Optimisation of soaking and thermal processing methods in reducing the trypsin, chymotrypsin and alpha-amylase inhibitors found in underutilised legumes for use as aquafeed

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ABSTRACT

In carnivorous fish farming industry, there are progressive increase demands for the finite resource of fish meal. A potential alternative to fish meal is to use legume meals which are free of enzyme inhibitors. In selected underutilised legumes the most effective processing method for eliminating trypsin (TIA), chymotrypsin (CIA) and α-amylase (AIA) inhibitors, without affecting the crude protein content, was investigated. These methods included soaking (S), wet heating (W), autoclaving (A) and dry freezing (D). No single method was effective at removing all the inhibitors. In all legumes tested, the combined processing methods which involved A were most effective in reducing CIA and AIA (p<0.05), but not TIA. However, in adzuki bean both TIA and CIA were reduced by the D+A combined method (p<0.05), whereas AIA of soybean and adzuki bean was decreased by combined methods of S+A (84.7 % and 99.3 % reduction respectively, p<0.05) or A+D (99.1 % and 72.6 % reduction respectively, p<0.05). All the processing methods retained 86.5 – 90.5 % of crude protein. Replacement of 10 % (w/w) of fish meal with D+A treated legume meal (either bambara groundnut or adzuki bean) for 28 days showed no significant difference in growth performance or inflammatory effects in *Danio rerio* or *Lates calcarifer*. Compared to *Lates calcarifer* given feed containing unprocessed adzuki bean meal, those on feed containing processed adzuki bean meal had increased hepatic gene expression of alanine aminotransferase (p<0.01), indicating an enhanced ability to utilise amino acids. The project identified specific food processing methods which are effective at removing enzyme inhibitors in legumes, thereby facilitating the application of legumes as aquafeed ingredients. Future studies are required to

examine what inclusion level of treated legume meal can promote growth performance in specific commercial fish species.

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LIST OF ABBREVIATIONS

ADG	Average daily gain
AIA	α-Amylase inhibitor activity
ALAT	Alanine aminotransferase
ANFs	Anti-nutritional factors
ANOVA	Analysis of variance
AOAC	Association of Analytical Communities
AWERB	Animal Welfare and Ethical Review Body
BAPNA	α-N-benzoyl-dl-arginine-p-nitroanilidehydrochloride
BBI	Bowman-Birk
bp	Base pair
BSA	Bovine serum albumin
BTEE	Benzoyl-l-tyrosine ethyl ester
cDNA	Complementary deoxyribonucleic acid
CIA	Chymotrypsin inhibitor activity
C_p/C_q	qPCR threshold cycle
CRP	C-reactive protein
$CuSO_4 \cdot 5\ H_2O$	Copper (II) sulphate pentahydrate
Da	Dalton
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNS	Dinitrosalicylic acid
dNTP	Deoxynucleotide
DOPA	Dihydroxyphenyl alanine
DW	Dry weight
EDTA	Ethylenediamine tetra-acetic acid
EFLα	Elongation factor-1α

FCR	Feed conversion ratio
gDNA	Genomic deoxyribonucleic acid
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HSI	Hepatosomatic index
HSP 70	Heat shock protein 70
il-1β	Interleukin 1 beta
il-8	Interleukin 8
k index	Fulton's condition factor
K_2SO_4	Potassium sulphate
KTI	Kunitz
leu-ser	Leucine-Serine
N/Protein	Nitrogen/Protein
NaCl	Sodium chloride
NACWO	Nominated Animal Care and Welfare Officer/Named Animal Care and Welfare Officer
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NFE	Nitrogen free extract
NH_4	Ammonia
NO_2	Nitrite
NO_3	Nitrate
NVS	Named Veterinary Surgeon
PCR	Polymerase chain reaction
PCR	Protein conversion ratio
PER	Protein efficiency ratio
psi	Pound-force per square inch

qPCR	Real time polymerase chain reaction
RIN	RNA integrity number
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error mean
ser-lys	Serine-Lysine
SGR	Specific growth rate
TAE buffer	Tris-Acetate-EDTA buffer
TIA	Trypsin inhibitor activity
TiO_2	Titanium dioxide
Tm	Melting temperature
β-actin	Beta-actin

CHAPTER 1

INTRODUCTION

1.1 Problem statement

Declining worldwide ocean fisheries stocks, together with the further expansion of human population that will approach 9.7 billion before 2050, are an incentive for the further growth of aquaculture (WWF, 2013a; Crab *et al.*, 2007; Hardy, 1999). Capture fisheries production has remained stable whilst aquaculture production continues to expand from 1995 to the present timeline (FAO, 2016). Asia is the world's largest contributor to world aquaculture and accounts for nearly 90% of the global production of freshwater finfish (FAO, 2016; FAO, 2012; Silva and Davy, 2010). This trend is expected to continue for the next decade.

Optimistically, aquaculture is a method to augment dwindling fish catches and thereby relieve the pressure on ocean fisheries (Deutsch *et al.*, 2007; Naylor *et al.*, 2000). However, farming high value carnivorous species such as cod, seabass, tuna, and salmon, has a devastating effect on wild fish populations. Large amounts of wild-caught or "trash" fish were used as aquafeed for carnivorous fish species (WWF, 2013b).

Typically production of 1 kilogram of these carnivorous species, uses up to 5 kilograms of wild-caught fish which is processed into fish meal and fish oil before being used as feed (Naylor *et al.*, 2000). The production of this type of aquafeed is not sustainable and has been widely criticised both for exploitation of the global fisheries resources and the misuse of high quality marine food sources that could

be used directly for human consumption (Hill *et al.*, 2013; Kaushik *et al.*, 2004). Therefore to sustain the steady increase in aquaculture production there is increasing investigation of substitute protein and oil sources for feeds (Pratoomyot *et al.*, 2010).

Legume are a potential sources to replace fish meal as it has relatively high protein content and favourable amino acid profile that fits the requirements of aquafeed (Gatlin III *et al.*, 2007). However, legumes also contain anti-nutritional factors (ANFs) such as enzyme inhibitors, that affect nutrient digestibility if substantial amount is consumed. Commercial legumes such as soybean are used to supplement in aquafeed for seabream, rainbow trout, European seabass, Atlantic cod, tilapia, and carps (Torrecillas *et al.*, 2017; Colburn *et al.*, 2012; Collins *et al.*, 2012; Lin and Luo, 2011; Santigosa *et al.*, 2008; Urán *et al.*, 2008; Barrows *et al.*, 2007).

Therefore to reduce over-reliance on major crops and to ensure future food security a sustainability and effective ANF-free plant-based aquafeed needs to be developed. The research described in this thesis sought to develop a processing strategy to remove ANFs present in underutilised legumes and investigate the effect of these processed legume-based feeds on the growth of zebrafish and Asian seabass ("*Siakap*"). This introduction describes the legumes that could be potentially used as a feed source and the range of anti-nutritional factors that occur in these legumes. It then describes processing methods which could be used to potentially reduce these factors, and lastly considers the characteristics of the fish species that could be used as animal models to assess the effectiveness of processing legumes on the biological availability of nutrients in feeds.

1.2 Aquaculture

Aquaculture refers to the farming of aquatic organism including finfish, molluscs, crustaceans, and aquatic plants in both coastal and inland areas has developed into a highly globalised trade-dependent industry (FAO, 2013a). Over the past three decades, aquaculture is the world's fastest growing food sector growing at an average annual rate of about 6 % to enhance the production (FAO, 2016; The World Bank, 2014). The production now accounts for 50 % of the world's fish that is used for food and it is expected to increase up to 70 % of seafood will be farm-raised in 2030 (FAO, 2018a; The World Bank, 2014).

The impressive growth rate in aquaculture industry is cause by the worldwide decline of ocean fisheries stocks combined with the increasing demand for fish and fish products of a specific type that is preferred by consumers in develop countries such as salmon, rainbow trout, cods and tuna (Golden *et al.*, 2016). Today, seafood harvest from the oceans is unlikely to increase further as 85 % of the global marine stocks are either fully exploited or overfished line (WWF, 2018). Therefore, fish farming provides a solution to fill the gap between declining natural production and increase market demand for protein source, and thereby contribute to food and nutritional security around the globe.

However, the rapid expanding of farming industry has come with the following impacts affecting wild fish populations, marine habitats, water quality and society (WWF, 2018):

a) One-third of the global harvest fish had been made into fish meal and fish oil as aquafeed

b) Using of excessive antibiotics and pesticides could have unintended consequences for marine organism and human health
c) High risk of transferable disease and parasites between farmed and wild species when many fish are grown in a confined space
d) The escaped farmed fish could interbreed with local wild stocks of the same population thereby altered the overall genetic diversity pool
e) Excess food and fish waste causing pollution in the water and lead to oxygen-deprived water that eventually threaten the aquatic life
f) Arising of conflict among the users of the shared coastal environment
g) Clearing of land for agriculture feed could affects high conservation value areas

Out of these seven impacts, one of the key challenges that arise, in the context of sustainable development of aquaculture industry especially in farming carnivorous fish, is the misuse of harvested fish that could be used directly for human consumption, this being an area that has received the most attention from researchers (Hill *et al.*, 2013; Kaushik *et al.*, 2004). The demand for fish meal in aquaculture sector has grown from virtually nothing to more than half of total production in the past 20 years (Lem *et al.*, 2014). The major concern now is that the wild trash fish inputs are larger than farmed fish outputs. This will eventually increase pressure on wild fisheries to make the aquafeeds sustainable for farmed carnivores.

1.2.1 Nutritional requirements for various type of cultured fish

Nutrition plays an important role in intensive aquaculture industry as it not only influences the production costs but also the fish health (Gatlin III, 2002). A low-cost balanced diet is needed for commercial cultured fish. Protein, lipid, and carbohydrate are the major nutrients in fish diet where the body can metabolises them to produce energy that needs for numerous physiological processes and physical activities (Gatlin III, 2010; AFCD, 2009). It is noteworthy that nutritional requirements of fish vary with different species, sizes and feeding habits (AFCD, 2009).

Basically, fish can be classified into three category based on their natural feeding habits which is herbivorous, omnivorous, and carnivorous (Craig, 2009). As showed in Table 1.1, herbivorous and omnivorous species required lesser protein but higher carbohydrate than carnivorous species. This is because carnivorous species are very efficient at using protein and lipid but limited capacity at using carbohydrate as energy (Gatlin III, 2010). The natural feeding habits of fish are believed to correlate closely to their digestive system. Herbivorous fish usually have no true stomach with long intestine, while omnivorous fish have true stomach with intermediate length of intestine, and carnivorous fish have a true stomach with short intestine (Smith, 1980). Digestive tract of fish vary from short and simple to complex multifunctional reflecting the variation in nutrient sources (Krogdahl *et al.*, 2005; Buddington *et al.*, 1997). This physiological characteristic means that fish differ in their ability to use the energy-yielding nutrients. Knowledge of the fish digestive system and nutrient requirement can help in aquafeed development and therefore selecting the most appropriate ingredients to be incorporated into the feed.

Table 1.1 The recommended nutrients composition for various type of cultured fish in grower size (10 – 200 g) (AFCD, 2009; Craig, 2009; Lall and Tibbetts, 2009)

Feeding habits	Example of cultured fish	Recommended nutrients composition		
		% Protein	% Lipid	% Carbohydrate
Herbivorous	Grass carp, Silver carp, Bighead carp	18 - 23	< 5	40 - 50
Omnivorous	Catfish, Tilapia, Common carp	24 - 33	5 - 6	25 - 40
Carnivorous	Salmonids, Grouper, Seabass, Seabream	38 - 50	> 10	< 20

1.2.2 Aquafeed for carnivorous fish

Traditionally, fish meal is one of the key ingredients used in recipes of farm-raised carnivorous fish aquafeeds to supply protein for optimal fish performance. Out of 3 major nutrients, protein is the most expensive component in aquafeed (Bhosalel *et al.*, 2010). Of all the protein sources, fish meal is the preferred protein source of choice in aquafeeds for several reasons, which include its high protein content, excellent amino acid profile, high nutrient digestibility, general lack of anti-nutritional factors (ANFs), and its wide availability (Gatlin III *et al.*, 2007). In aquaculture industry, about 30 – 50 % of the carnivores' aquafeed are made up by fish meal which has the consequence of making aquafeed 60 – 70 % of the total operational cost (Barrows *et al.*, 2008; FAO, 2002).

The growing demand, along with the limits in wild fish catch, have created an uncertain market for fish meal with prices rising by almost 300 % in the past 10 years, which is expected not to revert to lower price levels (Oil, 2014). The world price for fish meal ranged between USD 500 – 700 per tonne between the years of 2000 to 2005 (FAO, 2018b). Over the last decades, the fish meal price has drastically increased and peaked up to USD 1874 per tonne in year 2010 (Figure 1.1). And the price of fish meal is expected to rise by 90 % over period of 2010 to 2030 (The World Bank, 2014). In all regions of the world, the increasing price of fish

meal will directly affect the manufacture price of aquafeed by 20 – 40 % and result in increasing the market price of the fish. Thus, the development of cost-effective aquafeeds is relatively important for aquaculture industry expansion.

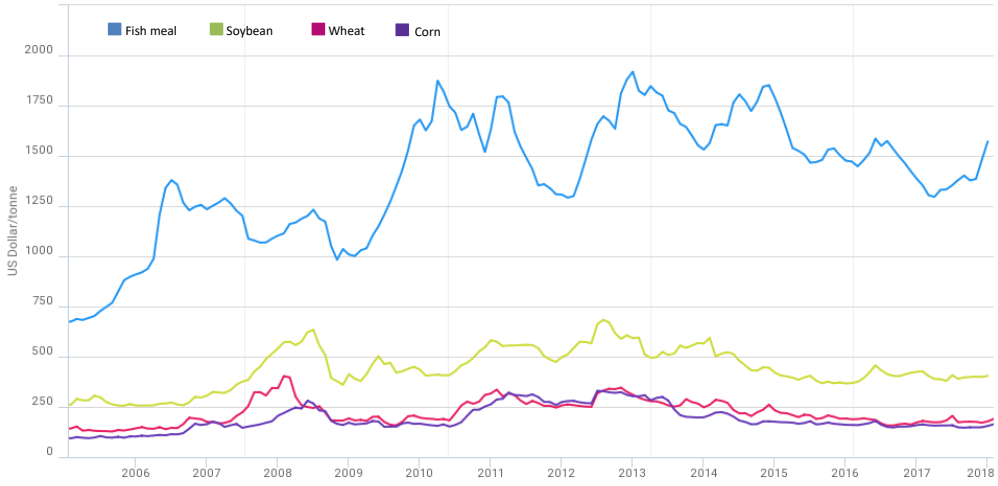

Figure 1.1 Commodity prices of main protein sources used in aquafeed (Adapted from FAO, 2018b).

Today, the supply of fish meal from wild harvest of marine stocks and associated growth of aquaculture industry is static (Salin *et al.*, 2018). The reliance on trash fish to produce fish meal ultimately worsens food security especially in poorer countries (FAO, 2012). The limitation in fish meal supply and the consequential increased price have led to an increase in the investigations to identify substitute animal protein for future aquaculture development. Hence, because of the limited availability, fluctuating price, and the growing concerns on the sustainability of fish meal, there has been an increasing trend to incorporate plant protein, especially soybean, as a substitute for animal protein in aquafeeds.

1.3 Legume as plant protein source

1.3.1 Commodity crop – Soybean

Soybeans has been grown as a commercial crop which account for 69 % of the total world production and approximately 90 % of the production is processed and crushed into soybean meal and oil for the usage in livestock and aquaculture industries whereas the remainder is consumed by human directly as food has become a major part of human diets (Salin *et al.*, 2018; Hartman *et al*., 2011). Over the years among the plant protein ingredients, soybean had been used either wholly or partially to substitute the fish meal in aquafeed due to its high protein (40%) and oil content (20%) not only to circumvent the shortage of fish meal but also to enhance the sustainability of aquaculture industry (Egbichi *et al.*, 2014; Mahmoud *et al.*, 2014; Rao and Reddy, 2010; Chou *et al.*, 2004; Dersjant-li, 2002; Refstie *et al.*, 1998; Dabrowski *et al.*, 1989).

Unfortunately, with the anticipated growth in livestock and aquaculture industries a shortage in soybean meal production is being predicted by 2020 (Wilson, 2008). This is because soybean meal is probably one of the most promising plant based protein used in livestock and aquaculture as feed in the world, despite its high price (Florou-Paneri *et al.*, 2014). However, the reliance of soybean in animal feed raises sustainability issues in terms of both economically and environmental (Lee *et al.*, 2016).

As indicated in section 1.2.2, production cost of aquafeed is one of the key aspects that will be considered when substituting fish meal by soybean. However, for the past two decades the world price of soybean has driven up more than 70 %. The world prices for soybean was ranged between USD 180 – 200 per tonne during the

years 2000 to 2005, whereas between the years of 2006 to 2018 the price ranged from USD 200 to 620 per tonne (Figure 1.1). The factors that contribute to the escalating of soybean price is not only the increasing demand of soybean for feed and food but also the fluctuation in production shortfalls due to climate change, global warming, scarcity of land and water, disease outbreaks, regional conflicts, and instabilities, as well as slow global economy growth rate (Salin *et al.*, 2018; USDA, 2009).

Environmentally, the main impact from farming soybean comes from clearing natural habitats, thereby threating forests and other natural ecosystems (Tim and Hassan, 2009). According to WWF (2017), in recent decades, millions of hectares of forest, savannah and grassland have been converted into soybean plantations, which has threated the biodiversity, depleted ecosystems services and emitted vast amounts of carbon dioxide. Deforestation has contributed to climate change, it is responsible for about 15 % of all the global greenhouse gas emissions caused by people (WWF, 2017; Lee *et al.*, 2016). The ongoing climate change could cut crop yields by more than 25 % and may impact the local food security (Iizumi and Ramankutty, 2015).

A key strategy required in order to adapt to a changing climate is to look for alternative plant protein sources that can used in aquafeed. Ideally these should be innovative resources, such as underutilised legumes, a these not only do not compete with existing sources used in aquaculture feed but also to mitigate the harmful impacts of aquaculture in long-term growth. Besides, in an attempt to solve problems of food security; an alternative plant based protein is essential to reduce the competition of soybean that used for human and animal diets since

soybean wasn't the only potential plant based protein (USDA, 2018; Florou-Paneri *et al.*, 2014).

1.3.2 Underutilised legumes

Legumes have been widely recognised as an important source of protein, as well as containing significant amounts of carbohydrates, lipids, crude fibre, minerals and vitamins in developing countries (Rehman and Shah, 2005; Alonso *et al.*, 2000; Kataria *et al.*, 1989). Apart from the commercial or major legume such as soybean and groundnut, there are a variety of underutilised legumes such as mung bean, adzuki bean, chickpea, hyacinth bean, rice bean, winged bean, marama bean, and bambara groundnut that have recently gained attention as nutritious pulses (Ebert, 2014; Katoch, 2013).

Underutilised legumes are considered as "minor crops" as they are less important than staple crops and agricultural commodities in terms of regional or global production and market value. Underutilised legumes have the potential to play a role in reducing the risk of over-reliance on very limited numbers of major crops (Mayes *et al.*, 2011). Globally, only 7000 out of 30,000 edible crops are being cultivated and less than 150 have been commercialised. It is estimated around 10,000 crops remain underutilised (Chivenge *et al.*, 2015). Legumes solely have about 30 species are cultivated and only a few of them are widely grown across the continents (Gowda *et al.*, 2007). Only 9 major crops including wheat, rice, maize, sorghum, millet, potatoes, sweet potatoes, soybeans and sugar (cane or beet) provided 75 % of the global plant-derived energy and estimated that approximately 95 % of the world's dietary energy comes solely from 30 crops (Kristof and Stacia, 2017).

The prime concern in cultivation of major crops have declined and continue to decline globally, even these crops offer greater genetic biodiversity but it is probably unsustainable in the long run due to global climate change (Chivenge *et al.*, 2015; Ebert, 2014). An added pressure is world's population is switching from animal-based protein diet to plant-based protein diet (Meyer and Reguant-Closa, 2017; Kumar *et al.*, 2010; FAO, 2003). This trend has created the demand for food legumes that exceeds the production as only a few legumes are traded in the global market (Bhat and Karim, 2009; Gowda *et al.*, 2007).

In this regard, underutilised legumes have been highlighted as an alternative to major commodity crops to meet the food legumes demand with several advantages. Underutilised legumes are cheap and able to adapt to a wide range of environment that contributed to agricultural sustainability with a relatively low input (Padulosi *et al.*, 2013; Polok *et al.*, 2008). They are able to withstand adverse environmental conditions and can thrive under extreme stress conditions such as droughts, floods, cyclone and heat waves (Bhat and Karim, 2009). Many of these species are maintained by poor farming communities under marginal environments such as high mountains, desert margins and poor soils (Padulosi *et al.*, 1999). These attributes make underutilised legumes worthy of investigation. In the future these crops could be used to help improve the current agriculture systems by providing genetic traits allow adaptation to the environment but also strengthen the resilience of agroecosystems through crop diversification (Padulosi *et al.*, 2011; Padulosi *et al.*, 1999). Thus, they have the potential to contribute in food and nutrition security, especially if the removal of enzyme inhibitors can increase the utilisation of nutrients such as protein.

1.3.3 Types of underutilised legumes

In this study, seven underutilised legumes, namely mung bean (*Vigna radiate* L.), adzuki bean (*Phaseolus angularis*), chickpea (*Cicer arietinum* L.), hyacinth bean (*Lablab purpureus* L.), black-eyed pea (*Vigna unguiculata* L.), pigeon pea (*Cajanus cajan* L.) and bambara groundnut (*Vigna subterranean* L.) were investigated as alternatives to replace major legumes as feed ingredients. These underutilised legumes have comparable amounts of protein (20 – 37 %) carbohydrate (40 – 60 %), minerals, dietary fibre, vitamins to major legumes. The macronutrient contents included protein, carbohydrate and fat of these underutilised legumes had summarised in Table 1.2. The above mentioned legumes except adzuki bean had been used as livestock feed for many years (Department of Agriculture and Fisheries (The State of Queensland), 2012; Abeke *et al.*, 2008; Teguia and Beynen, 2005; Aganga *et al.*, 2000). Even so, to-date the research on using these underutilised legumes as potential aquafeed ingredient is scarce.

a) Mung bean

Mung bean (*Vigna radiate* L.) (Figure 1.2 A), also known green bean, is one of the important warm season crops which native to the North-eastern India-Burma (Myanmar) region of Asia (Keatinge *et al.*, 2011; Yao *et al.*, 2008). It contains 27 % (w/w) of protein, 60 % (w/w) of carbohydrate and 1 – 2 % (w/w) of fat with a range of micronutrients (Butt and Batool, 2010; Mubarak, 2005; Kataria and Chauhan, 1988; Kataria *et al.*, 1988). Aside from its use as bean sprout, mung bean is also processed into noodles (Maneepun, 2003; Hoover *et al.*, 1997). In the Philippines mung bean is boiled and utilised in native desserts such as hopia, butse-butse and halo-halo (Barroga *et al.*, 1985).

b) Adzuki bean

Adzuki bean (*Phaseolus angularis*) (Figure 1.2 B) also known as small red bean is smooth and shiny in maroon colour with white hilum and is a popular ingredient in many confections in the Orient (Takahama *et al.*, 2013; Yoshida *et al.*, 2010; Sacks Frank, 1977). It is a rich source of carbohydrate (60 – 70 % (w/w)), protein (21 – 24 % (w/w)) and a range of micronutrients (such as minerals, vitamins) (Yousif *et al.*, 2007; Tjahjadi *et al.*, 1988). It can be cooked and consumed in paste form or whole bean is boiled and sweetened that is used in desserts, snacks and confectionery items (Yousif *et al.*, 2007; Yousif *et al.*, 2003; Yousif *et al.*, 2002). In China, red bean is used as soup ingredients for therapeutic purposes such as driving away dropsy, relieving diarrhoea and tonic to the viscera (Meng and Ma, 2001; Chau and Cheung, 1997).

c) Chickpea

Chickpea (*Cicer arietinum* L.) (Figure 1.2 C) is being cultivated throughout the world including Asia, West Asia, North Africa, East Africa, Southern Europe, North and South America and Australia (Noor *et al.*, 2003; Singh, 1997). It is an ancient legume believed to have originated in south-eastern Turkey and the adjoining part of Syria (Sreerama *et al.*, 2010). Chickpea has been divided into two groups “macrosperma” or “kalibu” and “mircosperma” or “desi” (Toker *et al.*, 2012). “Kalibu” chickpea seeds are large in size, have a thin salmon-white colour seed coat and is usually grown in temperate regions of the world. The “desi” chickpea seeds are smaller, with dark colour seed coat are grown on semi-arid land (Toker *et al.*, 2012; Roy *et al.*, 2010; Alajaji and El-Adawy, 2006; El-Adawy, 2002). Both types of chickpeas’ appearance is beaked, often angular and wrinkled on the seed surface (Singh, 1997).

Chickpea contains high levels of carbohydrate (47 % (w/w)) and protein (22 % (w/w)) (Alajaji and El-Adawy, 2006; El-Adawy, 2002). Chickpea and its flour (garbanzo flour or besan) is used extensively in food processing due to its ideal polysaccharides composition and high content of oil (Sreerama *et al.*, 2012). In South and Southeast Asian cuisines, chickpeas are served as a staple ingredient. Chickpea flour is the main ingredient in many Indian sweets, desserts, and savoury products. French and Italian cuisines also use it to make variety of desserts, noodles, snacks and main dishes (Alajaji and El-Adawy, 2006).

d) Hyacinth bean

Hyacinth bean (*Lablab purpureus* L.) (Figure 1.2 D) previously classified as *Dolichos lablab* is one of the lesser known tropical and subtropical legumes (Murphy and Colucci, 1999; Akpapunam, 1996). It is an ancient crop widely distributed in the tropics particularly in India and Southeast Asia, Egypt and Sudan (Lim, 2012; Maass, 2006). Hyacinth bean has many local names such as lablab, bonavist, Chinese flowering, Egyptian, Pharao, shink, val, wild field and Indian bean (USDA, 2012). The hyacinth bean exists in two very distinctive forms, one with deeply pigmented flowers, stems and foliage is a popular ornamental vine, whereas the common pulse form has white flowers and unpigmented stems and foliage (Akpapunam, 1996). The green seed of hyacinth bean will either turn into dark brown, black or pale tan or white, often speckled (Lim, 2012).

Hyacinth bean considered as non-oilseed legumes as it contained only 1 – 2 % (w/w) of fat with 20 – 28 % (w/w) of protein, 53 % (w/w) of carbohydrate and large amount of various vitamins and minerals (Lim, 2012). After soaking in water overnight the dry hyacinth beans are cooked together with rice for a protein

supply. It also used as raw material for tempeh, an Indonesian traditional fermented food that commonly made from soybeans (Subagio, 2006). It is eaten as a vegetable in Southeast Asia, whereas in India it is predominantly used as a pulse often as dhal (Lim, 2012).

e) Black-eyed pea

Black-eyed pea (*Vigna unguiculata* L.) (Figure 1.2 E) also known as cowpea is an underutilised legume in United States that has been consumed extensively in Africa, and to lesser extent in Asia (Sreerama *et al.*, 2012; Hallén *et al.*, 2004). It has a creamy skin with black dot and is medium oval in shape (Geil and Anderson, 1994). It is underutilised partly because changes with storage means it has to have a prolonged cooking time, and the presence of ANFs (Prinyawiwatkul *et al.*, 1997; Prinyawiwatkul, 1996).

Black-eyed pea is high in protein (18 – 35 % (w/w)) and consists of 50 – 60 % (w/w) of carbohydrate content and low in fat content (1 – 2 % (w/w)) that contain no cholesterol make it a potentially important nutritional component (Butt and Batool, 2010; Hallén *et al.*, 2004; Ragab *et al.*, 2004). They are often consumed alone in fried name akara or as steamed bean cakes name moin-moin in Nigeria (Carvalho *et al.*, 2017; Onimawo *et al.*, 2007; Giami, 1993; Kochhar *et al.*, 1988).

f) Pigeon pea

Pigeon pea (*Cajanus cajan* L.) (Figure 1.2 F) is one of the oldest food crops grown and consumed in the tropics and semi-arid tropics but is underutilised (Maninder *et al.*, 2007). It is also known as red gram, yellow dhal, Angola pea and Congo pea (Kaushal *et al.*, 2012; Saxena *et al.*, 2002). India alone contributes over 90 % of the

world pigeon pea production and it is becoming popular in several countries of Africa, Southeast Asia, Carribbean and Latin America (Torres *et al.*, 2007; Agunbiade and Longe, 1999). Pigeon pea is rich in protein (22 % (w/w)), carbohydrate (60 % (w/w)) (Tiwari *et al.*, 2011; Saxena *et al.*, 2002). It possesses low concentration of fat (1 – 2 % (w/w)), moderate amount of fibre, starch and a reasonably balanced range of all dietary essential minerals (Tiwari *et al.*, 2011; Torres *et al.*, 2007). In Ethiopia, not only pods but also young shoots and leaves are cooked and eaten. Whereas in India, dehusked split pigeon pea is consumed after boiling to make dhal (Liu *et al.*, 2010; Hoover *et al.*, 1993).

g) Bambara groundnut

Bambara groundnut (*Vigna subterranean* L.) (Figure 1.2 G) is an indigenous African crop that produced 330,000 tonnes annually even under conditions of drought and low soil fertility (FAO, 2015; Linnemann, 1991). Similar to groundnut, the pods and seeds are formed on or just below the soil surface (Bamshaiye *et al.*, 2011; Swanevelder, 1998). Their seeds are various colours from white to cream, red, black or brown and sometime mottled depending on the ripeness (Umar and Turaki, 2014). Bambara groundnut is a rich source of carbohydrate (49 – 63 % (w/w)), protein (16 – 25 % (w/w)), fat (4.5 – 7.4 % (w/w)) which is comparable to soybean (Murevanhema and Jideani, 2013; Mkandawire, 2007). People consumed bambara groundnut in numerous forms including fresh when it is semi-ripen as snacks and the mature seeds are cooked or grounded into flour (Umar and Turaki, 2014; Mkandawire, 2007).

Figure 1.2 Various type of underutilised legumes. A: Mung bean, B: Adzuki bean, C: “Kalibu” Chickpea, D: Hyacinth bean, E: Black-eyed pea, F: Pigeon pea, G: Bambara groundnut.

1.4 Anti-nutritional factors present in legumes

Although legumes are rich in nutrients, they also contain a range of anti-nutritional factors (ANFs) such as enzyme inhibitors, phytic acid, saponins, lectins, tannins and cyanogens. ANFs are defined as substances generated through metabolism that adversely affect the nutritional value in living systems by interfering with food utilisation (Novak and Haslberger, 2000; Kumar, 1992). Generally ANFs could be divided into four groups (Bora, 2014; Onder and Kahraman, 2009; Francis *et al.*, 2001):

a) Factors affecting protein utilisation and digestion such as protease inhibitors, tannins, lectins
b) Factors affecting mineral utilisation such as phytic acid, gossypol pigments, oxalates, glucosinolates
c) Antivitamins such as dihydroxyphenyl alanine (DOPA)
d) Miscellaneous substances such as mycotoxins, mimosine, cyanogens, nitrate, alkaloids, photosensitizing agents, phytoestrogens, saponins

ANFs in plant-based aquafeed ingredients remain a concern as less is known about the exact response of fish to these compounds (Hajra *et al.*, 2013). Studies by Krogdahl *et al.* (2010) and Francis *et al.* (2001) showed that the presence of ANFs can limit the protein utilisation in fish thereby affecting their growth rate as protein is not only essential for fish to grow but also for energy and life support.

1.4.1 Protease inhibitors

Protease inhibitors such as trypsin inhibitors and chymotrypsin inhibitors which have the serine group are found in abundance in raw legumes (Krogdahl *et al.*, 2010; Shahidi, 1997; Thompson, 1993; Van Der Poel, 1990). These protease inhibitors are proteins that form stoichiometric protease-inhibitor complexes with their respective enzymes and inhibit their activity in gastrointestinal tract. They will bind to the digestive enzyme either through the action of competitive or allosteric to render the enzyme inactivation (Glencross, 2015).

Traditionally, trypsin inhibitors are categorised into two groups Kunitz (KTI) and Bowman-Birk (BBI). KTI is a single headed inhibitor that binds to one enzyme molecule per inhibitor. The inhibitory site of KTI mainly inhibits trypsin with little inhibition on chymotrypsin. They are generally absent from *Phaseolus, Pisum* and *Vigna* species but they are found in soybeans and winged beans (Deshpande, 2005).

The BBI can simultaneously inhibit both trypsin and chymotrypsin as it contains two independent inhibitory binding sites (Chen *et al.*, 2014; Sessa and Wolf, 2001). As shown in Figure 1.3, the BBI inhibitors are a stabilise polypepetide which makes it relatively stable to proteolytic breakdown, acid denaturation as well as heat, which has a significant impact on the protein digestion in the intestine (Krogdahl *et al.*, 2010). Unlike trypsin inhibitors, chymotrypsin inhibitors have not been well categorised.

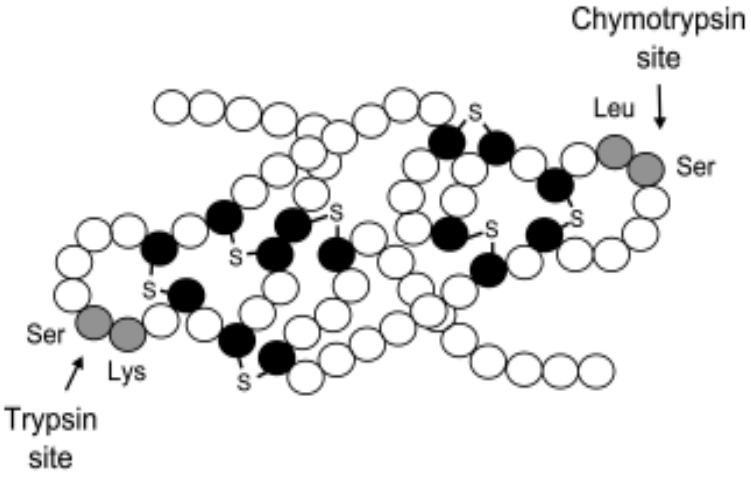

Figure 1.3 Bowman-Birk inhibitor structure from soybean. The amino acids interacting with trypsin (ser-lys) and chymotrypsin (leu-ser) are marked in grey and seven cysteine bridges are shown in black (Adapted with permission from Krogdahl *et al.*, 2010; license number: 4203270327989)

The presence of chymotrypsin inhibitors in animal diets will cause indigestion and abdominal pain, whilst pancreatic enlargement and growth depression are caused by the presence of trypsin inhibitors (Kumar *et al.*, 2013). The pancreatic enlargement (hypertrophy and hyperplasia) is because trypsin inhibitors suppress the negative feedback regulation of pancreatic secretions through an increased release of hormone cholecystokinin from intestinal mucosa. The hyper-secretion of pancreatic digestive enzyme leads to a loss of sulphur rich endogenous proteins (methionine and cysteine). As legume seed proteins are generally deficient in sulphur amino acids, the combination of the ANF effect and the quality of the protein in the legumes can then lead to depressed growth and contribute to the loss in body weight (Jezierny *et al.*, 2010; Guillamón *et al.*, 2008; Shahidi, 1997; Thompson, 1993). The effect of these inhibitors on pancreatic enlargement has been observed in pigs, chicks and rats when fed with legumes (Jezierny *et al.*, 2010; Clarke and Wiseman, 2005; Tavano and Inácio, 2005). It is expected the same phenomenon will be present in humans and fish and which could potentially lead to the onset of pancreatitis and pancreatic cancer in high legumes consumption. The

elevation of cholecystokinin produces a chronic trophic stimulus to pancreas that leads to the formation of pancreatic nodules and adenomas (Hatcock, 1991).

1.4.2 α-Amylase inhibitor

Similar to trypsin and chymotrypsin inhibitors, α-amylase inhibitors are widely distributed in legumes (Pusztai *et al.*, 2004). α-amylase inhibitors from legumes do not inhibit plant, fungal or bacterial α-amylase activity but do affect isoforms found in mammals and some insects (Yamada *et al.*, 2001). In total, there are seven types of proteinaceous α-amylase inhibitors found in nature. But only 1 type of α-amylase inhibitor namely legume lectin-like (isoform α-AI1) is widely distributed in legumes and has the anti-amylase activity in humans (Cuthbert Obiro *et al.*, 2008; Svensson *et al.*, 2004). α-AI1 will bind to the human and porcine pancreatic amylases through the action of mixed non-competitive inhibition mechanism (Santimone *et al.*, 2004; Le Berre-Anton *et al.*, 1997).

The inhibition activity of α-amylase has been categorised as ANF for human and livestock. Complex formation of α-amylase inhibitors with α-amylases cause reduction in starch digestion by inhibiting the hydrolysis of α-1,4-glycosidic bonds of starch (Singh *et al.*, 2010). Therefore presence of legume α-amylase inhibitor in human diet can cause impaired carbohydrate digestion and coeliac disease, leading to weight loss (Kumar *et al.*, 2013; Rekha and Padmaja, 2002).

Table 1.2 Summary of macronutrients and enzyme inhibitory activity in selected legumes

Legumes	% Protein (w/w)	% Carbohydrate (w/w)	% Lipid (w/w)	Trypsin inhibitory activity (TIA unit/mg)	Chymotrypsin inhibitory activity (CIA unit/mg)	α-amylase inhibitory activity (AIA unit/mg)	References
Mung bean	27	60	1 – 2	15.80	na	na	Butt and Batool, 2010; Mubarak, 2005; Kataria and Chauhan, 1988; Kataria *et al.*, 1988
Adzuki bean	21 – 24	60 – 70	na	na	na	na	Yousif *et al.*, 2007; Tjahjadi *et al.*, 1988
Chickpea	22	47	na	10.43	5.70 – 9.40	nd – 3.38	Shi *et al.*, 2017; Alajaji and El-Adawy, 2006; El-Adawy, 2002; Frias *et al.*, 2000; Singh, 1988; Skekib *et al.*, 1988
Hyacinth bean	20 – 28	53	1 – 2	28.96	na	na	Lim, 2012; Osman, 2007
Black-eyed pea	18 – 35	50 – 60	1 – 2	13.02	na	2.21	Butt and Batool, 2010; Hallén *et al.*, 2004; Ragab *et al.*, 2004; Egounlety and Aworh, 2003; Skekib *et al.*, 1988
Pigeon pea	22	60	1 – 2	15.40	2.10 – 3.60	na	Tiwari *et al.*, 2011; Torres *et al.*, 2007; Oloyo, 2004; Saxena *et al.*, 2002; Singh, 1988
Bambara groundnut	16 – 25	49 – 63	4.50 – 7.40	49.10 – 60.40	na	na	Murevanhema and Jideani, 2013; Mkandawire, 2007; Tibe *et al.*, 2007
Soybean	40	35	20	0.048	na	0.94	Yadav *et al.*, 2018; Shi *et al.*, 2017; Karr-Lilienthal *et al.*, 2005; Miyagi *et al.*, 1997

Notes:
nd represented not detectable
na represented not applicable (either not reported in published journal or reported in different unit)

1.5 Effect of ANFs on fish

In fish, the nutrient absorption is known to take place in pyloric caeca and anterior intestine; and to a lesser extend in posterior intestine (Pérez-Jiménez *et al.*, 2009). The pyloric caeca is finger-like organ that located near the junction of stomach and anterior intestine that responsible for the pancreatic enzymes secretion in response to the presence of nutrients in intestine lumen (Gulf and Science, 2015; Pérez-Jiménez *et al.*, 2009; Einarsson *et al.*, 1997; Liddle, 1997; Singer, 1993).

With the presence of ANFs in plant-based meal, these anti-nutrients might affect the nutrient absorption in fish and cause detrimental effects to the fish health. The presence of ANFs in aquafeed might alter the nutrient balances of the diets, reduce the palatability and bioavailability of nutrients for absorption by fish. When ANFs are in excess, it could cause inhibition of growth, intestinal dysfunction, altered gut microflora, modulation of immune responses, goitrogenesis, pancreatic hypertrophy, hypoglycaemia and liver damage in fish (Krogdahl *et al.*, 2010). It had been reported that the high value marine cultured finfish including flounders, turbot, seabass, rainbow trout, salmonids, and seabream are particularly sensitive to the nutrient quality of the ANFs present in plant-based meal (Gouveia and Davies, 2000). The ANFs in plant-based meal lead to histological alteration in salmonids and seabream intestines along with impair intestinal absorption (Santigosa *et al.*, 2008; Baeverfjord and Krogdahl, 1996).

The presence of protease inhibitors could reduce the proteolytic enzymes activity that are secreted into the intestine lumen and result in reduction of protein digestibility that ultimately lead to poor fish performance and growth (Alarcón *et al.*, 2001; Moyano López *et al*., 1999). In addition, the binding of protease inhibitors

to the proteases will suppress the signal that responsible to stop the pancreatic enzymes secretion and stimulate the pyloric caeca or pancreas to secrete larger amounts of digestive enzymes to overcome the inhibitors and digest the feed protein (Savoie *et al.*, 2011; Alarcón *et al.*, 2001). The elevation of pancreatic enzymes could lead to pancreatic enlargement and this had been observed in pigs, chicks and rats when fed with legumes (Jezierny *et al.*, 2010; Clarke and Wiseman, 2005; Tavano and Inácio, 2005).

Unlike mammals, the mechanism of inhibition of protease inhibitors has been extensively studied whilst the mechanisms of response in fish to protease inhibitors is limited in term of pancreatic enlargement. This is because the pancreas of carnivores' fish consists of scattered pancreatic cells in the mesenteric adipose tissue between the pyloric caeca thus the pancreas size could be hardly determined (Olli *et al.*, 1994).

Krogdahl *et al.* (2010) and Francis *et al.* (2001) reported than the presence of protease inhibitors at more than 5 mg/g in aquafeed may reduce the protein digestibility and utilisation in most of the cultured fish since the protease inhibitors decrease the proteolytic activity in the chyme of the mid-intestine. However, evidence has also shown low levels of trypsin inhibitor activity (less than 3 mg/g) and oligosaccharide content (up to 16 %) in soybean meal had no apparent adverse effect on protein digestibility and growth performance in rainbow trout and European seabass (Tibaldi *et al.*, 2006; Kaushik *et al.*, 1995).

1.6 Effect of processing methods on ANFs and protein content

Recent years, the growing interest in the usage of legume protein for incorporation into food and feed applications has spurred research into how the processing methods affect the nutrient, particularly protein and ANFs content. Published findings suggested that the application of some adequate and economic technologies that aimed to reduce or eliminate the ANFs present in legumes could improve the legume nutritional value (Cowieson, 2005; Mubarak, 2005; Vidal-Valverde *et al.*, 1998). The ANFs level of reduction differs with the variety of legume, the type of ANFs present in the legume, as well as the processing methods applied to the legume.

Several legume processing methods, such as dehulling, soaking, heat treatment, germination, fermentation, and enzymatic processing with different operation parameters are commonly applied attempt to increase the legume utilisation. Among the macronutrients protein is the major component of legumes, the increasing level of protein and reducing of ANFs had been noticed after the processed (Table 1.3). The maximum protein increment was observed in dehulled chickpea (18.8 %) followed by fermented mucuna bean and locust bean (17.8 % and 17.7 % respectively) and roasted kidney pea (17.1 %). Caution needs to be exercised when resorting to processing methods to avoid any unintended adverse effects on the nutritional quality of the legumes.

Other than processing methods, there are factors affecting the levels of nutrient and ANFs in raw legumes. They are much dependent upon genotype, the environment including soil pH, weather and climate, the growth location and year and may vary within legumes of the same species, seed maturity, fertilizer

applications, cultural management practices as well as postharvest handling and storage (Shi *et al.*, 2007; Hornick, 1992). In addition, the enhancement of nutrients with the removal of ANFs can be done through breeding and biotechnological method such as genetic modifications that have been achieved through the insertion of genes encoding entire metabolic pathways or through targeted alternations in existing pathways (Khokhar and Apenten, 2011; Kok and Kuiper, 2003).

1.6.1 Dehulling

Dehulling is one of the widely used mechanical processing that applies to legumes. Often the seed coat (hull) of legumes are indigestible and may have a bitter taste often caused by that contributed by tannins (Pal *et al.*, 2017). Dehulling has been reported to increase the palatability and taste of various legumes (Wang *et al.*, 2009; Van Der Poel *et al.*, 1991). Out of various types ANFs, tannin is one of the ANFs that can be eliminated efficiently (reduced up to 70 %) by using dehulling process (Khokhar and Apenten, 2011). This is because tannin is mainly located in the seed coat. Besides, the tannin contents are closely associated with the colour of seed coat (Punia *et al.*, 2000). The deeper the colour of the seed coats the higher the tannins content (Igwenyi *et al.*, 2013). Published studies indicated that dehulled green gram, cowpea, black bean and chickpea had reduced tannin ranged from 19.9 – 43.4 % with improved protein content ranged from 7.3 – 18.8 % (Akinjayeju and Ajayi, 2011; Ghavidel and Prakash, 2007; Egounlety and Aworh, 2003).

Almost all the phytic acid was located in the cotyledons instead of seed coat (Ariza-Nieto *et al.*, 2007). However, published literatures (Table 1.3) showed that the

reduction of phytic acid for green gram, cowpea, black bean and chickpea was up to 50 % may be attributed to the removal seed coat (Malunga *et al.*, 2012).

1.6.2 Soaking

Soaking has been defined as the preliminary step prior to cooking. Soaking involves in immersing legume kernels in water, thereby moistening them and dissolving soluble nutrients. The process of soaking legumes in water produces a swelling of tissue and water uptake without cell separation (Eyaru *et al.*, 2009). Soaking can be performed by using different soaking temperature, soaking time period, composition of soaking solution (water, acidic or basic) which could affect the ANFs of legumes. Studies showed that the quantity of phytic acid, tannins and enzyme inhibitors had reduced although there was not a complete removal in legumes (Table 1.3) regardless the soaking solution, soaking time period or soaking temperature.

Tannin was able to eliminate better compared to enzyme inhibitors and phytic acid in the soaking process, this is because tannin is a water-soluble phenolic compound that make it stand out from the other 2 types of ANFs (Bhat *et al.*, 2013; Kumar, 1992). The reduction might be due to the ANF components having leached into the soaking water. Besides, soaking could also enhance the hydrolysis of oligosaccharides into fructose, glucose, and galactose in the soaking legumes (Khattab and Arntfield, 2009).

Soaking also able to increase the protein content of legumes as reported by Wang *et al.* (2008), Alonso *et al.* (2000) and El-Adawy *et al.* (2000). They stated that the kidney bean, lupin, and field pea soaked for 12 h, 13 h and 24 h had increased

0.63 %, 2.3 % and 2.6 % respectively may be attributed to the loss of soluble solids during soaking.

1.6.3 Heat treatment

Heat treatment is the most frequent method used to remove the proteinaceous ANFs (such as protease inhibitors and lectins) leading to irreversible protein denaturation (Shimelis and Rakshit, 2007). ANFs such as protease inhibitors and lectins are heat sensitive. The common heat treatment methods such as boiling, autoclaving, microwave cooking, roasting, blanching and extrusion could potentially decrease trypsin inhibitor activity, chymotrypsin inhibitor activity, α-amylase inhibitor activity, oligosaccharides, phytic acids and lectins to a considerable extent in legumes (Table 1.3) (Olawepo *et al.*, 2014; El-sayed, 2011; Hefnawy, 2011; Shimelis and Rakshit, 2007; Martín-Cabrejas *et al.*, 2004; Abd El-Hady and Habiba, 2003; Egounlety and Aworh, 2003; Ibrahim *et al.*, 2002; Adeparusi, 2001; Kataria *et al.*, 1988).

Heat processing could also partially reduce phytic acid to petaphosphates and tetraphophates (Marzo *et al.*, 1998). However, this process is not very efficient because phytic acid is a heat stable component that is not easily degraded using thermal processing (Kumar *et al.*, 2010). Thermal processing of raw pigeon pea, chickpea, black gram and green gram brought about a 70 % decrease in their tannin contents (Rao and Deosthale, 1982). Pugalenthi *et al*. (2006) and Vijayakumari *et al.* (1996) working on in *Canavalia* and *Mucuna monosperma* seeds. Demonstrated the contents of oligosaccharides decrease when subjected to thermal treatment, this might be attributable to heat hydrolysis of oligosaccharides to simple monosaccharide.

Processing methods such as heat treatment, solvent extraction, flaking and grinding showed improvement in nutritional value of plant-based meal in carnivorous fish (rainbow trout and salmon) and omnivorous fish (tilapia, carp, and catfish). Heat treatment of raw pea seed meal and raw African yam bean improved the meal quality, which was associated with a great reduction in ANFs, particularly trypsin inhibitors, which then showed better growth performances in African catfish (Ogunji *et al.*, 2016; Davies and Gouveia, 2008).

Besides, the heat treatment process could increase the protein content slightly in faba bean, jack bean and kidney bean in the studies of Olanipekun *et al.* (2015), Agbede and Aletor (2005) and Alonso *et al*. (2000). Their studies reported that extruded of faba bean, autoclaved of jack bean and roasted of kidney bean could increase the protein content by 0.22 %, 3.4 % and 17.1 % respectively. This could be due to the break down the crude protein by unfolding the protein secondary, tertiary and quaternary structures to give primary structure which an apparent increase in crude protein content and potentially increases its digestibility (Awuah *et al.*, 2007).

1.6.4 Germination

Germination is one of the most simple, common, popular and effective method in Asia which results in the reduction of phytic acid, tannin, trypsin inhibitor, chymotrypsin inhibitor, α-amylase inhibitor and augmenting the levels of protein, carbohydrates, dietary fibre and other components (Bora, 2014). During the germination process of legume, several endogenous enzymes become active with the intention of degradation of proteinaceous ANFs (Savelkoul *et al.*, 1992).

The decreased in protease inhibitors content is due to the proteolytic activity that degrades the inhibitors during germination (Domash *et al.*, 2008; McGrain *et al.*, 1989). Germination will also increase the endogenous phytase activity might lead to the breakdown of phytic acid (Egli *et al.*, 2002). Ghavidel and Prakash (2007) reported that the phytic acid and tannin of green gram, cowpea, chickpea, and lentil were reduced up to 20 % and 18 % respectively after 1 day of germination. Whereas Aguilera *et al.* (2013) and Sathe *et al.* (1983) reported that trypsin, chymotrypsin, and α-amylase inhibitory activities were reduced up to 78 %, 73 % and 67 % respectively after 4 to 5 days of germination in *Phaseolus vulgaris*, *Vigna unguiculata* L., *Canavalia ensiformis* L., *Lablab purpureus* L. and *Stizolobium niveum* L. These studies showed that a prolonged germination of legumes could lead to a significant reduction of enzyme inhibitors and phytic acid.

Several studies showed that germination of bambara groundnut, mucuna bean, cowpea and soybean had increased the protein content from 1.2 % to 13.2 % (Joshi and Varma, 2016; Devi *et al.*, 2015; Mugendi *et al.*, 2010; Agbede and Aletor, 2005). The increment might due to the synthesis of enzyme proteins or a compositional change following by the degradation of other seed constituents (Bau *et al.*, 1997).

1.6.5 Fermentation

Fermentation is a common practice in Africa and Southeast Asia countries that believed to enhance the flavour, texture, taste and nutritional value of fermented foods in addition to increasing shelf life (Mohammed *et al.*, 2017; Annor *et al.*, 2014). Fermentation is a process carried out by fungi or bacteria which has the effect of declining of lectins, tannins, oligosaccharides (stachyose and raffinose), phytic acid and protease inhibitors, hydrolysing these complex stored proteins into

simpler and more soluble available products (Annor *et al.*, 2014; Martín-Cabrejas *et al.*, 2004; Ibrahim *et al.*, 2002). Through fermentation, the concentration of tannin, phytate, trypsin inhibitor activity and saponin in *Phaseolus vulgaris* L. were reduced up to 46 %, 100 %, 52 % and 60 % respectively after 4 days (Shimelis and Rakshit, 2008).

Fermentation process are capable to increase the protein content of bambara groundnut, yam bean seed, locust bean and mucuna bean for 2.4 %, 13.9 %, 17.7 % and 17.8 % respectively. The observation in protein increment might due to the action of extracellular enzymes produced by the fermenting microorganism through synthesis of new proteins during fermentation (Enujiugha, 2003; Akubor and Chukwu, 1999).

1.6.6 Enzymatic processing

The difference of between fermentation and enzymatic processing is fermentation utilises the endogenous activities of micro-organism borne on or inside the seeds, whilst the latter is the process of adding commercial exogenous enzymes that have isolated and cultured from fungi or bacteria (Shimelis and Rakshit, 2008). Belewu and Sam (2010) showed that the trypsin inhibitor, lectin, saponin and phytic acid of *Aspergillus niger* (fungus) treated *Jatropha curcas* seed were reduced by 68 %, 78 %, 95 % and 70 % respectively after for 7 days. The reduction of oligosaccharides in *Canavalia* using enzymatic processing by adding α-galactosidase treatment ranged from 67 % to 91 % (Pugalenthi *et al.*, 2006). The action of the enzyme is to convert oligosaccharides to monosaccharide and disaccharides by cleaving the α-galactosidic linkage between the sugar molecules (Somiari and Balogh, 1993).

Table 1.3 Effect of different processing methods on anti-nutrients and protein in legumes

Processing methods	Conditions	Legumes	Reduction of anti-nutrients	Increment of protein content	References
Dehulling	Mechanically	Green gram	52.5 % phytic acid 45.6 % tannin	7.3 %	Akinjayeju and Ajayi, 2011; Ghavidel and Prakash, 2007; Egounlety and Aworh, 2003;
		Cowpea	51.7 % phytic acid 46.8 % tannin	9.5 %	
		Black bean	49.4 % phytic acid 19.9 % tannin 28.2 % trypsin inhibitor activity	15.2 %	
		Chickpea	47.4 % phytic acid 43.4 % tannin	18.8 %	
Soaking	Water 12 h at room temperature	Green gram	19 % phytic acid 7 % trypsin inhibitor activity 23 % tannin	na	Grewal and Jood, 2006; Egounlety and Aworh, 2003; Ibrahim *et al.*, 2002; Alonso *et al.*, 2000
	0.03 % $NaHCO_3$ in water for 16 h at room temperature	Cowpea	10.1 % phytic acid 0.08 % tannin 24.2 % trypsin inhibitor activity	na	
	Water 12 h at 30°C	Kidney bean	5.66 % phytic acid 24.2 % tannin 5.48 % trypsin inhibitor activity 15.1 % chymotrypsin inhibitor activity 11.2 % α-amylase inhibitor activity	0.63 %	
	Water for 12 – 14 h at room temperature	Soybean	54.6 % tannin 2.4 % trypsin inhibitor activity	na	

Heat treatment	Cooking at 100°C for 35 min	Green gram	28 % phytic acid 67 % trypsin inhibitor activity 32 % polyphenol	na	Olanipekun *et al.*, 2015; Grewal and Jood, 2006; Agbede and Aletor, 2005; Alonso *et al.*, 2000
	Roasted in frying pan at 120°C until turn brown	Kidney bean	52.3 % tannin 40 % saponin 44.7 % phytohemagglutinin	17.1 %	
	Autoclaved at 105°C at 1.2 kg/cm^3 pressure for 30 min	Jack bean	37.8 % phytic acid 22.2 % tannin 47.6 % trypsin inhibitor activity 100 % lectin	3.4 %	
	Extrusion temperature (152 – 156°C), 25 % moisture 100 rpm	Faba bean	26.7 % phytic acid 54.4 % tannin 28.6 % polyphenol 98.9 % trypsin inhibitor activity 52.8 % chymotrypsin inhibitor activity 100 % α-amylase inhibitor activity 99.6 % hemagglutinating activity 19.9 % hydrocyanic acid	0.22 %	
Germination	72 h	Bambara groundnut	20.5 % tannin 7.3 % oxalate 16.4 % trypsin inhibitor activity	1.2 %	Joshi and Varma, 2016; Devi *et al.*, 2015; Mugendi *et al.*, 2010; Agbede and Aletor, 2005
	24 h	Cowpea	16.2 % phytic acid 28.5 % trypsin inhibitor activity	9.5 %	
	72 h	Mucuna bean	6.77 % phytic acid 43.78 % trypsin inhibitor activity	3.13 %	
	48 h	Soybean	36.2 % trypsin inhibitor activity 44.1 % tannin	13.2 %	

Fermentation	72 h at 37°C	Locust bean	59.8 % tannin 62.5 % phytic acid 89 % trypsin inhibitor activity	17.7 %	Chikwendu *et al.*, 2014; Mugendi *et al.*, 2010; Steve Ijarotimi and Ruth Esho, 2009; Esenwah and Ikenenbomeh, 2008
	96 h at room temperature	Bambara groundnut	25.6 % tannin 15.8 % oxalate 37.3 trypsin inhibitor activity	2.4 %	
	72 h at 32°C	Mucuna bean	45.3 % tannin 100 % trypsin inhibitor activity	17.8 %	
	48 h at 28°C	Yam bean seed	21.6 % tannin 49 % phytic acid	13.9 %	

Note:
na represent not applicable (not reported in published journal)

1.7 Plant-based aquafeeds

Over the last few decades, researchers had made many efforts in replacing portions of fish meal in aquafeeds with alternative plant protein source. Plant protein sources are attractive potential feed ingredient sources as they are more likely to be sustainable, less costly and abundant than other ingredients (Liu *et al.*, 2015). Naylor *et al.* (2000) stated that herbivorous and omnivorous freshwater fish utilise plant-based proteins and oils better than carnivorous marine fish as they require minimal quantities of fish meal to supply essential amino acids. Still, it is possible to replace the fish meal to plant-based fish feed for carnivorous fish. The replacement of fish meal in all carnivores' species including salmon, trout, seabream and seabass has increased from 25 % to 90 % depending on species (Glencross *et al.*, 2016; Hardy, 2010).

A wide variety of legumes including soybean, canola seed, rape seed, pea, mung bean, lupin, and broad bean have been investigated as alternative plant protein sources in aquafeed (Hernández and Roman, 2016; Jane *et al.*, 2015; Collins *et al.*, 2012; Gaber, 2006; Tibaldi *et al.*, 2006; Kaushik *et al.*, 2004; Boonyaratpalin *et al.*, 1998). The growth performance of these carnivorous fish is slightly low due to poor plant-based feed intake (Panserat *et al.*, 2009; Sitjà-Bobadillaa *et al.*, 2005; Kaushik *et al.*, 2004; Raso and Anderson, 2003).

1.8 Animal models

In the current study, zebrafish (*Danio rerio*) was first selected as a fish model to investigate on the effect of processed plant-based aquafeed before feed was investigated in commercially cultured fish – Asian seabass (*Lates calcarifer*). Zebrafish and Asian seabass are two different types of fish which are omnivore and carnivore respectively according to their natural feeding habits. Undeniable zebrafish is not a perfect fish model fish model due to the structural difference in digestive system. Zebrafish has long intestine without true stomach whilst Asian seabass has a true stomach with short intestine.

Even so, zebrafish is an established fish model which offers several benefits including ease in handling for breeding and experimentation due to its small size and short generation time, substantial genomic resources, as well as the ability to consume of a wide variety of food (Ribas and Piferrer, 2014; Ulloa *et al.*, 2014). Despite the fact of structural difference in digestive system, zebrafish still serve as a potential and reliable model organism in finfish aquaculture research to study the nutritional impact of alternative protein source. Zebrafish offers an opportunity to conduct the nutritional research at reduced cost, time, and space needed in research facilities (Rurangwa *et al.*, 2015). Besides, to fully understand the repercussion of new diets on fish physiology, the determination of molecular mechanisms fish in responses to different diets also provide some insights to solve the existing problems cause by the nutrition interventions in aquaculture industry; but does not replace the commercial species of interest that has its own gastrointestinal tract characteristic (Rurangwa *et al.*, 2015; Ulloa *et al.*, 2014).

1.8.1 Zebrafish

Compare to other fish species, zebrafish (*Danio rerio*) to larger extend fulfils the conditions listed below as a good fish model for research usage (Ribas and Piferrer, 2014; Ulloa *et al.*, 2013; Ulloa *et al.*, 2011):

a) Possessed the basic biological features and exhibits comparable physiological responses to most of the important cultured species
b) Short life cycle, easy and inexpensive to breed
c) Small and stable genome so little genetic variation between individuals
d) Numerous resources including genomic information and transgenic that facilitate research in most areas

Zebrafish is a freshwater teleost omnivores fish belongs to the family of *Cyprinidae* (Dahm and Geisler, 2006). They consumed a great variety of foods including fish meal and plant based diets (Ribas and Piferrer, 2014). They also have a large number of offspring, a single female can lay up to 200 eggs per week and the continuous eggs production distinguishes them from most of the cultured fish (Yoon *et al.*, 2013; Dahm and Geisler, 2006). Zebrafish has short generation interval allowing for performance of growth studies in a shorter time thereby giving savings on maintenance costs and space requirements.

Undoubtedly, zebrafish is a famous well-established model organism particularly for biologists to study the developmental, molecular, toxicological studies and immunological studies (Dahm and Geisler, 2006). Recent year, it has emerged as a potential model organism in aquaculture research which involved in different aspects including reproduction, stress, pathology, toxicology nutrition for inflammatory disorders of the digestive tract and growth to examine the

experimental diets (Fuentes-Appelgren *et al.*, 2014; Ribas and Piferrer, 2014; Ulloa *et al.*, 2014; Ulloa *et al.*, 2011).

There are very limited studies had been done on zebrafish that related to ANF. Out of so many different types of ANF, most the studies were only considering the effect of soybean saponin on the growth and innate immune system in zebrafish. Fuentes-Appelgren *et al.* (2014) and Hedrera *et al.* (2013) stated the presence of soybean saponin in the diet could triggered the immune response and lead to intestinal inflammation. Studied done by Liu *et al.* (2013) and Ulloa *et al.* (2013) reported that the substitution of fish meal with soybean meal had adverse effect on growth performance of zebrafish but with the supplement of phytase growth performance was improved. This could be atributed to the increased mineral bioavailability in the phytase treated diet. Besides, the presence of phytase will dephosphorylate phytic acid that are able to form complexes with proteins that alter the protein solubility, enzymatic activity and proteolysis in mono-gastric animals (Krogdahl *et al.*, 2010; Kumar *et al.*, 2010; Urbano *et al.*, 2000; Reddy and Pierson, 1994).

1.8.2 Asian seabass

Asian seabass (*Lates calcarifer*) also known as “Siakap” in Malaysia and barramundi in Australia is one of the important aquaculture species in Southeast Asia due to its high growth rate and consumer demand (Eusebio and Coloso, 2002). It is broadly distributed in the Indo-West Pacific region from the Arabian Gulf to China, Taiwan Province of China, Papua New Guinea and northern Australia (FIGIS, 2013).

Barramundi derived from the aboriginal word “barramundi” meaning large scales is the common name in Australia however it also known as giant perch and cock up. Other variations in nomenclature includes *anama* in Papua New Guinea, *kakap* in Indonesia, *bulgan* in Philippines, bhakti in India (Schipp *et al.*, 2007). It is the most commonly eaten and popular fish therefore among the most commercially valuable. Barramundi is carnivorous fish that farmed commercially in ponds, cages and recirculating tanks in Southeast Asia and Australia. They are fed on trash fish in Asia and pellets in Australia (Tian and Qin, 2003).

According to Department of Fisheries Western Australia (2013), barramundi live in both freshwater and saltwater and they eat almost anything including other barramundi and crustaceans. They can consume prey up to 60 % of their own length. Common size of barramundi is between 25 cm to 100 cm however they can grow up to 200 cm in length and 60 kg (FIGIS, 2013). Even though the species can grow up to 60 kg, the harvest size of barramundi is usually between 400 g to 4000 g depending on the market demand (Glencross, 2008). Barramundi can change sex from male to female during their lifecycle. By observing the body and fins colour, the freshwater and saltwater barramundi can be differentiated. Freshwater barramundi are greenish-blue on the upper part of the body and dark brown to black colour fins while saltwater barramundi have silvery body and yellow fins (FIGIS, 2013).

Barramundi is known to survive in water with a salinity over 50 ppt and at temperature 16°C to 35°C. Yet, juvenile barramundi tend to grow faster in lower salinities (Schipp *et al.*, 2007). They are widely known for their good taste and firm texture including tender, mild tasting as well as boneless fillets (Schipp *et al.*, 2007;

Peet, 2006). Barramundi are suited to aquaculture as they are hardy, fast-growing, feed well on pelleted diets and universally regarded as a fine table fish (FIGIS, 2013; Peet, 2006). They have the uncommon ability to synthesize long chain omega-3 fatty acids that contribute to human health (Peet, 2006).

According to FAO (2013), the major nutrients requirement for barramundi include protein, lipid and carbohydrate (Table 1.4). Diets for carnivorous species are mainly composed of protein, lipid and carbohydrate whilst diets for omnivorous species have less protein and more carbohydrate (Jobling, 2015).

Table 1.4 The typical diet composition for barramundi (Adapted from FAO, 2013)

Proximate composition (% Dry feed basis)	Life stages			
	Larvae (2 – 25 days)	Nursery (< 10 g)	Grower (10 – 200 g)	Grower (> 200 g)
Dry matter		70	70	
Crude protein, % min	50		45 – 50	40 – 45
Crude lipid, % min		15 – 18		19
Carbohydrate, % recommended			20	
Gross energy, min kJ/g	21			
Digestible energy, min kJ/g			15	17
Protein to energy ratio, mg/kJ			22.5 – 30.7	
Phosphorus, % min			0.5 – 1.0	

Over the past 20 years, feeds for barramundi have undergone considerable development from the use of baitfish or feed fish, to a simple pellet-pressed diet, to a modern, extruded, high-energy pellets (Glencross, 2006). There are concerns that the replacement of fish meal in aquaculture diets could adversely affect the marketability of the fish because of consumer perception of altered taste or reduced health benefit due to lowered omega-3 fatty acid content (Williams *et al.*, 2003). Therefore, there is growing interest in the specific effects of plant proteins on lipid synthesis and metabolism and their effects on flesh quality.

1.9 Aims of the project

Currently, farming of high value carnivorous fish is one of the most harmful aquaculture production systems. To overcome the negative impacts of farming carnivorous fish, the incorporation of underutilised legume protein as an alternative protein source in aquafeed is needed for more sustainable aquaculture production. The presence of ANFs particularly enzyme inhibitors can adversely affect nutrient absorption in the fish digestive system. It is therefore predicted that feeding carnivorous fish with untreated legume protein will result in lower growth performance when compared to treated legume protein feed. Little work has been done to develop a processing strategy to remove ANFs present in underutilised legumes and investigate on the effect of these processed legume-based feeds on the growth of zebrafish and Asian seabass ("*Siakap*").

The specific aims of this study were:

1. To determine the targeted ANFs present in underutilised legumes
2. To develop processing techniques to reduce ANFs present in underutilised legumes
3. To develop the formulation for the legume-based aquafeed
4. To investigate the effect of the ANF-reduced legume-based aquafeed on the growth performance of zebrafish and Asian seabass

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and reagents

All the chemicals and reagents used for the study were tabulated in Table 2.1. All reagents were of analytical grade. Water used throughout the analyses was purified by Milipore water purification system (Milipore Corporation, USA).

Table 2.1 List of chemicals and reagents

Chemicals/ Reagents	Molecular Weight (g/mol)	Supplier, Country
Sodium hydroxide	40.00	Merck, Germany
Quick Start™ Bradford Dye Reagent, 1x	-	Bio-Rad, USA
Bovine serum albumin	-	Sigma-Aldrich, USA
Sodium phosphate	380.12	Sigma-Aldrich, USA
α-N-benzoyl-dl-arginine-p-nitroanilidehydrochloride	434.88	Sigma-Aldrich, USA
Acetic acid glacial	-	Merck, Germany
Trypsin inhibitor	-	Amresco, USA
Tris HCl	157.60	Sigma-Aldrich, USA
Trypsin	-	Amresco, USA
Glycine HCl buffer	111.53	Sigma-Aldrich, USA
Chymotrypsin	-	Sigma-Aldrich, USA
Benzoyl-l-tyrosine ethyl ester	313.35	Sigma-Aldrich, USA
Methanol	32.40	Merck, Germany
Hydrochloric acid fuming 37 %	-	Merck, Germany
Petroleum ether 40 – 60°C	-	Sigma-Aldrich, USA
Boric acid	61.83	Sigma-Aldrich, USA
Titanium tablet	-	Buchi, Switzerland
β-mercaptoethanol	78.13	Sigma-Aldrich, USA
Formic acid 98-100%	46.03	Fisher Scientific, USA
Phenol	94.11	Fisher Scientific, USA
Sodium metabisulpite	190.11	Fisher Scientific, USA
Tri-sodium citrate, dihydrate	294.10	Fisher Scientific, USA
2,2'-Thiodiethanol	122.19	Fisher Scientific, USA
Physiological fluid chemical kit	-	Biochrom Ltd, UK
Hydrogen peroxide 30 %	34.01	Fisher Scientific, USA
Amino acid standard, 2.5 µmol/mL	-	Sigma-Aldrich, USA

Cysteic acid	187.17	Sigma-Aldrich, USA
Methionine sulfone	181.21	Sigma-Aldrich, USA
Norleucine	131.17	Sigma-Aldrich, USA
Benzocaine	165.19	Sigma-Aldrich, USA
Ethanol	46.07	Merck, Germany
Sulphuric acid 95-97%	98.08	Merck, Germany
RNeasy® Fibrous Tissue Mini kit	-	Qiagen, Netherlands
RevertAid RT kit	-	Thermo Scientific, USA
100 bp DNA ladder (500 µg/mL)	-	New England BioLabs, UK
6x loading dye	-	New England BioLabs, UK
50x TAE buffer	-	Thermo Scientific, USA
RNA*later*®	-	Ambion, USA
UltraPure DNase/RNase-Free water	-	Thermo Scientific, USA
LightCycler® 480 Sybr Green I Master	-	Roche, Switzerland
QuantiNova™ Reverse Transcription Kit	-	Qiagen, Netherlands
QuantiFast® Sybr® Green PCR kit	-	Qiagen, Netherlands

2.2 Instruments

All the instruments and apparatus were kept in proper condition before and after used to ensure quality work safety. Table 2.2 stated the instruments and apparatus used throughout the study.

Table 2.2 List of instruments and apparatus

Instruments/Apparatus	Model	Manufacturer/Supplier, Country
Centrifuge	5810 R	Eppendorf, Germany
Vortex mixer	SA8	Stuart, UK
Analytical balance	MS-204	Mettler Toledo, Switzerland
Hot plate stirrer	Labtech	Daihan Labtech Co Ltd, Korea
pH meter	pH 510	Sartorius, Germany
Micropipette (100 – 1000 µl)	Research plus	Eppendorf, Germany
Micropipette (10 – 100 µl)	Research plus	Eppendorf, Germany
Micropipette (0.5 – 10 µl)	Research plus	Eppendorf, Germany
Orbital shaker	Certomat IS	Sartorius, Germany
Microplate reader	Epoch	BioTek, USA
Freeze dryer	Alpha 1-4 LD plus	Christ, Germany
Soxhlet	EV6 AII/16	Gerhardt, Germany
Kjeldahl distillation unit	K-350	Buchi, Switzerland
Kjeldahl digester	K-446	Buchi, Switzerland

Scrubber	K-415	Buchi, Switzerland
Amino acid analyser	Biochrom 30+	Biochrom Ltd, UK
LightCycler® System	480	Roche, Switzerland
Mastercycler	Nexus gradient	Eppendorf, Germany
Real-Time PCR System	Eco	Illumina, USA
Crude Fibre digestion system	R16	Gerhardt, Germany
Oven	VO200cool	Memmert, Germany
Ashing furnace	AAF 12/18	Carbolite Gero Limited, UK
Fume hood	Hamilton Concept	Thermo Scientific, USA
Chiller	VC 100	ETS Bio freeze, Malaysia
Autoclave machine	HVE-50	Hirayama, Japan
-20°C freezer	E388L	Fisher & Payker, Australia
-80°C freezer	Platinum 340V	Angelantoni Lifescience, Italy
Flash N/Protein analyser	EA1112	Thermo Scientific, USA
Bomb calorimeter	6300	Parr, USA
Ice maker machine	ZBS50	Nuove Tecnologie del freddo, Italy
Gel doc	Gel Doc XR+	Bio-Rad, USA
Horizontal Electrophoresis system	Mini-Sub Cell GT system	Bio-Rad, USA
Dissecting Microscope	EZ4	Leica microsystems, Germany
NanoDrop	ND1000	Thermo Scientific, USA

2.3 Anti-nutritional factors analysis

2.3.1 Trypsin inhibitors assay

Trypsin inhibitor was determined using α-N-benzoyl-dl-arginine-p-nitroanilidehydrochloride (BAPNA) as the substrate for trypsin. A 1 g of ground freeze-dried legumes was extracted by soaking with 10 mL of 0.15 M sodium phosphate buffer pH 8.1 at 4°C for 12 h. The extracts were then centrifuge at 3000 *g* for 10 min at 4°C. Extracts (20 µL) was incubated with 40 µL of trypsin solution (0.004 % (w/v) trypsin in 0.025 M glycine HCl buffer) and diluted to 80 µL with pH 8.1 buffer phosphate were then incubated for 10 min at 37°C. A 100 µL of 0.001 M BAPNA solution in pH 8.1 sodium phosphate buffer, previously warmed to 37°C, will be added. After 10 min incubation at 37°C, 20 µL of 30 % (v/v) acetic acid was added to stop the reaction. Trypsin inhibitor activity (TIA), was expressed as trypsin inhibitor unit/mg sample, and calculated from the absorbance read at 410 nm against a reagent blank. One trypsin unit was defined as the increase by 0.01 absorbance unit at 410 nm of the reaction mixture (Marzo *et al.*, 1998).

There were a wide range of expression units had been used to measure the trypsin inhibitor activity that included trypsin inhibitor units per milligram sample, milligram trypsin inhibitor per gram of sample, trypsin units per gram protein, trypsin inhibited units per milligram protein, parts per million of Kunitz units, that could lead to confusion. The unit that being used in this study (TIA unit/mg) was the most commonly reported by researchers.

2.3.2 Chymotrypsin inhibitors assay

The enzyme inhibitory activity was determined in extracts as described by Marzo *et al.* (1998). A 1 g of ground freeze-dried legumes was extracted by soaking in 10 mL of 0.08 M Tris HCl buffer (pH 7.6) for 12 h at 4°C. The extracts were then centrifuge at 3000 *g* for 10 min at 4°C. Sample extracts (20 µL) was incubated with 40 µL of chymotrypsin solution (0.005 % (w/v) chymotrypsin in Tris HCl buffer pH 7.6) and diluted to 80 µL with pH 7.6 Tris HCl buffer and incubated for 10 min at 30°C. A 100 µL of 0.001 M benzoyl-l-tyrosine ethyl ester (BTEE), previously warmed to 30°C was added and mixed. After 10 min incubation at 30°C, 20 µL of 30 % (v/v) acetic acid was added to stop the reaction. The absorbance was read at 256 nm against the blank. Chymotrypsin inhibitor activity (CIA), was expressed as chymotrypsin inhibitor unit/mg sample, and calculated from the absorbance read at 256 nm against a reagent blank. One chymotrypsin unit was defined as the increase by 0.01 absorbance unit at 410 nm of the reaction mixture.

2.3.3 α-Amylase inhibitors assay

α-Amylase inhibitor activity (AIA) was evaluated according to the modified method of Deshpande *et al.* (1982). A 1 g of ground freeze-dried legumes was extracted by soaking with 10 mL of deionized water for 12 h at 4°C and the supernatants were tested for α-amylase inhibitory activity. A 25 µL sample solution containing the inhibitor was incubated with 25 µL of α-amylase enzyme solution (0.003 % (w/v) in 0.2 M sodium phosphate buffer, pH 7.0, and containing 0.006 M NaCl) for 15 min at 37°C. To this mixture was added 50 µL of 1 % (w/v) starch solution (preincubated at 37°C). At the end of 3 min, the reaction was stopped by the addition of 200 µL of 1 % (w/v) dinitrosalicylic acid (DNS) reagent and heating in a boiling water bath for 10 min. The absorbance was recorded at 540 nm. The α-amylase inhibitor activity

(AIA) was expressed as α-amylase inhibitor unit/mg sample and calculated from the absorbance read at 540 nm against a reagent blank. One unit of α-amylase activity inhibited was defined as one α-amylase inhibitory unit.

2.4 Nutritional analysis

2.4.1 Protein analysis

a) Quantification of protein content using Kjeldahl method

The analysis was carried out according to AOAC, 1990. A 0.1 g of ground freeze-dried legumes were weight into a sample tubes and 2 titanium tablets (3.71 g per tablet which consists of 3.5 g of potassium sulphate (K_2SO_4) / 0.105 g of copper (II) sulphate pentahydrate ($CuSO_4 \cdot 5\ H_2O$) /0.105 g titanium dioxide (TiO_2)) were added followed by 15 mL of concentrated sulphuric acid (H_2SO_4). The samples were digested at 380°C for 1.5 h. The digested samples were then allowed to cool down to room temperature for 10 to 15 min before subjected to distillation process. The digested sample were distilled by using the Kjeldahl distillation unit. To the digested sample was added 63 mL of 32 % (w/v) sodium hydroxide (NaOH) and 60 mL 4 % (w/v) boric acid and 60 mL of distilled water and allowed it to distil for 4 min. Ammonium sulphate used as standard. The distilled sample was then being titrated using 0.1N of H_2SO_4 to pH 4.65. The protein content was calculated using the following formula:

$$\% \text{ Protein} = (((\text{sample} - \text{blank}) \times \text{normality} \times 1.4) / \text{sample weight}) \times 6.25$$

Notes:
Normality – normality of titrated H_2SO_4
Molecular mass of nitrogen – 1.4
Conversion factor – 6.25

b) Quantification of protein content using Bradford method

The protein of legumes was carried out according to a modified method of Arifin *et al.* (2009) and Shen *et al.* (2008). The ground freeze-dried legumes were mixed with 0.03 M NaOH pH 12 solution (10:1 v/w) with continuous shaking at 150 rpm for 1 h at 25°C. The mixture was centrifuged at 6000 *g* for 10 min at room temperature. The supernatant was carefully collect for further filtration process. It was filtered through Whatman No. 1 filter paper to obtain a crude extract. The amount of protein was quantify using the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as the standard. A 5 µL of sample was added with 250 µL of Bradford reagent and allowed it to react for 5 min at room temperature. The solution was measured against the blank (Bradford reagent) at 595 nm.

2.4.2 Lipid analysis

Lipid content was extracted by using Soxhlet method (AOAC, 1990). A 5 g of ground freeze-dried legumes were weighed in a thimble and subjected to hot extraction with 300 mL of petroleum ether for 6 h in the Soxhlet apparatus. After extraction, the petroleum ether was rotary evaporated. Lard was used as standard. The lipid content was calculated using the following formula:

$$\% \text{ Lipid content} = [(M2 - M1) / \text{sample size}] \times 100$$

Notes:
M1 – initial weight of flat bottom flask
M2 – final weight of flat bottom flask

2.4.3 Crude fibre analysis

A 1 g of defatted freeze-dried sample was weighed into the fibrebag. The fibrebag was dipped into the extraction beaker which contained of 360 mL of 0.128 M sulphuric acid. The extraction beaker was boiled on the hot plate for 5 min then simmered for another 25 min. The acid was drained from the fibrebag and washed with 300 mL of hot distilled water. The fibrebag was washed until a neutral pH was obtained. Then, added 360 mL of hot 0.313 M sodium hydroxide (NaOH) into the extraction beaker and bring to boil. The solution was allowed to simmer for 25 min. The fibrebag was then washed with 300 mL of hot distilled water, 300 mL of 0.1 M hydrochloric acid (HCl) and 600 mL of hot distilled water, in succession. This wasfoloowed by the fibrebag being washed in diethyl ether. The residues in fibrebag were oven dried for 4 h at 105°C. The fibrebag was then cooled in the desiccator for 15 min and the weight of the fibrebag with residue was noted. The cooled fibrebag was placed in an ashing furnace and ashed for 4 h at 550°C. The fibrebag was then cooled in desiccator for 15 min and re-weighed. The ash was brushed out and the empty crucible re-weighed. The crude fibre was calculated as below:

$$\% \text{ Crude fibre} = ((\varepsilon - \alpha) - (\delta - \gamma)) / \beta \times 100$$

Notes:
ε – dry fibrebag weight (g)
α – empty dried fibrebag (g)
δ – crucible with ash weight (g)
γ – empty crucible weight (g)
β – sample size (g)

2.4.4 Ash analysis

A 5 g of freeze-dried sample was weighed in a crucible and placed the crucible in a ashing furnace. The sample was heated at 550°C for 12 h. Weighed the crucible again with the ash. The ash was calculated as below:

$$\% \text{ Ash} = (\beta - \alpha) / \delta \times 100$$

Notes:
β – weight of crucible with sample (g)
α – weight of crucible with ash (g)
δ – weight of sample (g)

2.4.5 Moisture analysis

The mositure content of the feed was determined by the weight difference between dry and wet material. A 2 g of ground diet was weight in a crucible and placed it in dyring oven at 105°C for 12 h. The sample was cool in the dessicator to room temperature (25°C) before weighing it.

$$\% \text{ Moisture} = (\beta - \alpha) / \delta \times 100$$

Notes:
β – total weight of sample (g)
α – dry weight of sample (g)
δ – total weight of sample (g)

2.4.6 Nitrogen free extract

NFE mainly composed of digestible carbohydrate, vitamins, and other non-nitrogen soluble organic compounds. The calculation was done by using the below formula:

% NFE = 100 - (% crude protein + % crude lipid + % crude fibre + % ash + % moisture)

2.4.7 Energy

The energy content of sample was determined using bomb calorimeter where gross energy is measured in terms of heat produced when a sample is completely combusted into carbon dioxide and water leaving remaining ash. A 1 g of freeze-dried sample was weighed into the crucible and was compressed with the compressing tool. The crucible was placed into the holder and attached with an ignition thread. The bomb head was inserted into the calorimeter and allowed a 20 s combustion in the chamber. Benzoic acid was used as a standard that consisted of 26.454 MJ/kg. The energy measurement provided by the bomb calorimeter was expressed as MJ/kg.

2.4.8 Amino acid quantification

A 10 mg nitrogen of sample was weight into a 100 mL bottle and placed the bottle in chiller for 2 h to cool. The oxidation solution was prepared by using 10 mL of 30 % (v/v) hydrogen peroxide with 90 mL of formic acid/phenol solution. The formic acid/phenol solution was prepared by taking 735 mL formic acid with 111 mL of distilled water and added with 4.73 g of phenol. The oxidation solution was incubated at 30°C for 1 h and chilled it for 2 h. A 5 mL chilled oxidation solution was added into the cold sample bottle. The sample bottle was returned to the chiller and allowed it to be oxidised for 18 h. The hydrolysis solution was prepared by taking 492 mL concentrated hydrochloride acid with 1 g of phenol making the volume to 1 L with distilled water. After oxidation, 0.84 g of sodium metabisulphite and 50 mL of hydrolysis reagent were added into the bottle. The sample bottle was placed in a 110°C oven and the lid was loosened. The lid was tighten after 1 h and left in the oven for a further 23 h. After hydrolysis, the sample bottle was placed in freezer for 45 min. The sample was removed from freezer and partly neutralised by adding 35 mL of 7.5 N sodium hydroxide into the sample and the samples were left in fume hood for 30 min to reach room temperature. The sample was then adjusted the pH to 2.2 using 7.5 N sodium hydroxide, 1 N sodium hydroxide and hydrolysis reagent.

A 4 mL of concentrated solution of the internal standard norleucine (10 µmol/mL) was added into the 200 mL volumetric flask. The hydrolysate was transferred to the volumetric flask and topped up to 200 mL using pH 2.2 tri-sodium citrate buffer. A 20 mL of hydrolysate was transferred into centrifuged tube and centrifuged at 3000 *x* g for 2 min. Collected the supernatant and filtered them through a 0.22 µm filter into a sterile sample vial. The sample was injected into the amino acid analyser. The

concentration of amino acid was expressed as gram per kilogram sample (g/kg). It is calculated as following:

$$\text{Amino acid (g/kg)} = (A \times MW \times F) / (W \times 50000)$$

Notes:
A – concentration of hydrolysate obtained by the instrument (ISTD-nmol / 50 µl)
MW – molecular weight
E – concentration of standard in mol/mL
W **–** sample weight (g)
F – total hydrolysate (mL)

2.5 Molecular techniques and methods

2.5.1 Extraction of RNA from fish tissue

The RNA was extracted using RNeasy® Fibrous Tissue Mini kit followed the instruction of the manufacturer. Less than 30 mg of sample was added with 300 µL of Buffer RLT and disrupted and homogenised using pellet pestle. The homogenised solution was added with 590 µL RNase-free water followed by 10 µL proteinase K (> 600 mAU/mL) and mix gently. The mixture was incubated at 55°C for 10 min. The mixture was the centrifuged at 10,000 *x* g for 3 min at room temperature. After centrifugation, the supernatant was collected and added 450 µL of 96 – 100 % (v/v) ethanol. The sample was transferred to RNeasy Mini column and centrifuged for 15 s at 8000 *x* g at room temperature. A 350 µL of Buffer RW 1 was added to RNeasy Mini column and centrifuged for 15 s at 8000 *x* g at room temperature, and the flow-through was discarded. An 80 µL of DNase I (1500 units) was added to RNeasy Mini column and allowed it to stand on benchtop for 15 min. After 15 min, another 350 µL of Buffer RW 1 was added to RNeasy Mini column and centrifuged for 15 s at 8000 *x* g at room temperature, and the flow-through was discarded. A 500 µL of Buffer RPE was added to RNeasy Mini column twice and centrifuged for 15 s at 8000 *x* g at room temperature, and the flow-through was discarded. The RNeasy Mini column was centrifuged at full speed (21,130 *x* g) for 1 min at room temperature. The RNeasy Mini column was placed into a new 1.5 mL tube and 30 µL of RNase-free water was added and centrifuged for 1 min at 8000 *x* g at room temperature to elute the RNA. The purified RNA was read on NanoDrop to obtain the concentration. The purified RNA was then stored at -80°C in RNase-free water.

2.5.2 RNA integrity for zebrafish

Purified RNA was subjected to gel electrophoresis to determine the integrity and size distribution of purified total RNA. The 28S and 18S ribosomal RNAs appeared as sharp bands. The expected apparent ratio of 28S to 18S rRNA was approximately 2:1.

2.5.3 RNA integrity for Asian seabass

Purified RNA was quantified using an Agilent® 2100 bioanalyzer to determine the integrity and size distribution of total purified RNA. The 28S and 18S ribosomal RNAs were expected to appear as sharp peaks and then used to give a RNA integrity number (RIN). A RIN value of 10 indicates no degradation has occurred whilst a value of 0 indicates a complete degrade in sample. A RIN value higher than 5 is recommended for RT-PCR.

2.5.4 Reverse transcription polymerase chain reaction (RT-PCR) for zebrafish

The RNA was reverse transcript using RevertAid RT kit followed the instruction of the manufacturer. A 5 µL of 100 ng/µL purified RNA was added with 1 µL Random Hexamer primer and 6 µL of RNase-free water. The mixture was incubated at 65°C for 5 min. The mixture was immediately chilled on ice. To the chilled mixture was added 5 µL of 5x Reaction buffer, 1 µL of RiboLock RNase Inhibitor (20 U/µL), 2 µL of 10 mM dNTP mix and 1 µL of RevertAid RT (200 U/µL) which was then gently mix and centrifuge briefly to collect the mixture. After centrifugation, the mixture was incubated at 42°C for 60 min and the reaction terminated by heating at 70°C for 5 min. The reverse transcription product (cDNA) was stored at -20°C until further use.

2.5.5 Reverse transcription polymerase chain reaction (RT-PCR) for Asian seabass

The RNA was reverse transcript using QuantiNova™ Reverse Transcription kit followed the instruction of the manufacturer. A 5 µL of 100 ng/µL purified RNA was added with 2 µL of gDNA Removal mix, 8 µL of RNase-free water. The mixture was incubated at 45°C for 2 min. The mixture was immediately chilled on ice. To the chilled mixture was added with 1 µL of Reverse Transcription Enzyme and 4 µL of Reverse Transcription Mix which was then gently mix and centrifuge briefly to collect the mixture. After centrifugation, the mixture was incubated at 25°C for 3 min and 45°C for 10 min. It was then heated at 85°C for 5 min to inactivate the Reverse Transcriptase Enzyme. The reverse transcription product (cDNA) was stored at -20°C until further use.

2.5.6 Real-time polymerase chain reaction (qPCR) for zebrafish

Forward and reverse primers (Table 2.3) were designed using Primer Express 3.0 to complement to the targeted DNA sequence at 5' and 3' end to allow annealing to take place during the reaction. The products size of each pair of the primers were less than 150 bp. The primers were designed for 2 gene transcripts of interest (interleukin 1 beta (il-1β) and interleukin 8 (il-8)) and 2 housekeeping genes (beta-actin (β-actin) and elongation factor-1α (EFLα)).

Table 2.3 Sequence of qPCR primers that used in this study

Primers	F/R	Sequence (5'-3')	GC content (%)	Tm (°C)	Accession number
il-1β	F	GGAATCTCCAAAAGTAACCTGTACCT	42.3	64.3	NM_212844.2
	R	GACCCGCTGATCTCCTTGAG	60	66.2	
il-8	F	CGCATTGGAAAACACATAAAGAGT	37.5	64.8	XM_001342570.5
	R	TGTCATCAAGGTGGCAATGATC	45.4	67	
β-actin	F	CACCCTGTCGTGCTCACTGA	60	67.9	NM_131031.1
	R	GTCTCGAACATGATCTGTGTCATCT	40	65.4	
EFLα	F	AATTCGAGACCAGCAAATACTACGT	40	64.7	NM_131263.1
	R	GTCAGCCTGAGAAGTACCAGTGATC	52	66.2	

Note:
F/R – Forward/Reverse primer
Tm – Melting temperature

The qPCR master mix was prepared according to Table 2.4. To 5 µL of cDNA template (section 2.5.4) was added 10 µL of master mix, and 5 µL of RNase-free water to each of the well in a multi-well plate. The plate was sealed and centrifuged at 250 *x* g for 1 min at room temperature. The plate was then placed in the LightCycler® System for qPCR reactions with the cycling program as shown in Table 2.5. The data was acquired at the 72°C step.

Table 2.4 Amount of each component needed per PCR reaction (master mix)

Components	Volume (µL)
LightCycler® 480 Sybr Green I Master	7.5
Forward primer 10 µM	0.45
Reverse primer 10 µM	0.45
RNase-free water	1.6

Table 2.5 qPCR program for LightCycler® System

Process	Temperature (°C)	Durations (s)	Number of cycles
Pre-denaturation	95	300	1
Denaturation	95	10	45
Annealing	60	15	
Extension	72	15	

2.5.7 Real-time polymerase chain reaction (qPCR) for Asian seabass

Forward and reverse primers (Table 2.6) were designed using NCBI Primer-BLAST to complement to the targeted DNA sequence at 5' and 3' end to allow annealing to take place during the reaction. The products size of each pair of the primers were less than 150 bp. The primers were designed for 3 gene transcripts of interest (heat shock protein 70 (HSP 70), C-reactive protein (CRP) and alanine aminotransferase (ALAT)) and 2 housekeeping genes (beta-actin (β-actin) and elongation factor-1α (EFLα)).

Table 2.6 Sequence of qPCR primers that used in this study

Primers	F/R	Sequence (5'-3')	GC content (%)	Tm (°C)	Accession number
HSP 70	F	TACCTCGGCCAAAAGGTGTC	55	57.3	HQ646109.1
	R	GTCTTTAGTCGCCTGACGCT	55	57.1	
CRP	F	CTGAGGGCCTTCACTCTGTG	60	57.6	HQ652974.1
	R	TGGTCTTCAGTTCGGTACGC	55	57.1	
ALAT	F	AACCAACTCGTTCAGTGCCA	50	57.1	XM_018693109.1
	R	AGGTGAGACGTGGGTATTGC	55	57.1	
β-actin	F	CAGCATCATGAAGTGCGACG	55	56.7	GU188683.1
	R	TGCCGGGGTACATAGTGGTA	55	57.7	
EFLα	F	GGTATTGGAACTGTCCCCGT	55	56.9	GU188685.1
	R	CAAAGGTGACGACCATGCCA	55	58.1	

Note:
F/R – Forward/Reverse primer
Tm – Melting temperature

The qPCR master mix was prepared according to Table 2.7. To 5 µL of cDNA template section (2.5.5) was added of 10 µL of master mix, and 5 µL of RNase-free water to each of the well in a multi-well plate. The sealed plate was centrifuged at 250 *x* g for 1 min at room temperature. The plate was then placed in the Illumina's Eco Real-Time PCR System for qPCR reactions with the cycling program as shown in Table 2.8. The data was acquired at the 72°C step.

Table 2.7 Amount of each component needed per PCR reaction (master mix)

Components	Volume (µL)
2x QuantiFast® Sybr® Green PCR Master Mix	7.5
Forward primer 10 µM	0.45
Reverse primer 10 µM	0.45
RNase-free water	1.6

Table 2.8 qPCR program for Illumina's Eco Real-Time PCR System

Process	Temperature (°C)	Durations (s)	Number of cycles
Pre-denaturation	95	120	1
Denaturation	95	5	40
Annealing & Extension	60	15	

2.6 Fish trials

2.6.1 Zebrafish

The juvenile zebrafish (AB wild type strain) were bred in house at The Institute of Integrative Biology aquarium facility, the University of Liverpool. The mixed sex of fish were approximately 2 months old at trial start. Throughout the study fish were housed in groups of 10 individuals in 25 identical 3 L zebrafish tanks, made by Aquatic Habitats, each 25 cm x 10 cm x 15 cm (L x W x H). The tanks were placed on the rank and connected to a centralised recirculating aquaculture system maintained by a sump filtration system and 50 % weekly water changes. Due to the small size of the fish, tanks were fitted with a 400 µm fry mesh baffle; cleaning was conducted weekly during the weighing of the fish to prevent further disturbances. Water quality was subsequently kept stable with the following parameters, ammonia (NH_4) at 0 mg/L, nitrite (NO_2) at 0 mg/L, nitrate (NO_3) at < 50 mg/L and pH 7.0. Fish were maintained at 28 ± 1°C and exposed to a 12 light : 12 dark h light cycle.

The study was conducted in a randomised design with 5 treatments and 5 replicates per treatment, 50 fish in total per diet. A palatability test had been carried out before the feeding trial to assure that the fish readily accepted the formulated diets. After each tank of fish had been weighed, one week's worth of daily feeds was pre-measured at 4 % body weight per day. This was repeated weekly to maintain a 4 % of body weight feed regime throughout 6 weeks trial, this encouraged maximum growth and health. On the days when fish were weighed, feed was given afterwards in order to gain accurate fish weights, on all other days feed was given in the morning.

a) Weighing fish and data collection

The body weight was taking weekly to record growth throughout and the length was only measured at the end of the study. Fish were weighed by tank; 10 fish as a whole due to time constraints. A separate 1.5 L tank was used, filled with 1 cm of system water, placed on a balance and tared. All 10 fish were caught in a small net, lifted from the housing tank, excess water was removed gently by dabbing the net on blue roll paper towel, the net preventing any physical harm to the fish, and then the fish were placed in the tank on the balance. The weight was recorded for each tank. While the fish were situated in the weighing tank, the housing tank and mesh baffle was cleaned before returning the fish.

b) Samples taken at termination

At the end of the study (6 weeks), the fish were humanely euthanized by over dosage of benzocaine, followed by pithing of the brain to confirm death. Once death, the intestines were removed and immediately immersed in RNA*later*®. The samples were initially stored at -80°C to avoid unwanted changes in the gene expression. The samples were then transported on dry ice to the University of Nottingham for further analyses.

2.6.2 Asian seabass

The juvenile Asian seabass (approximately 5 to 6 cm) were purchased from BD Aquaculture Sdn. Bhd., Johor and maintained in the Crop for Future Research Centre (CFF) aquarium according to standard protocols. Fish were housed in groups of 4 individuals in 9 identical 120 L aquaria throughout the study. The aquaria were placed on the rank, centralised recirculating aquaculture system equipped with a biological filter. At least 50 % of the water from the system was replaced weekly. Tanks cleaning were carried out 1 h before and after every feeding using siphon. Juvenile Asian seabass were housed in the aquarium maintained at 28°C ± 1°C on a 14 h light : 10 h dark photoperiod throughout the study. Water quality was subsequently kept stable with the following parameters, ammonia (NH_4) at 0 mg/L, nitrite (NO_2) at 0 mg/L, nitrate (NO_3) at < 50 mg/L and pH 7.0.

The study was conducted in a randomised design with 3 treatments and 3 replicates per treatment, 12 fish in total per diet. A palatability test had been carried out before the feeding trial to assure that the fish readily accepted the formulated diets. After each tank of fish had been weighed, 6 days' worth of daily feeds was pre-measured at 4 % body weight per day. This was repeated weekly to maintain a 4 % of body weight feed regime throughout 5 weeks trial, this encouraged maximum growth and health. On the days when fish were weighed, feed was given afterwards in order to gain accurate fish weights, on all other days feed was given in the morning.

a) Weighing fish and data collection

The body weight was taking weekly to record growth throughout and the length was only measured at the end of the study. Fish were weighed by tank; 4 fish as a

whole due to time constraints. A separate 3 L tank was used, filled with 10 cm of system water, placed on a balance and tared. All 4 fish were caught in a small net, lifted from the housing tank, excess water was removed gently by dabbing the net on paper towel, the net preventing any physical harm to the fish, and then the fish were placed in the tank on the balance. The weight was recorded for each tank. While the fish were situated in the weighing tank, the housing tank was cleaned before returning the fish.

b) Samples taken at termination

At the end of the study (5 weeks), the fish were humanely euthanized by over dosage of benzocaine, followed by pithing of the brain to confirm death. Once death, the liver was immediately immersed in RNA*later*®. The submerged liver samples were initially stored on ice then -80°C to avoid unwanted changes in the gene expression. The samples were then transported on dry ice to the University of Nottingham for further analyses. All the fish that had the viscera and kidney removed were towel dried to remove excess water and three fish from each tank were used for moisture determination (section 2.4.5). The remaining fish were frozen at -80°C then lyophilised for 24 h. The dried fish were then ground into powder for analytical analysis including crude protein and crude lipid (section 2.4.1 a and 2.4.2).

2.7 Statistical analysis

All data was analysed using IBM SPSS Statistics software (Version 22, IBM Corporation, USA) and graphs were constructed using GraphPad Prism (Version 6, GraphPad Software Inc, USA). The details of specific statistical tested used to check statistical significance are stated in each separate chapter.

CHAPTER 3

DETERMINATION OF THE ANTI-NUTRITIONAL FACTORS PRESENT IN UNDERUTILISED LEGUMES

3.1 Introduction

One of the major constraints for aquafeed production is the limited availability of feed ingredients to replace fish meal and soybean meal. Due to the booming prices of fish meal and soybean meal, it is crucial to look for alternative and more economical and nutritious legume sources that with comparable protein content as in fish meal and soybean. Therefore, current study was conducted to investigate the possibility of using underutilised legumes available in Malaysia and South-East Asia for this purpose, with an ultimate aim to promote the use of local resources for production of sustainable aquafeed. In this study, seven underutilised dried legumes, namely mung bean, adzuki bean, chickpea, hyacinth bean, black-eyed pea, pigeon pea, and bambara groundnut were investigated.

One of the challenges of using leguminous protein source is the endogenous anti-nutritional factors (ANFs), such as enzyme inhibitors, that affected the digestion of nutrients by animals. Although use of soybean as an alternative has met the high dietary protein requirement of fish, it contains numerous ANFs included trypsin inhibitors, lectins, tannins, phytic acids, saponins and oligosaccharides (Adeyemo and Onilude, 2013; Choct *et al.*, 2010; De Toledo *et al.*, 2007). These ANFs have the potential to diminish legumes' nutrient value resulting in decreased animal performance and health. Therefore, soybean which is currently used in feed industry, for example for the use in poultry feed, has to undergo effective heat-

treated process, such as roasting and extrusion, to avoid the reduction of animal performance (Newkirk, 2010; Stein *et al.*, 2008).

The specific aims of this study were:

- To evaluate the protein and lipid contents in the underutilised legumes
- To determine the presence of enzyme inhibitors via enzymatic assays in the underutilised legume - trypsin, chymotrypsin, and α-amylase inhibitor
- To determine the level of enzyme inhibitors and protein content present in six adzuki bean and bambara groundnut varieties

The hypothesis of this study was that relative soybean (control) underutilised legumes would have a lower macronutrient and higher anti-nutrient factor concentrations. The six variety of adzuki bean and bambara groundnut that obtained from different locations would have different level of protein and anti-nutrient factor concentration.

3.2 Experimental design

3.2.1 Various legumes preparation

For the study reported in section 3.3.1, the dried legumes (soybean, mung bean, adzuki bean, chickpea, hyacinth bean, black-eyed pea, and pigeon pea) were purchased from Giant Hypermarket Semenyih, Selangor. Whilst the bambara groundnut was provided by Crop for Future Research Centre (CFF).

For the study reported in section 3.3.2, the dried adzuki bean were purchased from Giant Hypermarket located in six states in Malaysia, namely Pahang (PHG), Perak (PRK), Johor (JHR), Pulau Pinang (PNG), Kuala Lumpur (KUL) and Selangor (SEL). Whereas, the bambara groundnut were provided by Dr Ajit Singh and CFF. These bambara groundnut were consist of four landraces from Nigeria (SOK, KAA, KAB and KAR), one from Thailand (SON), and one from Indonesia (GER).

These dried legumes with seed coat were ground into fine powder using a miller without going through dehulling process. The dehulling process was not carried out mainly due to lacking of dehulling machine in the lab and also the aim to reduce processing step that might add on additional cost to the final product. The finely ground legume powder was then to pass through a series of mesh sieves with the size of 1.68 µm, 1.18 µm and 0.85 µm. The powder that was stopped on 1.18 µm mesh sieve was collected for further analysis. All assay was carried out in triplicate.

3.2.2 Determination of ANFs and Macronutrients

The grounded legumes were subjected to the determination of ANFs, namely trypsin inhibitors (section 2.3.1), chymotrypsin inhibitors (section 2.3.2) and α-amylase inhibitors (section 2.3.3) as well as nutritional composition which were protein using Kjeldahl and Bradford methods (section 2.4.1a and 2.4.1b) and lipid content using Soxhlet method (section 2.4.2).

3.2.3 Statistical analysis

All the results were presented as mean ± standard error mean (SEM). IBM SPSS Statistics software (Version 22, IBM Corporation, USA) was used to perform one-way Analysis of Variance (ANOVA) on data sets and where this was statistical significant ($p<0.05$) subsequent post-hoc analysis was carried out using Duncan's Multiple Comparison test with confidence intervals of 95 % with threshold for significance when $p<0.05$. Data was checked for normality using Shapiro-Wilk test and for homogeneity of variance using the Levene test. Graphs were constructed using GraphPad Prism (Version 6, GraphPad Software Inc, USA).

3.3 Results and Discussion

3.3.1 Determination of macronutrients and ANFs

Both Kjeldahl and Bradford methods are the official analysis methods of Association of Official Analytical Chemists (AOAC International) and are used commonly in nutritional labelling and quality control. In this study, the Kjeldahl unit was only made available at the later stage of the study, thus Bradford method was used initially as an alternative method to determine the protein content in legumes. Bradford method determine the total protein concentration of a sample based on the binding of Coomasie dye to specific amino acids such as arginine, lysine, histidine, phenylalanine, tryptophan, and tyrosine that commonly present in protein sample (Moore *et al.*, 2010; Nielsen, 2010; Compton and Jones, 1985). Continued use of Bradford method in this study allowed further understanding on the changes of major amino acids during processing steps. While Kjeldahl method only allowed determination of crude protein content, and not specific amino acids. Kjeldahl method digested the sample with strong acid to transform all nitrogen in the sample into ammonium sulphate, followed by distillation and titration to determine the crude protein content in a sample. Hence, both methods are useful to provide further insights on the effect of processing steps on the specific amino acids and crude protein content of legume samples. Since the principles of both Kjeldahl and Bradford methods are different, hence is not advisable to compare the protein contents determined by these methods directly.

When the crude protein content was determined using Kjeldahl method, Figure 3.1 showed that soybean exhibited the 30 – 40 % higher crude protein per unit dry weight than the tested legumes. Among the underutilised legumes tested, their

protein contents were ranging from 33.81 and 39.43 g/100g DW with hyacinth bean and chickpea highest and lowest protein content respectively.

Compared to USDA (2017) database, crude protein content of soybean, mung bean, adzuki bean, chickpea, black-eyed pea and hyacinth bean were 33 to 47 % higher than current study. This large variation of crude protein content might due to the different cultivate field, genetic variation, and influence of environmental factors such as temperature and water availability. Crude protein content seems to be particularly sensitive to environmental stress (Wang and Daun, 2006). As reported in Nikolopoulou *et al.* (2007), the nutrients including protein, fat and starch content of field pea was affected by the interaction between the cultivation area and the cultivation year.

When protein content was determined using the Bradford method, Figure 3.2 showed that adzuki bean and mung bean exhibited the higher protein content of 13.21 g/100g DW and 12.75 g/100g DW respectively which were significantly higher (p<0.001) than the remaining legumes tested. Since the Coomassie Brilliant Blue dye used in Bradford methods binds strongly to basic amino acid residue which are arginine and lysine trough electrostatic attraction, and to a lesser extent histidine and aromatic amino acids, such as phenylalanine, tryptophan, and tyrosine through hydrophobic interaction (Nielsen, 2010, Compton and Jones, 1985). Hence current finding suggests that both mung bean and adzuki bean might have higher basic and aromatic amino acids than the remaining legumes tested. Wang *et al*. (2017) and Wilson (1986) reported that amino acids especially lysine and tryptophan are required for normal growth and metabolism of fish. Hence legumes with higher amount of amino acids will be preferable for use as aquafeed.

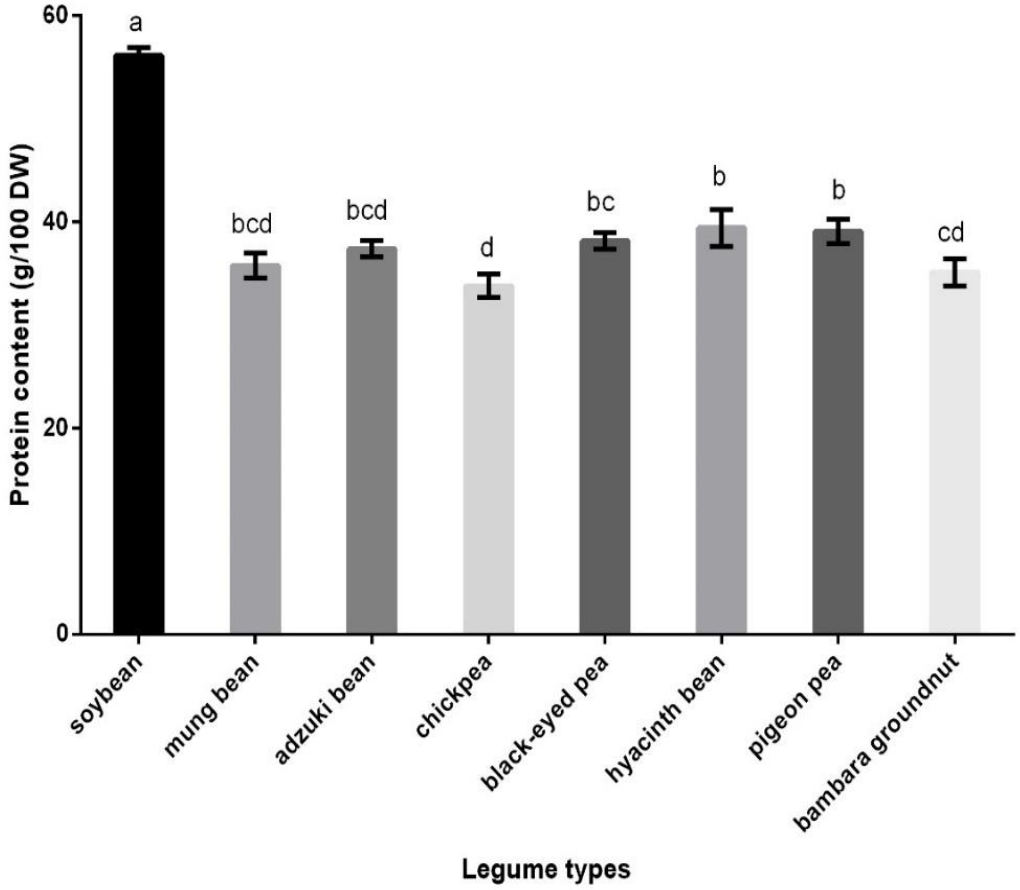

Figure 3.1 The protein content in selected legumes. The protein content of the indicated legumes were determined utilising the Kjeldahl method. There was significant difference (ANOVA, $p<0.001$). Values with different letters are significantly different ($p<0.05$), error bars represent +/- standard error mean, n=3.

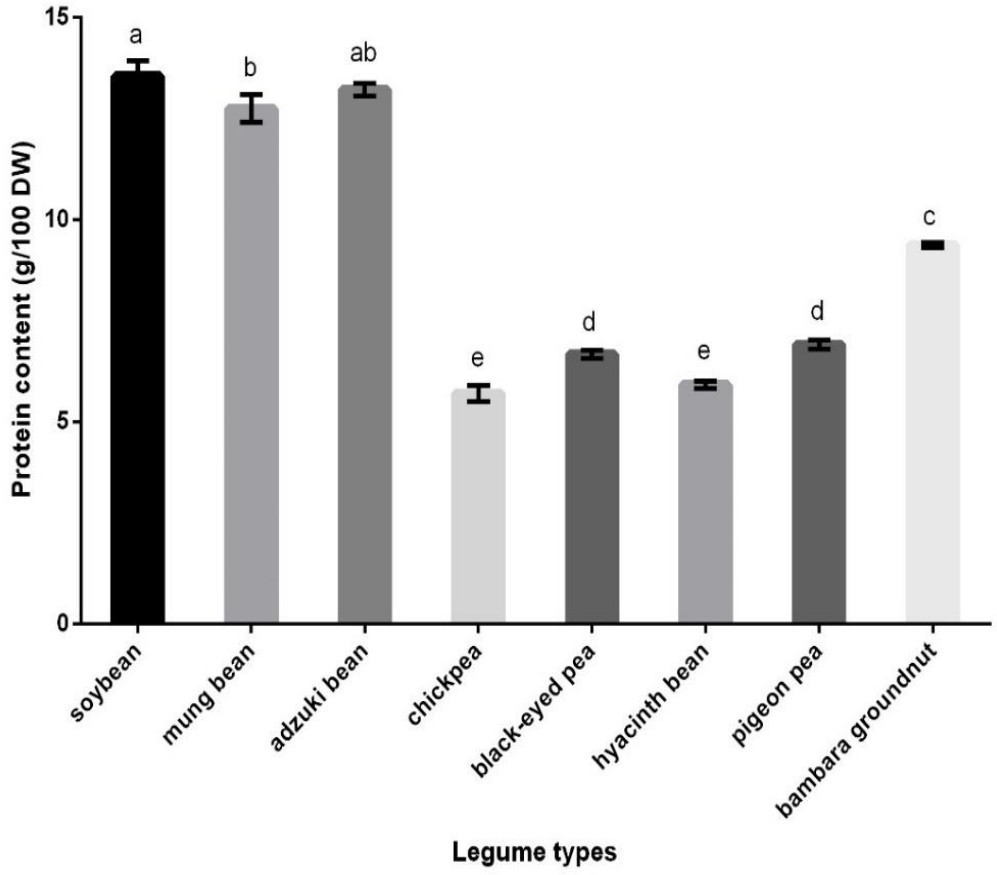

Figure 3.2 The protein content in selected legumes. The protein content of the indicated legumes were determined utilising the Bradford method. There was significant difference (ANOVA, $p<0.001$). Values with different letters are significantly different ($p<0.05$), error bars represent +/- standard error mean, n=3.

There was a significant difference in the concentration of lipid in the legumes (p<0.001). The lipid content (Figure 3.3) was found to be greater in soybean (27.67 g/100g DW) followed by bambara groundnut and the remaining legumes were within the range of 6.27 to 10.33 g/100g DW with no significant difference (p<0.001) amongst them.

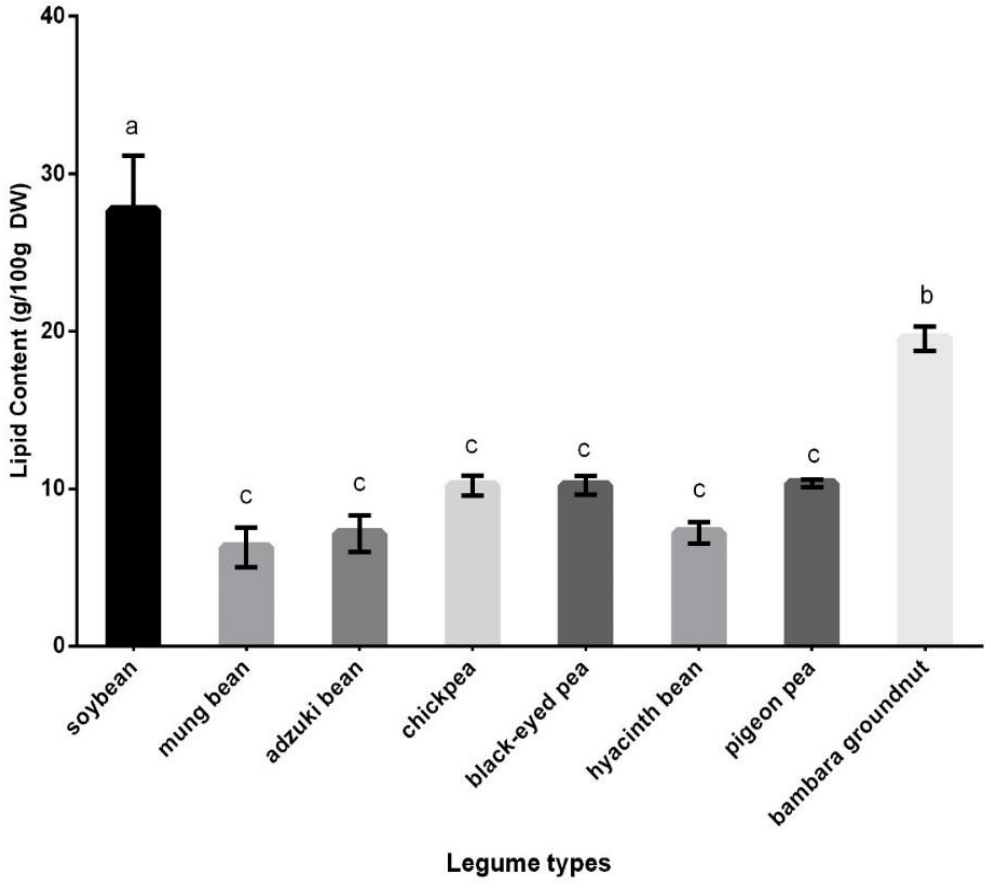

Figure 3.3 The total lipid content in selected legumes. The indicated legumes was determined utilising the Soxhlet method. There was significant difference (ANOVA, p<0.001). Values with different letters are significantly different (p<0.05), error bars represent +/- standard error mean, n=3.

According to USDA (2017) database, the lipid content of soybean, mung bean, adzuki bean, chickpea, black-eyed pea and hyacinth bean were ranged from 1.69 to 19.94 g/100g DW where hyacinth bean and soybean had the highest and lowest respectively. The lipid content variation of current study compared with USDA data was in agreement with Anwar *et al.* (2016) who reported that the lipid content of soybean was affected by the varieties.

From the data presented here, mung bean and adzuki bean could possibility be a good candidate as an alternative to soybean. Although their crude protein and lipid content was approximately 36 % and 77 % respectively lower than soybean. Yet, mung bean and adzuki bean had comparable amino acid of arginine, lysine, histidine, phenylalanine, tryptophan, and tyrosine residues to soybean. This made them stand out from the other underutilised legumes.

Even though the crude protein content of underutilised legumes were 30 – 40 % lower than soybean, some of them contain a comparable amino acids content as soybean, therefore this possibly makes them promising ingredients for aquafeed. A potential advantage of these legumes was their lipid content which was 29 – 77 % lower than soybean, large quantities of lipid not being ideal for aquafeeds. Thus, these underutilised legumes might not able to completely replace the soybean but could contribute as one of the protein sources along with incorporation of other oil seed or sources.

In order to enhance a protein sparing effect and decrease nitrogenous losses, high dietary lipid levels are commonly used in carnivorous fish as an important source of energy (Regost *et al.*, 2003). Published work from Glencross *et al.* (2016) showed

that it is possible to almost replace all the fish meal with soybean meal and poultry meal without losing the barramundi growth performance, up to and including diets with as little as 100 g/kg fish meal. While for fish oil, there has been more success in completely replacing it with soybean oil and rice bran oil without affecting the growth of salmonids and barramundi respectively (Glencross *et al.*, 2016; Hardy, 1987).

Proteolytic enzymes such as trypsin and chymotrypsin take affect the utilisation of protein and ultimately affect the growth of fish (Dabrowski and Glogowski, 1977). Trypsin cleaves polypeptides at the carboxyl side of arginine and lysine residues whilst chymotrypsin cleaves at the carboxyl side of tyrosine, tryptophan, phenylalanine, and leucine residues. Trypsin and chymotrypsin inhibitors bind to the respective active site of the enzyme thereby inhibiting digestive enzyme activity in the fish digestive tract (Krogdahl *et al.*, 2010). While the presence of α-amylase inhibitory activity will impair carbohydrate digestion by inhibiting the hydrolysis of α-1,4-glycosidic bonds (Singh *et al.*, 2010). The α-amylase inhibitors form complexes with α-amylases potentially preventing fish using carbohydrate as an energy source (Yengkokpam *et al.*, 2007).

There was a significant difference in the trypsin inhibitory activity in the legumes (p<0.001). As shown in Figure 3.4, mung bean had the highest trypsin inhibitory activity (0.171 TIA unit/mg). No trypsin inhibitor was detected in black-eyed pea, hyacinth bean and bambara groundnut. The trypsin inhibitory activity of underutilised legumes was ordered as mung bean > adzuki bean and chickpea > pigeon pea > black-eyed pea, hyacinth bean and bambara groundnut.

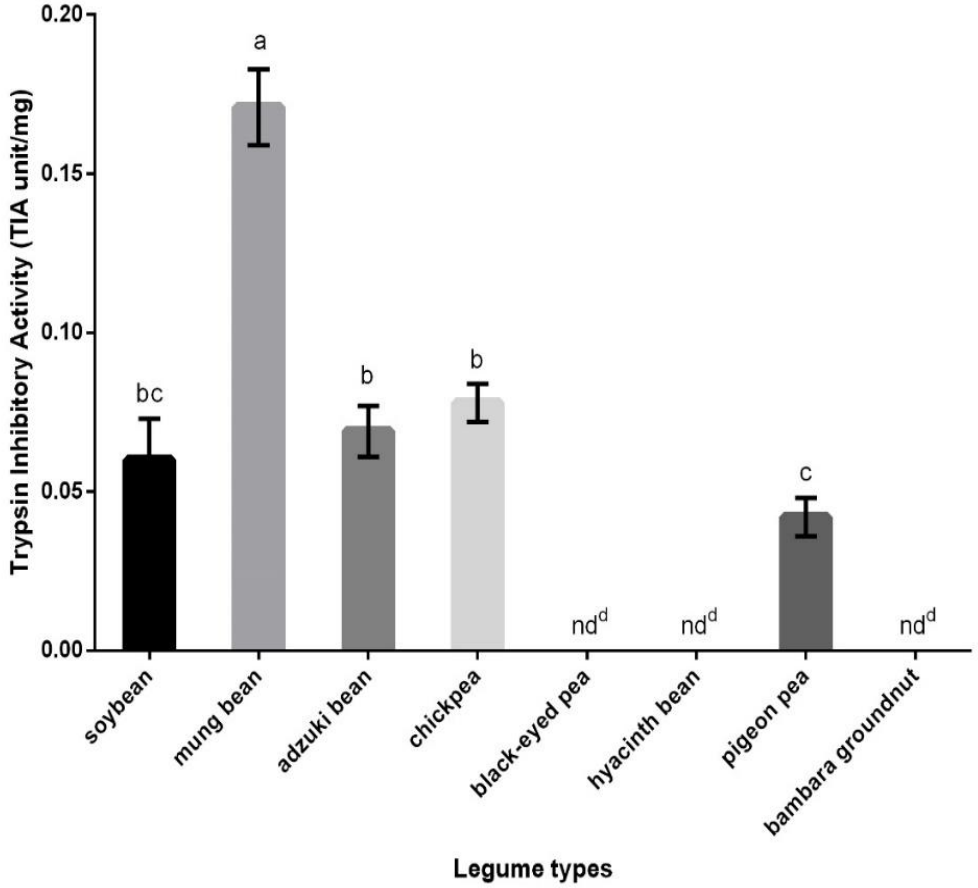

Figure 3.4 The trypsin inhibitory activity in selected legumes. There was significant difference (ANOVA, $p<0.001$). Values with different letters are significantly different ($p<0.05$), error bars represent +/- standard error mean, nd represent not detectable, n=3.

The unexpected undetectable trypsin inhibitor in black-eyed pea, hyacinth bean and bambara groundnut could be due to the sensitivity of the current biochemical technique that been applied in enzymatic assay. The plausible explanation of this could be that because the concentration of trypsin inhibitor was too low and beyond the detection limit. Current method is an inexpensive and common method that had been used to detect the trypsin inhibitors, but the detection of biochemical technique is relatively less sensitive compared to high-throughput technique. To date, there is no available of high-throughput assay to quantify the total enzyme inhibitors in legumes.

Miyagi *et al.* (1997) reported trypsin inhibitory activity of 0.048 TIA unit/mg for soybean which is 20 % lower than current study. Whereas the reported studies on mung bean, chickpea, black-eyed pea, hyacinth bean, pigeon pea and bambra groundnut were at least 94 – 100 % higher than current findings (Osman, 2007; Tibe *et al.*, 2007; Mubarak, 2005; Oloyo, 2004; Egounlety and Aworh, 2003; Frias *et al.*, 2000).

There was a significant difference in the chymotrypsin inhibitor activity in the legumes ($p<0.001$). Figure 3.5 shows that the chymotrypsin inhibitor content of mung bean (1.71 CIA unit/mg) was highest followed by soybean (1.53 CIA unit/mg), adzuki bean (1.07 CIA unit/mg) and black-eyed pea (1.15 unit/mg). Hyacinth bean, pigeon pea and bambara groundnut exhibited chymotrypsin inhibitor content with no significant difference among themselves ($p>0.05$) but were significantly higher ($p<0.05$) than chickpea (0.49 CIA unit/mg).

The chymotrypsin inhibitory activity of pigeon pea and chickpea has previous reported was ranging from 2.10 to 3.60 CIA unit/mg and 5.70 to 9.40 CIA unit/mg respectively, whilst soybean was 30.16 CIA unit/mg (Shi *et al.*, 2017; Singh, 1988). These values are at least 60 – 96 % higher than current findings.

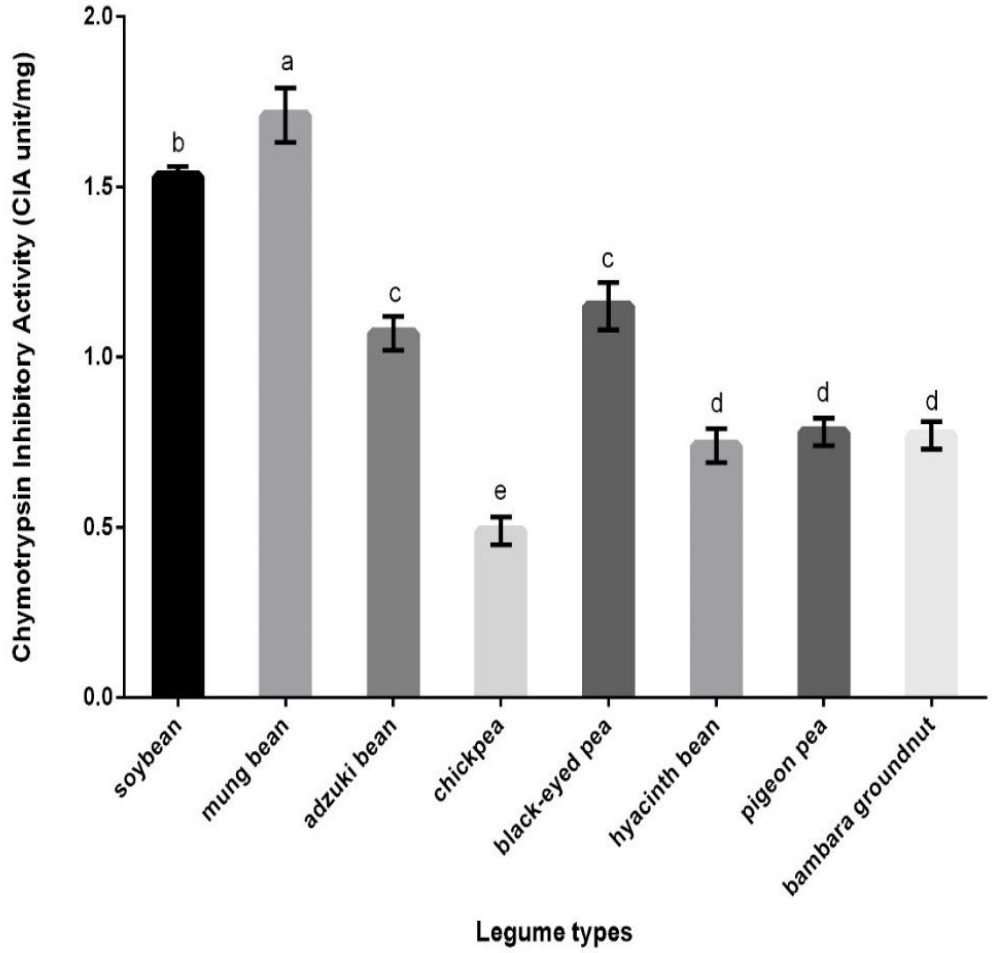

Figure 3.5 The chymotrypsin inhibitory activity in selected legumes. There was significant difference (ANOVA, $p<0.001$). Values with different letters are significantly different ($p<0.05$), error bars represent +/- standard error mean, n=3.

There was a significant difference in the α-amylase inhibitor activity in the legumes (p<0.001). Unlike for TIA and CIA, where mung bean had the highest activity, α-amylase inhibitor was highest in soybean (1.99 AIA unit/mg). The remaining legumes were not significantly different (p>0.05).

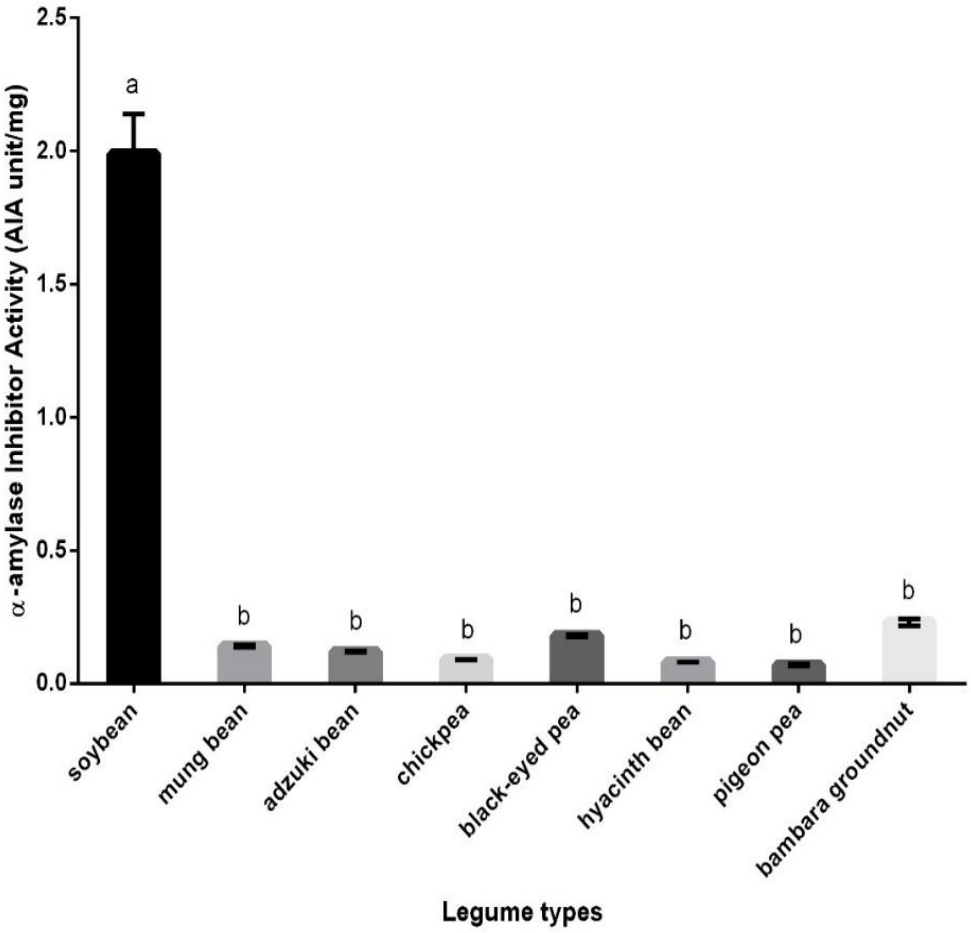

Figure 3.6 The α-amylase inhibitory activity in selected legumes. There was significant difference (ANOVA, p<0.001). Values with different letters are significantly different (p<0.05), error bars represent +/- standard error mean, n=3.

Shi *et al.* (2017) stated that the α-amylase inhibitory activity in soybean was high (0.94 AIA unit/mg) but was undetectable in peas, lentils, faba bean and chickpea. Whilst Shekib *et al.* (1988) showed that α-amylase inhibitory activity of chickpea and black-eyed pea were 2.21 AIA unit/mg and 3.38 AIA unit/mg respectively, which is approximately 95 % higher than current study. The variation in trypsin and α-amylase inhibitory activity detected in legumes is probably due to the genetic differences in legume varieties and cultivars as well as the interaction with environmental factors such as climatic conditions, location, soil type and crop year. However the large variation of chymotrypsin inhibitor might be attributed to the different chymotrypsin inhibitor assays used to those described in the literature.

The ANFs, including oligosaccharides, tannins and phytic acid, of field pea have been identified as being affected by cultivation area and cultivation year (Nikolopoulou *et al.*, 2007). It could be anticipated that the content of proteinaceous enzyme inhibitors might be affected as well. Other than that, the structure of the seed will affect the enzymatic inhibitory activity too. As reported by Shi *et al.* (2017) the split legume had higher enzymatic inhibitory activity than the whole seed. Compare to protease inhibitors, less data have been documented on the α-amylase inhibitor content of legumes.

3.3.2 Determination of the effect of ANFs and protein content in adzuki bean and bambara groundnut obtained from different sources

The study was conducted to investigate the impact of legume sources on the ANFs and protein content of underutilised legumes. Two legumes, adzuki bean and bambara groundnut were selected for this investigation. Both legumes were purchased from six sources at different locations. In general, the protein content, TIA, CIA and AIA of adzuki bean and bambara groundnut reported in section 3.3.1 were within the same range of value with those legumes from different sources reported in this section.

The protein content of adzuki bean and bambara groundnut from multiple sources were ranging from 28.59 to 37.39 g/100g DW and 30.71 to 37.02 g/100g DW, respectively. The protein content of adzuki bean (Table 3.1) was found to be greater in SEL (37.39 g/100g DW) whilst bambara groundnut (Table 3.2) were greater in SOK and GER (37.02 g/100g DW and 35.13 g/100g DW respectively) with no significant difference (p<0.001) amongst them.

When compare the trypsin inhibitory activity among the legumes tested, adzuki bean from PHG had the highest trypsin inhibitory activity of 0.083 TIA unit/mg, while PNG had the lowest trypsin inhibitory activity of 0.025 TIA unit/mg. While bambara groundnut from four sources exhibited trypsin inhibitory activity that ranged from 0.081 to 0.178 TIA unit/mg, except for SOK and GER that had no detectable level of trypsin inhibitor.

When compare the chymotrypsin inhibitory activity among the legumes tested, the adzuki bean from SEL and bambara groundnut from SON had the highest CIA of

1.06 CIA unit/mg and 0.91 CIA unit/mg, respectively. Chymotrypsin inhibitory activity of adzuki bean had the range of 0.32 to 1.06 CIA unit/mg whereas bambara groundnut had the ranged of 0.19 to 0.91 CIA unit/mg.

When compare the α-amylase inhibitory activity among the legumes tested, adzuki bean from PRK and SEL had the highest α-amylase inhibitory activity (0.121 AIA unit/mg and 0.123 AIA unit/mg respectively). Whilst bambara groundnut from GER had the highest α-amylase inhibitory activity (0.228 AIA unit/mg). The α-amylase inhibitory activity of adzuki bean had the range of 0.123 to 0.091 AIA unit/mg whereas bambara groundnut had the ranged of 0.228 to 0.082 AIA unit/mg.

Table 3.1 The protein content determined utilising the Kjeldahl method and enzyme inhibitors in adzuki bean from different location

Location	Protein content (g/100g DW)	Trypsin inhibitory activity (TIA unit/mg)	Chymotrypsin inhibitory activity (CIA unit/mg)	α-amylase inhibitory activity (AIA unit/mg)
PRK	28.59 ± 0.44 ^{d}	0.056 ± 0.008 ^{b}	0.40 ± 0.09 ^{bc}	0.121 ± 0.004 ^{a}
KUL	31.36 ± 0.68 ^{bc}	0.053 ± 0.009 ^{b}	0.56 ± 0.03 ^{b}	0.103 ± 0.006 ^{bc}
PNG	30.12 ± 0.58 ^{cd}	0.025 ± 0.006 ^{c}	0.41 ± 0.07 ^{bc}	0.108 ± 0.001 ^{b}
PHG	33.26 ± 0.84 ^{b}	0.083 ± 0.009 ^{a}	0.52 ± 0.04 ^{b}	0.094 ± 0.004 ^{cd}
JHR	29.84 ± 0.68 ^{cd}	0.067 ± 0.007 ^{ab}	0.32 ± 0.02 ^{c}	0.091 ± 0.003 ^{d}
SEL	37.39 ± 0.78 ^{a}	0.067 ± 0.008 ^{ab}	1.06 ± 0.05 ^{a}	0.123 ± 0.002 ^{a}

Notes:
Values are presented in mean ± SEM (n=3)
Within a column, values with different letters are significantly different at p<0.001

Table 3.2 The protein content determined utilising the Kjeldahl method and enzyme inhibitors in bambara groundnut from different location

Location	Protein content (g/100g DW)	Trypsin inhibitory activity (TIA unit/mg)	Chymotrypsin inhibitory activity (CIA unit/mg)	α-amylase inhibitory activity (AIA unit/mg)
SON	31.24 ± 0.83 ^{bc}	0.178 ± 0.005 ^{a}	0.91 ± 0.03 ^{a}	0.082 ± 0.005 ^{de}
KAA	30.71 ± 1.45 ^{cd}	0.117 ± 0.008 ^{b}	0.68 ± 0.04 ^{b}	0.092 ± 0.007 ^{d}
KAB	34.16 ± 1.13 ^{ab}	0.081 ± 0.008 ^{c}	0.19 ± 0.04 ^{d}	0.071 ± 0.002 ^{e}
KAR	29.81 ± 0.36 ^{c}	0.108 ± 0.004 ^{b}	0.30 ± 0.07 ^{cd}	0.127 ± 0.002 ^{c}
SOK	37.02 ± 0.88 ^{a}	nd ^{d}	0.37 ± 0.02 ^{c}	0.193 ± 0.006 ^{b}
GER	35.13 ± 1.31 ^{a}	nd ^{d}	0.77 ± 0.04 ^{b}	0.228 ± 0.004 ^{a}

Notes:
Values are presented in mean ± SEM (n=3)
Within a column, values with different letters are significantly different at $p<0.001$
nd represent not detectable

Aforementioned in section 1.6, the macronutrient (protein) and ANFs of legume could possibly have affected by cultivation area, cultivation year, maturity of seed, genetic variation, and influence of environmental factors such as temperature and water availability. The protein content of adzuki bean and bambara groundnut reported were not within the range of those reported in other studies (Murevanhema and Jideani, 2013; Mkandawire, 2007; Yousif *et al.*, 2007; Tjahjadi *et al.*, 1988). They reported that the protein content of adzuki bean and bambara groundnut was in the range of 21 – 24 g/100g and 16 – 25 g/100g respectively.

Studies described by Wang and Daun (2004) and Vollmann *et al.* (2000) the protein content in soybean and field peas was affected by environment factor. As an example, soybean that grown under moderately dry condition (19 mm rainfall) and high temperature (19.2°C) yield the highest protein content approximately 433.7 g/kg during the growing period compared to those that grown under humid condition. Nikolopoulou *et al.* (2007) stated that the pea that grown at different cultivation area could also influences the nutrient density, an average of 24 to

30.5 % of protein higher had been observed in pea cultivar in the same cultivation year. Three different variety chickpea which were “Evros”, “Amorgos” and “Gravia” that cultivated at the same area had 7 to 9 % difference in protein content which grown in two different cultivation year (Nikolopoulou *et al.*, 2006).

In addition, several researchers reported that the ANFs including phytic acid, trypsin inhibitor, tannin, oxalate, saponin and lectin content was resulting from legume genetic variation (Shang *et al.*, 2016; Dhole and Reddy, 2015; Ajibade *et al.*, 2005; Shim *et al.*, 2003). The trypsin inhibitor in the 56 genotypes of common bean ranged from 0.016 to 15.947 mg/g with the mean value of 1.60 mg/g, whilst 20 genotypes of African yam bean ranged from 17.11 to 33.56 TIU/mg with the mean value of 26.1 TIU/mg (Shang *et al.*, 2016; Ajibade *et al.*, 2005). It also reported that when the mung bean was grew under the same environment and climate, the level of phytic acid in 102 genotypes ranged from 5.85 to 20.02 mg/g with the average of 8.26 mg/g (Dhole and Reddy, 2015). The wide variation in trypsin inhibitory activity and phytic acid were cause by genetic variability.

Other than that, the changes in trypsin inhibitory activity related to cultivation year of common bean were being cultivated in the same environment was being observed over 3 years. A wide variation trypsin inhibitory activity was found within the cultivars across 3 years with an average of 27.67 TIU/mg DM in 1995, 25.31 TIU/mg DM in 1996 and 23.39 TIU/mg DM in 1997 (Piergiovanni and Pignone, 2003). Whereas the phytic acid content of chickpea that cultivated at different locations within the same cultivation year had varied from 0.2 % to 0.5 % (Nikolopoulou *et al.*, 2006).

In this study, adzuki bean that traded in Malaysia market were imported from other countries included Japan, China, Taiwan, and South Korea, but it was not distinguishable from the origin country. Whereas the bambara groundnut was imported from Nigeria, Thailand, and Indonesia. There was lacking the information such as cultivation area, cultivation year, maturity of seed, genetic variation as well as growing temperature and water availability which could affect the nutrient and anti-nutrient contents in these legumes. Thus, it is rather difficult to conclude that the variation of these legumes was cause by a particular factor.

3.4 Summary

In current findings, the mung bean and adzuki bean appear to be good alternative source of aquafeed than soybean. The mung bean and adzuki bean are high in crude protein and in selected amino acids such as arginine, lysine, histidine, phenylalanine, tryptophan and tyrosine in relative to other underutilised legumes tested. Unfortunately, these legumes are with substantial level of protease inhibitors which might affect the protein digestibility and availability to fish.

Besides, the present results also indicating the varietal differences exist in the protein and enzyme inhibitor contents of adzuki bean and bambara groundnut. The SEL adzuki bean and GER bambara groundnut had the highest protein content as well as substantial level of protease inhibitors. Therefore, it is essential to explore various processing methods that could be used to reduce these ANFs prior to application as aquafeed.

CHAPTER 4

EFFECT OF PROCESSING METHODS IN REDUCING ENZYME INHIBITORS PRESENT IN UNDERUTILISED LEGUMES

4.1 Introduction

Findings from Chapter 3 suggested that majority of the underutilised legumes tested contain substantial amount of enzyme inhibitors that might restrict their application as ingredients for aquafeed. These enzyme inhibitors such as trypsin, chymotrypsin and α-amylase enzyme inhibitors might cause detrimental effect to the digestion and nutrient absorption aquatic animals. Hence it is the aim of current study to develop effective thermal and non-thermal processing methods that could to reduce these enzyme inhibitors present in legumes.

The elimination of enzyme inhibitors for legumes is remains a challenge to researchers, as they need to ensure the processing methods is effective and will not deteriorate the nutritive value of the processed food (Hailu *et al.*, 2015; Luo and Xie, 2013; Singhal *et al.*, 2012; Wang *et al.*, 2008; Osman, 2007). The common processing methods used to remove trypsin and chymotrypsin inhibitors are through soaking and heat treatment which involves cooking or autoclaving (ElMaki *et al.*, 2007; Egounlety and Aworh, 2003). However, to the author's knowledge, no single processing method or strategy is effective in removing these enzyme inhibitors totally. Thus, current study will employ and optimise the food processing methods available including soaking, heat treatment, autoclaving, and dry freezing and develop a strategy to remove these enzyme inhibitors and without affecting the nutritional content of the legumes.

Dehulling, germination, and fermentation were not being used in this study due to several limitations. There is no dehulling machine available in the university. Besides, the ultimate aim for these processed legumes are to use as aquafeed ingredient, from the point of industrialisation and commercialisation; the processing methods need to be less laborious and cheap. Germination process will lead to additional labour cost for harvesting that need to be take into consideration whilst fermentation process involved in acid production that might alter the palatability of the legume could possibility reduce the feed intake in targeted fish.

The overall objective of this research on of underutilised legume is to enhance the use of plant protein in aquafeed thereby reducing the over-reliance of commercially important legume which are finite source. The presence of enzyme inhibitors that affect the utilisation of protein and carbohydrate could serve as a major drawback.

To overcome these challenges, the specific aim of this chapter was:

- To determine the optimal operating parameters such as soaking, wet heating, autoclaving and dry freezing as well as the effect of different time periods and temperatures in reducing enzyme inhibitors (trypsin, chymotrypsin, and α-amylase inhibitors) present in underutilised legumes whilst also attempting to retain their nutritive values

Current hypothesis suggested that the optimised wet heating and autoclaving will be the most effective methods, out of four processing methods, to reduce the enzyme inhibitors present in underutilised legumes, since enzyme inhibitors can be decomposed better with heat.

4.2 Methodology

4.2.1 Sample preparation

The sources of seven underutilised legumes, namely mung bean (MB), adzuki bean (AB), chickpea (CP), hyacinth bean (HB), black-eyed pea (BEP), pigeon pea (PP) and control soybean (SB) tested in this study were reported in section 3.2.1.

4.2.2 Legume processing methods

The dried legumes were purchased from the same single source. Each of the processing methods was performed in triplicate for each type of legume (single sample).

a) Soaking

A 10 g of dried legumes were soaked in 100 mL of distilled water for different durations of 0 h, 6 h, 12 h, 18 h and 24 h at room temperature (25°C) respectively. After removing the soaking medium, the soaked legumes were chilled overnight at 4°C before being frozen at -80^{o}C for 24 h.

b) Wet heating

A 10 g of dried legumes were immersed in 100 mL of distilled water and then immediately heated at either 50°C or 100°C for 30 min or 60 min. After decanting the water phase, the wet heated legumes were allowed to cool to room temperature and chilled overnight at 4°C before being frozen at -80^{o}C for 24 h.

c) Autoclaving

A 10 g of dried legumes were immersed in 100 mL of distilled water and then immediately autoclaved at 121°C, 15 psi for 15 min. After decanting the water phase, the autoclaved legumes were allowed to cool to room temperature and chilled overnight at 4°C before being frozen at -80°C for 24 h.

d) Dry freezing

The dried legumes (10 g) were frozen at -20°C and -80°C for 24 h at each temperature and proceed to the lyophilised process without allowing them to heat up to room temperature.

All the frozen samples were lyophilised for 24 h using freeze-dryer (Alpha 1-4 LD plus, Christ). The lyophilised legumes were then ground into powder form using a miller without going through dehulling process. The fine powder was then to pass through a series of mesh sieves with the size of 1.68 µm, 1.18 µm and 0.85 µm. The powder that was stopped on 1.18 µm mesh sieve was collected and stored in 4°C chiller up to 2 weeks prior to analysis for enzyme inhibitors and nutrients.

4.2.3 Enzyme inhibitors and nutrient determination

The trypsin, chymotrypsin and α-amylase inhibitors present in the raw and processed dried samples were determined according to sections 2.3.1 – 2.3.2. The protein content of raw and processed dried samples was determined according to section 2.4.1a – 2.4.1b. In this study, both Bradford and Kjeldahl protein determination methods were used to determine the soluble amino acids in sodium hydroxide (NaOH) and total crude protein respectively. All assay was carried out in triplicate, except for crude protein (Kjeldahl method) that only one replicate was being performed.

4.2.4 Statistical analysis

The results were presented as mean ± standard error mean (SEM) which had carried out in triplicate. Except for crude protein content determined using Kjeldahl method that presented as single analysis value. IBM SPSS Statistics software (Version 22, IBM Corporation, USA) was used to perform two-way Analysis of Variance (ANOVA) on data sets and where this was statistical significant (p<0.05) subsequent post-hoc analysis was carried out using Duncan's Multiple Comparison test confidence intervals of 95 % with threshold for significance when p<0.05. Data was checked for normality using Shapiro-Wilk test, where stated, none normally distributed data was transformed (square root) before statistical analysis was performed. Data was checked for homogeneity of variance using the Levene test.

4.3 Results and Discussion

In this chapter, the effect of operational parameters such as soaking, wet heating, autoclaving and dry freezing on the enzyme inhibitors (trypsin, chymotrypsin, and α-amylase inhibitors) and protein content present in underutilised legumes were investigated. Only 10 g of legumes in each replicate were subjected to processing methods due to the limitation of the equipment. The benchtop freeze dryer is designed for lyophilizing light sample loads and lead to the decision of processed only 10 g of legumes for each replicate. As mentioned before in section 3.3, Kjeldahl machine was not available during the early stage of the study and Bradford method was used as an alternative. Another challenge for Kjeldahl method was the limitation of chemical, this chapter had too much of samples and due to the constraint of research cost; Kjeldahl method was only performed in single replicate for verification purpose.

4.3.1 Soaking treatment

Soaking is a preliminary step prior to cooking which helps to soften the texture of dried legume and shorten the cooking time (Xu and Chang, 2008). The soaking treatment is the imbibition process involved in admission of water into the dried legume through the pores and the thinnest area of the seed coat , resulting in swelling of tissue and water uptake without cell separation (Eyaru *et al.*, 2009; Perissé and Planchuelo, 2004). The soaking medium usually becomes slightly coloured which indicates some of the soluble constitutes such as phenolic compounds had leached into soaking medium under the influenced of concentration gradient (Xu and Chang, 2008).

4.3.1.1 Effect of soaking on enzyme inhibitors

There was a significant difference in the trypsin inhibitory activity (TIA), chymotrypsin inhibitory activity (CIA) and α-amylase inhibitory activity (AIA) in the interaction between legumes and the soaking period (p<0.001). The effect of soaking treatment on trypsin, chymotrypsin and α-amylase inhibitory activity are shown in Table 4.1, 4.2 and 4.3. In general, the soaking treatment increased the trypsin and chymotrypsin inhibitory activity in tested legumes. In contrast, there was a reduction of α-amylase inhibitory activity in was observed in four soaked legumes, namely MB, AB, CP, and BEP but this was only consistently seen in the early part of the soaking period (6 to 12 h of soaking).

There was a significant difference of trypsin inhibitory activity (TIA) in the legumes with the soaking duration (p<0.001) as it had increased with the soaking duration. As shown in Table 4.1, the raw legumes possessed the lowest TIA ranging from 0.042 to 0.171 TIA unit/mg. Interestingly, no detectable TIA was found for BEP, HB and BG.

The TIA of soaked SB, AB and MB showed no significant difference with raw legumes after 6 h of soaking period. However, for the remaining five legumes, a significant increment of 87 % to 100 % when compared to raw legumes. AB (1.294 TIA unit/mg) and HB (0.181 TIA unit/mg) had the highest and lowest trypsin inhibitory activity after soaked for 12 h, respectively.

There was a significant difference of chymotrypsin inhibitory activity (CIA) in the legumes with the soaking duration (p<0.001) as it had increased with the soaking duration. As shown in Table 4.2, the raw legumes possessed the lowest CIA ranging

from 0.49 to 1.71 CIA unit/mg. All tested legumes, except for BG, showed a significant increase in CIA ranging from 40 % to 58 % after soaked for 6 h. The highest CIA after soaking for 18 h was in SB (5.32 CIA unit/mg), whilst the CIA of 24 h-soaked BG was not significantly different from raw BG.

There was a significant difference of α-amylase inhibitory activity (AIA) in the legumes with the soaking duration (p<0.001) as it had increased with the soaking duration. As shown in Table 4.3, the AIA of four legumes (MB, AB, CP, BEP) reduced significantly (p<0.001) after 6 h of soaking period, except for SB, HB, PP, and BG. Regardless the soaking duration, raw SB had showed the highest AIA (1.991 AIA unit/mg) which range from 8-fold to 27-fold higher when compared to the other legumes. Other than that, SB had increased the greatest of all the legumes with the soaking period. After soaking for 24 h, a 89 % increment of AIA was observed for SB, but after 24 h-soaked BG was only 18 % higher than raw BG. For HB, of the range for the increase was 13 % to 50 % when soaked for 12 – 24 h. Whilst for PP, of the range for the increase was 24 % to 62 % when soaked for 6 – 18 h. However, the 24 h-soaked PP showed significantly lower AIA than raw PP.

For the four legumes that showed reduction after 6 h of soaking period, the prolong soaking period to 12 h had increased the AIA of MB and CP. Whereas, the AIA of AB and BEP only increased after 18 h of soaking period. After 24 h of soaking treatment, the AIA of these four legumes was significantly increased by 14 % to 66 % when compared to raw legumes.

Table 4.1 The interaction of legume x soaking duration in the trypsin inhibitory activity

Legume types	Trypsin inhibitory activity (TIA unit/mg)				
	Raw	Soaked 6 h	Soaked 12 h	Soaked 18 h	Soaked 24 h
SB	0.060 ± 0.013^{lmn}	0.165 ± 0.011^{jklm}	0.350 ± 0.027^{fgh}	1.728 ± 0.135^{a}	0.989 ± 0.061^{c}
MB	0.171 ± 0.012^{jklm}	0.150 ± 0.011^{jklm}	0.637 ± 0.038^{d}	0.328 ± 0.026^{fghi}	0.891 ± 0.050^{c}
AB	0.069 ± 0.008^{lmn}	0.139 ± 0.008^{klm}	1.294 ± 0.075^{b}	0.469 ± 0.091^{efg}	1.039 ± 0.082^{c}
CP	0.078 ± 0.006^{lmn}	0.328 ± 0.018^{fghi}	0.363 ± 0.022^{fgh}	0.472 ± 0.036^{def}	0.287 ± 0.012^{ghij}
BEP	nd ^{n}	0.235 ± 0.030^{hijk}	0.359 ± 0.017^{fgh}	0.276 ± 0.030^{ghijk}	0.606 ± 0.012^{d}
HB	nd ^{n}	0.363 ± 0.053^{fgh}	0.181 ± 0.011^{ijkl}	0.380 ± 0.044^{fgh}	1.022 ± 0.043^{c}
PP	0.042 ± 0.006^{mn}	0.328 ± 0.025^{fghi}	0.367 ± 0.013^{fgh}	0.383 ± 0.016^{fgh}	0.943 ± 0.024^{c}
BG	nd ^{n}	0.330 ± 0.011^{fghi}	0.546 ± 0.019^{de}	0.363 ± 0.029^{fgh}	0.604 ± 0.023^{d}
P value Legume types			$p<0.001$		
P value Soaking duration			$p<0.001$		
P value Legume types x Soaking duration			$p<0.001$		

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root)
Values are presented in mean ± SEM (n=9), back transformed of square root values
Values with different letters are significantly different at $p<0.001$
nd represent not detectable

Table 4.2 The interaction of legume x soaking duration in the chymotrypsin inhibitory activity

Legume types	Chymotrypsin inhibitory activity (CIA unit/mg)				
	Raw	Soaked 6 h	Soaked 12 h	Soaked 18 h	Soaked 24 h
SB	1.53 ± 0.03 ^{mno}	2.53 ± 0.07 ^{fgh}	2.94 ± 0.07 ^{de}	5.32 ± 0.13 ^{a}	3.06 ± 0.15 ^{de}
MB	1.71 ± 0.08 ^{klmn}	3.19 ± 0.05 ^{d}	2.94 ± 0.07 ^{de}	4.28 ± 0.07 ^{b}	2.48 ± 0.07 ^{gh}
AB	1.07 ± 0.05 ^{p}	1.83 ± 0.07 ^{jklm}	2.09 ± 0.07 ^{ij}	3.00 ± 0.08 ^{de}	2.21 ± 0.08 ^{hi}
CP	0.49 ± 0.04 ^{r}	1.61 ± 0.05 ^{lmno}	1.64 ± 0.08 ^{lmno}	2.84 ± 0.06 ^{def}	1.49 ± 0.11 ^{no}
BEP	1.15 ± 0.07 ^{p}	3.10 ± 0.06 ^{de}	3.06 ± 0.07 ^{de}	3.83 ± 0.10 ^{c}	2.76 ± 0.07 ^{efg}
HB	0.74 ± 0.05 ^{q}	1.75 ± 0.07 ^{klmn}	1.94 ± 0.05 ^{ijk}	1.91 ± 0.09 ^{ijkl}	1.39 ± 0.08 ^{o}
PP	0.78 ± 0.04 ^{q}	1.60 ± 0.08 ^{mno}	1.39 ± 0.08 ^{o}	2.03 ± 0.09 ^{ijk}	1.03 ± 0.07 ^{p}
BG	0.77 ± 0.04 ^{q}	0.58 ± 0.07 ^{r}	1.06 ± 0.06 ^{p}	0.75 ± 0.04 ^{q}	0.94 ± 0.07 ^{pq}
P value Legume			p<0.001		
P value Soaking duration			p<0.001		
P value Legume x Soaking duration			p<0.001		

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root)
Values are presented in mean ± SEM (n=9), back transformed of square root values
Values with different letters are significantly different at p<0.001

Table 4.3 The interaction of legume x soaking duration in the α-amylase inhibitory activity

Legume types	α-amylase inhibitory activity (AIA unit/mg)				
	Raw	Soaked 6 h	Soaked 12 h	Soaked 18 h	Soaked 24 h
SB	1.991 ± 0.149 ^{d}	4.252 ± 0.096 ^{c}	4.583 ± 0.099 ^{c}	7.401 ± 0.272 ^{b}	18.060 ± 0.232 ^{a}
MB	0.144 ± 0.004 ^{lm}	0.085 ± 0.003 ^{qr}	0.109 ± 0.002 ^{p}	0.169 ± 0.005 ^{jk}	0.169 ± 0.005 ^{jk}
AB	0.124 ± 0.002 ^{no}	0.084 ± 0.002 ^{qr}	0.094 ± 0.003 ^{q}	0.185 ± 0.007 ^{j}	0.185 ± 0.007 ^{j}
CP	0.088 ± 0.001 ^{qr}	0.067 ± 0.002 ^{t}	0.091 ± 0.002 ^{q}	0.259 ± 0.006 ^{fg}	0.259 ± 0.006 ^{fg}
BEP	0.181 ± 0.004 ^{j}	0.129 ± 0.003 ^{no}	0.135 ± 0.002 ^{mn}	0.319 ± 0.008 ^{e}	0.319 ± 0.008 ^{e}
HB	0.080 ± 0.000 ^{rs}	0.084 ± 0.001 ^{qr}	0.092 ± 0.003 ^{q}	0.161 ± 0.005 ^{kl}	0.161 ± 0.005 ^{kl}
PP	0.074 ± 0.004 ^{s}	0.089 ± 0.001 ^{q}	0.087 ± 0.002 ^{qr}	0.121 ± 0.007 ^{op}	0.030 ± 0.000 ^{u}
BG	0.228 ± 0.013 ^{hi}	0.243 ± 0.002 ^{gh}	0.235 ± 0.003 ^{ghi}	0.214 ± 0.004 ^{i}	0.281 ± 0.002 ^{f}
P value Legume types			p<0.001		
P value Soaking duration			p<0.001		
P value Legume types x Soaking duration			p<0.001		

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root)
Values are presented in mean ± SEM (n=9), back transformed of square root values
Values with different letters are significantly different at p<0.001

The process of soaking is a common treatment for reduction of ANFs which included protease inhibitors, phytic acid, saponin and flatulence causing oligosaccharides in legumes, as all can be solubilised and eliminated with the discarded soaking medium (Frias *et al.*, 2000). Previous studies demonstrated that soaking could increase the inactivation of trypsin inhibitors in lentils, soybean and pea up to 3.6 % after soaked for 12 h (Hefnawy, 2011; Xu and Chang, 2008; Osman, 2007; Egounlety and Aworh, 2003).

Current findings of increased in trypsin and chymotrypsin inhibitory activity, and reduction in AIA after soaking period were in agreement with published studies of Embaby (2010) and Wang *et al*. (2008). They have reported a significant increment of trypsin inhibitors in soaked lupin and peas during prolonged soaking. Martín-Cabrejas *et al.* (2009) reported that the trypsin and chymotrypsin inhibitory activity of chickpea, lentil, white bean and pink-mottled cream bean significantly increased but α-amylase inhibitory activity a significantly reduced after soaking. This is because soaking might have caused retention of inhibitors in soaked legumes in which the structure of the intact seed could possibly limit the removal of inhibitors in an aqueous environment (Shi *et al.*, 2017; Wang *et al.*, 2008). The increased of inhibitors in soaked legumes could also due to low leaching-out effect during hydration, in which the loss of inhibitors to the soaking water was lower than other seed constituents such as soluble phenolic compounds (Shi *et al.*, 2017; Martín-Cabrejas *et al.*, 2009).

This had been observed in the soaking water of the legumes as the water was slightly coloured and the colour intensity was increased with the soaking period that indicated the leaching out of phenolic compounds. Besides, the above

plausible explanation of these results also showed that the removal of enzyme inhibitors is limited during soaking process regardless the soaking period, hence the soaking process could be taken as a pre-process before combine with other process to remove them effectively. The suitable soaking period varied from legumes to legumes, it could be determined by the softness of the cotyledon. The legumes should soak until soft as felt between the fingers to reduce the leaching out effect of soluble phenolic compounds.

4.3.1.2 Effect of soaking on protein content

In current study, the effect of soaking on the protein content of tested legumes was first conducted via Bradford method, and then verified with Kjeldahl method. Bradford method is a quick microassay used for sodium hydroxide soluble protein determination, however, this method only able to detect proteins containing amino acids such as arginine, lysine, phenylalanine, tryptophan, tyrosine, and histidine. Bradford method is based on protein-dye binding with direct detection of the colour change associated with the bound dye. Kjeldahl method involves the process of digestion, distillation and titration should be a more comprehensive determination of crude protein as this is based on the measurement of nitrogen content. A nitrogen conversion factor 6.25 is then used to estimate the crude protein content.

There was a significant difference in the protein content determined via Bradford method in the interaction between legumes and the soaking period (p<0.001). The protein content of the soaked legumes determined via Bradford method is presented in Table 4.4. The protein content of all soaked legumes was significantly reduced with a range of 63 % to 84 % after 6 h and 12 h of soaking.

Interestingly the values for all legumes at 6 h and 12 h were similar a range 1.96 – 2.14 g/100g DW whilst at the raw samples their range was 5.70 – 13.55 g/100g DW. This could be related to the limitation of using Bradford reagent as soaking might have changed the ion charged of the protein and losing its binding sensitivity (Olson and Markwell, 2007). Soaked SB and AB had the greatest reduction in protein content as they reduced from 13.55 g/100g DW to 0.67 g/100g DW and 13.21 g/100g DW to 0.54 g/100g DW respectively, while CP had the smallest reduction when compared to raw legumes. After prolong soaking to 24 h, only of 4 % to 21 % Bradford reagent detectable protein remained in soaked legumes.

Interestingly, current finding suggested that after declining to their lowest values at 18 h the protein content of legumes had increased by approximately 2-fold at 24 h, except for BG. To explore this finding further, due to the specificity of Bradford method the Kjeldahl method was conducted to verify the protein content of these soaked legumes. Aforementioned, Bradford reagent tends to bind to the selected amino acids such as arginine, lysine, phenylalanine, tryptophan and tyrosine rather than total crude protein.

To our knowledge, there is no studied reported on protein content of soaked legumes determined using Bradford method. In this study, the result suggested that amino acid such as arginine, lysine, histidine, phenylalanine, tryptophan, and tyrosine residues might have leached out into the soaking water.

Table 4.4 The interaction of legume x soaking duration in the protein content determined via Bradford method

Legume types	Protein content (g/100g DW)				
	Raw	Soaked 6 h	Soaked 12 h	Soaked 18 h	Soaked 24 h
SB	13.55 ± 0.38 ^{a}	2.14 ± 0.01 ^{h}	2.02 ± 0.01 ^{hij}	0.67 ± 0.02 ^{no}	1.29 ± 0.00 ^{k}
MB	12.75 ± 0.34 ^{b}	2.08 ± 0.00 ^{hij}	2.10 ± 0.01 ^{hi}	0.62 ± 0.01 ^{o}	1.28 ± 0.00 ^{k}
AB	13.21 ± 0.16 ^{a}	2.07 ± 0.00 ^{hij}	2.11 ± 0.01 ^{hi}	0.54 ± 0.01 ^{p}	1.29 ± 0.00 ^{k}
CP	5.70 ± 0.20 ^{g}	2.09 ± 0.01 ^{hi}	1.96 ± 0.01 ^{ij}	0.73 ± 0.01 ^{mn}	1.21 ± 0.00 ^{kl}
BEP	6.67 ± 0.10 ^{e}	2.09 ± 0.01 ^{hi}	2.13 ± 0.01 ^{h}	0.52 ± 0.01 ^{p}	1.30 ± 0.00 ^{k}
HB	5.92 ± 0.09 ^{f}	1.96 ± 0.02 ^{ij}	1.98 ± 0.01 ^{ij}	0.77 ± 0.02 ^{m}	1.21 ± 0.00 ^{kl}
PP	6.91 ± 0.11 ^{d}	2.08 ± 0.01 ^{hij}	2.08 ± 0.01 ^{hij}	0.64 ± 0.01 ^{o}	1.28 ± 0.00 ^{k}
BG	9.37 ± 0.06 ^{c}	1.96 ± 0.01 ^{ij}	1.98 ± 0.01 ^{ij}	1.94 ± 0.06 ^{j}	1.16 ± 0.01 ^{l}
P value Legume			p<0.001		
P value Soaking duration			p<0.001		
P value Legume x Soaking duration			p<0.001		

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root)
Values are presented in mean ± SEM (n=9), back transformed of square root values
Values with different letters are significantly different at p<0.001

When the crude protein content of soak legumes was further verified using Kjeldahl method (Table 4.5) an interesting observation was recorded. There are no replicates in this observation thus there is no statistical comparison has been done. Five of the eight soaked legumes demonstrated increased protein content relative to the raw legumes after soaking for 24 h, rather than a reduction. Soaked MB at 6 – 24 h showed 6.5 % to 12 % of increment in protein content, while AB showed 1.3 % to 5 % increment when soaked from 12 – 24 h. For HB, 2.3 % to 4 % of increment were only observed after soaking for 18 – 24 h. Soaked CP and BEP only showed 7.7 % and 13.8 % increment at 6 h and 12 h respectively, follow by reduction after prolong soaking treatment. Only PP and BG showed 3.6 % to 12 % reduction after soaking treatment.

Table 4.5 The crude protein in legumes soaked in water determined via Kjeldahl method

Legume types	Crude protein content (g/100g DW)				
	Raw	Soaked 6 h	Soaked 12 h	Soaked 18 h	Soaked 24 h
SB	56.15	54.74	56.26	56.34	56.48
MB	35.77	40.44	38.95	38.74	38.22
AB	37.39	36.20	39.27	37.90	39.13
CP	33.81	36.64	33.53	35.07	34.81
BEP	38.16	38.41	44.28	36.91	38.04
HB	39.43	38.54	27.80	40.36	41.08
PP	39.09	38.63	38.12	37.68	38.68
BG	35.13	30.94	33.21	32.80	34.00

Note:
Only one analysis was performed for verification purpose (n=1)

Majority of the legumes showed increase in crude protein content is in the agreement with studies done by Wang *et al.* (2008) and El-Adawy *et al.* (2000) on field pea and lupin soaked for 24 h and 13 h. These studies suggested that the crude protein content of lupin was increased 2.3 % after soaked for 13 h and further increased 2.6 % for 24 h-soaked field pea may be attributed to the loss of soluble solids during soaking.

In contrast, the crude protein content of PP and BG decreased with the increased of soaking period was in accordance to those reports by Agume *et al.* (2017) and Abd El-Hady and Habiba (2003). In their studies, the crude protein content of soybean were decreased by 18.8 % after soaked for 24 h and further decreased 19.5 % after soaked for 72 h suggested that the water soluble proteins had leached to the soaking water which lead to a reduction of protein content (Luo *et al.*, 2009; Mumba *et al.*, 2004).

There is conflicting published data on the crude protein content of soaked legumes from the above reported researchers (Agume *et al.* 2017; Wang *et al.*, 2008; Abd El-Hady and Habiba, 2003; El-Adawy *et al.*, 2000). Some of them reported the increased of crude protein under the process of soaking and vice versa. It can be noticed that the crude protein content of different legumes behaved differently even under the same soaking medium and soaking temperature.

4.3.2 Wet heating

Dry legumes are usually processed by common thermal treatment including wet heating, simple boiling, roasting or frying. These processes depletion of ANF thereby improving the nutritional quality (Khattab and Arntfield, 2009; Rehman and Shah, 2005). Wet heating could change various physical characteristic and alter the chemical compositions of dry legumes, such as protein denaturation and browning, losses of vitamin content and starch gelatinisation. These would potentially alter the sensory characteristic such as colour, flavour, and texture, either advantageously or adversely (Lewis and Jun, 2011; Aguilera *et al.*, 2009; Xu and Chang, 2008; De Almeida Costa *et al.*, 2006). In this section, the dried legumes were soaked in water and then heated at 50°C and 100°C for either 30 min or 60 min to reduce the enzyme inhibitors.

4.3.2.1 Effect of wet heating on enzyme inhibitors

There was a significant difference in the trypsin inhibitory activity (TIA), chymotrypsin inhibitory activity (CIA) and α-amylase inhibitory activity (AIA) in the interaction between legumes and the wet heating condition (p<0.001). The effect of wet heating treatment in reducing the trypsin, chymotrypsin and α-amylase inhibitory activity are shown in Table 4.6, 4.7 and 4.8. In general, the wet heating treatment had markedly increased the trypsin inhibitory activity in tested legumes. While the heating effect on chymotrypsin and α-amylase inhibitory was varied depend on the legumes tested.

There was a significant difference of trypsin inhibitory activity (TIA) in the legumes with the wet heating condition (p<0.001). As shown in Table 4.6, the trypsin inhibitory activity (TIA) of all tested legumes had increased significantly (p<0.001)

after wet heated at 50°C and 100°C for 30 min. The raw legumes possessed the lowest TIA ranging from 0.042 to 0.171 TIA unit/mg. Interestingly, no detectable TIA was found for BEP, HB and BG. When heating at 50°C was prolonged to 60 min, all tested legumes showed further reduction in TIA. For heated SB, MB, and CP their TIA were similar to the TIA raw legumes. When heating at 100°C was prolonged to 60 min, four legumes (MB, CP, HB, PP) were heat stable and showed no change in TIA. Whereas BG showed 58 % reduction in TIA, and three legumes (SB, AB, BEP) showed increased TIA.

There was a significant difference of chymotrypsin inhibitory activity (CIA) in the legumes with the wet heating condition (p<0.001). As shown in Table 4.7, the chymotrypsin inhibitory activity (CIA) of four legumes (MB, CP, BEP, HB) increased significantly (p<0.001) after heating at 50°C for 30 min, except for BG which decreased by of 35 %. Whereas no significant change in CIA was detected for SB, AB, and PP. When heating duration was prolonged to 60 min, only SB, AB and PP showed further increase in CIA whilst MB, BEP, and HB decreased were still significantly higher than raw legumes, however BG reduced to a not detectable level. No significant change was detected for the CP.

The majority of legumes heated at 100°C for 30 min decreased CIA activity compared to raw legumes with a range of a 12 % decrease to not detectable level. The except were for CP, BEP, and HB which had an increase in the range 1.4 % to 26 % relative to the raw legumes. When heating duration was prolonged to 60 min, the CIA of MB increased of 15 % whilst SB decreased of 41 % relative to the raw legumes. For the remaining legumes, no change in CIA was detected for prolonged heating duration.

There was a significant difference of α-amylase inhibitory activity (AIA) in the legumes with the wet heating condition (p<0.001). As shown in Table 4.8, the α-amylase inhibitory activity (AIA) of five legumes (MB, AB, CP, BEP, BG) reduced significantly (p<0.001) after heating at 50°C for 30 min, except for SB that showed an increment of 56 %. Whereas, no significant change in AIA was detected for HB and PP. When the heating duration was prolonged to 60 min, only SB, MB, and BG showed further increased in AIA whist PP decreased relative to 30 min. For the remaining legumes, no change in AIA was detected for prolonged heating duration.

When the heating temperature was increased to 100°C for 30 min, all tested legumes showed increased AIA in the range of 14 % to 76 % when compared to raw legumes, except for BG that which was reduced by 52 %. When heating duration was prolonged to 60 min, no significant changes was detected for all the legumes relative to 30 min.

Table 4.6 The interaction of legume x wet heating condition in the trypsin inhibitory activity

Legume types	Trypsin inhibitory activity (TIA unit/mg)				
	Raw	50°C, 30 min	50°C, 60 min	100°C, 30 min	100°C, 60 min
SB	0.060 ± 0.013^{klm}	0.198 ± 0.015^{gh}	0.050 ± 0.008^{klm}	0.217 ± 0.009^{fgh}	0.339 ± 0.014^{cd}
MB	0.171 ± 0.012^{hi}	1.387 ± 0.044^{a}	0.117 ± 0.009^{ij}	0.235 ± 0.006^{fgh}	0.198 ± 0.009^{gh}
AB	0.069 ± 0.008^{jkl}	0.335 ± 0.025^{cd}	0.174 ± 0.010^{hi}	0.185 ± 0.009^{h}	0.457 ± 0.039^{b}
CP	0.078 ± 0.006^{jkl}	0.281 ± 0.021^{def}	0.107 ± 0.014^{jk}	0.237 ± 0.010^{fgh}	0.267 ± 0.015^{efg}
BEP	nd^{m}	0.489 ± 0.021^{b}	0.120 ± 0.011^{ij}	0.211 ± 0.010^{fgh}	0.356 ± 0.009^{c}
HB	nd^{m}	0.231 ± 0.013^{fgh}	0.100 ± 0.011^{jk}	0.198 ± 0.009^{gh}	0.172 ± 0.015^{hi}
PP	0.042 ± 0.006^{lm}	0.256 ± 0.014^{efg}	0.107 ± 0.009^{jk}	0.200 ± 0.008^{gh}	0.198 ± 0.011^{gh}
BG	nd^{m}	0.311 ± 0.021^{cde}	0.231 ± 0.018^{fgh}	0.089 ± 0.010^{jkl}	0.037 ± 0.008^{lm}
P value Legume types			$p<0.001$		
P value Wet heating condition			$p<0.001$		
P value Legume types x Wet heating condition			$p<0.001$		

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root)
Values are presented in mean ± SEM (n=9), back transformed of square root values
Values with different letters are significantly different at $p<0.001$
nd represent not detectable

Table 4.7 The interaction of legume x wet heating condition in the chymotrypsin inhibitory activity

Legume types	Chymotrypsin inhibitory activity (CIA unit/mg)				
	Raw	50°C, 30 min	50°C, 60 min	100°C, 30 min	100°C, 60 min
SB	1.53 ± 0.03 ^{gh}	1.56 ± 0.04 ^{fgh}	1.84 ± 0.09 ^{def}	0.92 ± 0.11 ^{mno}	0.79 ± 0.05 ^{nop}
MB	1.71 ± 0.08 ^{fg}	3.45 ± 0.06 ^{a}	2.98 ± 0.05 ^{b}	1.35 ± 0.06 ^{hij}	2.02 ± 0.08 ^{d}
AB	1.07 ± 0.05 ^{klm}	1.16 ± 0.08 ^{jklm}	1.72 ± 0.05 ^{efg}	0.32 ± 0.03 ^{r}	0.96 ± 0.12 ^{lmno}
CP	0.49 ± 0.04 ^{q}	0.77 ± 0.09 ^{nop}	0.78 ± 0.09 ^{nop}	0.67 ± 0.07 ^{pq}	0.64 ± 0.07 ^{pq}
BEP	1.15 ± 0.07 ^{jklm}	2.41 ± 0.06 ^{c}	2.00 ± 0.05 ^{de}	1.16 ± 0.05 ^{jkl}	1.08 ± 0.05 ^{klm}
HB	0.74 ± 0.05 ^{nop}	1.47 ± 0.05 ^{ghi}	1.11 ± 0.06 ^{jklm}	0.97 ± 0.04 ^{lmn}	0.84 ± 0.06 ^{nop}
PP	0.78 ± 0.04 ^{nop}	0.83 ± 0.09 ^{nop}	1.26 ± 0.05 ^{ijk}	0.69 ± 0.04 ^{opq}	0.64 ± 0.05 ^{pq}
BG	0.77 ± 0.04 ^{nop}	0.50 ± 0.06 ^{q}	nd ^{s}	nd ^{s}	nd ^{s}
P value Legume			p<0.001		
P value Wet heating condition			p<0.001		
P value Legume x Wet heating condition			p<0.001		

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root)
Values are presented in mean ± SEM (n=9), back transformed of square root values
Values with different letters are significantly different at p<0.001
nd represent not detectable

Table 4.8 The interaction of legume x wet heating condition in the α-amylase inhibitory activity

Legume types	α-amylase inhibitory activity (AIA unit/mg)				
	Raw	50°C, 30 min	50°C, 60 min	100°C, 30 min	100°C, 60 min
SB	1.991 ± 0.149 ^{d}	4.553 ± 0.112 ^{c}	5.488 ± 0.101 ^{b}	8.445 ± 0.185 ^{a}	7.984 ± 0.059 ^{a}
MB	0.144 ± 0.004 ^{ij}	0.083 ± 0.001 ^{qrs}	0.099 ± 0.001 ^{no}	0.169 ± 0.005 ^{h}	0.169 ± 0.005 ^{h}
AB	0.124 ± 0.002 ^{kl}	0.089 ± 0.003 ^{pqr}	0.097 ± 0.001 ^{nop}	0.185 ± 0.007 ^{h}	0.185 ± 0.007 ^{h}
CP	0.088 ± 0.001 ^{pqr}	0.076 ± 0.004 ^{stu}	0.073 ± 0.003 ^{tu}	0.259 ± 0.006 ^{f}	0.259 ± 0.006 ^{f}
BEP	0.181 ± 0.004 ^{h}	0.127 ± 0.001 ^{kl}	0.136 ± 0.003 ^{jk}	0.319 ± 0.008 ^{e}	0.319 ± 0.008 ^{e}
HB	0.080 ± 0.000 ^{rst}	0.088 ± 0.001 ^{opqr}	0.094 ± 0.002 ^{opq}	0.161 ± 0.005 ^{hi}	0.161 ± 0.005 ^{hi}
PP	0.074 ± 0.004 ^{stu}	0.081 ± 0.001 ^{qrs}	0.069 ± 0.001 ^{u}	0.121 ± 0.007 ^{lm}	0.121 ± 0.007 ^{lm}
BG	0.228 ± 0.013 ^{g}	0.167 ± 0.004 ^{h}	0.213 ± 0.004 ^{g}	0.108 ± 0.001 ^{mn}	0.116 ± 0.001 ^{lm}
P value Legume types			p<0.001		
P value Wet heating condition			p<0.001		
P value Legume types x Wet heating condition			p<0.001		

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root)
Values are presented in mean ± SEM (n=9), back transformed of square root values
Values with different letters are significantly different at p<0.001

There is a general consensus that proper wet heating treatments could inactivate heat labile ANFs and increase the availability of protein thereby improve the protein quality (Hefnawy, 2011). In this study, the increased of heating temperature to 100°C had resulted in reduced chymotrypsin inhibitory activity. However, for trypsin and α-amylase inhibitory activities there was an increase at 100°C when using the assays described in this project. As described in Morrison *et al*. (2007) study these enzyme inhibitors could be heat stable. The rigid and compact trypsin inhibitor protein structure is stabilised by seven disulphide linkages which allows it to be resistant to being denatured at high temperature (Sierra *et al*., 1999).

Traditional wet heating process always focusses on simple boiling, using 100°C to process the dried legume before consumption. In this study, 50°C was also used with the aim of reducing energy usage whilst increasing the legumes' palatability and reducing the enzyme inhibitors. Unfortunately, 50°C increased TIA and CIA in the majority of the legumes particularly with shorter incubations (30 min). Wati *et al*. (2010) suggested when the temperature increased from 50 to 70°C, the heat causes the trypsin inhibitor to loosen its compact structure which is normally stabilised by numerous of disulphide bonds. However, these conformational changes are reversible when cooled down to 25°C. Therefore inhibitors possibly possess some degree of flexibility that allows them to regain their original conformation, and inhibitory activity immediately after being heated (Prasad *et al*., 2010).

In brief, the low temperature wet heating (50°C) with prolonged heating period (60 min) seems to be the best process method among the four to cook the dried

legume even though the trypsin inhibitors are higher than the unprocessed legumes. From a practical standpoint, the low temperature wet heating having more advantages than the high temperature (100°C) with prolonged heating period because high heat wet heating could induce chemical changes such as Maillard reaction which are associated with nutrient damage or formation of undesirable substances that maybe potentially problematic for health. This Maillard reaction between polysaccharides and protein can occur even under wet heating although the browning effect may not be seen (Han *et al.*, 2017). Study done by Zhang *et al.* (2014) showed that the degree of browning effect occurred in soybeans was increased with time at wet heating (90°C). The formation of Maillard reaction products such as high carboxymethyl lysine and acrylamide during processing not only affect protein digestibility but also could promote diabetes, cardiovascular disease and induce cancer (Tamanna and Mahmood, 2015; Seiquer *et al.*, 2006). Thus, the determination of appropriate wet heating temperature and duration is a key aspect to provide consumer some insight in improving the overall nutritional value of legumes.

4.3.2.2 Effect of wet heating on protein content

There was a significant difference in the protein content determined via Bradford method in the interaction between legumes and the wet heating condition (p<0.001). The protein content of wet heated legumes determined via Bradford method is presented in Table 4.9. Using this assay, the detectable protein content of all tested legumes was significantly reduced from 66 % to 85 % after heated at 50°C for 30 min. Whilst there is no significant change when heating duration was prolonged to 60 min. When heating temperature 100°C for 30 min was used, a significant reduction in the protein content by 72 % to 89 % was observed when

compared to raw legumes. All tested legumes showed further reduction to 73 % to 93 % after prolonged heating for 60 min. Aforementioned in soaking effect, the similar range of detectable protein content range 1.89 – 2.11 g/100g DW after heated at 50°C could cause by limitation of Bradford reagent as heating might have changed the ion charged of the protein and losing its binding sensitivity.

Current finding that heating treatment reduced protein content was in accordance with those reported by Candela *et al.* (1997) and Purcell and Wiliam (1982), who described how cooking led to a significant decrease in all amino acids in kidney bean, chickpea, lentils and sweet potatoes. During cooking, the heat will initiate a partial degradation of certain amino acid causing a reduction in protein content (Clawson and Taylor, 1993). Furthermore, severe heating can limit protein digestion and amino acid availability due to the modification of primary protein structure (Pedrosa *et al.*, 2015). The wet heating treatment could cause denaturation of protein and followed by aggregation of the unfolded molecules which eventually results in loss of protein structure and functionality (Carbonaro *et al.*, 1997).

Table 4.9 The interaction of legume x wet heating condition in the protein content determined via Bradford method

Legume types	Protein content (g/100g DW)				
	Raw	50°C, 30 min	50°C, 60 min	100°C, 30 min	100°C, 60 min
SB	13.55 ± 0.38 ^{a}	1.99 ± 0.01 ^{hijkl}	1.95 ± 0.01 ^{ijkl}	1.50 ± 0.01 ^{q}	1.14 ± 0.03 ^{s}
MB	12.75 ± 0.34 ^{b}	2.01 ± 0.01 ^{hijkl}	2.04 ± 0.01 ^{hijk}	1.89 ± 0.01 ^{klm}	0.89 ± 0.01 ^{t}
AB	13.21 ± 0.16 ^{a}	2.04 ± 0.01 ^{hijk}	2.09 ± 0.01 ^{hi}	1.91 ± 0.01 ^{klm}	1.51 ± 0.01 ^{q}
CP	5.70 ± 0.20 ^{g}	1.91 ± 0.01 ^{jklm}	1.89 ± 0.01 ^{lm}	1.59 ± 0.01 ^{pq}	1.30 ± 0.02 ^{r}
BEP	6.67 ± 0.10 ^{e}	2.11 ± 0.01 ^{h}	2.08 ± 0.01 ^{hi}	1.65 ± 0.01 ^{op}	0.87 ± 0.01 ^{t}
HB	5.92 ± 0.09 ^{f}	1.97 ± 0.01 ^{hijkl}	1.91 ± 0.01 ^{klm}	1.66 ± 0.01 ^{op}	1.60 ± 0.01 ^{pq}
PP	6.91 ± 0.11 ^{d}	2.06 ± 0.02 ^{hij}	2.02 ± 0.02 ^{hijkl}	1.75 ± 0.01 ^{no}	1.17 ± 0.01 ^{s}
BG	9.37 ± 0.06 ^{c}	1.96 ± 0.01 ^{hijkl}	1.92 ± 0.01 ^{jklm}	1.79 ± 0.01 ^{mn}	1.65 ± 0.01 ^{op}
P value Legume			p<0.001		
P value Wet heating condition			p<0.001		
P value Legume x Wet heating condition			p<0.001		

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root)
Values are presented in mean ± SEM (n=9), back transformed of square root values
Values with different letters are significantly different at p<0.001

When further study was carried out using Kjeldahl method (Table 4.10) the crude protein content is at least 4-fold higher than Bradford method. Again, there are no replicates in these values therefore no statistical comparison has been done. Only five legumes (SB, AB, BEP, PP, BG) showed reduction in crude protein content after heated at 50°C for 30 min, whereas the remaining three legumes showed increment of 2.3 % to 9.2 % after heat treatment. Prolonged heating duration to 60 min had caused reduction in protein content for all tested legumes, except AB.

When heating temperature was increased to 100°C for 30 min, a significant reduction in the protein content by 2.4 % to 12.8 % was observed for all tested legumes, except for MB and AB relative to raw legumes. When heating duration was prolonged to 60 min, all tested legumes showed increment in protein content by 0.4 % to 10.5 %, except for BG relative to 100°C for 30 min. Interestingly, only the crude protein content of mung bean was not affected by heat treatment at 50°C or 100°C for 30 min or 60 min.

Table 4.10 The crude protein of wet heated legumes determined via Kjeldahl method

Legume types	Crude protein content (g/100g DW)				
	Raw	50°C, 30 min	50°C, 60 min	100°C, 30 min	100°C, 60 min
SB	56.15	54.53	53.62	53.13	55.62
MB	35.77	37.32	36.56	38.51	41.47
AB	37.39	36.35	37.71	37.83	39.41
CP	33.81	34.62	33.12	29.47	32.93
BEP	38.16	37.32	36.49	36.76	40.20
HB	39.43	43.42	37.34	38.48	38.64
PP	39.09	35.60	38.91	35.20	37.68
BG	35.13	31.47	33.21	32.13	31.92

Note:
Only one analysis was performed for verification purpose (n=1)

The increment of crude protein content was in agreement with studies done by Olanipekun *et al.* (2015) and Doss *et al.* (2011) on kidney bean and jack bean. In their studies, the crude protein of jack bean was increased by 1.7 % after wet heated at 100°C for 20 min and further increased 13 % in kidney bean when prolonged to 3 h suggested that the wet heated treatment may break down the crude protein by unfolding the protein secondary, tertiary and quaternary structures to give primary structure which an apparent increase in crude protein content and potentially increases its digestibility (Awuah *et al.*, 2007).

4.3.3 Autoclaving

Autoclaving is one of the common moist heat treatment method involving high temperature (121°C) and high pressure (15 psi) (Habiba, 2002). The advantage of this pressure cooking is it requires less energy than boiling in an open pan and the heat can be distributed quickly and evenly (Güzel and Sayar, 2012). High pressure boiling and steaming is a promising technology and when applied to legumes has minor structural changes on small molecule nutrients such as amino acids, vitamins, and flavour compounds (He *et al.*, 2014). However, it can induce extensive changes in structure of macronutrients such as protein and carbohydrate that could improves their texture, palatability and may enhance the nutritional value by gelatinisation of starch, denaturation of proteins, increased nutrient availability as well as and inactivating heat labile toxic compounds and other enzyme inhibitors thereby provide high quality of food products (Wang *et al.*, 2009; Xu and Chang, 2008; Khatoon and Prakash, 2004). Besides, the use of high temperature under pressure also kills microorganisms and inactivates enzymes effectively to ensure legumes can be consumed safely (Verma *et al.*, 2012).

4.3.3.1 Effect of autoclaving on enzyme inhibitors

There was a significant difference in the trypsin inhibitory activity (TIA), chymotrypsin inhibitory activity (CIA) and α-amylase inhibitory activity (AIA) in the interaction between legumes and autoclaving (p<0.001). The effect of autoclaving treatment in reducing the trypsin, chymotrypsin, and α-amylase inhibitory activity are shown in Table 4.11, 4.12 and 4.13. In general, the autoclaving treatment had reduced the AIA in all tested legumes, and TIA and CIA in most of the legumes.

There was a significant difference of trypsin inhibitory activity (TIA) in the legumes with autoclaved (p<0.001). As shown in Table 4.11, only MB showed significant reduction of 30 % TIA after autoclaving. The TIA of five legumes (SB, AB, BEP, HB, PP) increased significantly (p<0.001) after autoclaved was unexpectedly. Initially, no detectable TIA was found in raw BEP, HB, and BG. However, after autoclaved, the TIA of BEP and HB had increased to 0.217 TIA unit/mg and 0.234 TIA unit/mg respectively. Only BG had no detectable TIA. Whereas the TIA of CP was not significantly affected by autoclaving.

Published studies reported that the autoclaved kidney bean, peas, lentils and tepary bean had either reduced or completely inactivated trypsin inhibitors (Hefnawy, 2011; Shimelis and Rakshit, 2007; Habiba, 2002; Osman *et al.*, 2002). These finding were in accordance to the inactivation of TIA in MB in the current study. Nevertheless, five autoclaved legumes tested in this study were detected with increased TIA. Dokka *et al.* (2015) suggested that the presence of crude fibre and phytic acid might safeguard the trypsin inhibitor against moist heat treatment.

There was a significant difference of chymotrypsin inhibitory activity (CIA) in the legumes with autoclaved (p<0.001). As shown in Table 4.12, five out of eight legumes showed significantly reduced of CIA after autoclaving the range of reduction being 24.55 % to 57.54 %, and for BG this was to non-detectable level. Only autoclaved CP showed increment in CIA by 49.66 %. Whereas the CIA of HB and PP were not affected by the autoclaving process.

The CIA content of tested legumes except for CP in current studied was in agreement with studies reported by Sathya and Siddhuraju (2015) and Tan *et al.* (1984) stated that autoclaved *Parkia* seeds and winged bean had either reduced or complete inactivated of chymotrypsin inhibitors.

There was a significant difference of α-amylase inhibitory activity in the legumes with autoclaved (p<0.001). As shown in Table 4.13, autoclaving process had successfully reduced the AIA in all tested legumes by a range of 29 % to 99 % relative to raw legume. Autoclaved BG had the highest AIA of 0.161 AIA unit/mg, whilst the rest had less than 0.042 AIA unit/mg.

Similar to CIA, several published studies reported that the autoclaved peas, mung bean, sorghum seeds and mucuna seeds had either reduced or complete inactivation of α-amylase inhibitors (Kavitha *et al.*, 2015; Habiba, 2002; Siddhuraju *et al.*, 1996; Mulimani and Supriya, 1993). These findings were in agreement to the reduction of AIA in the tested legumes.

Autoclaving process had reduced the CIA and AIA more effectively than TIA as majority of the tested legumes had decreased amount of CIA and AIA whilst TIA was only decreased in MB. The reduction in the inhibitors' activity could be because they are heat labile and degrade upon pressure cooking (Sathya and Siddhuraju, 2015).

Table 4.11 The interaction of legume x autoclaving in the trypsin inhibitory activity

Legume types	Trypsin inhibitory activity (TIA unit/mg)	
	Raw	Autoclaved
SB	0.060 ± 0.013^{gh}	0.092 ± 0.006^{def}
MB	0.171 ± 0.012^{c}	0.114 ± 0.014^{d}
AB	0.069 ± 0.008^{fgh}	0.099 ± 0.006^{de}
CP	0.078 ± 0.006^{efg}	0.096 ± 0.008^{def}
BEP	nd [i]	0.217 ± 0.013^{b}
HB	nd [i]	0.234 ± 0.011^{b}
PP	0.042 ± 0.006^{h}	0.407 ± 0.013^{a}
BG	nd [i]	nd [i]
P value Legume types	$p<0.001$	
P value Autoclaving	$p<0.001$	
P value Legume types x Autoclaving	$p<0.001$	

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root)
Values are presented in mean ± SEM (n=9), back transformed of square root values
Values with different letters are significantly different at $p<0.001$
nd represent not detectable

Table 4.12 The interaction of legume x autoclaving in the chymotrypsin inhibitory activity

Legume types	Chymotrypsin inhibitory activity (CIA unit/mg)	
	Raw	Autoclaved
SB	1.53 ± 0.03 [a]	0.98 ± 0.05 [cd]
MB	1.71 ± 0.08 [a]	1.18 ± 0.07 [b]
AB	1.07 ± 0.05 [bc]	0.60 ± 0.05 [gf]
CP	0.49 ± 0.04 [g]	0.98 ± 0.05 [cd]
BEP	1.15 ± 0.07 [bc]	0.49 ± 0.04 [g]
HB	0.74 ± 0.05 [ef]	0.56 ± 0.05 [g]
PP	0.78 ± 0.04 [e]	0.81 ± 0.05 [de]
BG	0.77 ± 0.04 [e]	nd [h]
P value Legume types	$p<0.001$	
P value Autoclaving	$p<0.001$	
P value Legume types x Autoclaving	$p<0.001$	

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root)
Values are presented in mean ± SEM (n=9), back transformed of square root values
Values with different letters are significantly different at $p<0.001$
nd represent not detectable

Table 4.13 The interaction of legume x autoclaving in the α-amylase inhibitory activity

Legume types	α-amylase inhibitory activity (AIA unit/mg)	
	Raw	Autoclaved
SB	1.991 ± 0.149^{a}	0.020 ± 0.001^{g}
MB	0.144 ± 0.004^{cd}	0.039 ± 0.002^{f}
AB	0.124 ± 0.002^{d}	0.042 ± 0.005^{f}
CP	0.088 ± 0.001^{e}	0.022 ± 0.001^{f}
BEP	0.181 ± 0.004^{bc}	0.042 ± 0.004^{g}
HB	0.080 ± 0.000^{e}	0.034 ± 0.002^{f}
PP	0.074 ± 0.004^{e}	0.037 ± 0.005^{f}
BG	0.228 ± 0.013^{b}	0.161 ± 0.002^{cd}
P value Legume types	$p<0.001$	
P value Autoclaving	$p<0.001$	
P value Legume types x Autoclaving	$p<0.001$	

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root)
Values are presented in mean ± SEM (n=9), back transformed of square root values
Values with different letters are significantly different at $p<0.001$

4.3.3.2 Effect of autoclaving on protein content

There was a significant difference in the protein content determined via Bradford method in the interaction between legumes and autoclaved (p<0.001). The protein content of autoclaved legumes determined via Bradford method is presented in Table 4.14. The protein content of all tested legumes was significantly reduced with a range of 88.56 % to 96.42 % after heated at 121°C for 15 min. Again, it is noticeable the autoclaved legumes had reduced to a similar concentration range 0.46 – 0.69 g/100g DW whilst at the raw samples their range was 5.70 – 13.55 g/100g DW. Aforementioned this is because the autoclaving have changed the ion charged of the protein and causing them to lose their binding sensitivity which is one of the limitation of Bradford reagent.

Hefnawy (2011) and Siddhuraju and Becker (2005) reported a 4.3 – 17.1 % reduction of amino acid particularly arginine, lysine, histidine, phenylalanine, tryptophan, tyrosine, methionine, and isoleucine residues in autoclaved lentils and mucuna seed. Therefore, in current study; the losses in protein content could be attributed to partial degradation of certain amino acid along with other nitrogenous compounds cause by the heat destruction.

Table 4.14 The interaction of legume x autoclaving in the protein content determined via Bradford method

Legume types	Protein content (g/100g DW)	
	Raw	Autoclaved
SB	13.55 ± 0.38^{a}	0.59 ± 0.01^{fgh}
MB	12.75 ± 0.34^{b}	0.54 ± 0.01^{hi}
AB	13.21 ± 0.16^{ab}	0.47 ± 0.01^{i}
CP	5.70 ± 0.20^{e}	0.64 ± 0.01^{fgh}
BEP	6.67 ± 0.10^{d}	0.46 ± 0.01^{i}
HB	5.92 ± 0.09^{e}	0.68 ± 0.01^{fg}
PP	6.91 ± 0.11^{d}	0.56 ± 0.01^{ghi}
BG	9.37 ± 0.06^{c}	0.69 ± 0.01^{f}
P value Legume types	$p<0.001$	
P value Autoclaving	$p<0.001$	
P value Legume types x Autoclaving	$p<0.001$	

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root)
Values are presented in mean ± SEM (n=9), back transformed of square root values
Values with different letters are significantly different at $p<0.001$

When further study was carried out using Kjeldahl method (Table 4.15) without replications, therefore no statistical comparison could be done. Only three legumes (HB, PP, BG) had reduced estimated crude protein with a range of 1.02 % to 7.52 % reduction. Whereas the remaining five legumes had an increase range of estimated crude protein of 0.29 % to 12.48 % after autoclaving.

Table 4.15 The crude protein of autoclaved legumes determined via Kjeldahl method

Legume types	Crude protein content (g/100g DW)	
	Raw	Autoclaved
SB	56.15	60.82
MB	35.77	40.87
AB	37.39	37.50
CP	33.81	34.30
BEP	38.16	40.27
HB	39.43	37.82
PP	39.09	36.15
BG	35.13	34.77

Note:
Only one analysis was performed for verification purpose (n=1)

To date, and to the extent of our knowledge, there was no existing research identifying an increasing of crude protein content in autoclaved legumes. A possible explanation for the increment of crude protein content could be due to the unfolding of protein secondary, tertiary, and quaternary structures making the more protein accessible for assessment of protein concentration. However the assessment of protein content had no replication therefore the increase seen after autoclaving could within the variance for the measurements, the values being within ~10 % of each other. The published studies either reported no significant changes or minimal reduction of crude protein content in autoclaved legumes (D'souza, 2013; Osman, 2007; Mubarak, 2005; Rehman *et al.*, 2001).

4.3.4 Dry freezing

Freezing serve as an ancient technology that have been used extensively to preserve foods such as meat, vegetables, fruits, and milk products (Chemat *et al.*, 2011; Archer, 2004). It plays a curial role in ensuring the safety of food products and retaining the quality such as taste, texture, and nutritional of foods better than any other preservation method over long storage periods (Mok *et al.*, 2015; George, 1993). This is because freezing not only prevents the growth of microbe but also avoid the usage of preservatives or additives in extending shelf life (Archer, 2004). If the freezing process has been done properly by controlling the freezing rate, least chemical changes will occur within the food and nutrients will be well preserved (Xue *et al.*, 2017). To our knowledge, there was no literature being published on the effect of dry freezing method towards the ANFs and nutritional component in legumes.

4.3.4.1 Effect of dry freezing on enzyme inhibitors

There was a significant difference in the trypsin inhibitory activity (TIA), chymotrypsin inhibitory activity (CIA) and α-amylase inhibitory activity (AIA) in the interaction between legumes and dry freezing treatment (p<0.001). The effect of 24 h dry freezing treatment at -20°C and -80°C in reducing the trypsin, chymotrypsin and α-amylase inhibitory activity are shown in Table 4.16, 4.17 and 4.18. In general, the effect of dry freezing on trypsin, chymotrypsin and α-amylase inhibitory activity varied according to the legumes tested.

There was a significant difference of trypsin inhibitory activity (TIA) in the legumes with dry freezing treatment (p<0.001). As showed in Table 4.16, the TIA of all tested legumes increased significantly (p<0.05) after stored at -20°C or -80°C,

except for CP and MB. The TIA of CP stored at -20°C was not significantly different from raw CP but was increased by -80°C and the TIA of MB stored at -80°C was not significantly different from raw MB, although after -20°C it was. As described previously early, no detectable TIA was found in raw BEP, HB, and BG. However, after dry-freezing storage, the TIA of BEP, HB, and BG had increased with values ranging from 0.159 TIA unit/mg to 0.243 TIA unit/mg after exposure to -20°C, and a range of 0.081 TIA unit/mg to 0.157 TIA unit/mg after -80°C.

There was a significant difference of chymotrypsin inhibitory activity (CIA) in the legumes with dry freezing treatment (p<0.001). As shown in Table 4.17, only SB and PP showed significant reduction of 12.27 % and 26.61 % CIA after stored at -20°C. After stored at -20°C MB, AB, BEP, and BG increased CIA in the range of 22.26 % to 52.91 %. Whereas, the CIA of CP and HB were not affected by the freeze storage. When temperature dropped to -80°C, the CIA of all treated legumes had no significant difference from the raw legumes, except for CP where CIA was detected.

There was a significant difference of α-amylase inhibitory activity (AIA) in the legumes with dry freezing treatment (p<0.001). As shown in Table 4.18, the dry freezing process significantly reduced the AIA in SB by 88 % irrespective of the freezing temperature. Whereas for BG stored at -20°C, no significant change in AIA was detected but at -80°C, it was reduced by 7.33 % when compared to raw BG. For the remaining legumes, the range of increases was in the range of 38 % to 76 % relative to raw legumes when considering the effects of both storage at -20°C and -80°C.

Table 4.16 The interaction of legume x dry freezing temperature in the trypsin inhibitory activity

Legume types	Trypsin inhibitory activity (TIA unit/mg)		
	Raw	-20°C	-80°C
SB	0.060 ± 0.013 ^{hi}	0.180 ± 0.018 ^{efg}	0.172 ± 0.011 ^{fg}
MB	0.171 ± 0.012 ^{fg}	0.546 ± 0.037 ^{a}	0.226 ± 0.010 ^{cd}
AB	0.069 ± 0.008 ^{hi}	0.191 ± 0.010 ^{defg}	0.319 ± 0.014 ^{b}
CP	0.078 ± 0.006 ^{hi}	0.091 ± 0.014 ^{h}	0.200 ± 0.008 ^{cdef}
BEP	nd ^{j}	0.176 ± 0.009 ^{efg}	0.156 ± 0.009 ^{g}
HB	nd ^{j}	0.243 ± 0.014 ^{c}	0.081 ± 0.005 ^{h}
PP	0.042 ± 0.006 ^{i}	0.219 ± 0.010 ^{cde}	0.283 ± 0.007 ^{b}
BG	nd ^{j}	0.159 ± 0.009 ^{fg}	0.157 ± 0.006 ^{fg}
P value Legume types		p<0.001	
P value Dry freezing temperature		p<0.001	
P value Legume types x Dry freezing temperature		p<0.001	

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root)
Values are presented in mean ± SEM (n=9), back transformed of square root values
Values with different letters are significantly different at p<0.001
nd represent not detectable

Table 4.17 The interaction of legume x dry freezing temperature in the chymotrypsin inhibitory activity

Legume types	Chymotrypsin inhibitory activity (CIA unit/mg)		
	Raw	-20°C	-80°C
SB	1.53 ± 0.03 ^{bc}	1.12 ± 0.10 ^{de}	1.23 ± 0.09 ^{d}
MB	1.71 ± 0.08 ^{b}	3.30 ± 0.09 ^{a}	2.99 ± 0.10 ^{a}
AB	1.07 ± 0.05 ^{de}	1.37 ± 0.11 ^{cd}	0.91 ± 0.08 ^{ef}
CP	0.49 ± 0.04 ^{hij}	0.52 ± 0.22 ^{ij}	nd ^{k}
BEP	1.15 ± 0.07 ^{de}	1.57 ± 0.07 ^{bc}	1.23 ± 0.07 ^{d}
HB	0.74 ± 0.05 ^{fgh}	0.63 ± 0.07 ^{ghi}	0.62 ± 0.08 ^{ghi}
PP	0.78 ± 0.04 ^{fg}	0.68 ± 0.07 ^{fghi}	0.33 ± 0.04 ^{j}
BG	0.77 ± 0.04 ^{fg}	1.64 ± 0.10 ^{bc}	0.59 ± 0.07 ^{ghi}
P value Legume		p<0.001	
P value Dry freezing temperature		p<0.001	
P value Legume x Dry freezing temperature		p<0.001	

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root)
Values are presented in mean ± SEM (n=9), back transformed of square root values
Values with different letters are significantly different at p<0.001
nd represent not detectable

Table 4.18 The interaction of legume x dry freezing temperature in the α-amylase inhibitory activity

Legume types	α-amylase inhibitory activity (AIA unit/mg)		
	Raw	-20°C	-80°C
SB	1.99 ± 0.15 [a]	0.23 ± 0.00 [fg]	0.23 ± 0.00 [ef]
MB	0.14 ± 0.00 [j]	0.27 ± 0.00 [d]	0.27 ± 0.00 [d]
AB	0.12 ± 0.00 [k]	0.25 ± 0.00 [de]	0.26 ± 0.00 [d]
CP	0.09 ± 0.00 [l]	0.21 ± 0.00 [gh]	0.21 ± 0.00 [h]
BEP	0.18 ± 0.00 [i]	0.30 ± 0.00 [b]	0.30 ± 0.00 [b]
HB	0.08 ± 0.00 [m]	0.29 ± 0.01 [bc]	0.27 ± 0.00 [cd]
PP	0.07 ± 0.00 [m]	0.31 ± 0.00 [b]	0.26 ± 0.00 [d]
BG	0.23 ± 0.01 [fg]	0.23 ± 0.00 [fg]	0.21 ± 0.00 [h]
P value Legume		$p<0.001$	
P value Dry freezing temperature		$p<0.001$	
P value Legume x Dry freezing temperature		$p<0.001$	

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root)
Values are presented in mean ± SEM (n=9), back transformed of square root values
Values with different letters are significantly different at $p<0.001$

Freezing can affect the food texture, especially in slow freezing encourages the formation of non-uniform ice crystal and produces larger ice crystals through condensation of solutes, this is to cell membrane breakage resulting in irreversible cell wall collapse and tissue breakage (Xue *et al.*, 2017; Mok *et al.*, 2015; Chassagne-Berces *et al.*, 2009). However, dried legumes have of relatively low water content and presumably there is a lower extent of structural damage caused by freezing (Li and Sun, 2002). Therefore, it is expected that the process of dry freezing might not be able to reduce the enzyme inhibitors effectively as the process tends to break open the structure of the material rather than denature it. The reasons for the unexpected large increase in TIA levels in dry frozen legumes of -20°C and -80°C remains obscure. The break open of legume structure might possibly allow more TIA was being extracted.

4.3.4.2 Effect of dry freezing on protein content

There was a significant difference in the protein content determined via Bradford method in the interaction between legumes and dry freezing treatment (p<0.001). The protein content of frozen legumes determined via Bradford method is presented in Table 4.19. The protein content of all tested legumes was significantly reduced in the range of 69 % to 86.96 % when examining storage of both at -20°C or -80°C. Only BG of all the legumes showed significant reduction in protein content when the temperature was reduced from -20°C to -80°C. The values for dry frozen legume at -20°C to -80°C were similar range 1.76 – 1.95 g/100g DW could be related to the limitation of Bradford reagent.

Surprisingly, the dry frozen legumes showed a drastic reduction of protein content availability particularly arginine, lysine, histidine, phenylalanine, tryptophan, and tyrosine regardless at temperature of -20°C or -80°C when compared to untreated legumes. This is because freezing is the pre-step of the freeze-drying process and it is known to be the best way to preserve almost all the nutrients in food (Zhang *et al.*, 2014). Theoretically, the process of dry frozen would not be expected to have such a big impact on the protein content, since the formation of ice crystallisation which will lead to structural damage not significant chemical damage such as strong denaturing of proteins.

Table 4.19 The interaction of legume x dry freezing temperature in the protein content determined via Bradford method

Legume types	Protein content (g/100g DW)		
	Raw	-20°C	-80°C
SB	13.55 ± 0.38 [a]	1.80 ± 0.01 [fg]	1.77 ± 0.01 [g]
MB	12.75 ± 0.34 [b]	1.87 ± 0.01 [fg]	1.91 ± 0.01 [fg]
AB	13.21 ± 0.16 [a]	1.93 ± 0.01 [fg]	1.95 ± 0.00 [f]
CP	5.70 ± 0.20 [e]	1.77 ± 0.01 [g]	1.78 ± 0.01 [fg]
BEP	6.67 ± 0.10 [d]	1.88 ± 0.01 [fg]	1.87 ± 0.01 [fg]
HB	5.92 ± 0.09 [e]	1.78 ± 0.01 [fg]	1.76 ± 0.01 [g]
PP	6.91 ± 0.11 [d]	1.88 ± 0.01 [fg]	1.92 ± 0.01 [fg]
BG	9.37 ± 0.06 [c]	1.84 ± 0.01 [fg]	1.77 ± 0.01 [fg]
P value Legume		$p<0.001$	
P value Dry freezing temperature		$p<0.001$	
P value Legume x Dry freezing temperature		$p<0.001$	

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root)
Values are presented in mean ± SEM (n=9), back transformed of square root values
Values with different letters are significantly different at $p<0.001$

Thus, further study was carried out using Kjeldahl method (Table 4.20); there are no replicates in this observation thus there is no statistical comparison has been done. Only two legumes (SB and AB) showed 3.47 % to 6.9 % of reduction in crude protein content after stored at -20°C. Whereas the remaining frozen legumes showed increases in the range of 0.12 % to 5.89 % crude protein content. When the temperature reduced further to -80°C, five legumes showed reduction in crude protein in the range of 3.33 % to 6 %. Whereas, MB, AB, and CP showed had an increase in the range 0.58 % to 2.25 % of crude protein content. Even so, the increased or decreased of crude protein content could be within the variance for the measurements since there is no replication in the crude protein assessment.

Table 4.20 The crude protein of dry frozen legumes determined via Kjeldahl method

Legume types	Crude protein content (g/100g DW)		
	Raw	-20°C	-80°C
SB	56.15	54.26	53.35
MB	35.77	36.63	36.36
AB	37.39	34.81	37.61
CP	33.81	33.85	34.59
BEP	38.16	38.91	35.87
HB	39.43	41.42	37.56
PP	39.09	39.92	37.51
BG	35.13	37.33	33.96

Note:
Only one analysis was performed for verification purpose (n=1)

Aforementioned, the nature of dried legume contained of low water content and presumably lower extend of structural damage caused by the formation of ice crystallisation. In this study, the nutritive value of crude proteins in most of the -20°C dry frozen legume and part of the -80°C dry frozen legume was found to be slightly higher than unprocessed legume. It was also noticeable that part of the dry frozen legumes had minor crude protein loss had been detected.

4.4 Summary

Current study revealed that the effective operational parameters that could reduce the enzyme inhibitors (trypsin, chymotrypsin, and α-amylase inhibitors) present in underutilised legumes are by soaking (6 h), wet heating (50°C for 60 min), autoclaving (121°C and 15 psi for 15 min) and dry freezing (-80°C), respectively. Under the optimum operational parameters, the underutilised legumes were able to retain their nutritive values in term of protein content. However, no single treatment was capable to completely eliminate the enzyme inhibitors in tested legumes.

CHAPTER 5

EFFECT OF COMBINED PROCESSING METHODS IN REDUCING ENYZME INHIBITORS PRESENT IN UNDERUTILISED LEGUMES

5.1 Introduction

In pervious Chapter 4, four operational parameters, namely soaking, wet heating, autoclaving and dry freezing were used to reduce enzyme inhibitors found in legumes. Nevertheless, findings suggested that the individual parameter were not able to reduce all enzyme inhibitors effectively, and very often the reduction efficiency was dependent on the legume. Hence, four processing methods tested in Chapter 4 had the strongest ability to reduce enzyme inhibitors and retain protein in legumes were chosen in current study:

(i) soaking for 6 h at 25°C

(ii) wet heating 50°C for 60 min

(iii) autoclaving at 121°C at 15 psi for 15 min

(iv) dry freezing at -80°C for 24 h

This chapter aim to optimise the removal of enzyme inhibitors while retaining nutrients by using combined processing method. For optimisation purpose, only three underutilised legumes (adzuki bean, mung bean, bambara groundnut) and soybean (control) were used.

The specific objectives of this chapter were:

- To investigate the combined processing method(s) based on any two of the above-mentioned methods to remove the enzyme inhibitors

- To investigate the effect of combine processing method on the protein content

The hypothesis for the research carried out in this chapter is the combined methods, which involve a combination of wet heating and autoclaving process, will completely eliminate the enzyme inhibitor in underutilised legumes without altering the protein content.

5.2 Experimental design

5.2.1 Combination of processing treatments

The dried legumes were purchased from the same single source. Similar to Chapter 3 and Chapter 4, all the dried legumes had not underwent dehulling process before subjected to the below combined processing method. Each of the combined processing methods was performed in triplicate for each type of legumes.

a) Soaking and wet heating (S+W)

The dried legumes were soaked in distilled water (ratio 1:10 (w/v)) for 6 h at room temperature (25°C) immediately followed by heating at 50°C for 60 min in the same soaking medium. After heating the associated liquid was poured away, the processed legumes were allowed to cool to room temperature and chilled overnight at 4°C, before being frozen at -80^{o}C for 24 h.

b) Soaking and autoclaving (S+A)

The dried legumes were soaked in distilled water (ratio 1:10 (w/v)) for 6 h at room temperature (25°C), immediately followed by autoclaving at 121°C, 15 psi for 15 min in the same soaking medium. After autoclaving the associated liquid was poured away, the processed legumes were allowed to cool to room temperature and chilled overnight at 4°C, before frozen at -80^{o}C for 24 h.

c) Soaking and dry freezing (S+D)

The dried legumes were soaked in distilled water (ratio 1:10 (w/v)) for 6 h at room temperature (25°C). The water was immediately drained, and the legumes were chilled overnight at 4°C before frozen at -80°C for 24 h.

d) Wet heating and autoclaving (W+A)

To dried legumes was added distilled water (ratio 1:10 (w/v)) which were then heated at 50°C for 60 min then immediately followed by autoclaving at 121°C, 15 psi for 15 min in the same medium. After autoclaving the associated liquid was poured away, the processed legumes were allowed to cool to room temperature and chilled overnight at 4°C, before frozen at -80^{o}C for 24 h.

e) Wet heating and dry freezing (W+D)

To dried legumes was added distilled water (ratio 1:10 (w/v)), this was then heated at 50°C for 60 min. The water was immediately drained, and the legumes were chilled overnight at 4°C before frozen at -80°C for 24 h.

f) Wet heating and soaking (W+S)

To dried legumes was added distilled water (ratio 1:10 (w/v)), this was then heated at 50°C for 60 min. The wet heated legumes in distilled water was allowed to cool to room temperature (25°C) under running tap water and followed by soaking for 6 h. After soaking the associated liquid was poured away and the processed legumes chilled overnight at 4°C before being frozen at -80^{o}C for 24 h.

g) Autoclaving and dry freezing (A+D)

To dried legumes was added distilled water (ratio 1:10 (w/v)), this was then autoclaved at 121°C, 15 psi for 15 min. The water was immediately drained, and the legumes were chilled overnight at 4°C before frozen at -80°C for 24 h.

h) Autoclaving and wet heating (A+W)

To dried legumes was added distilled water (ratio 1:10 (w/v)), this was then autoclaved at 121°C, 15 psi for 15 min. The autoclaved legumes in distilled water was allowed to cool to room temperature (25°C) under running tap water and followed by heating at 50°C for 60 min. After heating the associated liquid was poured away, the processed legumes were allowed to cool to room temperature and chilled overnight at 4°C before being frozen at -80^{o}C for 24 h.

i) Autoclaving and soaking (A+S)

To dried legumes was added distilled water (ratio 1:10 (w/v)), this was then autoclaved at 121°C, 15 psi for 15 min. The autoclaved legumes in distilled water was allowed to cool to room temperature (25°C) under running tap water and followed by soaking for 6 h. After soaking the associated liquid was poured away and the processed legumes chilled overnight at 4°C before being frozen at -80^{o}C for 24 h.

j) Dry freezing and soaking (D+S)

The dried legumes were frozen at -80°C for 24 h and immediately followed by soaking in distilled water (ratio 1:10 (w/v)) for 6 h at room temperature (25°C). After soaking the associated liquid was poured away and the processed legumes chilled overnight at 4°C before being frozen at -80^{o}C for 24 h.

k) Dry freezing and wet heating (D+W)

The dried legumes were frozen at -80°C for 24 h and immediately followed by heating in distilled water (ratio 1:10 (w/v)) at 50°C for 60 min. After heating the associated liquid was poured away and the processed legumes allowed to cool to room temperature and chilled overnight at 4°C before being frozen at -80^{o}C for 24 h.

l) Dry freezing and autoclaving (D+A)

The dried legumes were frozen at -80°C for 24 h followed by immediately autoclaving in distilled water (ratio 1:10 (w/v)) at 121°C, 15 psi for 15 min. After autoclaving the associated liquid was poured away and the processed legumes allowed to cool to room temperature and chilled overnight at 4°C before being frozen at -80^{o}C for 24 h.

All the frozen samples were lyophilised for 24 h using freeze-dryer (Alpha 1-4 LD plus, Christ). The lyophilised legumes were then ground into powder form using a miller without going through dehulling process. The fine powder was then to pass through a series of mesh sieves with the size of 1.68 µm, 1.18 µm and 0.85 µm. The powder that was stopped on 1.18 µm mesh sieve was collected and stored in 4°C chiller up to 2 weeks prior to analysis for enzyme inhibitors and nutrients.

5.2.2 Enzyme inhibitors and nutrient determination

The trypsin, chymotrypsin and α-amylase inhibitors present in the raw and processed dried samples were determined according to sections 2.3.1 – 2.3.2. The protein content of raw and processed dried samples was determined according to section 2.4.1a – 2.4.1b. In this study, both Bradford and Kjeldahl protein determination methods were used to determine the soluble amino acids in sodium hydroxide (NaOH) and total crude protein respectively. All assay was carried out in triplicate.

5.2.3 Statistical analysis

The results were presented as mean ± standard error mean (SEM) which had carried out in triplicate. IBM SPSS Statistics software (Version 22, IBM Corporation, USA) was used to perform two-way Analysis of Variance (ANOVA) on data sets and where this was statistical significant (p<0.05) subsequent post-hoc analysis was carried out using Duncan's Multiple Comparison test confidence intervals of 95 % with threshold for significance when p<0.05. Data was checked for normality using Shapiro-Wilk test, where stated, none normally distributed data was transformed (square root) before statistical analysis was performed. Data was checked for homogeneity of variance using the Levene test.

5.3 Results and discussion

For this chapter results are presented in two parts. Section 5.3.1 reports the effect of combined methods on enzyme inhibitors, whilst section 5.3.2 reported the effects of combined methods on protein content in underutilised legumes. The 4 optimised parameters had formed into 12 combined processing methods and these combined methods could possibility removed the enzyme inhibitors more effectively than the individual process. It is noteworthy that the processing sequences might be able to manipulate the efficiency in removal of anti-nutritional factors in legumes. Thus, the different sequence of combined processing method seems to be a necessity to examine their efficiency in removal of anti-nutritional factors in legumes.

5.3.1 Effect of combined methods on enzyme inhibitors

There was a significant interaction between combined treatments and legumes for the trypsin inhibitory activity (TIA), chymotrypsin inhibitory activity (CIA) and α-amylase inhibitory activity (AIA) (p<0.001). The effect of 12 combined methods on the trypsin, chymotrypsin and α-amylase inhibitors are shown in Table 5.1, 5.2, and 5.3. In general, the effect of these methods on the enzyme inhibitors are legume specific.

There was a significant difference of trypsin inhibitory activity (TIA) in the legumes with the combined treatments (p<0.001). Table 5.1 showed the TIA of the legumes treated with the 12 combined methods. For mung bean, eight methods have successfully reduced the TIA with a range of 31.6 – 83.6 %, with the greatest reduction by W+S method with only D+S method showed 21.2 % increase. For adzuki bean and bambara groundnut, majority of the treatments significantly

increased TIA with a range of 37.8 – 77.5 % and 2.2 – 15 %, respectively for each legume. Only D+A showed 100 % of reduction in TIA of adzuki bean to non-detectable activity, whilst no reduction of TIA was detected in bambara groundnut for this method. For soybean, only D+A showed reduction to non-detectable TIA, whilst S+A, S+D, W+A, W+D, D+S and D+W increased TIA with a range of 45.9 – 67.2 %. Whereas no significant change in TIA was detected for the remaining methods.

Table 5.1 The interaction of combine treatments x legumes in trypsin inhibitory activity

Treatments	Trypsin inhibitory activity (TIA unit/mg)			
	Soybean	Mung bean	Adzuki bean	Bambara groundnut
Raw	0.060 ± 0.013^{mnopq}	0.171 ± 0.012^{de}	0.069 ± 0.008^{klmnop}	nd^{r}
S+W	0.033 ± 0.008^{opqr}	0.083 ± 0.008^{ijklmn}	0.150 ± 0.008^{ef}	$0.100 \pm 0.012^{ghijklm}$
S+A	0.117 ± 0.017^{fghij}	0.072 ± 0.012^{jklmno}	0.061 ± 0.016^{lmnopq}	nd^{r}
S+D	0.183 ± 0.008^{bcde}	0.150 ± 0.000^{ef}	0.306 ± 0.026^{a}	0.139 ± 0.011^{efgh}
W+A	0.111 ± 0.016^{fghijk}	$0.100 \pm 0.012^{ghijklm}$	0.122 ± 0.009^{fghi}	nd^{r}
W+D	0.117 ± 0.024^{fghij}	0.178 ± 0.012^{cde}	0.200 ± 0.000^{bcd}	0.117 ± 0.014^{fghij}
W+S	0.033 ± 0.012^{opqr}	0.028 ± 0.009^{pqr}	0.111 ± 0.011^{fghijk}	nd^{r}
A+D	0.094 ± 0.013^{hijklm}	0.139 ± 0.018^{efgh}	0.144 ± 0.019^{efg}	nd^{r}
A+W	0.067 ± 0.014^{klmnop}	0.111 ± 0.011^{fghijk}	$0.100 \pm 0.008^{ghijklm}$	0.050 ± 0.012^{nopq}
A+S	0.061 ± 0.016^{lmnopq}	0.094 ± 0.013^{hijklm}	0.106 ± 0.010^{fghikl}	nd^{r}
D+S	0.150 ± 0.020^{ef}	0.217 ± 0.024^{bc}	0.228 ± 0.012^{b}	0.150 ± 0.014^{ef}
D+W	0.156 ± 0.051^{ef}	0.117 ± 0.017^{fghij}	0.172 ± 0.009^{cde}	0.033 ± 0.012^{opqr}
D+A	nd^{r}	$0.100 \pm 0.019^{ghijklm}$	nd^{r}	0.022 ± 0.009^{qr}
P value Treatments	$p<0.001$			
P value Legume	$p<0.001$			
P value Treatments x Legume	$p<0.001$			

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root); Values are presented in mean ± SEM (n=9), back transformed of square root values; Values with different letters are significantly different at $p<0.001$; nd represent not detectable.

Various combined treatments with different conditions have been attempted by researchers to inactivate the ANFs present in legumes (Luo and Xie, 2013; Hefnawy, 2011; Wang *et al.*, 2008; Shimelis and Rakshit, 2007; Vijayakumari *et al.*, 2007; Abd El-Hady and Habiba, 2003; Chau and Cheung, 1997). Most of these methods involve soaking legumes combined with another intervention, such as some sort of heating. Only limited number of studies were found which had used the S+A method, however due to the variation in treatment duration and medium used, it is hard to compare these findings directly. To author's knowledge, no relevant published literature is available for the remaining methods used in this study.

A study done by Abd El-Hady and Habiba (2003) showed TIA of various legumes reduced to undetectable level after a soaking and extrusion process. The studies reported by Luo and Xie (2013), Shimelis and Rakshit (2007) and Chau and Cheung (1997) showed that the trypsin inhibitors were reduced up to 50 % using the combined process of soaking and cooking and 75 % by using soaking and autoclaving. Using high heat, the TIA of pre-soaked legumes could be reduced effectively. This observation was in accordance with current finding in mung bean, adzuki bean and bambara groundnut under the treatment of S+A.

There was a significant difference of chymotrypsin inhibitory activity (CIA) in the legumes with the combined treatments (p<0.001). Table 5.2 shows the CIA of legumes treated with the 12 combined methods. Overall, six combined methods (S+A, W+A, A+D, A+W, A+S, D+A) were able to reduce the CIA of all four legumes however the range was 55.5 – 100 %. Only W+D increased CIA of all the legumes with a range of 17.4 – 37.9 %.

For adzuki bean, nine methods reduced the CIA significantly. Six methods, namely S+A, W+A, A+D, A+W, A+S and, D+A reduced the CIA completely, whereas three methods (W+S, D+S, and D+W) gave a reduction with a range of 28.9 – 49.5 %. Whereas S+W, S+D and W+D method did not change CIA. For bambara groundnut, seven methods reduced the CIA significantly. Four methods, namely W+A, A+D, A+W, and A+S reduced the CIA completely, whereas three methods (S+A, W+S, and D+A) had a 57.1 – 62.3 % range of reduction. Three methods (S+W, S+D, and W+D) showed increased CIA with a range of 37.9 – 51.3 %, whereas the remaining methods had no significant effect. For soybean, six methods reduced the CIA significantly. Only the A+D method reduced the CIA completely, whereas five methods (S+A, W+A, A+W, A+S, and D+A) exhibited 45.8 – 86.3 % range of reduction. Three methods (S+W, S+D, and D+W) increased CIA with a range of 23.8 – 49.2 %. Whereas the remaining methods had no significant change on CIA. For mung bean, seven methods (S+A, W+A, W+S, A+D, A+W, A+S, and D+A) reduced the CIA significantly with a range of 33.3 – 71.3 %. However, no complete reduction was detected. The methods S+D increased of CIA by 19.7 %. The remaining methods had no significant change on CIA.

Table 5.2 The interaction of combine treatments x legumes in chymotrypsin inhibitory activity

Treatments	Chymotrypsin inhibitory activity (CIA unit/mg)			
	Soybean	Mung bean	Adzuki bean	Bambara groundnut
Raw	1.53 ± 0.03^{efg}	1.71 ± 0.08^{cde}	1.07 ± 0.05^{hij}	0.77 ± 0.04^{klm}
S+W	2.01 ± 0.15^{bc}	1.61 ± 0.11^{def}	0.89 ± 0.08^{ijk}	1.58 ± 0.11^{ef}
S+A	0.47 ± 0.06^{opqrs}	0.65 ± 0.07^{klmno}	nd^{u}	0.29 ± 0.07^{rst}
S+D	3.01 ± 0.14^{a}	2.13 ± 0.13^{b}	0.81 ± 0.06^{jkl}	1.54 ± 0.12^{efg}
W+A	0.26 ± 0.07^{st}	0.73 ± 0.06^{klm}	nd^{u}	nd^{u}
W+D	1.88 ± 0.20^{bcde}	2.07 ± 0.13^{bc}	1.33 ± 0.11^{fgh}	1.24 ± 0.04^{gh}
W+S	1.74 ± 0.14^{cde}	1.14 ± 0.11^{hi}	0.54 ± 0.09^{mnopq}	0.33 ± 0.06^{pqrst}
A+D	nd^{u}	0.49 ± 0.10^{nopqr}	nd^{u}	nd^{u}
A+W	0.37 ± 0.09^{pqrst}	0.76 ± 0.12^{klm}	nd^{u}	nd^{u}
A+S	0.83 ± 0.09^{jk}	0.57 ± 0.12^{lmnop}	nd^{u}	nd^{u}
D+S	1.28 ± 0.14^{gh}	1.96 ± 0.11^{bcd}	0.67 ± 0.13^{klmno}	0.66 ± 0.13^{klmno}
D+W	2.03 ± 0.05^{bc}	1.96 ± 0.11^{bcd}	0.76 ± 0.11^{klm}	0.92 ± 0.10^{ijk}
D+A	0.21 ± 0.05^{t}	0.74 ± 0.13^{klmn}	nd^{u}	0.32 ± 0.06^{qrst}
P value Treatments		$p<0.001$		
P value Legume		$p<0.001$		
P value Treatments x Legume		$p<0.001$		

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root); Values are presented in mean ± SEM (n=9), back transformed of square root values; Values with different letters are significantly different at $p<0.001$; nd represent not detectable.

Cooking of pre-soaked legumes could completely remove the CIA (Shi *et al.*, 2017). In current study, the thermal processes used to heat the pre-soaked legumes were wet heating and autoclaving, but not ordinary cooking that boiled the legumes in water for a certain period until tenderness of seeds was achieved (Kaushik *et al.*, 2010). Under the treatment of S+A, the CIA of pre-soaked legumes had the maximum reduction especially in adzuki bean. Similar results have also been reported by Siddhuraju and Becker (2005) and Mulimani and Paramjyothi (1994) in pre-soaked red gram and mucuna beans, where the CIA was completely destroyed under the treatment of cooking and autoclaving.

There was a significant difference of α-amylase inhibitory activity (AIA) in the legumes with the combined treatments (p<0.001). Table 5.3 showed the AIA of the legumes treated with the 12 combined methods. Overall, six combine methods (S+A, W+A, A+D, A+W, A+S, D+A) were able to reduce the AIA of all four legumes with a range of 69.6 – 99.3 %. This finding was similar to CIA. However, no complete reduction of AIA was detected with any method with any legume. All legumes showed significant reduction with a range of 22.8 – 99.3 % after treatment with the 12 methods, except for soybean.

For soybean, six methods (S+A, W+A, A+D, A+W, A+S, and D+A) reduced the AIA significantly with a range of 98.4 – 99.3 %. Only S+D method had no significant change on AIA, whereas the remaining five methods increased AIA with a range of 22.7 – 59.7 %.

Table 5.3 The interaction of combine treatments x legumes in α-amylase inhibitory activity

Treatments	α-amylase inhibitory activity (AIA unit/mg)			
	Soybean	Mung bean	Adzuki bean	Bambara groundnut
Raw	1.991 ± 0.149 ^{c}	0.144 ± 0.004 ^{e}	0.124 ± 0.002 ^{ef}	0.228 ± 0.013 ^{d}
S+W	2.711 ± 0.266 ^{b}	0.058 ± 0.003 ^{j}	0.056 ± 0.001 ^{j}	0.031 ± 0.003 ^{nop}
S+A	0.014 ± 0.002 ^{u}	0.017 ± 0.002 ^{t}	0.019 ± 0.003 st	0.021 ± 0.001 ^{rs}
S+D	1.958 ± 0.138 ^{c}	0.080 ± 0.004 ^{h}	0.077 ± 0.004 ^{hi}	0.047 ± 0.008 ^{k}
W+A	0.028 ± 0.000 ^{opq}	0.027 ± 0.000 ^{opq}	0.027 ± 0.001 ^{opq}	0.029 ± 0.003 ^{opq}
W+D	2.953 ± 0.111 ^{b}	0.065 ± 0.004 ^{ij}	0.061 ± 0.003 ^{j}	0.068 ± 0.007 ^{ij}
W+S	2.576 ± 0.056 ^{b}	0.082 ± 0.004 ^{h}	0.059 ± 0.005 ^{j}	0.057 ± 0.003 ^{j}
A+D	0.018 ± 0.002 st	0.024 ± 0.002 ^{qr}	0.034 ± 0.002 ^{lmno}	0.023 ± 0.003 ^{r}
A+W	0.023 ± 0.002 ^{r}	0.036 ± 0.002 ^{klmn}	0.037 ± 0.002 ^{klmn}	0.029 ± 0.002 ^{opq}
A+S	0.025 ± 0.002 ^{pqr}	0.025 ± 0.002 ^{pqr}	0.038 ± 0.003 ^{klm}	0.031 ± 0.001 ^{nop}
D+S	4.944 ± 0.254 ^{a}	0.110 ± 0.003 ^{fg}	0.087 ± 0.003 ^{h}	0.066 ± 0.004 ^{ij}
D+W	4.231 ± 0.101 ^{a}	0.095 ± 0.007 ^{gh}	0.086 ± 0.006 ^{h}	0.037 ± 0.002 ^{klmn}
D+A	0.031 ± 0.003 ^{nop}	0.042 ± 0.003 ^{kl}	0.037 ± 0.002 ^{klmn}	0.038 ± 0.002 ^{klm}
P value Treatments	p<0.001			
P value Legume	p<0.001			
P value Treatments x Legume	p<0.001			

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root); Values are presented in mean ± SEM (n=9), back transformed of square root values; Values with different letters are significantly different at p<0.001.

Shi *et al.* (2017) reported an 80 to 93 % reduction of α-amylase inhibitory activity in a wide range of pre-soaked legumes with cooking. Whilst Chau and Cheung (1997) also found that cooking of pre-soaked legumes and autoclaving of pre-soaked legumes caused 15 to 20 % reduction in α-amylase inhibitory activity respectively. Data from current study in all the legumes under the treatment of S+A was in line with these results.

In general, S+A treatment was able to reduce the enzyme inhibitors (TIA, CIA, and AIA) in the tested legumes. This might because the process of soaking facilitates water imbibition and aiding the thermolabile inhibitors to be inactivated under high heat treatment. It is noticeable that the combination treatments which involved autoclaving process (S+A, W+A, A+D, A+W, A+S, and D+A) brought a significant lower or completely reduce of enzyme inhibitors than other combination methods. Out of these six combined methods, D+A was able to reduce the enzyme inhibitors effectively in all the tested legumes.

5.3.2 Effect of combined methods on protein content

The crude protein content of legumes treated with 12 combined methods via Kjeldahl method are shown in Table 5.4. There was no significant interaction between combined treatments and legumes on the crude protein content (p=0.590). Finding suggested that the 12 methods used had no significant effect on the crude protein of legume relative to raw legume.

The current finding of legume treated with soaking and autoclaving (S+A) was in accordance with the studies reported by various researchers (Ramadan, 2012; Hefnawy, 2011; Alajaji and El-Adawy, 2006; Siddhuraju and Becker, 2005). They stated that S+A process had no significant change in the detectable crude protein of soybean, lentils, mucuna beans and chickpea.

Table 5.4 The interaction of combine treatments x legumes in protein content of treated legumes determined via Kjeldahl method

Treatments	Crude protein content (g/100g DW)			
	Soybean	Mung bean	Adzuki bean	Bambara groundnut
Raw	56.15 ± 0.79	35.77 ± 1.19	37.39 ± 0.78	35.13 ± 1.31
S+W	58.24 ± 2.29	39.19 ± 1.26	34.48 ± 2.98	31.73 ± 0.90
S+A	55.76 ± 1.83	40.75 ± 0.92	36.03 ± 1.96	34.51 ± 0.98
S+D	54.44 ± 0.47	37.52 ± 0.12	37.44 ± 0.78	32.10 ± 0.96
W+A	55.78 ± 1.99	41.85 ± 0.95	40.74 ± 0.27	36.89 ± 1.78
W+D	52.17 ± 0.61	37.69 ± 0.62	36.17 ± 0.13	33.63 ± 1.58
W+S	55.45 ± 0.88	37.60 ± 0.32	37.63 ± 0.20	30.39 ± 0.30
A+D	58.78 ± 0.80	38.88 ± 1.44	37.03 ± 0.55	31.64 ± 1.28
A+W	55.03 ± 1.53	38.93 ± 1.56	37.89 ± 1.92	31.42 ± 1.91
A+S	53.00 ± 1.57	40.90 ± 3.25	36.61 ± 3.59	31.73 ± 2.48
D+S	52.15 ± 1.44	35.20 ± 2.76	32.81 ± 2.33	29.77 ± 2.92
D+W	50.84 ± 2.52	32.14 ± 1.00	35.88 ± 3.63	30.62 ± 2.67
D+A	60.40 ± 4.64	38.47 ± 1.52	35.56 ± 2.80	30.73 ± 3.10
P value Treatments	$p<0.001$			
P value Legume	$p<0.001$			
P value Treatments x Legume	$p>0.05$			

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root); values are presented in mean ± SEM (n=3), back transformed of square root values.

The protein content of legumes treated with 12 combined methods assessed via Bradford method are shown in Table 5.5. There was a significant interaction between combined treatments and legumes in the protein content (p<0.001). The 12 methods used significantly reduced the detectable protein content of soybean, mung bean, adzuki bean and bambara groundnut with a range of 61.2 – 95.8 %.

As discussed in Chapter 3 earlier, Bradford method uses Coomassie brilliant blue G-250 dye which only detects basic and aromatic type of amino acids arginine and lysine, histidine, phenylalanine, tryptophan, and tyrosine (Nielsen, 2010; Compton and Jones, 1985). Current finding suggested that this group of amino acids were affected by these 12 methods, and not the crude protein content (determined by Kjeldahl). Other than that, the Coomasie dye had been reported to bind with proteins and polypeptides > 3000 Da only (Moore *et al.*, 2010). Ten out of twelve combined methods used thermal processing, either wet heating or autoclaving, which could have hydrolysed the proteins into a small size peptide which is undetectable by using Bradford method.

Current results suggested that the combination of two processing methods had strengthened the removal of enzyme inhibitors, whilst retaining the crude protein content of the four legumes tested. Out of twelve combined methods, D+A method was the most efficient method to process the tested legumes. The retention of crude protein content after a combined treatment was one of the major considerations for selecting a process and ingredient(s) that could be used aquafeed. Thus, adzuki bean and bambara groundnut were determined as the best candidates for use as an aquafeed since D+A method did not significantly

affect the crude protein. Besides, using this combined method, both legumes possessed the lowest TIA, CIA, and AIA relative to soybean and mung bean.

Table 5.5 The interaction of combine treatments x legumes in protein content of treated legumes determined via Bradford method

Treatments	Protein content (g/100g DW)			
	Soybean	Mung bean	Adzuki bean	Bambara groundnut
Raw	13.55 ± 0.38 [a]	13.21 ± 0.16 [b]	12.75 ± 0.34 [c]	5.70 ± 0.20 [d]
S+W	2.16 ± 0.03 [e]	2.10 ± 0.01 [e]	2.14 ± 0.01 [e]	2.13 ± 0.01 [e]
S+A	1.21 ± 0.04 [f]	0.76 ± 0.01 [jk]	0.55 ± 0.03 [m]	0.60 ± 0.02 [m]
S+D	2.17 ± 0.01 [e]	2.09 ± 0.02 [e]	2.10 ± 0.03 [e]	2.18 ± 0.01 [e]
W+A	1.07 ± 0.08 [g]	0.77 ± 0.01 [jk]	0.53 ± 0.01 [m]	0.68 ± 0.01 [l]
W+D	2.18 ± 0.01 [e]	2.12 ± 0.01 [e]	2.17 ± 0.01 [e]	2.17 ± 0.02 [e]
W+S	2.16 ± 0.02 [e]	2.13 ± 0.01 [e]	2.18 ± 0.01 [e]	2.16 ± 0.01 [e]
A+D	1.05 ± 0.02 [g]	0.80 ± 0.02 [ijk]	0.58 ± 0.04 [m]	0.88 ± 0.01 [hi]
A+W	0.82 ± 0.05 [ij]	0.76 ± 0.02 [jk]	0.55 ± 0.02 [m]	0.75 ± 0.01 [jkl]
A+S	0.72 ± 0.03 [kl]	0.81 ± 0.01 [ij]	0.59 ± 0.01 [m]	0.68 ± 0.02 [l]
D+S	2.16 ± 0.01 [e]	2.10 ± 0.02 [e]	2.13 ± 0.01 [e]	2.06 ± 0.01 [e]
D+W	2.11 ± 0.01 [e]	2.13 ± 0.01 [e]	2.13 ± 0.01 [e]	2.16 ± 0.01 [e]
D+A	1.05 ± 0.02 [g]	0.80 ± 0.03 [ijk]	0.58 ± 0.01 [m]	0.89 ± 0.01 [h]
P value Treatments		$p<0.001$		
P value Legume		$p<0.001$		
P value Treatments x Legume		$p<0.001$		

Notes:
Not normally distributed, therefore statistics were performed on transformed data (square root); values are presented in mean ± SEM (n=3), back transformed of square root values.

5.4 Summary

This study was looking at the effect of combined two processing methods in reducing enzyme inhibitors (TIA, CIA, and AIA) whilst retaining the crude protein in the underutilised legumes. Compared to single methods of processing, it appeared that the combined methods had strengthened the removal of enzyme inhibitors with no effect on crude protein content. However, the efficiency of each combined method was influenced by the types of legumes tested. Under the treatment of dry freezing and autoclaving (D+A), adzuki bean and bambara groundnut possessed the lowest enzyme inhibitors content but maintained their crude protein content.

CHAPTER 6

SUBSTITUTION OF LEGUME MEAL ON THE GROWTH PERFORMANCE AND INTESTINAL INFLAMMATORY RESPONSE OF ZEBRAFISH

6.1 Introduction

The progressive increasing demands of the carnivorous fish farming industry for the finite resources of fish meal and fish oil has stimulated investigations into the potential of using plant-based aquafeed as an alternative (Hill *et al.*, 2013). Over the last few decades, to reduce the reliance of fish meal as a protein source, researchers had made many efforts in partially replacing fish meal in aquafeeds with alternative plant protein source such as soybean, canola seed, pea, and broad bean.

However, the prime concern with these major crops is the global production yield has been declining and in the long run production is probably unsustainable due to global climate change (Chivenge *et al.*, 2015; Ebert, 2014). In order to reduce the over-reliance on major crops and to ensure future food security and sustainability, the development of ANF-free plant-based aquafeed using underutilised legumes is essential.

In the preliminary study describe in this chapter, zebrafish were used as *in vivo* study as an established fish model. Zebrafish were used rather than a farmed fish species due to the feasibility of using greater number of fish with more treatments as the trials could be carried out at a lower cost and for shorter duration. Also there is a more substantial background literature on zebrafish than farmed fish, particularly at a molecular level (Dahm and Geisler, 2006). This level of information supported the

associated objective of study which sought to investigate the impact of altering the feed ingredients on the gene expression response of zebrafish.

The ultimate application of any changes in aquafeed would be in the commercially valuable farm species Asian seabass. Undeniably, zebrafish is not perfect for the evaluation of the immune response, due to the structural differences in digestive system of zebrafish and Asian seabass. Zebrafish has long intestine and no true stomach, while carnivorous fish have a true stomach and short intestine. Nevertheless, current study serves as a good baseline study to better understanding on the effect of these legume-based ingredients prior to Asian seabass trial.

Based on the findings in Chapter 5, two legumes – adzuki bean and bambara groundnut were selected as the potential pant-based ingredients for substitution in zebrafish aquafeed. This chapter aimed to investigate the effect of adzuki bean and bambara groundnut with reduced enzyme inhibitors on the growth performance and intestinal inflammatory response of zebrafish.

The specific aims of this study were:

- To investigate the growth performance of zebrafish fed with legume meals with reduced enzyme inhibitors
- To investigate the zebrafish intestinal inflammatory response via the gene expression of pro-inflammatory cytokines in the gut – interleukins il-1β and il-8

Current hypothesis proposed that the aquafeed containing legumes which are processed to reduce enzyme inhibitors will has higher digestibility compared to feed

containing unprocessed legumes. This will result in the zebrafish on these feeds performing better on the processed legume-based feeds than the unprocessed feeds, hence would have relatively enhanced growth. This enhanced growth will be associated with reduced expression inflammatory signal genes.

6.2 Experimental design

6.2.1 Ethical issues

This study was conducted in University of Liverpool. The zebrafish were housed using standard aquarium methods and fed on balanced diets formulated to meet nutritional requirements or exceed the minimum nutritional needs; therefore, there was no requirement for ethical clearance to carry out this trial according to the Animals (Scientific Procedures) Act 1986 (ASPA). Advice was sought from the Home Office of University of Liverpool who confirmed that these experiments were sub-threshold and did not require a project licence. Local AWERB (Animal Welfare and Ethical Review Body) approval was obtained before work started. Experiments were carried out by trained technical staff under direct supervision of an experienced supervisor and fish welfare was checked daily by the facility NACWO (Nominated Animal Care and Welfare Officer). The fish were bred at the University of Liverpool zebrafish facility and so endured minimal transport or housing stress prior to this experiment starting. However, throughout the experiment welfare was monitored and if reached an unacceptable level the fish affected were removed from the experiment.

6.2.2 Zebrafish

250 zebrafish (approximately 2 months old) were used for the study and were housed under the conditions listed in section 2.6.1 at University of Liverpool. Five replicate tanks were given the same experimental diet, 50 fish in total per diet consisting of mixtures of male and female were distributed equal across the tanks, n=10 per tank. The fish was acclimatised in the tanks for 1 week before the starting of fish feeding trial. The fish feeding trial was carried out for six weeks and the body weight was taking weekly to record growth throughout and the length was only

measured at the end of the study as described in section 2.6.1a. The fish were fed once at 4 % body weight per day. At the end of feeding trial, the fish were culled and tissue were collected as listed in section 2.6.1b.

6.2.3 Feed ingredients and diet formulation

The different experimental diets differed in the legume they contained, either adzuki bean or bambara groundnut which had be treated or not. The four diets being identified as untreated adzuki bean (RAB), treated adzuki bean (PAB), untreated bambara groundnut (RBG) or treated bambara groundnut (PBG). The PAB and PBG were treated by using the process of dry freezing and autoclaving (D+A). The dried legumes without underwent the dehulling process were frozen at -80°C for 24 h which were subsequently had distilled water (ratio 1:10 (w/v)) added to them before it reached room temperature. The legumes were then autoclaved at 121°C, 15 psi for 15 min. After autoclaving the associated liquid was poured away and the legumes were cooled to room temperature and chilled overnight at 4°C. The chilled legumes were frozen at -80°C for 24 h then lyophilised for 24 h using freeze-dryer (Alpha 1-4 LD plus, Christ). The lyophilised legumes were then ground into powder form using a miller. The fine powder was then to pass through a series of mesh sieves with the size of 1.68 µm, 1.18 µm and 0.85 µm. The powder that was stopped on 1.18 µm mesh sieve was collected and stored in 4°C chiller prior incorporated to the aquafeed.

The material chemical composition data and the amino acid content are presented in Table 6.1. Four experimental diets were formulated: T1, T2, T3, and T4 to reduce fish meal (FM) content by 10 % (w/w), but retain the same nutrient content, by replacing this one of the following ingredients RAB, PAB, RBG or PBG, respectively (Table 6.2).

As a control, a basal diet (T0) was formulated so that did not contain any legumes. The nutritional profile of diets was presented in Table 6.3. The diet was formulated using a model of best fit programme by Dr Iain Young and Mr Kieran Magee from Institute of Integrative Biology, University of Liverpool. The desired amounts of all ingredients required were mixed using a Hobart food mixer until thoroughly mixed. Water was added until the mixture achieved dough like consistency. The dough mixture was then spread out on trays and dried for 24 h at 50°C using a nine shelf Parallexx Excalibur food dehydrator. Once dry the diet was crushed and processed through a series of sieves with apertures of 850 µm and 425 µm. The desired pellet size fell between the two. All diets were stored at 4°C.

Table 6.1 Chemical composition of legumes used to substitute the fish meal in experimental diets (values are in % DW unless otherwise indicated)

	RAB[e]	PAB[f]	RBG[g]	PBG[h]
Proximate compositions				
Dry matter	85.88 ± 0.43	95.87 ± 1.05	88.34 ± 0.02	95.91 ± 0.09
Crude protein	22.01 ± 0.18	26.66 ± 0.13	18.90 ± 0.09	21.18 ± 0.18
Crude lipid	0.43 ± 0.04	0.49 ± 0.02	6.60 ± 0.09	7.52 ± 0.04
Moisture	14.12 ± 0.43	4.13 ± 1.05	11.66 ± 0.52	4.09 ± 0.09
Ash	3.61 ± 0.02	1.71 ± 0.03	3.31 ± 0.02	2.34 ± 0.03
Crude fibre	6.64 ± 1.31	5.71 ± 0.53	8.95 ± 1.69	4.81 ± 0.89
NFE[a]	53.18	61.30	50.58	60.06
Energy (MJ/kg)	16.58 ± 0.08	17.73 ± 0.02	18.19 ± 0.06	19.11 ± 0.06
Essential amino acids (g/kg DW)				
Lysine	18.56	22.80	14.15	17.35
Threonine	8.47	9.90	6.72	8.23
Methionine	2.57	4.11	2.30	2.62
Isoleucine	9.70	13.83	8.11	10.15
Leucine	18.87	26.15	15.13	19.10
Valine	11.90	15.62	9.76	11.08
Phenylalanine	14.32	18.92	10.32	13.59
Histidine	6.86	9.46	6.67	7.44
Arginine	17.72	21.20	15.36	16.75
Enzymatic proteinaceous inhibitors				
TI (TIA unit/mg)[b]	0.069 ± 0.008	nd	nd	0.022 ± 0.009
CI (CIA unit/mg)[c]	1.07 ± 0.05	nd	0.77 ± 0.04	0.32 ± 0.06
AI (AIA unit/mg)[d]	0.124 ± 0.002	0.037 ± 0.002	0.228 ± 0.013	0.038 ± 0.002

Notes:
nd represent not detectable
[a] NFE – Nitrogen free extract was calculated as the difference of ash, crude protein, crude lipid, moisture, crude fibre from 100
[b] TI – trypsin inhibitors
[C] CI – chymotrypsin inhibitors
[d] AI – α-amylase inhibitors
[e] RAB – untreated adzuki bean
[f] PAB – treated adzuki bean
[g] RBG – untreated bambara groundnut
[h] PBG – treated bambara groundnut

Table 6.2 Diets formulation of the experimental diets

Ingredients	Diets (g/kg)				
	T0	T1	T2	T3	T4
Fish meal (white trimmings)	387.40	344.20	349.70	348.80	350.80
Legumes powder	-	242.00	260.30	165.10	160.80
Rapeseed Oil	43.70	45.70	53.60	34.60	33.30
Vitamins	3.10	3.00	3.20	3.00	3.00
Minerals	4.20	4.00	4.30	4.00	4.00
Wheat Gluten	229.10	203.90	173.20	219.20	206.10
Corn Starch	318.20	135.70	131.90	206.70	221.40
Binder (CMC powder)	5.20	5.00	5.40	5.00	5.00
Lysine	9.00	11.30	11.30	10.40	10.90
Arginine	-	2.20	3.00	1.50	1.90
Leucine	-	3.00	4.10	1.70	2.60

Notes:
T0 – control basal diet without inclusion of legume
T1 – replacement of 10 % of fish meal in feed with untreated adzuki bean powder (RAB)
T2 – replacement of 10 % of fish meal in feed with treated adzuki bean powder (PAB)
T3 – replacement of 10 % of fish meal in feed with untreated bambara groundnut powder (RBG)
T4 – replacement of 10 % of fish meal in feed with treated bambara groundnut powder (PBG)

Table 6.3 The proximate analysis of various diets (values are in % DW unless otherwise indicated)

	T0	T1	T2	T3	T4
Dry matter	96.78±0.09	96.53±0.05	96.10±0.02	95.53±0.01	95.60±0.04
Crude protein	50.66±0.17	51.75±0.05	51.72±0.61	52.17±0.18	52.69±0.53
Crude lipid	7.57±0.02	7.95±0.04	8.57±0.01	7.82±0.02	7.41±0.16
Moisture	3.23±0.10	3.48±0.05	3.90±0.02	4.47±0.01	4.40±0.04
Ash	7.06±0.00	7.43±0.04	6.89±0.00	6.80±0.00	6.57±0.01
Crude fibre	0.00±0.00	1.66±0.25	2.41±0.33	1.40±0.20	1.37±0.11
NFE[a]	31.49	27.75	26.52	27.36	27.58
Energy (MJ/kg)	20.81±0.03	20.66±0.04	21.00±0.04	20.77±0.02	20.66±0.04

Notes:
T0 – control basal diet without inclusion of legume
T1 – replacement of 10 % of fish meal in feed with untreated adzuki bean powder (RAB)
T2 – replacement of 10 % of fish meal in feed with treated adzuki bean powder (PAB)
T3 – replacement of 10 % of fish meal in feed with untreated bambara groundnut powder (RBG)
T4 – replacement of 10 % of fish meal in feed with treated bambara groundnut powder (PBG)
[a] NFE – Nitrogen free extract was calculated as the difference of ash, crude protein, crude lipid, moisture, crude fibre from 100

6.2.4 Chemical analysis

All ingredients and formulated diet were analysed for dry matter, crude protein, crude lipid, ash, crude fibre, gross energy, and amino acid according to the procedure described in Chapter 2 (section 2.4.1 to 2.4.8).

6.2.5 Growth performance indices

a) Weight gain

Weight gain is defined as an increased in body weight and it was calculated as:

$$\text{Weight gain} = \text{Final weight of fish (g)} - \text{Initial weight of fish (g)}$$

b) Average daily gain (ADG)

Average daily gain is defined as the rate of weight gain (mg) per day over a specified period. It was calculated as:

$$\text{ADG (mg/day)} = (\text{Final weight of fish} - \text{Initial weight of fish}) / \text{feeding days}$$

c) Specific growth rate (SGR)

The specific growth rate is the growth that achieved per day during feeding period.

$$\text{SGR (\%)} = 100 \times (\text{Ln}W_2 - \text{Ln}W_1) \times (t_2 - t_1)^{-1}$$

Notes:
Ln – natural log
W_1 – initial weight
W_2 – final weight
t_1 – starting time point (day one)
t_2 – end time point (final day number)

d) Feed conversion ratio (FCR)

Feed conversion ratio is used to indicate the efficiency at which feed is converted into animal biomass, calculated as below:

$$FCR = \text{Feed consumed (g)} / \text{Weight gain (g)}$$

The assumption is that all the feed put in the tank is completely eaten by the fish since the feed was not given on an *ad lib* basis. The feed was given based on the weight of the fish which is 4 % of the fish body weight per day.

e) Protein conversion ratio (PCR)

Protein conversion ratio is the ratio of protein in the diet to total animal production and it was calculated as:

$$PCR = FCR \times (\%\ \text{Feed protein} / 100\ \%)$$

f) Fulton's condition factor (k index)

Fulton's conditions factor is commonly used in fisheries science to measure an individual fish's health that uses the relationship of fish weight and length to overall condition.

$$k = (\text{Weight (g)} / \text{Length}^3\ \text{(mm)}) \times 10^5$$

6.2.6 Gene expression analysis

For each treatment group (n=10 fish), the intestines (< 30 mg) from five fish were pooled and submerged in 500 µL of RNA*later*® and stored at -80°C until further analysis. The RNA was extracted from pooled intestines from each treatment group according to method as described in Chapter 2 (section 2.51 and 2.5.2). Expression of interleukins il-1β and il-8 were used as markers of an activated inflammatory response and these genes were determined by quantitative reverse transcription polymerase chain reaction (RT-qPCR) as described in Chapter 2 (section 2.5.4 and 2.5.6).

The sequences of il-1β (NM_212844.2), il-8 (XM_001342570.5) and β-actin (NM_131031.1) were obtained from NCBI GenBank Database. Each gene was tested in quintuplicate. The mean C_p values from each sample were normalised against the mean C_p value of a reference gene (β-actin, housekeeping gene). The melting curve was used to ensure that a single product was amplified and confirm the absence of primer-dimer. The relative quantification of each gene was obtained with Pfaffl's method using the fish fed with control diet (T0) as the reference, this reference was compared to the treatments (Pfaffl, 2004).

6.2.7 Statistical analysis

All the results were presented as mean ± standard error mean (SEM), tank replicated (n=5). IBM SPSS Statistics software (Version 22, IBM Corporation, USA) was used to perform one-way Analysis of Variance (ANOVA) on data sets and where this was statistical significant (p<0.05) subsequent post-hoc analysis was carried out using Duncan's Multiple Comparison test with confidence intervals of 95 % with threshold for significance when p<0.05. Data was checked for normality using Shapiro-Wilk test and for homogeneity of variance using the Levene test. Graphs were constructed using GraphPad Prism (Version 6, GraphPad Software Inc, USA).

6.3 Results and discussion

As mentioned in Chapter 1 (section 1.8), zebrafish was selected as fish model for quick analysis of alternative aquafeed ingredients with the potential use in nutritional studies. It is important to recognise that the conditions under which they were routinely bred and housed were strictly controlled and, as such caution should be shown in extrapolating the results to other fish species farmed under commercial conditions (Ulloa *et al.*, 2014). They are small in size, rapid growth rate and extensive knowledge of their genome, together with the ability to carefully control environmental conditions, make them a useful model for testing specific effects of nutrients. Therefore, they are a good model to study the inflammatory effect in gastrointestinal tract in response to different diets.

Fish diet has profound effect on the microbial composition and nutrient uptake in the gastrointestinal tract (Rurangwa *et al.*, 2015). The environment factors such as rearing water environment, temperature, feeding habitats and combination of diets could possibly modulate the normal gastrointestinal tract microbiota composition of fish (Betiku *et al.*, 2018). In this chapter, the study of microbial composition in zebrafish through 16s rRNA gene composition was not in the consideration due to the constraint of research facilities and cost.

Through 16s rRNA sequencing technique, it can expands the knowledge of the structure and diversity of bacterial communities within fish gastrointestinal tract may be important for improving health and production of aquaculture fish species (Betiku *et al.*, 2018). Although, molecular-based technique is not the only approach to study the microbial composition, but the conventional culture-dependent method is not recommended. Studies had been reported that the fish gastrointestinal tract

microbiota of fish was low cultivability, it only represented < 0.1 % of the total microbial community in the gastrointestinal tract (Wang *et al.*, 2017; Ghanbari *et al.*, 2015; Zhou *et al.*, 2014; Al-Harbi and Naim Uddin, 2004). As an alternative to study the impact of diet nutrition to fish health, the expression of inflammatory genes of gastrointestinal tract using qPCR were being targeted.

6.3.1 Growth performance of zebrafish

In this study, zebrafish were fed 6 weeks with one of the four experimental diets (T1 – T4) or the basal diet (T0) which served as a control. The initial body weight of zebrafish ranged from 40.4 – 44.4 mg (Table 6.5). The ADG for each week of fish on feed containing untreated adzuki bean (T1) was only significantly different in week 6, whilst for feed containing untreated bambara groundnut (T3) was significant higher on week 5 onwards (Table 6.4). Whereas for the fish being offered feed containing treated bambara groundnut (T4) or adzuki bean (T2) exhibited significant higher ADG on week 2 and week 3 onwards, respectively. The highest ADG was found in fish offered with treated adzuki bean diet (T2), which ranged from 0.716 mg/day at week 1 to 1.462 mg/day at week 6 even though it was not significantly different from the other feeds.

The fish being fed with treated adzuki bean had the highest average daily grow (ADG). The fish feeds were formulated to equal isonitrogenous and isocalorific relative to the control, therefore the better ADG could have been due to the higher essential amino acid content of treated adzuki bean than untreated adzuki bean, untreated bambara groundnut and treated bambara groundnut, especially lysine and methionine. This is in agreement with Lu *et al.* (2014) who reported the improvement of growth performance in juvenile black seabream with the

supplementation of lysine and methionine. Alternatively, a more plausible explanation could be that because there was no detectable level of protease inhibitors and low level of α-amylase inhibitors in treated adzuki bean. This potentially may mean the protein and energy content of this feed ingredient can be better utilised than the control feed.

Table 6.4 Effect on different legume-based meals on average daily gain (ADG)

Feeding period	ADG per fish (mg/day on a weekly basis)				
	T0	T1	T2	T3	T4
Week 1	0.656 ± 0.073^{d}	0.744 ± 0.158^{b}	0.716 ± 0.175^{b}	0.770 ± 0.116^{b}	0.544 ± 0.146^{b}
Week 2	0.886 ± 0.107^{c}	1.014 ± 0.182^{ab}	1.058 ± 0.185^{ab}	1.002 ± 0.117^{ab}	0.986 ± 0.110^{a}
Week 3	1.096 ± 0.061^{bc}	1.124 ± 0.149^{ab}	1.182 ± 0.180^{a}	0.990 ± 0.081^{ab}	0.904 ± 0.126^{a}
Week 4	1.086 ± 0.050^{bc}	1.106 ± 0.142^{ab}	1.198 ± 0.120^{a}	1.008 ± 0.071^{ab}	0.982 ± 0.127^{a}
Week 5	1.168 ± 0.057^{ab}	1.210 ± 0.141^{ab}	1.326 ± 0.116^{a}	1.194 ± 0.086^{a}	1.106 ± 0.088^{a}
Week 6	1.344 ± 0.069^{a}	1.360 ± 0.113^{a}	1.462 ± 0.124^{a}	1.264 ± 0.084^{a}	1.218 ± 0.119^{a}

Notes:
Values are presented in mean ± SEM, n=5
[a-b] Within a column, values with different letters are significantly different at $p<0.05$
Within a row, no significant difference ($p>0.05$) were found
T0 – control basal diet without inclusion of legume
T1 – replacement of 10 % of fish meal in feed with untreated adzuki bean powder (RAB)
T2 – replacement of 10 % of fish meal in feed with treated adzuki bean powder (PAB)
T3 – replacement of 10 % of fish meal in feed with untreated bambara groundnut powder (RBG)
T4 – replacement of 10 % of fish meal in feed with treated bambara groundnut powder (PBG)

When compare the growth of zebrafish fed with T1 – T4 diets with basal diet (T0), there was no significantly difference ($p>0.05$) for weight gain, ADG, SGR, PCR, and k index (Table 6.5). No significant difference was observed between raw legume and legume that with reduced enzyme inhibitors. Tibaldi *et al.* (2006) reported that the trypsin inhibitory activity less than 5.7 TIA unit/mg had no apparent adverse effect on growth performance in European seabass. As shown in Table 6.1, the trypsin inhibitors of processed and unprocessed adzuki bean and bambara groundnut was

0.069 TIA unit/mg to undetectable level. Thus, current findings suggested that these substitutions of legume-based meals have not caused detrimental effect on the digestion and growth performance of zebrafish.

Table 6.5 Zebrafish growth performance

Growth parameters	T0	T1	T2	T3	T4
Initial weight (mg)	40.40 ± 2.16	41.00 ± 4.17	44.40 ± 3.08	43.60 ± 3.92	41.80 ± 3.88
Weight gain (g)	0.057 ± 0.003	0.057 ± 0.005	0.061 ± 0.005	0.053 ± 0.004	0.051 ± 0.005
SGR (%)[1]	2.14 ± 0.10	2.14 ± 0.07	2.11 ± 0.03	1.96 ± 0.10	1.95 ± 0.04
FCR[2]	1.76 ± 0.09	1.77 ± 0.08	1.79 ± 0.04	1.96 ± 0.12	1.94 ± 0.04
PCR[3]	0.89 ± 0.05	0.91 ± 0.04	0.92 ± 0.02	1.03 ± 0.06	1.02 ± 0.02
k index[4]	1.63 ± 0.04	1.63 ± 0.05	1.65 ± 0.06	1.68 ± 0.04	1.59 ± 0.05

Notes:
Values are presented in mean ± SEM, n=5
No significant difference (p>0.05) were detected
[1] SGR – specific growth rate
[2] FCR – feed conversion ratio
[3] PCR – protein conversion ratio
[4] k index – Fulton's condition factor
T0 – control basal diet without inclusion of legume
T1 – replacement of 10 % of fish meal in feed with untreated adzuki bean powder (RAB)
T2 – replacement of 10 % of fish meal in feed with treated adzuki bean powder (PAB)
T3 – replacement of 10 % of fish meal in feed with untreated bambara groundnut powder (RBG)
T4 – replacement of 10 % of fish meal in feed with treated bambara groundnut powder (PBG)

According to Siccardi Iii *et al.* (2009), the lack of a significant difference (p>0.05) k index is considered a good indicator of fish health. A decreased in k factor indicates depletion of energy reserves, such as stored liver glycogen or body fat.

Aforementioned, only in recent years have zebrafish emerged as a model organism in aquaculture research. Very limited studies have been done on zebrafish to examine the effect of plant-based diets on the growth and toxicology nutrition for inflammatory disorders which could be caused by the enzyme inhibitors. Recent

studies done by Karga and Mandal (2016), Liu *et al.* (2013), Ulloa *et al.* (2013) and Siccardi Iii *et al.* (2009) disclosed that different feed ingredients such as soybean meal, corn gluten, and mustard oil cake could influence the growth performance of zebrafish. Karga and Mandal (2016) and Liu *et al.* (2013) showed that when zebrafish were fed with soybean at the inclusion rate of 250 – 500 g/kg with 35 – 46 % of crude protein in the experimental diet, they had a lower weight gain and SGR than control. The zebrafish fed with zooplankton had gained 0.057 g whilst those fed with soybean meal gained 0.028 g after 1 month of feeding. This is mainly because the crude protein in the diet supplied with zooplankton or soybean were different (54 % and 35 % respectively) (Karga and Mandal, 2016). In addition soybean is known to contain ANFs that retarded the growth performance, whilst zooplankton is a natural live food, that met the dietary requirements of zebrafish (Hedrera *et al.*, 2013; Watts *et al.*, 2012).

6.3.2 Intestinal inflammatory response of zebrafish

Like any other animals, gastro-intestinal tract of fish serves multiple functions including digestion and absorption, endocrine regulation of appetite and digestion, electrolyte balance (osmoregulation) and immune function (Buddington and Krogdahl, 2004). Since zebrafish have no true stomach, their intestine epithelium surface is the first site exposed to the nutrient including legume-based feed ingredients (Wang *et al.*, 2010). Hence the current study was carried to investigate whether these treatments diets (T1 – T4) are responsible for triggering the intestinal inflammatory response of zebrafish.

Figure 6.1 showed that no significant change in the gene expression of the pro-inflammatory cytokines il-1β (p=0.445) and il-8 (p=0.337) in response to the four experimental diets (T1 – T4) relative to control diet (T0). These results suggested that replacement of 10 % of fish meal with legume meal did not affect inflammation as indicated by a change in cytokine gene expression.

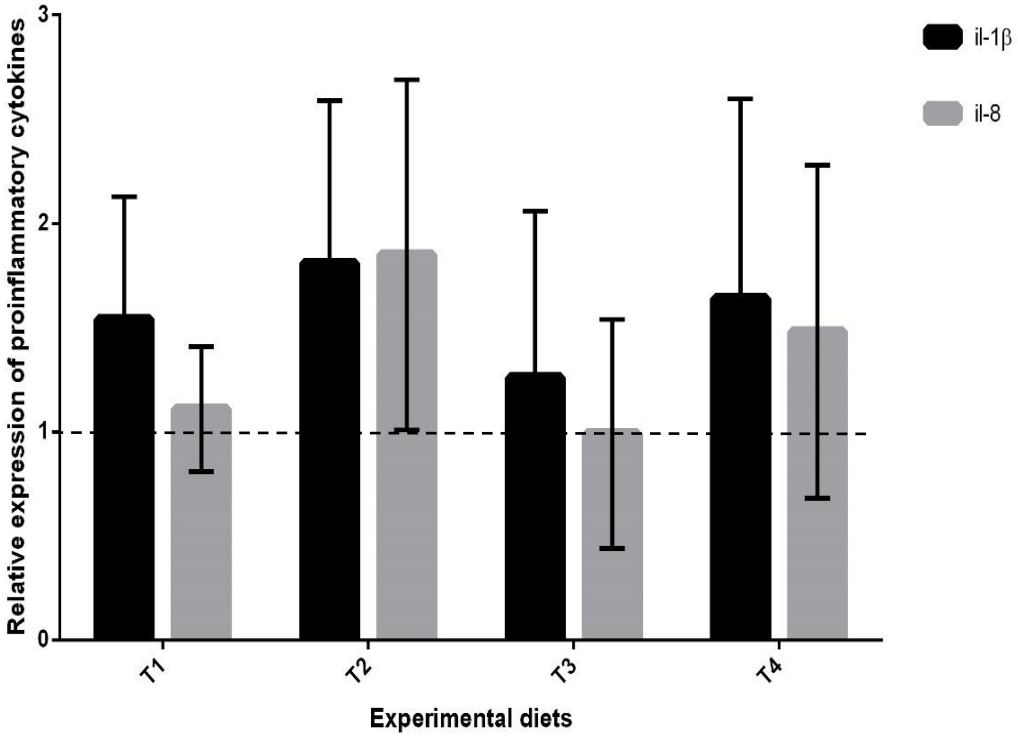

Figure 6.1 The immunological response in fish intestines after fed at different experimental diets. Transcription levels of pro-inflammatory cytokines, interleukin (il-1β) and interleukin (il-8) were quantified by quantitavite PCR. T0 – control basal diet without inclusion of legume; T1 – replacement of 10 % of fish meal in feed with untreated adzuki bean powder (RAB); T2 – replacement of 10 % of fish meal in feed with treated adzuki bean powder (PAB); T3 – replacement of 10 % of fish meal in feed with untreated bambara groundnut powder (RBG); T4 – replacement of 10 % of fish meal in feed with treated bambara groundnut powder (PBG). The error bars represent the standard error mean, n=5. No significant difference (p>0.05) were detected.

Current observation is in contrast with the studies of Fuentes-Appelgren *et al.* (2014) and Hedrera *et al.* (2013) that reported different inclusion level of soybean meal – that possibly consist of different level of ANFs – had caused an upregulation of pro-inflammatory cytokines of il-1β and il-8 in zebrafish. The cytokines' induction and severity of morphological alteration in fish gastrointestinal towards soybean meal is highly related to the fish variety, as some of the fish, such as Atlantic salmon, tend to be more sensitive to plant protein (Wang *et al.*, 2017; Hu *et al.*, 2016).

Although the experimental feeds contained ANF, the relative expression of the selected intestinal inflammatory signals genes was not upregulated in the zebrafish upon the ingestion of legume protein. The 10 % (w/w) inclusion level of legume may have been too low and not sufficient to observe the effect of inflammation triggered by the enzyme inhibitors at molecular level. A more sensitive indication of inflammation of intestines may have been histological observations for any changes in morphological. Using this method the shortening of primary and secondary intestinal mucosal folds, an increased in the number of goblet cells and infiltration of inflammatory cells are the typical signs of inflammation in intestines and directly indicative of phenotype indicating intestine damage (Rimoldi *et al.*, 2016). The general observations in the zebrafish trial describe in this thesis indicated the likely no consequences of replacing feed ingredients in fish feed. Therefore, this study did provided results which are potentially applicable to commercially important fish.

6.4 Summary

Current findings confirm that the legume meals with reduced enzyme inhibitors did not affected the growth performance of zebrafish and not induced inflammatory signals, at least in the intestine. Surprisingly, feeding zebrafish feed containing raw legumes for 6 weeks had no effect in the growth performance and intestine. In this study, a 10 % (w/w) replacement of fish meal with adzuki bean or bambara groundnut had comparable growth to basal diet. However, the average daily gain (ADG) of treated adzuki bean was the highest over 6 weeks. Thus, this legume had been chosen as the feed ingredient to be investigated with the growth and nutritional evaluation on the commercially important fish – Asian seabass as described in the next chapter.

CHAPTER 7

SUBSTITUTION OF LEGUME MEAL ON THE GROWTH PERFORMANCE AND HEPATIC RESPONSE OF ASIAN SEABASS

7.1 Introduction

Previous findings in Chapter 6 suggested that 10 % replacement of fish meal with adzuki bean or bambara groundnut had shown no significant difference in growth of zebrafish compared to the basal diet. In this chapter, the effect of substitution of legume meal on the growth of Asian seabass was investigated.

Asian seabass is a commercially importance carnivorous species and is extensively cultured in South East Asian and Australia due to the high consumer demand (Mohd-Yusof *et al.*, 2010). Over the past decades, researchers have been looking into the use of different raw feed ingredient materials such as soybean meal, poultry meal, canola meal, green pea, chicken waste, kernel meal and feather meal along with many potential sources of protein, to reduce the reliance on fish meal as protein source for cultured Asian seabass (Glencross *et al.*, 2016; Ganzon-Naret, 2013c; Glencross, 2011; Nandakumar *et al.*, 2013; Plaipetch and Yakupitiyage, 2011).

Substitution of animal protein with plant protein has detrimental effects on fish growth and performance, especially in carnivorous fish, where these effects can be directly related to the effects of enteritis (Fuentes-Appelgren *et al.*, 2014). However, studies done by Santigosa *et al*. (2008) as well as German and Horn (2006) discovered that carnivorous fish have a compensatory mechanism that allows them to modify and adapt their digestive and physiological system to a feeds containing high proportion of plant-derived ingredients.

This chapter aimed to investigate the effect of substituting into fish feed adzuki beans, treated to reduce enzyme inhibitors on the growth performance and hepatic response of Asian seabass.

The specific aims of this study were:

- To investigate the growth performance of Asian seabass fed with feeds containing adzuki bean meal treated to reduce enzyme inhibitors
- To investigate the effect of this feed on Asian seabass hepatic inflammatory genes heat shock protein 70 (HSP 70) and C-reactive protein (CRP)
- To investigate the Asian seabass hepatic amino acid metabolism via assessment of alanine aminotransferase (ALAT) gene expression

Current hypothesis proposed that fish feed containing adzuki bean meal treated to reduce enzyme inhibitors will increase the dietary protein availability compared to untreated adzuki bean meal-based feed in Asian seabass which will result in increased growth relative untreated adzuki bean. In addition, the relatively enhanced the growth of Asian seabass on treated adzuki bean meal based feed will be associated induced the inflammatory signals when compared to untreated adzuki bean meal based feed.

7.2 Experimental design

7.2.1 Ethical issues

The research reported in this chapter was conducted in compliance with Named Animal Care and Welfare Officer (NACWO) and Named Veterinary Surgeon (NVS) guidelines. The study received animal ethics approval from the Animal Welfare and Ethical Review Committee with approval number UNMC8.

7.2.2 Asian Seabass

36 Asian seabass (approximately 5 to 6 cm) were used for the study and were housed under the conditions listed in section 2.6.2 at Crop for Future Research Centre. Three replicate tanks were given the same experimental diet, 4 fish per tank a total of 12 fish per diet. The fish was not being acclimatised before the starting of fish feeding trial. The fish was not being acclimatised as the fish position was being switch every week after weighing to mitigate the lighting effect on the fish. The fish feeding trial was carried out for five weeks and the body weight was taking weekly to record growth throughout and the length was only measured at the end of the study as described in section 2.6.2a. The fish were fed once at 4 % body weight per day, 6 days per week. At the end of feeding trial, the fish were culled and tissue were collected as listed in section 2.6.2b.

7.2.3 Feed ingredients and diet formulation

The untreated adzuki bean (RAB) and treated adzuki bean (PAB) were included in the experimental diets. To produce PAB the adzuki bean were treated by using the processed of dry freezing and autoclaving (D+A). The dried legumes without underwent the dehulling process were frozen at -80°C for 24 h which were subsequently had distilled water (ratio 1:10 (w/v)) added to them before it reached room temperature. The legumes were then autoclaved at 121°C, 15 psi for 15 min. After autoclaving the associated liquid was poured away and the legumes were cooled to room temperature and chilled overnight at 4°C. The chilled legumes were frozen at -80°C for 24 h. The legumes were lyophilised for 24 h using freeze-dryer (Alpha 1-4 LD plus, Christ). The lyophilised legumes were then ground into powder form using a miller. The fine powder was then to pass through a series of mesh sieves with the size of 1.68 μm, 1.18 μm and 0.85 μm. The powder that was stopped on 1.18 μm mesh sieve was collected and stored in 4°C chiller prior incorporated to the aquafeed.

The material chemical compositions data and amino acids content were presented in Table 7.1. Two experimental diets were formulated: T1 and T2 to reduce fish meal (FM) content by 10 % (w/w), but retain the same nutrient content, by replacing this with either RAB or PAB (Table 7.2). As a control, a basal diet (T0) that did not contain any legumes was formulated. The nutritional profile of the diets was presented in Table 7.3.

The desired amounts of all ingredients required were mixed using a mixer until thoroughly mixed. A 40 % (v/w) of water was added into the mixture to achieve dough like consistency. The dough mixture was then processed into a spaghetti-like

product (3 mm Ø) using a meat grinder and was broke into pellet size pieces with the lengths of approximately 5 – 10 mm. The pellets were dried for 24 h at 50°C oven. The diets were stored at 4°C.

Table 7.1 Chemical composition of legumes used to substitute the fish meal in experimental diets (values are in % DW unless otherwise indicated)

	RAB[e]	PAB[f]
Proximate compositions		
Dry matter	85.88 ± 0.43	95.87 ± 1.05
Crude protein	22.01 ± 0.18	26.66 ± 0.13
Crude lipid	0.43 ± 0.04	0.49 ± 0.02
Moisture	14.12 ± 0.43	4.13 ± 1.05
Ash	3.61 ± 0.02	1.71 ± 0.03
Crude fibre	6.64 ± 1.31	5.71 ± 0.53
NFE[a]	53.18	61.30
Energy (MJ/kg)	16.58 ± 0.08	17.73 ± 0.02
Essential amino acids (g/kg DW)		
Lysine	18.56	22.80
Threonine	8.47	9.90
Methionine	2.57	4.11
Isoleucine	9.70	13.83
Leucine	18.87	26.15
Valine	11.90	15.62
Phenylalanine	14.32	18.92
Histidine	6.86	9.46
Arginine	17.72	21.20
Enzymatic proteinaceous inhibitors		
TI (TIA unit/mg)[b]	0.069 ± 0.008	nd
CI (CIA unit/mg)[c]	1.07 ± 0.05	nd
AI (AIA unit/mg)[d]	0.124 ± 0.002	0.037 ± 0.002

Notes:
[a] NFE – Nitrogen free extract was calculated as the difference of ash, crude protein, crude lipid, moisture, crude fibre from 100
[b] TI – trypsin inhibitors
[C] CI – chymotrypsin inhibitors
[d] AI – α-amylase inhibitors
[e] RAB – untreated adzuki bean
[f] RAB – treated adzuki bean

Table 7.2 Diets formulation of the experimental diets

Ingredients	Diets (g/kg)		
	T0	T1	T2
Fish meal	562.00	505.80	505.80
Untreated adzuki bean powder	-	56.20	-
Treated adzuki bean powder	-	-	56.20
Dextrin	148.00	148.0	148.00
Soybean meal	116.00	116.0	116.00
Corn meal	64.00	64.00	64.00
Fish oil	60.00	60.00	60.00
Vitamin premix	30.00	30.00	30.00
Mineral premix	20.00	20.00	20.00

Notes:
T0 – control basal diet without inclusion of legume
T1 – replacement of 10 % of fish meal in feed with untreated adzuki bean powder (RAB)
T2 – replacement of 10 % of fish meal in feed with treated adzuki bean powder (PAB)

Table 7.3 The proximate analysis of various diets (values are in % DW unless otherwise indicated)

	T0	T1	T2
Dry matter	91.30	90.70	90.20
Crude protein	47.20	44.50	44.10
Crude lipid	13.60	12.90	9.30
Moisture	8.70	9.30	9.80
Ash	8.80	8.40	8.10
Dietary fibre	7.50	7.90	6.50
Total carbohydrate	21.70	24.90	25.30
Energy (MJ/kg)	16.72	16.54	16.46

Notes:
T0 – control basal diet without inclusion of legume
T1 – replacement of 10 % of fish meal in feed with untreated adzuki bean powder (RAB)
T2 – replacement of 10 % of fish meal in feed with treated adzuki bean powder (PAB)

7.2.4 Chemical analysis

All ingredients were analysed for dry matter, crude protein, crude lipid, ash, crude fibre, gross energy, and amino acid according to the procedure described in Chapter 2 (section 2.4.1 to 2.4.8). While the analyses of proximate (Table 7.3) in experimental diets were outsourced to ALS Technichem (M) Sdn Bhd. The whole carcass was freeze dried and minced. The dried carcass was then analysed for crude protein and crude lipid according to section 2.4.1a and 2.4.2. Whereas the moisture content of whole carcass was determined according to section 2.4.5 after the fish being culled.

7.2.5 Growth performance indices

a) Weight gain

Weight gain is defined as an increased in body weight and it was calculated as:

$$\text{Weight gain} = \text{Final weight of fish (g)} - \text{Initial weight of fish (g)}$$

b) Average daily gain (ADG)

Average daily gain is defined as the rate of weight gain (g) per day over a specified period. It was calculated as:

$$\text{ADG (g/day)} = (\text{Final weight of fish} - \text{Initial weight of fish}) / \text{feeding days}$$

c) Specific growth rate (SGR)

The specific growth rate is the growth that achieved per day during feeding period.

$$SGR\ (\%) = 100 \times (LnW_2 - LnW_1) \times (t_2 - t_1)^{-1}$$

Notes:
Ln – natural log
W_1 – initial weight
W_2 – final weight
t_1 – starting time point (day one)
t_2 – end time point (final day number)

d) Feed conversion ratio (FCR)

Feed conversion ratio is used to indicate the efficiency at which feed is converted into animal biomass, calculated as below:

$$FCR = \text{Feed consumed (g)} / \text{Weight gain (g)}$$

The assumption is that all the feed put in the tank is eaten by the fish since the feed was not given on an *ad lib* basis. The feed was given based on the weight of the fish which is 4 % of the fish body weight per day.

e) Protein conversion ratio (PCR)

Protein conversion ratio is the ratio of protein in the diet to total animal production and it was calculated as:

$$PCR = FCR \times (\%\ \text{Feed protein} / 100\ \%)$$

f) Protein efficiency ratio (PER)

Protein efficiency ratio is an indicator of the conversion of feed ingredient protein into animal protein, calculated as below:

$$PER = FCR \times (\% \text{ feed protein} / \% \text{ protein in culture species})$$

g) Fulton's condition factor (k index)

Fulton's conditions factor is commonly used in fisheries science to measure an individual fish's health that uses the relationship of fish weight and length to overall condition.

$$k = (\text{Weight (g)} / \text{Length}^3 \text{ (mm)}) \times 10^5$$

h) Hepatosomatic index (HSI)

The organ index commonly liver is also used to assess fish health

$$HSI = (\text{Liver mass (g)} / \text{Fish weight (g)}) \times 100$$

7.2.6 Gene expression analysis

For each diet, three replicates of liver tissues (< 30 mg) were each independently submerged in 500 µL of RNA*later*® and stored at -80°C until further analysis. The RNA was extracted according to method as describe in Chapter 2 (section 2.51 and 2.5.3). Expression of heat shock protein 70 (HSP 70), C-reactive protein (CRP) were used as markers of an activated inflammatory responses. Whilst expression of alanine aminotransferase (ALAT) was used as an indicator of dietary protein availability.

These genes were determined by quantitative reverse transcription polymerase chain reaction (RT-qPCR) as described in Chapter 2 (section 2.5.5 and 2.5.7).

The sequences of HSP 70 (HQ646109.1), CRP (HQ652974.1), ALAT (XM_018693109.1) and β-actin (GU188683.1) were obtained from NCBI GenBank Database. Each gene was tested in nonuplicate. The mean C_q values from each sample were normalised against the mean C_q value of a reference gene (β-actin, housekeeping gene). The melting curve was used to ensure that a single product was amplified and confirm the absence of primer-dimer. The relative quantification of each gene was obtained with Pfaffl's method using the fish fed with control diet (T0) as the reference, this reference was compared to the treatments (Pfaffl, 2004).

7.2.7 Statistical analysis

All the results were presented as mean ± standard error mean (SEM), tank replicated (n=3). IBM SPSS Statistics software (Version 22, IBM Corporation, USA) was used to perform one-way Analysis of Variance (ANOVA) on data sets and where this was statistical significant (p<0.05) subsequent post-hoc analysis was carried out using Duncan's Multiple Comparison test with confidence intervals of 95 % with threshold for significance when p<0.05. Data was checked for normality using Shapiro-Wilk test and for homogeneity of variance using the Levene test. Graphs were constructed using GraphPad Prism (Version 6, GraphPad Software Inc, USA).

7.3 Results and discussion

In the study described in this thesis, a high inclusion rate of D+A processed adzuki bean protein was not used because the feeds were formulated to be isonitrogenous and isocalorific based on the basal diet to meet the standard requirements of nutritional quality of Asian seabass. To fulfil these criteria a 10 % fish meal replacement was the maximum amount that could be used in Asian seabass diet. Therefore, in the current study it was difficult to compare the growth performance between those fed on legume-based protein, processed to certain low enzymatic inhibitor activity, directly to those fed animal protein, as the fish were fed at different protein concentrations.

As mentioned in Chapter 6, zebrafish study serves as a preliminary study therefore the experimental design of Asian seabass should be an exact duplication from the previous chapter except substitute the fish model to farmed fish. However, the organ that collected for inflammatory effect in Asian seabass was liver while zebrafish was from intestines. The size of zebrafish is the major challenge in isolated the liver. It was too small to be isolated and the collected amount will not be able to reach the minimum requirement of using the RNA extraction kit even with the pooled sample. Besides, the difference of study in targeted genes in both chapters was due to the availability of gene sequence provided in NCBI. The information in GenBank for Asian seabass was not established as zebrafish. Thus, different genes which also encoded for inflammatory response had been chose.

7.3.1 Growth performance of Asian seabass

Juvenile Asian seabass at the length of 1 – 7 cm has an intense desire for cannibalism (Mathew, 2009). Although there was some cannibalistic behaviour in juvenile Asian seabass, with damage to fin and tail were being observed during the feeding period, the survival rate of fish was 100 % with no dead fish was being found in 5 weeks of feeding period.

In this study, Asian seabass were fed with two experiment meals (T1 and T2) and one control diet (T0). The initial body weight of Asian seabass ranged from 8.82 – 9.31 g (Table 7.5). After the 5 weeks feeding period, there was no significant difference of ADG found in all diets. Only in the first week did the fish being offered with T1 diet 20 % significantly higher ADG than T2 diet.

Table 7.4 Effect on different legume-based meals on average daily gain (ADG) on a weekly basis

Feeding period	ADG per fish (g/day on weekly basis)		
	T0	T1	T2
Week 1	0.263 ± 0.022^{ab}	0.307 ± 0.018^{a}	0.243 ± 0.003^{b}
Week 2	0.267 ± 0.020^{a}	0.250 ± 0.021^{a}	0.250 ± 0.006^{a}
Week 3	0.257 ± 0.020^{a}	0.250 ± 0.029^{a}	0.250 ± 0.009^{a}
Week 4	0.230 ± 0.026^{a}	0.247 ± 0.029^{a}	0.247 ± 0.017^{a}
Week 5	0.240 ± 0.030^{a}	0.237 ± 0.032^{a}	0.243 ± 0.012^{a}

Notes:
Values are presented in mean ± SEM, n=3
$^{a-b}$ Within a row, values with different letters are significantly different at $p<0.05$
Within a column, no significant difference ($p>0.05$) were found
T0 – control basal diet without inclusion of legume
T1 – replacement of 10 % of fish meal in feed with untreated adzuki bean powder (RAB)
T2 – replacement of 10 % of fish meal in feed with treated adzuki bean powder (PAB)

When compared with basal diet (T0) the Asian seabass fed with T1 and T2 meals showed good growth and were not significantly different ($p>0.05$) in terms of weight

gain, SGR, FCR, PCR, PER, HSI and k index (Table 7.5). No significant difference was observed between raw legume and legume that with reduced enzyme inhibitors and this observation was in accordance with zebrafish. Aforementioned in Chapter 6, it has been reported that if the trypsin inhibitor activity is less than 5.7 TIA unit/mg had no apparent adverse effect on growth performance in European seabass was observed (Tibaldi *et al.*, 2006). As shown in Table 7.1, the trypsin inhibitors of processed and unprocessed adzuki bean was 0.069 TIA unit/mg to undetectable level respectively.

The previously reported the critical threshold of replacing fish meal with plant protein concentrate for juvenile Asian seabass based on a balanced diet was approximately 15 % (weight) without affecting the growth performance and fish health (Glencross *et al.*, 2011). In agreement with this the current findings suggested that these substitutions of legume-based meals have not caused detrimental effect on the digestion and growth performance of Asian seabass. The growth rates of current study were in contrast to those reported by Katersky and Carter (2009) and Tantikitti *et al.* (2005) where Asian seabass were fed with lupin meal or soybean meal, respectively, and they found higher growth efficiency with the lower amounts of feed protein (40 % crude protein). This difference to the study described in this thesis was probably due to a different legume used as feed ingredient, and differences in rearing conditions, as current studies was carried out at fresh water whilst the other studies were in brackish water with 10 – 33 % salinity. In an experiment conducted with Asian seabass, it was shown fish being reared in saltwater resulted in better FCR and greater weight gain relative to those reared in freshwater (Harpaz *et al.*, 2005). Asian seabass is a freshwater-adapted fish, the salt in brackish water can satisfy the osmoregulatory requirements and thus spare energy

used for osmoregulation, leaving more energy available for growth (Gatlin *et al.*, 1992).

Despite the difference in the legume used and rearing condition, published literature suggested that the growth performance of carnivorous species, such as Asian seabass, Japanese seabass, rainbow trout, and Japanese flounder, are influenced by different feeding rate and frequency (Ye *et al.*, 2011; Cheng *et al.*, 2010; Soler-Vila *et al.*, 2009; Salama, 2008). As an example, Ganzon-Naret (2013b) stated that the growth of Asian seabass was greatly enhanced by feeding the fish 6 times daily at 12 % body weight per day. However, in this study, the fish was only fed once per day at 4 % of their body weight per day.

Table 7.5 Asian seabass growth performance

Growth parameters	T0	T1	T2
Initial weight (g)	9.21 ± 0.22	9.31 ± 0.42	8.82 ± 0.13
Weight gain (g)	8.45 ± 1.70	8.28 ± 1.89	8.61 ± 0.73
SGR (%)[1]	2.24 ± 0.36	2.19 ± 0.43	2.35 ± 0.14
FCR[2]	2.15 ± 0.37	2.24 ± 0.47	2.01 ± 0.14
PCR[3]	1.02 ± 0.10	1.00 ± 0.12	0.89 ± 0.04
PER[4]	1.57 ± 0.16	1.57 ± 0.19	1.34 ± 0.05
k index[5]	2.01 ± 0.21	1.95 ± 0.19	1.99 ± 0.15
HSI[6]	2.00 ± 0.38	1.83 ± 0.53	1.95 ± 0.35

Notes:
Values are presented in mean ± SEM, n=3
No significant difference (p>0.05) were detected
[1] SGR – specific growth rate
[2] FCR – feed conversion ratio
[3] PCR – protein conversion ratio
[4] PER – protein efficiency ratio
[5] k index – Fulton's condition factor
[6] HSI – hepatosomatic index
T0 – control basal diet without inclusion of legume
T1 – replacement of 10 % of fish meal in feed with untreated adzuki bean powder (RAB)
T2 – replacement of 10 % of fish meal in feed with treated adzuki bean powder (PAB)

Protein is the most expensive element in aquafeed. It is crucial to have good PCR and PER in aquafeed manufacturing and feeding practices (Boyd *et al.*, 2007). The PCR for fish fed with T2 diet indicated only 0.89 kg of crude protein could be applied in aquafeed in order to obtain 1 kg of fish, which was 11.0 – 12.7 % lower than basal (T0) and T1 diet. Whilst PER indicated the efficiency of converting the feed protein to aquatic animal protein for human consumption. The PER for fish fed with T2 diet indicated only 1.34 kg of crude protein must be applied in aquafeed in order to obtain 1 kg of fish protein, which was 14.6 % lower than basal and T1 diet.

As mentioned in Chapter 6, k index is important to determine the fitness and health of the fish population. In this study, no significant difference (p>0.05) was found in k index indicating that, generally, the fish were in relatively good condition on all the diets. HSI is a measurement of energy reserves and metabolic activity in fish (Humphrey *et al.*, 2007). There was no significant increased (p>0.05) in HSI among the experimental diets indicated the plant protein were not causing lipid accumulation in the liver.

Recently, various published research studies have investigated, not only the effect of using soybean, but other plant protein, such as green pea, canola and lupin, as an alternative fish meal replacement in barramundi feed (Glencross *et al.*, 2016; Van Vo *et al.*, 2015; Plaipetch *et al.*, 2014; Ganzon-Naret, 2013a; Plaipetch and Yakupitiyage, 2011). Although a recent studied had shown replacement of 90 % fish meal in barramundi did not affecting the growth performance, only 30 % was replaced with plant-based ingredient (soybean meal) the other 60 % was replaced by poultry meal (Glencross *et al.*, 2016). Whereas Van Vo *et al*. (2015) and Plaipetch and Yakupitiyage (2011) reported there was no adverse effect in barramundi growth performance

when their feed had an inclusion rate of fermented canola meal (50 %) or lupin meal (75 %), respectively. They were able to include a higher inclusion rate as ANFs were significantly reduced in these ingredients, in particularly phytic acid which had decreased by 87 % in fermented lupin and 17 % in fermented canola.

With 10 % fish meal replacement there was no significant difference in protein and lipid content indicating the nutritional values of the fish were not influenced by the diets. In terms of protein, lipid and moisture content, the carcass of Asian seabass was relatively consistent across all diets in this study (Table 7.6). This observation was in agreement with Ye *et al.* (2011) and Øverland *et al.* (2009) who stated that the fish carcass of Atlantic salmon and Japanese flounder was not influenced by feeds containing soybean meal and pea. This could be attributed to the similar nutrient composition in experimental diets to the basal diet and fish was able to utilise the plant protein in the same way they utilised the fish meal protein.

Table 7.6 Fish carcass that being fed with different diets across 5 weeks

Fish carcass (% DW)	T0	T1	T2
Crude protein	64.82± 1.03	63.40± 1.27	66.03± 2.16
Crude lipid	11.14± 0.42	12.80± 0.40	15.58± 0.76
Moisture	75.46± 1.65	75.37± 0.68	75.42± 0.19

Notes:
Values are presented in mean ± SEM, n=3
No significant difference (p>0.05) were detected
T0 – control basal diet without inclusion of legume
T1 – replacement of 10 % of fish meal in feed with untreated adzuki bean powder (RAB)
T2 – replacement of 10 % of fish meal in feed with treated adzuki bean powder (PAB)

7.3.2 Hepatic genes response

Fish liver is the main organ which not only plays an important role in toxicant and immune response but also involved in nutrient utilisation as the centre of intermediary metabolism (Panserat *et al.*, 2009; Tintos *et al.*, 2008). Therefore, liver is the target organ to study the experimental diet induced effects in fish.

Figure 7.1 shows that no significant change in the expression of the hepatic inflammation response genes HSP 70 and CRP between the treatments meals (T1 – T2). Whereas the transcriptions of the amino acid metabolism gene ALAT significant increased (p<0.01) by 1.4-fold in fish fed the T2 meal.

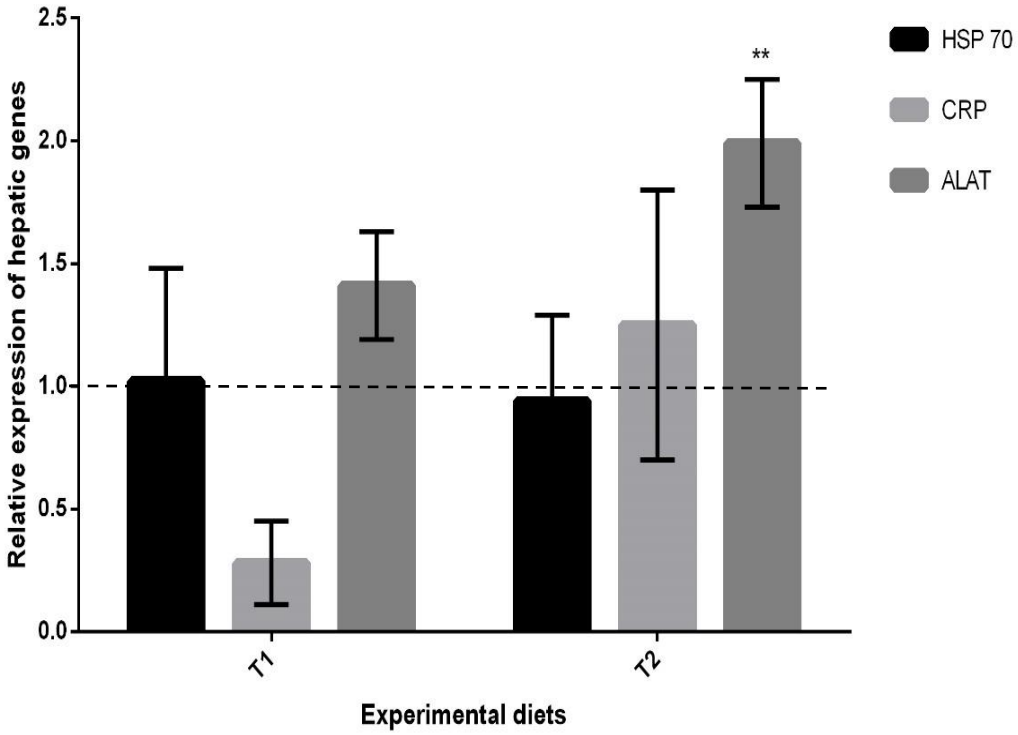

Figure 7.1 The hepatic genes expression of heat shock protein 70 (HSP 70), C-reactive protein (CRP) and alanine aminotransferase (ALAT) in fish liver after fed at different experimental diets. T0 – control basal diet without inclusion of legume; T1 – replacement of 10 % of fish meal in feed with untreated adzuki bean powder (RAB); T2 – replacement of 10 % of fish meal in feed with treated adzuki bean powder (PAB). The error bars represent the standard error mean, n=3. ** significant difference (p<0.05) were detected.

The current findings showed that the experimental diets had not appear to cause any inflammation effects in fish liver, as indicated by changes in gene expression of genes associated with this response, which was in accordance with recent study done by Glencross *et al.* (2016). They stated that Asian seabass had been offered with 100 % substitution of fish meal with the mixture of poultry meal and soybean meal have no induction of inflammatory markers of HSP 70 and CRP.

In nature, carnivorous fish have limited capacity to utilise carbohydrate and they are adapted to preferential use of dietary amino acid from protein as the main source of energy production (Gatlin III, 2010; Stone, 2003). ALAT is reliable marker of dietary protein utilisation in fish (Metón *et al.*, 2015). It plays an important role in linking amino acid, carbohydrate and energy metabolism by catalysing the reversible transamination between L-alanine and ketoglutarate to pyruvate and L-glutamate, thereby generating non-essential amino acids (Metón *et al.*, 2015; González *et al.*, 2012).

It is noteworthy that ALAT gene expression is upregulated in liver's of fish fed with T2, indicating the upregulation of transamination, even though T1 and T2 had a similar protein content (approximately 44 %). This differential in gene expression might indicate the differences in the efficient in using dietary amino acid for growth in the reduced enzyme inhibitors legume meal (T2), since on this diet yielded a better weight gain. The rise in the hepatic activity of protein-metabolising gene denote the usage of dietary amino acids for growth (Bibiano Melo *et al.*, 2006).

On the other hand, an interpretation could be that availability of amino acid in T2 diet is better than T1 which then might have caused the upregulation of ALAT gene.

As shown in Table 7.1, the essential amino acids in untreated adzuki bean powder (RAB) used in T1 diet was lower compared to treated adzuki bean powder (PAB). When the available amino acids are deficient, it will lead to the decrease of ALAT enzyme activity, indicated by the associated change in ALAT gene expression (Cheng *et al.*, 2010). The upregulation of amino acid metabolism ALAT in fish that fed with treated adzuki bean postulated there was a not deficiency of amino acids for juvenile Asian seabass, but they also used very few amino acids for energy. Therefore it could be suggested the amino acids were used for maintenance, health and synthesis of new structural proteins, which would result in maximum feed efficiency and growth (Ovie and Eze, 2013). Previously a study had shown upregulation of gene ALAT in liver was associated with high ALAT enzyme activity (Caballero-Solares *et al.*, 2015). It is assumed that this could have happened in the present study, even though the enzyme activity was not quantified.

Like any other studies, there were limitations in Asian seabass feeding trial. Some of these were associated with the lighting supply in the fish lab, as the quantity of light reaching the bottom aquaria was limited resulting in the fish in these aquaria being less active than those in other aquaria that were exposed to more light. To mitigate this effect, the position of the fish was switched every week after their weight was taken. The cannibalistic and aggressive behaviour of juvenile Asian seabass is another challenge of the study. They all started at the similar size (approximately 5 to 6 cm) however it was observed that some were more aggressive than others. During the feeding period, the aggressive fish tends to attack the less aggressive fish which reduced the feeding of the latter. Therefore, over the feeding period there was a differential in nutrient intake of the fish within a tank, however analysis was carried

out at the level of the tank rather than individual fish, the statistical replicate being the tank.

For future experiments it should ensure that lighting is more uniform across the tanks this would avoid the requirement to weekly switch the position of the fish in order to reduce the stress. To overcome the cannibalistic behaviour that might possibility affect the appetite of juvenile seabass; the density of fish in the aquarium should be increased. It has been suggested that with high density of fish could limited the resting space and breakdown of hierarchical territoriality (Kaiser *et al.*, 1995). Fish tend to form shoals and cannibals become perceptively confused and the ability of selecting a prey is low as the number of potential prey is too high (Baras and Jobling, 2002).

7.4 Summary

Current findings confirm that the adzuki bean meal with reduced enzyme inhibitors did not affected the growth performance of Asian seabass and not induced inflammatory signals, at least in the liver. However, there is an increasing effect on gene that associated amino acid metabolism which may indicate the adaptations of Asian seabass towards plant based aquafeed.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSION

The over-reliance on fish meal as protein source in aquaculture industry has caused pressure on ocean fisheries because farming carnivorous species required large amount of fish feed derived from wild-caught fish. To ensure the sustainability of aquaculture industry, plant protein sources such as underutilised legumes has evolved as a potential alternative aquafeed ingredient. However, the presence of enzyme inhibitors in legumes are known to reduce digestion and nutrient absorption in carnivorous fish. Hence, this study aimed to develop a processing strategy to remove trypsin, chymotrypsin, and α-amylase inhibitors present in underutilised legumes, followed by incorporating these processed legumes in aquafeed to replace fish meal and enhance the growth of zebrafish and Asian seabass.

The aim of the study has been achieved by using a combination method of dry freezing (D) at -80°C for 24 h and autoclaving (A) at 121°C at 15 psi for 15 min. This was the first study that has used the combination of D+A to reduce the enzyme inhibitors. Current findings showed that the D+A treatment was most effective on adzuki bean. The trypsin and chymotrypsin inhibitors in adzuki bean were reduced to non-detectable level, whilst up to 70 % of reduction in α-amylase inhibitor. The crude protein content was not affected by this D+A treatment. The outcome of this study had contributed to overcome the nutritional shortcomings of legume for use as a sustainable protein source in aquafeed. Furthermore, the D+A treatment is a simple and effective approach that could be upgraded to commercial scale to satisfy the need of aquaculture industry.

The concept of formulated aquafeed is to use available ingredients and processed them in the most economical way to provide the essential nutrient content and balance diet to the commercial cultured fish by taking the feed quality and palatability into consideration. A key aspect for sustainable production and profitable aquaculture industry is to reduce the cost that mainly contributes by aquafeed that represent up to 60 % of operational cost. Reductions in feeding costs can be done through substitution of fish meal using underutilised legume since protein is the most expensive element in formulated aquafeed. Thus, the application of D+A treatment by eliminating the enzyme inhibitors in these legume-based ingredients would possibly promote good fish growth, survival, production, and at the same time boost the income of farmers.

As described in Chapter 1 (section 1.3.1), a shortage in soybean meal is anticipated with increasing demand in aquaculture industry and in conjunction with fluctuation in production deficits and resulted in price escalating. This factor has also lead to the increase of market price for aquafeed and farmed fish. Hence, underutilised legumes that with sustainable production, lower market price and some that could even withstand to adverse climate and environmental conditions are the way forward for food and feed industries. Undeniable the crude protein content of adzuki bean (21 – 24 %) was not as high as soybean (40 %) and might limit the possibility of total replacement of soybean in aquafeed. However, adzuki bean combined with other underutilise legumes could still replace a substantial portion of fish or soybean meal and contribute to fulfil the protein requirement of farmed fish.

Current study with inclusion of 10 % (w/w) of treated adzuki bean had no effect on growth performance of zebrafish or Asian seabass. Current finding suggests that the

treated legume protein may potentially replace the fish meal without having negative effects the growth performance of carnivorous fish. However, the expression of amino acid metabolism gene ALAT was upregulated in fish fed with treated adzuki bean suggested the increment of accessibility in amino acid. As mentioned in Chapter 7 (section 7.3.2), ALAT gene is one of the principal pathways responsible for the synthesis and transamination of amino acids by transfer an amino acid to a ketoacid to form new amino acid, thereby, allowing interconversion between carbohydrate and protein metabolism to meet the energy demand of fish. The rise of this protein-metabolising gene denotes the usage of dietary amino acids were used for maintenance, health, and synthesis of new structural proteins in resulting maximum growth in Asian seabass.

The fish carcass always serve as an indicator of quality of the fish as a food product (Mumba and Jose, 2005). In this study, regardless of whether legume constituent of the diet was treated or untreated, the carcass protein and lipid composition in juvenile Asian seabass was not significantly different to the fish fed the control – animal protein (fish meal) based feed, suggesting they can utilise legume-based protein in the same way they utilised the fish meal protein. Investigation of the impact of legume-based protein on fatty acid composition, especially in terms of omega-3 long-chain polyunsaturated fatty acids of Asian seabass would also be of interest on flesh quality. Furthermore, the changes in fish diets often associated with changes in fillet characteristics in terms of texture, colour, and taste. Sensory evaluation may play a critical role in the evaluation of fish quality as well as the acceptance level of consumer.

In future, the incorporation level of treated D+A legume protein could increase from 10 % (w/w) to complete replacement of fish meal to examine the maximum incorporation rate in the feed without depressed the growth in carnivorous fish. Further investigation on the prolonged feeding period from weeks to months should be warranted since the juvenile fish (< 10 g) of Asian seabass required up to half year feeding period to reach marketable size (approximately 500 - 600 g). These studies could hopefully address the farmer's demand of farmed fish with good growth performance and meat quality.

Beside enzyme inhibitors, there are also wide range of ANFs including saponin, phytic acid, oligosaccharides, lectin, tannin, and cyanogen are naturally present in legumes which could possibly affect the fish digestive system and thereby influence their growth performance. The interaction of these ANFs especially phytic acid and tannin with macronutrient present in fish feed would also be of interest since the effect on digestive and absorptive process in carnivorous fish have not been well documented. The feed palatability may reduce with the presence of tannins due to their astringent flavour.

In summary, the D+A processing method is effective in reducing the enzymatic inhibitors present in underutilised legumes and could also be applied to any other legumes in an attempt to study their possibility as the alternative aquafeed ingredients. Shifting away from wild-harvested fish meal to using treated underutilised legume as protein source and ingredient in aquafeed provide a solution on dwindling fish catches, thereby creates a more sustainable aquaculture industry that relieve pressure on ocean fisheries.

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APPENDICES

APPENDIX A

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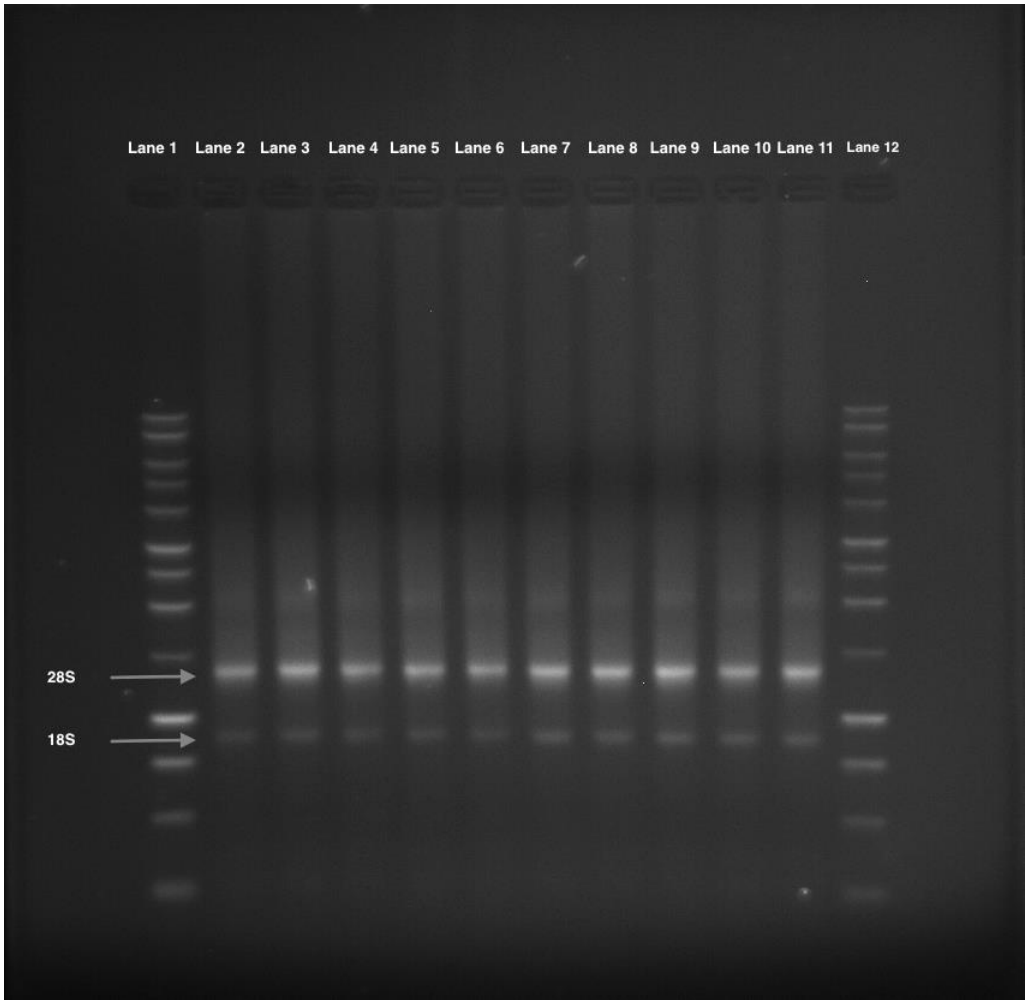
APPENDIX B

B1: Gel image of zebrafish feeding trial

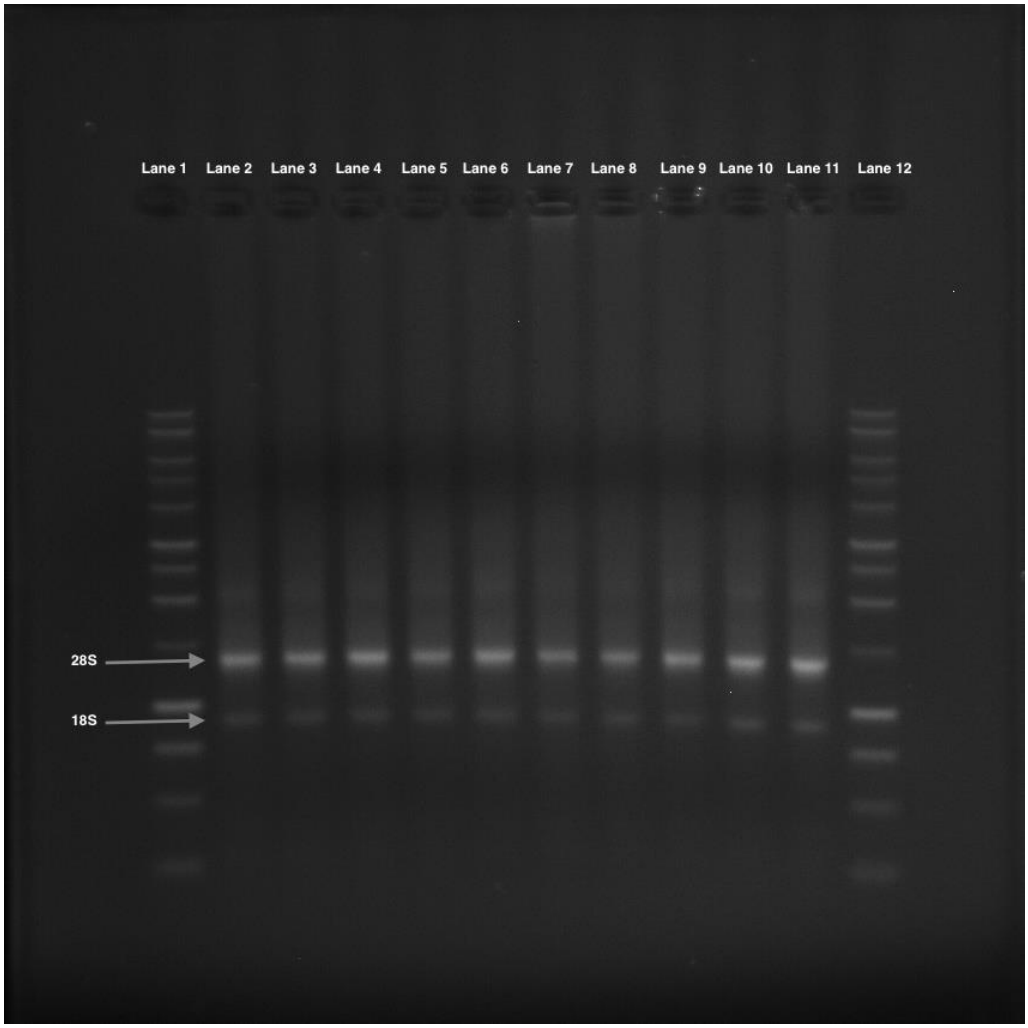


Lane 1 & 12: Promega 1kb DNA ladder

Lane 2 – 6: Fish fed with basal

Lane 7 – 11: Fish fed with untreated adzuki bean samples

B2: Gel image of zebrafish feeding trial

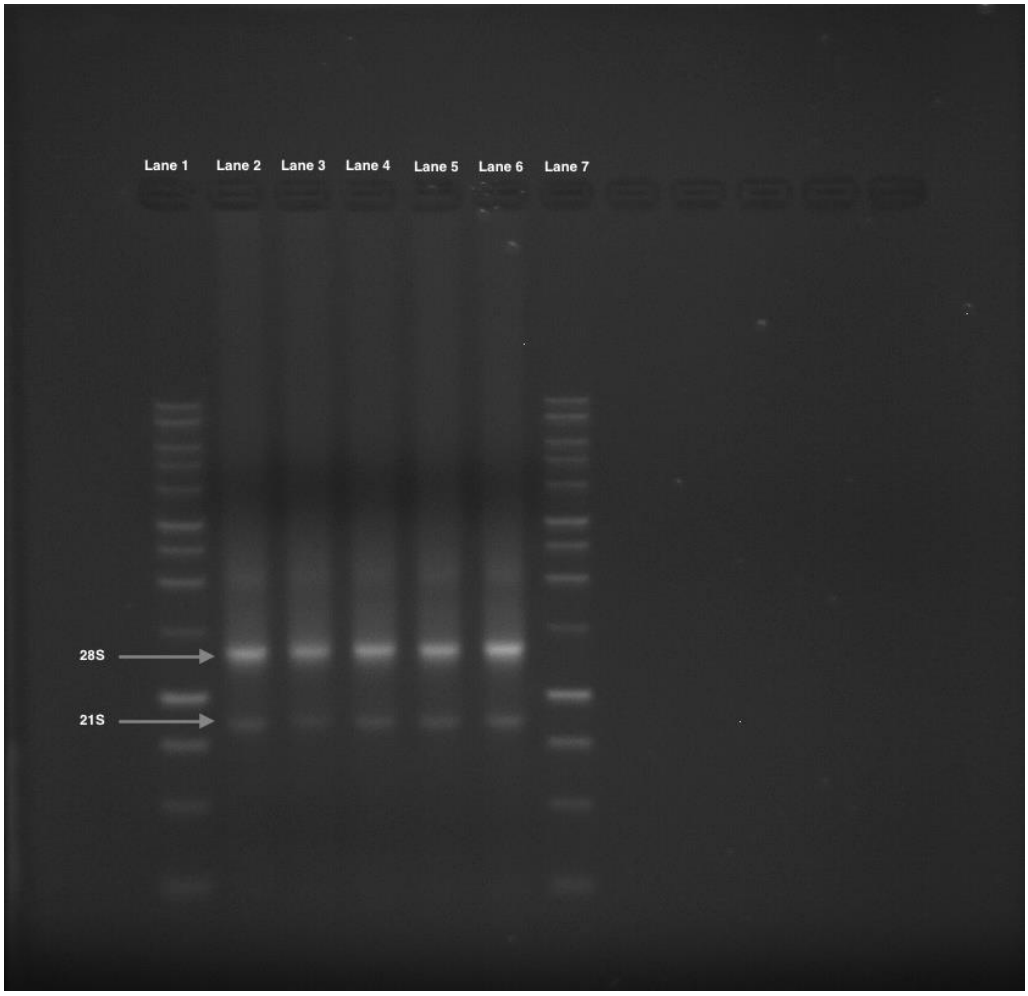


Lane 1 & 12: Promega 1kb DNA ladder

Lane 2 – 6: Fish fed with treated adzuki bean samples

Lane 7 – 11: Fish fed with untreated bambara groundnut samples

B3: Gel image of zebrafish feeding trial



Lane 1 & 7: Promega 1kb DNA ladder

Lane 2 – 6: Fish fed with untreated bambara groundnut samples

APPENDIX C

The animal ethic approval document for Asian seabass feeding trial from the Animal Welfare and Ethical Review Committee.

Animal Welfare and Ethical Review Body

Cover Form applicable to the use of animals in Non-Regulated procedures or overseas

Approval Ref. No. _____UNMC8_____

Title of Study: **Efficiency of plant-based ingredients to replace fish meal in the diet of Asian Seabass, *Lates calcarifer***

Name of Applicant: **Dr Yin-Sze Lim (UNMC), Professor Andrew Salter (UoN) and Professor Tim Parr (UoN)**

School: **Biosciences**

1. Requests to use animals in non-regulated procedures must incorporate the information required below in the format requested for project licence applicants. The completed form should be returned electronically to the bsu@nottingham.ac.uk. When approved a protocol reference number will be provided to the applicant and must be used when ordering animals for use in the non-regulated study.

Its purpose is to highlight the key points in the proposed programme of work and to provide an executive summary in non-technical language accessible to the lay members of the Animal Welfare and Ethical Review Body.

Study Title (max. 50 characters)	Efficiency of plant-based ingredients to replace fish meal in the diet of Asian Seabass, *Lates calcarifer*
Key Words (max. 5 words)	Plant-based ingredient, underutilised legume, fish meal, growth, Seabass
Expected duration of the study (yrs)	10 weeks
Purpose of the study (Indicate clearly which area applies).	Basic research: ; Translational and applied research: X; Regulatory use and routine production: ; Protection of the natural environment in the interests of the health or welfare of humans or animals: ; Preservation of species: ; Higher education or training: ; Forensic enquiries:
Describe the objectives of the study (Simply state the overall aim of your study and then specifically what you are wishing to achieve).	Reduce the finite marine ingredient fish meal inclusion level and increase fish health by plant-based ingredients. These plant-based ingredients are (i) underutilised legumes with removed anti-nutrients and (ii) resistant starches. This project aims to reduce the substantial level of fish meal in fish diet and improve gastrointestinal health of fish by using nutritionally rich underutilised legumes.

1

What are the potential benefits likely to derive from this study (how science could be advanced or humans or animals could benefit from the project)?	Underutilised legumes are nutritious and high in protein, and can be grown in a sustainable way to replace fish meal. Inclusion of legumes with removed anti-nutrients and inclusion of resistant starch can improve the digestion, nutrient absorption and gastrointestinal health of fish. Meeting the consumer demand for environment friendly fish feed. Reducing the dependency on unsustainable and expensive fish meal and commercial legumes.
What species and approximate numbers of animals do you expect to use over what period of time?	Fish, Asian Seabass (*Lates calcarifer*) – 250, for a period of 10 weeks.
What procedures will be conducted?	Feeding trial using diets prepared. Analysis of fish at the end of feeding trial.
In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end? If collection of tissues state 'humane killing by a competent person using a S1 method'	Diseases are common and inherent problem in any aquaculture project conducted outdoor or indoor. However, the chance of disease is reduced in our controlled laboratory. Fish samples will be collected at the end of the experiment following humane killing by a competent person using a S1 method.
Application of the 3Rs	
1. Replacement State why you need to use animals and why you cannot use non-animal alternatives	Fish meal is consumed by the aquafeed industry in order to feed commercially important fishes. To evaluate the potential of a substitute, we must carry out the feeding trials.
2. Reduction Explain how you will assure the use of minimum numbers of animals. (Short explanation required about how you have ensured that only the minimum numbers of animals are used. E.g. The method of statistical analysis and group sizes required).	Fish nutrition trial aims to maximize the growth by ensuring the optimum health of rearing fish. Thus, a minimum stocking density where the fishes can enjoy the feed in stress free environment will be followed. Fish will be randomly allocated to twelve experimental tanks and assigned to one of the experimental feeds. One way ANOVA. Our experimental tanks of 120L (0.12$m^{3)}$ capacity, can support 3~4 kg/m^3 of a biomass. Thus depending upon the individual fish weight and size, the number will be adjusted in order to maintain the above mentioned stocking density for Asian seabass reared in fresh water.

3. Refinement Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.	Seabass is widely known and popular among fish farmers due its hardy nature and resistance to disease. Being a euryhaline fish species, it is also able to tolerate a wide range of salinity fluctuation. Fish will be transported in oxygenated plastic bags and will be allowed to adjust to the laboratory conditions for one week and during the acclimatization period, water will be exchanged daily and fish will be fed a nutritionally well balanced diet.
4. Housing and Care Please confirm that animals will be housed and cared for according to the requirements of the UK Animals (Scientific Procedures) Act 1986 draft Code of Practice for the care and accommodation of animals (February 2013) **or** provide details and justification for alternative housing and care standards.	https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/148568/aspa-draft-code-of-practice.pdf Our indoor lab at Crops for Future research centre Malaysia is well equipped with water exchange, gentle aeration, air conditioning, water cooler and heater and illuminated lighting system to control all abiotic factors effecting fish growth. Dissolved oxygen will be maintained using aerator, to avoid the oxygen deficiency siphoning and tank cleaning will be carried out after one hour of every feeding. Care will also be taken to avoid the super saturation of oxygen by controlling the air supply through air stone. Water quality parameters such as ammonia, pH, nitrite and nitrate will be measured weekly using water analysis kit. Light will be maintained as the natural environment of 14:10 hours light and darkness.
5. Humane Killing Please confirm that animals will be humanely killed using an appropriate method of humane killing detailed in Schedule 1 of the UK Animals (Scientific Procedures) Act 1986 **or** provide details and justification for alternative methods of killing.	Appropriate method of anaesthesia will be employed using benzocaine to kill fishes after the termination of feeding trial. Afterwards, the intestine and liver will be dissected for analysis.

To be signed by the head of the research group:
I confirm that the procedures to be conducted will not result in any animal experiencing pain, suffering, distress or lasting harm and are therefore non-regulated under ASPA.

Humane killing (if applicable) will be in accordance with Schedule 1 (revised 1 Jan 2013) and will only be conducted by competent individuals named on the University of Nottingham register.

Signature..... *Date*...15th September 2016.....

Committee use only

Comments by NACWO:

This study is in line with a previously approved study UNMC 00005 (October 2015). The study involves a feeding trial using novel plant based ingredients to feed Sea Bass rather than fish derived protein.

It is not clear if this substitute diet will support the nutritional requirements of Sea Bass without any impact on the health of the fish. It is assumed that no impact on fish health is expected. This should be confirmed.

If this cannot be confirmed it is strongly suggested that pilot studies with small numbers of fish are undertaken to ensure that the nutritional requirements of the Sea Bass can be met ahead of the main study and a revised experimental plan submitted.

A figure of 250 fish is given with no indication of group sizes / control groups and number of diets to be assessed. The applicant is kindly asked to state group sizes and number of diets and explain the experimental design to support the use of the fish for the period of 10 weeks.

The stocking densities and environmental conditions would appear to be consistent with the information that supported the earlier study.

It is not clear if Tricaine methane sulfonate (MS-222) will be used to humanely kill the fish as a more general generic wording of benzocaine is used. This should be clarified.

Neil Yates
25/09/16

Comments by NVS:

The proposed study seems to have a worthwhile aim and it would appear that there are useful benefits if the work is successful. However, as noted above, there is no background detail as to how the diet will be analysed for nutritional status before the study commences, and the group sizes appear to be arbitrary. These points should be addressed before approval is given.

Ewan McNeill 26-09-16

Draft 2.
Thank you for this comprehensive clarification (below). This response satisfactorily addresses the points raised and the work is approved.
Neil Yates 20/10/16

Comments by primary reader for the Committee (if applicable):

Comments by Lay Person (If applicable):

Committee decision:

Approved

Communicated to applicant (date): 21/10/16

Response to comments from NACWO and NVS:

It is not clear if this substitute diet will support the nutritional requirements of Sea Bass without any impact on the health of the fish. It is assumed that no impact on fish health is expected. This should be confirmed.

If this cannot be confirmed it is strongly suggested that pilot studies with small numbers of fish are undertaken to ensure that the nutritional requirements of the Sea Bass can be met ahead of the main study and a revised experimental plan submitted.

The substitution of the Sea Bass's feed is to be fed with either a legume (LT test feed) or a resistant starch (RS test feed). In LT test feed the fish meal is to be replaced with legume at 10% weight of the fish meal component (there are two feeds to be tested LT1 and LT2), all the other constituents remain the same proportion (Table 1). For RS test feed (RST1) the resistant starch (Lens RS) is included at 2.5% with the all individual feed constituents being reduced by the same proportion of their weight (Table 2).

Table 1: LT test feed examining the effect of replacing fish meal with 10% legumes in Sea Bass feed

	Feed		
	Basal	LT1	LT2
Ingredients	(g/1.5kg)	(g/1.5kg)	(g/1.5kg)
Fish meal	843	758.7	758.7
Legumes	0	84.3	84.3
Dextrin	222	222	222
Soybean meal	174	174	174
Corn meal	96	96	96
Fish oil	90	90	90
Vitamin premix	45	45	45
Mineral premix	30	30	30
Total weight:	1500	1500	1500

Table 2: RS Test feed examining the effect of 2.5% inclusion of resistant starch in Sea Bass Feed. The basal feed is the same as in Table 1

	Feed	
	Basal	RST1
Ingredients	(g/1.5kg)	(g/1.5kg)
Fish meal	843	821.925
Lens RS	0	37.5
Dextrin	222	216.45
Soybean meal	174	169.65
Corn meal	96	93.6
Fish oil	90	87.75
Vitamin premix	45	43.875
Mineral premix	30	29.25
Total weight:	1500	1500

In the control (Basal) feed the proximate crude protein content is estimated as 45g/100g dry weight whilst in the test feeds the lowest protein content is 42g/100g dry weight (Table 1 feeds LT1 and LT2), the reduction in protein content of test feeds in Table 2 is less than Table 1 . Fish have a high protein requirement in their feeds and this is the predominant factor influencing growth. However the protein content in all the feeds listed in the trials does not drop below 40%, which has been described to be adequate for Sea Bass growth in experimental feeds (Katersky & Carter, 2009). Fish meal consists predominantly of protein (70% dry weight). Replacement of the fish meal with legumes in Table 1 inevitably results in the reduction in crude protein in the test diets but the legumes contain relatively high quantities of carbohydrate to protein therefore the feeds will not be energy deficient. The feeds also contain fish oils to ensure that the provision of essential fatty acids, this does not change. In Table 2 the inclusion of resistant starch (Lens RS) is at 2.5% of the feed. To compensate for this component the other feed ingredients are reduced by 2.5% of the weight in the control feed (Basal). Therefore the overall relative composition of the feed remains the same. This reduction is not sufficient to affect the growth of the fish.

As is standard practice fish are meal fed, with feed being fed at 4% of their body weight which enables growth to occur (Personal communication, Dr Iain Young, University of Liverpool). Therefore neither groups of feeds essential nutrient composition will impact on fish health as the feeds are designed to enable growth in all treatment groups.
The experiment is designed to assess variations in growth rate.

Recently we carried out a similar experiment at the University of Liverpool (collaboration with Dr Iain Young) examining the impact of the two feed additives (legumes and resistant starch) at similar inclusion rates on the growth of zebra fish. There was no apparent effect on palatability of the feeds and there no negative impacts on health or welfare.

Given the above we suggest that there is no requirement for a pilot study.

A figure of 250 fish is given with no indication of group sizes / control groups and number of diets to be assessed. The applicant is kindly asked to state group sizes and number of diets and explain the experimental design to support the use of the fish for the period of 10 weeks.

The trial will consist of 4 groups using testing the feeds shown in Table 1 and 2:

Basal Feed (identical in Table 1 and 2)
LT1 (Table 1). Legume sample 1; 10% by weight inclusion of Adzuki bean flour

LT2 (Table 1). Legume sample 2; 10% by weight inclusion of Adzuki bean flour treated to reduce anti-nutritional factor content
RST1 (Table 2). Resistant starch sample;

The basal feed group acts as a control for both the "LT" (Table 1) and "RS" (Table 2) feeds thereby reducing the need for two control groups. The aim of the experiment is compare the effects of the modified fish feeds represented by LT1, LT2 and RST1 relative to the Basal feed. The experiment will also allow us to determine the effect of including resistant starch relative to the Basal feed. In addition it will examine of the effect of inclusion of legumes in to fish feed relative to the Basal feed and the impact reducing ANF in legumes.

A 10 week feeding period is required as the effect of these feeds on growth will be assessed at the end trial by differences in weight (weight gain). As this is an end point measurement fish have to be grown for sufficient time (10 weeks) for small changes in weight gain to be detected. At the end of the trial fish will be killed and processed to assess body composition this is the main experimental outcome for the trial.

There are 12 experimental tanks available therefore there are n=3 tanks assigned to each test feed. In the original application the number of fish to be used was stated as 250. From these fish will be selected disease free individuals of near equal weight so that there are 8 fish per treatment tank, a total of 96 fish being used for the whole experiment.

As described in the original document the experimental tanks are each 120L (0.12m3). The recommended stocking density for sea bass is 30-40kg/m3 (http://www.dpi.nsw.gov.au/fishing/aquaculture/publications/species-freshwater/barramundi-aquaculture-prospects); apologies there was an error in the original application. Therefore each tank can support 3.6- 4.8kg of sea bass. The maximum mature weight of a sea bass is 500g after 6-12 months of growth from hatching, dependent on feed quality and temperature. Assuming the maximum growth rate, then a stocking density of 8 fish per tank will not exceed the recommended stock density when fish are grown for 10 weeks, as the animals will start the trial weighing less than 100g each.

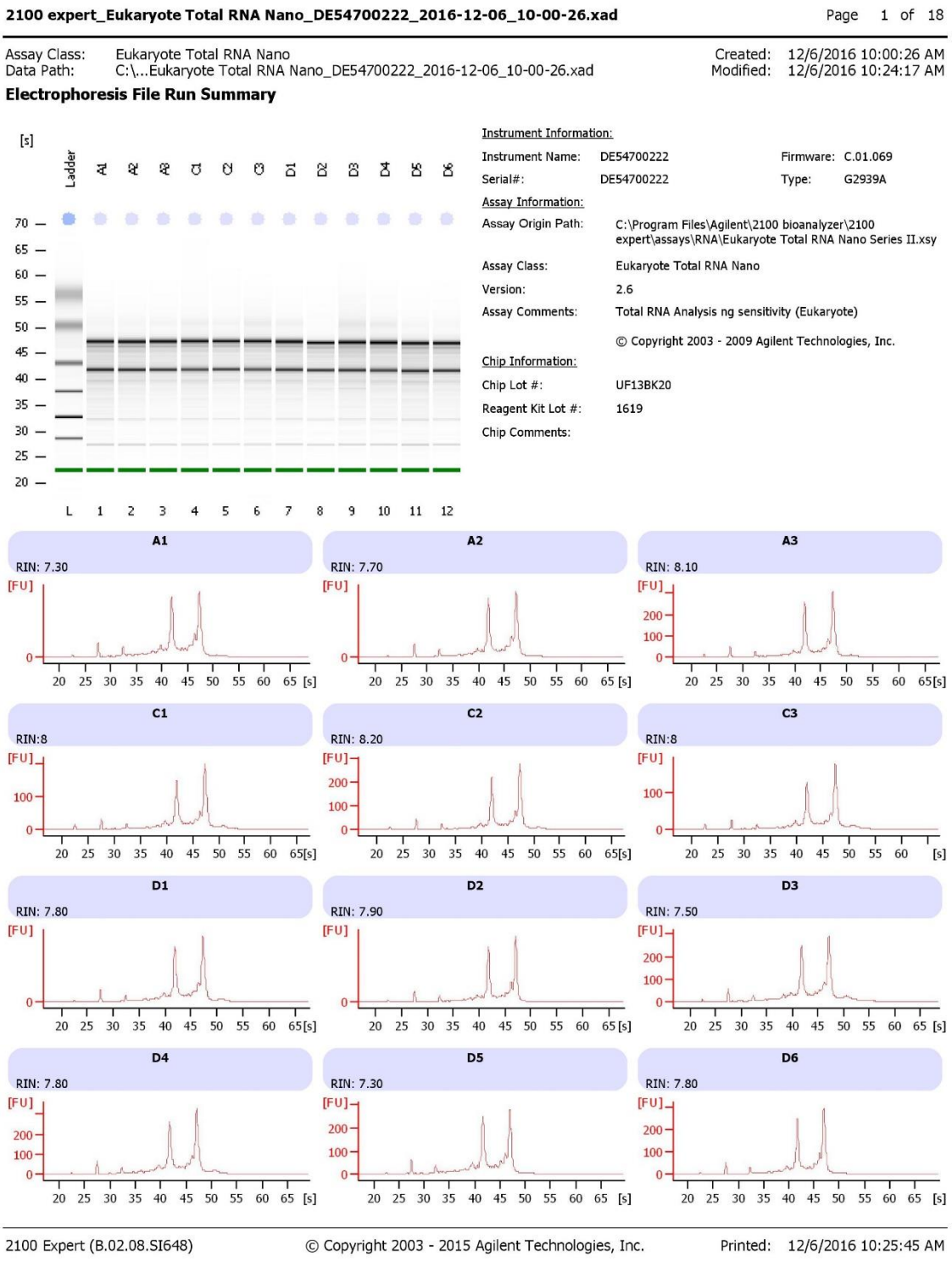
We hope the detail above is sufficient

It is not clear if Tricaine methane sulfonate (MS-222) will be used to humanely kill the fish as a more general generic wording of benzocaine is used. This should be clarified.

Both Tricaine methane sulfonate (MS-222) and Benzocaine (ethyl para-aminobenzoate) are anaesthetics primarily used to immobilize fish and to reduce stress and pain during various handling and sampling procedures. Recently we carried out a similar experiment at the University of Liverpool (collaboration with Dr Iain Young) examining the impact of the two feed additives at similar inclusion rates on the growth of zebra fish. In this experiment, the benzocaine was used to humanely kill the fish. This is the Home Office Inspector approved procedure which is utilsed at the University of Liverpool fish biology unit for the endpoint fish growth trials (Schedule 1 procedure). Hence to ensure consistency across experiments and taking into consideration that anaesthetics can alter tissue function, we have chosen to use the similar anaesthetic – benzocaine for this proposed study.

APPENDIX D

The gel image from bioanalyzer for Asian seabass trial



Assay Class: Eukaryote Total RNA Nano
Data Path: C:\...Eukaryote Total RNA Nano_DE54700222_2016-12-06_10-00-26.xad

Created: 12/6/2016 10:00:26 AM
Modified: 12/6/2016 10:24:17 AM

Electrophoresis File Run Summary (Chip Summary)

Sample Name	Sample Comment	Status	Result Label	Result Color
A1		✔	RIN: 7.30	
A2		✔	RIN: 7.70	
A3		✔	RIN: 8.10	
C1		✔	RIN:8	
C2		✔	RIN: 8.20	
C3		✔	RIN:8	
D1		✔	RIN: 7.80	
D2		✔	RIN: 7.90	
D3		✔	RIN: 7.50	
D4		✔	RIN: 7.80	
D5		✔	RIN: 7.30	
D6		✔	RIN: 7.80	
Ladder		✔	All Other Samples	

Chip Lot #	Reagent Kit Lot #
UF13BK20	1619

Chip Comments :