School of Molecular and Life Sciences

Physiological Responses of Juvenile Barramundi (*Lates calcarifer*) Fed Processed Animal Protein Diets

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This thesis is presented for the Degree of
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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_2015_41.

Signature: ……..

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PREAMBLE

The aim of this research was to investigate the effectiveness of dietary animal protein sources as fishmeal (FM) protein replacement. Tuna hydrolysate (TH) and poultry by product meal (PBM) were two sources of proteins tested.

The thesis consists of 8 chapters. Chapter 1 the Introduction, briefly highlights the importance of animal-derived proteins used for the substitution of FM, the limitations of animal based products for aqua-feed formulation and summarises the importance of fermentation/bioprocessing and FPH supplementation for improving the quality of animal-based ingredients. This chapter also states the aim, objectives and significance of the study.

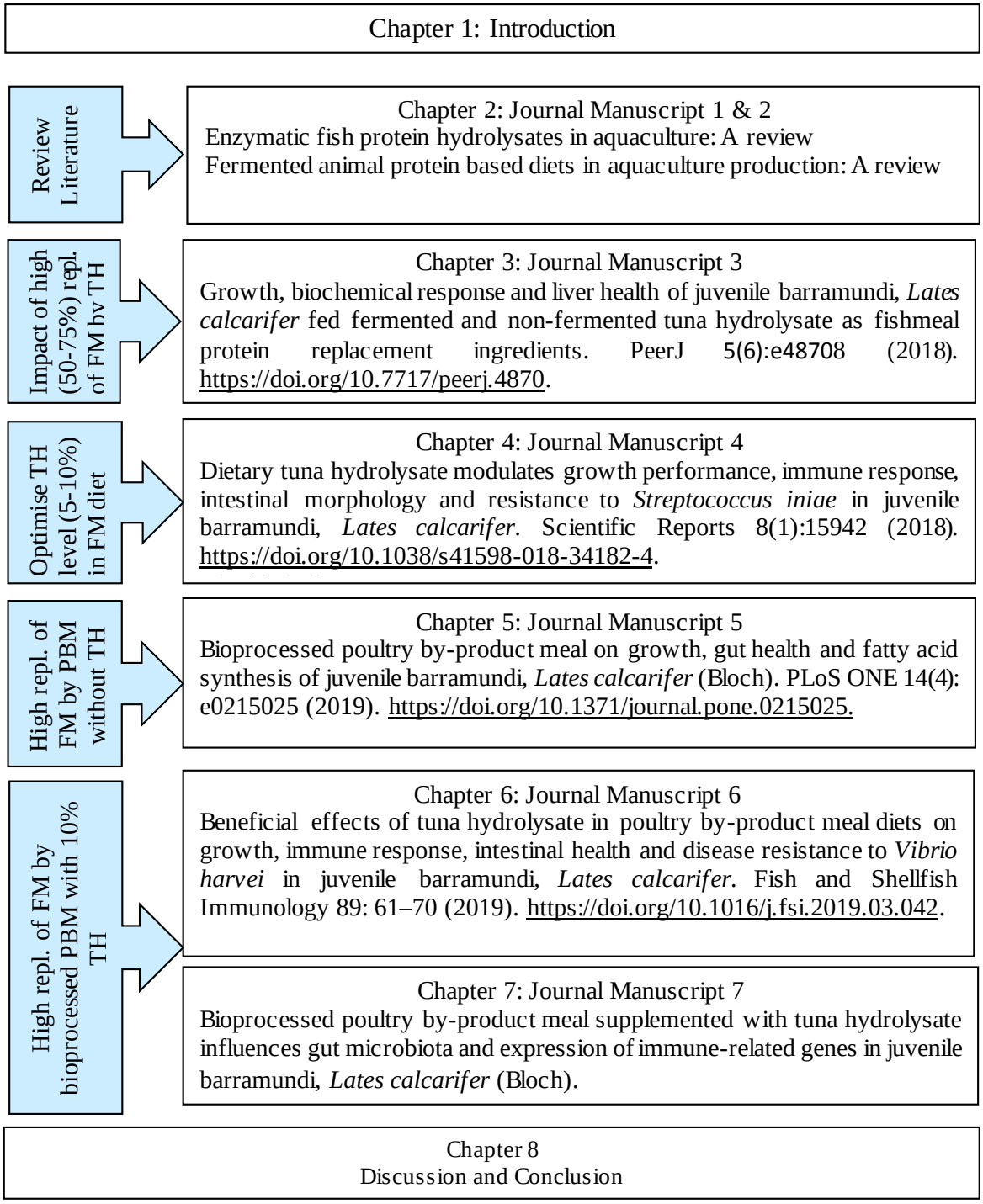
Chapter 2 presents literature reviews on the two animal-derived protein sources. Firstly, FPH literature is reviewed including attention to the source, production, nutritional composition and role in aquaculture production. Secondly, the bioprocessed/fermented PBM as an alternative protein source to replace FM in aquaculture production is reviewed.

Chapters 3 and 4 evaluates TH as replacement of FM at higher (50-75%) and moderate (5-20%) levels to optimise inclusion levels in FM based diets. Parameters for assessment included growth performance, immune response, histopathology, histo-micromorphology and disease resistance of juvenile barramundi.

Chapter 5 evaluates if bioprocessing of PBM can replace FM completely (100%) without compromising growth and health of juvenile barramundi. Chapters 6 and 7 produced from same experiment considers the potential benefits of using bioprocessed PBM and TH in combination on the performance of juvenile barramundi.

In chapter 8, the outcomes of the research are discussed holistically along with the final conclusions and limitations of the study. Also, this chapter provides links to scope for future research.

The summary and the publications arising from the research are summarized as:



Every chapter in this thesis is independently formatted due to journal requirements, including often a different style of citing the scientific name against the common name of fish. The terms fermentation and bioprocessing are used interchangeably in different chapters, though their meaning is the same.

ABSTRACT

Processed animal protein diets including fish protein hydrolysates (FPH) and bioprocessed poultry by-product meal (PBM) which can both substitute fishmeal (FM) and stimulate the defence mechanism of fish are a research priority for sustainable aquaculture development. FPH produced from fish by-products through enzymatic hydrolysis are being used for FM replacement as well as immunostimulant in aqua-feeds. Bioprocessed/fermented diets are also gaining attention for improving the utilization of dietary ingredients and thereby to improve the health of fish. To date most of the research on FPH has focused primarily on the growth performance of fish fed with FM, no information is available on the effect of FPH on FM-free diets in fish production. On the other hand, although some research has been conducted on PBM as a substitute of FM, information on the efficacy of the impact of using bioprocessed PBM on the growth and health of fish species is scarce. The present study was employed to understand if FM replacement with tuna hydrolysate (TH), bioprocessed PBM or a combination of TH and bioprocessed PBM could be used to enhance the growth performance and physiological response of juvenile barramundi, *Lates calcarifer*.

To facilitate continuous improvement of TH based diets, a series of laboratory experiments were conducted to assess different levels of dietary TH supplementation on growth performance, intestinal health, nutrient utilisation, histopathology of internal organs, non-specific immune response, immune-related gene expression and disease resistance of juvenile barramundi. The objective of the first experiment was to evaluate the efficacy of high levels (50-75%) of FM protein substitution by TH protein in diets on juvenile barramundi. The results indicated that these replacement levels negatively affect the growth performance, feed utilization and digestibility and also, increases the potential risk of hepatic failure in juvenile barramundi. The second experiment objective was to standardize the TH level in terms of growth, immune function and disease resistance of juvenile barramundi. The research demonstrated that replacement of 5 to 10% FM with TH improves growth, immune response, intestinal health and disease resistance in juvenile barramundi.

The third experiment was conducted to investigate if bioprocessed PBM can improve growth performance, gut morphology and fatty acid synthesis of juvenile barramundi. The outcomes revealed that juvenile barramundi fed 75% FM replacement both by bioprocessed and unprocessed PBM was not significantly different from the control whilst complete replacement of FM with PBM negatively affected the growth performance and gut health of barramundi. However, bioprocessed PBM improved the lipid nutritional quality when compared to the unprocessed PBM diets, which can be beneficial for human consumption. The fourth experiment was undertaken to investigate whether 10%TH supplemented with both bioprocessed and unprocessed PBM (100% FM replacement) improves growth performance, biochemical response, immune related-gene expression, intestinal health and microbiota, and disease resistance of juvenile barramundi. The results positively reflected growth, immune response, intestinal micromorphology and microbiota and disease resistance, along with immune-related cytokines gene expression with bioprocessed PBM supplemented with 10% TH. Therefore, this feed formulation strategy could be considered an ideal approach in maintaining higher growth and improved physiology of fish.

List of common abbreviations and acronyms

FM	Fishmeal
FPH	Fish protein hydrolysate
TH	Tuna hydrolysate
PBM	Poultry by-product meal
FPBM	Fermented poultry by-product meal
BPBM	Bioprocessed poultry by-product meal
FBW	Final body weight
SGR	Specific growth rate
FCR	Feed conversion ratio
ADC	Apparent digestibility coefficients
ANOVA	Analysis of variance
GLDH	Glutamate dehydrogenase
AST	Aspartate transaminase
CARL	Curtin Aquatic Research Laboratory
DO	Dissolved oxygen
EAA	Essential amino acid
FAO	Food and Agriculture Organization
SH	Shrimp hydrolysate
KH	Krill hydrolysate
CFU	Colony-forming unit

List of common fish species names mentioned in the research

Common name	Scientific name
Barramundi/Asian seabass	*Lates calcarifer*
Atlantic bluefin tuna	*Thunnus thynnus*
Japanese sea bass	*Lateolabrax japonicus*
Atlantic salmon	*Salmo salar*
Rainbow trout	*Oncorhynchus mykiss*
Red sea bream	*Pagrus major*
Persian sturgeon	*Acipenser persicus*
Olive flounder	*Paralichthys olivaceus*
Indian major carp	*Labeo rohita*
Florida pompano	*Trachinotus carolinus*
Red salmon	*Oncorhynchus nerka*
Round sardinella	*Sardinella aurita*
Cod	*Gadus morhua*
Pollock	*Theragra chalcogramma*
Ribbon fish	*Lepturacanthus savala*
Yellowfin sole	*Limanda aspera*
Herring	*Clupea harengus*

CHAPTER 1: Introduction

1.1 Background

Global fish production has been increasing steadily with the all-time high of 171 million tonnes produced in 2016 aligned with a record-high per capita fish consumption of 20.3 kg in 2016 (FAO 2018). Whilst global capture fisheries production is relatively static since the late 1980s, aquaculture is playing a pivotal role in fulfilling the increasing global demand for fish and fisheries products. From 1950 to 2016, aquaculture production increased at an annual production rate of 8.12% per year compared with 2.54% for capture fisheries (FAO 2018) (Figure 1.1). However, aquaculture production is over-dependent on fishmeal (FM), produced from wild harvested fish and fish by-products, as a protein source in aqua-diets. Although FM is considered the most nutritious and most digestible ingredient for aqua-feed, issues with supply, increasing prices and environmental concerns, is resulting in pressure on the aquaculture industry to reduce the levels of FM in diets (Ilham et al. 2016a) . As well, feed, as the highest cost in aquaculture production, accounts for around 50% of the production costs (FAO 2018). Therefore, investigation of economically viable, environmentally sustainable and readily available alternative protein sources is an area of priority research for the aquaculture industry.

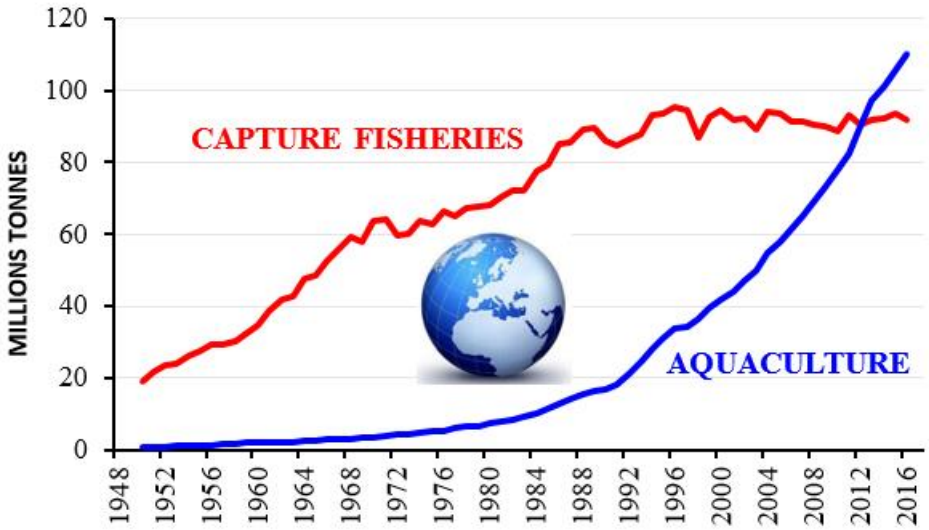


Figure 1. 1 Global aquaculture and capture fisheries production (FAO 2018).

Over the past decades, aquaculture scientists have examined the efficacy of various plant protein sources in aqua-feeds but less information is published regarding the

inclusion of animal by-products protein in fish feed. In recent years, a variety of low-cost animal by-products based diets, including ingredients such as poultry by-product meal (PBM), meat and bone meal, and fish offal meal have received attention as possible alternative protein sources in fish diets (Lewis et al. 2019; Mata-Sotres et al. 2018). Among them, PBM in particular has high protein levels and adequate amino acid profile and therefore has the potential to perform equally to FM with dietary supplementation (Sealey et al. 2011) . PBM is also readily available, has a low demand from other sectors currently and therefore is cheaper (Cruz-Suárez et al. 2007; Rawles et al. 2011). A number of studies have investigated the efficacy of PBM on aquaculture species with mixed results. PBM successfully replaced FM at 67% with rainbow trout, *Oncorhynchus mykiss* (Badillo et al. 2014), 70% with Florida pompano, *Trachinotus carolinus* (Riche 2015) and 100% for juvenile Asian seabass, *Lates calcarifer* with supplementation of amino acid (Glencross et al. 2016).

Although animal by-products are good source of protein and essential amino acid (EAA), some EAA limitations have been reported in animal products including leucine, isoleucine and methionine (Tacon 1993). Another important factor which lowers the quality of feed products produced from animal by-products is lipid oxidation, particularly during storage of diets. Animal by-products as aquaculture feed ingredients may also be constrained by high moisture, indigestible particles and microbial contaminants. (Samaddar et al. 2015). As an animal by-product, although PBM is a good source of protein and amino acids, its digestibility varies highly from batch to batch in the rendering process and between suppliers (Lewis et al. 2019). Another major impediment to incorporating substantial levels of PBM in aqua diets is their variable nutritive value due to poor quality issues and digestibility (Simon et al. 2019). According to Simon et al. (2019), the freshness of raw materials and their processing conditions are the critical factors determining the quality of the final product. The amount of heat and moisture applied to the material during the rendering process has major implications for feed quality and utilisation effectiveness of the finished meal (Lewis et al. 2019).

To overcome the aforementioned shortcomings, bioprocessing of animal by-products and/or supplementation of ideal ingredients with the animal by-products can be strategies for inclusion in fish diet formulations.

Fermentation and/or advanced processing are likely to be environmentally friendly, cost effective approaches to diminish many of the inherent problems concerned with the utilization of animal wastes in fish feed formulation (Kader et al. 2012; Mondal et al. 2008). Fermentation breaks down carbohydrates into a form that makes the innate energy and protein digestible (Gatlin et al. 2007), whilst improving the nutritional quality of animal by-products by producing low-molecular-weight compounds that potentially enhance mineral absorption (Hotz & Gibson 2007; Ilham et al. 2016b), amino acids profile (Lee et al. 2016), and reduce anti-nutritional factors (Barnes et al. 2015; Gatlin et al. 2007). (Fagbenro & Jauncey 1995) found that feeds made from fermented products tend to have higher stability in water, thereby allowing more time for fish to ingest the feed and maximise nutrient intake. Furthermore, fermented feeds are characterized by high amounts of lactic acid bacteria (Heres et al. 2003b) which can proliferate and produce high concentrations of lactic acid, as well as several volatile fatty acids including acetic acid, butyric acid, and propionic acid which are beneficial for microorganisms growth and reproduction (van Winsen et al. 2001). It is also reported that fermentation can also reduce the pH of feeds (Canibe & Jensen 2012), resulting in the inhibition of the development of pathogenic organisms in the feed (van Winsen et al. 2001) and thereby reduction of pH in the entire gastrointestinal tract (GIT) (van Winsen et al. 2001). This reduction in pH influences the ecology of bacteria (van Winsen et al. 2001) and prevent the proliferation of pathogens from developing in the GIT (Canibe & Jensen 2012; Missotten et al. 2015).

FPH is a highly nutritious product made from whole fish or fish by-products which may contain higher protein than that of the original fish depending on the production technique. FPH is now commonly produced using commercial enzymes (alcalase, neutrase, flavourzyme, protamex, papain and trypsin) as they are able to improve the nutritional value and functional properties of FPH. The favorable effects of FPH in aquaculture practices has already been established and FPH been used in many diets of commercially important fish species as a partial or total replacement of FM (Aksnes et al. 2006a; Kim et al. 2014; Ospina-Salazar et al. 2016), as a supplements in aqua-diets to improve immunity (Bui et al. 2014; Khosravi et al. 2015b), as an immune-stimulant to protect fish against environmental stress and pathogens (Bui et al. 2014; Jang et al. 2017; Tang et al. 2008; Zheng et al. 2012) and as an attractant to increase

diet palatability (Chotikachinda et al. 2013; Ho et al. 2014). The possible uses and functions of FPH are portrayed in Figure 1.2.

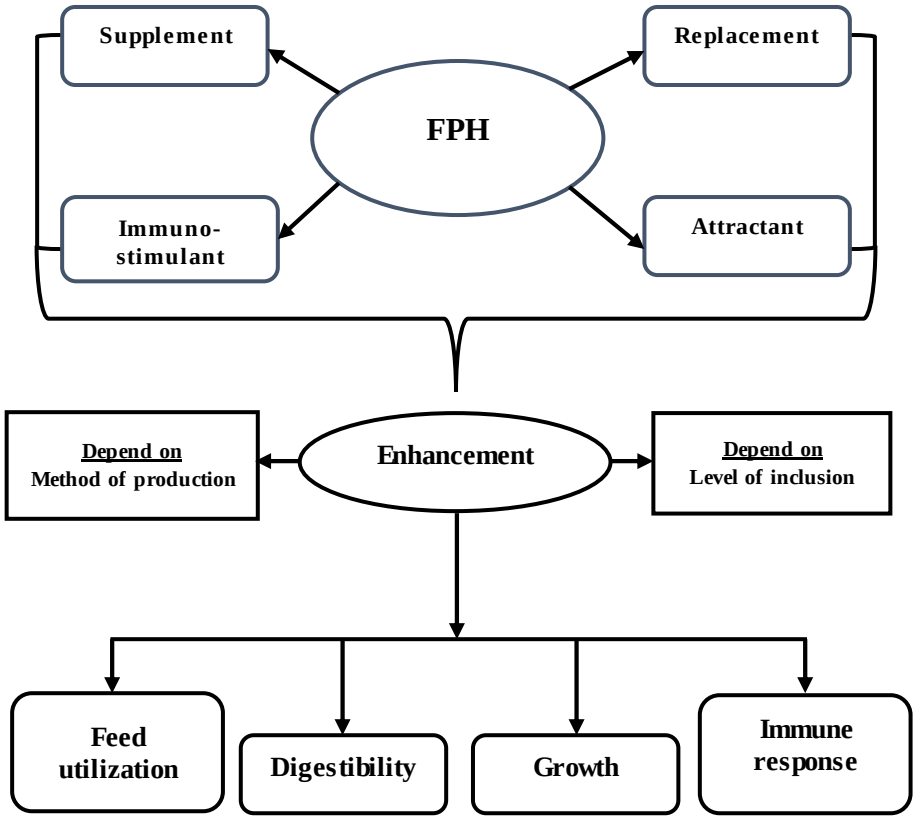


Figure 1. 2 Graphical representation to depict the possible uses and roles of FPH in aquaculture production.

Barramundi, *Lates calcarifer* is one of the economically important carnivorous marine finfish popularly cultured in many countries including Indonesia, Vietnam, Malaysia, Philippines, Taiwan, Bangladesh and Australia (Siddik et al. 2018a). This species is highly carnivorous and requires relatively higher protein levels in their diets (Table 1.1). Although the protein requirement varies with size, the highest requirement is in the early stages of life i.e. larvae and fry (NRC 2011). Like other carnivorous fish, FM is the main source of dietary protein in the diets for juvenile barramundi. Therefore, one of the major challenges to the development of barramundi aquaculture in Australia is the reduction of FM use in aqua-feeds through the provision of environmentally and economically sustainable diets that boost growth and survival. To this extent, research addressing efforts to replace FM with increased levels of animal protein ingredients for barramundi diets is gaining momentum. Until recently, most of the studies for FM

replacement have been based on untreated animal protein ingredients and the only indicators of the ingredient quality studied were fish growth performance and nutrient digestibility. Literature based on the impact of bioprocessing of these animal by-products or supplementation of nutritionally enriched FPH to animal by-products for substituting FM in marine carnivorous fish species is still very limited.

Table 1. 1 Protein requirement of barramundi, *Lates calcarifer* at different stages of life cycle.

Protein ranges estimated (% to %)	Optimal range (%)	Gross Energy range at Optima (MJ/kg)	Initial fish size (g)	Temp (ºC)	Authors
35–50	50	50	1.3	29	Catacutan & Coloso (1995a)
39–55	46–55	18.4–18.7	7.6	28	Williams & Barlow (1999)
44–65	60	20.9–22.8	8.0	28	Williams et al. (2003)
nd	46	20.2–29.6	11.8	30	Glencross et al. (2007)

nd: not defined

1.2 General and specific objectives of the study

1.2.1 General objective

The study was carried out to investigate whether processed animal protein based ingredients in diets were able to substitute FM completely or at varying levels in formulated diets without compromising the growth and health of juvenile barramundi. It was expected that the outcomes from this study would be of great importance in promoting sustainable barramundi aquaculture.

1.2.2 Specific objectives

In achieving the aims, the following specific objectives were considered:

i. To assess the efficacy of high levels of fermented and non-fermented tuna hydrolysate (TH) as a protein source on growth performance, antioxidant capacity, biochemical status and liver health in juvenile barramundi.

ii. To optimize inclusion levels and investigate whether TH is beneficial in terms of growth and immune functions for juvenile barramundi.

iii. To investigate the effect of optimized TH level with PBM on growth performance, immune response and disease resistance of juvenile barramundi.

iv. To examine the effects of improvement of PBM through fermentation on growth performance, gut health and fatty acid synthesis in juvenile barramundi.

v. To evaluate if fermentation of PBM and TH supplementation influence intestinal microbiota and immune-regulated cytokines gene expression in juvenile barramundi.

1.3 Significance

The outcomes of the proposed research will provide baseline information on the feasibility of using processed animal based protein as alternative protein sources in formulated diets for juvenile barramundi. The specific significances of the present research are detailed as follows:

i. The information on fermentation using *Saccharomyces cereviceae* (baker's yeast) and *Lactobacillus casei* (probiotic drink, Yakult®) will be useful for the refinement of practical diets for juvenile barramundi which can be used as baseline information by feed manufacturers.

ii. The research will assist in creating a better understanding of enzymatic FPH production, source and possible role in growth, feed utilization, innate immunity and disease resistances of fish.

iii. The research will contribute to increasing knowledge on barramundi growth, immunity and disease resistance under the influence of alternative animal protein based diets including TH and PBM.

iv. TH, acts as an immune stimulant, is expected to enhance growth and physiology (haematological and immune responses) of the farmed fish, may be a possible strategy to avoid/decrease antibiotic use in aquaculture production.

v. The research will support more FM to be replaced by animal protein options in aqua-feeds such as PBM, consequently relieving pressure on wild capture fisheries.

vi. Finally, the application of FPH in non-FM based diet will be considered a novel strategy for improving growth performance as well as health management of barramundi.

CHAPTER 2: Literature Review

Fish and fishery products play a critical role in food security, increasing protein supply and tackling malnutrition in a significant part of the world's population. To meet the ever-increasing global demand for fish protein, the aquaculture industry continues to expand. However, in intensive farming systems fish are exposed to higher densities that may increase stress, leading to susceptibility to diseases that may result in severe economic losses. Therefore, suitable feeding practices incorporating bioactive compounds that can stimulate the defence mechanisms of fish and achieve better growth are a priority for sustainable aquaculture development.

Recently, there are two innovative approaches have been in practice for the substituting fishmeal (FM) with animal protein based diets. The first innovative approach is to produce FPH from unutilized fish wastes or by-products in order to incorporate as concentrated protein in the aqua-feeds formulation. The second approach is to employ a fermentation technique for the selected animal protein ingredients to use as alternatives to FM.

2.1 Fish protein hydrolysates (FPH)

FPH produced from processing waste, has received considerable attention in recent years due to being environmentally friendly and safe compounds for fish production with the potential to improve immune functions and disease resistance (Bui et al. 2014; Siddik et al. 2018b). FPH produced from by-products of fish or different parts of fish viz. skin, heads, muscle, viscera, liver, bones, frames, or roe also have potential to be included in aquaculture feed as sources of amino acids, feed binding agents and palatability enhancers (Chalamaiah et al. 2012). A number of studies have demonstrated that FPH can have beneficial effects on growth, feed utilization and survival of fish at moderate inclusion levels (Hevrøy et al. 2005; Refstie et al. 2004; Siddik et al. 2018b). In addition, their bioactive compounds were reported to increase innate immune response, gut enzymatic activity, stimulation of digestibility and disease resistance (Bui et al. 2014; Murray et al. 2003). With interest in FPH and aquaculture, this review aims to summarize the sources, production processes and role

of FPH on fish growth, feed utilization, digestibility, innate immunity and disease resistance.

FPH can be a powdered or an inert liquid product obtained from fish waste or by-products by the action of enzymatic hydrolysis under accelerated conditions using proteolytic enzymes (Mackie 1982). These enzymes hydrolyse or break down the proteins into short chain peptides and free amino acids depending on the nature of the enzyme (Chalamaiah et al. 2012; Halim et al. 2016). In the hydrolysis process, the hydrophobic groups are converted to hydrophilic groups providing two-end carbonyl and amino groups that increased exposure of more polar and charged groups with water (Jemil et al. 2014). These interactions control the physicochemical properties of FPH such as solubility, emulsifying, oil binding capacity and water holding capacity as they are directly responsible for shaping the product quality. The biochemical mechanism of hydrolysis of macromolecules to peptides and amino acids is shown in Figure 2.1.1.

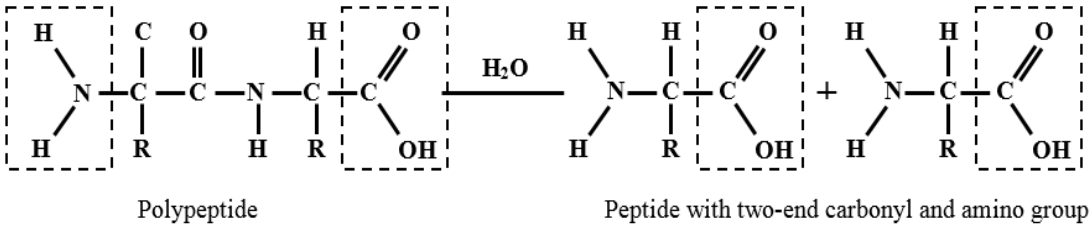


Figure 2.1.1 Schematic diagram of the major conversion pathway of macromolecules into small peptides and amino acids during hydrolysis.

2.1.1 Sources of FPH

Both marine and freshwater fish are being used to produce FPH and many studies have already been conducted using various parts of fish, including muscle, skin, fins, frames, heads, viscera, trimmings and roe, for FPH production. Dark muscle of fish rich in protein has limited consumption value due to the high possibility of oxidation and off-flavour, which may result in low consumer appeal and low market value of manufactured products (Chalamaiah et al. 2012). Therefore, many studies have been done to utilize this dark fish muscle into FPH as a highly valuable product (Nakajima et al. 2009; Naqash & Nazeer 2013; Ghassem et al. 2014; Nalinanon et al. 2011; Johannsdottir et al. 2014). Viscera from both fresh and saltwater fish have also been used to produce FPH. Numerous studies have been conducted on using this source as

raw material for FPH production (Je et al. 2009; Bhaskar et al. 2008; Aspmo et al. 2005; Ovissipour et al. 2014). Fish skin, rich of collagen and gelatin, has also been used in the fish processing industry as a good source of FPH. Several studies reported the use of fish skin from different fish species to convert into hydrolysates (Phanturat et al. 2010; Yin et al. 2010; Ngo et al. 2010). Fish heads generated from the fish processing industry are also a rich source of protein. Some previous studies have reported on the utilization of fish heads to convert into hydrolysates. These include black scabbard fish, *Aphanopus carbo* (Batista et al. 2010); red salmon, *Oncorhynchus nerka* (Sathivel et al. 2005); round sardinella, *Sardinella aurita* (Bougatef et al. 2010) and salmon, *Salmo salar* (Gbogouri et al. 2004). A considerable amount of fish bone and fish frame is discarded as by-product waste from the fish processing industry. The increasing concern of environmental pollution and decreasing trend of natural resources emphasize the need to develop potential products with this material. Several studies described the production of FPH from fish bones and fish frames of different species, such as Atlantic salmon, *Salmo salar* (Liaset et al. 2003); cod, *Gadus morhua* (Šližytė et al. 2009); pollock, *Theragra chalcogramma* (Zheng et al. 2012; Zheng et al. 2013a); ribbon fish, *Lepturacanthus savala* (Nazeer et al. 2011) and yellowfin sole, *Limanda aspera* (Rajapakse et al. 2005). Finally, some studies used the whole body parts of various fish for hydrolysates production, including herring, *Clupea harengus* (Hevrøy et al. 2005); Pacific hake, *Merluccius productus* (Ho et al. 2014) and pollock, *Theragra chalcogramma* (Refstie et al. 2004; Tang et al. 2008).

2.1.2 Production of FPH

There are several methods used to produce FPH, including enzymatic hydrolysis, chemical hydrolysis (acid and alkaline hydrolysis), autolysis and bacterial fermentation. Among them, enzymatic hydrolysis and chemical hydrolysis are the most commonly used by researchers due to a number of advantages. The chemical hydrolysis process is low cost, rapid and results in a high protein recovery. But this process operates with little control on the consistency of the hydrolysed products, with large variations in free amino acid profile and with non-specific breakdown of peptide bonds resulting in weak functional properties (Celus et al. 2007). The autolysis process regulated by the action of endogenous digestive enzymes in the fish. But these endogenous enzyme concentrations vary greatly within a species and between species, as well as being highly seasonal and age specific, resulting in end products of

inconsistent molecular profiles (Kristinsson & Rasco 2000). Bacterial fermentation favors the growth of lactic acid bacteria that produces acid and antimicrobial factors inhibiting competing bacteria, but under this method removal of lipid is quite impossible (Kristinsson & Rasco 2000). With the aim of producing better quality FPH, enzymatic processing is widely accepted to produce precise hydrolysates retaining the nutritive value of the source protein (Zamora-Sillero et al. 2017). This process works with a shorter reaction time and is beneficial for target specific peptide bond and amino acids with optimal activity at specific conditions. Furthermore, enzymatic hydrolysis does not produce any residual organic solvents and toxic chemicals in the end products (Najafian & Babji 2012). Due to these advantages, the present review has focussed on production of FPH based on enzymatic processes.

There are several proteolytic enzymes including alcalase, neutrase, flavourzyme, protamex, papain, pepsin, and trypsin are commonly used to produce FPH (Kristinsson & Rasco 2000) in which alcalase, an alkaline enzyme obtained from *Bacillus licheniformis*, has been found to be a highly efficient enzyme for FPH production due to its high extraction ability under mild conditions and able to produce FPH with small-sized peptides in a relatively short period (Kristinsson & Rasco 2000).

In the enzymatic process, fish by-products and wastes are minced and homogenized with water (2:1 w/w) before being transferred to the reactor vessel where it is heated to the appropriate temperature. FPH should have well-controlled fat content (< 0.5% w/w) as higher fat content may result in darkening of the final products due to lipid oxidation, producing brown pigments (Kristinsson & Rasco 2000). De-fatting is therefore required for fatty fish before mixing with water and commonly organic solvents are used for this purpose. Treatment with organic solvents reduces extra fat as well as minimizing bacterial degradation (Kristinsson & Rasco 2000). As enzyme type, enzyme concentration, temperature, pH and time are influential parameters affecting product quality and function (Srichanun et al. 2014), it is necessary to optimize these parameters during the production process. The suitable ranges of these parameters include temperature (35–60°C), time (10–600 min), pH (1.5–11), and enzyme concentration (0.01–5.00%) for the various enzymes used to produce FPH (Razali et al. 2015). The optimized enzymatic hydrolysis conditions stated in various studies are presented in Table 2.1.1 and Figure 2.1.2.

Table 2.1.1 Enzymatic process to produce FPH from different fish and parts of fish body using different enzymes

Fish species	Fish part to produce FPH	Enzyme to produce FPH	Optimized conditions				Outcomes	Reference
			Enzyme conc. (%)	pH	Time (min)	Temperature (^{0}C)		
Sturgeon, *Acipenser persicus*	Viscera	Alcalase	0.1 AU/g	8.5	30, 60, 120, 180, 205	35, 45 and 55	The highest degree of hydrolysis was observed at 55°C after 205 min	Ovissipour et al. (2009)
Beluga, *Huso huso*	Viscera	Protamex	14, 22, 34, 46, 55	-	53, 80, 120, 60, 187	33, 38, 45.5, 53, 58	The optimum conditions to reach the highest degree of hydrolysis were: 39.21°C, 114.2 min, and a protease (protamex) activity of 27.41 AU/kg protein.	Molla & Hovannisyan (2011)
Eel, *Monopterus* sp.	Flesh	Alcalase	1.8 0.5, 1.5, 2.5	9.00 7,8,9	-	55.76 40, 55, 70	The optimum conditions were temperature of 55.76°C, enzyme concentration of 1.80% and pH of 9.0.	Jamil et al. (2016)
Tuna	By-product	Alcalase Neutrase	1.0 0.5, 1, 1.5	8.50 7,8,9	60.00 40, 100, 160	55 40, 50, 60	The optimum condition with alcalase at temperature 55°C, time 60 min, 1% enzyme concentration and pH 8.5	Saidi et al. (2013)
Small-spotted catshark, *Scyliorhinus canicula*	Discards	Alcalase Esperase	0.5	6.0-12	37.3-80	60.8	The optimal conditions for the highest proteolysis were established with esperase in 60.8°C and pH 8.9	Vazquez et al. (2017)

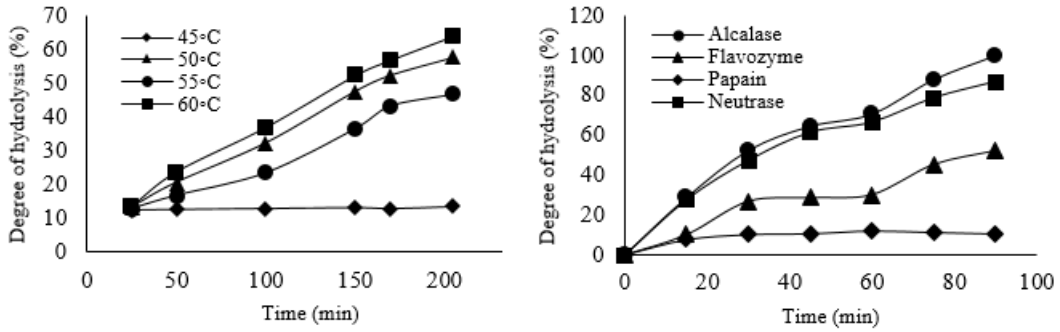


Figure 2.1.2 Effect of temperature and enzymes on degree of hydrolysis of fish by-product proteins by alcalase and papain enzymes

In the hydrolysation step, the mince water slurry is subjected to homogeneous mixing with the selected enzyme. The enzyme selection plays a crucial role in production process as it allows better control of the hydrolysis process as well as the resulting product. A number of studies have reported that enzymes with microbial origin have a close to neutral pH reaction range (7–9), such as alcalase, neutrase and flavourzyme, have been using extensively for producing FPH due to several advantages including temperature stabilities, greater pH range and a wide variety of catalytic activities resulting in products of high quality and nutritive value. Enzymes derived from animal sources such as pepsin, or plant sources such as papain, in the acidic pH range may lead to low protein recoveries with nutritional values due to the damaging of the essential amino acid and low functionalities due to excess hydrolysation (Kristinsson & Rasco 2000). The production of FPH also varies depending on the applied enzymes. For instance, the highest protein recovery of 70.5% was produced by alcalase compared to 57.6% with neutrase and 57.1% with papain from capelin, *Mallotus villosus*.

Hydrolyzation time and processing temperature are chosen according to the preferred protein recovery and functionalities of the final product. The size of the peptide fractions decreases with increased temperature and enzyme concentration until the temperature reaches the point of enzyme denaturation (Jamil et al. 2016). The hydrolyzation is terminated by inactivating the exogenous enzymes using heat at 85-95°C for 5-20 min (Ghassem et al. 2014). Liquid FPH may be dried in the recovery step to generate a powder form, because liquid hydrolysates can spoil quickly. FPH in

powder form can be stored for a longer period of time and is easier to transport (He et al. 2013). The hydrolysed sample is centrifuged before drying. Centrifugation (speed: 4000 rpm and time: 30 min) separates the sample into three layers: a semisolid layer at the bottom, the hydrolysed solution in the middle and a layer of fat on the top (Figure 2.1.3). After removing the fat layer the hydrolysed protein solution is transferred carefully without mixing with the bottom semi-solid layer. Then the protein hydrolysis solution is freeze dried and the creamy white final product is stored at 4°C or lower, occasionally with vacuum packaging. The process and optimized condition to produce FPH from fish by-products is presented in Figure 2.1.4.

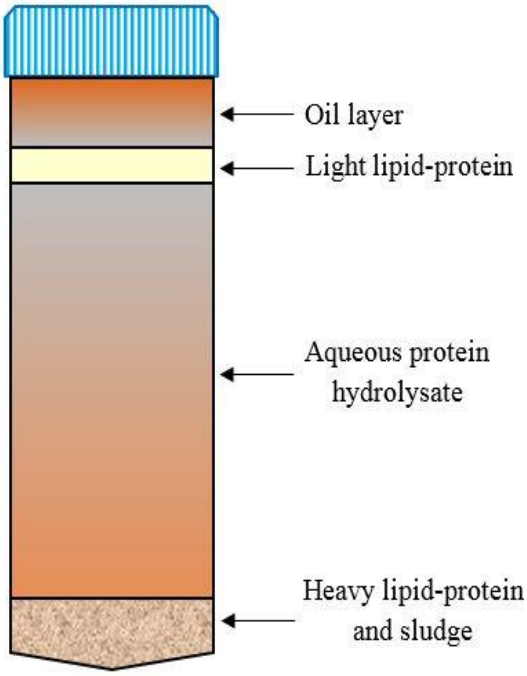


Figure 2.1.3 Fractions of soluble FPH produced from fish by-products using alcalase enzyme.

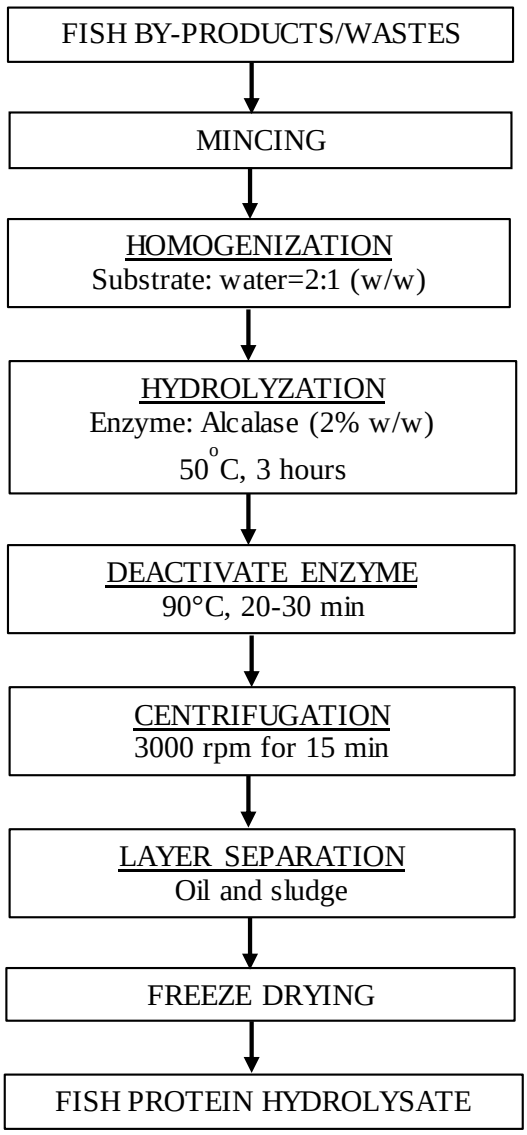


Figure 2.1.4 Schematic diagram of the processing methods of FPH from Australasian snapper, *Pagrus auratus* by-product using alcalase enzyme.

Size and molecular weight of peptides are considered to have significant effect on the biological activities of FPH. FPH with low molecular weight peptides are more easily absorbed and assimilated by the gastrointestinal tract of animals than FPH with higher molecular weight peptides (Chi et al. 2015). Several studies recommend gel filtration (GF), nanofiltration (NF) and ultrafiltration (UF) to refine hydrolysates and to increase

efficiency of by-products to make bioactive dietary ingredients for human and animal consumption (Picot et al. 2010). Pressure driven membrane separation is sometimes used to separate peptides into different size groups to increase the performance of specific activities (Bourseau et al. 2009). In line with the molecular weight cut-offs (MWCO), NF and UF of protein hydrolysates are recommended depending on the different outcome required. NF can be used to concentrate hydrolysed products, while high MWCO (20-100 kDa) of UF membranes can be used to separate hydrolysed peptides from native proteins and proteolytic enzymes. UF with intermediate MWCO (~4-8 kDa) allows fractionation of the peptide chain to enrich specific molecular sizes in the hydrolysate (Bourseau et al. 2009). Fast performance liquid chromatography (FPLC) may be used in GF to obtain small molecular weight (≤3 kDa) peptides (Centenaro et al. 2014). According to a report by Mahmoodani et al. (2014), fish waste derived bioactive peptides molecular weight ranges between 200 and 2000 Da. Similarly, Sarmadi & Ismail (2010) reported that most purified peptide molecules from fish by-products ranges from 2 to 20 amino acids in sequence and generally smaller in molecular size. However, Ngo et al. (2014) reported that the molecular weights for bioactive hydrolysate peptides is less than 3000 Da. A typical procedure for the isolation of bioactive peptide from FPH is showed in Figure 2.1.5.

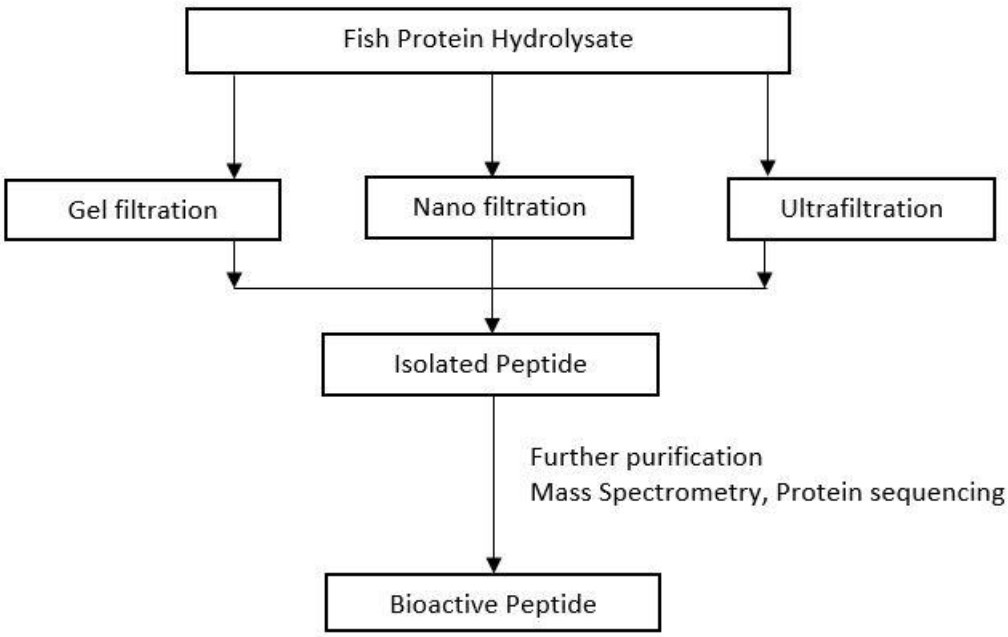


Figure 2.1.5 Diagram of representing procedures for isolation and characterisation of bioactive peptide from FPH.

A number of studies have revealed that fish derived peptides have a myriad of bioactive potential including antihypertensive, antioxidative, antimicrobial and anti-inflamatory activity depending on the molecular weights of the peptides (Ishak & Sarbon 2017; Zamora-Sillero et al. 2017). For instance, the angiotensin converting enzyme (ACE) inhibitory (antihypertensive) activity of (*Channa striatus*) protein hydrolysate increased from 0.058 mg/ml with a molecular weight 10 kDa to 0.033 mg/ml with a molecular weight 3 kDa (Ghassem et al. 2014), while the antioxidant activity of tuna by-product hydrolysates protein increased from 11% with a molecular weight <4 kDa to 75% with a molecular weight <1 kDa (Saidi et al. 2014). According to Najafian & Babji (2012) peptides derived from animal muscles which have a molecular weight below 10 kDa and less than 50 amino acids in sequence, exhibit antimicrobial activity. A study of Ahn et al. (2015) reported that the highest anti-inflamatory activity from salmon by-product protein hydrolysates was derived with the molecular weight between 1000 and 2000 Da.

2.1.3 Nutritional composition of FPH

The awareness on nutritional composition of any food materials is of vital importance in order to maintain good human health as well as for the assessment and application of food ingredients in a food production system. The proximate composition of FPH produced from various fish parts is displayed in Table 2.1.2. As shown in Table 2.1.2, the protein contents of FPH ranged from 60% to 90% in various fish species. The high protein content of FPH is due to the solubilisation of protein during enzymatic hydrolysis and the removal of insoluble fractions by centrifugation (Chalamaiah et al. 2012). However, the protein content of the FPH is varied with the temperature used for drying in the production process. According to Abdul-Hamid et al. (2002) the crude protein content of FPH was decreased to 23.9% when the drying temperature was increased from 150 ^{0}C to 180 ^{0}C.

There are a number of studies demonstrated that lipid content of the FPH is less than 5% of total composition (Abdul-Hamid et al. 2002; Bhaskar et al. 2007; Ovissipour et al. 2009; Pacheco-Aguilar et al. 2008). The low lipid content of FPH may be due to removal of fat and insoluble protein fractions by centrifugation (Chalamaiah et al.

2012). Several studies demonstrated that the ash content of FPH ranged between 0.45% and 27% of total composition (Chalamaiah et al. 2012; Choi et al. 2009; Yin et al. 2010). The high ash contents in FPH may be due to the addition of alkali for pH adjustment and/or largely contributed by bones in the raw material (Batista et al. 2010; Choi et al. 2009). Many scientists reported the moisture content of FPH below 10% (Bhaskar et al. 2008; Chalamaiah et al. 2010). The low moisture content of FPH may be because of sample type and the temperatures employed during freeze the drying process (Chalamaiah et al. 2012).

The amino acid contents of any food materials has a substantial role in various biological activities such as giving cells their structure, carriers of oxygen, CO_2, enzymes and serve as optimal storage of all nutrients including proteins, lipids, carbohydrates, minerals, vitamins and water. The essential and non-essential amino acids needed for good health have been found abundant in FPH (Sathivel et al. 2003; Yin et al. 2010). However, the FPH have been described in many studies to exhibit variation in their amino acid content (Wasswa et al. 2007). The disparity in amino acid contents of various FPH depends on several factors such as the raw material for producing the hydrolysate, enzyme used for hydrolysis, and the conditions and duration of hydrolysis (Klompong et al. 2007).

Table 2.1.2 The nutritional composition of FPH prepared from finfish and their body parts with various enzymes

Fish species	Fish parts to produce FPH	Applied enzymes	Nutritional composition (%)				Reference
			Protein	Lipid	Ash	Moisture	
Persian sturgeon, *Acipenser persicus*	Viscera	Alcalase 2.4 L	65.82 ± 7.02	0.18 ± 0.4	7.67 ± 1.24	4.45 ± 0.67	Ovissipour et al. (2009)
Atlantic salmon, *Salmo salar*	Head	Alcalase 2.4 L	82.3 ± 1.9	0.8 ± 0.02	10.4 ± 1.1	5.3 ± 0.2	Gbogouri et al. (2004)
Tuna	By-product	Flavourzyme	66.40 ± 0.27	2.37 ± 0.52	25.94 ± 0.04	7.25 ± 0.09	Nilsang et al. (2005)
Pacific whiting, *Merluccius productus*	Muscle	Autolysis	85.6 ± 2.3	0.3 ± 0.1	16.6 ± 0.3	2.5 ± 0.6	Mazorra-Manzano et al. (2010)
Small-spotted catshark, *Scyliorhinus canicula*	Muscle	Alcalase Esperase & Protamex	89 ± 0.46	0.35 ± 0.06	1.11 ± 0.06	7.79 ± 0.70	Vazquez et al. (2017)
Tuna, anchovy and wild fish	Tuna frame, anchovy & wild fish in a proportion of 5:4:1	Papain & Bromelain	69.94	1.77	17.5	3.03	Wu et al. (2018)

2.1.4 Role of FPH in aquaculture production

Based on published information, the potential effects of FPH on growth performance, feed utilization, digestibility, nutrient composition, hematological indices, immunological parameters and disease resistance in fish are summarized below.

2.1.4.1 Growth performance

A summary of studies carried out using FPH and their effect on growth performance indices such as final body weight (FBG), weight gain (WG) and specific growth rate (SGR) are illustrated in Table 2.1.3. The use of FPH for improving growth performance of fish has been well documented. Improved growth performance, when FM is replaced by fish hydrolysate, may be due to increased palatability of the feed (Hevrøy et al. 2005; Refstie et al. 2004; Siddik et al. 2019b). Aksnes et al. (2006b) demonstrated improved growth performance of Atlantic cod, *Gadus morhua* when one third of FM was replaced with fish hydrolysate. Wei et al. (2016) conducted an investigation to demonstrate the effect of ultra-filtered and non- ultra-filtered FPH in turbot, *Scophthalmus maximus* juveniles. Fish fed a FM diet and 10% ultra-filtered fish hydrolysate diet showed increased WG and SGR compared with the other diets of 5%, 15% and 20% ultra-filtered fish hydrolysate. Also, improvement in growth performance of red sea bream, *Pagrus major* larvae was observed using krill and shrimp hydrolysates in white FM diets compared to FM based diet as control (Bui et al. 2014). Similar results for the same species were also reported by Khosravi et al. (2015b) using protein hydrolysates in a low FM diet. For Japanese sea bass, *Lateolabrax japonicus* dietary inclusion of FPH at a moderate level (15%) achieved better growth performance than lower and higher levels (Liang et al. 2006). Moreover, the effects of FPH produced from pollock, *Theragra chalcogramma* on the growth performance of large yellow croaker, *Pseudosciaena crocea* demonstrated that the diets containing 10% and 15% hydrolysate showed a significant increase in weight gain and specific growth rate compared with the FM diet (Tang et al. 2008). Improved growth performance at moderate FPH inclusion level was also reported in turbot, *Scophthalmus maximus* by Zheng et al. (2013a). They found that 11% FPH added to the diet resulted in higher growth performance than low FM based diet and the same performance as those fed the high FM based control diet in Japanese flounder, *Paralichthys olivaceus*. Zheng et al. (2012) demonstrated the effect of FPH and ultra-filtered FPH on growth performance of juvenile Japanese flounder, *Paralichthys*

olivaceus using four experimental diets designed as FM diet, FPH (non- ultrafiltered) diet and two ultra-filtered diets (UF1 and UF2, contained small molecular compounds). Results of this investigation indicated that the diet containing the higher proportion of small molecular weight peptides UF1 attained the best overall growth of experimental fish compared to FM and other groups. The same authors also found the identical response of FPH and ultra-filtered FPH in turbot, *Scophthalmus maximus* juveniles i.e. that the small molecular weight peptides resulted in higher growth performance (Zheng et al. 2013a). The higher growth in ultra-filtered hydrolysate group suggests that small molecular weight fractions in fish hydrolysate are beneficial for growth response (Aksnes et al. 2006b).

However, Xu et al. (2016) in assessing the growth performance of juvenile turbot, *Scophthalmus maximus* showed that the replacement of 10% FM protein with FPH showed no significant effect on fish growth, but the replacement of 20% FM protein with FPH reduced the growth performance. Aksnes et al. (2006b) conducted an experiment to evaluate the effect of FPH and ultra-filtered FPH on different bio-functional parameters of rainbow trout and the findings showed significantly negative correlation with dietary inclusion of fish hydrolysates in substitute of FM. Findings in Atlantic salmon, *Salmo salar* also found no improvement on growth performance using FPH in partial replacement of regular FM (Berge & Storebakken 1996).

2.1.4.2 Feed utilization

The studies carried out using FPH and their effect on feed intake and utilization in various fish species is summarized in Table 2.1.3. Bio-functional properties of FPH and their active compounds in facilitating better feed intake and feed utilization have been reported in many fish species. The affirmative effects of fish hydrolysates on fish performance are related to palatability enhancing digestive enzymes and high biomass production (Aguila et al. 2007; Oliva-Teles et al. 1999). Feed palatability is often connected with the availability of small molecular weight peptides and free amino acids in hydrolysed protein (Kasumyan & Døving 2003). Hevrøy et al. (2005) studied Atlantic salmon, *Salmo salar* and reported that the dietary use of FPH at different inclusion levels of 0, 6, 12, 18, 24 and 30% hydrolysates, in exchange of low-temperature-dried FM influenced both feed intake and utilization. Fish fed with 18% and 24% FPH showed higher feed intake than fish fed with lower and higher levels of FPH. The significantly increased feed conversion ratio (FCR) and decreased protein

efficiency ratio (PER) were found in fish fed increased levels of FPH. According to Hevrøy et al. (2005), the inclusion of FPH can increase the absorption of amino acids and protein, but higher inclusion levels of FPH, resulted in oxidation and produced energy being stored in the body tissues, thus reducing anabolism availability and affecting feed utilization. The diverse results of hydrolysate diets may be related to the size of peptide fractions that originated from different filtration. Small fractions of low molecular weight peptides in fish diet are correlated with feed utilization. It has been demonstrated that the low molecular weight fractions of fish hydrolysates can increase the utilization of amino acids by reducing gluconeogenesis (Li et al. 2009; Wei et al. 2016). Zheng et al. (2013a) tested two ultra-filtered (UF) (molecular weight <1000 Da) and non-UF fish hydrolysate in turbot, *Scophthalmus maximus* and found the highest feed utilization in fish fed with higher dose of UF compared to non-UF fish hydrolysate and the control. The higher feed utilization in UF hydrolysate group suggests that small molecular weight fractions in fish hydrolysate are beneficial for feed utilization (Aksnes et al. 2006b; Aksnes et al. 2006c). In another study, turbot, *Scophthalmus maximus* fed a diet containing 12.4% graded level of FPH by the replacement of 20% FM protein showed an increase in feed intake compared to fish fed diets containing no FPH and 3.1% FPH. Besides, replacement of 10% and 20% FM with FPH significantly lowered feed efficiency ratio compared to turbot, *Scophthalmus maximus* fed with FM based diet. Though, compared to FM based diet, 5% and 10% replacement of FM with FPH showed no significant effect on protein efficiency ratio and protein retention, but 20% FM replacement showed a significant reduction (Xu et al. 2016). Refstie et al. (2004) reported that the post-smolt Atlantic salmon, *Salmo salar* showed higher feed consumption in fish groups fed 10% and 15% FPH than that of fish groups fed 0% FPH (FM based diet) and 5% FPH, with no remarkable differences on feed efficiency ratio among dietary groups. In another investigation, dietary UF FPH was significantly correlated with feed efficiency, protein productive value and protein efficiency ratio, but not significantly correlated with feed intake (Wei et al. 2016). In the study it was demonstrated that the high-level UF FPH showed positively lowest feed efficiency but the low-level UF FPH showed positive correlation with protein productive value and protein efficiency ratio.

Khosravi et al. (2015b) found significant improvement in feed utilization when red sea bream, *Pagrus major* were fed hydrolysate diets when compared to low FM diet.

However, mozambique tilapia, *Oreochromis mossambicus* fed a low level of FPH from marine by-products showed a lower FCR when compared to the fish fed FM based control diet (Goosen et al. 2015). Zheng et al. (2012) found no significant difference in feeding rate of Japanese flounder, *Paralichthys olivaceus* fed with FM, UF fish hydrolysate or non-UF fish hydrolysate based diet. But fish fed with UF fish hydrolysate (37 g/kg) and non-UF fish hydrolysate showed higher feed efficiency and protein efficiency ratio compared to fish fed with UF fish hydrolysate (12 g/kg), where, fish fed with UF fish hydrolysate (37 g/kg) had significantly higher protein retention efficiency compared to fish fed with UF fish hydrolysate (12 g/kg) and FM based diet. However, Oliva-Teles et al. (1999) did not find any improvement of using FPH on growth or feed utilization in juveniles turbot, *Scophthalmus maximus*.

Table 2.1.3 The effect of FPH inclusion on growth performance, feed utilization and digestibility on farmed finfish

Tested species	Source of hydrolysate	Enzyme used for preparing hydrolysate	Inclusion level (%)	Duration of growth trial	Responses	References
Turbot, *Scophthalmus maximus*	By-products of pollock, *Theragra chalcogramma*	-	UF 5, 10, 15, 20	68 days	(↓) Final weight, specific growth rate & protein efficiency ratio at 20% (↔) Feed intake, condition factor & survival (↑) ADC protein (↓) ADC dry matter at 20%	Wei et al. (2016)
Atlantic salmon, *Salmo salar*	Whole herring	Alcalase	6, 12, 18, 24, 30	68 days	(↑) Specific growth rate at 24 & 30% (↑) Thermal growth coefficient at 24% (↔) Final weight, weight gain, condition factor & survival (↑) Feed conversion ratio (↓) Protein efficiency ratio (↑) ADC protein (↔) ADC lipid & dry matter	Hevrøy et al. (2005)
Japanese flounder, *Paralichthys olivaceas*	Frames of pollock, *Theragra chalcogramma*	Alcalase & flavourzym	UF 3.7 & 1.2 NUF 3.7	60 days	(↑) Final body weight, specific growth rate at UF 3.5% (↑) Feed efficiency both at UF % & NUF 3.5% (↑) ADC protein (↑) ADC dry matter at UF 3.7% (↓) Protein efficiency ratio (↔) Survival & feeding rate per day	Zheng et al. (2012)

Japanese flounder, *Paralichthys olivaceas*	Frames of pollack, *Theragra chalcogramma*	Alcalase & flavourzym	6, 11, 16, 21, 26	63 days	(↑) Final body weight, Specific growth rate at 11, 16 & 21% (↑) Feeding rate & feed efficiency ratio at 26% (↑) ADC protein (↔) Survival	Zheng et al. (2013b)
Turbot, *Scophthalmus maximus*	Frames of pollock, *Theragra chalcogramma*	Alcalase & flavourzym	UF 3.7, 1.2 NUF 3.7	8 weeks	(↑) Final body weight, specific growth rate at UF 1.5% (↑) Feed efficiency at UF 3.5% (↑) ADC protein & dry matter at UF 3.7% (↑) Protein efficiency ratio at UF 3.7% (↔) Hepatosomatic index	Zheng et al. (2013a)
Turbot, *Scophthalmus maximus*	By-products of pollock, *Theragra chalcogramma*	-	5, 10, 20	12-week	(↓) Specific growth rate at 20% (↑) Feed intake at 20% (↑) ADC protein & dry matter at UF 3.7% (↑) Protein efficiency ratio at UF 3.7% (↔) Hepatosomatic index	Xu et al. (2016)
Atlantic salmon, *Salmo salar*	Raw body parts of pollock, *Theragra chalcogramma*	Protamex	5,10,15	68 days	(↑) Specific growth rate, thermal-unit growth coefficients, body weights & feed intake at 10 &15% (↑) ADC nitrogen, lipid & energy (↔)ADC protein & feed efficiency ratio	Refstie et al. (2004)

Sea bream, *Pagrus major*	Whole Antarctic krill, *Euphausia superba* Farmed shrimp, *Litopenaeus vannamei* co-products Farmed tilapia co-products	-	SH 4.80 TH 4.23 KH 4.03	12 weeks	(↑) Final body weight, specific growth rate, protein efficiency ratio SH & KH (↓) Feed conversion ratio (↑) ADC protein (↔) ADC dry matter, survival & feed intake	Bui et al. (2014)
Red sea bream, *Pagrus major*	Tuna co-products Whole Antarctic krill, *Euphausia superba*	-	TH 2% KH 2%	12 weeks	(↑) Final body weight with KH (↓) Feed conversion ratio with KH (↑) ADC protein (↔) Specific growth rate, feed intake, protein efficiency ratio, ADC dry matter & survival	Khosravi et al. (2015a)
Olive flounder, *Paralichthys olivaceus*	Fresh tuna co-products Whole Antarctic krill, *Euphausia superba*	-	TH 2% KH 2%	9 weeks	(↑) Final body weight with KH (↓) Feed conversion ratio with KH (↑) ADC protein & ADC dry matter (↔) Specific growth rate, feed intake, protein efficiency ratio & survival	Khosravi et al. (2015a)
Pike silverside, *Chirostoma estor*	Fillets, *Scomberomorus regalis* & shrimp tails, *Pennaeus* sp.	-	15, 30, 45	8 weeks	(↓) Weight gain & specific growth rates at 30 & 45% (↑) ADC lipid & ADC dry matter at 30% (↔) Feed intake, feed conversion ratio, protein efficiency ratio & ADC protein	Ospina-Salazar et al. (2016)

Japanese sea bass, *Lateolabrax japonicus*	Gut & head of pollock, *Theragra chaloogramma*	Protease	5, 15, 25	60 days	(↑) Final body weight at 15 & 25% (↑) Specific growth rate at 15% (↑) Feed conversion ratio at 15 & 25%	Liang et al. (2006)
Yellow croaker, *Pseudosciaena crocea*	Tissues of pollock, *Theragra chalcogramma*	Flavourzyme & Alcalase	5, 10, 15	8 weeks	(↑) Weight gain (↑) Specific growth rate at 10 & 15%	Tang et al. (2008)
Persian sturgeon, *Acipenser persicus*	Yellowfin tuna, *Thunnus albacares* viscera	Alcalase	10, 25, 50	54 days	(↑) Final body weight, weight gain at 10 & 25% (↔) Survival & condition factor	Ovissipour et al. (2014)
Olive flounder, *Paralichthys olivaceus*	Yellowfin tuna, *Thunnus albacares* & skipjack tuna, *Katsuwonus pelamis* by-products	-	5, 10, 20, 30, 40, 60, 80, 100	7 weeks	(↑) Weight gain (↑) Specific growth rate	Kim et al. (2014)

Note: Increase, ↑; decrease, ↓; no change, ↔; compared to the control diet ($P<0.05$). -; not mentioned. ADC, apparent digestibility coefficient; UF, ultrafiltered fish hydrolysate; NUF, non-ultrafiltered fish hydrolysate; SH, shrimp hydrolysate; TH, tilapia hydrolysate; KH, krill hydrolysate.

2.1.4.3 Nutrient digestibility

The studies carried out using FPH and their effect on nutrient digestibility of fish are illustrated in Table 2.1.3. A number of studies were conducted on the effect of FPH on nutrient digestibility at the juvenile or larval stages of fish since the digestible peptide fractions in hydrolysates are beneficial for the early stages of fish having less developed digestive system (Cahu & Zambonino Infante 1995; Cai et al. 2015; Delcroix et al. 2015; Masuda et al. 2013; Ovissipour et al. 2014). Apparent digestibility coefficients (ADC) of nutrients tends to increase with dietary inclusion of FPH containing small molecular weight fractions. Turbot, *Scophthalmus maximus* juveniles fed UF and non-UF FPH diets showed higher apparent digestibility coefficient of dry matter and protein than fish fed with FM based diet (Zheng et al. 2013a). Similarly, (Khosravi et al. 2015a) found improvement in dry matter, protein and lipid digestibility when red sea bream, *Pagrus major* was fed with hydrolysate diets when compared to low FM diet and the protein digestibility was the best in fish fed high FM diet and shrimp hydrolysate diet. With the same species, Bui et al. (2014) found significantly higher ADC of protein in dietary inclusion of tilapia hydrolysates, however, in case of ADC of dry matter, no significances were revealed among dietary groups. Enhanced ADC of nutrients by dietary inclusion of fish hydrolysates may be due to their higher absorption rate (Hevrøy et al. 2005; Kotzamanis et al. 2007; Liang et al. 2006; Zheng et al. 2012; Zheng et al. 2013a), as the functional properties of the supplementary proteins are improved by hydrolysis processes (Chalamaiah et al. 2012). Improved ADC of dry matter and protein in Japanese flounder, *Paralichthys olivaceus* due to dietary inclusion of FPH was reported by Zheng et al. (2012). They found that ADC of dry matter and protein were significantly higher in fish fed with UF fish hydrolysate (37 g/kg) diet compared to the fish group fed with the FM diet. Although more investigations are necessary to explain the particular mechanism for improved ADC of dry matter by dietary inclusion of hydrolysates, it could be predicted that the molecular form of protein in hydrolysate could positively affect the assimilation of dietary protein by increasing expression of intestinal amino acids and/or peptide transporter gene as presented in fish by Bakke et al. (2010) and in chicken by Gilbert et al. (2010). However, Oliva-Teles et al. (1999) found no significant effect between FM based diet and partially replaced FPH diets on apparent digestibility coefficients

of dry matter, protein and energy of juvenile turbot, *Scophthalmus maximus*. Tonheim et al. (2007) explained the negative relation of FPH on protein digestibility by suggesting that the nitrogenous compounds in FPH are not as digestible as in FM protein.

2.1.4.4 Body composition

The body proximate composition of cultured fish is affected by exogenous (e.g., diets, environmental conditions such as temperature, salinity) and endogenous (e.g., life cycle stage, sex, fish size) factors (Chatzifotis et al. 2010; Cook et al. 2000; Shearer 1994). Turbot, *Scophthalmus maximus* fed with UF FPH diet and non-UF FPH diet showed comparatively higher protein content than fish fed with FM, although no effect was observed on dry matter, lipid and ash contents of fish (Zheng et al. 2013a). The results of body composition of Persian sturgeon, *Acipenser persicus* larvae suggested that the groups fed tuna viscera hydrolysate diets substituted for 10% and 25% FM had the maximum protein content (Ovissipour et al. 2014). In contrary, Xu et al. (2016) reported that turbot, *Scophthalmus maximus* fed with 20% FM replaced by FPH diet had lower protein and lipid content and higher moisture content, but no effect was observed on ash content. However, Wei et al. (2016) found no significant effect of different dietary UF FPH on body moisture and protein content in the same species turbot, *Scophthalmus maximus*. Similarly, Bui et al. (2014) found no significant differences in proximate composition of juvenile red sea bream, *Pagrus major* fed hydrolysate based diets and FM based diet. Refstie et al. (2004) reported that post-smolt Atlantic salmon, *Salmo salar* showed higher whole-body energy content in fish groups fed 10% and 15% FPH than that of fish groups fed 0% FPH (FM based diet) and 5% FPH, and no significant differences on crude protein content among dietary groups were observed. A study on olive flounder, *Paralichthys olivaceus* showed no significant difference on fish body composition including moisture, protein, lipid and ash contents among different dietary treatments of FM and hydrolysates (Khosravi et al. 2017). Similarly, no significant effect of FPH on whole body composition of Japanese flounder, *Paralichthys olivaceus* in terms of dry matter, ash content and crude lipid was found, but, crude protein of fish fed with ultrafiltered fish hydrolysate (37 g/kg) diet was significantly higher ($P<0.05$) than control diet (Zheng et al. 2012).

2.1.4.5 Hematological indices

A summary of the effect of FPH on hematological responses of fish is presented in Table 2.1.4. Hematological parameters are considered as vital physiological indicators for assessing general health and nutritional status of fish (Siwicki et al. 1994b; Vazquez & Guerrero 2007). A number of studies reported that the improved functional properties of FPH result from the presence of biologically active peptides (Gildberg et al. 1996; Halim et al. 2016; Harnedy & FitzGerald 2012; Hermannsdottir et al. 2009; Kotzamanis et al. 2007; Ovissipour et al. 2014). A study on red sea bream, *Pagrus major* showed that replacement of low FM diet by fish hydrolysates led to an increase of hematocrit, hemoglobin, total protein and cholesterol levels, and the measured decrease of plasma glucose and triglyceride levels may indicate that the dietary inclusion of FPH leads to better absorption of the hydrolysed protein and enhancement of the general health condition of fish (Khosravi et al. 2015b). This contrasted with the results of another study on the same species, which found no significant differences in the hematological parameters of fish fed diets containing hydrolysates including assessment of total protein, haematocrit, hemoglobin, glucose, total cholesterol and triglyceride influenced by the experimental diets (Bui et al. 2014). Goosen et al. (2015) found no significant effect of FPH on hematocrit and total protein level in mozambique tilapia, *Oreochromis mossambicus*. A study on juvenile coho salmon, *Oncorhynchus kisutch* also found no significant differences on hematocrit, leucocrit and total plasma protein level between FM and hydrolysate dietary groups (Murray et al. 2003). This inconsistency in results might be due to a number of factors including fish size, experimental conditions and handling methods, as these factors may strongly affect the physiology of fish (Chatzifotis et al. 2010).

2.1.4.6 Immunological parameters

FPH stimulate various immune-hematological parameters in fish and a summary of these results are presented in Table 2.1.4. Lysozyme, phagocytes and complement activities are considered as important non-specific immune indices in fish (Murray et al. 2003). Particularly, lysozyme, the leucocytic origin's mucolytic enzyme, is considered as a vital indicator for the body immune response (Saurabh & Sahoo 2008). It acts against viral, bacterial and parasitic infections, and higher level of its activity is found in fish blood as a response to infection (Puangkaew 2004). It is also found as essential defense components for all vertebrates and invertebrates (Song et al. 2006).

Phagocytes acts as antibacterial response to remove dead and dying cells to maintain healthy tissues (Ellis 1999). Fish complement activity (ACH50) has been found to have capacity to fight against foreign organisms and lyse foreign cells for destruction (Gasque 2004). Immunoglobulins are considered as one of the major body protection parameters for animals and humans, particularly for the teleost (Cuesta et al. 2004; Ross et al. 1998; Watts et al. 2001).

Dietary inclusion of FPH in fish diets may trigger the immune system of fish (Khosravi et al. 2015b; Kotzamanis et al. 2007; Murray et al. 2003). Previous studies have demonstrated that the partial replacement of FM with FPH can improve the fish immunity (Bøgwald et al. 1996; Bui et al. 2014; Gildberg et al. 1995; Kotzamanis et al. 2007; Liang et al. 2006; Tang et al. 2008). A study on Japanese sea bass, *Lateolabrax japonicus* reported that the dietary inclusion of 15% and 25% FPH significantly increased the lysozyme activity and complement haemolytic activity, whereas the phagocytic activity was significantly higher at all levels of FPH (5%, 15% and 25%) (Liang et al. 2006). Immunoglobulin M, lysozyme activity and complement C4 were significantly higher in fish fed with diets containing 10% and 15% FPH when compared to fish fed with the basal diet or diet containing 5% FPH (Tang et al. 2008). A study on red sea bream, *Pagrus major* demonstrated an increase of nitro-blue tetrazolium activity and myeloperoxidase level in fish fed protein hydrolysate diets when compared to the low FM based diet, as well, comparatively higher total immunoglobulin level, lysozyme activity and antiprotease activity were observed in fish fed high FM based diet and hydrolysate diets when compared to low FM based diet (Khosravi et al. 2015b). Bui et al. (2014) found some improvement in antiprotease activity, lysozyme activity, nitro-blue tetrazolium activity and myeloperoxidase level in juvenile red sea bream, *Pagrus major* fed with hydrolysates when compared to the FM based dietary group, and immunoglobulin level was found to be significantly higher in hydrolysate groups. It has been suggested that the effect of hydrolysates on the fish immune system may be dependent on the size and concentration of peptides. FPH containing medium and small size peptides (molecular weight range 500–3000 Da) have been reported to stimulate the non-specific immunity of fish (Bøgwald et al. 1996; Gildberg et al. 1996). Superoxide anion production in Atlantic salmon, *Salmo salar* was reported to be stimulated by the peptides, size ranges from 500 to 3000 Da (Gildberg et al. 1996).

In some cases, no significant effect of FPH was found on fish immunological parameters. Zheng et al. (2013a) reported that the serum lysozyme activity, acid phosphatase activity and alkaline phosphatase activity of turbot, *Scophthalmus maximus* were not affected by the levels of FPH inclusion in fish diet. Similarly, Goosen et al. (2015) did not find any significant effect of FPH on serum lysozyme concentration and immunoglobulin level in mozambique tilapia, *Oreochromis mossambicus*. Murray et al. (2003) studied juvenile coho salmon, *Oncorhynchus kisutch* and found no significant differences on complement activity, lysozyme activity, total serum immunoglobulin level, myeloperoxidase level, nitro-blue tetrazolium activity and phagocytic activity in fish fed diets supplemented with hydrolysate bones removed, hydrolysate with dried bones added and/or cooked fish with bones compared to fish fed FM-based control diet.

Table 2.1.4 Effect of FPH on hematological and immunological responses of fish

Fish	Hydrolysate and inclusion level	Responses	References
Sea bream, *Pagrus major*	SH: 4.80% TH: 4.23% KH: 4.03%	(↔) Hematocrit, hemoglobin, glucose, total protein, cholesterol & triglyceride. (↑) Immunoglobulin (↑) Superoxide dismutase & antiprotease @ KH (↔) Lysozyme activity, nitro blue tetrazolium activity & myeloperoxidase level	Bui et al. (2014)
Coho salmon, *Oncorhynchus kisutch*	Boneless fish hydrolysate: 30.30% Hydrolysate with bones: 30.30% Cooked fish with bones: 29.13%	(↓) Hematocrit (↑) Leucocrit, @ cooked fish with bones (↓) Total plasma protein @ cooked fish with bones (↔) Lysozyme activity, complement activity, total serum immunoglobulin, myeloperoxidase, phagocytosis & nitro blue tetrazolium activity	Murray et al. (2003)
Red sea bream, *Pagrus major*	TH 2% KH 2%	(↔) Hematocrit, hemoglobin, glucose, total protein, total cholesterol & triglyceride. (↑) Lysozyme activity (↑) Nitro blue tetrazolium activity @TH (↔) Immunoglobulin, myeloperoxidase activity, superoxide dismutase & antiprotease	Khosravi et al. (2015a)
Olive flounder, *Paralichthys olivaceus*	TH 2% KH 2%	(↔) Hematocrit, hemoglobin, glucose, total protein, total cholesterol & triglyceride. (↑) Lysozyme activity@TH,@KH (↑) Nitro blue tetrazolium activity & superoxide dismutase @KH (↔) Immunoglobulin, antiprotease, myeloperoxidase activity	Khosravi et al. (2015a)
Olive Flounder, *Paralichthys olivaceus*	SH: 3.34 TH: 2.88 KH: 3.12	(↔) Hematocrit, hemoglobin, glucose, total protein, total cholesterol, triglyceride, aspartate aminotransferase activity & alanine aminotransferase activity (↑) Superoxide dismutase @SH	Khosravi et al. (2017)*

		(↔) Immunoglobulin, lysozyme activity, antiprotease activity, glutathione peroxidase activity, nitro blue tetrazolium activity & myeloperoxidase activity	
Japanese flounder, *Paralichthys olivaceas*	Frames of pollock, *Theragra chalcogramma* UF 3.7 & 1.2 NUF 3.7	(↑) Plasma IGF-I (insulin-like growth factor I) levels (↑) Liver IGF-I mRNA expression @ UF 3.7%	Zheng et al. (2012)
Turbot, *Scophthalmus maximus*	Frames of pollock, *Theragra chalcogramma* UF 3.7, 1.2 NUF 3.7	(↔) Lysozyme activity, acid phosphatase activity, total antioxidative capacity, alkaline phosphatase activity & superoxide dismutase activity	Zheng et al. (2013a)
Yellow Croaker, *Pseudosciaena crocea*	5, 10, 15	(↑) Lysozyme activity, complement activity & immunoglobulin at 10 & 15%	Tang et al. (2008)
Japanese sea bass, *Lateolabrax japonicus*	5, 15, 25	(↑) Phagocytic activity (↑) Lysozyme activity, complement haemolytic activity at 15 & 25% (↔) Number of nitroblue tetrazolium-positive cells	Liang et al. (2006)

Note: Increase, ↑; decrease, ↓; no change, ↔; compared to the control diet ($P<0.05$). * Compared with high FM based diet as control. ADC, apparent digestibility coefficient; UF, Ultrafiltered fish hydrolysate; NUF, non-ultrafiltered fish hydrolysate; SH, shrimp hydrolysate; TH, tilapia hydrolysate; KH, krill hydrolysate.

2.1.4.7 Antioxidant activities

Short chain peptides with lower molecular weight are considered more active compounds to play an important role as electron donors. These electron donors may prevent chain reactions by reacting with free radicals to make them more stable substances (Chi et al. 2014). Hydrolysation unfolds complex protein structure to produce low molecular weight peptides and amino acids, which improve antioxidant activity of hydrolysed protein in comparison with the intact protein (Sarmadi & Ismail 2010). FPH from Alaskan pollock, *Theragra chalcogramma* frames (Je et al. 2005); mackerel, *Scomber austriasicus* (Wu et al. 2003); yellowfin sole, *Limanda aspera* frame (Jun et al. 2004); capelin, *Mallotus villosus* (Amarowicz & Shahidi 1997) and herring, *Clupea harengus* (Sathivel et al. 2003) have been reported to possess antioxidant properties. A study on red seabream, *Pagrus major* demonstrated an increase of superoxide dismutase in fish fed protein hydrolysate diets compared to the low FM based diet, as well, comparatively higher glutathione peroxidase activity were observed in fish fed high FM based diet and hydrolysate diets when compared to low FM based diet (Khosravi et al. 2015b). With the same species, Bui et al. (2014) found improvement on the superoxide dismutase level in hydrolysate treatments when compared to the control group fed with FM based diet. However, Zheng et al. (2013a) reported that the superoxide dismutase activity of turbot, *Scophthalmus maximus* was not affected by the levels of fish hydrolysates inclusion. But turbot, *Scophthalmus maximus* fed with high amount of UF FPH diet presented highest total antioxidant capacity of fish groups fed with low amount of ultra-filtered FPH diet, non-UF FPH diet and FM diet.

2.1.4.8 Disease resistance

Studies demonstrating enhanced disease resistance in fish for the FPH addition in diets are summarized in Table 2.1.5. As described above, low molecular weight bioactive peptides in FPH may have immune-stimulating and antibacterial properties (Bøgwald et al. 1996; Kotzamanis et al. 2007). The improvement of cellular and/or humoral immune function with heightened disease resistance of various fish due to bioactive peptides in FPH has already been established (Kotzamanis et al. 2007; Liang et al. 2006; Murray et al. 2003; Siddik et al. 2019b). It is assumed that the fish hydrolysates having polypeptide fractions may stimulate some mechanisms in fish that are essential for disease resistance (Murray et al. 2003; Siddik et al. 2019b). Red sea bream, *Pagrus*

major fed hydrolysate diets exhibited significant improvement in disease resistance against *Edwardsiella tarda* compared to the group fed a low FM based diet, and hydrolysate treatments showed higher survival rate than high FM based treatment (Khosravi et al. 2015b). Similarly, Bui et al. (2014) found significant improvement in survival rate of juvenile red seabream, *Pagrus major* fed with fish hydrolysates during challenge trial with *Edwardsiella tarda*. However, higher levels of short peptide chains may introduce negative effects on fish health (Gildberg & Mikkelsen 1998). There were no significant differences in survival rate in Japanese sea bass, *Lateolabrax japonicus* fed with different levels of FPH when exposed to *Vibrio anguillarum* (Liang et al. 2006). The poor survival of juvenile coho salmon, *Oncorhynchus kisutch* fed diets containing FM, FPH and cooked fish following challenge with *Vibrio anguillarum*, suggested that the supplementary ingredients had no advantageous effect on the cellular defense mechanisms (Murray et al. 2003). Gildberg et al. (1995) reported that the survival rate of Atlantic salmon, *Salmo salar* fed with diets containing hydrolysed fish protein was poor when challenged with *Aeromonas salmonicida*. The variation in the effect of FPH on disease resistance may result from the variation in peptide profile of hydrolysates, such variation depending on the source of native protein, hydrolysis conditions, enzyme specifications, experimental period, level of dietary inclusion and species-specific differences (Klompong et al. 2007).

Table 2.1.5 Effect of FPH on disease resistances of fish

Species	Used hydrolysate	Pathogen (s) challenged and Dose (CFU/g)	Methods and duration of challenge trial	Response	Reference
Sea bream, *Pagrus major*	SH: 4.80% TH: 4.23% KH: 4.03%	*Edwardsiella tarda* 1×10^5 CFU mL^{-1}	Injection 21 days	KH and TH in diets exhibited higher disease resistance compared to control in fish against *Edwardsiella tarda*	Bui et al. (2014)
Persian sturgeon, *Acipenser persicus* L.	Tuna viscera protein hydrolysate 10, 25, 50%	*Aeromonas hydrophila* 10^9 CFU mL^{-1}	Immersion 5days	None of the FPH included levels resulted in higher survival compared to control in fish against *Aeromonas hydrophila*	Ovissipour et al. (2014)
Coho salmon, *Oncorhynchus kisutch*	Boneless fish hydrolysate: 30.30% Hydrolysate with bones: 30.30% Cooked fish with bones: 29.13%	*Vibrio anguillarum*, 7.71×10^5 bacteria mL^{-1}	Immersion 14 days.	No differences were observed in survival to control in fish among the dietary groups	Murray et al. (2003)
Red Sea bream, *Pagrus major*	TH 2% KH 2%	*Edwardsiella tarda* 1×10^5 CFU mL^{-1}	Injection 21 days	TH diet resulted in higher disease resistance to control in fish against *Edwardsiella tarda*	Khosravi et al. (2015a)
Olive flounder, *Paralichthys olivaceus*	TH 2% KH 2%	*Edwardsiella tarda* 1×10^3 CFU mL^{-1}	Injection 10 days	FPH groups exhibited higher disease resistance to control in fish but the differences were not significant among treatments.	Khosravi et al. (2015a)
Atlantic salmon, *Salmo salar*	Cod muscle protein: 10% Lactic acid bacteria: 10%	*Aeromonas salmonicida* 5.8×10^4 cells $fish^{-1}$	Injection 4 weeks	No difference was registered between death rates of fish fed FPH to the control	Gildberg et al. (1995)

Note: FPH, fish protein hydrolysate; CFU, colony-forming unit; SH, shrimp hydrolysate; TH, tilapia hydrolysate; KH, krill hydrolysate.

2.1.5 Conclusion and future perspectives

The potential effect of FPH on survival, growth, feed intake, feed utilization, nutrient digestibility, body composition, hematological parameters and disease resistance have been studied quite thoroughly over the years and their favorable effect in aquaculture practices has been well stated. Nowadays, some investigations have reported anti-pathogenic, antioxidative and immunomodulatory activities of FPH but their functional, biochemical and bioactive properties are not well examined in aquaculture production. Enhancement of the body immune system to prevent fish diseases is the most promising approach nowadays. Thus, more investigations on FPH are required to prove their effectiveness in aquaculture as a FM supplement which can provide enhanced protection of fish against pathogenic infection.

2.2 Fermented animal protein diets

As an alternative protein source for aquaculture, animal by-products, such as fish offal meal, PBM, feather meal, bone meal and slaughter house blood meal, are a good option because of their low price and wide availability. There are a number of studies which have been conducted over the decades on the utilization of animal by-products to replace FM in aqua-feeds. However, although animal by-products may be a good source of protein, the utilization of these products in aqua-feeds is still constrained by various factors including lack of some essential amino acids, high moisture, indigestible particles, microbial contaminates and the possibility of disease transmission (Mondal et al. 2008; Samaddar et al. 2015).

Microbial fermentation significantly improved the palatability, protein contents and bioavailability of minerals in feeds (Koh et al. 2002; Niba et al. 2009) as well as containing of beneficial bacteria and animal digestive enzymes which may help in balancing the intestinal flora resulting in better digestion of nutrients (Shi et al. 2017). Fermented animal feedstuffs have been successfully used to partially replace FM in the formulation of diet for Indian major carp, *Labeo rohita* (Samaddar et al. 2015), freshwater catfish, *Heteropneustes fossilis* (Mondal et al. 2008), Indian minor carp, *Labeo bata* (Mondal et al. 2011) and olive flounder, *Paralichthys olivaceus* (Sun et al. 2007). Fermented shrimp processing waste boosted antioxidant activity (Sachindra & Bhaskar 2008) and fermented plant products were reported to increase antioxidant and nonspecific immune responses of parrot fish, *Oplegnathus fasciatus* and Japanese flounder, *Paralichthys olivaceus* (Kim et al. 2009; Sachindra & Bhaskar 2008). In this section of the literature review, available information regarding the potential effects of fermentation on the nutritional profile of feeds, growth, feed utilization, hematology and immune response of aquaculture species are discussed.

2.2.1 Processing of animal by-products

Some forms of processing techniques like traditional bioprocessing (heat treatment and drying), advanced bioprocessing, fermentation, germination and enzyme treatment have been applied to change or convert raw foodstuffs into safe and more palatable foodstuffs (Vo et al. 2015). Among these techniques, fermentation is a cost effective and environmental affordable biotechnological technique that has been applied by many researchers to overcome the inherent problem of animal by-products and make

them suitable for inclusion in aqua-feeds(Siddik et al. 2019a). The animal by-products used in the study were fermented following a technique described elsewhere (Himawan et al. 2016). In short, the animal product was weighed and Baker's yeast, *Saccharomyces cereviceae* (Instant dried yeast, Lowan®) was added at 10% and *Lactobacillus casei* in the form of skim milk product (Yakult®) was added at 5% (cell density of 3×10^6 CFU mg^{-1}) of the weight of the product. Distilled water was then added at approximately 70% of the weight of the total meal mixture and all ingredients were thoroughly mixed in a food mixer. The mixture was then placed in an Erlenmeyer flask covered with aluminium foil and incubated at 30°C for 4 days. The fermented product was dried in oven at 60°C for 26 h and used as a feed ingredient.

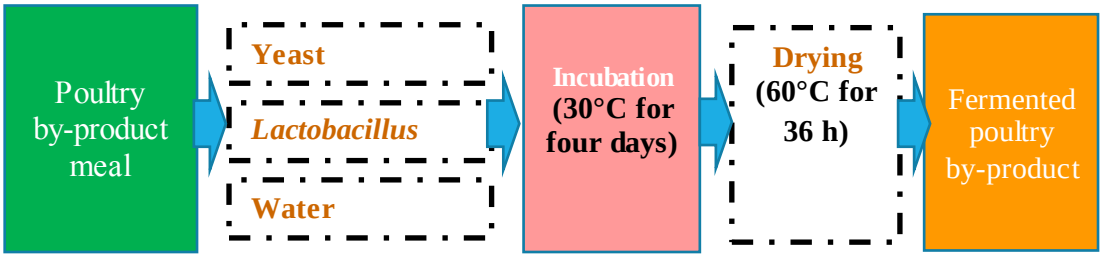


Figure 2.2.1 A schematic representation of the fermentation protocol of animal by-product with yeast and *Lactobacillus casei*.

2.2.2 Fermentation and its importance

Fermentation is an environmentally friendly approach which is conducted by microorganisms to modify the biochemical properties of raw materials (Caplice & Fitzgerald 1999). Microorganisms in fermented products display many functional properties including probiotic properties (Hill et al. 2014), antimicrobial properties (Meira et al. 2012), antioxidant activity (Perna et al. 2014), peptide production (de Mejia & Dia 2010) and degradation of anti-nutritional compounds (Tamang et al. 2016). Bacterial fermentation of raw materials can exert many beneficial effects by reducing anti-nutrients, improving bioavailability of minerals like phosphorus, calcium, magnesium and copper augmenting protein content in terms of lysine, histidine and methionine, and breaking down indigestible carbohydrates (Niba et al. 2009). Furthermore, fermented feeds are characterized by high numbers of lactic acid bacteria (LAB) (approximately 10^9 CFU/ml of feed) (Heres et al. 2003a; Niba et al. 2009) which can proliferate and produce high concentrations of lactic acid and several

beneficial volatile fatty acids including acetic acid, butyric acid, and propionic acid (van Winsen et al. 2001). Fermentation can also reduce the pH of feeds (Canibe & Jensen 2012), resulting in the inhibition of the development of pathogenic organisms in the feed (van Winsen et al. 2001) and subsequent reduction of pH in the entire gastrointestinal tract (GIT) (van Winsen et al. 2001). This reduction in pH may influence the ecology of endogenous bacteria (van Winsen et al. 2001) and prevent the proliferation of pathogens from developing in the GIT (Canibe & Jensen 2012; Missotten et al. 2015).

Fermented products may improve the growth performance of animals by controlling pathogenic organisms and activating endogenous enzymes which may enhance the digestibility and availability of certain nutrients (Brooks 2008). Improvements in the GIT morphology in terms of villus length and villus/crypt ratio has also been observed, and both these characteristics are associated with increased digestive capacity (Missotten et al. 2015; Siddik et al. 2019a; Siddik et al. 2018b). However, the quality of the final end product of fermentation is affected by some factors such as fermentation conditions, temperature and product to water ratio (Missotten et al. 2015), types of micro-organisms and substrate quantity and quality (carbohydrates, fibers, protein, amino acids and vitamins) (Canibe & Jensen 2012; Missotten et al. 2015; Niba et al. 2009). For example, improper fermented feed can produce higher concentrations of yeast, resulting in the production of off-flavours and taints due to the production of acetic acid, ethanol and amylic alcohols, leading to less palatable feed (Brooks 2008).

2.2.3 Nutritional changes of animal sourced feed ingredients due to fermentation

2.2.3.1 Protein and amino acid

Fermentation has significant effects on the physico-chemical properties of feed ingredients and feeds (Brooks 2008). Fermentation has been reported to increase the number of proteolytic bacteria and reduce the pH which accelerates the proteolysis resulting in higher protein levels in liquid wheat meal (Brooks 2008; Plumed-Ferrer et al. 2004). However, Ozyurt et al. (2016) stated that fermented products show very minor change in crude protein content. The study of Samaddar & Kaviraj (2014), the level of crude protein was reduced slightly in both *L. acidophilus* (1.15%) and whey fermented slaughterhouse blood (1.49%) when compared to fresh slaughterhouse blood. Fermentation of klunzinger's ponyfish, *Equulites klunzingeri* with

Lactobacillus plantarum reduced the protein content significantly (Ozyurt et al. 2016). The crude protein contents in fermented saltwater fish, fermented freshwater fish and fermented tilapia residues were lower than crude protein levels in the corresponding raw ingredients (Vidotti et al. 2003). This lower level of protein from fermented ingredients may result from the inclusion of the fermentation materials, i.e. *L. plantarum* (5%) and sugar cane molasses (15%) (Vidotti et al. 2003), which may proportionally decrease the levels of crude protein in the fermented products. A similar reduction in protein was also reported by Fagbenro & Bello-Olusoji (1997) using cane molasses or cassava starch to produce fermented shrimp head products and by Rangacharyulu et al. (2003) using molasses, propionic acid, sorbic acid and tertiary butyl hydroxyquinone to produce fermented silkworm pupae. Reduction of crude protein level in the fermented items could also be the result of the microbial utilization of protein as a nutrient source of microorganisms during the fermentation process. An increased level of free amino acids especially lysine, histidine and methionine has been reported in the fermented products (Niba et al. 2009) likely due to the breakdown of a portion of protein by LAB (Savijoki et al. 2006). Samaddar & Kaviraj (2014) observed free amino acids in both *L. acidophilus* and whey fermented slaughterhouse blood.

2.2.3.2 Lipid

Fermented products show very minor changes in crude lipid level (Ozyurt et al. 2016). *Streptococcus thermophiles* fermented klunzinger's ponyfish showed a marginal increase in lipid content, whilst with a *Lactobacillus plantarum* fermentation there was no noticeable effect on lipid content (Ozyurt et al. 2016). Mach & Nortvedt (2009) observed similar crude lipid level between raw materials and silages of lizard fish, *Saurida undosquamis* and blue crab, *Portunus pelagicus*. Samaddar & Kaviraj (2014) also observed minor changes in crude lipid levels in both *L. acidophilus* and whey fermented slaughterhouse blood.

2.2.3.3 Ash

The fermentation process increases the ash content of animal sourced dietary ingredients. Comparatively higher ash content was reported by Rangacharyulu et al. (2003) in fermented silkworm pupae than in the corresponding untreated silkworm pupae. Similarly, higher ash contents was found in fermented by-products and slaughterhouse wastes (Kherrati et al. 1998) and *L. acidophilus* and whey fermented slaughterhouse blood when compared to non-fermented products (Samaddar &

Kaviraj 2014). Ash content of klunzinger's ponyfish fermented with *Lactobacillus plantarum* or *Streptococcus thermophiles* showed a significant increase in ash in both fermented groups (Ozyurt et al. 2016).

2.2.3.4 Moisture

The fermentation process may change in the moisture content of animal sourced dietary ingredients. Fermentation of klunzinger's ponyfish, *Equulites klunzingeri* with *Streptococcus thermophiles* reduced the moisture significantly (Ozyurt et al. 2016). Lower moisture content was also reported in fermented silkworm pupae when compared with untreated silkworm pupae (Rangacharyulu et al. 2003). However, Samaddar & Kaviraj (2014) observed a significant increase in moisture level in both *L. acidophilus* and whey fermented slaughterhouse blood. Similarly, Kherrati et al. (1998) found comparatively higher contents of moisture in slaughterhouse wastes after fermentation.

2.2.3.5 Crude fibre

Generally, the fermentation process reduces the crude fibre content. A significant reduction was reported by Samaddar & Kaviraj (2014) for crude fibre content in slaughterhouse blood when fermented with *L. acidophilus* or whey. Fermentation process associated with cellulolytic microbial activity was reported to have a possible effect on the reduction of crude fibre content in fermented ingredients (Shi et al. 2006).

2.2.4 Role of animal sourced fermented ingredients on fish production

2.2.4.1 Effect on growth

A summary of the impact of fermented animal protein based diets on growth performance, feed utilization and body composition of different farmed fish are presented in Table 2.2.1. Microorganisms used in fermentation processes produce different enzymes, such as proteinase, amylase, cellulose and catalase which can breakdown the complex compounds into simple bio-molecules (Tamang et al. 2016). Such modulation of ingredients may enhance the digestion process and consequently increase the growth performance of fish fed fermented diets. Kaviraj et al. (2013) reported that *Labeo rohita* fed fermented mulberry leaf and fish offal at 50 and 75% replacement of FM revealed significantly higher growth performance in terms of weight gain, specific growth rate and feed conversion ratio. Similarly, significantly higher growth performance was observed in freshwater catfish, *Mystus vittatus* when

fed diets containing 25% FM and 25% fermented offal meal (FOM) or 20% FM and 30% FOM, when compared to the reference diet without FOM incorporation (Samaddar et al. 2011). In another study, Mondal et al. (2008) demonstrated that fermented fish-offal can replace FM successfully at up to 30% in the diet of *Heteropneustes fossilis*. However, Samaddar et al. (2015) found no significant difference in specific growth rate and weight gain of *Labeo rohita* fed with different levels of fermented fish offal and slaughter house blood protein up to 75% replacement level when compared to FM, but observed a significantly decreased performance in 100% replacement of FM with fermented protein. Growth rates were also reported to be significantly increased up to 30% replacement of FM with fermented soybean meal and scallop by-product blend (FP) of Japanese flounder, *Paralichthys olivaceus* but further increase in replacement levels (45% and 60%) significantly retarded the growth performance (Kader et al. 2012). Several studies have stated that higher replacement level of fermented animal by-products to FM protein may produce a more water stable pellet, thereby tying up the nutrients resulting in decreased availability of nutrients to the species. In addition, Argüello-Guevara & Molina-Poveda (2013) reported that lower digestibility was a consequence of the higher stability of the feed in the water. Similarly, Deng et al. (2006) and Uyan et al. (2006) reported that in increasing level of FM replacement by fermented products reduced feed intake, thereby lowering growth performance was recorded in Japanese flounder, *Paralichthys olivaceus*. The lowered growth performance might also be due to the depletion of some more easily digestible low-molecular-weight carbohydrates and loss of essential nutrients during fermentation (Niven et al. 2006).

Table 2.2.1 The effect of fermented animal protein based diets on growth performance, feed utilization and digestibility of different farmed fish

Raw materials/substrate	Microorganisms used for fermentation	Fish species trial carried out on	Outcomes	Reference
Fish-offal	Lactic acid producing Bacteria	Freshwater catfish, *Heteropneustes fossilis*	(↑) Growth performance, feed conversion and protein utilization at 30% (↔) Feed intake (↑) ADC protein at 30% (↑) Whole body protein	Mondal et al. (2008)
Soybean meal and squid by-product blend	*Bacillus* spp.	Japanese flounder, *Paralichthys olivaceus*	(↔) Growth performance up to 36% (↔) Whole body proximate composition (↓) Protein retention	Kader et al. (2012)
Slaughter house blood and fish offal	*Lactobacillus acidophilus*	Indian major carp, *Labeo rohita*	(↔)Weight gain, specific growth rate, feed conversion ratio, protein efficiency ratio and apparent net protein utilization up to 75% (↑) ADC protein at 50, 75 and 100% (↑) ADC lipid (↑) Amino acid absorption	Samaddar et al. (2015)
Fish offal	Microbial suspension (10^8 cells.mL^{-1})	*Labeo rohita*	(↑) Growth performance (↑) Carcass protein and lipid (↑) Feed intake (↔) ADC protein	Mondal et al. (2007)
Fish offal meal	*Lactobacillus* sp. *Rhodopseudomonas* sp. *Azotobacter* sp. and	Freshwater catfish, *Mystus vittatus* (Bloch)	(↑) Growth, ADC protein, and protein deposition (↓) Protease and amylase (↑) Lypase	Samaddar et al. (2011)

	Saccharomyces sp.			
Fish-offal meal	Suspension of effective microorganisms	Indian minor carp *Labeo bata*	(↑) Growth (↔) Feed intake (↑) Protein deposition	Mondal et al. (2011)
Fisheries by-products and soybean curd residues mixture	-	Juvenile olive flounder, *Paralichthys olivaceus*	(↔) Weight gain, hepatosomatic index and specific growth rate up to 30% (↑) Feed efficiency and protein efficiency ratio at 60% (↔) Condition factor and survival rate	Sun et al. (2007)
Mulberry leaf and fish offal	*Lactobacillus* sp. *Rhodopseudomonas* sp. *Azotobacter* sp. *Saccharomyces* sp	*Labeo rohita*	(↑) Weight gains, specific growth rate and feed conversion ratio (↔) Feed intake (↑) Whole body protein and lipid	Kaviraj et al. (2013)

Note: Increase, ↑; decrease, ↓; no change, ↔; compared to the control diet ($P<0.05$), - ; not mentioned.

2.2.4.2 Effect on feed intake

Feed intake of fish is highly associated with feed texture, palatability and flavor which all can be enhanced by fermentation (Gaggia et al. 2011). Mondal et al. (2007) reported that the inclusion of 25% and 30% fermented fish offal in the diet of *Labeo rohita* showed significantly higher feed intake rate than fish fed with a 40% FM based control diet. Previous studies have indicated that inclusions of fermented fish offal in formulated diets were well received by the fingerlings of *Labeo bata* (Mondal et al. 2011) and *Mystus vittatus* (Samaddar et al. 2011). Mondal et al. (2008) reported that juvenile *Heteropneustes fossilis* well accepted diets containing FM (40%) and fermented fish-offal (0%), FM (25%) and fermented fish-offal (25%) or FM (20%) and fermented fish-offal (30%). Such improvement in feed intake indicate that fermentation increased the palatability of the diets. As well, lactic acid produced by fermentation might have a positive effect on feed intake and feed efficiency (Missotten et al. 2015). An investigation by Kaviraj et al. (2013) found no significant variation in feed intake when fermented blend of mulberry leaf and fish offal was used as a partial replacement of FM for *Labeo rohita*. However, their observed intake rate was comparatively lower than the feed intake rate reported by Mondal et al. (2007) for the same species using a similar FM (40%) based reference diet and experimental diets comprising FM and fermented fish offal meal. Fagbenro & Jauncey (1995) observed no noticeable change on feed intake by juvenile catfish, *Clarias gariepinus* when fish were fed with diets formulated from FM or *Lactobacillus plantarum* fermented mixed-sex tilapias blended with meat and bone meal, hydrolysed feather meal, PBM or soybean meal. But in some studies it has been reported that reduction of dietary FM reduced the rate of feed intake in several fish (Kaushik et al. 2004). As compared to FM based dietary group, no significant effect was observed on the feed intake by *Labeo rohita* fed with a fermented blend of animal by-product as replacement of FM up to 75% (Samaddar et al. 2015), but the authors observed significant reduction of feed intake in the 100% FM replaced dietary group. Generally, it seems a portion of FM is required to ensure voluntary feed intake, but potentiality of other ingredients in aqua diets cannot be overlooked.

2.2.4.3 Effect on digestibility

Fermentation may improve digestibility of fish as it has been hypothesized that that microbial fermentation improves the feed quality by enhancing flavor, texture and nutritional value (Gaggia et al. 2011) and may also enrich the feed by producing some desirable metabolites (Cho and Kim, 2011). Mondal et al. (2008), working with a *H. fossilis* reference diet containing mustard oil cake, rice bran and 40% FM showed a 95.39% apparent protein digestibility, whereas inclusion of 30% fermented fish-offal positively replaced 50% dietary FM and a significantly higher protein digestibility was recorded. In another study with *Labeo bata*, the same authors reported that diet containing 40% FM exhibited 89% apparent protein digestibility (APD), which significantly improved to 91% and 94% in diets containing 37.5% and 50% fermented fish-offal meal as substitution of FM, respectively (Mondal et al. 2011). In a study with *Mystus vittatus*, Samaddar et al. (2011) reported that apparent digestibility of protein increased when a fermented blend containing fish-offal was used as a major source of dietary protein instead of FM. Samaddar et al. (2015) reported that *Labeo rohita* revealed better protein and lipid digestibility when fish were fed with a fermented blend of slaughter house blood and fish-offal. This might be related to the fermentation process augmenting the protein contents in terms of lysine, histidine and methionine levels and also increasing amino acid availability from dietary animal protein (Bertsch & Coello 2005). This, noting that the best digestibility of dietary protein is often correlated with the presence of adequate levels of essential amino acids (Wilson 2002). Increasing lipid digestibility was observed in *Labeo rohita* fed with increasing levels of fermented ingredients as dietary FM replacements, indicated that lipid digestibility is also positively correlated with dietary ingredients following fermentation (Samaddar et al. 2015). Fagbenro & Jauncey (1995) studied the dietary nutritional values of dried fermented fish silage for tilapia, apparent digestibility coefficients of energy (82.8%), dry matter (82.8%) and protein (84.5%) indicated that this fermented ingredient was a potential aqua-feed ingredient. Feed containing fermented ingredients has also been tested on different animals and it was reported that fermented feed increased the beneficial bacteria in the GIT of broiler chicks (Naji et al. 2015) and turkeys (Firman et al. 2013) and exerted beneficial effects on the intestinal morphology in terms of villus height and villus height to crypt depth ratio in turkey (Drazbo et al. 2018). These factors alone or combined increase the surface area of intestinal mucosa for nutrient absorption and nutrient utilization, which might also

be associated with the increased digestibility of fish. It is noticeable however that Mondal et al. (2007), Mondal et al. (2008) and Mondal et al. (2011) used almost similar dietary ingredients for *Labeo rohita*, *Heteropneustes fossilis* and *Labeo bata*, respectively but the dietary effect of fermented fish-offal on APD was negative for *Labeo rohita* and positive for *Heteropneustes fossilis* and *Labeo bata*, indicating a possible species specific variability in applying to feed formulation.

2.2.4.4 Effects on body composition

Fermented animal sourced by-products have a significant effect on fish body composition. Mondal et al. (2008) found significantly higher whole body protein content in both fermented dietary groups and significantly higher whole body crude lipid content in the 30% fermented fish-offal based dietary group as compared with the reference dietary group. Similar significant improvement of crude protein and lipid contents were reported by several authors using fermented ingredients in diets for *Labeo rohita* (Mondal et al. 2007), *Labeo bata* (Kaviraj et al. 2013; Mondal et al. 2011) and *Mystus vittatus* (Samaddar et al. 2011). In general, fish can easily absorb polyunsaturated fatty acids, followed by monounsaturated fatty acids and saturated fatty acids, and both polyunsaturated and monounsaturated fatty acids digestibility decreases with increasing the levels of saturated fatty acids (Menoyo et al. 2003). Microbial fermentation processes reduce the level of saturated fatty acids and consequently increase the availability of unsaturated fatty acids. However, Kader et al. (2012) found no significant change in total lipid, crude protein, body moisture and ash content, when Japanese flounder fed with fermented squid by-product and soybean meal blend based diets at replacement levels of 48, 36, 24 or 12% FM. Similarly, Samaddar et al. (2015) reported that *Labeo rohita* fed with FM based diet, or 25% or 50% FM replaced fermented animal bi-products based diets showed similar body crude protein and ash contents, but significant decreases were observed in 75% and 100% FM replaced with fermented fish offal and slaughter house blood by-products diet.

2.2.4.5 Effect on hematological parameters and immune response

A summary of the effect of fermented animal protein based diets on hematological parameters and immune response of farmed fish are presented in Table 2.2.2. Blood and immune parameters are commonly used to determine the physiological condition of fish (Bui et al. 2014; Rey Vazquez & Guerrero 2007; Siwicki et al. 1994a), and may

be assumed to be influenced by fermented products in the feed. Kader et al. (2012) observed significantly increased serum protein, antioxidant activity and bactericidal activity in fish when fed 36% and 48% of fermented soybean meal and squid by-product blends when compared to the control, a FM based diet. Sachindra & Bhaskar (2008) found improved antioxidant activity in feed containing fermented shrimp by-products when compared to the non-fermented formulation. The fermentation process was reported to hydrolyse bioavailable bioactive peptides from the original state of the parent protein sequence (Korhonen & Pihlanto 2003) and produce lower molecular weight small chain peptides and free amino acids (Feng et al. 2007; Tang et al. 2012), polysaccharides and bioactive compounds. As well enrichment of lactic acid bacteria was reported (Xue et al. 2009); such enrichment may have immunostimulatory potential (Sachindra & Bhaskar 2008). Other important functional properties reported in fermented feed products include antioxidant activity. For example 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity, and antimicrobial activities due to the production of antimicrobial compounds (bacteriocin and niacin) (Gaggia et al. 2011; Tamang et al. 2016). These positive changes as a result of fermented feed uptake could influence the health condition of fish. However, Kader et al. (2012) and Fagbenro & Jauncey (1995) found no significant effects on hematological parameters of Japanese flounder, *Paralichthys olivaceus* fed different levels of fermented soybean meal and squid by-product blend, and no effect on North African catfish, *Clarias gariepinus* fed fermented mixed-sex tilapia co-dried with meat and bone meal, hydrolysed feather meal, PBM or soybean meal, respectively. These variable findings might be attributed to some other factors such as fermentation time, temperature, microorganisms and different fish species.

Table 2.2.2 The effect of fermented animal protein based diets on hematological parameters and immune response of different farmed fish

Raw materials/substrates	Microorganisms used for fermentation	Fish species trial carried out on	Outcomes	Reference
Tuna hydrolysate	*Lactobacillus casei*	Barramundi, *Lates calcarifer*	(↓) haemoglobin, haematocrit	Siddik et al. (2018a)
Soybean meal and squid by-product Blend	*Bacillus* spp.	Japanese flounder, *Paralichthys olivaceus*	(↔) Haematocrit, albumin, bilirubin, glucose and triglyceride. (↑) Serum protein (↑) Lysozyme activity (↓) Bacterial count	Kader et al. (2012)
Fish-silage	*Lactobacillus plantarum*	Juvenile catfish, *Clarias gariepinus*	(↔) Haematocrit (↔) Haemoglobin	Fagbenro & Jauncey (1995)

Note: Increase, ↑; decrease, ↓; no change, ↔; compared to the control diet ($P<0.05$).

2.2.4.6 Effects on gut microbes

The intestine of fish contains specific intestinal microbiota including aerobic, facultative anaerobic and obligate anaerobic bacteria that are very sensitive to dietary changes (Ringo et al. 2006). A previous study revealed that incorporation of microorganisms through fermentation can modulate the gut microbes of animals (Missotten et al. 2015) and the most common changes in stomach and small intestine due to fermentation is an increase in the concentration of lactic acid bacteria (Canibe & Jensen 2012) and the number of yeast cells (Missotten et al. 2015). These microbiological changes are a strategy to protect the animals from enteropathogens (Missotten et al. 2015). Effects of fermented feed have been tested on different animals and they reported that fermented feed increase the levels of beneficial microbes (Canibe & Jensen 2012) and reduce the prevalence of pathogenic bacteria (van der Wolf et al. 2001). Similarly, feed containing fermented poultry by-product enriched the lactic acid bacteria belonging to the Firmicutes phylum in the intestine of juvenile barramundi (unpublished work). The aforementioned results indicate the potency of the application of fermentation in aqua-diets to modulate the gut microbiota of fish which can have beneficial effects on growth, digestibility and immune response.

2.2.4.7 Effects on survival rate

Rangacharyulu et al. (2003) studied the effect of fermented silkworm pupae on the survival rate of carps, namely *Labeo rohita*, *Cirrhinus mrigala* and *Catla catla* and found a significant increase in survival rate from the fermented dietary group (84.16%) than the corresponding dietary groups of unfermented silkworm pupae (65.83%) and FMl (67.5%). Samaddar et al. (2015) found no significant difference in the survival rate of *Labeo rohita* between dietary groups containing a fermented blend of fish offal and slaughter house blood, and a FM based dietary group. A similar result was also reported by several authors using fermented ingredients in diets for Florida pompano, *Trachinotus carolinus* (Rhodes et al. 2015), *Labeo rohita* (Mondal et al. 2007), *Labeo bata* (Mondal et al. 2011) and *Mystus vittatus* (Samaddar et al. 2011). Moreover, Fagbenro & Jauncey (1995) observed no mortality during experimental periods between fish fed lactic acid fermented tilapia into meat and bone meal, hydrolysed feather meal, PBM and soybean meal, and the control.

2.2.5 Conclusion and future perspective

The application of fermentation in animal by-products to produce feed for aquaculture production has not received much attention in the past. A very few studies, however, which have been conducted on different fish species, have reported a significant benefits in terms of growth performance, digestibility and health condition, presumed to be due to different functional properties in the fermented feed. Since fermentation has been reported to enhance the two key factors of growth performance and immune response in aquaculture, a modern and standard fermentation technique applied to alternate protein sources may help to sustain the aquaculture development by offering an option for replacement of FM.

Further studies on microbial fermented product performance should focus on other molecular biotechnology tools such as quorum sensing and high through genomes technologies to provide a clear understanding of the mode of action. Further research pertaining to the utilization of different microorganisms in fermenting animal by–products and their effects on immunology, gut microbes and gut morphology of different species are of high significance to investigate

Chapter 3: Growth, biochemical response and liver health of juvenile barramundi (*Lates calcarifer*) fed fermented and non-fermented tuna hydrolysate as fishmeal protein replacement ingredients

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Abstract

Conventional aquaculture feed materials available in Australia are expensive, which has prompted the search for alternatives that would be cost-effective and locally available. The present study was undertaken in order to maximize the use of a tuna hydrolysate (TH), which was produced locally from the tuna-processing discards. The growth performance, biochemical status, antioxidant capacity and liver health of juvenile barramundi (*Lates calcarifer*) were assessed. Two series of isonitrogenous and isocaloric diets labelled as $TH_{50,}$ TH_{75} (non-fermented tuna hydrolysate) and $FTH_{50,}$ FTH_{75} (fermented tuna hydrolysate) were formulated to replace FM at 50% and 75%, respectively. A basal diet without the TH supplementation was used as a control. The experimental diets were fed to the triplicate groups of fish three times a day for 56 days. The results of the experiment revealed that fish fed on both fermented and non-fermented TH-containing diets significantly reduced ($P<0.05$) the final body weight, weight gain and specific growth rate compared to the control. The highest apparent digestibility coefficients for dry matter, protein and lipid were obtained in the control group, and decreased with the increasing level of TH in the diets. However, the whole-body proximate compositions and the blood biochemical indices of fish were not affected by the TH inclusion in the diets. The fish fed on TH diets of TH_{50}, FTH_{50} and TH_{75} exhibited reduced ($P<0.05$) glutathione peroxidase (GPx) activity compared to the control; whereas, the FTH_{75} exhibited no difference with the control. The excessive inclusion of TH in the diets of TH_{75} and FTH_{75} resulted in cytoplasmic vacuolization, with an increased amount of lipid accumulation, and necrosis in the liver tissue. These results indicated that the replacement of the FM protein with TH at 50% and 75% inclusion levels negatively affected the growth performance, feed utilization, and digestibility in juvenile barramundi; and it also increased the potential risk of hepatic failure in the fish. Further investigation is, therefore, required in order to optimize the TH levels in the fish diets which would be suitable for the growth of

fish, as well as for maintaining the enhanced biochemical response in juvenile barramundi.

Keywords: Barramundi, GPx activity, fermentation, tuna hydrolysate, biochemical response.

3.1 Introduction

Fish-processing industries produce a large volume of fish waste across the globe as the fillets are often the only desired product in the market (Knuckey et al. 2004). As a result of the environmental concerns and the increased cost of waste disposal, options for waste utilization are being considered in order to maximize the use of the discarded fish waste, which will generate a more economic return from the same harvest, and cause less impact on the environment. In recent years, fish protein hydrolysates (FPH) obtained from a variety of low-value fish by-products, such as skin, fins, frames, heads, viscera, trimmings, and roe, have received significant consideration in the aqua feeds for their variety of uses, for example, as protein replacements (Kim et al. 2014; Ospina-Salazar et al. 2016), supplements (Bui et al. 2014; Khosravi et al. 2015a), attractants (Ho et al. 2014), palatability enhancers (Suresh et al. 2011), and immunostimulants (Bui et al. 2014). FPH, because of their short-chain peptides and well-balanced amino acids, are easily absorbed by animals and facilitate the biological nutrients uptake (Carvalho et al. 2004). Although beneficial effects, such as growth enhancement, feed utilization, and survival, have been reported for the inclusion of FPH at moderate levels (5% to 30%) in the fish diets, these bioactive substances have also been reported to result in an increase in the innate immune response (Liang et al. 2006), gut enzymatic activity (Cahu et al. 1999), disease resistance (Khosravi et al. 2015b), and stimulation of digestibility (Kousoulaki et al. 2013). In addition to good nutritive values and functional properties, some authors have reported that FPH plays a substantial role in the fish health as promoters for antioxidant and antimicrobial activities (Bougatef et al. 2010; Chalamaiah et al. 2012).

As an FPH, tuna hydrolysate (TH) is a nutrient-rich dietary supplement, which has been used in the aqua-feeds since a long time (Tacon et al. 2006). The availability of TH in Australia initiated this study, as several studies over the years have demonstrated that the TH could serve as an ideal protein source in the aqua diets for replacing FM, or it may be used as a feed palatability enhancer resulting in a higher feed intake, or

for the stimulation of the immune and digestive systems of the fish (Kolkovsksie et al. 2000). The optimum level of TH inclusion in the aqua diets is species-specific and most of the studies have reported inclusion levels of up to 30% (Kim et al. 2014; Ospina-Salazar et al. 2016). It has been reported previously that higher inclusion levels may lead to imbalanced absorption of the amino acids and thus, saturate the peptide transportation system in the fish (Carvalho et al. 2004). It is, therefore, of interest to search for the techniques which could possibly improve the nutritional values of TH, potentially allowing the incorporation of higher amounts of TH in the diets, in order to enhance the bioactive, antioxidant, and antimicrobial properties, on a species-specific level.

In order to improve the nutritional value of the feed ingredients, several techniques such as fermentation, advanced bioprocessing, soaking, and germination may be applied (Vo et al. 2015). Among these techniques, fermentation is the widely used one, for improving the nutritional quality of the feed ingredients, as well as for enhancing the palatability of the feed ingredients. Fermentation has also been associated with the reduction of ash content in the feed ingredients which may stabilize the pellet inside water (Fagbenro et al. 1994), improvement in the digestibility of various feed ingredients (Samaddar et al. 2015), and the removal of growth limiting factors in both plant and animal feed ingredients (Shamna et al. 2017). It has also been reported that fermentation by yeast and lactic acid bacteria induced immune modulation which may enhance the growth, non-specific immune response, and disease resistance in the host fish (Giri et al. 2013).

Barramundi (*Lates calcarifer*) is widely distributed across the coastal areas of the Asia-Pacific region, all the way up to Papua New Guinea and northern Australia (Glencross 2006; Siddik et al. 2016). This species has excellent attributes for use in aquaculture, including rapid growth rate, high consumer preference, a competitive market price, an accepted taste, and the ability to be cultured in a wide range of environments including ponds, sea cages, and recirculating aquaculture systems (Schipp et al. 2007b). The expansion of barramundi aquaculture has resulted in an increased pressure on the environment and on the sustainable supply of high-quality, less-expensive FM. This, in turn, has led a demand for further research in the area of finding alternatives to FM protein. The by-products of fish processing could serve as suitable alternatives to FM, because these are less expensive and locally available, in addition to being

environmentally safe. According to the research conducted to date, a maximum of 50% FM has been successfully replaced with tuna muscle by-product hydrolysate in juvenile Japanese flounder (*Paralichthys olivaceus*), without suppressing the growth performance and feed utilization (Uyan et al. 2006). However, no study has so far evaluated the suitability of fermented TH at a higher inclusion level, such as 50% and above, as an alternative to FM protein. We have been refining the fermentation technique for TH for several years now and our preliminary data with the other host species have provided us with a certain direction for increasing the inclusion levels of non-fermented and fermented TH. Therefore, the present study was conducted to assess the efficacy of fermented as well as non-fermented TH as a protein source, in terms of their effects on the growth performance, antioxidant capacity, biochemical status, and liver health in juvenile barramundi.

3.2 Materials and methods

3.2.1 Ethics statement

The growth trial of the fish was performed in accordance with the Australian code of conduct for the use and care of animals for scientific purposes. The procedures and protocols used in this experiment for treating fish were approved by the Animal Ethics Committee of Curtin University, Australia (Approval Number: AEC_2015_41).

3.2.2 Fish and rearing conditions

Juvenile barramundi, obtained from the Australian Centre for Applied Aquaculture Research, Fremantle, WA, Australia were used for the experiment. Prior to the commencement of feeding, the fish were acclimated to the laboratory conditions for one week, during that period they were fed on a commercial barramundi diet containing 470 g protein kg^{-1} diet and 20.0 MJ kg^{-1} dietary gross energy, three times a day. The barramundi juveniles, with a mean initial weight of 6.75 ±0.16 g $fish^{-1}$, were stocked randomly into fifteen tanks (capacity: 250 L each), at a stocking density of 20 fish $tank^{-1}$. The water in all the tanks used for this experiment was recirculated independently from an external bio-filter with a water refreshment capacity of 10 L min^{-1} and was constantly aerated to ensure sufficient levels of dissolved oxygen.

The water temperature, salinity, pH, and the level of dissolved oxygen in the water were monitored daily, and the levels of nitrite and ammonia were monitored twice a week. The water quality parameters maintained during the experimental period were

as follows: water temperature 27.8–29.7°C, salinity 31–38 ppt, pH 7.20–8.10, dissolved oxygen concentration 5.65–7.45 mg L^{-1}, nitrite <0.25 mg L^{-1}, and total ammonia nitrogen <0.5 mg L^{-1}. The room for the experiment was maintained under a 12-h light–12-h dark cycle, using *automatic indoor-light* switches (Clipsal, Australia). Prior to feeding, the daily feed ration for the fish was divided into three equal portions, and then the fish were hand-fed to satiation with the respective experimental diets, three times a day, at 0800 h, 1300 h, and 1800 h. After 0.5 h of each time, the uneaten feed was removed from the tank by siphoning, transferred to aluminum cups, and dried to constant weight, in order to evaluate the feed conversion ratio.

3.2.3 Preparation of fermented TH

Tuna hydroysate (TH) supplied by SAMPI, Port Lincoln, Australia, was fermented by following the technique proposed by Himawan et al. (2016). Baker's yeast *Saccharomyces cerevisiae* (Instant dried yeast, Lowan®), and *Lactobacillus casei* in the form of a skim-milk product (Yakult®), were used at 10% and 5% (cell density for *Lactobacillus casei* 3×10^6 CFU g^{-1} meal) of the total weight of the meal mixture, respectively, and distilled water was used at approximately 70% of the total weight of the meal ingredients; all these ingredients were homogenized together in a food mixer. The mixture was transferred into an Erlenmeyer flask, which was then covered with aluminium foil and incubated at 30 °C for 4 d. Following this, the fermented product was dried in an oven at 60 °C for 24 h, and then used as a feed ingredient.

3.2.4 Experimental diets

All the feed ingredients used in this study, except TH, were obtained from Specialty Feeds Pty. Ltd, Great Eastern Highway, Western Australia; TH was provided by SAMPI, Port Lincoln, Australia. The diets were formulated to fulfil the nutritional requirements of the juvenile barramundi set by the (NRC 2011). Two series of experimental diets, isonitrogenous and isocaloric, were formulated for the juvenile barramundi, containing approximate 47% crude protein (CP) and 20 MJ kg^{-1} gross energy (GE), respectively. The diets were labeled as TH_{50} and TH_{75} (non-fermented TH), and FTH_{50} and FTH_{75} (fermented TH). The diets were formulated to replace FM at 50% and 75% inclusion levels of TH/FTH. A basal diet with FM as the sole protein source was used as the control diet. The dried unprocessed tuna hydrolysate (TH) contains 58.4% protein, 1.05% lipid and 11.3% ash, whilst the bioprocessed TH contains 59.10% protein, 1.02% lipid and 12.58 % ash. Another set of diets was

formulated with the addition of 5 g chromic oxide (Cr_2O_3) per kg of diet as an inert marker for assessing the digestibility in the fish (Cr_2O_3; Thermo Fisher Scientific, Scoresby, VIC, Australia). All the test diets were processed with the addition of water to about 35% mash dry weight of the mixed ingredients, in order to form a dough. The dough was passed through a mincer in order to create pellets of desired diameter (3 mm). The moist pellets were oven dried at 60 °C for 48 h and then, cooled at room temperature, sealed in plastic bags, and stored at –15 °C until further use. The formulations and the proximate compositions of the experimental diets are presented in Table 3.1.

Table 3.1 Ingredients and proximate composition of the experimental diets.

Ingredients ((g kg^{-1})[a]	Control	TH_{50}	TH_{75}	FTH_{50}	FTH_{75}
Fish meal	610.00	305.00	152.50	305.00	152.50
Tuna hydrolysate	-	415.00	589.50	454.00	606.50
Wheat flour	266.00	152.00	110.00	113.00	75.00
Wheat starch	20.00	20.00	20.00	20.00	20.00
Fish Oil	30.00	30.00	30.00	30.00	30.00
Limestone ($CaCO_3$)	2.00	2.00	2.00	2.00	2.00
Salt (NaCL)	2.00	2.00	2.00	2.00	2.00
Vitamin Premix[b]	1.00	1.00	1.00	1.00	1.00
Casein	63.00	70.00	90.00	70.00	108.00
Cellulose	6.00	3.00	3.00	3.00	3.00
Proximate composition (% dry matter basis)					
Crude protein	47.42	47.31	47.20	47.08	47.29
Crude lipid	10.00	10.15	10.67	10.19	10.09
Ash	13.04	8.48	6.15	8.61	6.26
GE (MJ kg^{-1})	19.98	20.10	20.19	20.24	20.26

[a]Supplied by Specialty Feeds, Perth, Australia. [b]Contains the following (as g kg^{-1} of premix): iron, 10; copper, 1.5; iodine, 0.15; manganese, 9.5; zinc, 25; vitamin A retinol, 100 IU; vitamin D3, 100 IU; vitamin E, 6.25; vitamin K, 1.6; vitamin B1, 1; vitamin B2, 2.5; niacin, 20; vitamin B6, 1.5; calcium, 5.5; biotin, 0.1; folic acid, 0.4; inositol, 60; vitamin B12, 0.002; choline, 150; and ethoxyquin, 0.125. TH: tuna hydrolysate; FTH: fermented tuna hydrolysate. GE: gross energy; MJ kg^{-1}: mega joule per kilogram.

3.2.5 Digestibility assessment

In order to estimate the apparent digestibility coefficients (ADCs) for dry matter, protein, and lipid, fecal matter was collected using the stripping technique (Austreng 1978), 15 h post feeding, prior to terminating the feeding trial. All the fecal matters collected from each tank within each period were pooled and frozen at –20 °C immediately. Prior to commencing the analysis, the fecal samples were oven-dried to a constant weight at 105 °C. The chromium oxide content in the diet formulations and the fecal samples was analyzed by the method described by Cho et al. (1982). The ADCs for each nutritional component in the test diets were calculated based on the formula given below:

ADC = 100– 100*[marker in feces (%)/marker in diet (%)]*[nutrient in feces (%)/nutrient in diet (%)]

3.2.6 Sampling and chemical analysis

At the termination of the trial, the fish fasted for 24 h. The fish were then anesthetized with 5 mg L^{-1} of AQUI-S (Australia), followed by bulk weighing in order to assess the final body weight (FBW), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate for the fish. Blood samples were collected from the caudal veins of three fish from each tank, using a 1-mL plastic syringe and a 22 G × 1½″ straight needle. The extracted blood sample was transferred to heparinized tubes for the analysis of biochemical indices of blood, such as hemoglobin (Hb) content, hematocrit concentration, leucocrit concentration, and the glutathione peroxidase (GPx) enzyme activity. Hematocrit (Hct,%) and leucocrit (%) concentrations were determined by using the standard McLeay and Gordon's method (McLeay & Gordon, 1977), following a centrifugation at 2,000 rpm for 5 min. The hemoglobin (Hb,%) content was determined by using an Hb kit (Randox Laboratories, Antrim, United Kingdom). The GPx activity (GPx units g^{-1} Hb) in the red blood cells was assessed by using the Ransel RS–505 assay kit (Randox, Antrim, United Kingdom).

The moisture, crude protein, crude lipid, ash, and gross energy contents in the experimental diets were assessed using the methods and procedures given by the Association of Official Analytical Chemists (AOAC 2006). Moisture in the samples was determined by oven-drying to a constant weight at 105 °C; ash content was determined by combustion at 550 °C for 24 h in an electric furnace (Carbolite, Sheffield, UK); crude protein content (N × 6.25) was determined by following the

Kjeldahl method, using a Kjeltec Auto 1030 analyzer (Foss Tecator, Höganäs, Sweden); crude lipid was analyzed by following the Soxhlet technique, using a Soxtec System HT6 (Tecator, Höganäs, Sweden); and the gross energy content was determined by using a bomb calorimeter (Heitersheim, Germany). The composition of amino acids, except tryptophan, in the tested diets, was analyzed by using high-performance liquid chromatography (HPLC), following an acid hydrolysis.

3.2.7 Histopathology

In order to analyze the histopathological conditions, one liver segment from an individual fish, i.e., six liver segments from each treatment were sampled. The liver tissue samples were excised and preserved in 10% buffered formalin until they were processed using the standard histological procedures. The blocks of the designated samples were dehydrated in 100% ethanol, and then, embedded in paraffin wax. The sections of approximately 5 µm in size were cut and stained with Hematoxylin-Eosin (H&E) stain, for histological examination under a light microscope (BX40F4, Olympus, Tokyo, Japan). All the samples were prepared using the standard histological techniques (Luna 1968).

3.2.8 Calculations

The fish fasted for 24 h prior to weighing and sampling, and the following parameters were measured after the growth trial of 56 d:

Weight gain, WG = [(mean final body weight – mean initial body weight) / mean initial body weight].

Specific growth rate, SGR (%/day) = [(ln mean final body weight – ln mean initial body weight)/number of days] × 100

Feed intake, FI = dry feed consumed/number of fish.

Feed conversion ratio, FCR = dry feed fed/wet weight gain.

Condition factor, CF (g $(cm^3)^{-1}$) = [(body weight, g)/(length, cm)^3] × 100.

Hepatosomatic index, HSI (%) = [(liver weight, g)/(body weight, g)] × 100.

Viscerosomatic index, VSI (%) = [(visceral weight, g)/(body weight, g)] × 100.

Survival rate, SR = (final number of fish/initial number of fish) × 100

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

where Σ is the summation for all the observations (x_i) within a sample, and $\bar{x}$ is the sample mean.

3.2.9 Statistical analysis

The statistical analyses were performed using SPSS version 24 for Windows, IBM, Curtin University, Australia. The experimental data for growth performance, digestibility, hematological parameters, and body composition were subjected to one-way ANOVA, followed by Duncan's multiple-range test. The data on length and weight distribution represented through skewness were executed together with the normal distribution test. Data values were expressed as a mean ±standard error in the triplicate tanks, and the threshold of statistical significance was set at $P<0.05$.

3.3 Results

3.3.1 Growth performance, feed utilization and somatic indices

The growth performance, feed utilization, and somatic indices for the juvenile barramundi fed on diets containing different inclusion levels of fermented and non-fermented TH for 56 d are presented in Table 3.2. At the end of the growth trial, it was observed that the growth performance parameters, including FBW, WG, and SGR, decreased as the levels of inclusion of TH and FTH in the diets increased. The control treatment demonstrated best growth performance, and the lowest growth performance was observed for the 75% replacement level in both fermented and non-fermented treatments. Similarly, highest FI was observed in the control group of fish, which was fed on the FM-based diet. The FCR was significantly higher in the fish fed on TH_{75} diet compared to the fish which were fed on all the other diets, except for the fermented-TH group of the same replacement level– FTH_{75}. No significant difference was observed in the FCR among the groups of fish fed on the control, TH_{50}, and FTH_{50} diets. The body indices, such as VSI, HSI, and CF, and the survival rate of the fish were not affected by the inclusion of TH or FTH in the diets. The distributions of length and weight of the juvenile barramundi fed on different diets are presented in Figure 3.1 and 3.2, and the respective values for skewness are presented in Table 3.2. The variations in the length and weight of the fish that were fed on different diets were not significant compared to control. Negative skewness, in terms of length, was obtained for the control, TH_{50}, and FTH_{50} groups; whereas, negative skewness, in terms of weight, was observed for the control and TH_{50} groups.

Table 3.2 Growth performance, feed utilization and somatic indices of juvenile barramundi fed TH diets without or with fermentation for 56 days.

	Experimental diets					P-value
	Control	TH_{50}	TH_{75}	FTH_{50}	FTH_{75}	
Growth performance parameters						
FBW (g)	$31.53^{a} \pm 0.59$	$26.36^{b} \pm 1.00$	$19.05^{c} \pm 0.59$	$27.69^{b} \pm 1.14$	$21.25^{c} \pm 1.67$	<0.001
WG (g)	$25.17^{a} \pm 0.31$	$19.40^{b} \pm 1.10$	$12.38^{c} \pm 0.66$	$20.91^{b} \pm 1.15$	$14.25^{c} \pm 1.18$	<0.001
SGR (% day^{-1})	$2.87^{a} \pm 0.12$	$2.38^{b} \pm 0.16$	$1.78^{c} \pm 0.02$	$2.51^{b} \pm 0.01$	$2.07^{c} \pm 0.01$	<0.001
FI (g $fish^{-1}day^{-1}$)	$1.09^{a} \pm 0.59$	$0.98^{bc} \pm 0.59$	$0.92^{c} \pm 0.59$	$1.00^{b} \pm 0.59$	$0.94^{c} \pm 0.59$	<0.001
FCR	$2.46^{c} \pm 0.02$	$2.69^{c} \pm 0.10$	$4.36^{a} \pm 0.14$	$2.97^{bc} \pm 0.45$	$3.64^{ab} \pm 0.25$	<0.05
VSI	8.95 ± 0.34	10.05 ± 0.36	9.02 ± 1.13	9.92 ± 0.63	9.77 ± 0.79	0.142
HSI	1.49 ± 0.15	2.15 ± 0.26	1.97 ± 0.45	2.20 ± 0.33	2.49 ± 0.38	0.277
CF	1.22 ± 0.03	1.05 ± 0.03	1.02 ± 0.09	0.99 ± 0.13	1.01 ± 0.03	0.277
SR	98.33 ± 1.67	95.00 ± 2.89	93.33 ± 1.67	96.67 ± 1.67	93.33 ± 1.67	0.364
Size distribution statistics						
Skewness for length	-1.087	-0.210	0.694	-0.375	0.280	
Skewness for weight	-0.132	-0.048	1.010	-0.159	0.719	

Values are mean of three replicate tanks (n= 3) ± standard error. Different superscript letters (a,b,c) in the same row denote significant differences ($P<0.05, 0.001$) determined by one-way ANOVA followed by Duncan's post hoc multiple range test. FBW: final body weight; WG: weight gain; SGR: specific growth rate; FI: feed intake; FCR: feed conversion ratio; HSI: hepatosomatic index; VSI: viscerosomatic index; SR: survival.

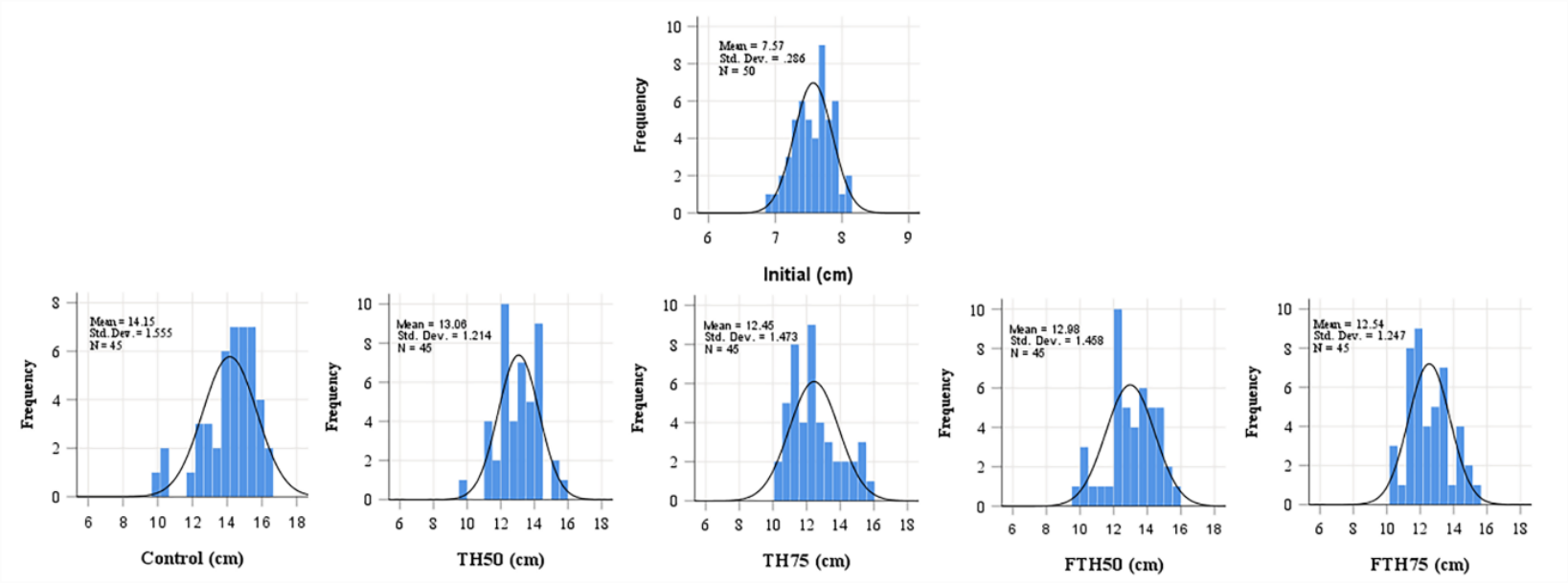

Figure 3.1 Length frequency distribution of initial fish (A) and fish fed on TH diets (B-F) without or with fermentation after 56 days. Frequency histograms of fish from different groups where control, TH_{50} and FTH_{50} fish showing negatively skewed curve indicate a higher proportion of large-body species, and the remaining groups of TH_{75} and FTH_{75} skewed positively indicate small-body species within the normal distribution. n=50 for initial fish (A), n=45 for each experimental treatment (B-F).

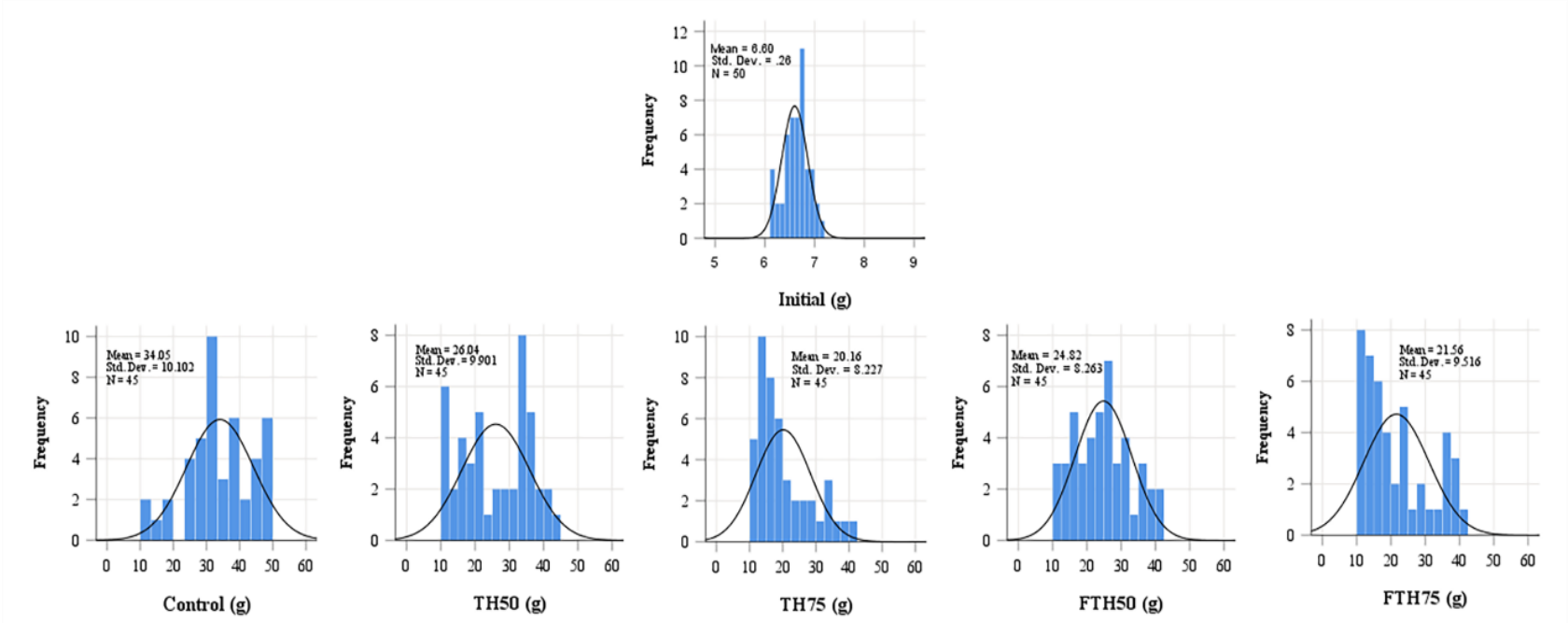

Figure 3.2 Weight distribution of initial fish and fish (A) and fish fed on TH diets (B-F) without or with fermentation after 56 days. Frequency distributions of control and TH_{50} and FTH_{50} fish skewed negatively indicates a higher proportion of large-body species and fish from TH_{75} and FTH_{75} groups skewed positively indicate small-body species within the distribution. n=50 for initial fish (A), n=45 for each experimental treatment (B-F).

3.3.2 Digestibility

The digestibility coefficients for dry matter (DM), protein, and lipid in the juvenile barramundi fed on the diets that contained TH and FTH at various inclusion levels are enlisted in Table 3.3 As seen in Table 3.3, the higher inclusion levels of TH and FTH in the diets resulted in a significant decrease ($P<0.05$) in the apparent digestibility coefficients (ADCs) for DM, protein, and lipid, with the lowest and the highest values of the ADCs for DM, protein, and lipid obtained for the TH_{75} and the control group, respectively.

Table 3.3 Apparent digestibility coefficients (%) of dry matter, crude protein and crude lipid of juvenile barramundi fed TH diets without or with fermentation for 56 days.

	Experimental diets					P-value
	Control	TH_{50}	TH_{75}	FTH_{50}	FTH_{75}	
Dry matter	$89.4^{a} \pm 0.32$	$86.07^{b} \pm 0.54$	$82.47^{c} \pm 0.52$	$87.73^{b} \pm 0.45$	$84.50^{c} \pm 0.27$	< 0.001
Crude protein	$93.97^{a} \pm 0.51$	$92.0^{b} \pm 0.76$	$90.01^{c} \pm 0.74$	$92.41^{ab} \pm 0.49$	$91.0^{bc} \pm 0.35$	< 0.05
Crude lipid	$95.90^{a} \pm 0.15$	$93.48^{bc} \pm 0.37$	$92.80^{c} \pm 0.47$	$94.29^{bc} \pm 0.38$	$93.43^{bc} \pm 0.47$	< 0.05

Values are mean of three replicated tanks (n= 3) ± standard error. Different superscript letters (a,b,c) in the same row denote significant differences ($P<0.05$, 0.001) determined by one-way ANOVA followed by Duncan's post hoc multiple range test. TH: tuna hydrolysate; FTH: fermented tuna hydrolysate.

3.3.3 Whole fish body composition

The whole-body proximate compositions (moisture, protein, lipid, and ash) and the energy content for the fish belonging to the different treatments are presented in Table 3.4. As seen in Table 4, the whole-body proximate composition and the gross energy of the juvenile barramundi were neither influenced by the TH types (fermented or non-fermented) nor by the levels of replacement ($P>0.05$).

Table 3.4 Whole body proximate composition of juvenile barramundi fed on TH diets without or with fermentation for 56 days.

	Experimental diets					*P*-value
	Control	TH_{50}	TH_{75}	FTH_{50}	FTH_{75}	
Moisture (%)	74.57 ± 2.34	75.40 ± 2.42	77.63 ± 1.74	76.53 ± 2.32	77.43 ± 1.74	0.819
Protein (% WW)	14.67 ± 0.77	14.62 ± 0.15	13.22 ± 0.27	14.31 ± 1.14	13.50 ± 0.40	0.974
Lipid (% WW)	4.08 ± 0.09	3.86 ± 0.21	3.50 ± 0.27	3.55 ± 0.26	3.56 ± 0.32	0.991
Ash (% WW)	3.89 ± 0.07	3.71 ± 0.22	3.69 ± 0.04	3.82 ± 0.05	3.87 ± 0.16	0.693
GE (MJ kg^{-1})	18.56 ± 0.55	19.34 ± 1.17	17.18 ± 0.59	19.66 ± 2.32	18.80 ± 1.15	0.720

Values are mean of three replicated tanks (n= 3) ± standard error. Values without superscript letters (a,b,c) in the same row are insignificant ($P<0.05$) determined by one-way ANOVA followed by Duncan's post hoc multiple range test. TH: tuna hydrolysate; FTH: fermented tuna hydrolysate; DM: dry matter; GE: gross energy.

3.3.4 Biochemical status

The blood biochemical indices and the glutathione peroxidase (GPx) enzyme activity for the juvenile barramundi are presented in Table 3.5. The dietary inclusion of TH and FTH exhibited no significant effect on the blood hemoglobin, hematocrit, and leucocrit levels in the fish. However, the incremental inclusion of TH and FTH exhibited significant effects on the glutathione peroxidase (GPx) enzyme activity in the juvenile barramundi. The fish fed on the FM-replacement diets TH_{50}, FTH_{50}, and TH_{75} exhibited significantly reduced GPx activity compared to control; whereas, the 75% FM-replacement diet FTH_{75} exhibited no significant difference in the GPx activity compared to the control. Furthermore, the GPx activity was significantly increased in the fish fed on fermented diet compared to the fish fed on the non-fermented diet, when the replacement level was 75%; however, at 50% replacement level, the difference in the GPx activity was not significant between the fermented and the non-fermented diets ($P>0.05$). The antioxidant glutathione peroxidase (GPx) activity values obtained for the juvenile barramundi belonging to different treatments are presented in Figure 3.3.

Table 3.5 Blood biochemical parameters of juvenile barramundi fed on TH diets without or with fermentation for 56 days.

	Experimental diets					*P*-value
	Control	TH_{50}	TH_{75}	FTH_{50}	FTH_{75}	
Hb (g dl^{-1})	73.0 ± 6.25	67.0 ± 7.88	62.33 ± 1.86	63.67 ± 3.00	79.67 ± 6.69	0.241
Hct (%)	27.67 ± 2.19	24.0 ± 2.00	25.0 ± 0.88	28.0 ± 0.58	29.67 ± 1.00	0.113
Leucocrit (%)	1.27 ± 0.04	1.24 ± 0.07	1.21 ± 0.07	1.19 ± 0.02	1.23 ± 0.06	0.328

Values are mean of three replicated tanks (n= 3) ± standard error. Values without superscript letters (a,b,c) in the same row are insignificant (P<0.05) determined by one-way ANOVA followed by Duncan's post hoc multiple range test. TH: tuna hydrolysate; FTH: fermented tuna hydrolysate; Hb: hemoglobin; Hct: hematocrit; gdL^{-1}: gram per deciliter.

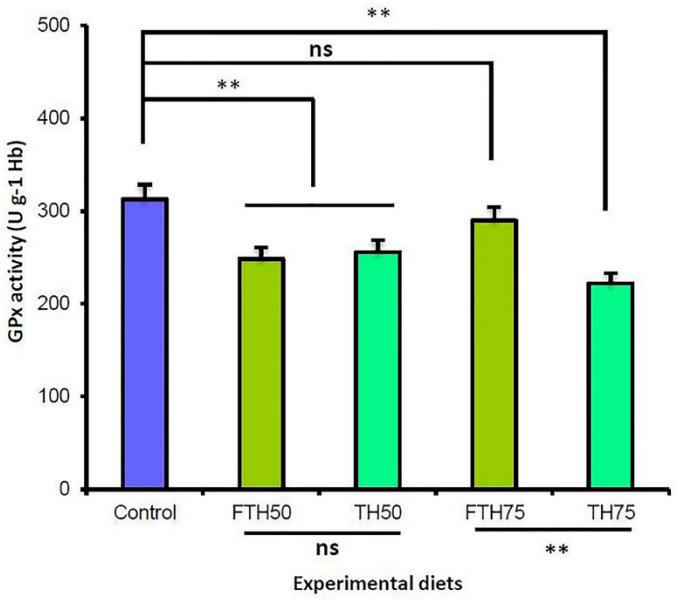

Figure 3.3 Glutathione peroxidase (GPx) activities of juvenile barramundi fed on TH diets without or with fermentation for 56 days. Post-ANOVA Duncan's multiple comparisons test was applied to compare GPx activities of fish fed on four experimental diets to the control. Values are mean of three replicate tanks per treatment ± standard error. The significant difference was considered at P<0.05. (**: significant; ns: non-significant).

3.3.5 Liver histopathology

The control fish exhibited a normal liver condition, which is characterized by hexagonal hepatocytes with a round, central nucleus, and a rare occurrence of cytoplasmic vacuolization or granules (Figure 3.4A). The excessive use of TH in the diets resulted in several alterations in the liver tissue of the fish. The alterations, including the occurrence of cytoplasmic vacuolization with lipid accumulation (steatosis), were observed in the fish fed on the TH_{75} diet (Figure 3.4B). In the fish fed on the FTH_{75} diet, severe and irreversible damages were observed in the liver, which were characterized by disorganized hepatic cordons, cellular deformation, and nuclear hypertrophy. In further damaged livers, in addition to the other alterations, the absence of nucleolus and the presence of necrotic foci were observed (Figure 3.4C).

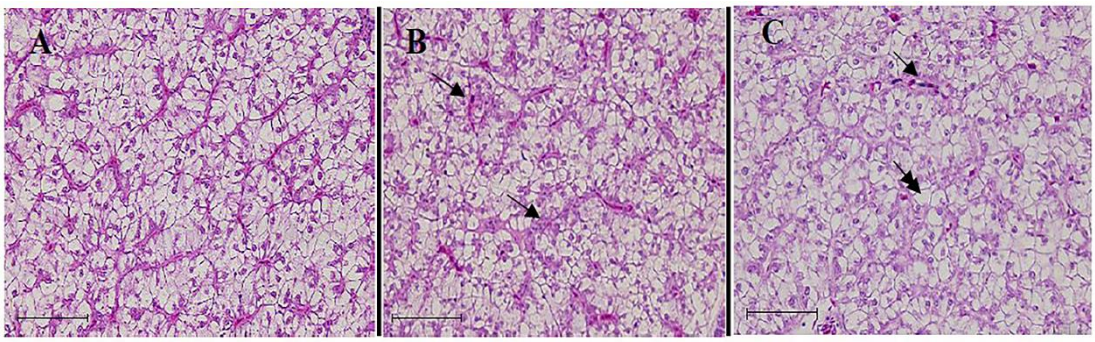

Figure 3.4 Liver histopathology of juvenile barramundi fed on TH diets without or with fermentation for 56 days. (A) Control group. The hexagonal hepatocyte with predominantly glycogen vacuoles. (B) Fish fed on TH_{75} diet. Arrows indicate hepatocytes containing lipid droplet and cellular degeneration. (C) Fish fed on FTH_{75} diet. Arrow indicates necrotic foci and double arrow at nucleus indicates disappearance in hepatic cells (H&E staining 400x magnification, scale bar = 50µm).

3.4 Discussion

The dietary inclusion of FPH produced from whole herring (*Clupea harengus*) at moderate levels (18% to 24%) has been reported to confer a positive effect to the growth performance, feed utilization, and digestibility in the Atlantic salmon (*Salmo salar* L.); whereas, the inclusion of the same FPH at higher (>24%) and lower (<12%) levels has been demonstrated to confer negative effects (Hevrøy et al. 2005). In a similar vein, Kim et al. (2014) reported that the replacement of FM in the fish feeds by more than 30%, with tuna by-product ingredients resulted in a decrease in the growth and feed utilization of juvenile olive flounder (*Paralichthys olivaceus*). Similar outcomes were observed by Cahu et al. (1999), where the addition of more than 19% of soluble protein hydrolysate negatively affected the enzymatic activities of trypsin, alkaline phosphatase, and aminopeptidase in the 41-days-old larvae of sea bass (*Dicentrarchus labrax*). The present study supports these findings; herein also, the feeding of juvenile barramundi with diets containing higher levels of TH (50% to 75%) resulted in detrimental effects on the WG and SGR of the fish. These deleterious effects on the growth performance of the fish could be due to an excessive number of short-chain peptides and free amino acids (FAA) present in the hydrolysed products (Ospina-Salazar et al. 2016), which might have caused saturation in the peptide transport mechanism (Carvalho et al. 2004). Furthermore, the higher amounts of FAA are able to alter the absorption of amino acids, leading to amino acid imbalances in the fish gut (Kolkovski & Tandler 2000a; Rønnestad et al. 2000); this might have been another reason for the poor growth performance of the fish fed on higher levels of protein hydrolysates.

The low-molecular-weight peptides released as a result of protein hydrolysis are often associated with improvement in feed palatability and attractability (Aksnes et al. 2006b; Kasumyan & Døving 2003), which may result in an increased consumption of feed by the fish (Refstie et al. 2004). In contrast, hydrolysis has also been reported to be responsible for creating a bitter taste in the feed, due to the release of certain peptides during the process that contains hydrophobic amino acid residues (FitzGerald & O'Cuinn 2006). In the present study, a significant reduction in the FI of the fish that were fed on the TH- and FTH-included diets, compared to control, indicated that the differences in the growth performance of the fish belonging to different treatments may be related to decreased palatability or increased bitterness of the diets. An increase

in the FCR values was observed with the increasing addition of TH in the diets. In accordance with the present study, a significant increase was observed in the FCR values, in juvenile Japanese sea bass (*Lateolabrax japonicus*) that were fed on diets containing 15%–25% FPH produced from the gut and head of Alaska pollock (*Theragra chalcogramma*) (Liang et al. 2006). Moreover, Hevrøy et al. (2005) demonstrated that the dietary inclusion of FPH procured from whole herring (*Clupea harengus*) at higher levels (> 30%), in the diets of Atlantic salmon (*Salmo salar* L.), significantly increased the FCR values for the salmon fish. However, Ospina-Salazar et al. (2016) observed no significant variation in the FCR values, despite feeding the juvenile pike silverside (*Chirostoma estor*) with diets containing up to 45% of fish soluble protein concentrates.

The CF of fish has been reported to reflect the nutritional status of the fish, and act as an indicator of the physiological condition of the fish (Vo et al. 2015). In the present study, the CF values were not influenced by the inclusion levels of TH and FTH in the diets of the juvenile barramundi. Similarly, Ovissipour et al. (2014) were not able to observe any significant difference in the CF of Persian sturgeon (*Acipenser persicus* L.) which was fed on a diet containing tuna-viscera protein hydrolysate at 50% inclusion level. In the present study, the replacement of FM protein by TH and FTH did not influence the HSI and VSI values for the fish. These results were in agreement with the findings reported by Khosravi et al. (2015a), a study which involved red sea bream (*Pagrus major*). However, the results of the present study for VSI values were in contrast with the findings of Xu et al. (2016), who reported a decreased performance in turbot (*Scophthalmus maximus*) which was fed on diets containing higher levels of FPH. The decreased HSI, observed in the present study, with increasing levels of FPH may indicate improper storage of the macro- and micro-nutrients in the fish body, an unhealthy condition of the liver, and clinically an unhealthy sign. Nevertheless, these indices might have been influenced by a variety of factors, including sex, life history, availability of food, and the experimental condition of the fish (Barton et al. 2002).

Several studies have reported that the inclusion of protein hydrolysates in fish diets improves the digestibility of fish (Hevrøy et al. 2005; Zheng et al. 2012); whereas, certain other studies have reported that the inclusion of FPH at higher levels may often cause adverse effects on the digestibility of the fish (Ospina-Salazar et al. 2016). In the present study, it was observed that the ADCs for dry matter, protein, and lipid

decreased with the increasing levels of TH and FTH inclusion in the diets, which might have occurred due to the availability of excess amounts of free amino acids and free nucleotides, which may, in turn, have disturbed the normal process of digestion and metabolism of the ingested diets, resulting in poor digestibility (Zheng et al. 2013a). In a study by Ospina-Salazar et al. (2016), it was observed that the replacement of FM by more than 30% with FPH (CPSP Special-G™) resulted in a significant reduction in the ADCs for dry matter and lipid; however, the ADC for protein remained unaltered by the inclusion levels of FTH in the diets. On the other hand, Bui et al. (2014) and Oliva-Teles et al. (1999) reported that the dietary inclusion of FPH in red sea bream (*Pagrus major*) and in turbot (*Scophthalmus maximus*), respectively, caused no influence on the digestibility of the fishes.

In the present study, the body composition of the juvenile barramundi remained unaffected by the addition of TH in the diets. Similarly, in the studies conducted by Khosravi et al. (2015b) on sea bream (*Pagrus major*), and Oliva-Teles et al. (1999) on turbot (*Scophthalmus maximus*), no differences were observed in the whole-body proximate composition of the fish that were fed on diets containing FPH at different inclusion levels. However, the results of the present study are contradictory to the findings of Ospina-Salazar et al. (2016), who reported a decrease in the lipid composition with an increase in the inclusion level of fish hydrolysate in the diets of juvenile pike silverside. In addition, Kim et al. (2014) reported that a moderate-level (>30%) inclusion of FPH in an FM-based diet significantly elevated the whole-body protein composition in juvenile olive flounder (*Paralichthys olivaceus*). Furthermore, Zeitler et al. (1984) reported that body composition was influenced by the species of the fish, the diet formulations, and the feeding method. It is possible that a number of factors, including the source of the hydrolysates, the varying inclusion levels of the hydrolysates, and the fish species, may have influenced the effects of FPH on the whole-body composition of the fish.

Hematological indices are used as important biological indicators for examining the physiological changes and the health condition of fish (Vazquez & Guerrero 2007). In the present study, no association was observed between the levels of replacement of the FM protein with TH diets and the modulations in the hematological indices, for juvenile barramundi. However, the Hb concentration obtained in the present study was comparable to that reported in a study on Atlantic salmon (*Salmo salar* L.), where the

Hb concentration (%) remained unaltered even after feeding the fish with diets containing hydrolysates from whole herring (*Clupea harengus* L.) at 30% inclusion level (Hevrøy et al., 2005). Similarly, the inclusion of hydrolysates from krill, shrimp, and tilapia in the diets did not alter the qualitative descriptions of Hb in red sea bream (*Pagrus major*) (Khosravi et al., 2015b). Furthermore, Ilham & Fotedar (2017) observed no significant variation in the Hb concentration in juvenile barramundi fed on fermented soybean meal supplemented with organic selenium. In the present study, the average hematocrit concentration ranged between 24% and 29.67%, which was below the normal range (30%–45%) suggested by Adams et al. (1993). It appears that a number of abnormalities, including stress and disease, in the farmed fish are directly or indirectly related to the physiological changes in the blood system (Ilham et al., 2016). For example, leucocrit content in the blood plays a vital role and serves as an indicator of health status in fish (Wedemeyerr et al. 1983), as malnutrition, as well as lower resistance to pathogens, are associated with lower levels of leucocrit (Ilham et al. 2018). In the present study, the leucocrit percentage was not influenced by the inclusion of fermented and non-fermented TH in the diets. There is no published information available to compare the differences in the leucocrit levels observed in this study in the juvenile barramundi fed on TH diets.

The enzymatic antioxidant GPx is more potent than any other antioxidant defense enzymes in fish (Ross et al. 2001). GPx plays a crucial role in accelerating the enzymatic defense system against the production of the extreme reactive oxygen species (ROS), as well as against lipid peroxidation (Kohen & Nyska 2002). In addition, it protects the cells from the oxidative damage by metabolizing the hydroperoxides (Arthur et al. 2003). The protein hydrolysates produced from fishes such as tuna (*Thunnus obesus*), mackerel (*Scomber australasicus*), and Alaska Pollack (*Theragra chalcogramma*) have demonstrated antioxidant activity (Je et al. 2005; Wu et al. 2003; Yang et al. 2011). In recent years, hydrolysed proteins from fish have been observed to possess antioxidant properties (Bougatef et al. 2009) and exhibit immunomodulatory effects (Liang et al. 2006). The antioxidant activity has been reported to be influenced by the inclusion levels of the hydrolysates and the composition of free amino acids and peptides in the protein hydrolysates (Wu et al. 2003). In the present study, increased antioxidant activity was observed in the fish fed on fermented TH compared to the fish fed on non-fermented TH, at 75% replacement

level, indicating that fermentation promotes the positive effects of augmenting the antioxidant capacity in the fish. This result is in line with the antioxidant GPx responses to the dietary inclusion of fermented soybean meal (SBM) reported in red sea bream (*Pagrus major*) by Kader et al. (2010). Similarly, a study by Azarm & Lee (2014) reported that fermented soybean meal prompted the enzymatic antioxidant GPx in blackhead sea bream (*Acanthopagrus schlegelii*), through the bioavailability of isoflavones produced by the microbial activity. Moreover, one of our previous studies suggested that an appropriate level of dietary inclusion of fermented lupin meal supports growth and the antioxidant GPx activity in juvenile barramundi (Ilham & Fotedar, 2017). However, in the present study, the reduced GPx activity in the fish fed on TH_{50}, FTH_{50}, and FTH75 replacement diets compared to control may have been caused as a result of a higher inclusion level of TH rather than due to the fermentation process. The decrease in the antioxidant capacity might have triggered a considerable consequence on the cellular structure of the studied fish. This circumstance might have accelerated the lipid accumulation in the hepatocytes, which could be the possible reason for the reduced growth and feed utilization reported in the juvenile barramundi.

The liver, which is an accessory digestive organ, is a good indicator of the nutritional condition of fish (Lozano et al. 2017; Raskovic et al. 2011). The most common changes observed in the fish liver due to nutritional disorders are lipid accumulation, vacuolization in the hepatocytes, changes in the size and shape of the nuclei, changes in liver parenchyma and the cell membranes, disappearance of the nucleolus, and in severe cases, formation of necrotic foci (Raskovic et al. 2011). In the present study, the fish fed on the TH_{75} and FTH_{75} diets revealed certain nutritional deficiency symptoms, including the presence of hepatocytes that contained lipid droplet, and the degeneration of vacuoles, along with necrotic loci and necrosis. It was assumed that an excess amount of FPH in the diets may have been able to boost the tocopheroxyl radicals in the tissues, thereby accelerating lipid peroxidation and tissue damage in the liver of the fish. According to Caballero et al. (1999), lipid deposition in the liver indicates a pathological process that may be considered an indication of hepatic failures in fat metabolism. Moreover, the hepatic alteration, due to excessive caloric ingestion which saturates the physiological competency of the liver, may also lead to lipid accumulation (Spisni et al. 1998). Apart from the lipid accumulation, distorted

nucleus, changes in the cell membranes, and mild necrosis in certain regions of the liver are the major symptoms of liver toxicity (Shiogiri et al. 2012).

3.5 Conclusion

Feeding the juvenile barramundi with high levels (50% and 75%) of TH-included diets resulted in declined growth and digestibility in the fish, and abnormal signs of liver histopathology were observed. When the FM protein was replaced with fermented and non-fermented TH at 50% and 75% inclusion levels in the diets, it did not improve the health and the antioxidant capacity of the juvenile fish, as indicated by the biochemical responses of blood and the GPx activity, respectively. However, it is unclear whether fermentation is able to improve the dietary efficacy of TH, which has already been subjected to intensive processing. Therefore, further studies are required to investigate the effects of fermentation and the optimum levels of TH inclusion in the diets for an improved growth performance and blood physiology.

Chapter 4: Dietary tuna hydrolysate modulates growth performance, immune response, intestinal morphology and resistance to *Streptococcus iniae* in juvenile barramundi, *Lates calcarifer*

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Abstract

This study investigated the effects of tuna hydrolysate (TH) inclusion in fishmeal (FM) based diets on the growth performance, innate immune response, intestinal health and resistance to *Streptococcus iniae* infection in juvenile barramundi, *Lates calcarifer*. Five isonitrogenous and isoenergetic experimental diets were prepared with TH, replacing FM at levels of 0% (control) 5%, 10%, 15% and 20%, and fed fish to apparent satiation three times daily for 8 weeks. The results showed that fish fed diets containing 5% and 10% TH had significantly higher final body weight and specific growth rate than the control. A significant reduction in blood glucose was found in fish fed 10%, 15% and 20% TH compared to those in the control whereas none of the other measured blood and serum indices were influenced by TH inclusion. Histological observation revealed a significant enhancement in goblet cell numbers in distal intestine of fish fed 5 to 10% TH in the diet. Moreover, fish fed 10% TH exhibited the highest resistance against *Streptococcus iniae* infection during a bacterial challenge trial. These findings therefore demonstrate that the replacement of 5 to 10% FM with TH improves growth, immune response, intestinal health and disease resistance in juvenile barramundi.

4.1 Introduction

Lates calcarifer, a euryhaline carnivorous fish species commonly known as barramundi or Asian sea bass, is widely cultured throughout the Indo-Pacific region and Australia (Ngoh et al. 2015; Siddik et al. 2018a). Currently, the commercial farming of this species is well established in ponds, net cages and recirculating aquaculture systems (RAS) in both fresh and saline water. However, the over-dependence on FM as a protein source in aqua-diets is regarded as one of the major threats for the sustainable development of aquaculture (Vo et al. 2015). Whilst FM is a highly effective protein source in aquatic feeds, issues with supply, increasing prices

and environmental concerns are putting pressures on the aquaculture industry to reduce the levels of FM in such diets (Ilham et al. 2016a). Further, mass production and intensive farming of barramundi may possibly result in disease outbreaks (Schipp et al. 2007a) and strategies to mitigate such events are necessary. Hence, suitable alternatives containing bioactive compounds which can both substitute FM and stimulate the defence mechanism of fish are a research priority for a sustainable barramundi industry. Fish protein hydrolysates (FPH) are a possible source of immunostimulants that offer potential growth and immunity benefits against stress and pathogens to the host fish (Bui et al. 2014; Ovissipour et al. 2014).

Fish processing industries produce large volume of by-products, including fins, skin, head, viscera and bones, that are commonly discarded as waste products (Yang et al. 2011). These by-products can potentially be used as dietary protein sources in the aquaculture industry following enzymatic hydrolysis, a protein pre-digestion process converting the native proteins into amino acids and peptides suitable for intestinal assimilation. Absorption of peptides through the intestine of vertebrates is a major path of transport and peptides of low molecular-weight are absorbed more rapidly than whole proteins (Tesser et al. 2005). Recent studies have found that protein hydrolysates with high digestibility, excellent viscosity, good texture, suitable polypeptide fractions and free amino acids, can increase nutrient uptake owing to enhanced biological functionality (Ospina-Salazar et al. 2016; Saidi et al. 2014).

The effects of dietary FPH have been evaluated in many commercially important fish species as a partial replacement or supplement to FM (Bui et al. 2014; Khosravi et al. 2015b; Kim et al. 2014), as immunostimulants to defend against stress and pathogens (Bui et al. 2014; Murray et al. 2003; Zheng et al. 2012) and as attractants to increase diet palatability (Ho et al. 2014). The findings of these studies suggest that dietary inclusion of FPH at an appropriate level can have beneficial effects on the feed intake, digestibility, growth performance, innate immunity and specific disease resistance of fish. In addition, most of the studies which have measured innate immune functions have suggested that the immune-reactive peptides in fish protein hydrolysates may play an important role in heightening the innate immunity. The immune-stimulating effects of FPH may therefore result in improved defense against pathogens (phagocytosis and pinocytosis), enhanced lysosomal enzyme activities, enriched alternate complement response, improved hematological defense parameters and

enhanced antioxidant activities (Bøgwald et al. 1996; Khosravi et al. 2015a; Murray et al. 2003).

Presently, several FPHs are effectively used in aqua-feeds for their versatile properties (Khosravi et al. 2017; Nurdiani et al. 2015; Wei et al. 2016) however, no information has been reported on the use of FPH in diets for barramundi. In an effort to diversify the use of FPH in aquaculture, the aim of our study was to investigate whether tuna hydrolysate (TH) is beneficial in terms of growth and immune functions for juvenile barramundi.

4.2 Materials and methods

4.2.1 Ethic statements

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Australia. The protocol was approved by the Ethics Committee in Animal Experimentation of the Curtin University (Approval number AEC_2015_41).

4.2.2 Experimental fish

Following a stringent size grading, a total of 300 healthy juvenile barramundi were sourced from the Australian Centre for Applied Aquaculture Research, Fremantle, Australia and transported to the Curtin Aquatic Research Laboratories (CARL), Bentley, Australia. The fish were then acclimated at CARL for 14 days. During the acclimation period, fish were fed twice a day with a commercially formulated diet (470 g protein kg^{-1} diet and 20.0 MJ kg^{-1} dietary gross energy).

4.2.3 Experimental diet

All ingredients, except TH were purchased from Specialty Feeds Pty. Ltd, Great Eastern Highway Western Australia. Liquid TH was provided by SAMPI, Port Lincoln, Australia. The dried TH contains 58.4% protein, 1.05% lipid and 11.3% ash. Five isonitrogenous and isocaloric diets were prepared for barramundi having 47% crude protein (CP) and 20 $MJ.kg^{-1}$ gross energy (GE). These diets were labelled as TH0, TH5, TH10, TH15 and TH20 to replace FM at 0%, 5%, 10%, 15% and 20%, respectively by TH. TH0 diet with no replacement was considered as the control. The formulation and proximate composition of the experimental diets are presented in Table 4.1. The experimental diets were prepared based on the standard method of

CARL (Ilham et al. 2016b). All test diets were processed with the addition of water to about 35% mash dry weight of mixed ingredients to form a dough. This dough was then passed through a mincer to create pellets of the desired size (3mm). The moist pellets were then oven dried at 60°C for 48 hours and then cooled at room temperature, sealed in plastic bags and stored at −15◦C until further use.

Table 4.1 Formulation and proximate composition of the experimental diets for juvenile barramundi.

Ingredients (g kg^{-1})[1]	Experimental diets				
	Control	TH05	TH10	TH15	TH20
Fish meal	610.0	579.5	549.0	518.5	488.0
Tuna hydrolysate	-	30.5	61.0	91.5	122.0
Wheat	266.0	260.0	254.0	248.0	240.0
Wheat starch	20.0	20.0	20.0	20.0	20.0
Fish Oil	30.0	30.0	30.0	30.0	30.0
Calcium carbonate	2.0	2.0	2.0	2.0	2.0
Salt (NaCl)	2.0	2.0	2.0	2.0	2.0
Vitamin premix[2]	1.0	1.0	1.0	1.0	1.0
Casein	63.0	69.0	75.0	81.0	89.0
Cellulose	6.0	6.0	6.0	6.0	6.0
Proximate composition (% dry matter)					
Dry matter	92.72	91.05	90.38	89.71	89.04
Crude protein	47.10	47.16	47.14	47.12	47.18
Crude lipid	9.99	9.88	9.96	9.94	9.92
Ash	13.04	12.56	12.09	11.61	11.14
NFE[3]	22.59	21.45	21.19	21.04	20.80
Gross energy ($MJkg^{-1}$)	19.98	19.97	19.96	19.95	19.97

[1]Supplied by Specialty Feeds, Perth, Australia. [2]Vitamin premix (g/kg): iron, 10; copper, 1.5; iodine, 0.15;manganese, 9.5; zinc, 25; vitamin A retinol, 100 IU; vitamin D3, 100 IU; vitamin E, 6.25; vitamin K, 1.6; vitamin B1, 1; vitamin B2, 2.5; niacin, 20; vitamin B6, 1.5; calcium, 5.5; biotin, 0.1; folic acid, 0.4; inositol, 60; vitamin B12, 0.002; choline, 150; ethoxyquin, 0.125. [3]Nitrogen free extracts (NFE) = dry matter - (crude lipid + crude ash + crude protein).

4.2.3 Experimental conditions and feeding

Following the aforementioned 14 day acclimation period, 300 uniformly sized juvenile barramundi (pool weight of 12.23 ± 0.11 g $fish^{-1}$) were randomly distributed into

fifteen independent tanks (300-L water capacity) at a stocking density of 20 fish per tank. Each tank was supplied with constant aeration and water was recirculated from an external bio-filter (Fluval 406, Hagen, Italy) at a rate of 10 L min^{-1}. The water quality parameters such as temperature (27.90 – 29.20 ^{0}C), salinity (32 - 36 ppt), dissolved oxygen (5.92 - 7.42 mgL^{-1}), ammonia nitrogen (< 0.50 mgL^{-1}) and nitrite (< 0.50 mgL^{-1}) were monitored daily and were always within the suitable range of fish culture in recirculating aquaculture systems. Fish were kept at 14:10 hr light: dark cycle using *automatic indoor light* switches (Clipsal, Australia). During the experimental period of 8 weeks, fish were fed the treatment diets to satiety three times a day at 0800, 1200 and 1700 h. Fish were starved for 24 h prior to being anaesthetised (AQUI-S®, 8 mgL^{-1}), weighed and taking blood samples.

4.2.4 Biochemical indices of blood and serum

At the end of the feeding trial, duplicate blood samples from two anaesthetized fish per tank (six fish per dietary treatment) were withdrawn by caudal vein puncture with a 1 mL non-heparinized syringe. The first set of extracted blood was transferred to heparinised tubes for the determination of haematocrit and blood glucose level. The second set of blood samples were transferred to non-heparinized tubes and allowed to clot overnight. The following day clotted blood samples were centrifuged at 3000 rpm for 15 min at 4 °C, serum was separated and then stored at −80 °C for later measurement of the serum biochemical parameters and immunological indices described below.

Hematocrit (Ht %) was determined by centrifugation of whole blood in glass capillary tubes at 2000 rpm for 5 min following the method of McLeay & Gordon (1977) and expressed as a percentage. A blood glucose meter kit (Accu-Chek, Australia) was used to measure the blood glucose level. Serum biochemical parameters, including aspartate aminotransferase (AST), glutamate dehydrogenase (GLDH), total protein and albumin were measured using an automated blood analyzer (SLIM; SEAC Inc, Florence, Italy) following the methods from Blanc et al. (2005). The total globulin content was determined by subtracting the albumin values from the total serum protein values. The albumin and globulin ratio (A/G ratio) was obtained by dividing albumin values by globulin values.

4.2.5 Histology and intestinal micromorphology analysis

In order to analyze the histopathological condition of liver, spleen, muscle, distal intestine, and histomorphological condition of the intestine, two fish from each replicate were examined (i.e. six juvenile barramundi per dietary treatment) from which blood had previously been extracted. Samples of all tissues were fixed in 10% buffered formalin, dehydrated in ethanol before equilibration in xylene and embedding in paraffin wax. Sections of approximately 5 µm were cut and stained with haematoxylin and eosin (H&E) for histological examination under a light microscope (BX40F4, Olympus, Tokyo, Japan). Digitalized histology images were analyzed using Image J software at different magnification for assessing the height of folds, enterocytes and microvilli according to the procedures described by Escaffre et al. (2007) with minor modifications. The number of goblet cells were counted in the highest 10 mucosal folds with the numbers expressed as average number of goblet cells per fold as described by Ramos et al. (2017). For gut sample, three cross-sections were quantified for GC, hF, hMV and ECS of the distal intestinal samples.

4.2.6 Bacterial challenge trial

S. iniae, a bacterium pathogenic for barramundi was obtained from the Bacteriology Laboratory, Department of Agriculture & Food, Perth, Australia. The bacteria were grown in trypticase soy broth (Oxoid, Basingstoke, UK) at 24°C for 24 h and the broth containing the culture was centrifuged at 5000g for 15 min. The supernatant was discarded and the pellets were washed twice in phosphate-buffered saline (pH 7.2).

At the end of growth trial, 10 average sized fish from each replicate tank were moved to each of 20 x 100 L capacity glass aquaria in separate room in CARL for 14 days bacterial challenge. Of the 20 aquaria, 15 were used for survival assessment counting and 5 were utilised for blood sampling after challenge. The experimental conditions were as follows: water temperature 28.2 °C, salinity 35 g L^{-1}, pH 7.6 and photoperiod 14:10 hr light: dark. Following the acclimation, fish were subjected to a bacterial bath challenge with *S. iniae* by removing the fish from the tank and adding them to a bath containing 1.8 X 10^3 CFU mL-1 of the bacteria for 1 minute according to Bromage and Owens (Bromage et al. 1999). After bathing, fish were returned to their respective aquaria and feeding continued on the treatment diets once per day and fish were closely monitored for bacterial infection. During the challenge period, fish were monitored for signs of infection counted twice daily at 0800 and 1700 h. Infected fish were counted

and then removed. Blood sampling for the immune parameters lysozyme and complement activity were conducted before challenge and then again 24 h and 7 d post challenge, and the fish were returned to the respective aquaria after bleeding. To avoid the repeated blood sampling from same fish, fish were tagged individually during stocking.

4.2.7 Lysozyme activity assay

Serum lysozyme activity was assessed by a turbidimetric assay described by Le et al. (2014) with slight modifications. Briefly, *Micrococcus luteus* (0.6 mg mL^{-1}) (Sigma) suspension at 0.2 mg $mL^{-1}$was suspended in sodium phosphate citrate buffer (pH 7.2, 0.05 M) and 30 µL of serum samples were placed into wells of a 96-well plate in triplicate. The mixture was incubated at 25^{o}C and its absorbance was monitored every 5 min for a total of 30 min at 450 nm with a plate reader. The results are presented as Unit mL^{-1}.

4.2.8 Alternative complement activity assay

The alternative complement activity was measured using a method modified from Yadav et al. (2014) using rabbit red blood cells. Briefly, the rabbit red blood cells (RaRBC) were washed 3 times in 10 mM EGTA-GVB buffer (ethylene glycol tetra-acetic acidmagnesium-gelatin veronal buffer) and then diluted to give 1% suspension containing 2 x 10^{8} cells mL^{-1} in the same buffer. The RaRBC suspension was standardized by adding 100 µL of the 1 % suspension to 3.4 mL of distilled water as a blank and the OD of the hemolysate was measured at 405 nm against distilled water to obtain the 100 % lysis value. For the blank, red blood cells were similarly mixed with the EGTA –GVB working buffer. A quantity of 100 µL aliquots of serially diluted serum in EGTA –GVB buffer were mixed with 20 µL of red blood cells in a 96 round bottom well plate. The plate was incubated for 90 min at room temperature with gentle shaking every 15 minutes to suspend the RaRBC. After incubation, the plate was centrifuged for 10 min at 800 g at 4 ^{o}C. The optical density (OD) of the supernatant was measured at 405 nm using a plate reader. The reciprocal of the serum dilution inducing 50% lysis of RBCs was determined as the ACH50 expressed as unit mL^{-1}.

4.2.9 Statistical analysis

The data were analysed using SPSS for Windows version 25, IBM Curtin University, Australia. Except lysozyme and complement activity all data was subjected to one-

way analysis of variance (ANOVA) followed by Turkey multiple range tests to compare the control diet against each test diet containing tuna hydrolysate (TH). Lysozyme and complement activity were analysed by multifactorial analysis of variance (ANOVA). All results are expressed as means and standard errors (S.E.) with p-values less than 0.05 were considered statistically significant. The FBW was subjected to quadratic regression analysis with TH inclusion levels. The survival graph was constructed using the Kaplan–Meier method and the differences among different dietary groups were performed using log-rank test.

4.3 Results

4.3.1 Growth performance

All tested diets were readily accepted by the juvenile barramundi during the 8 week feeding trial. Growth performance, feed intake (FI), food conversion ratio (FCR) and survival of barramundi fed the four experimental diets and the control are shown in Table 4.2. Among the dietary groups, significantly greater final body weight (FBW) and specific growth rate (SGR) were observed in the group fed TH05 and TH10 compared to the control, but they were not significantly different from the other treatments. The optimal levels of TH for FBW and SGR were investigated through the quadratic regression analysis (Figure 4.1), and the estimated TH inclusion level was 10.5% for the highest FBW. However, the feed utilization indices such as FI and FCR, and survival of fish were not affected by any dietary treatments.

Table 4.2 Growth performance and feed utilization of juvenile barramundi (initial body weight, 12.23 ± 0.41 g) fed tuna hydrolysate (TH) included diets at various levels for 8 weeks.

Parameters	Experimental diets					P-value
	Control	TH05	TH10	TH15	TH20	
FBW (g)	78.17^{b}±1.17	85.37^{a} ±1.79	85.05^{a}±1.08	81.72^{ab}±1.21	80.67^{ab}±1.60	0.021
SGR (%/d)	3.31^{b}±0.03	3.47^{a}±0.04	3.46^{a}±0.02	3.39^{ab}±0.03	3.37^{ab}±0.04	0.020
FI (g/fish/day)	1.46±0.01	1.47±0.02	1.49±0.01	1.47±0.01	1.46±0.01	0.831
FCR	1.24±0.03	1.13±0.03	1.13±0.03	1.19±0.02	1.20±0.03	0.056
Survival (%)	100.00±0.00	100.00±0.00	98.33±1.67	98.33±1.67	96.67±1.67	0.512

Different superscript letters (a,b,c) in the same row denote significant differences ($P<0.05$). Data were represented as mean ± SE.

FBW: mean final body weight (g).

SGR: specific growth rate = [(ln final body weight - ln (pooled initial body weight))/days] ×100

FI: feed intake = dry feed consumed/fish number.

FCR: feed conversion ratio = dry feed fed/wet weight gain.

Survival (%) = (number of final fish- number of initial fish)/number of initial fish×100

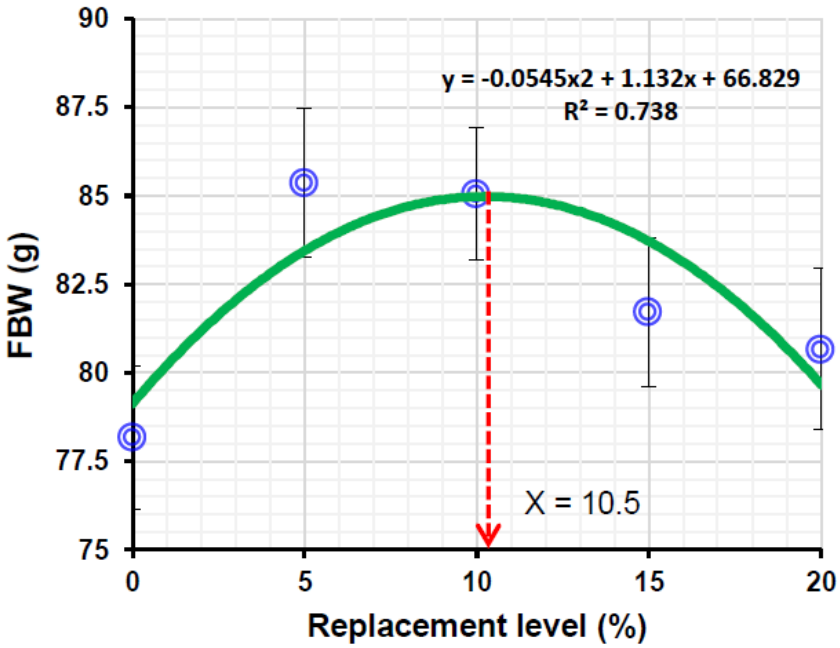

Figure 4.1 Quadratic regression analysis of final body weight (FBW) for juvenile barramundi fed diets at varying levels of tuna hydrolysate (TH) for 8 weeks. X-axis represents the TH inclusion levels of 0 (control), 5, 10, 15 and 20 are considered as experimental treatments. The multiplication sign 'X' represents the TH level for the highest FBW for juvenile barramundi. Each point in the graph represents one treatment with the mean of three replicate groups of fish. The optimal TH level obtained with the quadratic regression analysis for FBW was 10.5% in the diet, respectively.

4.3.2 Biochemical indices

With the exception of glucose, none of the measured blood and serum biochemical indices including hematocrit, aspartate aminotransferase (AST), glutamate dehydrogenase (GLDH), total protein, albumin, globulin, albumin and globulin ratio

(A/G ratio) were influenced by TH inclusion in diets due to large variation between the fish from within the same dietary treatments (Figure 4.2). Blood glucose decreased with increasing TH level, with those fish fed TH10, TH15 and TH20 having significantly lower blood glucose than those in the control.

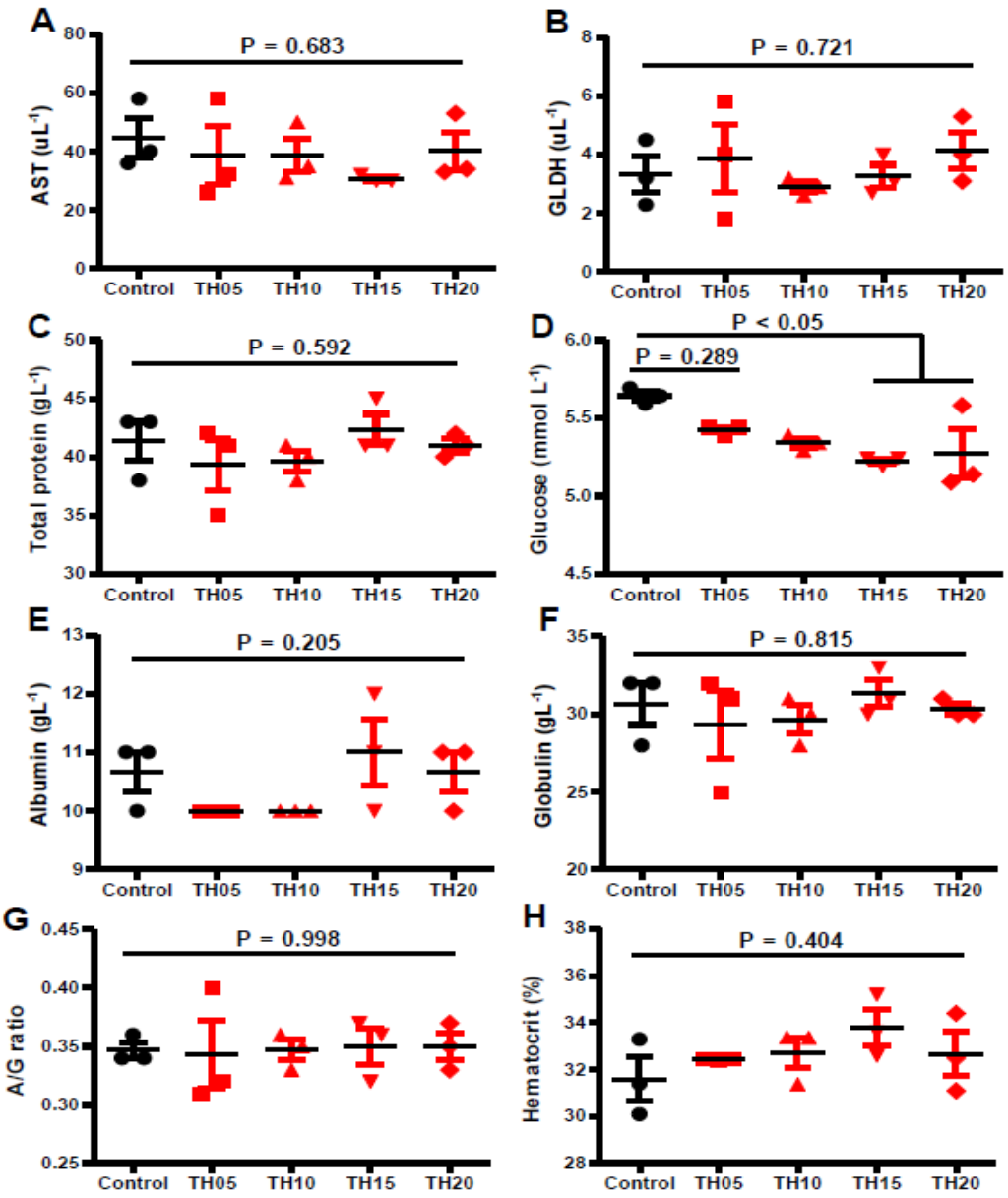

Figure 4.2 Blood and serum biochemical parameters of juvenile barramundi fed tuna hydrolysate (TH) included diets at various levels for 8 weeks. X-axis represents the TH inclusion levels of 0 (control), 5, 10, 15 and 20 are considered as experimental treatments. (A) AST, aspartate transaminase (B) GLDH, glutamate dehydrogenase

(C) total protein (D) glucose (E) globulin (F) albumin (G) A/G ratio (albumin/globulin ratio) and (H) hematocrit. Data were represented as mean ± S.E., n = 3. Post ANOVA Turkey multiple comparison test was applied to compare the mean value of each treatment with the mean value of the control. Mean values significantly different from the control are noted with $P<0.05$.

4.3.3 Histopathology and intestinal morphology

Histopathological investigation revealed that those juvenile barramundi fed with TH20 diet had mild to severe alterations in the liver, spleen, and intestinal tissues (Figure 4.3). The notable alterations including cytoplasmic vacuolization with an increased amount of lipid accumulation (steatosis) were found in liver of fish fed with TH20 and control diet. However, no histopathological hepatic alterations were observed in fish fed TH10 as indicated by balanced hexagonal hepatocytes with prominent nuclei and rare cytoplasmic vacuolization or granules. Histopathological observation of the spleen revealed higher and bigger melanomacrophage aggregates, increased white pulp and splenic cord in the corpuscles of the spleen of fish fed with TH20 diet than all other diets. The intestinal folds of fish fed TH20 diet were shorter and fewer in number and the lumen was wider, while fish fed all other diets showed histologically normal intestinal folds. No histopathological abnormalities such as muscular dystrophy, injury or necrotic fibres were observed in muscle tissues of fish fed the experimental diets.

The histological measurements of the distal intestine of juvenile barramundi fed diets with different levels of TH are presented in Figure 4.4.The micromorphology of intestinal parameters such as goblet cell number per fold (GC), fold height (hF), microvillous height (hMV) and external circumference of serosa (ECS) were altered with the inclusion of TH in diets. The significantly increased GC was found in fish fed 5 to 15% TH included diets whereas increased hMV and ECS were found in TH05 and TH10 diets compared to control. The increased hF was found in fish fed TH05 and TH10 while the decreased hF was observed in TH 20 diet compared to control.

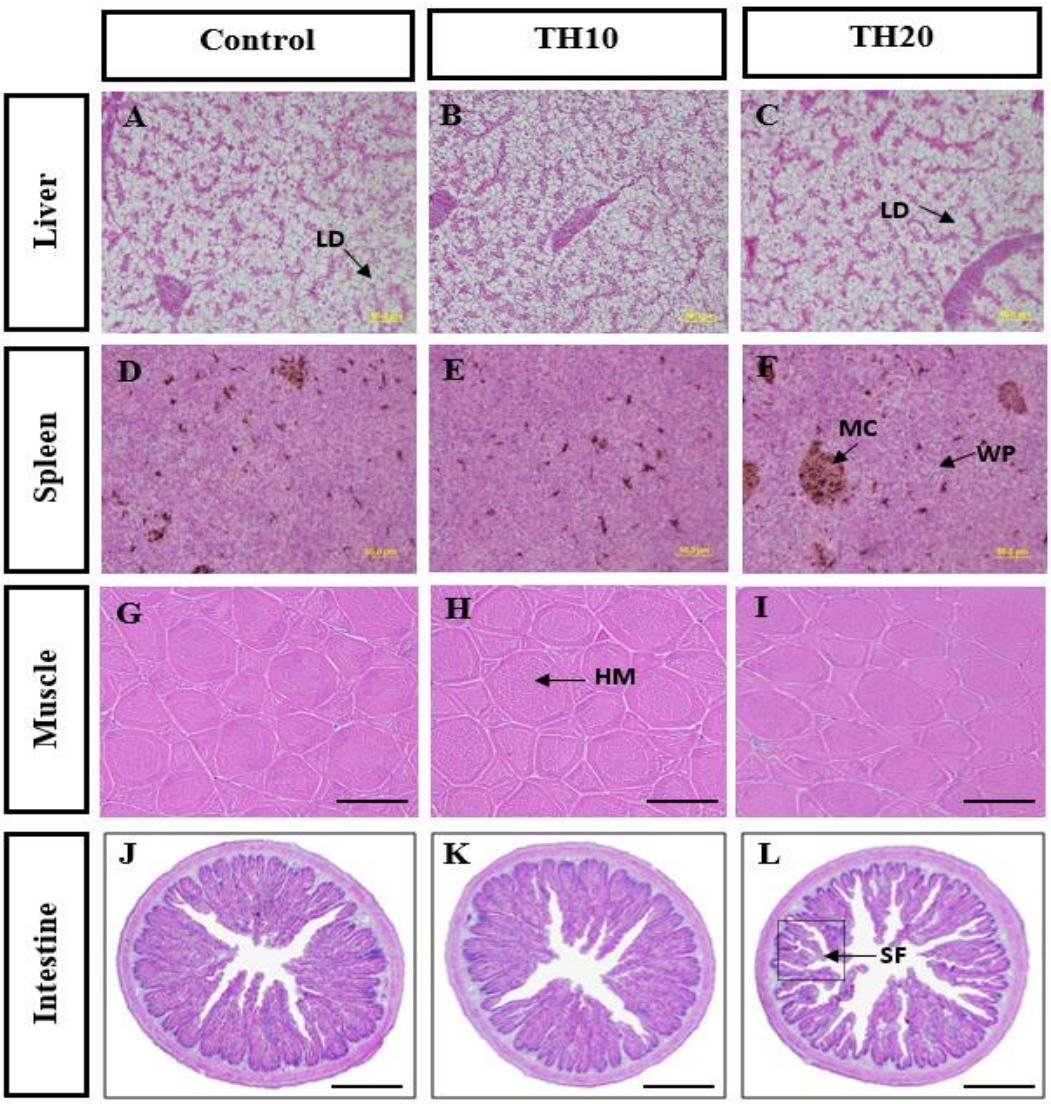

Figure 4.3 Representative micrographs of liver, spleen, muscle and intestine of juvenile barramundi after 8 weeks of being fed with control, TH10 and TH20. A-C: Liver histology from control (A) and TH20 (C) contain increased lipid deposition in hepatocytes while normal cells were observed in TH10 (B) fed fish. D-F: Light micrographs of spleen showing marked melanomacrophage aggregates in TH20 (F) whereas such cases were not observed in control (D) and TH10 (E) diets. G-I: Muscle tissues containing different diets showed healthy myotomes characterised by rounded, packed and uniformly identical muscle fibres. J-L: The distal intestine of fish fed TH20 (L) showing reduced mucosal fold lengths and loss of epidermal integrity whereas control (J) and TH10 (K) fed fish intestinal fold were appear to be healthy with no obvious signs of intestinal inflammation. (LD = lipid droplet; MC =

melanomacrophages complex; WP = white pulps; HM = healthy myotome; SF = short fold. All sections are stained with H&E. Scale bar, 50 µm.

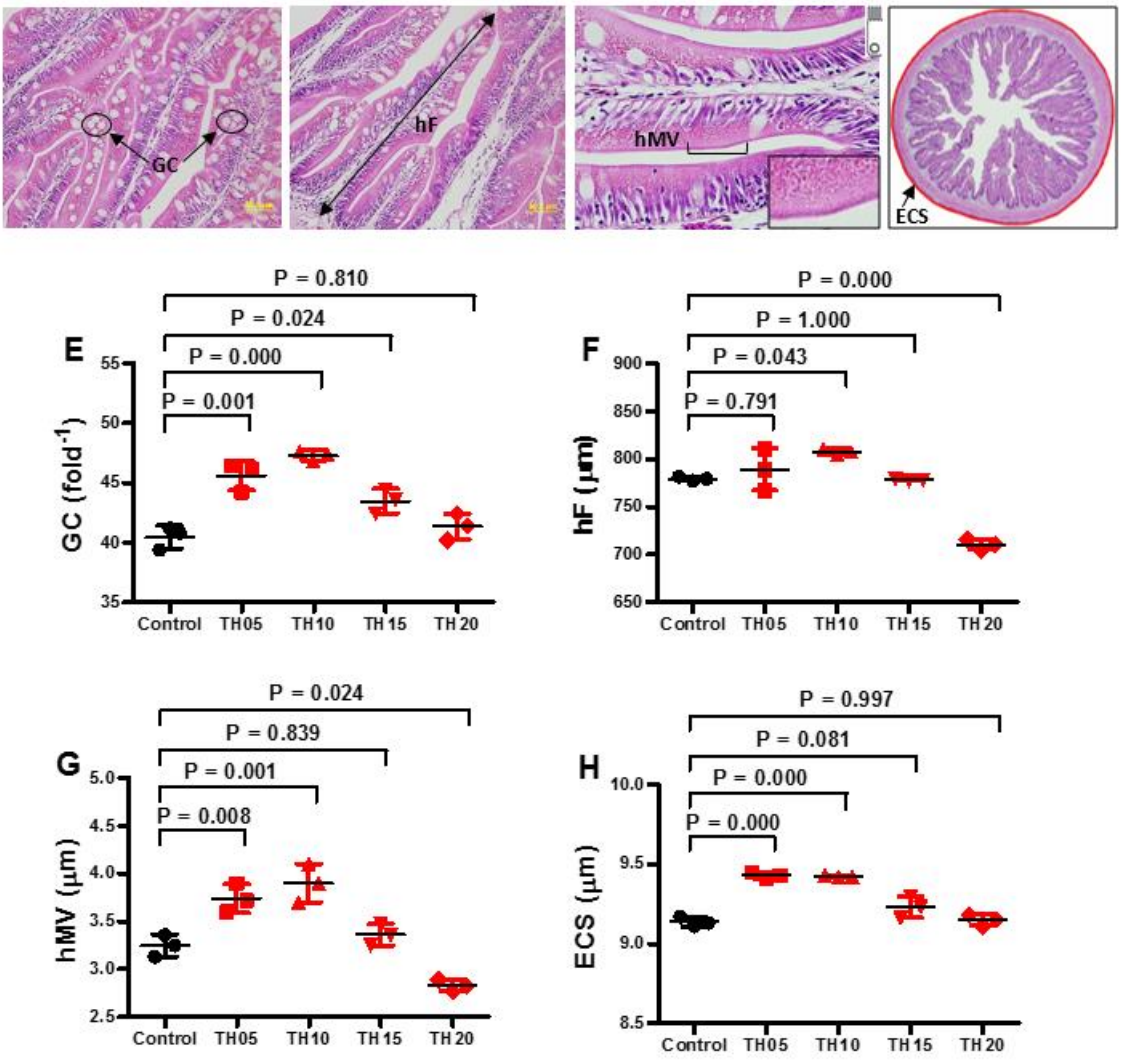

Figure 4.4 Transverse sections of distal intestine photomicrograph of the juvenile barramundi (Panel A-D). All sections are stained with H&E. Scale bar, 50 µm, inset 20 µm. X-axis represents the TH inclusion levels of 0 (control), 5, 10, 15 and 20 are considered as experimental treatments (E-H). The distal intestine of juvenile barramundi is influenced by the inclusion of tuna hydrolysate (TH) in diets at varying levels for 8 weeks. The different measurements include GC=Goblet cells (Panel E), hF=fold height (Panel F), hMV= microvillous height (Panel G), ECS= external circumference of serosa (Panel H). Arrow point and cartoon with bracket both indicate hMV (Panel C). Data were represented as mean ± S.E., n = 5. Post ANOVA Tukey

multiple comparison test was applied to compare the mean value of each treatment with the mean value of the control. Mean values significantly different from the control are noted with $P<0.05$, $P<0.01$ and $P<0.001$.

4.3.4 Lysozyme and complement (ACH50) activity

There was a significant variation observed in the serum lysozyme activities of pre-challenged and post-challenged fish. Fish at 24 hours post-challenge exhibited higher lysozyme activity compared to pre-challenged fish and those 7 days post challenge in all dietary treatments. However, serum lysozyme activity was not influenced by the different inclusion levels of TH in the diets (Figure 4.5). The highest complement activity was registered in fish 7 days post challenge compared to pre-challenge and post-challenge fish at 24 h in all dietary treatments. However, no significant difference was observed between pre-challenge and post-challenge fish at 24 h in all treatments. The complement activity of fish was not influenced by the different inclusion levels of TH in the diets (Figure 4.5). The interactive effects of experimental treatments and sampling period (pre, post-24 and 7 d of challenge) on serum lysozyme activity and complement activity of fish are shown in Table 4.3.

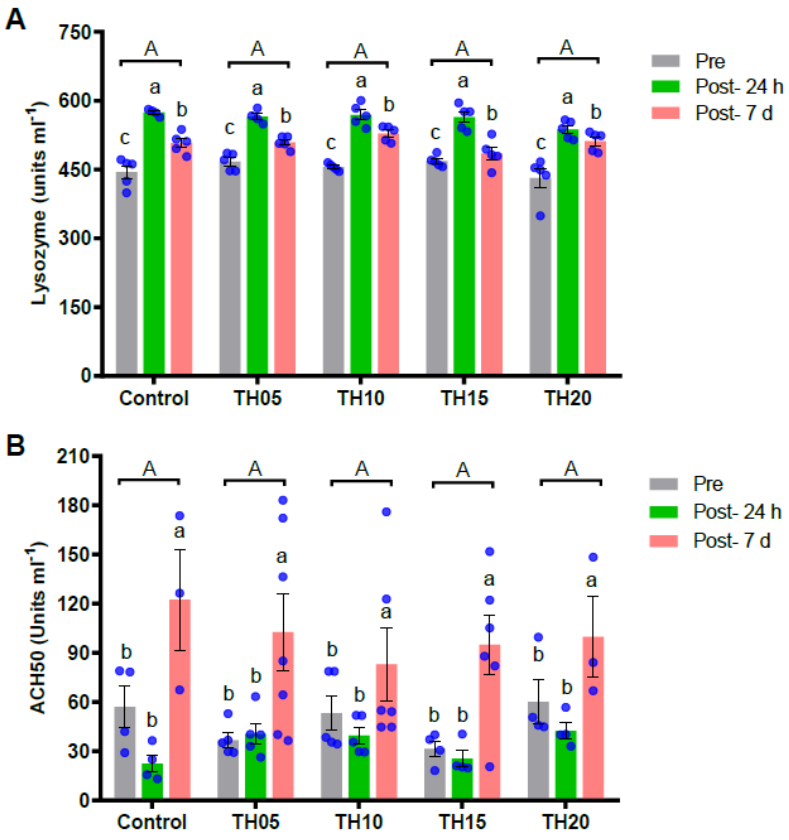

Figure 4.5 Serum lysozyme (A) and complement (B) activities of juvenile barramundi fed TH diets at different inclusion levels for 8 weeks. Data were expressed as mean ± SE. X-axis represents the TH inclusion levels of 0 (control), 5, 10, 15 and 20 are considered as experimental treatments. Different lowercase letters (a,b,c) denote statistically significant differences among pre-challenge, post-challenge-24 h and post-challenge- 7 d in the same treatment. Bar holding P-values denote significant level among the experimental treatments (Multifactorial ANOVA; Tukey post-hoc test; not significant $P>0.05$; significant $P<0.05$; $P<0.001$).

Table 4.3 Two-way ANOVA analysis on the effect of experimental diets and their challenge period (pre, post-24 and 7 d) and their respective interactions on lysozyme and complement activity.

Parameter	Factors		Interaction
	Challenge period	Diets	Challenge period × Diets
Lysozyme activity	0.000	0.063	0.068
Complement (ACH) activity	0.000	0.308	0.268

Multifactorial ANOVA; Tukey post-hoc test; not significant $P>0.05$; significant $P<0.05$; $P<0.001$.

4.3.5 Resistance to infection

Kaplan-Meier analysis revealed significant differences in survival between treatments (log- rank; χ^2 (4) = 10.23, $P<0.05$). Survival of barramundi following challenge with *S. iniae* was significantly higher in those fish fed TH05 and TH10 diets compared to the control (χ^2_{TH05} = 4.72, df =1, $P<0.05$ and χ^2_{TH10} = 8.09, df =1, $P<0.01$) whereas dietary groups of TH15 and TH20 exhibited no significant difference compared with the control (χ^2_{TH15} = 2.74, df =1, P = 0.098 and χ^2_{TH20} = 1.25, df = 1, P = 0.263) (Figure 4.6).

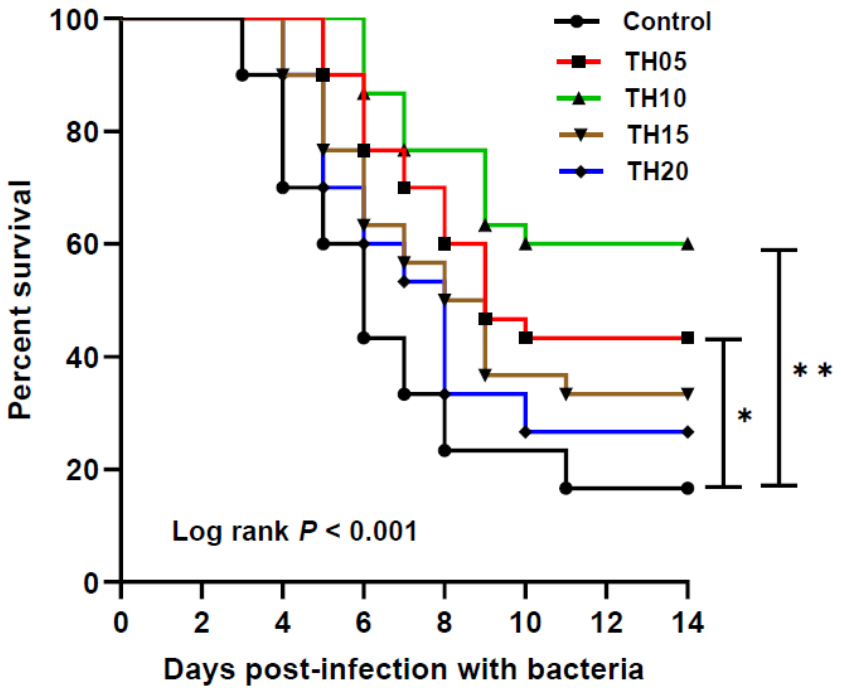

Figure 4.6 The Kaplan Meyer's survival analysis of juvenile barramundi after immersion challenge with *Streptococcus iniae*. Survival curves displaying the outcome of bacterial challenge where n = 30 for each treatment. Infection in control started at 3 days post challenge (dpc), 4 dpc in the TH15 and TH20, while infection started in TH05 and TH10 at 5 and 6 dpc, respectively. Significantly higher post challenge survival was found in TH05 and TH10 fish (p = 0.030 and 0.004, respectively) when compared with control. Asterisks * and ** indicate statistically significant difference between treated group and infected control at P<0.05 and P<0.01, respectively.

4.4 Discussion

Fish protein hydrolysates (FPH) derived from raw waste materials produced through enzymatic hydrolysis are regarded as promising aqua-feed ingredients due to their favorable functional (Liceaga-Gesualdo & Li-Chan 1999; Liu et al. 2014; Shen et al. 2012) and nutritional properties (Chalamaiah et al. 2012; Masuda et al. 2013). A number of studies have reported that fish hydrolysates are potent growth promoters in fish (Hevrøy et al. 2005; Kotzamanis et al. 2007; Refstie et al. 2004). However, FPH have not been previously studied in barramundi. In this study, tuna hydrolysate (TH) derived from processing by-products was tested in juvenile barramundi and it was found inclusion levels of 5 to 10% enhanced the FBW and SGR. Similar positive growth responses to dietary inclusion of fish hydrolysates have been found in many

fish species including olive flounder, *Paralichthys olivaceus* (Khosravi et al. 2017), yellow croaker, *Pseudosciaena crocea* (Tang et al. 2008) and Atlantic salmon, *Salmo salar* (Refstie et al. 2004) . The improved growth performance in the present study following moderate levels of hydrolysate inclusion may be a result of the improved availability and subsequent uptake of free amino acids and suitable peptide fractions produced during the enzymatic process which may be beneficial for the growth performance of fish (Xu et al. 2016). Amino acids are crucial for a wide variety of protein syntheses with major physiological functions, such as carriers of oxygen, carbon dioxide, vitamins, enzymes and structural proteins (Chalamaiah et al. 2012). FPH containing free amino acids and suitable peptides has a substantial role in maintaining good health of fish (Santos et al. 2009). However, the use of FPH in aqua-feeds must be at the appropriate level as higher inclusion of FPH may negatively influence the growth and feed utilization in fish (Raa 1996; Siddik et al. 2018a). In Japanese flounder, *Paralichthys olivaceus* 16% or higher inclusion of fish hydrolysate in the diet resulted in significant reduction in growth (Zheng et al. 2013b). Also, an inclusion level of 20% fish hydrolysate in turbot, *Scophthalmus maximus* resulted in significantly reduced specific growth rate (SGR) and feed utilization (Xu et al. 2016). In this study, growth performance was significantly elevated at 5 to 10% FM replaced by TH and at further higher replacements (15 to 20%) growth performance started to decline. The detrimental effects of hydrolysates at high inclusion level on fish physiological functioning could be due to an excessive amount of free amino acids (FAA) and peptides of low molecular weight, which may lead to an imbalance in amino acid absorption and saturation of peptide transportation systems (Carvalho et al. 2004; Kolkovski & Tandler 2000b).

Hematological indices have been considered as valuable biological indicators to assess the health status and physiological condition of fish (Adams et al. 1993). The results of the current study showed that dietary inclusion of TH in FM based diets had no significant effect on the hematological indices measured, with the exception of glucose. Likewise, Khosravi et al. (2017) found that the addition of protein hydrolysates in low FM diets did not alter most of the hematological indices in juvenile olive flounder, *Paralichthys olivaceus* while some of the health parameters (lysozyme activity, total immunoglobulin) were improved in hydrolysate supplemented groups. In the current study, the concentration of blood glucose was significantly lower in

juvenile barramundi fed with 10 to 20% TH included diets compared to those in the control. This result is in accordance with Khosravi et al. (2015b), who reported the same effect in red sea bream, *Pagrus major* where blood glucose levels were significantly reduced in those fish fed diets containing shrimp hydrolysate. However, another study with the same species found no significant differences in blood glucose levels when fed a diet containing fish hydrolysate (Bui et al. 2014). This difference therefore appears to be due to the different types of hydrolysates used between the two studies, but may be due to a number of factors including experimental conditions, fish size and handling methods, as they can strongly affect fish physiological condition (Chatzifotis et al. 2010). The enzymes AST and GLDH are normally measured in fish as the indicators of hepatocellular injury, to determine liver health status. In the present study, the lack of a significant increase in AST and GLDH suggest that the FPH did not cause liver damage. Similarly, Khosravi et al. (2015b) found no significant difference in serum AST level by the addition of FPH to the diet of red sea bream, *Pagrus major*. However, Cai et al. (2015) observed that yellow croaker, *Larimichthys crocea* fed a diet with 40% fish hydrolysates had higher AST levels than fed a control diet.

The intestine, a primary immune organ of the body, plays *a* major role in the ingestion and absorption of nutrients, and participates in the protection of the host body through a strong defence against pathogens, allergens and toxins (McGuckin et al. 2011). Some earlier studies have stated that the distal intestine of carnivorous fish is more sensitive in relation to diets and have larger absorptive surface area including villi, microvilli, and higher densities of goblet cells (GC) in the epithelium (Apper et al. 2016; Miao et al. 2018; Purushothaman et al. 2016). Furthermore, this part of the intestine has shown the highest variations when alternative protein sources are incorporated in the diets of fish (Gajardo et al. 2017). In the present study, the GC in the intestine were found scattered in order to protect the mucus membranes by secreting mucus (Allen et al. 2009). Fish fed TH05, TH10 and TH15 had higher numbers of mucus-secreting GC in the intestine compared to the control. A number of previous studies have reported that GC are positively correlated with the absorption of digestible substances and higher GC results in higher mucosal membrane protection (Domeneghini et al. 2005; Murray et al. 1996). The increment of GC in fish fed the TH05 and TH10 diets might be due to the improved innate immune function against invading microorganisms. These

observations are in agreement with an earlier study on red sea bream, *Pagrus major* where dietary inclusion of shrimp hydrolysate in a low FM diet resulted in an increased GC (Khosravi et al. 2015b). It is well known that dietary intake of fish has a marked effect on intestinal health, development and function. The longer fold and villus height of intestine are associated with the good health and high absorptive efficiency, whereas shorter fold and villus height are correlated with higher number of pathogenic bacteria in the digestive tract. Moreover, a shortening of the microvillus height can lead to poor nutrient utilization and absorption, reduced immune functions, thereby lower growth performance of fish (Farhangi & Carter 2001). According to Dimitroglou et al. (2009) good intestinal health in fish is of great importance not only to achieve target growth rates and feed efficiency but also improved the health status of the mucosal epithelium by providing an effective immune barrier against potential intestinal pathogens. In the current study, the histological evaluations in terms of hF, hMV and ECS were increased in fish fed TH05 and TH10 diets might be due to the greater nutrient absorption and utilization results in more surface area for nutrient uptake which was demonstrated by enhanced growth performance of fish. Novriadi et al. (2017) reported that the inclusion of 4% squid hydrolysate in the plant based diet partially restore the intestinal inflammation caused by the high inclusion of plant proteins in the diet of Florida pompano, *Trachinotus carolinus*.

When a fish is challenged with pathogens, it is the task of the innate defense system to protect or fight against the pathogens. In order to compensate for a deficiency in the adaptive immune system, fish lysozyme, in the absence of complement has substantial antibacterial activity compared with mammalian lysozymes, not only against Gram-positive bacteria but also against Gram-negative bacteria. Neutrophils and macrophages are the major sources for producing lysozyme (Saurabh & Sahoo 2008). The alternative pathway of complement activity is also an innate component of the immune system protecting fish from invasive pathogens (Muller-Eberhard 1988). Multiple studies have suggested that inclusion of FPH in fish diets may stimulate the non-specific immune responses, and this stimulant is strongly influenced by the amount of hydrolysate in the diet (Gildberg & Mikkelsen 1998; Kotzamanis et al. 2007). However, if the inclusion level of the hydrolysate is too high (>30%), it may have a negative effect in fish (Gildberg & Mikkelsen 1998; Raa 1996). The higher lysozyme activity in infected fish demonstrates the defense response to the *S. iniaie*

infection in 24 h post challenge and decline at 7 d post-challenge may be explained by granulocyte extravasation from the blood into the peripheral tissues. Interestingly, we observed an opposite pattern in the serum alternative complement pathway activity (ACH50) which had a lower response at 24 h post-challenge compared to 7 d post-challenge. Such an opposite regulation of the immune pathway may indicate that the components of the immune systems in fish species may be regulated in different directions. Since complement acts earlier than lysozyme, which breaks up the resistant layer, lipopolysaccharide, the reduction in alternative pathway may be related to temporary decrease in C3b (cleavage of complement component 3) in the first 24 h post challenge due to usage of this protein in the first hours of response post challenge. On the other hand, according to Ogundele (1998) lysozyme has anti-inflammatory action to inhibit the hemolytic activity of complement, particularly in pathological ranges. Furthermore, a peak response of ACH50 in 7 d post challenge is probably because of a positive feedback loop induced by activation of the classical or lectin pathways (Janeway et al. 2001). In the current study, fish fed the TH05 and TH10 diets showed higher resistance against infection, while control fish showed the lowest resilience during the 14 days of bacterial challenge. Similarly, dietary administration of FPH increased the disease resistance of various fish, such as red sea bream, *Pagrus major* and juvenile olive flounder, *Paralichthys olivaceus* against *Edwardsiella tarda* (Bui et al. 2014) and European sea bass larvae, *Dicentrarchus labrax* to *Vibrio anguillarum* (Kotzamanis et al. 2007).

In summary, based on the quadratic regression analysis of FBW level, the optimum TH for juvenile barramundi was estimated to be 10.5%. Although the immune parameters (lysozyme, ACTH50) were not affected by TH inclusion in the diets, the increased growth performance and intestinal micro-morphological parameters (GC, hF, hMV and ECS), and decreases in blood glucose level in fish fed TH included diets at moderation might be associated with the improved resistance of juvenile barramundi against *S. iniae* infection, resulting in higher survival during post-challenge. However, further studies on this subject are needed to connecting the linkage between FPH utilization and disease resistance of fish.

Chapter 5: Potential effects of bioprocessed poultry by-product meal on growth, gut health and fatty acid synthesis of juvenile barramundi, *Lates calcarifer* (Bloch)

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Abstract

Poultry by-product meal (PBM) has been utilised as a substitute of fishmeal (FM) in many aquaculture species. However, little information is known regarding the use of bioprocessed PBM (BPBM) in aquaculture production. This study was undertaken to investigate whether replacing FM with BPBM improved growth performance, gut morphology and fatty acid synthesis of juvenile barramundi, *Lates calcarifer*. The PBM was bioprocessed by baker yeast, *Saccharomyces cerevisae* and *Lactobacillus casei*. The BPBM was used to replace FM at 75% and 100% (75BPBM and 100BPBM) contrasting against unprocessed PBM (75PBM and 100PBM) at the same levels and FM based diets as the control. Juvenile barramundi with a mean initial weight of 3.78±0.16 g were stocked at a density of 20 fish per tank. After the 42 days of study, the final weight, specific growth rate and feed conversion ratios of fish fed 75PBM and 75BPBM were not significantly different from the control. However, 100% supplementation diets of 100PBM and 100BPBM resulted in reduced performance in all growth and feed variables except total feed intake and survival. The hind gut microvillus density was significantly higher ($P<0.05$) in fish fed 75BPBM, whereas the microvillus diameter remained unaffected with the other experimental diets when compared to the control. A reduction in eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids of fish muscles led to a lower Σn−3/Σn−6 ratio in all dietary groups when compared to the control. The percentage of Σn-3 PUFAs decreased in 100% FM replacement diets of 100PBM and 100BPBM, while Σn−6 PUFAs increased when both bioprocessed and unprocessed PBM protein was increased in the diets. Fish fed bioprocessed diets had higher fatty acid hypocholesterolemic/hypercholesterolemic ratios (HH), indicating improved suitability for human consumption.

Keywords: Bioprocessing, Yeast, *Lactobacillus casei*, poultry by-product, fatty acid, *Later calcarifer*.

5.1 Introduction

Fishmeal (FM) is one of the best dietary sources of protein in aqua-diets due to a high protein content, balanced amino acid profile, elevated omega-3 polyunsaturated fatty acid, high protein digestibility, and excellent palatability properties. Unfortunately, a shortage of raw materials due to the progressive depletion of fish stocks, coupled with soaring demand and economic issues has impacted the sustainable development of aquaculture production (Perez-Velazquez et al. 2018). Therefore, there is a growing interest in the aquaculture production sector to find alternative protein sources of FM that are locally available, sustainable and cost effective. Over the last decades, there has been an unprecedented number of studies conducted on plant protein sources as possible alternative feed ingredients for aquatic species (Rawles et al. 2011). The major limitation in plant feedstuffs is that they lack essential long chain omega 3 fatty acids and are naturally rich in carbohydrate which is poorly metabolized by carnivorous fish (Naylor et al. 2009; Zambonino-Infante et al. 2019). The potential use of rendered animal by-products, including PBM, meat and bone meal, feather meal and fish offal meal for aquatic feeds is not new, with such nutritional potential recognized at the very inception of fish farming. Over the years, significant research has been conducted on PBM due to its high protein content and favourable amino acid profile (Badillo et al. 2014; Glencross et al. 2016). PBM is also readily available and often cheaper due to the low demand from other sectors such as food and pharmaceutical companies (Cruz-Suárez et al. 2007; Rawles et al. 2011).

Many studies have investigated the efficacy of PBM on various fish species and success was generally only reported when PBM partially replaced FM in the diets (Dawson et al. 2018; Saadiah et al. 2010). Moreover, the utilization of high levels of PBM to replace FM has resulted in depressed growth performance of fish (Rossi & Davis 2012). Several studies have confirmed that higher inclusion levels of PBM in fish diets, also resulted in declines of methionine and lysine levels (Abdul-Halim et al. 2014; Fuertes et al. 2013), which are the major impediments to incorporating substantial levels of PBM into the diets of carnivorous fish. Another major concern with using PBM in aqua diets is its poor digestibility giving variable nutritive value (Simon et al. 2019). Simon et al. (2019) stated that freshness of raw materials and their processing conditions are the main factors determining the quality of the final product.

The amount of heat and moisture applied to the material during the rendering process has major implications to the feed quality and utilisation of the finished meal (Lewis et al. 2019). Like other terrestrial animal by-products, PBM is also deficient in adequate proportions of n-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) (Norambuena et al. 2015), potentially lowering the nutritional quality of fish meat intended for human consumption.

A possible strategy to circumvent these shortcomings is using a bioprocessing or fermentation technique - an environmentally suitable and cost effective method used to overcome many of the inherent problems of animal by-product protein (Brandelli et al. 2015; Kader et al. 2012; Kaviraj et al. 2013; Mondal et al. 2008) to make it suitable for inclusion in fish diet formulations (Bertsch & Coello 2005; Fagbenro et al. 1994; Samaddar & Kaviraj 2014). Fermentation breaks down carbohydrates into a form that makes the innate energy and protein digestible (Gatlin et al. 2007), whilst improving the nutritional quality of animal by-products by producing low-molecular-weight compounds that potentially enhance mineral absorption (Hotz & Gibson 2007; Ilham et al. 2016b), amino acids profile (Lee et al. 2016) , and reduce anti-nutritional factors (Barnes et al. 2015; Gatlin et al. 2007). Fagbenro & Jauncey (1995) found that feeds made from fermented products tend to have higher stability in water, thereby allowing more time for fish to ingest the feed and maximise nutrient intake. Furthermore, fermented feeds are characterized by high amounts of lactic acid bacteria (Heres et al. 2003b) which can proliferate in the gut and produce high concentrations of beneficial lactic acid, as well as several volatile fatty acids including acetic acid, butyric acid, and propionic acid (van Winsen et al. 2001).

Barramundi, *Lates calcarifer* (Bloch), a common species in the Indo-Pacific region and Australia, is gaining much attention from farmers and researchers (Ilham et al. 2016b). Due to its wide range of salinity tolerance, adaption capacity in versatile farming systems and highly appreciated meat, barramundi is progressively becoming a major commercial species in aquaculture (Simon et al. 2019). Thus, it is necessary to establish a cost-effective growth promoting diet for a continuing and feasible barramundi farming industry. To the authors' knowledge, growth studies looking at the potential of PBM to replace FM with emphasis on gut health and fatty acid

synthesis, remains unassessed. The present study therefore aimed to evaluate the effects of graded levels of bioprocessed and unprocessed PBM on growth performance, nutritional composition, gut and liver health, and fatty acid synthesis in juvenile barramundi.

5.2 Materials and methods

5.2.1 Ethics

This experiment was conducted under the guidance of the Care and Use of Laboratory Animals in Australia. The procedures and protocols of treating fish in this study were approved by the Animal Ethics Committee of the Curtin University, Australia (Approval Number: AEC_2015_41). In short, fish were starved for 24 h prior to being anaesthetised (AQUI-S, 8 mgL^{-1}), weighed and taking blood samples. After completion of the trial, remaining fish were euthanized according to CARL SOP Euthanasia of Fish, using AQUI-S.

5.2.2 Experimental diets

The formulation and nutrient composition of the experimental diets are presented in Table 5.1. All feed ingredients for this study were procured from Specialty Feeds, Glen Forrest Stockfeeders, Perth, Western Australia. The PBM was sieved through a 0.5mm size mesh sieve and was used as the raw material for bioprocessing. The fermentation of PBM was completed following a technique described in our earlier study (Siddik et al. 2018a). In short, PBM was weighed and Baker's yeast, *Saccharomyces cereviceae* (Instant dried yeast, Lowan) was added at 10% and *Lactobacillus casei* in the form of skim milk product (Yakult, cell density of 3×10^6 CFU ml^{-1}) was added at 5% of the weight of PBM. Distilled water was then added at approximately 70% of the weight of the total meal mixture and all ingredients were thoroughly mixed in a food mixer. The mixture was then placed in an Erlenmeyer flask covered with aluminium foil and incubated at 30°C for 4 days. The fermented product was dried in an oven at 60°C for 24 h and used as a feed ingredient. Five isonitrogenous and isocalorific diets having 48.0% crude protein and 20.0 MJ kg^{-1} gross energy were formulated based on bioprocessed and unprocessed PBM to replace FM at 75% (75PBM and 75BPBM) and at 100% (100PBM and 100BPBM). The control diet was formulated based on FM as the main protein source. The experimental diets were prepared based on the standard

protocol of Curtin Aquatic Research Laboratories (CARL) and met the nutrient requirements of juvenile barramundi according to NRC (2011). The amino acids (AAs) and fatty acids (FAs) composition of the experimental diets and tested unprocessed and bioprocessed PBM are presented in Table 5.2 and Table 5.3, respectively.

Table 5.1 Formulations and nutrient composition of experimental diets fed on juvenile barramundi.

Test Ingredients[1]	Experimental diets (g kg^{-1} DM)				
	Control	75PBM	75BPBM	100PBM	100BPBM
Fishmeal[2]	610.0	152.50	160.0	-	-
PBM[3]	-	429.0	-	575.0	-
BPBM[4]	-	-	439.0	-	595.0
Wheat flour	266.0	290.0	272.50	300.o	280.0
Wheat starch	20.0	20.0	20.0	20.0	20.0
Fish oil	30.0	30.0	30.0	30.0	30.0
Calcium carbonate	20.0	20.0	20.0	20.0	20.0
Salt (NaCl)	20.0	20.0	20.0	20.0	20.0
Vitamin premix	10.0	10.0	10.0	10.0	10.0
Casein	63.0	65.0	65.0	65.0	65.0
Cellulose	60.0	85.0	85.0	50.0	50.0
Nutrient composition (% dry weight)					
Crude protein	48.91	48.33	48.73	48.31	48.04
Crude Lipid	9.99	10.93	10.19	11.29	10.49
Ash	12.87	9.18	9.55	8.31	8.35
Moisture	17.93	14.20	11.86	13.98	11.50
NFE[5]	28.23	31.56	31.53	32.09	33.12
Gross energy (MJ kg^{-1})	19.84	20.62	19.97	20.35	20.21

[1]Supplied by Specialty Feeds, Perth, Australia. [2]Fishmeal: 64.0% crude protein, 10.76% crude lipid and 19.12% ash. [3]PBM (poultry by-product meal): 67.13% crude protein, 13.52% crude lipid and 13.34% ash. [4]BPBM (Bioprocessed poultry by-product meal): 66.98% crude protein, 11.70% lipid and 14.68% ash. [5]Nitrogen free extracts (NFE) = dry matter - (crude lipid + crude ash+ crude protein).

Table 5.2 The amino acid composition (g/100g) of the five experimental diets and tested unprocessed and bioprocessed PBM.

	Experimental diets					PBM	BPBM
	Control	75PBM	75BPBM	100PBM	100BPBM		
Essential amino acids							
Phenylalanine	2.05	2.02	2.02	2.19	2.02	2.65	2.65
Glutamic acid	7.70	8.42	8.54	8.67	8.54	9.55	9.84
Leucine	3.88	3.71	3.75	4.00	3.75	5.00	4.97
Lysine	3.25	2.83	2.95	3.01	2.95	4.17	4.21
Methionine	1.30	1.06	1.02	1.21	1.02	1.46	1.43
Isoleucine	2.12	1.99	2.06	2.20	2.06	2.67	2.66
Histidine	1.35	1.05	1.00	1.22	1.00	1.47	1.39
Threonine	2.21	2.00	1.97	2.20	1.97	2.81	2.86
Valine	2.41	2.34	2.41	2.55	2.41	3.08	3.11
Non-essential amino acid							
Arginine	2.92	3.49	3.18	3.39	3.18	5.43	5.24
Alanine	2.88	3.01	3.15	3.23	3.15	4.62	4.89
Taurine	0.11	0.19	0.12	0.21	0.12	0.31	0.31
Tyrosine	1.71	1.70	1.67	1.83	1.67	2.15	2.17
Glycine	3.11	4.19	4.14	4.04	4.14	6.53	6.79
Aspartic acid	4.43	3.98	4.15	4.34	4.15	5.95	6.03
Cysteine	0.44	0.50	0.53	0.57	0.53	0.66	0.69
Serine	2.32	2.34	2.26	2.48	2.26	3.01	3.08
Proline	3.48	5.72	4.37	5.67	4.37	5.63	5.94

Table 5.3 The fatty acid composition (% of total fatty acids) and total fatty acid content (mg/g of lipid) of the five experimental diets and tested unprocessed and bioprocessed PBM.

Fatty acids	Experimental diets					PBM	BPBM
	Control	75PBM	75BPBM	100PBM	100BPBM		
C12:0	0.06	0.07	0.08	0.08	0.10	0.09	0.08
C13:0	0.04	0.09	0.02	0.03	0.03	0.02	-
C14:0	2.85	1.92	1.73	1.65	1.66	0.97	0.85
C14:1n-5	0.04	0.12	0.12	0.15	0.14	0.21	0.18
C15:0	0.72	0.35	0.34	0.21	0.23	0.20	0.18
C15:1	0.11	0.07	0.06	0.01	0.01	0.03	0.03
C16:0	17.76	18.61	19.07	18.80	19.17	14.13	21.58
C16:1n-7	3.37	4.23	4.64	4.52	5.04	5.36	5.65
C17:0	1.09	0.51	0.53	0.31	0.34	0.38	0.36
C17:1	0.58	0.36	0.35	0.30	0.32	0.24	0.24
C18:0	5.71	6.21	6.58	6.35	6.71	9.38	8.53
C18:1cis or trans	16.43	31.92	32.16	36.97	37.31	49.39	42.25
C18:2 trans 9	0.13	0.09	0.10	0.10	0.08	0.15	0.12
C18:2n-6	5.61	13.47	13.28	15.62	14.59	13.68	13.72

C18:3n6	0.15	0.16	0.16	0.15	0.13	0.12	0.13
C18:3n3	1.33	2.32	2.15	2.55	2.29	1.6	1.85
C18:4n-3	0.98	0.77	0.62	0.68	0.67	0.06	0.06
C20:0	0.29	0.21	0.21	0.19	0.19	0.18	0.17
C20:1	2.31	2.01	1.7	1.95	1.99	0.67	0.59
C20:2	0.35	0.31	0.27	0.30	0.29	0.16	0.16
C20:3n-3	0.22	0.14	0.12	0.12	0.12	-	-
C20:3n-6	0.18	0.25	0.24	0.26	0.24	0.24	0.25
C20:4n-6	1.74	1.46	1.51	1.34	1.13	1.21	1.31
C20:5n-3 (EPA)	6.65	3.32	2.99	2.27	2.23	0.13	0.19
C21:0	0.12	0.09	0.08	0.07	0.07	0.08	0.07
C22:0	0.02	0.01	-	-	-	0.13	0.10
C22:1n-9	0.29	0.25	0.20	0.23	0.24	0.12	0.06
C22:2	0.03	0.03	0.02	0.02	0.02	-	-
C22:4n-6	1.96	0.54	0.60	0.12	0.11	-	0.06
C22:5n-3	1.92	1.3	1.13	1.1	1.04	0.41	0.35
C22:6n-3 (DHA)	26.31	8.48	8.63	2.99	2.94	0.24	0.45
C23:0	0.07	0.03	0.03	0.38	0.38	0.39	0.38
C24:0	0.16	0.07	0.07	0.03	0.03	-	0.03
C24:1	0.42	0.23	0.20	0.15	0.15	0.02	0.03
ΣSFA	28.89	28.17	28.74	28.1	28.91	25.95	32.33
ΣMUFA	23.55	39.19	39.43	44.28	45.2	56.04	49.03
ΣPUFA	47.56	32.64	31.82	27.62	25.88	18.0	18.65
Σn-3	37.41	16.33	15.64	9.71	9.29	2.44	2.90
Σn-6	9.64	15.88	15.79	17.49	16.2	15.25	15.47
Σn-3/Σ n-6	3.88	1.01	0.99	0.56	0.57	0.16	0.19
Total Fatty Acids (mg/g)	836.8	891.8	859.6	941.2	898.6	1019.7	1054.7

EPA: Eicosapentaenoic acid**,** DHA: docosahexaenoic acid, ΣSFA, sum of saturated fatty acids; ΣMUFA, sum of monounsaturated fatty acids; ΣPUFA, sum of polyunsaturated fatty acids; Σn-3 PUFA, sum of omega-3 polyunsaturated fatty acids; Σn-6 PUFA, sum of omega-6 polyunsaturated fatty acids; IA, index of atherogenicity; IT, index of thrombogenicity and HH, (hypocholesterolemic/hypercholesterolemic ratio, -, not detected.

5.2.3 Fish, experimental conditions and feeding

Juvenile barramundi were sourced from the Australian Centre for Applied Aquaculture Research, Fremantle, Australia. Fish were acclimatized to the laboratory conditions for 14 days. During the acclimation period, fish were fed twice daily with a basal formulated diet (48.0% crude protein and 20.0 MJ kg^{-1} dietary gross energy). Following acclimation, a total of 300 uniformly sized juvenile barramundi (mean initial weight of 3.78±0.16 g $fish^{-1}$) were randomly distributed into fifteen tanks (300-L water capacity) at a stocking density of 20 fish per tank. Each tank was connected with an aerator, water heater and external bio-filter (Fluvial 406, Hagen, Italy) exchanging water at a rate of 10 L min^{-1}. Fish were raised in saltwater medium with a salinity range of 31.0 to 34.0 ppt. The water quality parameters such as temperature,

salinity, dissolved oxygen, ammonia and nitrite were monitored daily and were within the suitable range for fish culture in a recirculating aquaculture system (Ardiansyah & Fotedar 2016). Fish were kept at a 12:12 light:dark cycle. Throughout the experimental period of 42 days, fish were fed to satiation the respective diets three times a day at 0800, 1200 and 1700 h. About 1.5 h after feeding, uneaten feed was removed carefully by siphoning, transferred to aluminium cups, and dried to a constant weight in order to calculate the feed conversion ratio. The growth performance, feed utilization and body indices parameters of juvenile barramundi were calculated using the equations describe previously (Siddik et al. 2018a).

5.2.4 Intestinal microvilli morphology

The potential effects of bioprocessed and unprocessed PBM on the ultrastructure of gut morphology were investigated by scanning electron microscopy (SEM). The SEM images (magnification × 30,000) were analysed to assess the microvilli density on the surface of enterocytes standardised to the 1 μm^2 region (Adeoye et al. 2016). The distal intestinal samples of three fish from each treatment (randomly selected one fish from each replicated tank) were considered for microscopic examination. For accuracy, at least 100 independent measurements were taken from the SEM images per treatment. The details of SEM sample preparation and data calculation were described previously in Ran et al. (2015).

5.2.5 Histopathology

After being fed for 42 days, three randomly selected fish from each treatment were dissected for liver histological analysis. Liver samples were dehydrated in ethanol, equilibrated in xylene and embedded in paraffin wax following standard histological techniques. The sections of approximately 5 mm in size were cut and stained with Hematoxylin-Eosin (H&E) stain, for histological examination under a light microscope (BX40F4, Olympus, Tokyo, Japan).

5.2.6 Biochemical analysis

Fish muscles and experimental diets were analysed for proximate composition based on the Association of Official Analytical Chemists procedures (AOAC 2006). After termination of the feeding trial, three fish from each tank (9 samples per dietary

treatment) were randomly selected, collected muscle tissues, freeze dried and grounded for biochemical analysis. The dry matter was determined by oven drying to constant weight at 105°C; crude ash by combustion at 550°C; crude protein content (N × 6.25) by the Kjeldahl digestion method; crude lipid content by the Soxhlet technique; and gross energy content of diets by an IKA oxygen bomb calorimeter (Heitersheim, Germany). The fatty acid composition of experimental diets and fish samples was performed following the method described by O'Fallon et al. (2007).

5.2.7 Indices of lipid quality

The lipid quality of fish fed bioprocessed and non-processed PBM was investigated by analysing the fatty acid profile of fish with three important indicators, namely, index of atherogenicity (IA), index of thrombogenicity (IT), and the fatty acids hypocholesterolemic/hypercholesterolemic ratio (HH) as follows:

(1) IA = [(C12:0 + (4 × C14:0) + C16:0)]/[(ΣMUFA + Σ(n-6) + Σ(n-3)] (Ulbricht & Southgate 1991)

(2) IT = (C14:0 + C16:0 + C18:0)/[(0.5×ΣMUFA + 0.5×Σ(n-6) + 3×Σ(n-3) + Σn-3/Σn-6] (Ulbricht & Southgate 1991)

(3) HH= (C18:1cis9 + C18:2n6 + C20:4n6 + C18:3n3 + C20:5n3 + C22:5n3 + C22:6n3)/(C14:0 + 16:0) (Santos-Silva et al. 2002)

5.2.8 Statistical analysis

Results of growth performance, nutritional composition and fatty acid profiles are compared by one way analyses of variance (ANOVA), using SPSS for Windows version 25, IBM Curtin University, Australia. Normality of the data was previously assessed by a Shapiro-Wilk's test and homogeneity of variances was verified using the Levene's test. If ANOVA of values were significant ($P<0.05$), Tukey or Duncan's post hoc tests for multiple pairwise comparisons was then applied. The principal component analysis (PCA) was applied to correlate variables to which fatty acid in fish muscles differed between dietary treatments of bioprocessed and unprocessed PBM using PAST 3.15 software.

5.3 Results

5.3.1 Growth and body indexes

The results of growth performance, feed utilization, body indices and gut micro-morphological indices of fish are shown in Table 5.4. A significant ($P<0.05$) decrease in final body weight (FBW), weight gain (WG) and specific growth rate (SGR) as well as increased feed conversion ratio (FCR), were found in fish fed 100PBM and 100BPBM when compared to the control, and the 75PBM and 75BPBM. The study also indicated that bioprocessing of PBM has no positive influence on the growth performance over unprocessed PBM. Total feed intake and survival was identical among the treatments. The percentage of hepatosomatic index (HSI) was significantly lower in the control and 75% replacement groups of 75PBM and 75BPBM when compared to the total replacement groups of 100PBM and 100BPBM, while the viscerasomatic index (VSI) and condition factor (CF) were not influenced in fish fed bioprocessed and unprocessed PBM at varying levels when compared to the control.

Table 5.4. Growth performance, feed utilization and gut micro-morphological indices of juvenile barramundi fed with different levels of bioprocessed and unprocessed PBM

	Experimental diets				
	Control	75PBM	75BPBM	100PBM	100BPBM
Growth performance					
FBW (g fish^{-1})	32.36 ± 1.94^{a}	32.72 ± 1.39^{a}	33.73 ± 1.28^{a}	26.56± 2.04^{b}	26.32 ± 2.43^{b}
WG (g fish^{-1})	28.81 ± 1.94^{a}	28.96 ± 1.22^{a}	29.84 ± 1.21^{a}	22.58± 2.45^{b}	22.59 ± 1.59^{b}
SGR (% day^{-1})	5.27 ± 0.15^{a}	5.15 ± 0.07^{a}	5.14 ± 0.05^{a}	4.52 ± 0.19^{b}	4.63 ± 0.25^{b}
Survival (%)	88.33± 4.41	91.67± 1.67	86.67± 1.67	90.0± 2.89	81.67± 3.33
Feed utilization					
TFI (g fish^{-1})	36.56±2.45	34.30± 0.35	34.17 ±1.58	31.20 ± 2.03	33.36 ± 2.05
FCR	1.27 ± 0.03^{a}	1.19 ± 0.09^{a}	1.15 ±0.07^{a}	1.41 ± 0.15^{b}	1.50 ±0.10^{b}
Body indexes					
CF (g/cm^{3})	1.26±0.05	1.22±0.05	1.24±0.04	1.18±0.04	1.16±0.06
HSI (%)	1.56±0.12^{bc}	1.77±0.07^{b}	1.49±0.10^{c}	1.99±0.07^{a}	1.83±0.05^{a}
VSI (%)	9.15±0.14	10.25±0.97	9.62±0.19	9.77±0.78	9.35±0.29
Gut micro-morphology					
Microvilli density (count/μm^{2})	125.27±2.01^{b}	125.80±1.71^{b}	134.80±1.75^{a}	116.73±1.75c	112.20±1.85^{c}
Microvilli diameter (μm)	0.11±0.00	0.12±0.00	0.11±0.00	0.10±0.00	0.11±0.00

Values are mean ± SE of three replicate tanks. Values in the same row with different superscript letters (a,b,c) are significantly different based on Duncan's multiple range test (One-way ANOVA, $P<0.05$).

Final body weight (FBW, g)

$$\text{Weight gain (WG, g fish}^{-1}) = \left[\frac{\text{mean final body weight} - \text{mean initial body weight}}{\text{mean initial body weight}}\right]$$

$$\text{Specific growth rate (SGR, \% day}^{-1}) = \left[\frac{\ln(\text{final body weight}) - \ln(\text{pooled initial body weight})}{\text{days}}\right] \times 100$$

$$\text{Feed intake (TFI, g)} = \left[\frac{\text{dry feed consumed}}{\text{fish number}}\right]$$

$$\text{Feed conversion ratio (FCR)} = \left[\frac{\text{dry feed fed}}{\text{wet weight gain}}\right]$$

$$\text{Survival (\%)} = \left[\frac{\text{number of final fish} - \text{number of initial fish}}{\text{number of initial fish}}\right] \times 100$$

$$\text{Condition factor (CF, g cm}^{-3}) = \left[\frac{\text{final body weight}}{\text{length}}\right] \times 100$$

$$\text{Hepatosomatic index (HSI, \%)} = \left[\frac{\text{liver weight}}{\text{body weight}}\right] \times 100$$

$$\text{Viscerosomatic index (VSI, \%)} = \left[\frac{\text{visceral weight}}{\text{body weight}}\right] \times 100$$

5.3.2 Gut microvilli morphology

SEM analyses showed that fish fed the 100% FM replacement diets of 100PBM and 100BPBM had lower gut microvilli density when compared to the control and the other two test diets. Significantly ($P<0.05$), the highest and the lowest microvilli density were found in fish fed 75BPBM and 100PBM, respectively, when compared to the control. However, the gut microvilli diameter of juvenile barramundi was not influenced, either by bioprocessed nor unprocessed PBM in the diets (Figure 5.1).

5.3.3 Liver histopathology

No histological alterations were observed in fish fed the control, and 75% replacement diets of 75PBM and 75BPBM. These treatments were characterized by normal structure with balanced hexagonal hepatocytes and rare cytoplasmic vacuolization. However, hepatocytes from fish fed the 100% replacement diets of 100PBM and 100BPBM showed irregular arrangement of liver samples and cytoplasmic vacuolization with lipid deposition (Figure 5.1).

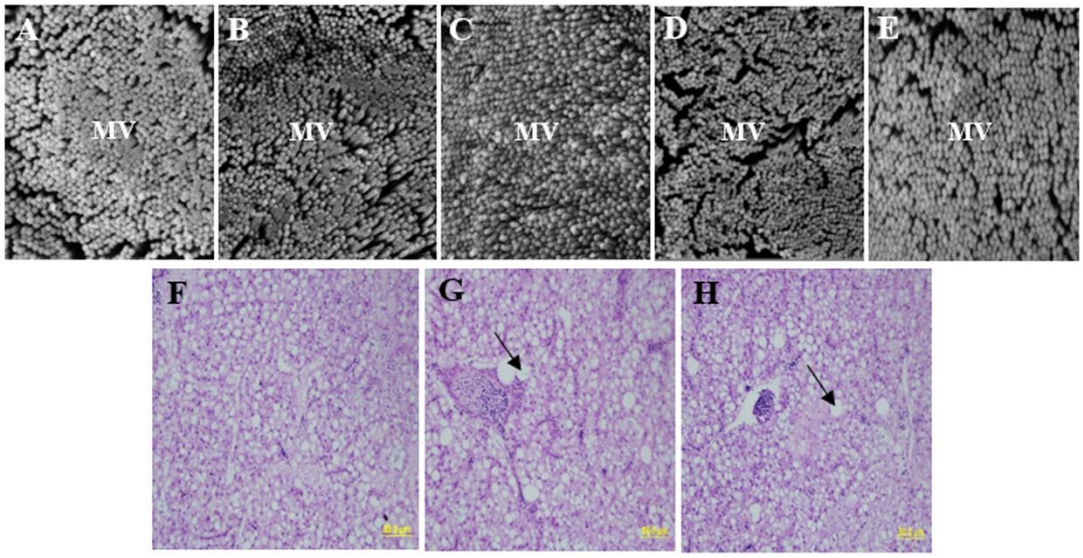

Figure 5.1. Gut microvilli and liver histological structure of juvenile barramundi. High magnification (x 30,000) electron micrographs showing microvilli in the distal gut of juvenile barramundi fed five experimental diets control, 75PBM, 75BPBM, 100PBM and 100BPBM (Panel A-E). MV=microvilli, Scale bar=2 µm, microvilli density count per µm². Histological structure of the liver of juvenile barramundi fed control, 100PBM and 100BPBM diets (Panel F-H). Black arrow in liver micrographs indicates large vacuoles in hepatic cells (H&E staining, 400x magnification, scale bar = 50µm).

5.3.4 Proximate composition and amino acid profile

The muscles proximate composition and amino acid profile of the juvenile barramundi fed the experimental diets are shown in Table 5.5. There were no significant ($P>0.05$) differences in moisture, protein, lipid and ash content of juvenile barramundi among the different dietary groups. The percentages of amino acids (AAs) such as Phenylalanine, Taurine, Cysteine and Serine content in muscles of fish fed PBM diets were similar to the control, whereas the remaining AAs were significantly different. The Methionine and Lysine content of fish fed control was significantly higher than the unprocessed diets but was similar to the fish fed bioprocessed diets (Table 5.5).

Table 5.5. Proximate composition (% wet weight basis) and amino acid profile (g/100g) of muscle tissue in juvenile barramundi at the end of feeding trial for 42 days.

	Experimental diets				
	Control	75PBM	75BPBM	100PBM	100BPBM
Proximate composition					

Moisture	73.81 ± 0.64	73.38 ± 0.72	74.47 ± 0.72	72.9 ± 0.78	74.28 ± 1.01
Crude protein	15.03 ± 0.34	15.12 ± 0.48	14.65 ± 0.47	14.57 ± 0.46	14.12 ± 0.30
Crude lipid	1.77 ± 0.22	1.87 ± 0.12	1.79 ± 0.07	2.07 ± 0.21	1.84±0.14
Ash	4.78±0.26	4.69±0.09	4.13 ± 0.07	4.74 ± 0.17	4.71 ± 0.06
Essential amino acid					
Phenylalanine	4.02±0.09	3.27±0.23	3.54±0.24	3.21±0.13	3.60±0.14
Glutamic acid	14.67±0.34^{a}	12.17±0.13^{c}	13.47±0.17^{b}	11.96±0.19^{c}	13.47±0.12^{b}
Leucine	7.56±0.09^{a}	6.32±0.10^{c}	6.94±0.09^{b}	6.13±0.10^{c}	6.96±0.15^{b}
Lysine	8.19±0.09^{a}	7.05±0.15^{b}	7.94±0.12^{a}	6.96±0.37^{b}	7.95±0.23^{a}
Methionine	2.73±0.10^{a}	2.23±0.07b^{c}	2.37±0.07^{b}	2.14±0.03^{c}	2.38±0.04^{b}
Isoleucine	4.22±0.17^{a}	3.39±0.20^{b}	3.83±0.04^{a}	3.34±0.13^{b}	3.90±0.08^{a}
Histidine	1.82±0.08^{a}	1.74±0.07^{ab}	1.62±0.03^{b}	1.43±0.03^{c}	1.62±0.06^{b}
Threonine	4.12±0.07^{a}	3.40±0.14^{b}	3.71±0.24^{ab}	3.36±0.11^{b}	3.72±0.09^{ab}
Valine	4.20±0.04^{a}	3.45±0.12^{b}	3.85±0.16^{ab}	3.41±0.03^{b}	3.82±0.31^{ab}
Non-essential amino acid					
Arginine	5.59±0.07^{a}	4.55±0.23^{b}	4.86±0.10^{b}	4.47±0.11^{b}	4.84±0.11^{b}
Alanine	5.82±0.13^{a}	5.07±0.23^{b}	5.15±0.11^{b}	4.92±0.12^{b}	5.14±0.17^{b}
Taurine	0.50±0.03^{b}	0.62±0.05^{a}	0.31±0.03^{c}	0.46±0.03^{b}	0.31±0.02^{c}
Tyrosine	3.14±0.01^{a}	2.65±0.19^{b}	2.77±0.14^{b}	2.52±0.05^{b}	2.76±0.06^{b}
Glycine	7.21±0.05^{a}	5.08±0.02^{c}	6.41±0.17^{b}	6.16±0.12^{b}	6.35±0.03^{b}
Aspartic acid	10.24±0.32^{a}	8.43±0.18^{c}	9.34±0.13^{b}	8.32±0.13^{c}	9.34±0.07^{b}
Cysteine	0.92±0.03	0.78±0.05	0.88±0.05	0.79±0.04	0.88±0.02
Serine	3.87±0.21	3.42±0.21	3.62±0.23	3.31±0.14	3.62±0.09
Proline	5.55±0.13^{a}	4.16±0.10^{b}	3.50±0.02^{c}	4.21±0.24^{b}	3.50±0.10^{c}

Values are expressed as the mean ± SE of three replicate groups. In the same row, means with different subscripts are significantly different (ANOVA and Tukey Post-Hoc Multiple Comparisons Test (P<0.05).

5.3.5 Fatty acid composition

The fatty acid (FA) composition (% of total fatty acids) of juvenile barramundi muscles was significantly influenced by the bioprocessed and unprocessed PBM at varying levels (Table 5.6). The total SFA was lower (P<0.05) in 100BPBM when compared to the control and other diets. Among the SFA, palmitic acid (C16:0) and stearic acid (C18:0) were the most abundant of total FAs in all experimental groups, whereas lauric acid (C12:0) and lignoceric acid (C24:0) were the least abundant among the groups. The lowest ΣMUFA values were observed in the control diet and the MUFA levels increased markedly as dietary FM substitution levels increased from

75% to 100% in both bioprocessed and unprocessed PBM diets. In MUFAs, oleic acid (C18:1n-9) was the predominant fatty acid in all groups, with the highest percentage observed in total FM replacement diets of 100PBM and 100BPBM, and the lowest value detected in the control. Total PUFAs decreased significantly in all dietary groups compared to the control and fish fed 100% replacement diets of 100PBM had the lowest levels of n-3 PUFAs. In the n-3 PUFAs, EPA and DHA levels in fish fed the control diet were significantly higher from the rest of the FM protein substituting diets. The highest level of n-6 PUFAs was found in fish fed 100BPBM in which linoleic acid-LA (C18:2n-6) was the predominant FA, representing 13.71% of total FAs in 100BPBM compared to 3.94% in the control.

Table 5.6. Fatty acid profile (% of total fatty acids) in muscles of juvenile barramundi after the completion of feeding trial for 42 days.

	Experimental diets				
	Control	75PBM	75BPBM	100PBM	100BPBM
C12:0	0.14±0.01^{a}	0.07±0.01^{b}	0.06±0.01^{b}	0.08±0.01^{b}	0.07±0.01^{b}
C13:0	0.04	-	-	-	-
C14:0	1.74±0.10^{a}	1.21±0.05^{c}	1.24±0.03^{c}	1.52±0.06^{ab}	1.34±0.12^{bc}
C14:1n5	0.02±0.00^{c}	0.05±0.00^{b}	0.09±0.01^{a}	0.10±0.02^{a}	0.05±0.01^{b}
C15:0	0.51±0.02^{a}	0.25±0.01^{b}	0.27±0.01^{b}	0.29±0.01^{b}	0.27±0.01^{b}
C15:1	0.06±0.01^{a}	0.03±0.00^{b}	0.04±0.00^{b}	0.06±0.01^{a}	0.03±0.01^{b}
C16:0	15.54±0.78^{bc}	17.99±1.29^{ab}	11.99±1.12^{d}	20.25±0.68^{a}	14.43±0.48^{cd}
C16:1n7	2.17±0.21^{c}	2.56±0.08^{c}	2.70±0.03^{b}	3.72±0.08^{a}	4.02±0.26^{a}
C17:0	0.82±0.02^{a}	0.37±0.01^{b}	0.42±0.03^{b}	0.38±0.02^{b}	0.31±0.01^{c}
C17:1	0.43±0.04^{a}	0.03±0.01^{c}	0.30±0.04^{b}	0.30±0.02^{b}	0.35±0.01^{b}
C18:0	9.11±0.77^{b}	18.60±2.46^{a}	9.13±0.61^{b}	9.37±0.63^{b}	6.93±0.67^{b}
C18:1cis+trans	18.29±1.90^{b}	17.81±1.49^{b}	29.51±1.17^{a}	28.13±1.03^{a}	31.57±0.45^{a}
C18:2 trans 9	0.25±0.02^{d}	0.45±0.02^{c}	0.39±0.02^{c}	0.55±0.03^{b}	1.05±0.01^{a}
C18:2n6	3.99±0.26^{d}	8.41±0.23^{c}	9.41±0.33^{c}	11.73±0.30^{b}	13.51±0.54^{a}
C18:3n6	0.23±0.02^{d}	0.40±0.02^{c}	0.44±0.01^{c}	0.62±0.60^{b}	1.15±0.50^{a}
C18:3n3	0.59±0.03^{e}	0.86±0.02^{d}	1.09±0.02^{c}	1.43±0.03^{b}	1.57±0.02^{a}
C18:4n3#	0.40±0.02^{b}	0.26±0.01^{c}	0.29±0.01^{c}	0.35±0.03^{b}	0.46±0.02^{a}
C20:0	0.33±0.02^{a}	0.23±0.01^{b}	0.25±0.02^{b}	0.26±0.01^{b}	0.24±0.02^{b}
C20:1	1.83±0.04^{a}	1.31±0.02^{c}	1.63±0.03^{b}	1.80±0.03^{c}	1.33±0.03^{a}
C20:2	0.27±0.02^{ab}	0.23±0.03^{b}	0.29±0.01^{ab}	0.28±0.02^{ab}	0.31±0.01^{a}
C21:0	-	0.07	-	-	-
C20:3n6 (DGLA)	0.18±0.01^{d}	0.46±0.01^{c}	0.47±0.01^{c}	0.54±0.02^{b}	0.76±0.01^{a}
C20:4n6 (ARA)	3.25±0.04^{a}	2.93±0.05^{b}	3.28±0.10^{a}	2.46±0.03^{c}	3.23±0.10^{a}
C20:3n3	-	-	-	-	-
C22:0	0.02	-	-	-	-
C20:5n3 (EPA)	4.64±0.46^{a}	2.96±0.07^{cd}	3.53±0.08^{bc}	2.78±0.09^{cd}	3.72±0.21^{b}
C22:1n9	0.21±0.01^{a}	0.16±0.01^{bc}	0.17±0.01^{bc}	0.19±0.01^{ab}	0.15±0.01^{c}
C22:2	-	-	-	-	-
C23:0	0.04	-	-	-	-
C22:4n6#	2.63±0.25^{a}	1.05±0.01^{bc}	1.30±0.03^{b}	0.63±0.02^{d}	0.73±0.02^{cd}
C24:0	0.27±0.02^{a}	0.07±0.01^{ab}	0.07±0.01^{ab}	0.07±0.01^{ab}	0.13±0.01^{ab}
C22:5n3#	2.40±0.12^{b}	8.35±2.71^{a}	2.66±0.12^{b}	2.13±0.05^{b}	2.67±0.06^{b}
C24:1	0.28±0.02^{a}	0.18±0.01^{b}	0.16±0.01^{bc}	0.172±0.02^{b}	0.13±0.02^{c}
C22:6n3 (DHA)	29.38±1.74^{a}	12.71±0.83^{c}	18.83±0.46^{b}	9.76±0.22^{d}	9.46±0.30^{d}

ΣSFA	28.48±1.47^{bc}	38.79±3.29^{a}	23.43±1.03^{c}	32.23±0.71^{b}	23.72±1.05^{c}
ΣMUFA	23.92±1.67^{c}	25.78±0.27^{bc}	30.50±0.32^{b}	36.35±0.70^{a}	35.09±0.75^{a}
ΣPUFA	47.96±2.65^{a}	38.84±2.50^{b}	41.69±0.84^{b}	32.99±0.57^{c}	38.32±1.18^{b}
Σn-3	37.41±2.12^{a}	25.15±2.49^{b}	26.40±0.54^{b}	16.46±0.39^{c}	17.89±0.50^{c}
Σn-6	10.29±0.56^{d}	13.24±0.16^{c}	14.90±0.43^{b}	15.99±0.40^{b}	19.38±0.68^{a}
Σn-3/Σn-6	3.64±0.09^{a}	1.90±0.23^{b}	1.78±0.06^{b}	1.03±0.04^{c}	0.93±0.01^{c}
ΣPUFA/ΣSFA	1.73±0.17^{a}	1.11±0.22^{b}	1.80±0.09^{a}	1.03±0.03^{b}	1.65±0.13^{a}
IA	0.26±0.02^{ab}	0.30±0.02^{a}	0.20±0.02^{b}	0.31±0.02^{a}	0.24±0.02^{ab}
IT	0.13±0.01^{c}	0.19±0.02^{b}	0.13±0.01^{c}	0.29±0.01^{a}	0.20±0.01^{b}
HH	3.69±0.25^{bc}	2.90±0.35^{cd}	5.37±0.40^{a}	2.70±0.11^{d}	4.21±0.20^{b}

Values are mean ± SE of three replicate fish from each treatment. Values in the same row with different superscript letters (a,b,c) are significantly different based on Tukey's test (One-way ANOVA, $P<0.05$). ΣSFA = sum of saturated fatty acids; ΣMUFA, sum of monounsaturated fatty acids; ΣPUFA, sum of polyunsaturated fatty acids; Σn-3 PUFA, sum of omega-3 polyunsaturated fatty acids; Σn-6 PUFA, sum of omega-6 polyunsaturated fatty acids; IA, index of atherogenicity; IT, index of thrombogenicity and HH, (hypocholesterolemic/hypercholesterolemic ratio; -, not detected.

5.3.6 Nutritional quality indices

Lipid nutritional quality indices of fish fed bioprocessed and unprocessed PBM at varying levels are shown in Table 5.6. The higher value of ΣPUFA/ΣSFA ratio was found in the bioprocessed feeds along with the FM-based control compared to the unprocessed feeds. The Σn-3/Σn-6 ratio was significantly higher ($P<0.05$) in the control diet and decreased with increasing levels of PBM in the diet with the lowest value recorded in complete FM replacement diets of 100PBM and 100BPBM. Fish fed the bioprocessed feeds of 75BPBM and 100BPBM, and the control showed the highest HH values compared to the unprocessed PBM diets of 75PBM and 100PBM. Fish fed the bioprocessed 100% FM replacement diet of 100BPBM showed significantly lower IA and IT values, and higher HH values when compared to the unprocessed diet of 100PBM. Whereas in the 75% FM replacement level, only the HH index was significantly different between bioprocessed and unprocessed diets. The higher HH value was found in the bioprocessed diet of 75BPBM compared to the unprocessed 75PBM.

5.3.7 Principal component analysis (PCA)

The most significant principal component (PC1 and PC2) and their statistical loadings (loading values ≥0.30) generated from selected FAs in the muscle tissue of barramundi were considered for the PCA analysis. Two principal components extracted in the current study explained 87.86% of the total variability in the dataset. According to the Kaiser (1974) rule, only eigenvalues >1.0 were considered significant elements for

data variance. The estimated eigenvalue for the first principal component (*i.e.*, PC1) was 5.44 and comprised 54.36% of the variance in the dataset, while the second (*i.e.*, PC2) had an eigenvalue of 3.35, and accounted for 33.35%. The mutual projections of loading vectors using PC1-PC2 are presented in Figure 5.2 to visualize the specific pattern of correlation between variables. Loading values ≥0.30 were considered significant according to Lombarte et al. (2012). Scores from PC1 indicated that the following FAs: ΣPUFA (0.40), Σn-3 (0.38), Σn-3/Σn-6 (0.33) and ΣPUFA/ ΣSFA (0.36), had a positive contribution while IT (-0.40) influenced PC1 in a negative manner. PC2 showed the highest positive loading for ΣSFA (0.41), Σn-3/Σn-6 (0.33) and IA (0.35), while Σ MUFA (-0.37), Σn-6 (-0.44) and HH (-0.37) influenced PC2 in a negative manner. The PCA analysis demonstrated the clear effects of dietary treatments on the fatty acids profile of juvenile barramundi where the control treated group was discriminated by the Σn-3 and Σn-3/Σn-6 and to a lesser extent by the ΣPUFA. The 75PBM diet was discriminated by an abundance of ΣSFA and IA, while the 75BPBM group was discriminated by an abundance of HH and ΣPUFA/ ΣSFA. 100PBM was mostly characterized by IT whereas 100BPBM treated groups were discriminated from other test groups by the contents of Σn-6 and ΣMUFA.

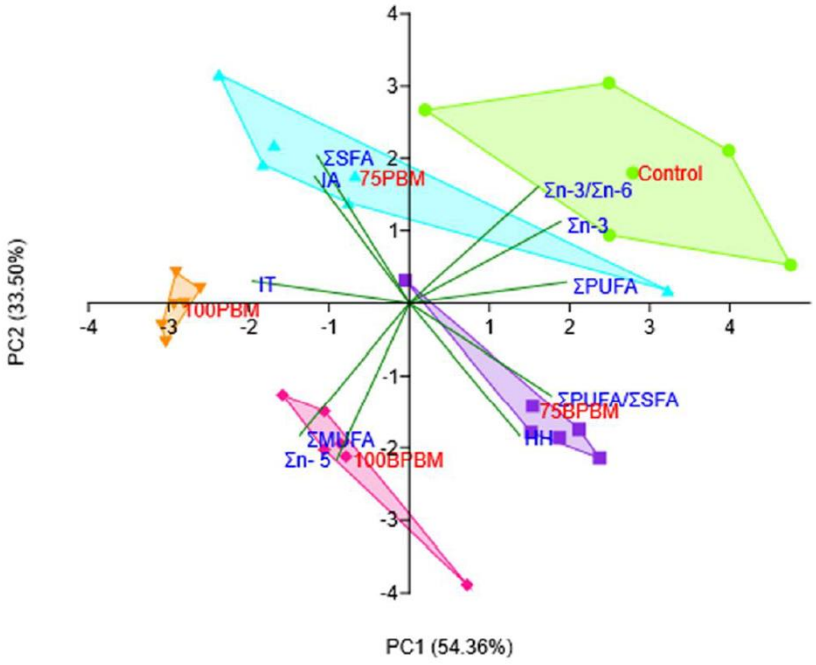

Figure 5.2. Graphical representation of first two principal components (PC1-PC2) biplot of fatty acid composition in juvenile barramundi fed with different levels of bioprocessed and unprocessed PBM. The figure depicts positive and negative association between variables and clustering between samples based on score obtained from analysis. Different markers represent samples from different dietary treatments used in the analysis and vectors indicate how the variables contributed to the formation of PC1 and PC2.

5.4 Discussion

In the present study, the inclusion of both bioprocessed and unprocessed PBM meal at levels of 75% in practical diets did not reduce growth performance of juvenile barramundi. These results are in agreement with a previous study with humpback grouper, *Cromileptes altivelis*, in which no significant differences in growth parameters were observed when FM was replaced with up to 75% of locally sourced feed-grade PBM (Shapawi et al. 2007). Zhou et al. (2011) reported replacement of FM with up to 60% of pet food-grade PBM did not decrease growth performance in terms of FBW, WG and FCR in juvenile cobia, *Rachycentron canadum*. In the present study, the total replacement groups of 100PBM and 100BPBM led to a significant decrease of FBW, WG and FCR in juvenile barramundi. The poorer growth performance in complete FM replacement diets of 100PBM and 100BPBM may be associated with the deficiency of some essential amino acids, and variability in biochemical composition, with high levels of ash and low digestibility in these diets (Cruz-Suárez et al. 2007; Subhadra et al. 2006). Wolters et al. (2009) also reported growth reductions in Atlantic salmon, *Salmo salar* due to increased accumulation of nitrogenous wastes. However, one of our previous study has found that up to 100% FM from a control diet was replaced by a combination of PBM and tuna hydrolysate meal, without altering growth performance of the juvenile barramundi (Siddik et al. 2019b).

The VSI and CF in the present study were not significantly influenced by the replacement of FM by PBM at various levels. These results were in agreement with Zhou et al. (2011) for juvenile cobia, *Rachycentron canadum* and Shapawi et al. (2007) for the humpback grouper, *Cromileptes altivelis*. The HSI showed a significant increase in fish fed 100BPBM and 100PBM compared to remaining diets, the results in agreement with previous studies reporting an increase in HSI with the increasing

inclusion of PBM in diets as noted in previous studies (Rawles et al. 2006; Takagi et al. 2000; Yang et al. 2006). The increased HSI with fish fed 100PBM and 100BPBM may be due to increased lipid deposition in the liver, resulting in hepatic alterations, including hepatic steatosis. The similar effects were reported in a study on juvenile barramundi when FM was replaced at 75% with tuna hydrolysate (Siddik et al. 2018a). However, Shapawi et al. (2007) and Hu et al. (2008) reported no significant variation in HSI even up to 100% replacement of FM with PBM in humpback grouper, *Cromileptes altivelis* and gibel carp, *Carassius auratus gibelio*, respectively.

The ingredients in fish feed has a marked effect on gut morphology as well as the overall health condition of fish (Dimitroglou et al. 2011; Tan et al. 2018). Longer fold and villus height of gut are associated with good health and the high absorptive efficiency of the diet, whereas shorter fold and villus height are indications of poor nutrient utilization and absorption, reduced immune functions, and subsequent lower growth performance of fish (Dimitroglou et al. 2009). The SEM images from this study revealed that fish fed the 75BPBM had higher microvilli density when compared to the control and the rest of the dietary groups. This could possibly be due to the effect of fermentation of the diet with probiotic bacteria *Lactobaccilus casei*, resulting in a superior beneficial effect than when PBM was used alone. This result is in agreement with Wang et al. (2016) who reported improved gut morphology (intestinal folds, enterocytes, and microvilli) of juvenile turbot, *Scophthalmus maximus*, fed soybean meal fermented with *Lactobacillus plantarum* when compared to a non-fermented diet. The significantly lower microvillus density in fish fed the 100PBM and 100BPBM diets may be attributed to the deleterious effect of sub-optimal feed on digestion and absorption of fish.

Both bioprocessed and unprocessed PBM diets had no significant effect in fish muscles moisture, protein, lipid and ash content of juvenile barramundi in the present study. This observation was in agreement with several studies demonstrated no effects of dietary PBM on the whole-body composition of fish (Gumus & Aydin 2013; Takagi et al. 2000). However, Shapawi et al. (2007) who reported that whole-body protein content was significantly lower in humpback grouper fed 100% replacement diet with PBM. In contrast, Yang et al. (2006) reported significantly higher whole-body protein with increasing dietary PBM in gibel carp, *Carassius auratus gibelio*. Such changes in

whole-body composition are likely to be associated with the species specific differences, compounding dietary and environmental factors, associated with the varying levels and quality of protein in PBM relative to FM (Shapawi et al. 2007). The amino acids (AAs) in barramundi muscles in this study were similar as reported by Riche (2015). Almost all the AAs (essential and non-essential) were depleted by the substitution of FM by PBM in the diets of barramundi which reflected in the test diets. Some AAs, including Arginine, Methionine, Glutamic acid, Alanine, Glycine and Proline were higher in PBM than fishmeal, but AAs in fish muscles were higher in fish fed control diet than the fish fed different levels of bioprocessed and unprocessed PBM suggesting that these AAs were not well utilized by the fish. In contrast, the Lysine in control diet was higher, but it was not different between control and bioprocessed PBM fed fish muscles.

Substitution of FM by PBM modified the fatty acid composition in the muscle tissue of fish. In the present study, FA analyses in the experimental diets showed a lower level of Σn-3 PUFA (notably 22:5n-3 and 22:6n-3), along with a higher volume of Σn-6 PUFA (notably 18:2n-6) when compared to the FM. This trend was reflected in the Σn-3 PUFA and Σn-6 PUFA contents of the fish muscles after 42-days of experimentation. Furthermore, the dietary n-3 PUFAs normally decrease and n-6 PUFA subsequently increase with the increasing substitution levels of FM protein by terrestrial animal derived protein, which are often deficient in n-3 PUFAs (Norambuena et al. 2015). In the present study, fish receiving 100PBM and 100BPBM diets had lower Σn-3 PUFA and higher Σn-6 PUFA in the fish muscles which aligns with the findings in fry of Nile tilapia, *Oreochromis niloticus*, fed 100% PBM protein-based diets (Aydin et al. 2015). However, an exception was observed for Σn-3 PUFA in fish fed 75BPBM, which was not lessened compared to the control, as the supplied 75BPBM diet possessed a higher EPA and DHA compared to the unprocessed one in the 75PBM diet. Human epidemiological studies have implicated that myristic acid (C14:0) positively correlated with higher cholesterol levels in plasma which increased the risk of cardiovascular disease (Briggs et al. 2017; Fernandes et al. 2014). Therefore, lower amounts of myristic acid in food may be beneficial for human health (Briggs et al. 2017). In the present study, the lowest amount of myristic acid (14:0) was found in the diets of 75PBM, 75FPBM and 100FPBM compared to the FM-based control diet.

According to Ulbricht & Southgate (Ulbricht & Southgate 1991), the indices of atherogenicity (IA) and thrombogenicity (IT) of a diet in humans indicate a state of coronary heart disease due to the obstruction of coronary vessels by atherosclerosis or thrombosis in the circulatory system. Therefore, the lower the indices of IA and IT values of a diet, the higher the protection for cardiovascular disorders. On the other hand, the HH ratio refers to the proportion of **Σ**hypercholesterolemic FA/**Σ**hypercholesterolemic FA and this value is related to cholesterol metabolism. In contrast to IA and IT, the higher HH values are more beneficial for human welfare. In this study, the lowest IT values were observed in 75BPBM and control diets compared to rest of the diets whereas the highest HH values were observed in bioprocessed diets of 75BPBM and 100BPBM compared to the remaining diets. The lower IT values and higher HH values found in these groups might be due to the higher levels of PUFA present in diets supplied by these fish groups. Approximately 2% lipid reduction in BPBM (11.70%) than PBM (13.50% lipid) may have a greater influence on improved nutrition and flesh quality of fish fed bioprocessed diets. Considering the lipid nutritional indices, the consumption of these species fed with bioprocessed as well as FM-based controlled diets may be beneficial to human health when compared to fish cultured with unprocessed PBM diets.

The PCA analysis was applied to test the influences of the different dietary inclusions of bioprocessed and unprocessed PBM on the FA profiles of fish and also indicating suitable FAs for human consumption. The loading plot of PC1-PC2 was explanatory to 87.86% of the total variation and showed a clear differentiation of samples based on their dietary inclusion levels of PBM. In the PCA biplot and convex hulls, fish fed control and 75BPBM were clustered in the positive site of PC1 with Σn-3, Σn-3/Σn-6, HH and ΣPUFA/ ΣSFA. These groups were separated from 75PBM, 100PBM and 100BPBM treated groups which clustered in the negative region of PC1 with IA, IT, ΣMUFA and Σn-6. The position of these factors and variables in the plot revealed that the 75BPBM diet positively influenced the HH and ΣPUFA/ΣSFA while 75PBM, 100PBM and 100BPBM had a negative correlation with IA and IT.

5.5 Conclusions

The results obtained in this study demonstrated that 75% FM replacement diets (both bioprocessed and unprocessed PBM) did not impair growth performance, gut morphology and liver health of juvenile barramundi. The nutritional quality indices of the fish muscle (IA, IT and HH) were improved with bioprocessed PBM diets compared to unprocessed PBM diets. These results may indicate that consumption of fish cultured in bioprocessed diets, and hence with different fatty acid profiles, may generate some human health benefits, potentially relating to reducing the risks of cardiovascular disease. However, further work, including clinical trials, would be required to fully determine a human health outcome.

Chapter 6: Beneficial effects of tuna hydrolysate in poultry by-product meal diets on growth, immune response, intestinal health and disease resistance to *Vibrio harveyi* in juvenile barramundi, *Lates calcarifer*

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Abstract

This study was conducted to investigate the effects that tuna hydrolysate (TH) supplementation in poultry by-product meal (PBM) diets would have on growth, immunity and resistance to *Vibrio harveyi* infection in juvenile barramundi, *Lates calcarifer*. Five isonitrogenous and isocaloric diets containing fishmeal (FM) without TH supplementation (control) and four diets with 10% TH supplementation viz. a FM protein diet (FMBD+TH), a 75% PBM protein diet (LPBM+TH) and two 90% PBM protein diets, either bioprocessed (BPBM+TH) or unprocessed (HPBM+TH), were formulated for juvenile barramundi, *Lates calcarifer*. The diets were fed to triplicate groups of juvenile barramundi (average pool weight 12.63 ± 0.11g) for 10 weeks. Significantly ($P<0.05$) higher final body weights and specific growth rates were noted in fish fed with FMBD+TH and BPBM+TH diets when compared to the control. Transmission electron microscopy observation of fish distal intestines revealed a significant enhancement of microvilli length in fish fed FMBD+TH and BPBM+TH whereas scanning electron microscopy analysis found no significant difference in microvilli density. A bacterial challenge with *Vibrio harveyi* was conducted for 14 days after the growth trial to test the immune response and survival of barramundi. In the pre-challenge condition, a significant reduction in blood glucose was found in BPBM+TH compared to the control, and fish in the post-challenge at 24 h had higher glucose levels compared to fish in the pre- and post-challenge conditions at 72 h. The serum lysozyme activity was significantly higher in FMBD+TH and BPBM+TH compared to the control and fish at 72 h post-challenge exhibited higher lysozyme activity in each treatment compared to all dietary groups in the post-challenge condition at 24 h and to HPBM+TH and BPBM+TH in the pre-challenge condition. Fish fed FMBD+TH, LPBM+TH and BPBM+TH diets had significantly higher survival to the bacterial challenge than fish in the control and HPBM+TH. These results showed that PBM supplemented with TH could successfully replace FM

without compromising growth, however, bioprocessed PBM supplemented with TH (BPBM+TH) may significantly improve growth performance, immune response, intestinal health and disease resistance in juvenile barramundi.

Keywords: Tuna hydrolysate, bioprocessed PBM, immune response, intestinal health, juvenile barramundi,*Vibrio harveyi*

6.1 Introduction

In recent years, there has been a growing disparity between the demand and supply of raw FM materials and the ecological and economic issues associated with its use, which has exerted pressure on aquaculture nutritionists to evaluate viable alternative protein sources to FM (Ilham et al. 2016a). With this aim, aquaculture scientists have tended to not only focus on finding suitable alternatives to FM but also on improving the applicability of the already existing alternative products through innovative approaches such as fermentation and advanced bioprocessing for nutritional enrichment (Vo et al. 2015). A number of studies have reported that fermentation, as an environmental friendly and cost effective method, improves the digestibility and amino acid profile of animal by-products (Bertsch & Coello 2005; Siddik et al. 2018a) and thus enhances the suitability of their inclusion in fish feed formulations (Bertsch & Coello 2005; Samaddar & Kaviraj 2014). Another advantage of the fermentation process is that it breaks down carbohydrates into lower molecular-weight compounds that can potentially enhance innate energy and mineral absorption (Hotz & Gibson 2007; Ilham & Fotedar 2017). Fagbenro et al. (1994) observed that feeds made from fermented products tend to have higher stability in water, thereby allowing more time for fish to ingest the feed and maximise nutrient intake. Fermentation is also used to overcome many of the other inherent problems of animal waste products including high moisture, indigestible particles and microbial contaminants (Kader et al. 2012; Mondal et al. 2008; Samaddar et al. 2015).

Among the animal waste by-products used in aqua-diets, PBM is a good source of protein but is limiting in some of the essential amino acids (Rawles et al. 2006). Another limiting factor in the use of PBM, as with many animal by-product meals, is that digestibility varies highly from batch to batch in the rendering process and among suppliers (Lewis et al. 2019). Therefore, success has only been reported when PBM

only partially replaced FM in the diet of fish. PBM successfully replaced FM at a level of 25% with juvenile tench, *Tinca tinca* (González-Rodríguez et al. 2016), 67% with both totoaba juveniles, *Totoaba macdonaldi* (Zapata et al. 2016), and rainbow trout, *Oncorhynchus mykiss* (Badillo et al. 2014), and 70% with Florida pompano, *Trachinotus carolinus* (Riche 2015). However, the complete replacement of FM by PBM has resulted in depressed growth performance of Florida pompano, *Trachinotus carolinus* (Rossi & Davis 2012). Therefore, to enable a qualitative improvement of PBM in fish diet, it may be refined through fermentation to potentially improve the biological value of the raw material as well as improve the utilisation of the finished product. Furthermore, PBM may be supplemented with a fish protein hydrolysate (FPH) during the preparation of diets to minimize the limiting amino acids as well as to improve the quality of the final meal. As a supplement, FPH is a highly nutritious product made from whole fish or fish by-products, and it has been used as a supplement in many aqua-diets to improve immunity (Bui et al. 2014; Khosravi et al. 2015b; Kousoulaki et al. 2013; Siddik et al. 2018b) and as an attractant to increase diet palatability (Chotikachinda et al. 2013; Ho et al. 2014). Many studies have reported that FPH consists of low molecular weight bioactive peptides that may have immune-stimulating and antibacterial properties (Bøgwald et al. 1996; Ha et al. 2019; Kotzamanis et al. 2007). The improvement of cellular and/or humoral immune function with heightened disease resistance of various fish species due to the bioactive peptides in FPH has already been established (Kotzamanis et al. 2007; Liang et al. 2006; Murray et al. 2003). For example, red sea bream, *Pagrus major*, fed different hydrolysate diets (krill, shrimp and tilapia hydrolysate) exhibited significant improvement in disease resistance against *Edwardsiella tarda* (Khosravi et al. 2015b).

Barramundi or Asian Seabass, *Lates calcarifer*, a catadromous species widely distributed in the Indo-Pacific region and Australia (Ilham et al. 2016b) is progressively becoming a major commercial species in aquaculture because of the species-wide range of salinity tolerance and adaptability to versatile farming systems, its tendency to readily consume pelleted feeds, and its highly appreciated meat among consumers (Simon et al. 2019). To date, no research has been done to prove whether fermentation and TH in combination further improve the inclusion level of PBM in fish diets. Therefore, the present study was designed to investigate the effect of complete replacement of FM with TH supplementation in PBM diets, on growth

performance, biochemical response, gut health and disease resistance of juvenile barramundi.

6.2 Materials and methods

6.2.1 Ethic statements

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Australia. The protocol was approved by the Ethics Committee in Animal Experimentation of the Curtin University (Approval number AEC_2015_41).

6.2.2 Diet preparation

Five experimental diets were prepared using Feed-soft Professional® providing 47.0% crude protein (CP) and 20.0 MJ kg^{-1} gross energy (GE) with commercial ingredients to fulfil the nutrient requirements of juvenile barramundi according to NRC (2011). The PBM was fermented following the technique described in our earlier study (Siddik et al. 2018a). In short, PBM was weighed and then Baker's yeast, *Saccharomyces cereviceae* (Instant dried yeast, Lowan®), and *Lactobacillus casei* in the form of skim milk product (Yakult® @ cell density of 3×10^6 CFU ml^{-1}), were added at 10% and 5% of the weight of PBM, respectively. Distilled water was then added to approximately 70% of the total weight of the meal mixture and ingredients were thoroughly mixed in a food mixer. The mixture was then placed in an Erlenmeyer flask covered with aluminium foil and incubated at 30°C for 4 days. Then, the fermented product was dried in the oven at 60°C for 24 h and used as a feed ingredient. All diets were prepared isonitrogenous and isocalorific, therefore, quantities of individual ingredients were adjusted so as to provide equal protein and energy contents. Wheat and wheat starch were used to provide NFE and binding strength respectively to the diets. Nevertheless, care is taken to minimise different ingredients concentration and therefore, casein, a pure source of protein, was used to balance the protein in the diet formulation.

All the experimental diets, except the control, were supplemented with 10% TH. Thus, the dietary treatments were: FM-based basal diet devoid of TH (control), FM-based

diet supplemented with 10%TH (FMBD+TH), a diet of 75% PBM and 15% FM supplemented with 10%TH (LPBM+TH), 90% PBM supplemented with 10% TH (HPBM+TH) and 90% bioprocessed PBM supplemented with 10% TH (BPBM+TH). The diet codes were allotted as described in Table 6.1. All dry ingredients were homogeneously mixed before adding fish oil and warm water in a food mixer (Hobart Food equipment, Australia) to form a dough. The dough was then passed through a mincer to produce 3mm pellets. The moist pellets were then dried in an oven at 60°C for 36 hours, cooled at room temperature, broken up by hand, sealed in plastic bags and stored in refrigerated conditions prior to use in the feeding trial. The proximate composition and amino acids (AAs) profile of the tested FM, PBM and BPBM are presented in Table 6.2. The formulation and proximate composition of the experimental diets are presented in Table 6.3.

Table 6.1 Dietary codes for feeds used in the article.

Codes	Diet
Control	Basal diet without tuna hydrolysate supplementation
FMBD+TH	Basal diet supplemented with 10% tuna hydrolysate
LPBM+TH	75% PBM and 15% FM supplemented with 10% tuna hydrolysate
HPBM+TH	90% PBM supplemented with 10% tuna hydrolysate
BPBM+TH	90% bioprocessed PBM supplemented with 10% tuna hydrolysate

Table 6.2 Nutrient composition (%) and amino acid contents (g 100 g^{-1} protein) of FM, PBM and BPBM used in the feed formulation.

	FM	PBM	BPBM
DM	94.32	97.7	95.40
Crude protein	64.00	67.13	66.98
Crude Lipid	10.76	13.52	11.70
Ash	19.12	13.34	14.68
Gross energy (MJ/kg)	20.30	21.05	20.42
Essential amino acid			
Arginine	4.35	5.43	5.24
Phenylalanine	2.68	2.65	2.65
Leucine	4.64	5.00	4.97
Lysine	4.77	4.17	4.21
Methionine	1.81	1.46	1.43

Isoleucine	3.04	2.67	2.66
Histidine	1.79	1.47	1.39
Threonine	2.53	2.81	2.86
Valine	3.20	3.08	3.11
Non-essential			
Alanine	4.42	4.62	4.89
Aspartic acid	6.20	5.95	6.03
Glutamic acid	8.25	9.55	9.84
Glycine	4.73	6.53	6.79
Proline	3.81	5.63	5.94
Serine	3.05	3.01	3.08

Table 6.3 Formulation and proximate composition of the experimental diets for juvenile barramundi.

Ingredients ($g\ kg^{-1}$)	Experimental diets				
	Control	FMBD+TH	LPBM+TH	HPBM+TH	BPBM+TH
FM	610.0	549.0	91.5	518.5	488.0
PBM	-	-	457.5	549.0	-
BPBM	-	-	-	-	549.0
TH	-	61.0	61.0	61.0	61.0
Wheat	266.0	264.0	268.0	267.0	265.0
Wheat starch	20.0	20.0	20.0	20.0	20.0
Fish Oil	30.0	30.0	30.0	30.0	30.0
Calcium carbonate	2.0	2.0	2.0	2.0	2.0
Sodium chlorite	2.0	2.0	2.0	2.0	2.0
Vitamin premix	1.0	1.0	1.0	1.0	1.0
Casein	63.0	65.0	61.0	63.0	64.0
Cellulose	6.0	6.0	6.0	6.0	6.0
Proximate composition (% dry matter)					
Dry matter	92.72	89.36	90.90	91.22	91.03
Crude protein	47.17	46.86	47.44	47.79	46.85
Crude lipid	9.99	9.40	10.69	10.94	9.53
Ash	13.04	12.06	9.26	8.70	8.71
Gross energy ($MJ\ kg^{-1}$)	19.98	19.92	19.89	19.90	19.91

FM: fishmeal, PBM: poultry by-product meal, BPBM: bioprocessed poultry by-product meal, TH: Tuna hydrolysate.

6.2.3 Fish and feeding trial

Juvenile barramundi were obtained from the Australian Centre for Applied Aquaculture Research, Fremantle, Australia, and reared in three fibreglass tanks (300L water capacity) to attain a desirable size of approximately 10g. The fish were then graded and 300 juvenile barramundi were selected for experiment purposes. Before commencing the feeding trial, fish were acclimated to experimental conditions at Curtin Aquatic Research Laboratories for two weeks. During the acclimation period, fish were fed three times a day with a commercially formulated diet (470g protein kg^{-1} diet and 20.0MJ kg^{-1} dietary gross energy). After that, juvenile barramundi with an initial pool weight of average pool weight 12.63 ± 0.11g were randomly distributed into fifteen independent tanks (300-L water capacity) at a stocking density of 20 fish per tank.

Each tank in the rearing facility was equipped with constant aeration, a water heater and an external bio-filter (Fluval 406, Hagen, Italy) to maintain water recirculation at a rate of 10L min^{-1} throughout the experimental period. Fish were reared in seawater with a salinity of 32.71 ± 2.17 ppt. The water quality parameters such as temperature, pH and dissolved oxygen were monitored daily using a portable multiparameter meter (YSI, USA) and were maintained at 26.0 ± 2.8°C, pH 6.51 ± 0.43 and 7.10 ± 0.6 mg L^{-1}, respectively. Total ammonia and nitrite were monitored using commercial kits twice a week with the resulting mean values of 0-2.0 mg L^{-1} and 0-1.0 mg L^{-1}, respectively. Fish were held under a 14-h light/10-h dark cycle using an automatic indoor light switch (Clipsal, Australia). Fish tanks were randomly assigned with three tanks for each individual diet. Throughout the 10-weeks of the feeding trial, fish were hand-fed to apparent satiation three times a day at 0800, 1200 and 1700 h. After 30 min of feeding, uneaten feed was removed from the bottom of the tank by siphoning, transferred to aluminium cups, and oven dried at 60°C for 36 h in order to determine the daily feed intake. At the end of the growth trial, total numbers of fish in each tank were counted and individually weighed after starving them for one day. Growth and

feeding performances of juvenile barramundi were assessed at the end of the feeding trial according to the equations described in (Siddik et al. 2018b).

6.2.4 Proximate analysis

After terminati6.on of the feeding trial, two fish from each tank (six samples per dietary treatment) were randomly selected for analysis of dry matter, protein, ash and gross energy whilst one fish from each tank (three samples per dietary treatment) were considered for lipid analysis. The muscles sample of fish were analysed for proximate composition based on the Association of Official Analytical Chemists procedures (AOAC 1995). Briefly, the carcass dry matter was determined by oven drying to constant weight at 105°C; crude ash by combustion at 550°C; crude protein content (nitrogen × 6.25) by the Kjeldahl digestion method; crude lipid content by the Soxhlet technique; and gross energy content by an IKA oxygen bomb calorimeter (Heitersheim, Germany).

6.2.5 Challenge with Vibrio harveyi

Vibrio harveyi is a virulent pathogen infecting wide range of marine vertebrates and invertebrates including fish. It is a primary pathogen for barramundi, *Lates calcarifer* too and has been used in many studies as a challenge pathogen to investigate the immune status of the species. The bacterial challenge study was performed by infecting juvenile barramundi with *Vibrio harveyi*, after the 10-week feeding trial. The pathogen, *Vibrio harveyi* was obtained from the Bacteriology Laboratory, Department of Agriculture & Food, Perth, Australia. The bacteria were grown in trypticase soy broth (Oxoid, Basingstoke, UK) at 24^{o}C for 24 h and the broth containing the culture was centrifuged at 5000g for 15 min. The supernatant was discarded and the pellets were washed twice in phosphate-buffered saline (pH 7.2) for experimental use. The LD_{50} of the bacteria was adjusted to 1.7×10^{8} cells/mL.

At the end of the growth trial, 10 fish from each replicate tank was relocated to each of 20 x 100 L capacity glass aquaria for 14 days of bacterial challenge. Of the 20 aquaria, five were used for blood sampling and 15 were utilized for survival counting. Following three days of acclimation, fish were subjected to intraperitoneal injection (IP injection) with *Vibrio harveyi* by removing the fish from the tank and bathing them

in mild anaesthetic (5-10ppm). A dose of 0.1 mL of *Vibrio harveyi* suspension in PBS media (1.7×10^8 CFU/mL) was administered to individual fish using a 1-mL syringe and 27-gauge needle according to the procedure described by (Talpur & Ikhwanuddin 2012). After IP injection all the challenged fish were returned to their respective rearing tanks and fed once daily for a further 2 week period with the same experimental diet that was assigned before the challenge. As a part of constant monitoring, fish with signs of infection were recorded immediately and the infected fish were removed from the tank and euthanised with AQUI-S at 175 mg/L for 20 minutes, following the protocol of the Curtin University Standard Operating Procedure CARL01 Euthanasia of Fish. Fish were euthanised as soon as clinical signs, including lethargic swimming, flared opercula, skin lesions, moribund and loss of equilibrium, were obvious. Under the bacterial challenge conditions, fish health and condition were monitored three times a day at around 7:00am, 2:00pm and 9.00pm.

6.2.6 Blood and serum biochemical indices

Blood and serum samples for assessing biochemical and immunological indices were conducted before the bacterial challenge and then again 24 h and 72 h post challenge. Two sets of blood from mildly anaesthetized fish (AQUI-S, 8 mg/l) were withdrawn by caudal vein puncture with a 1mL non-heparinised syringe at the end of the growth trial. The first aliquot of extracted blood sample was collected in heparinised tubes for the determination of blood glucose level. The second aliquot of the blood sample was collected in non-heparinised tubes and allowed to sit for 24 h. The clotted blood samples were then centrifuged at 3000 rpm for 15 min at 4°C for serum extraction and the extracted serum was stored at −80°C for the measurement of the serum biochemical indices including serum total cholesterol (TC), triglyceride (TG), total protein (TP) and albumin using an automated blood analyser (SLIM; SEAC Inc, Florence, Italy) and following the methods of Blanc et al. (2005). The total globulin content was determined by subtracting the albumin values from the total serum protein values. The albumin and globulin ratio (A/G ratio) was obtained by dividing albumin values by globulin values.

6.2.7 Biochemical assessment of liver damage

To investigate liver health, serum aspartate aminotransferase (AST) and glutamate dehydrogenase (GLDH) activities were examined at pre-challenge and post-challenge

conditions with an automatic biochemical analyser (Mindray BS-400, Mindray Medical International Ltd., Shenzhen, China) and attached kit (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan).

6.2.8 Serum immunological indices

Serum immunological indices, including lysozyme and bactericidal activity, were assayed in pre- and post-challenge fish at 24 h and 72 h. Serum lysozyme activity was examined based on the method previously described by Siddik et al. (2018b) and the bactericidal activity according to Le & Fotedar (2014).

6.2.9 Intestinal mucosal morphology

Three randomly selected fish per treatment after 10-weeks post feeding was considered for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analysis. For TEM analysis, intestinal samples were dissected into ~1 mm long transverse sections and then processed using a microwave-assisted protocol. Briefly, samples were excised from freshly collected specimens and then immediately bathed in 2.5% glutaraldehyde buffered in 1x PBS at pH 7.4 and stored at 4°C for over 24 h. Samples were then briefly rinsed in PBS prior to secondary fixation using 1% OsO_4 (80W 2 min on, 2 min off, 2 min on), dehydration in ethanol (50, 70, 95 and 100% at 250W, 40 s each) then acetone (100% 2x at 250W, 40 s each) and finally infiltration with epoxy resin in acetone (Procure 812, Proscitech) (1:3, 1:1, 3:1 ratios at 250W, 3 mins each) was undertaken using a laboratory microwave (Pelco, Biowave® with cold spot and vacuum chamber). Samples were left in the final 3:1 ratio overnight before two further overnight changes in 100% resin. Samples were then mounted in BEEM™ capsules and polymerized in an oven at 70°C overnight. Semi- and ultra-thin transverse sections of the gut were cut from trimmed blocks using an ultramicrotome (UC6, Leica Microsystems) at thicknesses of 1 µm and 120 nm using a Histo and Ultra diamond knife, respectively (Diatome, Switzerland). Semi-thin sections were mounted on glass slides and stained with toluidine blue, whilst ultra-thin sections were mounted onto carbon filmed copper finder TEM grids (EMS, Hatfield, USA). TEM imaging was carried out on a LaB_6 TEM (JEOL 2100, Japan) operating at 120 kV. Conventional bright field images were acquired on an 11 Mpx charge coupled device camera (Gatan

ORIUS1000, Pleasanton, USA). The electron micrographs were investigated with Photoshop CS6 (Adobe, USA), and ImageJ (National Institute of Health, USA) to determine microvilli length, as described elsewhere (Ran et al., 2015). TEM images (magnification × 30,000) were examined to measure the microvilli length.

For SEM analysis, the intestinal samples were removed and cleaned by dipping in normal saline water. The samples were then dissected, the interior cleaned and cut into pieces measuring 5mm. They were then washed in 1% S-carboxymethyl-L-cysteine for 30 s to remove mucus and then preserved in 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M pH 7.2). Samples were processed as described elsewhere (Merrifield et al. 2009) and screened with a JSM 6610 LV (Jeol, Tokyo, Japan) SEM or JEN 1400 (Jeol, Tokyo, Japan). The SEM images (magnification × 30,000) were analysed to assess the number of microvilli present on the surface of enterocytes standardised to 1 μm^2 region (Adeoye et al. 2016).

6.2.10 Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's tests was applied in growth and muscle tissues composition to compare the control diet against each dietary groups containing TH. The biochemical and immune responsive parameters were analysed by multifactorial analysis of variance (ANOVA). The survival graph was constructed using the Kaplan–Meier method and the differences among different dietary groups were performed using the log-rank test. All results were expressed as means and standard errors (SE), and p-values less than 0.05 were considered statistically significant.

6.3 Results

6.3.1 Growth performance and feed utilization

Dietary TH supplementation in fish diets had a significant effect on the growth performance and feed utilization of juvenile barramundi (Table 4). Fish fed FMBD+TH and BPBM+TH diets demonstrated the highest growth performance in terms of final body weight (FBW) and weight gain (WG) than the control ($P<0.05$). However, FBW and WG were not significantly ($P>0.05$) different with the treatment of HPBM+TH. The highest feed intake was observed in fish fed FMBD+TH when

compared to the control and the rest of the dietary groups. The survival rate, feed conversion ratio (FCR) and daily weight gain (DWG) of fish were not significantly (P>0.05) affected by any dietary groups.

Table 6.4 Growth performance and muscles nutrient composition of juvenile barramundi fed tuna hydrolysate (TH) included diets at various levels for 10 weeks.

Parameters	Experimental diets					P-value
	Control	FMBD+TH	LPBM+TH	HPBM+TH	BPBM+TH	
Growth performance						
Initial body weight (g)	12.71±0.11	12.65±0.01	12.69±0.07	12.49±0.14	12.61±0.09	0.526
Final body weight (g)	106.32±5.34^{b}	127.19±9.13^{a}	120.63±3.01^{ab}	105.27±6.49^{b}	127.06±0.53^{a}	0.046
Weight gain (g)	93.61±5.45^{b}	114.54 ±9.13^{a}	107.95±2.95^{ab}	92.78±6.60^{b}	114.46±0.58^{a}	0.048
Specific growth rate (%/d)	3.31±0.08	3.60±0.11	3.52±0.03	3.33±0.10	3.61±0.02	0.065
Feed intake (g/fish/day)	1.63±0.04^{bc}	1.70±0.01^{a}	1.68±0.01^{ab}	1.61±0.01^{d}	1.69±0.01^{ab}	0.028
Feed conversion ratio	1.12±0.07	1.13±0.07	1.13±0.03	1.19±0.07	1.20±0.03	0.152
Survival (%)	96.67±1.67	95.00±2.88	91.67±4.41	93.33±4.41	98.33±1.67	0.365
Muscles nutrient composition (% wet weight basis)						
Moisture (%)	72.42±0.37^{a}	73.22±0.26^{a}	72.63±0.13^{a}	75.07±0.37^{b}	75.40±0.32^{b}	0.000
Protein (% WW)	15.96±0.34	16.68±0.37	15.34±0.51	14.89±0.39	15.01±0.61	0.056
Lipid (% WW)	2.58±0.02^{a}	2.13±0.02^{b}	2.35±0.01^{ab}	1.88±0.01^{c}	1.87±0.01^{c}	0.000
Ash (% WW)	3.59±0.07	3.74±0.05	4.03±0.22	4.18±0.28	4.32±0.30	0.122
GE (MJ Kg^{-1})	0.61±0.04	0.56±0.00	0.59±0.04	0.55±0.00	0.56±0.00	0.501

Values are mean ± SE of three replicate tanks per treatment. Values in the same row with different superscript letters (a,b,c) are significantly different based on Tukey's multiple range test (One-way ANOVA, P<0.05).

Weight gain (g) = [(mean final body weight – mean initial body weight) / mean initial body weight].

Specific growth rate (%/d) = [(ln final body weight - ln (pooled initial body weight))/days] ×100

Feed intake (g/fish/day) = dry feed consumed/fish number.

Feed conversion ratio = dry feed fed/wet weight gain.

Survival (%) = (number of final fish- number of initial fish)/number of initial fish×100

6.3.2 Proximate composition

Fish fed with HPBM+TH and BPBM+TH had a higher lipid content than fish with the control and other diets. The moisture content of barramundi showed a tendency to decrease in response to increased lipids and hence significantly lower moisture levels were registered in HPBM+TH and BPBM+TH when compared to the control. No

significant differences were observed in protein, energy and ash content among the dietary groups (Table 6.4).

6.3.3 Blood and serum biochemical indices

The blood and serum constituents of juvenile barramundi fed the experimental diets are presented in Table 6.5. A significant decrease ($P<0.05$) in glucose with BPBM+TH and triglyceride levels occurred in all dietary groups at pre-challenge condition over the control. But the glucose and triglyceride levels among the treatments were not significantly different in the post-challenge condition at 24 h and 72 h. The highest glucose level was observed in the post-challenge condition at 24 h when compared to fish at the pre-challenge and post-challenge condition at 72 h. However, the triglyceride level was found to be significantly higher at the pre-challenge condition when compared to post-challenge fish at 24 h and 72 h. Although the cholesterol level remained consistent among the dietary groups in the pre-challenge condition, a significantly lower level was observed with the control and FMBD+TH when compared to the rest of the diets in the post-challenge condition at 24 h and 72 h. Neither dietary supplementation of TH nor fermentation had the significant effect on the total protein, albumin, globulin and A/G ratio in juvenile barramundi ($P>0.05$) in either the pre-challenge or post-challenge conditions.

Table 6.5 Blood/serum biochemical parameters of juvenile barramundi fed FM and PBM diets supplemented with tuna hydrolysate for 10 weeks pre-challenge, post-challenge at 24 h and post-challenge at 72h.

Parameter	Diets	Pre-challenge	Post-challenge-24h	Post-challenge-72h
Glucose	Control	5.40 ± 0.04^{a}	$6.97\pm0.13^{*}$	5.43 ± 0.22
	FMBD+TH	5.29 ± 0.07^{a}	$6.76\pm0.03^{***}$	5.21 ± 0.10
	LPBM+TH	5.35 ± 0.06^{a}	$6.84\pm0.05^{***}$	5.24 ± 0.05
	HPBM+TH	5.39 ± 0.04^{a}	$6.86\pm0.18^{*}$	5.33 ± 0.17
	BPBM+TH	5.11 ± 0.03^{b}	$6.67\pm0.10^{***}$	5.23 ± 0.13
Cholesterol	Control	$6.03\pm0.46^{**}$	3.20 ± 0.21^{ab}	$3.57\pm.49^{ab}$
	FMBD+TH	$5.67\pm0.70^{*}$	2.63 ± 0.30^{b}	$2.20\pm.00^{b}$
	LPBM+TH	5.37 ± 0.69	3.45 ± 0.15^{a}	4.27 ± 0.39^{a}
	HPBM+TH	5.20 ± 0.60	3.77 ± 0.07^{a}	3.80 ± 0.35^{a}
	BPBM+TH	$6.27\pm0.81^{*}$	3.70 ± 0.26^{a}	4.37 ± 0.48^{a}
Triglyceride	Control	$3.53\pm0.41^{a**}$	0.40 ± 0.06	0.30 ± 0.00
	FMBD+TH	$1.70\pm0.06^{b**}$	0.40 ± 0.12	0.90 ± 0.29
	LPBM+TH	$1.50\pm0.26^{b*}$	0.20 ± 0.00	1.10 ± 0.51
	HPBM+TH	$1.20\pm0.15^{b*}$	0.23 ± 0.09	0.70 ± 0.20
	BPBM+TH	$1.00\pm0.12^{b*}$	0.30 ± 0.06	$0.83\pm0.22^{*}$
Total protein	Control	43.33 ± 6.36	31.00 ± 2.08	37.00 ± 1.73

	FMBD+TH	38.33±2.33	33.33±3.84	35.00±1.00
	LPBM+TH	40.00±3.00	35.00±1.00	37.00±2.00
	HPBM+TH	41.00±1.53	34.00±1.15	37.00±5.13
	BPBM+TH	40.67±3.71	32.67±3.18	36.00±1.15
Albumin	Control	12.00±2.00	9.00±0.58	11.33±0.67
	FMBD+TH	10.67±0.88	9.33±0.88	9.67±0.33
	LPBM+TH	10.67±0.67	11.50±0.50	9.67±0.33
	HPBM+TH	12.33±0.33	8.67±0.88	10.00±1.53
	BPBM+TH	11.00±1.00	9.33±0.88	9.67±0.67
Globulin	Control	31.33±4.37	22.00±1.53	25.67±1.20
	FMBD+TH	27.67±1.45	24.00±3.00	25.33±0.88
	LPBM+TH	29.33±2.33	23.50±0.50	27.33±1.67
	HPBM+TH	28.67±1.33	25.33±0.88	27.00±3.61
	BPBM+TH	29.67±2.73	23.33±2.40	26.33±0.67
A/G	Control	0.38±0.01	0.41±0.01	0.44±0.03
	FMBD+TH	0.38±0.01	0.39±0.02	0.38±0.02
	LPBM+TH	0.36±0.01	0.49±0.01**	0.35±0.01
	HPBM+TH	0.43±0.02	0.34±0.04	0.37±0.01
	BPBM+TH	0.37±0.01	0.40±0.03	0.37±0.02

The values are expressed as mean ± SE of three replicate fish per treatment. Mean values bearing different lowercase (a,b,c) letters among different dietary treatments were statistically significant (P<0.05). *P<0.05, **P<0.01 and ***P<0.001 denote significant level among pre-challenge, post-challenge-24 h and post- challenge- 72 h in each treatment.

6.3.4 Assessment of liver damage

The serum AST activity of fish was not significantly affected, either by the tested diets or challenge periods (P>0.05), except in FMBD+TH where the AST level in post-challenge fish at 72 h was significantly lower when compared to post-challenge fish at 24 h (Figure 6.1A). However, except for FMBD+TH, the cumulative results (pre, post-24h and post-72h) of serum GLDH activity was significantly lower in the experimental diets when compared to the control. No significant changes were observed between challenge periods in each treatment for GLDH activity (Figure 6.1B).

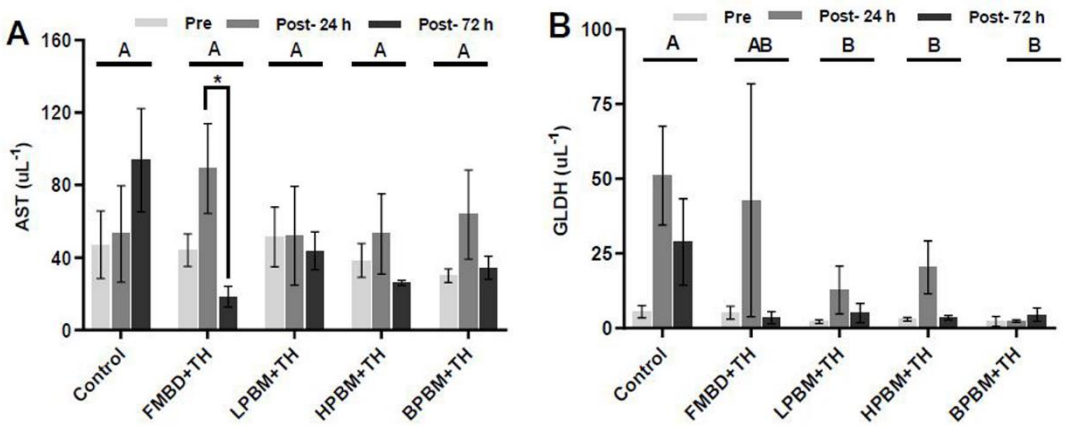

Figure 6.1. Serum aspartate aminotransferase (AST) and glutamate dehydrogenase (GLDH) in barramundi fed with different experimental diets supplemented with TH. Data were expressed as mean ± SE, n = 3. Different uppercase (A,B,C) letters among

different dietary treatments were statistically significant (two-way ANOVA; Tukey post-hoc test; $P<0.05$). *$P<0.05$ denotes significant level among pre-challenge, post-challenge-24 h and post- challenge- 72 h in each treatment (one-way ANOVA; Tukey post-hoc test).

6.3.5 Lysozyme activity

Serum lysozyme activity was significantly influenced, both by dietary groups and the duration of time after the challenge (Figure 6.2A). The highest overall lysozyme activity was found in fish fed FMBD+TH and BPBM+TH when compared to the control. Fish at 72 h post-challenge exhibited higher lysozyme activity in each treatment compared to the post-challenge condition at 24 h in all dietary groups, and to the pre-challenge condition in HPBM+TH and BPBM+TH.

6.3.6 Bactericidal activity

In terms of bactericidal activity, the higher the efficiency of immune cells to kill pathogens, the lower the bactericidal colonies observed in cells. In the present study, the accumulative serum bactericidal activity in all dietary groups was not significantly different ($P>0.05$) when compared to the control (Figure 6.2B). However, fish at 72 h post-challenge demonstrated the highest bactericidal activity compared to the pre-challenge condition in FMBD+TH and BPBM+TH. Fish that received the control, LPBM+TH and HPBM+TH diets showed no significant differences in bactericidal activities among the challenge periods.

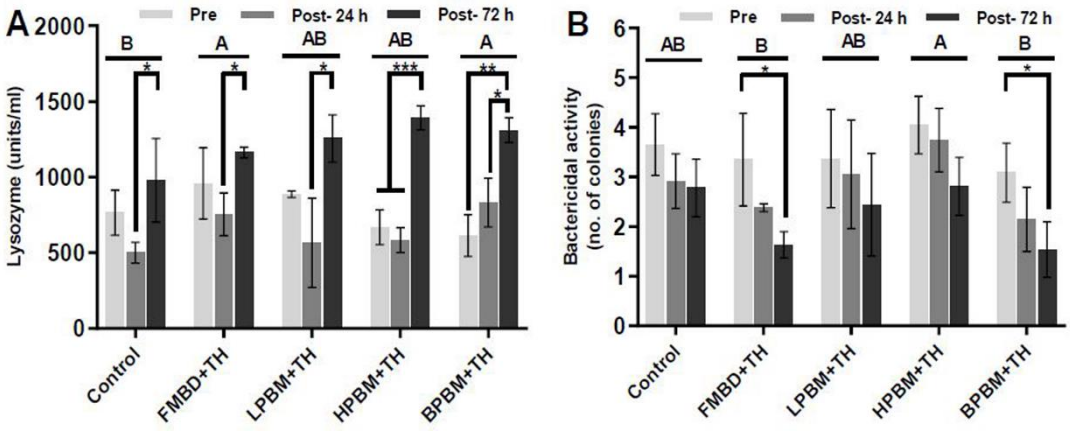

Figure 6.2 The serum lysozyme (A) and bactericidal (B) activity in barramundi fed with different experimental diets supplemented with TH. Data were expressed as mean ± SE, n = 3. Different uppercase (A,B,C) letters among different dietary treatments

were statistically significant (two-way ANOVA; Tukey post-hoc test; $P<0.05$). *$P<0.05$, **$P<0.01$ and ***$P<0.001$ denote significant level among pre-challenge, post-challenge-24 h and post- challenge- 72 h in each treatment (one-way ANOVA; Tukey post-hoc test).

6.3.7 Intestinal mucosal morphology

The intestinal mucosal morphology of juvenile barramundi fed the experimental diets was analysed by observation of TEM and SEM (Figure 6.3 and Figure 6.4, respectively) after 10 weeks of the feeding trial. TEM analysis revealed a significant difference ($P<0.05$) in the microvilli height of the distal intestinal tracts of barramundi reared in different dietary groups (Figure 6.5A). The highest microvilli height was found in the FMBD+TH and BPBM+TH, and the lowest value was observed in the HPBM+TH when compared to the control ($P<0.05$). However, the microvilli height of fish in the LPBM+TH group was similar to that of the control. SEM analysis of the distal intestine demonstrated that microvilli density was not significant among the dietary groups when compared to the control (Figure 6.5B).

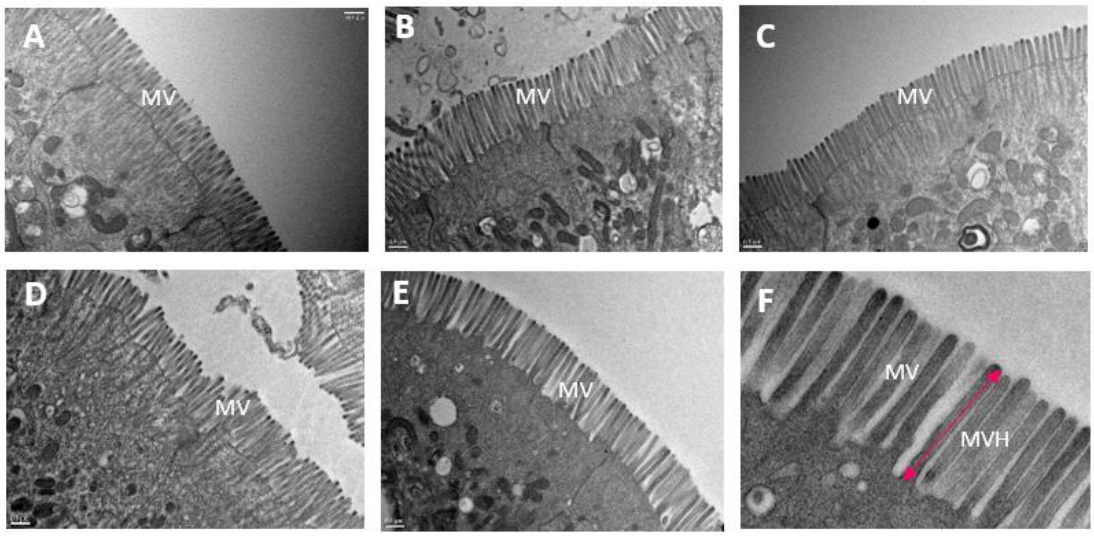

Figure 6.3 Comparative transmission electron micrographs (TEM) from the distal intestine of barramundi at the end of the feeding trial. TEM images for microvilli length; A: Control, B: FMBD+TH, C: LPBM+TH, D: HPBM+TH, E: BPBM+TH and F: measurement of microvilli length and diameter. MV: microvilli, MVD: microvilli diameter, MVH: microvilli height. Scale bar=0.5 µm.

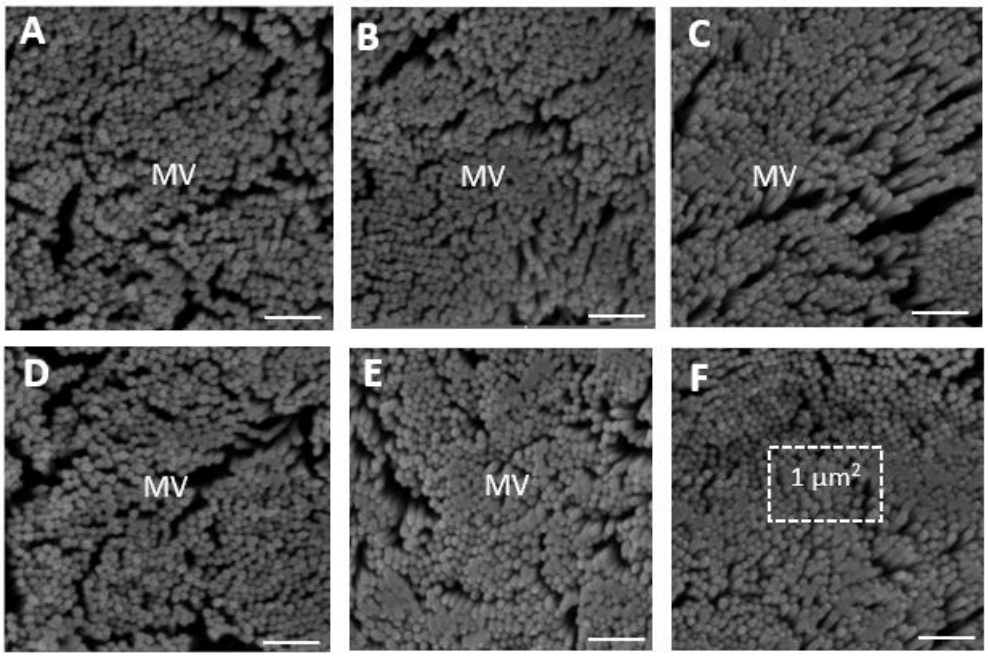

Figure 6.4 Scanning electron micrographs (SEM) from the distal intestine of barramundi at the end of the feeding trial. SEM images for microvilli density; A: Control, B: FMBD+TH, C: LPBM+TH, D: HPBM+TH, E: BPBM+TH and F: measurement of microvilli density in each square micrometer from the fish under different dietary groups. MV: microvilli. Scale bar=0.5 µm.

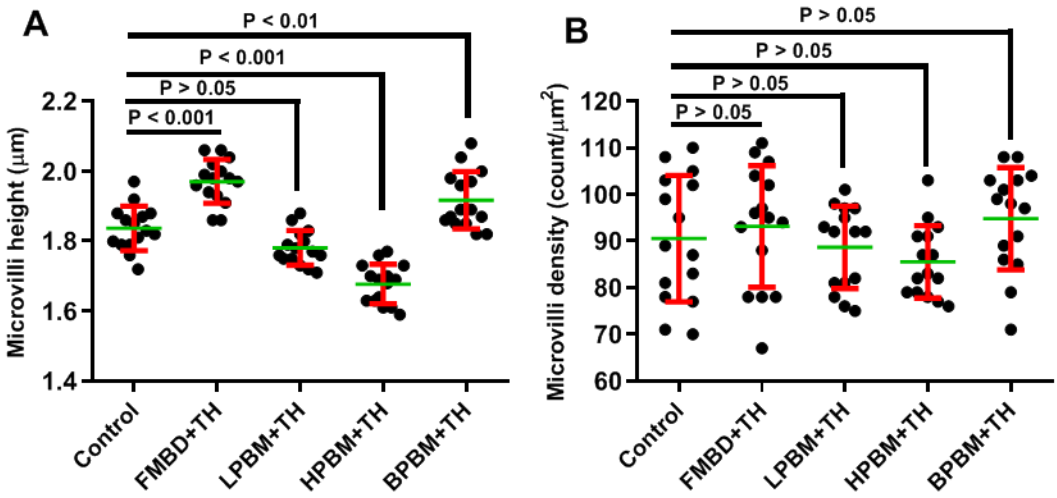

Figure 6.5 Intestinal microvilli height (A) and density (B) of barramundi fed with different experimental diets supplemented with TH. Data were expressed as mean ± SE, n = 15. Bar holding P-values denote significant level among the experimental treatments (one-way ANOVA; Tukey post-hoc test; not significant P>0.05; significant P<0.05; P<0.001).

6.3.8 Resistance to infection

The survival of *V. harveyi* challenged barramundi was significantly higher in fish fed all TH treated groups (FMBD+TH, LPBM+TH, BPBM+TH) when compared to the control ($\chi^2_{FMBD+TH}$ = 14.34, df =1, P<0.001, $\chi^2_{LPBM+TH}$ = 9.25, df =1, P<0.01 and $\chi^2_{BPBM+TH}$ = 22.72, df =1, P<0.001). However, the HPBM+TH dietary group exhibited no significant difference in survival compared to the control ($\chi^2_{HPBM+TH}$ = 3.63, df =1, P>0.05). After 14 days, the BPBM+TH had the highest cumulative survival percent with 90.3%, followed by 80.6%, 67.6%, 50.0% and 33.3% for the FMBD+TH, LPBM+TH, HPBM+TH and control, respectively (Figure 6).

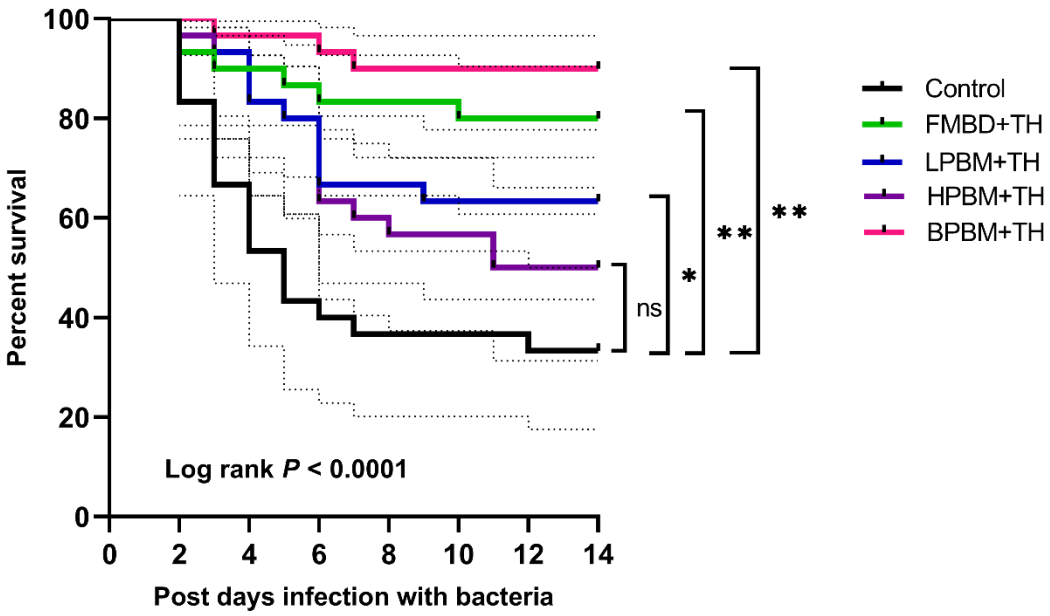

Figure 6.6 Survival curve (based on Kaplan Meier's estimates,) of barramundi after being challenged with *Vibrio harveyi*. The mortality (moribund condition) was recorded daily for 14 days after the bacterial challenge. Thirty individuals per group i.e.10 per replicate were considered for the survival analysis and comparison among groups were made at the same time point after completion of the challenge trial. Significantly higher survival was observed in all test diets when compared to the control. Asterisks * and ** indicate statistically significant difference between treated group and infected control at P<0.01 and P<0.001, respectively. ns indicates non-significant. The dotted lines indicate 95% confidence intervals.

6.4 Discussion

FPH has been reported as a promising aqua-feed ingredient for aquaculture, not only to improve fish growth but also to enhance immune status, potentially leading to increased disease resistance (Siddik et al. 2018b). The present study investigated the application of 10% TH in experimental diets, and was prompted by the outcomes of our previous study on the use of FPH in juvenile barramundi diets (Siddik et al. 2018b). The results suggest that supplementation of 10% TH in diets of FMBD+TH and BPBM+TH significantly improve the FBW and SGR of juvenile barramundi. These results are in agreement with past studies in which dietary inclusion of FPH consisting of low molecular weight peptides (<3000 Da) had positive effects on the growth performance of marine carnivorous species, including Atlantic salmon, *Salmo salar* (Refstie et al. 2004), yellow croaker, *Pseudosciaena crocea* (Tang et al. 2008), and olive flounder, *Paralichthys olivaceus* (Khosravi et al. 2017). The enhanced growth performance with FMBD+TH and BPBM+TH may be due to the supplementation of TH, which improved the palatability of the diets leading to feed intake of fish (Bui et al. 2014). Furthermore, the fermented bacteria, *Lactobacillus casei* and yeast could also have stimulated endogenous enzyme activity for digestion (Ray et al. 2012; Ray et al. 2010) and positively affected the gut microbiota through improved feed intake and enhanced nutrient absorption and assimilation (Rimoldi et al. 2018).

The lipid content of the muscle tissues of juvenile barramundi in the present study was influenced by TH inclusion which was in accordance with many previous studies where the proximate composition of the whole-body and/or individual organs or component parts of the fish were affected by the addition of various alternative animal protein sources in FM containing diets (Lee et al. 2012; Lee et al. 2010; Uyan et al. 2006). In particular, Wu et al. (2018) found that crude fat in the whole body and liver of yellow catfish, *Pelteobagrus fulvidraco*, were changed by the low, medium and high inclusions of hydrolysate stickwater at various levels in non-FM diets. Like the present study, protein, energy and ash content of fish were not found significantly different by the dietary inclusion of marine soluble proteins in FM diets (Khosravi et al. 2015b; Oliva-Teles et al. 1999). However, altered whole-body protein contents were reported in fish with the addition of FPH (Bui et al. 2014; Zheng et al. 2012). It is likely a number of factors, including sources of hydrolysates, varying inclusion levels and the

size of fish species, may have impacted the effects of protein hydrolysates on the whole-body composition of fish (Khosravi et al. 2015b; Wu et al. 2018).

Blood indices act as indicators of metabolic function, nutritional status and physiological condition of fish (Ilham et al. 2016b; Siddik et al. 2018a; Siwicki et al. 1994b). Previous studies showed that fish hydrolysate and fermented diets with animal protein blend resulted in a lowering of the blood glucose level in fish (Kader et al. 2010). Immunostimulants used in aquaculture production are capable of influencing the glucose levels of fish by increasing the insulin level (Sahu et al. 2007; Talpur & Ikhwanuddin 2012). The results presented here suggest that TH produced through enzymatic hydrolysis via the addition of several enzymes, such as alcalase, protease, neutrase, trypsin, α-chymotrypsin, pepsin, protamex and flavourzyme (Chalamaiah et al. 2012) may trigger insulin secretion, accordingly reducing the glucose level, which in turn favourably affects the wellbeing of the fish (Talpur & Ikhwanuddin 2012). In post bacterial challenges at 24 h, elevated glucose levels in all experimental groups were detected. This might have been due to the induced stress by pathogenic bacteria as well as reduced feeding from three times a day to once in the challenge condition. Ardiansyah & Fotedar (2016) also found increased blood glucose levels in juvenile barramundi after an ammonia stress challenge.

Cholesterol and triglycerides, important metabolites of blood, are widely used indicators for assessing the nutritional status of fish (Congleton & Wagner 2006; Jia et al. 2018). In the present study, the serum triglyceride levels were significantly reduced in all dietary groups at the pre-challenge condition compared to the control, as well as the cholesterol in the control and the FMBD+TH groups at post-challenge conditions compared to rest of the dietary groups. This result was in accordance with the study of Kader et al. (2010), who found lower triglyceride and cholesterol in fish fed with fish hydrolysate and an animal protein blend. Furthermore, serum triglycerides and cholesterol in fish varied with the feed depravation time and were usually compensated with body reserves during fasting (Takahashi et al. 2011; Zhu et al. 2018). Accordingly, in the current study, it can be hypothesised that serum triglycerides and cholesterol were decreased after the challenge due to the lower feed supply in the challenge condition.

AST and GLDH are normally measured in fish as indicators of hepatocellular injury, to thereby determine liver health status. Higher levels of these enzymes in serum might indicate cell damage in the liver or the detrimental effect of feeding regimes. In the present study, AST and GLDH values were found non-significant among the dietary groups, indicating that the tested feeding patterns do not damage the liver in barramundi. These results agreed with the findings of Khosravi et al. (2015b) who reported the serum AST level was not influenced by different FPH inclusions in fish diets.

The structure of the intestinal mucosal morphology is a good indication of the ability of fish to digest and absorb nutrients in the digestive tract (Tan et al. 2018; Torno et al. 2018). The improvements in intestinal micromorphology of fish, including microvillus height and density, is a positive indication of good intestinal health, which is important to boosting the health status of the mucosal epithelium as well as increasing the ability to prevent opportunistic microbial infection (Dimitroglou et al. 2009). TEM analysis in the present study revealed that microvillus height increased significantly in the distal intestines of the barramundi fed FMBD+TH and BPBM+TH diets. This result was in accord with our previous study in which dietary supplementation of TH in FM-based diets at 10% significantly increased the microvilli height in juvenile barramundi (Siddik et al. 2018b). Similarly, other than in fish, improvements in villus height have been reported in broiler chickens using a low-level (2%) of enzymatically hydrolysed scallop visceral protein (Xing et al. 2018). SEM analysis demonstrated that none of the dietary treatments appeared to affect microvillus density in the distal intestines of fish. Despite this, the improvement of microvilli morphology may lead to improved apical brush border integrity which may prevent harmful bacteria and their toxins from impacting intestinal epithelial cells. This, in turn, may improve the growth performance and disease resistance of fish. The longer microvillus of fish fed diets FMBD+TH and BPBM+TH supplemented with TH was also consistent with the observed feed intake and growth performance, indicating that more nutrients may have been digested and absorbed in the intestine. Therefore, the overall analysis of the results obtained from the TEM and SEM images indicate that TH added to diets can induce a significant increase in the absorptive capacity of the intestinal mucosa, with no evidence of morphological alterations.

It has been reported that antimicrobial peptides such as lysozyme may assist in preventing colonisation of micro-organisms in the host body, thereby resulting in the prevention of pathogens (Chen et al. 2018; Zhang et al. 2018). Serum lysozyme is one of the antimicrobial enzymes produced by immune cells to fight infection (Misra et al. 2006). The bactericidal activity is a mechanism of killing pathogenic microorganisms in fish (Ellis 1999). Previous studies suggested that dietary inclusion of FPH in fish diets may stimulate the innate immune responses, and this stimulant is strongly influenced by the amount of hydrolysate in the diet (Gildberg & Mikkelsen 1998; Kotzamanis et al. 2007).The overall improvement of serum lysozyme and bactericidal activities in FMBD+TH and BPBM+TH are an indication of enhanced immune response with these diets in fish when compared to the control. These results are comparable with the increased serum lysozyme activity noted in large yellow croaker fed with FPH with the inclusion of 10 and 15% in a floating case system (Tang et al. 2008). In addition, Japanese sea bass fed with a 15% FPH diet had significantly improved lysozyme and complement activities after feeding for 60 days (Liang et al. 2006).

In the present study, the cumulative mortality of barramundi after the 14-day challenge with *V. harveyi* was significantly lower in all experimental diets when compared with the control and the HPBM+TH (Figure 6.5). This may indicate that 10% TH supplementation in FM (FMBD+TH), 75% PBM (LPBM+TH) and 90% bioprocessed PBM (BPBM+TH) diets could protect fish from the impact of bacterial infection. Similarly, dietary administration of FPH increased the disease resistance of various fish, such as red sea bream, *Pagrus major*, and juvenile olive flounder, *Paralichthys olivaceus*, against *Edwardsiella tarda* (Bui et al. 2014) and European sea bass larvae, *Dicentrarchus labrax*, to *Vibrio anguillarum* (Kotzamanis et al. 2007). In one of our previous studies, it was demonstrated that TH is able to improve survival rates in barramundi against *Streptococcus iniae* infection (Siddik et al. 2018b). A number of studies in the past have reported that enzymatic FPH with low molecular weight peptides (<3000Da) have a number of bioactive properties with the potential to influence antioxidative, antimicrobial and anti-inflammatory activity, which may enhance the disease resistance of fish (Zamora-Sillero et al. 2017). The reduction of infection in the TH supplemented groups of FMBD+TH, LPBM+TH and BPBM+TH

in our present study could be explained either by an improvement in the overall fish health or by the stimulation of the immune system.

6.5 Conclusion

In conclusion, the present study revealed that the dietary supplementation of TH in FM (FMBD+TH) and bioprocessed PBM (BPBM+TH) diets in barramundi improved the growth performance and feed intake whilst significantly modulating the gut morphology, lysozyme activity, and disease resistance against *V. harvei*. Although growth performance, biochemical response and disease resistance of barramundi were not significantly improved by the HPBM+TH diet, this diet provided equal performance when compared to the FM based control diet. Therefore, the application of TH in non-FM based diets may present a novel strategy for enhancing growth performance as well as assisting in fish health management in barramundi aquaculture.

Chapter 7: Tuna hydrolysate supplemented fermented poultry by-product meal influences gut microbiota and expression of immune-related genes in juvenile barramundi, *Lates calcarifer* (Bloch)

Abstract

A 10-week feeding trial was conducted to investigate the supplemental effects of tuna hydrolysate (TH) in dietary fishmeal (FM) and poultry by-product meal (PBM) diets on gut microbiota composition and expression of immune-responsive genes in the distal gut of juvenile barramundi, *Lates calcarifer*. Fish were fed with four isonitrogenous and isoenergetic diets: Control (a FM based diet without TH supplementation), FMBD+TH (a FM based diet with 5%TH supplementation), and FPBM+TH and PBM+TH (fermented and non-fermented PBM diet with 10%TH supplementation). The results of the trial revealed that FPBM+TH significantly influenced the gut microbiota composition and regulated cytokine gene expression in juvenile barramundi. The 16s sRNA analysis using V3-V4 region evidenced the ability of FPBM+TH to modulate the distal intestinal gut microbiome, augmenting the richness of Firmicutes and Fusobacteria phyla. Altogether 19024 operational taxonomic units (OTUs) ranging from 3504 to 6316 were identified in which Proteobacteria, Firmicutes, and Tenericutes were the most predominant phyla. The result of the RT-qPCR revealed that FPBM+TH significantly up-regulated the expression level of interleukin1β (IL-1β), interleukin 8 (IL-8), interleukin 10 (IL-10) and interleukin 17F (IL-17F) cytokine genes in the distal gut. The aforementioned results revealed that fermentation and TH supplementation concurrently enhance gut immunity of juvenile barramundi by modulating microbial populations and up-regulating immune-related cytokines.

Keywords: Fishmeal, fermented PBM, tuna hydrolysate, gut microbiota, gene expression, *Lates calcarifer*.

7.1 Introduction

Gut microbes in fish play an important role in stimulating epithelial proliferation, immune response, nutrient assimilation and overall physiological development (Huyben et al. 2017; Semova et al. 2012) and also assist in preventing pathogen or other foreign factors from colonization (Ringø et al. 2016). Hence, understanding interactions between fish health/immune response and gut microbiota is an important research area due to the aligned importance for fish health and aquaculture. It has been demonstrated that protein sources, dietary lipid, chitin and cellulose, probiotics, prebiotics, synbiotics, immunostimulants (Ringø et al. 2016) and fermentation (Catalan et al. 2018) modulate the intestinal microbiome of fish. Although the role of fermentation on the gut microbiota of fish is still unclear and not well-understood, Catalan et al. (2018) found that fermented soybean meal boosted the growth and physiology of Atlantic Salmon *Salmo salar* by augmenting the number of lactic acid bacteria. If the intestinal microbial compositions and its assembly is well understood, there is the possibility to manipulate the microbial population to promote the host health (Roeselers et al. 2011) by reducing the levels of opportunistic bacteria and stimulating immunological response (Dimitroglou et al. 2011). Thus, with the possibility to provide alternate feed ingredients to FM, more studies are needed pertaining to the application of fermentation in aqua-feed and their subsequent effects on the fish intestinal microbiota.

Utilization of small quantities of supplements (enzymes, herbs, prebiotics, probiotics, and FPH) in aqua-feed is of importance in aquaculture with the potential to enhance the feed quality and hence boost growth performance, gut health, immune response and disease resistance. Among supplements, FPH with good functional properties and bio-active peptides have been evaluated for palatability enhancement and feed attractant (Kolkovski & Tandler 2000b; Refstie et al. 2004), immunostimulation (Liang et al. 2006) and antimicrobial or antioxidant properties (Chalamaiah et al. 2012). The nutritive value of FPH supplements in the diets of Atlantic salmon *Salmo salar* (Kousoulaki et al. 2012), Persian sturgeon *Acipenser persicus* (Ovissipour et al. 2014), Asian seabass *Lates calcarifer* (Chotikachinda et al. 2013), European sea bass *Dicentrarchus labrax* (Delcroix et al. 2015), red sea bream *Pagrus major* (Khosravi et al. 2015b), turbot *Scophthalmus maximus* (Xu et al. 2016) and pike silverside

Chirostoma estor (Ospina-Salazar et al. 2016) have been evaluated. These studies have confirmed that inclusion of FPH at appropriate levels has beneficial impacts on growth performance, digestibility, feed intake, nutrient utilization, oxidative status, immune response and disease resistance of fish. Apart from those positive impacts, FPH plays an important role in modulating the intestinal bacteria of fish (Ringø et al. 2016). Protein hydrolysis releases single amino acids, short peptide and glycopeptide which can improve immune responsive gene expression (Yang et al. 2009a) and control key immune regulatory pathway (Kiron 2012) and can also modulate the intestinal cells condition and activity as well as the residing bacteria by providing suitable substrate for bacterial proliferation (Delcroix et al. 2015).

Although fermentation has been practiced in several studies, there are no published data available regarding the effect of fermented PBP simultaneously supplemented with TH in marine carnivorous fish like barramundi. Hence, the present study was designated to evaluate if the PBM fermentation and TH supplementation in combination with affects the intestinal microbiota and immune-regulated cytokines gene expression in the intestine of juvenile barramundi.

7.2 Materials and methods

7.2.1 Ethic statement

This experiment was conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Australia. The protocol was approved by the Ethics Committee in Animal Experimentation of the Curtin University (Approval number AEC_2015_41). All fish handling procedures were performed using recommended doses of aquatic anaesthetic (Aqui-S®, Lower Hutt, New Zealand Ltd).

7.2.2 Experimental diets

Four experimental diets were formulated providing approximately 47.0% crude protein and 20.0 MJ kg^{-1} gross energy to fit the nutrient and amino acid requirements of juvenile barramundi according to NRC (2011) as shown in Table 7.1. A basal diet without supplementation of TH was served as the control. The other three diets were as follows: full FM based diet supplemented with 10%TH (diet FMBD+TH), 90%

PBM supplemented with 10%TH (diet PBM+TH) and fermented PBM supplemented with 10%TH (diet FPBM+TH). The PBM was fermented following the technique described in our earlier study (Siddik et al. 2018a). All diet ingredients were finely ground and mixed thoroughly and then passed through a laboratory scale mincer to produce 3 mm pellets using standard CARL extrusion protocols. The pellets were dried at 60°C for 36 h and then cooled at room temperature before being bagged and labelled.

Table 7. 1 Formulation and proximate composition of the experimental diets for juvenile barramundi.

Ingredients (g kg^{-1})[1]	Experimental diets			
	CONTROL	FMBD+TH	PBM+TH	FPBM+TH
Fishmeal	610.0	549.0	518.5	488.0
PBM	-	-	549.0	-
FPBM	-	-	-	549.0
Tuna hydrolysate	-	61.0	61.0	61.0
Wheat	266.0	264.0	267.0	265.0
Wheat starch	20.0	20.0	20.0	20.0
Fish Oil	30.0	30.0	30.0	30.0
Calcium carbonate	2.0	2.0	2.0	2.0
Salt (NaCl)	2.0	2.0	2.0	2.0
Vitamin premix	1.0	1.0	1.0	1.0
Casein	63.0	65.0	63.0	64.0
Cellulose	6.0	6.0	6.0	6.0
Nutrient composition (% dry matter)				
Dry matter	92.72	89.36	91.22	91.03
Crude protein	47.17	46.86	47.79	46.85
Crude lipid	9.99	9.40	10.94	9.53
Ash	13.04	12.06	8.70	8.71
NFE[2]	22.48	20.87	23.47	24.70
Gross energy (MJkg^{-1})	19.98	19.92	19.90	19.91

[1]Supplied by Specialty Feeds, Perth, Australia. [2]Nitrogen free extracts (NFE) = dry matter - (crude lipid + crude ash + crude protein). PBM contains 67% protein, 13.5% lipid and 14.0% ash. FPBM consists 65% protein, 10.7% lipid and 14.1% ash and TH contains 58.4% protein, 1.05% lipid and 11.3% ash.

7.2.3 Experimental setup

The feeding trial was conducted at Curtin Aquatic Research Laboratory (CARL) in the facilities of the Curtin University, Australia. A total of 240 juvenile barramundi were acclimated to experimental conditions for 2 weeks and then fish with an initial pool weight of 12.63 ± 0.41 g were randomly distributed into twelve independent tanks (300-L water capacity) at a stocking density of 20 fish per tank. Fish tanks were randomly assigned having three tanks to each of the experimental diets to allow the triplicate measurements. All experimental tanks in the rearing facility were equipped with constant aeration, water heater and external bio-filter (Fluval 406, Hagen, Italy) to maintain water recirculation at a rate of 10 L min^{-1} throughout the experimental period. The light regime was set at 14-h light/10-h dark cycle using an automatic indoor light switch (Clipsal, Australia). During the whole experimental period of 10-weeks, fish were hand-fed up to apparent satiation three times a day at 0800, 1200 and 1700 h. Tanks were cleaned daily after the last feeding of the day by siphoning out uneaten feed and faecal matter.

7.2.4 Gene expression analysis

At the termination of the 10 week feeding trial, the mRNA expression of innate immune-related genes encoding interleukin1β (IL-1β), interleukin 8 (IL-8), interleukin 10 (IL-10) and interleukin 17F (IL-17F) in the distal gut collected from six fish per treatment (two fish/replicate) were analysed with RT-qPCR (Table 7.2). Following collection, these tissues were stored in liquid nitrogen and then transferred to -80°C until use. Total RNA from 5 mg of each sample was isolated via homogenization in a polytron homogenizer (Kinematica) using the protocol of RNeasy Mini Kit (Qiagen, Hilden, Germany). The quality of the extracted RNA was checked using 1% agarose gel electrophoresis and the quantity was detected by a nano-drop spectrophotometer measuring at 2000c (Thermo Fisher Scientific, USA). 2 µg of total RNA as template was used to synthesize cDNA for RT-qPCR using the TransScript cDNA Synthesis SuperMix (TransGen Biotech, AT301, Beijing, China) according to manufacturer's protocols.

Real-time qPCR for IL-1β, IL-8, IL-10, IL-17F, and β-actin was carried out using cDNA samples of the distal intestine in 7500 Real-Time PCR System (Applied Biosystems, USA) employing PowerUp™ Cyber Green Master Mix (Thermo

Scientific, USA) following the manufacturer's protocols after standardization. The total volume of PCR reaction was 20µl consisting of 10µl TransStart Top Green qPCR SuperMix (2×), 0.6µl of each primer, 1µl cDNA, and 7.8µl RNase-free H_2O, 4µM of each forward and reverse gene-specific primer (Table 2). Thermal cycling parameter included 95°C for 3 min and then 40 cycles of 95°C for 30s, 58°C for 20s, and 72°C for 20s followed by 40 cycles of denaturation at 95°C for 15s, annealing at 60°C for 20s and extension at 72°C for 30s. geNorm (v3.5) was used to normalize the quantitative real-time PCR data and the expression levels of IL-1β, IL-8, IL-10, IL-17F were calculated using $2^{-\Delta\Delta CT}$ method.

Table 7. 2 List of gene primers used for qRT-PCR assay

Primer name	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	Accession number (AC)
IL-1β	5′-ATCTGGAGGTGGTGGACAAA-3′	5′-AGGGTGCTGATGTTCAAACC-3′	AM490063.1
IL-8	5′-GTCTGAGAAGCCTGGGAGTG-3′	5′-GCAATGGGAGTTAGCAGGAA-3′	AM490063.1
IL-10	5′-CGACCAGCTCAAGAGTGATG-3′	5′-AGAGGCTGCATGGTTTCTGT-3′	AM268529.1
IL-17F	GTCTCTGTCACCGTGGAC	TGGGCCTCACACAGGTACA	Awaiting for AC
β-actin	TTGAGCAGGAGATGGGAACCG	AGAGCCTCAGGGCAACGGAAA	AB039726

7.2.5 DNA extraction, 16S amplification and high-throughput sequencing

Around 200 mg of the pellet from the pooled homogenized distal intestine (each pool was made of two samples from the same tank) content was taken into a 1.5-mL Eppendorf tube for DNA extraction (Dahlhausen et al. 2018). Total bacterial DNA was extracted from the distal intestine sample using commercial DNeasy Blood and Tissue Kit (Qiagen, Crawley, UK) following the manufacturer's instructions. Nano-Drop spectrophotometer 2000c (Thermo Fisher Scientific, USA) was used to measure the concentration of DNA. To make the final concentration to 30 ng/µL, the extracted DNA was subsequently diluted with nuclease-free water. V4-V5 hypervariable regions of bacteria were used to perform PCR amplification and the master mixture consisting of 50 µL reaction contained 25 µL of Hot Start 2X Master Mix (BioLab Inc., Australia), 2µL of template DNA, 1µL of each forward and reverse primers, and 21 µL of nuclease-free water was prepared. After several trials, the optimized conditions are as follows: initial denaturation temperature of 95°C for 5 min followed by 29 cycles of 95°C for 30s, 55°C 40s, 72°C for 30s, and the final extension at 72°C for 10 min. Then, BioRad S100 Gradient Thermal Cycler (Bio-Rad Laboratories, Inc., Foster City,

California, USA) was used for 30 cycles of amplification reactions. To separate the amplified products in 1% agarose gel and pictured under gel doc (FujiFilm LAS-4000 Image Analyzer, Japan), a horizontal gel electrophoresis system (Bio-Rad Laboratories Inc., USA) was used. Purification of PCR products was performed using bead methods and sequencing libraries were prepared in accordance with the Illumina standard protocol (Gajardo et al., 2016). The pooled samples were then sequenced up to 20000 reads on an Illumina MiSeq apparatus using a v3 kit (600 cycles).

7.2.6 Sequence and taxonomic data analysis

The resulting raw sequence data from Illumina MiSeq were analysed by MICrobial Community Analysis (MICCA) (v1.6.1) workflow (Albanese et al. 2015) and trimmed by sickle program and FASTQ file to remove low quality and polyclonal sequences (Joshi & Fass 2011). MICCA megapairs (micca mergepairs -i fastq/*_R1*.fastq -o merged.fastq) were used to merge overlapping pair end sequences to get a single consensus FASTQ file. MICCA merge (micca merge -i *.fasta -o merged.fasta -f fasta) was used to assemble the two FASTA sequence. Sequences were analysed in MICCA (v1.6.1) to pick operational taxonomic units (OTUs), which was identified at 97% similarity from the SILVA database for the identification of the microbial community in the sample (Quast et al. 2013). MICCA classify (micca classify -i otus.fasta -o taxa.txt) was used to assigne the respective taxonomy for each of the bacterial genera. OTUs picking for each phylum of bacteria was perfomed from the taxtable2 file and the genus level information of the most abundant bacteria (<1%) was picked from the taxtable6.

7.2.7 Statistical analyses

Normality of all obtained data were tested by Kolomogorow-Smirnov test, using IBM SPSS statistic *ver*. 25. Statistical differences in expression levels of immune-related genes among tested diets was determined by one-way ANOVA with Turkey multiple range test at $0.05 < P < 0.001$ significance level. The correlation between genes and expression pattern was visualised in a heatmap using the package “gplots” in R studio.

7.3 Results

7.3.1 Gene expression

In this study, the mRNA expression level of 4 selected immune-regulatory genes IL-1β, IL-8, IL-10 and IL-17F in the distal gut of fish fed FMBM+TH and PBM+TH were quantified by qPCR and the results were analysed based on two approaches: 1. heatmap hierarchical cluster analysis (Figure 7.1); and 2. Column graph by estimating mean of triplicate values of expression (Figure 7.2). The hierarchical cluster analysis of heatmap reveals that IL-1β and IL-8 represent the first group, while IL-10 and IL-17F represent the second group with a similar expression and clustering. As Figure 7.2, the mRNA expression of all genes revealed a significant up-regulation in the fish feeding on FPBM+TH diet when compared to fish feeding on the control and FMBD+TH and PBM+TH diets after a 10 week of feeding trial. The expression of genes encoding IL-1β, IL-10 and IL-17F were significantly increased in fish fed FPBM+TH when compared to PBM+TH treatment, except for the expression of IL-8, which did not reveal significant variation when compared to PBM +TH.

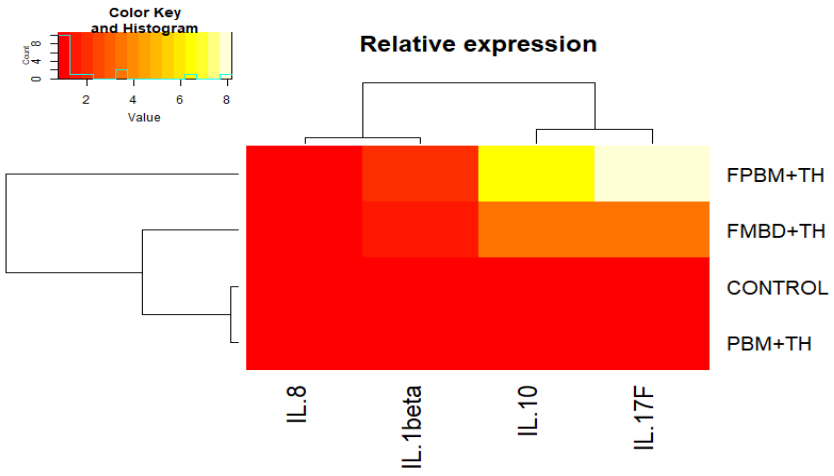

Figure 7.1 Heatmap plot shows the relative gene expression pattern of the distal intestine in juvenile barramundi in response to four different experimental diets. The colour intensity reveals the relative values for gene expression with legend indicated at the top and left sides of the heatmap, light yellow color reveals the high expression levels while red color reveals the low expression levels. Expression values of target genes were expressed relative to β-actin expression.

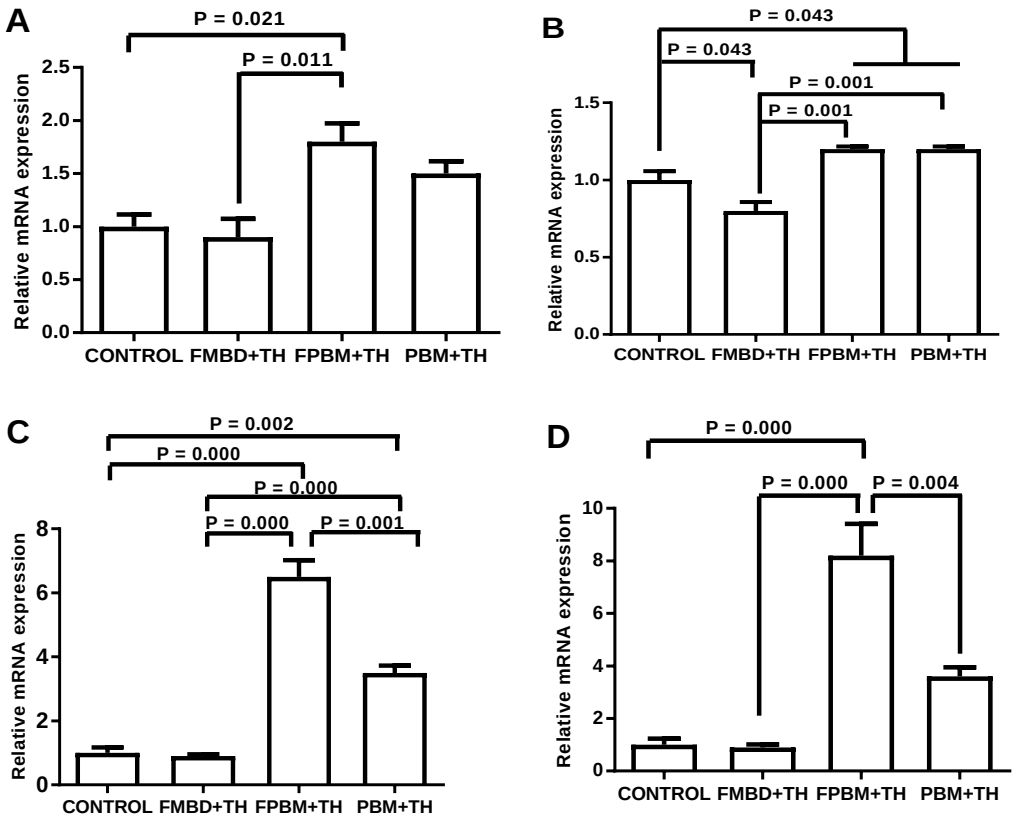

Figure 7.2 Expression levels of immune-related interleukin genes encoding IL-1β (A), IL-8 (B), IL-10 (C) and IL-17F (D) quantified by RT-qPCR in distal gut of barramundi fed four experimental diets following 1 10-week of feeding trial. The bars denote the mean of three replicates per group with error bars representing standard error of the mean.

7.3.2 Intestinal microbiota

The high-throughput sequencing analysis of bacterial 16S rRNA V3-V4 region generated from the barramundi intestine (performed on 4 samples, each sample made of a pool of 3 fish) yielded 49,072 sequence reads representing 19024 OTUs. Figure 7.3 shows the most bacterial abundant phyla in the intestine of fish fed either the control or experimental diets. A total of 19024 OTUs ranging from 3504 to 6316 were observed in the present study. The most prevalent bacteria associated with the intestine of barramundi were assigned to Proteobacteria (3451) and Firmicutes (3361). Firmicutes were the most representative phylum in the fish fed FPBM+TH, whereas Proteobacteria were abundant in the FMBD+TH treated groups. Control fish showed

the highest abundance of Bacteriodetes and Tenericutes were the dominant phylum in the PBM+TH treated groups. The core microbiota at genus level was calculated on the basis of the presence of respective OTUs using venn diagram. Altogether 4 OTUs were the commonly shared gut microbiota among the four treatments while the unique OTUs number for Control, FMBD+TH, FPBM+TH and PBM+TH groups were 3, 113, 54 and 6, respectively (Figure 7.3).

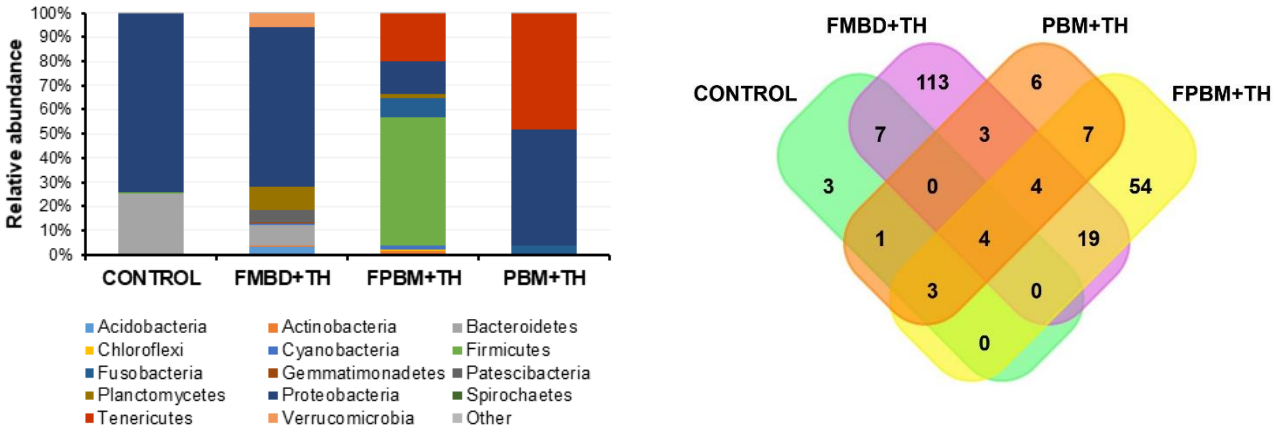

Figure 7.3 Comparison of the relative abundance of gut microbiota composition of barramundi after 10 weeks of feeding trial. Venn diagrams representing the common and specific bacterial phylum (accounting for >0.01%) identified in the distal intestine of fish fed control and other test diets.

7.4 Discussion

Cytokines are defined as small glycoprotein messengers that form a group of proteins that assist in intercellular communication to aid innate and adaptive immune response and host defense mechanism against pathogens such as bacteria, virus, and parasites (Bruce & Brown 2017). Families of cytokines include interleukins (ILs), interferons (IFN), lymphokines, tumor necrosis factor (TNF), chemokines and transforming growth factors (TGF) (Secombes et al. 2009). IL-1β, an important immune-related pro-inflammatory cytokine in fish and was been shown to enhance lypmphocyte activation, migration of leucocytes, macrophage proliferation, phagocytosis, chemotaxis, bactericidal activities and lysozyme synthesis (Giri et al. 2015; Goetz et al. 2004) and can also regulate production of other cytokines (Wang et al. 2015; Zou et al. 2003) and immune-related genes (Buonocore et al. 2005; Peddie et al. 2001). IL-8 is another

important pro-inflammatory cytokine which contributes to migrate chemotaxis in target cells to the infected site in mammals and host defence mechanism in regards to invasion or bacterial colonization (Kim & Austin 2006). IL-17F is also an essential pro-inflammatory cytokine enhancing the host defence mechanism in response to bacteria and fungi. The results of qRT-PCR demonstrated that dietary inclusion of FPBM+TH significantly upregulated the IL-1β expression in the intestine of barramundi. A similar trend was observed for other pro-inflammatory cytokines encoding IL-8 and IL-17F, pointing to an elevation of pro-inflammation cytokines in the intestine of juvenile barramundi. To date, there have been no published studies pertaining to the influence of fermented PBM supplemented with TH on fish intestinal immune relevant gene expression. However, immune-related genes encoding lysozyme and superoxide dismutase were significantly elevated in Pacific white shrimp, *Litopenaeus vannamei* fed 5% and 15% fermented duckweed (Flores-Miranda et al. 2014). Another study has demonstrated that dietary administration of 10 g kg^{-1} fermentable fibre induced immune-related expression (TNFα and lysozyme) in rainbow trout (Yarahmadi et al. 2014). Irrespective of fermentation, FPH has been considered in several fish species as a strong antioxidant (Khosravi et al. 2015b) and immunostimulant (Bui et al. 2014; Liang et al. 2006) which could stimulate gene expression. (Duarte et al. 2006) reports that dietary administration of FPH significantly up-regulated the IL-4, IL-6 and IL-10 gene expression as well as some pro-inflamattory cytokines encoding IFNγ and TNFα in mice. Similarly, up-regulation of some immune-relevant genes such as IL-2, IFN-c, IL-5 and IL-6 was observed in mice when fed with chum salmon hydrolysate (Yang et al. 2009b). The elevation of IL-1β may induce the secretion of mucus and activation of macrophage as well as up-regulating a number of NF-kB-related genes (Lindenstrøm et al. 2004). Thus, it is postulated that upregulation of IL-1β along with other pro-inflammatory cytokines such as IL-8 and IL-17F in barramundi reveals the stimulation of early inflammatory response and fermentation might have stimulated the immune responsive genes in this experiment.

IL-10 produced by a variety of cells (T cells, B cells, monocytes, dendritic cells, macrophages, and natural killer) is an anti-inflammatory cytokine which inhibits and regulates pro-inflammatory cytokines and endogenous anti-cytokines, and also plays a pivotal role in suppressing antigen presentation and preventing inflammatory

pathologies (Iyer & Cheng 2012). Deficiency of IL-10 has been hypothesized to support the colonisation of some particular microorganisms that cause inflammatory bowel disease in the gut of mice (Saraiva & O'Garra 2010). In the present study, feeding with FPBM+TH significantly upregulated the IL-10 expression in the intestine, indicating that fermentation has anti-inflammatory signalling effects. To our knowledge, the present study represents the first evidence of IL-10 up-regulation in the intestine of barramundi after the feeding of fermented products. However, upregulation of IL-10 was found in the head kidney and gut of European sea bass, *Dicentrarchus labrax* L. treated with lipopolysaccharide (Buonocore et al. 2007) and in the kidney of carp, *Cyprinus carpio* fed diet with spirulina, *Spirulina plantensis* (Watanuki et al. 2006). This expression between fish species seems to be pretty variable, as Zou et al. (2003) reported very low levels of constitutive expression in all tissue sampled of puffer fish, *Fugu rubripes*, whilst Inoue et al. (2005) observed a high level of expression in the gills of rainbow trout, *Oncorhynchus mykiss*. Further research should be conducted to answer how fermentation modulates the pro-inflammatory and anti-inflammatory cytokines through signalling pathway.

Fish generally harbour a diverse group of microbial communities, consisting of bacteria, virus, fungi, protista, yeast, and protoctista (Merrifield & Rodiles 2015) however the dominant group of microbiota in the fish intestine is bacteria (Egerton et al. 2018; Rombout et al. 2011). It is well established that modulation of diet significantly influences the fish intestinal microbiota (Catalan et al. 2018). The most dominant phyla harboured by fish are Proteobacteria, Firmicutes, and Actinobacteria, though gut microbial composition seems to vary among fish species. In addition, different studies conducted on microbiota thus far comprise 90% of Proteobacteria in addition to Firmicutes and Bacteroidetes in the intestine of fish (Ghanbari et al. 2015). In the present study, 3 phyla including Proteobacteria, Firmicutes and Bacteroidetes were the most representative phyla in barramundi fed control and other experimental diets. To our knowledge, there are no studies conducted to date regarding the concurrent effects of fermented animal by-products and TH supplementation on the intestinal microbiota of fish. However, these results are in agreement with the findings of Xia et al. (2014) who found the dominance of Proteobacteria and Firmicutes in Asian seabass, *Lates calcarifer* reared in freshwater. Similarly, Sullam et al. (2012) applied a recent meta-analysis and reported 5 major phyla namely Proteobacteria,

Firmicutes, Fusobacteria, Actinobacteria, and Bacteroidetes in the gut of marine carnivorous fish. Although different genera belonging to the Firmicutes phylum is a normal constitutional part of fish (Roeselers et al. 2011), Firmicutes in the present study were further enriched in the intestine of fermented PBM treated fish when compared with the other test diets. Firmicutes, of which many strains are potent probiotics due to their beneficial effects with a few minor exception, including assisting in the digestion process of fish by releasing different kinds of enzymes (Smriga et al. 2010), adjusting the intestinal environment, maintaining intestinal barrier function, preventing gastro intestinal tract (GIT) associated disease by inhibiting the adhesion of fish as well as regulating intestinal mucosal immunity. At the genus level, *Lactococcus* and *Bacillus* were the unique groups in the intestine of fermented treated groups. *Bacillus* belonging to the phylum Firmicutes is considered probiotic microorganism which can exert a range of beneficial effects on aquaculture systems including the modulation of gene expression in the intestinal epithelial cells (Ma et al. 2017), improvement of food absorption by accelerating the protease level (Irianto & Austin 2002) and enhancement of immunity of animals against pathogenic bacteria (Zhang et al. 2010). Similarly, most of the *Lactococcus* species are generally recognized as safe microorganisms exerting beneficial effects on fish development and health (Avella et al. 2012; Sequeiros et al. 2015) as well as protecting against pathogens by producing antimicrobial compounds (Balcazar et al. 2007). The results presented here are in agreement with Catalan et al. (2018) who stated that fish fed fermented soybean meal had higher levels of Firmicutes phylum, namely under the genera of lactic acid bacteria such as *Lactobacillus, Lactococcus, and Pediococcus* in comparison with fish fed either non-fermented or FM-based diet. Similarly to Firmicutes, the relative abundance of Fusobacteria was enriched in FPBM+TH treated groups but barely found in the intestine of control and FMBM+TH treated groups. Gram-negative anaerobic bacilli belonging to phylum Fusobacteria can synthesize vitamins (Navarrete et al. 2012; Roeselers et al. 2011), and produce butyric acid which acts as a main substrate for intestinal microbial respiration and also shows anti-inflammatory and immunomodulatory properties (Rimoldi et al. 2016; Terova et al. 2016; Udayangani et al. 2017). The genus *Cetobacterium* belonging to Fusobacteria was only found in the FPBM+TH treated group. This genus was previously isolated from fish intestine (Li et al. 2015) reported contribute to fermenting peptides and

carbohydrate to produce vitamine B12 (Finegold et al. 2003). Hence, it could be conclude that upregulation of cytokines related to immune response might be influenced by the modulation of intestinal bacteria in barramundi.

7.5 Conclusion

In summary, this study represents the first reporting of concurrent effects of fermentation and TH supplementation in PBM on immune related cytokines and intestinal microbiota in juvenile barramundi. The results from the present study suggested that the dietary supplementation of TH in fermented PBM up-regulated the expression of immune-related interleukin genes of IL-1β, IL-8, IL-10 and IL-17F. Notably, TH supplemented fermented PBM greatly modified the composition of the intestinal microbial community with an enrichment of the Firmicutes and Fusobacteria in the distal intestine of juvenile barramundi. These positive results merit further study focusing on how fermentation and TH supplementation influence the immune related cytokines and intestinal microbiota and the mode of action of fermentation and supplementation on gut microbiota and immune response.

CHAPTER 8: General discussion, conclusions, recommendations and limitations

8.1 General discussion

Aquaculture production largely relies on quality and availability of aqua-feeds in which FM is still considered the important protein source (FAO 2018). As a result, every year approximately 20 million tonnes of wild harvest fisheries catch is used in the production of aqua-feeds (FAO 2018). This indiscriminate use of marine pelagic fisheries which has ecological and environmental consequences has resulted in concerns being raised over the sustainability of the aquaculture industry. Environmental concerns on its usage as well as the broadening gap between demand and supply of FM has resulted in extensive investigations focusing on identifying viable aqua-feed alternatives to FM.

Fish processing by-products could be suitable FM alternatives that not only are environmentally acceptable, but are less-expensive and locally available. In recent years, a variety of low value fish by-products such as skin, fins, frames, heads, viscera, trimmings and roe converted to FPH have received significant consideration in aqua-diets as FM protein replacements (Kim et al. 2014; Ospina-Salazar et al. 2016). For instance, tuna muscle by-product hydrolysate has been used successfully to replace FM at 50% without suppressing growth performance and feed utilization in juvenile Japanese flounder, *Paralichthys olivaceus* (Uyan et al. 2006). Also, FPH has been used at lower levels as a supplement to improve immunity and subsequently the disease resistance of fish (Bui et al. 2014). In red sea bream, *Pagrus major* fed different hydrolysate diets (krill, shrimp and tilapia hydrolysate at 3.12%, 2.88% and 3.34%, respectively) exhibited significant improvement in disease resistance against *Edwardsiella tarda* (Khosravi et al. 2015b). Similarly, Bui et al. (2014) found significant improvement in the survival rate of juvenile red sea bream, *Pagrus major* fed tilapia and krill hydrolysate during a challenge trial with *Edwardsiella tarda*. A number of studies have confirmed that FPH consists of short chain peptides and well balanced amino acid profiles which are easily digested and absorbed by animals (Carvalho et al. 2004). Such fish derived peptides have a myriad of bioactive potential including antihypertensive, antioxidative, antimicrobial and anti-inflamatory activity depending on the molecular weights of the peptides (Ishak & Sarbon 2017; Zamora-Sillero et al. 2017). FPH also have excellent physicochemical properties including solubility, emulsifying properties, foaming properties, water holding capacity and fat

binding capacity, which, in turn increase feed palatability and simplifies the biological nutrient uptake (Bhaskar et al. 2007; Kasumyan & Døving 2003; Nilsang et al. 2005).

As an alternative protein source for aquatic feeds, PBM may also be a good option because of its high protein content (Sealey et al. 2011) . However, this product may have some limitations including variable amino acid composition and indigestible particles (Simon et al. 2019). Furthermore, the nutrients may not be in an available form for efficient utilisation by fish (New & Wijkstrom 2002). Bioprocessing or advanced fermentation by microorganisms as a cost effective and environmental acceptable biotechnological technique has been applied by many researchers to overcome the inherent problems of ingredients and make them more suitable for inclusion in aqua-feeds (Vo et al. 2015). Microbial fermentation may significantly improve the palatability, protein contents and bioavailability of minerals in feeds (Koh et al. 2002) and may increase levels of beneficial bacteria and animal digestive enzymes which may help in balancing the intestinal flora and hence better digestion of nutrients (Shi et al. 2015; Shi et al. 2017). It was hypothesized therefore that bioprocessing PBM may assist in improving PBM as an aqua-feed ingredient.

In the present study, a series of laboratory based studies were conducted to evaluate the efficacy of processed animal protein diets to replace FM in juvenile barramundi. Through the first two experiments, TH level was optimised in a FM diet for the highest growth performance and immune response. The third experiment was conducted on PBM with and without bioprocessing to understand whether growth performance of juvenile barramundi would match the outcomes of the previously optimised TH diets in FM. The final experiment was conducted using a combination of TH and processed and unprocessed PBM to maximise growth, biochemical response, gut micromorphology and microbiota and immune related gene expression in juvenile barramundi.

8.1.1 Higher replacement (50-70%) of FM by TH and FTH in juvenile barramundi

Juvenile barramundi fed with higher replacement of FM (50 and 75%) with TH and FTH diets resulted in a decreased growth performance, feed intake and digestibility than the FM control (Table 8.1). The possible causes of reduced growth performance may be due to an excessive number of short chain peptides and free amino acids in these hydrolysed products (Ospina-Salazar et al. 2016), which could cause saturation of the peptide transport mechanism (Carvalho et al. 2004). Furthermore, the higher amount of free amino acids could alter the absorption of amino acids leading to amino acid imbalances in the fish gut (Kolkovski & Tandler 2000b). The significant reduction of feed intake in fish fed TH and FTH included diets compared to the control may also be related to decreased palatability or increased bitterness of diets. In this study, the decreased digestibility of protein, lipid and dry matter with increasing levels of TH and FTH may be due to the availability of excess amount of free amino acids and free nucleotides which may disturb the normal digestion and metabolic process resulting in poor digestibility (Zheng et al. 2013b). This result is in agreement with the study of Ospina-Salazar et al. (2016), in which it was reported that more than 30% FM replacement by FPH (CPSP Special-G^{TM}) resulted in significant reduction of digestibility of dry matter and lipid in juvenile pike silverside, *Chirostoma estor*.

The body composition of juvenile barramundi was not influenced by any of the experimental diets (Table 8.1). Similarly, Khosravi et al. (2015b) and Oliva-Teles et al. (1999) found no differences in whole-body proximate composition of sea bream, *Pagrus major* and turbot, *Scophthalmus maximus*, respectively fed diets containing FPH at different inclusion levels. A number of studies reported that the body composition of barramundi is likely to be influenced by total dietary protein levels and the size of fish rather than the source of protein in diets (Catacutan & Coloso 1995b; Vo et al. 2015). The FM protein replaced by TH and FTH at 50 and 75% did not improve the health and antioxidant capacity of the juvenile barramundi as indicated by biochemical responses of blood and GPx activity, respectively. Furthermore, fish fed 75% TH and FTH diets revealed some nutritional deficiency symptoms including lipid accumulation and necrotic loci in hepatocytes which may be the possible reason for the reported lower growth and feed utilization of juvenile barramundi. This experiment

clearly demonstrated that juvenile barramundi fed with 50 to 75% FM replacement diets with TH and FTH declined growth performance, feed intake and digestibility. Furthermore, these inclusion levels did not improve the health and antioxidant capacity of fish and experimental animals showed abnormal signs of liver histopathology. This study also proved that fermentation of TH diets has no role in increasing overall fish performance. Therefore, further study was required to optimise TH inclusion level for improved growth performance and biochemical responses of juvenile barramundi.

Table 8.1 Impact on growth performance, digestibility, whole body composition and biochemical indices of juvenile barramundi fed TH and FTH diets for 56 days when compared to a FM based control diet.

Parameters investigated	Level of FM replaced by TH			
	TH50	TH75	FTH50	FTH75
Growth indices				
FBW (g)	↓	↓	↓	↓
SGR (% day^{-1})	↓	↓	↓	↓
FI (g $fish^{-1}day^{-1}$)	↓	↓	↓	↓
FCR	↔	↓	↔	↓
ADC (%)				
Crude protein	↓	↓	↓	↓
Crude lipid	↓	↓	↓	↓
Body proximate composition				
Protein (% WW)	↔	↔	↔	↔
Lipid (% WW)	↔	↔	↔	↔
GE (MJ kg^{-1})	↔	↔	↔	↔
Biochemical indices				
GPx (U g^{-1} Hb)	↓	↓	↓	↔

Note: Increase, ↑; decrease, ↓; no change, ↔; compared to the control diet ($P<0.05$). TH, tuna hydrolysate; FBG, final body weight; SGR, specific growth rate; FI, feed intake; FCR, feed conversion ratio; ADC, apparent digestibility coefficient; GPx, glutathione peroxidase.

8.1.2 Moderate replacement (5-20%) of FM by TH in juvenile barramundi

Juvenile barramundi fed with moderate (5%, 10%) TH inclusion levels in FM diets improved the growth performance and gut micromorphology when compared to the control (Table 8.2). Further, fish fed diets containing 5% and 10% TH had significantly higher final body weight and specific growth rate than the control. The improved growth performance with 5-10% inclusion levels may be a result of the improved availability and subsequent uptake of free amino acids and suitable peptide fractions produced during the enzymatic process which may be beneficial for the growth performance of fish (Xu et al. 2016).

The measured blood and serum indices were not negatively influenced by TH inclusion at different levels in fish diets (Table 8.2) suggesting that the TH inclusion levels (5-20%) did not cause any negative effect on fish health. AST and GLDH enzymes are measured in fish as the indicators of liver damage of fish (Abdollahi-Arpanahi et al. 2018). In the present study, the lack of a significant increase in AST and GLDH suggested that the liver of experimental barramundi was not affected or functionally damaged with 5-20% TH in their diets.

The histological observation revealed a significant enhancement in goblet cell (GC) numbers in the distal intestine of fish fed 5 to 10% TH in the diet. The increase of GC number in fish fed the TH05 and TH10 diets might suggest an improved innate immune function against invading microorganisms. A number of previous studies have reported that GC numbers are positively correlated with the absorption of digestible substances and higher GC results in higher mucosal membrane protection (Domeneghini et al. 2005; Siddik et al. 2018b). As well, the histological evaluations in terms of hF, hMV and ECS which were increased in fish fed 5 to 10% TH might be due to the greater nutrient absorption and utilization results leading to more surface area for nutrient uptake, and a concomitant enhanced growth performance. Also, fish fed 10% TH exhibited the highest resistance against *Streptococcus iniae* infection during a bacterial challenge trial (Figure 8.4).

These findings demonstrated that the dietary replacement of FM at 5% and 10% with TH resulted in improved growth performance, intestinal health and disease resistance

of juvenile barramundi. The optimised TH level in this study may potentially be used as the basis for the further development of an alternate animal protein based diets for barramundi and other marine carnivorous fish.

Table 8.2 Growth performance, biochemical indices and gut micromorphology of juvenile barramundi fed tuna hydrolysate (TH) included diets at various levels for 56 days

Parameters investigated	Inclusion level of TH in FM based diet			
	TH05	TH10	TH15	TH20
Growth indices				
FBW (g)	↑	↑	↔	↔
SGR (% day^{-1})	↑	↑	↔	↔
FI (g $fish^{-1}day^{-1}$)	↔	↔	↔	↔
Biochemical indices				
AST (uL^{-1})	↔	↔	↔	↔
GLDH (uL^{-1})	↔	↔	↔	↔
Glucose (mmol L^{-1})	↔	↓	↓	↓
Total protein (g L^{-1})	↔	↔	↔	↔
Gut micromorphology				
GC ($fold^{-1}$)	↑	↑	↑	↔
hF (µm)	↔	↑	↔	↓
hMV (µm)	↑	↑	↔	↓
ECS (µm)	↑	↑	↔	↔

Note: Increase, ↑; decrease, ↓; no change, ↔; compared to the control diet ($P<0.05$). TH, tuna hydrolysate; FBG, final body weight; SGR, specific growth rate; FI, feed intake; FCR, AST, aspartate transaminase; GLDH, glutamate dehydrogenase; GC, Goblet cell; Fh, Fold height; hMV, microvillous height; ECS, external circumference of serosa.

8.1.3 Growth performance of juvenile barramundi fed bioprocessed and unprocessed PBM diets

Juvenile barramundi fed 75% FM replacement diets of 75PBM and 75BPBM resulted in the same growth performance when compared to control, whereas 100% supplementation diets of 100PBM and 100BPBM resulted in reduced performance in all growth and feed variables except total feed intake and survival (Table 8.3). The lower growth performance in complete FM replacement diets of 100PBM and 100BPPB may be associated with the deficiency of essential amino acids, and

variability in biochemical composition, with high levels of ash and low digestibility in these diets (Cruz-Suárez et al. 2007; Subhadra et al. 2006). The increased HSI with fish fed 100PBM and 100BPBM may be due to increased lipid deposition in the liver as observed in this study, resulting in hepatic alterations, including hepatic steatosis.

The SEM images revealed the significantly lower microvillus density in fish fed the 100PBM and 100BPBM diets. This may result in sub-optimal digestion and absorption of feed of fish. However, fish fed the 75BPBM had higher microvilli density when compared to the control and the rest of the dietary groups. This could possibly be due to the effect of fermentation on the animal protein in the diet with probiotic bacteria *Lactobaccilus casei,* resulting in a superior beneficial effect than when PBM was used alone. This result is in agreement with Wang et al. (2016) who reported improved gut morphology (intestinal folds, enterocytes, and microvilli) of juvenile turbot, *Scophthalmus maximus* fed soybean meal fermented with *Lactobacillus plantarum.*

The human nutritional quality indices of IA and IT in experimental fish were decreased and HH was increased with 100% bioprocessed PBM diets when compared to unprocessed PBM diets (Figure 8.1). This results may indicate that consumption of fish reared with bioprocessed PBM at different levels, may be beneficial to human health, particularly when related to the lowering the risks of cardiovascular disease (Ulbricht & Southgate 1991). The lower IT values and higher HH values found in these bioprocessed groups might be due to the higher levels of PUFA present in diets supplied by these fish groups. Approximately 2% lipid reduction in bioprocessed PBM (11.70%) than unprocessed PBM (13.50% lipid) may have a greater influence on improved nutrition and flesh quality of fish fed bioprocessed diets. However, in comparison of these indices with the FM-based control, the IA value was not significantly different in fish fed both 75PBM and 100BPBM, whereas IT value was higher in the 100BPBM diet. The HH value was higher in fish fed 75BPBM while no difference was observed with 100BPBM (Figure 8.2).

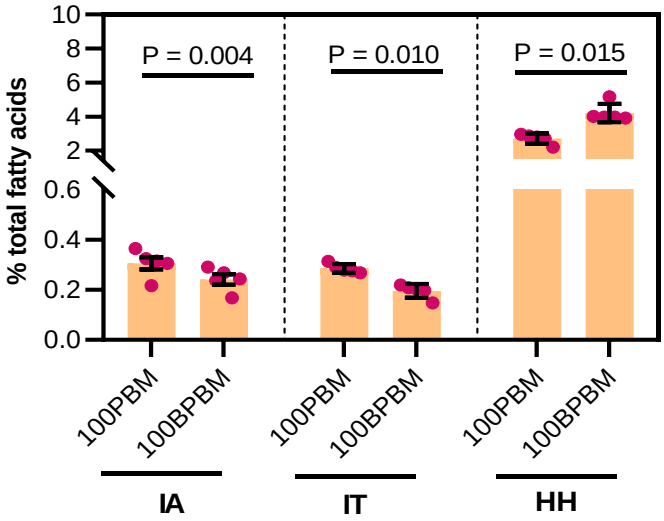

Figure 8.1 Comparison of fatty acids indices (IA, IT, HH) of fish fed bioprocessed and unprocessed PBM at 100% replacement level. PBM, unprocessed poultry by-product meal; BPBM, bioprocessed poultry by-product meal; IA, index of atherogenicity; IT, index of thrombogenicity and HH, hypocholesterolemic / hypercholesterolemic ratio. Bar holding P-value denote significant different between bioprocessed and unprocessed PBM (two-tailed *t* test; significant at P<0.05).

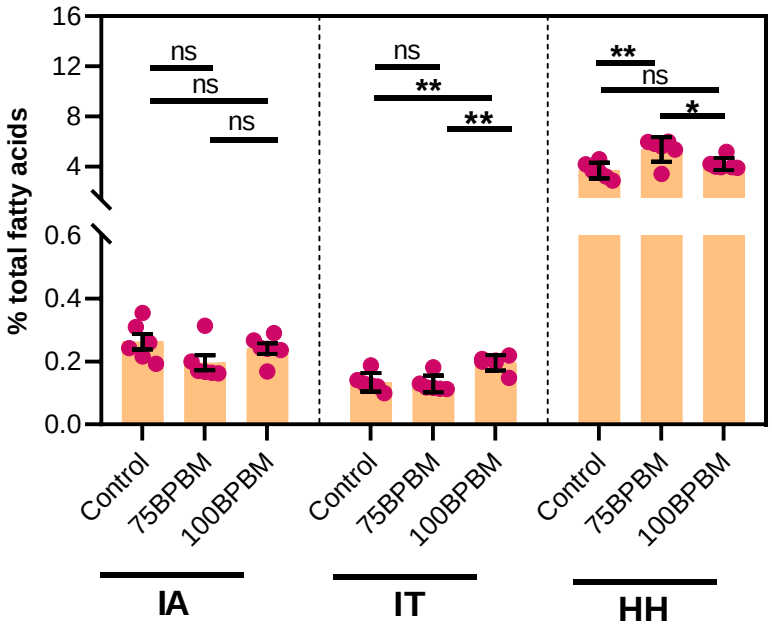

Figure 8.2 Comparison of fatty acids indices (IA, IT, HH) of fish fed bioprocessed PBM at varying levels when compared to control. PBM, poultry by-product meal; BPBM, bioprocessed poultry by-product meal; IA, index of atherogenicity; IT, index

of thrombogenicity and HH, hypocholesterolemic / hypercholesterolemic ratio. Bar holding P-value denote significant different between bioprocessed and unprocessed PBM (Turkey multiple comparison test; significant at $P<0.05$, $P<0.01$).

The findings of this part of the study indicated that complete replacement (100%) of FM both by bioprocessed and unprocessed PBM negatively affected the growth performance and gut health of juvenile barramundi. However, bioprocessed PBM improved the lipid nutritional quality than the unprocessed PBM diets, which can be beneficial for human consumption.

Table 8.3 Growth performance, digestibility, whole body composition and biochemical indices of juvenile barramundi fed PBM diets without or with fermentation for 56 days.

Parameters investigated	Level of FM replaced by PBM			
	75PBM	75BPBM	100PBM	100BPBM
Growth indices				
FBW (g)	↔	↔	↓	↓
SGR (% day^{-1})	↔	↔	↓	↓
FCR	↔	↔	↓	↓
Body composition				
Protein (% WW)	↔	↔	↓	↔
Gut micromorphology				
Microvilli density (per 1 µm^2)	↔	↑	↓	↓

Note: Increase, ↑; decrease, ↓; no change, ↔; compared to the control diet ($P<0.05$). TH, tuna hydrolysate; FBG, final body weight; SGR, specific growth rate; TFI, total feed intake; FCR, feed conversion ratio; DW, dry matter basis; GE: gross energy.

8.1.4 Beneficial effects of 10%TH in PBM diet for juvenile barramundi

To develop a nutritionally improved and complete FM devoid diet for juvenile barramundi, PBM was fermented and added with 10% TH. The findings confirmed that that bioprocessed PBM (BPBM) with 10%TH diets significantly improved the FBW and SGR of juvenile barramundi when compared to the control and those fed unprocessed PBM diet (Table 8.4). A comparison of SGR values of TH and PBM diets in each chapter is compared to the control in Figure 8.3. The enhanced growth

performance with SGR may be due to the supplementation of TH, which increases the presence of associated peptide fractions produced during the enzymatic hydrolysis. These peptides are likely to be beneficial for stimulating growth performance of fish (Khosravi et al. 2017; Siddik et al. 2018b). The increased feed intake observed with BPBM may also be due to improved palatability of the diet containing TH (Bui et al. 2014). The improved growth performance may be attributed to the ability of fermented bacteria to stimulate endogenous enzyme activity for digestion (Ray et al. 2012; Ray et al. 2010) as well as the hydrolysate capacity to increase the availability of suitable substrates for probiotic action (Bedford & Cowieson 2012). The enhanced growth performance with BPBM diet may also be due to the addition of yeast during bioprocessing (Sheikhzadeh et al. 2012). Furthermore, the fermenting *Lactobacillus casei* could also positively affect the gut microbiota through improved feed intake and enhanced nutrient absorption and assimilation (Rimoldi et al. 2018).

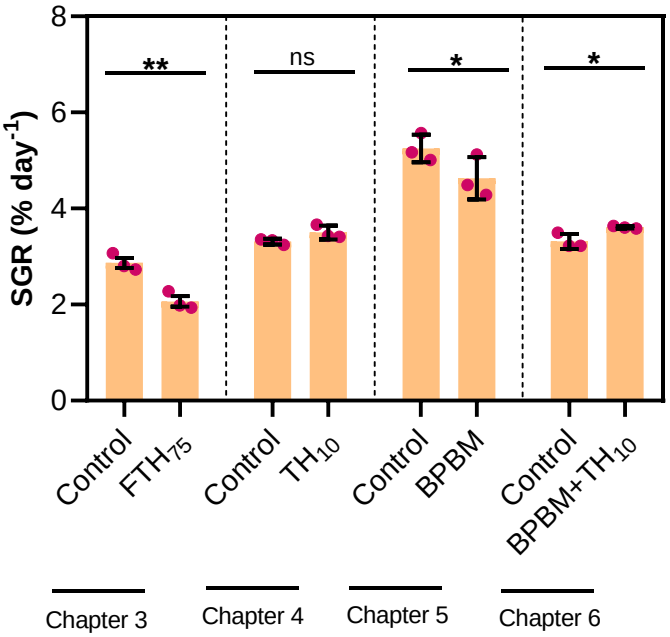

Figure 8.3 Specific growth rate (SGR) of juvenile barramundi fed FM and TH diets in different chapters. The SGR was significantly decreased in fed 75% TH, no change with 10% TH and significantly increased with BPBM supplemented with 10% TH. The bars with P-value in the same chapter indicate statistically significant ($P<0.05$).

Table 8.4 Growth performance, body proximate composition, biochemical indices and gut micromorphology of juvenile barramundi fed tuna hydrolysate (TH) included diets at various levels for 10 weeks.

Parameters investigated	Experimental diets			
	FMBD+TH	LPBM+TH	HPBM+TH	BPBM+TH
Growth indices				
FBW (g)	↑	↔	↔	↑
SGR (% day^{-1})	↔	↔	↔	↔
FI (g fish^{-1}day^{-1})	↑	↔	↔	↓
Body proximate composition				
Moisture (%)	↔	↔	↓	↓
Lipid (% DM)	↓	↔	↓	↓
Biochemical indices				
AST (uL^{-1})	↔	↔	↔	↔
GLDH (uL^{-1})	↔	↓	↓	↓
Glucose (mmol L^{-1})	↔	↓	↓	↓
Gut micromorphology				
Microvilli length (µm)	↑	↔	↓	↔

Note: Increase, ↑; decrease, ↓; no change, ↔; compared to the control diet ($P<0.05$). TH, tuna hydrolysate; FBG, final body weight; SGR, specific growth rate; FI, feed intake; DW, dry matter basis; AST, aspartate transaminase; GLDH, glutamate dehydrogenase.

The study revealed that blood glucose and serum triglyceride were significantly decreased in fish fed FPBM diet indicating that TH produced through enzymatic hydrolysis may trigger insulin secretion, accordingly reducing the glucose level, which in turn favourably effects the wellbeing of fish (Talpur & Ikhwanuddin 2012). The overall improvement of serum lysozyme activity in BPBM may also be an indication of enhanced immune response with these diets when compared to the control. TEM analysis revealed that microvillus height increased significantly in the distal intestine of the barramundi fed BPBM+TH diets. This result was in accord with our previous study in which dietary supplementation of TH in a FM based diet at 10% significantly increased the microvilli height in juvenile barramundi (Siddik et al. 2018b). The cumulative mortality of barramundi 14-days after challenge with *Vibrio harveyi* was

significantly lower in BPBM when compared with the control. This result validated the earlier finding that juvenile barramundi fed the 10%TH diet resulted in the highest resistance against *Streptococcus iniae* infection (Siddik et al. 2018b) (Figure 8.4).

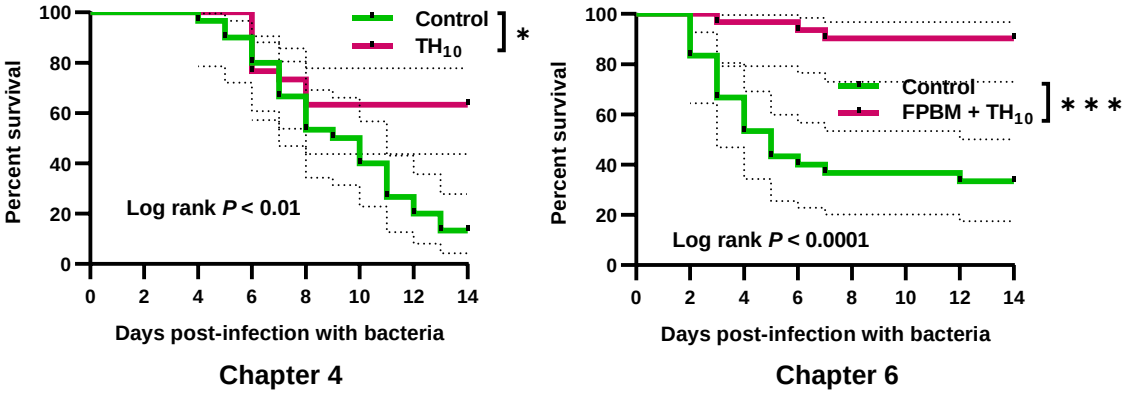

Figure 8. 4 Survival curve of barramundi after being challenge with *Streptococcus iniae* (immersion bath) and *Vibrio harvei* (IP injection) with the best diets in chapter 4 and chapter 6, respectively. The infection (moribund condition) was recorded daily for 14 days. Chapter 4 showed the highest disease resistance with TH10, while BPBM+TH supplementation demonstrated the maximum resilience against disease in chapter 6. Asterisks * and *** indicate statistically significant difference between control and TH treated group at P<0.05 and P<0.001, respectively.

The result of the RT-qPCR analysis revealed that bioprocessed PBM supplemented with TH significantly up-regulated IL-1β, IL-8, IL-10 and IL-17F gene expression. Furthermore, Firmicutes were the most representative phylum in the fish fed FPBM+TH (Chapter 7). *Bacillus* belonging to Firmicutes is used as a probiotic, and can exert a range of beneficial effects on finfish aquaculture systems including the modulation of gene expression in the intestinal epithelial cells Ma et al. (2017), improvement of food absorption by accelerating the protease level (Irianto & Austin 2002) and enhancement of immunity of animals against pathogenic bacteria (Zhang et al. 2010). Similarly, most of the *Lactococcus* species under Firmicutes are generally recognized as safe microorganisms exerting beneficial effects on fish development and health (Avella et al. 2012; Sequeiros et al. 2015) as well as protecting against pathogens by producing antimicrobial compounds (Balcazar et al. 2007). These results elucidate the accumulated benefits of both the bioprocessing of PBM and the supplementation of TH. Therefore, the application of TH in non-FM based diets may

present a novel strategy for growth performance as well as health management in barramundi aquaculture.

8.2 Conclusions

Based on this study, the following conclusions are drawn:

i. Juvenile barramundi fed diets containing higher levels of TH (50 to 75%) resulted in reduced growth performance, feed intake and digestibility, and show abnormal signs of liver histopathology. Furthermore, these replacement levels did not improve biochemical responses of blood and GPx activity of juvenile barramundi.

ii. Following supplementation of 0-20% TH, the optimum TH for juvenile barramundi was estimated to be 10.5% in the diet to enhance growth and intestinal micro-morphological parameters including goblet cell, fold height, microvillous height and external circumference of serosa. Fish fed 10% TH exhibited the highest resistance against *Streptococcus iniae* infection 14-days after bacterial challenge.

iii. Without TH addition, FM protein cannot be replaced by more than 75% both by bioprocessed and unprocessed PBM in terms of growth and gut morphology of juvenile barramundi. However, lipid nutritional quality indices including IT and HH were improved with fish fed bioprocessed PBM diets when compared to unprocessed PBM indicating that consumption of barramundi reared on bioprocessed PBM may generate some human health benefits, mostly related to the risks of cardiovascular disease.

iv. With the supplementation of optimised TH level (10%TH) in PBM, 100% FM protein can be replaced with PBM protein in juvenile barramundi. Furthermore, 10%TH in bioprocessed PBM diet is recommended to enhance gut morphology, lysozyme activity, and disease resistance against *Vibrio harvei* infection of juvenile barramundi.

v. PBM bioprocessed by *Saccharomyces cerevisiae and Lactobacillus casei* in combination with 10%TH stimulates beneficial gut microbiota, concomitant gut health and immune-related cytokines genes expression in juvenile barramundi.

8.3 Recommendations

i. Further research on bioprocessed PBM based diets comprising TH supplementation needs to be trialled with other species and/or under commercial farming conditions to ensure the findings of the present study.

ii. Future research should consider whether TH supplementation could also be successfully used in combination with other animal protein replacement options.

iii. There is a need to analyse the secretion of digestive enzymes in the supplementation of bioprocessed and unprocessed animal-derived proteins.

iv. Research to understand a signalling pathway focussed on how fermentation and TH supplementation influence the immune response, immune related gene expression and disease resistance in juvenile barramundi is recommended.

8.4 Limitations

i. The PBM fermentation process was undertaken in the laboratory. A number of factors can affect the fermentation process, which in turn can determine the quality of the animal-derived proteins. These factors include pH, temperature, fermenting agents and their concentrations and the absence of oxygen. These factors are easy to control at laboratory scale but with commercial scale-up, issues of contamination and /or quality deterioration maybe involved in fermentation process. Such differences must be considered when applying the results in a commercial context.

ii. Tuna hydrolysate was supplied by a commercial operation. Therefore, it was not subjected to size fractioning as secondary processing which may further enhanced immune and growth stimulation impact.

iii. The initial size of fish, duration and parameters tested in the growth trials were not similar in all experiments conducted for this study. Thus, the results obtained after the feeding trials of each experiment cannot be compared.

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AUTHORS CONTRIBUTIONS IN PUBLISHED ARTICLES

Paper 1 (Chapter 3)	Title of paper	Growth, biochemical response and liver health of juvenile barramundi (*Lates calcarifer*) fed fermented and non-fermented tuna hydrolysate as fishmeal protein replacement ingredients
	Authors name and Contribution	Muhammad A.B. Siddik Janet Howieson: Co-supervisor Ilham Ilham: <5% Ravi Fotedar: Supervisor
	Content of contribution	Muhammad A.B. Siddik: Conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, drafted and reviewed of the paper Janet Howieson: Contributed reagents/materials/analysis tools, reviewed drafts of the paper, approved the final draft. Ilham Ilham: Help in formulating feed, revised drafts of the paper. Ravi Fotedar: Contributed reagents/materials/analysis tools, reviewed drafts of the paper, approved the final draft.
Paper 2 (Chapter 4)	Title of paper	Dietary tuna hydrolysate modulates growth performance, immune response, intestinal morphology and resistance to *Streptococcus iniae* in juvenile barramundi, *Lates calcarifer*
	Authors name and Contribution	Muhammad A.B. Siddik Janet Howieson: Co-supervisor Gavin J. Partridge: Associate supervisor Ravi Fotedar: Supervisor Hosna Gholipourkanani: <5%
	Content of contribution	Muhammad A.B. Siddik: Conceived and designed the experiment, conducted the experiment, analyzed data, prepared figures, tables and wrote the paper. Janet Howieson: Funded the study, revised the final draft. Ravi Fotedar: Revised the final draft. Gavin J. Partridge: Revised the final draft. Hosna Gholipourkanani: Help in analyzed the samples of lysozyme and complement activity.
Paper 3 (Chapter 5)	Title of paper	Bioprocessed poultry by-product meal on growth, gut health and fatty acid synthesis of juvenile barramundi, *Lates calcarifer* (Bloch)

	Authors name and Contribution	Muhammad A.B. Siddik Patience Chungu: <10% Ravi Fotedar: Supervisor Janet Howieson: Co-supervisor
	Content of contribution	Muhammad A.B. Siddik: Conceptualization and design the study, data curation, formal analysis, investigation, methodology, writing original draft Ravi Fotedar: Project administration, supervision, review and editing Patience Chungu: Help in iinvestigation and data curation Janet Howieson: Review and editing
Paper 4 (Chapter 6)	Title of paper	Beneficial effects of tuna hydrolysate in poultry by-product meal diets on growth, immune response, intestinal health and disease resistance to *Vibrio harvei* in juvenile barramundi, *Lates calcarifer*.
	Authors name and Contribution	Muhammad A.B. Siddik Ravi Fotedar: Supervisor Janet Howieson: Co-supervisor
	Content of contribution	Muhammad A.B. Siddik: Designed research, performed experiment, collected and processed data, analyzed data, wrote the paper Ravi Fotedar: Reviewed and revised paper Janet Howieson: Reviewed and revised paper

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