Department of Environment and Agriculture

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Physiological responses of juvenile barramundi, *Lates calcarifer* (Bloch, 1790) when fed bioprocessed plant base diets

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

The research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes 8th edition (2013). The proposed research study received animal ethics approval from the Curtin University Animal Ethics Committee, Approval Number: AEC_2014_14/25

Signature: 

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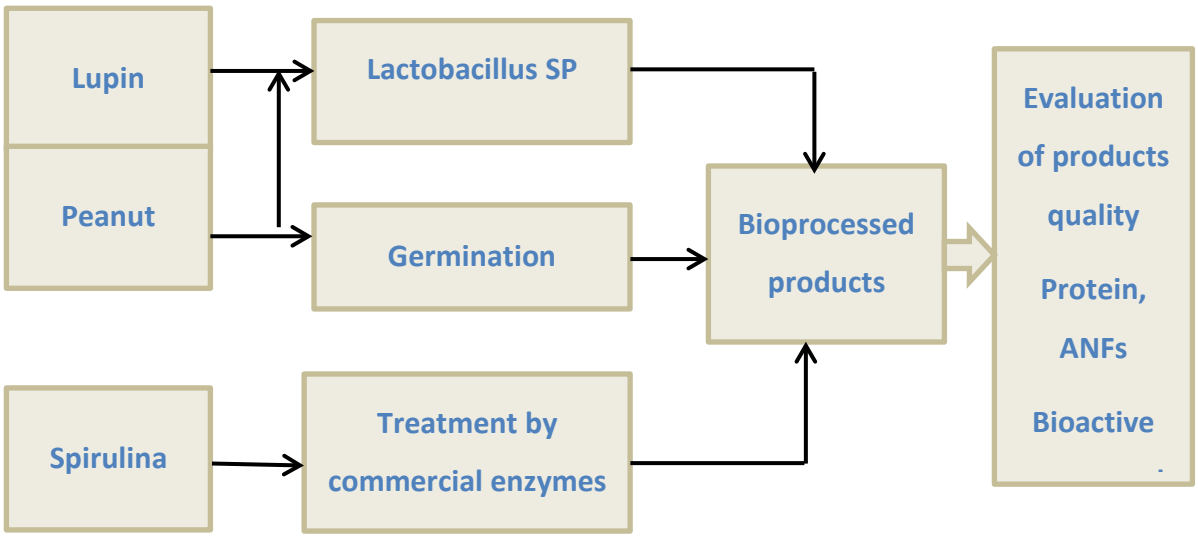
PREAMBLE

The overall goal of this study is to select appropriate plant products and/or their bioprocessed form that contain suitable quantity and quality of protein to replace fishmeal in juvenile barramundi (*Lates calcarifer*) diets. The bioprocessing techniques of the selected plant products include fermentation, germination and enzymatic (celluloses and proteinases) treatment to improve their quality before using them as fishmeal replaced ingredients. To achieve this goal, the research has adopted two phases (Figure 1). In the first phase, sweet lupin (*Lupinus angustifolius*), peanut meals (*Arachis hypogaea*) and blue-alga (*Spirulina platensis*) (SP) were selected and either treated with fermentation, germination or enzymes before including into the juvenile barramundi diets. The treated plant products were then evaluated for their quality by examining antinutritional factors (ANFs), bioactive compounds, protein structure, and amino acids contents. In the second phase, treated plant-derived proteins were used to replace fishmeal in juvenile barramundi diets. Physiological responses, growth performance, digestibility, biochemical haematological parameters, carcass composition of the fish, and nutrients discharged into the surrounding environment were evaluated against the control diet where fishmeal was the sole protein source.

Phase 1: Bioprocessed plant products and evaluation of their quality



Phase 2: Included bioprocessed plant products into juvenile barramundi diets to evaluate the fish performances

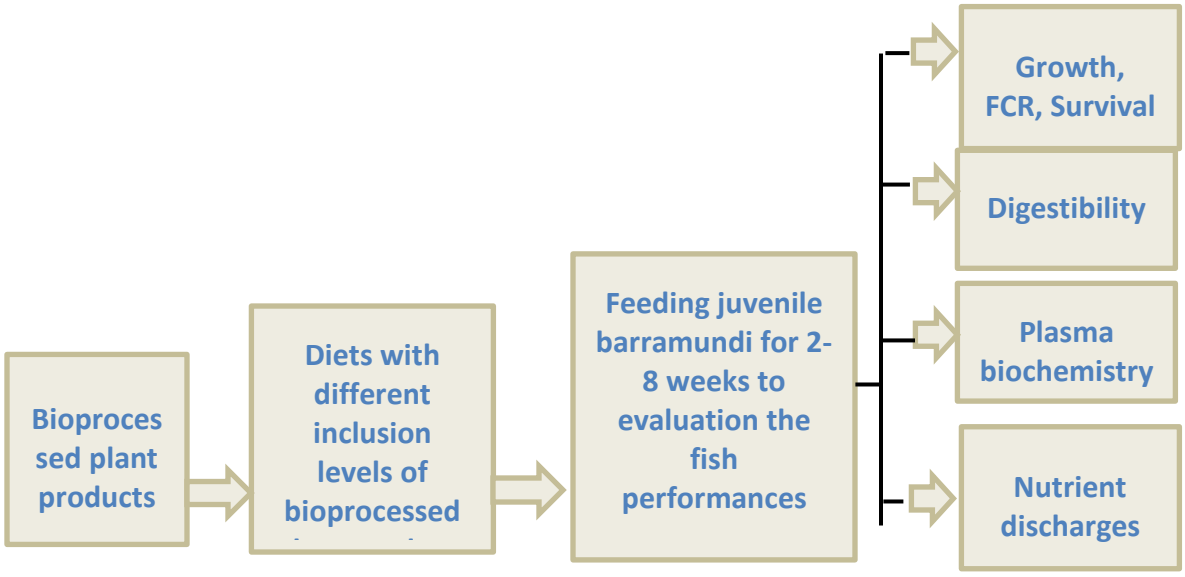


Figure 1. Lupin, and mechanical oil extracted peanut were grounded followed by Lactobacilli fermentation, while germinated peanut seed were mechanical oil extracted and grounded. Blue-alga was treated by two enzymes, Celluclast®1.5L and Alcalase® 2.4L. The grounded and enzyme treated products were nutritionally evaluated (Phase 1) and then included in juvenile barramundi diets as alternative ingredients to fishmeal. The growth performance, feed utilization, digestibility, plasma biochemistry and nutrient discharges into the rearing environment of the feed fed these diets were assessed against the fish fed control (fishmeal based) diet (Phase 2).

This thesis consists of nine chapters. Chapter 1 is the introduction, briefly highlights the comprehensive knowledge of plant-derived proteins used as a fishmeal substitution, the constraints in the use of the plant products for aquadiet and suggests methods for improving the quality of the plant ingredients. This chapter also justifies the aim, objectives and significance of the research. Chapter 2 is literature review that highlights the important aspect of aquaculture, aquadiets, the plant-derived protein used in the

fish diet, bioprocessing, the biology and the nutritional requirement of barramundi. Although the target species in this research is juvenile barramundi, relevant information from other species is included to demonstrate or compare the nutritional requirements of the fish. The key information of the test ingredient consisting of the lupin the peanut and the blue-alga is also discussed in this chapter.

Chapter 3 emphasis if the bioprocessing can improve quality of plant-derived protein ingredients and if yes then to what levels? The indicators of the nutritional improvement are the reduction of ANFs, the increase of some limiting amino acids, the protein structure and the content of certain bioactive compounds. Chapter 4 details the evaluation of growth and digestibility performance of juvenile barramundi when fed fermented lupin based diet. The result of this chapter was published in Aquaculture journal. Chapter 5 describes the plasma physiological responses of juvenile barramundi when fed fermented lupin before and after the fish were challenged with fluctuating temperature. Data of this chapter was formed to manuscript and being reviewed by Journal of Fish Physiology and Biochemistry. Chapter 6 assesses growth performance and haematological parameters when juvenile barramundi fed fermented peanut meal, germinated peanut meal and mechanical processed peanut meal while Chapter 7 examines the effects of these peanut meals on digestibility of the fish and nutrient discharges into rearing environment. The results of these chapters have been submitted to Journal of Food Quality and Aquaculture Research. Chapter 8 is details of growth, digestibility, rearing water quality when juvenile barramundi fed enzyme treated blue-alga. Data of this chapter is being submitted in Aquaculture Journal. As these chapters are designed for publication in separate journals, there are some minor repetitions such as in ‘Introduction’ and ‘Materials and methods’ sections.

Chapter 9 summarises and discusses the effectiveness of bioprocessing on the plant-derived protein quality and the efficacy of using the bioprocessed plant ingredients in the juvenile barramundi diets. Afterward, important conclusions are highlighted. Finally, the limitation, challenges and recommendation of using bioprocess plant protein are also discussed in this chapter.

ABSTRACT

Rapid growth in global aquaculture production is placing pressure on fully exploited fisheries-supplied fishmeal. Sustainable growth of aquaculture will depend on finding nutritional alternatives to fishmeal in aquadiets. Plant ingredients which are rich protein sources, are becoming important feedstock. Among these, soybean, lupin, peanut meals, and microalgae are good candidates for fishmeal replacement. However, presence of anti-nutrient factors and imbalanced amino acids are some of the limitations of using proteins derived from plants in aquadiets. Treatments of plant ingredient before use in aquadiets, are widely practised of which fermentation is proven to reduce/eliminate antinutritonal factors (ANFs) balance amino acid profile and provide vitamins, while germination has demonstrated to increase bioactive compounds. Similarly, enzymatic treatment has shown to improve the protein quality by reducing the peptide sizes and thereby increase digestibility.

A series of experiments were conducted to investigate the quality of bioprocessed ingredients including fermented sweet lupin meal (*Lupinus angustifolius*) and peanut meals (*Arachis hypogaea*), germinated peanut meal and enzyme treated blue-alga (*Spirulina platensis*). These bioprocessed ingredients were then evaluated for their effectiveness by feeding juvenile barramundi (*Lates calcarifer*). The effectiveness was measured by evaluating the growth performances, feed utilization, digestibility, physiology responses of barramundi juveniles and surrounding water quality. Five experiments were conducted independently with inclusion levels of 30%, 45%, 60% and 75% for lupin; 15%, 30% and 60% for peanut meal; and 10%, 20% and 40% for blue-alga (SP). The reference diets for these experiments were diets with fishmeal as a sole protein source.

The results showed that fermented lupin reduces 87.0% and 17.6 % of phytic acid and tannins contents respectively, while fermented peanut meal decreased 45.4% and 60.9% of alkaloids and tannins respectively. Similarly, alkaloid and tannins reduced 85.6% and 30.4% in germinated peanut meal. Fermentation increased methionine

(30.7%) and tryptophan (9.6%) in peanut meals. The bioprocessing improved bioactive compounds by increasing vitamin E and flavonoids contents in fermented peanut meal at 25.0% and 22.1%, respectively and in germinated peanut meals at 148.9% and 28.9% respectively. The peptide sizes of protein in peanut reduced to about 80% of short peptide (<35 kDa) in fermented peanut meal, and 74 % in germinated peanut meal as compared to 45% in mechanical pressed peanut meal. Similar results were observed in SP and fermented lupin. Fermentation of lupin and peanut meals increased their inclusion level in the diets, up to 60% and improved the feed conversion ratios (FCR) to less than 1.0 in juvenile barramundi. Fermentation also significantly increased digestibility of protein (96.6%) and phosphorus (96.2%) in the fish as compared to those from control (91.4% and 49.1% respectively). Similarly, the germinated peanut meal increased growth and survival at 30% inclusion level while produced adverse results when the diet containing 60% of this meal. Enzyme treated SP increased digestibility and improved FCR at 10% and 20% inclusion level, but growth was reduced when 40% of SP included in diet. There was also an improvement in physiological homeostasis under stress exposure in the fish fed fermented lupin and peanut meals, and germinated peanut meal based diet. Feeding the fish with fermented lupin and peanut meals, and SP improved rearing water quality by reducing nitrogen waste concentration.

The study suggests that the bioprocessing can improved the plant-derived proteins quality by reducing ANFs and peptide sizes, increasing contents of some bioactive compounds and limiting amino acids. Consequently, the diets contained fermented lupin or peanut improved the fish health by maintaining the fish with physiological homeostasis under stress exposure and in turn increase their inclusion level, digestibility, feed utilization and improve rearing water quality overall.

Keywords: bioprocessed, plant-derive proteins, barramundi, physiological responses

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LIST OF COMMON ABBREVIATIONS

ADC	Apparent Digestibility Coefficients
ANF	Anti-nutritional Factors
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate transaminase
CARL	Curtin Aquatic Research Laboratory
CEDMA	Centre of Environment and Disease Monitoring in Aquaculture
COD	Chemical oxygen demand
DO	Dissolved oxygen
EAA	Essential amino acid
FAO	Food and Agriculture Organization
FCR	Feed conversion ratios
EPS	Enzyme treated spirulina platensis
FL	Fermented lupin
FPM	Fermented peanut meal
GPM	Germinated peanut meal
MNM	Mechanical pressed peanut meal
NBC	National Brood stock Centre
PM	Peanut meal
RIA1	Research Institute for Aquaculture No.1
RPS	Raw spirulina platensis

SDS	Sodium dodecyl sulfate
SGR	Specific growth rate
SP	Spirulina platensis
TSS	Total suspended solids
TAN	Total ammonia nitrogen

LIST OF COMMON SPECIES NAMES MENTIONED IN THE RESEACH

Common name	**Scientific name**
Fish	
Atlantic cod	*Gadus morhua*
Barramundi/Asian Seabass	*Lates calcarifer*
Beluga	*Huso huso*
Black sea bream	*Acanthopagrus schlegeli*
Channel catfish	*Ictalurus punctatus*
Chinook salmon	*Oncorhynchus tshawytscha*
Common carp	*Cyprinus carpio*
Cuneate drum	*Nibea miichthiodes*
Discus	*Symphysodon aequifasciata*
Indian catla	*Catla catla*
Nile tilapia	*Oreochromis niloticus*
Rainbow trout	*Oncorhynchus myskiss*
Red drum	*Sciaenops ocellatus*
Rohu	*Labeo rohita*
Salmon	*Salmo salar*
Sturgeon	*Acipenser baeri*
Plants	
Black cumin	*Nigella sativa*
Blue-algae	*Spirulina platensis*

Cowpea	*Vigna sinensis*
Duckweed	*Lemna polyrhiza*
Kidney bean	*Phaseolus vulgaris*
Jack bean	*Canavalia ensiformis*
Lima bean	*Phaseolus lumnatus*
Mung bean	*Phaseolus aureus*
Peanut	*Arachis hypogaea*
Pigeon pea	*Cajanus cajan*
Rapeseed (canola)	*Brassica napus*
Sorghum	*Sorghum bicolor*
Soybean	*Glycine max*
Sweet lupin	*Lupinus angustifolius*
Wheat grain	*Triticum aestivum*

LIST OF PUBLICATIONS

Papers

Vo Van Binh, Bui Phan Dien, Nguyen Quang Huy, Ravi Fotedar, 2015. Optimised fermented lupin (*Lupinus angustifolius*) inclusion in barramundi juvenile diets. Aquaculture 444, 62-69 (Chapter 4).

Vo Van Binh, Susan G. Low, Nguyen Quang Huy, Ravi Fotedar, 2017. The survival and the plasma biochemical changes of juvenile barramundi (*Lates calcarifer*) fed various inclusion levels of fermented lupin (*Lupinus angustifolius*): Effects of fluctuating temperature (being reviewed by Fish Physiology and Biochemistry journal; Chapter 5).

Vo Van Binh, Ravi Fotedar, Nguyen Quang Huy, 2017. Evaluating bioprocessed peanut (*Arachis hypogaea*) meal to replace fishmeal in barramundi (*Lates calcarifer* Bloch) diets (submitted to Journal of Food Quality; Chapter 6).

Vo Van Binh, Bui Phan Dien, Nguyen Quang Huy, Ravi Fotedar, 2017. Digestibility and water quality investigations on the use of fermented, germinated and mechanical pressed peanut (*Acachis hypogaea*) meals as protein sources alternative to fishmeal in juvenile barramundi (*Lates calcarifer*) diet (submitted to Aquaculture Research jounal; Chapter 7).

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Conferences

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Vo Van Binh, Ravi Fotedar, 2016. Physiological responses of juvenile barramundi (*Lates calcarifer*) when fed bioprocessed plant based diets (3rd International

Conference on Fisheries and Aquaculture 24th- 25th August, 2016) Negombo-Sri Lanka.

Chapter 1: INTRODUCTION

1.1 Background

Aquaculture is one of the fastest growing industries in food production (FAO, 2014) and is expected to continue this upward trend. FAO (2014) has projected that total output of aquaculture and wild catches will increase by 15% to 172 million tonnes in 2020, with the expansion largely by growth in aquaculture sector. However, the future of sustainable aquaculture will strongly depend on the development of alternative protein sources to fishmeal in aqua-diets (Tacon and Metian, 2008).

There are various plant ingredients that can be tested as candidates to replace fishmeal as protein sources in aqua-diets. This includes oilseeds: soybean (*Glycine max*), peanut (*Arachis hypogaea*), sesames (*Sesamum indicum*) and canola (*Brassica napus*); legumes: cottonseed (*Gossypium hirsutum*) and lupin (*Lupinus* sp); and cereal grains: corn (*Zea mays*) and wheat (*Triticum sp*) (Hansen, 2009). The agricultural crops, having highest global production and thereby highly available are soybeans, followed by corn, wheat, canola and lupin (FAOSTAT, 2011).

To be viable alternative-protein source to fishmeal in aqua-feeds, a plant candidate must possess certain nutritional characteristics such as high protein content, favourable amino acid profile, high nutrient digestibility, low levels of fibre, starch, especially non-soluble carbohydrate and absence of antinutrients, and at the same time should be reasonable palatability. However, raw plant ingredients (both agricultural crops and microalgae), although cheaper than fishmeal do not meet all of these requirements.

Many plant proteins have high levels of undesirable components known as antinutritional factors (ANFs) (Storebakken, 1985; Refstie *et al.*, 1999; Francis *et al.*, 2001). Soybean, Lupin are ones of the most studied plant protein for several ANFs, such as; saponins, protease inhibitors, lectins and phytic acid (Hansen, 2009). ANFs are

known to affect performance of salmonid fish by altering its gut histology (Sanden *et al.*, 2005), inducing inflammations (Bakke-McKellep *et al.*, 2000) and decreasing nutrient digestibility (Krogdahl *et al.*, 2003).

Addition to the limited digestibility of plant protein due to ANFs, is the unbalanced amino acid profile in the plant proteins than the fishmeal, especially the shortage of methionine and/or lysine (Hansen *et al.*, 2007). Past trials have shown large variation in requirement for lysine (3.7-6.2% of dietary protein) and methionine (2.1-3.3% of dietary protein) in Atlantic salmon (Espe *et al.*, 2007; Espe *et al.*, 2008). These requirement levels are higher than that found in most plant proteins. Consequently, the first limiting amino acid follows Liebig's Law (Liebig, 1842) that restricts protein synthesis and feed utilization.

One of the greatest challenges is to increase amount of plant derived protein in the diet of carnivorous fish (Burr *et al.*, 2011). Characteristics of a carnivorous fish species are short digestive tract, limited amylase activity, rapid gastrointestinal passage and only minor microbial activity in the hindgut (Buddington *et al.*, 1987). High inclusion level with primary plant protein in diet can result in poor growth performance and digestibility which in turn increase the losses of nutrient such as nitrogen and phosphorus that discharge into rearing environment.

So far, most of the tests for fishmeal replacement are based on "untreated" plant protein ingredients (Table 1.1). The raw materials are ground and used directly, or they are oil extracted before grinding. Plant rich protein ingredients undergone with these physical treatments are unlikely going to reduce any ANFs, or increase bioactive compounds. Thus, they may require further treatment, including bioprocessing, to improve their quality and thereby their inclusion level can be increased.

Thermal and mechanical processes, fermentation, socking, and germination/malting (all these processing are termed as bioprocessing in this thesis) are used to increase bioactive compounds and enhance the bioavailability of micronutrients in plant products (Hotz and Gibson, 2007). For improvement of plant protein in aquadiets, the

fermentation, germination and enzymatic treatment are expected to improve quality and bioavailability/value of plant ingredients that in turn are expected to increase the nutrient utilization in carnivorous fish, and consequently decrease the pollution load from dietary nutrition.

Table 1. 1 Summary of studies on fishmeal replaced by plant protein in aquafeeds.

Fish species	*Products tested*	*Parameters tested*	*Results*	*References*
Angel fish	Canola meal	Growth, FCR, protein digestibility.	Accepted at 16% inclusion level	(Erdogan and Olmez, 2010)
Atlantic salmon	Soybean	Intestinal morphology	Reduced micivilli, severity of enteritis	(Uran *et al.*, 2009)
Atlantic cod	Microalgae (Nanno and Iso)	Growth, FCR, viscerosomatic indices	Accepted at 15%, but starved at 30% inclusion levels	(Walker and Berlinsky, 2011)
Flounder	Vegetable product	Superoxide generation, leukocytes	Enhanced immunity	(Ashida and Okimasu, 2005)
Parrot fish	Meju, fermented soybean	Growth, antioxidatant activitiy, red blood count	Improved non-specific immunity	(Kim *et al.*, 2009)
Sturgeon	Spirulina	Growth, FCR, fatty acid	Accepted 50% inclusion, give higher content of fatty acids in fillet	(Giovanni *et al.*, 2005)
Tilapia	Spirulina	Growth, FCR, protein utilization	Accepted at 40% inclusion level	(Olvera-Novoa *et al.*, 1998)

Barramundi or Asian sea bass (*Lates calcarifer*) is a carnivore and an economically farmed species in Indo-Pacific waters such as Southeast Asian countries, Taiwan and Australia. It is widely consumed in the Europe and United States (Schipp *et al.*, 2007). The nutritional studies on barramundi diets have been widely conducted. These included requirements for most nutrients, energy demand, special amino acids requirements, ingredient utilization and the effects on dietary nutrition on flesh quality (Glencross, 2006; Glencross *et al.*, 2013). Optimal protein content of diets has been shown to vary with diet energy, stocking density and also the size of fish (Boonyaratpalin and Williams, 2001; Williams *et al.*, 2001; Williams *et al.*, 2003a; Robin *et al.*, 2009; Yabaya *et al.*, 2009; Catacutan and Coloso, 1995). However, bioprocessed

products of plant derived protein have not been tested in barramundi diets as an alternative to fishmeal.

To evaluate quality of a plant ingredient, the content of ANFs and amino acid profile usually examined. The quality of the ingredient is also reflected in the amount of bioactive compounds as antioxidant vitamins or protein structure (Hong *et al.*, 2004). An acceptance of plant derived protein by fish could be evaluated by feeding the fish with the diet containing the plant ingredient. Indicators such growth performances, feed utilization and digestibility are commonly used for the assessment. Additionally, responses of haematological physiology by the fish when fed the plant based diets can be useful tool to fully assess the level that the ingredient can be utilized for fish diet. Thus, in this thesis, plant derived protein ingredients are bioprocessed following by the quality assessment. These bioprocessed ingredients then use to replace fishmeal in barramundi diets. Growth performances, digestibility, plasma chemistry and nutrient discharge are indicators to evaluate the diet acceptance by the fish.

1.2 Aims and Objectives

1.2.1 Aim

The study aims to evaluate replacing fishmeal with bioprocessed plant meal products in juvenile barramundi formulated diets.

1.2.2 Objectives

To achieve the above aim following objectives can be met:

Objective 1. To evaluate and select suitable plant products (for example, lupin, sesame, peanuts, soybean, canola and microalgae) which can be used as fishmeal replacements for juvenile barramundi formulated diets.

Objective 2. To standardise bioprocess techniques involving fermentation, germination and commercially enzymatic treatment of selected plant meals by supplementing

Lactobacillus spp. in order to improve their nutrient profile as assessed from the literature.

Objective 3. To formulate and evaluate the performance of juvenile barramundi diets based on partial or full replacement of fishmeal with the product(s) obtained from the previous objectives. These diets will be evaluated by meeting the following sub-objectives:

3.1. Evaluating growth performance, feed conversion ratio and digestibility of juvenile barramundi when fed these customised diets.

3.2. Investigating physiological and/or immunological responses of juvenile barramundi when fed these customised diets.

3.3. Analysing nutrient discharges into rearing environment when juvenile barramundi are fed these customised diets.

1.3 Significance

Results of this study will provide evidence for efficient utilization of bioprocessed plant protein-rich products including microalgae in aqua-feeds.

Utilization of fermented lupin and peanut, germinated peanut and enzyme treated *Spirulina* in aquafeeds has never been evaluated in fish; therefore it can provide reference for aquafeeds manufacturers on the alternative use of fishmeal replaced ingredients.

Bioprocessed products, which will be expected to enhance non-specific immune responses and disease resistances of the cultured fish, will be a solution to avoid the use of antibiotic in aquaculture industry. Finally, the thesis results will support for more fishmeal to be replaced by plant protein, consequently pressures on wild capture fisheries will be lessen. The carbon dioxide, the cause of global warming, would be eased when more feedstocks derived from plants is used instead of fishmeal.

Chapter 2. LITERATURE REVIEW

2.1 Global aquaculture production

In the last decade, while the production by capture fisheries sector remained unchanged (about 91-93 million tonnes), worldwide aquaculture production increased from 55.7 million tonnes by 2009 to 73.8 million tonnes by 2014 (FAO, 2016). The production of finfish and crustaceans (two major aquaculture groups use formulated feed) shares up to 67.5% and 9.3%, respectively.

Globally the capture fisheries production is higher than aquaculture production, however in the Asia the aquaculture production contributes 55% of total volume and the production of finfish shares about 87.7% of total finfish (49.8 million tonnes) produced in the world (FAO, 2016). Although the major part aquaculture production comes from finfish, only small part is contributed by marine finfish (about 6.3 million tonnes in 2014) mainly by Asian aquaculture (3.3 million tonnes). In the Europe about 1.8 million tonnes of marine finfish were produced in the 2014 (FAO, 2016) and the common marine finfish with active feeding grew faster than none-fed groups (FAO, 2016).

Diadromous aquaculture production is dominated by salmon (*Salmo salar*) with 44% (1.5 million tonnes) in 2008 followed by milkfish (*Chanos chanos*) 20.4%, rainbow trout (*Oncorhynchus mykiss*) 17.4% and eels (*Anguilla sp*) with 7.9%. Norway and Chile are the world leading producers of salmon, accounting for 36.4% and 28.0% of global production of diadromous fish (FAO, 2010). Barramundi/Asian seabass (*Lates calcarifer*) contributes a small volume, but has significantly increased from 21.0 thousand tonnes to 69.1 thousand tonnes during 2000-2011 (FAO, 2013). In the same period, aquaculture of barramundi in Australia has increased in 530 % from 814 to 4352 tonnes (Harrison *et al.*, 2014).

2.2 Fishmeal

2.2.1 Characteristics

Fishmeal is a protein-rich ingredient that is mostly made from marine pelagic fish which are generally not useable for human nutrition. Fishmeal can be also derived from by-products of marine product processing. There are a number of commercially available fishmeal types that can be distinguished according to the origin of raw materials and processing methods. High quality of fishmeal requires a sophisticated way of processing that includes cooking, pressing and fine grinding of raw fresh marine fish. Majority of fishmeal production is for aquadiet, that occupying 46% in 2006 (Hardy, 2010). Fishmeal contains about 45% to 75% protein and, thus, is an ideal protein source for formulated feed. Fishmeal is a highly digestible protein, rich of long chain omega-3 fatty acids, full and balanced amino acids and abundant in essential vitamins as well as minerals. Fishmeal is known as palatable feed to fish and thereby optimizes the feed intake and feed utilization. Fishmeal has now becomes scare and expensive due to the increased demand for the use in aquaculture (Tacon and Metian, 2008; Hardy, 2010).

2.2.2 Demand and concerns

About 61% global fishmeal is used for aquadiets (FAO, 2012). In aquaculture, five major aquatic groups use formulated feeds including crustaceans, marine fish, salmon, carp and tilapia (FAO, 2016). Although, growth percentage of aquaculture production is quite stable, the volume of formulated feed has steeply increased, from 7.6 million tonnes in 1995 to 28.9 million tonnes in 2008 and estimated to 70.0 million tonnes by 2020 (Tacon *et al.*, 2011). Inclusion of fishmeal in aquadiets varies among species, that is low level in tilapia diet (2.5%), medium in carps diet (8.5%) and high levels in shrimp and marine fish diet (27% and 36% respectively) (Chiu *et al.*, 2013). Thus, amount of fishmeal required for aquadiets will be approximately 5-7 million tonnes by 2020 provided the reduction of fishmeal proportion by alternative ingredients is not achieved.

The over use of fishmeal raises environmental concerns as strong reliance on fishmeal increases pressure on wild fish stocks and overfishing of certain form of fisheries with consequent influences on the stocks of other wild fisheries (Naylor et al., 2000). Another concern of using fishmeal is the contamination of chemicals including organochlorine. Hites *et al.* (2004) reported that higher concentration of organochlorine in the farmed salmon than wild one. The important sources of organochlorine are mercury, polycyclic artomatic hydrocarbons (PAHs), dichlorophenyltrichloroethanes (DDTs), polychlorinated biphenyl (PCBs) and polybronminated diphenyl ethers (PBDEs) (Guo et al., 2009; Suominen et al., 2011). These pollutants can be bio-accumulated and biomagnified through the food chains and become a serious risk if a large proportion of fishmeal is used in aqua-feed.

2.3 Alternative protein sources

The widening gap between supply and demand of fishmeal coupled with the environmental concerns on its usage in aquaculture industry has led to increased research focussing on finding any alternative ingredient(s) to fishmeal. There have been a number of researches conducting on the trials of protein utilization from bacteria, by–products of animal and plant sources (Gatlin *et al.*, 2007; Glencross *et al.*, 2007; Walker and Berlinsky, 2011; Couto *et al.*, 2016).

2.3.1 Animal proteins

Proteins from terrestrial animal by-products including blood meal, poultry by-product meal, meal-bone meal and feather meal, are widely recognized. These protein sources are cheaper than fishmeal and thus have been evaluated on various fish species including Rainbow trout (*Oncorhynchus mykiss*) and Pacific threadfin (*Polydactylus sexfilis*) (Bureau et al., 2000; Erturk and Sevgili, 2003; Cheng et al., 2004; Forster et al., 2005). However, using animal protein in aquadiets raises a risk of food safety such as bovine spongiform encephalopathy and food-borne bacterial infections (Londhe *et al.*, 2012)

2.3.2 Plant proteins

Plant derived proteins are potential candidate for substitution of fishmeal. Various plant ingredients including lupin, canola, peanut and soybean, cereals, corn etc have been tested to replace fishmeal in aquadiets (Glencross, 2006; Gatlin *et al.*, 2007) . Depending on processing technology, different forms of plant ingredients including ground whole seed, dehulled seed, concentrated protein products (soybean), and meals (oil extracted seed)are used.

2.3.2.1 Soybean

Among the plant sources, soybean has the largest world production and has become the most available for animal feed (FAO, 2013). The largest producers of soybean are United States, Argentina, Brazil and India, and of course the United States of America shares the biggest amount of crop (338 million metric tonnes in 2016) (data from Global Soybean Production website) Two forms, soybean meal and soybean concentrate are generally used in aquadiets.

2.3.2.2 Lupin

A number of lupin species are planted worldwide including *Lupinus angustifolius, L. albus, L. luteus, L. mutabilis, L. arboreus* and *L. polyphyllus.* While *L angustifolius* species is dominant in Australia, *L. luteus and L. albus* species are main cultivates in the Eastern Europe (Lucas *et al.*, 2015). In Mediterranean countries including Spain, Morocco, Portugal, Egypt and Greece *L. albus* and *L. luteus* are important lupin producing countries. Before 1980, the largest producers of lupin were Poland and the former East Germany; however, recently about 85% world lupin production is from Australia mainly with *L. angustifolius* (sweet or narrow-leaf lupin). Western part of Australia is suitable for lupin growth and the largest production comes from this region, accounting for 80% of the country production (ABARES, 2012).

Protein content and other components of lupin vary from species to species. *L. angustifolius* has lower protein content, but less bitter than *L. luteus* (AGOGTR, 2013).

The less bitter taste has an advantage when the lupin is used for human nutrition or animal feed because of improvement in palatability (Lucas *et al.*, 2015). Protein content of lupin is relatively high, reaching 36-52% and thus they are mainly used by stockfeed manufacturers for production of animal feed in Australia (ABARES, 2012). Ruminants such as cows and sheep are the largest consumers of lupin followed by swine and poultry. Although there is a small proportion of lupin used for aquaculture, the use of this protein source has dramatically increased (white *et al.*, 2008; Lucas *et al.*, 2015), especially in South East Asia, where aquaculture is significantly expanding and requiring a cheaper protein source for fishmeal replacement (Hishamunda *et al.*, 2009).

2.3.2.3 Peanut

Peanut (*Arachis hypogaea*), also known as groundnut, is a tropical and subtropical legume crop of global importance. Worldwide peanut production is more than 35.6 million MT, mainly by China, India and South American countries (INC, 2014) . Most peanut production is for oil extraction for humans, butter, roasted peanut, snacks and desserts. The remaining part of peanut, after oil extraction is rich in protein which can be used for humans food and animal feed.

The peanut protein owns a good capacity of foaming, stability and activity of emulsifying (Wu *et al.*, 2009). It also had an excellent water retention and high solubility which are advantage characteristics for product formulation and protein fortification in industry (Wu *et al.*, 2009). Furthermore, peanut protein also is classed as high quality as that from meat or egg (FAO, 1991), thus, it was considered as most attractive and promising plant-derived protein (Zhao *et al.*, 2012).

However, peanut contains a significant amount of antinutritional factors (ANFs) such as tannins, alkaloids and trypsin inhibitor (Ejigui *et al.*, 2005), limiting its inclusion level in animal feed. Processing methods can improve functional properties of peanut protein. For example protein solubility, emulsifying and water/oil binding capacities and

foaming capacity and viscosity improved when peanut protein was fermented (Yu *et al.*, 2007).

2.3.2.4 Spirulina

Spirulina, also termed as blue-green algae is a prokaryotic cyanobaterium that has been marketed worldwide for over thirty years (Bishop and Zubeck, 2012). Spirulina is used as functional food to supplement vitamins and minerals in humans' diet. In aquaculture, Spirulina is very important in nursing of aquatic animal at early life stages when its use is in live form. Currently, about 3000 tons of dry weight are produced annually in the USA, Thailand, Taiwan, China, India and Myanmar (Bishop and Zubeck, 2012).

Spirulina contains high protein contents, up to 60-70% (by dry weight), depending on culture and nutritional conditions (Ogbonda *et al.*, 2007). It also has a high content of vitamins (B, E), phycocyanin, omega 6 fatty acids and a number of mineral (Gershwin and Belay, 2007). It is worth to note that content of β-carotenes in *Spirulina* is 10 times higher than in carrots (Belay *et al.*, 1993). Thus, this algae seem to be a supper food and feed for human and animals (Bishop and Zubeck, 2012). However, the production cost of *Spirulina* is quite high that is limiting factor in its application.

2.3.2.5 Challenges of alternative plant based fishmeal

Compared to fishmeal, plant ingredients are readily available, have a lower price and lesser adverse environmental impacts (Davidson *et al.*, 2013). However, plant products contains large amount of anti-nutrients (Farhangi and Carter, 2001; Gatlin *et al.*, 2007) and lower content of some amino acids such as lysine and methionine, thus reducing their values in aquadiet formulations. Additionally, in the plant ingredients there is a large proportion of carbohydrate which is generally inefficiently used by carnivorous fish (McMeniman, 2003). Processing technology of the plant ingredients is important to improve their quality and thereby increase feed utilization (Drew *et al.*, 2007). Processing strategies aim at partly removing ANFs (Ejigui *et al.*, 2005) and the reducing

of carbohydrate using alcohol in the process of de-hulled and defatted soybean was reported to partly remove saponins (Ingh *et al.*, 1996).

2.4 Determination of the feed efficiency and nutritional requirements

Growth/net nutrient composition, survival and feed conversion ratio (FCR) are important indicators in studying the acceptance of feed and nutritional requirement of fish. These indicators are the most accurate tools to evaluate the feed efficiency (Belal, 2005). The growth rate is measured based on the difference of length and weight of target fish before and after the fish are fed the test diet. FCR is the relationship of amount of intake feed that the target receives and the fish weight gain. Almost all types of research and farming use growth and FCR to estimate the benefit of using feed and cost involve with culture operation (Belal, 2005).

Another important tool to evaluate the quality of a diet and nutrient requirement is digestibility. Digestibility is the measurement of nutritional composition in a diet and in faeces of fish ingesting the diet. Faecal collection methods for digestibility widely used are, faecal sedimentation (Austreng, 1978; Cho and Slinger, 1979) and from striping (Glencross *et al.*, 2007; Tabrett *et al.*, 2012). While faecal sedimentation is deemed to be overestimating the digestibility values, the stripping method involved with stress to the studied fish (Blyth *et al.*, 2015).

To assess a nutritional balanced diet, physiological responses of the fish to a diet under environmental changes are measured as these responses are more sensitivity than mortality (Heath, 1995). There are several plasma chemical parameters that have been widely analysed depending on the availability of resources. These parameters include liver enzyme aspartate amino transferase (AST) and alanine amino transferase (ALT), glucose, cortisol, total plasma protein, albumin, globulin, and exchange ions (Na+, K+, Cl-). The plasma biochemistry can be evaluated under experimental conditions (Ngo *et al.*, 2016) or together with stressors such as acute changes in dissolved oxygen and

temperature (Vanlandeghem *et al.*, 2010), transportation or high density (Ardiansyah and Fotedar, 2016).

Feed utilization by fish is also reflected in the amount of nutrient discharge through faecal wastes. Levels of FCR and digestibility are hypothesized to link with nutrient waste releasing into rearing water and water quality. Additionally, Davidson *et al.* (2013) reported that feeding rainbow trout (*Oncorhynchus mykiss*) with grain-based diet increased nitrogen waste in rearing water than fishmeal based diet. Thus, parameters of water quality can be useful tool to evaluate the quality of a diet and level of acceptance of the diet by fish, even though this tool is not commonly applied.

2.5 Improvement of nutritional value of plant ingredients

Almost all raw materials for animal feed have to undergo some forms of processing before they are ready to be used as feed (Hotz and Gibson, 2007). Processing is defined as a variety of operation by which raw foodstuffs became suitable for consumption, cooking or storage. Food processing involves the transformation of raw ingredients by physical, chemical or biological methods that changes or converts them into safe, edible and more palatable foodstuffs. To be an alternative fishmeal to aquadiet, a plant ingredient must obtain some important characteristics such as high protein content, condensed energy and high nutrient digestibility. These characteristics rarely exist in raw plant materials, and therefore they need to be processed (Drew *et al.*, 2007).

2.5.1 Heat treatment (traditional bioprocessing)

Heat treatment is the oldest method for quality improvement of plant ingredients. Almost all plant ingredients for human food are cooked or partly cooked before use. Animal feed and aquadiet have undergone extrusion that involves of heat treatment. Thermal treatment is observed to decrease ANFs, improve digestibility thus the materials become more ready assessable (Liener, 1979). Srihara and Alexander (1984) reported that heat treatments by microwave and extruded process improved protein quality of many plant blends, corn, wheat, rice, oats, white beans, soybean, peanut and

sesame. By treating plant ingredients with heat, Liener (1979) found that protein of the plant ingredients were denatured with an inactivated ANFs thereby increased the digestion of protein in plant food. Similarly, physiochemical properties of flaxseed was improved when the seed is extruded by high temperature under high pressure (Min *et al.*, 2015). However, many vitamins or bioactive compounds do not tolerance against high temperature, thus these compounds may be quickly destroyed during heat treatment (El-Adawy, 2002).

2.5.2 Drying (traditional bioprocessing)

An Important step to improve and maintain high quality plant ingredients is drying. The drying also assists the extending storage, reducing transportation cost and for further processing. Raw materials, that are sensitive to heat, care need to be taken during drying. Conventional drying by means of direct hot air application can be detrimental to maintain the bioactive compounds in the ingredients (Mujumdar and Law, 2010).

In drying, the water content in the plant ingredients is reduced to a level that biological reactions such as enzyme activity, microbial growth and other deteriorative processes are inhibited (Mujumdar and Law, 2010), limiting the ingredients with spoilage or biochemical change such as germination or fungi development. Drying can be in the form of vacuum drying, vacuum-freeze drying, flash drying, spray drying, sun drying, rotary or tunnel drying (Mujumdar and Law, 2010).

2.5.3 Advanced Bioprocessing

The advanced bioprocessing can increase the protein concentration that is important to carnivore fish diets. By bioprocessing, plant ingredients such as soybean meal and canola meal, become more efficient to utilise and increasing digestibility (Drew *et al.*, 2007). Bioprocessing also reduces any antinutritional factors (Nnam and Obiakor, 2003; Lopez-Amoro´s *et al.*, 2006; Khamal and Ahmad, 2014) that could be common in plant ingredients (Farhangi and Carter, 2001; Gatlin *et al.*, 2007). Bioprocessing also improves protein quality by reducing peptide sizes (Hong *et al.*, 2004).

2.5.3.1 Fermentation

Fermentation is considered as a process conducted by microorganisms which results in biochemically modified raw material(s) (Caplice and Fitzgerald, 1999). Bacterial fermentation of the ingredients plays a role as probiotic or prebiotic supplementation thus increases digestibility and growth by enhancing the expression of immune regulation genes (Munir *et al.*, 2016). Fermentation also predigest the toxins and ANFs in plant based diet (Park *et al.*, 2003), making it easier for the fish to absorb and maintain overall good health. Lactic acid (*Lactobacillus* sp.) fermentation of oil-extracted soybean meal partly eliminates and inactivate ANFs restricting the absorption of lipids by Atlantic salmon which then leads to higher digestibility of total dietary energy, and subsequently improves the feed efficiency (Refstie *et al.*, 2005). The fermentation eliminates sucrose, reduces the level of raffinose, and lowers the trypsin inhibitor activity in the fermented white flacks soybean and it also induces less pathological changes in the intestine of Atlantic salmon (Refstie *et al.*, 2005). Lactic acid fermentation has been shown to give a significant reduction of phytic acid in cereal and sesame seed (Marklinder *et al.*, 1996; Mukhopadhyay and Ray, 1999; Skrede *et al.*, 2002). In addition a reduction of the antigenicity, resulting in less pronounced pathological changes in the distal intestine is found when fish is fed with the fermented product ingredient based feed. Skrede *et al.* (2002) indicated that Atlantic salmon can use native starch more efficiently when the wheat ingredient was fermented than unfermented form thus improving the digestibility.

2.5.3.2 Germination

Germination is a biological process that takes place in plant seeds when the environmental conditions, such as humidity, temperature, nutrients are favourable for the optimum survival, growth and development (Sangronis and Machado, 2007). During germination, the complex processes occur, changing biochemical and physiological characteristics of the seeds that allows the plant to absorb nutrients

stored in the seed to grow. Those changes vary among the plant species and the conditions of germination (Bau *et al.*, 1997; Sangronis and Machado, 2007).

Germination increases protein, bioactive compounds and reduces carbohydrates, fat and ANFs in many plant seeds (Table 2.1). If germinated seeds are used in aquadiets, they support the maximised growth, as they provide important nutrients and bioactive compounds for growth and health of fish. Germination product such as carod seed germ meal was evaluated on meagre juvenile (*Argyrosomus regius*). The growth of the fish was unchanged at 225g kg^{-1} inclusion, but higher content of this product led to decrease in digestive enzymes and protein retention (Couto *et al.*, 2016).

Table 2. 1 Chemical and nutritional changes by germination and fermentation of plant protein ingredients.

Plant ingredients	***Chemical and nutritional changes***	***Processes***	***References***
Beans (*Phaceolus vulgarsis* and *Cajanus cajan*)	Decreased trypsin inhibitors, phytic acid, tannins Increased ascorbic acid, thiamine, digestibility	Germination	(Sangronis and Machado, 2007)
Lupin (*Lupinus angustifolius*)	Decreased Glutathione Increased SOD-like activity	Germination	(Fernandez-Orozco *et al.*, 2006)
Wheat grain (*Triticum aestivum*)	Increased vitamins C, E and beta carotenoids	Germination	(Yang *et al.*, 2001)
Sorghum (*Sorghum bicolor*)	Decreased tannins Increased digestibility	Fermentation	(Hassan and Tinay, 1995)
Cowpea (*Vigna unguiculata*)	Decreased tannins, phytates, trypsin inhibitors, raffinose, starchyose	Cooking/germination and fermentation	(Ibrahim *et al.*, 2002)

2.5.3.3 Enzyme treatment

Enzyme is a group of complex protein or conjugated proteins acting as catalysts in specific biochemical reactions. Enzymes are produced by living cells and help break down larger molecules to smaller molecules such as starch, lipids, protein during the digestion (Hong *et al.*, 2004). The use of enzymes in industry is well established over a century and continues to develop (DiCosimo *et al.*, 2013). Enzymes treatments of

ingredients prior to use were widely practised with food and feed (Fraatz *et al.*, 2014). Despite it, quite a few studies on the use of enzymes to treat plant ingredients in aquadiets are found in literature; only some trials on the use of phytase supplementation to increase the availability of phosphorus in fish diets (Von Danwitz *et al.*, 2016) are available.

2.6 Barramundi

2.6.1 Biology

Barramundi (Figure 2.1), also named as Asian seabass, was described first by Bloch (1790). There has been long controversy over the taxonomy of the species (Pethiyagoda and Gill, 2013), but nowadays its taxonomy as described by Pethiyagoda and Gill (2013) is broadly accepted and used:

Phylum: *Vetebrata*

Class: *Osteichthyes*

Subclass: *Teleostomi*

Order: *Perciformes*

Suborder: *Percoidae*

Family: *Latidae*

Genus: *Lates*

Species: *Lates calcarifer* Bloch, 1970

Figure 2. 1 Morphology of juvenile barramundi (*Lates calcarifer*).

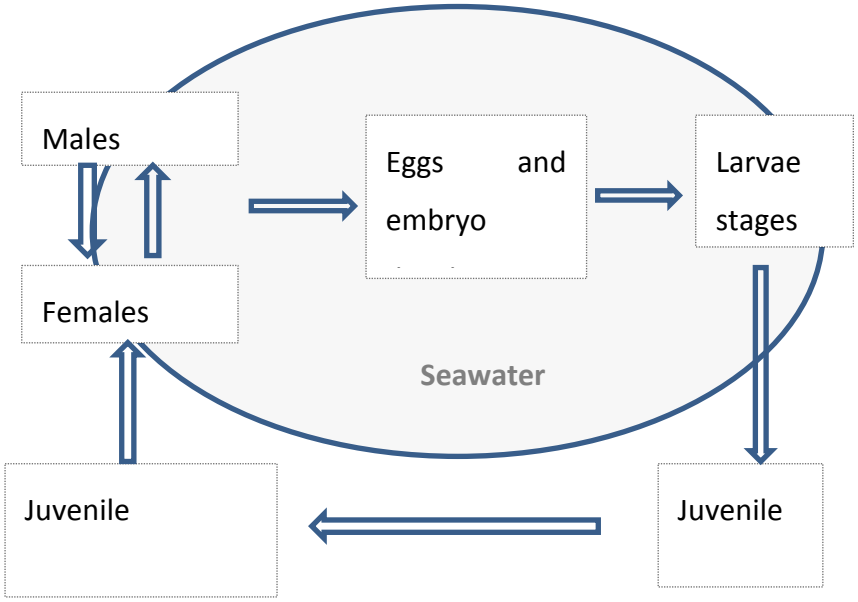

Figure 2. 2 The life cycle of barramundi. From matching and spawning to larvae stage, the fish have to be in marine water where salinity is 30-35‰. Later life of cycle could be in freshwater, seawater or brackish water.

Barramundi is a diadromous species (Figure 2.2), naturally distributed in Indo-Pacific regions which range from the Arabian Gulf to China, Pagua New Guinea and North of Australia. This species is also natively present in Australia, in the tropical zone from Exmouth Gulf, the North of the continent and Nary River in Queensland (Marshall,

2005). In aquaculture, barramundi has been successful in artificial spawning and can be easily cultured in ponds, cages and pen, therefore it is widely introduced into many regions including Southeast Asian nations, Japan, American subcontinents and other countries. In Vietnam, barramundi is an introduced species for aquaculture. Today, this fish is widely propagated throughout the country, but concentrating in Haiphong, Quangninh, Nhatrang, and Vungtau provinces (Vi *et al.*, 2013).

This species is hermaphroditic that they are males when are small size but then become female at large size (Jerry *et al.*, 2013). In Northern Australia, fish with size at 500-600 mm body length are males and when they reach to 850-900 mm body length the sexual organ is changed to female (Jerry *et al.*, 2013). In aquaculture, fish with about 2 kg (male) and 3 kg (male or female) each can be selected for induced spawning.

2.6.2 Nutritional requirement

Being a carnivore species, barramundi contains a digestive system characterised for animal diets from hunted preys (Jesus-Ayson *et al.*, 2014). The intestine of barramundi is short, well designed for more efficiency in utilisation of dietary protein and lipid, but less for dietary carbohydrates (McMeniman, 2003). Therefore, the formulated diet for barramundi should meet these inherent characteristics. Additionally, it should be taken into consideration when formulate diet for juvenile barramundi as in early life stages that the fish require higher protein content in diet than when they are adult (Glencross, 2006; Saavedra et al., 2016).

2.6.2.1 Protein

The requirement of protein for barramundi varies and depends on the growth stages. At larvae stage, the protein requirement in the diet is as high as 55% (Catacutan and Coloso, 1995); when the fish grow to the bigger size, the level of protein required can be reduced to about 40%. Formulated diets containing lower than 40% protein have proven to result in poor growth performance (Glencross, 2006).

Optimised protein level in diet for growth of barramundi has been evaluated by Cozon (1988). The author designed a series of diets with protein levels from 350 g to 550 g kg^{-1}. The results of this experiment indicated that juvenile barramundi required protein level of 450-550 g kg^{-1}, although these levels did not take account the isocaloric values in the test diets. In other tests, where fishmeal was used as protein source for diets, fish with initial size at 1.3 g or 7.5 g each required protein level of 500 g kg^{-1} feed (Sakaras 1988; Catacutan 1995).

Dietary protein is the only source for constructing amino acids building up for the body. However, protein also plays a role in the production of energy. Therefore, the ratios of protein to energy in diet can influence the protein requirement of the fish. When a low level of fat is in the diet having inefficient energy, the fish is in need of high protein level to compensate for the loss of energy. Several studies (Glencross *et al.*, 2013; Ngo *et al.*, 2016; Catacutan and Coloso, 1995) suggest that a protein level of 45-50% is sufficient for optimum growth of juvenile barramundi.

2.6.2.2 Amino acids

The exact requirement of amino acid by barramundi has not been known yet. Some specific amino acids are quantitatively evaluated such as tryptophan, methionine, lysine and arginine (Coloso et al., 1993; Milamena, 1994) with the requirement levels in dietary protein as 5 g kg^{-1}, 22 g kg^{-1}, 49 g kg^{-1}, and 38 g kg^{-1}, respectively. Deficiency of any amino acids can lead to limiting factor and follows the Liebig law (Liebig, 1842) that restricts protein utilization. In contrast, the excess of some amino acids could have adverse effect such as the excess of tryptophan resulted in kidney malefaction in the fish (Boonyaratpalin, 1997). Thus, it is important that the suitable level of amino acids provided in the diet that is similar to the levels found in the fish (Table 2.2).

Table 2. 2 Essential amino acid in muscle and whole body of juvenile barramundi.

Amino acids	*Muscle (g kg^{-1})*	*Whole body (g kg^{-1})*
Arginine	6.4	6.9
Histidine	2.4	1.5
Isoleucine	4.3	3.6
Leucine	6.8	7.1
Lysine		6.2
Methionine	2.9	3.0
Phenylalanine	3.7	4.2
Threonine	4.1	4.5
Valine	4.4	4.4

Note: Data are combined from Vo-Binh Van et al., (2015) and Glencross (2006)

2.6.2.3 Lipids

Lipids are nutrient source rich in energy content which carnivorous fish species can well utilise. The lipids supply essential fatty acids (EFA) and play an important role in the transportation of the fat-soluble vitamins (Reboul and Borel, 2011). Energy from lipids can be utilized two fold than from proteins and carbohydrates. Simple lipids are fatty acids and triacylglycerols. Generally, fish needs fatty acids in the form of omega3 and 6 (n-3 and n-6) (Sargent *et al.*, 1999). The optimum level of lipids for fish, depend on many factors and it was reported that temperature is strongly influenced factor (Tocher, 2003); the lower the temperature results the high the level of lipids requirement.

Lipids requirement for barramundi is reviewed by Glencross (2006). The author summarised that the optimum level of lipids required by barramundi is in the range of 50-180 g kg^{-1}. In the initial study by Sakaras *et al.* (1988), the optimum lipids levels were estimated between 150-180 g kg^{-1}. Another investigation by Tucker *et al.* (1988) reported at 90-130 g kg^{-1} lipid in diets did not alter the growth, but significantly reduced feed conversion ratios (FCR) with the higher lipid level in the experimental range. Catacutan and Coloso (1995) evaluated lipids levels of 50 g, 100 g and 150 g kg^{-1} in barramundi with different protein levels of 350 g, 425 g and 500 g kg^{-1} and found

that highest level of lipids and protein in the test ranges resulted in the highest growth performance. Williams *et al.* (2003a) reported that barramundi at size of 230 g each required 190 g kg^{-1} lipids in diet containing 500 g kg^{-1} protein to have optimum growth performance.

2.6.2.4 Carbohydrate

Carbohydrates (starches and sugars) are the most economical and inexpensive sources of energy for fish diet. However, the intestine of carnivore fish is not designed for digestion of high content of carbohydrate diet. Energy requirement of these species is mainly from lipids as they digest lipids much more efficiently than carbohydrates (McMeniman, 2003).

The study by Catacutan and Coloso (1997) showed that carbohydrates in the form of gelatinized bread flour contributed dietary energy to barramundi in an experiment with two levels of carbohydrate, 150 and 200 g kg^{-1}, respectively. The 200g kg^{-1} carbohydrate in diet resulted in better performance than 150g kg^{-1}. As compared with tilapia, barramundi performed relatively poor in clearing intraperitoneal injection of glucose (Anderson, 2003). This author also reported that metabolism of starches, dextrins and maltose was limited in barramundi, but quickly absorbed free glucose. To evaluate the digestibility of carbohydrate in barramundi, McMeniman (2003) examined different inclusion levels of various forms of carbohydrates from 150 to 300g kg^{-1} and the author observed that glucose, maltose and different starch sources decreased significantly when the inclusion level increased. Thus, to the formulated diet for barramundi, it should be important to reduce proportion of carbohydrate as much as possible, although carbohydrates play another role of gelatinized, forming the diet for floating.

2.6.2.5 Vitamins and minerals

Vitamins are necessary organic compounds for normal growth and health of animals. Like other animals, fish requires a small amount of vitamins, but lack of vitamins in

dietary nutrients can cause serious growth performance. For instance, malformation developed during skeletogenesis is due to the lake of vitamins C or/and D (Cahu *et al*., 2003; Lall and Lewis-McCrea, 2007). Three distinct levels of vitamins in diets shall be considered for optimum growth, for adaptive response under changed environment and access level (Figure 2.3). Variations of vitamin requirement of different fish species are not large (Antony Jesu Prabhu et al., 2014).

Many researches dealing with dietary vitamins required by barramundi have been conducted (Table 2.3). Vitamins A, D, E, K, thiamine, phdoxine and ascorbic acid have been evaluated and the combination of these vitamins in diets has been widely used and termed as vitamin premix. The premix can be from different vitamins or in combination of vitamins and minerals.

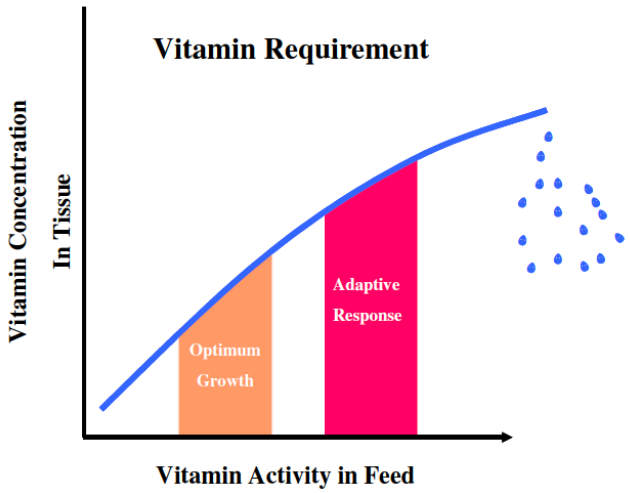

Figure 2. 3 Vitamins requirement by fish. Source: Lecture material by Mark Newman, 2004 (ASAIM, USA).

Table 2. 3 Summary of vitamins requirements (mg/kg of diet) for barramundi. Adapted from (Glencross, 2006).

Vitamins	*Requirement*	*Deficiency signs*	*References*
Thiamine	R	Poor growth, high mortality, stress	Boonyatpain and Wanokowat (1993)
Riboflavin	R	Erratic swimming, cataract	Boonyatpain and Wanokowat (1993)
Pyridoxine	5-10	Erratic swimming, high mortality, convulsions	Wanadakowat et al. (1989)
Patothenic acid	15-90	High mortality	Boonyarapalin et al. (1993)
Nicotinic acid	n/a	Fin haemorrhaging and erosion, clubbed gill, high mortality	Boonyarapalin et al. (1993)
Inositol	R	Poor growth, abnormal bone formation	Boonyatpain and Wanokowat (1993)
Vitamin C	25-30	Gill haemorrhages, scoliosis, lordosis, broken black syndrome, fatty liver, muscle degeneration, poor gill development, bone deformations	Boonyaratpalin at al. (1989; 1992) Phoromkunthong et al. (1997)
Vitamin E	R	Muscular atrophy, increased disease susceptibility	Boonyarapalin et al. (1993)

Note: R is requirement but the quantity is not identified. n/a is the data not identified by the author(s).

Minerals are inorganic elements necessary in the food to maintain the body's functions. There are two groups of minerals, macro-minerals and micro-minerals. Common micro-minerals are sodium, chloride, potassium and phosphorus, while micro-minerals are iron, zinc, manganese and copper. The requirement of mineral by fish is reviewed by Antony Jesu Prabhu *et al.* (2014). The requirement of vitamins and minerals by barramundi or other fish is low and the fish diets are usually designed with higher amount than the actual need. For barramundi, the level of some vitamins and minerals are referred from other fish as those not different among fish (Antony Jesu Prabhu *et al.*, 2014). The requirement of phosphorus has evaluated and fixed to be the optimum level of 5g kg^{-1} (Boonyaratpalin and Phongmaneerat, 1990). Effects of trace element such as selenium (Ilham *et al.*, 2016) was 2-3 g kg^{-1}.

2.7 Physiological responses of fish against plant based diets

Common aquaculture feed is primarily based on fishmeal which as stated earlier has a limited availability. Access to alterative protein source(s) to fishmeal is a key factor for sustainable aquaculture development in the future (Tacon and Metian, 2008). There have been many investigations focused on the use of terrestrial and aquatic plants to replace fishmeal. Plant-derived proteins have been tested and are increasingly being used in fish feed.

2.7.1 Growth performance

Growth performance of fish is the first key parameter for the evaluation of the test diets. In the case of fast growth the test diet is considered as acceptable and thus the test ingredient is believed to be as good enough for fishmeal replacement. A variety of species have been used to evaluate the quality of plant ingredients, most commonly are salmon (Skrede *et al.*, 2002; Uran *et al.*, 2009), rainbow trout (Farhangi and Carter, 2001; Cheng *et al.*, 2003; Drew *et al.*, 2005; Glencross, 2011), and barramundi (Katersky and Carter, 2009; Glencross *et al.*, 2012). These species may response differently in term of growth performance with even the same ingredient. Additionally, other factors such as environment conditions (temperature salinity and water quality), size of the fish (Eldridge *et al.*, 2015) and feed processing methods (Drew *et al.*, 2007) also affect the growth performance. Usually, if high proportion of fishmeal is replaced by plant-derived protein ingredients, it can result in reduced growth rate, increases feed conversion ratios (FCR) and mortality (Ilham *et al.*, 2016).

Some terrestrial plant ingredients such as soybean or lupin are well utilized by many fish species. However, aquatic plant, like microalgae (*Spirulina sp*) can only be efficiently used by some fish. Carps, for example *Catla catla* and *Labeo rohita* can use protein from *Spirulina platensis* in their diet without compromising growth rate and FCR (Nandeesha *et al.*, 2001). However, in the case of Atlantic Cod (*Gadus morhua*) any

inclusion levels of *S. platensis* could reduce growth rate and increase FCR (Walker and Berlinsky, 2011).

2.7.2 Digestibility

One of the important aspects of introduction of a new alternative ingredient to fishmeal is digestibility. Several investigations dealing with the digestibility of plant-derived protein from soybean (Ngandzali *et al.*, 2011), lupin (Tabrett *et al.*, 2012), microalgae (Riche *et al.*, 2016) canola meal (Ngo *et al.*, 2015) by fish were carried out. They reported that relative high protein digestibility, up to 75%-99%, fat 65%-90%, energy 45%-85% and dry matter 30%75% are documented. Digestibility of an ingredient is dependent on the processing method and fish species (Table 2.4).

Table 2. 4 Apparent digestibility of plant derived protein in comparison with fishmeal. Incl.= inclusion; SBM = soy bean meal; PC = protein concentrate.

Ingredients	*Incl. level*	*Protein*	*Lipid*	*Reference*
Bio processed SBM	20	+	-	(Refstie *et al.*, 2006)
Corn Gluten	20	=	=	(Aslaksen *et al.*, 2007)
Faba, dehulled bean	20	=	=	(Aslaksen *et al.*, 2007)
Faba, whole bean	20	=	=	(Aslaksen *et al.*, 2007)
Fermented ESBM	20	-	-	(Refstie *et al.*, 2005)
Lupin PC	30	+	+	(Refstie *et al.*, 2006)
Lupin	20	=	+	(Aslaksen *et al.*, 2007)
Lupin kernel meal	20	-	=	(Refstie *et al.*, 2006)
Oat	20	-	=	(Aslaksen *et al.*, 2007)
Pea	20	+	=	(Aslaksen *et al.*, 2007)
Rapeseed	20	-	+	(Aslaksen *et al.*, 2007)
SBM	20	-	=	(Aslaksen *et al.*, 2007)
SBM	30	=	na	(Refstie *et al.*, 2006)
Soy PC	60	=	na	(Glencross *et al.*, 2004a)
Soy PC	30	=	na	(Glencross *et al.*, 2004a)
Soy protein isolate	30	=	na	(Glencross *et al.*, 2004a)
Sunflower	20	+	=	(Aslaksen *et al.*, 2007)

Incl. means inclusion; SBM means soy bean meal; PC means protein concentrate; + means increased digestibility; - means decreased digestibility; = means no changed; na means data is not available.

Protein from soybean meal or corn gluten meal are well digested by pompano (*Trachinotus carolinus*), giving apparent digestibility coefficients of 10%-20% higher than that of fishmeal (Riche and Williams, 2010). On the other hand, these protein ingredients are not very well digested by barramundi meaning less digestible than fishmeal. The treatment of bioprocess can improve digestibility of plant ingredient (Table 2.5)

Table 2. 5 Improvement of digestibility plant ingredient by adding enzyme in some species.

Treatment	***Species and ingredient***	***Digestibility***	***References***
Exogenous protease for soybean	Gible carp (*Carassius auratus gibelio*)	+ (dry matter and protein)	(Shi *et al.*, 2016)
Protease for flax: pea	Rainbow trout (*Oncorhynchus mykiss*)	+ (protein)	(Drew *et al.*, 2005)
Protease for canola:pea		+ (protein, lipid, energy and dry matter)	(Drew *et al.*, 2005)
Anlkaine serine endipeptidase for soybean and groundnut meals	Tilapia (*Oreochromis niloticus*) White shrimp *Litopenaeus vanamei*)	+ (dry matter and protein)	(Li *et al.*, 2015)
Phytase for rapeseed	Turbot *Psetta maxima*)	+ (protein at 2000 FTU kg^{-1})	(Von Danwitz *et al.*, 2016)

+ increased digestibility

2.7.3 Carcass composition

The different dietary composition may affect the fish quality as expressed in the carcass composition, especially when the fish is ready for market. Carcass lipid of cuneate drum (*Nibea miichthioides),* red drum (*Sciaenops ocellatus*), discus (*Symphysodon aequifasciata*), and barramundi has been reported to be reduced when the fish was fed with plant inclusion diets (Chong *et al.*, 2003; Tantikitti *et al.*, 2005; Wang *et al.*, 2006). In freshwater prawn (*Macrobranchium rosenbergii*) the replacement of fishmeal by microalgae (*Arthospira platensis*) resulted in significantly higher carcass protein, and lipid (Radhakrishnan *et al.*, 2016). However, as observed in sea bream (*Acanthopagrus schlegelii*) carcass protein seems to be not affected when fishmeal was replaced by plant protein from soybean (Ngandzali *et al.*, 2011).

2.7.4 Water quality

Nutrient discharge from feed in aquaculture is an increasing concern because more intensive production is achieved in the limited clean water. Thus, reduction of nutrient discharge is important for sustainable aquaculture. The feed utilization can be evaluated through the levels of nutrients discharge into the water environment. A diet containing plant-derived protein is considered well utilized when the nutrient retention is high and the load of nutrients such as phosphorus or nitrogen is low. Plant based diet has shown to increase the nutrient load as compared to fishmeal based diet (Davidson *et al.*, 2013). However, nutrient load could be ease when enzyme phytase is added to the feed (Ngandzali *et al.*, 2011).

2.7.5 Blood biochemistry

Biochemistry of blood is an indicator for health of animals including fish. Highly variation in blood chemistry of fish could be a signal of homeostasis disorder. To evaluate an acceptance of feed ingredients, chemical parameters of blood such as plasma glucose, cortisol, alanine transaminase (ALT), creatinine kinase (CK), alanine aminotransferase (ALAT), and glutamate dehygrogenase (GLDH) are used as indicators of physiological homeostasis (Suski *et al.*, 2003; Glencross *et al.*, 2011a). Each parameter has individual threshold levels. For instance, the large variation of ALT out of normal range indicates liver damage; the changes in levels of Na+, K+ or Cl- are the signal of osmo-regulation. Besides the effects of environmental condition, the chemistry of blood was also affected by dietary composition (Waagbo *et al.*, 1994).

Glencross *et al.* (2016) reported that in the case of barramundi, diets replaced almost all fishmeal with soybean and poultry meal did not alter the physiological parameters of blood such as creatinine kinase (CK), alanine aminotransferase (ALAT) and glutamate dehydrogenase (GLDH). The diets containing canola meals extracted by solvent and expeller methods also had similar effects (no changes of the concentration of plasma protein, glucose and haem) and reported by Ngo *et al.* (2016).

Stress is defined as chemical and physical disturbances causing body reactions that may contribute to disorder, disease and/or death (Randall and Perry, 1992). Stress is described by two phases (Selye, 1973): a) eustress is a response of the body against situations of physiological changes to optimize biological performance, b) distress happens when the organism faces with physiological changes that disorder or compromised the body homeostasis. In studies of effect of stress, various stressors are applied including challenges with pathogen, environment shock such as dissolved oxygen deprivation, and increase toxic chemical from nitrogen compound or transportation stressor (Martínez-Porchas *et al.*, 2009). Fish response to the stressors by changing their plasma biochemistry to adapt and tolerance with a slight and short stress, however severe stress or prolonged exposure to the eustress could lead to behaviour disorder as observed in swimming performance, reduced growth, and impaired metabolic rate (Hughes, 1973; Diaz and Rosenberg, 1995; Herbert and Steffensen, 2005).

Dietary composition has influenced the level of responses when fish is challenged with a pathogen (*Streptococcus inae*) that in turn increases the number of erythroblasts and haematocrit values if the diets is not supplemented with enough vitamin C level (500 mg kg^{-1}) (Garcia *et al.*, 2007). When rainbow trout exposed to 5 hours transportation stress, skin mucosa and the skin-associated bacteria increases by 10-50 fold as compared to trout without stressor (Tacchi *et al.*, 2015).

A large variation of biochemistry was observed when largemouth bass (*Micropterus salmoides*) exposed to stress by temperature dissolved oxygen shocks (Vanlandeghem *et al.*, 2010). Plasma glucose, cortisol and lactate dehydrogenase activity significantly increased when the fish was subjected to heat and cold shocks (from $20^{o}C$ to $8^{o}C$) and hypoxic (from normal to 2 mg L^{-1}) and hyperoxic (from normal to 18 mg $^{L-1}$). Similarly, stress caused by high stocked density can increase the level of plasma glucose and cortisol concentrations in barramundi raised in RAS system (Ardiansyah and Fotedar, 2016). The authors recommended to stock at 18.57 kg m^{-3} to reduce stress on fish.

2.7.6 Responses of barramundi

There is some evidence dealing with the influence of processing method on the performance of barramundi (Figure 2.4). Ngo et al. (2016) reported that processing methods by solvent extraction and expeller extraction of canola meal resulted the same level of growth and did not modify any changes of the biochemistry and gene expression. However, at 300 g kg^{-1} inclusion level of expeller extraction reduced growth, feed intake and increase FCR as compared with that of the same inclusion level of solvent extracted product.

In some cases, the growth performance and feed utilization by barramundi are not influenced by dietary nutritional composition under optimum temperature condition from 25^{o}C to 32^{o}C (Glencross and Bermudes, 2010). However, when the temperature is outside of the optimum ranges, different diets could affect to the growth, feed utilization (Glencross and Rutherford, 2010). This means that some key factors for evaluation of the capacity of plant-derived protein to replace fishmeal are not presented at normal condition, however, the differences or influences appear when the fish got stress from surrounding environment.

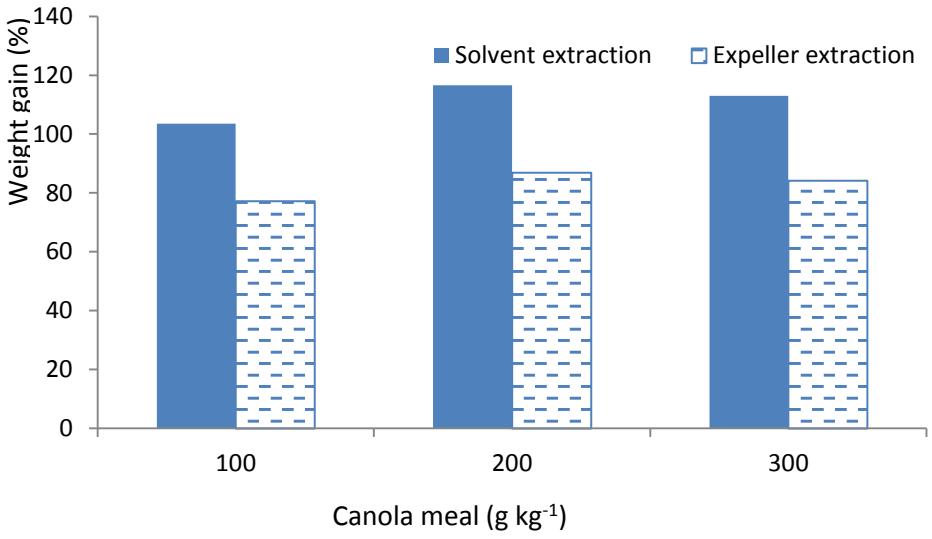

Figure 2. 4 Weight gain (%) compared to control of barramundi fed canola meal (solvent and expeller extraction) at different inclusion levels (modified from Ngo *et al.* (2016)).

2.8 In conclusion

Aquaculture industry has been expanding and thus requires a larger amount of aquafeed. Traditional aquadiet based on fishmeal as major protein source is no longer sustainable. Other resources of protein for aquafeed such as from plant-derived proteins show a potential for sustainable aquaculture development in the future. Most fish species accept plant protein in fish diets at low inclusion levels, generally 30% inclusion of a plant ingredient, except for some specific cases that the inclusion can be up to 85% (Glencross *et al.*, 2011a). A major reason for limiting the utilization of plant-derived protein ingredients in aquadiet is the presence of ANFs (Francis *et al.*, 2001; Gatlin *et al.*, 2007) which adversely influence to growth and health performance by reducing absorptions of nutrients, and digestibility. Another limitation of using plant ingredients is the low concentration of some amino acids such as lysine or methionine (Gatlin *et al.*, 2007; Hansen, 2009).

Proper bioprocessing of the plant ingredients is deemed to enhance the feed utilization and thereby increase inclusion level in the fish diet. Evaluation of growth performances and physiological responses of fish fed bioprocessed plant based diets are necessary to provide aquafeed producers with the technical knowledge in preparation, processing and utilization of plant-derived protein ingredients for cost-effective feed formulation.

Chapter 3: BIOPROCESSING OF PLANT-DERIVED PROTEIN INGREDIENTS

3.1 Introduction

Biological and/or mechanical processing, including heat treatment or soaking followed by heat treatment of the plant based aqua-feed ingredients is believed to improve the protein quality (Wu *et al.*, 1996; Hong *et al.*, 2004; Yabaya *et al.*, 2009) and reduce the anti-nutrients in aquadiet ingredients (Gatlin *et al.*, 2007). However, there are concerns of costs and reduction in protein quality maillard reaction (Seiquer *et al.*, 2006) or the loss of vitamins when heat is applied. Bioprocessing including fermentation and germination (Hotz and Gibson, 2007), therefore are considered environmental friendly and cheaper methods. Fermentation is deemed to improve the protein quality by reducing the peptide sizes of the protein molecule (Hong *et al.*, 2004) and thus increases the digestibly of the ingredient (Hassan and Tinay, 1995). The germination also increases the availability of the vitamins and antioxidant compounds (Lopez-Amoro´s *et al.*, 2006), thereby improving the performance of the host fish (Narra *et al.*, 2015). Thus, this study examines the effect of bioprocessing by lactobacilli fermentation of lupin and peanut meal (PM), germination of peanut and enzymatic treatment of *Spirulina platensis* (SP) on the quality of plant-derived protein ingredients which reflect in the content of proximate composition, amino acids, antinutritional factors (ANFs), bioactive compounds and protein structure.

3.2 Materials and Methodology

3.2.1 Materials

Twenty kg of sweet lupin (*Lupinus angustifolius*) kernels, were provided by Co-operative Bulk Handling Grain, Western Australia. Whole dehulled peanut seeds of 40

kg were purchased from a local store in Hungloc-Nghean, Vietnam. Ten kg of *Spirulina* was purchased from Vinh Hao Co, Vietnam in the form of whole cells dried paste.

Lactobacilus acidoophilus, *L. aporogenes* and *L. kefir*, were obtained from a commercial product BIOLAC, BIOPHARCO, Vietnam. *Lactobacillus* growth medium, MRS (De Man-Rogosa-Sharp) broth and Soy Extract were purchased from Kim Nguu Chemical Co. Hanoi, Vietnam. Commercial enzymes cellulolase (Celluclast®1.5L) and proteinase (Alcalase® 2.4L), in aliquots forms, were donated by Novozyme Australia Pt Ltd, North Rocks, NSW, Australia. Chemicals used to make phosphate buffer were kindly provided by Environment and Disease Monitoring Centre, Research Institute for Aquaculture No.1, Vietnam.

3.2.2 Fermentation of lupin

The lupin kernels were grounded to less than 200 µm before fermenting by *Lactobacilli* spp. The *Lactobacilli were* mass incubated in MRS broth medium (Merck KgaA Germany) containing polysorbate, acetate, magnesium and manganese, which are known to act as special growth factors for *Lactobacilli spp*. To each 1000-ml of distilled water was added with 55 g MRS broth and 250-ml soy extract. The combination was autoclaved at 121^{o}C for 15 minutes prior to the lactobacilli species were added. The incubation was carried out in a black glass jar with minimum oxygen for 24 hours at 37^{o}C in a refrigerated incubator (Scientifica, VELP). After incubation the solution was mixed with autoclaved lupin in a plastic bag where commercial N_2 gas (obtained from Hai Duong Gas Company, Vietnam) was filled to increase anaerobic conditions. The lupin fermentation was conducted under 37^{o}C for 72 hours. After fermentation, the samples of the Lupin were collected to count bacterial density and nutritional profile.

3.2.3 Enzymatic treatment of SP

The two enzymes, Celluclast®1.5L and Alcalase® 2.4L were separately diluted in phosphate buffer (pH=7) with a ratio of 1:20. The phosphate buffer with Celluclast®1.5L was added with SP at 10% (v/v) for 1 hour followed by the addition of the phosphate

buffer with Alcalase® 2.4L at 10% (v/v) for another hour to form a dough of SP, which was termed as enzyme-treated SP (ESP). ESP and RSP were then sampled for nutritional analyses.

3.2.4 Bioprocessing of peanuts

The peanut seed were divided into two equal portions. One portion was used for germination while the other for oil extraction. The extraction of oil was performed by steaming the seed at 90^{o}C for 2 hours before grinding to about 500 µm; then the grounded seed was pressed mechanically (6YL165A, China) until most of the oil was extracted. Oil extracted product was then grounded again to 200µm size and was ready to be used as an alternative ingredient to fishmeal. This oil extracted peanut by mechanical method was termed as mechanically pressed peanut meal (MPM).

Half of the MPM was further fermented by Lactobacilli *spp.*as described as with fermentation of lupin. After incubation, samples were collected to check bacterial density, while the remaining part was mixed with autoclaved MPM in a plastic bag, filled with commercial N_2 gas (from Hai Duong Gas Company, Vietnam) to enhance the anaerobic conditions. The MPM fermentation was conducted at 37^{o}C for 72 hours. The final fermented MPM product was termed as fermented peanut meal (FPM).

Germination was performed at 25-27^{o}C and 90% humidity. The whole peanut seed was rinsed under dark conditions in a 70-l aluminium container by fresh 37^{o}C warm water for 12 hours. The water in the container was then drained out. Germination took place in the same container where the seed was soaked with 33-37^{o}C water after every 6 hours. After germination, seed with about 2 mm sprouts was selected for oil extraction. The oil extraction and grinding were performed identically as outlined earlier in this section to obtain the germinated peanut meal (GPM). FPM and GPM were defined as bioprocessed PM. Finally, 50g (9 samples) of each processed PM was collected for protein extraction and electrophoresis analyses.

3.2.5 Protein extraction

Protein extraction and SDS-polyacrylamide gel electrophoresis was carried out in Vietnam Institute of Biotechnology following the method described by Faurobert (1997) . The proteins in PMs and SP were extracted by grinding 10g samples with a laboratory glass homogenizer (30 ml Dounce Glass Tisue Grinder, China). Afterwards, 50mg of the grounded samples were then homogenized for 5 minutes with 2 mL of 20 mM Tris-HCl buffer, pH 7.6 (model Polytron 1200B, Brinkmann, Westbury, NY) on ice. The Tris-HCl buffer was prepared with 0.1% sodium dodecyl sulfate (SDS), 5 mM dithiothreitol, and 5 µg/mL of protease inhibitor cocktail (Promega, Madison, WI). The samples were then centrifuged at 10,000 rpm for 15 minutes at 4°C (ELMI FlufaMix High Speed Lab Centrifuge (w/o Rotor), 1000-15000, CM-50M, USA). Sedimentation after centrifuge was mixed with acetone with a ratio of 1:4 and diluted by distilled water. The supernatants were transferred to 1.5-mL microcentrifuge tubes which were ready to be used for the protein analysis. Protein content in each sample was examined using a Bio-Rad Protein Assay Kit (Bio-Rad DuoFlow, Chromatography, F40 Tubing Kit NIB, USA) by measuring absorbance on a spectrophotometer (uv-vis spectrophotometer lab equipment 200-1000 nm 2 nm 220/110v n4 c4, China) at 595 nm. Bovine serum albumin (product no. 500-0007, Bio-Rad) was used as a protein standard in the protein determination.

3.2.6 SDS-polyacrylamide gel electrophoresis (PAGE)

Soluble proteins were analysed by SDS-PAGE in a Bio-Rad method using a Bio-Rad Mini-Protean 3 Electrophoresis System with 12% polyacrylamide separating gels containing 0.1% SDS in 1xTris-glycine buffer. Amount of 5-10 µg of extracted protein was taken to load for each well. The sample was then separated at 80 mV for 180 minutes. Every sample was loaded and replicated three times. After electrophoresis, the gel was stained for 40 minutes using Comassi Blue Brillant 0.2%. De-stained for final visual check was carried out in a solution of 450 ml methanol, 50 ml acetic acid and 500 ml distilled water for 12 hours.

3.2.7 Nutritional chemical analyses

ANFs and bioactive compounds in lupin, fermented lupin and in all types of peanut meals were analysis in Lareal, HCM city, Vietnam. Proximate nutritional parameters of diets were analysed in National Institute for Food Control, Hanoi, Vietnam. Tannins and alkaloids beta-carotene, vitamin E (alpha, gamma-tocophenol) and flavonoid (quercetin) were determined by HPLC (Indyk, 1988). Crude protein (Kjeldalh) and hydrolysed fat (ISO 6492) were analysed following procedures described by AOAC (1990).

Lupin, fermented lupin, MPM, FPM and GPM were nutritionally analysed in National Institute for Food Control, Hanoi, Vietnam. Nutritional parameters were analysed in accordance with AOAC (1996). These consisted of crude protein (Kjeldalh), hydrolysed fat (ISO 6492:1999), crude fibres (OACS Ba-6a-05), phosphorus (AOAC 965.17), and amino acid profile (HPLC).

3.2.8 Statistical analysis

The data were analysed using IBM SPSS for Windows version 20 at Curtin University. The results are expressed as the means and standard deviations unless otherwise specified. Paired-sample T test was used to compare means of single nutritional parameters of the lupin before and after fermentation. Orthogonal Contrasts was performed to compare nutrient profiles of different processed PMs.

3.3 Results

3.3.1 Effect Fermentation on nutritional composition of lupin

Bacterial density found in fermented product was 3.10^8 CFU/g. There were differences in anti-nutrients and amino acid profile before and after the lupin was fermented. While two anti-nutrients, tannins and phytic acid were significantly reduced to 87.04% and 17.64% respectively, the amino acids, lysine, methionine, as well as phosphorus availability were increased (Table 3.1, Table 3.2).

Table 3. 1 Nutritional composition of fermented lupin and lupin (g $100g^{-1}$) (n=3).

Proximate composition	*Fermented lupin*	*Lupin*
DM	83.00±0.0	89.50±0.02
CP	40.00±1.1	38.55±0.6
Lipid	5.87±1.1	7.80±1.3
Fibre	2.58±0.2[a]	4.20±0.6[b]
Ash	2.58±0.4	2.60±0.8
Amino acids		
Arginine	3.90±0.03	4.14±0.13
Histidine	1.33±0.13	0.78±0.11
Isoleucine	1.84±0.06	1.42±0.12
Leucine	2.93±0.11	2.55±0.10
Lysine	2.23±0.05[a]	1.73±0.04[b]
Methionine	0.32±0.06[a]	0.25±0.02[b]
Phenylalanine	1.70±0.00	1.40±0.13
Threonine	1.63±0.24	1.34±0.09
Tryptophan	0.33±0.12	0.33±0.05
Valine	1.90±0.07	1.40±0.10
Calcium	0.30±0.04[a]	0.20±0.01[b]
Available Phosphorus	0.23±0.06[a]	0.09±0.16[b]

Table 3. 2 Antinutritional factor (ANFs) presence (%) in lupin and its fermentation product (n=3).

ANFs	*Lupin*	*Fermented lupin*	*Pooled S.E.M*
Phytic acid (phytate salt)	0.54±0.01[a]	0.07±0.00[b]	0.006
Tamins	0.17±0.00[a]	0.14±0.00[b]	0.003

Note: Within rows, values followed by the same letters are not significantly different ($p<0.05$, pair T test)

3.3.2 Effect of bioprocess on ANFs and bioactive compounds of peanut

The total lactobacilli bacterial count in final product of FPM was greater than 10^8 $CPUg^{-1}$. There were significant differences in ANFs and bioactive compounds profiles among FPM, GPM and NPM (Table 3.3). Fermentation and germination resulted in lower concentration ($p<0.05$) of tannins and alkaloids and higher levels of vitamin E and flavonoid than the physical (none bioprocess) oil extraction (NPM). The tannins

concentration was significantly removed at higher rate by fermentation method than that by germination. In contrast, alkaloid concentration was significantly decreased ($p<0.05$) by germination than by fermentation. There was no significant difference of vitamin E concentration between FPM and GPM, however flavonoid level in GPM was as two-fold higher ($p<0.05$) than in FPM. Beta-carotene was undetected in NPM and hardly found in FPM, but was about 0.01 mg g-1 in GPM (Table 3.3).

Table 3. 3 ANFs (%) and bioactive compounds (mg/100g) concentrations in peanut meal processed in different methods (n=3).

	FPM	***GPM***	***NPM***	***Pooled SE***
Tannins	0.27[a]	0.48[b]	0.69[c]	0.62
Alkaloids	4.64[a]	1.22[b]	8.49[c]	1.06
Beta-Carotene	0.03*	Undetected	1.00	-
Vitamin E **	8.50[a]	8.30[a]	6.80[b]	0.32
Flavonoid (quercetin)	11.30[a]	5.80[b]	4.50[c]	1.09

Note: Within rows, values followed by different letters are significantly different ($p<0.05$, Orthogonal Contrasts, Bonferroni test).
(*) n=1, value was detected in only one sample.
(**) the values are total of alpha and grama-tocophenol

3.3.3 Effects of bioprocess on nutritional composition and protein structure of peanut

Mechanical processing of peanut meal resulted in approximately 46.3% protein which was significantly ($p<0.05$) lower than the FPM, 48.0% (Table 4). GPM had significantly highest protein content, 49.0% than MPM and FPM. In contrast, hydrolysed fat, carbohydrate and phosphorus levels in GPM were significant lower than in MPM and FPM. Fermentation resulted in higher lipid and carbohydrate contents (12.2%, and 17.1%, respectively) than the germination (6.9% and 8.7% respectively). The Phosphorus content was higher in FPM (0.8%) than in the GPM and MPM (0.4% and 0.5%, respectively).

Table 3. 4 Nutritional compositions of three types of peanut meal (g 100g^{-1}) (n=3).

Proximate nutrients	***MPM***	***FPM***	***GPM***
Dried mater	82.3±0.5^{a}	75.8±0.2^{b}	89.3±0.5^{a}
Crude protein	46.3±0.3^{a}	48.1±0.2^{b}	49.1±0.1^{c}
Lipid	11.2±0.8^{a}	12.2±0.3^{a}	6.9±0.3^{b}
Carbohydrate	17.8±0.4^{a}	17.2±0.7^{a}	8.7±0.1^{b}
Fibbers	4.3±0.3^{a}	3.7±0.2^{a}	2.9±0.3^{b}
Phosphorus	0.4±0.0^{a}	0.8±0.0^{b}	0.5±0.1^{a}
Amino acids			
Arginine	3.02±0.14^{a}	3.13±0.21^{a}	2.31±0.10^{b}
Histidine	1.25±0.02	1.28±0.08	1.10±0.10
Isoleucine	1.66±0.05	1.77±0.03	1.76±0.04
Leucine	3.06±0.19	3.08±0.07	2.71±0.16
Lysine	1.57±0.05	1.72±0.12	1.67±0.03
Methionine	0.72±0.02^{ab}	0.82±0.12^{a}	0.60±0.06^{b}
Phenylalanine	1.66±0.14	1.54±0.23	1.52±0.05
Threonine	0.33±0.05	0.38±0.12	0.39±0.01
Tryptophan	2.38±0.11^{ab}	2.67±0.09^{a}	2.17±0.09^{b}
Valine	1.97±0.10	2.04±0.07	2.12±0.10

Note: within rows, values followed by different superscript letters are significantly different ($p<0.05$, Orthogonal Contrasts, Bonferroni test)

Different processing of PM did not significantly alter histidine, isoleucine, leucine, lysine, threonine, phenylalanine, and valine (Table 3.4), but resulted in significant differences in arginine, methionine and tryptophan contents. Arginine was significantly decreased by germination which was 2.31% in GPM than 3.02% in MPM and 3.13% in FPM. Met and Try were 0.82 and 2.67%, respectively and were significantly lower in FPM and 0.60% and 2.17% respectively in GPM.

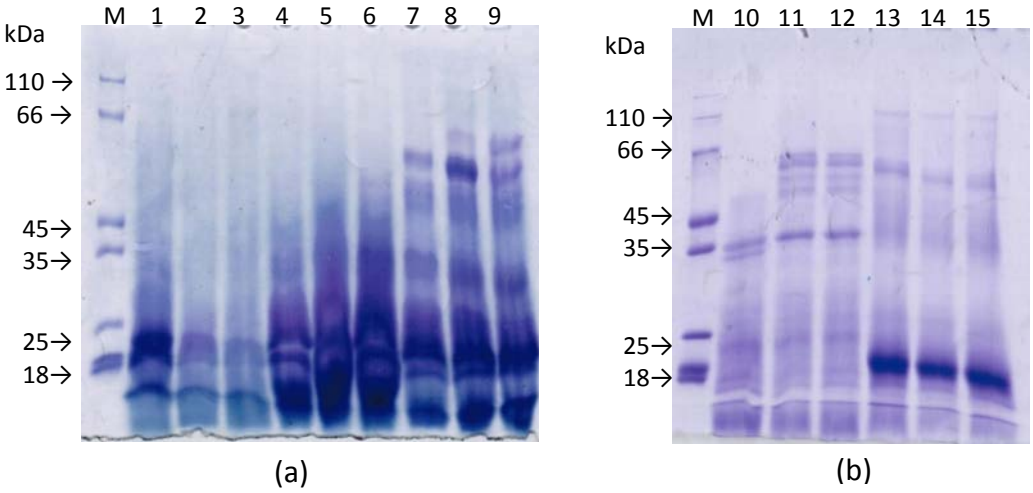

Figure 3. 1. SDS-PAGE Electrophoresis of FPM, MPM, GPM (a) and SP (b). Bands 1, 2, 3 are peptide size of GPM; bands 4, 5, 6 are of FPM; bands 7, 8, 9 are of MPM; bands 10, 11, 13 are of RSP and bands 13, 14, 15 are of ESP. M is standard bands (kDa)

Peptide size of protein due to different processing of PM ranged from <18 kDa to 110 kDa (Figure 3.1a) with significant difference in the peptide size distribution. While more than 80% peptides of FPM and GPM were shorter than 35kDa, large proportion of peptide, 74% peptides from MPM was long than 35 kDa (Table 3.5). Similarly, large proportion of short peptide size (<18 kDa) was observed in FPM and GPM, being 38% and 21.5% respectively than MPM, 8.5%. GPM had a few peptides (less than 3%) larger than 35 kDa, whereas FPM had about 20% of total peptide ranged in size of 45-66 kDa.

3.3.4 Effects of Enzymatic treatment of SP

ESP had larger proportion of small peptide than RSP (Figure 3.1b). Most of the peptides in ESP protein fell in the range of 18-25 kDa (82.5%), while RSP protein contained majority of larger peptide sizes of 35-45 kDa (78.2%). There was no significant difference in proximate composition between ESP and RSP (Table 3.6).

Table 3.5 Estimated peptide size proportions of different processed peanut meals and SPs (n=3).

Peptide size (kDa)	*MPM*	*FPM*	*GPM*	*RSP*	*ESP*
110-66					2.0±0.0
66-45	28.3±0.6[b]	12.0±0.6[a]	1.0±1.0[c]	6.6±0.1[b]	13.3±0.4[b]
35-45	17.63±0.7[b]	7.2±0.4[a]	1.3±1.5[c]	78.2±0.8[b]	1.0±0.1[c]
25-35	28.6±0.6[a]	27.3±0.8[a]	12.1±0.3[b]	9.8±0.5[a]	
18-25	17.0±0.6[b]	15.3±0.9[a]	64.0±3.4[c]	1.1±1.1[b]	82.5±1.3[c]
<18	8.5±0.8[b]	38.1±0.2[a]	21.5±1.7[c]	4.3±0.2[b]	1.2±0.1[c]

Note: Within rows, values followed by the same letter are not significantly different ($p<0.05$, Orthogonal Contracts, Bonferroni test)

3.4 Discussion

A number of ANFs are presented in protein-rich plants (Francis *et al.*, 2001) including lupins (Dupont *et al.*, 1994). Sweet Australia lupin is low in alkaloids (Dupont *et al.*, 1994) however phytates and tannins are major factors influencing the digestibility and thus reduces growth performance in aquatic species. Tannins contents are 1.17 and 2.64 µg g^{-1} in sweet and bitter lupins respectively (Dupont *et al.*, 1994) that influence the protein utilization and digestion (Francis *et al.*, 2001). These ANFs are rather stable under heat treatment (Boland *et al.*, 1975) but can be efficiently removed by fermentation (Nnam and Obiakor, 2003). Lactic acid fermentation has been shown to give a significant reduction in phytic acid in cereals and sesame seed (Marklinder *et al.*, 1996; Mukhopadhyay and Ray, 1999; Skrede *et al.*, 2002). Bartkiene *et al.* (2013) Indicated that Lacto-fermentation of sweet lupin (*L. angustifolius*) could reduce acrylamide in enriched bread with high quality protein. Phytic acid and tannins in fermented lupin were reduced by 27.3% and 10.7%, respectively after 9 hours of fermentation by traditional method (Dhankher and Chauhan, 1987). Fermentation is the most effective way in decreasing the 56-96% of phytic acid than soaking and germination of brown rice (Liang *et al.*, 2008).

Hassan and Tinay (1995) reported that tannins content in Sorghum (*Sorghum bicolor*) was reduced by 30% after 14 hours under natural fermentation. Alkaloids are degraded during germination, for instance alkaloids in black cumin (*Nigella sativa*) seed reduced significantly to undetected levels when germinated at day 10 (Khamal and Ahmad, 2014). In our study, both alkaloids and tannins in peanut meals were reduced ($p<0.05$) after fermentation and germination. While tannins were efficiently removed by fermentation (60.9%), alkaloids content were remarkably reduced by germination (85.6%).

To replace fishmeal in marine fish diet, a plant ingredient should have relatively high protein content to meet a balanced formulation. Well-known plant ingredients such as soybean meal and lupin do have high protein levels, 45-67% and 36-47% respectively, depending on how they are processed. The protein in processed PM in this study was also high which ranged from 46.3% in MPM to 49.0% in GPM (Table 3.4). These protein levels are acceptable to replace protein from fishmeal. Protein content was significant higher in GPM than the other forms of peanut meals. Similar results were previously recorded by Ejigui *et al.* (2005) where dehulled germinated seeds had 33.34% and none germinated seeds which had 31.19% protein. The fermentation process also increased the protein content of PM and was similar to result of fermented lupin kernel in this study. All these elevated levels of protein become essential in the dietary formulation of the carnivore fish diet given that fishmeal usually has protein content greater than 60%.

The different bioprocessing resulted in high variation in the fat and carbohydrate levels of PM, wherein mechanical pressing and fermentation almost doubled these levels compared to germinated process (Table 3.4). When the peanut is germinated, its fat level gets reduced as Ejigui *et al.* (2005) reported that the germination in the dehulled peanut significantly reduced the fat level from 45 % to 41 %. Fat and carbohydrate in the PMs are important source of energy in the fish diet. Additionally, as fishmeal contains minimum carbohydrate, the reduction of this nutrient in PMs is an advantage

as it provides a larger room in the formulation for the inclusion of other nutrients to achieve a complete nutritional profile of the diet.

Except significant increases in arginine, methionine and tryptophan in FPM the essential amino acids in the three processed PM were not significantly different. Elevation of methionine in FPM is important because relative to fishmeal, plant ingredients usually contain lower levels of methionine (Gatlin *et al.*, 2007; Hansen, 2009) and becomes limiting factor in plant based diets. As a result, according to Liebig's Law (Liebig, 1842), it restricts the protein synthesis and feed utilization. Therefore, fermentation, by increase methionine content, balances the amino acid profile of FPM, narrowing it to that of the fishmeal. The remaining essential amino acids were not significantly changed by the processing, however the digestibility of these amino acids could be strongly influenced by processing methods. For example, the digestibility of raw kidney bean (*Phaseolus vulgaris* L) improved from 8-28% to 80-83% after cooking (Wu *et al.*, 1996). Similarly, soy bean fermented by yeast (*S. cerevisiae*) raised the essential amino acids, methionine and lysine from 1.24 to 5.67 and 2.02 to 6.57 (Yabaya *et al.*, 2009)..

Further, fermentation and germination influenced the characteristics of protein by increasing the amounts of small-sized peptides in the present study. This is similar to soybean and soybean meal fermented by fungus (*Aspergillus oryzae*) (Hong *et al.*, 2004). Reducing peptide size by fermentation helps digestion as fish could reduce the usage of the enzyme to cut peptide in the intestine and thereby increases protein digestibility. In this study, the increase in the proportion of shorter peptides by fermentation enhanced digestibility of protein.

When the algae were treated by enzyme cellulolase and proteinase, digestibility of protein in juvenile barramundi significantly increased. Pre-treatment of sweet lupin (*Lupinus angustifolius*) with lactobacilli fermentation significantly increased the protein and phosphorus digestibility (Vo-Binh *et al.*, 2015). The enzyme generated from fermentation process was proved to reduce peptide sizes, for instance, large peptides

(> 60 kDa) were reduced from 39% to 4% while small peptides (< 20 kDa) increased from 23% to 82% when soybean was fermented by the fungus (Hong *et al.*, 2004).The greater proportion of shorter peptides gained from fermentation or enzyme treatment assist in increasing the protein digestibility as the digestion of protein in intestine involves protein degradation by proteolytic enzymes to oligopeptides, tripeptides, dipeptide and then amino acid (Berg *et al.*, 2002). Therefore, the larger proportion of short peptides can save degraded enzymes, thereby increase digestibility by shorten degradation time.

Chapter 4: OPTIMISED FERMENTED LUPIN (*Lupinus angustifolius*) INCLUSION IN BARRAMUNDI *(Lates calcarifer)* JUVENILES DIETS

(*This chapter was published in Aquaculture journal*)

4.1. Introduction

The dependence of fishmeal based protein source for aqua-feed has long been realized as a significant limitation for sustainable development of aquaculture (Tacon, 1997; Tacon and Metian, 2008). Therefore, alternative high protein raw materials from animal by-product or plants are currently getting attention (Wanga *et al.*, 2006; Gatlin *et al.*, 2007). Lupins (*Lupinus* spp.), have been successfully tested as potential fishmeal replacements for salmonids and several other marine species (Carter and Hauler, 2000; Glencross *et al.*, 2004a; Glencross *et al.*, 2004b; Glencross and Hawkins, 2004; Glencross *et al.*, 2005; Glencross *et al.*, 2008; Katersky and Carter, 2009) and now are used in commercial diets (Glencross and Hawkins, 2004). Lupins at 40% inclusion level also produced unchanged growth and nitrogen retention in barramundi (*Lates calcarifer*) (Williams, 1998).

Although lupin and other legume seeds (*Phaseolus aureus, Cajanus cajan, Canavalia ensifomis*) contain a high amount of protein, their uses in food and aqua-feed are still limited due to their low protein digestibility and the presence of several anti-nutritional factors (ANFs) (Mubarak, 2005). Sweet lupin (*Lupinus angustifolius*), contain large amounts of soluble and insoluble non-starch polysaccharides, oligosaccharides, phytates, and tannins that have anti-nutritional effects including reduced digestion and absorption of amino acids (Barneveld, 1999; Glencross *et al.*, 2003). It has been suggested that lupins may also affect the structure of the gastrointestinal tract of salmonids (Farhangi and Carter, 2001; Refstie *et al.*, 2005) which might potentially affect amino acid flux and subsequent protein metabolism.

To enhance bioavailability of micronutrients in plant based diets by eliminating ANFs, several methods such as thermal and mechanical processes, fermentation, soaking and germination/malting can be applied (Hotz and Gibson, 2007). For improving utilization of plant protein in aqua-feed, fermentation seems to be cost effective method due to its simplicity and requirements for low operational energy and investment (Kang *et al.*, 2010). It is expected that *lactobacilli* fermentation of sweet lupin could improve its quality by reducing ANFs, improving amino acid balance and increasing digestibility thereby could increase its inclusion levels in the feed. However, pretreatment of lupin by fermentation to use as a source of protein for fish diets has never been investigated. Therefore, this study aims to evaluate the digestibility, growth performance and body composition of barramundi juveniles when fed different inclusion levels of lupin fermented by *Lactobacilli* spp.

4.2. Materials and methods

4.2.1. Experimental design

Barramundi (*Lates calcarifer*) juveniles were obtained from Northern National Marine Broodstock Centre, Vietnam and shipped to National Freshwater Breeding Centre (NBC), Haiduong, Vietnam where the juveniles were raised until they were adapted to 5 ppm. The fish were then acclimated for two weeks by feeding with Uni-President-Vietnam feed (45% protein, 12% fat). The juveniles were then graded, and those within the weight range of 7.0±1.6 g were selected and randomly delivered into fifteen tanks of 3.5 m^3, each attached to independent recirculating water system. The culture systems were set up out-doors in an open shed with a roof to protect from rain and direct sunlight. The natural temperature and photoperiod ranged between 28 – 31^oC and 12 hours of light respectively. After acclimation the experimental fish were fed for 61 days with 5 different pre-designed diets (Table 4.2). Every diet was fed in triplicate and three times daily (8 am, 12 am and 4 pm). After 61 days, the experiment was continued for another 7days to determine digestibility by feeding with the same diets

after 1% Cr_2O_3 as an inert biomarker was added to them. Feeding was modified to 90% ASA-IM satiation technique of which fish were fed to satiety for 20 minutes; the uneaten feed was collected immediately and measured in a calculation to determine amount of the feed intake; this amount was used for next 5 days and continued with another amount determined as outlined.

4.2.3 Diets preparation

Diets were designed based on the nutritional composition of raw materials (Table 4.1) to meet 45% protein and 13% lipid levels. The five experimental diets having five inclusion levels, *viz*. 0%, 30%, 45%, 60% and 75%, of fermented lupin (FL) (as described in Chapter 3) replacing fishmeal were prepared and labelled as 0FMR (control), 30FMR, 45FMR, 60FMR and 75FMR respectively (Table 4.2). Two sets of diets were prepared; one set was without chrome oxides (Table 4.2), and in the other set 1% of chrome oxide as an inert marker was added. The chrome oxide was added by replacing apart of cassava meal and wheat flour (for 75FMR) in the formulation thus protein content in diets was not affected (Table 4.2). The diet 0FMR contained 630 g kg^{-1} fishmeal. Diets were processed by addition of water to about 35% mash dry weight with well mixing to form a dough. This dough was then screw pelleted by a laboratory pelletizer to 1.2 – 2 mm pellets. These moist pellets were oven dried at 60^{o}C for 12 hours followed by cooling at room temperature before storing at – 20^{o}C till further use.

Table 4. 1 Ingredients and diets' chemical analyzed.

Parameters (%)	***Fishmeal***	***0FMR***	***30FMR***	***45FMR***	***60FMR***	***75FMR***
DM	93.20	88.76	87.46	87.70	86.51	98.84
Ash	21.80	20.69	17.37	14.82	13.71	12.74
Gross energy [+]	20.50	21.26	21.65	21.85	22.17	22.37
Digestible energy [+]	18.08	17.80	17.63	17.47	17.12	16.96
Crude protein	62.10	44.77	44.55	43.97	44.21	44.01
Digestible crude protein	55.90	38.98	39.20	39.02	37.97	37.86
Lipid	5.70	13.04	14.72	13.62	13.83	13.04
Fibre	1.50	1.32	1.50	1.59	1.68	1.77
LOA (18:2n-6) *	0.02	0.58	0.79	0.91	1.01	1.11
LNA (18:3n-3) *	0.04	0.12	0.17	0.20	0.22	0.24
ARA (20:4n-6) *	0.14	0.14	0.12	0.11	0.09	0.08
EPA (20:5n-3) *	0.40	0.87	0.85	0.84	0.84	0.83
DHA (22:6n-3) *	1.43	1.70	1.55	1.45	1.33	1.24
Total n-3 *	1.87	2.70	2.57	2.48	2.39	2.31
Total n-6*	0.16	0.71	0.91	1.01	1.11	1.19
n3:n6 *	11.66	3.78	2.84	2.45	2.16	1.93
Total phospholipid *	2.00	2.84	2.98	3.04	3.00	3.04
Cholesterol *	0.06	0.09	0.08	0.08	0.08	0.08
Arginine	3.68	2.56	2.83	2.96	2.98	3.09
Histidine	1.53	1.20	1.32	1.41	1.56	1.65
Isoleucine	3.03	2.00	1.92	1.84	1.63	1.56
Leucine	4.82	3.57	3.61	3.65	3.77	3.82
Lysine	4.81	3.39	3.25	3.15	3.08	3.02
Methionine	1.90	1.26	1.05	0.92	0.74	0.63
Phenylalanine	2.66	1.99	2.03	2.07	2.15	2.18
Threonine	2.69	1.91	1.90	1.88	1.87	1.86
Tryptophan	0.72	0.52	0.50	0.48	0.47	0.46
Valine	3.34	2.47	2.47	2.47	2.54	2.56
Ca *	6.10	3.87	3.02	2.45	1.70	1.21
Available P	4.65	2.96	2.28	1.84	1.25	0.87

Note: * The data of Lupin obtained from Kevin William, CSIRO 2007-2008; [+] calculated as MJ/kg

Table 4. 2 Ingredients composition of diets' formulation for growth, FCR and feed intake determination.

	Formula %				
Ingredient	***0FMR***	***30FMR***	***45FMR***	***60FMR***	***75FMR***
Fish meal	63.00	48.00	38.00	25.00	16.50
Lupin	0.00	20.00	31.00	40.00	49.50
Fish oil, Salmon	8.20	8.80	9.20	9.90	10.20
Wheat flour	12.00	10.00	10.00	6.50	5.00
Blood meal	4.50	6.50	8.60	14.00	16.00
Cassava meal	10.44	4.84	1.34	2.74	0.94
Soy lecithin	1.00	1.00	1.00	1.00	1.00
Vitamin PMX-F2	0.50	0.50	0.50	0.50	0.50
Mineral PMX-F1	0.25	0.25	0.25	0.25	0.25
Mold Inhibitor	0.05	0.05	0.05	0.05	0.05
Stay C - 35%	0.03	0.03	0.03	0.03	0.03
Antioxidant	0.02	0.02	0.02	0.02	0.02

Notes: Vitamin and mineral premix per kg: Vitamin A (UI) 1335000, vitamin D3 (UI) 500000, vitamin E (UI) 16670, vitamin K3 (mg) 3335, vitamin B1 (mg) 6670, vitamin B2 (mg) 5835, vitamin B6 (mg) 6670, vitamin B12 (mg) 3.35, folic acid (mg) 835, d-calpan (mg) 20000, vitamin C mono-phosphate (mg) 33335, inositol (mg) 45000, iron (mg) 8335, zinc (mg) 16670, manganese (mg) 3000, copper (mg) 8335, cobalt (mg) 670, iodine (mg) 167.5 and selenium (mg) 67.5.

4.2.3 Fish handling and sampling

Before the commencement of the experiments, nine (9) fish were randomly selected and pooled into 3 groups for initial carcass analyses. The body parts, without tail, fins, intestine and head were collected for body composition analyses. The body parts were dried at 105^{o}C for 24 hours in a vacuum oven (model 1445-2, USA) at Environment and Disease Monitoring in Aquaculture, Vietnam, before sending to analyze crude protein and fat, energy and amino acid profile.

All fish handling activities were performed according to the Australian Code of Practice for the care of animals for science purposes, Approval No AEC_2014_14. Measurement of weight and length was carried out under an application of 2-phenoxyethanol anesthetic with a dose of 0.2 ml/l and 0.5ml/l to humanely kill the fish for body composition analyses (Tsantilas *et al.*, 2006). To evaluate growth, daily specific growth rate, feed conversion rates, and feed intake, all the fish at the beginning were

measured for individual length and weight. At the end of experiments, 20 fish in each tank were randomly selected to measure length and weight. The digestibility analyses was performed by using faecal sedimentation method (Cho and Slinger, 1979). In every tank, a feeding tray was installed to collect all uneaten feed and if any escaped feed into the water column was siphoned immediately. After one hour of feeding, settled faeces at the tank bottom were collected by siphoning, and frozen to -20^{o}C until further analyses. After 61 days of feeding test diets, one (1) fish from every tank was randomly selected to get 15 fish samples (3 samples/treatment) for final carcass analyses.

4.2.4 Calculations

Specific growth rate was calculated as:

$$SDR = 100 * \frac{(\ln W_2 - \ln W_1)}{(t2 - t1)}$$

where W_1 and W_2 are body weight at start and at the end of the experiments, respectively and t_2-t_1 is the culture period (days).

Condition index (K) was determined based on length (L) and weight (W) using Fulton (1904) formulas as:

$$\mathrm{K} = 100 * \frac{L}{W^3}$$

Skewness value was statistically calculated as:

$$S = [1/n\sum^{n}_{i=1}(x_i-\bar{x})^3]/[\,[1/n\sum^{n}_{i=1}(x_i-\bar{x})^2]^{3/2}$$

where x_i and n denote for the individual and observation, $\bar{x}$ was the sample mean.

Apparent digestibility coefficient (ADC) of each nutritional component of the diets was calculated as:

$$ACD\ (diet) = 100 - 100 * \frac{Marker\ in faeces\ (\%)}{Marker\ in\ diet\ (\%)} * \frac{Nutrient\ (i) in\ faeces\ (\%)}{2!Nutrient\ (i) in\ diet\ (\%)} \qquad (1)$$

where (i) is a single nutrient like crude protein or hydrated fat.

Ingredient ADC was calculated as described by Forster (1999) and Glencross *et al.* (2007) as:

$$ADC(ing) = \frac{(100-j)*(Nutrre+Nutring*j)*ADC\ test-(100*j)*(Nutrre*ADCre)}{Nutring*j} \quad (2)$$

where j is percentages of FL replaced fishmeal proportion, $Nutr_{re}$ and $Nutr_{ing}$ are given nutrients in reference diet and FL, respectively and ADC_{ing}, ADC_{re} and ADC_{test} are digestibility coefficients of FL, reference diet and test diet respectively.

4.2.6. Statistical analysis

The data were analyzed using SPSS for Windows version 18 and Stata SE 12, USA with the results expressed as the means and pooled standard errors of the mean (S.E.M). Paired-sample T Test was used to compare means of single nutritional parameter of the lupin before and after fermentation. One-way analysis variance (ANOVA) was used to compare effects of diets without and with different fermented lupin inclusions into the diets. Size distribution presented in the skewness values was performed together with normal distribution test. Levels of significance were determined for length and weight (Bonferroni), condition index, body composition (Tukey's HSD), digestibility, and growth performance (LSD planned comparisons), with significant limits being set at $p<0.05$.

4.3. Results

4.3.1. Growth performance

There were some significant differences in final weight and length among fish fed the different diets (Table 4.3).

Fish grew to a higher weight ($p<0.05$) when fed diets 45FMR and 60FMR than the control diet (0FMR), while those fed 30FMR and 75FMR did not show any growth increases. The juvenile barramundi length increased significantly when they were fed 60FMR and decreased when fish fed 75FMR than 0FMR, whereas 30FMR and 45FMR resulted in unchanged growth of the fish.

Table 4. 3 Growth performance, SGR and feed intake of fish fed fishmeal diet and fishmeal partly replaced by fermented lupin diets.

Parameters	*0FMR*	*30FMR*	*45FMR*	*60FMR*	*75FMR*	*Pooled S.E.M*
Initial weight (g)	6.80[a]	7.23[a]	6.91[a]	6.93[a]	6.93[a]	0.68
Initial length (cm)	7.73[a]	7.89 [a]	7.85[a]	7.85[a]	7.84[a]	0.45
Final weight (g)	30.35[a]	33.29 [a]	34.61[b]	34.62[b]	31.37[ab]	0.38
Final length (cm)	13.09[a]	13.76 [ab]	13.80[ab]	14.01[b]	12.95[c]	0.58
SGR (%)	2.45	2.50	2.61	2.63	2.47	0.33
Feed intake (g)	25.87[a]	27.48[ab]	28.31[bc]	29.34[c]	29.36[c]	0.36
FCR	1.11[a]	1.06[a]	1.05[a]	1.08 [a]	1.21[b]	0.28
Survival (%)	96.00[a]	98.00[a]	96.00 [a]	93.00[b]	93.00[b]	0.07
Size distribution statistics						
Skewness for weight	-0.179	-0.096	-0.091	-0.124	0.062	
Skewness for length	-0.126	0.169	-0.360	-0.262	-0.408	

Note: Within rows, values followed by the same letter are not significantly different ($p<0.05$, LSD test)

The survival of all diets was more than 93% (Table 4.3). Among them, diet 30FMR yielded the highest survival (98%) and was significantly higher than the control and other test diets. In contract, feed conversion rates (FCR) were not significantly different among any diets except the 75FMR which produced the higher FCR ($p<0.05$).

The length and weight distribution (Figure 4.1, Figure 4.2) and skewness (Table 4.3) showed the various patterns in sizes of fish groups fed different diets. The variations in length and weight were similar to the control and test diets, despite the fact that the shorter and lighter fish were found in 75FMR diet. In contrast, the K indices, indicating a fatness of the fish, were significantly different among fish fed various diets. The fatness calculated from condition indices of fish fed FL diets were not significantly different with the control diet. However, in general, fish were significantly less fat when fed diets 30FMR, 45FMR and 60FMR than fed 75FMR.

3.3. Digestibility

In general, the higher inclusion levels of FL as protein source in test diets resulted in the higher apparent digestibility coefficients (ADC) of nutrients (Table 4.4). Protein

digestibility of fish fed 45FMR, 60FMR and 75FMR diets were higher than fish fed 0FMR, while that of 30FMR was lower. But there were no significant differences of ADC of protein among diets. Similarly, there was an increase in the digestibility of hydrolyzed fat, energy, fiber and phosphorus. The ADC of fat was lower ($p<0.05$) in 30FMR than 75FMR diets. Except 30FMR, all test diets resulted in no change in ADC of fat. There was no change in ADC of energy when fishmeal was partly replaced by FL, except 75FMR which resulted in significantly higher digestibility. Digestibility of phosphorus significantly increased when inclusion levels of FL increased in fish diets (Table 4.4).

Table 4. 4 Digestibility (%) of diets containing different FL inclusion levels and FL ingredient in test diets

	Diets					***Pooled***
AD (%)	***0FMR***	***30FMR***	***45FMR***	***60FMR***	***75FMR***	***S.E.M***
Diets						
Protein	91.37^{a}	89.79^{a}	94.79^{a}	94.78^{a}	96.59^{a}	1.82
Hydrolyzed fat	92.14^{a}	89.20^{b}	94.96^{ac}	96.22^{ac}	97.81^{c}	1.54
Energy	88.25^{a}	87.57^{b}	93.63^{abc}	94.43^{ac}	96.48^{c}	1.09
Fiber	40.53^{a}	47.14^{a}	54.76^{a}	48.07^{a}	89.10^{b}	4.90
Phosphorus	49.09^{a}	69.70^{b}	89.81^{c}	92.23^{c}	96.19^{c}	4.75
Ingredient-FL						
Protein		86.14	68.10	98.32	97.72	6.47
Fat		59.24^{a}	67.28^{a}	98.94^{b}	99.18^{b}	5.62
Energy		86.00^{a}	70.43^{b}	98.54^{c}	99.21^{c}	3.64
Fiber		62.42^{a}	58.71^{a}	53.10^{a}	108.64^{b}	6.78
Phosphorus		117.85	123.73	120.96	111.82	1.83

Note: within rows, values followed by the same letter are not significantly different ($p<0.05$, Tukey's HSD test)

4.3.3. Body composition

Some significant differences were found in body compositions between fish fed before and after the test diets (Table 4.5). Proximate protein content (%) in initial fish and fish fed control diet were higher ($p<0.05$) than fish fed FL diets. All FL diets resulted in the same carcass proximate protein levels while the fish fed the control diet did not change

in carcass protein compared to the fish before the experiment commenced. The initial carcass fat and energy levels had significantly higher than the fish fed any test diets. No significant difference in carcass fat and energy levels were found in any fish fed test diets.

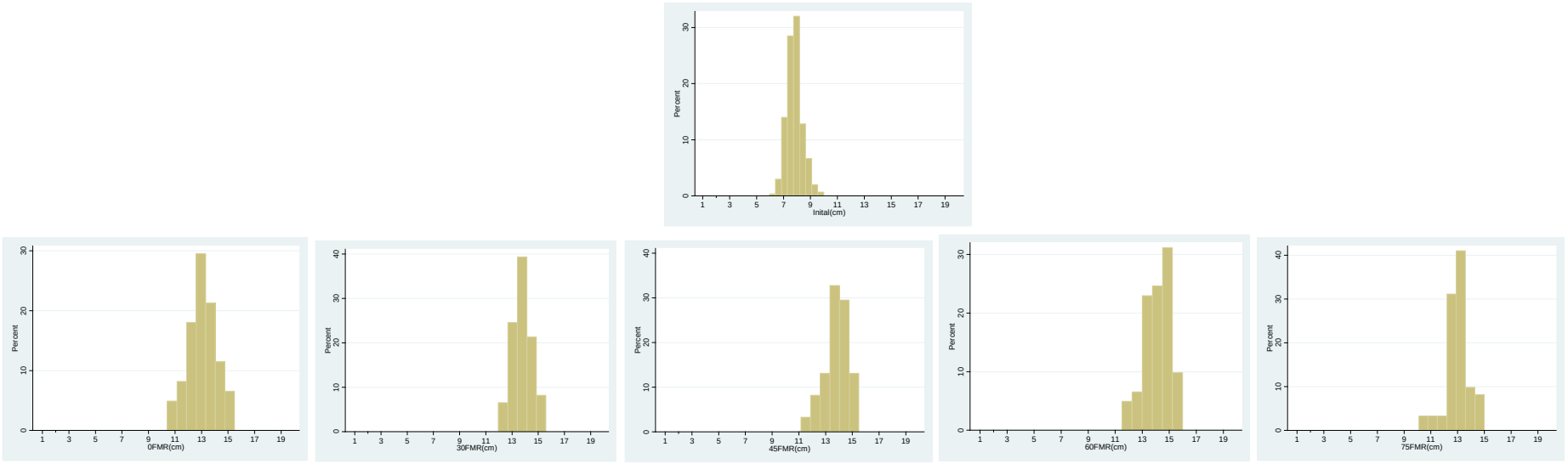

Figure 4. 1 Histogram of length distribution of initial fish (N=600) and their (N=60) after 61 days fed different FL inclusion levels in diets.

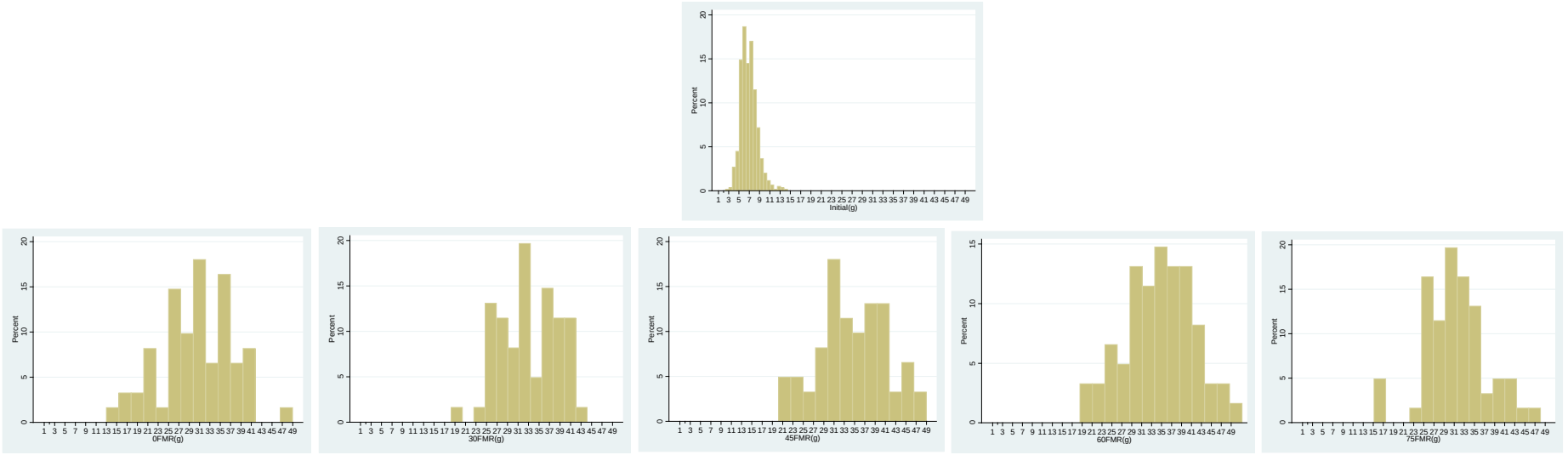

Figure 4. 2 Weight distribution of initial fish (N=600) and their (N=60) after 61 days fed different FL inclusion levels in diets.

Table 4. 5 Body composition (%) of initial fish and fish fed test diets after 61 days.

Diets	***Body proximate***				***Essential amino acids***									
	Moisture	***Protein***	***Fat***	***Energy***	***His***	***Thr***	***Arg***	***Val***	***Met***	***Lys***	***Iso***	***Leu***	***Phe***	***Try***
Initial	78.27	17.21^{a}	1.00^{a}	0.79^{a}	2.52	4.78^{a}	7.13^{a}	6.88^{abcde}	4.61^{a}	11.31^{abf}	6.22^{ade}	9.47^{ae}	10.65^{a}	1.31^{a}
0FMR	79.00	16.38^{a}	0.50^{b}	0.70^{a}	2.99	6.39^{b}	7.39^{ab}	4.61^{abcef}	8.24^{b}	14.34^{be}	7.02^{b}	10.56^{abe}	13.42^{b}	1.26^{a}
30FMR	78.07	15.40^{b}	0.43^{b}	0.67^{b}	2.55	5.76^{b}	7.93^{ab}	6.29^{abcef}	4.47^{a}	11.26^{acf}	5.95^{cde}	9.17^{ce}	12.36^{c}	1.30^{a}
45MFR	75.53	14.43^{b}	0.40^{b}	0.62^{b}	2.65	5.69^{b}	6.40^{c}	6.01^{adef}	4.68^{a}	20.73^{d}	5.54^{de}	7.57^{d}	7.25^{d}	1.09^{b}
60FMR	78.60	15.10^{b}	0.46^{b}	0.65^{b}	2.93	6.13^{b}	7.11^{abcd}	6.90^{abcde}	5.11^{a}	13.72^{be}	6.24^{abcde}	7.61^{C}	9.10^{e}	1.30^{a}
75FMR	79.23	15.73^{b}	0.52^{b}	0.67^{b}	2.85	2.57^{c}	8.26^{ce}	6.49^{bf}	4.58^{a}	11.18^{acf}	5.87^{e}	9.38^{e}	12.22^{c}	1.25^{a}
Pooled S.E.M	0.53	0.23	0.05	0.01	0.05	0.32	4.48	0.19	0.33	0.83	0.13	0.27	0.52	0.02
p<F	0.47	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.01

Note: Within columns, values followed by the same letter are not significantly different (p<0.05, Tukey's HSD test)

Table 4. 6 Nutrient retention (%) in different FL inclusion levels in diets.

Diets	*Protein*	*Lipid*	*Energy*
0FMR	37.07[a]	0.478	2.98
30FMR	31.71[b]	0.54	2.64
45MFR	29.27[b]	0.51	2.62
60FMR	30.47[b]	0.63	2.77
75FMR	31.70[b]	0.71	3.03
Pooled S.E.M	0.75	0.28	0.68
p<F	0.00	0.33	0.42

Note: within columns, values followed by the same letter are not significantly different (p<0.05, Tukey's HSD test)

The percentages of essential acid amine (EAA), His and Try were the same between initial fish and after fish were fed test diets, whereas the remaining EAA had significant differences. Met of fish fed 0FMR was higher than initial fish and fish fed test diets. However, when FL inclusion increased, these significant differences in amounts of Arg, Val, Lys, Iso, Leu and Phe were not correlated. In all carcass analyzed, the lowest amount of Try was found in fish fed 45FMR.

There were no significant differences in nutrient retention among fish fed test diets (Table 4.6). However, higher protein retention was seen in fish fed 0FMR diet than fish fed FL inclusion diets. Different inclusions of FL in diets resulted in no change of protein, fat and energy retentions in any fish.

4.3.4. Interactions

There was no significant interaction between inclusion levels of FL and blood meal; tannins and phytates; and FL and cassava. A closed significant (p=0.07) interaction was observed between FL and inclusion levels of wheat flour. The variations in FCR and ADC of phosphorus were significantly related to FL inclusion levels and concentration of ANFs (Table 4.7, Figure 4.3).

Table 4. 7 Regression relationships betweentwo phytates level and FCR, ADC of protein, ADC of fat, ADC of energy, ADC of fiber and ADC of phophorus. In equations, y denotes for phytates and x denotes for the parameters in the same row.

Parameters	*Equations*	R^2	*P*
FCR	$y = 0.112x^2 - 0.189x + 1.1028$	0.96	0.03
Protein	$y = 0.0002x^2 - 0.0334x + 2.1754$	0.77	0.2
Hydrolyzed fat	$y = 2.6042x^2 - 2.1167x + 91.594$	0.76	0.2
Energy	$y = 1.5655x^2 + 1.1773x + 87.685$	0.85	0.1
Fiber	$y = 15.483x^2 - 13.733x + 42.574$	0.77	0.2
Phosphorus	$y = -6.7333x^2 + 37.566x + 48.054$	0.96	0.04

4.4. Discussion

Lactobacilli fermentation of sweet lupin (*L. angustifolius*) resulted in the elimination and/or inactivation of ANFs that restrict the absorption of nutrients by barramundi juvenile. This led to higher digestibility of crude protein, hydrolyzed fat and phosphorus which in turn resulted in an improved feed efficiency. The fermentation also improved lupin quality, reflecting in the acceptance of high inclusion level of the FL in test diets. Even though the protein retention in reference diet was higher than in test diets, increase levels of FL inclusion did not change the retentions of protein, fat and energy. In addition, the body composition was the same among any fish fed any test diet, suggesting that high inclusion of FL, up to 60% could result in higher growth in barramundi juveniles.

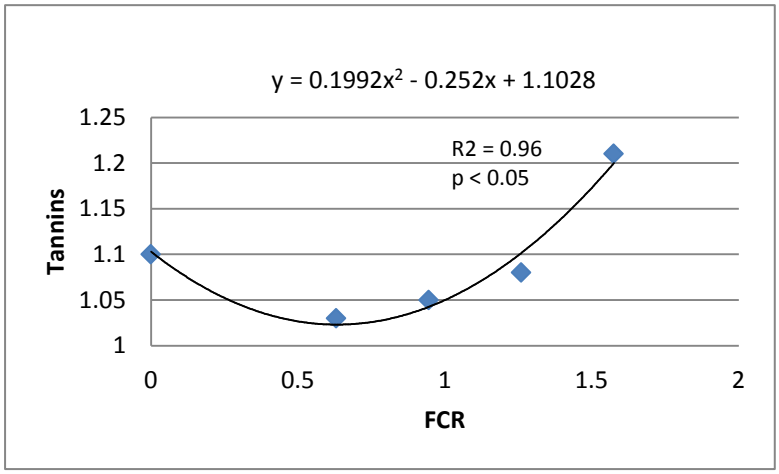

Figure 4. 3 Regression of Tannins concentration in diets and FCR. The concentration was calculated based on the Tannins concetration in the lupin and inclusion levels of each test diets.

A number of ANFs are presented in protein-rich plants (Francis *et al.*, 2001) including lupins (Dupont *et al.*, 1994). Sweet Australia lupin is low in alkaloids (Dupont *et al.*, 1994) however phytates and tannins are major factors influencing the digestibility and thus reduces growth performance in aquatic species. Tannins contents are 1.17 and 2.64 μg g^{-1} in sweet and bitter lupins respectively (Dupont *et al.*, 1994) that influence the protein utilization and digestion (Francis *et al.*, 2001). These ANFs are rather stable under heat treatment (Boland *et al.*, 1975) but can be efficiently removed by fermentation (Nnam and Obiakor, 2003). Lactic acid fermentation has been shown to give a significant reduction in phytic acid in cereals and sesame seed (Marklinder *et al.*, 1996; Mukhopadhyay and Ray, 1999; Skrede *et al.*, 2002). Bartkiene *et al.* (2013) Indicated that Lacto-fermentation of sweet lupin (*L. angustifolius*) could reduce acrylamide in enriched bread with high quality protein. Phytic acid and tannins in fermented lupin were reduced by 27.3% and 10.7%, respectively after 9 hours of fermentation by traditional method (Dhankher and Chauhan, 1987). Fermentation is the most effective way in decreasing the 56-96% of phytic acid than soaking and germination of brown rice (Liang *et al.*, 2008).

In this study, the fermentation by *Lactobacilli spp.* significantly decreased the levels of phytic acid and tannins by 87.04% and 17.64% respectively. These reductions are crucial to increase the inclusion levels of FL diets as high concentration of these ANFs can be detrimental to growth, for instance 0.5% purified phytic acid supplemented in feed can reduce 10% growth rate in rainbow trout (Spinelli *et al.*, 1983). Although other ANFs such as saponins, oxalate and cyanogenic glycosides, were not evaluated in the present study, they are too reduced when raw materials are fermented (Ketiku *et al.*, 1978; Eka, 1980; Fenwick and Oakenfull, 1983).

Sweet lupin had a little effect on the palatability of fish. The mixture of lupin kernel and lupin concentrate in barramundi juvenile diets did not influence to palatability (Katersky and Carter, 2009). Similarly, Glencross *et al.* (2011b) demonstrated that a threshold where diets' palatability was maintained at 150 g kg^{-1} fishmeal with lupin, contributed 425 g kg^{-1} diet. In the present study feed intake was not reduced in any fish, with the highest inclusion level of FL was at 495 g kg^{-1} diet. This could be due

to low alkaloids presence in the lupin, as the alkaloids result in a bitter taste and fermentation of lupin can improve aroma for the diets (Schindler *et al.*, 2011b).

After 61 days of culture, the barramundi juveniles fed all diets gained greater than 30 g from an initial average of 7g. Unfermented lupin as a fishmeal replacing single ingredient has been evaluated in other marine species. In rainbow trout 50% inclusion of lupin (*L. angustifolius*) in the diet resulted in significant reduction in the growth (Farhangi and Carter, 2001). In Atlantic salmon, the same replacement at a inclusion levels of 25 – 33% , resulted in lower utilization (Carter and Hauler, 2000). In this study, up to 60% of fishmeal was replaced by FL which resulted in higher growth than the control diet where only fishmeal was a main source of protein.

Dependence on only fishmeal source presents considerable risks associated with supply, price and quality fluctuations (Glencross *et al.*, 2007). Therefore, proportion of fishmeal should be reduced in a diet while maintaining a balanced nutritional formulation and thereby producing an acceptable good growth and low FCR. When fishmeal is replaced by a lower protein sources such as lupin, the blood meal has been used in accordance with the levels of FL included into diets. Concomitantly, the wheat flour and cassava meal were also used to balance the nutrients in the diets. Blood meal can be well utilized by barramundi and its added levels in these test diets were in the range that did not negatively influence to growth and FCR (Williams *et al.*, 2003). In contrast, the carbohydrate derived from wheat flour and cassava meal could influence the growth performance as carbohydrate was used limitedly by only marine fish (McMeniman, 2003). In the present study, interaction among ingredient inclusion levels was not observed, proving better growth rate and high digestibility of test and control diets. This suggests that all formulated diets in the current study were nutritionally balanced.

Length-weight composition and K indices are important to determine the fitness and health of the fish population (Fulton, 1904), which is also referred as a return rate of operation cost in fish culture (Engle *et al.*, 2011). Size composition reflected by the fitted or skewed frequencies of the size (Ohlberger *et al.*, 2013) are strongly influenced by food quantity and quality (Fuiman, 2002), and feeding regime (Wang *et al.*, 1998). The fish in this study were more uniform when fed all FL inclusion

diets than the control diet which is desirable from marketing viewpoint. As high inclusion level of FL in the diet formulation can reduce the feed production cost, a minimum size variation in the harvested fish size is critical for feed producers to reduce the feed costs.

Very few studies have attempted to evaluate the nutrient digestibility of FL. However, the bio-processed pre-treatment for plant ingredients have proven to increase digestibility. Lactic acid (*Lactobacillus* sp) fermentation of oil-extracted soybean meal partly eliminates and inactivate ANFs restricting the absorption of lipids by Atlantic salmon which then leads to a higher digestibility of total dietary energy, and subsequently improved feed efficiency (Refstie *et al.*, 2005). The addition of lupin protein concentrate and wheat gluten, exposed to certain extent of bio-processing, increases protein digestibility in diets for Atlantic salmon (Storebakken *et al.*, 2000; Refstie *et al.*, 2006). The digestibility in this study was higher than the study of Carter and Hauler (2000) partly due to the fecal collection method by sedimentation which can overestimate the digestibility of the nutrients (Glencross *et al.*, 2007), however the main reason for the increase could be attributed to the fermentation process that reduced tannins and phytates, and others ANF's (Refstie *et al.*, 2005), improved amino acid profile (Yabaya *et al.*, 2009) and aroma (Schindler *et al.*, 2011a).

The results in this study were in agreement with Carter and Hauler (2000) where inclusion of sweet lupin resulted in a significant increase in digestibility of crude protein but no changes in energy levels. An combination of different plant ingredients also increased digestibility in juvenile barramundi (Glencross *et al.*, 2011b). Apparent digestibility of phosphorus was affected by the inclusion levels of FL in diets with a strong regression (R^2 = 0.97). This was explained by the content of digestible phosphorus which was high in lupin ingredient and the fermentation process leads to increase in digestibility of phosphorus as shown in pigs (Almeida and Stein, 2012).

Body composition of barramundi was not affected by the test diets in this study. There were significant differences in crude protein, fat and energy between the carcass of initial and final fish fed on the test diets. There was no change in

proximate compositions among fish fed test diets. The results in this study were similar to the finding on cuneate drum (*Nibea miichthioides*) fed soybean substituting fishmeal (Wang *et al.*, 2006) where carcass protein also remained unchanged. The higher level of protein, fat and energy contents in initial carcass in the present study could be explained by the age and the diets. Initial fish were smaller and were fed on both trash fish and commercial feed before they were procured to the test facility. Further, some essential amino acid (EAA) in diets was well utilized by the fish, reflecting in the amount of His, Thre and Val in carcass which were higher in diets as FL inclusion increased. On the other hand, Leu and Phe were not well utilized, especially Met which was the highest in carcass of fish fed control diet than those of other fish fed test diets. When FL inclusion increased, Met in diets decreased, indicating that Met became limiting EAA under the current regime of EAA supplementation by FL.

Chapter 5. THE SURVIVAL AND THE PLASMA BIOCHEMICAL CHANGES OF JUVENILE BARRAMUNDI (*Lates calcarifer*) FED VARIOUS INCLUSION LEVEL OF FERMENTED LUPIN (*Lupinus angustifolius*): EFFECTS OF FLUCTUATING TEMPERAUTRE

(*This chapter was under reviewed by Fish Physiology and Biochemistry journal*)

5.1 Introduction

Increasing demand for aquafeed to support the rapid expansion of aquaculture along with concerns on the availability and environmental impact of the use of fishmeal has led the search for alternative protein sources. Plant-derived proteins have been widely evaluated as possible fishmeal replacement in fish diets (Glencross *et al.*, 2004b; Glencross *et al.*, 2008; Walker and Berlinsky, 2011; Ngo *et al.*, 2016); these investigations have provided information on fish growth, nutrient digestibility, feed utilization and fish physiology. No significant effects on growth and physiological homeostasis have been recorded at inclusion rates of plant-derived protein at 30% inclusion level or less; performance has been affected at inclusion rates (Wang *et al.*, 2006; Ngo *et al.*, 2016).

Fermented lupin products improve the digestibility of protein and phosphorus and the fermented materials can be included at higher rates compared to the unfermented plant (Vo-Binh *et al.*, 2015). Fermentation uses enzymes from microorganisms to alter the nutritional properties such as the reduction of peptides sizes in soybean protein (Hong *et al.*, 2004) to improve digestibility. Fermentation has altered the amino acid profiles in soybean cake to more closely resemble fishmeal (Yabaya *et al.*, 2009), increased antioxidant vitamins in cowpea (Dueñas *et al.*, 2005) and decreased the level of antinutritional factors (ANFs) in baobab or lupin (Nnam and Obiakor, 2003; Vo-Binh *et al.*, 2015).

Under most experimental conditions, environmental factors such as temperature and dissolved oxygen are maintained in optimal ranges for fish growth. Under

production conditions temperature is not stable but changes through the day and from season to season. Fish need to be healthier under fluctuating temperature conditions to cope with the changing environmental conditions (Eldridge *et al.*, 2015).

Barramundi is a euryhaline species and farmed in many regions including Australia and Southeast Asian. The use of plant-derived protein in barramundi diets as a fishmeal replacement was evaluated under constant temperature conditions (Glencross *et al.*, 2016). In a previous study, Vo-Binh *et al.* (2015) reported that fermented lupins can be included at 60% without compromising growth of juvenile barramundi. There was no effect of dietary compositions with different sources of plant proteins on fish survival and plasma biochemical parameters when the fish grown in constant temperature (Kaushik *et al.*, 2004). However, under conditions that induce stress in fish, nutritional effect could include growth, survival and changes in physiological parameters. Decreased growth rates and increased plasma cortisol were recorded in fish grown in fluctuating temperatures, compared to fish grown under constant temperature (Eldridge *et al.*, 2015). In this study, fermented lupin meal (at 4 inclusion rates) was used to replace fishmeal in juvenile barramundi diets under constant and fluctuating temperature conditions; survival rates and plasma biochemical parameters were measured as indicators of performance and ability to cope with stress.

5.2 Materials and Methods

5.2.1 Materials and formulated feed

All facilities including composite tanks, water and 630 juvenile barramundi used for the experiment were from National Brood-stock Centre, Hai Duong, Vietnam. Fishmeal, fish oil and premix were obtained from Curtin University, Australia (originally purchased from Speciality Feeds, Glenforrest, WA). Lupin (*Lupinus angustifolius*) kernels were donated by Co-operative Bulk Handling Grain, Western Australia. The remaining ingredients were purchased from a local supplier in Camau town, Vietnam. Fermentation of lupin kernels was performed as per the

protocol outlined in Chapter 3. Isonitrogenous and isocaloric diets were formulated as for the previous study (Vo-Binh *et al.*, 2015). There were 5 diets with inclusion levels of 0%,30%, 45%, 60% and 75% dry matter fermented lupin; diets were formulated to contain 45% protein, 13% lipid, and 20 MJ/kg and labelled as 0FL (control), 30FL, 45FL, 60FL, and 75FL respectively. The diet with 0% FL contained 630 g kg^{-1} fishmeal was served as control diet. Diets were mixed and pelleted using a laboratory pelletiser (BT 25 Lab Pelletiser, Taiwan) to produce 2.00 mm pellets; pellets were dried at 60^{o}C for 12 hours, cooled to room temperature and stored at -20 ^{o}C until required.

5.2.2 Experimental description

The tanks were set up indoors as a block design with a recirculation system. Water temperature was controlled at 29±0.7^{o}C by a heater system inside the tanks. Water used in the tanks was a mixture of freshwater and seawater to a concentration of 5‰ salinity and followed by chlorine (0.15 mg L^{-1}) treated under aeration for 4 hours before use. The fish were fed a grower commercial diet (Uni President 45% protein, 13% lipid and 21 MJ/kg energy) at 8 am and 4 pm for two weeks. During this period all dead, unhealthy or fish damaged by handling were removed. At the end of the initial 2 week period fish with size of 7.9-8.2g of weight were selected and randomly assigned to 15 composite tanks with a volume of 3.5 m^3. Each tank was stocked with 40 fish. Fish were fed trial diets to appetite 3 times daily at 8 am, 4 pm and 6 pm.

Table 5. 1 Fluctuating temperature ranged in 6 days exposing the fish to semi-outdoor rearing culture system.

	Day0	***Day1***	***Day2***	***Day3***	***Day4***	***Day5***	***Day6***
Temperature (^{o}C)							
At 3pm	29±7	25±3	24±4	26±3	23±4	24±4	23±3
At 6am	29±7	19±3	19±3	20±3	19±5	18±4	18±5
Variation	0	6	5	6	4	6	5

Note. Temperature in Day0 was indoor temperature and was controlled constantly at 29±0.7^{o}C.

After 8 weeks 5 fish from each tank were randomly selected for collection of blood samples; the remaining fish were adjusted to 30 fish per tank and then transferred into a series of recirculating tanks (included control tanks) of similar size and had the same water supply that were set up semi-out door with a roof to protect from rain and direct sunlight. Fish were fed for 6 days with the same feeding regime as for trial diets; when the water temperature in the tanks fluctuated in a range of 18-26^{o}C (Table 5.1) during this period. Temperature was measured manually at 3 pm and 6 am using a thermometer. Mortality was recorded every day. After 6 days under fluctuating temperature, five fish from each tank were randomly selected for blood samples.

5.2.3 Fish handling and blood sampling

All fish handling activities were performed in accordance with the Australian Code of Practice for the care of animals for science purposes and under Curtin University Ethics Committee Approval No AEC_2014_14. Blood sampling was carried out under an application of 2-phenoxyethanol anaesthetic with a dose of 0.2 ml L^{-1} of fresh cleaned water (Tsantilas et al., 2006). After blood collecting, the fish were killed by using the same anaesthetic at a higher dose of 0.5ml L^{-1}. Blood was collected from the caudal tail vein using a 1 mml syringe and 18 G needle. Blood from the five fish was pooled into a labelled Eppendorf tube (Eppendorf, North Ryde, NSW, Australia). Tubes with blood were then centrifuged at 1000 g for 5 minutes to settle the erythrocytes; plasma was transferred to a new labelled Eppendorf tube before freezing (-12^{o}C) and sending for biochemical analyses.

5.2.4. Plasma analyses

Plasma analysis was performed at MELATEX hospital, Hanoi, Vietnam. Plasma was analysed for aspartate amino transferase (AST) and alanine amino transferase (ALT), glucose, cortisol, total plasma protein, albumin and globulin. ALT and AST were analysed using ALT Activity Assay (MAK052) and AST Activity Assay (MAK055) (Sigma-Aldrich New Zealand) respectively. Plasma AST, ALT, total protein, globulin and albumin are considered to be indicators of liver function and nutritional status (Carpentier *et al.*, 1982; Arrieta *et al.*, 2010). Plasma glucose and cortisol were

analysed using protocols described for largemouth bass (Suski *et al.*, 2003). Total protein was analysed using a test kit (Olympus OSR 6132). Albumin was determined using the method described by Busher (1990). Total globulin fraction was determined by subtracting the albumin from total protein (Fuhrman et al., 2004). Cortisol was measured by competitive protein binding using a commercially available kit (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, CA, USA); glucose levels were quantified enzymatically as using a 96-well microplate reader and commercially available spectrophotometer (Specitra Max Plus 384, Model 05362,USA). Plasma glucose and cortisol are used as immunological indicators and were used to evaluate stress in fish fed the test diets for 8 weeks at constant temperature and for 6 days in fluctuating temperature conditions.

5.2.5 Statistical analysis

The data were analysed using SPSS for Windows (version 18) with the results expressed as the means $\pm$ standard errors (SE). One-way analysis variance (ANOVA) was used to compare effects of inclusion of fermented lupin on survival and plasma biochemistry of juvenile barramundi. Factorial interaction between temperature and diets was determined using General Linear Model (Univariate), with significant limits being set at $P<0.05$.

5.3 Results

After 8 weeks feeding test diets, the survival rate of fish grown in constant temperature varied from 94.2% for 0FL (control) diet to 96.7% for 60FL diet (Figure 5.1). There was no significant difference on survival fish between test diets. Survival of fish exposed to fluctuating temperature was significantly (P=0.00) reduced compared to those grown in constant temperature. The survival rate of fish exposed to fluctuating temperature was significantly lower for fish fed 75FL diet (65.7%) (P=0.00) compared to control (76.7%) or other test diets (77.7.3%-82.3%) (Figure 5.1).

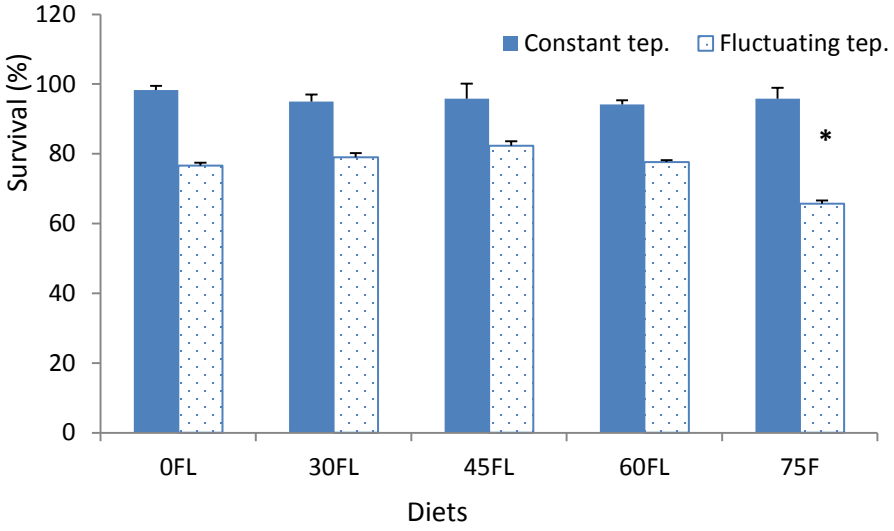

Figure 5.1 Survival rate (%) of fish fed fermented lupin (FL) at different inclusion levels for 8 weeks in constant temperature (29±7°C) and in fluctuating temperature conditions (mean ± SE). Bar with asterisk indicates significant difference (p<0.05) with control (One-way ANOVA, Tukey HSD test).

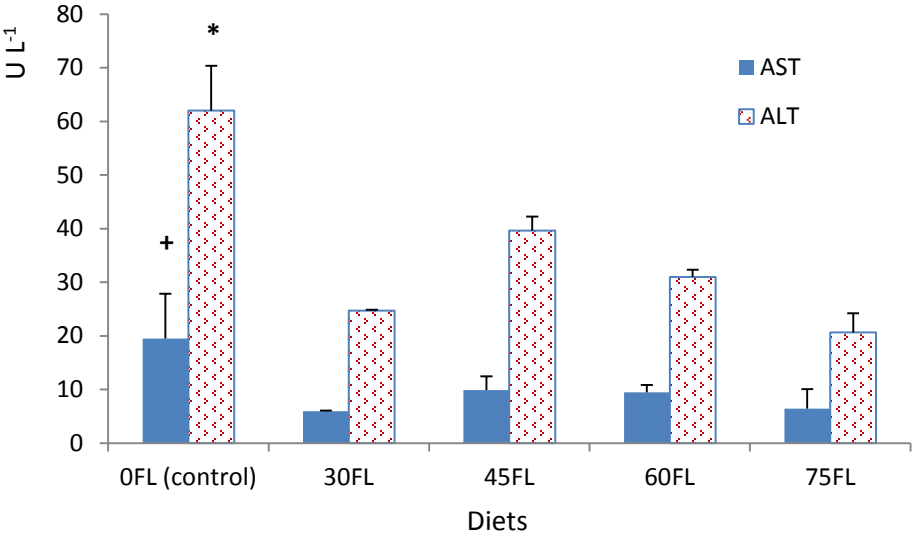

Figure 5.2 Plasma ALT (alanine amino transferase) and AST (aspartate amino transferase) concentrations (UL^{-1} mean + SE) of fish fed fermented lupin at different inclusion rates and grown in constant temperature (29±7°C). Asterisk (*) or plus (+) indicates significant difference between control and other test diets (One-way ANOVA, Tukey HSD test).

After 8 weeks feeding test diets and grown in constant temperature, plasma ALT of fish fed control diet (62.0 U L^{-1}) was significant (P=0.01) higher than all fermented lupin-based diets. ALT concentration in fish fed 75FL diet was the lowest at 20.1 U L-

[1] (Figure 5.2). Similarly, all fermented lupin based diets resulted in significant (P=0.01) lower plasma AST level (5.9-9.8 U L^{-1}) than control diet (19.5 U L^{-1}). There was no significant difference in the level of plasma AST between the fermented lupin based diets.

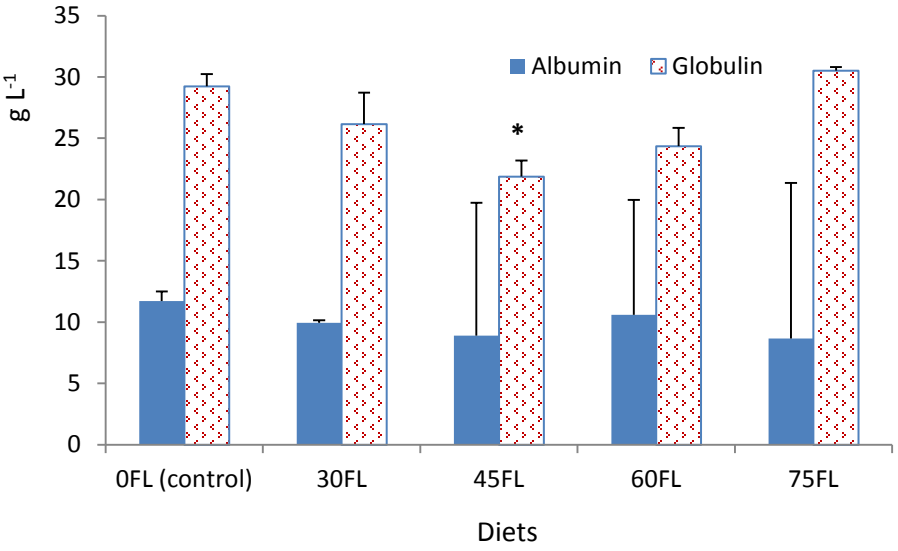

Figure 5. 3 Plasma Globulin and Albumin concentrations (g L^{-1}) of fish fed fermented lupin (FL) at various inclusion levels and grown in constant temperature.

Total plasma protein of fish grown in constant temperature ranged from 30.7 g L^{-1} for 45FL diet to 40.9 g L^{-1} for 0FL (control) diet. There was no significant difference in the levels of total plasma protein between control and test diets or between the test diets (ie diets containing fermented lupin). Similarly, plasma albumin varied between 8.6-11.7 g L^{-1} with no significant difference of between treatment diets (Figure 5.3). Plasma globulin was significant lower in fish fed 45FL diet (21.9 g L^{-1}) than that in fish fed control diet (29.2 g L^{-1}) and 75FL diet (30.5 g L^{-1}). Plasma globulin concentrations were 26.2 and 24.4 g L^{-1} for diets containing 30FL and 60FL respectively; these were not significantly different when compared to control diet.

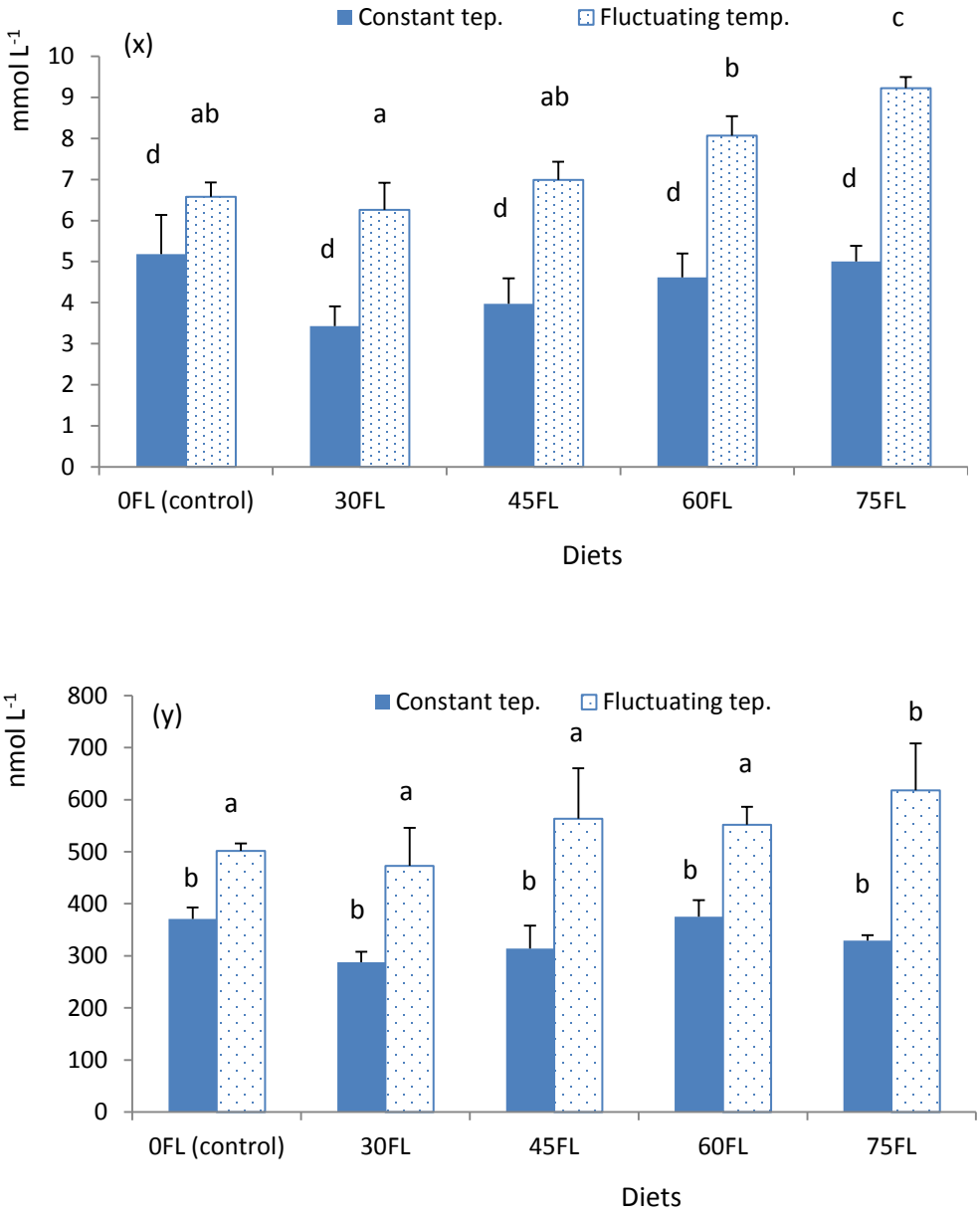

Figure 5. 4 Plasma glucose (x) and cortisol (y) concentrations (mmol L^{-1}, mean ± SE) of fish grown in constant temperature (Constant temp.) and in fluctuating temperature (Fluctuating temp.) fed diets with various inclusion levels of fermented lupin (FL). Bars with different letters are significantly different ($p<0.05$).

When the fish were grown in constant temperature, plasma glucose concentration of fish fed diets containing fermented lupin ranged from 3.4 mmol L^{-1} to 5.0 mmol L^{-1} and control diet was 5.3 mmol L^{-1} (Figure 5.4x). At constant temperature, there was no significant difference in plasma glucose concentration between test (fermented lupin) and control diets or between the fermented lupin diets. Plasma glucose concentration of fished grown under fluctuating temperature significantly

(P=0.00) increased. Fish fed 75FL diet had higher plasma glucose (9.2 mmol L^{-1}) than fish fed control diet (6.6 mmol L^{-1}).

Under constant temperature conditions, plasma cortisol concentration of fish ranged between 287.8 nmol L^{-1} for 30FL diet and 375.3 nmol L^{-1} for 60FL diet (Figure 5.4y). Under these conditions there was no significant difference in the plasma cortisol level between control diet and fermented lupin inclusion diets, or between fermented lupin diets. When fish were grown under fluctuating temperature, plasma cortisol significantly (P=0.00) increased in all treatment diets. Plasma cortisol concentration in fish fed 75FL diet (617.8 nmol L^{-1}) was higher than the fish fed all other test diets (473.2-563.5 nmol L^{-1}).

5.4 Discussion

Together with growth, survival of fish is very important indicator to evaluate quality and the level of acceptance of a diet. High quality and nutritional balanced diets generally result in optimal growth performance and survival of the fish over time and under environmental stress. For instance diets with balanced amino acids have been shown to increase survival, growth, feed intake, feed utilisation, digestibility and improve immunity in fish (Li *et al.*, 2009). In our study, there was no significant difference in survival rate between control and fermented lupin inclusion diets when fish were grown in constant temperature. At controlled temperature 28-30^{o}C, previous results (Vo-Binh *et al.*, 2015) reported similar high survival rates (>93%). However, exposure to fluctuating temperature decreased the survival rate in fish fed 75% fermented lupin inclusion diet (65.7% survival). This suggests that the diet containing 75% of fermented lupin is either poor quality or nutritional unbalanced and thus is not well accepted by the fish. In fact, 75% fermented lupin inclusion resulted in increase of food conversion ratio in juvenile barramundi (Vo-Binh *et al.*, 2015).

Barramundi have been shown to grow well within a temperature range of 21-37^{o}C (Katersky and Carter, 2007; Glencross and Rutherford, 2010). However temperature was constant at each experimental point in these studies. Under constant

temperature fish are less sensitive than under fluctuating temperatures (Eldridge *et al.*, 2015) with the latter resulting in increased mortality. In the present study, the temperature fluctuated from 18-26^{o}C between day and night and resulted in higher mortality rates, suggesting that fish may be more stressed under fluctuating temperatures; stress may have increased when fish were fed diets containing high inclusion level of fermented lupin (75%).

The liver is required for the metabolism of nutrients such as lipids and proteins and converts chemically toxic materials into harmless forms. AST and ALT are two enzymes generally produced by liver and kidney cells and a small amount in muscle cells (Ozer *et al.*, 2008) and can be used as indicators of liver damage. In healthy bodies, ALT and AST concentrations are low; however, if liver is damaged or affected by toxins concentrations of these enzymes may increase (Park *et al.*, 2000; Ozer *et al.*, 2008). In the present study, concentrations of ALT and AST were lower when fishmeal was replaced by fermented lupin at all inclusion levels. There have been very few studies on the levels of ALT, AST and other plasma biochemical parameters of barramundi in order to establish normal ranges that could be compared with data in our study. However the lower concentrations of ALT and AST suggest that the liver was not damaged or functionally impaired when juvenile barramundi were fed diets contained fermented lupin. Based on this data it could be inferred that liver function for fish fed diets containing fermented lupin is equal to or better than liver function of fish fed fishmeal based diets.

Total protein level can be used to assess nutritional status and as an indicator of kidney and liver disorders. Abnormal plasma total protein concentration may indicate poor quality diets is malnutrition or nutritional unbalance (Dempsey *et al.*, 1988). In our study, there was no significant difference in total plasma protein concentration between test and control diets, suggesting that fermented lupin diets provided similar nutritional balance as the fishmeal diet. Globulin and albumin are the two main components of plasma protein. While albumin contains a large proportion of small molecule amino acids for maintaining osmotic pressure in blood, globulins assist in immune function. Although plasma globulin was significantly lower in fish fed 45% fermented lupin diet than fish control diet, there

was no significant difference in plasma albumin between the diets indicating that nutritional status was in normal range for diets containing fermented lupin at any of the levels.

Plasma glucose and cortisol concentrations of barramundi can be indicators of stress such as dropping dissolved oxygen levels in water (our un-published data) or high stocking density (Ardiansyah and Fotedar, 2016). Under stress conditions, plasma cortisol levels in fish increase and induce increased plasma glucose concentration as a means of coping with high demand of metabolic energy against stress (Martínez-Porchas *et al.*, 2009). Atlantic salmon, (*Salmo salar*) was showed to have effect to plasma and cortisol concentrations when fed different dietary lipid levels, (Waagbo *et al.*, 1994). In the present study, fish grown in constant temperature and fed fermented lupin diets at any inclusion level were unlikely to be stressed compared with fish fed fishmeal based diet; this is supported by no significant difference in plasma levels of glucose and cortisol between fermented and fishmeal based diets.

However, when the fish were exposed to fluctuating temperatures for 6 days, plasma glucose and cortisol levels significantly increased in both control and fermented lupin containing diets. Similar results were reported with largemouth fish (*Micropterus salmoides*) when the temperature was reduced (Vanlandeghem *et al.*, 2010). Significant difference in the level of glucose and cortisol were observed in fish fed control diet and in fish fed 75% fishmeal replacement (75FL), indicating that fish fed high inclusion level of fermented lupin were more physiologically sensitive under temperature stress than fish fed fishmeal based diet.

In conclusion, juvenile barramundi fed with diets containing various levels of fermented lupin had similar survival rates and plasma biochemical indicators as fish fed diets containing fishmeal when reared in constant temperature. However, juvenile barramundi were sensitive to fluctuating temperature, as indicated by reduced survival rates of the fish. Under conditions of fluctuating temperature, fish fed a diet containing 75% fermented lupin had significantly reduced survival rates and were under stress as indicated by plasma biochemical indicators.

Chapter 6. EVALUATING BIOPROCESSED PEANUT (*Arachis hypogaea*) MEAL TO REPLACE FISHMEAL IN JUVENILE BARRAMUNDI (*Lates calcarifer*) DIETS

(*This chapter was under reviewed by Journal of Food Quality*)

6.1 Introduction

The increase in fish production through aquaculture has been accompanied by rapid growth of aqua-feed production. While fishmeal, a primary protein source in aqua-feed, has become limited and expensive, the plant-derived protein ingredients as potential alternatives to fishmeal have gained recognition (Gatlin *et al.*, 2007; Hardy, 2010). A wide range of products from oilseeds, legumes and cereal grains have been tested to determine their optimal inclusion levels to replace fishmeal in fish diets (Carter and Hauler, 2000; Farhangi and Carter, 2001; Glencross *et al.*, 2005; Katersky and Carter, 2009).

The failure to use higher inclusion levels of plant derived ingredients is due to the presence of high levels of undesirable antinutritional factors (ANFs) (Francis *et al.*, 2001) that adversely influence the fish growth performance and/ or fish health. Legumes such as lupins and soybeans contain large amounts of ANFs as soluble and insoluble non-starch polysaccharides, oligosaccharides, alkaloids, and tannins. All these ANFs decrease the growth and survival rates (Hendricks *et al.*, 1981), digestibility (Barneveld, 1999), palatability and absorption of amino acids (Hansen, 2009) of the host aquaculture species. Therefore, the bioprocessing of plant-derived protein are practiced to increase the quality of the ingredients (Gatlin *et al.*, 2007). Bio-processed methods such as fermentation and germination have proven to decrease ANFs and increase the bioactive compounds such as antioxidant vitamins (Lopez-Amoro´s *et al.*, 2006; Vo-Binh *et al.*, 2015), thereby have led to increased dietary inclusion levels, enhanced growth performance and health of the target species.

Peanut (*Arachis hypogaea*), a worldwide important crop, ranks second in term of cropped area after rapeseed (FAS USDA web 2013). Most peanuts are used for oil

extraction for human consumption (Zhao *et al.*, 2012), and the rest, peanut meal (PM) which has a rich protein source can be an ideal ingredient in aqua-feed but PM as an alternative to fishmeal in fish diets has never been investigated. The PM, as other plant rich protein sources, contains ANFs such as tannins (Ejigui *et al.*, 2005), which can have adverse effects on the fish performance.

Barramundi (*Lates calcarifer*), a carnivore fish is an important cultured species in Australia and Southeast Asia. A number of studies on barramundi, using protein sources originated from soybean meal (Tantikitti *et al.*, 2005), canola and wheat gluten (Glencross *et al.*, 2011a) and lupins (Katersky and Carter, 2009; Vo-Binh *et al.*, 2015) have been published. These studies reported that the growth performance of barramundi is not influenced by a low dietary inclusion levels of plant-derived protein, however, further bioprocessing of the selected plant protein source may be necessary in order to include higher dietary levels into the aqua-feed (Vo-Binh *et al.*, 2015). Thus, this study assessed different processed forms of peanut meals (PM) including in juvenile barramundi diets by evaluating growth, mortality, feed utilization efficiency, and physiological parameters of the fish. The physiological parameters of the fish fed PMs based diets were reassessed by challenging the fish to acute hypoxic conditions.

6.2 Materials and methods

6.2.1 Diets preparation

All ingredients, except the peanut were obtained from Speciality Feeds, 3150 Great Eastern Highway, Glen Forrest, WA 6071, Australia. Mycotoxin binder, mould inhibitor and stay C were purchased from Feed Company, Ca Mau, Vietnam. Ten diets from various inclusion levels (0%, 15%, 30%, and 60%) of NPM and FPM, and GPM replacing the fishmeal were prepared and labelled as, 15FPM, 30FPM, 60FPM (FPM based diets), 15GPM, 30GPM, 60GPM (GPM based diets), 15NPM, 30NPM, 60NPM (NPM based diets), and 0PM (Table 6.1). The diet 0PM contained 630 g kg^{-1} fishmeal and no PM which was considered as a reference diet. Diets were processed by addition of water to about 35% mash dry weight with well mixing to

form the dough. This dough was then screw pelleted by a laboratory pelletiser to 1.2-2 mm pellets. These moist pellets were oven dried at 60^{o}C for 12 hours followed by cooling to room temperature before storing at – 20^{o}C till further use.

Table 6. 1 Composition of test and reference diets. 0PM (reference), 15NPM, 30NPM, 60NPM, 15FPM, 30FPM, 60FPM, 15GPM, 30GPM and 60GPM denote for diets contained 0% peanut, 15%, 30% and 60% of NPM, 15%, 30% and 60% of FPM, and 15%, 30% and 60% of GPM, respectively.

Ingredient	*15NPM*	*30NPM*	*60NPM*	*15FPM*	*30FPM*	*60FPM*	*15GPM*	*30GPM*	*60GPM*	*0PM*
Fishmeal	53.55	44.1	25.2	53.55	44.1	25.2	53.55	44.1	25.2	63
NPM	9.45	18.9	37.8							
FPM				9.45	18.9	37.8				
GPM							9.45	18.9	37.8	
Fish oil, Salmon	9.1	9	8.8	9.1	9	8.8	9.1	9	8.8	9.2
Wheat flour	12.9	12.9	10.18	12.9	12.9	10.18	12.9	12.9	10.18	12.9
Blood meal	3	3	3	3	3	3	3	3	3	3
Cassava meal	4.58	2.28		4.58	2.28		4.58	2.28		6.98
Corn gluten	5.5	7.9	13.1	5.5	7.9	13.1	5.5	7.9	13.1	3
Soy lecithin	1	1	1	1	1	1	1	1	1	1
Mycotoxin binder	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin Premix	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mineral Premix	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Mold Inhibitor	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Stay C - 35%	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Total	100	100	100	100	100	100	100	100	100	100
Diet protein	45.58	45.55	45.58	45.58	45.55	45.58	45.58	45.55	45.58	45.53
Diet fat	13.73	13.76	13.76	13.73	13.76	13.76	13.73	13.76	13.76	13.71

Notes: Vitamin and mineral premix per kg: Vitamin A (UI) 1335000, vitamin D3 (UI) 500000, vitamin E (UI) 16670, vitamin K3 (mg) 3335, vitamin B1 (mg) 6670, vitamin B2 (mg) 5835, vitamin B6 (mg) 6670, vitamin B12 (mg) 3.35, folic acid (mg) 835, d-calpan (mg) 20000, vitamin C mono-phosphate (mg) 33335, inositol (mg) 45000, iron (mg) 8335, zinc (mg) 16670, manganese (mg) 3000, copper (mg) 8335, cobalt (mg) 670, iodine (mg) 167.5 and selenium (mg) 67.5.

6.2.2 Experimental design

Juvenile barramundi were obtained from Northern National Marine Broodstock Centre, Vietnam and shipped to National Freshwater Breeding Centre (NBC), Vietnam where the juveniles were raised until they were adapted to salinity of 0‰. The fish were then acclimated for two weeks and fed Uni-President-Vietnam feed (45% protein, 13% fat). These fish were then graded, and those within the weight range of 6 -6.5 g each were selected randomly into thirty tanks of 3.5 m^3 each. Every tank was stocked 40 fish. The flow-through culture systems were set up in an open outdoor shed with a roof to protect from rain and direct sunlight. The natural temperature and photoperiod ranged between 28-31^oC and 12 hours light respectively. After acclimation the experimental fish were fed for eight weeks with the ten different pre-designed diets (Table 6.1). Every diet was fed in triplicate and three times daily (8 am, 12 am and 4 pm) till satiation which reached within 20 minutes. Dead fish were recorded every day to calculate mortality rates. After eight weeks of feeding, all fish in every tank were weighted to determine growth rate and feed conversion ratios (FCR).

The challenge test to depict acute exposure to reduced dissolved oxygen (DO) was performed to the fish after eight weeks of feeding with 10 test diets. To perform the acute challenge test, five fish were randomly collected from every tanks and kept in closed 5-l plastic bags of freshwater where DO was reduced from 5.0 mg L^{-1} to 2±0.2 mg L^{-1} for 4 hours. The water used to DO exhaust was taken from the same tank that the fish were collected. The reduction of dissolved oxygen was carried out by pumping continuously pure N_2 gas, purchased from Hai Duong Gas Company (Vietnam) into the water. DO was measured by HI9146 Portable Dissolved Oxygen Meter (HANNA Instruments).

6.2.3 Fish handling and sampling

All fish handling activities were performed according to the Australian Code of Practice for the care of animals for science purposes, Approval No. AEC_2014_14/25. Blood samples were carried out under an application of 2-phenoxyethanol with a dose of 0.2 ml L^{-1}; fish were killed with a dose of 0.4 ml L^{-1}

after blood sampling (Tsantilas *et al.*, 2006). Fish blood samples were collected at the end of feeding trial and after the fish were challenged by reduced DO. In every tank, one blood sample was collected from five fish using a 1-mL syringe and an 18G needle via the caudal tail vein. Blood was stored in single Eppendorf tube (Eppendorf, North Ryde, NSW, Australia). The tubes were then centrifuged at 1000 g for 5 min to settle the erythrocytes and then plasma was transferred to a new Eppendorf tube prior to freezing and then sent for plasma chemical analyses.

6.2.4 Sample analyses

ANFs and bioactive compounds in all types of PM and proximate nutritional parameters of diets were analysed in National Institute for Food Control, Hanoi, Vietnam. Tannins and alkaloids beta-carotene, vitamin E (alpha, gamma-tocophenol) and flavonoid (quercetin) were determined by HPLC (Indyk, 1988) (Table 1). Crude protein (Kjeldalh) and hydrolysed fat (ISO 6492) were analysed following procedures described by AOAC (1990) (Table 2). All blood samples were analysed at Laboratory of Melatec Hospital, Hanoi, Vietnam. Blood chemical parameters consisted of sodium, chloride, alanine aminotransferase (ALT), cortisol, and glucose were analysed as described by Suski et al. (2003). The plasma concentrations of sodium and chloride were measured by using a flame photometer (Model 2655-00) and a chloridometer (Model 4435000) respectively. The plasma concentration of cortisol was measured by competitive protein binding using a commercially available kit (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, CA, USA) whereas ALT and glucose concentrations were qualified enzymatically following the methods of Lowry and Passonneau (1972) in a 96-well microplate read with commercially available spectrophotometer (Specitra Max Plus 384, Model 05362,USA).

6.2.5 Statistical analysis

The data were analysed using IBM SPSS for windows version 20 at Curtin University and STATA 14, USA with the results expressed as the means and pooled standard errors unless otherwise specified. Orthogonal Contrasts test was performed to compare ANFs and bioactive compounds between processed PM samples. One-way

analysis variance (ANOVA) was used to compare effects of diets containing different levels of NPM, FPM, and GPM with reference diet. Interaction between types of peanuts in diets and inclusion levels was performed by Wald tests (t) on regression model in Stata SE 14. Levels of significance were determined by weight, feed conversion ratio (FCR), survival and physiological parameters using Bonferroni tests, with significant limits being set at $p<0.05$.

6.3 Results

6.3.1 Growth performance

At the end of the feeding trial, final body weight of juvenile barramundi was in range of 29-32g (Table 6.2). At all inclusion levels, the fish fed FPM and NPM diets grew as fast as the fish fed reference diet. Feeding GPM based diets showed a significant difference in growth performance among the inclusion levels. The fish fed the diet containing 15% of GPM had the highest growth while feeding 60% of GPM based diet resulted in significantly lower growth. The fish fed 60GPM diets also grew significantly slower than the fish fed reference diet and all other test diets.

Table 6. 2 Effects of different diets on growth (g) and survival (%) rates, and FCR. Data are expressed in means and standard errors of the mean of 3 samples.

Diets	*Final body weight (g)*	*FCR*	*Survival (%)*
15FPM	30.24^{a}	0.92^{a}	96.7^{ab}
30FPM	29.15^{ac}	1.00^{a}	95.8^{ab}
60FPM	29.12^{ac}	1.03^{a}	91.7^{ab}
15GPM	32.78^{b}	1.09^{a}	97.5^{a}
30GPM	29.61^{a}	0.87^{c}	100.0^{a}
60GPM	27.12^{c}	1.44^{b}	81.7^{b}
15NPM	29.85^{a}	1.06^{a}	90.0^{ab}
30NPM	30.16^{a}	0.98^{a}	91.7^{ab}
60NPM	28.80^{ac}	0.95^{a}	85.8^{ab}
0PM (ref)	30.60^{ab}	0.96^{a}	90.8^{ab}
Pooled SE	0.34	0.03	1.03
p-values	0.026	0.000	0.000

Note: Within columns, values followed by the same letter are not significantly different ($p<0.05$,One-way ANOVA , Bonferroni test).

Survival of fish in all dietary treatments was greater than 90% except for the fish fed diet containing 60% GPM and NPM, where survival rates were 82.8% and 85.7% respectively. Feeding FPM and NPM diets at different inclusion levels did not result in the differences of survival rate. However, fish fed GPM diets at various inclusion levels resulted in different mortalities (Table 6.2). The diet contained 60% GPM showed significantly higher mortality (11.2%) than the diets containing 15% and 30% inclusion levels. The diet containing 60% GPM also resulted in significantly reduced survival rate when compared with reference and other diets. The fish fed 30GPM diet had the highest survival rate, with no mortalities.

Feed conversion ratio (FCR) varied from 0.87 to 1.44 (Table 6.2). Feeding with diets containing FPM and NPM at different inclusion levels resulted in FCR values of 1.0 or less and no significant difference was observed among these diets. However, GPM included in diets resulted in a variation of FCR values ($p<0.05$). Fish fed 60GPM diet had a significant higher FCR value (1.44) than reference diet (0.96). In contrast feeding 30GPM diet resulted in significantly decreased FCR (0.87) compared to the reference diet and this value was the lowest one among the test diets.

Table 6. 3 Growth performances of juvenile barramundi at different inclusion levels and types of PM. FW and FCR denote for final weight and feed conversion ratios, respectively.

	Inclusion levels (%)*			***Types of peanut processed****			***control***	***P***
	15	30	60	FPM	GPM	NPM		***values***
FW (g)	31.0±0.57^{a}	29.6±0.55^{a}	28.3±0.44^{b}	29.6±0.49^{a}	29.5±0.55^{a}	29.8±0.83^{a}	30.6±0.51^{a}	0.019
FCR	1.03±0.04	0.95±0.03	1.14±0.08	1.00±0.02	0.98±0.03	1.14±0.09	0.96±0.04	0.238
Sur (%)	94.7±1.59	95.9±1.82	86.6±2.05	89.2±1.91	94.5±1.76	93.5±2.77	91.4±2.47	0.018

Note: Within rows, values followed by the same letter are not significantly different ($p<0.05$, Orthogonal Contrasts, Bonferroni test).
(*) n=9; (**) n=3

Regardless of the types of PM, the growth of fish fed 15% and 30% PM inclusion levels was significantly higher than the fish fed 60% PM diet. A dietary inclusion of 60% PM resulted in reduced growth compared with the reference diets. However, when inclusion levels were ignored, different types of bioprocessed PM included in diets resulted in the same fish growth, FCR and fish survival (Table 6.3) as without PM (reference) diet.

6.3.3 Effect of diets on physiological responses

Sodium and chloride concentrations in plasma of the fish at the end of feeding trial ranged from 143-157 mmol L^{-1}, and 135-145 mmol L^{-1}, respectively. After DO stress was induced, the plasma sodium and chloride concentrations changed (127-148 mmol L-1, 117-130 mmol L-1, respectively) (Figure 6.1a, Figure 6.1b). However, these changes in the physiological parameters were not significantly different due to the feeding different diets or when exposed to DO reduction.

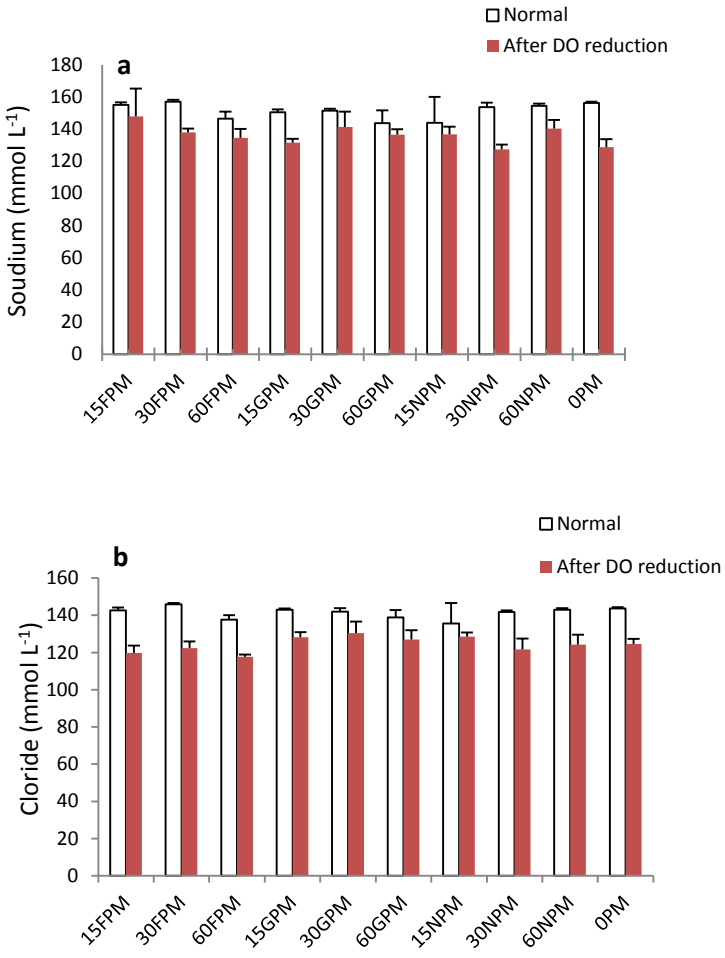

Figure 6. 1 Concentration of plasma sodium (a), and chloride (b) of juvenile barramundi when fed test and reference diets subjected to dissolved oxygen drop in 4 hours. Error bars show ± standard deviation (SD).

The ALT concentrations of plasma of fish groups fed different diets before and after DO reduction were in the range of 78-159 U L^{-1} and 83-154 U L^{-1}, respectively. Before and after hypoxic exposure to the fish, average levels of ALT concentrations showed a decreasing trend, (but not significant, ($p>0.05$) for the fish fed diets

15FPM, 30FPM, 60FPM, 60GPM, 15NPM, 30NPM and 0PM while increased for the fish fed diets 15GPM and 30GPM (Figure 6.2a).

Before juvenile barramundi were subjected to DO reduction, the concentration of cortisol in the plasma was 68-177 nmol L^{-1} with the highest level in fish fed 30GPM diet and the lowest in fish fed 60GPM diet (Figure 6.2b). However, there was not significant difference in the plasma cortisol level between fish fed test and reference diets. In contrast, exposure to reduced DO significantly increased plasma cortisol level in fish fed diets contained 60% of GPM, 30% of NPM and 60% of NPM compared to the reference diet.

After 8 weeks of feeding test diets, the glucose concentrations in plasma of fish were in the range of 4.9-8.4 mmol L^{-1}. Under hypoxic stress conditions, the fish fed all test diets resulted in significantly four-fold increase in plasma glucose concentration (19-31 mmol L-1) compared to the fish without stress (Figure 6.2c). The increased magnitude of plasma glucose levels before and after DO reduction was significantly higher in fish fed 60GPM and 60NPM diets than in fish fed reference and 15NPM diets.

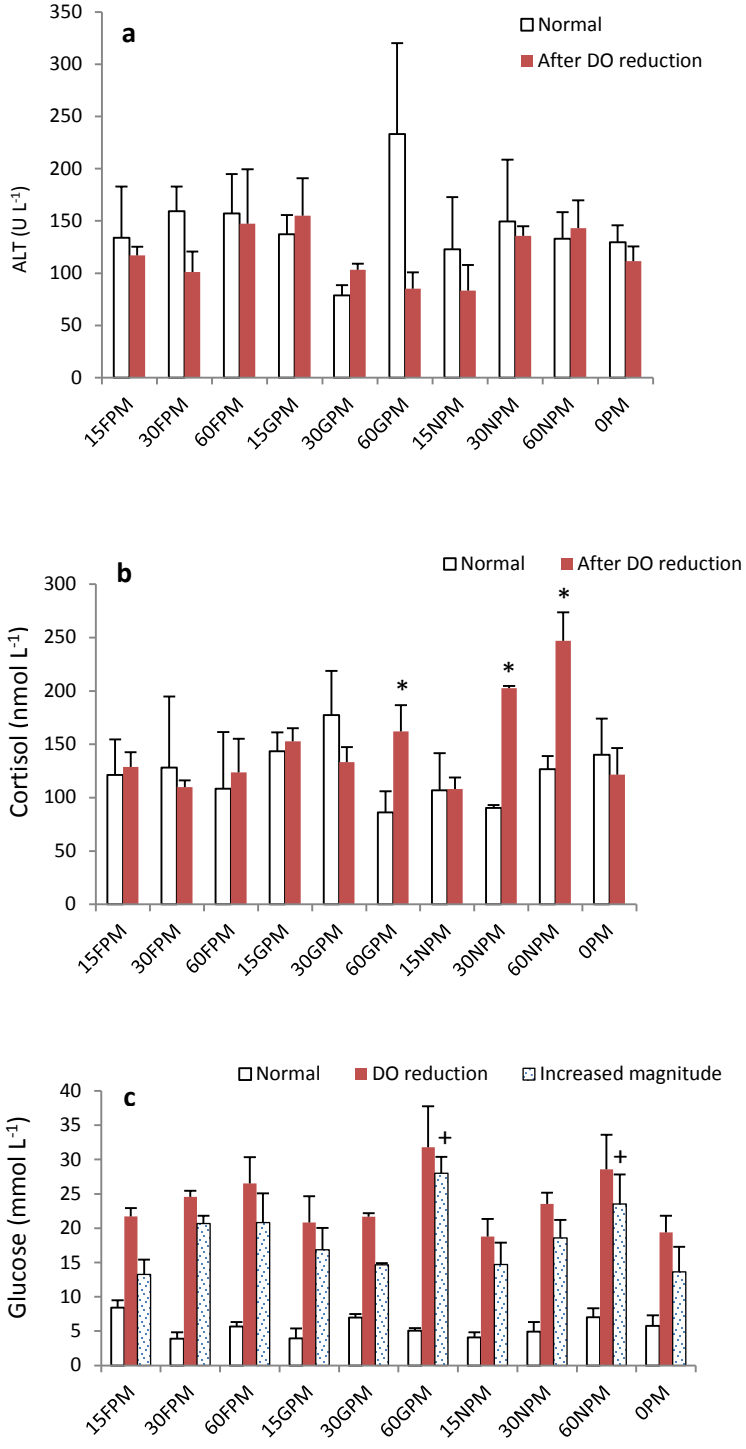

Figure 6. 2 Concentration of plasma ALT (a), cortisol (b) and glucose (c) of juvenile barramundi fed test diets and reference diet subjected to oxygen reduction shock (from 5.6 mg L^{-1} to 2.0 mg L^{-1}). An asterisk denotes a statistically significant differences in plasma cortisol level between before and after DO reduction, and plus signs indicate the differences in plasma glucose concentration from 0PM (reference) and 15FPM. Error bars show ± standard deviation (SD).

6.3.4 Interaction

In terms of growth, survival rates and FCR, no significant interactions were observed among dietary inclusion levels of PM and bioprocessed PM. Inclusion levels and nature of bioprocessing of PM also did not result in any interaction related to the f physiological parameters before hypoxic stress. Similarly, interaction between DO reduction and the test diets on physiological parameters were not observed.

6.4 Discussion

Many ANFs, tannins and alkaloids are commonly present in protein rich plants (Francis *et al.*, 2001) including peanuts (Ejigui *et al.*, 2005). The ANFs are rather stable under heat treatment (Boland *et al.*, 1975) but can be efficiently removed by fermentation (Nnam and Obiakor, 2003). Hassan and Tinay (1995) reported that tannins content in Sorghum (*Sorghum bicolor*) was reduced by 30% after 14 hours under natural fermentation. Fermentation by *Lactobacilli* is able to reduce phytic acids and tannins in sweet lupin (*Lupinus angustifolius*) by 87.04% and 17.64%, respectively (Vo-Binh *et al.*, 2015). Meanwhile, alkaloids are degraded during germination, for instance alkaloids in black cumin (*Nigella sativa*) seed reduced significantly to undetected levels when germinated at day 10 (Khamal and Ahmad, 2014). In our study, both alkaloids and tannins were reduced ($p<0.05$) by fermentation and germination. While tannins were efficiently removed by fermentation (60.9%), alkaloids content were remarkably reduced by germination (85.6%).

Tannins included in a diet reduced palatability and growth in common carp (*Cyprinus carpio*) (Becker and Makkar, 1999) as they can interfere to decrease the concentration of digestive enzymes as observed in different India carps, rohu (*Labeo rohita*), catla (*Catla catla*), and mrigala (*Cirrhinus mrigala*) (Mandal and Ghosh, 2010). Similarly, pyrrilizidine alkaloids extracted from tansy ragwort (*Senecio jacobaea*) included in rainbow trout's (*Salmon gairdneri*) diet at a concentration of 100mg kg^{-1} resulting in severe reduction in growth and survival (Hendricks *et al.*,

1981); and at a lower concentration or 2 mg kg^{-1} it resulted in hepatic lesions consisting of necrosis, megalocystis, fiber tissue scarring, and occlusion of the hepatic veins.

After 8 weeks of feeding, the barramundi juveniles fed all diets in the present study gained greater than 29 g from an initial average of 6.2g with FCR values were around 1.0 or less, except in fish fed 60GPM diets which resulted in reduced growth of 27 g and increased FCR of 1.44. So far there is no published research available on the use of the PM as fishmeal replacement diet in order to compare with this study. Furthermore, FPM and GPM are never tested as a diet ingredient for barramundi or any other marine species. In comparison to other plant-derived protein ingredient tested for the same species, our previous investigation (Vo-Binh *et al.*, 2015) reported that ANFs of sweet lupin were significantly removed by fermentation, resulting in an increase of fishmeal replaced proportion in juvenile barramundi diets. This study is similar where 60% fermented peanut inclusion diets resulted in no changes in growth rate and FCR compared to the reference diet. This is additionally supported by the significant correlation between growth, survival and reduced levels of these ANFs in diets due to fermentation (Figure 6.3a, b).

Germination elevates antioxidant vitamins in wheat (Yang et al., 2001) and antioxidant activities in peas (*Pisum sativum* L.variety Elsa) and beans (*Phaseolus vulgaris L.variety* La Granja) as demonstrated by Lopez-Amoro´s et al. (2006). Similarly, phenolic content increases in germinated lupins too (Fernandez-Orozco *et al.*, 2006; Dueñas *et al.*, 2009). On the other hand, fermentation of cowpea (*Vigna sinesis* L.) by bacteria (*Lactobacillus plantarum*) in 48 hours at $37^{o}C$ increased quercetin concentration from undetected to 22 µg g^{-1} (Dueñas *et al.*, 2005). This elevation of antioxidant vitamins and flavonoid are useful for fish growth and especially the improvement of survival rate (Tocheri *et al.*, 2002; Zhou *et al.*, 2012; Narra *et al.*, 2015).

The survival of the fish in our study were very high in diets contained 15% and 30% GPM, but got reduced at 60% inclusion level. The higher survival rate in low inclusion levels of GPM based diet could be explained by the presence and balance

of antioxidant compounds that were generated in GPM. However, if these compounds are in excess, there could be adverse effects on fish health (Garcia *et al.*, 2007). Additionally, during germination, total lipid decreased as reported in lima bean seeds (*Phaseolus lunatus*) (Dibofori *et al.*, 1994) and this study; there are other negative compounds could be increased as well, for instance cyanide level in sorghum (Ahmed *et al.*, 1996). These adverse effects could be attributed to the reduced survival in fish fed 60% GPM based diet.

Dietary compositions can influence the physiological responses of the target species (Waagbo *et al.*, 1994). The inclusion of fermented vegetables increases non-specific immunity of fish (Ashida and Okimasu, 2005). Likewise, antioxidant activity is increased by feeding the fish germinated lupin (Dueñas *et al.*, 2009) which in turn, enhances the resistance to certain stressors. In the present research, the diets containing FPM at any inclusion levels did not change the plasma glucose. However, 60% GPM or 60% NPM based diets significantly increased the glucose level compared to reference diet when the fish were exposed to reduced DO levels. Also, plasma cortisol concentrations were significantly increased in fish fed 30% and 60% NPM, supporting the hypothesis that bioprocessed PMs provide immunological stimulants into diets and thus partly explain the improvement the fish health and enhancement of the resistance under stress condition.

The monovalent sodium and chloride ions are involved in neuromuscular excitability, acid-base balance and osmotic pressure (Verma *et al.*, 1981). Ion regulation is energy demanding and disturbances in ionic balance can reduce growth rates and impair swimming performance (Wilson and Wood, 1992). On the other hand, elevated levels of ALT, a liver enzymes, in general, signify some form of liver damage or injury (González *et al.*, 2012). In the present study, there were no significant changes in the concentration of plasma sodium,, chloride and ALT, indicating that the diets and DO reduction stressor did not induce changes in osmotic regulation and liver dysfunctions.

Concentrations of sodium and chloride in plasma in this study were similar to those found by Glencross *et al.* (2011a), where seawater juvenile barramundi were fed

various plant ingredients and similar levels of these immunological parameters were recorded in fish nursed in saline groundwater fed commercial diet (Partridge and Lymbery, 2008). However, ALT concentrations in our experiment were five-fold higher than that reported by Glencross *et al.* (2011a). This difference could be explained by the difference in the rearing water salinity where the fish were cultured. In the present study the fish were cultured in freshwater while (Glencross *et al.*, 2011a) carried out the experiment in seawater.

The hypothalamus of the stressed fish releases corticotropin-releasing factor and other chemicals in blood circulation which finally activate the release of cortisol by the interrenal tissue. Additionally, the increase in plasma glucose is to cope with the demand of energy as the response of the fish fight against the stressor. Barton *et al.* (1988) reported that diets with different levels of lipid can affect the levels of plasma glucose and cortisol. Dietary supplementation of chitosan oligosaccharides at 40mg kg^{-1} improved phagocytic activities, respiratory burst activities and reduced the level of cortisol when exposure to bacterial pathogen (Lin Luo *et al.*, 2009). In this study, NPM included at medium and high levels (30% and 60%) in diet and under reduced DO stressor, significantly increased the cortisol level, while these fish exhibited normal performances of growth, survival and FCR. Plasma cortisol concentration was also significantly higher in fish fed GPM where they showed reduced growth, increased mortality.

To reduce feed production cost, proportion of fishmeal should be decreased in diets while ensuring a balanced nutritional formulation that provides the fish with good growth performance and health. Protein concentration in the processed PMs is lower than in fishmeal thus high protein ingredients, blood meal and corn gluten have been used in accordance with levels of the PMs included into the diets. Meanwhile the cassava meal and wheat flour portions were changed to keep the diets with isonitrogenous and isocloric. Barramundi used efficiently blood meal and the Inclusion of this ingredient did not affect to the fish growth and FCR (Williams *et al.*, 2003), but the carbohydrate is less utilized by marine fish (McMeniman, 2003). The ingredients' portion changes in this study did not generate an interaction among the test ingredients, suggesting balanced nutrition of diets is acceptable.

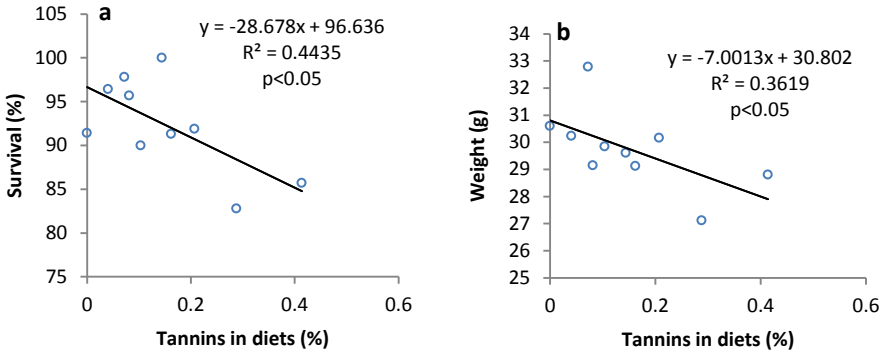

Figure 6. 3 Correlation between concentrations of ANFs (tannins and alkaloids; a, b) in diets and growth performance (survival and growth rate). Data were generated from Table 3.3 (Chapter 3) and Table 6.2.

In conclusion, fermentation and germination of peanut resulted in the significant reduction of tannins, alkaloid by 60% and 40%, and 86% and 45%, respectively and increase of vitamin E and flavonoids as well. The inclusion level of fermented peanut meal in the diet could be increased up to 60%, without compromising the growth performance and affecting blood biochemical homeostasis. On the other hand, dietary inclusion of 60% germinated peanut meal or none-bioprocessed peanut meal in the barramundi feed either showed a reduced performance or had a larger variations in physiological response.

Chapter 7: DIGESTIBILITY AND WATER QUALITY INVESTIGATIONS ON THE USE OF FERMENTED, GERMINATED AND MECHANICAL PRESSED PEANUT (*Arachis hypogaea*) MEALS AS PROTEIN SOURCES ALTERNATIVE TO FISHMEAL IN JUVENILE BARRAMUNDI (*Lates calcarifer*) DIET

(*This chapter was submitted to Aquaculture Research*)

7.1 Introduction

Fishmeal, a primary protein source in aqua-feeds, is becoming increasingly scarce and uncertain (Tacon and Metian, 2008; Olsen and Hasan, 2012). Consequently, many investigations to evaluate the efficiency of alternative to fishmeal ingredients have been conducted. Plant-derived proteins have gained more interest due to their ease of availability and the lower cost. A number of studies have focussed on the replacement of fishmeal by lupin (*Lupinus sp*), rapeseed (*Brassica napus*), and soybean (*Glycine max*) meals which have achieved some success (Tantikitti *et al.*, 2005; Glencross *et al.*, 2011a; Ngo *et al.*, 2016). However, the main barrier for the use of plant protein is the presence of anti- nutritional substances in plant based ingredients (Francis *et al.*, 2001) that adversely affect the growth, ingredient digestibility (Barneveld, 1999), the amino acid absorption (Hansen, 2009) in the host fish and increase the discharge of organic matter in the culture environment.

Barramundi (*Lates calcarifer*), a carnivore species, requires relatively higher dietary protein (45 – 50 %) (Glencross, 2006) which traditionally is sourced from the fishmeal. The replacement of fishmeal by cheaper plant-based protein sources is critical to the sustainability of barramundi farming. Peanut (*Arachis hypogaea*) is a legume which has a high content of oil and protein. The oil of the peanut is extracted for human use (Zhao *et al.*, 2012) and the remainder becomes protein-rich and relatively cheap source to substitute fishmeal. Our previous study (un-published data) showed that 60% fermented peanut meal can be included in

juvenile barramundi feed without compromising the growth performance whereas, the germinated peanut meal could increase the survival at a low inclusion level of 30% and less. In the current study, three different processed peanut meals, namely mechanical pressed peanut meal (MPM), lactobacilli fermented MPM (FPM) and peanut meal derived from germinated peanut seed (GPM) were included at 15%, 30% and 60% in diets to evaluate their effectiveness on digestibility in juvenile barramundi and nutrients discharged into the rearing environment.

7.2 Materials and Methodology

7.2.1 Diet preparations

All ingredients, except the peanut were obtained from Speciality Feeds, 3150 Great Eastern Highway, Glen Forrest, WA 6071, Australia. Mycotoxin binder, mould inhibitor and stay C were purchased from Feed Company, Ca Mau, Vietnam. Ten diets with different inclusion levels (0%, 15%, 30%, and 60%) of MPM and FPM, and GPM and with the inner marker is Cr2O3 were prepared (Table 7.1). These diets were labelled as 15FPM, 30FPM, 60FPM (FPM based diets), 15GPM, 30GPM, 60GPM (GPM based diets), 15MPM, 30MPM, 60MPM (MPM based diets), and 0PM. The diet 0PM contained 630 g kg^{-1} fishmeal and no peanut meals and was considered as a reference diet. Diets were processed by addition of water to about 35% mash dry weight with well mixing to form the dough. This dough was then screw pelleted by a laboratory pelletiser to 1.2-2.5 mm pellets. These moist pellets were oven dried at 60^{o}C for 12 hours followed by cooling to room temperature before storing at -20^{o}C till further use.

Table 7. 1 Compositions of test and reference diets *.

Parameters	***0PM***	***MPM15***	***MPM30***	***MPM60***	***FPM15***	***FPM30***	***FPM60***	***GPM15***	***GPM30***	***GPM60***
Fish meal	63	53.55	44.1	25.2	53.55	44.1	25.2	53.55	44.1	25.2
MPM		9.45	18.9	37.8						
FPM					9.45	18.9	37.8			
GPM								9.45	18.9	37.8
Fish oil	9.2	8.9	8.8	8.6	9.1	9	8.8	9.25	9.3	9.4
Wheat flour	12.9	12.9	12.9	9.22	12.9	12.9	9.22	12.9	12.9	9.22
Cassava meal	6.02	3.97	1.82	0.8	3.77	1.62	0.6	3.62	1.32	
Corn gluten	3	5.5	7.9	13.1	5.5	7.9	13.1	5.5	7.9	13.1
Cr2O3	1	0.85	0.7	0.4	0.85	0.7	0.4	0.85	0.7	0.4
Analysed diets' composition (%)										
Dry matter	92.77	92.79	92.83	92.98	91.51	90.25	87.81	92.65	92.53	92.37
Ash	14.37	12.68	10.98	7.73	12.67	10.97	7.72	12.66	10.95	7.69
Gross energy (MJ/kg)	21.21	21.12	21.07	20.96	21.18	21.14	21.04	21.08	20.94	20.66
Crude protein	45.85	46.01	46.08	46.60	46.06	46.20	46.88	46.13	46.35	47.18
Lipid	14.22	14.33	14.64	15.44	14.60	14.98	15.93	14.22	14.24	14.44
Fibre	1.01	1.29	1.56	2.1	1.23	1.44	1.87	1.15	1.29	1.57
Arginine	2.57	2.54	2.51	2.44	2.55	2.53	2.49	2.48	2.38	2.18
Histidine	1.16	1.16	1.15	1.14	1.16	1.16	1.15	1.14	1.12	1.08
Isoleucine	2.07	2.00	1.92	1.77	2.00	1.94	1.81	2.00	1.94	1.81
Leucine	3.76	3.84	3.90	4.05	3.84	3.91	4.06	3.80	3.84	3.92
Lysine	3.33	3.04	2.75	2.17	3.05	2.78	2.22	3.05	2.77	2.20
Methionine	1.28	1.20	1.11	0.94	1.21	1.13	0.97	1.19	1.09	0.89
Phenylalanine	2.04	2.10	2.15	2.26	2.12	2.21	2.37	2.08	2.11	2.18
Threonine	1.92	1.87	1.81	1.70	1.86	1.79	1.65	1.91	1.90	1.87
Tryptophan	0.51	0.48	0.45	0.38	0.49	0.46	0.40	0.49	0.46	0.41
Valine	2.47	2.41	2.34	2.19	2.41	2.35	2.22	2.42	2.36	2.25
Available P	3.00	2.59	2.18	1.38	2.62	2.25	1.51	2.59	2.19	1.40

(*) The diet formulation consisted of 30g kg^{-1} of blood meal (98%), 10g kg^{-1} of soy lecithin, 5g kg^{-1} of vitamin premix, 2.5g kg^{-1} of mineral premix, 0.5g kg^{-1} of mould inhibitor, and 0.3g kg^{-1} of stay C (35%). Per 1kg Vitamin premix consisted of: Vitamin A (UI) 1335000, vitamin D3 (UI) 500000, vitamin E (UI) 16670, vitamin K3 (mg) 3335, vitamin B1 (mg) 6670, vitamin B2 (mg) 5835, vitamin B6 (mg) 6670, vitamin B12 (mg) 3.35, folic acid (mg) 835, d-calpan (mg) 20000, vitamin C mono-phosphate (mg) 33335, and inositol (mg) 45000. Per 1 kg mineral premix consisted of iron (mg) 8335, zinc (mg) 16670, manganese (mg) 3000, copper (mg) 8335, cobalt (mg) 670, iodine (mg) 167.5 and selenium (mg) 67.5.

7.2.2 Experiment design

A 4x3 factorial design with triplicates was used where factors were inclusion levels (0%, 15%, 30% and 60%) of PM based diets and different processed PM (mechanical processed, fermented and germinated). Juvenile barramundi were adapted to salinity of 5‰. These fish were then graded, and those within the weight range of 30-32 g each were selected and randomly distributed into thirty tanks of 3.5 m^3 each. Every experimental tank was stocked with 30 fish. A circulating culture system was set up under out-door conditions in an open shed with a roof to protect from rain and direct sunlight. The natural temperature and photoperiod ranged between 25-31^{o}C and 12 hours light respectively. The experimental fish were fed with 10 different pre-designed diets (Table 7.1) at two times daily (8 am, and 14 pm) to satiation for 20 minutes every feeding time. The faecal samples were collected to determine the digestibility after 10 days of feeding the diets and till day 18, a total for 8 days. After faecal sampling was completed, water circulation was stopped for 6 days; during this time no water in tanks was exchanged for the determination of nutrient discharged into the rearing medium. Feeding regime and other factors of the experiment were kept identical as described earlier. Samples of unchanged water were collected from every tank at 6 am in day2, day4 and day6.

7.2.3 Fish handling and sampling

All fish handling activities were performed according to the Australian Code of Practice for the care of animals for science purposes, Approval No. AEC_2014_14/25. Faecal samples were collected by sedimentation method (Cho and Slinger, 1979) as described in Vo-Binh *et al.* (2015). In every tank, a feeding tray was installed to collect all uneaten feed and if any feed escaped into the water column, it was siphoned out immediately. After one hour of feeding, settled faeces at the tank bottom were collected by siphoning, and frozen to -20^{o}C until further analyses. Samples of water were collected by taking one bottle (0.5 litters) of water from a depth of 10 cm beneath the surface from every tank. Once the water samples were collected, they were immediately sent to Environment and Disease Monitoring Centre (CEDMA) Bacninh, Vietnam for chemical analyses.

7.2.4 Sample analyses and calculation

Parameters including NH4-N (ammonium), $PO4^{-}$ (ionic phosphate), TSS (total suspended solids) and COD (chemical oxygen demand) were analysed using APHA, (1998) protocols. Apparent digestibility coefficients of each nutritional component were calculated as described by Glencross *et al.* (2007) for diet (1) and for ingredients (2)

$$ACD\ (diet) = 100 - \frac{Marker\ in faeces\ (\%)}{Marker\ in\ diet\ (\%)} * \frac{Nutrient\ (i) in\ faeces\ (\%)}{2!Nutrient\ (i) in\ diet\ (\%)}$$

(1)

Where ADC is apparent digestibility coefficients; (i) is a single nutrient like crude protein or hydrated fat.

$$ADC(ing) = \frac{(100-j)*(Nutrre+Nutring*j)*ADC\ test-(100*j)*(Nutrre*ADCre)}{Nutring*j} \qquad (2)$$

Where j is percentages of fishmeal replaced by PM proportion, Nutrre and Nutring are given nutrients in reference diet and PM, respectively and ADCing, ADCre and ADCtest are digestibility coefficients of peanut, reference diet and test diet, respectively.

7.2.5 Statistical analysis

The data were analysed using IBM SPSS for Windows version 20 at Curtin University. The results are expressed as the means and standard deviations unless otherwise specified. One-way analysis variance (ANOVA) was used to compare effects of diets containing mechanical processed, fermented and germinated PM with reference diets. Factorial analyses were performed using General Linear Model (Univariate). Levels of significance were determined nutrient digestibility coefficients and water quality using Turkey HSD tests, with significant limits being set at $p<0.05$.

7.3 Results

7.3.1 Digestibility of diets

Juvenile barramundi fed diets at any inclusion levels of FPM, MPM and GPM had no change in diet apparent digestibility co-efficiency (ADC) of dry matter, hydrolysed fat, energy and phosphorus which were in the range of 62.3-70.4%, 90.1-93.8%, 77.4-85.9%, and 78.8-91.2% respectively (Table 7.2). However, the diet ADC of protein and fibre were significantly different among the test diets. Fermented PM based diets at any inclusion level resulted in significantly higher diet ADC of protein than the reference diet, MPM and GPM based diets. MPM and GPM diets at all three inclusion levels did not alter the diet ADC of protein relative to the reference diet, except for GPM60 diet which had significantly lowest diet ADC of protein. Meanwhile, MPM60 diet resulted in significantly reduced diet ADC of fibres than FPM60, although there was no significant difference of diet ADC of fibres between reference diet and all test diets (Table 7.2). Factorial analyses showed that processing had a significant effect on the level of diet digestibility of protein and phosphorus, whereas different inclusion levels significantly influenced the diet digestibility of protein. Fermentation resulted in higher diet digestibility of the diet with 93.93%, followed by mechanical process, 88.91% and germination 80.26% (Table 7.3). Lower inclusion level of PM resulted in a higher diet digestibility of the protein. There was no interaction between processed peanut meals and inclusion levels in the digestibility of the dietary dry mater, fat, energy, fibre and phosphorus. However, the interaction was observed in the protein digestibility of the diet.

7.3.2 Digestibility of ingredient

Ingredient ADC of energy (60.0-79.0%) and fibres (38.8-76.5%) was not significantly different among test diets (Table 7.2). In contrast, significantly higher ingredient ADC of protein was observed in fish fed FPM based diets which ranged from 97.1% for FPM60 diet to 101% for FPM30 diet than fish fed all other diets except MPM15 and MPM30. GPM based diets were poor in ingredient digestibility of protein, wherein the ADC in GPM60 diet was the lowest at 44.2%. Significant higher ingredient ADC of hydrolysed fat was observed in FPM15, MPM15 and MPM30

diets (113.2%, 109.2 and 100.3% respectively) than FPM30, FPM60, MPM60, GPM30 and GPM60 diets (93.9%, 92.7%, 95.4%, 97.3% and 96.2% respectively). Ingredient ADC of phosphorus was higher in diet contained FPM than diets included MPM and GPM at any inclusion level. GPM60 diet had the lowest ingredient ADC of phosphorus among the test diets, with value of 71.4% (Table 7.2).

Table 7. 2 ADC (%) of diets containing various inclusion levels of different forms of PM and ADC (%) of PM ingredients in test diets.

Diets digestibility	***Dry matter***	***Protein***	***Hydrolysed fat***	***Energy***	***Fibre***	***Phosphorus***
FM	64.4±0.7	90.5±0.7^{a}	90.1±0.6	85.9±1.5	45.6±1.5^{ab}	83.0±3.7
FPM15	66.4±2.5	94.2±0.8^{b}	93.6±0.4	84.1±2.8	46.1±0.9^{ab}	87.2±2.1
FPM30	69.5±0.6	93.1±1.2^{b}	91.3±0.4	81.4±0.2	48.8±0.3^{ab}	87.3±1.1
FPM60	70.4±1.8	94.5±1.2^{b}	91.2±0.9	81.8±0.8	50.2±0.8^{a}	91.2±1.4
MPM15	65.5±0.8	90.3±0.2^{a}	93.0±0.2	82.1±0.9	46.2±3.1^{ab}	87.4±1.7
MPM30	63.6±7.0	89.6±0.6^{a}	93.2±2.2	80.5±1.3	44.4±2.1^{ab}	89.5±2.4
MPM60	66.8±0.8	85.6±0.6^{c}	93.4±0.3	79.4±0.8	42.3±3.9^{b}	87.1±1.1
GPM15	70.7±1.1	89.6±0.4^{a}	91.3±0.5	82.1±1.5	49.2±1.8^{ab}	84.8±3.3
GPM30	62.3±8.6	88.7±1.6^{a}	92.3±2.2	81.0±3.2	48.4±0.2^{ab}	85.4±1.5
GPM60	62.3±1.2	62.4±2.2^{d}	93.8±0.2	77.4±3.4	46.4±2.1^{ab}	78.8±2.7
Ingredient digestibility						
FPM15		100.6±4.0^{a}	113.2±1.3^{a}	73.3±13.4	48.3±14.2	106.5±4.7^{a}
FPM30		101.0±1.5^{a}	93.9±0.2^{b}	70.6±6.5	53.9±5.4	108.9±6.2^{a}
FPM60		97.1±0.3^{a}	92.7±1.7^{b}	79.0±4.7	51.8±4.7	99.2±2.9^{a}
MPM15		89.4±5.9^{ab}	109.2±2.3^{a}	60.4±3.9	48.7±11.2	86.8±5.8^{b}
MPM30		87.6±3.7^{ab}	100.3±8.4^{a}	67.6±4.3	42.5±4.3	89.3±3.7^{b}
MPM60		82.3±0.7^{b}	95.4±0.1^{b}	75.3±0.7	41.2±0.7	90.3±4.4^{b}
GPM15		84.9±6.5^{b}	98.3±1.3^{ab}	60.0±17.6	66.9±9.0	84.1±5.2^{b}
GPM30		84.5±3.8^{b}	97.3±8.3^{b}	69.7±6.2	53.6±7.3	92.8±1.2^{b}
GPM60		44.2±3.5^{c}	96.2±0.7^{b}	71.6±4.7	46.7±2.0	71.4±10.8^{c}

Note: within columns, values followed by different superscript letters are significantly different ($p<0.05$, One-way Anova, Bonferroni multiple test)

and 100.3% respectively) than FPM30, FPM60, MPM60, GPM30 and GPM60 diets (93.9%, 92.7%, 95.4%, 97.3% and 96.2% respectively). Ingredient ADC of phosphorus was significantly higher in diet containing FPM than the diets with MPM and GPM at any inclusion level. GPM60 had the lowest ($p<0.05$) ingredient

ADC of phosphorus among the test diets, with value of 71.4% (Table 7.2). Fermented PM showed to have better digestibility of protein and phosphorus than germinated PM (Table 7.3)

Table 7. 3 Factorial analysis for digestibility of fish fed different types of PM at various inclusion levels.

Factors	*DM*	*Protein*	*Fat*	*Energy*	*Fibre*	*Phosphorous*
PM type						
Fermentation	68.73	93.93^{x}	92.05	82.41	48.36	88.55^{x}
Mechanical	65.30	88.91^{y}	92.45	80.69	43.78	88.00^{xy}
process						
Germination	65.12	80.26^{z}	93.20	80.15	48.53	82.99^{x}
Inclusion						
0	64.38	90.50^{x}	90.11	85.96	45.6	83.00
15	67.51	90.49^{x}	92.67	82.76	47.7	86.47
30	65.14	91.38^{x}	92.27	80.93	46.7	87.38
60	66.49	81.23^{y}	92.77	79.55	46.3	85.68
Anova p values						
PM type	ns	0.000	ns	Ns	Ns	0.012
Inclusion	ns	0.000	ns	Ns	Ns	ns
PM*Inclusion	ns	0.000	ns	Ns	Ns	ns

Note: Within columns, values followed by the same letter are not significantly different ($p<0.05$, Univariate, Turkey HSD test)' ns denotes for not significant.

7.3.3 Effects of diets on water quality

The means pH and temperature in all tanks ranged from 7.4-7.6 and 25-27.4^{o}C respectively, and were neither significantly different among the dietary treatments nor sampling days. All diets resulted in significant increase in NH4-N and COD, and reduction in PO4- after 2, 4 and 6 days after water circulation was stopped (Figure 7.1). However, there was no change of PO4-, COD, and TSS concentrations between reference and test diets. TSS levels which ranged at 8-12 mg L^{-1} were neither affected by the sampling time or by the dietary treatments.

NH4-N level varied from 0.25 to 0.55 mg L^{-1} at day2, and significantly increased as the experiment progressed and reached to 0.95-1.50 mg L^{-1} at day4 and 1.40-2.10 mg L^{-1} at day6. NH4-N at day2 and day4 were not significantly different between reference and PM based diets and within the PMs containing diets. However, there

was a significant difference in NH4-N among the diets measured at day6. Tanks fed FPM diets had significant lower concentration of NH4-N than reference tanks, while tanks fed MPM diets had significant higher. GPM diet at 15% and 30% inclusion levels did not significantly alter NH4-N concentration compared to reference diet, but the diet contained 60% GPM had significant higher level of NH4-N (Figure 7.1).

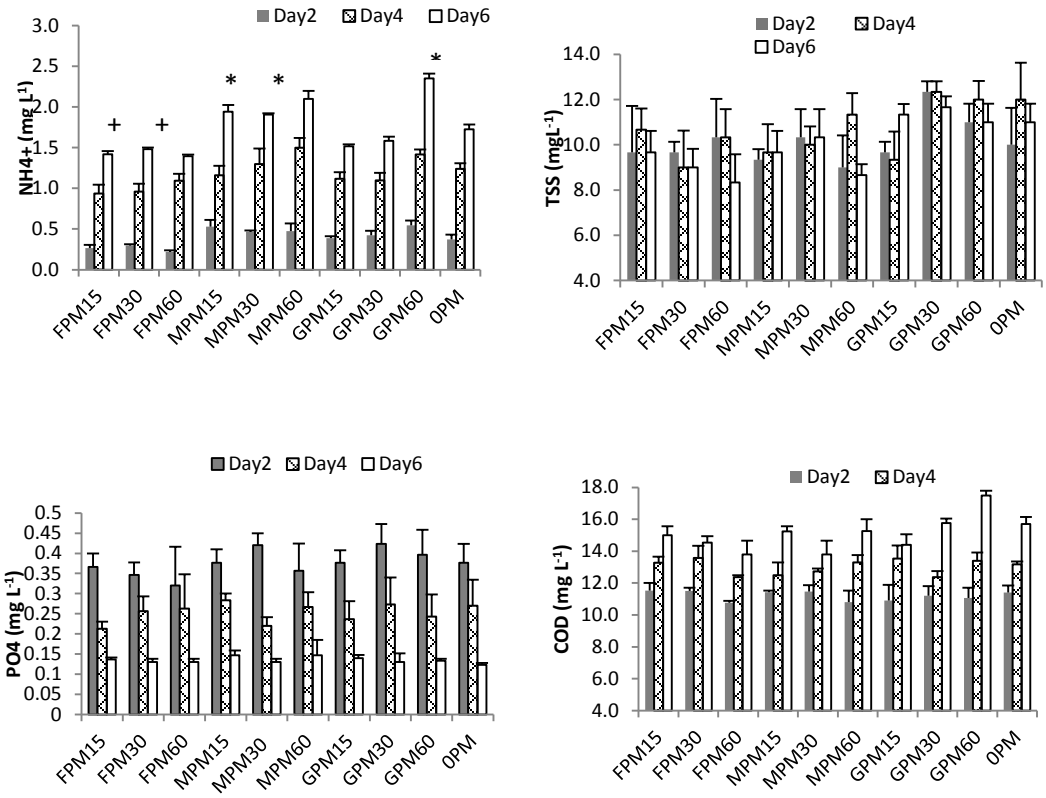

Figure 7. 1 Concentration of rearing water quality indicators after day2, day4 and day6 of no water exchanged. Bars are means±SE. (+) denote for significant lower than reference and (*) denote for higher than reference at day6.

Factorial analyses showed that there were effects of different processed PMs at different levels in NH4-N, SST and COD at different levels. Significantly lower NH4-N was recorded at day 2, day4 and day6 and TSS at day6 of feeding juveniles with fermented than mechanically processed or germinated diets. In contrast higher concentration of COD was observed with FPM at day2 and day4 than mechanical and germination processing (Table 7.4). There was interaction between different processed PM and inclusion levels in the COD of the rearing environment, but that interaction was not observed in NH4-N and SST.

Table 7. 4 Factorial analysis for water quality indicators of tanks fed different PM at various inclusion levels.

	NH4-N			*SST*	*COD*		
Factors	***day2***	***day4***	***day6***	***day6***	***day2***	***day4***	***day6***
PM type							
Fermentation	0.26^{x}	0.99^{x}	1.43^{x}	9.00^{x}	11.27^{x}	13.07^{x}	14.44
Mechanical	0.49^{y}	1.32^{y}	1.98^{y}	9.56^{x}	11.23^{x}	12.84^{xy}	14.77
process							
Germination	0.45^{y}	1.21^{y}	1.57^{z}	11.33^{y}	9.44^{y}	10.89^{y}	14.02
Inclusion							
0	0.37	1.24^{xy}	1.73	11.00	11.4^{x}	13.17	15.70^{x}
15	0.39	1.07^{y}	1.63	10.22	9.68^{y}	10.89	13.01^{y}
30	0.40	1.12^{xy}	1.66	10.33	11.39^{x}	12.89	14.70^{x}
60	0.41	1.33^{x}	1.71	9.33	10.88^{x}	13.02	15.52^{x}
Anova p values							
PM type	0.000	0.005	0.000	0.003	0.000	0.000	0.086
Inclusion	NS	0.016	NS	0.000	0.000	0.000	0.000
PM*Inclusion	NS	NS	NS	0.094	0.000	0.000	0.000

Note: Within columns, values followed by the same letter are not significantly different ($p<0.05$, Univariate, Turkey HSD test). NS denotes for not significant different.

There was significant correlation between ADC of protein and the concentration of NH4-N (Figure 3), higher ADC values resulted in a lower concentration of NH4-N, but no correlation between ADC of phosphorus and $PO4^{-}$, and ADC of dry matter and COD were found.

7.4 Discussion

The use of bioprocessing of the plant based ingredients in aqua-diets, including fermentation and germination has gained popularity over the mechanical methods to reduce the ANFs, to balance the amino acids profiles, to improve bioactive useful compounds (Yang *et al.*, 2001; Vo-Binh *et al.*, 2015) and also to reduce the peptide sizes (Hong *et al.*, 2004). These improvements of plant ingredient lead to increase in digestibility, growth and enhance health of the host fish (Kim *et al.*, 2009; Vo-Binh *et al.*, 2015). In spite of these advantages, there are few studies on the bioprocessing of the plant prior to its use as fishmeal replacement ingredients in fish diets. Peanut seeds after the oil extraction contain concentrated protein

which can be a suitable candidate to substitute the fishmeal. Though the peanut cultivation worldwide is increasing (Zhao *et al.*, 2012), limited attention has been given to its quality after bioprocessing and its role as potential fishmeal replacer.

It is obviously that juvenile barramundi had relatively higher capacity to digest protein from PMs. In comparison to the published information, our results showed that the digestibility of protein from PM (85-94%) is higher than meat meal (53-63%), poultry offal meal (78%), canola meal (81%) (McMeniman, 1998), but lower than lupin (92-94%) and sorghum (109%) (Glencross *et al.*, 2012; Tabrett *et al.*, 2012). Digestibility capacity of an ingredient is dependent on the host species and their sizes (Blyth *et al.*, 2015), and environment conditions including temperature. The ADC in the present study could be overestimated due to the faecal sedimentation collection method (Glencross *et al.*, 2007; Blyth *et al.*, 2015) used in the present study. However, higher digestibility of FPM than FM and other types of PM suggests that it can still be accepted as a fishmeal replacement ingredient.

Although no research attempting to evaluate the nutrient digestibility of bio-processed PMs, fermentation used to pre-treat plant ingredients has proven to increase digestibility. Lactobacilli fermented lupin removed phytic acid and tannins in sweet lupin and thus increased digestibility (Vo-Binh *et al.*, 2015). Fermentation of soybean meal by lactic acid (*Lactobacillus* sp) partly eliminated and inactivated ANFs, in turn improved the absorption of lipids and increased the digestibility of dietary energy in Atlantic salmon (Refstie *et al.*, 2005). The present study showed that among processed PM, FPM seemed to have greater advantage in digestibility of protein. Fermentation process reduced ANFs (Refstie *et al.*, 2005; Vo-Binh *et al.*, 2015), improved amino acid profile (Yabaya *et al.*, 2009), fragrance (Schindler *et al.*, 2011a), and immunostimulant capacity as reported in soybean (Kim *et al.*, 2009) which are probable reasons for increased digestibility of FPM diet, suggesting that the fish can digest protein better in PM if it is fermented. Diet contained FPM had higher digestibility of protein than diets included MPM and GPM compared at the same inclusion levels. This also was similar with ingredient digestibility. The digestibility of protein was higher in FPM than MPM and GPM which is in agreement with the result of fermented lupin (Vo-Binh *et al.*, 2015).

As for GPM, higher protein content was observed compared to MPM or FPM, but it had poorer ADC of protein though the ADC of energy, hydrolysed fat and fibres were unchanged relative to fishmeal based diet (Table 7.3). The data in this study are not sufficient to explain why high inclusion level of GPM has resulted in poorer digestibility than other types of PM exception that it had lower contents of carbohydrate and hydrolysed fat. A higher phosphorus digestibility of GPM than FPM could be explained by the content of digestible phosphorus that was more availability due to the fermentation, leading to an increased digestibility, similar to the results shown in pigs (Almeida and Stein, 2012).

The water chemical parameters in all tanks fed test diets were within the accepted levels in recirculating aquaculture systems (Colt, 2006) at the day2 after water circulation was stopped. However, nitrogen load increased significantly after 4 and 6 days of the water getting stagnant. The concentration of NH4-N increased from 0.22-0.55 mg L^{-1} for day2 to 0.94-1.50 mg L^{-1} for day4, and then to toxic level of 1.42-2.10 mg L^{-1} at day6. Concentration of NH4-N is correlated to toxic NH3 depending on pH and temperature (Colt, 2006) and at high concentration of NH3 can lead to reduced growth and survival, similarly to what was reported in channel catfish (*Ictalurus punctatus*) (Colt and Tchobanoglous, 1978).

In the present study, the excretion of NH4-N was significantly affected by the diets which contained protein from different processed PM and inclusion levels had also an effect on the concentration of NH4-N in the water. Fishmeal based diet resulted in lower level of the NH4-N than MPM based diet but was higher than that of FPM based diet (Figure 2, Table 6). At low inclusion level, 15% or 30%, GPM did not alter the concentration of NH4-N, however, when the inclusion increased to 60%, GPM based diet resulted in significantly elevated NH4-N concentration. The higher NH4-N level in MPM diets could be explained by the different source of protein that was from fishmeal in the reference diet and PM from MPM diet. Similar trend is reported by Davidson *et al.* (2013) and Cheng *et al.* (2003) where fishmeal was replaced by combination of soy bean protein concentrate and corn protein concentrate, and partly soy bean meal, respectively. The lower concentration of NH4-N in tank fed FPM diets in this study could be due to the fermentation when

the bacteria had pre-digested the material and increased digestibility, similar to above discussion. NH4-N can also be absorbed by microalgae (Lananan *et al.*, 2014) existing in rearing water and thus partly influenced to the results of the present study.

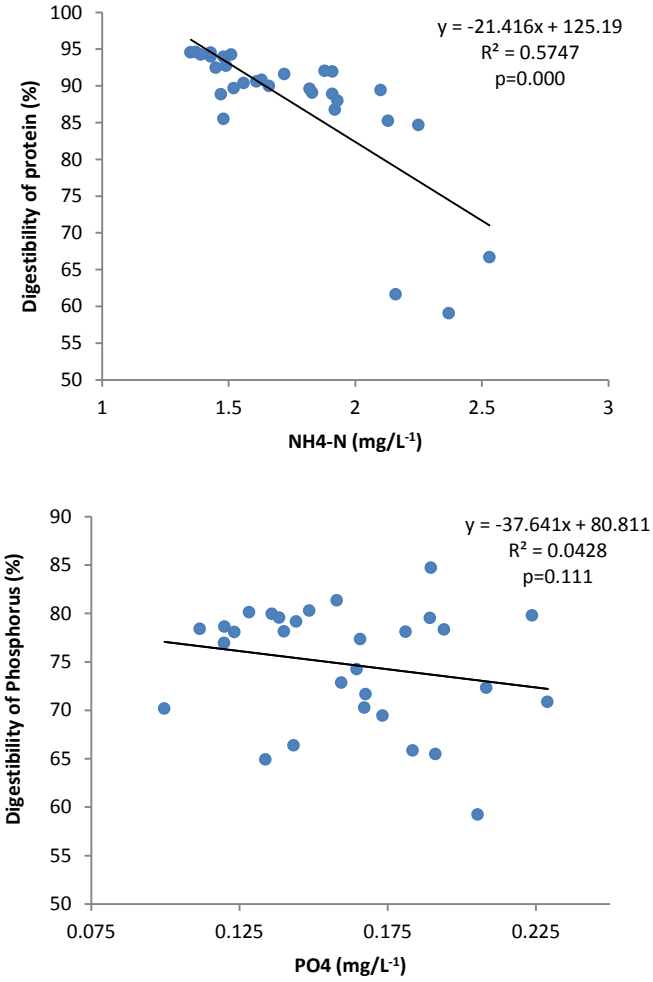

Figure 7. 2 Correlation between digestibility of diet protein and nutrient discharges (NH4-N and $PO4^-$) in water cultured juvenile barramundi.

The $PO4^-$ concentration was significantly reduced over time. The level of $PO4^-$ measured after two days of water getting stagnant was around 3.5 to 4.0 mg L^{-1}. As the water in tanks was not exchanged or circulated, the $PO4^-$ accumulation, from the fish excreta, is thought to increase but this level was reduced by approximately 70% to less than 1.5 mg L^{-1} at day 6. This reduction in phosphate could be partly attributed to the natural process of microalgae growth which uses up nutrients including PO4. However, there was an unknown process involved which removed

the large proportion of $PO4^-$ from the rearing water. Meanwhile, COD and TSS concentration were not affected by the diets which contained fishmeal or differently processed PM.

Relations between levels of protein in diet, the sources of the protein and the amount of nitrogen exerted have been studied previously (Beamish and Thomas, 1984) but correlation between digestibility of dietary nutrient and nutrient discharged through fish excretion, to a certain extent, has not been published yet. In this study, FPM included in diet was digested better than MPM and GPM and this digestibility had a significant negative correlation with NH4-N in tanks (Figure 7.2), suggesting that when an ingredient has a higher digestibility, it also releases less organic matter into the rearing environment.

In conclusion, processing the PM resulted in various levels of nutrients and the inclusion of the PMs in juvenile barramundi diets showed a variation in digestibility and water quality. Lactobacilli fermented PM resulted in higher quality of the diet that improved the digestibility of the fish and reduced NH4-N concentration in rearing water. The mechanically processed PM or PM processed from germinated seed, however, did not show these advantages.

Chapter 8. REPLACEMENT OF FISHMEAL BY ENZYME TREATED ALGA (*Spirulina platensis*) IN JUVENILE BARRAMUNDI (*Lates calcarifer*) DIETS

(*This chapter was submitted to Aquaculture*)

8.1 Introduction

Rapid development of aquaculture along with a limited fishmeal production has currently increased the demand for alternative protein sources in aqua-feeds. Proteins from grains and legumes have been successfully evaluated to replace fishmeal, but increasing utilisation of these products as human food and other animal feed has reduced their availability for aqua-diets. Recently, attention has been focused on the use of microalgae characterized by their rapid growth (Krzemińska *et al.*, 2014) and high protein levels (Becker, 2007) as an alternative to fishmeal in aqua-diets.

Microalgae, due to their high protein content (Greenwell *et al.*, 2010; Güroy *et al.*, 2011), immunomodulation capacities (Cerezuela *et al.*, 2012), improvement in pigmentation (Gouveia *et al.*, 1996), and as a source of rich vitamins and minerals (Kay and Barton, 1991), have been used as additives in aqua-feeds to enhance the growth and health of the host fish (Xu *et al.*, 2014). The different inclusions of whole cell microalgae to replace fishmeal were evaluated in Nile tilapia (*Oreochromis niloticus*) (Olvera-Novoa *et al.*, 1998), India carps (*Catla catla* and *Labeo rohita*) (Nandeesha *et al.*, 2001), Atlantic cod (*Gadus morhua*) (Walker and Berlinsky, 2011), sturgeon, (*Acipenser baeri*) (Giovanni *et al.*, 2005), and salmon, (*Salmo salar*) (Burr *et al.*, 2011). Though, microalgae including green (*Chlorella sp.*) and blue (*Spirulina sp.*) contain high protein content of 50-70% (Becker, 2007), the utilization of these plant ingredients by fish has shown mixed results. Increased growth of Indian carp was reported at any inclusion level of blue alga (*Spirulina platensis*) (Nandeesha *et al.*, 2001) while decreased growth of Atlantic cod was

observed at all its inclusion levels (Walker and Berlinsky, 2011). Limitation of microalgae utilization in fish diets could be due to their limited digestibility (Burr *et al.*, 2011) as the microalgal cells are surrounded by rigid cell walls (Domozych *et al.*, 2007) that are harder to dissolve by the digestive enzymes in the digestive track of the host fish. Hence, the enzymatic treatment of microalgae prior to their inclusion in aqua-diets should aid the digestibility (Fraatz *et al.*, 2014) by the host fish.

Barramundi (*Lates calcarifer*) is widely cultured in southeast Asia and Australia. The barramundi requires higher dietary protein that is mainly sourced from the fishmeal. Replacement of fishmeal by lupin (*Lupinus albus*) (Tabrett *et al.*, 2012), canola (*Brassica napus*) (Ngo *et al.*, 2016), soybean (*Glycine max*) (Tantikitti *et al.*, 2005; Ilham *et al.*, 2016) in barramundi diet has been evaluated but no research has been conducted on any microalgae as alternatives to fishmeal in barramundi diets. Besides, high protein content, dietary inclusion of microalgae could improve the functional properties such as the elevated content of EPA and DHA fatty acids, as shown in the muscle tissues of rainbow trout (*Oncorhynchus mykis*) (Dantagnan *et al.*, 2009).

Barton *et al.* (1988) has shown that handling stress of Chinook salmon (*Oncorhynchus tshawytscha*) fed different dietary compositions can elicit different plasma glucose response. Similarly, the concentrations of nutrient discharge is different when protein from fishmeal is replaced by plant protein in rainbow trout, culture (Cheng *et al.*, 2003). However without any exposure to stress, the inclusion of dietary plant did not change the chemical parameters of the plasma in barramundi (Glencross *et al.*, 2011), even though the replacement diets can alter the growth, feed utilization and digestibility (Ngo *et al.*, 2015). Therefore, the current study examined if enzymatic (cellulase and proteinase) treatment can alter the protein quality of blue alga and how juvenile barramundi response to dietary raw and enzyme-treated algae. Growth rate, feed conversion ratios (FCR), survival rate, digestibility, water quality and weight loss by feed deprivation and plasma biochemical variations pre or post challenged with transportation stressor, were used as indicators to evaluate the fish performances.

8.2 Materials and methods

8.2.1 Materials

Two form of blue alga (*Spirulina platensis*) (SP) as described in Chapter 3 were prepared and term as raw SP (RSP) and enzyme treated SP (ESP). Local fishmeal, cassava meal and wheat flours were obtained from Ca Mau Aqua-feed Company, Vietnam while all other ingredients (Table 8.1) were purchased from Speciality Feeds, Great Eastern Highway, Glen Forrest, WA 6071, Australia. Juvenile barramundi were purchased from local hatchery in Tien Hai, Thai Binh, Vietnam and shipped to National Freshwater Breeding Centre, Vietnam where they were acclimated to salinity of 5‰ for 2 weeks. All other experimental facilities including tanks, water and laboratory pelletiser were from National Brood-stocks Centre, Vietnam.

8.2.2 Diets formulation

Isonitrogeous and isocaloric diet formulation were adapted from the diet formulation used by American Soybean Association (USA) to meet 47% protein and 14% lipid (Table 8.1) for barramundi. Two series of diets were formulated. The diets used to evaluate the growth performance, biochemical variations, and weight loss due to the food deprivation, were formulated without inert marker, Cr2O3 (Table 8.1). Similar diets for assessment of digestibility were formulated but 1% Cr2O3 was added which replaced the cassava portions. Diets were processed by adding water to about 15-30% mash dry weight and then mixed well to form a dough. This dough was then screw pelleted by a laboratory pelletiser to 2.0-3.0 mm pellets. These moist pellets were oven dried at 50^{o}C for 12 hours followed by cooling to room temperature before storing at -20^{o}C till further use.

Table 8. 1 Diets' formulation of experimental diets (g kg^{-1} dry matter).

	Diets						
Ingredients	***ESP10***	***ESP20***	***ESP40***	***RSP10***	***RSP20***	***RSP40***	***FM(ref.)***
Local fishmeal	630	560	420	630	560	420	700
Enzyme-treated SP	70	140	280				0
Raw SP				70	140	280	0
Fish oil	89	91	95	89	91	95	87
Wheat flour	130	130	130	130	130	130	130
Blood meal	20	19	17	20	19	17	20
Cassava meal	43.2	42.2	40.2	43.2	42.2	40.2	45.2
Soy lecithin	10	10	10	10	10	10	10
Vitamin premix[1]	5	5	5	5	5	5	5
Mineral premix[2]	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Stay C (35%)	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Proximate composition							
Moisture	93.16	93.03	92.75	92.05	93.12	91.39	93.29
Protein	47.02	47.10	47.24	47.18	17.22	47.21	46.93
Lipid	14.14	14.12	14.08	13.89	14.00	14.15	14.16
Energy	22.01	22.59	23.08	22.66	22.19	22.98	21.40

([1]) Vitamin premix (per kg): Vitamin A (UI) 1335000, vitamin D3 (UI) 500000, vitamin E (UI) 16670, vitamin K3 (mg) 3335, vitamin B1 (mg) 6670, vitamin B2 (mg) 5835, vitamin B6 (mg) 6670, vitamin B12 (mg) 3.35, folic acid (mg) 835, d-calpan (mg) 20000, vitamin C mono-phosphate (mg) 33335, and inositol (mg) 45000.
([2]) Mineral premix (per kg): Iron (mg) 8335, zinc (mg) 16670, manganese (mg) 3000, copper (mg) 8335, cobalt (mg) 670, iodine (mg) 167.5 and selenium (mg) 67.5.

8.2.3 Experimental design

All fish handling activities were performed in accordance to the Australian Code of Practice for the care of animals for the scientific purposes, Approval No. AEC_2014_14/25. During acclimation, the fish were fed Uni-President-Vietnam feed (45% protein, 13% fat). The fish were then graded, and those within the weight range of 9-9.5 g were selected and randomly stocked into 21 tanks of 3.5 m^3 each under a 4x2 factorial design where factors were 4 inclusion levels (0%, 10%, 20% and 40%) of algae and 2 forms were raw (RSP) and enzyme-treated (ESP) algae. Each dietary treatment was triplicated and every tank was stocked with 40 fish. The recirculating aquaculture systems were set up in an open outdoor shed with a roof to protect from rain and direct sunlight. The natural temperature and photoperiod

ranged between 28-31^{o}C and 12 hours of light respectively. The experimental fish were fed for eight weeks with the seven different pre-designed diets (Table 8.1). Fish were fed three times daily (8 am, 12 am and 4 pm) till satiation which reached within 20 minutes. Uneaten feed, if any was siphoned out immediately after feeding. Dead fish were recorded every day to calculate mortality and then removed.

At the end of feeding period, all fish were weighed to determine growth rates and feed conversion ratios. One fish from every tank was randomly sampled out while the remaining fish were restocked to the same tanks. The sampled fish were individually weighed and then stocked in a foam box (60x40x35 cm) with 40 litres of clean water. These fish were not fed for three weeks and were weighed individually every week.

The restocked fish were fed continuously for 7 days before blood sampling. Blood samples were taken from six randomly selected fish from each tank of which three fish were used to collect blood samples while the other three were subjected to transport stressor prior to the blood sampling. Transport stress was performed by placing the fish in plastic bags with 5 litres of water with oxygen pumped until the bags were fully stretched and placed in a van (Ford Ranger) for travelling around rural area for 3 hours. The number of fish after blood samples was adjusted to 26 fish per tank and the water in the tanks was replaced by the same inlet source of water as previously used. Afterwards, the water circulation was stopped for a week to analyse the accumulated nutrients in the water and water quality. Feeding regime and other culture conditions of the experiment were kept identical as described earlier. Samples of water were collected from every tank at 6 am on day0, day2 and day4 of the days after water circulation was stopped.

Digestibility was determined in another set of the experiment, where 20 fish were randomly selected and reused from the above experiment for water quality determination. Experimental conditions and procedures were similar as mentioned previously, except the diets containing Cr_2O_3 were used. Fish were fed for 7 days

before faeces were collected. Faeces were collected by sedimentation method (Cho and Slinger, 1979) after one hour of feeding.

8.2.4 Sampling and data collection

Fish weights and blood samples were carried out under application of 2-phenoxyethanol with a dose of 0.2 ml L^{-1}. (Tsantilas *et al.*, 2006). At the end of feeding experiment, all the fish in every tank were weighed once and this sum was divided by the number of survived fish to get an average fish weight. In every tank, one blood sample from pooling blood of three fish was collected via the caudal tail vein using a 1-mL syringe and an 18G needle. Blood samples were stored in Eppendorf tubes (Eppendorf, North Ryde, NSW, Australia). The tubes were then centrifuged at 1000 g for 5 min to settle the erythrocytes and the plasma was transferred to a new Eppendorf tube prior to freezing. The blood samples were analysed for plasma biochemical parameters in Melatec hospital, Hanoi, Vietnam.

8.2.5 Samples analyses and calculations

Blood biochemistry

Analysed blood chemical parameters consisted of sodium, chloride, alanine aminotransferase (ALT), cortisol, and glucose which were analysed as described by Suski et al. (2003). The plasma concentrations of sodium and chloride were measured by using a flame photometer (Model 2655-00) and a chloridometer (Model 4435000) respectively. The plasma concentration of cortisol was measured by competitive protein binding using a commercially available kit (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, CA, USA) whereas ALT and glucose concentrations were qualified enzymatically following the methods of Lowry and Passonneau (1972) in a 96-well microplate read with commercially available spectrophotometer (Specitra Max Plus 384, Model 05362,USA).

Water quality and digestibility calculation

Water quality parameters including TAN (total ammonia nitrogen), PO_4 (phosphate ion), and COD (chemical oxygen demand) were analysed using American-Public-

Health-Association (2005) protocols. Apparent digestibility coefficients (ADC) of each nutritional component of the diets were calculated as described by Glencross *et al.* (2007) for diet:

$$ACD\ (diet) = 100 - \frac{Marker\ in faeces\ (\%)}{Marker\ in\ diet\ (\%)} * \frac{Nutrient\ (i) in\ faeces\ (\%)}{2!Nutrient\ (i) in\ diet\ (\%)}$$

Where ADC is apparent digestibility coefficients; (i) is a single nutrient like crude protein or hydrated fat.

8.2.6 Statistical analysis

The data were analysed using IBM SPSS for Windows version 20 at Curtin University with the results expressed as the means and standard deviations. Factorial analyses were performed using General Linear Model (Univariate) to compare effects of inclusion level and types (raw or enzymatic treatment) of SP. One-way analysis variance (ANOVA) was used to compare effects of diets containing SP at different inclusion levels with control diets. Levels of significance were determined nutrient digestibility coefficients and water quality using Turkey HSD test, with significant limits being set at $p<0.05$.

8.3 Results

8.3.2 Growth performance and feed utilization

After 8 weeks of feeding the experimental diets, the final body weight of the fish fed test diets ranged from 36 to 48g while fish fed the control diet grew to 45 g (Figure 8.1a). Fish fed ESP diets, at all inclusion levels, resulted in similar growth rates as the fish fed the controlled diet. In contrast, fish fed RSP40 diet had a significantly ($P<0.05$) lower growth than the fish fed the control diet, while RSP10 and RSP20 diets did not alter the fish growth. RSP40 diet resulted in the lower growth rate among the test diets, with the lowest final weight being 36g.

Feed conversion ratios (FCR) varied between 1.17 and 1.48 (Figure 8.1b); with the highest FCR found in fish fed diet containing 40% RSP. Except RSP40 diet, fish fed all the test diets had a similar FCR to those fed control diet. Different inclusion levels

of ESP in diets did not alter FCR, whereas, low dietary inclusion (10%) of RSP resulted in significantly lower FCR than higher (40%) inclusion level. At the same inclusion level, there were no significant differences in FCR between raw and

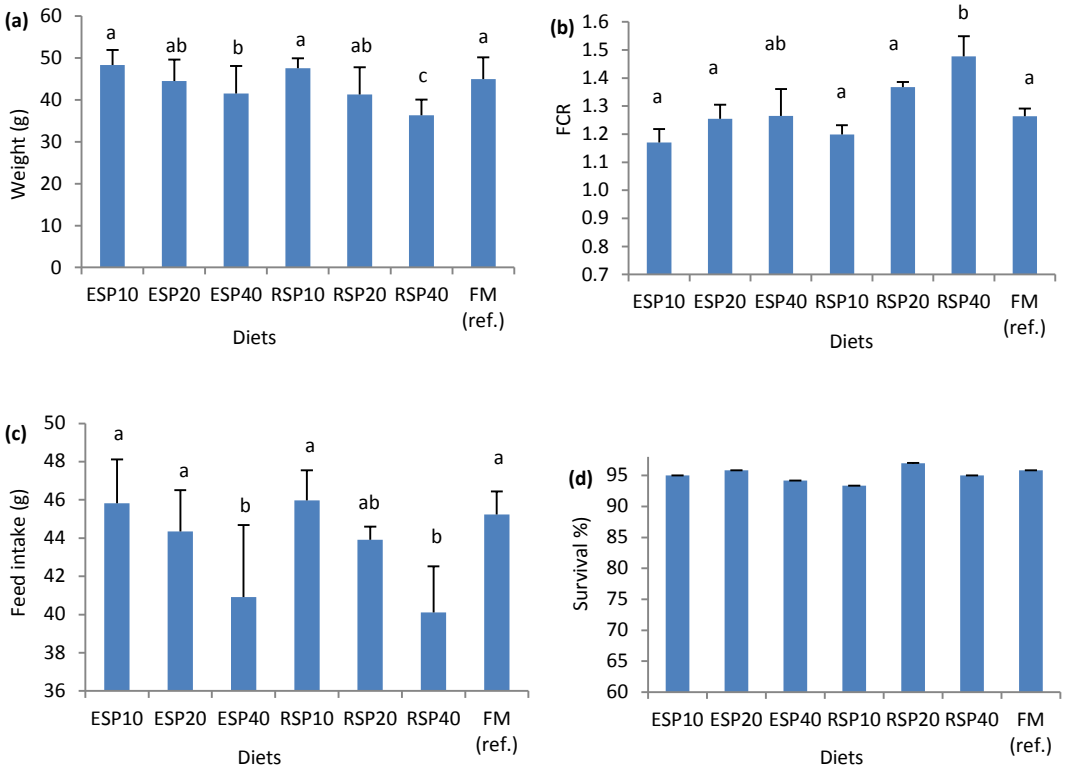

Figure 8. 1 Means of final weights (a), FCR (b), feed intake (c) and survival (d) of juvenile barramundi fed different inclusion levels of enzyme-treated and untreated SP. Bars followed by the same letter are not significantly different (P<0.05, Turkey HSD test)

enzyme-treated SP. Feed intake showed an opposite trend with FCR, which ranged from 40.9 to 46.0g per fish. At 10% and 20% inclusion levels, there was no significant difference (P>0.05) in feed intake of fish fed RSP, ESP and control diets. At higher inclusion level (40%), feed intake of fish fed test diets was significantly (P<0.05) lower than the fish fed control diet regardless to if the raw or enzyme-treated algae (Figure 8.1c). Survival ranging from 93-97% (Figure 8.1d) was relatively high in all fish fed any diet without any significant differences among them.

Factorial analyses showed that no significant difference in growth, feed intake and survival between enzymes-treated SP based diet and raw SP based diet (Table 8.2). However enzyme-treated SP based diet resulted in significantly lower FCR (1.23)

than raw SP diet (1.34). Inclusion levels influenced the fish growth, feed intake and FCR. Fish fed diet including the control diet did not grow as fast as fish fed 10% inclusion level of SP diet, while 40% inclusion level of SP diet reduced the growth rate, feed intake and increased the FCR. There was no factorial interaction between enzyme treatment and dietary inclusion of algae with respect to growth, feed intake and FCR (Table 8.2).

Table 8. 2 Factorial analysis for growth, FCR, feed intake and survival.

Factors	*Growth*	*FCR*	*Feed intake*	*Survival*
Enzyme				
Treated	44.8	1.23^{x}	43.3	95.0
Raw	41.7	1.34^{y}	43.7	95.0
Inclusion				
0	45.0^{xy}	1.26^{xy}	45.2^{x}	95.8
10	48.0^{x}	1.18^{x}	45.9^{x}	94.2
20	42.9^{xy}	1.31^{y}	41.1^{xy}	94.9
40	38.9^{y}	1.37^{y}	40.5^{y}	96.3
p values				
Enzyme	ns	0.003	ns	Ns
Inclusion	0.023	0.001	0.003	Ns
Enzyme*Inclusion	ns	ns	ns	Ns

Note: ns denotes for not significant different; within columns, values followed by the same letter are not significantly different (P<0.05, Turkey HSD test)

8.3.3 Digestibility

Apparent digestibility co-efficiency (ADC) of diet protein varied between 92.2% for control diet and 85.7-92.7% for SP included diets (Table 8.3). Fish fed control diet had higher digestibility of protein than fish fed 40% of ESP and 20% and 40% of RSP inclusion levels. Increase inclusion level of SP in diets resulted in poorer digestibility of protein, being lowest in 40% SP diet (Table 8.3). All diets resulted in greater than 90% ADC of lipid. Fish fed ESP40, RSP20 and RSP40 diets had lower ADC of lipid than fish fed control diet. ADC of energy was not significantly different among the test and control diets which were in the range of 64.8-71.1%.

Considering factorial effect, diets contained ESP had significantly higher digestibility of protein (92.2%) than RSP- diets (88.3%). Similarly, ADC of lipid was higher in treated (95.6%) than in untreated algal diets (92.9%). Forty percent inclusion level

resulted in lower ADC of protein (85.7%) than lower inclusion levels (89.0-91.0%) (Table 8.3). However, inclusion level did not affect to ADC of lipid. Factorial effect was also not observed in ADC of energy. Significant factorial interaction between enzymatic treatment and inclusion level was observed with the ADC of protein, however, it was not found with ADC of lipid or energy.

Table 8. 3 ADC of protein, lipid and energy of fish fed ESP and RSP at various inclusion levels.

Diets	*ADC protein*	*ADC lipid*	*ADC energy*
ESP10	92.7±1.0^{a}	94.6±1.5^{ab}	66.5±3.5
ESP20	91.7±0.4^{a}	95.8±1.1^{ab}	66.4±5.4
ESP40	89.9±0.3^{ab}	90.9±0.3^{c}	65.7±6.9
RSP10	88.4±1.9^{b}	94.3±1.1^{ab}	64.8±3.2
RSP20	86.5±1.5^{c}	93.6±2.0^{b}	67.9±3.6
RSP40	85.7±1.6^{c}	91.8±1.8^{bc}	66.0±4.3
FM (ref.)	92.2±0.4^{a}	96.4±0.2^{a}	71.1±2.5
Factorial analysis			
Enzyme			
Treated	92.2^{x}	95.6^{x}	66.1
Raw	88.3^{y}	92.9^{y}	67.9
Inclusion			
0	91.0^{x}	91.8	66.0
10	90.6^{x}	93.7	68.4
20	89.0^{x}	94.5	65.6
40	85.7^{y}	94.6	67.2
p values			
Enzyme	0.030	0.001	Ns
Inclusion	0.000	ns	Ns
Enzyme*Inclusion	ns	0.015	Ns

Note: ns denotes for not significant different; within columns, values followed by the same letter are not significantly different (P<0.05, Turkey HSD test).

8.3.4 Variations in Blood Biochemistry

Pre-exposed to transportation stress, plasma glucose of the fish fed test diets ranged from 2.9-6.2 mmol L^{-1}. Fish fed SP diets (except RPS10 diet) had significantly lower plasma glucose concentration than fish fed the control diet with the lowest level observed in fish fed EPS20, RPS20 and RPS40 diets (Figure 8.2a). Post- stress-exposure resulted significantly increased plasma glucose level to 8.4-12.1 mmol L^{-1},

except the fish fed RPS10 diet. While RPS40 diet resulted in significantly increased plasma glucose concentration than the fish fed the control diet, RPS10 diet showed a significantly lower concentration. Similarly, the highest variation of plasma glucose between pre and post stressor exposure was observed in fish fed RPS40 diet, whereas plasma glucose was lowest in fish fed RPS10 diet.

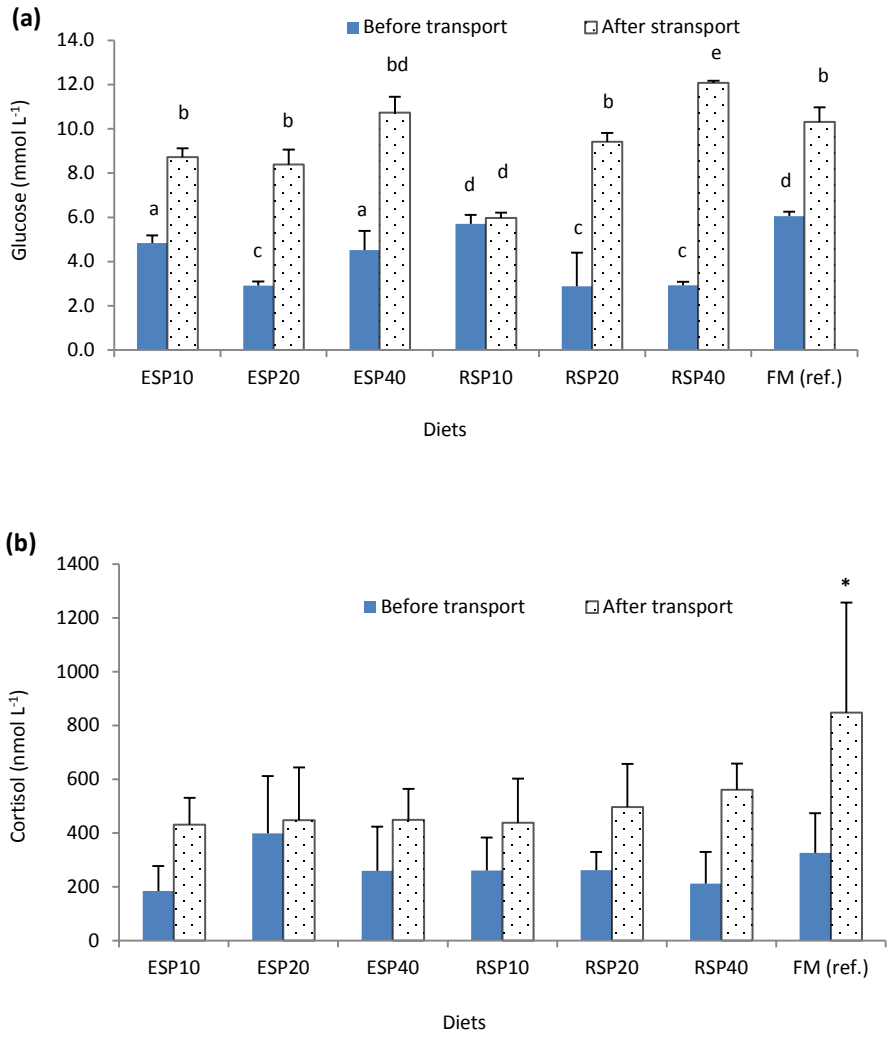

Figure 8. 2 Variation of plasma glucose (a) and cortisol (b) of fish fed various SP diets before and after transportation stress. Bars followed by the same letter are not significantly different (P<0.05, Turkey HSD test). Bar with asterisk (*) indicates the significant difference between control and test diets.

Cortisol level in plasma of fish varied between 185-398 nmol L^{-1} before exposure to the transportation stress and 431.10-848.07 nmol L^{-1} after the exposure to transportation stress (Figure 8.2b). There were no significant difference of plasma cortisol among the pre-stressed fish fed the test and controlled diet. However,

post-stressed the fish fed control diet had significantly increased plasma cortisol than any other fish.

Enzymatic treatment did not significantly affect the level of plasma glucose and cortisol. However, inclusion level of algae influenced the plasma glucose concentrations (Table 8.4). Pre-transport stressed fish, inclusion level of 0% and 10% of algae resulted in significantly higher plasma glucose concentrations, (6.1 and 5.3 mmol L^{-1}) than high inclusion levels of 20% and 40%, (2.9 and 3.7 mmol L^{-1} respectively). Post- stressed fish, inclusion of 10% and 20% algae resulted in significant lower plasma glucose (7.5 and 8.9 mmol L^{-1} respectively) than at inclusion of 0% and 40% inclusion level (10.3 and 11.5 mmol L^{-1} respectively).

Table 8. 4 Factorial analysis for plasma glucose and cortisol.

	Glucose (mmol L^{-1})		***Cortisol (nmol L^{-1})***	
Factors	***Before***	***After***	***Before***	***After***
Enzyme				
Treated	4.1	9.1	281.1	442.7
Raw	3.8	9.3	244.8	498.6
Inclusion				
0	6.1^{x}	10.3^{x}	326.9	848.1^{x}
10	5.3^{x}	7.5^{y}	222.8	434.8^{y}
20	2.9^{y}	8.9^{z}	330.3	472.6^{y}
40	3.7^{y}	11.4^{w}	235.6	504.7^{y}
p values				
Enzyme	ns	ns	ns	Ns
Inclusion	0.000	0.000	ns	0.025
Enzyme*Inclusion	0.027	0.000	ns	Ns

Note: ns denotes for not significant different; within columns, values followed by the same letter are not significantly different (P<0.05, Turkey HSD test)

Before the fish were subjected to the stressor, plasma cortisol level was not affected by the inclusion of the algae. After the fish were exposed to transportation stress, plasma cortisol in fish fed diet without algae was higher than the fish fed diet containing algae. Factorial interaction between enzymatic treatment and inclusion was observed in concentrations of plasma glucose, but was not found in cortisol level (Table 8.4).

8.3.5 Weight loss due to feed deprivation

Feed deprivation resulted in significant weight loss in all fish (Figure 8.3). However, fish fed SP (raw and enzyme-treated forms) based diets showed significantly lesser weight loss than the fish fed control diet. After the first week, fish fed control diet lost 11.49% of body weight, while fish fed SP contained diets lost 3.79-6.75%. Similar trend was found after second and third weeks when fish fed the control diet lost 15.71% and 18.49% of body weight respectively whereas the fish fed SP contained diet lost 6.32-8.94% and 8.67-11.73% respectively. Different inclusion levels of the raw algae and enzyme treated algae showed no significant difference in the rate of weight loss due to the feed deprivation. Factorial interaction between types of SP and inclusion levels was not observed.

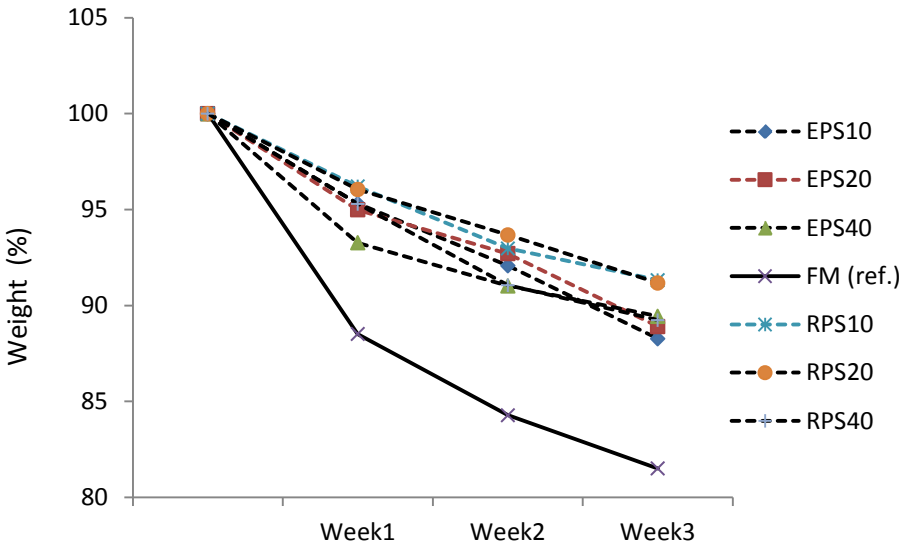

Figure 8. 3 Body weight loss (%) of experimental fish after stopped feeding 1,2 and 3 weeks. Data is expressed by means of 3 samples.

8.3.6 Water quality

Concentration of TAN of inlet water used for recirculation system was 0.099 mg L^{-1}. At day2 after the water circulation was stopped, the levels of TAN in rearing water were in the range in 0.111-0.143 mg L-1 (Figure 8.4). There was no significant difference in TAN of water measured at day2 between tanks fed control and the test diets or among the test diets. However, at day4 of the stagnant water, TAN

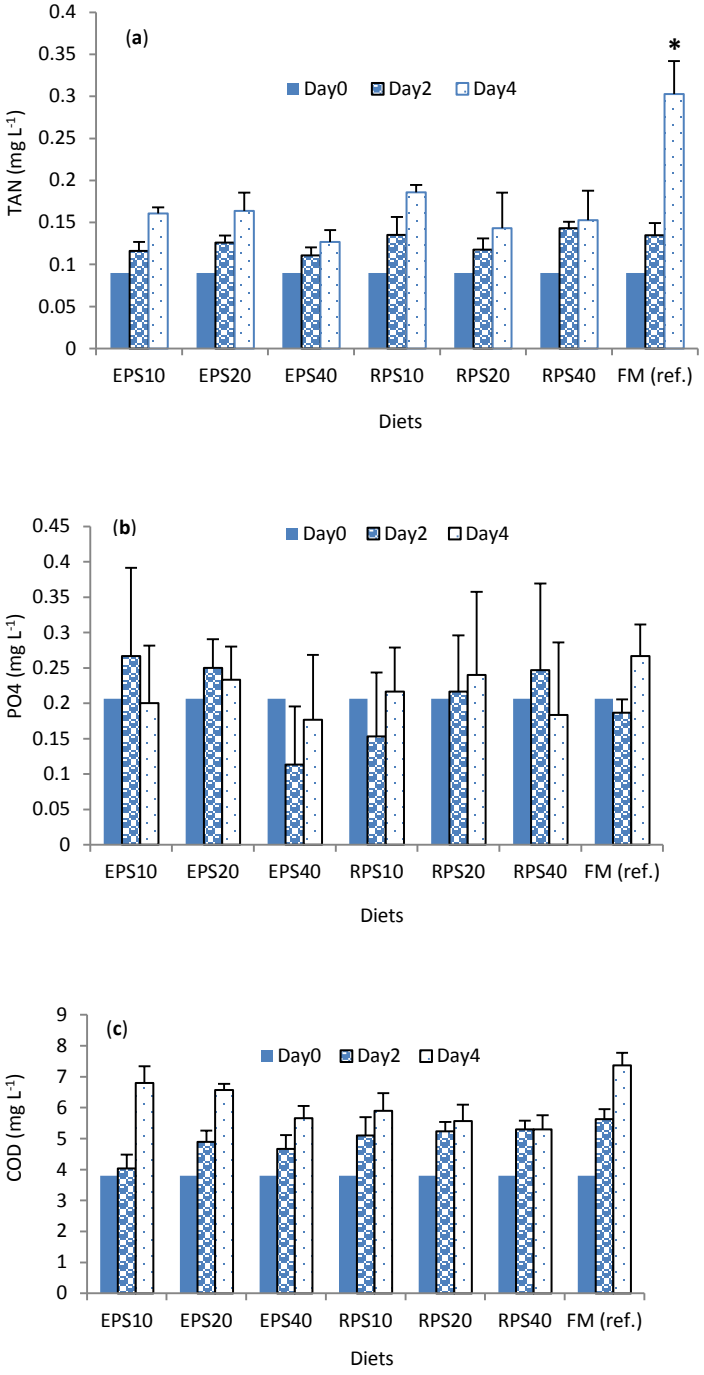

Figure 8. 4 Concentration of TAN (a), PO_4^- (b) and COD (c) in tanks fed fishmeal and SP diets after 2 and 4 days when the water recirculation was stopped. Bar with asterisk (*) indicates the significant difference between control and test diets.

level in tank fed control diet significantly increased (0.303 mg L^{-1}) than from day2 (0.135 mg L^{-1}). Similarly, TAN level increased between day2 and day4 in tanks rearing fish fed SP contained diets which ranged from 0.127 to 0.164 mg L^{-1}. All tanks fed SP based diets resulted in lower concentration of TAN than tanks fed

control diet at day4. Level of TAN was not different in tanks fed ESP and RSP or between different inclusion levels of SP. There was neither difference in the levels of $PO4^-$ and COD between tanks fed control and SP based diets nor among the days of measurement.

Enzymatic treatment of algae significantly reduced the COD levels (4.5 mg L^{-1} and 5.6 mg L^{-1}) compared to raw algae (5.2 mg L^{-1} and 6.3 mg L^{-1}) at day2 and day4 respectively (Table 8.5), but it did not affect the level of TAN and PO4-. Inclusion level of SP also did not affect the TAN, $PO4^-$ and COD concentrations in the rearing water. Factorial interaction between enzyme treatment and algae inclusion levels was not found to alter rearing water quality parameters.

Table 8. 5 Factorial analysis for TAN, PO_4^- and COD.

	TAN		***PO4-***		***COD***	
Factors	***day2***	***day4***	***day2***	***day4***	***day2***	***day4***
Enzyme						
Treated	0.12	0.15	0.21	0.20	4.5^x	5.6^x
Raw	01.3	0.16	0.20	0.21	5.2^y	6.3^y
Inclusion						
0	0.14	0.30^x	0.21	0.27	5.6	7.4^x
10	0.13	0.17^y	0.23	0.21	4.6	6.3^{xy}
20	0.12	0.15^y	0.19	0.24	5.1	6.1^y
40	0.13	0.14^y	0.18	0.18	5.0	5.5^y
Anova p values						
Enzyme	ns	ns	ns	ns	0.012	0.012
Inclusion	ns	0.000	ns	ns	ns	0.049
Enzyme*Inclusion	ns	ns	ns	ns	ns	Ns

Note: Within columns, values followed by the same letter are not significantly different (P<0.05, Turkey HSD test); ns means not significant

8.4 Discussion

Microalgae as a fishmeal replacement ingredient has been evaluated on various species (Olvera-Novoa *et al.*, 1998; Nandeesha *et al.*, 2001; Giovanni *et al.*, 2005; Burr *et al.*, 2011; Walker and Berlinsky, 2011). Growth rate of silver seabream, (*Rhabdosargus sarba*) was unchanged when 50% of fishmeal was replaced by alga

(*Spirulina maxima*) (El-Sayed, 1994) while Atlantic cod (*Gadus morhua*) showed a poor growth if this alga was included in the diet (Walker and Berlinsky, 2011). In our study with barramundi, SP had some effects on fish performances (Table 8.6). The inclusion of SP at 10% or 20% did not alter the growth performance; however 40% inclusion level resulted in reduced growth and increased FCR. In contrast, SP was reported to be well utilized by two carps (*Catla catla* and *Labeo rohita*) where fishmeal was completely replaced by SP (Nandeesha *et al.*, 2001), indicating that the SP utilization efficiency is species-dependent. The carnivorous species including Atlantic cod and barramundi have less ability to perform at optimum levels when fed SP as shown by Walker and Berlinsky (2011) and this study. This suggests that SP contains high protein level though, high inclusion level in carnivorous fish diets is not recommended. To increase the utilization of SP for carnivores, properly process before use this protein ingredient as fishmeal replacement is necessary.

Table 8. 6 Performance of juvenile barramundi fed treated and raw SP at different inclusion levels compared to that of control fish.

Treatment	*Growth*	*Survival*	*FCR*	*ADC Protein*	*ADC Lipid*	*TAN*	*COD*	*PO4*	*Weight lost*	*Glucose*	*Cortisol*
10% ESP	#	#	#	#	#	+	#	#	+	+	#
20% ESP	#	#	#	#	#	+	#	#	+	+	#
40% ESP	#	#	#	#	-	+	#	#	+	+	#
10% RSP	#	#	#	-	#	+	#	#	+	#	#
20% RSP	#	#	#	-	-	+	#	#	+	+	#
40% RSP	-	#	-	-	-	+	#	#	+	+	#

Note: # denotes for no change, + denotes for positive performance and - denotes for negative performance.

So far, protein digestibility of SP is not previously evaluated on the juvenile barramundi. However, other plant derived proteins have been tested on barramundi. For example digestibility of protein from peanut meal was 82-94% (our unpublished data, Chapter 7), lupin 92-94% and sorghum 109% (Glencross *et al.*, 2012; Tabrett *et al.*, 2012). Another species of Spirulina (*S.maxima*) was tested on Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*) with protein digestibility of 85% and 82%, respectively (Burr *et al.*, 2011). In our study, juvenile barramundi had a capacity to digest SP, giving an apparent digestibility value of

85%-93%. ADC of protein was affected by inclusion levels and the enzymes treatment.

Dietary nutrients were reported to affect the blood biochemistry of Atlantic salmon (Waagbo *et al.*, 1994), but when an optimum growth performance was achieved, dietary compositions did not alter the blood biochemical parameters as reported in barramundi (Glencross *et al.*, 2011) or grouper (Yu-Hung Lin and Shi-Yen Shiau, 2003). In the current study, dietary SP influenced the level of plasma glucose. High level of glucose in plasma is not good for human health (Inoguchi *et al.*, 2000), although its effect on fish is not yet studied.

Barton *et al.* (1988) reported that low fat content (7%) in chinook salmon (*Oncorhynchus tshawytscha*) had lower plasma glucose than fish fed high fat content (19%) after 3,6,12 and 24 hours of handling stress. Chitosan oligosaccharides supplementation at dietary 40 mg kg^{-1} decreased plasma cortisol of rainbow trout (*O. mykiss*) when the fish were challenged with bacterial pathogen (*Aeromonas hydrophila*) (Lin Luo *et al.*, 2009). In the present study, there was no effect of SP inclusion on the plasma cortisol before the fish was subjected to transportation stress. However, after the fish exposed to the stress, diet with algae at any level were shown to have lower cortisol level. As reported by Kay and Barton (1991) SP is rich in vitamins and minerals. Vitamin E at 50 and 200 mg kg^{-1} in diet reduced variation of plasma glucose under handling stress in juvenile beluga (*Huso huso*) and these fish were shown to have optimal growth (Falahatkar *et al.*, 2012). The lower level of cortisol for fish fed SP based diets in this study suggesting that SP in diets can enhance the resistance of the juvenile barramundi under stress condition.

The body weight of starved chinook salmon gets progressively reduced (Barton *et al.*, 1988) and this happened in our study too. However, less body weight was lost in the fish fed algae inclusion diets. Similar results have been reported in rainbow trout fed alga (*Spirulina maxima*) (Güroy *et al*., 2011) and on black sea bream, fed macroalgae, Ulva meal (Nakagawa *et al.*, 1987). The mode of action explaining the difference of weight loss due to diets is unknown, but Nakagawa *et al.* (1987) and

Güroy *et al.* (2011) reported that preferential lipid mobilization to generate energy providing from microalgae in muscle that is presented in the algae could be the reason in weight loss reduction. Under many commercial environments, the juvenile fish experience some short-term holdings under starvation conditions, prior to their sales, and the fish with lesser weight reduction under these holding conditions, would mitigate risk to the farmers.

Different sources of protein have been reported to affect the nitrogen excretion in fish. Davidson *et al.* (2013) observed that fishmeal based diet produces less nitrogen waste than the combination of soybean and corn protein concentrate based diet. Similar trend was also reported by Cheng *et al.* (2003) when the fishmeal was partly replaced by soybean meal. Excess dietary protein or the original protein sources such as from plant product could affect to protein metabolisms which increases the excretion of ammonia (Tibbetts *et al.*, 2000; Cheng *et al.*, 2003). In our study, the tanks fed fishmeal diet had higher level of nitrogen excretion than tanks fed SP based diets. Additionally, increased SP inclusion resulted in the reduced level of COD, suggesting that the rearing environment can be improved by replacing the fishmeal with SP.

In conclusion, the inclusion of SP can be up to 20% in juvenile barramundi diets without adverse changes in growth performance, inclusion level can be improved if the SP is treated with the two enzymes. The inclusion of SP in diet resulted in less reduction of body weight than without SP diet under starvation conditions. The enzymatic treatment of SP did not change the survival rate of weight loss and biochemical parameters, but it improved the growth, FCR and digestibility at 40% algae inclusion level. These indicate that enzyme treated PS has a potential to partly replace fishmeal in barramundi diet.

Chapter 9: GENERAL DISCUSSION, CONCLUSIONS, CHALLENGES AND RECOMMENDATIONS

9.1 General discussion

Among animals, marine carnivore fish requires higher dietary protein to optimise growth and physiological performance. Fishmeal is a primary protein source in aquadiet but this essential component is becoming less available and expensive. Thus, replacement of fishmeal by other sources of protein including plant-derived protein ingredients is deemed to sustain the aquaculture of the future (Gatlin *et al.*, 2007; Hardy, 2010). Plant products with high content of protein have been widely used, but antinutrients, imbalance amino acids, poorer digestibility relative to fishmeal are some of the main concerns.

In general, plant products need some sort of pre-treatment(s) before they can be used ingredient(s) in aquadiets (Drew *et al.*, 2007). One of the pre-treatment, heating is usually applied to kill pathogenic bacteria, fungi (Fink-Gremmels, 2012) and partly elimination of anti-nutritional factors (ANFs) (Gatlin *et al.*, 2007). The thermal application is still used, although there are concerns on the loss of vitamins, mineral and the reduction of protein quality due to the maillard reaction (Seiquer *et al.*, 2006) or severely affecting the digestibility of the ingredients, if over heat-treatment (Glencross *et al.*, 2004c). The more environmental friendly method is bioprocessing, which can improve the plant ingredient by reducing or eliminating ANFs, supplementing bioactive compounds and improving the nutritional (mainly amino acid profiles) composition. Bioprocessing of plant products using fermentation, germination or enzymatic treatments, is widely practised with terrestrial animals such as swine and poultry feeds (Niba *et al.*, 2009; Almeida and Stein, 2012; Fraatz *et al.*, 2014), but there is a little information available on the use of bioprocessed plant-derived protein ingredient in fish diets.

With highly adapted with large range of salinity and climate, barramundi (*Lates calcarifer*) is widely distributed and grown outside its natural range (Harrison *et al.*,

2014) in many regions including Australia, Southeast Asians and Americans. Barramundi is a carnivore species, requiring protein content of 45-50% in their dietary nutrition (Glencross, 2006). The use of fishmeal as a sole protein source in barramundi diet has been an environmental concern and burden to captured fishery. Thus alternative protein source, especially from renewable sources such as plant-derived products has gained attention (Gatlin *et al.*, 2007). A wide range of plant protein-derived products have been tested to replace fishmeal in barramundi diets. These fishmeal replacement evaluations suggest that, protein originated from plants can only partly replace fishmeal. However, increased inclusion level of the plant protein could adversely affect the growth or retard the physiological performances of the target fish. Fermentation, germination and enzymatic treatments have shown to improve the nutritional profiles of plant ingredients (Bau *et al.*, 1997; Hong *et al.*, 2004; Bartkiene *et al.*, 2013) and this have enhanced the growth and physiological performance of the fish fed the bioprocessed plant-derived protein based diet (Vo-Binh *et al.*, 2015).

8.1.1 Impacts of bioprocessing on ingredient nutrients

The previous studies (Table 9.1) have demonstrated that bioprocessing reduces/eliminates ANFs. In our studies, sweet lupin, peanut meal and *Spirulina* were bioprocessed before their use as fishmeal replacement ingredients. The current research has demonstrated that the bioprocessing has positively affected the quality of ingredients by partly removing the ANFs, improving amino acid profiles and enhancing the bioactive compounds and reducing peptide sizes (Chapter 3).

Tannins, phytates and alkaloids are very common in plant-derived proteins (Farhangi and Carter, 2001) and they were all significantly reduced when lupin and peanut meal and germinated peanut meal were bioprocessed (Chapter 3). Fermentation of soybean has reduced trypsin inhibitor activity, level of raffinose and sucrose concentrations making it easier to digest for Atlantic salmon (*Salmo salar*) (Refstie *et al.*, 2005). Similarly, the fermentation of peanut meal has removed tannins and alkaloids with rates of 60 and 70% respectively (Chapter 3). The

reduction of ANFs supported the improved digestibility as showed in the present studies. Blyth *et al.* (2015) reported that high variation of digestibility was observed in lupin cultivars showing in their bitter levels which correlated with the presence of alkaloids (Wysocka and Jasiczak, 2004). The higher level of bitter resulted in lowering of the digestibility.

Table 9. 1 Bioprocess reduced ANFs in some plant-derived proteins.

Plant products	Treatment methods	ANFs removal	References
Soybean (*Glycine max*)	Fermentation	Tannins	(Hong *et al.*, 2004)
Soybean (*G. max*) meal	Fermentation		(Hong *et al.*, 2004)
Peanut (*Arachis hypogaea*)	Fermentation	Reduced tannins (60%) and alkaloids (85%)	Chapter 3
	Germination	Reduce tannins (46%) and alkaloids (31%)	
Lupin (*L. angustifolius*)	Fermentation	Reduced tannins (84%) and phytic acid (16%)	(Vo-Binh *et al.*, 2015)
Lupin (*L. angustifolius*)	Germination	Glutathione (79%)	(Fernandez-Orozco *et al.*, 2006)
Pigeon pea (*Cajanus cajan*)	Germination	Reduced a-galactosides (83%), phytic acid (61%) and trypsin inhibitor (36%)	(Torres *et al.*, 2007)

Bioprocessing has reported to improve amino acid profiles in plant ingredients by increasing contents of their limiting amino acids, including methionine and lysine (Garcia *et al.*, 2007). Soy bean fermented by yeast (*S. cerevisiae*) can rise these amino acids, methionine and lysine from 1.24 to 5.67 and 2.02 to 6.57, respectively (Yabaya *et al.*, 2009) consequently it improved amino acid profiles to close to fishmeal's. Similar results have been reported in the present studies where fermentation increased contents of methionine, tryptophan and arginine 36.7%, 23.0 and 35.5% respectively in peanut meal and germination raised these contents at 20.0%, 30.7 and 9.6% respectively (Chapter 3). Together with improvement of amino acid profile, fermentation and enzymatic treatment have also changed the protein structure by reducing peptide sizes of peanut meal's protein from majority of 25-65 kDa to less than 25 kDa (Chapter 3).

The germination has improved the bioactive compounds including antioxidants, vitamins and phenolic content in plant derived ingredients, for example, in wheat (*Triticum aestivum)* (Yang et al., 2001), in beans (*Phaseolus vulgaris*) (Lopez-

Amoro´s *et al.*, 2006). Fermentation was reported to increase flavonoids in cowpea (*Vigna sinensis*) (Dueñas *et al.*, 2005). The fermentation and germination, in our studies, have increased the content of vitamin E and flavonoids in peanut meal of which fermentation has increased vitamin E and flavonoids by 25% and 150% respectively, while germination has increased these vitamins by 23% and 29%, respectively (Chapter 3). Narra *et al.* (2015) and Zhou *et al.* (2012) stated that diets with supplemented antioxidant vitamins or other bioactive compounds can improve the survival and growth performance of freshwater cat fish (*Clarias batrachus*).

Nutritional composition of plant-derived protein products processed by different methods has shown to have a variation, such as protein and fat levels in peanut (Yu *et al.*, 2007). It was reported that canola meal processed by solvent extraction method has favourable nutritional values for barramundi than processed by expeller extraction (Glencross *et al.*, 2004c), in turn, the barramundi showed higher performance in terms of growth and feed utilisation, when the diet contained solvent extracted canola meal (Ngo *et al.*, 2016). Nutritional variation was also found in the same plant species of the canola but grown in different region (Spragg and Mailer, 2007) or *Spirulina* cultured under different nutritional conditions (Ogbonda *et al.*, 2007).

9.1.2 Physiological responses of barramundi fed bioprocessed plant based diets

There has been a number of plant derived ingredients tested in various fish species. In barramundi, plants with high level of protein content tested included lupin (Katersky and Carter, 2009), canola (Ngo *et al.*, 2015; Ngo *et al.*, 2016), soybean (Williams, 1998; Tantikitti *et al.*, 2005), and cereal grain (Glencross *et al.*, 2012). These ingredients have shown that they can ONLY partly replace the fishmeal. The inclusion of plant ingredients at 50% or less in these investigations has not altered the growth performance of barramundi relative to when fed fishmeal ingredient. However, when diet contained 60% lupin, the growth was reduced while FCR was increased (Williams, 1998). Similarly, 50% inclusion level of soybean reduced growth rate and feed intake (Ilham *et al.*, 2016). In another carnivore fish, Atlantic

cod (*Gadus morhua*), reduced growth was observed when fishmeal was replaced by a mixture of plant-derived protein ingredients at 50% level (Hansen *et al.*, 2007).

The impact of various inclusions of bioprocessed plant-derived protein in juvenile barramundi diet in the present studies is summarised in Table 9.2. Growth performance of juvenile barramundi was dependent on the selected ingredients that were used to replace the fishmeal at various inclusion levels. The highest

Table 9. 2 Intensity impact of bioprocess on juvenile barramundi performances.

Ingredients	*Growth*	*FCR*	*ADC*	*Plasma biochemical parameters*							*Rearing water quality*		
				Na+	*Cl-*	*K+*	*Cortisol*	*Glucose*	*ALT*	*AST*	*TAN*	*COD*	*PO4-*
FL	++	++	++	-	-	-	+	+	++	++	na	na	na
FMP	++	++	++	-	-	-	++	++	+	+	+	+	-
GPM	-	-	-	-	-	-	++	++	na	Na	-	-	-
MPM	-	-	-	-	-	-	-	-	na	Na	-	-	-
ESP	-	-	+	-	-	-	-	-	na	Na	++	-	-
RSP	- -	- -	- -	-	-	-	-	-	na	Na	++	-	-

++ denote for high intensity impact; + denote for medium intensity impact; - denote for no impact; - - denote for negative impact; and na denote for data is not available

FL, FPM, GPM, MPM, ESP and RSP denote for fermented lupin, fermented peanut meal, germinated peanut meal, mechanical pressed peanut meal, enzyme treated Spirulina, and raw Spirulina.

FCR and ADC denote for feed conversion ratio and apparent digestibility coefficient.

inclusion levels of the ingredient-replacing fishmeal were determined by their protein content that was able to replace the protein requirement of barramundi. *Lactobacillus* fermented lupin increased growth and reduced FCR at 30% or 45% respectively and kept growth unaltered at 60% inclusion than fishmeal (Vo-Binh *et al.*, 2015). On the other hand unfermented lupin at 60% inclusion level, reduced the growth (Williams, 1998). *Lactobacillus* fermented peanut resulted in superior growth than the unfermented peanut (Chapter 6). However, enzyme treated SP or germinated peanut did not increase the growth than the untreated ones (Chapter 6, 8) due probably to the properties of protein in germinated or SP which are not suitable to the fish's digestion.

Estimation of the effect of bioprocessing on the digestibility of the plant ingredients is difficult due to the non-availability of the precise data. However, the fermentation of plant ingredients has been demonstrated to increased digestibility of ingredients in animals such as or cows (Agle *et al.*, 2010; Missotten *et al.*, 2015). In aquaculture, there have been a few trials conducted on the use of fermented diets or fermented feed ingredients. For example, the fermentation of duckweed (*Lemna polyrhiza*) leaves by *Bacillus* sp isolated from common carp (*Cyprinus carpio*) increased digestibility of rohu (*Labeo rohita*) than fishmeal based diets or diets containing the same amount of the raw leaves (Bairagi *et al.*, 2002).

Nutritional properties in terms of reduced peptide sizes and changed amino acid due to bioprocessing have been confirmed in the present study (Vo-Binh *et al.*, 2015 , Chapter 6, 8). These changes in turn have supported the increase in digestibility of juvenile barramundi as explained in Figure 9.1. Compared to fishmeal based diets, *lactobacillus* fermented lupin (at 60% inclusion level) based diet increased the digestibility of 3.4% protein, 4.08% lipid, 7.5% fibre and 43.1% phosphorus. Our studies did not compare unfermented lupin with fermented lupin, but relative to fishmeal based diet, diet contained the same lupin at 30% was not different in digestibility in juvenile barramundi (Tabrett *et al.*, 2012). Meanwhile, *lactobacillus* fermented peanut meal showed a higher digestibility than unfermented one. At inclusion level of 15%, 30% and 60%, fermented peanut meal

resulted in increase of 3.7%, 2.9% and 4.0% digestibility of protein respectively. Additionally, diets contained fermented peanut meal had a higher (3.5%-8.9%) digestibility than diet contained fishmeal. Similar results were observed with SP treated by Celluclast®1.5L and Alcalase® 2.4L. The enzyme treated SP increased the digestibility of protein by 4.3%, 5.2% and 4.2% at 10%, 20% and 40% respectively than untreated ones. However, the digestibility of enzyme treated SP was poorer than fishmeal, fermented peanut and fermented lupin.

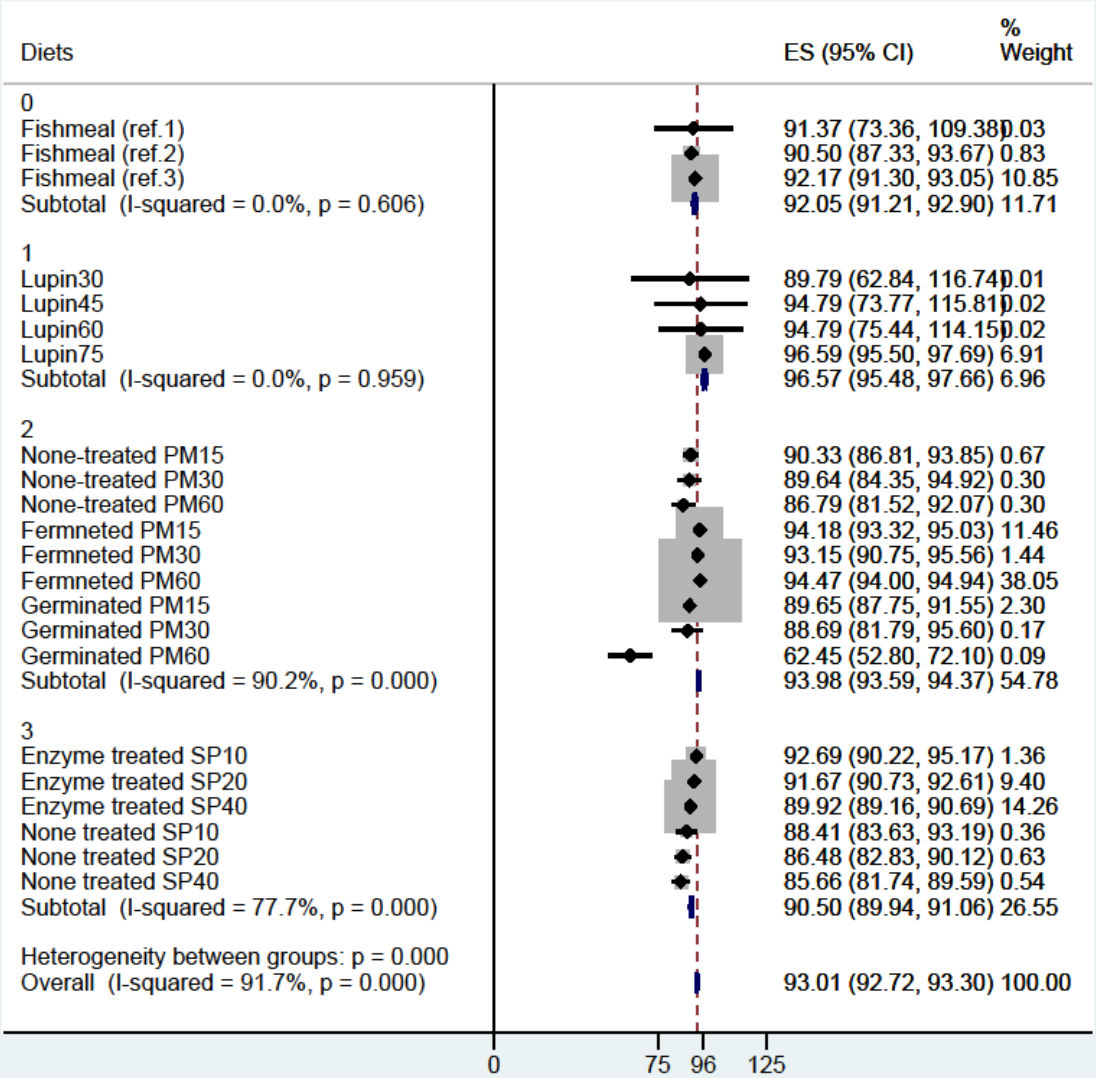

Figure 9. 1 Meta-analysis of protein digestibility corresponding 95% estimated size (ES) confidence interval (CI) of test diets in juvenile barramundi fed bioprocessed plant-derived protein based diets. Digestibility of about 96% was estimated for most test diets. Ref 1, 2,3 denote for reference diets 1,2,3.

The present investigations used faecal sedimentation method (Cho and Slinger, 1979) to collect the faecal matter of barramundi to calculate the nutrient digestibility. This method may lead to an over estimation of nutrient digestibility (Glencross *et al.*, 2007). Although Blyth *et al.* (2015) suggested to use stripping method, the results showed that there was no an significant difference between the sedimentation and stripping methods for determining the protein digestibility. The experimental fish in our studies were small and therefore handling involved during stripping would have stressed and thus potentially would have influenced the results.

Bioprocessing by fermentation of plant based diets were known to affect the non-specific immunity of fish (*Paralichthys olivaceus*) by increasing phagocytic and consequently enhancing the stress resistance (Ashida and Okimasu, 2005). Diet contained different level of lipid caused an increase in hyperglycemia in chinook salmon (*Oncorhynchus tshawytscha*) but did not affect the plasma cortisol level (Barton *et al.*, 1988). In the present study, fermentation and germination improved the plasma glucose and cortisol homeostasis in juvenile barramundi under stressful fluctuating temperature, acute hypoxic and transportation conditions (Chapter 5, 6, 8).

Total plasma protein which consists of globulin, albumin, transferrin and prealbumin is known to be linked to the nutritional status in humans (Fuhrman *et al.*, 2004) that low level of hepatic protein in blood is related to the consumption of dietary protein. Total plasma protein level was not different in fish fed fishmeal, bioprocessed or untreated plant-derived protein ingredient in our studies (Chapter 5, 6). Similar results were reported with barramundi fed a mixture of plant ingredients (Glencross *et al.*, 2011a; Ngo *et al.*, 2016). This indicates that the none-bioprocessed or bioprocessed plant ingredients has no effect on the nutritional status of juvenile barramundi.

ALT and AST are known to be indicators of liver health (Ozer *et al.*, 2008), their increase signifying liver-damage. In our studies these enzymes were significantly decreased in the fish fed fermented lupin based diet than the fishmeal based diet

(Chapter 5). Level of ALT was not significantly different among the fish fed fermented and germinated peanut or fishmeal based diets, suggesting that fish's liver was not affected or functionally damaged when juvenile barramundi were fed bioprocessed plant derived protein based diets.

The bioprocessing improved the chemical composition of the ingredients that were vitamins, antioxidant compounds (Chapter 3) and further investigations on juvenile barramundi showed to have an improved homeostasis of blood chemistry (Vo-Binh et al., 2015 and Chapter 6). Plasma glucose and cortisol levels were more stable under stressful conditions of dissolved oxygen depletion (Chapter 6) and transportation (Chapter 8).

Feeding the fish bioprocessed plant-derived protein did not affect the carcass protein of the discus fish (*Symphysodon aequifasciata*) (Chong *et al.*, 2003), cumeate drum (*Nibea miichthioides*) (Wang *et al.*, 2006) and juvenile barramundi (Raso and Anderson, 2003; Tantikitti *et al.*, 2005) and in our study (Vo-Binh *et al.*, 2015). Carcass composition of barramundi is likely to be influenced by dietary protein levels and the fish sizes rather than the source of protein (Vo-Binh *et al.*, 2015) as again reported by Catacutan and Coloso (1995) in barramundi too. Fermentation and germination of the peanut meal and sweet lupin resulted in increase in protein levels and a similar amino acid profile to fishmeal. However, there is no evidence of this change in nutritional properties of the ingredients can lead to any changes in the carcass compositions of the fish.

When fishmeal was replaced by plant-derived protein ingredients, the nitrogen waste in rearing water was increased (Cheng *et al.*, 2003; Davidson *et al.*, 2013), which was similar in our studies with the case of fermented peanut (Chapter 7), but opposite result when SP was included in the diet (Chapter 8). Fermentation of the ingredients resulted in reduced nitrogen and organic particles into the rearing water. Fermentation also increased digestibility of plant-derived ingredients (Vo-Binh et al., 2015, Chapter 8), suggesting that there is a link between digestibility and waste from the fish released into the rearing environment.

ANFs in plant derived protein ingredients is a barrier for digestibility, feed intake and growth performance (Francis *et al.*, 2001; Gatlin *et al.*, 2007), therefore, plant derived protein cannot be included at high dietary inclusion levels. In our studies, fermentation and germination resulted in reduction in ANFs and peptide sizes of protein in sweet lupin and peanut, consequently increasing inclusion levels. Up to 60% of fermented sweet lupin or peanut can replace the fish meal (Vo-Binh *et al.,* 2015, Chapter 6), while fishmeal in diet can be substituted with 40% of enzyme treated SP (Chapter 8). Meanwhile without bioprocessing, the inclusion of the lupin and peanut was 45% and 30%, respectively and SP was 20% (William *et al*., Chapter 6, 8). The processing of oil extracted canola with solvent (Ngo *et al.*, 2016) and alcohol defatted soybean (Ingh *et al.*, 1996)were also proven to increase the inclusion level. Germination reduced the ANFs and peptide size of protein in peanut (Chapter 3), however, inclusion of germinated peanut meal was not increased similar to fermented lupin or fermented peanut (Vo-Binh *et al*., 2015 and Chapter 6), although with 30% inclusion level, it increased the survival of juvenile barramundi (Chapter 6)

Vitamin E has an ability to reduce the stress levels in fish (Falahatkar *et al.*, 2012) and is essential for protection of erythrocytes, and can play a role of immunostimulants (Garcia *et al.*, 2007), thus reducing the requirements of antibiotics (Defoirdt *et al.*, 2011). This is important as dependence on the antibiotic can increases the risk of antimicrobial resistance (Akinbowale *et al.*, 2006; Smith, 2008). *Lactobacillus* spp. played a role as probiotics, occupying nutrients and sites in the intestine, and producing antibacterial compounds (Sanders and Klaenhammer, 2001). Thus, the dominance of *Lactobacillus* sp. in the ingredients due to fermentation can reduce the pathogenic bacteria. In our studies (Vo-Binh et al., 2015 and Chapter 6) total *Lactobacillus acidophilus, L. aporogenes, and L. kefiri* were greater than $3*10^7$ CFU g^{-1}. Missotten *et al.* (2015) reported that the pathogens such as *coliforms* and *Salmonella* could be prevented if the pigs are fed Lactobacillus fermented diets. Although the properties of antibiotics in bioprocessed plant-derived protein ingredients were not focused in these studies,

these potential effects are important since the antibiotic resistance is becoming a problem (Sapkota *et al.*, 2008).

9.2 Conclusions

Fermentation, germination and enzymatic treatment improved the overall quality of the plant protein ingredients and thereby bring numerous growth and physiological benefits to the juvenile barramundi when fed these ingredient based diets. After bioprocessed, plan-derived protein ingredients are easier digestible, ANFs reduced, more bioactive compound available and balanced amino acid profile. Consequently, they result in increased growth, survival and feed utilisation of juvenile barramundi, and can be included in the fish diet at higher percentage as compares with none-bioprocessed ingredients. The following conclusions can be highlighted based on the current research:

1. Lactobacillus fermentation reduced anti-nutritional factors, phytic acid and tannins, in sweet lupin by 87.04% and 17.64% respectively (objective 2).
2. Lactobacillus fermentation increased digestibility of protein, lipid, fibre and phosphorus of sweet lupin (objective 3.1).
3. The 60% of lactobacillus fermented-lupin in juvenile barramundi diet does not compromise the growth performance (objective 3.1).
4. Fermentation and germination reduced the amount of anti-nutritional factors including alkaloids and tannins, reduced the peptide sizes and enhanced the bioactive compounds of peanut meals. Additionally, germination reduced content of lipid in peanut (objective 2).
5. Fermentation increased the digestibility of protein, fibre and phosphorus of the peanut meal (objective 3.1).
6. The germinated peanuts had high levels of bioactive compounds that were associated with improved survival of juvenile barramundi when they were included in the diet at 30% or 15% (objective 3.1).
7. Treatment of SP with commercial enzymes, cellulose (Celluclast®1.5L) and proteinase (Alcalase® 2.4L) reduced the peptide sizes, increased the

digestibility of protein. However, high inclusion level of SP was not favourable diet for juvenile barramundi as with20% inclusion level started reduce growth rate (objective 2 and 3.1).

8. The stress resistance of juvenile barramundi increased when they were fed fermented lupin and peanut, and germinated peanut based diets (objective 3.2).
9. The nitrogen discharge into the rearing environment was reduced when juvenile barramundi fed fermented lupin or peanut meal, or SP (objective 3.3).

9.3 Limitation and challenges associated with using bioprocess plant proteins in fish diet

There are several factors that can influence the fermentation process, which in turn can determine the quality of the plant-derived proteins. These factors include duration of the fermentation, the use of bacterial or fungi species, the nutrient media, the fermentation environment such as pH, temperature and the absence of oxygen (Ibanoglu *et al.*, 1995; Beltran *et al.*, 2008). At laboratory scale, these factors are easy controlled but in reality, with manufactory scales, the challenges is the risk of contamination involved in fermentation process (Formenti *et al.*, 2014). Another challenge is the cost involve with germination. To have optimised germination, plant derived protein seeds should thoroughly handling during harvesting, storing and processing. All these would increase the final product cost if germinated seed is used for aquadiet.

9.4 Recommendation

Each of bioprocessed plant products has advantage when being a fishmeal replacer. For example, germinated peanut meal has increased growth and survival rates at 30% inclusion level, while fermented peanut meal has increased inclusion level up to 60%. On the other hand, SP has shown an improved rearing water quality. Therefore, a combination of these bioprocessed ingredients in juvenile barramundi

might produce more benefit to the fish growth performance and optimize the utilization of the ingredients. In fact, a mixture of different none-bioprocessed plant-derived protein ingredients in diets showed to have better growth performance in barramundi (Glencross *et al.*, 2011a). Thus it is recommended to combine fermented lupin, fermented and germinated peanut meal, and SP together and proportion of each of these ingredients should be determined to optimise fishmeal replacement.

When a plant ingredient is tested to replace fishmeal, its protein content and quality are concern, and this was the case of the present studies. However, in these plant plant-rich protein ingredients, content of carbohydrates is also high, for example 30% in soy bean (Knudsen, 1997) or 17.8% in peanut meal (Chapter 5). Fermentation has not only improved quality of protein in lupin and peanut meal (Chapter 5) but it could also improve quality of carbohydrates as found in soybean (Refstie *et al.*, 2005). Thus, after the plant-derived protein ingredients are bioprocessed quality of carbohydrate, reflecting in the fish digestibility, should be evaluated.

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