soybean produced (Rittler, Bykova 2022). This has particularly been reported as a problem in Brazil, where land and water use, and deforestation is prevalent due to the high export. Because of this, the growth and use of soybean is not inherently unsustainable, local consumption would not have the same negative results. The presence of antinutritional factors within the seeds is also a limiting factor, especially for unprocessed consumption; and the consumer perceptions of using genetically modified feed sources is increasingly low. All of these factors drive the search for alternative protein sources (Taherzadeh, Caro 2019; Vagadia et al. 2017).

However, when considering soybean in comparison to other alternative crops, the selection pressures applied to soybean should be considered. Soybean crops have had a long period of domestication, thought to be 6000-9000 years (Sedivy et al. 2017). This period of time has allowed the selection of favourable traits such as a high protein and oil content, and a reduction in other traits such as antinutritional factors (Sedivy et al. 2017; Zhao et al. 2015). Reducing the amount of antinutritional factors in this way means that less post-harvest processing needs to be applied in order to mitigate any potential negative effects. Alternative and underutilised crops are considered at the start of this development process, and so it is difficult to directly compare them to the soybean crop that we see today.

1.4 Current alternatives to soybean

It is a combination of these circumstances that drives the need to find alternative feed sources, and in particular, alternative protein sources. There are many points of research in this area that have gained increasing attention in recent years; including insects such as crickets, mealworms and black soldier flies (van HUIS, Arnold van Huis 2022; Shah et al. 2022), microalgae proteins (Amorim et al. 2021) single cell proteins (Sharif et al. 2021), and underutilised crops (Mudau et al. 2022.). It is unlikely that these research areas will be able to immediately replace the use of soybean meal, due to years of research that has gone into the modification of soybean meal into the feed ingredient that it currently is. Other research areas are being approached in their natural form, without any modification. However, there is an aim to partially replace the use

of soybean meal until modifications can be made to bring alternatives to the same level as soybean meal.

1.4.1 Underutilised crops

The three dominant cereal crops in human diets are maize *(Zea mays),* wheat (*Triticum aestivum)*, and rice *(Oryza sativa).* These are used extensively alongside oil crops such as soybean, with 20 plant species making up most of the worlds calories and contributing towards a more uniform global diet where crops are considered (Massawe et al. 2016a). These major crops have extremely successful agricultural outputs, with intensive techniques used to maximise their annual output. However, this is not the most sustainable of productions, with breeding work for desirable traits resulting in a selection of major crops which only have a high output in the right climates and with suitable soil types. With climate change resulting in more extreme and unpredictable weather conditions, there are expected to be worldwide impacts on major crop yields (Kamenya et al. 2021). The production of crops with a high protein concentration has already started to intensify, with the pulse and legume production already increasing in recent years, but European interest is still low compared to other parts of the world and still relies on only a few specific crops such as beans, peas and chickpeas (Parisi et al. 2020).

Despite global dependence on major crops, there are more than 50,000 known crops that would be suitable for human consumption. A wider variety of these need to be introduced to the global market in order to help feed the population and tackle climate change (Massawe et al. 2016). Further utilisation of protein

rich crops in particular, will be a valuable tool in increasing sustainability and decreasing costs of feed production for animal feeds (Parisi et al. 2020).

Many suitable crop species are known as underutilised crops, also described as orphan, forgotten or minor crops. These are crops which are not extensively traded globally, are often under-researched, and usually have a lack of genetic resources and knowledge (Massawe et al. 2016a; Kamenya et al. 2021). They are often only grown and cultivated within small populations, with the knowledge base kept local. They are often highly valued within local communities for both food and income, with many having a long history and becoming a staple crop (Padulosi et al. 2002). For example, indigenous food plants in Africa are often fundamental to the cultural identity of a group, as well as for diversifying their diets. However, knowledge of the harvest, preparation, management and nutritional value of these foods remains local with little records kept. This lack of outside awareness means that that their utilisation cannot become more widespread and their potential economic value remains limited (Akinola et al. 2021). Public perception can also limit the expansion of the use of these alternative crops. They often have low consumer opinions when outside of the local area in which they are utilised, with many being referred to as 'poor mans crops' when compared to a diet containing major crops (Gerrano et al. 2022).

Some underutilised crops gaining research traction include bambara groundnut (*Vigna subterranea*) (Chibarabada et al. 2017), mezquite (*Prosopis*) (Andrade-Montemayor et al. 2011), marama bean (*Tylosema esculentum*) (Omotayo,

Aremu 2021), winged bean (*Psophocarpus tetregonolubus*) (Tanzi et al. 2019), finger millet (*Eleusine coracana*), pearl millet (*Pennisetum glaucum*) (Saini et al. 2021), common vetch (*Vicia sativa*) (Huang et al. 2017) and cowpea (*Vigna unguiculata*) (Gerrano et al. 2022). All of these crops are valued due to their nutritional profiles, with high, and usually good quality, levels of protein (Table 1.2).

Table 1.2. The profiles of some commonly researched underutilised crops.

Under-utilised crop	Crude Protein (g/100g DW)	Limiting amino acid	Fat (g/100g)	Climate resistance	Sources
Bambara groundnut seeds	16-25	Methionine	6.5	Low rainfall, poor soils	(Oyeyinka et al. 2019) (Tan et al. 2020)
Mezquite pods	7-34	Methionine	1-6	Low rainfall, poor soils	(Ruiz-Nieto et al. 2020) (Ellsworth et al. 2018)
Marama bean seeds	34.71	Methionine	32-36	Low rainfall, high temperatures	(Omotayo, Aremu 2021) (Bower et al. 1988)
Winged bean seeds	29-27	Methionine	15-18	Low or high rainfall, high temperatures	(Salunkhe, Kadam, 1984) (Ekpenyong, Borchers, 1982)
Finger millet	5-8	Lysine	1.3-1.8	Low rainfall, high temperatures	(Devi et al. 2014) (Gebre, 2019)
Pearl millet	14.5	Lysine	5-7	Low rainfall, high temperatures	(Devi et al. 2014) (Anitha et al. 2019) (Shrestha et al. 2023)
Common vetch	24-32	Methionine	1.5-2.7	Low rainfall. High temperatures	(Nguyen et al. 2020) (Grela et al. 2020)
Cowpea	25.4-27.6	Methionine	1-1.5	Low rainfall	(Gondwe et al. 2019) (Vasconcelos et al.2010)

However, it is not only nutritional value that characterises many of these crops, but also their adaptability to environmental stresses (Parisi et al. 2020). They often show adaptations to grow in niche environments where major crops may not. This includes growth in areas of extremely poor soil conditions, high heat or drought, making them a valuable asset when planning climate change adaptations (Massawe et al. 2016a; Kamenya et al. 2021; Azam-Ali{ et al. 2019).

1.4.1.1 Crops of interest

In this thesis, two underutilised crops of interest will be the focus; bambara groundnut and mezquite. Bambara groundnut was selected due to the fact that it is currently gaining research interest because of its nutritional profile and potential use as a human food source, and within animal feeds. The bambara groundnut intended to be the sole focus of this thesis, prior to the covid-19 pandemic, which halted the work. Following this, work continued looking at mezquite as a potential feed and food crop. This crop was selected due to the fact that mezquite trees are the focus of eradication plans in countries where they are not native; and so this body of work hopes to evaluate alternative uses for the pods.

1.4.1.2 Bambara Groundnut

Bambara Groundnut (*Vigna subterranea* (L.) Verdc.) is an underutilised leguminous crop of current interest. It can be found in West Africa, Asia, South America and Oceania. The Bambara groundnut is herbaceous, low-growing and self-pollinating (Gulzar, Minnaar 2017). The seeds are produced underground,

making Bambara groundnut a difficult crop to harvest mechanically, discouraging large-scale commercial harvesting. However, it is valued as it produces a reasonable yield on poor soils and has a high drought tolerance. This means the crop has the potential to assist in providing food security in dry areas (Bonny et al. 2019). Additionally, it can improve other local crop yields through nitrogen fixation; which can be achieved with the rotation of crop growth in an area (Azam-Ali{ et al. 2019).

1.4.1.3 Mezquite

Mezquite is the common name for plants in the genus *Prosopis,* which is a group of leguminous trees belonging to the *Fabaceae* family. This group contains 44 species, predominantly distributed in Africa, Asia and the Americas (Díaz-Batalla, Hernández-Uribe, et al. 2018). The mezquite trees are valued due to their ability to fix nitrogen in the soil and their growth in areas of little rainfall (Ruiz-Nieto et al. 2020). The success of mezquite in soil conditioning led to its introduction into new countries, with the aim to enhance productivity of the soils as well as provide a food and fuel source for the local people. mezquite trees also produce fruit pods twice per year, which are valued as a local food source due to their nutritional composition (Almanza, Moya 1986). However, limited knowledge about germination, cultivation, usage and harvesting, means mezquite trees are largely underutilised (Barba De La Rosa et al. 2006).

1.5 Alternative Foods, Summary

To summarise, proteins are an essential part of both human and animal diets. With a growing population, there is a need for increased production of both livestock (Speedy, 2003) as human protein sources, and plant-based protein sources as animal feed. Underutilised crops have the potential to be a sustainable animal feed ingredient. They often provide good yields in extreme weather conditions, where major crops such as wheat and soybean would not grow (Massawe et al. 2016a). However, there is usually little knowledge on the potential utilisation of these crops, due to small scale production and local cultivation (Akinola et al. 2021). As with many plant-based feed sources, the use of underutilised crops may also be limited by anti-nutritional factors (ANFs).

1.6 Antinutritional Factors

ANFs are one of the most commonly limiting factors in the consumption of plant materials. ANFs can be defined as biological compounds present in human or animal foods that reduce nutrient utilisation or feed intake, thereby contributing to impaired gastrointestinal (GI) and metabolic performance (Arendt, Zannini 2013). ANFs found in plant materials are also known as plant secondary metabolites (PSMs). PSMs are a secondary compound produced as a by-product of the processes leading to primary metabolite synthesis; they have been evolved to be a defence mechanism in plants and play a role in natural protection against some diseases or predation (Kong et al. 2022;

Gemede, Ratta 2014). They can be classified as either proteinaceous, being a protein and therefore temperature sensitive, or non-proteinaceous ANFs (Hamid et al. 2017).

ANFs are particularly prominent in cereals and legumes, meaning that they are commonly found in both human and animal diets. Because they interfere with the release or absorption of nutrients, they reduce the bioavailability of these nutrients within the food. This can actually have both positive and negative effects for the consumer, depending on the levels and frequency of ingestion (Kong et al. 2022) and also depending on the body system of the animal, for example, monogastric or ruminant. This is because some ANFs, such as protease inhibitors, are degraded in the rumen; meaning that higher concentrations are required to exert adverse effects on ruminant animals (Akande, Alabi 2021). In this respect, the same ANFs may have positive effects in some animals and negative effects in others or may have positive effects when consumed in low amounts or toxic effects when ingested regularly in high amounts (Kong et al. 2022). Some of the more commonly found and researched antinutritional factors are phytate, tannins, trypsin inhibitors, saponins and lectins.

Table 1.3. Antinutrient content of a range of underutilised crops.

Crop	Tannins (%)	Phytate (%)	Trypsin Inhibitors (Units/mg)	Lectins (+ or -)	Saponins (%)	References
Quinoa	0.53	1.05-1.31	4-7.3	+	2.05	Chauhan *et al.* 1992 Filho *et al.* 2017. Jancurová *et al.* 2009 Silva *et al.* 2015
Bambara Groundnut	0.02-0.62	1.44	5-73	NR	1	Olaleye *et al.* 2013 Brough and Azam-Ali, 1992 Unigwe *et al.* 2018
Pearl Millet	0.01	0.65-0.8	7.1-7.8	NR	NR	Osman, 2011 Sharma and Kapoor, 1996 Jalgonkar *et al.* 2016
Mesquite	1.5	0.33-0.89	17.5	+	NR	Sawal *et al.* 2004 Andrade-Montemayor *et al.* 2011 Retana-Márquez *et al.* 2016
Marama Bean	0.38	NR	250-617.4	NR	0.08	Maruatona *et al.* 2010 Tibe *et al.* 2016 Nyembwe *et al.* 2015 Richards *et al.* 2014
Wing Bean	0.13-0.68	0.77-1.2	0.09-0.14	+	NR	Kotaru *et al.* 1987 Sathe and Salunkhe, 1981

NR= Not Recorded

1.6.1 Phytic Acid

1.6.1.1 Mechanisms responsible for anti-nutritional effects

Phytic acid (Figure 1.5), also known as inositol-6-phosphate, is the major storage form of phosphorous (Kaplan et al. 2019) and represents 50-85% total phosphorous in plants. Sources of phytic acid include cereals, legumes, oilseeds and nuts; making it a common ANF in both human and animal diets (Kishor Gupta et al. 2015) (Phytic acid has six reactive phosphate groups, giving it strong chelating properties. Consequently, it can bind to minerals, particularly divalent metals, forming insoluble complexes (Yasothai 2016). Within seeds, these bound compounds are known as phytates, which are broken down by phytase enzymes when the seed sprouts, but as humans and animals lack this digestive enzyme, phosphorus in this form is not utilised in the body. When ingested as phytic acid, the chelating properties means they also bind to minerals within the body, making them unavailable. This can cause inhibition of the absorption of iron, zinc, calcium and magnesium in the body, leading to mineral ion deficiencies (Yasothai 2016). As well as this, phytic acid has been reported to bind to proteins in a similar way, with the phosphoric acid groups of phytate bind with the cationic group of amino acids. This reaction can reduce protein solubility and digestibility. The negative impacts of these complexes, resulting in deficiencies, are what categorises phytic acid as an antinutritional factor, though it also has reported beneficial qualities as well (Feizollahi et al. 2021).

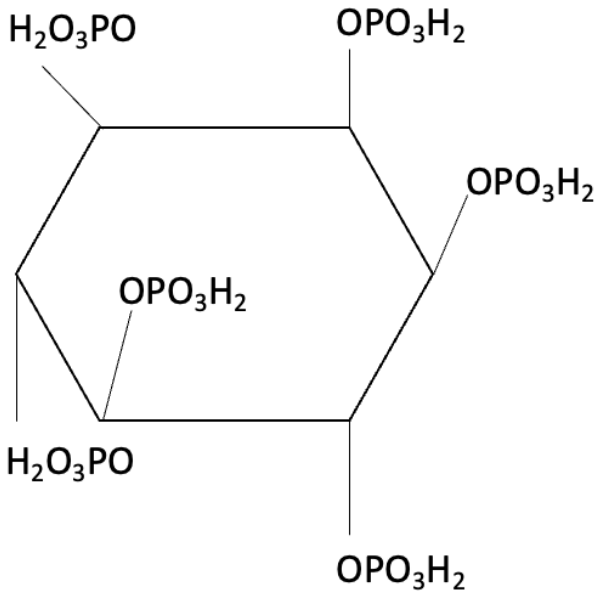


Figure 1.5. The structure of phytic acid, adapted from (Higuchi 2014).

1.6.1.2 Effects in Animal diets

Phytic acid has varying effects in different animal species. The rumen gut microbiota found in ruminants are able to break down the complexes formed by phytic acid in the body, making the phosphate bioavailable. However, monogastric species cannot, meaning that the bioavailability of phosphate is reduced, and excess is excreted (Sureshkumar et al. 2022). Phosphorus is an essential nutrient for skeletal mineralisation, protein synthesis and cell metabolism, it is therefore an essential component of the diet (Humer et al. 2014). Deficiencies of phosphorus in poultry can restrict growth and reduce bone density, in turn increasing bird mortality. Production is also reliant on mineral availability in the diet, which can be reduced with high dietary levels of phytic acid. For example, layer hens are heavily reliant on both sufficient

phosphorus and calcium levels to maintain egg production (Javadi et al. 2021). While increasing the concentration of phosphorus in the feed via supplementation may be one solution, this has been shown to also increase the excretion of phosphorus in animal manure. This phosphorus in turn builds up in the soil, limiting future crop production (Rosemarin et al. 2021).

The most common solution to this is the supplementation of exogenous enzyme phytase into animal diets (Cowieson et al. 2009). This was originally considered as a method of reducing phosphorus excretion in intensive animal production systems but has now been widely commercially accepted in feed both to reduce phosphorus excretion and to increase phosphorus bioavailability in diets, in turn improving animal health and production (Selle, Ravindran 2007). (Liu, Ru 2010) found that the inclusion of phytase into broiler chicken diets decreased ($p<0.05$) ileal flows of iron (21%), magnesium (8%) and potassium (13%), suggesting the absorption may have been improved.

1.6.1.3 Effects in Human diets

In the UK, cereals and cereal products are the main sources of phytate in human diets; predominantly coming from breakfast cereals, breads, vegetables and savoury snacks. The intake of these foods varies between different age groups and between men and women, giving a variation of daily phytate intakes within the population (Amirabdollahian and Ash, 2010). However, phytate intake also varies between countries, with people in developed countries showing a lower daily dietary intake than those in developing countries (Ma *et al.* 2007).

Iron is an essential element in haemoglobin and myoglobin but currently its deficiency is recognised as one of the most prevalent nutritional problems in the world and is commonly linked to phytate intake (Perera et al. 2018). The effect of phytate on iron is supported by (Hallberg et al. 1989) who found that eating wheat rolls with phytate added significantly ($p<0.001$) inhibited the absorption of iron supplemented in additional rolls, in a dose dependent manner; with 2mg inhibiting absorption by 18%, 25mg by 64% and 250mg by 82%. It has also been reported that a phytic acid to iron ratio of 10:1 is required to majorly inhibit iron absorption in the body (Bohn et al. 2008). Zinc is also an essential micronutrient, as it is a co-factor for more than 300 enzymes. Its deficiency causes growth retardation and immune dysfunction and is the second mineral where the inhibition of absorption is most commonly linked to high a phytate diet (Perera et al. 2018). Deficiencies in calcium is less pronounced than other mineral deficiencies, though a long-term deficiency in adults can severely affect teeth and bone health. Fredlund et al. (2006) found that the addition of phytate in bread rolls fed to adults significantly decreased both zinc absorption and calcium retention compared to rolls with no added phytate.

Despite these negative effects, phytate has also been reported to have some beneficial health effects in humans. One of these positive effects is providing protection against diabetes mellitus, with studies suggesting that dietary phytate can reduce the levels of glucose in the blood (Yoon et al. 1983). Phytic acid has also shown strong anti-cancer properties in both *in vitro* and *in vivo*

studies. This includes cell line growth inhibition in ovary, breast and liver cancer cells(Norhaizan et al. 2011). The complexes formed with iron in the body also give phytic acid antioxidant properties, which has been shown to be beneficial when consumed as part of a diet also containing animal proteins (Cornforth 2001).

1.6.1.4 Removal from diets

The most common method of removing phytic acid from grains is milling, however, this also has the disadvantage that it also removes parts of minerals and dietary fibres. Heating, fermentation and germination have also been shown to be effective biological methods of reducing phytic acid, particularly when combined with long soaking periods (Gupta et al. 2015). These processing techniques reduce phytic acid content by increasing the phytate degrading activity of naturally occurring phytase within the plants by releasing bound phytase. These therefore are most commonly used for deactivation methods prior to human consumption. For animal feeds, the supplementation of exogenous phytase is more commercially viable (Cowieson et al. 2009).

1.6.2 Tannins

1.6.2.1 Mechanisms responsible for anti-nutritional effects

Tannins are one of the most commonly occurring PSMs, they are a group of high molecular weight polyphenolic compounds, occurring naturally in leguminous seeds, cereals, fruits and vegetables. This makes them extremely common compounds in both human and animal diets. They are commonly

classified into two groups, hydrolysable tannins and condensed tannins (Figure 1.6). Hydrolysable tannins can be further classified as either gallotannins or ellagitannins, which have gallic acid as their base unit; these are water soluble and are decomposed by high temperatures. Condensed tannins are the most commonly found PSM and are formed by the condensation of flavan-3-ol. Under oxidative conditions they depolymerise into anthocyanidins (Nath et al. 2022; Barreca et al. 2017). The anti-nutritional effect comes from tannins ability to bind or precipitate proteins, meaning when consumed they can make dietary proteins partially unavailable (Gemede, Ratta 2014). Tannin-protein complexes are formed as a result of the formation of multiple hydrogen bonds between the tannin hydroxyl group and the protein carbonyl group (Nikmaram et al. 2017). Tannins have also been reported to inhibit digestive enzymes by binding to them, as well as interfering with dietary iron absorbance (Gemede, Ratta 2014).

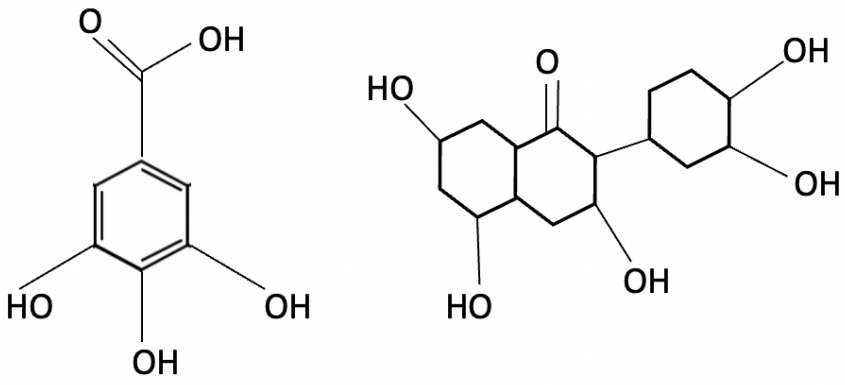


Figure 1.6. The structure of hydrolysable tannins (left) and condensed tannins (right), adapted from Ghosh (2015).

1.6.2.2 Effects in Animal diets

In animal diets, tannins, particularly condensed tannins, have been studied as both antinutrients and beneficial additives (Soldado et al. 2021). As an antinutrient, the negative effects are more pronounced in monogastric species. Reduction in protein digestibility, lower body weight gain and reduced feed intake are the expected impacts of a high tannin diet for monogastric animals. These effects limit the use of many plant-based feed ingredients (Xin et al. 2021; Gemede, Ratta 2014). For example, Vilariño et al. (2009) separated and re-combined faba beans to create reconstituted faba beans with high, medium or low tannin contents of 9.9, 5.8 and 1.3 g/kg, to assess their effect in broiler chicken diets. They found that apparent metabolisable energy content and protein digestibility was significantly lower ($p<0.001$) for high tannin faba beans then low tannin faba beans; supporting the idea that tannin content reduces nutritional bioavailability. Similarly, Nyamambi et al. (2000) found that high-tannin sorghum significantly ($p<0.05$) depressed weight gain, feed intake and feed efficiency in broiler chickens compared to maize-based diets. The safe level of tannins has been reported as 1.5-2mg/g, before negative effects can be observed (Farinde et al. 2018).

Tannins are also astringent and bitter compounds, which can have an impact on palatability, therefore reducing feed intake in all species, including ruminants (Méndez-Ortiz et al. 2018; Gemede, Ratta 2014). However, for ruminants this only seems to have an effect when tannin levels in the diet are high. In sheep, it is generally accepted that a dietary condensed tannin intake

of greater than approximately 50g condensed tannin/kg dry matter will decrease dry matter intake, while lower concentrations do not affect dry matter intake (Méndez-Ortiz et al. 2018). Salem et al. (2005) found that lambs fed hay had a significantly greater dry matter intake than lambs fed a high tannin acacia diet. Similarly, (Méndez-Ortíz et al. 2012) found that short-term feeding of a tannin rich fodder reduced dry matter intake as well as dry matter digestibility in sheep. Adejoro et al. (2020) also found that a diet supplemented with tannin extracts at 42g/kg DM showed significantly reduced ($p<0.001$) daily weight gain in lambs from 47.2g to 91.1g after 72 days, suggesting that some negative effects do carry over into young ruminants.

However, a low concentration of tannins in the diet can have positive effects in ruminants, including increased growth and increased milk production (Hoste et al. 2006). The protein binding ability of tannins means tannin-protein complexes are formed when digested, as in monogastric species. However, these complexes are stable and can reduce ruminal protein degradation in the rumen by slowing their digestion rate, therefore increasing the amount of intestinal metabolisable protein (Yanza et al. 2021; Houdijk et al. 2012; Butter et al. 2000). Tannins also inhibit the growth of ruminal cellulolytic and proteolytic bacteria by the formation of complexes with the cell wall membrane of bacteria, causing morphological changes (Bodas et al. 2012). This can reduce ruminant methane emissions, both by the inhibition of methane-producing bacteria known as methanogens, or by changes in the bacterial

population which affects the fermentation process (Vargas et al. 2022; Molina-Botero et al. 2019).

Condensed tannins have also been found to act as an anthelmintic compound, reducing faecal egg counts and halting larval development within the host (Brunet, Hoste 2006). However, the mechanisms of action behind this effect are uncertain. It has been suggested that tannins may work directly to prevent the hatching and development of larvae (Athanasiadou et al. 2000a; Athanasiadou et al. 2000b) or alternatively that they work indirectly by improving the hosts response to infection (Brunet, Hoste 2006; Butter et al. 2000).

1.6.2.3 Effects in Human diets

In human diets tannins are not highly detectable in staple crops such as corn, rice or wheat, but are found in minority crops such as legumes, nuts, sorghum and barley. They are also present in many commonly consumed fruits as well as fruit juices, wine, tea and beer (Serrano et al. 2009). They can sometimes be detected by their astringency, causing a tongue drying sensation when consumed (Nath et al. 2022).

In human diets, tannins have been found to have many beneficial effects. Tannins have been found to show inhibitory action on HIV-1 replication and HIV-1-mediated cell fusion (Lü et al. 2004). Condensed tannins in wines have been linked to an inhibition of atherosclerosis by the suppression of endothelin-1 and inducing blood vessel dilation (Corder et al. 2006).

Hydrolysable tannins have been found to inhibit Herpes simplex virus directly by attaching to and penetrating the cells, as well as showing viral inactivation at high concentrations (Cheng et al. 2002).

1.6.2.4 Removal from diets

Various processing methods can be applied to food sources high in tannins to negate the negative effects. Cooking and germination have both been shown to significantly reduce the level of tannins in cowpea seeds, while soaking and fermentation is seemingly ineffective (Ibrahim et al. 2002). An often-simpler processing method to reduce the tannin content is dehulling. In legumes, the majority of the tannin content is contained in the seed hulls, which can be manually removed before consumption or further processing. However, dehulling may also cause the removal of beneficial nutrients (Nikmaram et al. 2017).

1.6.3 Enzyme Inhibitors

1.6.3.1 Mechanisms responsible for anti-nutritional effects

Enzyme inhibitors are prevalent in legumes, usually in the form of protease inhibitors, α-Amylase inhibitors (Das et al. 2022) or lipase inhibitors. They are specific to their target enzyme and so are grouped based on the enzymes that they inhibit. They, like other antinutrients, have been researched for both harmful and beneficial effects (Nath et al. 2022).

Protease inhibitors are proteins, which can be characterised as Kunitz inhibitors with a molecular weight of 18-24kDa or Bowman- Birk inhibitors with a molecular weight of 7-9kDa (Das et al. 2022). They are responsible for slowing or inhibiting the catalytic action of digestive protease enzymes, thereby reducing bioavailability by reducing the production of peptides and amino acids that can be absorbed (Nikmaram et al. 2017). The protease inhibitors trypsin and chymotrypsin Inhibitors are common in legume seeds (Gemede, Ratta 2014) and inhibit the activity of the pancreatic enzymes' trypsin and chymotrypsin (Avilés-Gaxiola et al. 2018). By forming indigestible and irreversible enzyme-inhibitor complexes, this reduces enzyme activity, protein digestion and therefore dietary protein absorption (Vagadia et al. 2017; Gemede, Ratta 2014). These are the most commonly studied, particularly for their harmful effects, as trypsin inhibitors are found in high quantities in soybean (Nath et al. 2022).

Amylase inhibitors can also be present in feed materials, such as legumes. These enzyme inhibitors inhibit the activity of digestive enzyme amylase, preventing the conversion of starch and glycogen into simple sugars (Nikmaram et al. 2017) this means that they are not digested, which can in turn lead to weight loss (Das et al. 2022).

1.6.3.2 Effects in Animal diets

Trypsin inhibitors are common in soybean, which is not often processed before inclusion in animal diets. They reduce protein digestibility, the most important aspect of animal feeds. As a result, high levels of trypsin inhibitors in the diet,

or a high trypsin inhibitor activity (TIA), can reduce weight gain and negatively impact performance. Palliyeguru et al. (2011) and Rada et al. (2016) both found that an increase in non-toasted/ raw full fat soya bean, with increasing TIA levels, significantly reduced protein digestibility, weight gain and feed conversion efficiency in broiler chickens. Similarly, the feeding of transgenic peas expressing an α-amylase inhibitor significantly reduced body weight, feed conversion ratio and ileal starch digestibility coefficient in broiler chickens (Li et al. 2019) and starch digestibility in pigs (Collins et al. 2006), when compared to a control diet.

However, this negative effect only appears to be prominent in monogastric animals. Hoffmann et al. (2003) and (Ortega (2021) have both conducted *in vitro* studies to see how trypsin inhibitors were affected by incubation in bovine rumen fluid. They found that trypsin inhibitors were extensively degraded in the rumen fluid, suggesting that they are unlikely to have any negative effects *in vivo*.

1.6.3.3 Effects in Human diets

Enzyme inhibitors in human diets usually come from legume seeds and in cereals at relatively low levels. Their presence can interfere with digestion, leading to pancreatic hypertrophy and reduced utilisation of amino acids. This is predominantly the case for protease inhibitors which, when binding to protease, slows the digestion of protein in the small intestine; meaning it is more readily excreted from the body (Nikmaram et al. 2017; Adeyemo and Onilude 2013) This could have a pronounced impact on growth in young

children when consumed in high enough doses, something which may become more of a concern as soybean-based meat and dairy alternatives become increasingly common (Nath et al. 2022).

However, there are also positive effects associated with enzyme inhibitors. The inhibition of amylase delays the rate of carbohydrate digestion, which in turn reduces the rate of glucose absorption in the body. This effect will delay the normal postprandial plasma glucose level rise and so amylase inhibitors can be used as a method of controlling diabetes (Ali et al. 2006; Bhutkar, Bhise 2012). Bowman-Birk protease inhibitors have also been shown to have an inhibitory effect on the entry of HIV into macrophages, suggesting that they could be used as a prevention and treatment method for HIV and other viral infections (Ma et al. 2018).

1.6.3.4 Removal from diets

Physical processing with heat is widely accepted as the most effective way of improving the nutritional value of legume seeds containing enzyme inhibitors, while soaking is the simplest and most economic method for enzyme inhibitor deactivation and promotes the removal of a wide range of water-soluble compounds. Chemical processes, such as the addition of reducing agents, can work by altering the molecular structure of trypsin inhibitors. However, these processes have the major disadvantage that the final products may have chemical residues. Biological methods, such as germination and fermentation; have the advantage of a low energy expenditure, although they are not often favoured as they can be time consuming (Avilés-Gaxiola et al. 2018).

1.6.4 Lectins

1.6.4.1. Mechanisms responsible for anti-nutritional effects

Lectins are proteins which can reversibly bind to monosaccharides and oligosaccharides, which can reduce their absorption. They can also bind to other molecules, including sugars on red blood cells which have known specificities; they can also therefore be known as haemagglutinins (Nath et al. 2022). Lectins and haemagglutinins have at least one non-catalytic domain that irreversibly binds to specific monosaccharides or oligosaccharides (Kwan Lam, Bun Ng, 2011). They are commonly present in many plant foods found in both human and animal diets (Vasconcelos, Oliveira 2004).

1.6.4.2 Effects in Animal diets

In animal diets, lectins are present in soybean, corn and wheat germ (Vasconcelos, Oliveira 2004). The addition of lectin containing kidney beans into the diet has been shown to significantly ($p<0.05$) increase intestine weight and decrease weight gain in pigs, chickens and rats, as well as induce pancreas growth in rats and chickens. These effects were not observed when the kidney beans undergo processing such as heat treatment or extrusion; which would inactivate the lectins (Huisman, Der Poel, Leeuwen, et al. 1990; Huisman, Der Poel, Mouwen, et al. 1990). However, as kidney beans also contain other ANFs including tannins, phytic acid and enzyme inhibitors, it cannot be said with certainty that it is the lectins that caused these negative effects.

1.6.4.3 Effects in Human diets

In human diets lectins are present in many commonly consumed plant foods, including tomato, potato, peas, carrots, rice, avocado and nuts (Vasconcelos, Oliveira 2004). An excess of active lectin consumption can cause three identified physiological reactions. The first is that they can cause severe intestinal damage, this disrupts digestion which in turn causes nutrient deficiencies. Secondly, they can activate IgG and IgM antibodies, causing allergy responses. Lastly, they can bind to erythrocytes, leading to hemagglutination and anaemia (Gemede, Ratta 2014). The initial symptoms of this acute toxicity in humans include nausea, bloating, vomiting and diarrhoea (Vasconcelos, Oliveira 2004). However, the level of binding of lectins from pulses in an average diet is likely too low to show an agglutinating effect, particularly to erythrocytes (Cortés-Giraldo et al. 2012).

As well as these negative effects, lectins have also been associated with health benefits when consumed. Lectins from different plant species have been found to induce apoptosis in different cancerous cells (Jiang et al. 2015; Pryme et al. 2002). Lectins extracted from mistletoe are particularly well researched in this area, with Pryme et al. (2002) finding that the feeding of mistletoe lectins to rats significantly decreased tumour weight and reduced the number of blood capillaries in the tumours, compared to a control diet. However, this effect has only currently been researched in rats and it is unknown if the effect would also be observed in human subjects.

1.6.4.4 Removal from diets

As lectins are proteins, they are inactivated by any processing methods that lead to protein denaturing, this is predominantly heating or cooking. For most legume seeds, heating under pressure is a common processing method which will inactivate the legumes (Lajolo et al. 2002). Dehulling and soaking have both been found to have no effect on the lectin content of peas (Patterson et al. 2017), likely because lectins, as proteins, will be more common in the inner seed rather than the hulls.

1.6.5 Saponins

1.6.5.1 Mechanisms responsible for anti-nutritional effects

Saponins are heat-stable amphipathic glycosides that can be classified as either steroid or triterpenoid saponins, based on their structure. They can bind to, and enhance the permeability of, cell membranes. As a result, dietary consumption can increase the permeability of intestinal mucosal cells, inhibit mucosal transport and facilitate the uptake of substances that would not normally be absorbed by the body (Couto et al. 2014.). The word ‘saponin’ is derived from the Latin word sapo which means ‘soap’; this is because saponin molecules form soap-like foams when they are shaken with water. Because of this, saponins are widely used in beverages, confectionary, cosmetics and pharmaceutical products (Gemede, Ratta 2014).

They are considered to be antinutrients as the hydrophobic part of a saponin molecule can bind to cholesterol within inside bio-membranes while the

hydrophilic sugar side chain part of the molecule binds to external membrane proteins. This alters the fluidity of the membrane which can affect its stability. If holes are formed in the membrane, leakage can result in cell death. Membrane fluidity also alters the intake of the cell, allowing the intake of substances that are not usually transported into the cell (Nikmaram et al. 2017). This can also inhibit the effects of dietary enzymes, trypsin and chymotrypsin, which in turn reduces protein digestibility (Shimoyamada et al. 1998).

1.6.5.2 Effects in Animal diets

The major limiting factor in the use of dietary plants that are high in saponins is the bitter taste. In animals, this taste can lead to a reduced feed intake (Gemede, Ratta 2014). As an antinutritional factor, the effect of saponins has predominantly been studied in fish species. For example, diets high in saponins, including in soybean meal, have been found to significantly decrease weight gain and induce intestinal inflammation in Atlantic salmon (Chikwati et al. 2012).

In ruminants, dietary saponins can lead to removal of specific protozoa from the rumen microbiome; this is known as defaunation. This occurs due to the interaction between the saponins with cell membrane cholesterol, causing membrane ruptures. This effect can increase nitrogen utilisation, in turn leading to an increase in growth and performance (Wina et al. 2005). In support of this, (Klita et al. 1996) found that intraruminal administration of saponins significantly decreased protozoal populations in sheep. They also found that

saponins increased volatile fatty acid concentrations and lowered ruminal pH after two days of intraruminal administration.

1.6.5.3 Effects in Human diets

There are many positive effects associated with the consumption of saponins in the diet. For example, they have been found to lower blood cholesterol by inhibiting cholesterol absorption and bile acid reabsorption (Leterme 2002). By lowering cholesterol levels, saponins also help to reduce the risk of coronary heart disease. They interact directly with cholesterol in the body, forming insoluble complexes and hence decreasing its absorption from the gut (Singh et al. 2017).

The anticancer properties of saponins are also a point of research interest, and includes anti-proliferation, anti-metastasis and anti-angiogenesis. These effects have been seen to be the effect of induction of apoptosis, promotion of cell differentiation and modulation of the immune system (Olalekan Elekofehinti et al. 2021; Nath et al. 2022).

1.6.5.4 Removal from diets

Sivajumaran et al. (2017) concluded that autoclaving and soaking are the most effective methods of reducing saponins in legume seeds. While heating, pressure cooking and germination have all also been shown to reduce saponin levels in legumes and pulses, they are more effective at removing saponins when combined with soaking (Singh et al. 2017).

Table 1.4. Summary of the impact of antinutritional factors on livestock health and growth.

ANF	Effect on monogastric species	Effect on ruminant species	Sources
Phytic Acid	Phosphorus deficiencies can lead to reduced growth rates and reduce bone density.	Rumen microbiota breaks down phytic acid complexes- no negative effects.	Sureshkumar et al. 2022 Javadi et al. 2021
Tannins	Reduced protein digestibility leads to reduced growth rates and feed intake.	They have been seen to reduce feed intake due to being bitter and astringent.	Gemede, Ratta 2014 Méndez-Ortiz et al. 2018
Enzyme Inhibitors	Decrease weight gain due to reduced protein and starch digestibility.	Enzyme inhibitors are extensively degraded in the rumen.	Hoffmann et al. 2003 Palliyeguru et al. 2011
Lectins	Agglutination of erythrocytes can lead to anaemia and the binding of dietary sugars can lead to depressed growth.	Lectins are not fully degraded in the rumen though effects may be lessened.	Baintner et al. 1993 Gemede, Ratta 2014 Huisman et al. 1990
Saponins	Induces intestinal inflammation by increasing epithelial permeability, reducing digestibility and growth rates. Bitter taste also reduces feed intake.	Effects are largely seen to be positive due to modulation of rumen microbiota increasing nitrogen utilisation.	Wina et al. 2005 Couto et al. 2014 Gemede, Ratta 2014 Chikwati et al. 2012

1.6.6 Environmental factors affecting PSMs

The levels of PSMs seen in crops can vary significantly between different species, this can be down to the location in which they were grown. As PSMs evolved as a plant defence mechanism, they can be affected by the levels of

environmental stress faced during growth (Goyal et al. 2016.). Howard et al. (2002) found that spinach grown through winter and early spring had significantly higher levels of total phenolics than spinach grown in autumn. They theorised that this may be due to the differences in growing temperature, light intensity and disease challenge. Adenubi and Oluyede (2015) found that when grown across three locations, African yam bean seeds had the highest phytic acid content when grown in an area with soil that had the best organic and inorganic matter content, including phosphorus. They also found that tannin content varied significantly across the locations, suggesting an importance of growing location.

It is possible to genetically alter crops using a gene knockout in order to remove the ANFs. Gene knockouts are alleles that disrupt a genes molecular function (Monroe et al. 2020). Using this method to reduce the ANFs produced by the plants means that the plants could be utilised in their raw form, without the requirement for processing before consumption. This would naturally improve the nutrient digestibility, improving the usage potential of many underutilised crops for both human and animal diets. However, ANFs in plants are natural protection against environmental stress, gene knockout to reduce their production can therefore have negative effects on plant growth as a result of reducing their stress tolerance. This can include the plants tolerance to salinity and ion toxicity (Sathee et al. 2022). Knocking out the responsible genes directly is therefore not currently feasible, but targeted knockout has the potential to be effective in reducing the accumulation of ANFs within the parts

of plants that are likely to be consumed with future work (Monroe et al. 2020). However, these current limitations with gene knockout emphasises the need for the research described in this thesis; finding alternative crops and methods of improving their nutrient utilisation.

1.7 Work conducted in this thesis

The use of underutilised crops as a food source in both human and animal diets is currently a research area of high interest and importance globally. With the increased attention, it is important to focus on specific crops of interest and evaluate them in depth to see their suitability. While many academic research papers are being published about bambara groundnut and mezquite, there are still gaps in the literature which this thesis aims to fill. The utilisation of bambara groundnut and mezquite is limited by the need to demonstrate variation between species and different geographic locations, particularly in respect to antinutritional factors. Where antinutritional factors are prevalent, it is important for the knowledge of how they may effect animals and how their effects can be mitigated are more widespread. This, in particular, will be of benefit to the local communities that grow and harvest the crops. The four experimental chapters presented in this thesis will look at (1) the nutritional and antinutritional composition of different species of bambara groundnut, (2) the nutritional and antinutritional composition of different species of mezquite pod flour, from different geographical locations, (3) the use of mezquite pod flour in broiler chicken diets and (4) the use of processing to mitigate the

negative effects of mezquite pod flour from distinct geographical locations in broiler chicken diets.

1.7.1 Impact log

Year two

The work carried out in this thesis has been impacted by some unavoidable events. The first of these was the COVID-19 pandemic. The first national lockdown in March 2020 occurred towards the start of the second year of my PhD, this timing, and the fact that I had undertaken 6 months of laboratory rotations before beginning my project, meant that very little laboratory work had been conducted prior to this. This meant that there was very little data analysis that could be conducted during the 5 months that the laboratory was closed. When the laboratory was reopened in August 2020, there was limited access due to the low occupancy rules, with no training available on new equipment or protocols and issues in the supply of general laboratory consumables.

Year three

The second lockdown, in November 2020, coincided with my first chicken trial. While this was allowed to go ahead, limitations in occupancy reduced the number of samples we were able to take. Additionally, the backlog of people waiting to use the analytical equipment, and the strain placed on technical staff, meant that the subsequent analysis of samples was significantly delayed. The third lockdown, from January 2021, did not further impact laboratory work,

though low occupancy rules remained in place. However, during this lockdown my mental health suffered, due to isolation and the loss of three family members.

During this year, there was further impact caused by the major renovations of laboratory facilities. The North Laboratory, School of Biosciences, was closed in September 2018 and the occupants were moved into temporary accommodation. These facilities were restricted, which already reduced research output prior to the pandemic safety precautions. The refurbishment of North lab was completed in March 2021 and the laboratories in the temporary accommodation were closed towards the end of March. Due to complications, including flooding, the new laboratories did not open until mid-June with the analytical equipment not moved back into the laboratory for several more months. Since the opening, several more floods, heating problems and last-minute contractor work have also temporarily closed the laboratories and prevented the use of certain equipment.

Year four

As a further impact from the pandemic, while planning for the second chicken trial, we faced major delays, both in feed formulation and delivery of the feed; this was as a result of company backlogs and staff shortages. There was also a major impact on the communities in Mexico, which delayed the harvest and shipment of the mezquite pod flour needed for the trial. The trial was therefore repeatedly pushed back until a start date of January 4th 2022; the ethical approval had been granted for the trial to originally begin in September 2021.

Due to contracting COVID-19 over Christmas, both myself and my supervisor would be unavailable for the trial start date. After reaching out to the director of the university of Nottingham Bio Support Unit for help with this, it was concluded that there was a problem with the ethical approval and the trial was cancelled until a home office licence was in place. Due to lack of time remaining, it was therefore agreed that the trial would take place in collaboration with SRUC, in Edinburgh, beginning on March 1st 2022 and ended on the 4th April 2022. This involved me travelling to and from Edinburgh each week to observe and help out at the unit, reducing other research output. Due to vehicle issues the day before the scheduled bird cull, we were unable to attend the sample collection, meaning less samples could be taken and they could not be transported back to Nottingham for several weeks.

Finally, in August 2022, I was faced with a period away from Nottingham due to having to move out of my rental property and being unable to find another place within Nottingham due to a high demand for rental properties and an increase in costs. The university was unable to provide accommodation during this period meaning that I spent some time in hotels, which was unaffordable longer term and resulted in me having to stay with family in Somerset for 6 weeks during an important period of lab work.

1.8 Thesis Aims

1. To investigate the nutritional and antinutritional composition of different species of Bambara groundnut seeds (Chapter 3)

2. To investigate the nutritional and antinutritional composition of both different species, and different growth locations, of mezquite pod flour (Chapter 4)

3. To assess the effect of a 15% inclusion of mezquite pod flour from Kenya on broiler chicken performance and amino acid digestibility (Chapter 5)

4. To assess the effect of a 15% inclusion of raw or processed mezquite pod flour from two distinct regions of Mexico on broiler chicken performance and amino acid digestibility (Chapter 6)

1.9 Thesis Hypothesis

Bambara groundnut and mezquite will have varying nutritional and antinutritional profiles between variants, which will affect their potential as feed ingredients.

2 Materials and Methods

2.1 Materials

All chemicals were obtained from Sigma-Aldrich (Dorset) unless otherwise stated. Major equipment and consumables are shown in Table 2.1.

Table 2.1 Commonly used equipment mentioned, and the associated manufacturer.

Equipment	Details	Manufacturer
Fume hood	Extracting fume cupboard	Premier, Glasgow
Freeze dryer	<0.4 mbar, -55°C	Christ, Germany
Dust mask	9320+ FFP2	3M Aura
Elemental analyser	EA 1112	Thermo Scientific, MA, USA
Bomb calorimeter	6300 calorimeter	Parr, Illinois, USA
Analytical scales	4 decimal places, GR-200	A&D Weighing, Abington
Platform scales	Max 60kg, precision 20g	Kern, Germany
Microplate reader	680 XR	Bio Rad, Oxford
Microplate	96 Well	Sarstedt, Germany
Spectrophotometer	LKB Ultrospec III	Pharmacia, Sweden
Waterbath	20-90°C	Grant, Cambridge
Vortex	Yellowline TTS2	IKA, USA
Pippettes	1.5µl- 1000µl	Sartorius, Germany
Pippette tips	1-200µl, 1000µl	Fisher Scientific, Loughborough
Coffee grinder	KSM2, Braun Aromatic	Braun, Mexico

Plant materials consisting of bambara groundnut seeds and mesquite pod flour were shipped to the university of Nottingham for use throughout this thesis. The details of this are described in the subsequent chapters. Mezquite

pod flour use was done in compliance with Nagoya protocols SGPA/DGGFS/712/0668/20 and SGPA/DGGFS/712/1426/22.

2.1 Nutritional Analysis

2.1.1 Drying

All analysed samples were fully dried prior to proximate nutrient analysis. For all proximate nutrient analysis, freeze-drying (Christ, Germany) was used as the drying method. Either plastic pots or foil trays, both with the lids removed, were used to dry the samples in.

Containers were weighed prior to adding the samples, then again with the samples added, to give the total weights of both the containers and samples. Samples were then placed in the freeze-dryer and left for 48 hours (<0.4 mbar, -55°C). At this point samples were removed and re-weighed before returning to the freeze-dryer. This was repeated until the samples were at a constant weight. The dry matter (DM) (g/100g) is shown in equation 2.1.

$$DM\ g/100g = \frac{ds}{s}\ \times 100 \quad (2.1)$$

Where s= sample weight and ds= dried sample weight.

2.1.2 Crude Protein

The crude protein content was determined using an EA 1112 elemental analyser (Thermo Scientific, MA, USA). This obtained the nitrogen content

(g/100g) of each sample. For calculations in this thesis, it was assumed that protein contains 16% (w/w) nitrogen; therefore, the nitrogen value was multiplied by 6.25 to obtain the total protein content (g/100g). This elemental analyser uses a combustion reactor to combust samples at 1000°C. The products of this are carried by helium through reagents that produce CO_2, H_2O, N_2 and NO_x. Copper was then used to reduce NO_x to N_2 and samples pass through H_2O and CO_2 traps, before entering a GC column, and then N_2 was detected by a Thermal Conductivity Detector.

Aspartic acid (10.52 (w/w) N%) (D5055, Elemental Microanalysis, Devon) was used as the bypass, standards and quality control. These, and the samples, were all prepared by weighing out 50mg into a tin capsule (Elemental Microanalysis, Devon) which was sealed and pressed into a disc using a sealing device and cylindrical pressing tool. These were stored into a desiccator prior to use. Samples were all dry weight and ground to a powder prior to use.

An initial bypass of 50mg aspartic acid was run at the start of each batch of samples. This was followed by two empty tin capsules, used as negative controls and recorded as blanks. Two aspartic acid samples used as nitrogen standards and then two aspartic acid samples as quality controls were then run. Samples were then loaded into the auto-sampler and a chromatograph was produced for each sample, showing the nitrogen peak. After every 10 samples two aspartic acid samples were run as quality controls and the values checked.

2.1.3 Gross Energy

Gross energy is the total chemical energy, determined by the complete combustion of a food source. Metabolisable energy is more typically used in nutrition and is calculated from the gross energy of a food, minus the energy lost in the urine and faeces (Hall et al. 2013).

The energy content of samples was determined by bomb calorimetry. This determines the enthalpy change of combustion within a high pressure and constant volume reaction chamber (6300 calorimeter, Parr, USA). Calorific grade benzoic acid pellets (1g, Parr, USA) were used as a standard with a known energy value of 26.454 MJ/kg. Energy efficiency can be calculated using equation 2.2.

$$W\ °\mathrm{C} = \frac{Hm+e1+e2+e3}{T} \quad (2.2)$$

Where:

W= Energy equivalent of the calorimeter (°C)

H= Heat of combustion of the benzoic acid standard (cal/g)

m= Mass of the sample (g)

T= Temperature rise (°C)

e1= Correction for heat of formation of nitric acid (cal)

e2= Correction for sulphur

e3= Correction for heating wire and the combustion of cotton ignition thread

1g of each sample was weighed our and flattened into a crucible. This was then placed into the bomb head and a 10cm cotton ignition thread (Parr, USA) was tied on, touching the sample. The exact weight of the sample was recorded, and the bomb head and sample were placed into the bomb calorimeter. When started, the sample was ignited and the increase in temperature was proportional to the amount of energy in the sample. Energy values were given and recorded in MJ/kg DM.

2.1.4 Total Fat

The total fat content of samples was determined using extraction with Gerhardt's Soxtherm system, where the lipid soluble components were separated from the water-soluble components using an organic solvent.

Dried samples were ground into a fine powder prior to this method. 1g sample was weighed onto two layers of filter paper (qualitative grade, 11cm diameter, Fisherbrand). The filter paper was folded to enclose the sample, this was then placed into an extraction thimble and cotton wool was added to secure it. Three boiling stones (Gerhardt, Brackley) were added to each extraction flask before weighing, and the weights were recorded. 130-140ml petroleum ether (Fisher Chemical, MA, USA) was then added to each flask and the thimbles were placed in using holders, so that the petroleum ether surrounded the thimble. Flasks were then attached to the Soxtherm extraction apparatus, and it was switched on.

During the extraction process, the flasks are firstly lowered on to hot plates to heat the solvent to boiling point; this liberates the extractable material within the filter paper. The solvent level is then lowered to below the extraction thimble and excess is collected into a recovery tank. The material is extracted by the refluxing, condensed solvent and is collected in the solvent below the extraction thimble. The bulk of the solvent is then distilled over into the rear storage tank. The beakers are then raised off the heating unit and the flasks can be removed from the apparatus.

After removing the flasks from the apparatus, the metal holders and thimbles were removed from the flasks. The filter paper and contained sample were left in the fume hood to dry for 24 hours. The flasks were removed and placed in an oven at 100°C for 1 hour to evaporate any remaining petroleum ether. After 1 hour the flasks were removed from the oven and placed in a desiccator to cool. Once cool, the flasks were weighed and the total crude fat, per g dried matter, was calculated as shown in equation 2.3.

$$Crude\ fat\ g/100g = \frac{(flask + fat\ weight\ (g)) - Flask\ weight\ (g)}{Sample\ weight\ (g)} \quad (2.3)$$

2.2 Antinutritional Factor Analysis

2.2.1 Total Phenolic Content

This method was adapted from Makkar (2003) (1g tannic acid (TA) was weighed out and diluted in 10ml distilled water. This was vortexed until dissolved and further diluted 1:10 (v/v). From this, a standard curve was created using

distilled water from 0-0.1mg/ml (table 2.1). Tannic acid was used as the standard to give an estimate of the tannin equivalent.

Table 2.2. The standard curve of tannic acid.

TA (mg)	0	0.2	0.4	0.6	0.8	1.0
Water (ml)	1.0	0.8	0.6	0.4	0.2	0
Concentration (mg/ml)	0	0.02	0.04	0.06	0.08	0.1

Ground samples were diluted 1:50 (w/v) in 0.1M NaOH and vortexed for 60 seconds to mix. The solution was then left at 4°C overnight. The following day the sample was vortexed again for a further 60 seconds. A colour change of the solution can be observed from the original clear solution, and the new colour was noted down for each sample. 1ml of each sample was taken and placed into 1.5ml tubes. These were centrifuged (accuSpin Micro, Fisher Scientific, Loughborough) at 9,520xg for 10 minutes at room temperature, the supernatants were then transferred into clean 1.5ml tubes and stored at 4◦C until used. Supernatants then may be further diluted with 0.1M NaOH in order to fit the standard curve, as detailed in each chapter.

25µl of each standard or sample were dispensed into a 96 well plate in triplicate. 200µl distilled water, 25µl Folin-ciocalteu reagent (2N) (1: 3 (v/v) in distilled water) and 25µl sodium carbonate (20% w/v) were added to each well, resulting in a colour change from clear to blue. The plate was then covered in foil to avoid light exposure and left at room temperature for 1 hour before the

absorbance was read at 655nm at room temperature. The tannic acid equivalent (TAE) (mg/g) was calculated by converting the µg/ml value given by the spectrophotometer. This was done by multiplying by multiplying by the µl of NaOH added to the original sample (5000µl), dividing by g sample used (0.1g), multiplying by any dilution factor used and then dividing by 1000 to give the µg/ml value.

2.2.2 Phytic Acid Assay

The measurement of phytic acid/ total phosphorus was measured using the Megazyme phytic acid assay kit (Megazyme, Bray, Ireland), which quantifies total phosphorus released for phytic acid following an incubation with phytase and alkaline phosphatase.

1g of sample was weighed out into a beaker, 20ml of 0.66M hydrochloric acid (HCL) was added and stirred overnight with foil covering the beaker. The following day, 1ml of the extract was transferred into a clean 1.5ml tube and centrifuged at 16,060xg for 10 minutes at room temperature. 0.5ml of the supernatant was then taken and neutralised with 0.5ml of 0.75M sodium hydroxide. This sample extract was then used for the following assay.

Following the sample extraction, an enzyme dephosphorylation reaction assay was carried out. This was run using two reactions simultaneously; a free phosphorus pathway measuring the phosphorus not within the phytate molecule, and a total phosphorus pathway using phytase to release all the

phosphorus from phytate. For this, the following solutions were used, as provided within the Megazyme kit:

- Solution 1: buffer (pH 5.5) with sodium azide (0.02% w/v)
- Suspension 2: phytase suspension (1.2ml)
- Solution 3: buffer (pH 10.4) with magnesium chloride, zinc sulphate and sodium azide (0.02% w/v) as a preservative
- Suspension 4: alkaline phosphatase suspension
- Solution 5: Phosphorus standard solution (50 µg/ml) and sodium azide (0.02% w/v) as a preservative

Table 2.3 Two-way pathway methodology for the enzyme dephosphorylation reaction assay.

	Volume added to 1.5ml tube	
	Free phosphorus	**Total phosphorus**
Distilled water	0.62ml	0.6ml
Solution 1	0.20ml	0.2ml
Sample extract	0.05ml	0.05ml
Suspension 2	-	0.02ml
Mixed by vortex and incubated in a water bath set at 40°C for 10 minutes. After 10 minutes, the reaction was started by the addition of:		
Distilled water	0.02ml	-
Solution 3	0.02ml	0.20ml
Suspension 4	-	0.20ml
Mixed by vortex and incubated in a water bath set at 40°C for 15 minutes. After 15 minutes, the reaction was stopped by the addition of:		
Trichloroacetic acid (50% w/v)	0.30ml	0.3ml
The terminated reaction was centrifuged at 16,060xg for 10 minutes at room temperature. The supernatant was carefully removed and used for the colourimetric determination of phosphorus.		

Following this, a colorimetric determination of phosphorus was completed. For this, a colour reagent was made. This was prepared from two solutions as follows. Solution A was 10% ascorbic acid (w/v) and 1M sulphuric acid, where 10g ascorbic acid was added to 90ml distilled water and 5.35ml concentrated sulphuric acid was added. This solution was then made up to 100ml with distilled water. Solution B was 5% ammonium molybdate (w/v), where 1.25g of ammonium molybdate was dissolved in 20ml distilled water. This was then made up to 25ml with distilled water. The colour reagent was then made by mixing 1 part of solution B with 5 parts of solution A. Standard phosphorus solutions were then prepared as shown in table 2.4.

Table 2.4. Preparations of phosphorus standard solutions for use in the phytic acid assay.

	Standard				
	0 (0µg)	1 (0.5µg)	2 (2.5µg)	3 (5µg)	4 (7.5µg)
Distilled water (ml)	5.00	4.95	4.75	4.50	4.25
Phosphorus standard solution (Solution 5)	-	0.05	0.25	0.50	0.75

Following the preparation of the standard solutions, 1ml of each sample supernatant or phosphorus standard was pipetted into a 1.5ml microfuge tube and 0.5ml of the colour reagent was added. This was then vortexed and incubated in a water bath at 40°C for 1 hour. Following this the solutions were

mixed by vortex and 1ml was transferred to a semi-micro cuvette and the absorbance was read at 655nm on a spectrophotometer at room temperature.

The absorbance of standard 0 was then subtracted from the absorbance of each phosphorus standard (1-4), giving a value for the change in absorbance of phosphorus ($\Delta A_{phosphorus}$). M was then calculated for each standard (1-4) as in equation 2.4.

$$M = \frac{P(\mu g)}{\Delta A phosphorus} \quad (2.4)$$

The obtained M values were then averaged to obtain a mean M value as in equation 2.5.

$$mean\ M = \frac{M_{STD1} + M_{STD2} + M_{STD3} + M_{STD4}}{4} \quad (2.5)$$

For the samples the free phosphorus absorbance was then subtracted from the total phosphorus absorbance to obtain a $\Delta A_{phosphorus}$ value. The concentration of phosphorus (c) was then calculated using equation 2.6.

$$C = \frac{mean\ M \times 20 \times 55.6}{10{,}000 \times 1.0 \times 1.0} \times \Delta A_{phosphorus} \quad (2.6)$$

Where:

- mean M= the mean value of phosphorus standards (µg/ $\Delta A_{phosphorus}$)
- 20= the original sample extract volume (ml)
- 55.6= dilution factor
- ΔA= absorbance change of sample
- 10,000= conversion from µg/g to g/100g
- 1.0= weight of original sample material (g)
- 1.0= sample volume (used in the colourimetric determination)

The value for phosphorus was then converted into phytic acid using equation 2.7.

$$Phytic\ acid\ \left[\frac{g}{100g}\right] = \frac{c}{0.282} \quad (2.7)$$

Where 0.282 is used on the assumption that the change in phosphorus concentration is due to exclusive release from phytic acid, and that this comprises 28.2% of phytic acid.

2.2.3 Trypsin inhibitor activity

The trypsin inhibitor activity was considered one of the major antinutritional factors of interest in this study. The measurement of this was attempted for both bambara groundnut seeds and mezquite pod flour using the

absorbance-based assay as described by Kakade et al. (1974). This was unsuccessful for the samples used in this thesis, due to a background absorbance of the samples interfering with the assay absorbance, leading to negative trypsin inhibitor activity being measured.

2.3 Broiler Chicken Trials

2.3.1 Feed Formulation

Broiler chicken diets were supplied by Target Feeds (Whitchurch, UK). For both broiler trials a mash feed was used. A wheat and soybean meal feed was used as a control diet and the basal feeds were supplied by Target Feeds to allow for inclusion of mezquite pod flour on site. The basal diets were altered to give isonitrogenous and isoenergetic feeds once each mezquite pod flour variety was added. The components of each basal feed formulation will be defined in the corresponding chapters. All diets also contained titanium dioxide (Feed grade, Azelis, Belgium) at 5g/kg, used as an indigestible marker to determine apparent ileal digestibility, this was added on site.

Table 2.5 Formulation of ingredients of the control diets used in both broiler chicken trials.

Raw Material	g/100g (w/w) of Ingredient
Barley Raw Ground	10.5
Wheat Raw Ground	50
Soya Ext Hipro	26
Full Fat Soya MASHAM	5
L Lysine Batch	0.4
DL Methionine Batch	0.4
L Threonine Batch	0.15
Soya Oil	4
Limestone Trucal 52	1.25
Monocalcium Phosphate	1.5
Salt	0.25
Sodium Bicarbonate	0.15
Broiler trial starter premix[1]	0.4

[1]Supplying: retinoic acid 4.8 mg/kg, cholecalciferol 75 µg/kg, α-tocopherol75 mg/kg, thiamine 3 mg/kg, riboflavin 10 mg/kg, pyridoxine 3 mg/kg, cobalamin 15 µg/kg; nicotinic acid 60 mg/kg, pantothenic acid 14.7 mg/kg, folic acid 1.5 mg/kg, biotin 125 µg/kg, choline chloride 25 mg/kg, Fe 20 mg/kg, Cu 10 mg/kg, Mn 100 mg/kg, Co 1.0 mg/kg, Zn 82.222 mg/kg, I 1 mg/kg, Se 0.2 mg/kg and Mo 0.5 mg/kg.

2.4.2 Feed Mixing

All feeds for both trials were subjected to mixing, this was done on site at the University of Nottingham. For the control diets, one scoop of feed (approximately 1.2kg) was taken out of each 25kg bag and mixed with 125g of

titanium dioxide in a separate bag, a further 4 scoops were added to this with continual manual mixing. The remainder of the 25kg bag of feed was added to a screw mixer (100L capacity, Gardner, Gloucester), followed by the feed and titanium dioxide mix. This was repeated for another 25kg bag of feed, giving 50kg of feed and 250g titanium dioxide in the screw mixer, which was mixed for 5 minutes after all components had been added. The mixed feed was then split back into two 25kg bags which were then sealed. This was repeated until all the control feed was mixed.

Experimental diets were also mixed on site. 21.25kg of basal feed was supplied of each diet to allow for 3.75kg of mezquite pod flour to be added in for a 15% (w/w) inclusion. The same procedure for the addition of titanium dioxide was repeated and the 3.75kg of mezquite pod flour was added per 22.5kg basal feed. This was repeated twice and mixed for 5 minutes once all components were added, as for the control diets. After mixing, the 50kg was split into two 25kg bags and sealed. All sealed bags of feed were then stored in a cool dry room until needed.

2.3.3 Subjects and Husbandry

One day old Ross 308 Broiler chicks were used in both trials, spare birds were ordered above the required to allow for extra in case of any birds suffering from ill health or perishing during the first few days of the trial. Specifics of the birds used in each trial will be detailed in the corresponding chapters.

The litter of each pen consisted of wood shavings, which were topped up to meet welfare considerations. Feed and water in trays were supplied *ad libitum.* Temperature and ventilation were monitored for appropriateness for the birds depending on age. The birds were inspected at least twice per day to monitor environmental conditions and bird welfare. Birds were weight and their feed intake was recorded twice weekly.

2.3.4 Sample Collection

In both trials, bird weights and feed intakes were recorded twice per week. Specific details of the performance data collection will be explained for each trial in the corresponding chapters. Both trials ran for 35-36 days before birds were culled and samples were taken. Details of the samples collected can be found in the respective chapters. For both trials, sample collection was limited due to COVID-19 social distancing restrictions in trial one and issues relating to the location, the number of people available, and transport of samples in trial 2. Analysis of faecal samples for cell sloughing was considered, as was the measurement of pancreas weights as an indication of trypsin inhibitor activity. While both of these would have been useful measurements to take, they were not possible for this thesis.

2.4.5 Apparent Ileal Digestibility

2.4.5.1 Sample Preparation

Ileal digesta samples from both broiler chicken trials were freeze-dried to a constant weigh, as described in 2.1.1, in plastic containers with the lids

removed. Ileal samples were combined to give one sample per pen (4 birds). These samples were ground using a pestle and mortar to give a fine powder.

2.4.5.2 Titanium dioxide measurement

The titanium dioxide (TiO_2) content of both ileal digesta and feed samples was determined using a method adapted from Short et al. (1995). All feed samples and ileal samples from both broiler trials were measured.

Firstly, the following reagents were prepared:

- Titanium solution (0.5mg/ml), 250mg TiO_2 was weighed into a 600ml beaker and 100ml concentrated sulphuric acid (98%) was added. This was placed on a hot plate in a fume hood and heated until simmering. This was left until the solid had dissolved, approximately 2 hours, and then left to cool on a heat resistant surface. Distilled water (200ml) was then added into a 500ml volumetric flask and place in a bucket of cold water, then slowly the sulphuric acid and TiO_2 solution was added. 100ml concentrated sulphuric acid was then added and the solution was taken up to 500ml with distilled water.

- 7.4 M sulphuric acid solution- 400ml distilled water was added to a 1L bottle and placed in a bucket of cold water in a fume hood. 400ml concentrated sulphuric acid was added and the solution was taken up to 1L with distilled water.

0.2g dry weight ileal digesta or broiler feed was weighed out, with each sample being measured in duplicate, into porcelain crucibles (60mm, FSE). A weight for the crucible and the crucible containing the sample was recorded. The samples were incinerated to an ash overnight in a muffle furnace (Carbolite, Derbyshire).

After ashing, 10ml 7.4M sulphuric acid solution was dispensed into each crucible and they were placed on a hotplate in a fumehood until dissolved, without allowing the samples to boil, this was approximately 250°C for 1 hour. The samples were then removed from the hotplate and left in the fumehood to cool. 25ml distilled water was added to a beaker per sample and the crucible contents was added, rinsing the crucible twice with distilled water. This solution was then poured into a labelled 100ml volumetric flask per sample through a quarter folded filter paper (Whatman 541, hardened ashless) using a funnel. The beaker was then rinsed with 25ml distilled water and this was also added through the funnel. The funnel was then removed, and 10ml 30% (v/v) hydrogen peroxide (Merck) was added and the solutions made up to 100ml with distilled water. The contents of the flask were then mixed, giving a yellow/ orange colour.

A calibration curve of TiO_2 samples was prepared as in table 2.6, in 100ml volumetric flasks (Table 2.6)

Table 2.6. Preparation of titanium dioxide standards to give a calibration curve.

	Standard										
	0	1	2	3	4	5	6	7	8	9	10
0.5mg/ml Titanium solution (ml)	0	1	2	3	4	5	6	7	8	9	10
Concentrated sulphuric acid (ml)	10	9	8	7	6	5	4	3	2	1	0
Distilled water (ml)	10	10	10	10	10	10	10	10	10	10	10
30% hydrogen peroxide (ml)	10	10	10	10	10	10	10	10	10	10	10

Following the preparation of the standards, the absorbance of all standard and sample solutions was measured at 410 nm using a spectrophotometer at room temperature, which was zeroed on the blank calibration setting. A calibration curve can then be drawn from the absorbance of the standards. The amount of TiO_2 (mg) per mg of the samples can then be calculated using equation 2.7.

$$TiO_2(mg) = \frac{absorbance}{X\ Coefficient \times mass\ of\ sample\ (mg)} \quad (2.7)$$

Where the X Coefficient is taken from the line equation of linearity of the calibration curve.

2.4.5.3 Amino Acid Analysis

The amino acid content of samples was analysed by LCMSMS. Samples were prepared for this using a hydrolysis by oven crimp tube digestion method. This method was optimised in house by Dr. Dongfang Li, technical specialist in the school of Biosciences, who also completed all sample analysis on the LCMSMS.

Before beginning the procedure, the following reagents were prepared:

- Formic acid/ Phenol solution: 735ml formic acid was mixed with 111ml of Milli Q water (Quantum Tex) and 4.75g phenol was added

- Oxidation solution: 10ml 30% (v/v) hydrogen peroxide was dispensed into a 100ml volumetric flask and made up to volume with formic acid/ phenol solution. This was incubated at 20-30°C for 1 hour, the solution then turned yellow. This was then cooled at 4°C before adding to samples. This solution was prepared fresh approximately 2 hours before beginning the procedure.

- 4M ammonium formate in water: 252.4g of ammonium formate (99%) (BDH Chemicals) was placed into a 1L bottle and made up to 1L with Milli Q water.

- 100mM ammonium formate in water: 6.31g of ammonium formate (99%) (BDH Chemicals) was weighed out, placed into a 1L bottle and made up to 1L with Milli Q water.

- 20mM ammonium formate buffer, pH 2.75: 200ml of 100mM ammonium formate in water was added to a 1L beaker, 2ml of formic acid and 700ml of water was added and mixed thoroughly. The pH was then adjusted to 2.8 with formic acid, it was transferred to a 1L bottle and made up to 1L with water.

A sample containing an estimated 5mg nitrogen was weighed out, which on average is 30g of sample. The weight was recorded, and the sample was then placed into 20ml crimp top tubes, ensuring the sample was deposited at the bottom of the tubes. Two samples of soy flour (NIST, MD, U.S) were also run per batch of samples as a standard reference material, the procedure for these followed the same as the experimental samples. 2.5ml of chilled oxidation solution was then added to each sample and they were stored at 4°C for 16-18 hours.

Following this, 0.42g of sodium metabisulphite (Fisher Scientific) was added to each sample to decompose excess oxidation reagent. 2.5ml 12M hydrochloric acid (HCL) and 0.5ml hydrolysis reagent was then added to each tube. Each tube was then crimp topped (chromatography direct) using a 20mm crimper (Supelco), ensuring a tight seal, and placed in the oven at 110°C for exactly 24 hours.

Once removed from the oven, the samples were left to cool for 10 minutes and the solution was transferred into 50ml tubes (Sarstedt), using ammonium formate buffer (20mM) to rinse the contents into the falcon tubes. The samples were partially neutralised by the addition of 10ml 4M ammonium Formate

solution. The pH of each sample was then adjusted, using a pH meter (Fisherbrand, Hydrus 400), to 2.75 using 4M ammonium formate and formic acid (pH 2.1) as needed. The volume was then made up to 50ml with ammonium formate buffer (20mM) and the tubes were centrifuged (Thermo Scientific, Heraeus Megafuge 40) at 3000rpm for 10 minutes. Approximately 1ml of the resulting supernatant was then passed through a 0.22µm filter (chromatography direct), using a syringe (BD Discardit™ II), into labelled 1.5ml tubes and frozen at -80°C until needed. Before running on the LC-MSMS, the 1.5ml tubes containing the sample solution were defrosted and 100µl of the sample was transferred into a labelled HPLC vial (chromatography direct). 100µl of 20mM ammonium formate buffer (pH 2.75) was then added, followed by 200µl of cell free stable isotope label (CFSIL) to give a final dilution factor of 4. The samples were then ready for analysis but could be kept at this stage at 4°C for 2 weeks or at -20°C for up to 1 year.

All samples were then separated and analysed using a Thermo-Fisher Vanquish (uHPLC) and Altis Triple Quadrupole Mass Spectrometer (MS/MS) with heated electrospray ionisation (H-ESI) spouse. For all amino acids, the positive ion mode was used.

1µl was injected on to a Thermo Scientific™ Acclaim™ Trinity P1 mixed mode column at 30°C. Mobile phases consisted of ammonium formate (pH 7.5) in water for phase A and the same mixed with acetonitrile (80:20 (v/v)) for phase B. Chromatographic separation was achieved by gradient elution under the conditions described in Table 2.7.

Table 2.7 Gradient elution for Thermo Vanquish uHPLC system.

Time (mins)	Flow Rate (ml/min)	%A	%B
0	0.3	100	0
5	0.3	100	0
7	0.3	0	100
11	0.3	0	100
12	0.3	100	0
12.5	0.45	100	0
14.5	0.45	100	0
15	0.3	100	0
18	0.3	100	0

Compounds were detected on a Thermo Scientific™ TSQ Altis™ triple quadrupole mass spectrometer equipped with a Thermo Scientific™ Easy-Max NG ion source with a heated electrospray ionization probe. Sheath gas was set at 45 arbitrary units, auxiliary gas at 15 arbitrary units and spray voltage at 3500 V for positive ionisation and at 2700 V for negative ionisation. The vaporiser temperature was set at 370°C and transfer tube temperature at 70°C. Source fragmentation was applied at 15 V. Data was acquired in Selected Reaction Monitoring (SRM) mode using a resolution of 0.7 full width at half maximum (FWHM) for both quadrupoles. All compounds were detected at positive mode.

Peak detection was carried out in the software: TraceFinder 4.1 General Quantification (Thermo Fisher Scientific, MA, US). Where a raw data file was used to detect each target peak and the retention times adjusted to peaks are detected for each amino acid in all standards and samples. Each peak was manually checked on the software to ensure the correct peak was detected and the retention time of the detected peak and internal standard match. The peaks were also adjusted to ensure that only the correct peak area was highlighted before they were quantified by the software. This gave the amino acid content in g/kg for each sample and the standard reference material (soy flour) for each batch.

2.4.5.4 Apparent Ileal Digestibility Calculations

The apparent ileal digestibility percentage was calculated for both crude protein and amino acids. This was calculated using equation 2.8, as seen in O'Neill et al. (2012):

$$Digestibility = 100 - (\frac{Ti_D \times N_1}{N_D \times Ti_I} \times 100) \quad (2.8)$$

Where:

N= Nutrient

Ti= Titanium dioxide

N_I= Nutrient in the ileal digesta

N_D= Nutrient in the diet

Ti_I= Titanium in the ileal digesta

Ti_D= Titanium in the diet

2.5 Statistical Analysis

Statistical analysis was conducted using Genstat 20th Edition software to determine significant differences. All significant differences were determined by a one, two or three-way ANOVA, as stated in individual chapters. Significance was taken at $p<0.05$. All graphs were drawn using Graph Pad Prism 8.

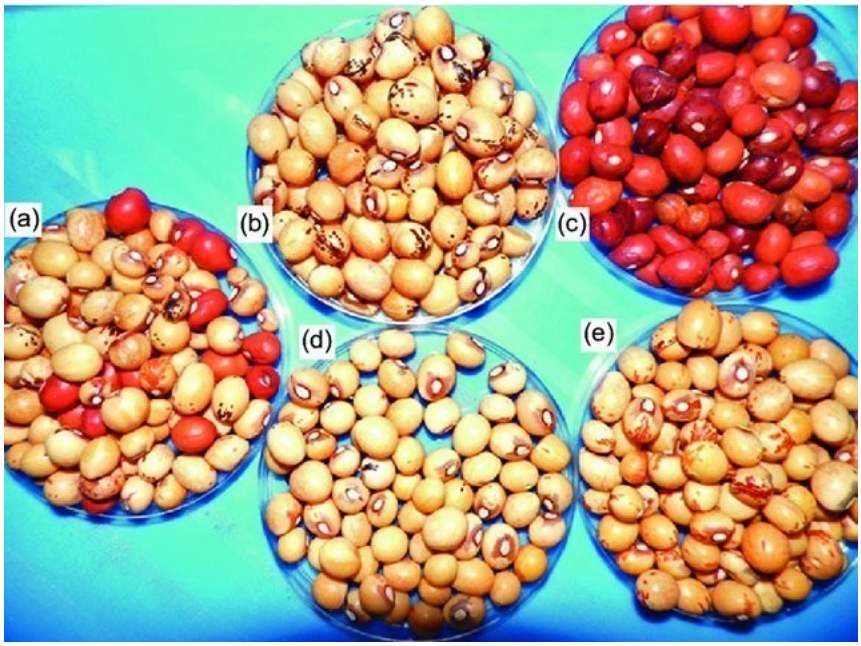
3 Bambara groundnut as a potential alternative crop for human and animal consumption

3.1 Introduction

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is considered the third most important legume crop in much of Africa (Gulzar, Minnaar 2017). Named after the Bambara tribe, it is considered to be a pulse crop, a subset of legumes harvested only for their seeds. This is because it is the seeds of bambara groundnut crops which are focussed on as the potential edible component (Halimi et al. 2019). Despite local importance as a plant-based protein for the rural poor of sub-saharan Africa, bambara groundnut crops are under-utilised largely due to the fact that they remain cultivated from landraces rather than as specific landraces (Massawe et al. 2016a, Halimi et al. 2019). Its harvest and processing rely on local knowledge, which is not exchanged throughout different, very localised, regions; meaning there is no widespread knowledge about the crops uses (Azam-Ali et al. 2001).

Although morphological variability within bambara landraces have been reported, there is little information available about genotypic variation (Massawe et al. 2005). Varieties are often chosen to be continually grown based on their drought tolerance, due to the hot and dry growing locations (Tan

et al. 2020). However, there are visible differences in seed varieties produced from a landrace, characterised by their size and seed coat colour (Figure 3.1). There are also seed variations of length, width, thickness and geometric diameter. Having lots of phenotypical variation increases the likelihood of a wider genetic variation within an area of growth. This acts as insurance; providing stress tolerance, local adaptation and yield stability (Tan et al. 2020). Darker coloured seeds are also reported to show semi-impermeability to water, giving them greater weather resistance compared to lighter coloured seeds (Mandizvo, Odindo 2019). This may be why lighter coloured seeds are reported to have shorter cooking times (Murevanhema, Jideani 2013). Different coloured seeds are favoured in different places, with cream seeds reportedly being more sought after in Ghana and red seeds being more popular, as well as reaching a higher price, in Zimbabwe (Hillocks et al. 2011).



Source: Chibarabada et al. 2014).

Figure 3.1. Bambara groundnut seeds from different landraces can be characterised by variable seed coat colour. (a) Mix of coat colours, (b) black-speckled colour, (c), red colour, (d) cream colour and (e) brown-speckled colour (Chibarabada et al. 2014).

The edible bambara groundnut seeds are reported as being a 'complete food', as they contain sufficient concentrations of proteins, carbohydrates, fats and minerals for a nutritionally balanced adult diet (Gulzar, Minnaar 2017). Amarteifio, Moholo (1998) estimated the mineral content for 100g seeds as 59mg Iron, 1240mg potassium, 296mg phosphorus, 3.7mg sodium and 78mg calcium. The protein content is variable, with 19-27% dry weight (DW) reported (Oyeyinka, Oyeyinka 2018), which although sufficient, is lower than many pulse seeds which contain between 20-40% protein (Azman Halimi et al. 2019). However, the seeds do contain high levels of lysine (6.5-6.8% DW) and

methionine (1.8-2.84% DW), which are often limiting amino acids in cereals or legumes respectively, suggesting a high-quality protein (Oyeyinka, Oyeyinka 2018). Soluble fibre is reported at 2-12%, there is limited experimental data on the insoluble dietary fibre concentrations, but Yao et al. (2015) reported that it is approximately half that reported for chickpea but follows the general pattern for pulses.

The carbohydrate component of bambara groundnut includes a high proportion (up to 53% whole weight) of starch. This is comparable to the reported level in chickpea and over four times the maximum reported levels for soybean (Azman Halimi et al. 2019). Starch modification techniques have been performed on the bambara groundnut with the intention of eliminating the problems faced with native starches, such as poor resistance to extreme processing conditions (Oyeyinka, Oyeyinka 2018). Bambara groundnut starch has previously been modified using carboxymethylation (Afolabi 2012), annealing and moist heat (Adebowale et al. 2002). Physical methods, such as annealing, moist heat and more recently, microwaving, are the most commonly favoured methods of starch modification as they are generally considered safer and more environmentally friendly (Oyeyinka, Oyeyinka 2018).

In sub-Saharan Africa, there are already many nutritional uses for the bambara groundnut. Fresh seeds can be processed by grilling or boiling before consumption but can also be processed further in order to produce more marketable products (Mubaiwa et al. 2018). Dried seeds can be ground into flour to be used in baking and the creation of porridge (Olatunde et al. 2021),

soup and a substitute for coffee(Mubaiwa et al. 2018). The seeds can also be soaked, wet milled and strained in order to produce a vegetable milk, comparable to soya milk (Azam-Ali et al. 2001; Murevanhema, Jideani 2013). Milk extracted from legumes is not commonly marketable, due to the associated beany flavour. However, this taste can be removed by dry-frying (Brough et al. 1993) or otherwise heat treating the bambara seeds before milk extraction (Nti 2009). While processed bambara groundnut seeds may be used alone to create such products, they can also be blended with traditional ingredients to improve nutritional value. For example, Akpapunam and Darbe (1994) found that cookies prepared with blended maize flour and bambara groundnut flour contained 10.9-21.65% protein. They also received good sensory evaluation scores and were found to be acceptable to consumers due to the mixing of flours. However, storage of bambara groundnuts can cause an issue in developing countries, as storage at high heats and relative humidity's can cause the hard-to-cook (HTC) phenomenon. HTC seeds require a longer boiling time of 3-4 hours compared to fresh seeds, before they become edible. While this is not a major problem in developed countries, this high energy expenditure needed for cooking is a significant limiting factor in the countries where bambara is commonly utilised (Mubaiwa et al. 2017).

3.1.1 Bambara groundnuts in animal feed

Due to their nutritional composition, the bambara groundnut has been trialled in animal feed studies to see how it affects production. Oloyede et al. (2010) fed four experimental diets to sixty one-day old broiler chicks; a control

soybean-based diet, a diet containing 35g/100g w/w raw dehulled bambara groundnut, a diet containing 35g/100g w/w roasted bambara groundnut, and a diet containing 35g/100g w/w fermented bambara groundnut. All diets were balanced for dry weight crude protein concentration. They found that all bambara groundnut diets significantly decreased the final live weight and the feed intake. However, of the bambara treatment groups, raw bambara performed the best, with a similar feed conversion ratio (FCR) to the control group. (Teguia, Benyen 2005) also found that the inclusion of bambara in broiler feed decreased overall growth rates throughout a trial, compared to a control diet.

(Ubua et al. 2018) studied the effects of bambara groundnut seed meal on the performance of grower rabbits in Nigeria. Bambara seed meal was included at 25g/100g DW in four treatment groups: unprocessed, boiled, toasted and water-soaked seeds. Results showed that there were no statistically significant differences in total weight gain in the rabbits when fed differently processed bambara groundnut seed meal. However, rabbits fed the soaked diet showed the best feed conversion ratio and highest weight gain; suggesting that soaking is the most effective processing method to be used before feeding bambara groundnut. Ani (2007) also conducted a similar experiment in weaner rabbits fed diets with inclusions of 0, 10, 20 and 30% raw bambara groundnut waste from bambara product production. They found that rabbits fed the 20 and 30% inclusions had significantly lower ($p<0.05$) feed intake and final body weights than those fed the other diets. While this evidence suggests that bambara

groundnut seeds have a negative effect on monogastric species, likely due to the presence of anti-nutritional factors (ANFs), there does not appear to be any literature exploring its effects in ruminant species which may not be as highly affected by ANFs.

3.1.2 Health benefits of the bambara groundnut

As well as the nutritional value, the bambara groundnut has also been suggested to provide human health benefits. In African countries, bambara seeds are reportedly used as a treatment of diarrhoea and are believed to help prevent colon cancer due to their levels of soluble fibre. The chewing of immature seeds is also reportedly used to treat morning sickness during pregnancies, as it is believed to relieve nausea and vomiting (Koné et al. 2011). Bambara groundnut milk also has a strong nutritional profile, which reportedly may be able to sustain the growth of probiotics. For example, the milk could potentially be fermented with lactic acid bacteria in order to make a probiotic beverage, which can be beneficial to GIT health (Murevanhema, Jideani 2013). Balogun et al. (2018) also found that bambara offered significant protection against mucosal ulceration in rats with aspirin-induced gastric mucosal injuries. The gastric submucosal layer showed a decrease in leucocyte infiltration compared to rats not fed bambara. However, much of this information is anecdotal with little experimental data to back up the claims.

3.1.3 Anti-nutritional factors in bambara groundnut

Plant secondary metabolites (PSMs) in bambara groundnut are a contributing factor to its apparent disease and pest resistance shown in drier areas. They may act as biocides and contribute to yield stability against disease while the seed hull protects against insects (Hillocks et al. 2011) However, antinutritional factors are one of the limiting factors in the upscale of underutilised crop consumption. Bambara groundnut seeds have been found to contain trypsin inhibitors (17.3 Tiu/mg), phytic acid (18.5 mg/g) and tannins (1.8g/100g) (Gulzar, Minnaar 2017). Although the presence of these PSMs can limit their consumption and use, particularly in animal feed, they are presumed to be largely contained in the hulls of the seeds. The hulls can therefore be removed before consumption and possibly utilised in other ways, as PSMs can be an inexpensive source of nutraceuticals (Harris et al. 2018).

Aims

1. To determine the nutritional potential of different landraces of bambara groundnut seeds

2. To determine the levels of antinutritional factors present in different landraces of bambara groundnut seeds

3. To assess the protein and total phenolic content of bambara groundnut milk

Hypothesis

1. Different landraces of bambara groundnut seeds will have significantly different levels of nutrients and antinutrients

2. Phenolic compounds will predominantly be found in the seed hulls

3. Milk produced from bambara groundnut seeds will have protein levels comparable to currently marketed vegetable milks

3.2 Materials and Methods

3.2.1 Bambara groundnut crops

Two separate batches of bambara groundnut seeds were used in this study. For batch one, seven landraces (Table 3.1) (Figure 3.2) of bambara groundnut seeds were used. For batch 2, five landraces (Table 3.2) (Figure 3.3) were used. All seeds were harvested in Malaysia and shipped to the University of Nottingham for this analysis. These same two batches were used throughout this chapter of work. The two batches of seeds cannot be compared to one another, as they were grown at different times, and were received and analysed separately. While all seeds were grown in a controlled environment in Malaysia, growth conditions and genetic variability is unknown. The different landraces are considered as different due to original locations and are characterised as separate due to the physical attributes such as hull colouration and eye colouration.

Table 3.1 Seven landraces of bambara groundnut seeds used in batch one for this study.

Bambara Landrace- Batch 1	Seed hull colour
DipC	Cream
Kano2	Cream
IlTA x LunT LI20	Tan
R/G	Brown
R/R	Red
S19-3	Black
IITA-686	Black



Figure 3.2. The seven landraces of bambara groundnut used for batch one, with varying hull colours shown.

Table 3.2 Five landraces of bambara groundnut seeds used in batch two for this study.

Bambara Landrace- Batch 2	Seed hull colour
Cream light brown eye (CLBE)	Cream
Exsokoto	Cream
Songkhla	Red
Cikur 2.1	Black
IPB Bam 6	Black



Figure 3.3. The five landraces of bambara groundnut used for batch two, with varying hull colours shown.

3.2.2 Seed Preparation

3.2.2.1 Seed dehulling

Seeds were dehulled in order to assess differences in the antinutrient content between the hulls and seeds without hulls, as indicated in the literature. 5g bambara seeds were soaked in 35ml distilled water for 24 hours before manually removing the seed hulls by hand, as per Alobo (1999) and Bulus et al. (2022). This left the separated hulls and their corresponding dehulled seeds (seeds with no hulls), these will be referred to as hulls and dehulled seeds. Manual dehulling was a difficult process, and not all seeds could be dehulled in a uniform way, meaning that the amounts available were unpredictable. A non-manual way of dehulling the seeds would have beneficial for this study to maximise the potential.

The seeds and corresponding dehulled seeds were then spread out in foil trays and placed in the oven for 12 hours at 60^{o}C to dry them out. These were then ground to a fine flour using an electric coffee grinder (Braun, Mexico).

3.2.2.2 Sample extracts

0.1g finely ground flour samples were extracted 1:50 (w/v) in 0.1M NaOH and mixed by vortexing for one minute. Solutions were then incubated at 4^{o}C for 24 hours before vortexing for one minute again. 1ml of the resulting solution was then transferred to a 1.5ml tube and centrifuged at 16,060xg for 10 minutes and the supernatants taken.

3.2.2.3 Bambara milk production

The method for milk production from bambara groundnut seeds was adapted from (Brough et al. 1993). Dehulled seeds were soaked in distilled water for 24 hours before adding to warm water 1:2 (w/v) and blending in a coffee grinder (Braun, Mexico) to give a pulpy consistency which was then vortexed at a high speed. This was strained through filter paper (Whatman) and the resulting milk was collected.

3.2.2.4 Freeze drying

A sample of each bambara landrace was freeze dried until a constant weight as described in Chapter 2.1.1. These were dried as whole seeds in foil containers with no lid. Once dried, the whole seeds were ground to a fine flour in a coffee grinder (Braun, Mexico).

3.2.3 Nutrient analysis

3.2.3.1 Crude Protein

The crude protein was determined using an EA 1112 elemental analyser (Thermo Scientific) to obtain the nitrogen content per sample, as described in Chapter 2.1.2.

3.2.3.2 Soluble Protein

Soluble protein was determined by method of Lowry against a BSA standard curve from 0.2-1.2mg/ml as per Lowry et al. (1951). Sample extracts from hulls

were diluted 1:4 (v/v) and extracts from dehulled seeds were diluted 1:2 (v/v) in 0.1M NaOH prior to running this assay. Bambara milk was assayed at a 1:10 (v/v) dilution in distilled water.

50ul standard or 10ul sample was pipetted into wells of a 96 well plate and 150/ 190ul 0.1M NaOH added. 50ul reagent 1 (5ml 2% (w/v) Na2CO3, 0.5ml 1% CuSO4, 0.5ml 2% KNa Tartate) was then added to all wells and mixed. After five minutes 50ul reagent 2 (0.5ml Folin Ciocalteu reagent, 5ml 0.1M NaOH) was added. The plate was then left at room temperature for 20 minutes before being read at 655nm.

3.2.3.3 Gross Energy

The energy content of the dry weight samples was determined by bomb calorimetry (6300 calorimeter, Parr). Calorific grade benzoic acid pellets (1g, Parr) were used as a standard. This is further described in Chapter 2.1.3.

3.2.4 Antinutritional content analysis

3.2.4.1 Total Phenolic Content

25ul of each sample extract were added to a 96 well plate alongside a tannic acid standard curve, as described in Chapter 2.2.1. Sample extracts from hulls were diluted 1:4 (v/v) and extracts from dehulled seeds were diluted 1:2 (v/v) in 0.1M NaOH prior to running this assay. Bambara milk was assayed at a 1:10 (v/v) dilution in distilled water. The absorbance was read at 655nm.

3.2.4.2 Phytic Acid

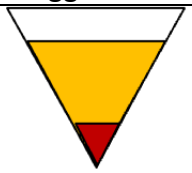
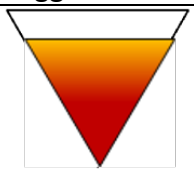
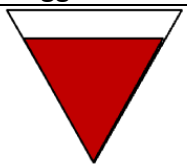
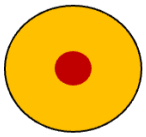
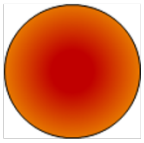
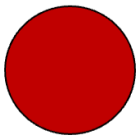
A phytic acid assay kit (Megazyme) was used to measure the phytic acid/ total phosphorus content of the bambara seeds. This used 1g DW of each bambara sample. The full method is described in Chapter 2.2.2.

3.2.4.3 Haemagglutination Assay

Blood samples from pigs were collected on site at the university of Nottingham as part of a trial unrelated to this thesis. These were collected in EDTA coated blood collection tubes (Vacuette, Greiner Bio-One, Austria) and centrifuged immediately (Beckman GS-6R) at 1900xg (4°C) until the plasma and cells separated. The plasma was removed, and cells including erythrocytes were suspended at 4% (w/v) in PBS buffer (Cortés- Giraldo et al. 2012).

A haemagglutination assay was adapted from (Cortés-Giraldo et al. 2012). Extracts from dehulled seeds and hulls, as section 3.2.2.2, were used for this assay and serial diluted in 0.1M NaOH from 1:2- 1:16 (v/v). A U-shaped 96 well plate was used; to each well, 25µl of suspended erythrocytes in PBS was added, with 25µl of a decreasing concentration of sample extracts from the hull or dehulled seed and 25µl of PBS. This was incubated for one hour at room temperature and the agglutinating activity was read as negative, intermediate or positive for each well (Table 3.3).

Table 3.3 The visual classification of negative, intermediate and positive agglutination of erythrocytes, as observed in the wells. Adapted from Cortés-Giraldo et al. (2012)

	Negative agglutination	Intermediate agglutination	Positive agglutination
Side view of wells			
Overhead view of wells			

3.2.5 Amino Acid Analysis

The amino acid content of the seeds was analysed by LCMSMS following a hydrolysis by oven crimp tube digestion. This method is described in Chapter 2.2.5.3.

3.2.6 Data Analysis

Statistical analysis was conducted using Genstat 20th Edition software to determine if there were any significant differences found between the different bambara landraces. A one-way analysis of variance (ANOVA) was used to determine any possible significant differences between landraces. Significance was taken at $p<0.05$ and errors reported as standard error of the mean (SEM). All graphs were drawn using Graph Pad Prism 8.

3.3 Results

3.3.1 Nutritional Analysis

There was a significant difference ($p<0.001$) (Table 3.3) in the crude protein content between different landraces of bambara groundnut analysed in batch 1. A Bonferroni post hoc test determined that this was due to the crude protein content of Uniswa RG being significantly higher than all other samples. There was also a significant difference in the gross energy content of the different landraces ($p=0.002$) (Table 3.3) with a Bonferroni post hoc test showing that this is due to Kano 2 seeds having significantly lower gross energy than IITA-686, LI20, DipC and Uniswa RR seeds. There were no significant differences ($p=0.760$) in the dry matter content of the bambara samples (Table 3.4).

Table 3.4 Nutritional analysis of bambara groundnut seeds in batch 1.

	Crude Protein (g/100g)	Gross Energy (mJ/kg)	Dry Matter (g/100g)
DipC	20.21[a]	19.02[b]	91.7
Kano 2	19.49[a]	18.57[a]	91.3
LI20	21.06[a]	19.00[b]	87.8
R/G	25.86[b]	18.82[ab]	91.4
R/R	21.32[a]	19.13[b]	90.3
S19-3	22.44[ab]	18.83[ab]	91.1
IITA-686	20.27[a]	18.93[b]	99.7
SED[1]	1.024	0.0657	7.08
P Value[2]	**<0.001**	**0.002**	0.760

[1] SED- standard error of the differences of the means, n=3 technical replicates for each sample.

[2]P values determined by a one-way ANOVA. Different letters within columns indicate a significant difference.

For batch two, there was a significant difference (p=0.002) in the crude protein content of the different bambara landraces (Table 3.4), this was due to a significantly lower crude protein content in Songkhla compared to the other landraces. There was a trend a significant difference between the different landraces in batch two for the energy content (p=0.05) (Table 3.5), with Exsokoto having the lowest value with CLBE and IPB Bam 6 having the highest.

Table 3.5 Nutritional analysis of bambara groundnut seeds in batch 2.

	Crude Protein (g/100g)	Energy (mJ/kg)
CLBE	21.98[b]	18.36
Exsokoto	24.41[b]	18.06
Songkhla	15.82[a]	18.10
Cikur 2.1	22.09[b]	18.18
IPB Bam 6	21.98[b]	18.36
SED[1]	1.201[b]	0.103
P Value[2]	**0.002**	0.050

[1] SED- standard error of the differences of the means, n=3 technical replicates for each sample.

[2]P values determined by a one-way ANOVA. Different letters within columns indicate a significant difference.

For the seeds in batch 1, there was a significant interaction ($p<0.001$) (Figure 3.4) between the type of sample (hulls or corresponding dehulled seed) and the bambara landrace. For landraces IITA-686, R/G and L120, there was no difference between hulls and dehulled seeds, whilst for all other landraces, the dehulled seed contained significantly more protein than the hull ($p<0.05$).

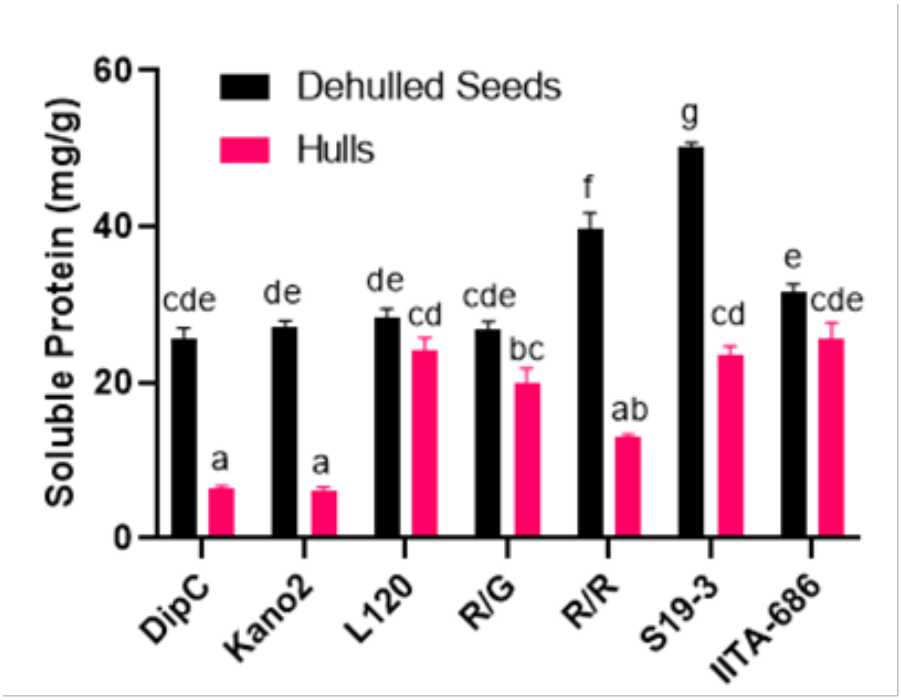


Figure 3.4. The soluble protein content (mg/g) of the separated dehulled seeds and hulls of each analysed batch 1 landrace of bambara groundnut. Data shown as means with SEM error bars, calculated using n=3 technical replicates. Significance is taken at $p<0.05$, determined using a two-way ANOVA. Different letters indicate a significant difference according to a Bonferroni post-hoc test ($p<0.05$).

For the seeds in batch 2, there was also a significant interaction ($p<0.001$) (Figure 3.5) between the type of sample (hull or dehulled seed) and the bambara landrace. Only Exsokoto had no difference between hull or dehulled seeds. The other seeds of this batch had significantly ($p<0.05$) higher protein in the dehulled seeds compared to the hull apart from Songkhla who's hulls uniquely had significantly ($p<0.05$) higher soluble protein content within the hulls.

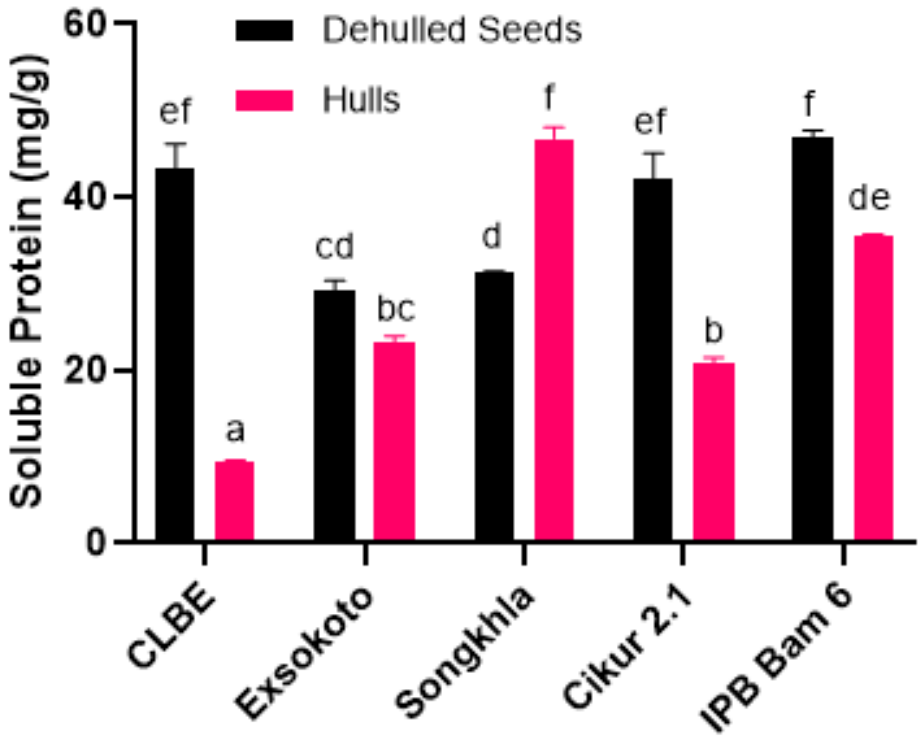


Figure 3.5. The soluble protein content (mg/g) of the separated dehulled seeds and hulls of each analysed batch 2 landrace. Data shown as means with SEM error bars, calculated using n=3 technical replicates. Significance is taken at $p<0.05$, determined using a two-way ANOVA. Different letters indicate a significant difference according to a Bonferroni post-hoc test ($p<0.05$).

3.3.1.1 Amino Acid Analysis

There was a significant difference ($p<0.05$) (Figure 3.6) in the valine and histidine content if bambara seeds in batch one, but not for the other essential amino acids. The valine and histidine content were the lowest in DipC with R/R having the highest concentration.

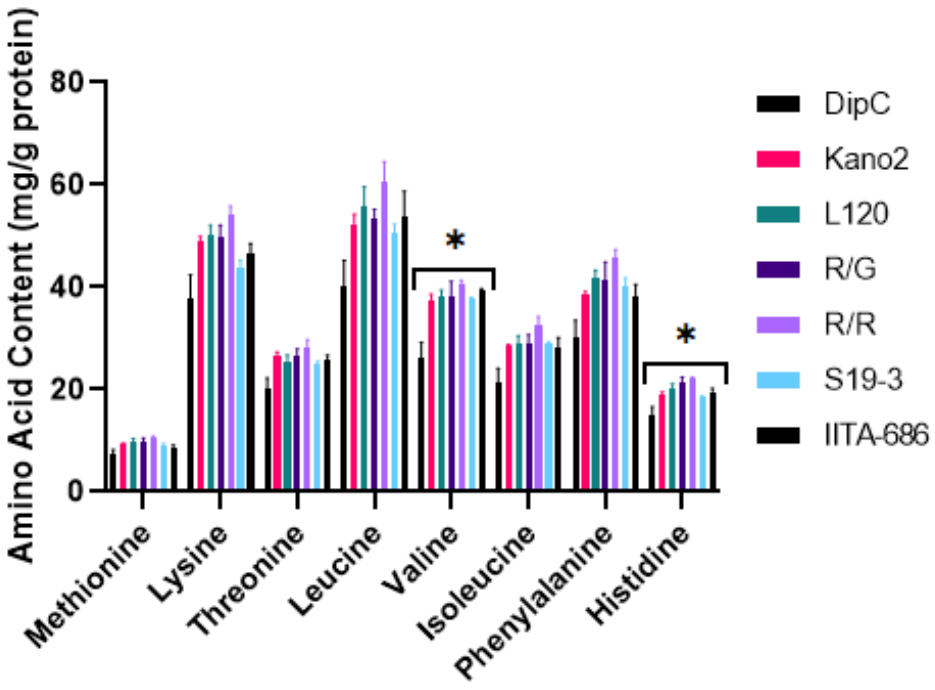


Figure 3.6. The amino acid content (mg/g protein) of the seven landraces of bambara groundnut seeds from batch 1. Data is shown as means plus SEM, n=2 technical replicates per landrace. Significance determined by one-way ANOVA *p<0.05.

There was a significant difference (p<0.05) (Figure 3.7) in methionine (p<0.01), threonine, valine and histidine (p<0.05) in the bambara seeds in batch two. In this batch, it was landrace CLBE that had the lowest value for all of these essential amino acids.

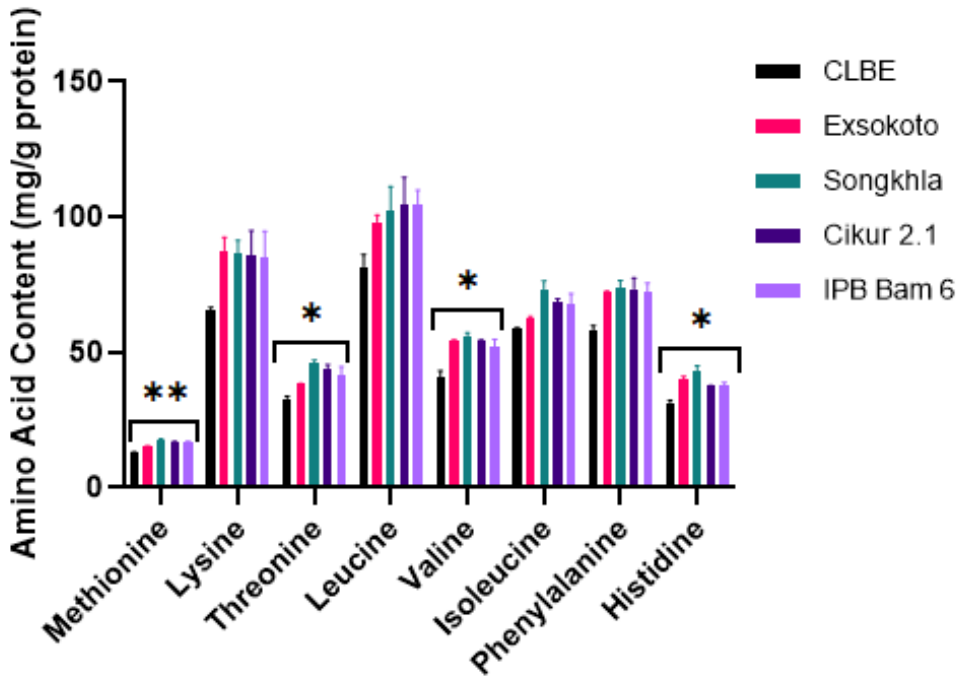


Figure 3.7. The amino acid content (mg/g protein) of the seven landraces of bambara groundnut seeds from batch 2. Data is shown as means plus SEM, n=2 technical replicates per landrace. Significance determined by one-way ANOVA *p<0.05

The range of amino acid contents observed is comparable to the literature reported results for soybean (Table 3.6). For most essential amino acids, the range observed for bambara encompasses the reported soybean values.

Table 3.6. Amino acid contents (g/100g protein) of both batches of bambara groundnut seeds in comparison to reported values for soybean.

Amino Acid	Soybean[1] (g/100g protein)	Bambara batch one [2] (g/100g protein)	Bambara batch two [2] (g/100g protein)
Methionine	0.6	0.7-1.1	1.3-1.8
Lysine	6.5	3.8-5.0	6.6-8.8
Threonine	1.4	2.0-2.8	3.2-4.6
Leucine	8	4.0-6.1	8.2-10.5
Valine	5	2.6-4.1	4.1-5.6
Isoleucine	5	2.2-3.3	5.9-6.9
Phenylalanine	4	3.0-4.2	5.8-7.4
Histidine	1.2	1.5-2.2	3.1-4.3

[1]Soybean amino acid content values taken from the literature as a source of comparison to values generated in this study (Kudelka et al. 2021).

[2]Values for each amino acid indicates the range of values obtained from the landraces assessed within these batches. n=2 technical replicates per landrace within each batch.

3.3.2 Antinutritional Analysis

There was a significant interaction ($p<0.001$) between the bambara landrace and the type of sample (hulls or corresponding dehulled seeds) for the total phenolic content (Figure 3.8) in batch one. The hulls had a significantly

higher total phenolic content than the dehulled seeds for all samples except DipC and Kano2.

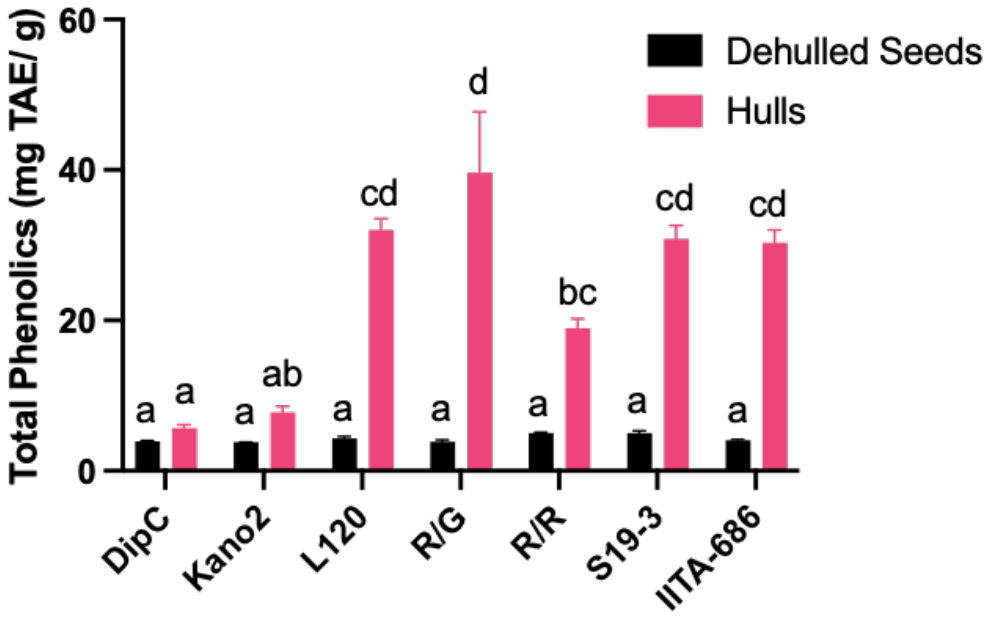


Figure 3.8. The total phenolic content of batch one bambara groundnut seeds and hulls as tannic acid equivalent (mg TAE/g). Data shows means with SEM, calculated using n=3 technical replicates. A two-way ANOVA showed a significant interaction between the different bambara groundnut landraces and the type of sample (seed or hull) for the total phenolic content ($p<0.05$). Different letters indicate a significant difference according to a Bonferroni post hoc test ($p<0.05$).

There was a significant interaction ($p=0.017$) between the bambara landrace and the type of sample (hulls or corresponding dehulled seeds) for the total phenolic content (Figure 3.9) in batch two. In this batch only two samples show significantly ($p<0.05$) higher values in the hulls than dehulled seeds; Excokoto and Cikur 2.1.

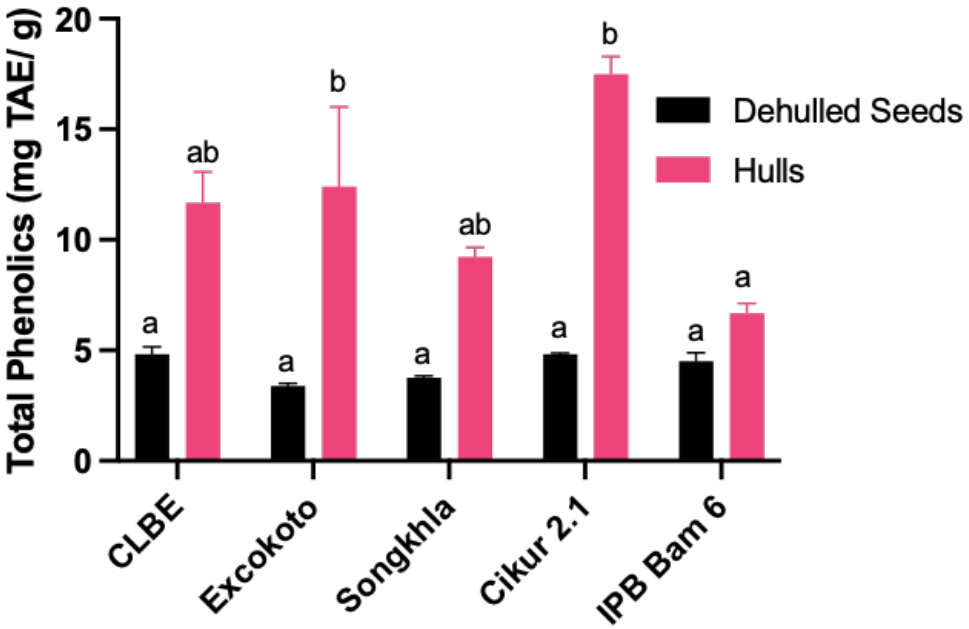


Figure 3.9. The total phenolic content of batch two bambara groundnut seeds and hulls as tannic acid equivalent (mg TAE/g). Data shows means with SEM, calculated using n=3 technical replicates. A two-way ANOVA showed a significant interaction between the different bambara groundnut landrace and the type of sample (hull or corresponding dehulled seed) for the total phenolic content ($p<0.05$). Different letters indicate a significant difference according to a Bonferroni post hoc test ($p<0.050$.

There were no significant differences in the phytic acid content between the different landraces of bambara groundnut seeds in batch one (p=0.237) (Table 3.7)

Table 3.7 Phytic Acid content of bambara groundnut seeds from batch one.

Bambara Landrace	Phytic Acid (g/100g)
DipC	0.747
Kano2	0.797
R/R	1.111
R/G	0.512
S19-3	0.673
IITA-686	0.566
L120	0.874
SED[1]	0.210
P Value[2]	0.237

[1] SED- standard error of the differences of the means, n=2 technical replicates per landrace.

[2]P values determined by a one-way ANOVA. Different letters indicate a significant difference.

There were no significant differences in the phytic acid content between the different landraces of bambara groundnut seeds in batch two (p=0.103) (Table 3.8).

Table 3.8 Phytic Acid content of bambara groundnut seeds from batch two.

Bambara Landrace	Phytic Acid (g/100g)
CLBE	0.634
Exsokoto	1.011
Songkhla	1.162
Cikur 2.1	1.151
IPB Bam 6	1.315
SED[1]	0.182
P Value[2]	0.103

[1] SED- standard error of the differences of the means, n=2 technical replicates per landrace.

[2]P values determined by a one-way ANOVA. Different letters indicate a significant difference.

Haemagglutinating analysis showed positive results for the presence of lectins, predominantly seen in the dehulled seeds as opposed to the seed hulls (Table 3.9). The lowest seed haemagglutinating was seen in Exsokoto, while this landrace also showed the highest level of hull haemagglutinating.

Table 3.9. The results of the haemagglutinating assay, showing positive, intermediate or negative for the presence of lectins.

	Dilution	Exsokoto	Cikur 2.1	IPB Bam 6	Songkhla	CLBE
Seeds	0	+	+	+	+	+
	1:2	+	+	+	+	+
	1:4	+/-	+	+	+	+
	1:8	-	+/-	+/-	+/-	+/-
	1:16	-	-	-	-	-
Hulls	0	+	+/-	+/-	+/-	+
	1:2	+/-	-	-	-	-
	1:4	-	-	-	-	-
	1:8	-	-	-	-	-
	1:16	-	-	-	-	-

Where + indicates blood cell agglutination, +/- indicates partial agglutination and – indicates no agglutination. n=1 replicate.

3.3.3 Bambara groundnut milk

In the bambara groundnut milk, there were no significant differences in the soluble protein content between the different bambara landraces (p=0.536) (Figure 3.10).

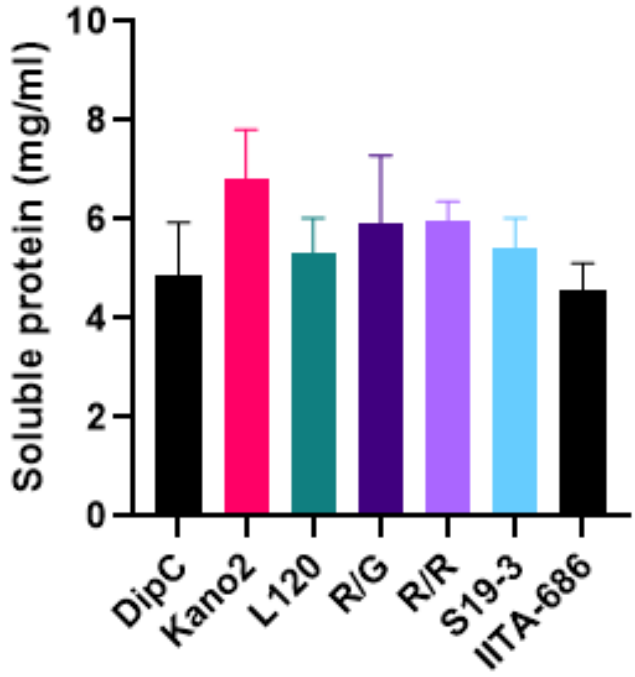


Figure 3.10. The soluble protein content (mg/ml) of milk produced from the 7 landraces of bambara groundnut seed in batch one. Data is shown as means with SEM, calculated using n=3 technical replicates. No significant differences were determined.

However, there was a significant difference in the total phenolic content of the bambara milk from different seed landraces (p=0.041) (Figure 3.11). Though a Bonferroni post-hoc test showed no significant differences.

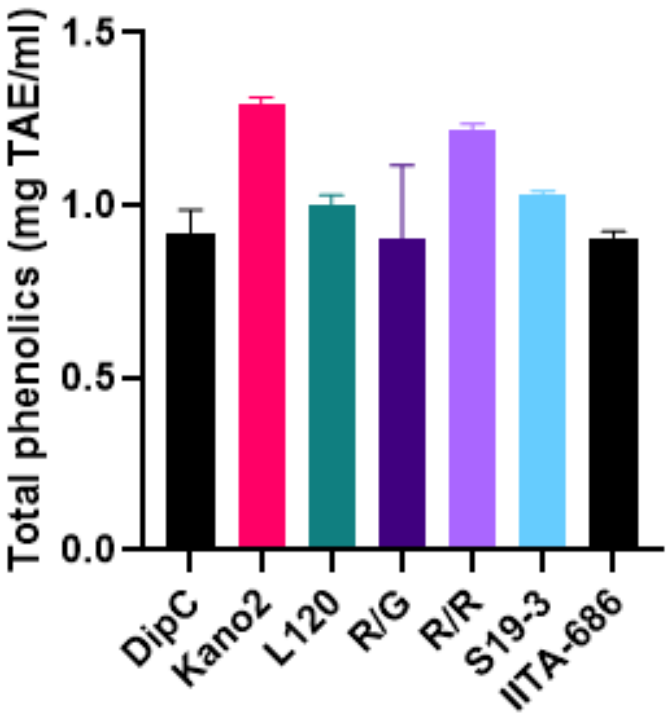


Figure 3.11. The total phenolic content (mg TAE/ml) of milk produced from the 7 landraces of bambara groundnut in batch one. Data is shown as means with SEM, calculated using n=3 technical replicates. A one-way ANOVA showed a significant difference ($p<0.05$) between the different bambara milks but a Bonferroni post hoc test showed no differences.

There was a significant difference ($p<0.001$) found in the protein content of the different milks produced from the bambara groundnut seeds in batch two (Figure 3.12). Songkhla contained the lowest values which was significantly different ($p<0.05$) from the rest of the landraces with Cikur 2.1 and IPB Bam 6 being significantly higher ($p<0.05$) than other batch two landraces.

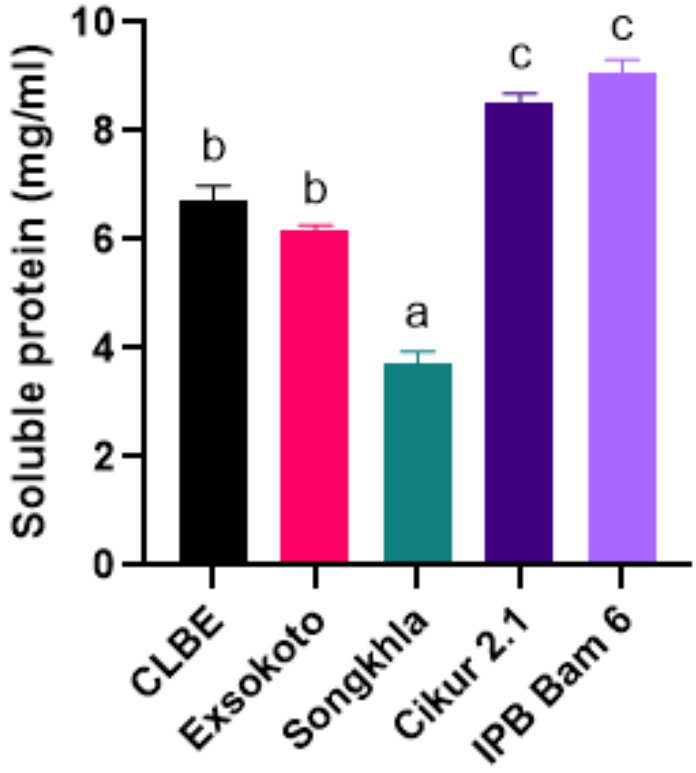


Figure 3.12. The soluble protein content (mg/ml) of milk produced from the 5 landraces of bambara groundnut in batch two. Data is shown as means with SEM, calculated using n=3 technical replicates. A one-way ANOVA showed a significant difference between the different bambara milks ($p<0.001$). Different letters indicate a significant difference determined using a Bonferroni post-hoc test ($p<0.05$).

There was a significant difference ($p<0.001$) between the total phenolic contents of the bambara groundnut milk produced from the five landraces of seeds in batch 2 (Figure 3.13). As with soluble protein, Songkhla contained the lowest value, which was significantly different ($p<0.05$) from the rest of the landraces, with Cikur 2.1 and IPB Bam 6 being significantly higher ($p<0.05$) than the Exsokoto landrace.

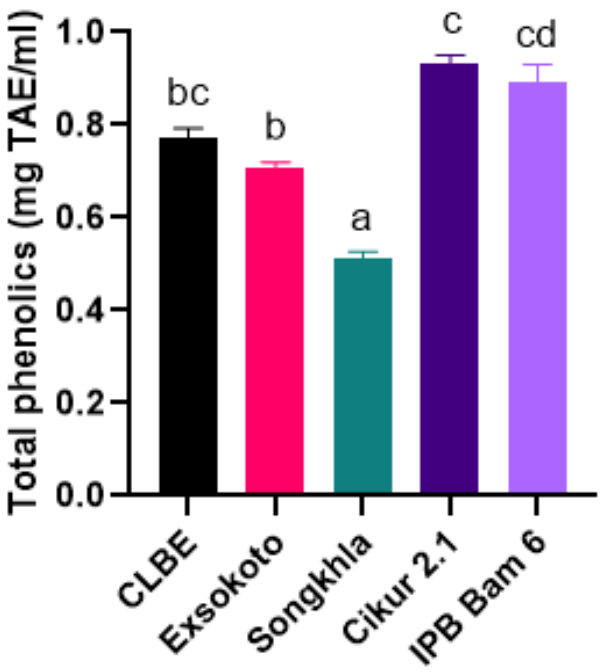


Figure 3.13. The total phenolic content (mg TAE/ml) of milk produced from the 5 landraces of bambara groundnut in batch two. Data is shown as means with SEM, calculated using n=3 technical replicates. A one-way ANOVA showed a significant difference between the different bambara milks ($p<0.001$). Different letters indicate a significant difference determined using a Bonferroni post-hoc test.

3.4 Discussion

Different landraces of bambara groundnut have significantly different nutritional and antinutritional profiles.

The results of this work show that the crude protein content differs significantly between different landraces of bambara groundnut. The brown landrace Uniswa R/G has a significantly higher protein content than all other landraces

except the black landrace S19-3. The crude protein content determined here ranges from 19.49- 25.86% which fit the reported ranges of 19- 26.3% in a data review completed by Ramatsetse et al. (2022). The lowest crude protein is observed in the cream landraces DipC and Kano2 which show 20.21% and 19.49% respectively, though the data reported by Ramatsetse et al. (2022) shows that the crude protein does not seem to correlate to hull colour, with cream landraces showing 19.5-23.51%, brown landraces showing 19-24.3%, red landraces showing 19.5-20.57% and black landraces showing 21.7-26.3%. This crude protein content is lower than that of soybean, which is reported at approximately 36.5% (Sakkas et al. 2019).

The crude protein content does not seem to correlate with the energy content; where the cream landrace Kano 2 has a significantly lower energy content than DipC, L120, Uniswa RR, and IITA-686. This variation appears to be specific to landraces rather than hull colour differences, as the energy content of the two cream landraces are not similar. However, a wider variety of seeds from different landraces would need to be tested to validate this.

The soluble protein content was measured as a way of determining the protein composition between the seed hulls and the corresponding dehulled seeds. The results show that the soluble protein found in the hulls of the bambara seeds is usually significantly lower than the corresponding dehulled seed. This suggests that the hulls can be removed before use in animal feed without a major loss of protein content. This is supported by the work of Mahala et al. (2010), who found that the crude protein content of bambara groundnut seeds

was significantly higher in the seeds compared to the hulls. However, this result was not uniform across all landraces and so the protein concentration of each landrace should be checked before incorporation as a dehulled feed ingredient. These results are also not proportional to the seed, as they are measured in mg/g of sample despite there being less weight of hulls than dehulled seed when considered per seed. It should also be considered that the removal of the hulls may also remove other nutritional components. (Gwala et al. 2020) found that the dehulling of bambara seeds from Zimbabwe decreased the concentration of calcium in the seed by 33%, though did not significantly change the concentration of magnesium, iron or zinc. However, the hull colour of the bambara seeds used was not discussed and so it is unknown if this may be variable between landraces.

The amino acid content of the seeds in batch one shows a significant difference for each essential amino acid except for valine, while batch two showed a significant difference for all essential amino acids. The seeds in batch two showed a trend of higher amino acid contents, though it is difficult to determine if this is down to variation within the seeds or the methods, as the analysis was completed on separate occasions. While the essential amino acid content was variable, the range for each amino acid encompassed the reported value for soybean (Kudełka et al. 2021), except for methionine, threonine and histidine which appear to be lower in soybean. It is difficult to make true comparisons as the methods used for determining the amino acid content may be variable, and within the same method variation can be observed. For lysine,

leucine, valine and isoleucine, the reported values for soybean fit within the highest values within the seeds of batch two. For phenylalanine, the value reported for soybean sits within the reported values for batch one of bambara groundnut. For methionine, threonine and histidine, the reported values for soybean are lower than the lowest value reported in batch one of the bambara seeds, suggesting that bambara groundnut seeds tend to be higher in these essential amino acids than soybean. This fits with literature reported in a review by Oyeyinka, Oyeyinka (2018) who stated that within the protein, bambara groundnut seeds contain 6.5-6.8% lysine and 1.8-2.84% methionine, considered to be high values for a legume seed.

Variations in the nutritional composition within seeds is important in order to make assessments on which parts of the seeds can be recommended for consumption and whether processing is needed. Variations between landraces is important information to consider when planning for potential widescale production of bambara groundnut crops. It is important to understand which landraces possess the desired traits for feed and food so that these are the seeds which can be focussed on when amplifying production.

Dehulling is a commonly discussed method of removing antinutritional factors, particularly tannins, from leguminous seeds (Nikmaram et al. 2017). As can be seen in this study, the total phenolic content is higher in the hull than the dehulled seed. This finding is supported by (Harris et al. 2018) who found that the tannin content in black, brown and red bambara groundnut seeds is higher in the hulls than dehulled seeds. This can be a low-cost method of processing,

which is extremely beneficial when considering bambara groundnut seeds as a feed and food ingredient for rural communities across the world with little disposable income.

It can be also be seen from the results that the total phenolic content varies significantly between the different bambara landraces. While there are no significant differences in the total phenolic content of the dehulled seeds, the lightest coloured seeds from batch one, Dipc and Kano2, show the lowest hull total phenolic content of the batch. The tan and brown landraces L120 and Uniswa R/G show the highest hull total phenolic content of batch one. These findings are supported by the work of Tibe et al. (2007), who also reported variable tannin content in different bambara groundnut seeds; with the highest content found in brown, tan and red nuts and the lowest content in cream-coloured seeds. The tannin content within a plant has been linked to the colour of plant organs. As in bambara groundnut seeds, and other bean seeds, these tannins are found in the seed hulls; they can determine the colour of the seed hulls (Díaz et al. 2010). As stated above, in bambara groundnut seeds it appears to be the tan and brown landraces that show the highest tannin levels while the cream landraces show the lowest. This is also supported in other species, with Guzman-Maldonado et al. (2003) finding significantly higher tannin levels in black *Phaseolus vulgaris* variants compared to beige variants. Similarly, (Wang et al. 1998) found that grass pea (*Lathyrus sativus* L.) seeds with darker seed coats contained higher levels of condensed tannins. This correlation is due to the oxidation of tannins, anthocyanins and other phenolic compounds

during the ripening of seeds causing colouration (Halloin 1982). However, the seeds in batch two do not appear to follow this same pattern, with the highest total phenolic content found in the hulls of black landrace Cikur 2.1 and cream landrace Excokoto and the lowest content found in black landrace IPB Bam 6. This suggests more of an importance of variation compared to hull colour. Being able to relate the levels of tannins to coat colour would be extremely beneficial for bambara groundnut seeds. As the seeds are often characterised by phenotypic variations such as coat colour instead of genetics, this information could allow for easy screening of variants that would be the most suitable for consumption.

The phytic acid content did not vary significantly between the different landraces in either of the seed batches, though it ranged from 0.566g/100g to 1.315g/100g across both batches. This fits within the wide reported range of 1.10-15.11mg/g in bambara seeds (Tan et al. 2020). In this study the phytic acid assay was measured in flour produced from the grinding of whole seeds, giving a whole seed estimate rather than a composition between hulls and dehulled seeds. This was due to a lack of hull material available to complete the phytic acid assay. However, it is reported that phytic acid is predominantly located within the dehulled seeds of bambara (Tan et al. 2020). This is also reported in faba beans (Luo et al. 2009) and in soybean (Anderson, Wolf 1995).

Lectins in seeds can be difficult to quantify, though their presence can be detected through their ability to agglutinate red blood cells. In this study, it can be seen that the agglutinating lectins are predominantly found in the seeds,

rather than within the hulls as seen for tannins. As lectins are proteins (Sharon and Lis, 2004), this stands to reason, as this is the area of the seed with higher protein levels within bambara seeds. This is supported by Sharma and Sehgal (1992)who reported that the haemagglutinating lectins in faba beans (*Vicia faba*) were not removed by complete dehulling. There does not appear to be a relationship between the haemagglutinating content and the total phenolics, due to the compounds being located in different parts of the seed. The haemagglutinating content is noted by eye and so is a very subjective measurement, however, as this data cannot be quantified there is not a more objective way of currently measuring this.

Bambara groundnut milk has a variable protein content, which is comparable to other popular vegetable milks; but also has an associated total phenolic content.

The milk produced from bambara groundnut seeds was evaluated to see how it could compare to other commonly marketed vegetable milks. For human consumption, this could be a way for the seeds to be utilised by more consumers, without the need for the seeds to be incorporated into a meal or bakery product.

Vegetable milks are a growing alternative to bovine milk for consumers with dietary requirements such as vegans, those with lactose intolerance and those with religious requirements (Murevanhema, Jideani 2013). Poulter and Caygill (1980) first evaluated bambara groundnut seeds as a potential for vegetable milk production, finding good stability and a low trypsin inhibitor content.

(Brough et al. 1993) then tested the palatability of bambara groundnut milk compared to milks from cowpea, pigeon pea and soybean, all prepared using the same method. They found that panellists preferred the lighter colour of bambara and cowpea milks compared to the more yellow soybean and pigeon pea milks. Bambara milk was voted last for the viscosity of the milks but first for the taste; which along with the colour, meant that it scored the highest overall of the four milks.

(Scholz-Ahrens et al. 2020) looked at the protein content in a variety of different milks. Two landraces of cow milk were analysed, finding 3.3 and 3.4g/100g protein. For the protein content of some popular vegetable milks, soy milk was found to have 3.7g/100g, almond 0.8g/100g, oat 1g/100g and rice 0.3g/100g. However, this may vary between different brands of these milks, according to location, production methods and potential processing or fortification. In the case of this study, the lowest soluble protein content was seen in the Songkhla milk at 3.7mg/ml. This is comparable to that of the soy milk studied by Scholz-Ahrens et al. (2020) and higher than bovine milk or other vegetable milk alternatives. In IPB Bam 6 the milk soluble protein content was 9.1mg/ml, considerably higher than observed in soymilk. The soluble protein content of the milk does not correspond to the crude protein content within the whole seeds (g/100g DW), though the landrace with the lowest crude protein content in batch two, Songkhla, also had the lowest milk soluble protein content.

However, variability in protein content may not be important if the protein is not digestible. The digestibility of the protein may be reduced by the tannin content of the milk. From the content of the analysed milks, it can be seen that the soluble protein and total phenolic content seem to follow the same pattern, implying that they may be correlated. However, the total phenolic content is much lower than seen in the hulls or even the dehulled seeds when considered as raw seeds. It could also be further reduced by heat treatment of the seeds prior to milk production, something which is already recommended by (Brough et al. 1993) reduce the bean taste associated with the milk.

3.5 Conclusions

This analysis was completed in order to evaluate if the nutritional and antinutritional profile of bambara groundnut seeds is variable between different seed landraces. As bambara seeds are locally produced on small scales, different seed variations are cultivated without widespread knowledge about their properties. Significant differences were observed between different landraces within all factors studied. These differences predominantly seem specific to the landrace studied, making conclusions on the potential of the seeds as feed ingredients difficult to draw. While the crude protein content is lower than that of soybean, the amino acid content is similar for the majority of amino acids and higher for methionine which is often limiting in legume crops.

It was observed that dehulling appears to be an effective way of reducing the total phenolic, and hence tannin, content of the seeds. This will reduce the potential for negative effects on animal performance. It is also observed that dehulling is unlikely to have a significant effect on the overall protein content of the seed. In conclusion of these factors, bambara groundnut seeds appear to be a good potential feed ingredient for production animals. However, the recommendation from this study should be that specific landraces are profiled before use, and that the hulls should be removed prior to feed formulation.

The production of bambara groundnut milk, both from this study and previous literature, can be recommended as the protein content is comparable to other marketable vegetable milks currently on offer. However, processing to further reduce the total phenolic content associated with the protein content of the milks should be carried out prior to production. A heat processing method such as roasting or dry frying of the seeds would likely be effective for this.

4 The differences in nutritional and antinutritional concentrations between different species of mezquite

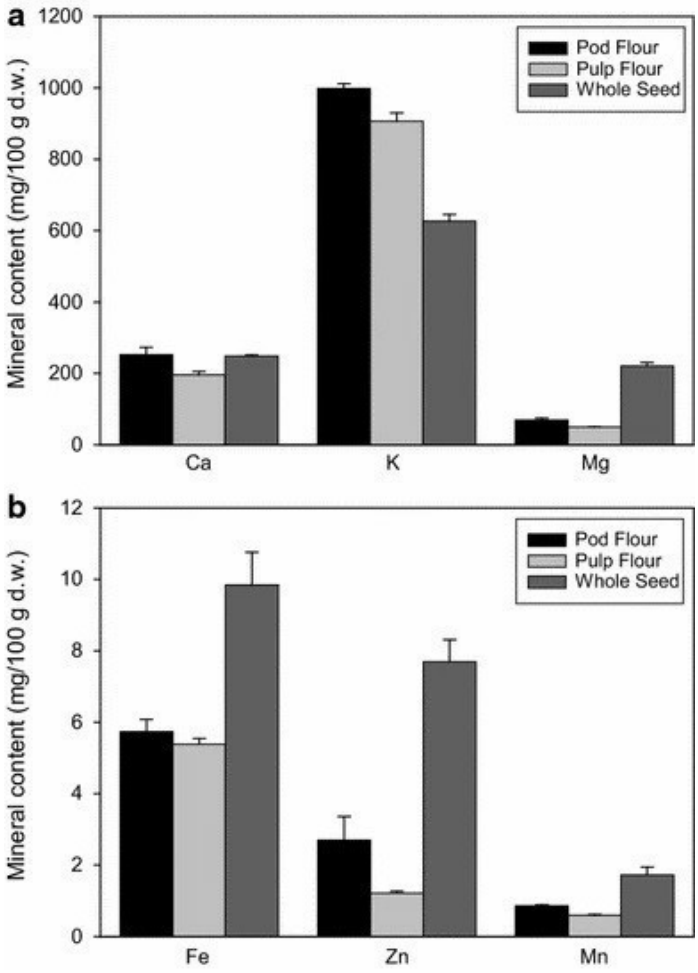
4.1 Introduction

Mezquite (*Prosopis*) trees are leguminous trees found in arid or semi-arid areas of Africa, Asia and the Americas (Díaz-Batalla, Hernández-Uribe, et al. 2018; Ruiz-Nieto et al. 2020). Forty of the 44 known species of mezquite are native to the Americas, with only four globally spread. As they are leguminous trees, they have been actively propagated beyond their native environment in an attempt to condition soils via nitrogen fixing (Ruiz-Nieto et al. 2020). The trees have stems ranging between 6-9m in height and produce fruits twice a year which are pods of around 15cm long (Sawal et al. 2004). These pods are formed of an exocarp, fleshy mesocarp and a woody endocarp which protects a seed (Díaz-Batalla, Hernández-Uribe, et al. 2018). They are a crop of interest as they can grow in areas of little rainfall which is often unsuitable for cultivation. The pods can potentially provide a food source for local communities and animals, as well as the wood being a useful fuel source (Ruiz-Nieto et al. 2020). However, due to a lack of competition, fast growth rate, high seed yield and hardiness to environmental conditions; the mezquite trees in many areas spread faster than possible utilisation, leading to them being considered an invasive species

(Patnaik et al. 2017). This, alongside a limited knowledge about germination, cultivation, usage and harvesting, means mezquite trees are largely underutilised when considered in a worldwide context (Barba De La Rosa et al. 2006).

In their native countries, mezquite trees are a longstanding and much valued part of local communities. The fruit pods have an average yield per year of around 10-50 kg pods, which begins 3 years after planting (Almanza, Moya 1986). The estimated worldwide pod availability is 2-4 million metric tonnes, with a two million tonne production estimate in India (Sawal et al. 2004). The pods are valued as a local food source due to their nutritional composition (Almanza, Moya 1986). They are predominantly used by grinding the whole pod into a flour, which can reportedly contain 7-34% protein, while the seed alone contains 34-39% protein (Ellsworth et al. 2018; Sawal et al. 2004; Ruiz-Nieto et al. 2020). The whole pods also contain 27-32% dietary fibre, which is reported to be predominantly insoluble fibre (Bravo et al. 1998) and 1-6% fat (Ruiz-Nieto et al. 2020). The pulp of the pods is also rich in saccharose (20-25%) and reduced sugar (10-20%) (Sawal et al. 2004), which can be used to produce sugar syrups for use in coffee roasting and some beverages; although, the dark brown colour of these syrups limits their commercial use (Larrauri Garcia, Saura Calixto 2000). Because of this sugar content, mezquite flours can also be substituted into pastries and biscuits (Sciammaro et al. 2016). Mezquite is also a source of minerals, the major minerals are reportedly observed in high levels, particularly potassium which is reportedly higher than the concentration

reported in soybean (Sciammaro et al. 2016; Batal et al. 2010). Calcium and magnesium, although lower than potassium, still show higher levels than many other flours (Sciammaro et al. 2016), including white and whole grain bread flours (Ciudad-Mulero et al. 2021). Trace minerals, iron and zinc, were also seen in high proportions, at similar levels to other legume sources, such as soybean (Sciammaro et al. 2016). While manganese is found in a minor proportion compared to the other minerals (Sciammaro et al. 2016).



Source: Sciammaro et al. (2016)

Figure 4.1 Mineral content of *P. alba* pod and pulp flour and whole seeds, profiled by Sciammaro *et al.* (2016).

As well as being a food source, mezquite trees are valued as a source of fuel for people in arid regions. Wood from *P. juliflora* trees reportedly burns well, with low ash production and a calorific value of 4000 kCal kg^{-1} (Patnaik et al. 2017). The exudate gum that is released from the trunk and branches of *P. juliflora* is also utilised both as an emulsifying agent, and anecdotally, as a medicinal aid to treat eye infections (Dave, Bhandari 2013; Patnaik et al. 2017). This forms part of the history of mezquite uses in ethnomedicine, with anecdotal reports that various parts of the tree have traditional uses in treating gastritis, aiding digestion and treating skin lesions (Almanza, Moya 1986; Patnaik et al. 2017).

The use of mezquite pods in the food and feed industries are limited by the presence nutrient limiting antinutritional factors (ANFs). In mezquite samples, tannins, phytic acid, lectins and protease inhibitors have been reported (Andrade-Montemayor et al. 2011). Polyphenols are one of the most common components reported in mezquite, with the highest levels found in the leaves, pods and flowers. Polyphenols have exhibited both antioxidant and antinutritional capacities, with tannins being one of the most commonly studied (Sciammaro et al. 2016). In the pods of *P. juliflora* trees, tannins have been found in the whole pods at 1.5% dry weight (DW) (Sawal et al. 2004). Trypsin inhibitors, a form of protease inhibitor, have also been identified in raw pods from *P. laevigata* trees at 17.5 Trypsin Inhibitor Units/g (Andrade-Montemayor et al. 2011). 0.33-0.89% phytic acid has also been quantified within the pods (Retana-Márquez et al. 2016). It is difficult to quantify lectins

within plant material, but their presence is identified within mezquite pods by their ability to agglutinate red blood cells (Andrade-Montemayor et al. 2011).

While work has been conducted looking at the proximate nutrient and antinutrient concentrations in mezquite pod flour, there are minimal studies comparing different species of mezquite. As different species are endogenous to different regions, it is important to generate this information to help inform local communities of the potential differences between species. Building up a profile of the nutrient and antinutrient concentrations of different species may help to inform recommendations for or against the use of the pod flour from different species of mezquite for both human use and use within animal feeds.

Aims

1. To determine the proximate nutrient concentrations of flour from the pods of different species of mezquite

2. To determine the levels of antinutritional compounds present in flour from the pods of different species of mezquite

3. To see how the species and different growth conditions may affect the nutritional and antinutritional composition of mezquite pod flour

Hypothesis

Different species of mesquite will have significantly different nutritional and antinutritional concentrations

4.2 Materials and Methods

4.2.1 Mezquite material

For the following Chapter, mezquite species identification was carried out by Dr. Arturo Castro-Castro (Centro Interdisciplinario de Investigacion para el Desarrollo Integral Regional, Durango) and Dr. Socorro Gonzalez Elizondo (Instituto Politecnico Nacional, campus CIIDIR Durango). Community liason and sample collection was carried out by Xochitl Soto-Luzania, Karina Fernandez Baurista (Universidad Technologica de San Luis Rio Colorado, Sonora), Dr. Yolanda Lopez-Franco (Centro Interdisciplinario para la Alimentacion y el Descarrollo, Sonora), Dr. Julio Rios Saucedo (National Institute of Forestry, Agriculture and Livestock Research), Dr. Nuria Rocha Guzman, Dr. Ruben Gonzalez Laredo (Instituto Tecnológico de Durango), Dr. Oscar Koech (University of Nairobi), Dr. Charles Kilawe and Professor. George Kajembe (Sokoine University of Agriculture). Collection was also completed by members of local communities from San Juan de Guadalupe, Durango (Mexico), Estacion Doctor, Sonora (Mexico), the Lang'ata bora peoples in Tanzania and the Il Chamus and Tugen communities from Kenya.

48 mezquite pod samples were collected from four different mezquite species, from four distinct locations (Table 4.1). The samples could be categorised into two groups per species, based on brief descriptions of the environmental conditions surrounding the trees. No more information was recorded about the environmental conditions, due to collection during the COVID-19 pandemic. As

each species is from a distinct location, with different environmental conditions for each; it is not possible to say whether differences described in this study are actually due to species or environment. Mezquite pod flour was used in this study, rather than whole pods or seeds, due to restrictions in shipping over whole material due to the potential risks of insects, disease or spread of non-native plants.

Table 4.1. The four different species of mezquite used in this study, along with their growth location and environmental conditions.

Mezquite Species	No. Of Samples	Location	Growth Conditions
P. velutina	8	Sonora, Mexico 31º55'59.88"N 114º42'00" W	Under irrigation,
P. velutina	4	Sonora, Mexico 31º55'59.88"N 114º42'00" W	Wild trees, saline soils
P. legaeviata	9	Durango, Mexico 24.6326° N 102.7776° W	Semi-arid soil
P. legaeviata	3	Durango, Mexico 24.6326° N 102.7776° W	Arid soil
P. juliflora	6	Baringo, Kenya 0° 29' 3''N 36° 3' 15''E	Lower slides, under irrigation
P. juliflora	6	Baringo, Kenya 0° 29' 3''N 36° 3' 15''E	Higher grounds, near roads
P. pallida	6	Kilimanjaro, Tanzania 3° 39' 27" N 37° 26' 29" W	Near buildings
P. pallida	6	Kilimanjaro, Tanzania 3° 39' 27" N 37° 26' 29" W	Near a lake

4.2.2 Drying

A sample of each mezquite pod flour was dried via freeze-drying (Christ) as per Chapter 2.1.1. Samples were weighed into foil trays and placed in the freeze-dryer for 48 hours before removing, re-weighing and returning to the freeze-dryer for a further 24 hours to confirm that they remained at a constant weight.

4.2.3 Nutrient analysis

Dry weight samples were used for all proximate nutrient analysis. The mezquite pod flours used in this chapter were analysed for crude protein via elemental analyser (FlashEA 1112 Series, Thermo Fisher Scientific), gross energy via bomb calorimetry (6300 calorimeter, Parr) and total fat (Gerhardt's Soxtherm system). These methods are described in Chapter 2.1.

4.2.4 Mineral content via Acid Digestion and ICPMS analysis

This work was carried out by Dr Jon Stubberfield, technician in the Division of Food, Nutrition and Dietetics.

Acid digestion and inductively coupled-plasma mass spectrometry (ICPMS) was used to evaluate the mineral content of dry weight mezquite pod flour samples. For the acid digestion, 200mg dry weight sample was placed into a digestion tube, and 6ml of 65% nitric acid (Merck, Germany) was added. The tubes were stoppered, screwed hand-tight and placed into the microwave rotor. The microwave (Anton Paar) was then turned on, the rotor placed inside and

programme P1 was selected and run to digest the samples. Once finished, the contents were decanted into 10ml tubes.

Samples were then diluted 1:10 (v/v) in pure water before ICPMS analysis. Standard multi-element analysis was completed on the ICPMS (Thermo-Fisher iCAP-Q) to give results for Ag, Al, As, B, Ba, Be, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti, Tl, U, V and Zn. Samples were introduced to an argon plasma, which dries the droplets and dissociates the molecules. Single charged ions are then formed by oxidation which pass through a mass spectrometer which filters and sorts the ions according to their mass-charge-ratio. Upon exiting the spectrometer, the ions are detected by the pulse caused as the ions impacting on the detector. Sample processing is undertaken using 'Qtegra software' (Thermo-Fisher Scientific), which compares pulses to a standard in order to determine the concentration of each measured mineral.

4.2.7 Amino Acid Concentrations

This work was carried out by Dr Jon Stubberfield, technician in the division of Food, Nutrition and Dietetics. Amino acid concentrations were analysed by method of hydrolysis by oven crimp top tube digestion, following analysis by LCMSMS, as described in Chapter 2.4.5.3.

The work in this chapter was amongst the first amino acid analysis carried out within the division, at the time of this analysis a soy flour standard reference material was not run alongside these samples. This practice has since been put

into place due to progression in the method optimisation since this work was carried out. This means that for these results there is no point of reference to adjust the samples to.

4.2.6 Antinutritional content analysis

Sample extracts were taken by the dilution of 0.1g dry weight material in 5ml 0.1M NaOH (1:50 w/v). The total phenolic content was measured as Chapter 2.2.1, at a 1:4 (v/v) dilution of sample extracts in 0.1M NaOH.

The phytic acid concentration was measured using the phytic acid assay kit (Megazyme), as described in Chapter 2.2.2. For this study, four samples per species were randomly selected using a random number generator, used to represent the species. Two samples from each environmental condition were used.

4.2.6 Data Analysis

Statistical analysis was conducted using Genstat 20th Edition software to determine if there were any significant differences found between or within the different mezquite species. A one-way analysis of variance (ANOVA) was used to determine any possible significant differences between or within species. Significance was taken at $p<0.05$ and errors reported as standard error of the mean (SEM). All graphs were drawn using Graph Pad Prism 8.

4.3 Results

4.3.1 Nutrient analysis

There was a significant difference seen between the four different species of mezquite for crude protein ($p<0.001$), fat ($p<0.001$) and energy ($p=0.022$) (Table 4.2). *P. juliflora* flour had the highest protein, fat and energy content. *P. velutina* had the lowest protein and energy content but *P. pallida* had the lowest fat content. The full analysis for each sample can be found in Appendix 1.

Table 4.2. Average crude protein, fat and gross energy content of four different species of mezquite.

Mezquite species	Protein (g/100g DW)	Fat (g/100g DW)	Gross Energy (MJ/Kg)
P. velutina	10.78^{a}	1.68^{b}	17.45^{a}
P. legaeviata	13.56^{b}	2.08^{bc}	17.56^{ab}
P. juliflora	13.72^{b}	2.51^{c}	17.91^{b}
P. pallida	11.01^{a}	0.90^{a}	17.48^{ab}
SED[1]	0.638	0.235	0.156
P Value[2]	**<0.001**	**<0.001**	**0.022**

[1] SED = standard error of the differences of the means, n= 2 technical replicates per sample, 12 samples per species.

[2]P values determined by a one-way ANOVA. Different letters indicate a significant difference, determined with a Bonferroni post-hoc test.

Within each species of mezquite, there were differences in environmental conditions, as provided by those who collected the material. However, this was not always associated with significant differences in nutrient content (Table 4.3). There was a significant difference ($p<0.05$) in the crude protein and fat content in *P. juliflora* pod flour associated with the different environmental conditions. *P. juliflora* pods had a higher protein and fat content when harvested from trees grown on higher ground, with no irrigation. For *P. pallida* pod flour there was a significant difference in the crude protein content, with pods harvested from trees grown in a built-up area surrounded by buildings having a higher protein content than those grown in a natural setting, near a lake.

Table 4.3. The differences in crude protein, fat and gross energy content within the four different species of mezquite, grouped according to environmental differences.

Mezquite species	Environmental conditions		Protein (g/100g DW)	Fat (g/100g DW)	Energy (MJ/kg)
P. velutina	Under Irrigation		11.11	1.87	17.51
	Saline soils		10.12	1.27	17.34
		SED[1]	0.978	0.498	0.184
		P Value[2]	0.334	0.260	0.373
P. legaeviata	Semi-arid soil		13.46	1.98	17.776
	Arid soil		13.13	2.43	17.52
		SED	1.110	0.498	0.251
		P Value	0.773	0.385	0.362
P. juliflora	Low ground, under irrigation		12.80	1.98	18.00
	High ground, no irrigation		14.64	3.05	17.82
		SED	0.696	0.161	0.256
		P Value	**0.024**	**<0.001**	0.498
P. pallida	Built up area		11.89	0.88	17.68
	Near water		10.13	0.92	17.29
		SED	0.736	0.150	0.227
		P Value	**0.038**	0.828	0.121

[1] SED = standard error of the differences of the means, n= 2 technical replicates per sample, 12 samples per species. Unbalanced design, environmental grouping detailed in Table 4.1.

[2]P values determined by a one-way ANOVA.

4.3.2 Amino Acid Concentrations

There was a significant difference ($p<0.05$) observed between the four analysed species of mezquite for the amino acid content (g/kg DW) of the mezquite pod flour, in all analysed essential amino acids. A Bonferroni post hoc test showed that for methionine, lysine, isoleucine, phenylalanine and histidine *P. pallida* and *P. velutina* had a significantly lower content than *P. juliflora* and *P. legaeviata*. For threonine, only *P. velutina* flour had a significantly lower content than *P. juliflora* and for valine, it was significantly lower than *P.* pallida. For leucine, *P. pallida* and *P. velutina* had a significantly lower content than *P. legaeviata*.

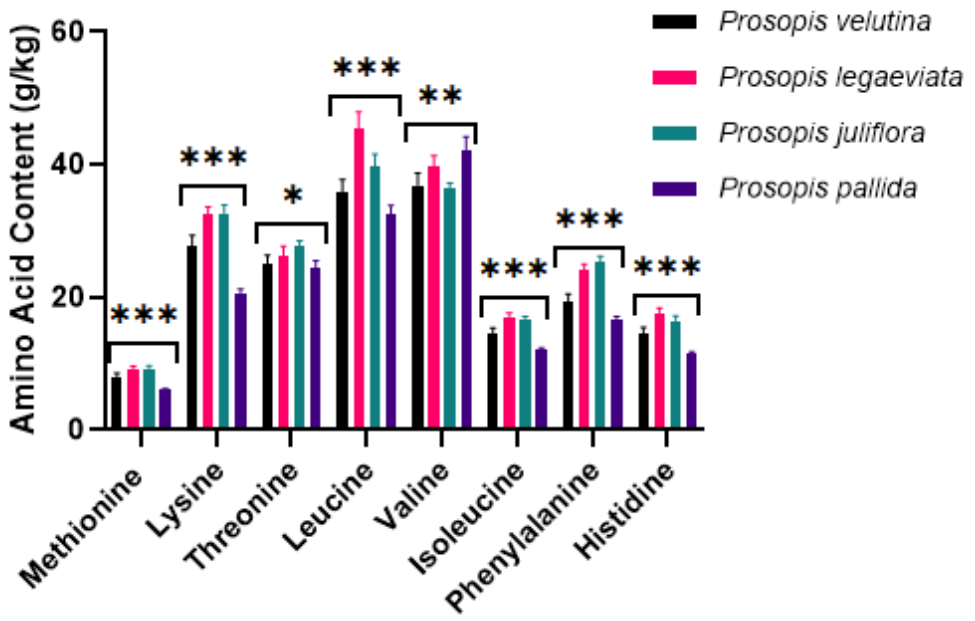


Figure 4.2. The amino acid content (g/kg) in the mezquite pod flour. Data is shown as means plus SEM, n=2 technical replicates per sample, 12 samples per species, analysed on a dry weight basis. Significance determined by one-way ANOVA *p<0.05 **p<0.01 ***p<0.001. Tryptophan was not analysed.

4.3.3 Mineral Concentrations

There was a significant difference (p<0.05) between all the minerals analysed in Table 4.6, the remaining analysed minerals and significant differences are shown in Appendix 2.

Table 4.6. The mineral concentrations (mg/kg) within the pod flour from four mezquite species.

Mineral	*P. velutina* (mg/kg)	*P. legaeviata* (mg/kg)	*P. juliflora* (mg/kg)	*P. pallida* (mg/kg)	SED[1]	P Value[2]
Na	461.8^{b}	71.99^{a}	110.1^{a}	822.7^{c}	61.06	<0.001
K	19808^{c}	17828^{b}	19060^{c}	16530^{a}	439.3	<0.001
Mg	932^{a}	1401^{c}	835^{a}	1076^{b}	46.59	<0.001
Ca	2056^{a}	4242^{c}	2828^{b}	2551^{ab}	212.1	<0.001
P	1283^{a}	1849^{b}	1863^{b}	1800^{b}	66.95	<0.001
Fe	23.12^{a}	44.47^{ab}	62.63^{bc}	78.94^{c}	8.891	<0.001
Cu	5.11^{c}	4.07^{b}	2.79^{a}	6.38^{d}	0.335	<0.001
Zn	18.56^{a}	28.23^{a}	15.77^{a}	19.41^{b}	1.516	<0.001
Mn	9.53^{a}	15.35^{b}	15.47^{b}	13.64^{b}	0.944	<0.001
Se	0.092^{a}	0.226^{a}	1.598^{c}	0.636^{b}	0.133	<0.001
Cd	0.002^{a}	0.019^{b}	0.011^{ab}	0.002^{a}	0.005	0.002
Pb	0.043^{b}	0.019^{a}	0.024^{a}	0.033^{ab}	0.007	0.003

[1] SED = standard error of the differences of the means. N= 2 technical replicates per sample, 12 samples per species.

[2]P values determined by a one-way ANOVA. Different letters indicate a significant difference, determined with a Bonferroni post-hoc test.

4.3.4 Antinutritional Factors

The total phenolic content and the phytic acid content were significantly different ($p=0.003$ and $p<0.001$ respectively) between the different mezquite pod flours (Table 4.4). *P. velutina* pod flour had the highest total phenolic content and *P. legaeviata* the highest phytic acid content. *P. pallida* showed the lowest concentrations for both.

Table 4.4. The antinutritional content of pod flour from four mezquite species.

Sample	Total Phenolics (mg TAE/g)	Phytic Acid (g/100g)
P. velutina	8.96[b]	0.1892[b]
P. legaeviata	7.46[ab]	0.3034[c]
P. juliflora	7.40[ab]	0.0329[a]
P. pallida	5.75[a]	0.0260[a]
SED[1]	0.786	0.021
P Value[2]	**0.003**	**<0.001**

[1] SED = standard error of the differences of the means. For total phenolics n= 3 technical replicates per sample, 12 samples per species. For phytic acid n= 2 technical replicates, 4 randomly selected samples per species.

[2]P values determined by a one-way ANOVA. Different letters indicate a significant difference, determined with a Bonferroni post-hoc test.

There was a significant difference ($p<0.05$) in the total phenolic content in *P. juliflora* pod flour associated with the different environmental conditions with an increased concentration in pods harvested from trees grown on higher ground, with no irrigation (Table 4.5). There was also a significant difference seen for the phytic acid content within *P. velutina* ($p=0.012$), with a higher phytic acid content seen in the pods harvested from trees grown in saline soils compared to under irrigation (Table 4.5).

Table 4.5. The differences in the total phenolic and phytic acid concentrations within the four different species of mezquite, grouped according to environmental differences.

Mezquite species	Environmental conditions		Total Phenolics (mg TAE/g)	Phytic Acid (g/100g)
P. velutina	Under Irrigation		8.42	0.230
	Saline soils		10.04	0.148
		SED[1]	1.661	0.023
		P Value[2]	0.352	**0.012**
P. legaeviata	Semi-arid soil		7.72	0.276
	Arid soil		6.88	0.331
		SED	1.695	0.041
		P Value	0.633	0.051
P. juliflora	Low ground, under irrigation		6.72	0.027
	High ground, no irrigation		8.08	0.039
		SED	0.474	0.005
		P Value	**0.017**	0.072
P. pallida	Built up area		5.84	0.024
	Near water		5.65	0.028
		SED	0.589	0.005
		P Value	0.758	0.461

[1] SED = standard error of the differences of the means. For total phenolics n= 3 technical replicates per sample, 12 samples per species with an unbalanced design, environmental grouping detailed in Table 4.1. For phytic acid n= 2 technical replicates, 2 randomly selected samples per environmental condition. [2]P values determined by a one-way ANOVA.

4.4. Discussion

The nutrient concentrations of mezquite pod flour are variable between different species, and within species depending on environmental conditions.

Four different species of mezquite were analysed in this study. While this study looks at the differences between these species, it cannot be said with certainty that species is the variate responsible for these changes. In this study, each species was from a distinct location, with varying environmental conditions both within and between species. This is a limiting factor on this study, ideally the study would allow for multiple of the same species grown in a range of different locations and monitored environmental conditions; or a range of species grown in the same location and environmental conditions. This would allow testing of how the nutritional and antinutritional concentrations in mezquite pod flour vary depending on species or environmental conditions during growth. However, the mezquite species used in this study are the predominant ones found in the regions they were harvested. This means that the results of this study could have an impact on the communities living in the areas of studies by advising on whether the endogenous species in the local area should be recommended for human or animal consumption.

The crude protein, energy and fat content all vary significantly between the different mezquite species. *P. juliflora* pod flour was found to have the highest protein, fat and energy content. The crude protein content ranges from 10.78-13.72%, this content is considerably lower than soybean, which is reported to

have around 36.5% dry weight (Sakkas et al. 2019). Within species there was a difference in growth conditions before the pods were harvested. In *P. juliflora* pods harvested from higher ground, with no irrigation had a significantly higher crude protein and fat concentration. This fits with the findings of Feng et al. 2020, who reported that a water deficit increased the protein content in sunflower seeds, compared to those under irrigation. The use of poor-quality water for irrigation could also cause a reduction in protein and fat content. This is because poor water quality irrigation can increase salinity, or salt content, in the surrounding soil (Muscolo et al. 2011). Saline soils have been shown to significantly reduce protein and fat content, with (Abbas et al. 2013) finding a 22.97% decrease in the protein content and a 22.97% decrease in the fat content of wheat grain when grown in saline soils compared to non-saline soils. However, in our study we do not know details of the irrigation level applied, the water quality, or if there is a difference in rainfall between the high and low ground; so, it is difficult to conclude whether the irrigation is the cause of the observed differences. There is also a reported change of elevation, with the higher protein and fat contents being shown in the *P. juliflora* pods from trees grown on higher ground. The growth of soybean plants at 846-963m compared to 336-480m has been shown to increase the protein content by 6.15% (Capelin et al. 2022). However, (Xue-jun et al. 2013) found a negative correlation between elevation during growth and the crude fat content (DW) in maize. It is therefore difficult to conclude the cause of the change in protein and fat content.

For *P. pallida* pod flour, the crude protein content was higher when harvested from trees in an area surrounded by buildings, in comparison to a more natural area near a lake. This difference is more difficult to investigate as no more details have been recorded and there are many variables that may have affected the results.

The amino acid concentration varied significantly between the different species of mezquite pod flour for all analysed essential amino acids. *P. velutina* showed the lowest amino acid concentration for all essential amino acids. However, this data is presented as g/kg of the dry weight flour, rather than of the protein. As *P. velutina* has the lowest crude protein concentration this is not surprising. The amino acid concentration results appear to follow the pattern of the crude protein concentration for most of the essential amino acids, expressing the data as amino acids within the protein may therefore negate the significant effects. However, as explained in Chapter 4.2.5, the amino acid results discussed in this study could not be normalised to a standard reference material, as done in the rest of this thesis. However, when considered as a percentage of dry matter, the amino acid concentration of the studied mezquite pod flours are comparable to those of soybean. For methionine, which is a limiting amino acid in soybean; mezquite pod flour contains 0.6-0.9%, while soybean is reported to contain 0.5-0.67%. Soybean is highest in the essential amino acids' lysine and leucine. For lysine, mezquite pod flour is shown to contain 2.1-3.3%, while soybean has a reported 2.5-2.66. In leucine, mezquite pod flour shows 3.2-4.5%, while soybean has a reported range of 2.2-

4% (Banaszkiewicz, Banaszkiewicz 2011). This implies that overall, mezquite pod flour has a comparable amino acid profile to soybean.

The mineral concentration varies significantly for all displayed minerals. Na, K, Mg, Ca and P were analysed as they are considered essential in a broiler chicken diet; in which mezquite pod flour has a potential utilisation. The percentage specifications for Ross 308 broiler chickens states the predominant mineral requirements as 0.65-0.95% Ca, 0.36-0.50% P, 0.05-0.3% Mg, 0.18-0.23% Na and 0.60-0.90% K (Aviagen, 2022). The percentage concentration seen in all species of mezquite pod flour is lower than these specifications however, as mezquite pod flour is being investigated as only a feed ingredient, these mineral concentrations would likely be higher in the final formulated feed. In soybean the percentage mineral concentrations are reported as 0.67% P, 2.05% K, 0.31% Ca, 0.28% Mg and 0.012% Na (Batal et al. 2010). In mezquite pod flour the percentage mineral concentrations are lower than soybean for P and Mg, but very close for K and the highest value observed in Ca is higher for *P. legaeviata* pod flour then observed in soybean and the Na concentration is higher in *P. velutina* pod flour and *P. pallida* pod flour. The Cd and Pb concentrations are low, at <0.05mg/kg for all mezquite pod species. For Cd, doses above 10mg/kg have been shown to leas to toxicity symptoms in poultry (Olgun et al. 2020) and in lead trace levels of 1mg/kg have been shown to result in reduced growth (Puschner et al. 2013); both above the levels seen in any of the studies mezquite pod flour species. Pb and Cd is usually elevated in plants due to high levels found in the soil. Heavy metals in the soil can come from

natural sources such as rocks, ores or volcanoes, or as contaminants from human activities such as mining, waste combustion, fertilisers and industrial wastewater from manufacturing plants Dinu et al. (2021) *P. laevigata* seedlings cultured in a media containing 3mM Pb showed a bioaccumulation factor of 21(Buendia-Gonzalez et al. 2010). This suggests that the low Pb and Cd concentrations seen in this study are likely due to no environmental pollution of these minerals. This is important information for people who may wish to harvest mezquite pods, as environmental testing can be completed to ensure that Pb and Cd concentrations are not at toxic levels.

The anti-nutrient concentrations of mezquite pod flour is variable between different species, and within species depending on environmental conditions.

The total phenolic content ranged between 5.75-8.96 mg TAE/g DW. These results fit with the findings of (Díaz-Batalla, Hernández-Uribe, et al. 2018) reported 6.68 mg gallic acid equivalent (GAE)/g DW in raw *P. legaeviata* pod flour. However, it would be interesting to see how these results vary when considering different parts of the pods or seeds. For example, in Chapter 3 it is shown that the total phenolic content of bambara groundnut seeds is significantly higher in the seed hulls compared to the seeds with hulls removed. In mezquite, Correa Uriburu et al. (2022) found that the total phenolic content in *P. alba* seed flour ranged from 5.05-8.58 mg GAE/g DW, similar to the results seen in the whole pod flour in this study. However, (Díaz-Batalla, Hernández et al. 2018) found that in *P. legaeviata* pods the total phenolic content of mesocarp flour was higher at 8.87 mg GAE/g DW than mesocarp seed flour at

8.36 mg GAE/g DW or seed flour at 6.57 GAE/g DW. This suggests that the total phenolic content within the pod is higher than within the seed, which may explain why the range of results seen in the mezquite pod flour is higher than the reported range of 3.70-5.22mg GAE/g (Yusnawan 2018). This information would be of value for the local communities consuming the pods, so they know which parts of the pods have potential to be consumed raw and which parts should be processed.

Similar to the protein and fat results, *P. juliflora* pods had a higher total phenolic content when harvested from trees grown on higher ground, with no irrigation. (Alonso-Amelot et al. 2007)studied the phenolic compounds in *Pteridium arachnoideum* fronds grown at different elevations, during the dry or rainy season. They found that the content of both low and high molecular weight phenolics increased as elevation increased. This occurred in both the rainy and dry season, but the increase was greater during the dry season. This suggests that a combination of elevation and less water resulted in increased total phenolics.

The phytic acid content was significantly different between species and ranged between 0.026g/100g and 0.3034g/100g, this is below the reported range of 0.33-0.89% (Retana-Márquez et al. 2016). Within species, the phytic acid content was significantly different only in *P. velutina* which showed a higher concentration in pods harvested from trees grown in saline soils compared to under irrigation. This is supported by (Ibrahem, Abd EL-Mohsen (2010) who found that saline soils significantly increased the phytic acid concentration in

faba beans compared to non-saline soils. However, small population sizes were used for this analysis, as not all samples were measured. The results therefore should not be generalised to assume the same pattern for all samples.

4.5 Conclusions

The analysis in this study was conducted in order to evaluate differences in the nutritional and antinutritional profiles of different mezquite species. Different species of mezquite are predominant in different regions, meaning the impact of this work is to contribute to the knowledge of which areas can utilise the mezquite pods grown on trees in their local area. As mezquite trees are considered invasive in Africa, assisting in the knowledge of potential methods of utilisation can help to prevent eradication of the trees. Significant differences were observed between species for the proximate nutrient concentrations, total phenolic content, mineral concentrations and essential amino acid concentrations.

The different species in this study all come from different regions, meaning it is difficult to draw conclusions about the causation of observed differences; species or environmental conditions. This could be considered a limitation of this study. However, as different environmental conditions were seen within the same species', samples were grouped to provide some analysis on differences within species depending on environmental conditions. This was conducted for the proximate nutrient analysis, finding differences in crude protein for *P. juliflora* and *P. pallida* flour, and in fat for *P. juliflora*. It was also conducted for the antinutrient concentrations, finding differences in the total

phenolic content for *P. juliflora* and the phytic acid content for *P. velutina*. Additionally, the study allowed more information to be gained about the species grown in specific environments. For the local communities this information may be extremely beneficial for understanding their local species, regardless of species and environment being confounding factors.

Overall mezquite pod flour has a lower crude protein content and lower mineral concentrations then soybean when looking at literature values. However, the amino acid profile is comparable, suggesting that partial replacement in animal feeds may be possible.

5 The effect of dietary mezquite pod flour (*Prosopis juliflora*) on the growth and amino acid digestibility of broiler chickens.

5.1 Introduction

While some mezquite species are native to Africa and Asia, many species of the trees were introduced to these areas as a means of improving soil qualities through nitrogen fixing, due to them being legumes. However, they spread rapidly, and their utilisation was not up taken; resulting in them now being considered an invasive species (Patnaik et al. 2017). Finding suitable methods of utilising the trees, and the pods they produce, therefore could be beneficial to the local communities.

P. juliflora is one of the dominant invasive species, native to Argentina but now widespread in Africa and Asia (Patnaik et al. 2017). In Kenya, its introduction aimed to control soil erosion with its extensive root system (Choge et al. 2022). The first documented introduction was in 1973, with the aim of rehabilitating quarries (Choge et al. 2007). However, it has rapidly spread across the arid and semi-arid areas, uncontrolled due to lack of knowledge of its utilisation. Strategies were initially put in place for it to be utilised as firewood, but now the focus is on eradicating the trees (Choge et al. 2022). It currently is considered to be the most notable invasive species in Kenya (Okul 2021).

The use of *P. juliflora* pods as a novel feed ingredient for animals is still relatively unexplored. With mezquite trees being so widespread, it is not unexpected that in many areas, mezquite pods have become a readily available potential grazing material for livestock (Almanza, Moya 1986). There is lots of recorded anecdotal evidence of livestock grazing on the pods, with Mwangi and Swallow (2008) reporting that this is one of the major causes of the spread of the trees; as the pods are consumed and the seeds passed back out in the faeces, resulting in further colonisation by this invasive species. This therefore is contributing to the negative aspects of the trees, rather than being a positive method of utilisation. They also found that the local communities perceived the hard pods, and high sugar content, of *P. juliflora* as the causation of jaw and dental problems in their farmed goats, causing economic losses due to decreased lifespan (Mwangi, Swallow 2008).

However, there are a limited number of controlled studies looking at the effects of the inclusion of flour as a feed ingredient on growth, particularly in monogastric species. In ruminant species, there does not appear to be any negative effects related to the consumption of mezquite pods (Koech et al. 2010; Cook et al. 2008; de Oliveira Moraes et al. 2016). In broiler chickens however, it is suggested that higher inclusions of mezquite pod flour may have negative effects on bird weight gain. An inclusion of below 30% (w/w) does not reportedly have a significant effect (Odero-Waitituh et al. 2016; Girma et al. 2011).

With this in mind, the study described in this chapter aimed to look at the effect of a 15% (w/w) inclusion of *P. juliflora* mezquite pod flour on broiler chicken performance and amino acid digestibility. This inclusion level was chosen as previous studies suggest it to be well below the level that had previously caused an impact on growth. The *P. juliflora* pods are utilised as a flour due to anecdotal evidence suggesting consumption of whole seeds by livestock is likely to increase the spread of the trees; and to ensure palatability for poultry throughout the whole study.

Aims

This study aimed to assess the effect of a commercial feed with a 15% (w/w) inclusion of *P. juliflora* pod flour, from Kenya, on:

1. Broiler chicken performance via growth and feed efficiency
2. Ileal crude protein and amino acid digestibility

Hypothesis

The hypothesis of the study was that a 15% inclusion of *P. juliflora* pod floor within broiler feed would not negatively impact animal performance.

5.2 Materials and Methods

5.2.3 Mezquite pod collection and preparation

Mezquite species identification was carried out by Dr. Arturo Castro-Castro (Centro Interdisciplinario de Investigacion para el Desarrollo Integral Regional, Durango) and Dr. Socorro Gonzalez Elizondo (Instituto Politecnico Nacional, campus CIIDIR Durango). Community liason and sample collection was carried

out by Dr. Oscar Koech (University of Nairobi) and collection was assisted by the Il Chamus and Tugen communities from Kenya.

The Mezquite species used in this study was *P. juliflora,* the pods from which were collected from the Baringo area in Kenya. The *P. juliflora* pods were ground to a fine flour, dried, and shipped to the university of Nottingham. Once the flour had arrived, 100g was weighed out and freeze-dried in an open topped tin foil container until a constant weight, as described in chapter 2.1.1, for the determination of proximate nutrient composition.

Mezquite pod flour was used in this study, rather than whole pods or seeds, due to restrictions in shipping over whole material due to the potential risks of insects, disease or spread of non-native plants.

5.2.4 Nutrient analysis

Dry weight (DW) samples were used for all proximate nutrient analysis. The *P. juliflora* pod flour used in this chapter was initially analysed for crude protein via elemental analyser (FlashEA 1112 Series, Thermo Fisher Scientific), gross energy via bomb calorimetry (6300 calorimeter, Parr) and total fat (Gerhardt's Soxtherm system). Broiler feeds and ileal digesta samples were also analysed for crude protein via elemental analyser. These methods are described in chapter 2.1.

5.2.3 *P. juliflora* pod flour antinutritional analysis

The total phenolics and phytic acid content were analysed as described in chapter 2.2.

The trypsin inhibitor activity was analysed externally by Sciantec (Yorkshire, UK) by spectroscopy. Inhibitors were extracted from the samples using dilute sodium hydroxide (pH 9.5) and an aliquot of the extract was incubated with a standard amount of trypsin. The trypsin concentration remaining after incubation was measured by reaction with substrate Benzoyl-DL-arginine-P-nitroanilide for a specified time and temperature, causing the formation of yellow coloured o-nitroaniline. The solutions were filtered and the absorbance measured at 410nm (information provided by Sciantec, 2022).

5.2.4 Mycotoxin analysis of *P. juliflora* pod flour

The mycotoxin levels in the *P. juliflora* pod flour were analysed externally by Sciantec (Yorkshire, UK) via quantification of LC/MS-MS using Immunoaffinity. Homogenised samples were extracted with 1% formic acid in methanol. An aliquot of the extract was diluted in PBS and cleaned up using liquid chromatography alongside mass spectrometry (LC/MS-MS). Samples were run alongside an in-house quality control sample (information provided by Sciantec, 2022).

5.2.5 *P. juliflora* broiler trial

5.2.5.6 Ethical approval

This trial was carried out at the University of Nottingham, Bio Support Unit, this was approved by the internal ethical review committee (ethical approval number: 000171). The ethical committee assessment was that no 'regulated procedures' were carried out and therefore the study was carried out under DEFRA regulations and did not require a Home Office license.

5.2.5.7 Experimental Diets

Two mash feeds were used for this diet, with broilers being kept on either the control or the *P. juliflora* pod flour-based feed throughout the trial intervention period. The control feed and basal experimental feed were formulated and supplied by Target Feeds (Whitchurch, UK). A wheat and soybean-meal based control diet (Control) was used. The experimental feed (*P. juliflora*) contained a 15% inclusion of *P. juliflora* pod flour, replacing a portion of the wheat. The *P. juliflora* diet also had 3% less soya oil to balance the energy content of the feeds (Table 5.1). Titanium dioxide was added at 5g/kg as an indigestible marker of ileal digestibility.

The feeds were mixed on site as described in chapter 2.4.2, this combined titanium dioxide with the supplied control feed and combined the *P. juliflora* pod flour and the titanium dioxide with the supplied experimental diet. 225kg of each diet were mixed, which was split into 9 x 25kg bags per diet.

Table 5.1. The % (w/w) composition of raw materials in the control feed vs the *P. juliflora* pod flour-based feed, generated by Target Feeds.

Raw material	Control diet (g/100g)	*P. juliflora* diet (g/100g)
Barley, raw ground	10.5	10.5
Wheat, raw ground	**50.0**	**38.0**
***P. juliflora* pod flour**	**0.00**	**15.0**
Soya ext hipro	26.0	26.0
Full fat soya masham	5.00	5.00
L Lysine	0.40	0.40
DL Methionine	0.40	0.40
L Theonine	0.15	0.15
Soya Oil	**4.00**	**1.00**
Limestone Trucal	1.25	1.25
Monocalcium phosphate	1.50	1.50
Salt	0.25	0.25
Sodium Bicarbonate	0.15	0.15
Broiler starter premix[1]	0.40	0.40
Crude protein	25.83	24.74

[1]Supplying: retinoic acid 4.8 mg/kg, cholecalciferol 75 µg/kg, α-tocopherol75 mg/kg, thiamine 3 mg/kg, riboflavin 10 mg/kg, pyridoxine 3 mg/kg, cobalamin 15 µg/kg; nicotinic acid 60 mg/kg, pantothenic acid 14.7 mg/kg, folic acid 1.5 mg/kg, biotin 125 µg/kg, choline chloride 25 mg/kg, Fe 20 mg/kg, Cu 10 mg/kg, Mn 100 mg/kg, Co 1.0 mg/kg, Zn 82.222 mg/kg, I 1 mg/kg, Se 0.2 mg/kg and Mo 0.5 mg/kg.

Differences between diets shown in bold.

5.2.5.8 Subjects and Husbandry

A total of 90 one-day-old male Ross 308 Broiler Chickens were delivered to the University of Nottingham, Sutton Bonington Campus bio support unit from P.D. Hook Hatcheries Ltd (Cote, Oxfordshire, UK). 90 birds were ordered to allow for 10 spares above the 80 birds needed, this allowed for extra in case of any birds suffering ill health or perishing during the trial.

The birds were group housed in two groups of 45 for the first six days of the trial, during which, all birds were fed on the control diet. Birds were wing tagged and randomly allocated to pens on day six, with 20 pens of four birds, ensuring no significant difference ($p>0.05$) in the starting weights of the birds in each treatment group.

All birds were housed within one room, with treatment groups alternating between pens. The litter of each pen consisted of wood shavings, which were topped up to meet DEFRA welfare requirements. Feed and water in trays were supplied *ad libitum.* Temperature and ventilation were monitored for appropriateness for the birds depending on age. The birds were inspected at least twice per day to monitor environmental conditions, feed supply and bird welfare.

5.2.5.9 Data Collection

Bird performance was measured by body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR). The BWG was measured by the weighing of the birds in each pen twice per week throughout the trial. This was then divided

by the number of birds in each pen to account for any losses and normalised to the number of days over which this weight gain took place; giving a value per bird, per day, for each time period. Feed intake was measured per pen, by monitoring how much feed was given and the weighing back of the feed remaining twice per week. This measurement was taken per pen and divided by the bird number for a 'per bird' FI estimate. FCR was calculated by dividing the average feed intake per bird per pen, by the average BWG per bird per pen. A FCR closer to "1" represents a more efficient feed to BWG conversion.

5.2.5.10 Sample collection

Birds were culled on day 35 and 36 of the trial, with pens 1-10 being culled on day 35 and 11-20 on day 36. Birds were culled by schedule 1 procedure of anaesthetic overdose (Animals (Scientific Procedures) Act 1986). After confirmation of death, the digestive tract of each bird was exposed and samples of ileal digesta was taken, this was the full ileal content from Meckel's diverticulum. The ileal samples were bagged and snap frozen in liquid nitrogen before storage at -80°C prior to analysis. The right-hand breast muscle (*pectoralis major*), and the liver, were also dissected from each bird and weighed. These weights were then also normalised to the body weight of that bird to give a proportional weight.

5.2.5.11 Apparent Ileal Digestibility

Ileal digesta samples were freeze-dried to a constant weight, in plastic containers, as described in chapter 2.1.1; and ground to a find powder using a

pestle and mortar. Ground samples were combined on a per pen basis, giving 10 samples per treatment group. These were analysed for crude protein, as in chapter 2.1.2, titanium dioxide and amino acids, as per chapter 2.4.5.

Feed samples (n=3 per diet) were also freeze-dried to a constant weight, in tin foil containers, as described in chapter 2.1.1; and ground to a fine powder using a pestle and mortar. These were analysed for crude protein, as in chapter 2.1.2, titanium dioxide and amino acids, as per chapter 2.4.5. Data generated for the titanium dioxide concentrations was used alongside the ileal crude protein and amino acid contents to generate values for the apparent ileal crude protein digestibility ($AIDC_{CP}$) and the apparent ileal amino acid digestibility ($AIDC_{AA}$), as described in chapter 2.4.5.4.

5.2.6 Data Analysis

Statistical analysis was conducted using Genstat 20th Edition software to determine if there were any significant differences. All significant differences in the following chapter were determined using a one-way ANOVA, except for the effect of diet on BWG over time; which was analysed using a two-way ANOVA (time x diet). Significance was taken at $p<0.05$. All graphs were drawn using Graph Pad Prism 8.

5.3 Results

5.3.1 *P. juliflora* pod flour analysis

The *P. juliflora* pod flour received from Kenya was analysed to determine its nutritional composition prior to feed formulation. This consisted of the crude protein, energy and fat content (Table 5.2). This was to ensure that the experimental feed could be formulated to be isonitrogenous and isoenergetic to the control feed.

Table 5.2. Nutritional composition of the *P. juliflora* pod flour used for feed formulation. N=2 technical replicates.

Crude Protein (g/100g DW)	**Energy (mJ/kg)**	**Fat (g/100g DW)**
12.3	18	3.8

The amino acid content of the *P. juliflora* flour was also measured, on a DW, as fed basis (table 5.3).

Table 5.3. Amino Acid composition of the *P. juliflora* pod flour. N=2 technical replicates.

Amino Acid	Amino Acid Content (g/100 DW)
Alanine	5.55
Arginine	9.11
Aspartate	15.86
Cysteic Acid	0.52
Glutamate	15.16
Glycine	5.88
Histidine	3.80
Isoleucine	2.63
Leucine	7.74
Lysine	5.36
Methionine Sulfone	1.30
Phenylalanine	4.96
Proline	12.27
Serine	5.41
Threonine	3.84
Tyrosine	4.89
Valine	6.04

The *P. juliflora* pod flour was also analysed to determine the antinutritional content. This consisted of the total phenolics content, the phytic acid content and the trypsin inhibitor activity (Sciantec, Yorkshire, UK) (Table 5.4).

Table 5.4. Antinutrient content of the *P. juliflora* pod flour. N=3 technical replicates.

Total Phenolics (mg TAE/g)	Phytic Acid (g/100g)	Trypsin Inhibitor Activity (mg/g)
8.37	0.32	0.8

A sample of the *P. juliflora* flour was analysed (Sciantec, Yorkshire, UK) for mycotoxin concentrations, as seen in Table 5.5. This was to ensure that there was no build-up of toxins that would affect the animals when consuming the mezquite diet.

Table 5.5. The levels of 6 mycotoxins contained in the *P. juliflora* pod flour used for the chicken trial.

Toxin	T2 Toxin	Aflatoxin	Deoxy-nivalenol	Ochra-toxin	Total Fumonisin	Zeara-lenone
Amount	25 ug/kg	4.8 ug/kg	0.37 mg/kg	<10 ug/kg	<0.22 mg/kg	216 ug/kg

5.3.2 Feed Analysis

There were no significant differences in the analytical replicates determining the dry matter content (p=0.817), crude protein content (p=0.616), gross

energy (p=0.278) or ash content (p=0.500) of the two broiler feeds used in this study (Table 5.6).

Table 5.6. The nutritional analysis of control and *P. juliflora* feeds used in the broiler trial. N= 2 technical replicates.

	Dry Matter (g/100g)	**Crude Protein (g/100g)**	**Gross Energy (MJ/kg)**	**Ash (g/100g DM)**
Control Feed	91.96	25.83	17.49	5.81
***P. juliflora* Feed**	91.70	24.74	17.01	6.62
SED[1]	0.889	1.860	0.225	0.808
P Value[2]	0.817	0.616	0.278	0.500

[1] SED- standard error of the differences of the means. N=

[2]P values determined by a one-way ANOVA.

There were no significant differences (p>0.05) in the amino acid content between the control and *P. juliflora* feeds, as seen in Figure 5.1.

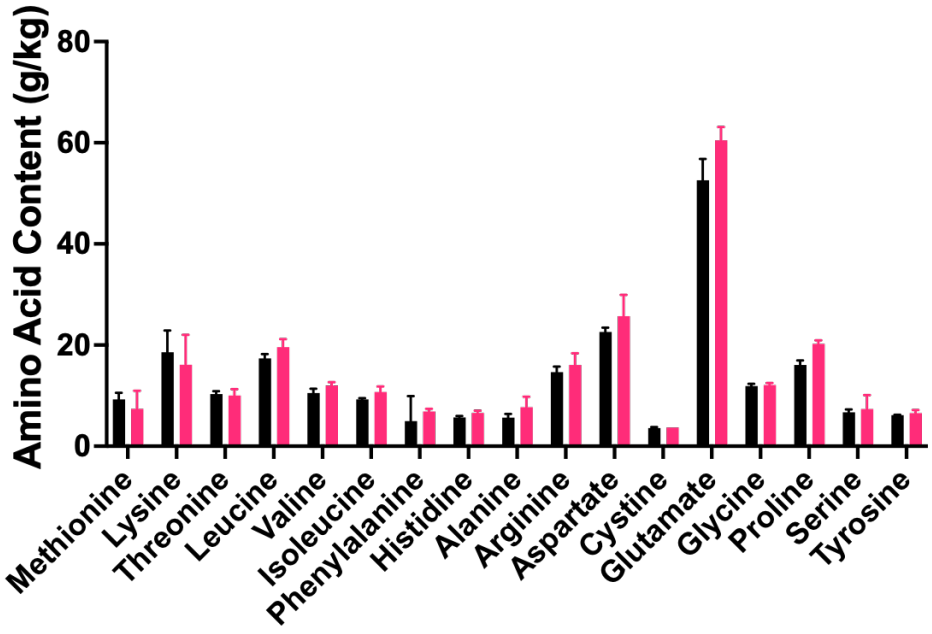


Figure 5.1. The Amino Acid content (g/kg) of the control and *P. juliflora* pod flour feeds. A one-way ANOVA showed no significant difference (p>0.05) between the content of the two feeds for any of the amino acids. Data shows means plus SEM, n=2 technical replicates per group.

There was a significant difference ($p<0.05$) in the analytical replicates determining the total phenolic content of the control and *P. juliflora* feeds (Table 5.7), with the *P. juliflora* feed having a higher content than the control feed. There was no difference ($p>0.05$) in the phytic acid content.

Table 5.7. The antinutritional analysis of control and *P. juliflora* feeds used in the broiler trial.

	Total Phenolics (mg TAE/g)	**Phytic Acid (g/100g)**
Control Feed	8.11	2.01
Mezquite Feed	13.12	1.79
SED[1]	1.324	0.089
P Value[2]	**0.019**	0.240

[1] SED- standard error of the differences of the means. N=3 technical replicates per group.

[2]P values determined by a one-way ANOVA.

5.3.3 Bird Performance

As seen in Table 5.7, there was no significant difference ($p=0.286$) in the initial live bird weights, taken at day 6 of the trial before switching to the experimental feeds. There was, however, a significant difference ($p<0.001$) seen in the final weights of the birds taken at day 36, after 30 days on the experimental diets (Table 5.8). There was not a significant difference in the

total feed intake per pen (p=0.847) but the significantly lower weights of the mezquite fed birds resulted in a significantly higher FCR (p<0.001) in the mezquite group (Table 5.8).

Table 5.8. The effect of inclusion of *P. juliflora* pod flour on broiler chicken performance, expressed as an average per bird at 36 days.

	Day 6 BWt (kg)	**Final BWt (kg)**	**Total Feed Intake (Kg)**	**FCR**
Control	0.129	2.19	2.93	1.34
P. juliflora	0.132	1.57	2.90	1.86
SED[1]	0.002	0.07	0.13	0.08
P-value[2]	0.286	**<0.001**	0.847	**<0.001**

[1] SED = standard error of the differences of the means, n =40 birds per treatment group.

[2] P Values determined using a one-way ANOVA.

BWt = Body weight, wt= weight.

There was a significant interaction between time and feed for body weight gain ($p<0.001$). BWG per bird, per day, significantly increased over time for both treatment groups, but the increase was significantly higher for birds fed the control feed (Figure 5.2).

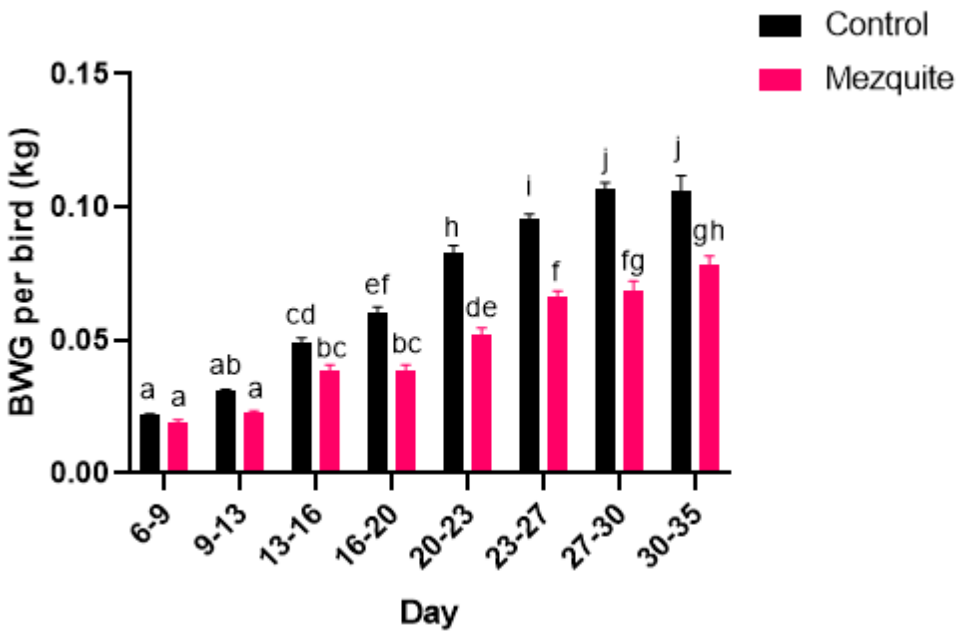


Figure 5.2. The effect of inclusion of *P. juliflora* pod flour on broiler chicken body weight gain. The graph shows the average body weight gain per bird, per day, on a pen basis for both treatment groups during each period between weighing. Error bars shown are SEM, n=10 pens per treatment group. A two-way ANOVA showed a significant interaction ($p<0.001$) between time and feed for BWG. Different letters indicate a significant difference, determined by a Bonferroni post-hoc test ($p<0.05$).

5.3.4 Bird Body Composition

The inclusion of *P. juliflora* pod flour had no significant effect on the liver weights of the birds after 30 days on the experimental feeds. This was also true when expressed as a percentage of live body weight (Table 5.9). However, the birds fed the mezquite diet did have significantly lower weight breast muscles ($p=0.009$) than the control fed birds. This was also true when expressed as a percentage of body weight ($p<0.001$), as seen in Table 5.9.

Table 5.9. The effect of inclusion of *P. juliflora* pod flour on broiler chicken body composition.

		Control Feed	*P. juliflora* Feed	SED[1]	P Value[3]
Liver	**Gross weight (g)**	0.06	0.07	0.03	0.674
	%BW [2]	2.51	2.41	0.11	0.240
Pectoralis major	**Gross weight (g)**	0.14	0.10	0.01	**0.009**
	%BW	6.12	5.39	0.18	**<0.001**

[1]SED- standard error of the differences of the means

[2]BW = Body weight

[3]P Values determined using a one-way ANOVA. N=40 birds per treatment group.

5.3.5 Digestibility

5.3.5.1 Crude Protein digestibility

There was a significant difference in the apparent ileal crude protein digestibility coefficients ($AIDC_{CP}$) ($p<0.001$) (Table 5.10) between the two treatment groups with the mezquite group being significantly lower than the control group.

Table 5.10. The effect of inclusion of *P. juliflora* pod flour on the broiler chicken apparent ileal crude protein digestibility coefficient.

	Control Feed	***P. juliflora* Feed**	**SED [1]**	**P Value[3]**
$AIDC_{CP}$[2] (%)	73.2	62.1	2.12	**<0.001**

[1]SED- standard error of the differences of the means

[2]$AIDC_{CP}$- Apparent ileal digestibility coefficient for crude protein

[3] P Values generated by one way ANOVA. N=10 pens per treatment group.

5.3.5.3 Amino Acid digestibility

The inclusion of *P. juliflora* pod flour had a significant effect on the apparent ileal amino acid digestibility coefficient ($AIDC_{AA}$), with several amino acids having a lower $AIDC_{AA}$ in birds fed the *P. juliflora* diet compared to the control diet. These were aspartate ($p=0.037$), cystine ($p<0.001$), glutamate ($p=0.022$),

glycine (p=0.009), leucine (p=0.019), lysine (p<0.001), methionine (p<0.001), proline (p=0.048), threonine, (p<0.001), tryosine (p<0.001) and valine (p=0.033) (Figure 5.5). The other amino acids did not show significant differences between treatment groups (p>0.05) (Figure 5.5). The $AIDC_{AA}$ ranged from 45.3% for phenylalanine in the control group, to 92.3% for methionine in the control group.

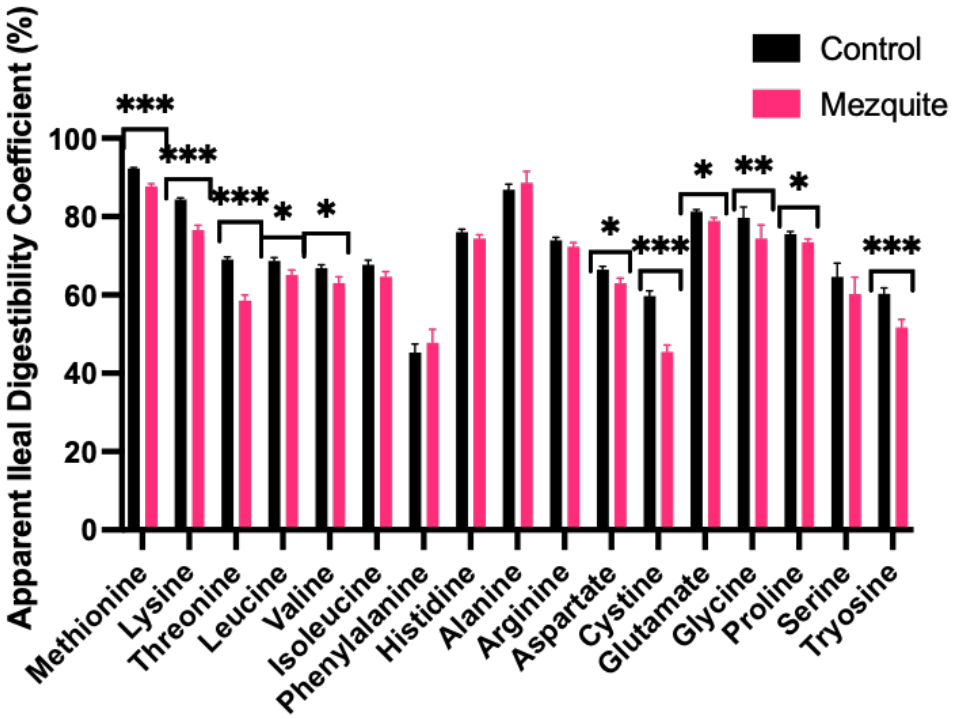


Figure 5.5. The effect of inclusion of *P. juliflora* pod flour on the apparent ileal amino acid digestibility coefficients. Digestibility was determined after 30 days on experimental feeds. Data is shown as means plus SEM, with data analysed through one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001.

5.4 Discussion

The experimental diet containing a 15% inclusion of P. juliflora pod flour was formulated to be nutritionally similar to the wheat-based control diet.

The feed analysis conducted prior to this study showed that the feeds were formulated to be isonitrogenous and isoenergetic. There were no significant differences in the crude protein, dry matter, gross energy or ash contents of the two feeds. The crude protein requirement for Ross 308 broilers is 23% in the starter phase, 21.5% in the grower phase and 19.5% in the finisher phase (Aviagen, 2022). In this trial the feeds were formulated to be fed throughout the whole trial, so both feeds had a greater protein content than needed for all phases at 25.83% for the control feed and 24.74% for the *P. juliflora* feed. The energy content required for Ross 308 broilers is 12.4 MJ/kg in the starter phase, 12.8 MJ/kg in the grower phase and 13 MJ/kg in the finisher phase (Aviagen, 2022). The energy content for the feeds in this trial were 17.49 MJ/kg for the control feed and 17.01 MJ/kg for the *P. juliflora* feed.

There were also no significant differences in the amino acid content of the feeds; importantly, this includes the essential amino acids. Both the feeds and the *P. juliflora flour* had higher percentage concentrations of all the essential amino acids analysed than is required in the starter, grower or finisher phase for Ross 308 broilers (Aviagen, 2022). With these things being balanced within the two feeds, it was expected that the birds in each treatment group would perform the same.

A 15% inclusion of P. juliflora pod flour into wheat-based broiler chicken diets significantly reduced body weight gain without changing the feed intake, increasing the feed conversion ratio.

The results of this study show that a 15% *P. juliflora* pod flour inclusion into broiler feeds inhibited growth rates. The final bird liveweight for *P. juliflora* fed birds was 28% lower than control birds. This suggests that something within the feed is interfering with nutrient digestion.

This result went against the findings of previous studies. In ruminant species, the inclusion of mezquite pod flour, including *P. juliflora,* has been shown to have a positive effect on body weight gain. Koech et al. (2010), found that the addition of *P. juliflora* to the diet increased weekly body weight gain in goats, compared to a control diet. This became significant during weeks 5-10 (end) of the trial. Cook et al. (2008) also found that goats could be fed up to a 90% inclusion of *P. glandulosa* mezquite pods in an alfalfa pellet diet for 12 days with no negative impact on feed intake or serum metabolite levels. Finally, de Oliveira Moraes et al. (2016) found that the inclusion of *P. juliflora* pod flour into a corn-based diet at 25, 50, 75 and 100% for dairy steers did not significantly alter feed intake or animal performance. However, if ANFs are a possible cause of the decreased weight gain seen in the current study, it is known that these are degraded in the rumen, resulting in less negative effects (Méndez-Ortiz et al. 2018).

In monogastric animals, the results of feeding experiments using mezquite pod flour show slightly different results. Odero-Waitituh et al. (2016) replaced

maize with *P. juliflora* pod flour at 10, 20 and 30% in broiler chicken diets from day 29-57 of life. They found that the body weight of the birds significantly decreased with higher inclusions of the *P. juliflora* pod flour. Similarly, Girma et al. (2011) found that a 30% *P. juliflora* inclusion negatively affected growth in Hubbard classic broiler chickens, while a 10 or 20% inclusion had no significant effect. These studies suggest that *P. juliflora* pod flour may be able to be fed to monogastric species in low amounts but may have a negative impact with higher inclusion levels. It was on these results that the chosen inclusion rate of 15% was based. However, our findings do not match up to those of these studies. This could be due to the breed of broiler chickens used in the studies. Both of the previous broiler chicken studies mentioned, appear to use broiler chickens with a slower growth rate than the Ross 308 broilers used in our study. Ross 308 broilers are selectively bred for extremely fast growth and optimal performance on diets similar to the control feed used in this study. This means that they will be much more sensitive to any negative effects caused by changes in their feed or environment, causing an amplified effect. This could also be seen as a limitation of this study, as it is not representative of the poultry that may be fed the *P. juliflora* pod material in the countries where it is harvested. The birds owned by small rural communities in Kenya, where the benefits of *P. juliflora* pod utilisation will be greatest, are not likely to be as fast growing as our broiler chickens. As these birds will not be optimised to such a fast growth rate, the growth inhibition seen in this study is likely to be less obvious.

The growth rate of the *P. juliflora* fed birds had not plateaued at 36 days, the data shows that the birds were still gaining weight through the study, suggesting that they possibly would have continued a regular growth curve if allowed to continue on the feed. However, this slower rate of growth means that in westernised countries, where demand is high, this would not be a viable.

The feed intake, measured on a pen basis, was not significantly different between the two treatment groups. At a 15% inclusion, this is similar to previous results, with Girma et al. (2011) finding that a *P. juliflora* inclusion rate of below 20% had no effect on feed intake. Though they did find that at the 30% inclusion level, where growth was negatively impacted, the feed intake was also affected. Feed intake in broilers has been shown to be affected by the crude protein content and amino acid profile of the feed (Ferket and Gernat, 2006). In this study, the feeds were balanced for crude protein and no significant differences were found in the amino acid profile of the feeds. The similar feed intakes also suggests that there were not any palatability issues with the *P. juliflora* feed.

The FCR seen in the control group of this study shows very efficient growth, with a FCR of 1.34 coming in below the ideal performance objective FCR of 1.41 for Ross 308 broilers at 36 days of age (Aviagen, 2022). This means that the birds fed the control feeds, in the conditions of this study, were performing at maximum capacity; making the effect of the dietary change caused by the *P. juliflora* pod flour inclusion more pronounced.

A 15% inclusion of P. juliflora pod flour into wheat-based broiler chicken diets had no effect the liver weights of the birds but did significantly reduce breast muscle weights.

The liver of each bird was taken and weighed as a measure of the effect on metabolically active tissue. In this study, a 15% inclusion of *P. juliflora* pod flour had no effect on the average liver weights of the birds, which was reflected when expressed as a percentage of body weight. However, there was an effect on breast muscle weight, with *P. juliflora* fed birds having both a significantly lower average breast weight and percentage of body weight. While the breast weight may be expected due to the smaller average live weight of the *P. juliflora* fed birds, a smaller breast muscle percentage indicates a problem. This has also been observed by (Biesek et al. 2020) who found that a 25% replacement of soybean meal with yellow lupin resulted in a significantly smaller breast muscle percentage in geese compared to a control feed. As the breast is the most economically valuable portion of a commercial chicken carcass, a smaller breast yield would have a significant impact (Marcu et al. 2013). As broiler chickens have been selectively bred for maximum muscle growth in a short period of time, particularly in the highly economically valuable breast muscles. However, this is not a function that is vital to the life of the birds. This may be why the breast muscle percentage was smaller in the *P. juliflora* birds, but the liver remained unchanged, as the reduction in muscle production relieved energy expenditure without harming the health of the birds or increasing the FI.

The *P. juliflora* pod flour used in the trial was analysed externally to test the levels of common mycotoxins. This could be reflected upon to ensure that no toxins were the cause of the reduced growth and breast muscle weights. However, most mycotoxins would predominantly affect the liver, which we found to have no significant differences in this trial. The analysed mycotoxins were T2, aflatoxins, deoxynivalenol, ochratoxin, fumonsins and zearalenone.

T2 is a toxic secondary metabolite belonging to the trichothecenes group. It is produced by fungi of the following species, *F. acuminatum*, *F. poae* and *F. Sporotrichioides.* The toxin is most commonly found in cereal grains. The toxic effects in mammals can include reduced production, feed refusal and gastroenteritis. In poultry the toxin can affect the digestive system and liver and reduce bird performance. The accepted rate in poultry feeds varies between different countries, with limits ranging between 25-100 ug/kg (Azizi et al. 2014). Aflatoxins are the most studied type of mycotoxin due to 25% of total agricultural products having contamination (Ditta et al. 2018). Toxic levels cause aflatoxicosis, which is a major problem in poultry production in the tropics. Doses of 30ug/kg and above can start to cause symptoms, predominantly affecting the liver (Mgbeahuruike et al. 2018). Deoxynivalenol is a mycotoxin produced by the *Fusarium* species. It is a trichothecene and is found in all kinds of grains. It is the most common worldwide contaminant of poultry feed material and has a natural occurrence of 0-5 mg/kg, which starts to increase with long term storage in improper conditions. Poultry are considered less susceptible to toxicity than other species, due to the low level

of absorption into tissues and rapid clearance. Though contamination with 10 mg/kg in broiler chicken diets has been shown to reduce feed intake and body weight gain (Awad et al. 2013). Ochratoxin is produced by some *Aspergillus ochraceus* and *Penicillium verrucosum* species. Contamination in poultry feedstuffs usually occurs due to the harvest of insufficiently dried cereals. Signs of toxicity in poultry include a reduction in weight gain and poor FCR, studies suggest that this occurs at around 0.5mg/kg (Denli, Perez 2010). Fumonsins are a group of fungal metabolites produced by *Fusariumproliferatum* and *F. Verticillioides.* In poultry they block the synthesis of complex sphingolipids which have protective effects within the body, causing them to accumulate in tissues. In broilers, the recommendation is to keep concentrations in feed below 20 mg/kg of feed (Grenier, Masching 2015). Zearalenone is produced by some *Fusarium* species and is often found alongside Deoxynivalenol in grains. High levels can cause a reduction in bird performance due to effects on the liver and is often an indicator of other mycotoxins being present (Liu, Applegate 2020). Around 2 mg/kg has had a reported negative impact on bird performance (Wu et al. 2021).

It can be seen from this that the levels of these mycotoxins found in the *P. juliflora* pod flour used for this study were lower than the levels previously reported to cause negative effects. As there was also no reduction in the average liver weights of birds eating the *P. juliflora* feed, it can be concluded that there was likely no significant mycotoxin contamination.

A 15% inclusion of P. juliflora pod flour into wheat-based broiler chicken diets had no effect on the ileal crude protein or amino acid content but significantly decreased the ileal crude protein and multiple amino acid digestibility coefficients.

Titanium dioxide was added into the broiler feeds as an inert marker of digestion. This is a commonly done practice in broiler chicken trials in order to enable the determination of diet digestibility. In this trial there was a significant decrease in the average ileal titanium content from 15.07 g/kg in the control birds to 11.14 g/kg in the *P. juliflora* birds. As the same quantity of titanium dioxide was mixed into both the control and *P. juliflora* feeds, the ileal contents should be similar. This indicates a reduced digestion in the *P. juliflora* fed birds. These titanium dioxide content measurements were used to calculate the AIDC for both crude protein and individual amino acids.

The crude protein content of the two feeds were not significantly different from one another, showing that a lack of protein in the feed was not the cause of the reduced weight gain in the *P. juliflora* birds. The crude protein content found in the ileal digesta was also similar between both treatment groups. However, the lower ileal titanium dioxide content in the *P. juliflora* birds means that the $AIDC_{CP}$ in the *P. juliflora* fed birds is significantly lower at 62.1% than the control birds at 73.2%. These results show that the *P. juliflora* feeds have a lower crude protein digestibility.

Similarly, there were no significant differences between the ileal amino acid content of the control and *P. juliflora* birds. However, the inclusion of the *P.*

juliflora pod flour significantly reduced the $AIDC_{AA}$ for multiple amino acids. Of the essential amino acids analysed in this study, the AIDC for methionine, lysine, threonine, leucine and valine were all significantly lower in the *P. juliflora* birds than the control birds. The proper levels and ratios of AAs are essential to maintain the high level of growth and muscle mass production that broilers have been selectively bred for; this variation could therefore have played a major role in the reduction seen in in the body weight and breast muscle proportion seen in the *P. juliflora* birds.

Methionine plays an important role in the optimum growth performance of poultry. It is often one of the first limiting amino acids in broiler chicken diets, meaning additional supplementation is essential (Alagawany et al. 2020). Methionine deficiency has been seen to result in a significantly lower relative breast muscle weight (Corzo et al. 2006).

Lysine represents approximately 7% of the protein in broiler chicken breast meat (Dozier et al. 2008) and alongside methionine is one of the limiting amino acids in broiler diets (Alagawany et al. 2020). Tesseraud et al. (2001) found that broilers fed a lysine deficient diet had significantly lower body weights and a 30% decrease in breast muscle weight relative to body weight compared to a lysine adequate diet.

Threonine is the third limiting amino acid in broiler diets. It is involved in protein synthesis and maintenance in the body, particularly within the gut. The presence of threonine within lumen speeds up the synthesis of mucosal protein and mucin; aiding proper gut functioning (Alagawany et al. 2020). Estalkhzir et

al. (2013) found that a diet containing 115% higher dietary threonine than required, resulted in a significantly higher broiler chicken daily weight gain a significantly higher breast muscle percentage at 42 days, when compared to diets containing the dietary requirement or 105% or 110% higher than the dietary requirement.

Leucine, alongside isoleucine and valine, is a branched-chain amino acid. Leucine is one of the essential amino acids most strongly considered to affect protein synthesis in muscle production. It acts as a signal to regulate protein synthesis and inhibit protein degradation in skeletal muscle production (Hm, Kratei n.d.; Kimball, Jefferson 2006). Erwan et al. (2008) found that adding 0.5g additional L-Leucine per kg of a diet without altering the protein or energy content significantly increased carcass weight in Cobb broiler chickens. The breast muscle also seems to be the most sensitive to changes in dietary lysine perhaps due to the differences in muscle fibers with a total dietary lysine content of 9.8 to 12.5g/kg significantly increasing daily weight gain and breast muscle weight compared to a total lysine content of 8g/kg (Leclercq 1998).

Valine is another essential branched-chain amino acid, which is often limiting in broiler chicken feeds (Kim et al. 2022). As it is a branched-chain amino acid, its primary protein synthesis role is involved in muscle mass production (Kimball, Jefferson 2006); deficiency in valine would therefore impact growth performance.

From this, it can be seen that all of the essential amino acids with reduced apparent ileal digestibility as a result of the *P. juliflora* pod flour inclusion could

have played a role in the observed reduction in weight gain and breast muscle weight reduction. Of these, it is the branched-chain amino acids that are largely responsible for the production of muscle mass, meaning that the difference in the apparent ileal digestibility of Leucine and Valine may be responsible for the smaller breast muscle percentage seen in the *P. juliflora* birds. The biggest significant difference in the apparent ileal digestibility for the essential amino acids was seen in threonine. Ahmed et al (2020) found that broilers fed diets with 10 or 20% higher dietary threonine than the NRC recommendation resulted in a higher apparent ileal digestibility of crude protein. A lower digestibility of the dietary threonine caused by the *P. juliflora* pod flour could therefore have contributed to the lower $AIDC_{CP}$ observed in the *P. juliflora* fed birds, contributing to the live weight difference.

It is well documented in the literature that mezquite pods contain ANFs in the form of tannins, phytic acid, lectins and protease inhibitors (Andrade-Montemayor et al. 2011; Retana-Márquez et al. 2016; Barba De La Rosa et al. 2006). These have also been a focus throughout this thesis. For the results of this trial, ANFs are considered as a possible explanation. Total phenolics as a tannic acid equivalent were found in the *P. juliflora* flour at 8.37mg/g. These are one of the most commonly found and studied ANF (Barreca et al. 2017) and so it was expected that these would be detected. In comparison, the total phenolic content of raw soybean seeds in the literature varies between around 1.13-8.07 mg/g; varying depending on cultivar and growth location (Lee et al. 2011; Malenčić et al. 2007; Xu, Chang 2008). In general, the total phenolic

content of soybean appears to be often lower than the result found for the *P. juliflora* used in this trial, likely correlating to a lower tannin content. As tannins bind to dietary proteins (Nikmaram et al. 2017), high levels in the diet have been recorded to have significant effects in poultry; reducing body weight gain due to a reduction in protein digestibility (Gemede, Ratta 2014; Vilariño et al. 2009; Nyamambi et al.2000) This reflects the results of this trial, suggesting that tannins are a likely contributor to the results. The safe limit of tannins is considered to be 1.5-2mg/g (Farinde et al. 2018), which is considerably lower than the levels seen in both feeds used in this trial. The first chapter of this thesis demonstrates that Bambara groundnut seeds contain most of their tannins within the seed hulls, this has also been demonstrated in other leguminous seeds, meaning that dehulling is often an effective method of reducing the negative effects caused by tannins (Nikmaram et al. 2017). This is a disadvantage of feeding the whole pods in this trial, tannins were likely highest within the hulls of the mezquite seeds contained within the pods, but their removal would be laborious in comparison to other processing techniques such as heat treatment and germination; which have also been shown to reduce tannin levels (Ibrahim et al. 2002).

Similarly, phytic acid was found in the *P. juliflora* flour at 0.32 (g/100g). Phytic acid has strong chelating properties, meaning that it can bind to dietary minerals, making them unavailable (Yasothai 2016). Mineral deficiencies such as low phosphorus, can result in a reduced growth rate in birds (Wei et al. 2021). However, in this study it is difficult to evaluate if phytic acid had a

negative effect on the birds consuming the *P. juliflora* diet, as mineral digestibility was not evaluated. There was also no significant difference observed in the phytic acid content between the two diets (p=0.240). The most common solution to high levels of phytic acid in a broiler chicken diet is the supplementation of the exogenous enzyme phytase (Cowieson et al. 2009). Commercially, this is a reasonably common practice to increase the availability of phosphorus and other minerals in the diet (Selle, Ravindran 2007; Liu, Ru 2010).

The trypsin inhibitor activity of the *P. juliflora* flour was found to be 0.8mg/g. Trypsin inhibitors inhibit the activity of the pancreatic enzymes trypsin and chymotrypsin, resulting in a reduction in the digestion of dietary protein (Vagadia et al. 2017; Gemede, Ratta 2014). This, in turn, has been shown to result in reduced weight gain in broiler chickens (Palliyeguru et al. 2011; Rada et al. 2016), matching the results of this study. Trypsin inhibitor activity therefore also must be a consideration when thinking about the causes of the reduced weight gain seen in the birds consuming the *P. juliflora* diet in this study. Heat processing and soaking of seeds have been shown to be the most effective way of reducing trypsin inhibitor activity, therefore mitigating their effects (Avilés-Gaxiola et al. 2018).

5.5 Conclusions

This broiler chicken trial was carried out to evaluate the effect of a 15% inclusion of *P. juliflora* pod flour into a wheat-based broiler feed on performance and apparent ileal digestibility of crude protein and amino acids.

The results of this study showed that a 15% inclusion of *P. juliflora* flour reduced body weight gain and proportional breast muscle weights despite showing no difference in feed intake or liver weights. This significantly increased the FCR for the *P. juliflora* birds. The *P. juliflora* diet also decreased ileal crude protein digestibility and the digestibility of several essential amino acids.

These results suggest that while there was likely no immediate toxicity or palatability issues with the *P. juliflora* pod flour inclusion, something within the flour interfered with protein digestion; resulting in a reduction of body weight gain and proportion breast muscle weights. Reviewing the literature, it seems likely that this could be a result of ANFs; namely one, or a mix of, tannins, phytic acid and trypsin inhibitors. All of these ANFs have been found in mezquite pod flour and have been shown to cause similar effects as seen in this trial.

Further studies into *P. juliflora* pod flour as an alternative production animal feed source should look further into the negative effects of the antinutritional factors and whether post-harvest processing, or the supplementation of exogenous enzymes, could improve nutrient utilisation.

6 The effect of two mezquite varieties from different geographical regions of Mexico on broiler chicken performance and amino acid digestibility.

6.1 Introduction

In Mexico mezquite trees have a rich history as an important source of food and fuel for the communities that utilize them (Almanza and Moya, 1986). There are many species of mezquite native to Mexico, with wide distribution in both arid and semiarid areas (Franco et al. 1996). Two varieties commonly found are *P. velutina,* also known as velvet mesquite (Schade, Hobbie 2005), and *P. articulata,* also known as bitter mezquite (de la Luz et al. 2005). While valued for their nutritional content, mezquite pods have varying nutrients depending on the species. For example, the protein content in the pods from different *P.* species have been reported to vary between 7 and 34% dry weight (Ellsworth et al. 2018; Sawal et al. 2004; Ruiz-Nieto et al. 2020) whilst the fat content is between 1 and 6% (Ruiz-Nieto et al. 2020). While their abundance and protein content mean that mezquite pods can potentially be utilised as grazing material for ruminant livestock (Almanza, Moya 1986; Koech et al. 2010), the presence of antinutritional factors (ANF) may be a limiting factor in their use for monogastric species.

The presence of ANFs in plants, as secondary metabolites, has evolved as a method of potentially preventing disease and has been suggested to reduce animal predation (Gemede, Ratta 2014). In leguminous plants, such as mezquite, the ANFs tannins, phytic acid, lectins and trypsin inhibitors can all have a negative impact on nutrient bioavailability for the animals consuming them (William, Jafri 2015). The content of these ANFs can vary significantly between species of the same plant and can be altered by the environment in which they were grown. It has been suggested that growth in a high stress environment can induce higher levels of ANFs than a low stress environment (Goyal et al. 2016).

Post harvest processing can be used as a method of reducing the negative effects of antinutritional factors. These methods include dehulling of legume seeds (Nikmaram et al. 2017) heat processing, soaking, fermentation (Avilés-Gaxiola et al. 2018) and applying high pressures (Lajolo et al. 2002). Of these, cooking, soaking and dehulling are all common practices before human consumption of plant materials which may be high in ANFS.

Tannins, a group of phenolic compounds, have the ability to bind to dietary proteins, minerals and digestive enzymes; making them partially unavailable or inhibiting their functions. In animal diets they cause a reduction in protein digestibility, resulting in reduced body weight gain (Gemede, Ratta 2014). Tannins have been shown to be heat-labile, meaning heat processing has been shown to be an effective method of reducing tannin concentrations; in turn reducing their negative effects (Ibrahim et al. 2002; Olanipekun et al. 2015).

For example, (Khattab, Arntfield 2009) found that roasting at 180^{o}C for 10 minutes significantly reduced the tannin content of several varieties of cowpea and pea. Similarly, Mesfin et al. (2021) found that roasting at 150^{o}C for 30 minutes or at 180^{o}C for 15 minutes significantly reduced the tannin content in two varieties of chickpea.

Phytic acid is the major storage form of phosphorus in plants, it binds to minerals, particularly divalent metals, forming insoluble complexes known as phytates (Kaplan et al. 2019; Yasothai 2016). These phytates are broken down by phytase enzymes within plants, but animals lack these digestive enzymes; meaning consumed phytic acid reduces the bioavailability of essential minerals from the diet (Kumar et al. 2010; Yasothai 2016). Soaking, roasting, boiling and fermentation have all been found to significantly reduce phytic acid (Khattab, Arntfield 2009; Mesfin et al. 2021). However, the most commonly utilised method of breaking down phytates in commercial monogastric production is the use of the exogenous enzyme phytase. The first commercial phytase products were released in 1991 and are now commonplace in commercial practice and in animal research studies (Konietzny 2006). Phytase enzymes catalyse the phosphate splitting of phytate to lower inositol phosphate esters and inorganic phosphate (Lei, Porres 2003). Phytase levels are measured in phytase units (FTU), with one FTU being the amount of enzyme required to release 1 µmol of monocalcium phosphate in one minute under the test conditions of pH 5.5 and 37^{o}C (FAO, 2012).

A previous study, described in Chapter 5, found that a 15% inclusion of *P. juliflora* pod flour from Kenya into broiler chicken feeds, significantly ($p<0.05$) reduced body weight gain, while not affecting feed intake. The *P. juliflora* diet also decreased ileal crude protein digestibility and the digestibility of several essential amino acids. It was concluded that the presence of ANFs may have been responsible for this reduction in feed digestibility, in particular tannins. It has also been shown in Chapter 4 that different species of mezquite can have significantly different ($p<0.05$) nutritional and antinutritional profiles. This study therefore aimed to see how different mezquite species affect broiler chicken growth and crude protein and amino acid digestibility, both when included raw or when processed to reduce ANFs. This study focusses on the reduction of tannins, measured as total phenolics, and phytic acid, as they are the most easily measurable and have both been shown to reduce body weight gain in poultry. The *P. juliflora* used in Chapter 5. is one of the most researched mezquite species, meaning findings are often generalised to other mezquite species. For the following study, two different species of mezquite were selected in order to see how the effects *in vivo* can vary between species. This work will help to inform communities in Mexico as to whether all mezquite species can be considered similarly for animal feeds, and whether there is potential for improvement by processing. However, it should be considered that mezquite species and geographical region are confounded, meaning that it cannot be said whether any potential differences are due to species or the region and associated environmental conditions. This was unavoidable due to different species of mezquite being endogenous to different regions.

Aims

1. To assess the effect on broiler performance of the combination of post-harvest processing (heat treatment) and phytase supplementation on mezquite pod flour from two species from distinct geographical areas of Mexico.

2. To assess the effect on crude protein and amino acid digestibility of the combination of post-harvest processing (heat treatment) and phytase supplementation on mezquite pod flour from two distinct geographical areas of Mexico.

Hypothesis

The hypothesis of this study was that difference mezquite varieties from different geographical regions within Mexico would influence broiler chicken production and that these feeds would be differentially affected by subjecting them to processing which would be associated with growth improvement.

6.2 Materials and Methods

6.2.1 Mezquite pod collection and preparation

Mezquite species identification was carried out by Arturo Castro-Castro (Centro Interdisciplinario de Investigacion para el Desarrollo Integral Regional, Durango, Mexico). Community Liason was carried out by Xochitl Soto-Luzania, Karina Fernandez Baurista (Universidad Technologica de San Luis Rio Colorado, Sonora, Mexico), Omar Mancera Gonzalez (Universidad Autonoma de Sinaloa,

Sinaloa, Mexico) and Carolina Ceballos-Bernal (Centro Interdisciplinario para la Alimentacion y el Descarrollo, Sinaloa, Mexico). Collection was completed by members of the Yoreme May peoples of El Fuerte Sinaloa, Mexico and from rural communities from Estacion Doctor and Mesa Rica, Sonora, Mexico. Grinding of the samples were completed by Xochitl Soto-Luzania and Karina Fernandez Baurista (Universidad Technologica de San Luis Rio Colorado, Sonora, Mexico). Shipping of the samples was completed by Xochitl Soto-Luzania (Universidad Technologica de San Luis Rio Colorado, Sonora, Mexico) and Yolanda Lopez-Franco (Centro Interdisciplinario para la Alimentacion y el Descarrollo, Sonora, Mexico).

Mezquite pods were harvested from the Sinaloa and Sonora regions of Mexico. In Sonora, whole *P. velutina* pods were collected on the 12th July 2021 from the Estacion Doctor area. These trees were grown under irrigation, near to water, with salty soil and near to domestic animals. In Sinaloa, whole *P. articulata* pods were collected on the 15th and 16th June from the Los Capomos area. These trees were located near to roads and water reservoirs.

The whole pods were collected and were finely ground into flour before being shipped to the University of Nottingham. Mezquite pod flour was used in this study, rather than whole pods or seeds, due to restrictions in shipping over whole material due to the potential risks of insects, disease or spread of non-native plants.

6.2.2 Mezquite pod flour processing

Due to a delay in the receipt of mezquite pod flour from Mexico, initial processing was completed using the *P. juliflora* pod flour from Kenya, used in the trial detailed in Chapter 5. This flour was processed in order to decide which processing method should be used in the following trial. Processing of the *P. juliflora* pod flour was carried out by Joseph Godrich (PhD student within the division of Food, Nutrition and Dietetics). This was used to determine the appropriate preparative methods, which was then repeated on the *P. articulata* and *P. velutina* flours used in the subsequent analysis.

Soaking, boiling, roasting and popping were chosen due to them being common methods used in order to reduce antinutritional factors in previous literature studies of other legume species. Germination and autoclaving were also considered but germination could not be used due to only being able to obtain flour samples and autoclaving could not be completed at the time of study due to technical issues with the autoclave, making it too unreliable to use on a larger scale for the animal trial. With the exception of popping, these techniques can be easily completed in Mexico where the flours were collected.

6.2.2.1 Soaking

500ml ultrapure water was added to 100g of finely ground mezquite pod flour (1:5 w/v) and left for 24 hours at room temperature. A 106-micron sieve was then used to sieve out excess water. The remaining flour was then collected and freeze-dried until at a constant weight, as described in Chapter 2.1.1. This

was then broken down with a pestle and mortar to ensure the product was evenly ground.

6.2.2.2 Boiling

500ml ultrapure water was added to 50g of finely ground mezquite pod flour (1:10 w/v) in a 1L glass beaker. This was placed on a hotplate and covered with foil and the solution was brought to a boil. A 106-micron sieve was then used to sieve out excess water. The remaining flour was then collected and freeze-dried until at a constant weight, as described in Chapter 2.1.1. This was then broken down with a pestle and mortar to ensure the product was evenly ground.

6.2.2.3 Roasting

50g finely ground mezquite pod flour was evenly spread in a foil tray with no lid. This was placed into a preheated convection oven for 10 minutes at 70^{o}C, 100^{o}C, 140^{o}C, 170^{o}C or 200^{o}C for five separate roasting treatments.

6.2.2.4 Popping

Popping is a novel food processing technique of current research interest. The process involves the application of high heat and pressure to grains or pulses until the internal moisture expands. It is of interest as it is a sustainable and cost-effective snack preparation technique (Godrich et al. 2023). This method was chosen in this work as it is a novel technique, to allow comparison of the antinutritional factor change to commonly use techniques.

1.8g finely ground mezquite pod flour was popped using a manual popping head (4.5 cm diameter, custom build, University of Nottingham, UK) (Godrich et al. 2023) for 4 seconds at 4 bar pressure, this was repeated 25 times to give 45g of sample. This was done at either 180^{o}C or 200^{o}C for two separate popping treatments. The produced popped mezquite cakes were then collected and ground back into a fine flour using a pestle and mortar.

6.2.4 Nutrient analysis

Samples were freeze-dried until a constant weight, as described in Chapter 2.1.1, prior to proximate nutrient analysis. The mezquite pod flour used in this Chapter was initially analysed for crude protein via elemental analyser (FlashEA 1112 Series, Thermo Fisher Scientific), gross energy via bomb calorimetry (6300 calorimeter, Parr) and total fat (Gerhardt's Soxtherm system). Broiler feeds and ileal digesta samples were also analysed for crude protein via elemental analyser. These methods are all described in Chapter 2.1.

6.2.3 Mezquite pod flour antinutritional analysis

The total phenolics and phytic acid content were analysed as described in Chapter 2.2

The trypsin inhibitor activity was analysed externally by Sciantec (Yorkshire, UK) by spectroscopy. Inhibitors were extracted from the samples using dilute sodium hydroxide (pH 9.5) and an aliquot of the extract was incubated with a standard amount of trypsin. The trypsin concentration remaining after incubation was measured by reaction with substrate Benzoyl-DL-arginine-P-

nitroanilide for a specified time and temperature, causing the formation of yellow coloured o-nitroaniline. The solutions were filtered, and the absorbance measured at 410nm (information provided by Sciantec, 2022).

6.2.4 Mycotoxin analysis of Mezquite pod flour

The mycotoxin levels in the *P. juliflora* pod flour were analysed externally by Sciantec (Yorkshire, UK) via quantification of LC/MS-MS using Immunoaffinity. Homogenised samples were extracted with 1% formic acid in methanol. An aliquot of the extract was diluted in PBS and cleaned up using liquid chromatography alongside mass spectrometry (LC/MS-MS). Samples were run alongside an in-house quality control sample (information provided by Sciantec, 2022).

6.2.5 Broiler chicken trial

This trial was completed at Allermuir poultry unit, SRUC, Edinburgh. The experimental manager was Professor Jos Houdijk (SRUC, Edinburgh) and the research project manager was Dr Farina Khattak (SRUC, Edinburgh) with technical support from Fraser Whyte (SRUC, Edinburgh) and stock workers at the poultry unit.

6.2.5.1 Ethics

This trial was completed under ASPA project license PPL P87B76570 held by Farina Khattak and personal licence ASPeL- IBA985EF8 held by Jos Houdijk.

6.2.5.2 Subjects and Husbandry

A total of 144 one-day-old male Ross 308 Broiler Chickens were delivered to the Allermuir poultry unit, SRUC, Edinburgh. The birds were randomly allocated to each of 37 pens, with 4 birds per pen. The pens were set across three rooms in a randomised block design. There were six pens on each side of each of the three rooms ensuring each treatment group on each side of each room; with a balanced treatment design across the six room-side combinations. The birds were all fed the control diet for the first six days to allow them to acclimatise to the environment. On day six the pens were allocated to one of six dietary treatment groups to ensure no statistically significant difference in weight across each treatment group.

The litter of each pen consisted of wood shavings, which were topped up to meet welfare considerations. Feed from trays and water from drinker lines were supplied *ad libitum.* Temperature and ventilation were monitored for appropriateness for the birds depending on age. The birds were inspected at least twice per day to monitor environmental conditions and bird welfare.

6.2.5.3 Experimental Diets

This trial has six treatment groups, as follows:

Diet 1 (CON)- a wheat-based control diet

Diet 2 (CONPRO)- a wheat-based control diet with 15% (w/w) substitution of processed wheat and phytase inclusion at 1500 FTU/ Kg

Diet 3 (SON)- a wheat-based diet with 15% (w/w) inclusion of *P. velutina* pod flour from Sonora, Mexico

Diet 4 (SONPRO)- a wheat-based diet with 15% (w/w) inclusion of processed *P. velutina* pod flour from Sonora and phytase inclusion at 1500 FTU/ Kg

Diet 5 (SIN)- a wheat-based diet with 15% (w/w) inclusion of *P. articulata* pod flour from Sinaloa, Mexico

Diet 6 (SINPRO)- a wheat-based diet with 15% (w/w) inclusion of processed *P. articulata* pod flour from Sinaloa and phytase inclusion at 1500 FTU/ Kg

To process the wheat, *P. velutina* pod flour and *P. articulata* pod flour, these ingredients were individually roasted at 140^{o}C for 10 minutes, cooled to room temperature and stored at room temperature in plastic containers until mixing into the feeds. In addition, quantum blue phytase from AB Vista (Wiltshire, UK) was added to the diet at a superdose of 1500 ftu/kg. This was mixed into the basal diets before the heat-treated ingredients were mixed in. The control diet and basal experimental diets were formulated and supplied by Target feeds (Whitchurch, UK) (Table 6.1). The processed wheat, raw mezquite pod flour and processed mezquite pod flour were mixed in on site at Sutton Bonington campus, university of Nottingham, alongside 5 g/kg titanium dioxide as an indigestible marker of ileal digestibility, as described in Chapter 2.4.2.

All diets were fed as a mash feed throughout the whole trial. 200kg of the control diet and 150kg of the other diets were mixed and split into 25kg bags.

Table 6.1. The composition of raw materials in the control feed and each experimental feed, generated by Target Feeds.

Raw Material	CON diet (g/100g)	CONPRO diet (g/100g)	SON diet (g/100g)	SONPRO diet (g/100g)	SIN diet (g/100g)	SINPRO diet (g/100g)
Barley Raw Ground	10.5	10.5	10	10	11.75	11.75
Wheat Raw Ground	50	35	40	40	40	40
Processed Wheat	0	15	0	0	0	0
Mezquite	**0**	**0**	**15**	**15**	**15**	**15**
Soya Ext Hipro	26	26	24	24	23	23
Full Fat Soya MASHAM	5	5	5	5	5	5
L Lysine Batch	0.4	0.4	0.4	0.4	0.4	0.4
DL Methionine Batch	0.4	0.4	0.4	0.4	0.4	0.4
L Threonine Batch	0.15	0.15	0.15	0.15	0.15	0.15
Soya Oil	**4**	**4**	**1**	**1**	**0.75**	**0.75**
Limestone Trucal 52	1.25	1.25	1.25	1.25	1.25	1.25
Monocalcium Phosphate	1.5	1.5	1.5	1.5	1.5	1.5
Salt	0.25	0.25	0.25	0.25	0.25	0.25
Sodium Bicarbonate	0.15	0.15	0.15	0.15	0.15	0.15
Broiler trial starter premix	0.4	0.4	0.4	0.4	0.4	0.4
Quantum Blue (ABVista)	**0**	**0.03**	**0**	**0.03**	**0**	**0.03**
Processing	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**
Crude Protein	22.68	23.14	23.30	25.17	22.88	22.16

[1]Supplying: retinoic acid 4.8 mg/kg, cholecalciferol 75 µg/kg, α-tocopherol75 mg/kg, thiamine 3 mg/kg, riboflavin 10 mg/kg, pyridoxine 3 mg/kg, cobalamin 15 µg/kg; nicotinic acid 60 mg/kg, pantothenic acid 14.7 mg/kg, folic acid 1.5 mg/kg, biotin 125 µg/kg, choline chloride 25 mg/kg, Fe 20 mg/kg, Cu 10 mg/kg, Mn 100 mg/kg, Co 1.0 mg/kg, Zn 82.222 mg/kg, I 1 mg/kg, Se 0.2 mg/kg and Mo 0.5 mg/kg.
Differences between diets shown in bold.

6.2.5.4 Data Collection

Bird performance was measured by body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR). The BWG was measured by the weighing of the birds in each pen twice per week throughout the trial. This was then divided by the number of birds in each pen to account for any losses and normalised to the number of days over which this gain took place. Feed intake was measured per pen, by monitoring of how much feed was given and the weighing back of the feed remaining twice per week. This measurement was taken per pen and divided by the bird number for a 'per bird' FI estimate. FCR was calculated by dividing the average feed intake per bird per pen, by the average BWG per bird per pen. A FCR closer to "1" represents a more efficient feed to BWG conversion.

6.2.5.5 Sample collection

Birds were culled on day 35 of the trial by cervical dislocation. At this point birds were weighed to give the final weight; and the right breast muscle (*pectoralis major*), and the liver, were dissected from each bird and weighed. These weights were then normalised to the body weight of that bird. Ileal digesta samples were also taken from each bird and pooled on a per pen basis. This was the full ileal content from Meckel's diverticulum and was collected by flushing through the ileum with water. Ileal digesta samples were frozen and transferred to the university of Nottingham where they were stored at –80^{o} until analysis.

6.2.5.6 Apparent Ileal Digestibility

Ileal digesta samples were freeze-dried to a constant weight, as described in Chapter 2.1.1, in plastic containers, and ground to a find powder using a pestle and mortar. As ileal digesta samples were on a per pen basis, there were six samples per treatment group. These were analysed for crude protein, as per Chapter 2.1.2, titanium dioxide and amino acids, as per Chapter 2.4.5.

Feed samples (n=3 per diet) were also freeze-dried to a constant weight, in foil containers, as described in Chapter 2.1.1; and ground to a fine powder using a pestle and mortar. These were analysed for crude protein, as per Chapter 2.1.2, titanium dioxide and amino acids, as per Chapter 2.4.5. Data generated for the titanium dioxide concentrations was used alongside the ileal crude protein and amino acid contents to generate values for the apparent ileal crude protein digestibility ($AIDC_{CP}$) and the apparent ileal amino acid digestibility ($AIDC_{AA}$), as described in Chapter 2.4.5.4.

6.2.6 Data Analysis

Statistical analysis was conducted using Genstat 20th Edition software to determine if there were any significant differences found between the diets with respect to BWG, FI, FCR. A one-way, two-way or three-way analysis of variance (ANOVA) test was used to determine any possible significant differences between treatment groups. Significance was taken at $p<0.05$. All graphs were drawn using Graph Pad Prism 8.

6.3 Results

6.3.1 Processing of *P. juliflora* pod flour from Kenya

Percentage yield was calculated as the yield of material remaining from the starting weight, after completion of the associated processing method. The moisture content was the moisture removed from the samples during the freeze-drying process following the associated processing method. These factors were calculated to help inform the suitability of the processing method when considering the ease of putting it into practice considering low mezquite pod material shipment weights for the feed formulation.

Table 6.2. The percentage yield and moisture content of *P. juliflora* pod flour after processing with one of five processing methods.

Processing Method	Yield (%)	Moisture Content (%)
Soaking	65.13	2.89
Boiling	57.88	1.04
Roasting 170°C	95.5	2.01
Popping 180°C	95.62	3.22
Popping 200°C	92.87	2.52

A one-way analysis of variance showed a significant difference ($p<0.001$) in the analytical replicates of total phenolic content of the *P. juliflora* flour either raw or following one of the processing methods, to assess the difference in techniques (Table 6.3). A bonferroni post-hoc test indicated that the flour popped at 180°C was the only sample significantly higher than the raw flour. No result was generated for the flour roasted at 200°C due to the flour burning after 6 minutes.

Table 6.3. The total phenolic content of the *P. juliflora* pod flour following a selection of different processing methods.

Processing Method	Total Phenolic Content (mg TAE/g)
Raw	7.41^{ab}
Soaking	8.53^{ab}
Boiling	6.13^{a}
Roasting 70°C	9.23^{ab}
Roasting 100°C	8.00^{ab}
Roasting 140°C	7.68^{ab}
Roasting 170°C	9.23^{ab}
Popping 180°C	15.20^{c}
Popping 200°C	12.16^{bc}
SED[1]	1.520
P Value[2]	**<0.001**

[1] SED- standard error of the differences of the means, n= 3 technical replicates per group.

[2]P values determined by a one-way ANOVA.

6.3.1 Mezquite pod flour analysis

The *P. velutina* pod flour received from Sonora and the *P. articulata received* from Sinaloa, was analysed to determine its nutritional composition prior to feed formulation. This was to ensure that the experimental feeds could be formulated to be isonitrogenous and isoenergetic. The crude protein, energy and fat content is shown in Table 6.4. There were no significant differences seen between the two flours for crude protein (p=0.055), energy (p=0.056) or fat (p=0.060) (Table 6.4), using two experimental replicates per treatment group to determine differences.

Table 6.4. Nutritional composition of the *P. velutina* and *P. articulata* used for feed formulation.

Mezquite	**Crude Protein (g/100g)**	**Energy (MJ/kg)**	**Fat (g/100g)**
P. velutina (SON)	11.87	18.20	1.91
***P. articulata* (SIN)**	18.89	18.51	1.46
SED[1]	1.726	0.075	0.117
P Value[2]	0.055	0.056	0.060

[1] SED- standard error of the differences of the means, n= 2 technical replicates per group.

[2]P values determined by a one-way ANOVA.

The mezquite pod flour was also analysed to determine the antinutritional content, as seen in Table 6.5. This was completed before and after processing by roasting at 140°C for 10 minutes and consisted of the total phenolics content, the phytic acid content and the trypsin inhibitor activity. There was a significant interaction ($p<0.05$) between mezquite variety and processing for the total phenolic content of the mezquite pod flours (Table 6.5). This is due to a significant difference between the two raw flours, which was not present after roasting. There were no significant differences between the flours ($p>0.05$) for the phytic acid content, for either variant or processing (Table 6.5).

Table 6.5. Antinutrient content of the *P. velutina* and *P. articulata* pod flour.

Mezquite	Total Phenolic content (mg TAE/g)	Phytic Acid (g/100g)
***P. velutina* (SON)**	6.33^{a}	0.24
***P. articulata* (SIN)**	9.37^{c}	0.21
Roasted *P. velutina* (SON)	7.23^{ab}	0.23
Roasted *P. articulata* (SIN)	8.50^{bc}	0.22
SED[1]	0.483	0.046
P Value[2]	**0.041**	0.847

[1] SED- standard error of the differences of the means, n= 3 technical replicates per group for total phenolic content and n=2 technical replicates per group for phytic acid content.

[2]P values determined by a two-way ANOVA.

A sample of each of the two raw flours were also analysed for the trypsin inhibitor activity (Sciantec, Yorkshire, UK) (Table 6.6).

Table 6.6. The trypsin inhibitor activity of the *P. velutina* and *P. articulata* pod flour.

	Trypsin Inhibitor Activity (mg/g)
***P. velutina* (SON)**	1.0
***P. articulata* (SIN)**	1.1

A sample of each flour was analysed (Sciantec, Yorkshire, UK) for mycotoxin concentrations (Table 6.7). This was to ensure that there was no build-up of toxins that would affect the animals when consuming the experimental diets containing mezquite.

Table 6.7. The levels of 6 mycotoxins contained in the mezquite pod flour used for the chicken trial.

Toxin	*P. velutina* (SON)	*P. articulata* (SIN)
T2 Toxin	17 ug/kg	18ug/kg
Aflatoxin	3.0 ug/kg	6.9 ug/kg
Deoxynivalenol	0.62 mg/kg	0.67 mg/kg
Ochratoxin	<10 ug/kg	<10 ug/kg
Total Fumonisin	<0.22 mg/kg	<0.22 mg/kg
Zearalenone	112 ug/kg	201 ug/kg

6.3.2 Feed Analysis

There were no significant differences in the dry matter (p=0.914), crude protein content (p=0.782) or ash content of the feeds (p=0.941) (Table 6.8). However, there was a significant difference in the energy content (p=0.033) (Table 6.8). A bonferroni post-hoc test showed that this was due to the gross energy content of the SON feed, which was the lowest, feed being significantly lower than the CONPRO feed, which was the highest. However, all energy contents are still higher than required for a Ross 308 broiler chicken (Aviagen, 2022).

Table 6.8. The nutritional analysis of the control and experimental feeds used in the broiler trial.

	Dry Matter (g)100g	Crude Protein (g/100g DM)	Gross Energy (MJ/kg)	Ash (g/100g DM)
CON	86.3	22.68	17.41[ab]	5.32
CONPRO	90.7	23.14	17.62[b]	5.10
SON	89.6	23.30	16.96[a]	6.15
SONPRO	89.6	25.17	17.28[ab]	6.56
SIN	97.3	22.88	17.33[ab]	6.33
SINPRO	89.9	22.16	17.41[ab]	7.61
SED[1]	9.98	1.960	0.123	2.77
P Value[2]	0.914	0.782	**0.033**	0.941

[1] SED- standard error of the differences of the means, n=2 technical replicates per treatment group.

[2]P values determined by a one-way ANOVA.

A significant interaction between variant and processing ($p<0.05$) was observed for all of the essential amino acids, apart from phenylalanine, as shown in Figure 6.1. In cases where a Bonferroni post hoc test showed significant differences, these were due to the evaluated amino acid being significantly lower in the SON diet compared to the control diet. The full amino acid content for each feed is shown in appendix 3.

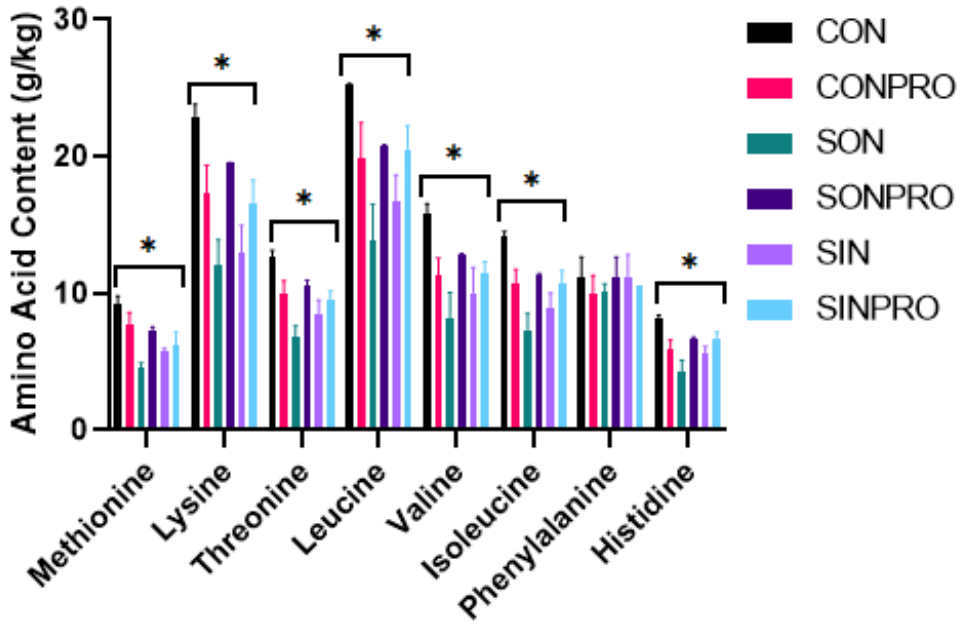


Figure 6.1. The Essential Amino Acid content (g/kg) of each experimental diet. Data shown as means with SEM with n= 2 experimental replicates per diet. *Significance of $p<0.05$, determined using a two-way ANOVA. Tryptophan was not determined.

There was no significant two-way interaction ($p>0.05$) between feed variant and processing for the total phenolic content (Table 6.9). However, there was a significant effect of processing ($p=0.012$), with processed feeds having a lower total phenolic content than raw feeds.

Table 6.9 The antinutritional analysis of the control and experimental feeds used in the broiler trial.

	Total Phenolic Content (mg TAE/g)	Phytic Acid (g/100g)
CON	4.80	1.96
CONPRO	4.27	0.15
SON	5.33	0.65
SONPRO	4.73	0.96
SIN	5.65	2.01
SINPRO	4.72	1.32
SED[1]	0.389	1.78
P Value[2]	0.744	0.717

[1] SED- standard error of the differences of the means. N=2 technical replicates.

[2]P values determined by a two-way ANOVA.

6.3.3 Bird Performance

As seen in Table 6.10, there was no significant difference (p=0.877) in the initial live bird weights for any treatment group; taken at day 6 of the trial, before switching to the experimental feeds. There was, however, a significant interaction (p=0.006) seen between feed variant and processing for the final weights of the birds taken at day 36, after 30 days on the experimental diets (Table 6.10). For the unprocessed feed both the SON and SIN were lower than

CON ($p<0.05$). However, for the processed feed CONPRO and SONPRO were not significantly different from CON. SINPRO was lower ($p<0.05$) than CON but not different from SIN (Figure 6.2).

Table 6.10. The effect of inclusion of mezquite pod flour from two distinct regions, either raw or processed, on broiler chicken growth; expressed as an average per bird at 36 days.

Treatment	Day 6 BWt (kg)	Final BWt (kg)	Total Feed intake (kg)	FCR
CON	0.119	2.08^{d}	2.86	1.46^{a}
CONPRO	0.116	1.98^{cd}	2.88	1.55^{a}
SON	0.117	1.68^{bc}	3.47	2.27^{bc}
SONPRO	0.115	2.08^{d}	3.41	1.74^{ab}
SIN	0.116	1.22^{a}	3.42	3.11^{d}
SINPRO	0.117	1.37^{ab}	3.51	2.80^{cd}
SED[1]	0.006	0.10	0.20	0.17
P-value[2]	0.877	**0.006**	0.864	**0.049**

[1] SED = standard error of the differences of the means.

[2]P Values determined by two-way ANOVA. Different letters within columns indicate a significant difference determined via a Bonferroni post hoc test.

BWt = Body weight, wt= weight. N= 6 pens per treatment group, with 4 birds per pen.

There was no interaction between variant and processing (p=0.864) for feed intake (Table 6.10), there was also no effect of processing. However, there was a significant effect of variant on feed intake (p<0.001), with birds fed the mezquite diets consuming significantly more feed in total than birds fed the control feeds.

There was a significant interaction (p=0.049) between feed variant and processing for the average total FCR per bird from day 6-35 (Table 6.10). For the unprocessed feed both the SON and SIN groups had a higher average total FCR than CON (p<0.05). However, for the processed feed CONPRO and SONPRO were not significantly different from each other, nor CON. SONPRO had significantly lower average total FCR than both SIN and SINPRO, with these not being different from one another (p<0.05). Therefore, it appeared that processing only had an effect on SON, but not SIN and CON.

There was a significant interaction between time, feed variant and processing (p=0.002) for the average BWG per bird per day. There was an increase in BWG per day as the broilers became older. However, the BWG for the feeds containing unprocessed mezquite (SON or SIN) was lower than the control (CON). In addition, there was a differential effect of processing on the feeds, with SONPRO being higher than SON for BWG per day (Figure 6.2).

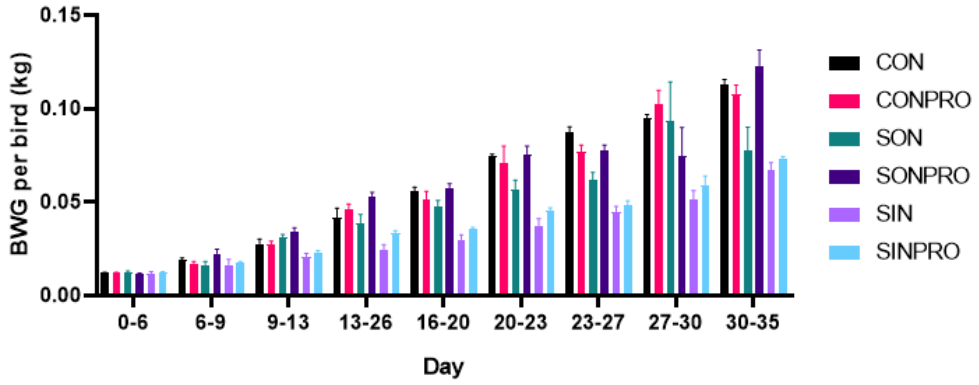


Figure 6.2. The effect of each dietary treatment group on broiler chicken body weight gain, throughout the trial. Data shows the average body weight gain per bird, per day, for each treatment group. Error bars show SEM, n=6 pens per treatment group, with 4 birds per pen. There was a significant interaction between variant, processing and time (P=0.002) (three-way ANOVA).

There was a trend of increased BWG over time. The earliest significant difference shows at days 20-23 with SIN significantly lower than both control diets and the SONPRO diet, at earlier time points there are no significant differences in variant processing. In days 27-30 and 30-35, both diets containing *P. articulata* pod flour (SIN and SINPRO) give a significantly lower BWG than both control diets and the SONPRO diet, showing an effect of feed variant. In days 30-35 there is also an effect of processing, with SONPRO giving a significantly higher BWG than SON. Data taken as the average weight gain (plus SEM) for each bird in each treatment group, per day, during the days between weighing; as indicated on the x-axis.

6.3.4 Bird Body Composition

There was a significant interaction between variant and processing (p=0.004) (Table 6.11) for the average breast muscle weight per treatment group, after 30 days on the experimental feeds. For the unprocessed feeds, CON (highest breast muscle weight), SON and SIN (lowest breast muscle weights) were all significantly different from each other (p<0.05). However, for the processed feeds CONPRO and SONPRO were not significantly different from CON. SINPRO was not significantly different to SIN, but was significantly lower than CON.

When expressed as a percentage of body weight, there was no significant interaction between variant and processing (p=0.160) (Table 6.11) and no effect of processing. However, there was a significant effect of feed variant (p<0.001), with diets containing *P. articulata* (SIN and SINPRO) having birds with a significantly lower breast muscle as a percentage of body weight than birds fed the diets containing *P. velutina* (SON and SONPRO) or control diets (CON and CONPRO).

There was no interaction between feed variant and processing (p=0.363) (Table 6.11) for the average liver weight per bird, after 30 days on the experimental feeds. There was also no effect of processing. However, there was a significant effect of feed variant (p=0.014), as birds fed a diet containing *P. articulata* (SIN and SINPRO) had significantly lower liver weights than birds fed a diet containing *P. velutina* (SON and SONPRO).

When the liver weights were expressed as a percentage of body weight, there was no significant interaction (p=0.981) between feed variant and processing (Table 6.11). However, there was a significant effect of processing (p=0.009), as the proportional liver weights of birds fed diets containing processed ingredients were lower than the diet containing unprocessed ingredients.

Table 6.11. Bird body composition, as liver and breast muscles, presented as an average per treatment group after 30 days on experimental diets.

Treatment	Breast muscle (g)	Breast muscle (%BW[2])	Liver (g)	Liver (% BW)
CON	0.173[c]	8.12	0.046	2.20
CONPRO	0.162[bc]	7.92	0.040	2.03
SON	0.137[b]	7.93	0.066	2.15
SONPRO	0.182[c]	8.54	0.042	2.00
SIN	0.086[a]	6.69	0.027	2.21
SINPRO	0.102[a]	7.16	0.028	2.04
SED[1]	0.01	0.33	0.01	0.11
P-value[3]	**0.004**	0.160	0.363	0.981

[1]SED- standard error of the differences of the means

[2]BW = Body weight

[3]P Values determined using a two-way ANOVA. N=6 pens per treatment group with 4 birds per pen.

6.3.5 Digestibility

6.3.5.1 Crude Protein Digestibility

There was no interaction (p=0.105) between variant and processing for the apparent ileal crude protein digestibility coefficients (Table 6.12). However, there was a significant difference between variants (p<0.001) with control feeds giving significantly higher AIDC than any mesquite feeds. There was also a significant difference (p=0.024) between the AIDC for processed vs non-processed feeds, which appears to be due to the higher AIDC for SONPRO compared to SON.

Table 6.12. Apparent ileal crude protein digestibility coefficient after 30 days on the experimental diets.

	CON	CON PRO	SON	SON PRO	SIN	SIN PRO	SED [1]	P Value[2]
$AIDC_{CP}$[3] (%)	81.90	81.63	74.06	80.14	72.35	74.93	2.038	0.105

[1]SED- standard error of the differences of the means

[2] P Values generated by one way ANOVA. N=6 pens per treatment group.

[3]$AIDC_{CP}$- Apparent ileal digestibility coefficient for crude protein

6.3.5.3 Amino Acid Digestibility

There was a significant two-way interaction between variant and processing ($p<0.05$) for the apparent ileal amino acid digestibility coefficients ($AIDC_{AA}$) for many amino acids. For the essential amino acids (Figure 6.5), there was a two-way interaction ($p<0.05$) between feed variant and processing for all except phenylalanine, which showed a significant ($p<0.05$) difference between variants.

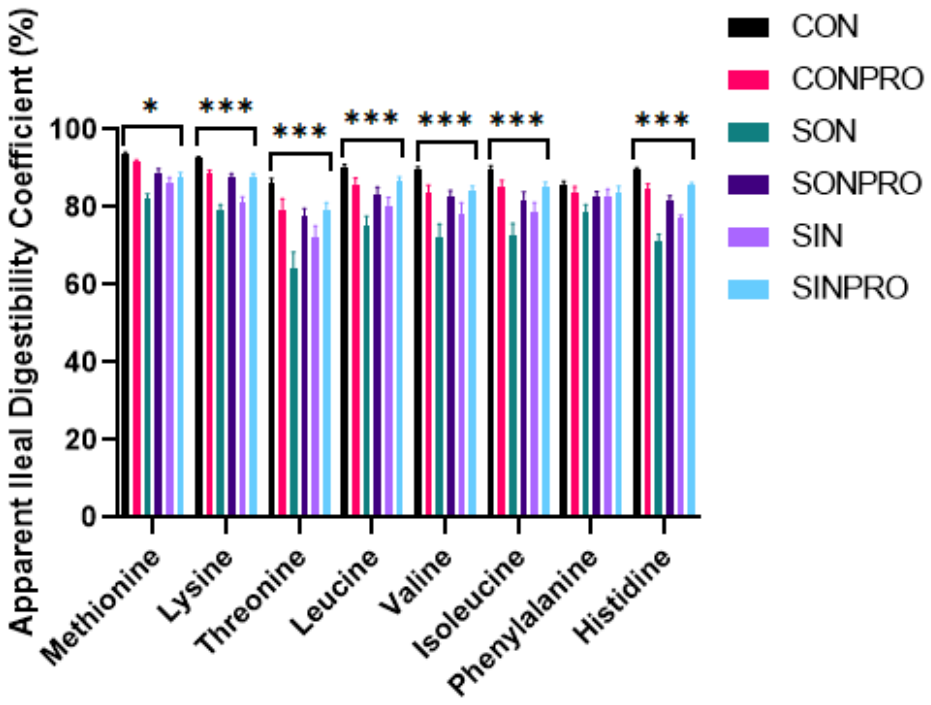


Figure 6.5. Apparent Ileal amino acid digestibility coefficients of broilers after 30 days on experimental feeds. The ileal amino acid content of broiler chickens after being on the experimental diets for 29 days. Data is shown as means plus SEM, n=6 pens per treatment group. Data analysed through two-way ANOVA. * $p<0.05$ *** $p<0.001$. Tryptophan was not determined.

6.4 Discussion

The experimental diets were formulated to be nutritionally similar to the wheat-based control diet.

The first step in designing this trial included choosing the processing method that would be used on half of the experimental diets. Due to delays in the shipment of the mezquite flours from Mexico used in this study, the *P. juliflora* flour used in Chapter 5 was used for the preliminary processing study. A portion of the flour underwent four processing methods: soaking, boiling, roasting and popping; with the roasting and popping each done at a selection of temperatures. These were chosen as they are commonly used methods in the literature to reduce antinutritional factors and improve digestibility (Avilés-Gaxiola et al. 2018; Lajolo et al. 2002; Khattab, Arntfield 2009; Mesfin et al. 2021).

It can be seen from the results that the flour yield is the lowest when soaking or boiling are used as the processing method. Shipping a large quantity of mezquite pod flour for use in this study is already difficult for the communities in Mexico that collected the pods and so only a limited amount could be sent. This meant that a high loss of flour during processing would result in not being able to formulate as much feed for the trials. These two methods were therefore not chosen as the method to be used in this study. The *P. juliflora* flour popped at 180^{o}C had a significantly higher content than the raw flour. As the popping at both temperatures seemed to increase the total phenolic

content, roasting was chosen as the processing method for this trial. This decision also factored in the ease of the processing method for communities that usually harvest and utilise *P. velutina* or *P. articulata* pods. These communities would not have the equipment necessary for popping samples, whereas roasting the samples is achievable. All of the roasted samples showed a slightly higher total phenolic content than the raw flour, though the increase was not significant. As roasting at 140^{o}C resulted in the smallest increase, this was the temperature chosen for this trial.

Processing using heat has been documented to reduce the levels of antinutrients including tannins (Ibrahim et al. 2002), enzyme inhibitors (Avilés-Gaxiola et al. 2018), lectins (Lajolo et al. 2002) and phytic acid (Gupta et al. 2015). However, in this processing study it can be seen that the total phenolic content seemed to increase with heat processing. This is speculated to be due to the heat breaking down polyphenols with a high molecular weight, making them more easily extractable (Duodu 2014). This has also been observed by (Zou et al. 2015) who found that roasting at 180^{o}C for 5 minutes increased the total phenolic content of wheat germ from 16.49 to 17.53 mg gallic acid equivalent (GAE)/g, which was not significant. However, roasting for 10 or 20 minutes significantly increased the total phenolic content to 18.62 and 30.24 mg GAE/g respectively. Similarly, it has been observed in other Mezquite species, with (Díaz-Batalla, Hernández-Uribe, et al. 2018) finding that baking *P. laevigata* pod mesocarp flour at 190°C for 20 minutes significantly increased the total phenolic content from 8.87g GAE/ kg to 12.4g GAE/kg.

The *P. articulata* pod flour from Sinaloa had a significantly higher total phenolic content then the *P. velutina* pod flour from Sonora. When the chosen heat treatment of 140^{o}C for 10 minutes was applied, the total phenolic content of the Sonora flour increased while the Sinaloa decreased, though neither change was significant. This resulted in the roasted flours also not having a significantly different total phenolic content from one another. Despite the differences in the total phenolic content of the two raw flours, there was no interaction between variant and processing for the total phenolic content of the six feeds. However, processed feeds did have a significantly lower total phenolic content than the raw feeds.

There were no significant differences between the phytic acid content of the flours, either raw or roasted, with the contents ranging from 0.21g/100g to 0.24g/100g. (Walk, Rama Rao 2020) reported that a feed containing more than 0.25% phytic acid can significantly reduce FCR and BWG. As the roasting of the flour did not have a significant effect on the phytic acid content, a super-dose at 1500 FTU of the exogenous enzyme phytase was added to the processed feeds (CONPRO, SONPRO and SINPRO) with the aim of bringing the phytic acid levels of the feed below this. A dose of 1500 FTU/ kg was a level seen in the literature to improve body weight gain. (Shirley, Edwards 2003) found that adding 1500 phytase units/kg into a corn-soybean meal diet significantly increased cobb x cobb broiler chicks BWG from 287g to 459g in the first 16 days of life. Similarly, Gautier et al. (2018) also found that the addition of phytase at 1400 units/kg significantly increased BWG of Ross 308 broilers from 2 to 23

days of age. There were no significant differences between the phytic acid content of the six feeds, with regards to variant or processing: despite the phytase supplementation. However, the results of this assay when analysing the feeds were extremely variable, with the percentage coefficient of variation ranging from 10% for CONPRO to 138% for SIN. It is difficult to say if this is due to technical error when performing the assay, or an interference within the feeds; as the variation was not present when measuring the flour samples.

The feed analysis conducted prior to this study showed that the feeds were formulated to be isonitrogenous. The crude protein requirement for Ross 308 broilers is 23% in the starter phase, 21.5% in the grower phase and 19.5% in the finisher phase (Aviagen, 2022), giving an average content of 21.3% throughout the life; which is lower than the protein content of all feeds in this trial.

However, the SON feed was found to have a significantly lower energy content than the CONPRO feed. The energy content required for Ross 308 broilers is 12.4 MJ/kg in the starter phase, 12.8 MJ/kg in the grower phase and 13 MJ/kg in the finisher phase (Aviagen, 2022). The energy content for the feeds in this trial were all higher than these values with the lowest at 16.96 MJ/kg and the highest at 17.62 MJ/kg.

It was found that the amino acid content in the SON feed was significantly lower than in the CON feed for multiple essential amino acids. However, all six feeds had higher percentage concentrations of all the essential amino acids

analysed than is required in the starter, grower or finisher phase for Ross 308 broilers (Aviagen, 2022).

Different mezquite varieties and the processing of raw mezquite pod flour both have a significant effect on broiler chicken weight gain, with raw mezquite having a negative impact and processing having a species dependent impact. The inclusion of any of the mezquite pod flours also increased feed intake, increasing the feed conversion ratio for all mezquite diets.

The results of this study show a significant effect of mezquite variety and processing on broiler chicken performance. Birds fed the CON, SON and SIN diets all had significantly different final body weights, showing a clear effect of variant. Birds fed the SONPRO diet weighed significantly more than birds fed the SON diet, showing an effect of processing. The results seen from both varieties of raw mezquite follow a similar pattern to the previous trial described in Chapter 5, with the final weights of birds fed either of the raw mezquite feeds being significantly lower than birds fed the control feed. This further reinforces that raw Mezquite pod flour is not a suitable ingredient for substitution into poultry feeds at 15%.

The results described show that different species of mezquite can have significantly different effects when consumed. As discussed in Chapter 4 of this thesis, the nutritional and antinutritional composition of mezquite pods can vary significantly between different species. There is little nutritional classification of different species of mezquite available. However, it can be seen from this study that the *P. velutina* pods from Sonora are lower in crude

protein, though not significantly, and have a lower total phenolic content than the *P. articulata* pods from Sinaloa. This may also have been affected by climate conditions such as the temperature, rainfall and proximity to water (Ruiz-Nieto et al. 2020).

The pods from both regions were grown from March- June/ July, with the *P. velutina* pods from Sonora collected mid-July and the *P. articulata* pods from Sinaloa collected mid-June. In Sonora, the average temperatures rise from 22^{o}C in March to 26^{o}C in July, with the average rainfall ranging from 15mm in March to 243 mm in July. In Sinaloa, the average temperatures rise from 20^{o}C in March to 27^{o}C in June, with the average rainfall ranging from 1mm in March to 4mm in June (Weatherspark, 2023). While both variants were grown in the vicinity of water the *P. velutina* trees were grown under irrigation, which the *P. articulata* tree were not. Combined with the higher rainfall in Sonora, the *P. velutina* had greater water access, though were also grown in an area with salty soil.

Saline, or salty, soils are common in semi-arid and arid regions due to high evaporation of saline underground water or poor quality of water used for irrigation (Muscolo et al. 2011). High salinity can cause an increased uptake of Na^{+} and Cl^{-} ions into the plant. Enhanced synthesis of plant secondary metabolites can then occur to protect cells from oxidative damage caused by these ions (Sytar et al. 2018) Mimicking this, (Parida et al. 2004)that hydroponic NaCl treatment at 250mM significantly increased the total phenolic content in *A. corniculatum* leaves after 7 days of treatment, with the

total phenolic content increasing over time through a 30-day study. Water stress is considered one of the most important environmental stresses when considering plant secondary metabolites (PSMs). Drought results in the induction of reactive oxygen species, which can damage cells within the plant. Increasing the production of PSMs is one of the methods a plant may react to this stress (Yadav et al. 2021). This can be the case in plants grown only in drought, with Jabeen et al. (2021) finding that growing turnips (*Brassica rapa* L.) in conditions of drought stress from two weeks after seed germination until 4 weeks, resulted in significantly higher total phenolic content than control plants. However, it can also be seen to be affected by season, with(Alonso-Amelot et al. 2007) finding that low molecular weight phenolics found in *P. arachnoideum* fronds growing in the Venezuelan Andean range increased from 23.7 mg/g^{-1} in the rainy season to 47.7 mg/g^{-1} in the dry season. However, a plants reaction to stress can vary between species (Yang et al. 2018). For example, de Oliveira et al. (2015) found that the condensed tannin content in *P. juliflora* leaves increased from 25.4 g/ kg^{-1} in the dry season to 38.8g/ kg^{-1} dry matter in the rainy season. This is not unexpected considering the ability of mezquite trees to still grow in conditions of drought. In this trial, *P. articulata* pod flour showed a higher total phenolic content and had a lower rainfall, with very little average rainfall during the months grown. As the trees were also not grown under irrigation it can be assumed that a lack of water may have contributed to the higher total phenolic content. While this does not fit with the results found for *P. juliflora* (de Oliveira et al. 2015), the findings of that study are from leaves rather than pods and are

likely to vary between different species. Another consideration is the temperature difference between the two areas. Higher temperatures are suggested to increase the production of PSMs (Yang et al. 2018). Allegretti et al. (2012) found that the total phenolic content of *P. flexuosa* leaves decreased from 4.2mg/g in Autumn to 3.2mg/g in Winter. While this fits with the current study which shows higher temperatures in Sinaloa, where the higher total phenolic content *P. articulata* trees were grown, it is hard to separate this from the low rainfall.

In the control diets, there was no significant effect of processing. The birds fed the processed control feed have slightly lower final weights on average, though not significantly. The lack of change may be due to the fact that the control feed has already been optimised for maximum broiler growth, meaning that there is unlikely to be room for improvement. However, a significant effect of processing can be seen in the *P. velutina* (Sonora) fed birds, bringing the final weights of the birds in the SONPRO treatment group level with those fed the control feed. This suggests that either heat treatment, the addition of phytase, or a combination of both has had a significant improvement on the digestibility of the feed. There is also a slight trend of higher final weights of birds fed SINPRO compared to SIN; though this was not significant. As the birds fed Sinaloa Mezquite based feeds grew at a slower rate than those fed the Sonora treatments, it is possible that a greater effect would have become more pronounced if the birds had been left for longer. Similarly, as discussed in Chapter 5.5, the Ross 308 broilers used in this trial are extremely efficient and

fast growing, meaning a small shift in their diet will show the maximum negative effects. In slower growing birds, such as those more likely to be used in Mexico, the differences between the raw feeds are not as likely to be so pronounced and a small increase caused by the processing in the broilers may be a larger increase in native birds.

The feed intake in this trial was significantly higher in all Mezquite treatment groups compared to the two control groups. This suggests a deficiency in nutrient digestibility, leading to the birds consuming more feed. As the FIs were increased, rather than decreased, there is no suggested palatability issues. These FI results are the opposite than could possibly be expected when compared with the growth results. The increase in FI also contrasts our previous study (Chapter 5), which found statistically similar intakes in the control and mezquite fed birds despite a similarly depressed final body weight. It also contrasts the findings of Girma et al. (2011), who found that the lowest inclusion of *P. juliflora* pod flour (30%) that resulted in a decreased final body weight; also caused a decrease in feed intake. This would be the usual expected result with a high presence of antinutrients, as previous literature suggests that their presence depresses feed intake rather than increases it (Ferket and Gernat, 2006). All birds were kept in the same environmental conditions, with the same *ad libitum* access to water, another factor that can affect feed intake (Ferket and Gernat, 2006). As broiler chickens are selected for maximum growth their FI is less impacted by dietary factors than other birds may be. Dietary energy content has the greatest effect, with lower energy contents in

feeds resulting in higher feed intake to support their metabolic energy requirement (Ferket and Gernat, 2006). However, in this trial, only the SON diet had a significantly decreased energy content.

This change in FI resulted in a significantly higher FCR for all the Mezquite diets. However, due to the higher growth the FCR for SONPRO birds was not significantly different from the CON and CONPRO groups. This makes the SONPRO diet a more commercially viable option than the other mezquite feeds, despite the higher FI.

Different mezquite variants and the processing of the flour both had a significant effect on the breast muscle weights of the birds but had no effect on the liver weights.

Birds fed either of the *P. articulata* (Sinaloa) diets has significantly lighter *pectoralis major* muscles than birds fed a *P. articulata* (Sonora) or control diet, this was the effect of variant. The effect of processing was due to birds fed a SONPRO diet having significantly heavier breast muscles than birds fed a SON diet. These results are as expected when considered alongside the body weights of the birds, as they follow the same pattern. When considered as a percentage of body weight there was no significant interaction, but there was a significant effect of variant; with birds fed a SIN or SINPRO diet having a smaller breast percentage than the other treatment groups.

There was no interaction between variant and processing for the average liver weight per bird, but there was a significant effect of variant ($p=0.014$) due to

the birds that consumed a *P. articulata* (Sinaloa) diet having a significantly lower liver weight than the *P. velutina* (Sonora) fed birds. This matches up to the body weights of the birds. When normalised to body weight there was a significant effect of processing (p=0.009) due to lower proportional liver weights in the processed diet groups compared to raw diets. This may correlate to the lower total phenolic content seen in the processed feeds. However, (Marzo et al. 2002), found that a 25g/kg inclusion of tannic acid into a 20% protein heated soybean diet significantly reduced both body weight from 12.1g per day to 4.4g per day, and proportional liver weight from 4.3g/100 body weight to 2.2g/100g body weight; with no change in feed intake. In the case of this trial, the liver weights have decreased where body weights have increased. This suggests that a higher tannin or tannin equivalent level should result in lower liver weights, the opposite of the observed results in this trial. Similarly, the addition of phytase at 1500 units/ kg diet has not been shown to affect liver weight in Ross 308 broilers (Gautier et al. 2018). This suggests that another component of the feeds has been affected by the processing, which has reduced the liver weights while not affecting the body weight.

The lower amino acid content of the processed control feed compared to the raw control feed, particularly in Lysine, suggests that the heat processing may have resulted in a Maillard reaction. Maillard reaction can occur when heat is applied due to the reaction of reducing sugars with the amino group of amino acids, particularly Lysine; resulting in the reacted amino acids becoming unavailable (Oliveira et al. 2021). Díaz-Batalla, HernándezUribe, et al. (2018)

found evidence that baking *P. laevigata* pod mesocarp flour at 190°C for 20 minutes resulted in a higher DPPH radical scavenging capacity, associated with the products of a Maillard reaction. This correlated with a higher total phenolic content in the thermal treated flour, as seen in the current study. It is reported that *P.* pod flour may be particularly prone to Maillard reaction development due to the high sugar content including monosaccharides glucose, fructose and xylose, all present in the pods (Díaz-Batalla, HernándezUribe, et al. 2018; Bigne et al. 2018). The occurrence of Maillard reactions between glucose and Lysine is well categorised (Moreno et al. 2003; Lee et al. 1984), if this was the case in the feeds used in the current studied this would lead to lower feed glucose levels. As glucose is converted into glycogen for storage in the liver, and glycogen storage is associated with water storage in the liver (Mcgarry et al. 1987; Mackay, Rgman 1934), it stands to reason that a Maillard reaction reducing the glucose content of the processed diets could result in lower glycogen and hence water stores within the liver. This could explain the recued proportional liver weights seen in the birds fed a processed feed. Analysis of the glycogen levels within the livers would be a useful source of information to support this but was not possible within this trial.

As also discussed in Chapter 5.4, the mycotoxins analysed within the mezquite material were not found at high enough levels to account for the reduction in bird growth seen in the birds fed the raw mezquite.

The mezquite variant used had a significant effect on the ileal crude protein and amino acid content. Both variant and processing had a significant effect on the ileal crude protein and multiple essential amino acid digestibility coefficients.

The ileal titanium dioxide concentration was significantly different between variants ($p<0.001$) with the CON, SON and SIN feeds all significantly different from one-another, despite the same amount being mixed into all six feeds. Processed feeds also had a significantly higher ileal titanium concentration than non-processed feeds ($p=0.022$). This suggests that digestion is inhibited in mezquite feeds compared to the control feeds, more so in the Sinaloa feeds; but that the processing improves it. These titanium dioxide content measurements were used to calculate the AIDC for both crude protein and individual amino acids.

Both *P. articulata* (Sinaloa) diets resulted in a significantly lower ileal crude protein content. This could be a direct result of tannins binding to protein and making it less digestible or it could also be due to diluted digesta as a consequence of increased intestinal mucus, which has been shown to be associated with tannins (Choi et al. 2022). For the $AIDC_{CP}$, there was a significant effect of variant ($p<0.001$) and of processing ($p=0.024$). The birds fed the control feeds had a significantly higher $AIDC_{CP}$ than any other groups, with the $AIDC_{CP}$ being improved for the SONPRO diet compared to the raw SON diet. As the control feeds had a significantly higher $AIDC_{CP}$ than the other groups, this may be reflective of the lower FI in the control birds. Decreased protein

has been seen to cause an increased feed intake in order to compensate (Ferket and Gernat 2006).

There was a significant effect of variant ($p<0.05$) for all the evaluated essential amino acids. The birds fed the Sinaloa diet had the lowest ileal amino acid content for each amino acid. There was a two-way interaction between variant and processing ($p<0.05$) for the $AIDC_{AA}$ for all the essential amino acids except phenylalanine, which only showed a significant difference between variants. As discussed in Chapter 5, all of the essential amino acids have been seen to affect body weight gain. The branched-chain amino acids; leucine, isoleucine and valine, are the most strongly linked with muscle synthesis (Hm, Kratei n.d.; Kimball, Jefferson 2006). However, in all cases of significance the SON diet resulted in the lowest $AIDC_{AA.}$ This is due to the lower amino acid content seen in the SON feed. However, this does not seem to fit with the titanium dioxide concentrations which indicates a higher level of digestion in the SON birds than the SIN birds, or the ileal amino acid content results, which are higher in the SON birds than the SIN birds. This makes it likely that an error occurred when measuring the amino acid content of the feeds. One replicate of the amino acid content for the SON diet is lower than expected in comparison to the other feed results, with a percentage coefficient of variance between technical replicates of 78.6% for Alanine.

6.5 Conclusions

This broiler chicken trial was carried out to evaluate the effect of two mezquite pod flour variants when fed either raw or when processed with heat and the

addition of exogenous enzyme phytase, on broiler chicken performance and crude protein and amino acid digestibility. The results of this study show that both mezquite variant and processing had a significant effect. There was in interaction effect of both variant and processing for the final body weight of the birds, the body weight gain per bird per day throughout the trial, the FCR, the breast muscle weights and the $AIDC_{AA}$ for multiple essential amino acids. However, multiple elements of the study also showed an effect of just variant or just processing; showing the importance of considering both.

It is difficult to determine if the effect of variant is due to the specific species used in this study, or the environment in which they were grownIt is likely that the factors of species and environment interlink with each other, and that different *P.* species thrive in different environments, changing what would be considered stress. It is therefore difficult to make recommendations for the use of any mezquite pod flour in broiler chicken feeds, as trees should be considered on an area-by-area basis.

Processing was only seen to have a significant effect on any variables for the *P. velutina* (Sonora) feeds. This may be due to the already lower total phenolic content of the flour. The processing of the *P. velutina* feed resulted in a statistically similar average final body weight and FCR as the control feed. While the slightly lower FCR due to the increased FI, and the decreased liver weights in this treatment group, may mean that the diets performance would not make it commercially viable for fast growing broilers; this diet would likely be very efficient in slower growing native birds.

7 Discussion

Prior to the work carried out in this thesis, underutilised crops were a widely researched topic, both for human and animal consumption. For bambara groundnut, previous research has looked at the proximate nutrient composition, palatability of bambara groundnut milk, methods of reducing the antinutritional content and the use of the seeds in animal feeds. However, there has been limited research looking at the variability of the nutritional and antinutritional content within different seed variants and their milk. Mezquite is a less researched crop in comparison to bambara groundnut, but its prevalence across Africa, Asia and the Americas makes it a crop of high impact. *P. julifllora* is the most commonly researched mezquite species, with little research showing the differences between species. While previous studies looking at the effect of mezquite pod flour have taken place, these are limited and predominantly in ruminant species.

The first hypothesis of this thesis was that different varieties of the same crop would have significantly different nutritional and antinutritional profiles. This hypothesis can be accepted as significant variation has been observed between different landraces of bambara groundnut seeds, and between the different species of mezquite studied in this thesis. In bambara groundnut, there was variation observed between landraces for the crude protein, gross energy, essential amino acids and total phenolic concentrations. In mezquite pod flour differences were observed between species for the crude protein, gross

energy, fat, total phenolics, phytic acid, minerals and essential amino acid concentrations. While both underutilised crops studied in this thesis show lower crude protein concentrations when compared to soybean, the amino acid concentrations appear to be comparable. However, it should be considered that soybean crops have been domesticated over a period of what is believed to be 6000-9000 years (Sedivy et al. 2017). During this time, selection pressures and breeding programs have reduced genetic diversity and resulting in the increase of favourable traits, which have spread throughout the domestic population (Sedivy et al. 2017; Zhao et al. 2015). When considering underutilised crops such as bambara groundnut or mezquite, this is not the case. These crops are being considered in their natural state, without the influence of artificial selection or high levels of research. It should also be noted that the reduction of variability in cultivated soybean crops has led to a reduced capacity for adaptability to changes in the environment (Zhao et al. 2015) this is also the case for wheat crops (Asseng et al. 2019; Hussain et al. 2018). With the currently changing climate, this is the reason that underutilised crops are such an important research topic.

The second hypothesis of this thesis was that the antinutritional factors can be reduced by post-harvest processing such as dehulling or heating. For bambara groundnut, it was found that the total phenolic content, used as an estimate of tannin concentration, was predominantly found in the seed hulls. This implies that the dehulling of the seeds would significantly decrease the overall total phenolic content. However, it was found that antinutritional protein lectins are

predominantly located within the seeds, suggesting that they would not be reduced by the dehulling of the seeds. In *P. juliflora* mezquite pod flour, it was found that of the post-harvest processing by soaking, boiling, roasting or popping, only boiling reduced the total phenolic content; though not significantly. It seems likely that this is due to the processing methods used breaking down long chain phenolics as well as releasing more bound phenolics, resulting in the soluble total phenolic content increasing (Duodu 2014).

Variation in the nutritional and antinutritional concentrations between species or variations is important to characterise in order for those utilising the crops. It is important on a local scale for people to be able to pick or grow variations with the desired nutritional profile, such as those with the highest protein or lowest phenolics. For wider implications, forming a database in which the nutritional and antinutritional compositions of underutilised crops is documented, can allow knowledge for potential breeding selections in order to prioritise the desired traits.

The third hypothesis was that feeding mezquite pod flour at a 15% inclusion would be unlikely to have any negative effects on bird performance. This hypothesis was based on previous literature from Odero-Waitituh et al. (2016) and Girma et al. (2011) looking at the effect of *P. juliflora* pod flour on broiler chicken performance. However, this hypothesis must be rejected, as this thesis finds that a 15% inclusion of *P. juliflora* pod flour had a negative effect on broiler chicken performance and ileal crude protein and amino acid digestibility. These results could have differed from the results seen in the

literature for multiple reasons. The birds used in this study showed extremely efficient growth, meaning that any changes in the diet will show the maximum negative effect. Additionally, the same mezquite grown in different locations may show different effects due to differing environmental stresses. Although this result was not as originally expected, this trial allows communities which hope to utilise *P. juliflora* the knowledge that the pod flour of this species is not suitable for broiler chicken consumption in its raw state. This could allow further research to be prioritised, such as utilising the crops in a processed form, or simply to recommend the trial of a different mezquite species.

The final hypothesis of this thesis was that any negative effects observed by the feeding of a 15% inclusion of mezquite pod flour could be mitigated by utilising post-harvest processing or the use of exogenous enzymes. This hypothesis can only partially be accepted. The results from the final study in this thesis show that roasting at 140^{o}C and the addition of phytase only improved bird performance compared to the raw mezquite pod flour in one species of mezquite, and not the other. As phytase was added to all processed feeds at the same level, and there were no significant differences in the phytic acid content of the flours, raw or roasted; it is assumed that the phytase activity reduced the phytic acid content of all the feeds by the same amount. It is therefore suspected that the roasting had the more significant improvement in the *P. velutina* feed, likely due to the lower total phenolic content in the raw flour, compared to *P. articulata.* The main impact of this trial is for the local communities that wish to utilise these species. For them, it may not be possible

to utilise a different mezquite species due to their location and the costs of travel. Therefore, the knowledge of whether or not their local species can be improved may play a large role in the future of the species. From this trial, the main take home message is that not all species of mezquite have the same effect, and not all can be improved.

Main conclusions from this thesis

- Bambara groundnut seeds can be dehulled to remove a significant amount of the total phenolic content, considered as tannin equivalent, making dehulling a useful tool for processing before consumption.

- Milk produced from bambara groundnut is comparable in protein content to other marketable vegetable milks. However, increased protein content is associated with increased milk total phenolic content, and so it is recommended that the seeds be heat treated prior to milk production.

- The nutritional and antinutritional profile of mezquite pod flour is variable between different species from distinct geographical locations, and within the same species due to environmental differences.

- A 15% inclusion of *P. juliflora* pod flour negatively affected broiler chicken growth, FCR and crude protein and amino acid digestibility.

- Processing of *Prospopis juliflora* pod flour by boiling reduced the total phenolic content, though not significantly. Processing by soaking, roasting or popping all increased the total phenolic content, though only popping at 180^{o}C increased the content significantly. However, this is considered to be due to a breakdown of larger bound phenolics, making them more soluble and therefore easily extractable.

- The impact of mezquite pod flour on broiler chicken performance and ileal crude protein and AA digestibility is variable depending on the species of mezquite pod flour, with *P. articulata* pod flour performing worse than *P. velutina* pod flour.

- A feed containing *P. velutina* pod flour roasted at 140^{o}C with phytase at 1500 FTU/Kg resulted in statistically similar BWG and FCR results to the control feed.

- Meezquite species behave differently to one-another. Different species can have different effects *in vivo* and while some may be improved by processing, this is not uniform.

Potential Future work

Future work should be considered to improve knowledge in this area, this could include:

- Profiling of the phenolics in mezquite pod flour before and after heat processing in order to see how they are changing. This would allow us to better understand the effects that these phenolics would have *in vivo.* This is important in order for us to have a better understanding of the results of Chapter 6.

- The use of *in vitro* digestion methods to study how both mezquite pod flour and bambara groundnut seeds are digested. This would be particularly useful for further understanding of the differences between digestibility of mezquite species with high and low ANF concentrations.

- Testing mezquite pod flour at a 15% inclusion rate in the feeds of slower growing birds. This would allow for a more realistic view of how mezquite pod flour may affect birds used in local communities where mezquite can be utilised. This would give a more practical application for the results of Chapters 5 and 6.

- The profiling of mezquite pod flour from a selection of mezquite species grown in the same area or similar environmental conditions, or alternatively the same species but from multiple areas and environmental conditions. This would allow more understanding of

the impact of species compared to environment on the nutritional and antinutritional content.

- Conduction a trial in which a variety of a select few mezquite species could be grown in controlled field conditions to allow for species specific comparisons without environment as a confounding factor. This would need to be a long length study to allow the trees to reach maturity for pod harvesting.

- Separation of whole mezquite pods to determine the components with the highest nutrients and antinutritional factor content. This may provide an alternative, low cost, processing method that could reduce the antinutritional factor content.

- Determination of the protein and total phenolic content of bambara groundnut milk produced from heat treated bambara seeds, it is expected that this will reduce the total phenolic content and produce a more marketable milk.

- Determination of the trypsin inhibitor activity in bambara groundnut seeds, both for the total content and the location within the seeds.

Final Conclusion

Underutilised crops have potential for the partial replacement of major crops used in both human diets and animal feeds. While bamabara groundnut seeds have a variable nutritional content, the amino acid concentrations are

comparable to that of soybean, with methionine concentrations seemingly higher in bambara groundnut seeds than soybean. This is important as methionine is a limiting amino acid in soybean. Bambara groundnut milk also has a protein content similar to other marketable vegetable milks, including soy milk; with further study this could be a new marketable milk. Mezquite pod flour also has a variable nutritional and antinutritional content, both within and between species. While it's difficult to determine the cause of this variation, knowledge of species profiling can help identify potential uses for trees in specific communities. Inclusion of *P. juiflora* pod flour into broiler chicken diets at 15% were found to have negative effects on growth, FCR and crude protein and amino acid digestibility. It therefore cannot be recommended as a potential commercial feed ingredient. However, different mezquite pod flour species show different effects on broiler chicken performance and crude protein and amino acid digestibility with *P. velutina* pod flour showing better results than then *P. articulata* pod flour, both raw and processed. This information can help to inform the communities that some species may be improved enough by processing methods that they can safely be used in poultry feeds, providing a cheap feed ingredient and a utilisation for the mezquite pods grown in their communities. The knowledge that not all mezquite species behave in the same way *in vivo* can reiterate the importance of communities learning more about the specific species they have available to them, rather than relying on generalised knowledge of one mezquite species.

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Appendix 1

Appendix 1. Nutrient analysis for all 48 mezquite pod flour samples analysed in Chapter 2 of this thesis.

Sample code	Species *(Prosopis)*	Protein (g/100g DW)	Fat (g/100g DW)	Gross Energy (MJ/kg)
FVSS1	*Velutina*	9.82	1.14	17.60
FVSS2	*Velutina*	10.44	1.60	17.41
FVSS3	*Velutina*	7.52	2.15	17.26
FVSS4	*Velutina*	10.86	0.64	16.66
FVSS5	*Velutina*	8.88	1.13	17.59
FVSS6	*Velutina*	13.23	1.17	17.83
FVSS7	*Velutina*	12.14	1.36	17.47
FVSS8	*Velutina*	9.82	3.83	17.69
FVSS9	*Velutina*	11.56	2.35	17.58
FVSS10	*Velutina*	11.20	1.30	17.49
FVSS11	*Velutina*	12.46	1.87	17.56
FVSS12	*Velutina*	11.48	1.49	17.25
FLDL1	*Legaeviata*	15.88	1.12	17.68
FLDL2	*Legaeviata*	13.92	1.74	17.80
FLDL3	*Legaeviata*	15.25	1.49	16.74
FLDL4	*Legaeviata*	14.05	3.11	17.58
FLDL5	*Legaeviata*	11.75	1.37	17.09
FLDL6	*Legaeviata*	11.65	1.36	17.33
FOLDL1	*Legaeviata*	11.95	1.95	17.66
FOLDL2	*Legaeviata*	15.21	2.97	17.91
FK1	*juliflora*	12.47	3.66	18.21
FK2	*juliflora*	14.06	2.79	17.33
FK3	*juliflora*	14.96	3.21	18.55
FK4	*juliflora*	11.98	2.63	18.39
FK5	*juliflora*	12.38	2.80	17.45
FK6	*juliflora*	11.22	3.18	18.05
FK7	*juliflora*	16.13	2.01	18.27
FK8	*juliflora*	15.57	2.09	18.27
FK9	*juliflora*	14.99	1.85	17.55
FK10	*juliflora*	14.00	1.93	17.68
FK11	*juliflora*	12.42	2.10	17.36
FK12	*juliflora*	13.75	1.91	17.77
FPT1	*Pallida*	13.49	1.13	18.11
FPT2	*Pallida*	12.04	1.10	17.82
FPT3	*Pallida*	12.41	0.41	17.52
FPT4	*Pallida*	11.91	1.03	17.34

FPT5	*Pallida*	11.20	0.77	17.71
FPT6	*Pallida*	10.27	0.86	17.56
FPT7	*Pallida*	8.64	0.90	17.19
FPT8	*Pallida*	9.39	0.92	17.33
FPT9	*Pallida*	8.81	1.19	17.58
FPT10	*Pallida*	11.91	0.63	16.44
FPT11	*Pallida*	10.27	0.66	17.90
FPT12	*Pallida*	11.74	1.20	17.31
EL2-4	*Legaeviata*	15.83	2.29	17.25
SP3 SP5	*Legaeviata*	12.23	2.37	17.72
D72	*Legaeviata*	11.93	2.99	18.15
TM3	*Legaeviata*	14.58	1.70	17.73
D5A	*Legaeviata*	12.12	2.92	17.61

Appendix 2

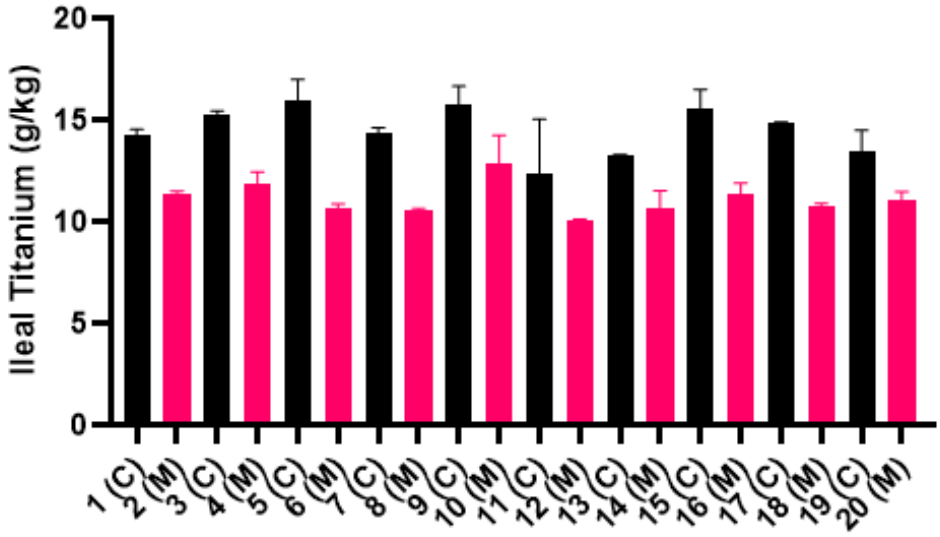
Appendix 2. Full mineral analysis for the four mezquite species analysed in Chapter 4.

Species	*Prosopis juliflora*	*Prosopis Legaeviata*	*Prosopis Pallida*	*Prosopis Velutina*
B	12.90820215	20.79903914	20.78293787	22.38681906
Na	110.0513886	70.9919473	822.6709062	461.7632238
Mg	834.5871791	1400.61921	1076.454829	931.5192372
P	1862.859971	1849.498822	1799.68762	1283.071835
S	2024.672499	2827.737344	2122.662748	1856.095679
K	19060.11505	17827.71956	16529.74224	19807.59345
Ca	2827.642344	4241.95732	2550.560427	2056.357258
Li	0.047942538	0.596650042	0.119185267	0.84242741
Be	-0.001528774	-0.001167243	0.003363118	-0.002426393
Al	17.36276616	17.7053375	14.2135887	14.29704176
Ti	2.303302765	2.393420693	0.810886473	0.892711309
V	0.041706043	0.012769033	0.008616196	0.003752866
Cr	-0.002400703	0.359326759	-0.037962216	0.055841849
Mn	15.46714377	15.35403341	13.6433036	9.527031038
Fe	62.63146305	44.47256065	78.94007793	23.11864565
Co	0.01110661	0.02236105	0.046275138	0.019705717
Ni	0.400776725	0.573956386	1.738708677	0.362677467
Cu	2.784781672	4.069928611	6.375820323	5.110729122
Zn	15.76535125	28.2329425	19.41044642	18.5569079
As	0.006327985	0.015755741	0.005888926	0.007769603
Se	1.598143674	0.225679775	0.635555296	0.092199727
Rb	12.9982942	17.00758524	5.245439206	7.536483848
Sr	14.94191803	20.98818238	60.54912385	27.73696574
Mo	1.36196935	0.543954465	0.705143567	0.498552622

Ag	-0.507044849	-0.514603823	-0.505177132	-0.494222215
Cd	0.011377036	0.019998693	0.002204801	0.002262671
Cs	0.000781082	0.109615296	0.001395524	0.019092602
Ba	2.46725664	4.994387003	7.47989046	4.544592147
Tl	0.002584179	0.003726606	5.93273E-05	0.001886425
Pb	0.02440982	0.018866208	0.033134941	0.042570925
U	-0.001550798	-0.001331795	-0.002435973	-0.00195096

Appendix 3

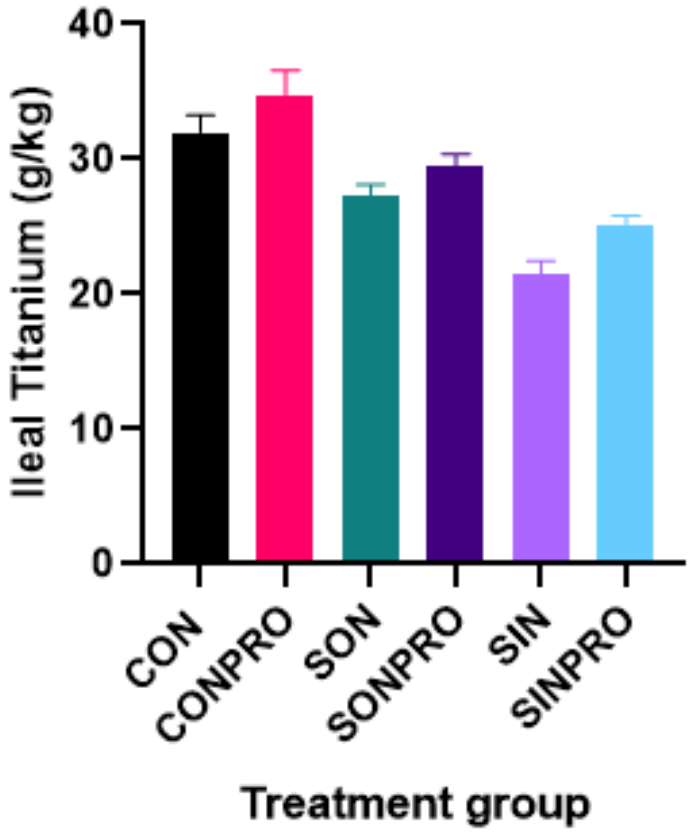
Appendix 3. The effect of inclusion of *Prosopis juliflora* pod flour on the average ileal titanium concentration in Chapter 5. Average ileal titanium concentration displayed on a pen basis after 30 days on the experimental diets per unit digesta dry matter. Error bars are shown as SEM for n=2 technical replicates per pen. Where (C) indicates a control diet and (M) indicates a *Prosopis juliflora* diet. A one-way ANOVA indicated a significant difference between the two treatment groups ($p<0.001$) where control birds average at 15.07 g/kg and mezquite birds average at 11.14 g/kg.



The ileal titanium dioxide content was significantly lower ($p<0.001$) in the birds fed the mezquite diet compared to the control diet, as seen in figure 5.3.

Appendix 4

Appendix 4. The effect of each dietary treatment on the average ileal titanium dioxide concentration per unit dry matter, from Chapter 6. Average ileal titanium concentration displayed per treatment after 29 days on experimental diets, where n=6 pens per treatment, error bars are shown as SEM. A two-way ANOVA showed no significant interaction between feed variant and processing ($p=0.896$), but there was a significant difference ($p<0.001$) between feed variants and between processed and non-processed diets ($p=0.022$).



There was no significant interaction between feed variant and processing ($p=0.896$) for the average ileal titanium dioxide content per unit dry

matter (Figure 6.3). However, there was a significant difference ($p<0.001$) between feed variants, with the control diets (CON and CONPRO) being significantly higher than feed containing *P. velutina* (SON and SONPRO) and both being significantly higher than feeds containing *P. artiulata* (SIN and SINPRO). There was also a significant difference between processed and non-processed feeds ($p=0.022$) with processed feeds having a higher titanium dioxide content.

Appendix 5

Appendix 5. The full amino acid content (g/kg DW) of each experimental diet used in Chapter 6 of this thesis.

	Feed							
	CON	**CON PRO**	**SON**	**SON PRO**	**SIN**	**SIN PRO**	**SED[1]**	**P-value**
Alanine (g/kg)	15.43	11.46	6.84	16.4	9.77	12.21	1.72	**0.011**
Arginine (g/kg)	20.07	14.84	12.65	16.95	10.15	17.37	1.95	**0.02**
Aspartate (g/kg)	29.45	20.5	21.07	25.6	16.16	25.42	3.22	0.053
Cysteic Acid (g/kg)	2.6	2.2	1.9	2.1	1.6	2.3	0.18	**0.013**
Glutamate (g/kg)	63.8	50.2	43.1	50.1	35.5	50.3	6.21	**0.046**
Glycine (g/kg)	13.53	9.29	8.44	9.4	6.25	10.27	1.33	**0.02**
Histidine (g/kg)	7.97	5.84	5.45	6.54	4.22	6.56	0.74	**0.026**
Isoleucine (g/kg)	10.61	8.02	6.72	8.07	5.5	8.53	0.94	**0.018**
Leucine (g/kg)	16.51	12.96	10.86	13.4	9.02	13.57	1.73	**0.05**
Lysine (g/kg)	19.84	14.95	11.26	14.4	10.38	15.87	1.96	**0.021**
Methionine Sulfone (g/kg)	12.3	10.3	7.74	8.25	6.01	9.63	1.15	**0.015**
Phenylalanine (g/kg)	2.62	2.66	2.93	2.77	2.53	2.93	0.43	0.891
Proline (g/kg)	17.8	15.13	13.34	15.77	11.07	16	1.67	0.062
Serine (g/kg)	13.46	10.80	6.81	8.32	4.04	8.48	2.07	**0.040**
Threonine (g/kg)	11.11	8.73	7.37	8.27	5.97	9.25	0.96	**0.02**
Tyrosine (g/kg)	4.94	3.8	3.11	4.57	2.78	4.81	0.46	**0.013**
Valine (g/kg)	11.55	8.3	7.34	8.39	6.02	9.38	1.32	**0.039**

[1] SED- standard error of the differences of the means, n=2 technical replicates. Significant differences highlighted in bold, taken at $p<0.05$, determined by two-way ANOVA.

Appendix 6

Appendix 6. The average amino acid concentration (g/kg DW) for each treatment group in Chapter 6 of this thesis.

Amino Acid (g/kg)	CON	CON PRO	SON	SON PRO	SIN	SIN PRO	SED [1]	P Value
ALANINE	2.32	2.01	1.42	1.61	0.97	1.39	0.41	0.44
ARGININE	5.42	5.35	5.02	5.13	4.46	4.50	0.39	0.87
ASPARTATE	9.49	9.51	8.63	9.39	7.42	7.59	0.68	0.64
CYSTEINE	1.18	1.24	1.11	1.15	0.94	0.92	0.08	0.90
GLUTAMATE	12.31	12.68	12.04	13.01	10.33	10.24	0.96	0.89
GLYCINE	4.44	4.91	4.05	4.79	4.61	4.10	0.45	0.23
HISTIDINE	2.94	2.94	2.72	2.94	2.59	2.50	0.17	0.39
Isoleucine	4.68	4.49	4.20	4.70	3.98	4.07	0.30	0.27
LEUCINE	5.81	5.77	5.20	5.67	4.73	4.84	0.40	0.65
LYSINE	5.17	5.31	4.91	5.11	4.46	4.58	0.34	0.99
METHIONINE	1.92	1.87	1.77	1.88	1.64	1.70	0.09	0.37
PHENYLALANINE	3.88	3.68	3.46	3.62	3.05	3.63	0.28	0.52
PROLINE	5.48	5.75	5.46	5.76	5.02	4.88	0.37	0.92
SERINE	4.40	4.53	4.11	5.28	3.73	3.48	0.92	0.61
THREONINE	4.74	4.88	4.18	4.47	3.80	3.75	0.37	0.95
TYROSINE	2.33	2.30	2.07	2.24	1.94	1.97	0.15	0.65
VALINE	4.62	4.61	4.09	4.42	3.86	3.94	0.31	0.72

[1] SED- standard error of the differences of the means, n=6 replicates per treatment (pen).
Significant differences taken at $p<0.05$, determined by two-way ANOVA.