School of Molecular and Life Sciences

Supplementing insect meal and fish protein hydrolysates in barramundi, *Lates calcarifer* diet improves the inclusion efficiency of poultry by-product meal: a physiological approach

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Doctor of Philosophy

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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.

Animal Ethics: 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 #ARE_2018_37.

Human Ethics: The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the Curtin University Human Research Ethics Committee (EC00262), Approval Number #HRE_2020_0689.

Signed | Md Reaz Chaklader

Dated | September 2021

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ABBREVIATION AND ACRONYMS

FM, Fish meal
FO, Fish oil
FPH, Fish protein hydrolysates
PBM, Poultry by-product meal
CH, Carp hydrolysate
KH, Kingfish hydrolysate
TH, Tuna hydrolysate
TPA, Texture profile analysis
HI, *Hermetia illucens*
FHI, full-fat *Hermetia illucens*
DHI, defatted *Hermetia illucens*
RAS, Recirculatory aquaculture system
RTP, Research Training Program
ACAAR, Australian Centre for Applied Aquaculture Research
FRDC, Fisheries Research and Development Corporation
EU, European Union
SFA, Saturated Fatty acid
MUFA, Monounsaturated Fatty acid
PUFA, Polyunsaturated fatty acid
EPA, Eicosapentaenoic acid
DHA, Docosahexaenoic acid
NRC, Nutritional Research Council
Ca, Calcium
Cu, Copper
Mg, Magnesium
Na, Sodium
Mn, Manganese
Fe, Iron
Zn, Zinc
AMP, Antimicrobial peptides
GIT, Gastrointestinal tract
CPC, Corn protein concentrate
ADC, Apparent digestibility coefficients
AA, Amino acid
CP, Crude Protein
CL, Crude lipid
FBW, Final body weight
SGR, Specific growth rate
SR, Survival rate
WG, Weight gain
DGI, Daily growth index
FI, Feed Intake
DFI, Daily feed intake
FCR, Feed conversion ratio
HSI, hepatosomatic index
CF, Condition factor
VSI, Viscerosomatic index
PER, Protein efficiency ratio
IFI, Intraperitoneal fat index
SI, Spleen index
RGL, Relative gut length
DM, Dry matter
PC, proximate composition
CAT, Catalase
MDA, Malondialdehyde
SOD, Superoxide Dismutase
GSH-Px, Glutathione Peroxidase
GPx, Glutathione Peroxidase
AST, Aspartate aminotransferase
GLDH, Glutamate dehydrogenase
Cho, Cholesterol
TG, Triglyceride

Glu, Glucose

ALB, Albumin

PCV, Packed cell volume

WBC, White blood cell

MCV, Mean corpuscular volume

MCHC, mean corpuscular hemoglobin concentration

GPT, Glutamic-pyruvic transaminase

LDL-c, Low-density lipoprotein cholesterol

LPS, Lipopolysaccharides

LAB, lactic acid bacteria

SCFA, Short-chain fatty acid

AOAC, Association of Official Analytical Chemists

TEM, Transmission electron micrograph

HM, Healthy myotome

SL, Secondary lamellae

BF, Broken fold

MVH, Microvilli height

MVD, Microvilli diameter

APB, animal protein blend

GCs, Goblet cells

MW, Muscular wall

NM, Neutral Mucin

IQF, Individual quick freezing

TP, Total protein

GLB, Globulin

HB, Haemoglobin

RBC, Red blood cell

MCH, Mean corpuscular haemoglobin

ALT, Alanine aminotransferase

HDL-c, High-density lipoprotein cholesterol

DAO, Diamine Oxidase

FFA, Free Fatty acid

MCFA, Medium-chain fatty acids

OTU, Operational taxonomic unit

DO, Dissolved oxygen

PAS, Periodic Acid-Schiff

NM, necrotic myotome

HSL, Hyperplasia in secondary gill lamellae

MV, Microvilli

TJ, Tight Junction

HSP, Heat shock protein

MN, multifocal necrosis

DPIRD, Department of Primary Industries and Regional Development

FH, Fold height

AM, Acidic mucin

LIST OF ANIMALS

Barramundi, *Lates calcarifer*

Carp, *Cyprinus carpio*

Southern bluefin tuna, *Thunnus maccoyii*

Black Sea Bass, *Centropristis striata*

Florida Pompano, *Trachinotus carolinus*

Red sea bream, *Pagrus major*

Gilthead sea bream, *Sparus aurata*

Chinook salmon, *Oncorhynchus tshawytscha*

Humpback grouper, *Cromileptes altivelis*

European sea bass, *Dicentrarchus labrax*

Hybrid tilapia, *Oreocromis niloticus* x *O. Mozambique*

Juvenile grass carp, *Ctenopharyngodon idellus*

African catfish, *Clarias gariepinus*

Meagre, *Argyrosomus regius*

Eurasian Perch, *Perca fluviatilis*

Yellow catfish, *Pelteobagrus fulvidraco*

Jian carp, *Cyprinus carpio* var. Jian

Mirror carp, *Cyprinus carpio var. specularis*

Japanese flounder, *Paralichthys olivaceus*

Black sea turbot, *Psetta maeoticus*

Silver seabream, *Rhabdosargus sarba*

Red drum, *Sciaenops ocellatus*

Black carp, *Mylopharyngodon piceus*

Largemouth bass, *Micropterus salmoides*

Sibel carp, *Carassius auratus gibelio*

turbot, *Scophthalmus maximus* L.

Black soldier fly, *Hermetia illucens* (HI)

Yellowtail kingfish, *Seriola lalandi*

Japanese Sea Bass, *Lateolabrax japonicas*

Totoaba, *Totoaba macdonaldi*

Cobia, *Rachycentron canadum*

Spotted rose snapper, *Lutjanus guttatus*

Black sea turbot, *Psetta maeoticus*

Grouper juveniles, *Epinephelus fuscoguttatus*

Atlantic salmon, *Salmo salar*

Siberian Sturgeon, *Acipenser baerii*

Rice field eel, Monopterus albus

Rainbow trout, *Oncorhynchus mykiss*

Penaeid prawns and Pacific White Shrimp, *Litopenaeus vannamei*

Zebrafish, *Danio rerio*

Nile Tilapia, *Oreochromis niloticus*

Turbot, Psetta maxima

Channel catfish, *Ictalurus punctatus*

Clownfish, *Amphiprion ocellaris*

Spotted rose snapper, *Lutjanus guttatus*

Penaeid prawns, *Marsupenaeus japonicas*

Australian snapper, *Pagrus auratus*

Tiger grouper juveniles, *Epinephelus fuscoguttatus*

Sunshine bass, *Morone chrysops* × *Morone saxatilis*

Hybrid grouper, *Epinephelus fuscoguttatus*♀× *Epinephelus lanceolatus*♂

Tenches, *Tinca tinca*

Hybrid striped bass, *Morone chrysops* x *M. saxatilis*

yellow croaker, *Larimichthys crocea*

Japanese eel, *Anguilla japonica*

Coho salmon, *Oncorhynchus kisutch*

yellowfin tuna, *Thunnus albacares*

American catfish, *Rhamdia quelen*

Largemouth bass, *Micropterus salmoides*

Olive flounder, *Paralichthys olivaceus*

Persian sturgeon, *Acipenser persicus* L.

Silver catfish, *Rhamdia quelen*

Atlantic halibut, *Hippoglossus hippoglossus* L.

Atlantic mackerel, *Scomber scombrus*

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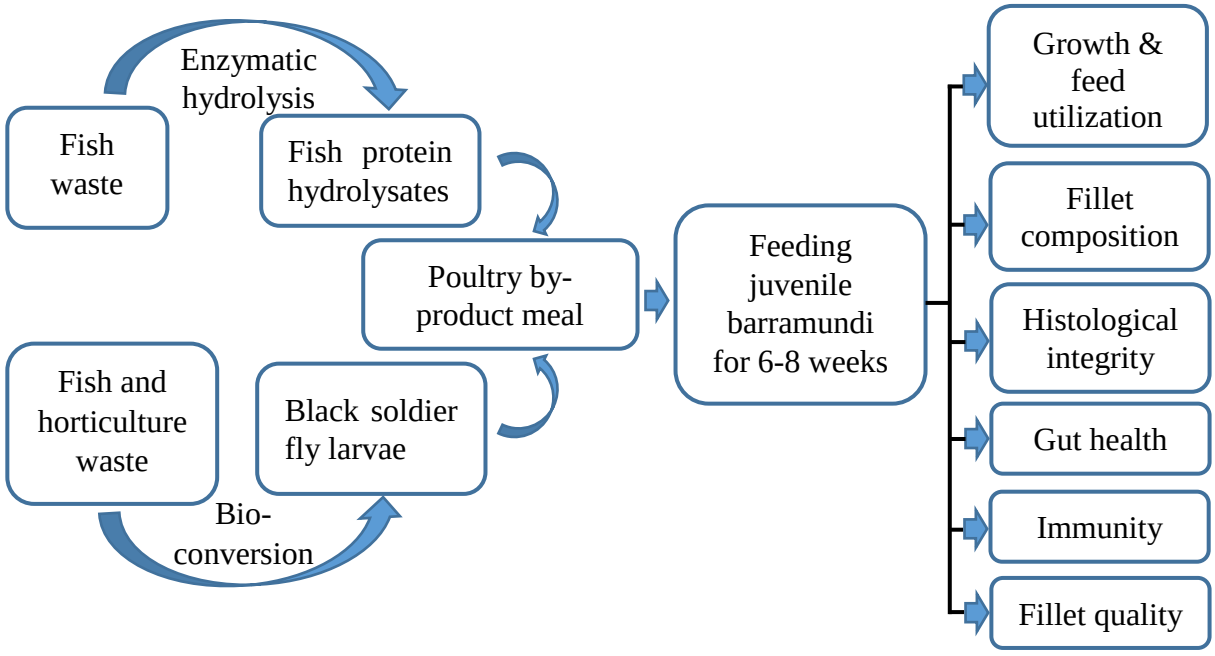
ACKNOWLEDGEMENT OF COUNTRY

We acknowledge that Curtin University works across hundreds of traditional landsand custodial groups in Australia, and with First Nations people around the globe. We wish to pay our deepest respects to their ancestors and members of their communities, past, present, and to their emerging leaders. Our passion and commitment to work with all Australians and peoples from across the world, including our First Nations peoples are at the core of the work we do, reflective of our institutions' values and commitment to our role as leaders in the Reconciliationspace in Australia.

PREAMBLE

Developing next-generation protein and lipid aquafeed sources from a combination of recycled food waste and other multiple protein sources could provide benefit from subsequent complementary nutritional profiles. This approach can be used to develop nutritionally balanced and functional aquafeed which may support the continuous development of a disease-resistant, chemotherapeutant-free, and sustainable aquaculture for carnivorous species, in particular, barramundi aquaculture. Whilst most previous aligned sustainable aquafeed research conducted has focused on the inclusion of single protein sources or with supplementation of immunostimulants or deficient nutrients. However, the present study was a first attempt to transform food waste into black soldier fly, *Hermetia illucens* (HI) larvae meal, and fish protein hydrolysates (FPH) which were then supplemented with poultry-by-product meal to replace fishmeal exclusively or completely from the barramundi diet. This approach is illustrated below in the schematic diagram. The efficacy of supplementing HI larvae meal and FPH with PBM was investigated by a multidisciplinary approach, including growth and biometry indices, a panel of serum metabolites, histopathology of different organs, mucosal barrier functions of gut and skin, gut microbiota, antioxidant responses, immunity, fillet proximate composition, and final product quality of barramundi.



A schematic representation of the present study plan

This thesis is structured with twelve chapters. Chapter 1 briefly describes the contribution of the aquaculture industry to world fish production and highlights the limitation of using fishmeal in aquafeed and the potentiality of using transformed HI larvae and FPH to complement rendered PBM and prevent the previously reported negative effect caused by exclusive or 100% inclusion of PBM. The aim, objectives, and significance of the present study are also included in this chapter.

Chapter 2 (submitted to "Review in Aquaculture") is a comprehensive review of existing literature sourced from the scientific databases Scopus® and Web of Science® on the three main ingredients used in the present research. A detailed review on the opportunities and challenges of dietary inclusion of HI larvae meal in aquafeed, with an especial focus on the nutritional composition of HI larvae meal and aquafeed inclusion effects on growth and digestibly, proximate composition, final product quality, serum metabolites, antioxidant activity, immune response and gut health of various commercially important aquaculture species, followed by a brief review regarding the use of FPH and PBM as immunostimulants and alternative protein ingredients in aquafeed. This chapter has ended up with a summary diluting all information.

Chapter 3 (published in "PLOS ONE") describes the effects of using a PBM-based diet on the growth, fillet composition, histopathology, intestinal barrier function, stress response, and immunity of barramundi. This chapter represents a negative control for comparison with other test diets in the following chapters.

Chapter 4 (Published in "Scientific Reports") and Chapter 5 (Published in "Frontiers in Nutrition") explains the efficacy of the supplementation of full-fat HI larvae (10%) meal, reared on fish waste with graded levels of PBM in barramundi diet and the effects were investigated using growth and biometry indices, fillet composition, adipocyte cell size, histopathology of different organs, the mucosal barrier function of intestine and skin, and immunity.

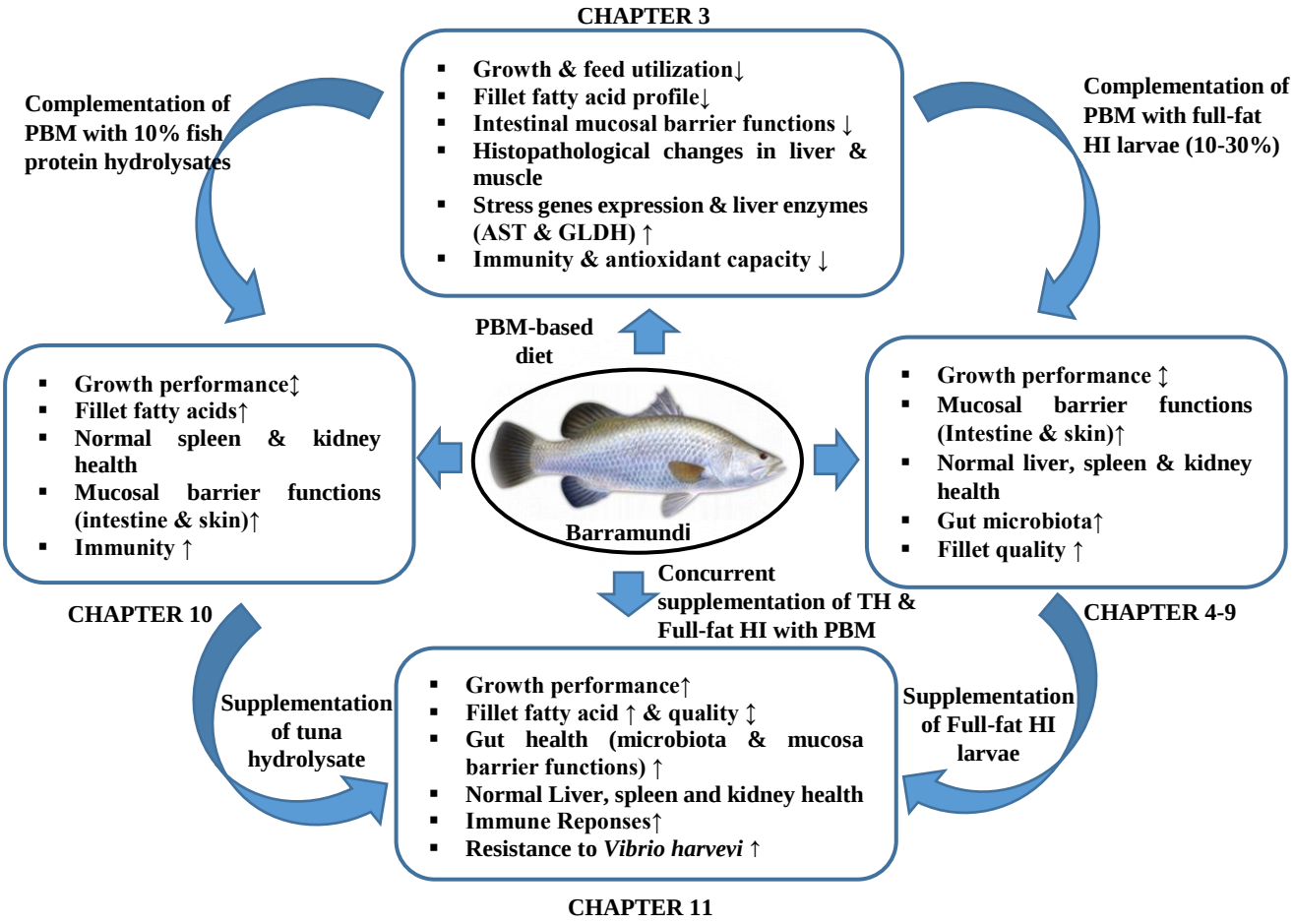
Chapter 6 to Chapter 9 describes the performance of barramundi when fed a mixture of graded levels of full-fat and defatted HI larvae meal (15-30%) and PBM (85-70%). Whilst Chapter 6 (published in "Science of the Total Environment") explains the positive influence of full-fat HI larvae (15-25%) with PBM in enhancing health status, Chapter 7 (under review in "Fronteirs in Nutrition") describes the enhancement of fillets quality traits under simulated retail display in response to diets used in Chapter 6. Chapter 8 deals with an experiment using full-fat and defatted HI larvae meal (30%) with PBM (70%) in barramundi diet (published in "Aquaculture") while enhancement of fillet quality attributes in response to the same diets used in Chapter 8 is described in Chapter 9 (under review in "Food Control").

Chapter 10 elucidates the positive effect of 10% supplementing various FPHs inclusions levels including kingfish hydrolysate, Bluefin tuna hydrolysate, and carp hydrolysate extracted from the relevant waste source with PBM (90%) on barramundi growth, fillet composition, gut health, immune-relevant cytokine expression, and disease resistance to *Vibrio harveyi*. This Chapter has been published in "Fish and Shellfish Immunology".

Chapter 11 describes the ability of concurrent supplementation of full-fat HI larvae and tuna hydrolysate (chosen based on functional properties) to improve the functionality of PBM by improving the growth, gut mucosal barrier functions, gut microbiota, and immunity of barramundi without changing the fillet quality traits. This chapter has been published in "Scientific Reports".

The last Chapter reanalyses and presents all findings in summary by discussing the most significant set of results to find out the efficacy of supplementation of HI larvae and FPH on its own or concurrently on physiological responses of barramundi and attributes of its fillet quality. The main summary, future recommendations, and limitations of the present study are also presented in this chapter.

GRAPHICAL ABSTRACT



ABSTRACT

With aquaculture currently providing 46% of total world fish production, the historic reliance on nutritional components of fishmeal protein (FM) and fish oil (FO), mainly sourced wild-captured forage fish, is not sustainable to continue predicted expansion in aquaculture production. Hence the additional predicted demand of 37.4 million tons of aqua feeds by 2050 has driven academia and the aquaculture industry to seek sustainable and in-expensive alternative protein sources to reduce the reliance on FM and FO in aquafers. Utilization of multiple protein sources from processed animal proteins, particularly recovering functional ingredients from the recycling of food waste by bio-conversion and enzymatic hydrolysis and subsequent utilization in conjunction with by-products such as poultry by-product meal (PBM) is a suggested strategy to underpin FM and FO free aquafeeds.

To support this strategy, in this study a total of seven feeding trials were carried out to investigate the supplementation of black soldier fly, *Hermetia illucens* (HI) reared on fish waste and horticulture waste and various fish protein hydrolysates (FPHs) recovered from three different fish waste including carp, *Cyprinus carpio*, yellowtail kingfish, *Seriola lalandi*, and southern bluefin tuna, *Thunnus maccoyii* to improve inclusion efficacy of PBM in juvenile barramundi, *Lates calcarifer* diet. Effects were monitored on growth and biometry indices, fillet amino acid fatty acid composition, serum metabolites, histopathology of different organs, adipocyte cell size, skin mucosa barrier functions, gut health in terms of mucosal barriers and microbiota, immune responses, and disease resistance against *Vibrio harveyi*, along with product fillet quality attributes.

The first feeding trial objective was to examine the efficacy of complete replacement of FM protein with PBM in juvenile barramundi diet. The results demonstrated that barramundi cannot rely on PBM-only-based diets, as supported by the growth depression, lower survival rate, less synthesis of essential fatty acids, pathological changes in liver, muscle, and gill, the disintegration of the intestine, and poor immune response. The second and third feeding trials objective was to investigate if supplementation of 10% full-fat HI (FHI) larvae could prevent the negative effects caused by exclusive or total inclusion of PBM (45-90%) in the juvenile barramundi diet. The results showed that up to 75% of FM protein can be replaced with PBM with an improved or equal growth performance together with no negative effects on the liver, muscle, and heart health, whilst decreasing fat deposition and improving skin and intestinal mucosal barrier functions and immunity. However, inclusion rates of 90% of PBM impacted the growth, liver health, and immunity of test fish. The fourth and fifth trials were conducted to delineate the gap between 75 and 90% of PBM by progressive inclusion of FHI and defatted HI (DHI) larvae meal (15-30%) in

combination with graded levels of PBM (75-85%). The results showed that FHI larvae meal is more functionally active than DHI larvae meal when mixed with PBM, which was reflected by equal growth performance, no changes in liver health and immune responses while improving antioxidant capacity, intestinal mucosal barrier functions, and enriching bacterial community coupled with increasing the abundance of beneficial bacteria including Lactobacillus, *Clostridium*, and *Ruminococcus*. DHI larvae meal in PBM were aligned with growth depression, hepatic steatosis, abnormal production of liver enzymes, and poor immune response. The HI larvae meal either fatted or defatted combined with PBM improved the sensory quality of the fillets with no changes in amino acid profile, colour, and texture profile, and delayed the lipid oxidation, during simulated retail displayed chilled storage. The sixth trial was performed to examine the supplementation efficacy of 10% FPHs, recovered from fish waste rather than feeding HI larvae to include 90% of PBM protein. Supplementation of various FPHs with PBM can replace FM entirely with equal growth performance with no changes in spleen and kidney health but enhanced the retention of monounsaturated and polyunsaturated fatty acids, skin, intestinal mucosal barrier functions, and immunity. The objective of the final trial was to examine if the concurrent supplementation of 10 and/or 5% FHI larvae and TH (chosen as the test FPH based on optimal peptide size) with PBM could further improve the functionality of juvenile barramundi diets. The results were promising since growth performance, fatty acids profile, serum metabolites, intestinal barrier functions, intestinal microbiota, immune response, and disease resistance against *V. harveyi* were improved in juvenile barramundi.

In summary, separate or concurrent supplementation of FPH and HI larvae, valorised from food waste, could be a novel strategy to complement alternative animal protein sources with an ability to exclude FM and FO from the diet of juvenile barramundi. Also, the improvement in fillet quality would help in the acceptance of FM-free diets by consumers. Larger scale testing and potential commercial implementation of the outcomes of the present study could help in ensuring a socially and environmentally sustainable future for the aquaculture industry.

CHAPTER 1: INTRODUCTION

1.1 Background

The predicted population growth to 9.7 billion by 2050 will require an additional supply of food by 25%-70% (Nations, 2019). Global food fish consumption per capita has increased significantly at an average rate of about 1.5 percent per year, rising from 9.0 kg (live weight) in 1961 to 20.5 kg in 2018 (FAO, 2020) and increased aquaculture production will fill the predicted consumption gap. Approximately 46% of the total world fish production has been reported to derive from aquaculture (FAO, 2020) which has historically depended on fishmeal (FM) and fish oil (FO) as standard protein and lipid sources for the production of most aquaculture species (Hua et al., 2019). Unfortunately, sourcing forage fish to manufacture FM and fish oil for the expanded aquaculture industry leads to overfishing and destructive fishing practices on already stressed wild capture fishery, causing a steady decline in the production of FM and FO over the last 20 years (Hua et al., 2019; Turchini et al., 2019). Consequently, this ingredient has become unsustainable and expensive. In addition, it has been predicted that aquafeed production will increase by 75% from 49.7 million tons in 2015 to 87.1 million tons 2025 (Figure 1. 1B). Hence, realisation of growing issues including depletion of wild fisheries stocks, escalated price (Figure 1. 1A) and an additional requirement of 37.4 million tons of aquafeeds by 2025 has driven academia and aquaculture industries to investigate sustainable and cost-effective renewable alternative protein sources. Whilst a number of plant-based protein sources including soybean, corn gluten and rapeseed meal, are being used for aquafeed production, such ingredients have significant limitations, particularly, imbalanced nutritional profile together with anti-nutritional factors (Hua et al., 2019). The use of plant-based ingredients in directly compete with humans for food, and even less potential to expand production without environmental issues such as land and water use, fertilizer use and deforestation (Hua et al., 2019). Alternative sustainable aquafeed ingredients such as rendered animal by-products, valorised fish protein hydrolysates (FPH) and insect's protein originating from circular bioeconomy have a high potential to develop and supply renewable protein sources required for current and future aquafeeds production.

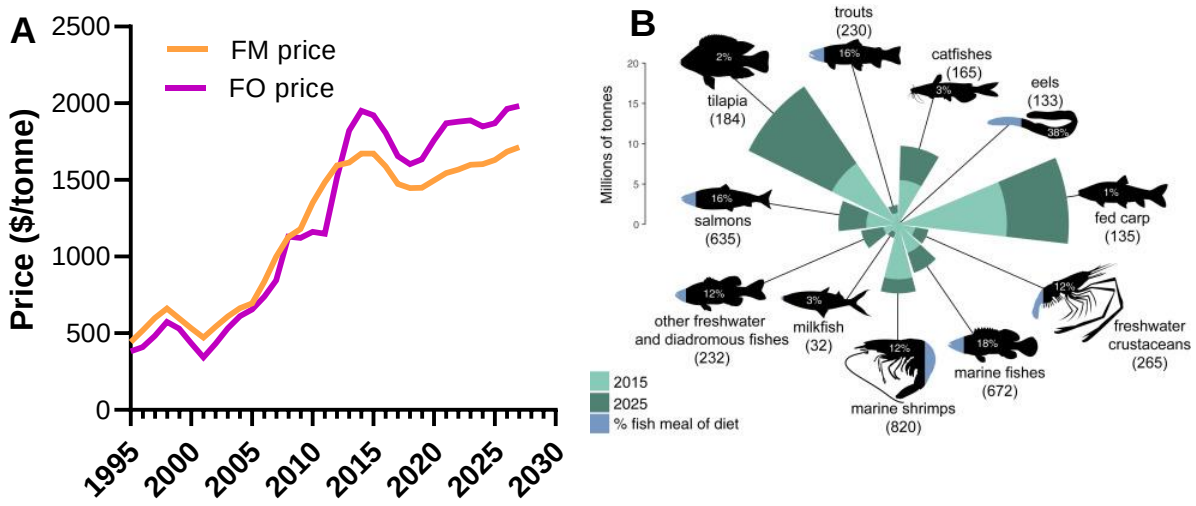


Figure 1. 1 Global FM and FO price trend (A) (Kok et al., 2020; OECD-FAO, 2018) and estimated demand for FM in aquafeeds formulation (Hua et al., 2019). The estimated aquafeed demand (millions of tons) for major aquaculture species in 2015 and 2025. The percentage and blue portion in each animal represent the use of FM in the diet of each group in 2015. The values in brackets underneath each species group are the estimated volume of FM included in the diets in 2015.

Poultry by-product meal (PBM) is one of the rendered animal by-products, consisting of inedible waste and by-products (clean flesh, skin, head, and feet but excluding feathers and intestine) of slaughtered poultry (Dawson et al., 2018), which is more economical and sustainable than FM. This by-product is characterised by a higher concentration of protein (> 65%), comparable amino acid profile to FM, and is a good source of vitamins and minerals, making this ingredient compatible with the requirement of carnivorous fish (Cruz-Suárez et al., 2007; Galkanda-Arachchige et al., 2020; Gunben et al., 2014). However, the nutritional composition of PBM is commonly inconsistent because of variability in raw material composition and processing conditions such as temperature, time, and pressure which affects certain essential amino acids, ash content and digestibility (Dawson et al., 2018; Galkanda-Arachchige et al., 2020; Garza de Yta et al., 2012; Kureshy et al., 2000; Tacon et al., 2006). PBM has been used to successfully replace FM protein in finfish with various successes: ≥ 50% replacement in Juvenile Black Sea Bass, *Centropristis striata* (Dawson et al., 2018), Japanese Sea Bass, *Lateolabrax japonicas* (Wang et al., 2015), Totoaba, *Totoaba macdonaldi* (Zapata et al., 2016) and Florida Pompano, *Trachinotus carolinus* (Rossi and Davis, 2012), cobia, *Rachycentron canadum* (Zhou et al., 2011), red sea bream, *Pagrus major* (Takagi et al., 2000), spotted rose snapper, *Lutjanus guttatus* (Hernández et al., 2014), gilthead sea bream, *Sparus aurata* (Nengas et al., 1999), black sea turbot, *Psetta maeoticus* (Yigit et al., 2006) and Chinook salmon, *Oncorhynchus tshawytscha* (Fowler, 1991)

and up to 100% replacement in tiger grouper juveniles, *Epinephelus fuscoguttatus* (Gunben et al., 2014) and humpback grouper, *Cromileptes altivelis* (Shapawi et al., 2007) without impacting growth performance. However, it has been reported that the inclusion of more than 50% PBM or even inclusion of small quantity of poultry-based protein impacted the welfare of barramundi, *Lates calcarifer* (Glencross et al., 2016; Siddik et al., 2019a; Simon et al., 2019). A number of studies have been able to replace FM with plant protein, either raw or fermented and supplemented with selenium (Ilham et al., 2016a; Ilham et al., 2016b; Ilham and Fotedar, 2017; Ilham et al., 2018; Van Vo et al., 2020a; Vo et al., 2020) and PBM complemented with fish protein hydrolysates (FPH) in barramundi (Siddik et al., 2019b). However none of the studies on feed ingredient variation in barramundi have considered human nutritional factors, full sensory profiles, and shelf-life in relation to physiological and immunological responses, though fillet quality attributes are strongly linked with dietary modifications and consumer preference and acceptance. Hence, developing FM-free aquafeed with a mixture of valorised alternative protein sources and functional material that will be cost-effective, maximise growth and support rigorous disease-resistant sustainable aquaculture while improving fillet quality is a big challenge for the aquaculture industry.

Valorisation of food waste by bio-conversion into renewable protein sources could also be an important strategy to develop sustainable alternative protein ingredients for aquafeed production. In this regard, insect meals have received growing attention in many countries as a potential aquafeed ingredient due to a number of advantages: short life cycle with an ability to grow on a wide range of wastes, high productivity, and feed conversion factor (Berggren et al., 2019; IPIFF, 2018), being non-vectors of pathogens and non-invasive (Wang and Shelomi, 2017), and does not compete with human food resources or human food production. Seven insect species have already been approved by the European Union for use of meal in aquafeed after processing (EU 2017/893 from July 2017). Black soldier fly , *Hermetia illucens* (HI), belonging to the Stratiomyidae family and Diptera order has appeared as a promising insect species as it can convert low-value by-products and wastes into high nutritional profile biomass (~60% protein and 10-30% lipid) suitable for fish and other livestock production (NRC, 2011). Also, this species accumulates a comparable amount of dispensable amino acid to FM, and a variety of vitamins and minerals (Henry et al., 2015; Van Huis, 2013) and includes functional molecules such as bioactive peptides and polysaccharides including choline, silkrose/dipterose. However, the disadvantage of HI application in aquafeed formulation is the richness of saturated fatty acids and omega-6 fatty acids, and the presence of negligible amounts of polyunsaturated fatty acids, particularly omega-3 fatty acids when compared to FM (Barroso et al., 2014; Hua et al., 2019). It is however noteworthy that

the fatty acid profile can be manipulated by substrates. For example, feeding fish offal has been reported to enrich black soldier fly larvae with eicosapentaenoic acid and docosahexaenoic acid (Sealey et al., 2011). The production level of HI larvae meal are not currently sufficient for constant supply (Arru et al., 2019; IPIFF, 2018) but the increase in global production from 7,000 to 8,000 tons in 2014–2015 to 14,000 tons in 2016 has resulted in a forecast for the HI larvae product to be price competitive with that of FM by 2023 (Hilkens et al., 2016).

HI larvae in the form of full-fat and defatted meal, have been evaluated on a variety of omnivorous and carnivorous fish species with various successes (please see the aligned review). For instance, promising results in terms of growth have been reported in Atlantic salmon, *Salmo salar* (Belghit et al., 2019b; Stenberg et al., 2019), European sea bass, *Dicentrarchus labrax* (Abdel-Tawwab et al., 2020), Siberian Sturgeon, *Acipenser baerii* (Rawski et al., 2020; 2021) Hybrid tilapia, *Oreocromis niloticus* x *O. Mozambique* (Yildirim-Aksoy et al., 2020), Rice field eel, Monopterus albus (Hu et al., 2020), Juvenile grass carp, *Ctenopharyngodon idellus* (Lu et al., 2020), Rainbow trout, *Oncorhynchus mykiss* (Renna et al., 2017) *and* African catfish, *Clarias gariepinus* (Fawole et al., 2020) but not in Siberian sturgeon and postmolt salmon (Caimi et al., 2020a; Lock et al., 2016). It is worthy to mention that full-fat HI larvae have been reported to prevent negative effects induced by exclusive inclusion of plant-protein (Kumar et al., 2020) and animal protein in rainbow trout (Randazzo et al., 2021b). Also, HI larvae meal has been reported to improve intestinal mucosal barrier functions (Rawski et al., 2021) and reduce the steatosis in different parts of the gut (Li et al., 2019; Li et al., 2020), and also reshape the gut microbial composition by improving beneficial bacteria in fish (Bruni et al., 2018; Terova et al., 2019) and egg laying hens (Borrelli et al., 2017), implying that HI larvae work as a potential prebiotic. However, the complementary effect of HI larvae alone and or concurrently with FPH on improving the inclusion efficiency of PBM in barramundi diets has not yet been investigated.

FPH are functional ingredients, recovered from inedible fishery and aquaculture by-products (consisting of viscera, heads, skin, bones, and blood) after industrial-scale processing through enzymatic hydrolysis (Chalamaiah et al., 2012). Valorisation of such by-products provides several benefits including supporting environmental sustainability of the industry, economic and social benefits, and sustainable supply of functional ingredients for aquaculture. FPH is characterized by several functional molecules, and therefore can be incorporated into aquafeed at certain levels as immunostimulants, attractants, and palatability enhancers (Kasumyan and Døving, 2003; Önal and Langdon, 2009; Ospina-Salazar et al., 2016). Beneficial effects of FPH have already been reported on numerous finfish and shellfish, as described in detail in a review from our laboratory (Siddik et al., 2021). FPH has also been reported to prevent negative effects induced by the exclusive

inclusion of alternative protein ingredients such as PBM and plant protein in barramundi (Siddik et al., 2019b) and salmon (Egerton et al., 2020). However, FPH supplementation alone or in combination with insect larvae and animal by-products such as PBM, on barramundi physiological response in relation with the final product quality has not yet been thoroughly investigated.

Barramundi, also commonly known as Asian sea bass, is distributed in the Indo-pacific region, is one of the most common marine aquaculture finfish, and is popularly cultivated in tanks, cages, and ponds in both saltwater and freshwater environments in Australia and Asian countries including Indonesia, Southern China, Philippines, Malaysia, Thailand and Taiwan (Tian and Qin, 2003). Barramundi is an economically desirable food fish due to of rapid growth, market demand, flesh texture, and taste. Barramundi, as a carnivorous fish, requires a high amount of protein (450 to 550 g kg^{-1}) which is generally derived from FM (Simon et al., 2019). Barramundi production in Australia has been reported to rely on imported FM which reduces the profitability and sustainability of barramundi production (Williams et al., 2003a). In addition, disease outbreaks due to bacterial infection could be a major limiting factor in the intensification of barramundi aquaculture. Vibriosis due to *Vibrio harveyi* has been reported in the net-cages culture of barramundi, causing economic loss to fish farmers (Ransangan and Mustafa, 2009; Ransangan et al., 2012). To improve the immunocompetency, whilst supporting sustainability, there have been numerous studies to replace FM with plant-based proteins and animal-based proteins alone or supplemented with limiting nutritional factors or immunostimulants in barramundi (Ilham et al., 2016b; Ilham and Fotedar, 2017; Ilham et al., 2018; Siddik et al., 2019b). Also, a number of studies have been performed adding plant herbs to enhance disease resistance against bacteria in barramundi (Talpur, 2014; Talpur and Ikhwanuddin, 2012; 2013; Talpur et al., 2013). Limited studies are available, however, on the complete replacement of FM with the mixture of processed animal proteins such as PBM, HI larvae meal, and FPH on barramundi physiological and immunological responses in relation to final eating quality.

1.2 Aim and specific objectives

1.2.1 Aim

The study was aimed at evaluating the supplementation effects of HI larvae and various FPH valorised from fish waste and horticulture waste with PBM to investigate if the supplementation of HI larvae and FPH could complement PBM and improve the inclusion efficacy of PBM to replace FM exclusively or completely from the barramundi diet without impacting growth, health status, and fillet quality.

1.2.2 Specific objectives

The below specific objectives were set to achieve the aim:

I. To evaluate the effect of complete replacement of FM with PBM protein on growth, fillet fatty acid composition, hepatic health, gill and muscle structure, intestinal mucosal barrier function, and immune function of juvenile barramundi.

II. To investigate the efficacy of full-fat HI larvae supplementation with graded levels of PBM as a replacement of FM on growth, intestinal and skin mucosal barrier function, hepatic health, adipocyte cell size, serum metabolites, and immune function of barramundi.

III. To investigate the efficacy of complete replacement of FM with progressive inclusion level of full-fat HI larvae meal on growth, fillet fatty acid, and amino acid composition, gut mucosal barrier function and microbiota and immune function of juvenile barramundi.

IV. To evaluate the efficacy of full-fat vs defatted HI larvae as a complete replacement of FM on juvenile barramundi growth, fillet fatty acid, and amino acid composition, hepatic steatosis, serum metabolites, antioxidant capacity and immune function.

V. To evaluate the potential effect of the mixture of full-fat and defatted HI larvae meal and PBM on final product quality of juvenile barramundi in terms of sensory evaluation and other food aspects quality including physical analysis (drip loss, quality index, texture, colour, and microstructure of muscle) and chemical analysis (pH and rancidity production) during shelf life.

VI. To examine the efficacy of various FPHs supplementation in PBM on the growth, fillet fatty acid composition, spleen and kidney health, intestinal and skin mucosal barrier function, serum metabolites, and immune function of juvenile barramundi.

VII. To evaluate the effect of concurrent supplementation of full-fat HI larvae meal and /or tuna hydrolysate in PBM on juvenile barramundi growth, fillet quality, liver, kidney, and spleen health, mucosal barrier functions, gut microbiota, and immunity of juvenile barramundi.

1.3 Significance

The present research provides the first insight on the complete replacement of FM with a mixture of valorised animal protein ingredients in the diets of juvenile barramundi. The specific significances achieved from the present research are described as follows-

I. Transformation of fish waste and horticulture waste by bio-conversion and enzymatic hydrolysis into insect protein and FPH, with different functional properties will contribute to the long-term sustainable metric of aquaculture and also provide environmental advantages by reducing waste disposal pressure.

II. The toxic effect of a PBM-based diet on barramundi growth and health in the present finding will be important to feed manufactures in preventing the utilization of PBM as a sole protein source.

III. The complementary effect of full-fat HI larvae meal in PBM will provide a new strategy to replace FM completely from the diet of barramundi with an improved growth and physiology.

IV. The more beneficial effects of full-fat HI larvae than defatted HI larvae meal in PBM will reduce the additional labour and processing cost.

V. Full-fat HI and TH could be used as a prebiotic in the barramundi diet, as proven by improving the abundance of beneficial bacteria in the barramundi gut.

VI. Full-fat and defatted HI incorporation will provide a new solution to resolve blue-greyish in farmed barramundi, manifested by the enhancement of brightness in barramundi flesh in response to full-fat and defatted HI larvae meal supplemented PBM-based diets.

VII. Full-fat and defatted HI could be used as fish quality enhancers since the inclusion of full-fat and defatted HI with PBM enhanced the sensory attributes of barramundi fillet.

VIII. The immunostimulatory effect of full-fat HI larvae meal and various FPHs in PBM will support rigorous disease-resistant sustainable aquaculture and develop zero synthetic antibiotic-based aquaculture.

CHAPTER 2: LITERATURE REVIEW

The research efforts over the last 30 years have brought a revolution in the aquaculture sector by shifting reliance on FM and FO to predominantly terrestrial plant materials and animal by-products in the aquafeed formulation which could be named "Aquafeed 2.0" (Cottrell et al., 2021). This puts heavy dependence on terrestrial agriculture products which directly compete with human food streams (Colombo and Turchini, 2021). Besides, this product has certain nutritional challenges for farmed aquatic species and also their own sustainability issues, such as freshwater use, deforestation, areal footprint, pesticide and fertilizer use, irrigation, and polluting runoff (Colombo and Turchini, 2021). Now it is time to conceive, plan and develop "Aquafeed 3" that will use aquafeed ingredients orientating from circular bioeconomy which can improve aquaculture's sustainability by declining its environmental footprint (Colombo and Turchini, 2021). Insects and aquaculture processing by-products are good examples of aquafeed ingredients produced through a circular bioeconomy (Colombo and Turchini, 2021). Hence, the mixture of multiple ingredients produced through circular bioeconomy could be a novel strategy to develop "Aquafeed 3" which is the opportunity for sustainable and resilient aquaculture.

2.1 Opportunities and challenges of black soldier fly meal as a renewable protein source in aquaculture: a comprehensive review

(Submitted to Reviews in Aquaculture)

Abstract

Developing next-generation protein and lipid resources via circular economy frameworks that do not deplete natural resources and instead, may have positive impacts to help control climate change is the opportunity for sustainable and resilient aquaculture. Black soldier fly, *Hermetia illucens* (HI) larvae is a good example of such aquafeed ingredient that contains high protein and amino acids resemble fishmeal but higher than soybean meal. HI larvae has been found to have lauric acid, novel antimicrobial peptides, and polysaccharides such as silkrose, dipterose, and choline with anti-inflammatory and antimicrobial properties. These functional properties have made it a functional material in the aquafeed formulation. However, the main shortcoming of HI use in aquafeed is the presence of a high amount of saturated fatty acids and a negligible amount of essential polyunsaturated fatty acids, though the lipid profile of HI larvae meal can be manipulated. A good number of studies have been carried out to investigate the feasibility of the use of HI larvae meal in aquafeed. Hence, the present comprehensive review explored the current state of knowledge on the opportunities and challenges of dietary HI larvae meal on farmed aquatic animals in terms of growth, feed utilization, proximate composition, final product quality,

antioxidant responses, gut health, and immune responses, and disease resistance against pathogens. As well, this review pointed out the potentiality of using HI larvae as prebiotic in aquafeed. According to the findings of all studies discussed in this review, some species can rely on HI larvae-based diets but not true for other species. The improvement in fillet quality, immune responses, disease resistance against the pathogen, and ability to enhance beneficial bacteria signals the potentiality of using HI larvae as final product quality enhancers, immunostimulants, and prebiotic in aquafeed formulation for certain species.

Key words: Chitin, lauric acid, antimicrobial peptide, immunostimulation, prebiotic

2.1.1 Introduction

Global per capita food fish consumption is growing by about 1.5 percent per year (FAO, 2020). The estimated 20.5 kg in 2018 is almost twice the level when compared to the per capita consumption in 1961(9 kg) (FAO, 2020). In 2018, Aquaculture supplied 52 percent of fish for human consumption, an estimated 82 million tonnes valued at USD 250 billion (FAO, 2020). During the early development of the aquaculture industry (1980-1990), fishmeal and fish oil mainly sourced from wild forage fish were predominantly incorporated to formulate aquafeed because FM is a nutrient-dense and palatable source of protein and lipid compatible with the requirement of farmed fish (Turchini et al., 2019). However, due to stagnation in the global captures of wild forage fish and rapid expansion of aquaculture production over the past three decades (Turchini et al., 2019), there is a substantial shift towards agricultural crops such as soybean, canola, maize, wheat, and nuts occurred to formulate aquafeed by reducing reliance on marine ingredients (Colombo and Turchini, 2021). Agricultural crops other than forage fish in aquafeed have their own environmental sustainability concerns and directly compete with human food streams (Colombo and Turchini, 2021), even the use of plant protein in aquafeed can introduce a suite of problems such as imbalanced in essential amino acid and presence of biologically active antinational factors (Glencross et al., 2020). As a result, researchers and industry must develop innovative practices that will present new opportunities for next-generation nutritional resources for aquafeed. One such opportunity is the development of protein and lipid sources through the circular bioeconomy concept.

Insects are a good example of aquafeed ingredients produced through circular bioeconomy which have been appeared as one of the most economic, and environmentally sustainable protein source alternatives to terrestrial plant and animal protein for aquaculture and animal feeds (St-Hilaire et al., 2007; Surendra et al., 2016; Van Huis, 2013). Amongst seven insect species, recently authorised by a recent EU directive (Regulation (EU) No 2017/893) for use in aquafeeds

(Guerreiro et al., 2020), black soldier fly, *Hermetia illucens* (HI) belonging to Diptera of the Stratiomyidae family, is an important candidate due to the circular bioeconomy and environmental advantages. This species possesses the excellent capacity to valorise low-value organic waste or by-products into highly nutritious biomass whilst requiring less arable land and water consumption as well as reduced CO_2 production (Henry et al., 2015). The final biomass contains high protein and lipid but they can vary depending on the substrate and processing method (Wang et al., 2019b). Moreover, HI larvae are known to possess macro and micro-minerals and valuable vitamins as well as different functional molecules including choline, polysaccharides such as silkrose and dipterose, chitin, and antimicrobial peptides. Such products have been demonstrated to produce antioxidant and immunostimulatory properties in fish (Chaklader et al., 2021a; Chaklader et al., 2021b). Such positive attributes have made HI a novel and renewable protein source for animal feed, in particular for fish feed. A number of recent studies have reported chitin as a preferential substrate for beneficial bacteria in the intestine of farmed animals (Borrelli et al., 2017; Bruni et al., 2018; Huyben et al., 2019; Terova et al., 2019), but tolerance to chitin and its derivatives varies among fish species. Although HI larvae usually lack essential fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) together with a higher concentration of n-6 polyunsaturated fatty acids (PUFAs), these levels can be manipulated by choosing appropriate rearing substrate (Liland et al., 2017; Meneguz et al., 2018). It is noting also that medium-chain fatty acid, particularly lauric acid is predominant in HI larvae (Spranghers et al., 2017), exhibiting antimicrobial and anti-inflammatory properties (Henry et al., 2015; Spranghers et al., 2018). These multiple attributes have made HI larvae meal a potential ingredient in aquaculture and other livestock animal production.

Hence, this review is aimed at summarizing the influence of the different substrates on the nutritional quality of HI larvae meal and also, how different stages influence the meal quality. The main novelty of this review is to illustrate the feasibility of HI utilization in a circular bioeconomy context, focusing on a capacity to valorise organic waste into valuable animal biomass and reviewing opportunities and challenges in regard to the utilization in aquaculture diets. All the information relating to the review was sourced from the scientific databases Scopus® and Web of Science®, with emphasis on the following keywords: black soldier fly larvae meal, essential and non-essential amino acid, antimicrobial peptides, lipids, and fatty acids, aquaculture.

2.1.2 Nutritional composition of black soldier fly larvae

2.1.2.1 Protein and lipid

Insects are a good source of protein which is higher than soybean meal but often lower than FM (Barroso et al., 2014). HI larvae are rich in protein and lipid but the concentration is largely influenced by the substrate where they grow (Table 2. 1) and the stage of HI larvae (Figure 2. 1A, B). Crude protein varied from 29.9 % to 58.80% while crude lipid varied from 9.5 % to 49.00% in HI larvae meal, making this insect a potential ingredient in aquafeed. Lipid levels can be reduced by defatting which may allow an increase in the inclusion level of HI meal. The variability in protein and levels in HI depending on the substrates suggests the possibility of enriching protein and lipid by manipulating growth substrate.

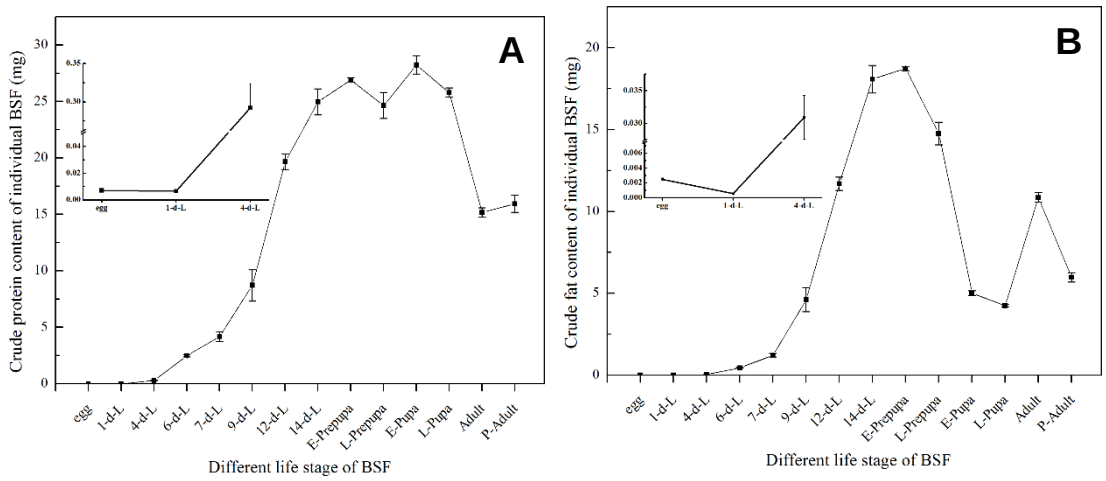


Figure 2. 1 Variations of crude protein (A) and fat content (B) in individual HI dry mass during life phases (Liu et al., 2017). All the results were calculated by dry mass. d-L: day-larvae. E-prepupa: early prepupa. L-prepupa: late prepupa. E-pupa: early pupa. L-pupa: late pupa. P-adult: postmortem adult.

Table 2. 1 Proximate composition of different stages of HI fed on various kind of substrates

Types of substrate	Proximate composition (%)				Authors
	Protein	Fat	Moisture	Ash	
Spent barley, spent malted barley, spent malted corn & spent sorghum and barley supplemented with water, brewer's yeast &	29.9-45.7	9.5-49.0	8.4-12.1	6.7-15.4	Chia et al. (2020)
Vegetables & fruits	41.88 & 30.75	-	-	-	Meneguz et al. (2018)
Winery and brewery	34.43 & 52.96	-	-	-	
Almond by-product	40.30-51.17	3.1-6.8	-	-	Palma et al. (2020)
Apple	31.12	36.1	-	-	Scala et al. (2020)
Banana	36.50	27.9	-	-	
Apple + Banana	35.60	33.4	-	-	
Spent gain	47.83	22.5	-	-	
Apple + spent gain	48.01	20.1	-	-	
Banana + spent gain	45.61	23.1	-	-	
Vegetable mixed	39.42	35.62		7.08	Cappellozza et al. (2019)
Hen diet	52.8			11.7	Bava et al. (2019)
Okara	51.2			5.91	
Maize distillers	53.4			4.94	
Brewer's grains	54.1			7.41	
Swine manure	43.6	33.1			St-Hilaire et al. (2007)
	52.00	11.30			Marono et al. (2015)

Animals acquired from different companies: Enviroflight LCC, OH, USA; Laboratory of Entomology, Wageningen University, the Netherlands.	51.8 58.80 58.4 49.90 50.50	11.30 12.9 29.00 28.40 24.90	
Organic waste from food designed for human consumption (different methods of obtaining the final product)	52.20 58.30	25.50 17.00	Lock et al. (2016)
Chicken feed Digestate Vegetable waste Restaurant waste	41.20 42.20 39.90 43.10	33.60 21.80 37.10 38.60	Spranghers et al. (2017)
Brewery solid waste, water, wheat bran, yeast slurry, processing wastes from fish feed factory	41.60	23.44	Devic et al. (2018)
Vegetable–fruit waste Fresh chicken manure	41.88 47.00	26.28 17.77	 Xiao et al. (2018)

2.1.2.2 Amino acid composition

The quality of protein for aquafeed is defined by the presence of ten balanced essential amino acids, which are available in FM at adequate levels with high digestibility, making FM the traditional primary protein source for aquafeed production. Insects have received attention over the years as alternative protein ingredients to FM because of a similar nutritional profile. However, the nutritional profile of insect meal is taxonomy dependent, as determined by the study of Barroso et al. (2014) which summarised the potentiality of 16 different insects belonging to three different taxonomic groups including Coleoptera, Diptera, and Orthoptera, and found that the essential and limiting amino acids in insects belonging to Diptera was superior to soybean meal and almost similar to FM (Figure 2. 2). Specifically, the essential and limiting amino acids in HI larvae were almost similar to FM though methionine was limiting in HI larvae (Figure 2. 3A). The methionine was higher in HI prepupae when compared to HI larvae, and higher than FM (Figure 2. 3B), suggesting the influence of the stage development (larvae, pupae, prepupae, and imago) on HI composition. The amino acids composition of different stages of HI larvae is summarized in Table 2. 2.

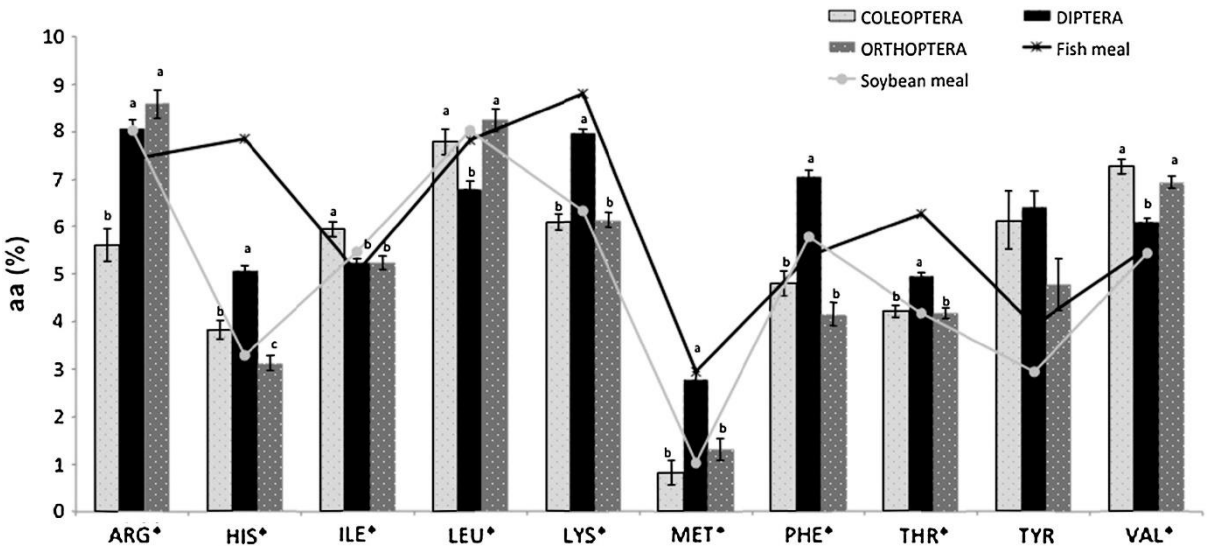


Figure 2. 2 Comparison in the essential amino acid composition of the different taxonomic groups of insects (Barroso et al., 2014) in comparison with commonly used ingredients such as FM and soybean meal in the aquafeed formulation.

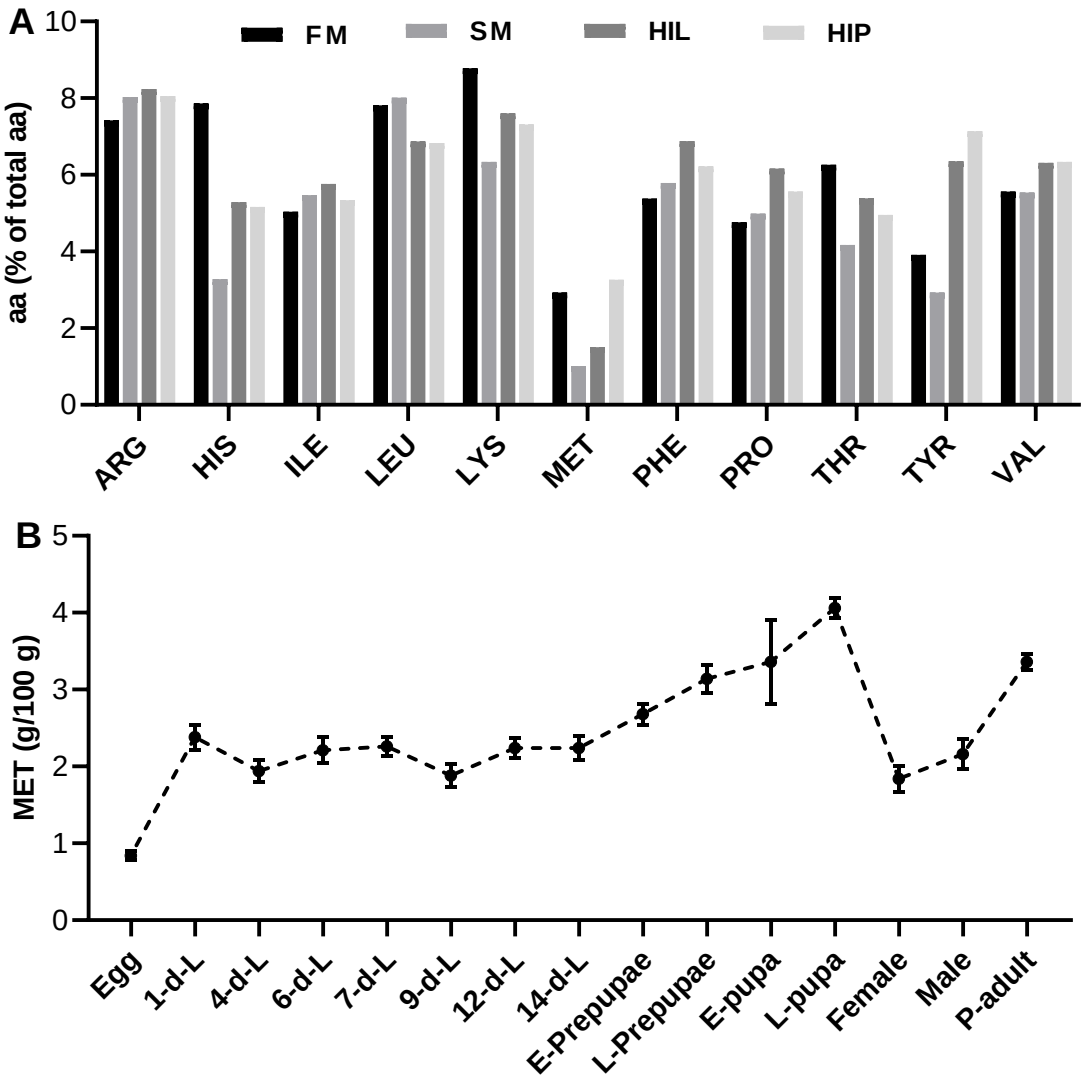


Figure 2. 3 Comparison in essential amino acid composition of HI larvae and prepupae in comparison with commonly used ingredients such as FM and soybean meal in aquafeed formulation (A) and fluctuation of methionine, a most limiting essential amino acid in HI meal depending on different stages (B).

Table 2. 2 Essential and non-essential amino acid composition of different stages of HI used for aquafeed production

	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Ala	Asp	Cys	Glu	Gly	Pro	Ser	Tyr	Tau	Authors
Larvae	1.65	0.88	1.48	1.9	2.12	0.63	1.48	1.34	0.77	1.90	2.45	3.18	0.25	4.25	1.53	1.92	1.23	1.84		Weththasinghe et al. (2021b)
Larvae	1.42	1.11	1.37	2.17	2.28	0.57	1.43	1.26	0.12	1.62	2.04	2.90	0.26	4.12	1.53	1.97	1.29	2.77		
Larvae	3.9	2.20	3.30	5.20	3.80	2.10	3.00	3.10		4.90	6.20	6.70	0.10	8.80	4.20	5.50	3.70	4.80		Renna et al. (2017)
Larvae	2.38	1.18	1.72	2.76	2.08	0.65	1.59	1.61		2.39								2.29		Wang et al. (2021a)
Larvae	5.47	3.25	4.73	7.83	6.82	2.12	7.76	4.43		6.79	8.21	7.30	0.76	13.1	6.15	6.68	4.88	6.71		Rawski et al. (2020)
Larvae	2.04	1.12	2.15	2.75	2.46	0.64	1.53	1.54	0.67	2.24	2.45	3.32	0.15		2.28					Abdel-Tawwab et al. (2020)
Larvae	3.00	1.90	2.20	4.20	3.80	1.20	2.30	2.40	0.9	3.7	3.70	5.90	0.80	6.40	3.50	3.40	2.40	3.80		Fisher et al. (2020)

Larvae	1.05	1.32	0.66	0.8	1.38	0.40	1.08	0.77		1.22	2.27	2.68		2.83	1.58	1.39	1.21	1.41		Melenchón et al. (2020)
Larvae	2.70	1.72	3.86	2.46	2.30	0.85	2.23	3.14		3.77	4.38	5.52		6.25	3.21	4.32	3.61	3.21		Guerreiro et al. (2020)
Larvae	4.12	2.73	3.86	6.24	5.42	1.24	3.45	3.57	1.31	5.35			0.80					7.04		Dietz Liebert (2018)
Larvae	3.17	1.53	1.96	3.12	3.07	0.87	1.95	1.76	0.75	3.32	3.14	4.25	0.26	5.08	2.36	2.63	1.81	3.12	0.03	Finke (2013)
Larvae	1.94	1.13	1.72	2.40	2.23	0.91	1.44	1.52	-	2.20	3.03	3.22	1.38	3.85	1.91	3.73	1.84	2.16	-	De Marco et al. (2015)
Larvae	2.65	1.62	2.23	3.61	3.72	1.16	2.35	2.24	-	3.78	3.34	5.28		5.57	2.63	5.55	2.02	3.40		Li et al. (2017a)
Larvae	2.15	1.23	1.85	2.86	2.10	0.65	1.66	1.72		2.72	3.45	3.72	0.01	4.87	2.35	3.06	2.03	2.64		Schiavone et al. (2017)
Larvae	2.70	1.63	12.40	3.67	2.52	0.86	2.18	2.18		3.45	4.37	4.88	0.02	6.37	3.03	3.27	2.68	3.41		

Larvae	2.00	1.18	1.84	2.9	2.70	0.75	1.75	1.72		2.63									Devic et al. (2018)
Larvae	2.19	1.06	1.92	3.00	2.79	0.63	1.79	1.67	0.54				0.42					0.37	Dumas et al. (2018)
Prepupae	2.03	1.36	1.72	2.86	2.34	0.76	1.70	1.64	0.67	2.41	2.52	3.78	0.25	4.19	2.26	2.25	1.66		Schiavone et al. (2017)
	2.03	1.35	1.84	2.95	2.57	0.87	1.87	1.68	0.62	2.49	2.43	3.36	0.24	3.98	2.56	2.21	1.55		
	2.00	1.24	1.73	2.80	2.26	0.76	1.63	1.54	0.58	2.48	2.42	3.59	0.21	4.13	2.22	2.14	1.50		
	1.99	1.38	1.91	3.06	2.30	0.71	1.64	1.62	0.54	2.82	2.78	3.69	0.22	4.58	2.52	2.51	1.59		
Prepupae	1.78	0.76	1.83	2.66	2.05	0.77	1.83	1.58		2.99	2.45	4.09		4.42	1.72		1.37	2.22	Sealey et al. (2011)
	1.89	0.82	1.89	2.72	2.12	0.79	1.82	1.60		3.06	2.55	4.14		4.17	1.80		1.42	2.57	
Prepupae	2.65	1.18	2.03	3.10	2.62	0.74	2.00	1.78		2.79	3.02	3.72		3.78	2.28	2.39	1.68	3.08	St-Hilaire et al. (2007)
Larvae	1.29	1.68	2.36	3.43	3.91	1.08	2.07	1.84	0.97	2.94	3.78	4.16	2.43	5.64	2.63	2.82	2.23	4.35	Adeoye et al. (2020)

Larvae	5.93	3.34	5.19	7.10	7.16	2.64	3.99	4.12		4.81			0.87						Kroeckel et al. (2012)
Larvae	3.44	1.42	2.64	3.25	4.80	1.62	2.59	2.91	0.84	3.10			0.49				1.89		Cummins et al. (2017)
Prepupae	5.01	3.05	4.94	7.22	4.80	1.93	3.98	4.59		5.97	7.73	9.30	2.43	11.43	5.07	5.91	7.13	5.81	0.15	Magalhães et al. (2017)

2.1.2.3 Fatty acid composition

The fatty acid profile in insects is more likely the reflection of the growth diet composition, suggesting the possibility to manipulate the fatty acid profile. The most abundant types of fatty acids in HI larvae meal is SFA (53.6 - 81.89%), in particular, lauric acid (C12:0) (21.00 - 60.89%), myristic acid (C14:0) (2.40 – 10.10%), palmitic acid (C16:0) (5.30 – 21.60%) and stearic acid (C18:0) (0.98 – 43.50%) (Table 2. 3), with variation depending on the stage and feeding substrate. Lauric acid, the predominant SFA in HI larvae meal has been reported to possess anti-inflammatory and immunostimulatory properties (Henry et al., 2015; Spranghers et al., 2018). Such properties may suggest that the inclusion of a smaller quantity of HI larvae meal or oil may improve the functionality of aquafeeds. However, a high abundance of SFA has been associated with pathological changes in the liver of fish for some (Bruni et al., 2020b; Zarantoniello et al., 2018) but not all species. The MUFA content is the second most abundant group due to the presence of palmitoleic acid (16:1n7) (1.60 -7.60%) and oleic acid (18:1n9) (5.66 – 22.70%) (Table 2. 3). In contrast, HI larvae contain a negligible amount of n-3 PUFA due to the absence of EPA and DHA (Table 2. 3), limiting use in aquafeed protein source alternatives to FM especially for seawater fish (Barroso et al., 2014). Nevertheless, lipid profile could be manipulated in HI larvae by the enrichment method, which is reflected in a number of studies in which adding fish offal, fish waste and corn bran, *Schizochytrium* sp and coffee by-product and brown algae *Ascophyllum nodosum* to the growth media enriched PUFA concentration of HI larvae (Agbohessou et al., 2021; Liland et al., 2017; Sealey et al., 2011; Zarantoniello et al., 2020b). The presence of a high concentration of 18:2n-6 increased the total amount of n-6 PUFA in HI larvae meal (Table 2. 3).

Table 2. 3 Fatty acid composition of different stages of HI

	12:0	14:0	16:0	18:0	20:0	SFA	16:1 n-7	18:1 n-9	MUFA	18:2 n-6	18:3 n-3	20:4 n-6	20:5 n-3	22:6 n-3	PUFA	n-3	n-6	n-3/n-6	Authors
Larvae	43.4	7.9	13.2	2.8	-	67.1	2.3	14.6	16.9	15.2	0.7	-	-	-	15.9	0.7	15.2		Barroso et al. (2014)
Prepupae	-	2.4	16.6	43.5	-	65.8	2.2	-	32.6	-	-	-	-	-	1.1	-	1.1		
Larvae	36.1	8.75	21.6	2.81	-	69.6	2.85	14.8	17.8	10.6	0.72	-	-	-	11.63	1.03	10.6	0.10	Guerreiro et al. (2020)
Larvae	42.91	8.33	14.74	2.69			2.44	15.35		8.87	0.06								Melenchón et al. (2020)
Defatted larvae	51.75	5.93	9.75	0.08						8.35	0.09	-	0.02	-					Abdel-Tawwab et al. (2020)
Larvae	21.00	3.70	5.30	0.80			1.60	6.10		3.80	0.50	-	-	-					Rawski et al. (2020)
Larvae	47.00	6.50	15.00	2.2		71.6		14.00	17.3	9.4	0.8	<0.1	<0.1	<0.1	10.2				Kroeckel et al. (2012)

Larvae	54.59	10.14	12.03	1.77	0.10	80.28			12.88	5.98	0.79	-	-	-	6.84	0.79	6.05	0.13	(Renna et al., 2017)
Larvae	28.58	5.48	16.15	2.95		53.16	2.11	18.87	21.32	22.11	2.04	0.03	0.08	0.01	25.53	2.93	22.59		Zhou et al. (2018)
Prepupae	23.60	5.10	19.80	6.50		55.00	6.30	22.70	29.00	6.80	-	-	0.10	1.70	6.90	0.10	6.80	0.01	Sealey et al. (2011)
	37.10	6.30	17.30	2.00		62.70	7.60	18.80	26.40	5.90	0.50		3.50	0.10	12.10	6.20	5.90	1.05	
Larvae	36.337	6.25	14.71	2.37		59.7	2.01	16.13	19.8	19.33	0.15	0.25	-	-	19.73	0.15	19.58	0.01	Li et al. (2016b)
Prepupae	57.35	7.34	9.65	1.36		77.13	1.97	7.54	9.51	11.55	0.70		0.60	0.10	13.45	1.90	11.55	0.16	Spranghers et al. (2017)
	43.65	6.87	10.12	1.75		63.56	7.58	7.93	15.51	7.90	0.83		0.11	0.20	9.69	1.79	7.90	0.23	
	60.89	9.48	8.70	1.11		81.81	2.93	5.66	8.59	4.52	1.37		0.01	0.01	6.78	2.26	4.52	0.50	
	57.56	7.14	10.29	0.98		78.00	3.34	7.97	11.43	7.83	1.10		0.23	0.01	9.67	1.84	7.83	0.23	
Larvae	52.60	8.54	10.9	1.53		75.00	1.98	6.16	8.14	11.6	1.03	0.29	-	-	12.60	1.01	11.89	0.08	Schiavone et al. (2017)

Larvae	40.60	8.50	14.80	2.50	66.40	2.00	8.80	10.80	17.90	1.40			19.30	1.40	17.90	0.08	Liland et al. (2017)
	23.90	6.70	16.60	4.10	51.30	2.50	17.90	20.40	18.60	1.60	2.10	1.00	23.30	2.60	20.70	0.13	
Larvae	50.16	8.82	15.39	1.80	77.57	3.85	10.62	15.01	7.13	0.12	0.02	0.00	0.00	0.7	7.14	0.09	Agbohessou et al. (2021)
	43.93	8.49	13.71	2.77	69.73	2.28	12.97	15.41	13.58	1.19	0.05	0.00	0.00	1.23	13.62	0.08	
	43.35	9.13	15.00	3.09	71.26	2.79	14.81	18.20	8.29	0.23	0.03	1.38	0.37	2.21	8.32	0.26	

2.1.2.4 Vitamins and minerals

Vitamins and minerals are key nutrients in feed ingredients. The vitamin profile in HI larvae is relatively similar to other insects (Finke, 2013). Most of the water-soluble B vitamins are adequately present and compatible with the requirements of animals (Finke, 2013). Similar to other insects, fat-soluble vitamins A (retinol), D3 (cholecalciferol), and E (α-tocopherol) in HI are deficient and reported less than 50% of the NRC requirements (Finke, 2013). Fat-soluble vitamin levels can be manipulated by changing the feeding substrate. For example, Liland et al. (2017) reported that HI larvae grown on seaweed, *Ascophyllum nodosum*-enriched media produced more than sixfold vitamin E (248.8 mg/kg) when compared to larvae grown on processed wheat (53.3 mg/kg).

HI larvae are characterized by different minerals including Ca, Cu, Mg, Na, Mn, Fe, and Zn which are within fish nutritional requirements (Chia et al., 2020; Davis and Gatlin III, 1996). Ca and P are the predominant macro minerals in HI larvae which are often deficient in animal feed and needs to be supplemented to meet requirements (Chia et al., 2020; McDowell, 2003; Suttle, 2010). The Ca/P ratio of HI is similar to the Ca/P ratio of FM and higher than other insects and soybean meal. However, the Ca/P ratio has been reported to be impacted by feeding substrates. For example, utilization of brewer's yeast supplemented with brewers' spent grain yielded lower Ca/P values in HI larvae meal than those reared on substrates supplemented with water or brewer's yeast plus molasses (Chia et al., 2020).

2.1.2.5 Antimicrobial peptides

Currently, over 1500 proteins with antimicrobial peptides (AMPs) have been reported in a variety of organisms including plants, fungi, bacteria, and animals (Bulet et al., 1999; Yi et al., 2014). Biologically active AMPs are very common in insects, stimulating the specific innate immune responses and exerting immunomodulatory effects resulting in antimicrobial activity in livestock animals (Jozefiak and Engberg, 2017). HI larvae, as an ecological decomposer, are often exposed to a relatively high concentration of a variety of microorganisms such as bacteria, viruses, and fungi, enhancing activation of the innate immune system to produce various functional components, such as AMPs to protect against microbial infection (see summary in Table 2. 4). Elhag et al. (2017) screened and purified seven new gene fragments including ecropinZ1, sarcotoxin1, sarcotoxin (2a), sarcotoxin (2b), sarcotoxin3, stomoxynZH1, and stomoxynZH1(a) from fifth instar HI larvae, with three types of AMPs and 189-basepair gene (stomoxynZH) among seven genes showed inhibitory activity against bacteria and fungi. Similarly, Park et al. (2015) identified defensin-like peptide4, a new AMP of 40 amino acids purified from the immunized hemolymph of HI larvae with bactericidal activity against bacteria. Hence, HI larvae due to the presence of such natural antibacterial peptides could provide a novel solution to synthetic antibiotic resistance bacteria in aquaculture or livestock production.

Table 2. 4 Potential antimicrobial peptides extraction from HI.

Peptide	Amino Acid Sequence	Immune-Induced Strains	Authors
Defensin			
Defensin-like peptide 1 (DLP1)	MRSVLVLGLIVAAFAVYTSAQPYQLQ YEEDGLDQAVELPIEEEQLPSQVVEQH YRAKRATCDLLSPFKVGHAACALH CIALGRRGGWCDGRAVCNCRR	*Staphylococcus aureus* KCCM 40881	Park et al. (2015)
Defensin-like peptide 2 (DLP2)	MRSILVLGLIVAAFAVYTSAQPYQLQY EEDGPGYALELPSEEEGLPSQVVEQH YRAKRATCDLLSPFKVGHAACALH CIAMGRRGGWCDGRAVCNCRR	*Staphylococcus aureus* KCCM 40881	Park et al. (2015)
Defensin-like peptide 3 (DLP3)	MRSILVLGLIVAVFGVYTSAQPYQLQ YEEDGPEYALVLPIEEEELPSQVVEQH YRAKRATCDLLSPFGVGHAACAVHC IAMGRRGGWCDDRAVCNCRR	*Staphylococcus aureus* KCCM 40881	Park et al. (2015) and Park Yoe (2017)
Defensin-like peptide 4 (DLP4)	MVHCQPFQLETEGDQQLEPVVAEVD DVVDLVAIPEHTREKRATCDLLSPFK VGHAACAAHCIARGKRGGWCDK RAVCNCRK	*Staphylococcus aureus* KCCM 40881	Park et al. (2015)
Defensin 1 (HiDef1)	unknown	*Lactobacillus casei*	Lee et al. (2020)
Cecropin			
CecropinZ1	GWLKKIGKMKFILGTTLAIVIAIFGQCQ	*Escherichia. coli* and *Staphylococcus aureus*	Elhag et al. (2017)

	AATWSYNPNGGATVTWTANVAATAR		
Cecropin 1 (Hicec1)	unknown	*Lactobacillus casei*	Lee et al. (2020)
Cecropin-like peptide 1 (CLP1)	MNFTKLFVVFAVVLVAFAGQSEAGWRKR VFKPVEKFGQRVRDAGVQGIAIAQQGA NVLATARGGPPQQG	*Staphylococcus aureus* KCCM 40881	Park Yoe (2017)
Cecropin-like peptide 2 (CLP2)	MNFAKLFVVFAIVLVAFSGQSEAGWWKR VFKPVEKLGQRVRDAGIQGLEIAQQGAN VLATARGGPPQQG	*Staphylococcus aureus* KCCM 40881	Park Yoe (2017)
Cecropin-like peptide 3 (CLP3)	MNFTKLFVVFAVVLIAFSGQSEAGWW KRVFKPVERLGQRVRDAGIQGLEIAQ QGANVLATVRGGPPQQG	*Staphylococcus aureus* KCCM 40881	Park Yoe (2017)
Attacin			
Hermetia illucens-attacin	MASKFLGNPNHNIGGGVFAAGNTRS NTPSLGAFGTLNLKDHSLGVSHTITPG VSDTFSQNARLNILKTPDHRVDANVFN SHTRLNNGFAFDKRGGSLDYTHRAG HGLSLGASHIPKFGTTAELTGKANLW RSPSGLSTFDLTGSASRTFGGPMAG RNNFGAGLGFSHRF	*Escherichia. coli* KCCM 11234	Shin Park (2019)
Sarcotoxin			
Sarcotoxin1	GWLKRKIGMKFILGTTLAIVVAIFGQCQA ATWSYNPNGGATVTWTANVAATAR	*Escherichia. coli* and *Staphylococcus aureus*	Elhag et al. (2017)

Sarcotoxin (2a)	GWLKRKIGKKFILGTTLAIVVAIFGQCQAA TWSYNPNGGATVTWTANVAATAR	*Escherichia. coli* and *Staphylococcus aureus*	Elhag et al. (2017)
Sarcotoxin (2b)	GWLKRKIGKKFILGTTLAIAVAIFGQCQA ATWSYNPNGGATVTWTANVAATAR	*Escherichia. coli* and *Staphylococcus aureus*	Elhag et al. (2017)
Sarcotoxin3	GWLKRKIGMMMKNSNFNSTEEREAAKK NYKRKYVPWFSGANVAATAR	*Escherichia. coli* and *Staphylococcus aureus*	Elhag et al. (2017)
Stomoxyn			
StomoxynZH1 (a)	RGFRKHFNNLPICVEGLAGDIGSILLGVG SDIGALAGAIANLALIAGECAAQGEAG AAVVAAT	*Escherichia. coli* and *Staphylococcus aureus*	Elhag et al. (2017)

2.1.2.6 Chitin

Aquatic insects in the adult and/or larval stages belonging to Diptera, Trichoptera, Odonata, Hemiptera, Coleoptera, and Ephemeroptera are the prey of many omnivorous and carnivorous fish species (Abilhoa et al., 2016; Badola and SIngh, 1980; Costa and Soares, 2015; Henry et al., 2015; Neves et al., 2015; Nogales-Mérida et al., 2018; Ribeiro et al., 2014; Severo-Neto et al., 2015). The exoskeleton of insects is built primarily of chitin fibres, a polysaccharide of glucosamine and N-acetylglucosamine having strong linkage with proteins/AAs, thereby making proteins unavailable for digestion (Jonas-Levi and Martinez, 2017; Nogales-Mérida et al., 2018). However, many fish species naturally consume shrimp, crabs, crayfish, and insects, indicating the presence of chitinolytic activity from enzymes including chitinase and chitobiase in the gastrointestinal tract (GIT) of fish to digest proteins/AAs bound with chitin (Gutowska et al., 2004; Lindsay and Gooday, 1985). It could be hypothesized that the chitin in HI comprised of 9.5% for larvae, 9.1% for prepupae, 10.3% for pupae 31.1% for HI shedding, 23.8% for HI cocoon, and 5.6% for HI flies (Soetemans et al., 2020), respectively should be digested by those fish that consume insect as a natural part of their diet. These chitinase activities have been associated with beneficial effects on the fish host body. For example, up to 2 g/kg of chitin improved the growth and feed efficiency coupled with enhancing the villus and microvillus length in the intestine of sea bass (Zaki et al., 2015). Also, feeding chitin from shrimp meal decreased the abundance of *Escherichia coli* and caecal *Salmonella* sp with an increase in *Lactobacillus* in broilers (Gutowska et al., 2004; Lindsay and Gooday, 1985). Recently, it has been reported that chitin in HI larvae or pupae works as a preferential substrate for enhancing the beneficial bacteria, particularly, lactic acid bacteria in the intestine of fish (Bruni et al., 2018; Terova et al., 2019). However, it is important to optimize the inclusion levels of HI larvae in the fish diet since a high amount of chitin may work as an anti-nutritional factor.

2.1.3 Effect on growth performance and digestibility

Tolerance to HI larvae meal inclusion varies considerably among various fish species, as summarized in Table 2. 5. HI larvae meal haemolymph is known to contain free amino acids and also have high levels of low molecular weight protein, suggesting a higher proportion of free amino acids which have been considered as fish attract (Kanost, 2009; Mintah et al., 2020). Amino acids in particular alanine and valine are higher in HI larvae than FM, reported to act as a strong taste stimulant for Siberian sturgeon, *Acipenser baerii* (Kasumyan, 2018; Rawski et al., 2020). Hence, the inclusion of full-fat HI larvae meal (5-30%) increased the growth performance of Siberian sturgeon with improved protein conversion ratios and feed acceptance rates (Rawski et al., 2020). Similarly, there were no negative effects of 15% inclusion of full-fat HI larvae meal in sturgeon

(Józefiak et al., 2019). However, negative results in terms of growth and digestibility were reported in sturgeon when added at more than 18.5% defatted HI larvae meal (Caimi et al., 2020b). Similar to the response of sturgeon to full-fat HI larvae meal, growth performance in zebrafish increased when fed full-fat prepupae meal for short-term feeding trials (Lanes et al., 2021; Zarantoniello et al., 2020b) but decreased when fed for longer periods (Zarantoniello et al., 2019).

Meanwhile, growth performance and body indices were unaffected in clownfish, *Amphiprion ocellaris* fed defatted larvae meal (25-75%) (Vargas-AbúNdez et al., 2019), Atlantic salmon, *Salmo salar* fed full-fat HI larvae meal (33-100%) (Belghit et al., 2019b; Stenberg et al., 2019), rainbow trout, *Oncorhynchus mykiss* Walbaum fed defatted larvae meal (25 & 50%) (Renna et al., 2017), Jian carp, *Cyprinus carpio* var. Jian fed full-fat and defatted larvae meal and oil (25-100%) (Li et al., 2017a; Li et al., 2016b; Zhou et al., 2018), Nile tilapia, *Oreochromis niloticus* fed full-fat HI larvae (10-100%) (Tippayadara et al., 2021), European sea bass, *Dicentrarchus labrax* fed full-fat HI larvae meal (25-50%) (Abdel-Tawwab et al., 2020) and Japanese seabass, *Lateolabrax japonicus* fed larvae meal (16-64%) (Wang et al., 2019b). Fisher et al. (2020) reported that up to 20% full-fat larvae meal could be included in salmon without affecting growth and feed utilization, which contradicts the aforementioned studies on salmon (Belghit et al., 2019b; Stenberg et al., 2019). Another study was able to include up to12.5% full-fat HI larvae meal and 6.7% HI larvae meal paste in salmon without affecting growth, however, 25% full-fat HI larvae inclusion retarded the growth performance (Weththasinghe et al., 2021a).

It is worthy to mention that HI larvae oil reduced VSI and IFI in Jian carp due to the high share of lauric acid that enhanced the lipid deposition by reducing the intraperitoneal fat size (Li et al., 2016b). Likewise, meagre, *Argyrosomus regius* can tolerate up to 20% defatted HI larvae meal with an apparent negative effect on growth but body indices such as VSI and HSI were unaffected by any inclusion levels (Guerreiro et al., 2020). Catfish, in particular, African catfish, *Clarias gariepinus* fed 50% defatted HI larvae meal (Fawole et al., 2020) and yellow catfish, *Pelteobagrus fulvidraco* fed 48% full-fat HI larvae meal (Xiao et al., 2018) showed an increase in growth performance whilst > 50% inclusion of HI larvae impacted the growth of both catfish. In contrast, the inclusion of up to 40% HI meal showed similar growth and FCR but 60% inclusion impacted the growth and FCR in Eurasian Perch, *Perca fluviatilis* (Stejskal et al., 2020). The possible reasons for the extensive variability in the growth performance of fish in response to HI larvae meal could be due to different fish species, culture systems, stage of HI larvae meal, the different substrates used for HI larvae culture, and chitin share. It has been reported that tolerance to HI larvae chitin, increased by defatting varied greatly among various fish species thereby influencing the digestibility.

The growth of fish with alternative protein ingredients is largely influenced by the presence of digestible nutrients. The nutritional content of HI meal was more digestible than commercially used plant-based protein sources such as corn protein concentrate (CPC) and SBM (Fisher et al., 2020). Dry matter and gross energy and mineral digestibility in HI meal were higher than CPC and SBM (Fisher et al., 2020). HI is rich in phosphorus, magnesium, calcium, manganese, and zine which may explain greater mineral digestibility than plant-based protein sources (Fisher et al., 2020). The apparent digestibility coefficients (ADC) of HI meal differed among various studies in Atlantic salmon. For instance, the digestibility of crude protein and lipid was over 80% in sweater cultured salmon (Belghit et al., 2019b) and 90% in freshwater cultured salmon (Belghit et al., 2018). However, a gradual increase of inclusion level of full-fat HI larvae meal and full-fat HI larvae paste linearly decreased the digestibility of protein and lipid in salmon (Weththasinghe et al., 2021b). Similarly, ADC of crude protein, lipid and ash, and essential and non-essential AAs decreased 1-3% in freshwater salmon fed HI larvae meal and oil (Belghit et al., 2018) but was comparable to or even higher than the digestibility of other alternative protein sources including wheat gluten meal, bacterial protein meal or poultry by-product meal fed to salmon (Aas et al., 2006; Burr et al., 2012; Storebakken et al., 2000).

However, complete replacement of FM with HI meal did not affect the ADC of protein, lipid, amino acids, and fatty acids in salmon (Belghit et al., 2019b; Lock et al., 2016). Digestibility of crude protein and AAs, except for hydroxyproline, in another salmonid, rainbow trout fed HI larvae meal and HI oil showed no variation, though digestibility of dry matter and lipid was low in HI larvae meal fed rainbow trout (Dumas et al., 2018). It is important to note that hydroxyproline, a limiting or low AA in plant products used aquafeed formulation (Aksnes et al., 2008), from HI larvae meal and oil was better digested in rainbow trout. The chitin and lauric acid, two abundant nutrients HI larvae and oil have been reported to stimulate the hydrolysis and absorption of proline and hydroxyproline in the small intestine by promoting prolidase activity (Wu et al., 2011). The superior digestibility of hydroxyproline from HI larvae meal and oil may reduce the supplementation of vitamin C in a low FM diet to convert proline to hydroxyproline (Brody, 1999) and is also associated with connective tissue health, in particular, skin, cartilage, and bone in vertebrates (Aksnes et al., 2008; Wu et al., 2011). In contrast, ADC of dry matter and crude protein decreased in rainbow trout fed 50% HI larvae meal while ADC of ether extract and gross energy were unchanged by 25 and 50% of HI larvae meal (Renna et al., 2017). The discrepancies in the digestibility of HI larvae nutrients among salmonids might be influenced by the diet formulation, culture environment, and size difference of salmon. There was a general increase in the ADC of protein, lipid, dry matter, organic matter, and energy, with a concomitant

increase in the ADC of arginine, histidine, and valine in European seabass fed prepupae meal (Magalhães et al., 2017). Similarly, dietary inclusion of graded level full-fat HI larvae meal (5-30%) did not affect the crude protein and fat digestibility in Siberian sturgeon (Rawski et al., 2020) while the inclusion of highly defatted HI larvae meal (25 and 50%) negatively impacted the crude protein digestibility in the same species (Caimi et al., 2020b). The variable results in the same species suggested that defatted HI larvae meal may increase the chitin share and consequently decrease the protein digestibility. Similarly, defatted HI larvae (17-76%) decreased the ADC organic matter, crude protein, crude lipid, and gross energy and it was mainly due to the absence of chitinase activity or chitinolytic active bacteria in the midgut of turbot (Kroeckel et al., 2012).

A common discussion point for the worst digestibility is the presence of chitin, a long-chain polymer of an N-acetylglucosamine commonly found in the exoskeleton and endoskeleton of all insects, including HI meal (Finke, 2007; Fisher et al., 2020). It has been reported that HI meal contains around 1.6% to 7.3% chitin (Craig Sheppard et al., 1994; Diener et al., 2009) which have been reported to have a negative correlation with CP digestibility (Renna et al., 2017). The proteins, lipids and other compounds strongly linked to chitin existed in insect cuticles (Chapman and Chapman, 1998; Kramer et al., 1995) may reduce the access and activity of digestive enzymes, in particular, proteolytic enzymes such as the brush border enzyme, leucine aminopeptidase that break down peptides into AA (Belghit et al., 2018) and lipase enzyme by reducing bile acid levels in the pylorus that helps to absorb lipid efficiently (Hansen et al., 2010). These disruptions in enzymatic activity may thus negatively impact the ADC of protein and lipid in the above-mentioned studies, suggesting that chitin is an energy sink. The presence of chitin in HI meal does not necessarily mean that dietary inclusion of HI meal exclusively are not digestible and reduce fish performance since chitin content can be modified by culture methods and processing methods which may be advantageous in removing indigestible nutrients such as chitin from HI meal (Belghit et al., 2018; Fisher et al., 2020). For example, the mantle was separated from HI biomass before processing into protein meal and oil resulting in a low amount of chitin in HI meal which allowed the addition of 600 g kg^{-1} HI meal in the salmon diet without affecting growth performances (Belghit et al., 2018).

Table 2. 5 Growth, biometry indices and feed utilization of different fish species fed different stage of HI larvae

Common name	Scientific name	Stage of BSF	Inclusion levels (%)	Duration (days)	Growth and body indices	Authors
Juvenile barramundi	*Lates calcarifer*	Defatted larvae meal	0, 30 meal & oil	42	FBW, WG, FCR, SGR & SR↕ when % inclusion ↑	Hender et al. (2021)
Hybrid tilapia	*Oreocromis niloticus* x *O. mozambique*	black soldier fly larvae frass	0. 5, 10 & 20	84	↑WG & ↕SR when % inclusion ↑ ↕FI, ↑PER & ↑APU when % inclusion ↑	Yildirim-Aksoy et al. (2020)
Rice field eel	*Monopterus albus*	Full-fat prepupae meal	0, 5.26, 10.52 & 15.78	70	↑FBW, WG & FCR when % inclusion ↑ ↕HSI, ↕VSI & ↑CF when % inclusion ↑	Hu et al. (2020)
Juvenile grass carp	*Ctenopharyngodon idellus*	Defatted larvae meal	0, 25, 50, 75 & 100	56	↕FBW, WG, SGR, SR & FCR when % inclusion ↑ ↕CF & VSI when % inclusion ↑ but ↑HSI at >25%	Lu et al. (2020)
Juvenile Pacific White Shrimp	*Litopenaeus vannamei*	Defatted larvae meal	0, 4.5, 7.5 & 10.5	28	FBW, WG, SGR↑ & FCR↓ when % inclusion ↑	Richardson et al. (2021)
Rainbow trout	*Oncorhynchus mykiss*	Full-fat larvae meal	0, 15 & 30	46	↕FBW, SGR, DFI & FCR when % inclusion ↑ ↕CF, HSI & VSI when % inclusion ↑	Melenchón et al. (2020)

African catfish	*Clarias gariepinus*	Defatted larvae meal	0, 25, 50 & 75	60	↑FBW, WG, SGR, PER, FCR & PPV at 50% ↕HSI, VSI & DP	Fawole et al. (2020)
Meagre	*Argyrosomus regius*	Defatted larvae meal	0, 10, 20 & 30	63	↕FBW & DGI at up to 20% but ↓FBW & DGI at 30% ↕HSI & VSI when % inclusion ↑	Guerreiro et al. (2020)
Zebrafish	*Danio rerio*	Full-fat prepupae meal	0, 25, 50, 75 & 100	21	↑ SGR when % inclusion ↑	Zarantoniello et al. (2020b)
Atlantic salmon	*Salmo salar*	Full-fat larvae meal	0, 10, 20 & 30	112	↕FBW, WG, SGR & TGC at up 20% but ↓FBW, WG, SGR & TGC at 30% ↕FC but ↑FCR at 30%	Fisher et al. (2020)
European sea bass	*Dicentrarchus labrax*	Full-fat Larvae meal	0, 25, 35 & 50%	56	↕FBW, WG, SGR & FI when % inclusion ↑ ↕CF, HSI, VSI, SSI & KSI when % inclusion ↑	Abdel-Tawwab et al. (2020)
Atlantic salmon	*Salmo salar*	Full-fat larvae meal & paste	0, 6.25, 12.5 & 25 3.7 & 6.7%	42	↕FBW & SGR at up to 12.5 % but ↓FBW & SGR at 25% inclusion of full-fat BSFL ↕FBW & SGR for paste ↕FCR & FI	Weththasinghe et al. (2021a)
Eurasian Perch	*Perca fluviatilis*	Defatted larvae meal	0, 20, 40 & 60	84	↕FBW, WG & SGR at up to 40% but ↓FBW, WG & SGR at 60%	Stejskal et al. (2020)

					↕FCR & FR at up to 40% but ↑FCR & FR at 60% ↕CF, SSI, VSI & PFL but ↓HSI at 60%	
Siberian Sturgeon	*Acipenser baerii*	Full-fat Larvae meal	0, 5, 10, 15, 20, 25 & 30	50	↑BW, BWG, SGR & WG when % inclusion ↑ ↕FI, ↓FCR & ↑PER when % inclusion ↑	Rawski et al. (2020)
Siberian Sturgeon	*Acipenser baerii*	Full-fat Larvae meal	0 & 15%	60	↕FBW & SR	Józefiak et al. (2019)
Nile Tilapia	*Oreochromis niloticus*	Full-fat Larvae meal	0, 10, 20, 40, 60, 80 & 100	84	↕FBW, DWG, WG, SGR, RGR, HPA & SR when % inclusion ↑ ↕FI, FCR & FE when % inclusion ↑ ↕FTL, CF, HSI & VSI when % inclusion ↑	Tippayadara et al. (2021)
Siberian Sturgeon	*Acipenser baerii*	Full-fat Larvae meal	0, 5, 10, 15, 20, 25 & 30	50	↑CF, ↕HSI & ↑VSI when % inclusion ↑	Rawski et al. (2021)
zebrafish	*Danio rerio*	Defatted larvae & prepupae meal	0 & 100	60	↑FTL, FSl, FBW, WG, DGR, SGR & CF at 100%	Lanes et al. (2021)
Siberian sturgeon	*Acipenser baerii*	Defatted larvae meal	0, 25, 50 & 100	118	↓FBW, WG & SGR when % inclusion ↑	Caimi et al. (2020b)

					↓FC, ↕FCR & ↕PER when % inclusion ↑ ↕CF, HSI & VSI when % inclusion ↑	
Japanese seabass	*Lateolabrax japonicus*	Larvae meal	0, 16, 32, 48 and 64	56	↕FBW, WG, SGR & SR when % inclusion ↑ ↑FI & ↕FCR when % inclusion ↑ ↕CF, HSI & VSI when % inclusion ↑	Wang et al. (2019b)
Yellow catfish	*Pelteobagrus fulvidraco*	Full-fat Larvae meal	0, 13, 25, 37, 48, 68, 68, 85 & 100	65	↑WGR, ↑SGR & ↕FCR at up to 48% but ↓WGR, ↓SGR & ↑FCR at 85 & 100% ↕CF, HSI & VSI when % inclusion ↑	Xiao et al. (2018)
Nile Tilapia	*Oreochromis niloticus*	Defatted larvae meal	0, 25, 50 & 100	56	↕FBW when % inclusion ↑ ↕SGR, FCR & PER at up to 50% but ↓SGR, FCR & PER at 100%	Dietz Liebert (2018)
Rainbow Trout	*Oncorhynchus mykiss*	Normal & enriched prepupae meal	0, 25 & 50	56	↓ WG when % inclusion of normal HI↑ but ↕WG when % inclusion of enriched HI↑ ↕FCR & FC when % inclusion of normal & enriched HI↑ ↓IFR & HSI when % inclusion of normal & enriched HI↑	Sealey et al. (2011)

Zebrafish	*Danio rerio*	Full-fat prepupae meal	0, 25 & 50	180	↓FBW at 50% but ↕FBW at 25%	Zarantoniello et al. (2019)
Barramundi	*Lates calcarifer*	Full-fat larvae meal	0, 25, 50, 75 & 100	56	↓WG &SGR when % inclusion ↑ ↑FCR at 100%	Katya et al. (2017)
European seabass	*Dicentrarchus labrax*	Prepupae meal	0, 15, 30 and 45%	62	FBW, WG, DGI, FI, FE & SR↕ but PER↓ when % inclusion ↑	Magalhães et al. (2017)
Atlantic salmon	*Salmo salar*	Full-fat larvae meal	25, 50 & 100 (IM A) and 25 & 100 (IM B)	105	↓FBW at A100, B25 & B100 ↓FCR at A50, A100 & B25 but ↑FCR at B100 ↓CF at B100 but ↑HSI & VSI at A100, B25 & B100	Lock et al. (2016)
Turbot	*Psetta maxima*	Prepupae meal	0, 17, 33, 49, 64 & 76%		FBW↓, SGR↓ & FCR↑ when % inclusion ↑	Kroeckel et al. (2012)
Jian carp	*Cyprinus carpio* var. Jian	Larvae oil	0, 25, 50, 75 & 100	59	↕FBW& SGR when % inclusion ↑ ↕FCR & FI when % inclusion ↑ ↕CF, ↓VSI, ↕HSI & ↓IFI when % inclusion ↑	Li et al. (2016b)
Nile tilapia	*Oreochromis niloticus*	Full-fat larvae meal	0, 30, 50 & 80	32	↕FBW, WG, SGR, FCR & PER when % inclusion ↑	Devic et al. (2018)

Yellow Catfish	*Pelteobagrus fulvidraco*	Full-fat meal	larvae	0, 10, 15, 20, 25 & 30	56	↓FBW & WGR when % inclusion ↑ ↑FCR at 100%	Hu et al. (2017)
Atlantic salmon	*Salmo salar*	Full-fat meal	larvae	0, 66 & 100	56	↕FBW, SGR, FCR, FI, HSI & VSI when % inclusion ↑	Stenberg et al. (2019)
Channel catfish	*Ictalurus punctatus*	Larvae frass		0, 5, 10, 20 & 30	70	↑FBW, WG, FI at 10, 20 & 30%	Yildirim-Aksoy et al. (2020)
Jian carp	*Cyprinus carpio* var. Jian	Full-fat meal	larvae	0, 25, 50, 75 & 100	56	↕FBW, WG, SGR, FI & FCR when % inclusion ↑ ↕CF, IFI,KI, VSI & SI but ↑HSI when % inclusion ↑	Zhou et al. (2018)
Jian carp	*Cyprinus carpio* var. Jian	Defatted meal	larvae	0, 25, 50, 75 & 100	59	↕FBW, WG, SGR, FI & FCR when % inclusion ↑ ↕CF, IFI,KI, VSI & SI but ↑HSI when % inclusion ↑	Li et al. (2017a)
Rainbow trout	*Oncorhynchus mykiss* Walbaum	Defatted meal	larvae	0, 25 & 50	78	↕FBW, WG, SGR, SR, FCR, PER & FR when % inclusion ↑	Renna et al. (2017)
Atlantic salmon	*Salmo salar*	Full-fat meal	larvae	0, 33, 66 & 100	114	↕FW, DGI, SGR, FI, FCR, CF, HSI & VSI when % inclusion ↑	Belghit et al. (2019b)
Juvenile mirror carp	*Cyprinus carpio var. specularis*	BSF pulp		0, 25, 50, 75 & 100	56	↕FBW, SGR, FCR, PER, PPR & SR when % inclusion ↑ ↕CF, VSI, & HSI when % inclusion ↑	Xu et al. (2020)

Clownfish	*Amphiprion ocellaris*	Defatted larvae meal	0, 25, 50 & 75	106	↕SGR & FCR when % inclusion ↑	Vargas-AbúNdez et al. (2019)
Rainbow trout	*Oncorhynchus mykiss* Walbaum	Defatted larvae meal & oil	0, 6.6, 13.2& 26.4 BSFLM 2.5, 5 & 10 BSFLO	90	↕FBW, WG, TGC at 6.6, 13.2, 2.5, 5 & 10 but ↓FBW, WG, TGC at 26.4	Dumas et al. (2018)

FBW, Final body weight; WG, Weight gain; SGR, Specific growth rate; SR, Survival rate; WG, Weight gain; DGI, Daily growth index; FI, Feed Intake; DFI, Daily feed intake; FCR, Feed conversion ratio; CF, Condition factor; HSI, hepatosomatic index; VSI, Viscerosomatic index; PER, Protein efficiency ratio; APU, apparent protein utilization; PPV, Protein productive value; TGC, Thermal growth coefficient; KSI, Kidney-somatic index; SSI, Spleen-somatic index; RGR, Relative growth rate; HPA, Harvest (kg per aquaria); DGR, Daily growth rate; IFR, Intraperitoneal fat ratio; IFI, Intraperitoneal fat index;

↑ increased significantly in comparison with control; ↓ decreased significantly in comparison with control and ↕ unchanged in comparison with control

2.1.4 Effect of HI larvae meal on proximate composition and final product quality

Proximate and FA composition of fish as a reflection of administered diets is summarised in Table 2. 6. Proximate compositions in terms of protein, lipid, dry matter, ash, and energy were unaffected in various fish by HI larvae inclusion, with the exception of African catfish, juvenile turbot, rainbow trout, and yellow catfish. Crude protein content increased in African catfish fed 50% of HI larvae meal and it was predicted that 50% of HI larvae contain more available free amino acid thereby enhancing muscle protein accretion (Fawole et al., 2020). However, rainbow trout (Dumas et al., 2018), and yellow catfish (Xiao et al., 2018) showed a low production of protein in muscle or whole fish when HI larvae inclusion increased gradually in the diet which may be due to the presence of chitin. Chitin strongly binds with protein thereby reducing the access of digestive enzymes to break down the protein (Belghit et al., 2018) and consequently may reduce the synthesis of protein for the above-mentioned species.

Saturated fatty acids (SFA) mainly due to the enhancement of lauric acid (C12:0), myristic acid (C14:0), and palmitic acid (C16:0) increased in all fish species fed either fatted or non-fatted HI regardless of the body parts used for analysis (Table 2. 6). In all studies, C12:0, C14:0, and C16:0 were significantly higher in HI larvae-based diets. This is an expected result since it is well established that HI larvae are a rich source of C12:0, C14:0, and C16:0. MUFA concentration decreased in the muscle or whole body in Jian carp (Zhou et al., 2018), Zebrafish (Zarantoniello et al., 2020b), Atlantic salmon (Belghit et al., 2019b), and rainbow trout (Renna et al., 2017) at any HI inclusion levels. Another study found that adding more than 50% HI decreased the MUFA content in the whole body of Clownfish. In contrast, feeding HI larvae meal enhanced the synthesis of MUFA in zebrafish (Zarantoniello et al., 2021a), Siberian sturgeon (Caimi et al., 2020b), and Atlantic salmon (Lock et al., 2016) but no changes in the MUFA concentration were found in meagre (Guerreiro et al., 2020) and Nile tilapia (Devic et al., 2018). However, adding HI larvae meal resulted in a reduction of n-3 PUFA, mainly because of a decrease in essential fatty acids, in particular, EPA and DHA in most of the fish which was aligned with the presence of a negligible amount of essential fatty acids in HI larvae meal. These results are shortcomings for the utilization of HI larvae in aquafeeds, however, it is noteworthy to mention that essential fatty acids in HI larvae meal can be improved by enriching feeding substrates. Currently, adding *Schizochytrium* sp. with coffee by-product (Zarantoniello et al., 2020b) and fish offal (Sealey et al., 2011) as feed substrates enriched the PUFA concentration of HI larvae meal. Among all studies conducted so far (Table 2. 6), two studies have reported elevated levels of n-PUFA in salmonids when fed HI larvae-based diets (Belghit et al., 2019b; Lock et al., 2016). The disparity among all studies may

be attributed to different fish species, culture methods of HI larvae, feeding habits of fish, and duration of feeding trial.

Assessing final product quality in relation to other health parameters of aquaculture fish is important during evaluating the potentiality of new ingredients since it has been reported that dietary modifications may influence the flesh quality of the final product. Extensive literature is available on the inclusion of HI larvae meal in the diet of fish, mainly focusing on health aspects, but very few studies have focused on how HI larvae feed inclusion influences fillet quality of aquaculture fish. Feeding 25 and 50% normal full-fat HI larvae meal or enriched HI larvae meal did not change the sensory attributes of rainbow trout, evaluated by 30 untrained panellists (Sealey et al., 2011). Similarly, complete replacement of FM with HI larvae meal resulted in no variation in the sensory attributes of Atlantic salmon (Bruni et al., 2020a; Lock et al., 2016). In contrast, similar inclusion levels of HI larvae meal in rainbow trout affected the fillet sensory profile, in particular, aroma, flavour, and texture (Borgogno et al., 2017).

Interestingly, the inclusion of HI larvae, either fatted or defatted, in our studies (unpublished works) enhanced the sensory attributes of the barramundi fillet. Despite having a negative effect on FA composition, lack of significant variation in salmonids or improved fillet sensory attributes in barramundi is quite unexpected, suggesting that other components in HI larvae meal may influence the sensory quality. For instance, chitin in HI larvae could be one of the factors since chitosan, the derivative of chitin has been reported to improve the sensory quality of seafood (Fan et al., 2009; Farajzadeh et al., 2016; Mohan et al., 2012). Moreover, the digestion of chitin by chitinolytic enzymes with the help of chitinoclastic bacteria has been demonstrated in marine fish.

Besides sensory evaluation, physio-chemical analysis of fillet is also important during shelf-life studies. Colour is important as it can be associated with the acceptance or rejection of aquaculture products by the consumer. HI larvae meal had minimal or no effect on the colour of rainbow trout (Bruni et al., 2020b) and European seabass (Moutinho et al., 2020) fillet while barramundi fed HI larvae based PBM diets showed brighter fillets when compared to the barramundi fed a commercial diet (Unpublished works).

Similar to colour, texture profile used to evaluate the fillet firmness and elasticity (in terms of adhesiveness, chewiness, cohesiveness, gumminess, hardness, and springiness) is a rarely studied physical parameter, associated with consumer preference of fish fed HI larvae based diets. However, feeding HI larvae meal with PBM improved the texture of barramundi, manifested (as by the instrumental method) in our studies. Similarly, muscle texture improved significantly in the eel, *Monopterus albus* after 10 weeks of feeding of 2.5, 5, and 7.5% of HI larvae meal (Hu et al.,

2020). The improvement in texture might be associated with lipid oxidation, a factor that is associated with the shelf life of fish. It has been reported that HI larvae has the ability to retard the lipid oxidation in European seabass (Moutinho et al., 2020) and rainbow trout (Bruni et al., 2020b) fillet. Even, adding HI larvae meal in PBM inhibited the lipid oxidation in barramundi fillet. The possible mechanisms for the reduction of lipid oxidation could be the presence of chitin or antimicrobial peptides in HI larvae meal. The possible mechanisms of chitosan, a derivative of chitin to inhibit the lipid oxidation is the bonding of positively charged NH_3^+ group of chitosan and the negatively charged microbial cell membranes, blocking the passage of nutrients and oxygen important for cellular metabolism via changing the cell permeability and thus leading to cell death (Kong et al., 2010; Tsai et al., 2002). In addition, the defensive power of antimicrobial peptides in HI larvae against bacteria and fungus has already been documented in previous studies (Elhag et al., 2017; Park et al., 2015; Park et al., 2014). Such positive effects in different fish species suggested that HI larvae could be used as a functional material to delay the oxidation process thereby increasing the shelf life of fish.

Table 2. 6 Proximate composition of different parts of fish including whole body and muscle when fed full-fat and defatted HI larvae

		Stage of BSF	Inclusion levels (%)		Analysed part	Proximate composition	Authors
African catfish	*Clarias gariepinus*	Larvae meal	0, 25, 50 and 75	60	Whole body	PC: moisture↑ at 75%, CP↑ at 25 & 50%, CL↕ and ash↕	Fawole et al. (2020)
Jian carp	*Cyprinus carpio* var.Jian	Larvae meal	0, 25, 50, 75 and 100	56	Whole body & muscle	PC: moisture, CP, CL, and ash↕ for whole body & muscle Whole body FA: ∑SFA↑ (C12:0 & C14:0), ∑MUFA↓ (16:1n-7 & 18:1n-9), ∑n-3PUFA↓ (18:3n3, & 22:6n-3) and Σn-6PUFA↓ (18:2 n-6 & 22:4 n-6) when % inclusion ↑	Zhou et al. (2018)
Zebrafish	*Danio rerio*	Full-fat prepupae meal	0, 25, 50, 75 & 100	21	Whole body	FA: ∑SFA↑ (C12:0) when % inclusion↑, ∑MUFA↓ at 75 & 100%, ∑n-3PUFA↓ (18:3n3, 20:5n-3 & 22:6n-3) when % inclusion ↑ and Σn-6PUFA↑ when % inclusion ↑	Zarantoniello et al. (2020b)
Eurasian perch	*Perca fluviatilis*	Defatted larvae meal	0, 20, 40 & 60	84	Whole body	PC: DM↕, CP↕, CL↕, ash↑ and gross energy↓ at 60% FA: ∑SFA↑ (C12:0 and C14:0) and ∑MUFA↓ (C18:1n9, C18:1n7 and	Stejskal et al. (2020)

						C20:1n9) when % inclusion, ∑n-3PUFA↓ (18:3n3, 20:5n-3 & 22:6n-3) and Σn-6PUFA↓ (C18:2n6, C18:3n6 and C20:3n6) when % inclusion ↑	
Zebrafish	*Danio rerio*	Full-fat prepupae meal	0, 25, 50, 75 & 100	21	Whole body	FA: ∑SFA↑ (C12:0) and, ∑MUFA↑ when % inclusion↑, ∑n-3PUFA↓ (18:3n3, 20:5n-3 & 22:6n-3) when % inclusion ↑ and Σn-6PUFA↑ when % inclusion ↑	Zarantoniello et al. (2021a)
Siberian sturgeon	*Acipenser baerii*	Defatted larvae meal	0, 25, 50 & 100	118	Whole body	PC: DM↑ at 50%, CP, CL, ash and energy ↕ when % inclusion ↑ FA: ∑SFA↑ (C12:0 and C14:0), ∑MUFA↑ (C16:1c9, C18:1t9-11, C18:1 c12, C20:1 c9 & C20:1c11), ∑n-3PUFA↑ (C20:5n3, C22:5 n3 & C22:6 n3) and Σn-6PUFA↑ (C18:2 n6, C18:3 n6, C20:2 n6, C20:3 n6 & C20:4 n6) when % inclusion ↑	Caimi et al. (2020b)
Zebrafish	*Danio rerio*	Full-fat prepupae meal	0, 25 & 50	180	Whole body	FA:∑SFA↑ (C12:0, C14:0 and C16:0), ∑MUFA↓ (18:1n7 & 20:1n9), ∑n-3PUFA↓ (18:3n3,	Zarantoniello et al. (2019)

						20:5n3 & 22:6n3) and Σn-6PUFA↑ when % inclusion ↑	
Jian carp	*Cyprinus carpio* var.Jian	Larvae meal	0, 25, 50, 75 and 100	59	Muscle & whole body	PC: moisture, protein, lipid, ash and energy↕ when % inclusion ↑	Li et al. (2017a)
Juvenile Jian carp	*Cyprinus carpio* var. Jian	Larvae oil	0, 25, 50, 75 and 100				
Meagre	*Argyrosomus regius*	Larvae meal	0, 10, 20 and 30%		Whole body	PC: moisture, CP, CL, ash and energy ↕ when % inclusion ↑ FA: ∑SFA↑ (C12:0 and C14:0), ∑MUFA↕, ∑n-3PUFA↓ (20:5n-3 & 22:6n-3) and Σn-6PUFA↕ when % inclusion ↑	Guerreiro et al. (2020)
European sea bass	*Dicentrarchus labrax*	Larvae meal	0, 25, 35 & 50%		Whole body	PC: moisture, protein, lipid, ash and energy ↕ when % inclusion ↑	Abdel-Tawwab et al. (2020)
Japanese seabass	*Lateolabrax japonicus*	Larvae meal	0, 16, 32, 48 & 64		Whole body	PC: DM↕, CP↕, CL↕, and ash↓ when % inclusion ↑	Wang et al. (2019b)
Pacific white shrimp	*Litopenaeus vannamei*	Larvae meal	0,7, 14, 28 & 36	63	Whole body	PC: moisture, protein, lipid, ash and energy↕ when % inclusion ↑	Cummins et al. (2017)
Atlantic salmon	*Salmo salar*	Larvae meal	0, 33, 66 and 100		Whole body	∑SFA↑ (C14:0 and C16:0) and ∑MUFA↓ (C18:1n9 & C18:1n7), ∑n-3PUFA↑ (20:5n-3, 22:5n-3 &	Belghit et al. (2019b)

						22:6n-3) and Σn-6PUFA↓(C18:2n6) when % inclusion ↑	
Juvenile turbot	*Psetta maxima*	pre-pupae meal	0, 17, 33, 49, 64 & 76	56		PC: Moisture↑, CP↕, CL↑, ash↕ and GE↓ when % inclusion ↑	Kroeckel et al. (2012)
Rainbow trout	*Oncorhynchus mykiss*	Defatted larvae meal and larvae oil	0, 6.6, 13.2, 26.4% meal 2.5, 5.0 & 10% oil	84	Whole body & skinless fillet	Body PC: moisture↕, protein↓, lipid↑, and ash↕ when % inclusion ↑ for meal Fillet PC: moisture, protein, lipid, ash and energy↕ when % inclusion ↑ for meal and oil	Dumas et al. (2018)
Clownfish	*Amphiprion ocellaris*	Defatted larvae meal	0, 25, 50 & 75	106	Whole body	PC: Dry weight and lipid↕ when % inclusion ↑ FA: ∑SFA↑ (C12:0 and C14:0) & ∑n-3PUFA↓ (18:3n3, 20:5n3 & 22:6n3) when % inclusion ↑, ∑MUFA↓ (20:1n9 & 22:1n9) & Σn-6PUFA↑ (18:2n6) 50 & 75%,	Vargas-AbúNdez et al. (2019)

Rainbow trout	*Oncorhynchus mykiss* Walbaum	Defatted larvae meal	0, 25 & 50	78	Fillet	PC: DM↑, CP↕, CL↑, and ash↓ when % inclusion ↑ FA:∑SFA↑ (C12:0 and C14:0) and ∑MUFA↓ (C18:1c9, C18:1 c11 & C20:1c11), ∑n-3PUFA↓ (20:5n-3, 22:5n-3 & 22:6n-3) and Σn-6PUFA↓(C20:2 n6, C20:3 n6 &C20:4 n6) when % inclusion ↑	Renna et al. (2017)
Yellow Catfish	*Pelteobagrus fulvidraco*	Larvae meal	0, 10, 15, 20, 25 & 30	56	Whole body & muscle	Body PC: moisture, CP, CL, and ash↕ when % inclusion ↑ Muscle PC: moisture↕, protein↕, lipid↕, and ash↑ when % inclusion↑	Hu et al. (2017)
Jian carp	*Cyprinus carpio* var. Jian	Larvae oil	0, 25, 50, 75 & 100	59	Muscle	FA:∑SFA↑ (C12:0, C14:0 and C16:0) and ∑MUFA↓ (18:1n-9), ∑n-3PUFA↑ (22:6n-3) and Σn-6PUFA↓(18:2n-6) when % inclusion ↑	Li et al. (2016b)
Atlantic salmon	*Salmo salar*	Full-fat larvae meal	25, 50 & 100 (IM A) and 25 & 100 (IM B)	105	Whole body	PC: protein, total fat and energy↕ when % inclusion↑ FA:∑SFA, ∑MUFA, ∑n-3PUFA and Σn-6PUFA↑ when % inclusion ↑	Lock et al. (2016)

Barramundi	*Lates calcarifer*	Full-fat larvae meal	0, 25, 50, 75 & 100	56	Whole body	PC: Moisture, CP, CL, ash and fibre↕ when % inclusion↑	Katya et al. (2017)
Rainbow trout	*Oncorhynchus mykiss*	Normal & enriched prepupae meal	0, 25 & 50	56	Muscle	PC: Moisture↑, CP↕, CL↓, ash↕ and energy↓ when % inclusion FA:∑SFA↑ (C12:0 and C14:0), ∑MUFA↓ (18:1n9), ∑n-3PUFA↑ (18:3n3 & 20:5n3) and Σn-6PUFA↓ (18:2n6) when % inclusion ↑	Sealey et al. (2011)
Rainbow trout	*Oncorhynchus mykiss*	prepupae meal	0, 25 & 50	63	Whole body & muscle	PC: Moisture↑, CP↕, CL↓, ash↕ and energy↓ when % inclusion↑ Muscle FA: ∑SFA↑ (C12:0), ∑MUFA↓ (16:1n7), ∑n-3PUFA↑ (18:3n3 & 20:5n3) and Σn-6PUFA↓ (18:2n6, 20:5n3, 22:5n3 & 22:6n3) when % inclusion ↑	St-Hilaire et al. (2007)
Yellow catfish	*Pelteobagrus fulvidraco*	Full-fat larvae meal	0, 13, 25, 37, 48, 68, 68, 85 & 100	65	Whole body	PC: Moisture↑, CP↓, CL↓, and ash↑ when % inclusion↑	Xiao et al. (2018)
Zebrafish	*Danio rerio*	Full-fat prepupae meal					

Eel	*Monopterus albus*	Larvae meal	0, 2.5, 5 and 7.5%	70	Whole body	PC: Moisture, CP, & ash↕ when % inclusion while CL↓ at 7.5%	Hu et al. (2020)
Juvenile grass carp	*Ctenopharyngodon idellus*	Defatted larvae meal	0, 25, 50 & 100%	56	Muscle	PC: Moisture, CP, CL & ash↕ when % inclusion↑	Lu et al. (2020)
Nile tilapia	*Oreochromis niloticus*	Full-fat larvae meal	0, 30, 50 & 80	32	Whole body	PC: DM, CP, CL, ash and CF↕ when % inclusion↑ FA: ∑SFA↑, ↕∑MUFA, ∑n-3PUFA↓ (20:5n-3& 22:5n-3) & Σn-6PUFA↕ when % inclusion↑	Devic et al. (2018)

PC, proximate composition; DM, Dry matter; CP, Crude protein; CL, Crude lipid; SFA, Satuarted fatty acid; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid.

↑ increased significantly in comparison with control; ↓ decreased significantly in comparison with control and ↕ unchanged in comparison with control

2.1.5 Effects on antioxidant activity

A number of recent studies have been conducted on the inclusion of HI, and the impact on the antioxidant activity of different fish. These studies are summarised in Table 2. 7. CAT-SOD are important cellular antioxidant enzymes, including mechanisms that are the first line of defense against oxidative stress. Oxidative stress is caused by the production of reactive oxygen species during the normal metabolic process if not suppressed by the antioxidant mechanism. Partially defatted HI meal showed an antioxidant potential, manifested by a decrease in SOD and increase in CAT activity with lower concertation of MDA in the serum of African catfish, *Clarias gariepinus* (Fawole et al., 2020). Such CAT-SOD mechanisms were not observed in the serum of juvenile Jian carp, *Cyprinus carpio* var. Jian although CAT activity elevated in 75 and 100% DBSFL fed groups (Li et al., 2017a). Similarly, CAT activity increased in the serum of juvenile grass carp (Lu et al., 2020) and mirror carp (Xu et al., 2020) when fed graded levels of HI larvae and pulp. In addition, Abdel-Latif et al. (2021) observed an elevation in different antioxidant markers including MDA, SOD, CAT, and GSH-Px in the serum of European seabass fed 25, 35, and 50% of HI larvae meal. The positive changes in antioxidant markers in different fish species suggested the antioxidant potential of HI larvae meal which might be accredited to chitin, bioactive peptides, and lauric acid present in HI larvae. Chitin and its derivatives have been reported to have antioxidant properties which have supported the scavenging activity of chitin against free radicals such as reactive oxygen species (Ngo et al., 2008; Ngo et al., 2009). Also, seventeen novel sequences of well-known antioxidant proteins were identified from hydrolyzed HI larvae, and showed the best scavenging activity to superoxide radicals and hydroxyl radicals (Zhu et al., 2020). Lauric acid has been well-documented for antioxidant properties. In contrast, a higher SOD in Siberian sturgeon was associated with lower palatability due to a higher inclusion of HI larvae, and concomitant lower consumption of HI larvae meal (Caimi et al., 2020a).

The weakening of GPx and activity of SeGPx at different levels of HI larvae meal in the liver and kidney of rainbow trout (Elia et al., 2018; Melenchón et al., 2020) and Siberian sturgeon (Caimi et al., 2020a) is not surprising since the polysaccharide chitin has an ability to bind with both inorganic (selenite) and organic (selenomethionine and selenocysteine) forms present in formulated or supplemented diets (Elia et al., 2014; Pacini et al., 2012; Pacini et al., 2013). Hence, it is feasible that chitin present in HI larvae or prepupae meal may have bound to selenocysteine, resulting in an unavailable or suboptimal level of Se available for physiological functions. These mechanisms might have impaired selenoprotein function, consequently decreasing the GPx or SeGPx levels in tissues of the above-studied fish species. SeGPx enzyme structure demonstrates that the GSH binding site contains one lysine and four arginine residues and selenocysteine in the

catalytic centre (Elia et al., 2018), indicating the importance of the presence of arginine and lysine in diet to maintain SeGPx activity. Though some studies reported the presence of low levels of arginine and lysine in HI larvae (Melenchón et al., 2020; Weththasinghe et al., 2021b), their concentration was higher in other HI larvae fed on alternate substrates (Dietz and Liebert, 2018; Rawski et al., 2020).

2.1.6 Effect on haematological and biochemical metabolites

Haematological metabolites are commonly used as an important tool to understand the physiological and immunological status and can also provide an indication of the internal environment of fish (Chaklader et al., 2021a; Chaklader et al., 2021b). Minimal effects of HI inclusion in aquafeed have been reported on the haematological responses of various fishes, as summarised in Table 2. 8. However, serum total protein, albumin, and globulin decreased in African catfish when fed more than 25% of HI larvae meal, suggesting the negative effects of HI larvae on the immune function of African catfish, however, total bilirubin, ALT, AST, TG, and ALP decreased with increasing inclusion levels in the serum of African catfish (Fawole et al., 2020). The decrease in these serum metabolites implies a hypocholesterolemic effect of HI larvae inclusion possibly due to the presence of chitin and also indicates that exclusive inclusion of HI larvae meal did not affect hepatic health. A similar hypocholesterolemic effect was found in Japanese seabass (Wang et al., 2019b) and Jian carp (Li et al., 2017a). An improvement in TB, ALT, and GLDH activity associated with liver, spleen, and kidney health was found in the serum of barramundi in our previous studies when a smaller quantity of full-fat HI larvae was supplemented with PBM meal (Chaklader et al., 2020b; Chaklader et al., 2021c). Haematological metabolites were unchanged when HI was included in Jian carp (Li et al., 2017a; Zhou et al., 2018), European sea bass (Abdel-Tawwab et al., 2020) and juvenile mirror carp (Xu et al., 2020). The variation in the haematological responses among different studies may be ascribed to different fish species, different inclusion levels and stages of HI larvae, the capability of chitin digestion of fish, different substrates used for HI larvae culture, and feeding habits of fish.

Table 2. 7 Antioxidant activity of different fish fed on various inclusion of level of HI larvae meal

Common name	Scientific name	Stage of BSF	Inclusion levels (%)	Antioxidant activity	
European seabass	*Dicentrarchus labrax*	Larvae	0, 25, 35 and 50%	Serum: MDA, SOD, CAT and GSH-Px ↑ when % inclusion↑	Abdel-Latif et al. (2021)
African catfish	*Clarias gariepinus*	Larvae meal	0, 25, 50 and 75	Serum: SOD↓, CAT↑ and MDA↕ when % inclusion↑	Fawole et al. (2020)
Rainbow trout	*Oncorhynchus mykiss*	Larvae	0, 15 & 30	Liver: SOD↑, MDA↓, CAT↕, & G6PDH↕ when % inclusion↑ while GPx, and GR↓ at 30%	Melenchón et al. (2020)
Siberian Sturgeon	*Acipenser baerii*	Larvae meal	0, 25 and 50	Liver: MDA↕, SOD↑ at 50%, CAT↕, GPx↓ at 50%, GR↑ at 50%, EROD↕ and GST↕ Kidney: MDA↕, SOD↑ at 50%, CAT↕, GPx↓ at 50%, GR↑, EROD↑ and GST↕ at 50%	Caimi et al. (2020a)
Rainbow trout	*Oncorhynchus mykiss*	Larvae meal	0, 25 and 50	Liver: MDA↕, SOD↕, CAT↕, SeGPx↓, totGPx↕, EROD ↑ at 50%, GST↕, GR↕, GSH+2GSSG↑ Kidney: MDA↕, SOD↕, CAT↕, SeGPx↓, totGPx↕, EROD↕, GST↑, GR↕, GSH+2GSSG↕	Elia et al. (2018)
Jian carp	*Cyprinus carpio* var.Jian	Larvae meal	0, 25, 50, 75 and 100	Serum: MDA↕ and T-AOC↓ at 100%	Zhou et al. (2018)

Jian carp	*Cyprinus carpio* var.Jian	Larvae meal	0, 25, 50, 75 and 100	Serum: SOD↕, CAT↑ at 75 and 100% and MDA↕	Li et al. (2017a)
Eel	*Monopterus albus*	Larvae meal	0, 2.5, 5 and 7.5%	Serum: CAT & MDA↑ at 7.5% and SOD↕ when % inclusion ↑ Liver: SOD, CAT & MDA↕ when % inclusion ↑	Hu et al. (2020)
Juvenile grass carp	*Ctenopharyngodon idellus*	Defatted larvae meal	0, 25, 50 & 100%	Serum: CAT↑ & SOD↕ when % inclusion ↑	Lu et al. (2020)
Juvenile mirror carp	*Cyprinus carpio* var. specularis	Pulp	0, 25, 50, 75 & 100%	Serum: SOD↕, CAT↑ & MDA↓ when % inclusion ↑	Xu et al. (2020)

MDA, Malondialdehyde; CAT, Catalase; SOD, Superoxide Dismutase; GSH-Px, Glutathione Peroxidase; GPx, Glutathione Peroxidase; GR, Glutathione reductase; GST, Glutathione S-transferase and EROD, Ethoxyresorufin O-deethylase. ↑ increased significantly in comparison with control; ↓ decreased significantly in comparison with control and ↕ unchanged in comparison with control.

Table 2. 8 Haematological biochemical metabolites of different fish fed on various inclusion of level of HI larvae meal

Common name	Scientific name	Stage of BSF	Inclusion levels (%)	Haematological biochemical metabolites	Authors
Juvenile barramundi	*Lates calcarifer*	Defatted larvae meal	0 & 30 meal & oil	Serum: AST, GLDH, Cho, TG, creatinine Glu, TP, ALB, GLB & ALB/GLB↕ at 30 meal and/or oil while Urea↑ at 30% oil	Hender et al. (2021)

African catfish	*Clarias gariepinus*	Larvae meal	0, 25, 50 and 75	Blood: PCV↕, HB↕, WBC↕, RBC↕, MCV↕, MCH↕, MCHC↕, Neutrophil↕, lymphocyte↕ and monocyte↕ when % inclusion ↑ Serum: TP↓ at 50%, ALB↕, GLO↓ at 50%, A/G ratio↕, TG↓, Chol↕, GLU↓, total antioxidant↕, TB↓, creatinine↕, AST↓, ALT↓ at 50 and 75%, and ALP↓	Fawole et al. (2020)
Juvenile Dusky Kob	*Argyrosomus japonicus*	Larvae meal	0, 5, 10 and 20	Serum: urea↕, creatinine↕, TP↕, ALB↕, GLOB↕, ALB/GLB↕, ALT↑, AST↑, CHOL↕ and TG↑ when % inclusion ↑	Madibana et al. (2020)
Jian carp	*Cyprinus carpio* var.Jian	Larvae meal	0, 25, 50, 75 and 100	Serum: GPT↕, GOT↕, TP↕, ALB↕, GLO↕, A/G↕, GLU↕, T-chol↕, TAG↕, HDL-c↕ and LDL-c↕ when % inclusion ↑	Zhou et al. (2018)
Jian carp	*Cyprinus carpio* var.Jian	Larvae meal	0, 25, 50, 75 and 100	Serum: ALT↕, AST↕, TP↕, ALB↕, GLO↕, A/G↕, GLc↕, Chol↓ and TG↕ when % inclusion ↑	Li et al. (2017a)
juvenile Jian carp	*Cyprinus carpio* var.Jian	Larvae oil	0, 25, 50, 75 and 100	Serum: ALT↕, AST↕, TP↕, ALB↕, GLO↕, A/G↕, GLU↕, Chol↕, TG↕, HDL-c↕ and LDL-c↕ when % inclusion ↑	
Meagre	*Argyrosomus regius*	Larvae meal	0, 10, 20 and 30%	Plasma: GLU↕, Chol↕, TG↕, TP↕ when % inclusion ↑ but TL↓ at 30%	Guerreiro et al. (2020)

European sea bass	*Dicentrarchus labrax*	Larvae meal	0, 25, 35 and 50%	Blood: RBC↕, Hb↕, Hct↕, MCV↕, MCH↕, MCHC↕, WBC↕, lymphocytes↕, monocytes↕ and neutrophils↕ when % inclusion ↑	Abdel-Tawwab et al. (2020)
European seabass	*Dicentrarchus labrax*	Prepupae meal	0, 15, 30 and 45%	Plasma: GLU, TP, TG & Chol ↕ when % inclusion ↑	Magalhães et al. (2017)
Japanese seabass	*Lateolabrax japonicus*	Larvae meal	0, 16, 32, 48 and 64	Serum: TCHO↓, TG↓, GLO↕, GLU↕, BUN↕, AST↕, ALT↕, LDL-c, HDL-c↓, DAO↕, LPS↕ and D-LA↕ when % inclusion ↑	Wang et al. (2019b)
Atlantic salmon	*Salmo salar*	Larvae meal	0, 33, 66 and 100	Plasma: ALT↕, AST↕, GLU↑ at 66%, FFA↕, TG↕, Chol↕, TP↕ and Na^+↕ when % inclusion ↑	Belghit et al. (2019b)
Eel	*Monopterus albus*	Larvae meal	0, 2.5, 5 and 7.5%	Serum: GOT ↑ at 7.5% while GPT, GLU, TG & TC↕ when % inclusion ↑	Hu et al. (2020)
Juvenile grass carp	*Ctenopharyngodon idellus*	Defatted larvae meal	0, 25, 50 & 100%	Serum: TP, ALB, GLB, A/G, ALT, AST, GLU, Chol, HDL-C & LDL-C↕ when % inclusion ↑ while TG↑ at 100%	Lu et al. (2020)
Juvenile mirror carp	*Cyprinus carpio* var. specularis	Pulp	0, 25, 50, 75 & 100%	Serum: ALT, AST, TP, ALB, GLO, GLU, T-cho, TG, HDL & LDL↕ when % inclusion ↑	Xu et al. (2020)

AST, Aspartate aminotransferase; GLDH, Glutamate dehydrogenase; Cho, Cholesterol; TG, Triglyceride; Glu, Glucose; TP, Total protein; ALB, Albumin; GLB, Globulin; PCV, Packed cell volume; HB, Haemoglobin; WBC, White blood cell; RBC, Red blood cell; MCV, Mean corpuscular volume; MCH, Mean corpuscular haemoglobin; MCHC, mean corpuscular hemoglobin concentration; ALT, Alanine aminotransferase; GPT, Glutamic-pyruvic transaminase; HDL-c, High-density lipoprotein cholesterol and LDL-c, Low-density lipoprotein cholesterol. ↑increased significantly in comparison with control; ↓ decreased significantly in comparison with control and ↕ unchanged in comparison with control.

2.1.7 Effect on gut health and gut microbiota

Gut mucosal barrier functions have been strongly linked to nutrient uptake, digestion, and immunity of fish (Ángeles Esteban, 2012). Dietary changes can improve or disturb the mucosal barrier function (Ángeles Esteban, 2012). Our previous studies demonstrated an improvement in intestinal mucosal barrier functions in terms of villi width, microvilli height, acid and neutral mucin-producing goblet cells, and microvilli density in barramundi when fed full-fat HI larvae based PBM diets (Chaklader et al., 2019; Chaklader et al., 2021c). Similarly, feeding full-fat HI larvae improved microstructures of pyloric caeca and proximal intestine, particularly, villus height, width, and area in Siberian Sturgeon (Rawski et al., 2021) but did not affect the intestinal health in the same species when fed partially defatted HI larvae meal (Caimi et al., 2020a), suggesting that such variable effects were dose-dependent or FHI larvae work better that DHI larvae meal. Li et al. (2020) reported that complete replacement of FM with HI larvae meal increased submucosa cellularity in the proximal intestine and was also linked with lower degree steatosis in the proximal intestine and higher relative weight of the distal intestine. Li et al. (2019) found that the inclusion of 85% of protein from HI larvae meal reduced the hyper-vacuolization in pyloric caeca mucosa, caused by the reference diet. The predicted underlying reason was the presence of bioactive components. Up to 12.5% of HI larvae meal or 6.7% of raw HI larvae paste reduced enterocyte steatosis in pyloric caeca in salmon and choline in HI larvae has been suggested as a contributing factor to reducing the steatosis (Weththasinghe et al., 2021a) since it has been reported that choline can prevent excessive lipid accumulation in the proximal intestine of post-smolt salmon (Hansen et al., 2020; Krogdahl et al., 2020). Besides the contribution of choline, other functional molecules may play role in preventing steatosis in different parts of the gut in the aforementioned studies but may not be ascribed for other species. Up to 50% of defatted HI larvae meal did not impact the intestinal health but more than 50% of HI larvae meal (75 and 100 %) caused a tissue disruption in the intestine of Jian carp (Li et al., 2017a). However, gut health was unaffected in clownfish (Vargas-AbúNdez et al., 2019), Japanese seabass *Lateolabrax japonicus* (Wang et al., 2019b) and rainbow trout (Elia et al., 2018) when fed HI larvae based diet.

A good number of studies have been performed to describe the resident microbial communities in fish (Austin, 2006; Austin and Al-Zahrani, 1988; Cahill, 1990; Horsley, 1977; Liston, 1957; Ringø et al., 1995; Ringø et al., 2016; Trust and Sparrow, 1974) and currently, GI microbiota research has been conducted in numerous fish species, as described in the review of Wang et al. (2018). The microbiota has been reported to be involved in different functions including nutrition by producing an extensive range of enzymes such as amylase, cellulase, lipase, proteases, chitinase, and phytase (Clements et al., 2014; Ray et al., 2012), pathogen protection, host metabolisms, and

immune modulation, thus maintaining gut homeostasis (Laparra and Sanz, 2010; Rombout et al., 2011; Sutili et al., 2018). However, the structure and function of GIT microbiota in fish have not yet been fully elucidated, limiting the application of knowledge in terms of selection of probiotics, prebiotics, and other functional materials to improve fish health as an alternative to antibiotics that have been prohibited for food animal application (Dawood and Koshio, 2016; Hoseinifar et al., 2015).

As described in detail in the review of Wang et al. (2018), the diet has been documented as one of the factors that influence the GI microbiota. Recently, the inclusion of HI larvae in aquafeed has been demonstrated as a prebiotic with an ability to reshape the gut microbiota of finfish (Bruni et al., 2018; Chaklader et al., 2021c; Terova et al., 2019), crustaceans (Foysal et al., 2019) and livestock animals (Borrelli et al., 2017) because of the presence of chitin, antibacterial peptides and high amounts of medium-chain fatty acids (MCFA), in particular, lauric acids (C12:0). Although chitin, a mucopolysaccharide polymer that exists in insects, is hardly digested by many fish, the inclusion of chitin at lower levels could trigger the growth of beneficial bacteria and hinder the proliferation of pathogens in the intestine of fish. Dietary inclusion of HI prepupae meal (10 to 30%) has been reported to reshape the intestinal bacteria in rainbow trout by influencing the abundance of *Actinobacteria* and *Proteobacteria* (Terova et al., 2019). At the genus level, lactic acid bacteria (LAB) belonging to the *Aerococcaceae*, *Enterococcaceae*, *Lactobacillaceae*, and *Leuconostocaceae* and many butyrate producers such as *Ruminococcaceae*, *Lachnospiraceae*, *Clostridiaceae*, and *Eubacteriaceae under* Clostridiales order were enriched in trout fed BSF prepupae meal (Terova et al., 2019). Butyrate, an important short-chain fatty acid (SCFAs) is well-documented for its numerous positive effects on the intestinal tract and peripheral tissue in vertebrates, including fish (Mátis et al., 2013). Butyrate has also been reported to have anti-inflammatory properties and regulate the immune system (Canani et al., 2011; Hamer et al., 2008; Terova et al., 2016; Terova et al., 2019; Wong et al., 2006). When defatted HI larvae meal instead of soybean meal was added to the diet of laying hens, the production of butyrate and propionate, two important SCFAs increased possibly due to the modulation of SCFAs producing bacteria (Borrelli et al., 2017). Likewise, a study on rainbow trout reported that partially defatted HI larvae-based diets increased intestinal mucosa- and digesta-associated bacterial community diversity with a concomitant increase in Firmicutes phylum and *Pseudomonas* spp and *Carnobacterium divergens* that has been reported as probiotics (Bruni et al., 2018). Similarly, administration of larval and pre-pupae stages of HI positively influenced the abundance of *Firmicutes* and *Actinobacteria* with a lower abundance of *Proteobacteria* and also stimulated the LAB and *Bacillaceae* in the distal intestine of rainbow trout (Huyben et al., 2019). Our recent two studies

found that supplementation of a smaller quantity of HI larvae (10-30%) with PBM improved the bacteria diversity and simultaneously increased the abundance of beneficial bacteria with a concomitant decreased in *Vibrio* in the intestine of barramundi (Chaklader et al., 2021c). Hu et al. (2020) found a gradual increase in bacteria diversity in the gut of rice field eel, *Monopterus albus* fed graded levels of HI larvae (5.26-15.78%).

The improvement in the richness in bacteria diversity in the above studies on different fish species may be an indicator of a healthy gut since an increase in bacteria diversity has been reported to resist pathogen invasion and intestinal infection by outcompeting pathogens for nutrients and colonization (Cerezuela et al., 2013; Huyben et al., 2019; Levine and D'Antonio, 1999). Concomitantly a decrease in diversity is often linked with dysbiosis of the gut environment thereby enhancing the risk of disease in fish (Terova et al., 2019; Zarantoniello et al., 2020b). Chitin in HI meal is not a typical material in aquadiet might have enhanced the colonization and growth of less common bacteria to digest chitin as a source of nutrients which may elucidate the enrichment of bacterial diversity in the above studies. However, defatted HI larvae meal did not change community diversity and richness but decease the abundance *Aeromonas* and *Shewanella* in the intestine of juvenile grass carp, *Ctenopharyngodon idellus* (Lu et al., 2020). The unchanged bacterial diversity in grass carp might be due to the utilization of defatted HI larvae since full-fat HI larvae increased the OTUs of lipase-producing bacteria, particularly, *C. variabile* belonging to Corynebacterium in rainbow trout (Huyben et al., 2019). Also, the addition of graded level of BSF larvae meal negatively influenced the presence of *Vibrio* in the gut of Zebrafish (Zarantoniello et al., 2020b). The abundance of *Cetobacterium* increased with increasing BSF inclusion levels in the zebrafish diet. The reported decrease in the abundance of pathogenic bacteria in the gut of several fish is not surprising since HI larvae contain novel antimicrobial peptides which have been demonstrated defence mechanisms against Gram-positive and Gram-negative bacteria and also against virus and fungus (Elhag et al., 2017; Park et al., 2015).

2.1.8 Effect on immune response and disease resistance

HI larvae meal due to the presence of different functional molecules such as lauric acid, novel antimicrobial peptides, chitin, and other polysaccharides including choline, silkrose, and dipterose could be considered as a functional ingredient in the aquafeed formulation. The functionality of HI larvae has been previously reported by Elhag et al. (2017), Park et al. (2014), and Park et al. (2015) who screened and purified antimicrobial peptides with an ability to defend Gram-positive and Gram-negative bacteria including *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, and fungi including *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*. The possible mechanism could be the bindings of AMPs to lipopolysaccharides (LPS) of Gram-negative

bacteria and teichoic and lipoteichoic acids of Gram-positive bacteria (as illustrated in Figure 2. 4, PART I). The binding protein penetrates the cell wall before changing structure and subsequent insertion into the membrane to form pores, finally leading to damage to the bacteria cell envelope and cell death (as illustrated in Figure 2. 4, PART II) (Jozefiak and Engberg, 2017). Also, AMPs may exhibit antibacterial activity by interactions with intracellular targets including heat shock protein (DnaK), DNA, and RNA (as illustrated in Figure 2. 4, PART III) (Jozefiak and Engberg, 2017). Besides AMPs, Ali et al. (2018) identified a novel polysaccharide (silkrose) from an insect and subsequent feeding of the novel compound stimulated the disease resistance of penaeid prawns such as *Litopenaeus vannamei* and *Marsupenaeus japonicas* against *Vibrio penaecida.*

Feeding HI larvae meal to European seabass improved the resistance to *Vibrio alginolyticus* possibly by enhancing non-specific immune responses including phagocytic activity, phagocytic index, lysozyme, and respiratory burst activity, and immune-relevant genes (Abdel-Latif et al., 2021). Tippayadara et al. (2021) found an elevated level of skin mucus lysozyme and peroxidase activity in Tilapia, *Oreochromis niloticus* when fed full-fat HI larvae meal (10-100%). Up to 48% of HI larvae meal elevated the level of lysozyme activity and phagocytic ratio in yellow catfish, *Pelteobagrus fulvidraco* (Xiao et al., 2018). Our earlier studies found that the inclusion of a smaller quantity of full-fat HI larvae meal enhanced the resistance against *Vibrio harveyi* (Chaklader et al., 2019; Chaklader et al., 2020b; Chaklader et al., 2021c). In contrast, another study from our laboratory found no variation in the serum lysozyme and bactericidal activity of barramundi fed 30% of partially defatted HI larvae meal and oil separately or concurrently for 42 days (Hender et al., 2021). Similarly, our recent study found no variation in lysozyme and bactericidal activity of barramundi but resistance to *V. harveyi* was significantly lower when fed 30% defatted HI with 70% PBM in comparison (Chaklader et al., 2021a).

Variable results for immune responses and disease resistance in barramundi fed full-fat and defatted HI larvae meal suggest that fat separation may reduce the functionality of subsequent defatted HI larvae meal. Saviane et al. (2021) found that HI oil is active against bacteria and suggested that lauric acid alone or synergically with antimicrobial peptides showed potential antimicrobial activity. It has also been reported that a variety of putative antimicrobials such as AMPs, polymers, and chemical complexes present in insects act alone or concurrently with fatty acids (Nesa et al., 2020; Tzompa-Sosa et al., 2019; Tzompa-Sosa et al., 2014; Vogel et al., 2018) to demonstrate antibacterial activity. Also, the inclusion of full-fat HI larvae meal and HI larvae oil with plant protein improved the serum lysozyme and peroxidase activity in rainbow trout (Kumar et al., 2020). Another study found stimulation in serum lysozyme and plasma alternative complement pathway activity in Nile tilapia when fed enriched or non-enriched HI larvae meal

(Agbohessou et al., 2021). Even, dietary utilization of frass, produced from HI larvae culture, improved complement activity and the disease resistance against both *Flavobacterium columnare* and *Streptococcus iniae* in channel catfish, *Ictalurus punctatus*, and the possible reason was the presence of chitin content or other HI larvae components in the frass (Yildirim-Aksoy et al., 2020).

Such immunostimulatory effects of HI larvae have not been demonstrated in juvenile Japanese seabass, *Lateolabrax Japonicus* (Wang et al., 2019b) and eel, *Monopterus albus* (Hu et al., 2020), and also in *Litopenaeus vannamei* (Wang et al., 2021a), supported by no alteration in serum acid phenol oxidase, acid phosphatase, alkaline phosphatase, lysozyme, complement 3 and 4, and immuno-globulin.

Alongside other functional molecules, chitin may also act as an immunostimulant since immunostimulatory effects of a smaller quantity of chitin have previously been reported in fish (Esteban et al., 2001; Gopalakannan and Arul, 2006). The possible mechanism of chitin is the proliferation of beneficial bacteria which have been proven in different fish (Bruni et al., 2018; Terova et al., 2019). In addition, it has been reported that the positive charge NH_3^+ group of chitosan, the derivative of chitin can bind with the carboxyl groups (COO^-) situated on the outer part of the membrane or the cell wall of bacteria and fungi, leading to cell death by blocking the passage of nutrients and oxygen important for cellular metabolism (Figure 2. 5). Likewise, the inclusion of dietary chitosan (5-20%) stimulated the disease resistance against *Vibrio anguillarum*, with an aligned higher haematological and innate immune response in Asian seabass or barramundi (Ranjan et al., 2014). Based on the aforementioned promising findings, it can be concluded that a smaller quantity of HI larvae or different functional molecules of HI could be used as a potential functional ingredient as an alternative to antibiotics.

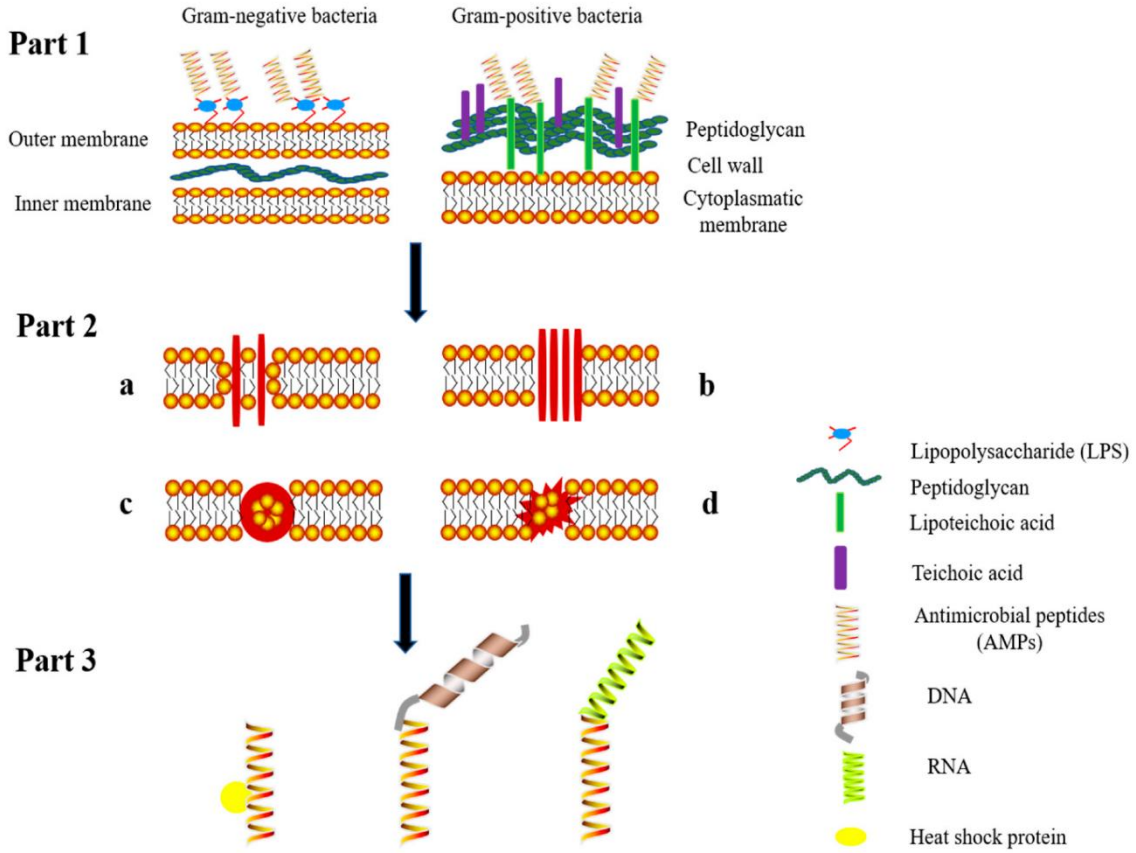


Figure 2. 4 The possible mode of action of AMPs from HI to exhibit antimicrobial activity (Xia et al., 2021). Binding of AMPs to lipopolysaccharides (LPS) of Gram-negative bacteria and to lipoteichoic or teichoic acid of Gram-positive bacteria and thus penetrate the cell wall (Part 1) and destroy the membrane structure of bacteria. The membrane damaging process takes place through four pathways including toroidal model (a), carpet-like model (b), barrel-stave model (c), and unstructured ring pores (d). AMPs antibacterial activity is facilitated by interactions with heat shock proteins, DNA and RNA.

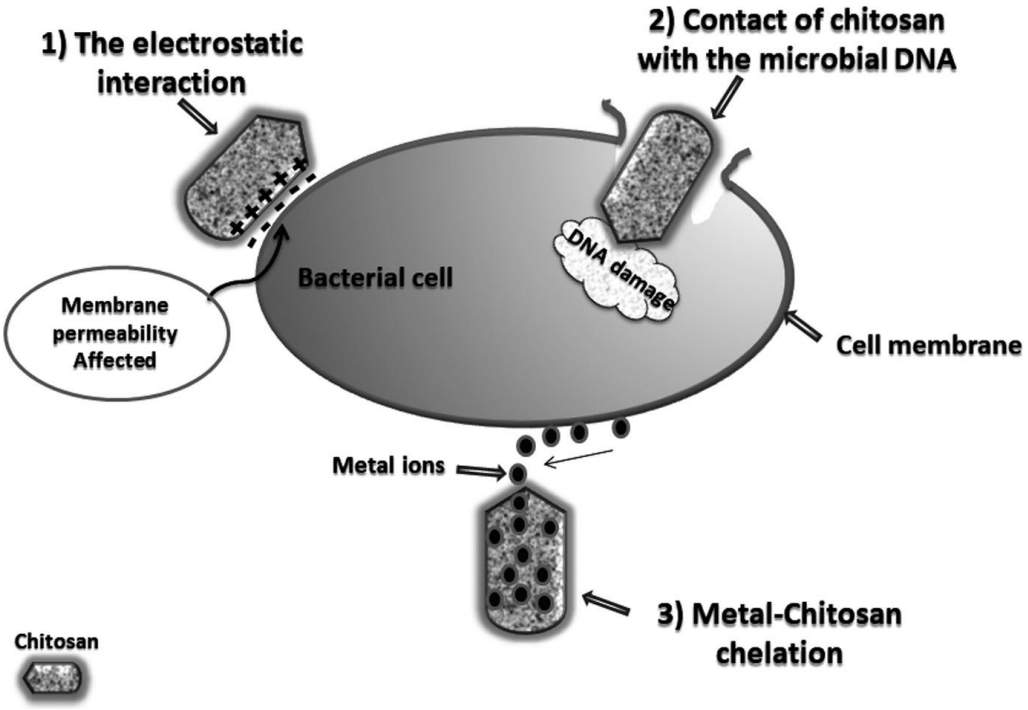


Figure 2. 5 The possible mode of action of chitosan of HI involves three different mechanisms (Abdel-Ghany and Salem, 2020): 1. Electrostatic forces between the protonated amine groups of chitosan and negative residue of bacteria leading to changes in membrane wall permeability by impacting internal osmotic imbalances and consequently hinder the growth of microorganisms. 2. Penetration of chitosan into the nuclei of the microorganisms via the contact of chitosan with the microbial DNA, leading to the inhibition of the mRNA and protein synthesis. 3. Chelation of metals, essentials nutrients required for microbial growth.

2.1.9 Conclusion and future perspectives

This review summarised the main results achieved in the last 15 years, hence highlighting ample scientific and technical scope to utilize HI larvae meal, either full-fat or defatted, as a replacement of FM and FO or plant protein sources partially and completely. The published results indicate that adding a small quantity of HI larvae has good potentiality for use as a complementary protein source for certain species and also can work as a prebiotic. However, in most of the studies, there was a measurable increase in SFA and a drop in the health benefiting essential fatty acid content in the edible portion of fish fed HI meal. This shortcoming may be resolved by incorporating limiting fatty acids or enriching HI larvae by manipulating feed substrates. Future studies should address the following issues:

I. The production of HI larvae has doubled in 2016 when compared to the production in 2014-2015 but it is important to scale up the production further with a continuous

quantity and quality to decrease the cost of HI larvae production in order for to be price competitive with currently used protein sources such FM and soybean meal.

II. Although chitin has been reported as an immunostimulant and preferential substrate for beneficial bacteria in the gut of some aquaculture fish, chitin has been reported to work as an anti-nutritional factor for some other species. Hence, further study is needed to developing a processing technology to remove the indigestible parts from HI meal which may open a new way to utilize HI meal for other species which cannot digest HI chitin.

III. It is important to identify the best substrates either single or mixed substrates (e.g. agriculture wastes and by-products and fish waste or by-products from aquaculture or seafood industry) to grow HI efficiently along with ensuring enriched essential fatty acid levels comparable to FM.

IV. HI larvae meal at smaller quality has been reported to work as a potential prebiotic for some but not all species. These results should be extended, at species-specific levels, to find out the optimum levels and stage of HI larvae which may be used as complementary protein sources in aquafeed to enhance gut health.

V. HI larvae contain some novel functional molecules such as antimicrobial peptides or polysaccharides (*e.g.* silkrose or dipterose) which have high potentiality for use as functional materials to enhance the functionality in aquafeeds. For example, silkrose, a novel polysaccharide recently extracted from an insect, stimulated disease resistance against bacteria in penaeid shrimps fed silkrose supplemented experimental diets (Ali et al., 2018). In addition, antimicrobial peptides extracted from HI larvae showed defensive mechanisms against bacteria, fungus, and viruses (Elhag et al., 2017; Park et al., 2015). Hence it is important to extract these functional molecules from HI larvae and investigate subsequent supplementation in aquafeeds to investigate if these molecules could be used as immunostimulants and as an alternative to conventional antibiotics.

VI. Though a number of studies have not found any significant effects of HI inclusion on fillet quality of fish, more studies are suggested to assess consumer perceptions and willingness-to-pay for fish produced on HI meal.

VII. Lastly, there is a lack of information currently on the effect regarding the use of HI meal through the food supply chain, from rearing substrates to fish which requires further study focusing on bioaccumulation of organic and inorganic contaminants and potential pathogens contamination from rearing substrates, to evaluate the quality, safety and security of fish fed on HI meal.

2.2 Fish protein hydrolysates and poultry by-product in aquafeed

2.2.1 Fish protein hydrolysates

Aquaculture by-product wastes are discarded by the fish processing industry in the form of head, skin, trimmings, fins, frames, viscera, and roes which have been estimated by more than 60% (Dekkers et al., 2011). Such a huge amount of discarded fish waste would be associated with different environmental issues in both developed and developing countries. It is estimated that the Australian seafood industries produce 100,000 tonnes of by-products per year (Peter and Clive, 2006) which need AUD 15 million annually on disposal (He et al., 2013). While some of the fish waste, rich in protein usually convert to FM (Hsu, 2010), a number of biotechniques have been developed to extract essential nutrients and functional components to be used for protecting several diseases and maintaining good health of human (Chalamaiah et al., 2012). In addition, some of this waste can be converted to fish protein hydrolysates by enzyme hydrolysis for subsequent use in aquafeed as immunostimulants, attractants, and palatability enhancers (Siddik et al., 2021). Enzymatic hydrolysis cleavage protein into small fragments of bioactive peptides containing 2-20 amino acids (Chalamaiah et al., 2012) which are important for various physiological functions, depending on inclusion levels, of aquaculture fish species. Dietary inclusion of > 15% FPHs have been reported to impact the growth performance of barramundi (Siddik et al., 2018a), Japanese flounder, *Paralichthys olivaceus*, turbot (Zheng et al., 2014), *Scophthalmus maximus* (Xu et al., 2016) which could be due to an excessive level of free amino acids associated with the saturation of peptide in transport mechanisms (Carvalho et al., 2004; Ospina-Salazar et al., 2016) and increase in amino acid oxidation and decline in the retention of dietary protein (Aragão et al., 2004; Kolkovski and Tandler, 2000). In contrast, a good number of studies have proven the ability of smaller inclusion of FPHs to enhance the biochemical response, immune responses, and disease resistance against pathogens in various farmed fish such as barramundi and other fish species, which has been described in detail in the review of Siddik et al. (2021). Furthermore, FPHs prevented the negative effects caused by the exclusive inclusion of animal and plant-based protein and improved the functionality by growth and immune responses of barramundi (Siddik et al., 2019b), Atlantic salmon (Egerton et al., 2020), and European sea bass (Gisbert et al., 2018) [*On this topic, a review article* (Siddik et al., 2021) *has been published from our laboratory elucidating production of various FPHs and their potential application as functional ingredients to improve the functionality of aquafeed*].

2.2.2 Poultry by-product meal

Rendered animal by-products such as poultry by-product meal (PBM), blood meal, feather meal, meat, and bone meal are commonly used ingredients in aquafeed formulation for finfish and crustaceans due to consistent availability and relatively cheaper compare to conventional protein sources (*i.e.,* FM) (Galkanda-Arachchige et al., 2020; Simon et al., 2019). PBM is generally comprised of discarded ground rendered parts of the carcasses but not including feathers and intestines considered ill-favoured for human consumption (Galkanda-Arachchige et al., 2020; Lewis et al., 2019). PBM is rich in protein and contains essential amino acids almost similar to FM with little or no presence of anti-nutritional factors when compared with terrestrial plant-derived protein sources (NRC, 2011; Simon et al., 2019). However, the confidence of aquafeed manufacturers to include PBM as a protein source is currently prevented by variable nutritional composition and digestibility. These variabilities have been linked to raw material freshness, cooking condition (*e.g.,* temperature and duration), and excessive processing conditions (e.g., extreme heat) which may cause nutritional degradation, generation of Maillard reaction products, oxidation of protein and lipid, formation of biogenic amines, amino acids cross-linking and racemisation (Galkanda-Arachchige et al., 2020; Lewis et al., 2019). Modern rendering processing through a computerized process control in time and temperature of the cooking process and laws and regulations in the selection of raw material plays a vital role in counteracting these challenges (Bureau et al., 1999; Cruz-Suárez et al., 2007; Garza de Yta et al., 2012). The suitability of different levels of PBM has been tested on a variety of commercially important aquaculture finfish and shellfish species and tolerance levels varied significantly among fish species (Galkanda-Arachchige et al., 2020). Dietary inclusion of PBM in different marine fish such as Juvenile Black Sea Bass, *Centropristis striata* (up to 90%) (Dawson et al., 2018), Japanese Sea Bass, *Lateolabrax japonicus* (up to 80%) (Wang et al., 2015), Totoaba, *Totoaba macdonaldi* (up to 67%) (Zapata et al., 2016) and Florida Pompano, *Trachinotus carolinus* (up to 67%) (Riche, 2015; Rossi and Davis, 2012), cobia, *Rachycentron canadum* (up to 60%) (Zhou et al., 2011), red sea bream yearlings, *Pagrus major* (up to 59%) (Takagi et al., 2000) spotted rose snapper, *Lutjanus guttatus* (up to 50%) (Hernández et al., 2014), gilthead sea bream, *Sparus aurata* (up to 50%) (Nengas et al., 1999), black sea turbot, *Psetta maeoticus* (up to 50%)(Yigit et al., 2006), Chinook salmon, *Oncorhynchus tshawytscha* (up to 50%) (Fowler, 1991), silver seabream, *Rhabdosargus sarba* (up to 25%) (El-Sayed, 1994), Australian snapper, *Pagrus auratus* (up to 21%) (Quartararo et al., 1998), red drum, *Sciaenops ocellatus* (up to 14%) (Kureshy et al., 2000), tiger grouper juveniles, *Epinephelus fuscoguttatus* (100%) (Gunben et al., 2014) and humpback grouper, *Cromileptes altivelis* (100%) (Shapawi et al., 2007) showed equivalent growth in comparison with those fish

fed FM-based diet. From the aforementioned studies, it is suggested that PBM alone was not able to replace FM completely for most of the marine fish species. [*In this issue, a remarkable meta-analysis of 47 published articles on PBM inclusion targeting 33 different species mainly freshwater, marine water fish, and crustaceans is available, describing the opportunities and challenges of PBM inclusion as an alternative to FM in aquafeed formulation* (Galkanda-Arachchige et al., 2020)]. Hence, complementation of PBM with other functional ingredients could be a strategy to increase the inclusion efficiency of PBM. A study by Siddik et al. (2019b) supplemented FPH to improve the functionality of PBM and found that FPH supplemented PBM can replace FM completely from the barramundi diet with an improvement in growth and immune responses.

2.3 Summary

In aquaculture, a multitude of ingredients is used to formulate aquaculture feeds, and therefore it is unlikely, nor necessary, that FM will be exclusively or fully replaced with a single protein source. Multiple protein sources and feed supplements can be used to facilitate the replacement of FM with alternative protein ingredients thereby improving the functionality of aquafeed. Hence, the mixture of PBM, HI larvae meal, and FPHs produced through a circular bioeconomy approach could allow a revolution in the aquaculture sector by achieving ‘Aquafeed 3.0’.

CHAPTER 3: Total replacement of fishmeal with poultry by-product meal affected the growth, muscle quality, histological structure, antioxidant capacity and immune response of juvenile barramundi, *Lates calcarifer*

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Abstract

The present study investigates if the total replacement of dietary fishmeal (FM) with poultry by-product meal (PBM), supplemented with methionine influences the muscle fatty acids composition, normal gut morphology, histological traits of the liver, muscle, and gill, liver enzymes, immune and antioxidant response, and stress-related gene in juvenile barramundi, *Lates calcarifer* in relation to growth and feed utilization. Barramundi (3.58±0.01g) were randomly distributed into six 300 L seawater recirculating tanks (25 fish/tank) and fed two formulated isonitrogenous and isolipidic diets for 6 weeks. The control diet had FM as the sole animal protein source, whereas other test diet had only PBM as an animal protein source. Dietary PBM affected the fish performance and feed utilization. Regarding muscle fatty acid profile, total saturated fatty acids and monounsaturated fatty acids elevated while total PUFA particularly n-3 LC-PUFA and EPA decreased in PBM fed fish than control diet fed fish. Liver, muscle, gill, and intestinal histology showed no obvious alteration in control diet fed fish, however, more lipid droplets and hepatic vacuolization in the liver, necrotic myotome in muscle, hyperplasia in secondary lamellae in gill and short and broken folds in the intestine were observed in PBM fed fish. Similar to light microscopy observation of intestinal morphology, the transmission electron microscopy (TEM) analysis revealed shorter and smaller microvilli in fish fed PBM. Histopathological alterations in the liver of PBM fed fish were further associated with the elevated levels of aspartate aminotransferase (AST) and glutamate dehydrogenase (GLDH) and the significant upregulation of stress-related genes, HSP70 and HSP90. Also, a negative influence on lysozyme activity, and antioxidant enzymatic activities were recorded in fish fed PBM. Overall, it can be concluded that a total substitution of FM protein by methionine supplemented PBM negatively influenced the growth performance, liver health, histological traits of different organs, immune and antioxidant response, and expression of stress-related genes in juvenile barramundi.

3.1 Introduction

One of the major bottlenecks for carnivorous aquafeed production is the inconsistence supply of global fishmeal (FM) and escalated prices. Therefore, efforts have been exerted over several decades to investigate the feasibility of alternative dietary protein sources replacing FM in carnivore finfish aquaculture (Gatlin et al., 2007; Hardy, 2010; Pham et al., 2020; Tacon and Metian, 2008). Presently, three main categories of FM replacements including terrestrial plant meals, rendered animal by-products, and seafood processing wastes are commercially available and used (Klinger and Naylor, 2012). However, more than 50% of substitution of FM is now regularly achieved commercially in most carnivorous species (Klinger and Naylor, 2012), including barramundi or Asian sea bass, *Lates calcarifer* a commercial important carnivorous fish species (Van Vo et al., 2020a). Barramundi has good meat quality, ability to tolerate a wide range of salinity, and ability to adapt to the versatile farming environment (Siddik et al., 2019a). It is popularly cultivated both in freshwater and seawater in Malaysia, Thailand, Taiwan, Indonesia, Saudi Arabia, and Australia, contributing USD 320 million globally (Simon et al., 2019; Van Vo et al., 2020b). In Australia, barramundi farming is heavily dependent on imported FM resulting in incurring around 40% diet related cost which is the main impediment to increase the profitability (Williams et al., 2003a). Hence, nutritional studies on barramundi have commenced since the 1980s (Glencross, 2006) and many of the studies have dedicated to replacing the FM with rendered animal meals (Chaklader et al., 2019; Glencross, 2011; Glencross et al., 2011; Siddik et al., 2019a; Siddik et al., 2019b; Siddik et al., 2019c; Williams et al., 2001; Williams et al., 2003a; Williams et al., 2003c) or plant meals (Glencross, 2011; Glencross et al., 2011; Ilham et al., 2016a; Ilham et al., 2016b; Irvin et al., 2015; Ngo et al., 2015; Van Vo et al., 2015; Vo et al., 2020).

Poultry by-product meal (PBM), an economical and easily available ingredient compared to FM contains a higher level of protein and most of the indispensable amino acids except for lysine and methionine (González-Rodríguez et al., 2016; Gupta et al., 2020; Zhou et al., 2011). Although, significant research in Australia and New Zealand has been conducted to commercially utilize PBM in various industries, its worldwide utilization is controlled by several regulations, for example the ban in European Union that has been recently lifted to allow the utilization of non-ruminant processed animal protein for aquaculture species (Klinger and Naylor, 2012). Like other animal-based protein, another major limitation regarding the utilization of PBM is the variable digestibility due to variability in its composition and quality (Galkanda-Arachchige et al., 2020). There have been several studies examining the effect of PBM on barramundi but results are mixed. For instance, Glencross et al. (2016) reported that the inclusion of poultry meals up to 338g/kg does not influence the growth but beyond this level had a deleterious effect. Besides, a recent study

of Simon et al. (2019) found that barramundi growth was impaired despite feeding balanced poultry protein concentrate (5-20%) while PBM along with supplementation of tuna hydrolysate could replace 90% of FM without impairing the growth (Siddik et al., 2019b). In our earlier study, regardless of full-fat black soldier fly larvae supplementation, barramundi fed 90% PBM impacted the growth performance (Chaklader et al., 2019). Similarly, the utilization of PBM above 50% affected the welfare of some marine fish species (González-Rodríguez et al., 2016; Karapanagiotidis et al., 2019; Shapawi et al., 2007; Yigit et al., 2006; Zhou et al., 2011). Imbalanced dietary essential amino acid (EAA) particularly methionine and lysine in PBM based diets are one of the major causes resulting growth depression in many fish (Galkanda-Arachchige et al., 2020). The methionine requirement for barramundi was reported to be 2.2% (Glencross, 2006). In this study, methionine was supplemented to PBM to investigate if supplementation of deficient EAA in PBM could substitute FM totally.

In addition to assessing the growth performance related to non-FM protein ingredients, health aspects including the changes in serum biochemical assays, immune responses, and stress-related oxidative biomarkers are also crucial parameters of interest to aquaculturists. Nutritional factors can influence the production of oxidative enzymes (Martínez-Álvarez et al., 2005). Oxidative stress is characterized by an increase in malondialdehyde (MDA) and a decrease in glutathione peroxidase (GPx) (Sinha et al., 2014). The imbalance between antioxidant defences and free radical generation may cause cell damage, which may provoke the leaking of liver enzymes particularly ALT and GLDH in fish (Hoseini et al., 2019), associated with liver cell damage. Although published data are available on the effects of plant protein on oxidative biomarkers of barramundi (Ilham et al., 2016a; Ilham et al., 2016b; Ilham and Fotedar, 2017; Ilham et al., 2018), there is less information on the effects of animal protein inclusions. The dietary inclusion of PBM impacted the liver health of barramundi by increasing the levels of ALT and GLDH (Chaklader et al., 2019), and animal protein ingredients elevated the levels of AST and ALT in hybrid grouper, *Epinephelus fuscoguttatus*♀× *Epinephelus lanceolatus*♂ (Ye et al., 2019a). Moreover, the equilibrium between oxidants and antioxidants is also important for immune cell function since it preserves the integrity and functionality of the cell membrane. Hence, it is crucial to understand the correlation between the antioxidants, liver enzymes, and immune response when entire dietary FM is replaced by any alternate- protein ingredients.

During dietary modification, it is important to consider that replacement of FM with potential ingredients do not exert adverse effects on the welfare of the tested species, as the welfare of fish in captive condition has been a growing concern over the decades (Huntingford and Kadri, 2014; Saraiva et al., 2015; Tort et al., 2011). The histological approach is one of the important frontline

tools applied to assess the health status that can be achieved by evaluating the morphological status of different organs. The liver is the biggest organs involved in nutrient metabolism and producing biochemical compounds required for digestion. Muscle structure is also important as it reflects the nutritional condition and agility of the fish. The intestine is a primary immune organ in fish participated in digestion and absorption of nutrients as well as defence mechanism against microbes (Siddik et al., 2018b). The evaluation of histopathological changes in these organs is important to assess the non-FM diet. Therefore, the present study aimed to investigate whether methionine supplemented PBM based diet has an ability to substitute FM completely without compromising growth performance, fatty acids composition, histological traits of different organs, serum biochemical response, stress-related genes expression, and antioxidant activities in barramundi.

3.2 Materials and methods

3.2.1 Animal ethical statement

The experiment was conducted at Curtin Aquatic Research Laboratory (CARL) in Curtin University, Australia in compliance with relevant guidelines and regulations set by the Australian Code of Practice for the care and use of animals for scientific purposes. All methods involving fish were reviewed and approved by the Curtin University Animal Ethics Committee (ARE2018-37). Prior to handling fish, AQUI-S® was used as anaesthesia and an overdose of AQUI-S was used as euthanasia to minimise stress, pain, and discomfort to the fish following the protocol of the Curtin Research Laboratories standard operating procedure (SOP) of anaesthetizing and euthanizing of fish.

3.2.2 Experimental diets

Except PBM, all the ingredients required for formulating test diets were purchased from the Special Feeds, 3150 Great Eastern Hwy, Glen Forrest, WA. Two isonitrogenous and isolipidic containing approximately 48% crude protein and 13% crude lipid were prepared to meet the nutritional requirement of barramundi (NRC, 2011). FM and PBM were used as the main protein source and canola oil and cod liver oil were used as lipid sources. A control diet was prepared based on FM and another diet was formulated by replacing 100% of FM with PBM supplemented with 0.40% methionine (Table 3. 1) to meet the established methionine requirement for normal growth of barramundi (Glencross, 2006; Poppi and Glencross, 2018). The diets were formulated in compliance with the standard protocol of CARL. Briefly, all the dry ingredients were mixed homogeneously using a food mixture (Hobart Food equipment, Australia) before blending with fish oil and distilled warm water to make a stiff dough. The dough was passed through a mincer

to make 3 mm pellets, then spread out and dried in an oven at 60°C for 36 hours. After drying, pellets were sealed in plastic bags before refrigerating at 4°C until used in the feeding trial. The fatty acid and amino acid profile of test diets and PBM is shown in Table 3. 2 and Table 3. 3, respectively.

Table 3. 1 Formulation and proximate composition of test diets for barramundi.

Ingredients[a] *(g/100g DM)*	Control	100PBM
†FM	72.00	0.00
‡PBM[b]	0.00	69.50
Canola oil	1.00	3.00
Cod liver oil	0.50	6.00
Corn/wheat starch	7.00	7.00
wheat (10 CP)	16.90	11.50
Lecithin - Soy (70%)	1.00	1.00
Vitamin C	0.05	0.05
Dicalcium Phosphate	0.05	0.05
Methionine	0.00	0.40
Vitamin and mineral premix	0.50	0.50
Salt (NaCl)	1.00	1.00
Proximate composition (% dry weight)[c]		
Moisture	14.96	13.98
Crude Protein	47.88	47.86
Crude Lipid	12.59	12.71
Ash	9.67	10.24
Gross energy (MJ/kg)	20.23	19.95

[a] Specialty Feeds, Glen Forrest Stockfeeders, 3150 Great Eastern Highway, Glen Forrest, Western Australia 6071

[b]Kindly provided by Derby Industries Pty Ltd T/A, Talloman Lot Lakes Rd, Hazelmere WA 6055

[c]Analysed according to Association of Official Analytical Chemists (AOAC), (AOAC, 1995)

† FM (Fishmeal): 64.0% crude protein, 10.76% crude lipid and 19.12% ash.

‡ PBM (Poultry by-product meal): 67.13% crude protein, 13.52% crude lipid and 13.34% ash

Table 3. 2 Fatty acids (mg/100g of dry sample) composition of control and test diet replacing FM totally with PBM in barramundi.

	Experimental diets		
Fatty acids	Control	100PBM	PBM
C12:0	2.73	7.11	9.39
C14:0	131.63	342.45	73.69
C16:0	1161.21	2090.88	2336.27
∑SFA[1]	1981.40	3216.29	3344.73
C14:1n5	1.52	11.32	16.26
C16:1n7	165.22	435.58	540.27
C18:1cis+trans	1158.94	3800.14	4410.64
C20:1	79.86	483.71	60.84
∑MUFA[2]	1482.21	4873.58	5057.95
C18:3n3	120.20	285.67	260.39
C20:5n3 (EPA)	178.50	278.99	16.79
C22:5n3#	63.30	64.60	36.67
C22:6n3 (DHA)	908.53	455.23	27.47
∑n-3 PUFA[3]	1309.12	1240.95	358.96
C18:3n6	8.84	10.58	23.07
C20:3n6	15.50	18.76	56.76
C20:4n6	112.83	43.18	180.13
C22:4n6#	91.14	16.39	4.78
∑n-6 PUFA	228.31	88.91	264.74
∑n-3/n-6	5.73	13.96	1.36
∑PUFA[4]	2184.01	2437.48	2386.97
∑n-3 LC-PUFA	1158.61	806.57	85.36

[1]Contains 10:0, 13:0, 15:0, 17:0, 18:0, 20:0, 21:0, 22:0 and 23:0

[2]Contains C15:1, C17:1, C22:1n9 and C24:1

[3]Contains C18:4n3, C20:3n3

[4]Contains C18:2 trans, C18:2 cis, C20:2, C22:2

Poultry by-product meal, PBM; saturated fatty acids, SFA; monounsaturated fatty acids, MUFA; polyunsaturated fatty acids, PUFA

Eicosapentaenoic acid, EPA; DHA, docosahexaenoic acid, sum of saturated fatty acids, ∑SFA; sum of monounsaturated fatty acids, ∑MUFA; sum of polyunsaturated fatty acids, ∑PUFA; sum of omega-3 polyunsaturated fatty acids, ∑n-3 PUFA; sum of omega-6 polyunsaturated fatty acids,

∑n-6 PUFA and LC-PUFA, long-chain polyunsaturated fatty acids (sum of 20:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3)

Table 3. 3 Amino acids (g/100g on dry matter basis) composition of test diets and PBM.

	Experimental diets		
Amino acids[a]	Control	100PBM	PBM
Hydroxyproline	1.7	3.2	3.2
Histidine	2.4	1.8	1.8
Taurine	0.5	0.5	0.4
Serine	5.3	5.0	5.0
Arginine	4.5	4.8	5.1
Glycine	13.2	16.4	16.5
Aspartic acid	8.8	7.8	7.8
Glutamic acid	11.7	12.1	11.5
Threonine	4.9	4.2	4.3
Alanine	9.4	9.1	9.1
Proline	6.1	7.3	7.0
Lysine	6.2	5.3	5.5
Tyrosine	2.0	1.8	2.0
Methionine	2.4	2.2	1.8
Valine	5.6	5.2	5.1
Isoleucine	4.3	3.8	3.8
Leucine	7.6	6.4	6.9
Phenylalanine	3.3	3.0	3.0

[a]Determined including hydroxyproline and taurine analysis following our earlier study (Chaklader et al., 2020c)

3.2.3 Fish husbandry and management

Three hundred and fifty barramundi were obtained from the Australian Centre for Applied Aquaculture Research (ACAAR), Fremantle, Australia in oxygenated plastic bags. Prior to commencing the trial, all fish were stocked into two fiberglass tanks (300 L) filled with ocean water and fed a commercial diet (470 g protein kg^{-1} diet and 20.0 MJ kg^{-1}dietary gross energy) twice daily for two weeks to acclimate them to CARL experimental facilities and conditions. Following acclimation, 150 normal and visually healthy fish averaging (3.58±0.01g) were randomly distributed into six 300-L tanks, containing 250 L water in each tank. Therefore,

stocking number of barramundi in each tank was 25. Each tank was equipped with an aerator, electric heater, and external bio-filter (Astro® 2212, China) to maintain DO, temperature, and other water quality parameters at an optimal level. Hence, the temperature was maintained at 27.90–29.20 °C, dissolved oxygen (DO) at 5.92–7.42 mgL^{-1}, salinity at 32–36 ppt, and photoperiod as 14:10 h LD. Commercial test kits were used to test ammonia nitrogen (<0.50 mgL^{-1}) and nitrite (<0.50 mgL^{-1}) level regularly. Each test diet had three replicates and fed by hand twice daily at 8.00 am and 6.00 pm to visual satiety levels for 42 days. Uneaten feed, if any, was collected by siphoning to calculate feed intake, and the number of dead fish were monitored daily to assess the fish survival rate. After 42 days, all fish were starved for 24 h prior to weighing total biomass to analyse the growth performance.

3.2.4 Fatty acids profile

Fish muscles in the form of three samples per dietary treatment were used for fatty acids analysis. Four fish muscle was filleted, wrapped with aluminium foil, freeze-dried, and pooled together. The fatty acids profile of experimental diets and fish flesh was carried out following the protocol of O'Fallon et al. (2007), and Siddik et al. (2019a). Approximately 0.5g of sample was hydrolysed at 55˚C for 1.5 hrs with 0.1ml of internal standard (1.2g nonadecanoic acid in 100ml chloroform), 0.7ml of 10N KOH and 5.3ml of methanol. The sample was then methylated at 55˚C for 1.5hrs with 0.6mL of 24N of sulphuric acid. The FAMES was extracted into 1ml of hexane and then quantified gas chromatography with flame ionization detection. The column used was a capillary column HP INNOWax GC column (60m x 0.25mm ID film 0.50 micron) with hydrogen as the carrier gas. Each sample were run in triplicate and results are expressed as an average.

3.2.5 Histological and transmission electron micrograph (TEM) analysis

After 42 days of feeding, one fish from each tank was randomly euthanized with AQUI-S at 175 mg/L to excise liver, muscle, gill, and intestine for histological and TEM evaluation in response to test diets. For histological analysis, samples of all organs were fixed immediately in 10% buffered formalin, subsequently dehydrated with series of ethanol, infiltrated in xylene, and embedded in paraffin wax, as per standard histological protocols. Section of approximately 5 µm thickness was stained with Periodic Acid-Schiff (PAS) and digitally photographed under a light microscope (BX40F4, Olympus, Tokyo, Japan).

For TEM analysis, freshly collected intestinal samples washed in 2.5% glutaraldehyde buffered in 1x PBS at pH 7.4 before performing secondary fixation in 1% OsO4 (80 W 2 min on, 2 min off, 2 min on), dehydrating in ethanol (50, 70, 95 and 100% at 250 W, 40 seach) and infiltrating finally with epoxy resin in acetone (Procure 812, Proscitech) (1:3, 1:1, 3:1ratios at 250 W, 3 min each).

Samples were processed as described in the earlier study in our lab (Siddik et al., 2019b) and screened a LaB6 TEM (JEOL2100, Japan) at 120 kV. The electron micrographs obtained from TEM analysis at 30,000 magnification were analysed using ImageJ (National Institute of Health, USA) to determine microvilli length and diameter.

3.2.6 Antioxidant status assessment

The enzyme activities of serum malondialdehyde (MDA) was determined using commercial assay kits following the manufacturer's instructions (Bockit, BIOQUOCHEM SL, 33428 Llanera-Asturias, Spain) and glutathione peroxidase (GPx) was measured with the Randox Laboratories test combination (Ransel, Antrim, United Kingdom) following the protocol of earlier study in our laboratory (Ilham et al., 2016a).

3.2.7 Serum biochemistry and immunity

Fish were captured gently at 42 days post-feeding, immediately dipped in a bucket containing 8 mg l^{-1} of AQUI-S®, and blood was taken by puncturing caudal vessels using 1 mL non-heparinized syringes (22G). Blood was allowed to clot for 24 h at 4 °C, centrifuged for 15 min at 3000 rpm and 4 °C, the serum collected and stored immediately at - 80°C for the analysis of serum biochemical parameters, oxidative biomarkers, and immune parameters. Serum clinical chemistry and immune-related parameters were analysed according to the protocol of our earlier study (Chaklader et al., 2019; Chaklader et al., 2020c).

3.2.8 RNA extraction and qRT-PCR analysis

Liver from control and PBM fed fish were aseptically collected after euthanizing (AQUI-S, 175 mg l^{-1}) the fish and preserved in RNA Later (Sigma-Aldrich, Germany) at - 80°C until RNA extraction. Five milligrams of liver tissue stored in RNA Later was used for RNA extraction using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer protocol. The quality of RNA was checked by gel electrophoresis and, the purity and quantity were determined by gel electrophoresis before synthesizing complementary DNA (cDNA) from 1 µg of total RNA using Omnicript RT kit (Qiagen, Hilden, Germany) following the instruction of manufacturer's company. qRT-PCR on stress-related genes were performed by PowerUp™ Cyber Green Master Mix (Thermo Scientific, USA) with 7500 Real-Time PCR System (Applied Biosystems, USA) and data were normalised against housekeeping genes, *18S rRNA* and *Ef1-a*, (Table 3. 4) and analysed using REST© software (Mohd-Shaharuddin et al., 2013).

Table 3. 4 Primers of qPCR used in the experiment

Genes	Sequences (5′ - 3′)	Product size	Tm (◦C)	
Heat shock protein kDa70, HSP70	F: AAGGCAGAGGATGATGTC R: TGCAGTCTGGTTCTTGTC	186	59	Mohd-Shaharuddin et al. (2013)
Heat shock protein kDa90, HSP90	F: ACCTCCCTCACAGAATACC R: CTCTTGCCATCAAACTCC	197	59	Mohd-Shaharuddin et al. (2013)
18S rRNA, 18S	F:TGGTTAATTCCGATAACGAACGA R: CGCCACTTGTCCCTCTAAGAA	94	59/60	Mohd-Shaharuddin et al. (2013)
Elongation factor-1α, ef1α	F: AAATTGGCGGTATTGGAAC R:GGGAGCAAAGGTGACGAC	83	59/60	Mohd-Shaharuddin et al. (2013)

3.2.9 Calculation and statistics

Specific growth rate (SGR), feed conversion ratio (FCR) and total feed intake (TFI) were calculated using the following equations-

Weight gain (WG, g) = [(Mean final weight-Mean initial weight)/(Mean initial weight)]

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

Feed conversion ratio (FCR) = [(dry feed fed)/(wet weight gain)]

Feed intake (FI,g/fish d^{-1}) = [(Dry diet given-Dry remaining diet recovered)/days of experiment)/ no. of fish]

All data were represented as mean ± SE. The differences between control and PBM fed fish in all data were determined by unpaired student *t*-test at the significance level of $0.05 < P < 0.001$. Percent survival at the termination of the feeding trial was plotted using the Kaplan-Meier survival method with the Log-rank (Mantel-Cox) test.

3.3 Results

3.3.1 Growth performance, feed utilization and survival

Fish growth, feed intake, and survival rate in response to 42 days feeding trial are presented in Table 3. 5 and Figure 3. 1. The mean final body weight (FBW) and specific growth rate (SGR) of fish fed PBM were significantly lower than the FBW and SGR of fish fed the control diet. FCR in PBM fed fish increased with lower feed intake in PBM fed fish. Survival rate (Figure 3. 1), as drawn by Kaplan-Meier survival analysis with 95% confidence at the end of the 42 days trial decreased significantly in PBM fed fish (81.33%) than the control (93.33%) ($\chi^2_{100PBM} = 4.514$, df = 1, $P = 0.034$).

Table 3. 5 Fish performance including Final Body Weight (FBW), Weight Gain (WG), Specific Growth Rate (SGR), Feed Intake (FI), and Feed Conversion Ratio (FCR) of barramundi when fed control and PBM based diet over a period of 42 days. Results are expressed as mean ± SE (standard error) (n = 3).

Growth performance	Experimental diets		Unpaired t-test	
	Control	100PBM	*t*-value	*P*-value
IW (g)	3.52±0.02	3.49±0.06	0.82	0.46
FBW (g)	54.91±0.55^a	32.67±0.23^b	37.38	0.00
WG (g)	51.39±0.53^a	29.18±0.23^b	38.93	0.00
SGR (%/d)	6.54±0.01^a	5.33±0.02^b	55.36	0.00
FI (g/fish d^{-1})	1.21±0.12^a	0.86±0.01^b	3.051	0.04
FCR (FCR)	0.99±0.15^a	1.24±0.01^b	-2.97	0.04

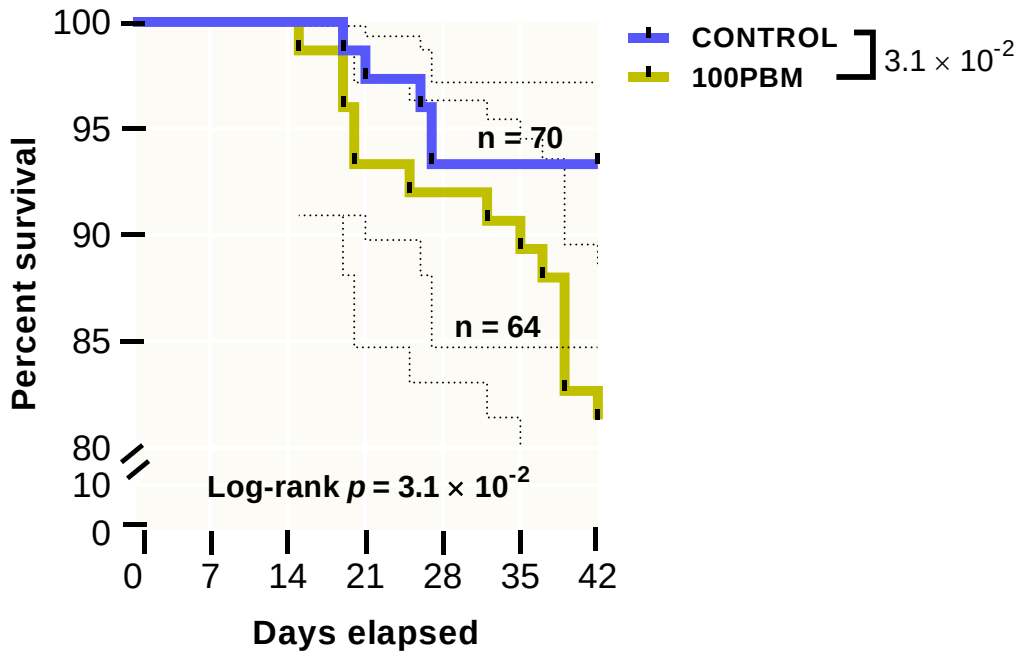

Figure 3. 1 Survival rate based on Kaplan-Meier survival analysis with Log-rank (Mantel-Cox) test of barramundi after 42 days feeding with either basal diet or PBM based diet. Dotted line in survival plot indicates 95% confidence interval and *P* value indicate significant at 0.05.

3.3.2 Muscle fatty acids composition

The FAs profile of barramundi muscle at the termination of 42 days trial was influenced by the PBM based diet (Table 3. 6). The dietary inclusion of PBM significantly augmented total SFA. All SFA including capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), margaric acid (C17:0), stearic acid (C18:0), arachidic acid (C20:0), heneicosylic acid (C21:0), behenic acid (C22:0) and tricosylic acid (C23:0) except for tridecylic acid (C13:0), and pentadecylic acid (C15:0) were significantly higher in the muscles of fish fed PBM. Similarly, total MUFA concentration increased in the muscle of PBM fed fish. PUFA differed significantly between the test diets, with lower concentration of n-3 LC-PUFA and C22:4n6 in PBM fed fish than the fish fed the control diet. Similar result was recorded in ∑n-3/∑n-6 ratio.

Table 3. 6 Fatty acids (mg/100g on dry matter basis) of barramundi muscle when fed control and PBM based diet over a period of 42 days. Results are expressed as mean ± SE (standard error) (n = 3).

Fatty acid	Experimental diets		Unpaired *t*-test	
	Control	100PBM	*t*-value	*P*-value
C12:0	1.02±0.09[a]	291.96±1.27[b]	-228.29	0.00
C14:0	67.46±0.77[a]	213.36±0.48[b]	-158.64	0.00
C16:0	713.65±10.19[a]	1253.24±68.93[b]	-7.75	0.00
∑SFA[1]	1153.45±15.15	2320.11±65.41	-17.38	0.00
C16:1n7	130.78±1.90[a]	286.88±2.21[b]	-53.82	0.00
C20:1	36.62±0.38[a]	111.68±2.97[b]	-25.07	0.00
C14:1n5	0.97±0.03[a]	6.30±0.06[b]	-84.46	0.00
C18:1cis+trans	859.76±5.73[a]	2961.30±69.99[b]	-29.93	0.00
∑MUFA[2]	1066.66±8.25[a]	3412.55±74.01[b]	-31.50	0.00
C18:3n3	55.12±0.38[a]	213.57±3.10[b]	-50.94	0.00
C20:5n3 (EPA)	109.31±1.78	113.69±1.31	-1.97	0.12
C22:5n3	71.50±0.95[a]	78.82±0.44[b]	-7.14	0.00
C22:6n3 (DHA)	683.13±12.43[a]	370.84±1.99[b]	24.82	0.00
∑n-3 PUFA[3]	940.67±15.93[a]	821.03±11.05[b]	5.24	0.01
C20:3n6	25.68±0.57[a]	49.16±2.84[b]	-8.07	0.00
C20:4n6	92.29±1.90[a]	114.06±3.54[b]	-5.43	0.01
C18:3n6	17.51±1.42[a]	51.44±6.52[b]	-5.08	0.01
C22:4n6	62.67±1.07[a]	23.06±0.26[b]	36.20	0.00
∑n-6 PUFA	198.15±3.86[a]	237.72±12.61[b]	-9.15	0.00
∑n-3/∑n-6	4.75±0.04[a]	3.47±0.18[b]	6.78	0.00
∑PUFA[4]	1485.98±24.56	1311.18±9.36	2.86	0.05
∑n-3 LC-PUFA[3]	868.55±15.25	569.45±2.78	19.29	0.00

[1]Contains 10:0, 13:0, 15:0, 17:0, 18:0, 20:0, 21:0, 22:0 and 23:0

[2]Contains C15:1, C17:1, C22:1n9 and C24:1

[3]Contains C18:4n3, C20:3n3

[4]Contains C18:2 trans, C18:2 cis, C20:2, C22:2

Poultry by-product meal, PBM; saturated fatty acids, SFA; monounsaturated fatty acids, MUFA; polyunsaturated fatty acids, PUFA and LC-PUFA, long-chain polyunsaturated fatty acids (sum of 20:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3)

Different superscripts letter indicate significant difference at $P < 0.05$, 0.01 and 0.001, followed by an unpaired student t-test.

3.3.3 Histopathology of liver, muscle, gill and intestine

Total replacement of FM with PBM dysregulated the histological structure of liver, muscle, gills, and intestine **(**Figure 3. 2A-H**).** The liver of control (Figure 3. 2A) fed fish showed higher pigmentation of hepatocyte cytoplasm, indicating a higher amount of glycogen, while the liver of PBM fed fish (Figure 3. 2B) showed less hepatocyte cytoplasm pigmentation, indicating less amount of glycogen with more lipid vacuolization. Healthy and normal myotome were observed in the muscle of the fish fed the control diet (Figure 3. 2C) but necrotic myotome was found in the fish fed PBM diet (Figure 3. 2D**)**. Control fed fish showed normal gill structure (Figure 3. 2E**)** but hyperplasia in secondary lamellae was recorded in PBM fed fish (Figure 3. 2F**)**. All the examined fish fed control (Figure 3. 2G**)** presented normal intestinal structure whilst broken and short fold were found in fish fed PBM (Figure 3. 2H**).**

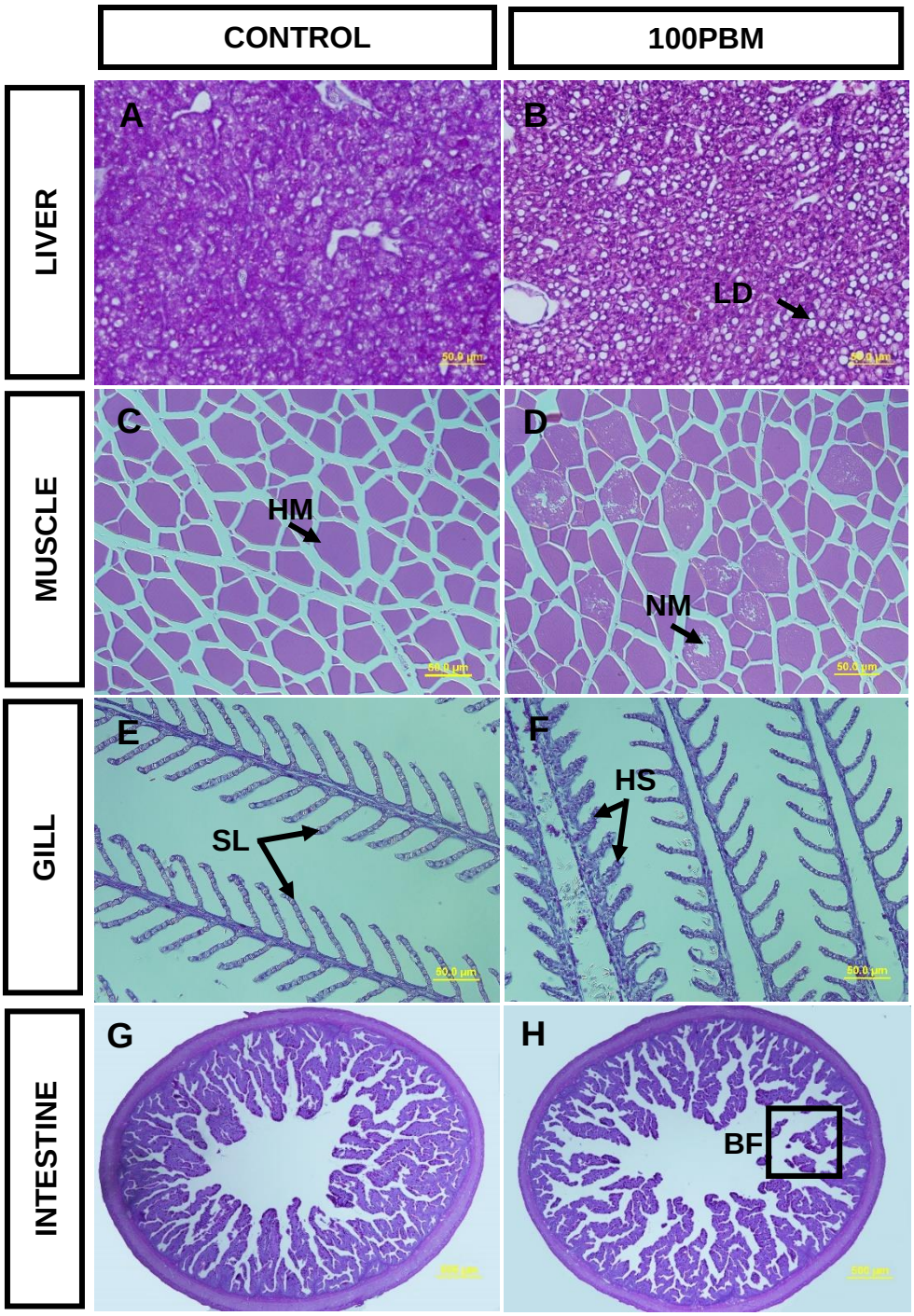

Figure 3. 2 Liver (A-B), muscle (C-D), gill (E-F) (PAS stain; 40 × magnification; scale bar = 50 µm) and distal intestine (G-H) (PAS stain; 4 × magnification; scale bar = 500 µm) sections of barramundi fed control and PBM based diet at the end of 42 days of feeding trial. Lipid droplet, LD; healthy myotome, HM; necrotic myotome, NM; secondary lamellae, SL; hyperplasia in secondary gill lamellae, HSL and broken fold, BF.

3.3.4 Intestinal morphology

The distal intestine of barramundi fed control (Figure 3. 3A) and PBM (Figure 3. 3B) were examined by transmission electron microscope. Microvilli height (Figure 3. 3D) (t = 6.727, df = 28, P < 0.0001) and diameter (Figure 3. 3E) (t = 3.494, df = 28, P = 0.0016) of barramundi fed PBM diet was significantly lower than barramundi fed control diet.

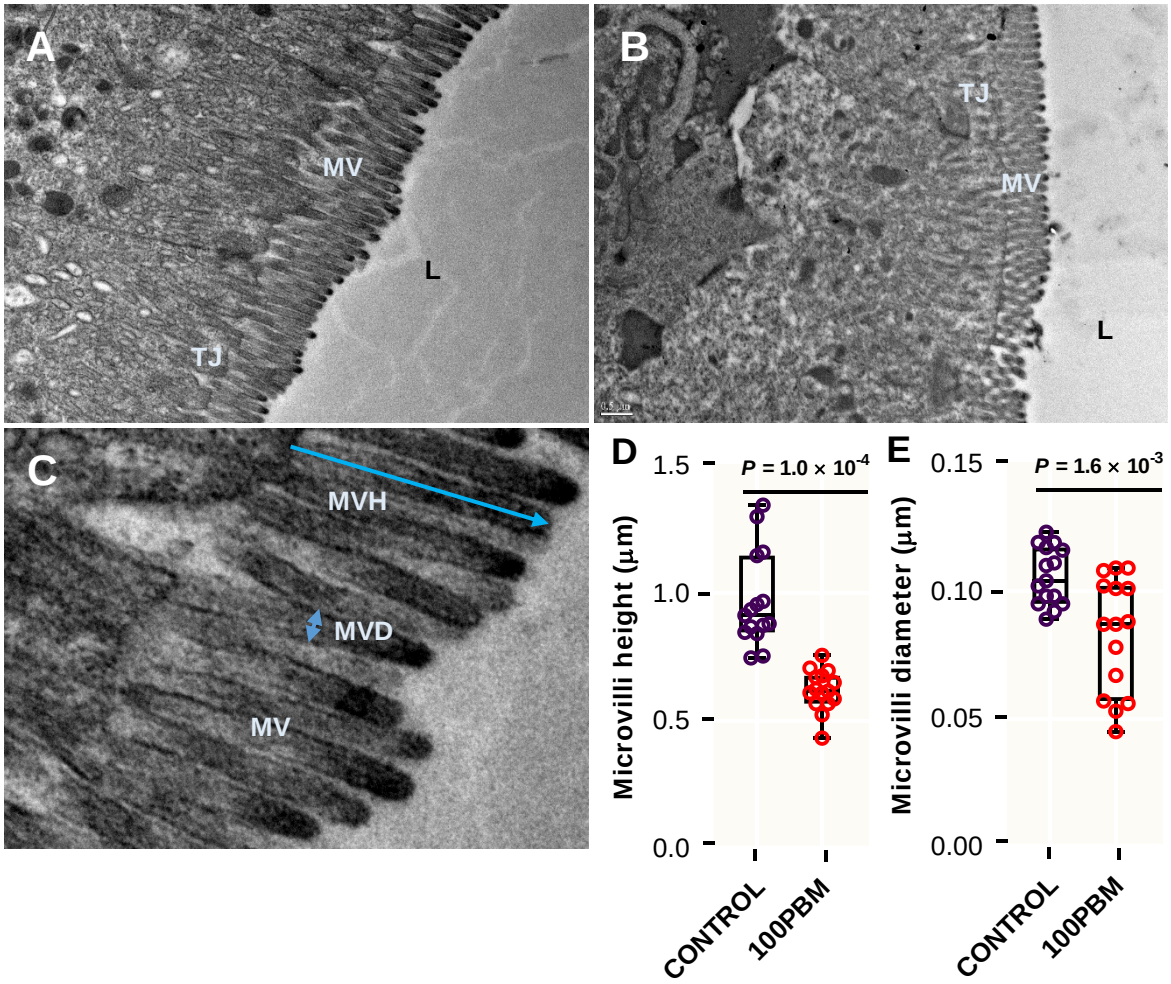

Figure 3. 3 Observation of TEM in the intestine of barramundi fed Control (A) and PBM (B) at the end of 42 days of feeding trial. (C) Microvilli height and diameter measurement and comparison of microvilli height and diameter (panel D & E), performed by an unpaired student t-test at P <0.05 and 0.01. Microvilli, MV; Microvilli height, MVH; tight junction, TJ; microvilli diameter, MVD.

3.3.5 Liver enzymes, immunity and stress related genes

Liver enzymes (AST and GLDH), immune response including serum lysozyme and bactericidal activity and stress related genes (HSP70 and HSP90) were significantly induced by the experimental diets (Figure 3. 4). AST and GLDH in PBM fed fish was significantly higher than

the control (t = 2.268, df = 10, P = 0.047 and t = 3.199, df = 10, P = 0.010) (Figure 3. 4A, B), while serum lysozyme decreased significantly in PBM fed fish compared to control (t = 2.842, df = 10, P = 0.018) (Figure 3. 4C). Meanwhile, none of the diets had significant effects on bactericidal activity (t = 1.572, df = 10, P = 0.147) (Figure 3. 4D). In line with liver enzymes, similar results were observed in HSP70 and HSP90 when compared with control (t = 2.905, df = 10, P = 0.016 and t = 5.102, df = 10, P = 0.001) (Figure 3. 4E, F).

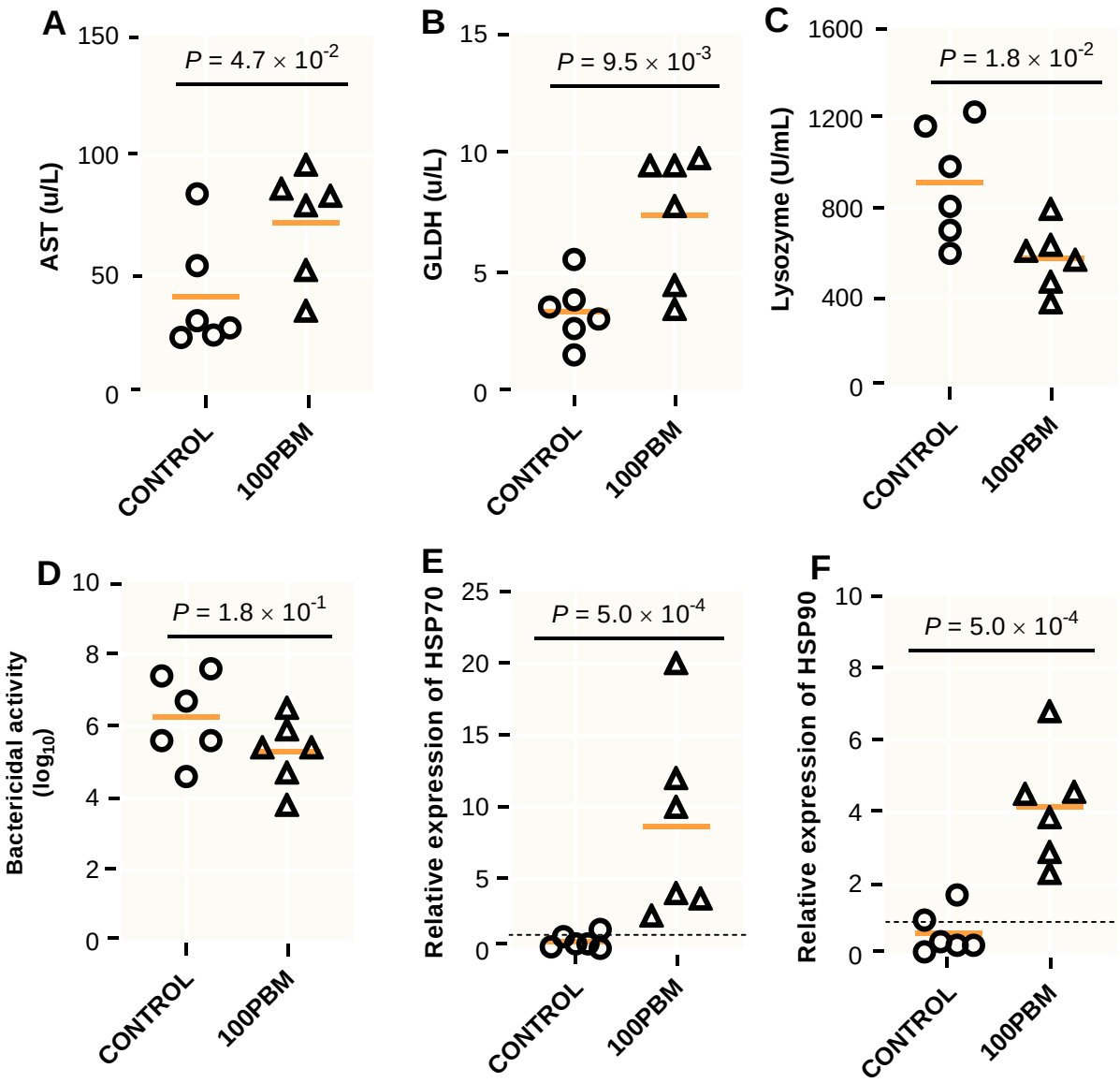

Figure 3. 4 AST, aspartate aminotransferase (A) and GLDH, glutamate dehydrogenase (B), lysozyme (C), bactericidal activity (D) and heat shock related gene including HSP70 (E) and HSP90 (F) in barramundi after 42 days feeding with either control diet or PBM based diet. P values indicate significant at $P < 0.05$, 0.01 and 0.001, followed by an unpaired student t-test.

3.3.6 Antioxidant activity

Antioxidant activities of blood serum were significantly affected by total inclusion of PBM. Serum GPx activity declined significantly in PBM fed fish (t = 2.833, df = 10, P = 0.017) (Figure 3. 5A), while MDA increased significant in PBM (t = 2.251, df = 10, P = 0.048) (Figure 3. 5B) with respect to control.

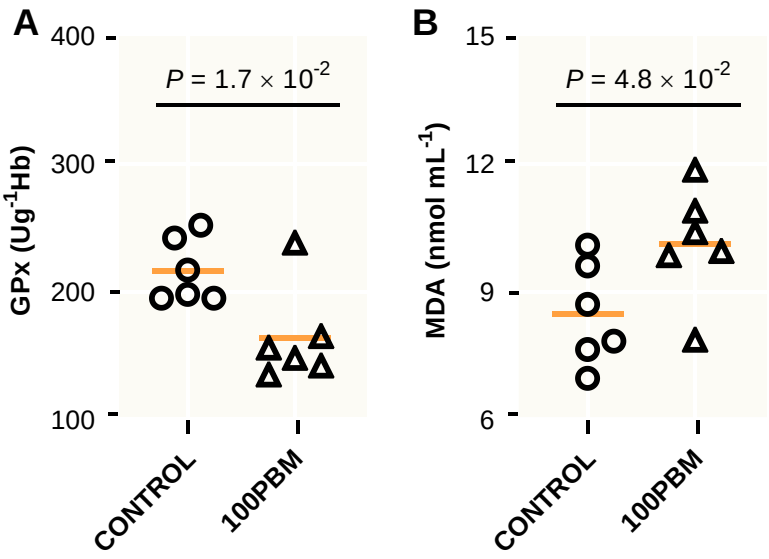

Figure 3. 5 (A) GPx (Ug^{-1} Hb) and (B) MDA (nmol mL^{-1}) in the serum of barramundi after 42 days feeding with either control diet or PBM based diet. P values indicate significant at $P < 0.05$, 0.01 and 0.001, followed by an unpaired student t-test.

3.4 Discussion

A good number of studies have been devoted over the years to incorporate different levels of PBM, at the expense of FM in the diet of finfish and shellfish aquaculture (Galkanda-Arachchige et al., 2020) but most of the studies were performed on the basic nutritional aspects including proximate composition, amino acids, and fatty acid content, and its potential effect on the growth performance of the host fish (Ayadi et al., 2012; Barreto-Curiel et al., 2016; Gümüş and Aydin, 2013). In depth investigations are still lacking pertaining to the effects of PBM on the integrity of different organs, stress-related genes expression, or antioxidative responses in barramundi.

Establishing protein derived from animal industry as an ideal feed for finfish aquaculture, a series of studies have been conducted in Australia especially on barramundi. For instance, dietary inclusion of high-quality poultry protein concentrate from 5 to 20% demonstrated a reduced growth performance despite providing balanced amino acids in the diet but the reverse trend was observed when supplemented with phosphorous (Simon et al., 2019). On the contrary, Siddik et

al. (2019b) were able to replace 90% FM with either bioprocessed or unprocessed PBM along with supplementation of fish protein hydrolysate with no apparent effects on the growth performance. In the present study, barramundi fed PBM supplemented with methionine affected the growth, feed utilization, FCR, and survival rate. Similarly, feeding barramundi non-FM based diet containing 450 g kg^{-1} PBM and 285 g kg^{-1} soybean meal impacted the growth, feed intake, and FCR despite supplementing taurine and the presumable reasons were palatability (Glencross et al., 2016). Deterioration in the growth performance of gibel carp, *Carassius auratus* gibelio was also observed when fed 100% animal protein containing PBM and meat and bone meal despite supplementation with methionine and lysine (Hu et al., 2008). The possible reasons were the nutritional superiority or enhanced palatability in FM that could not be met up by PBM and MBM. Methionine, lysine and arginine levels in the 100PBM diet were at optimum level for barramundi growth (Glencross, 2006) but histidine, isoleucine, and phenylalanine were lower compare to FM based diet which may suppress the growth performance. Similarly, deficiency of histidine, methionine, isoleucine, lysine, and phenylalanine were identified to reduce the growth performance of spotted rose snapper, *Lutjanus guttatus* with higher inclusion of PBM (Hernández et al., 2014). Moreover, the abundance of MUFA and n-6 PUFA coupled with a deficiency of EFA particularly n-3 LC-PUFA, EPA and DHA in PBM were highlighted as one of the reasons for the reduced growth in Totoaba (Zapata et al., 2016), catfish, *Ictalurus punctatus* (García-Pérez et al., 2018), black sea turbot, *Psetta maeotica* (Yigit et al., 2006) and gilthead sea bream, *Sparus aurata* L (Nengas et al., 1999). Similarly, higher MUFA content and lower levels of PUFA, in particular, n-3 LC-PUFA and EPA contents were found in 100PBM diets that could be responsible for the negative influence on growth, survival, feed utilization and FCR. In addition, n-3 PUFA have been reported as an indispensable FAs for optimum growth and survival of many marine fish species (Galkanda-Arachchige et al., 2020; Nengas et al., 1999). However, these findings contradict with the results of Panicz et al. (2017), Gunben et al. (2014) and Shapawi et al. (2007) who reported no adverse effects of 100PBM on the growth and biometry indices of female tenches, *Tinca tinca*, tiger grouper juveniles, *Epinephelus fuscoguttatus and* humpback grouper, *Cromileptes altivelis*. This heterogeneity might be due to use different fish species and culture system or variability in nutritional composition palatability, and digestibility of PBM as it varies from batch to batch or among supplier companies (Chaklader et al., 2019; Galkanda-Arachchige et al., 2020).

FAs composition of diet affects the FAs composition of fish muscle or meat which have been reported in many fish species (Emre et al., 2016; González-Félix et al., 2016; Xu et al., 2015). In the present study, FAs of barramundi fillet were affected by the PBM diet. Total muscle SFA concentration was significantly higher in PBM fed fish may be due to the abundance of palmitic

acid and myristic acid in fish muscle that are reflected in the PBM diet. A higher concentration of total SFA due to a high abundance of palmitic acid was observed in juvenile black sea bass fed 100PBM (Dawson et al., 2018). Muscle MUFA content in the present study increased in PBM fed fish which could be due to higher proportion MUFA in the diet. This finding was similar to our earlier study (Chaklader et al., 2020c). Lower concentration of n-3 LC-PUFA and adrenic acid in fish muscle resulted in low total PUFA and n-3/n-6 ratio which are similar to the findings in barramundi fed high levels of PBM (Siddik et al., 2019a). Similarly, 100PBM was lacking in essential fatty acids (EFAs) and also worsened the EFAs in the muscle of totoaba juveniles, *Totoaba macdonaldi* (Zapata et al., 2016). These results demonstrated that the total substitution of FM with PBM decreased PUFA levels in barramundi, which may consequently affect the nutritional value in terms of fatty acids available for human consumption.

It is well known that AST and GLDH are two important enzymes which primarily exist in liver at lower levels under normal condition but can leak into the blood rapidly when liver cells are damaged due to various stressors (Sabbagh et al., 2019; Wells et al., 1986). In the present study, the PBM diet significantly increased the levels of AST and GLDH in the serum of barramundi, concomitant with the histopathological damage of liver tissue. Likewise, plasma ALT was negatively impacted by the inclusion of animal protein blend (APB) (20% to 80%) in the diet of hybrid grouper while AST augmented significantly in 80% APB fed fish (Ye et al., 2019a). However, Panicz et al. (2017) reported no effects on blood biochemical parameters of juvenile tenches, *Tinca tinca* fed graded levels of PBM (25.7 to 100%).

To further clarify the effects of PBM on the liver function of barramundi, heat shock-related genes including HSP70 and HSP90 were examined. HSP70 and HSP90 are two important stress-related protein and their expression level elevate significantly when fish are exposed to different stressors, including pathogenic infection, crowding, poor water quality, and nutritionally deficient diet (Huang et al., 2015; Ming et al., 2012; Rollo et al., 2006). In the present study, both HSP70 and HSP90 upregulated significantly in the liver of barramundi that received 100PBM, indicating that 100% inclusion of PBM could act as a stressor.

In fish, immune functions of immune organs are strongly associated with the presence and activity of a unique array of molecules including lysozyme, complement proteins, immunoglobulins (Burgos-Aceves et al., 2016; Lauriano et al., 2016; Lazado and Caipang, 2014), and bactericidal activity that are influenced by the dietary modifications. Serum lysozyme was negatively triggered by PBM diet that support the findings of (Subhadra et al., 2006a); Subhadra et al. (2006b) who reported aggravated levels of complement and lysozyme activity in PBM fed largemouth bass, *Micropterus salmoides*.

Substitution of 100% FM with PBM resulted in lipidosis with clearly visible inflammation in the liver of juvenile tenches, *Tinca tinca* (Panicz et al., 2017), supporting our present findings as hepatocyte lipid vacuolization with less amount of glycogen was observed in the liver tissue of barramundi fed PBM. The excessive amount of fat deposition in the liver negatively impacted the growth and immune response of fish (Kang-Le et al., 2014) that are synchronous with the immunological results in the present study. Similarly, Siddik et al. (2019a) fed juvenile barramundi with different levels of PBM for 42 days and reported irregular liver arrangement with lipid deposition in the 100% PBM and bioprocessed PBM groups. Furthermore, higher administration of animal protein blend affected the morphology of the liver of hybrid grouper, characterized by hepatic vacuoles and a high amounts of lipid droplets which is a sign of hepatic steatosis (Ye et al., 2019a). The lipid accumulation in the liver may occur when dietary lipid exceeds the capacity of the hepatic cells to oxidize which lead to synthesize and deposit larger amounts of triglyceride in vacuoles (Kang-Le et al., 2014; Spisni et al., 1998; Ye et al., 2019a).

Muscle structure is the determinant of fish growth and can be affected by nutritionally-deprived diet (Alami-Durante et al., 2018). For example, nutritional deficiency altered the muscle structure of Atlantic salmon, *Salmo salar* including myodegeneration (Rodger et al., 1991). Similarly, fish fed PBM diet showed necrosis and fibre degeneration in muscle. Gill is one of the important immune organs in fish and its structure can be affected by stress and diet (Anderson Brunetti et al., 2009). In the present study, hyperplasia in secondary gill lamellae was in PBM fed fish but the possible reasons are not well understood, deserving further study.

Evaluating intestinal morphology in response to dietary changes is important to determine the health status and welfare of fish. Intestinal morphology, in particular, villous structure, and microvillus height and diameter is related to absorption and assimilation of nutrient and immunological function (Nicholson et al., 2012; Siddik et al., 2019b; Urán et al., 2008)**.** Histological analysis showed that broken and short fold in the present study in PBM fed groups are in line with TEM results, showing significantly smaller with a shorter diameter of microvilli, which are responsible for the lower efficiency of nutrient uptake, thus suppressing the growth and survival. Similar results were reported by Siddik et al. (2019b) who found significantly lower microvilli height in the distal intestine of barramundi after 56 days post-feeding with 10% supplemented 90PBM. Hence, total replacement of FM with PBM impacted the welfare of barramundi, as reflected by the histological and TEM analysis.

Antioxidant status in fish, as determined by several antioxidant enzymes including CAT, SOD, GPx, and MDA have been considered as the first line of defensive biomarkers to protect cells and tissues from oxidative damage, caused by some free radicals such as superoxide anion (O^{2-}),

hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) (Devi et al., 2019; Zheng et al., 2019). Glutathione peroxidase, GPx is an important antioxidant enzyme showing strong radical-scavenging capacity against free radicals and lipid peroxides (Wang et al., 2009). The present study detected a significantly lower activity of GPx in the serum of barramundi fed with PBM, which may due to the lower levels of n-3 LC-PUFA and DHA in 100PBM diet. Liu et al. (2015) reported that marine fish are susceptible to oxidative stress due to their high demand for LC-PUFA. However, elevated serum GPx activity in barramundi fed 90% fermented PBM supplemented with tuna hydrolysate (Siddik et al., 2019c) might be due to the antioxidant capacity of fish protein hydrolysate (Thiansilakul et al., 2007). It has been reported that the GPx activity is well correlated with the concentration of MDA (Sabzi et al., 2017). MDA is a natural biomarker and main ending product of lipid peroxidation (Nugroho and Fotedar, 2014; Satoshi et al., 1989) and its elevation indicates oxidative injury (Halliwell and Gutteridge, 2015) and associates with the pathological state of animals including cell structure damage and function (Nugroho and Fotedar, 2014; Satoshi et al., 1989). A lower activity of GPx with the higher level of MDA indicates that PBM based diet may provoke the oxidative damage of barramundi which was further proven by the presence of hepatocyte lipid vacuolization.

In summary, regardless of methionine supplementation, the total replacement of FM with PBM is not nutritionally adequate for barramundi, as indicated by depressed growth performance and immune response. An unfavourable effect of a PBM based diet was observed on antioxidant enzymes. Also, adding PBM induced the lipid droplet in the liver for barramundi via affecting the expression levels of heat shock related genes and liver enzymes. Feeding PBM not only triggered the fiber degeneration and necrosis in muscle and hyperplasia in gills but also induced the intestinal villus morphology by decreasing intestinal microvilli morphology, which may suggest that high levels of PBM could impair the welfare of barramundi. Further studies need to be conducted along with supplementation of other EAA and/or EFA with PBM to investigate the welfare of farmed barramundi.

CHAPTER 4: Insect larvae, *Hermetia illucens* in poultry by-product meal for barramundi, *Lates calcarifer* modulates histomorphology, immunity and resistance to *Vibrio harveyi*

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Abstract

This study investigated the effects of replacement of fishmeal (FM) with poultry by-product (PBM) protein, supplemented with black soldier fly, *Hermetia illucens* (HI) larvae on growth, histomormhology, immunity and resistance to *Vibrio harveyi* in juvenile barramundi. Two hundred and twenty five barramundi averaging 3.51± 0.03g were randomly allocated into three groups and fed isonitrogenous and isocalorific diets containing different levels of PBM supplemented with HI as follows: Control (FM based diet), 45PBM+ HI (45% PBM supplemented with 10% HI), and 90PBM+ HI (90% PBM supplemented with 10% HI) for 6 weeks. Results showed that dietary inclusion of 45PBM+HI significantly improved the growth performance than control whereas growth inhibition occurred in the 90PBM+HI. The 45PBM+HI groups demonstrated significant increases in histometric measurements (villus and enterocyte width, and microvilli height) and acidic mucins. The impaired growth in 90PBM+HI groups was further associated with multifocal necrosis in the liver, an upregulation of the stress related genes (HSP70 and HSP90) and increase in the levels of liver enzymes. When 45PBM+HI was fed, survival against *V. harveyi* increased significantly and also an increase in serum immunity and immune-related genes in the head kidney was observed after infection.

4.1 Introduction

For many decades, fishmeal (FM) has been used as an optimal protein source in aquafeed due to highly digestible protein, balanced amino acid composition and good palatability (Elia et al., 2018; Oliva-Teles et al., 2015). However challenges such as limited supply and increasing price of FM, burgeoning global demand for fish protein and the impact of FM production on wild marine fish stocks have motivated the aquaculture nutritionist to search and find sustainable protein rich ingredients for aquafeeds (FAO, 2016). Rendered animal by-product meals consisting of discarded parts of farmed animals not being suitable for human consumption have been used for many decades in aquadiets (Lewis et al., 2019) due to the good source of amino acids, higher protein content, and energy (Bureau et al., 1999; Zhou et al., 2011; Zhou et al., 2004). PBM, a rendered by-product from the poultry processing industry can be a viable protein source to be incorporated in the diet of carnivorous fish (Shapawi et al., 2007) as it has high protein content and favourable indispensable amino acid profiles (Castillo-Lopez et al., 2016; Riche, 2015; Tacon, 1993). In addition, it is a sustainable source of animal protein having a lower price than FM (Cruz-Suárez et al., 2007). However, nutritional composition and digestibility of PBM vary from batch to batch and among supplier companies, which is one of the limiting factors in the utilization of PBM in aquadiets (Lewis et al., 2019). PBM as a FM replacer has been evaluated on number of marine fish species (El-Sayed, 1994; Fowler, 1991; Goto et al., 2001; Kureshy et al., 2000; Nengas et al., 1999; Wang et al., 2006; Yigit et al., 2006) and success has been achieved in recent years when up to 100% PBM was able to replace FM in gilthead seabream, *Sparus aurata* L. (Nengas et al., 1999), red sea bream, *Pagrus major* (Takagi et al., 2000) and hybrid striped bass, *Morone chrysops* x *M. saxatilis* (Gaylord, 2005). However, higher inclusion of PBM levels in the diet of fishes has also been reported to result in a number of problems including deficiencies one or more essentials amino acids (methionine and lysine) (Fuertes et al., 2013), inadequate proportion of favourable fatty acids (EPA; 20:5n-3 and DHA; 22:6n-3) (Norambuena et al., 2015), depressed growth performance (Rossi and Davis, 2012), reduced digestibility (Tibbetts et al., 2006) and palatability issues (Fuertes et al., 2013). In particular, deficiencies of amino acids and fatty acids is one of the main shortcomings to including higher levels of PBM in the diet of carnivorous fish.

Recently, interest has turned to insects as a promising alternative protein source in aquaculture (Nogales-Mérida et al., 2018; Stamer, 2015) particularly since insects are the main prey for many omnivorous and carnivorous fish in their natural environment (Henry et al., 2015). Insects have received attention for animal feed production when compared to conventional animal protein sources due to an ability to grow in harsh environments, often commonly infested with a wide variety of microorganisms. Such environment may induce the target insects to produce many

native bioactive peptides with anti-microbial, anti-fungal and anti-viral functions (Elhag et al., 2017; Park et al., 2015). Insects have been reported to contain biologically active antimicrobial peptides, which not only work against pathogenic bacteria, but may also boost species specific innate immune responses and promote some immunomodulatory effects (Nogales-Mérida et al., 2018). Subsequently in veterinary and livestock production, these peptides have been considered as an alternative to antibiotics (Jozefiak and Engberg, 2017). Nogales-Mérida et al. (2018) reported that insect derivate products including protein concentrates, chitins, oils and antimicrobial peptides not only enhance the growth performance, but may also boost the fish immunity. These authors recommended to include insect meal in fish diets at very low quantities to promote fish performance and immune system function. Black soldier fly, *H. illucens* (HI) belonging to the Diptera order is one of the promising source of insect protein due to containing from 40 to 54% crude protein and 15 to 49% crude lipid (dry matter basis), well-balanced amino acids similar to FM (Lock et al., 2016; Makkar et al., 2014), and being a good source of minerals and a variety of vitamins (Henry et al., 2015). Another important attribute of HI is the presence of antibacterial activity (Park et al., 2015; Park et al., 2014) which, in low doses, may boost the immunity similarly to low doses dietary antibiotics (Gadde et al., 2017). In particular, PBM lacks certain functional components and therefore, supplementation of HI larvae with PBM could be an effective way to stimulate the growth, immunity and disease resistance against pathogens in juvenile barramundi.

Evaluation of histological changes in different organs is an effective way to assess the health status of fish. Hence, the relationship between dietary modification and the internal architecture of various tissues and cells has been a research priority in finfish production. Dietary effects of HI larvae on the histology of intestine and liver of rainbow trout, *Oncorhynchus mykiss*(Elia et al., 2018), Jian carp, *Cyprinus carpio* var. Jian (Li et al., 2017a) clownfish, *Amphiprion ocellaris* (Vargas-AbúNdez et al., 2019) and Zebrafish, *Danio rerio* (Vargas et al., 2018; Zarantoniello et al., 2019) have been investigated, however, information on the histological structure of juvenile barramundi when fed HI remains largely unknown.

Barramundi, *Lates calcarifer* is a highly valued commercial species because of the fillet flavour and rapid growth (Liu et al., 2018). It is popular for both freshwater and saltwater aquaculture and barramundi culture technology in ponds, tanks and cages is well established in Australia, Indonesia, Philippines, Malaysia, Thailand and Taiwan (Tian and Qin, 2003). Intensive production of barramundi results in bacterial disease outbreaks namely Vibriosis, caused by *V. harveyi* which is the hindrance for sustainable barramundi production and causes a huge financial losses to fish farmers (Ransangan et al., 2012; Talpur and Ikhwanuddin, 2013). Head kidney, a large active immunocompetent organ in teleost fish contain reticular cells, macrophages, plasma cells and

lymphocytes involving in antigen trapping, phagocytosis and immunologic memory (Dannevig et al., 1994; Press et al., 1996). Thus maintaining the immune function of head kidney is of importance in fish production. However, a significant effort has been given over the decades for barramundi nutrition to develop functional feed but to date, no information is available relating to the supplemental effects of HI larvae in partially or completely replacement of FM protein with PBM protein based diets. Hence, the aim of the present study was to investigate the effects of HI supplementation with PBM on growth, biometry indices, histological structure, immune response, immune-related genes and resistance to *V. harveyi* of juvenile barramundi.

4.2 Materials and methods

4.2.1 Ethics statements

The experiment was performed in a recirculatory aquaculture system (RAS) at Curtin Aquatic Research Laboratory (CARL) in Curtin University, Australia in accordance with the Australian Code of Practice for the care and use of animals for scientific purposes, and all protocols was reviewed and approved by the Curtin University Animal Ethics Committee (ARE2018-37). During fish handling, AQUI-S® (8 mg/L) was used for anaesthesia and an overdose of AQUI-S® (175 mg/L) was used as euthanasia to achieve humane endpoint when fish reached a moribund condition, following the protocol of the Curtin Research Laboratories statement of purpose (SOP) of anaesthetizing and euthanizing of fish. All experimental efforts were dedicated to minimise stress, pain and discomfort to the fish.

4.2.2 Diets

Three test diets to be nearly isonitrogenous (47% crude protein) and isolipidic (13% crude lipid) containing different levels of PBM and supplemented with HI larvae meal (Table 4. 1) were formulated from the ingredients procured from Specialty Feeds, Glen Forrest Stockfeeders, 3150 Great Eastern Highway, Glen Forrest, Western Australia 6071. The control diet contained 100% FM and the other two diets contained 45 and 90% PBM along with the 10% of HI larvae supplementation, abbreviated as 45PBM+ HI and 90PBM+ HI, respectively. All test diets were pelletized to 2.5 mm long and packed by Specialty Feeds, delivered to CARL and stored at -20◦ C prior to the commencement of the trial. Trial ingredients and formulation are summarized in Table 4. 1.

Table 4. 1 Ingredients formulation and proximate composition of three test diets fed to juvenile barramundi for 6 weeks.

Ingredients[a]	Test diets		
	Control	45PBM+ HI	90PBM+HI
PBM[b]	0.00	31.00	63.00
Canola oil	1.00	2.40	3.00
HI larvae (full-fat)[c]	0.00	12.00	12.00
FM[d]	72.00	33.00	0.00
Corn/wheat starch	7.00	7.00	7.00
Lecithin - Soy (70%)	1.00	1.00	1.00
Vitamin C	0.05	0.05	0.05
Dicalcium Phosphate	0.05	0.05	0.05
Wheat (10 CP)	16.90	12.00	9.90
Vitamin premix	0.50	0.50	0.50
Salt (NaCl)	1.00	1.00	1.00
Cod liver oil	0.50	0.00	2.50
Proximate composition (% dry weight)			
Crude protein	47.88	47.86	47.97
Crude lipid	12.59	12.33	12.03

[a] Specialty Feeds, Glen Forrest Stockfeeders, 3150 Great Eastern Highway, Glen Forrest, Western Australia 6071

[b] PBM, poultry by-product meal: crude protein (67.13%),crude lipid (13.52%) and ash (13.34%)

[c] HI, *Hermetia illucens* larvae: crude protein (40.43%) and crude lipid (17.23 %)

[d] FM, fishmeal: crude protein (64.0%), crude lipid (10.76%) and ash (19.12%).

4.2.3 Fish husbandry and management

The experimental design and analytical procedures are presented in Figure 4. 1. A total of 350 barramundi (mean weight = 1g) were provided by the Australian Centre for Applied Aquaculture Research (ACAAR), Fremantle, Australia, transported in an oxygenated plastic bag and adapted to the rearing condition and facilities for two weeks at CARL in 300L seawater tanks. The fish were fed a commercially formulated diets (470 g protein kg^{-1} and 20.0 MJ kg^{-1} gross energy) three times daily. Following acclimatization and size grading, 225 juvenile barramundi weighing 3.40 ± 0.03 g (mean ± SE) were randomly split into 9 independent tanks (25 fish/tank). An aerator and electric heater was equipped with each tank and also an external bio-filter (Astro® 2212, China) was set up to filter the water. Water quality parameters in term of temperature (27.90–29.20 °C), dissolved oxygen (5.92–7.42 mgL^{-1}), salinity (32–36 ppt), ammonia nitrogen (<0.50 mgL^{-1}) and nitrite (<0.50 mgL^{-1}) were checked every day and maintained within the range suitable for barramundi production (Siddik et al., 2018b). Fish were reared under a 14:10 h light: dark, with the light period from 08:00 am to 10:00 pm using an automatic indoor timer (Clipsal, Australia). Each diet was hand fed in triplicate until apparent satiation twice a day (8.00 am and 6.00 pm) for 6 weeks. In order to calculate feed intake, uneaten feed was collected by siphoning from the bottom of the tank one hour after each meal, oven dried in aluminium cups for 36 h at 60°C and then weighed. Throughout the 6 weeks of trial, mortality, if any, were checked daily to assess the fish survival rate and dead fish were weighed. After 6 weeks feeding, the fish in each tank were food deprived for 24 h, anaesthetized with AQUI-S® at 8 mg/L prior to bulk weigh and then individually counted to evaluate the growth performance and survival.

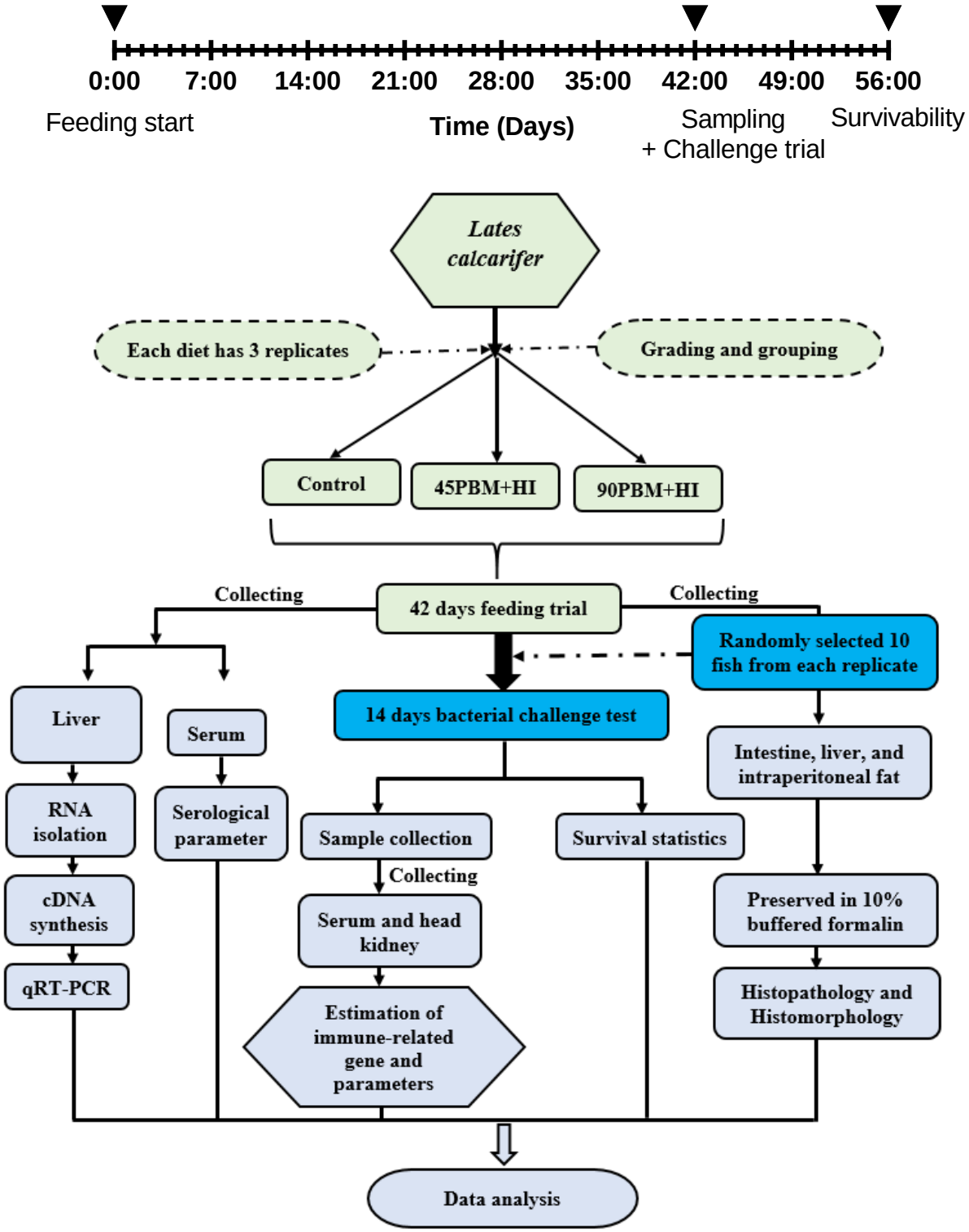

Figure 4. 1 The framework represents the experimental design and analysis procedure after 42 days of feeding trial and 14 days of challenge trial.

4.2.4 Calculation

At the beginning and end of the 42 days feeding trial, all fish were weighed to nearest to 0.1 g and fish growth performance (WG, SGR, FCR and survival) and feed intake were calculated as follows:

$$\text{Weight gain (WG, g)} = \left[\frac{\text{Mean final weight-Mean initial weight}}{\text{Mean initial weight}}\right]$$

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

$$\text{Feed intake (FI, g/fish d}^{-1}\text{)} = \left[\left(\frac{\text{Dry diet given-Dry remaining diet recovered}}{\text{Days of experiment}}\right) / \text{No. of fish}\right]$$

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

$$\text{Survival (SR, \%)} = \left[\frac{\text{Final number of fish}}{\text{Initial number of fish}}\right] \times 100$$

Nine fish per treatment (3 fish/replicate) were collected randomly to calculate biometry indices by the following formula:

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

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

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

$$\text{Spleen index (SI, \%)} = \left[\frac{\text{Weight of spleen (g)}}{\text{Whole body weight (g)}}\right] \times 100$$

$$\text{Intraperitoneal fat index (IFI, \%)} = \left[\frac{\text{Intraperitoneal fat weight (g)}}{\text{Whole body weight (g)}}\right] \times 100$$

$$\text{Relative gut length (RGL, \%)} = \left[\frac{\text{Length of intestine (cm)}}{\text{Length of fish (cm)}^3}\right] \times 100$$

4.2.5 Histomorphology and histopathology

At the end of 6 weeks trial, 6 fish per treatment were selected randomly and euthanized with an overdose of AQUI-S® to excise liver, intraperitoneal fat and intestine for histological analysis. Fragments of all tissue samples were fixed in 10% neutral buffered formalin, dehydrated with a series of ethanol concentrations before infiltrating in xylene and embedding in paraffin wax, and finally sectioned at approximately 5 µm using a rotary microtome machine for staining with hematoxylin and eosin (H&E) following the standard histological procedures. Histological slides were digitally photographed under a light imaging microscope (BX40F4, Olympus, Tokyo, Japan).

Intestinal section were further subjected to two different stains including Periodic Acid-Schiff (PAS) and Alcian Blue (AB) pH 2.5 staining to identify neutral mucins and acidic mucins, respectively. Both type of mucins were counted from ten intact randomly selected villi, as described earlier by Elia et al. (2018). Ten intact villi were randomly selected to measure the intestine histometric in terms of villi height and width, enterocyte width, muscular wall, submucosa thickness and microvilli height and diameters of adipocyte were measured using ImageJ software.

4.2.6 Challenge trial with *V. harveyi*

The pathogenic bacteria, *V. harveyi* used in the challenge trial was provided by Diagnostic and Laboratory Services, Department of Primary Industries and Regional Development (DPIRD), 3 Baron-Hay Court, South Perth WA 6151. Cultures were incubated for 24 h at 24^{o}C in trypticase soy broth (Oxoid, Basingstoke, UK) culture medium. The culture was centrifuged for 15 minute at 5000g and the discarded pellets were suspended in PBS (phosphate-buffered saline, pH 7.2) for challenge trail. The bacterial suspension was adjusted to 5.4 x 10^7 cells/mL for challenge test.

The challenge trail in this study were conducted following an established CARL protocol (Siddik et al., 2019b). Briefly, after 6 weeks of growth trial, 10 fish from each treatment group including the control maintaining the same replicate were selected at random and distributed to another 12 glass aquaria having 100 L capacity. Out of 12 aquaria, 9 were used to analyse the probability of survival and the remaining 3 were utilized for blood collection. Following acclimation for three days, fish were injected intraperitoneally by using a 1-mL syringe and 27-gauge needle with a lethal dose of 0.1 mL of *V. harveyi* suspension containing 5.4 x 10^7 cells/mL and fed with same experimental diets once daily after returning the challenged fish into the respective aquaria. The signs of fish infection were monitored and recorded three times a day (7:00am, 2:00pm and 9:00pm) for 14 days and infected fish were subjected to euthanasia with AQUI-S® at 175 mg/L for 20 minutes according to the protocol of the CARL SOP of euthanizing of fish.

4.2.7 Blood and serological parameters

At the end of the feeding and challenge trial, blood and serum samples were collected to determine biochemical and immunological indices. Six fish from each replicate (18 fish/treatment) were anaesthetized randomly using AQUI-S® (8 mg l^{-1}) and blood from these fish were collected from the caudal vein using 1 mL heparinized and non-heparinized syringes and pooled. Then blood in non-heparinized tubes were kept in room temperature for 24 h until coagulation followed by centrifugation (3000 rpm, 15 min) at 4 °C to obtain serum and stored at - 80°C for the analysis of immunological indices such as lysozyme and bactericidal activity, and serum biochemical indices

including aspartate aminotransferase (AST), glutamate dehydrogenase (GLDH), triglyceride and cholesterol.

Both serum lysozyme and bactericidal activity were analysed in before-challenged and after-challenged fish, following the method earlier described in Le Fotedar (2014a). Serum biochemical indices including AST, GLDH, TG, and cholesterol were assessed according to the method of Siddik et al. (2019b).

4.2.8 RNA extraction and qRT-PCR analysis

Liver and head kidney tissues from six euthanized (AQUI-S®, 175 mg l^{-1}) fish (two/replicate) each from control and HI supplemented groups were harvested after 42 days feeding of the trial and 24 h post infection, preserved in RNA Later (Sigma-Aldrich, Germany) and stored at - 80°C prior to RNA extraction. For RNA extraction, the harvested frozen tissues were thawed, homogenized and ground into a fine powder. The total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) from approximately 5 mg of various tissue samples and RNase free DNase-I (Qiagen, Hilden, Germany) was used to treat extracted RNA to remove DNA contamination. After checking the quality and quantity of RNA with gel electrophoresis and NanoDrop spectrophotometer 2000c (Thermo Fisher Scientific, USA), 1 µg of RNA for each sample (liver and head kidney) was used to synthesize complementary DNA (cDNA) Omnicript RT kit (Qiagen, Hilden, Germany) following the instruction of manufacturer's company. qRT-PCR was carried out using PowerUp™ Cyber Green Master Mix (Thermo Scientific, USA) with 7500 Real-Time PCR System (Applied Biosystems, USA) and mRNA expression level of selected genes were normalised to the *18S rRNA* and *Ef1-a*, housekeeping genes (Table 4. 2) and calculated using $2^{-\Delta\Delta ct}$ method.

Table 4. 2 Primers of qPCR used in the experiment

Genes	Sequences (5′ - 3′)	Product size	Tm (◦C)	
Heat shock protein kDa70, HSP70	F: AAGGCAGAGGATGATGTC R: TGCAGTCTGGTTCTTGTC	186	59	Mohd-Shaharuddin et al. (2013)
Heat shock protein kDa90, HSP90	F: ACCTCCCTCACAGAATACC R: CTCTTGCCATCAAACTCC	197	59	Mohd-Shaharuddin et al. (2013)

Complement 3, C3	F:GCAATCCTCCACAACTACAG R: ACTCTGACCTCCTGACGATAC	11	59	Mohd-Shaharuddin et al. (2013)
Complement 4, C4	F: TTGCTTCTTCCCTACAGTG R: GGTCCAACCCTCCTTTAC	185	59	Mohd-Shaharuddin et al. (2013)
MHC class IIb, MHC-IIb	F: GTTGGATACACTGAGTTTGG R: GAGGGTTTGACTGACTTAGAC	152	60	Mohd-Shaharuddin et al. (2013)
18S rRNA, 18S	F:TGGTTAATTCCGATAACGAACGA R: CGCCACTTGTCCCTCTAAGAA	94	59/60	Mohd-Shaharuddin et al. (2013)
Elongation factor-1α, ef1α	F: AAATTGGCGGTATTGGAAC R:GGGAGCAAAGGTGACGAC	83	59/60	Mohd-Shaharuddin et al. (2013)

4.2.9 Statistical analysis

Group of fish/tank were used as experimental unit for data on growth, while individual fish were used as experimental unit for data on biometry indices, histomorphology, serum biochemical parameters, immune parameters and gene expression, as no tank-relevant effect was observed during the trial. The results of growth performance, biometry indices, histomorphology and immune parameters were expressed as mean ± standard error of mean, and subjected to normality and homogeneity of variances with Shapiro-Wilk's and Levene's tests. When both tests were satisfied, an ordinary one-way ANOVA with Dunnett's multiple comparisons test was applied to test the statistical significant difference at $0.05 < P < 0.001$ where diet was used as explanatory variable. Survival curve of barramundi at the end of the trial and after being challenged with *V. harveyi* were estimated by Kaplan-Meier method, followed by pairwise multiple comparison Log-Rank (Mantel-Cox) test. A paired student t-test was used to determine the significant difference between before and after challenge test groups.

4.3 Results

4.3.1 Fish performance and survival

Feeding juvenile barramundi with different levels of PBM supplemented with HI larvae significantly influenced the growth performance, biometry indices and feed utilization (Table 4. 3). When compared to the control, growth performance in terms of FBW, SGR and WG increased significantly ($P < 0.05$) in fish fed 45PBM+HI, while growth performance was significantly lower in 90PBM+HI groups. There was no significant difference ($P > 0.05$) in FI between control and 45PBM+HI but decreased significantly ($P < 0.05$) in 90PBM+ HI when compared with control.

Biometry indices including HSI, VSI, SI and RGL with the exception of CF and IFI were not influenced by different diets (Table 4. 3). CF differed significantly ($P < 0.05$) with the lowest value observed in 90PBM+HI fed fish, whilst significantly lower value of IFI was observed in 45PBM+HI than the control and 90PBM+HI. At the end of the trial, survival rate in response to 90PBM+HI diet decreased significantly ($\chi^2_{90PBM+HI} = 3.69$, df = 1, $P = 0.035$) than the control, though there was no significant difference observed between control and 45PBM+HI ($\chi^2_{45PBM+HI} = 0.58$, df = 1, $P = 0.447$) (Figure 4. 2).

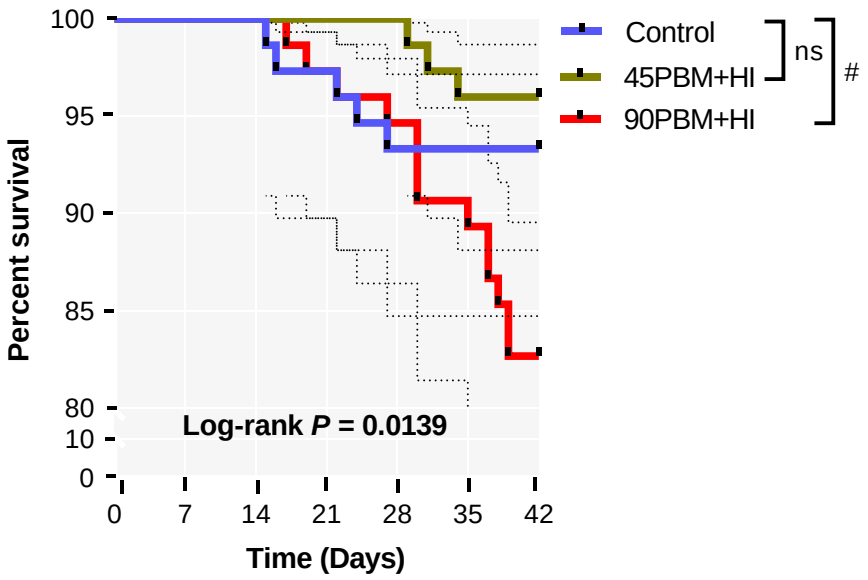

Figure 4. 2 Kaplan-Meier survival rate based on Log-rank (Mantel-Cox) test of juvenile barramundi after 6 weeks feeding either control diet or different levels of PBM supplemented with HI. # indicates significant difference at $P < 0.05$.

Table 4. 3 Fish performance, biometry indices and survival of juvenile barramundi after six weeks feeding with test diets contain various level of PBM supplemented with HI larvae.

	Test diets			ANOVA - *P*
Parameters	Control	45PBM+HI	90PBM+HI	
IBW (g)	3.48±0.03[a]	3.50±0.06[a]	3.53±0.05[a]	0.731
FBW (g)	50.07±3.54[b]	62.39±2.37[a]	31.24±1.92[c]	0.001
WG (g)	46.59±3.57[b]	58.88±2.41[a]	27.71±1.90[c]	0.001
SGR (%/d)	6.33±0.19[a]	6.85±0.12[a]	5.18±0.13[b]	0.001
FI (g/fish d^{-1})	0.78±0.05[ab]	1.16±0.15[a]	0.62±0.63[b]	0.049
FCR	0.71±0.06[a]	0.83±0.07[a]	0.96±0.22[a]	0.518
Biometry indices				
HSI (%)	1.82±0.26[a]	1.52±0.12[a]	1.41±0.05[a]	0.043
VSI (%)	8.43±0.42[a]	7.61±0.44[a]	7.98±0.80[a]	0.612
CF (g/cm^3)	1.18±0.05[ab]	1.48±0.14[a]	1.08±0.12[b]	0.224
SI (%)	0.09±0.02[a]	0.10±0.02[a]	0.11±0.02[a]	0.667
IFI (%)	1.01±0.18[ab]	0.67±0.09[b]	1.12±0.06[a]	0.043
RGL (%)	46.40±2.85[a]	45.16±2.61[a]	40.75±1.77[a]	0.251

IBW = initial body weight; FBW = final body weight; WG = weight gain; SGR = specific growth rate; FI = feed intake; FCR = feed conversion ratio; HIS = hepatosomatic index; VSI = viscerasomatic index; CF = condition factor; SI = spleen index; IFI = intraperitoneal fat index and RGL = relative gut length.

Data are means ± SE. Means with different letters, within row, indicate statistical significant difference at $P < 0.05$, followed by Dunnett's multiple comparisons test.

4.3.2 Histometric measurements and histochemistry in intestine

At 42 days of feeding trial, HI larvae supplementation significantly modulated the intestinal morphology and histochemistry where a significant increase in villi width (Figure 4. 3F) ($P < 0.01$), enterocyte width (Figure 4. 3G) ($P < 0.05$) and microvilli height (Figure 4. 3J) ($P < 0.05$) was observed in 45PBM but declined significantly in 90PBM+HI than the control. Acidic mucin per fold in 45PBM+HI increased significantly (Figure 4. 3L) ($P < 0.05$), though an insignificant difference was observed between control and 90PBM+HI ($P > 0.05$). However, none of the diets imposed significant effects on villi height (Figure 4. 3E), muscular wall thickness (Figure 4. 3H), submucosa thickness (Figure 4. 3I) and neutral mucins (Figure 4. 3K) ($P > 0.05$).

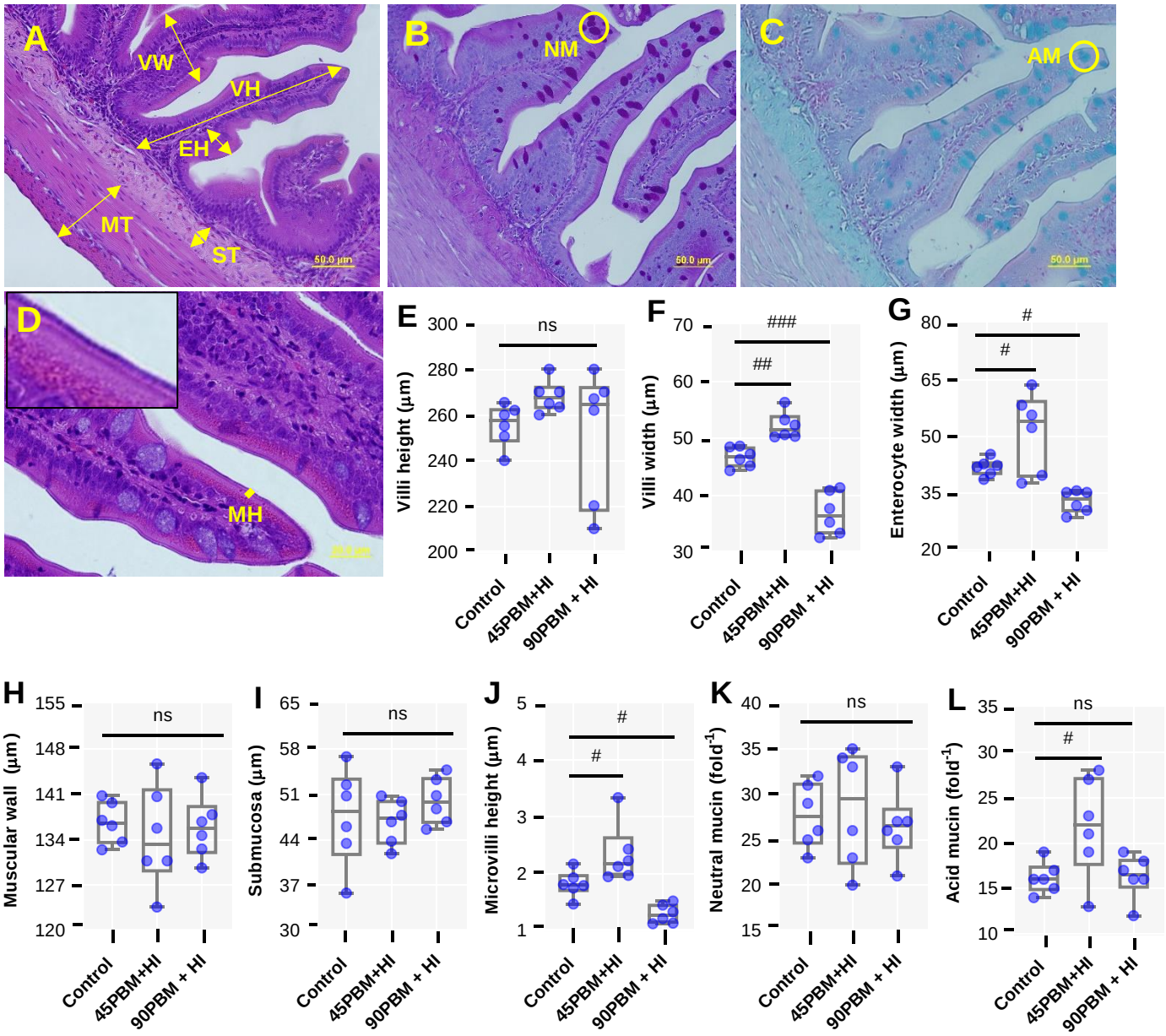

Figure 4. 3 Distal intestine histometric and histochemical findings of juvenile barramundi fed control and test diets containing different levels of PBM supplemented with HI for 6 weeks. (A) histometric measurements (H & E stain, 40 × magnification) including villi height (E),villi width (F), enterocyte width (G), musculosa wall (H) and submucosa thickness (ST); (B) goblet cells reacting to Periodic Acid-Schiff (PAS) stain (PAS stain, 40 × magnification) represents neutral mucins (K); (C) goblet cells reacting Alcian Blue (AB) pH 2.5 represents acidic mucins (L); (D) microvilli measurements represents the height of microvilli (J). Data of panel (E-L) are expressed as mean ± SE (n = 6) from one representative experimental diet. ns, not significant; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ by Dunnett's multiple comparisons test.

4.3.3 Morphology of intraperitoneal adipose tissue

Adipocytes sizes in intraperitoneal fatty tissue in the different experimental treatments are shown in Figure 4. 4(A-D). The adipocytes size in fish fed 45PBM+HI decreased significantly ($P < 0.01$) than the control, whereas no significant difference was observed between control and 90PBM+HI (Figure 4. 4D).

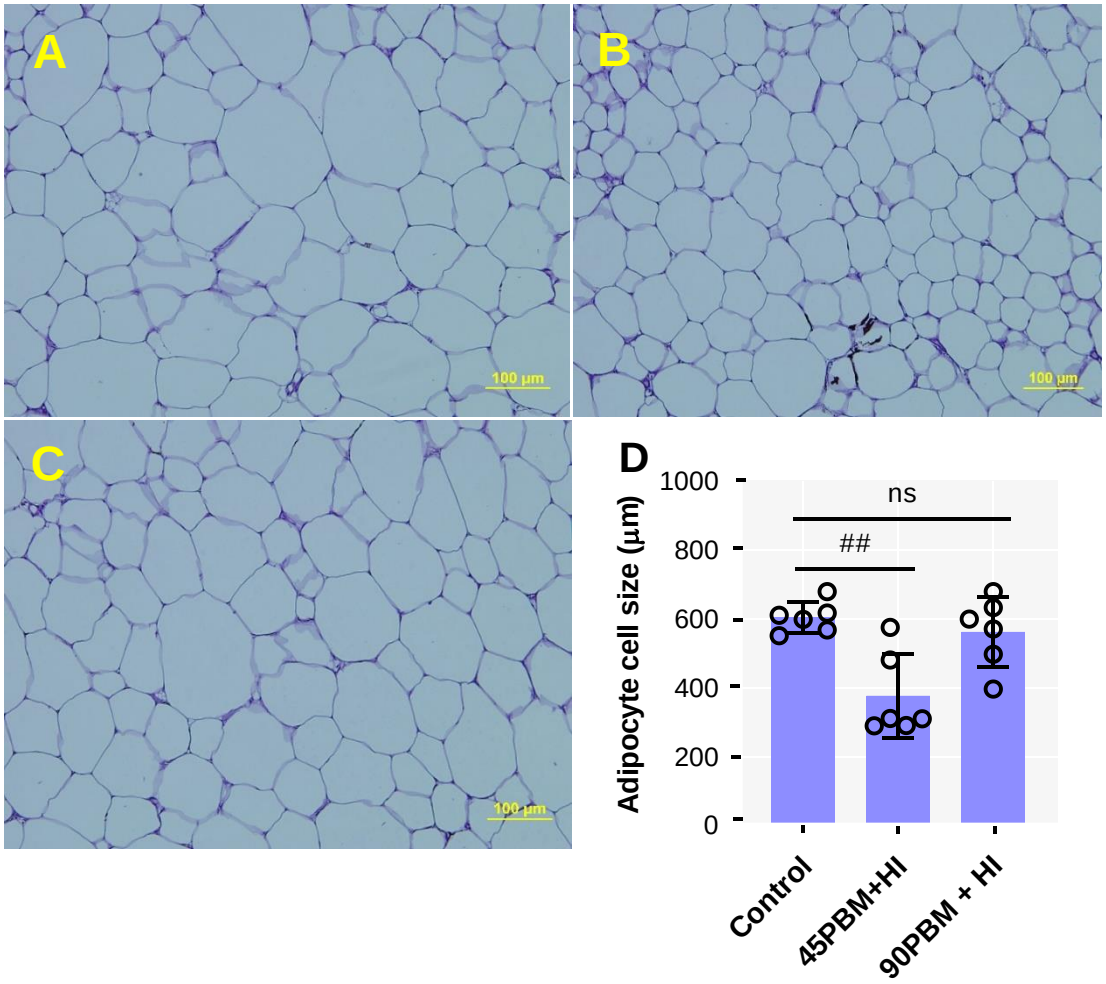

Figure 4. 4 Representative adipocyte cell size structure of intraperitoneal fatty tissue of fish fed control and test diets supplemented with HI larvae after 6 weeks of feeding trial (PAS stain, 40 × magnification). (A) Intraperitoneal fatty tissue from fish fed 0% PBM and HI larvae supplementation (Control); (B) intraperitoneal fatty tissue from fish fed 45% PBM and 10% HI larvae supplementation (45PBM+HI); (C) intraperitoneal fatty tissue from fish fed 90% PBM and 10% HI larvae supplementation (90PBM+HI). (D) Variation of adipocyte cell size of intraperitoneal fatty tissue in response to different levels of PBM supplemented with HI. Data of panel (D) are expressed as mean ± SE (n = 6) from one representative experimental diet. ns, not significant and $^{\#\#}P < 0.01$ by Dunnett's multiple comparisons test.

4.3.4 Serum biochemical indices

Serum biochemical parameters including AST, GLDH, cholesterol and triglyceride are shown in Figure 4. 5(A-D). AST and GLDH increased significantly in fish fed 90PBM+HI ($P < 0.05$) while no significant difference was observed between control and 45PBM+HI ($P > 0.05$) (Figure 4. 5A, B). Meanwhile, fish fed control and HI supplemented PBM diets had no significant effects on cholesterol and triglyceride ($P > 0.05$) (Figure 4. 5C, D).

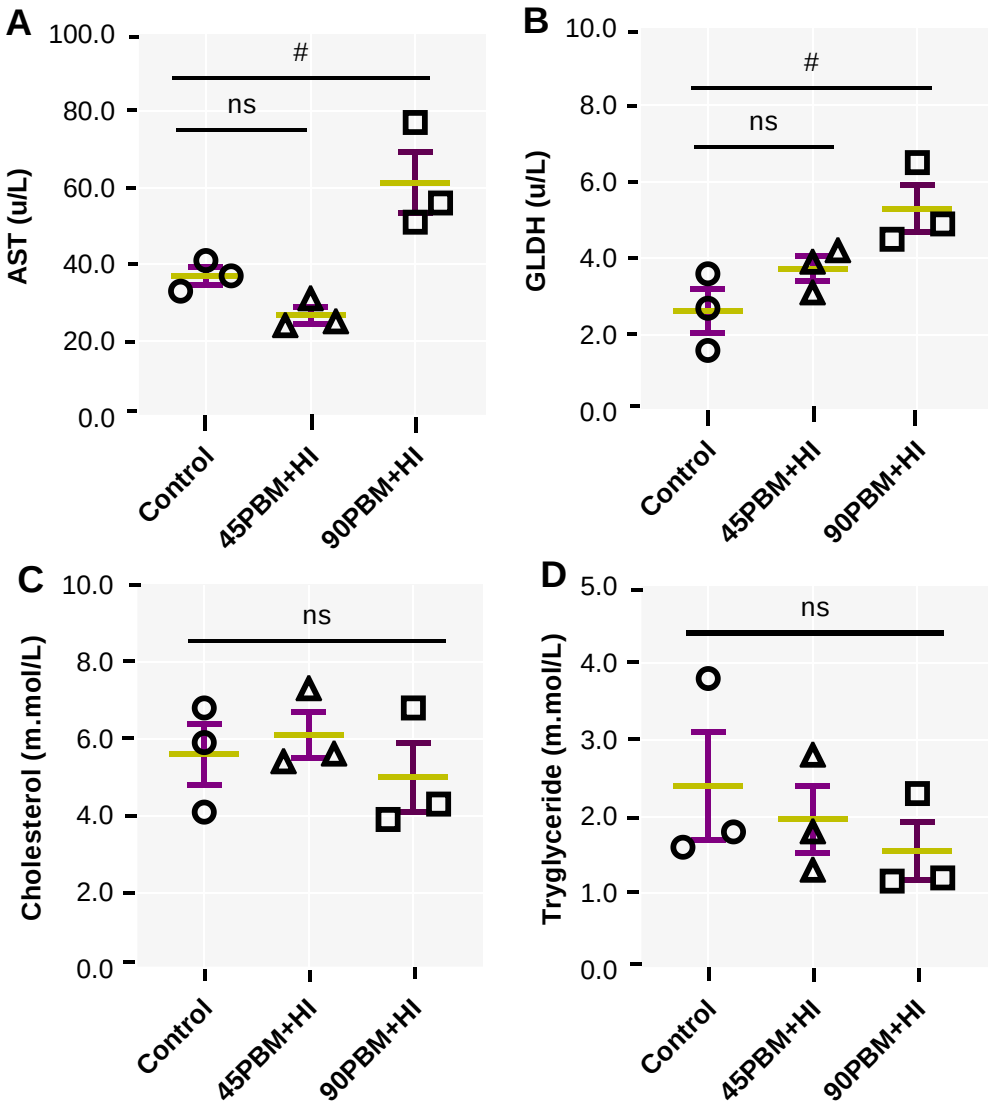

Figure 4. 5 Serum AST, aspartate aminotransferase (A), GLDH, glutamate dehydrogenase (B), cholesterol (C) and triglyceride (D) of juvenile barramundi after six weeks feeding with test diets containing various level of PBM supplemented with HI. Data represent means ± SE of three values per treatment. ns, not significant and $^{\#}P < 0.05$ by Dunnett's multiple comparisons testing.

4.3.5 Histopathology and expression of HSP in liver

Histopathological analysis revealed that fish fed control and 45PBM+HI showed no obvious alterations with normal hepatocyte morphology and exocrine pancreas with zymogen in liver (Figure 4. 6A, B), however multifocal necrosis was observed in fish when fed 90PBM+HI (Figure 4. 6C). Both mRNA expression levels of HSP70 and HSP90 upregulated in 90PBM+HI groups ($P < 0.05$) with no significant difference between control and 45PBM+HI ($P > 0.05$) (Figure 4. 6D, E).

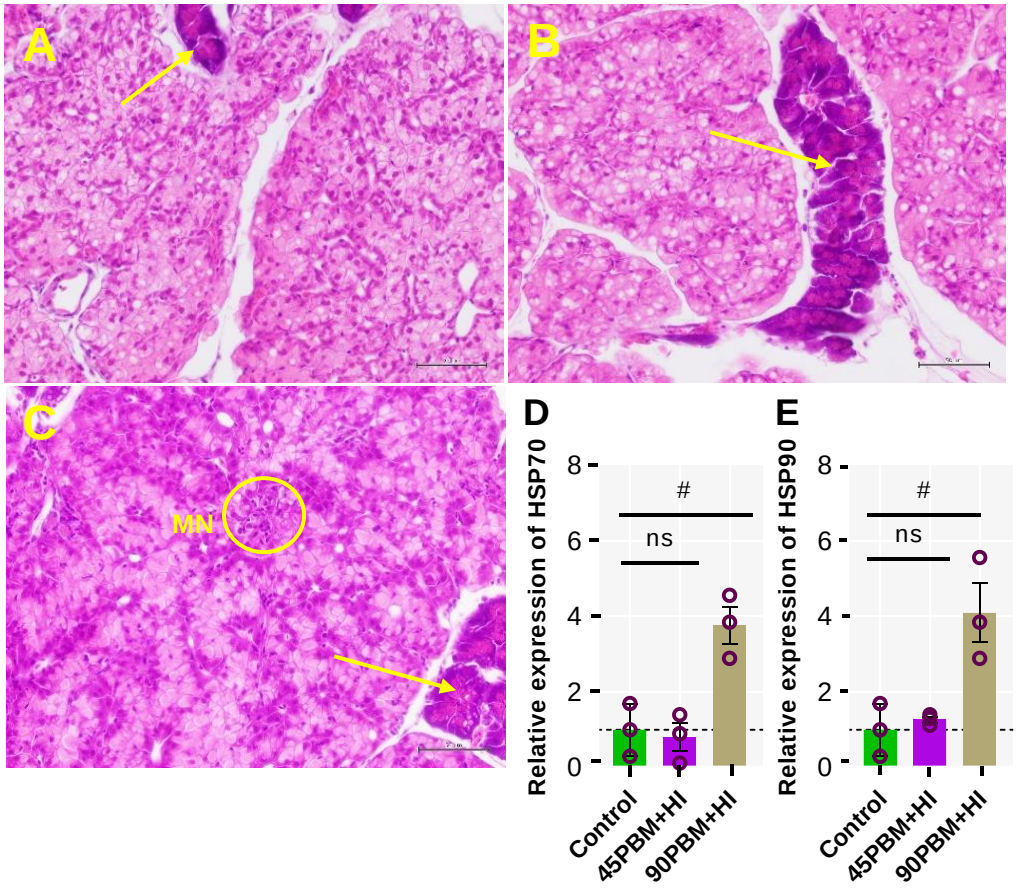

Figure 4. 6 Representative histopathological features of liver sections of fish fed diets supplemented with HI larvae after 6 weeks of feeding trial (H & E stain, 40 × magnification). (A) liver from fish fed 0% PBM and HI larvae supplementation (Control), showing normal hepatocyte structure and exocrine pancreas with zymogen (arrow); (B) liver from fish fed 45% PBM and 10% HI larvae supplementation (45PBM+HI), exhibiting normal hepatocyte structure and exocrine pancreas with zymogen (arrow); (C) liver from fish fed 90% PBM and 10% HI larvae supplementation (90PBM+HI), revealing multifocal necrosis (yellow circle) and exocrine pancreas with zymogen (arrow). (D and E) variation in the expression of hepatic HSP70 and HSP90 in response to different levels of PBM supplemented with HI. Data of panel (D) are

expressed as mean ± SE (n = 3) from one representative experimental diet. ns, not significant and $^{\#}P < 0.05$, by Dunnett's multiple comparisons testing.

4.3.6 Resistance and immunity against *V. harveyi*

The survival rate of barramundi when challenged with *V. harveyi* was significantly influenced (Kaplan-Meier, log-rank (Mantel-Cox); χ^2 (2) = 31.34, $P < 0.001$) by dietary supplementation of HI larvae and higher inclusion of PBM. At the end of the 14 days challenge trial, in comparison with the control, survival rate against *V. harveyi* in fish fed 45PBM+HI increased significantly ($\chi^2_{45PBM+HI}$ = 5.48, df = 1, $P = 0.019$), while a significant decrease in survival rate was observed with 90PBM+HI ($\chi^2_{90PBM+HI}$ = 110.71, df = 1, $P = 0.001$) (Figure 4. 7A). Serum lysozyme (t=3.416, df=10, $P = 0.006$) and bactericidal activity (t = 3.398, df = 2, $P = 0.007$) elevated in after-challenged 45PBM+HI groups when compared with before challenged 45PBM+HI groups, however there was no significant difference between before and after challenged groups fed control (t = 0.4378, df = 2, $P = 0.6708$) and 90PBM+HI diet (t = 1.940, df = 2, $P = 0.0811$) (Figure 4. 7B, C). Relative expression of complement C3 (t = 4.783, df = 2, $P = 0.041$) and C4 (t = 17.46, df = 2, $P = 0.003$) in response to *V. harveyi* upregulated significantly in 45PBM+HI compared to before challenge (Figure 4. 7D, E), while the expression levels of C3 and C4 between before and after challenged control and 90PBM+HI groups showed no significant difference. Feeding fish with control and HI larvae supplemented diet had no significant effect on MHC-IIb both in before and after challenged groups (Figure 4. 7F).

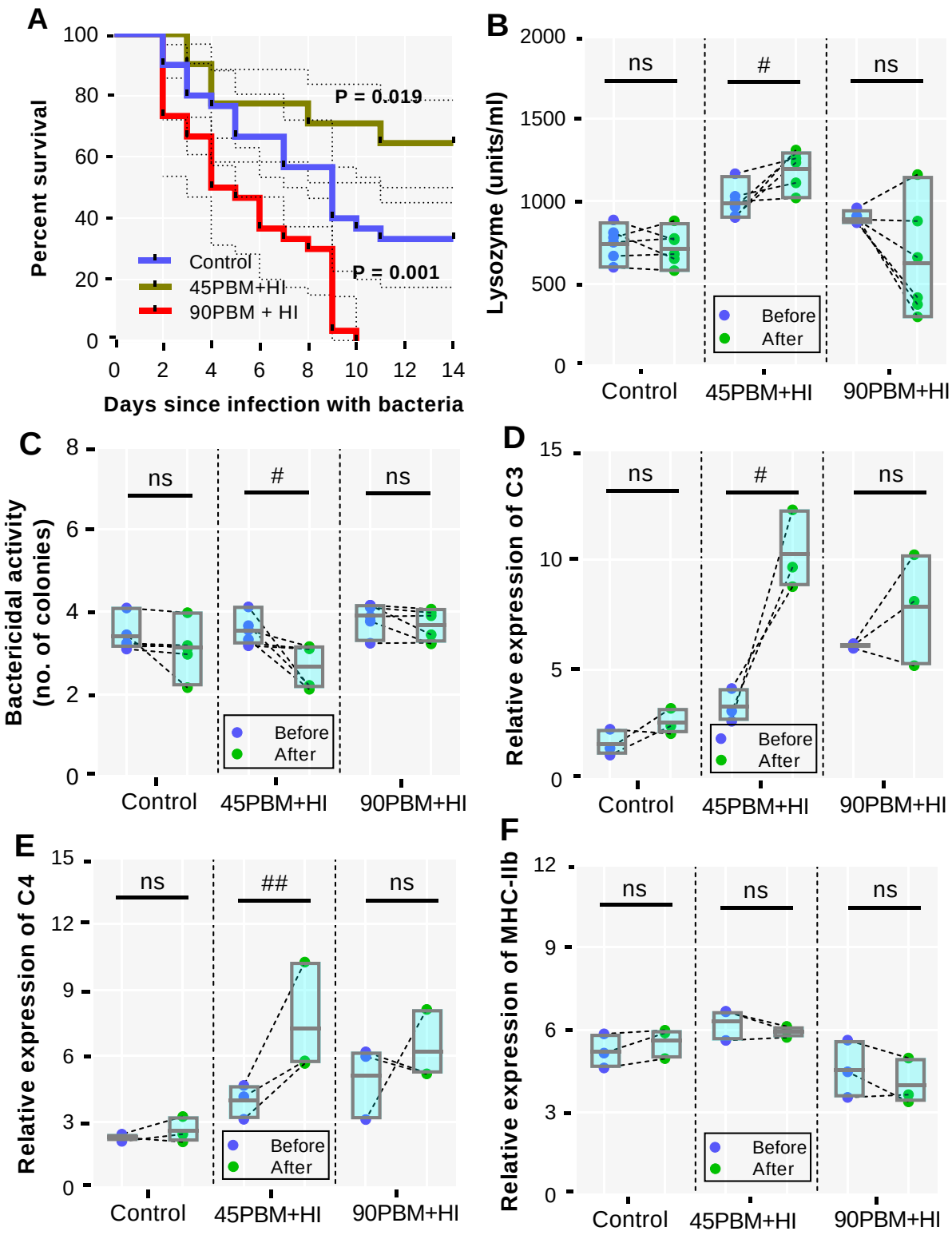

Figure 4. 7 Kaplan-Meier survival (A) following a 14 days *V. harveyi* challenge, serum lysozyme (B) and bactericidal activity (C), relative expression of complement C3 (D), C4 (E) and MHC-IIB (F) in the head kidney of juvenile barramundi before and after 24 h of challenge test. Data of panel (D) are expressed as mean ± SE (n = 3) from one representative experimental diet. ns, not significant; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ by a paired student t-test.

4.4 Discussion

Recently, HI larvae has received much attention in aquafeed production and different inclusion levels have been successfully evaluated on variety of fish species (Henry et al., 2015). However, no research has been carried out to investigate the supplementation of HI with PBM in the diet of any marine carnivorous species. The results of the present study revealed that supplementation of 10% HI with 45% PBM significantly improved the growth performance of juvenile barramundi when compared with control. Inclusion of HI larvae as a replacement of FM enhanced the growth performance of juvenile turbot, *Psetta maxima* (Kroeckel et al., 2012), yellow catfish, *Pelteobagrus fulvidraco* (Xiao et al., 2018) . However, growth performance and FI decreased significantly in fish fed 90PBM+HI compared to control, which are in line with the earlier study in our lab, where inclusion of 100PBM either non- bioprocessed or bioprocessed significantly depressed the growth performance (FBW, WG and SGR) and increased FCR in juvenile barramundi, *Lates calcarifer* (Siddik et al., 2019a). The possible reason for poorer growth at high PBM levels could be a reduction of palatability, deficiency in some essential amino acids and fatty acids, inconsistency in biochemical composition and low digestibility in these feeds (Cruz-Suárez et al., 2007; Fuertes et al., 2013). Similarly, reduced palatability resulting from low feed consumption and a higher FCR have been reported in some marine carnivorous fish (Fowler, 1991; Webster et al., 2000; Yigit et al., 2006) when fed with the diets containing higher levels of PBM (> 30%). However, one of the previous studies in our laboratory was able to include 90% PBM along with 10% tuna hydrolysate supplementation in the diet of juvenile barramundi, without altering the growth performance and FCR (Siddik et al., 2019b).

AST and GLDH are important liver specific enzymes in aquatic organisms, elevated rapidly in blood serum following liver cell damage and disinfection (Kim and Kang, 2014). Serum AST and GLDH activity in the current study increased significantly in 90PBM+HI fed fish compare to control, implying that higher inclusion of PBM affected the functions of the liver, leading to the impairment of immune response in juvenile barramundi. However, Siddik et al. (2019b) reported no significant influence on serum AST and GLDH activity between control and higher replacement of PBM treated juvenile barramundi.

HI supplementation with PBM significantly influenced the IFI with significantly lower value found in 45PBM+HI, while 90PBM+HI had no significant effect on IFI in comparison with control. The lowest IFI value in 45PBM+HI might be due to dietary medium-chain fatty acids, mostly C_6–C_{12}, which are the main fatty acids of HI meal (Han et al., 2003) and have been considered physiologically active compounds utilizing as an energy source and reducing the adipose tissue deposition (Hashim and Tantibhedyangkul, 1987). Histological investigation of IFI

can provide a valuable insight about the adipose cell size. In the current study, adipose cell size of intraperitoneal fat tissue significantly decreased in fish fed 45PBM+HI. Similar results were observed by Li et al. (2016b) who reported a decrease level of IFI and adipose cell size with the concurrent upregulation of mRNA level of PPARα, a lipid hydrolysis gene in juvenile Jian carp, *Cyprinus carpiovar*. Jian when fed with 75 and 100% of HI oil.

The distal intestine is an important part of the gastrointestinal tract of fish, which is more sensitive to dietary modulation and has shown highest variations in the histometric measurements including villi, microvilli and number of goblet cells (GC) in response to dietary administration of alternative protein sources (Gajardo et al., 2017; Miao et al., 2018). In the present study, villi and enterocyte width and microvilli height increased significantly in fish fed 45PBM+HI, indicating the enlargement of digestion and absorption surface area. The observed shortening of villi and enterocyte width and microvilli height might be associated with poor growth performance and immune response in the 90PBM+HI. An earlier study reported that good growth performance and absorption efficiency are highly correlated with the longer villus height and width and height of microvilli (Siddik et al., 2018b). Goblet cells (GCs) secret and synthesize acid and neutral mucins which are known to play an important role in lubricating, trapping and eradicating pathogens (Padra et al., 2014; Sklan et al., 2004). The presence of greater number of acidic GCs in the present study in 45PBM+HI groups signals the protection of fish by binding and preventing the adherence of pathogenic bacteria in the intestinal epithelium. A possible explanation of such positive alterations in intestine may be related to the presence of higher amount of SFA (especially lauric acid, C12) in insects, these have been demonstrated to have positive effects on gut health because of their intestinal anti-inflammatory, antiviral (Henry et al., 2018; Vargas et al., 2018) and antibacterial activity (Gasco et al., 2018).

Heat shock proteins (HSP) consisting of HSP70 and HSP90 are two important conserved cellular proteins elevate significantly in response to environmental stressors and feed factors (Lin et al., 2014; Zhang et al., 2011). Irrespective of HI supplementation, both HSP70 and HSP90 upregulated significantly when fish were fed with increasing level of PBM, revealing that higher replacement of PBM imposed stress on juvenile barramundi. Similar results were observed in histological observation in the liver of juvenile barramundi where multifocal necrosis was observed in 90PBM+HI groups. Similar to our findings, Siddik et al. (2019a) reported large vacuoles and irregular arrangement of liver in fish fed 100% of PBM and bioprocessed PBM.

Insects possess a wide spectrum of novel antimicrobial peptides which can exhibit activity against microbial-related disease (Elhag et al., 2017). Elhag et al. (2017) identified seven new gene (cecropinZ1, sarcotoxin1, sarcotoxin (2a), sarcotoxin (2b), sarcotoxin3, stomoxynZH1,and

stomoxynZH1(a)) and three types of antimicrobial peptides in HI larvae, exhibiting diverse inhibitory activity against Gram-positive bacterium, Gram-negative bacterium and fungus, suggesting a potentially important role in controlling antibiotic-resistant pathogens. Similarly, Park et al. (2014) extracted low molecular weight antimicrobial factors from the HI larvae demonstrating a broad spectrum of antifungal and antibacterial activity. In the present study, fish fed 45PBM+HI diet revealed significantly higher survival rate against *V. harveyi* than other barramundi. Many earlier studies reported that dietary inclusion of insects at very low doses can enhance the disease resistance against pathogenic bacteria. For instance, Ming et al. (2013) reported a higher protection rate against *Aeromonas hydrophila* in black carp, *Mylopharyngodon piceus* after 60 days of feeding 2.5% maggot, *Musca domestica* meal. In red sea bream, *Pagrus major*, dietary inclusion of 5% housefly pupae protected 100% fish from *Edwardsiella tarda* while all fish fed control diet died within 10 days following bacterial challenge (Ido et al., 2015). Interestingly, dietary intake of HI larvae modulated the phylum of *Firmicutes* and *Proteobacteria* in the intestine of rainbow trout, *Oncorhynchus mykiss* (Bruni et al., 2018) and prebiotic bacteria in the gut of lying hens (Borrelli et al., 2017). However, Li et al. (2019) did not find significant difference in antibody response against infectious pancreatic necrosis virus (IPNV) between reference and HI larvae treated Atlantic salmon, *Salmo salar*. The increased resistance to *V. harveyi* might be due to the presence of chitin in HI larvae. Esteban et al. (2001) and Gopalakannan Arul (2006) reported that dietary inclusion of crustacean chitin at low levels can enhance immune response and disease resistance against pathogen in Gilthead seabream, *Sparus aurata* and common carp, *Cyprinus carpio*. However, regardless of HI supplementation, resistance to *V. harveyi* significantly declined in 90PBM+HI, revealing that higher inclusion of PBM has negative effects on immune function of barramundi. Similarly, higher replacement of FM with PBM impacted the immune function of sunshine bass, *Morone chrysops* × *Morone saxatilis* (Rawles et al., 2011) and largemouth bass, *Micropterus salmoides* (Subhadra et al., 2006a). Enhanced disease resistance against *V. harveyi* deserve further study to evaluate how HI larvae influence the pathogenic microbes in fish.

Several serum activities including lysozyme and bactericidal is an important indicator of innate immunity in teleost fish. Lysozyme, a low molecular weight alkaline protein is an important non-specific defense molecule in fish immune system (Wang et al., 2019a; Wu et al., 2019a) and can protect the fish from the infectious disease by decomposing 1, 4 glycosidic bonds in the peptidoglycan of Gram-positive and Gram-negative cell wall (Wu et al., 2019b) and its level has been reported to be enhanced in many fish species when expose to bacterial infection (Yao et al., 2008). In the present study, HI supplementation significantly influenced the immune response of

barramundi with significantly higher values of lysozyme activity observed in 45PBM+HI treated groups after being challenged with *V. harveyi*. Similarly, serum lysozyme activity enhanced markedly in yellow catfish, *Pelteobagrus fulvidraco* when fed with 31.9% black soldier fly larvae protein (Xiao et al., 2018). Also, dietary inclusion of 10% of yellow mealworm, *Tenebrio molitor* significantly elevated lysozyme activity against Gram-negative bacteria in European sea bass, *Dicentrarchus labrax* (Henry et al., 2018). Bactericidal activity, a nonspecific response play an important role by inhibiting and killing infectious microorganisms (Siddik et al., 2019b; Yano, 1996). Earlier studies reported that dietary inclusion of partially defatted HI larvae can influence the beneficial bacteria in rainbow trout, *Oncorhynchus mykiss* and also HI larvae can eradicate *Escherichia coli* O157:H7 and *Salmonella enterica* (Erickson et al., 2004).

Complement system consisting of 40 difference protein molecules functioning either as enzymes or as binding proteins is an important part of innate and adaptive immune system (Gasque, 2004), playing a pivotal role in killing pathogens directly (Morgan et al., 2005) and promoting humoral immune responses (Beutler, 2004). Ohta et al. (2014) identified a novel bioactive immune-activating polysaccharide composed of nine monosaccharides in the pupae of the melon fly belonging to Diptera, which induced proinflammatory cytokines and interferon β (IFNβ) in mouse against various pathogenic microorganisms and viral infections through the TLR4 signalling pathway. Complement C3 and C4 in the present study induced significantly in post-challenged 45PBM+HI groups. In line with the present study, upregulation of inflammatory genes including interleukin 1 β (*IL-1β*), *IL-8*, *IL-10* and tumor necrosis factor α (*TNF-α*) was observed in the head kidney leukocytes of salmon fed 66 and 100% of HI larvae (Stenberg et al., 2019). However, the stimulation of immune response against *V. harveyi* might also be due to either secretion of aintimicrobial peptides or due to presence of chitin in insects (Henry et al., 2018; Lee et al., 2008; Nogales-Mérida et al., 2018) or by other insects component (Ido et al., 2015). Therefore, it is reasonable to hypothesize that supplementation of HI larvae with PBM can modulate the immune response of juvenile barramundi.

To conclude, PBM supplemented with 10% HI larvae effectively replaced 45% FM in feed for juvenile barramundi, supporting good growth with positive effects on histometric measurements. This results is also further supported by decrease in the size of adipose tissue cell size, enhancement of disease resistance against *V. harvei*, and elevated level of serum immune response (lysozyme and bactericidal activity) and complement systems (C3 and C4) following 24 hours of challenge test. However, 90PBM+HI significantly depressed the growth performance, reflected by the elevation of AST and GLDH activity and presence of multifocal necrosis in liver. The supplementation of HI larvae influenced the health of juvenile barramundi. However, the specific

role(s) of chitin, antimicrobial peptide and/or bioactive polysaccharides of HI larvae on fish health need further investigations.

CHAPTER 5: Supplementation of *Hermetia illucens* larvae in poultry by-product meal based barramundi, *Lates calcarifer* diets improves adipocyte cell size, skin barrier functions, and immune responses

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Abstract

A six-week feeding trial was performed to examine the effects of supplementing *Hermetia illucens* (HI) larvae meal when fishmeal (FM) was replaced with poultry by-product meal (PBM) in juvenile barramundi, *Lates calcarifer* diet. The effect was evaluated in terms of barramundi growth, fillet quality, internal tissue structure, serum biochemistry, skin neutral mucins, immune response, and resistance to *Vibrio harveyi*. Three isonitrogenous (48% crude protein) and isolipidic (18% crude lipid) diets: an FM-based diet (Control) and two diets containing 60 and 75% of PBM supplemented with 10% HI larvae (60PBM+HI and 75PBM+HI) were formulated. A total of 225 barramundi with an average weight of 15.87±0.14g were randomly distributed into nine tanks each holding 25 fish. There were no significant effects of test diets on growth, but feeding HI supplemented PBM diets significantly increased the survival rate. A significantly reduced intraperitoneal fat index in HI supplemented PBM fed fish was correlated to a decreased size of peritoneal adipocytes. The observation of no histopathological alteration of the liver in the HI supplemented PBM fed fish was further supported by significant alterations in the serum biochemistry, in particular, a decreasing trend of alanine transaminase (ALT), glutamate dehydrogenase (GLDH) and total bilirubin (TB). A 14-day challenge with *V. harveyi* indicated that HI supplemented PBM diets reduced the infection rate in barramundi. After 24h of infection, increased serum (lysozyme) and skin barrier functions and down-regulation of interleukin-1beta (*IL-1β*) and upregulation of interleukin-10 (*IL-10*) were found in HI supplemented PBM fed fish.

Keywords: Insect larvae, neutral mucins, adipocyte cell, *Vibrio harveyi*, immune function, barramundi

5.1 Introduction

Insects are the prey of many carnivorous and omnivorous fish in their natural environment (Henry et al., 2015) and hence, could be a nutritionally suitable and potential source of sustainable alternative animal protein ingredients for aquacultured species. Insects are characterized by high quantity (60-80%) and quality of protein, ability to grow on waste and by-products, fast growth with a lower risk of transmission of zoonotic disease and may contain native bioactive peptides with anti-microbial, anti-fungal and anti-viral functions (Elhag et al., 2017; Gasco et al., 2020b; Henry et al., 2015; Park et al., 2015; Van Huis et al., 2013; Zarantoniello et al., 2019). In particular, insect larvae belonging to the Diptera family contain essential amino acid compositions that are similar to fishmeal (FM) (Henry et al., 2015).

Hermetia illucens (HI) larvae of the Diptera family, commonly known as black soldier fly, have begun to play an important role in aquafeed production. HI larvae can dwell in harsh environments, infested with bacteria and fungi, leading to the production of low molecular weight antimicrobial factors, which have antifungal and antibacterial effects against Gram-positive and Gram-negative bacteria (Park et al., 2015; Park et al., 2014). HI larvae are also characterized by the presence of chitin and high levels of medium-chain fatty acids especially lauric acid (C12:0) (Gasco et al., 2018). Chitin and chitin derivatives are reported to have the immunomodulating capacity in fish (Bruni et al., 2018), and lauric acid is well documented for antibacterial and antiviral activity (Lieberman et al., 2006). Therefore, complete HI larvae as a supplement could provide a crucial value-added role in the diet of aquaculture species, especially for carnivorous fish.

Over the last decades, significant efforts have been made by commercial feed producers and fish farmers to investigate the use of animal by-products meal, in particular, poultry by-products meal (PBM) to replace fishmeal (FM), an expensive and often considered unsustainable source of protein in the diet of carnivorous fish (Rimoldi et al., 2018; Rossi and Davis, 2012; Siddik et al., 2019a; Siddik et al., 2019b). PBM, an economical, and arguably a more sustainable aquafeed ingredient contains high-quality protein (58–65%), amino acids similar to FM and is also a good source of vitamins and minerals (Badillo et al., 2014; Cheng and Hardy, 2002; Yu, 2004). A number of studies regarding the inclusion of PBM in aquadiets have been published over the last decade, though the findings are still controversial in terms of inclusion levels of PBM and have tended to investigate the replacement of FM protein with PBM protein without any supplementation. Some studies were able to substitute up to 100% FM protein with PBM in humpback grouper (Shapawi et al., 2007), Nile tilapia, *Oreochromis niloticus* (El-Sayed, 1998; Hernández et al., 2010) and hybrid striped bass, *Morone chrysops*× *Morone saxatilis* (Rawles et al., 2011) without deleterious effects on growth. In contrast, inclusion levels of 50% and greater

were reported to impose adverse effects on many fish species including tench, *Tinca tinca* (González-Rodríguez et al., 2016), black sea turbot, *Psetta maeoticus* (Yigit et al., 2006) and cobia, *Rachycentron canadum* (Zhou et al., 2011). In addition, replacing FM with PBM negatively influenced the immune response of largemouth bass, *Micropterus salmoides* (Subhadra et al., 2006a; Subhadra et al., 2006b).

Lates calcarifer, commonly known as barramundi is one of the highly valued euryhaline carnivorous fish species used for commercial farming both in marine and freshwater environments, particularly in Australia and some other southeast Asian countries including Indonesia, Philippines, Malaysia, Thailand and Taiwan (Tian and Qin, 2003). Intensive farming of barramundi in marine net-cages and in freshwater ponds has accelerated disease outbreaks in South East Asia (Talpur and Ikhwanuddin, 2013). Vibriosis caused by *V. harveyi* is one of the major problems in barramundi farming, causing a major loss through mortalities (Ransangan et al., 2012; Talpur and Ikhwanuddin, 2013). Much research has been conducted to develop alternative therapeutics for barramundi including the use of plant herbs and fish protein hydrolysate (Siddik et al., 2019b; Siddik et al., 2018b; Talpur and Ikhwanuddin, 2012; 2013). However, disease resistance against bacterial pathogens in the aquatic system in response to insect larvae supplementation with animal by-products remains unknown.

The mucosal surface of fish, such as gill, skin, and gastrointestinal tracts are important sites for microbial exposure, forming a thin biochemical and physical barrier between the external environment and the internal milieu to protect fish from any pathogenic invasion (Ángeles Esteban, 2012). Amongst all mucosa-associated lymphoid tissue, skin associated mucosa, serving as an anatomical and physiological barrier against encountering pathogenic microorganisms, plays a crucial role in the immune system of fish (Ángeles Esteban, 2012)**.** Recently research on gut-associated lymphoid tissue has been intensified in relation to dietary parameters (Fuglem et al., 2010; Rombout et al., 2011), however, fish skin mucosal immunity is a new interest and rarely studied in finfish aquaculture (Ángeles Esteban, 2012). Therefore, it is of interest to study how dietary alteration and challenge with pathogens may induce skin relevant immunity in fish.

To date, insect supplementation with animal by-products in the diets of carnivorous fish has not been intensively assessed. Hence, the present work was aimed to evaluate the effect of incorporating PBM as a replacement of FM along with supplementation of HI larvae meal on the biological and physiological response of barramundi through a multidisciplinary approach integrating biometric, histological, biochemical, molecular, immunological and bacterial challenge analyses.

5.2 Materials and methods

5.2.1 Ethics statements

All experimental procedures involving fish were performed in a recirculatory aquaculture system (RAS) at Curtin Aquatic Research Laboratory (CARL) in Curtin University, Australia in strict accordance with the Australian Code of Practice for the care and use of animals for scientific purposes, and following review and approval by the Curtin University Animal Ethics Committee approval number: ARE2018-37. Experimental procedures were dedicated to minimizing stress, pain, and discomfort to the fish including using an anaesthetic (AQUI-S®, 8 mg/l) and, for euthanasia an overdose of AQUI-S® (175 mg/l). Such practices were outlined in the protocol of the CARL standard operating procedures (SOP) of anaesthetizing and euthanizing of fish.

5.2.2 Diets

Three isonitrogenous (48% crude protein) and isolipidic (18% crude lipid) diets were formulated based on the locally available ingredients to meet the nutritional requirement of barramundi (Table 5. 1). All the ingredients were procured from Specialty Feeds, Glen Forrest Stockfeeders, 3150 Great Eastern Highway, Glen Forrest, Western Australia 6071 with the exception of HI larvae and PBM. Six day old HI larvae were received from Future Green Solution (FGR) having been cultivated in plastic trays, filled with a mixture of carp mince and grain waste (70:30) for 6 days. HI larvae were collected, dried in an oven for 48 h at 60 ˚C, and ground into fine powder by a digital blender (Anko, XJ-12412, China). PBM was kindly provided by Derby Industries Pty Ltd T/A, Talloman Lot Lakes Rd, Hazelmere WA 6055. All the dried ingredients were weighed, mixed homogenously in a food mixer (Hobart Food equipment, Australia), and then oil and distilled water were added to make a dough. Furthermore, 3 mm long pellets were produced by passing the dough through a mincer, the pellets were then dried in an oven for 36 h at 60 ˚C, broken up by hand after cooling at room temperature and sealed in plastic bags before storing in the refrigerator until use in the feeding trial. The fatty acids profile of the HI larvae, PBM, and experimental diets are presented in Table 5. 2.

Table 5. 1 Feed formulation and nutritional composition of test diets containing two different levels of PBM supplemented with full-fat HI larvae

Ingredient (g/100g)	Control	60PBM+HI	75PBM+HI
[a]FM[†]	72.00	22.50	11.00
[b]PBM[‡]	0.00	42.00	52.50
HI larvae[†]	0.00	12.00	12.00

[a]Cod liver oil	0.50	2.50	2.50
[a]Canola oil	1.00	3.00	3.00
[a]Wheat	16.90	8.40	9.40
[a]Wheat starch	7.00	7.00	7.00
[a]Vitamin C	0.05	0.05	0.05
[a]Vitamin premix	0.50	0.50	0.50
[a]Dicalcium phosphate	0.05	0.05	0.05
[a]Lecithin – Soy (70%)	1.00	1.00	1.00
Salt (NaCl)	1.00	1.00	1.00
[c]Nutritional compositions (%)			
Dry matter	90.21	91.10	90.15
Crude protein	47.88	47.93	47.93
Crude lipid	17.59	17.69	17.77
Ash	13.25	11.85	10.98

[a]Purchased from Specialty Feeds, Glen Forrest Stockfeeders, 3150 Great Eastern Highway, Glen Forrest, Western Australia 6071

[b]Kindly provided by Derby Industries Pty Ltd T/A, Talloman Lot Lakes Rd, Hazelmere WA 6055

[†] Fishmeal (FM): 64.0% crude protein, 10.76% crude lipid and 19.12% ash.

[‡]PBM (Poultry by-product meal): 67.13% crude protein, 13.52% crude lipid and 13.34% ash

[†]HI (*Hermetia illucens*) larvae: 40.43% crude protein and 17.23 % crude lipid

[c]Determined according to Association of Official Analytical Chemists (AOAC), (AOAC, 1995)

Table 5. 2 Fatty acids (mg/100g on dry matter basis) composition of formulated three experimental diets and HI larvae and PBM.

	Control	60PBM+HI	75PBM+HI	HI larvae	PBM
C8:0	1.39	1.27	1.19	4.01	0.90
C10:0	0.59	59.86	65.25	539.10	4.10
C11:0	0.00	0.60	0.48	2.00	0.33
C12:0	2.73	990.02	1143.19	10058.58	9.39
C13:0	1.53	1.95	1.49	3.03	0.54
C14:0	131.63	367.55	355.86	1588.87	73.69
C14:1n5	1.52	11.20	11.78	19.27	16.26
C15:0	41.42	32.58	25.44	34.35	15.14
C15:1	1.19	7.04	4.29	0.92	1.92

C16:0	1161.21	2019.55	2085.29	2596.67	2336.27
C16:1n7	165.22	437.70	413.84	361.93	540.27
C17:0	118.84	88.15	70.13	124.60	48.17
C17:1	31.53	39.92	27.38	31.52	19.73
C18:0	448.54	596.51	605.39	819.58	762.41
C18:1cis+trans	1158.94	3812.07	4097.72	3750.13	4410.64
C18:2 trans	6.95	10.19	10.78	18.92	6.52
C18:2 cis	624.01	1876.79	2067.26	2856.81	1736.53
C18:3n6	8.84	13.78	18.57	18.31	23.07
C18:3n3	120.20	400.99	420.75	385.84	260.39
C18:4n3	30.30	71.28	64.87	5.63	13.21
C20:0	22.44	30.48	30.53	21.99	25.98
C20:1	79.86	228.40	202.35	21.16	60.84
C20:2	14.57	19.62	18.46	4.02	19.64
C21:0	9.09	12.58	12.40	0.00	10.61
C20:3n6	15.50	35.96	38.95	4.45	56.76
C20:4n6	112.83	132.99	127.41	12.00	180.13
C20:3n3	8.29	8.94	7.12	1.63	4.43
C22:0	14.10	15.15	14.79	5.76	10.01
C20:5n3	178.50	193.65	134.95	11.30	16.79
C22:1n9	9.44	22.90	19.72	1.38	5.73
C22:2	1.05	1.08	0.90	0.00	0.59
C23:0	27.89	37.60	37.47	58.53	47.20
C22:4n6	91.14	35.44	22.67	0.00	4.78
C24:0	0.00	0.00	0.00	0.00	0.00
C22:5n3	63.30	60.47	50.83	2.02	36.67
C24:1	34.51	31.02	24.94	0.00	2.56
C22:6n3	908.53	473.15	310.50	2.60	27.47
∑SFA	1981.40	4253.84	4448.89	15857.06	3344.73
∑MUFA	1482.21	4590.25	4802.02	4186.31	5057.95
∑PUFA	2184.01	3334.31	3294.01	3323.54	2386.97
∑n-3	1309.12	1208.46	989.03	409.03	358.96
∑n-6	228.31	218.17	207.59	34.76	264.74
∑n-3/∑n-6	5.73	5.54	4.76	11.77	1.36

Hermetia illucens, HI; poultry by-product meal, PBM; saturated fatty acids, SFA; monounsaturated fatty acids, MUFA and polyunsaturated fatty acids, PUFA.

5.2.3 Fish husbandry and management

Four hundred barramundi averaging 7.25 g were collected from the Australian Centre for Applied Aquaculture Research (ACAAR), Fremantle, Australia, and transported to CARL in an oxygenated esky. Prior to the start of the trial, the fish were housed in two 300L seawater tanks and fed a commercial diet for two weeks twice a day to adapt them to the CARL experimental conditions. Thereafter, 225 barramundi, fasted for 24 h, were divided equally into nine tanks (25 fish /tank), each filled with 250 L of seawater. Each diet was hand-fed until apparent satiation twice a day at 8.00 am and 6.00 pm for 42 days, with each treatment repeated in triplicate. The rearing conditions and facilities during experimentation were maintained as described in the earlier studies at CARL (Siddik et al., 2019b; Siddik et al., 2018b). Excess diet from each tank was siphoned off one hour after feeding, oven-dried for 36 h at 60°C and then weighed to calculate feed intake. Feed consumption was recorded daily, and dead fish, if any, were weighed and recorded. At the end of the trial, all fish were starved for 24 h and thereafter anaesthetized with 8 mg/l AQUI-S® before determining the number of fish and the total fish biomass in each tank to calculate survival rate and growth performance. Length and weight, viscera, and liver were collected from five randomly chosen fish from each tank to determine the biometry indices such as condition factor, viscerosomatic and hepatosomatic indices.

5.2.4 Fatty acids profile

Fish muscles from four biological replicates and three technical replicates were used for fatty acids analysis. Fish were filleted to produce muscle which was wrapped with aluminium foil and freeze-dried for three days at -48.4°C and 1.9×10^{-1} mB. The fatty acids profile of experimental diets and fish muscle was carried out following the protocol of O'Fallon et al. (2007) and Siddik et al. (2019a). Approximately 0.5g of the sample was hydrolysed at 55°C for 1.5 hrs with 0.1ml of internal standard (1.2g nonadecanoic acid in 100ml chloroform), 0.7ml of 10N KOH, and 5.3ml of methanol. The sample was then methylated at 55°C for 1.5hrs with 0.6mL of 24N of sulphuric acid. The fatty acid was extracted into 1ml of hexane and then quantified using gas chromatography with flame ionization detection. The column used was a capillary column HP INNOWax GC column (60m x 0.25mm ID film 0.50 micron) with hydrogen as the carrier gas. Each sample was analysed in triplicate and results were expressed as an average.

5.2.5 Challenge test with *V. harveyi*

The challenge trial was conducted according to the protocol of our earlier study (Chaklader et al., 2019). Briefly, at the end of the six weeks feeding trial and after collecting all samples, Ten (10) fish from each dietary treatment were back to respective tanks and injected intraperitoneally by 1-mL syringe fitted with the 27-gauge needle with 0.1 mL of pathogenic *V. harveyi* suspension (LD_{50} = 1.1 x 10^8 cfu/ml), which was supplied by Diagnostic and Laboratory Services, Department of Primary Industries and Regional Development (DPIRD), 3 Baron-Hay Court, South Perth WA 6151. *V. harveyi* were grown in trypticase soy broth (Oxoid, Basingstoke, UK) for 24 h at 24ºC and the culture was centrifuged (5000 × g, 15 min) at 4ºC before suspending pellets in PBS (phosphate-buffered saline, pH 7.2) for the challenge trial. Clinical signs of vibriosis in terms of the thick layer of mucous on the body surface, congestion of the fins, and haemorrhages and ulceration of the skin and muscle tissue were observed three times a day (7:00 am, 2:00 pm and 9:00 pm) for 14 days and fish with such symptoms of vibriosis were subjected to euthanasia with AQUI-S at 175 mg/L for 20 minutes according to the protocol of the CARL SOP for euthanizing of fish.

5.2.6 Histological analysis

Liver, intraperitoneal fat, heart, and muscle from six euthanized (AQUI-S®,175 mg l^{-1}) fish/treatment (two/replicate) at the end of the trial and also a similar set of skin samples following 24 h post-challenge, were collected and immediately preserved in 10% neutral buffered formalin. Following preservation, fragments of all tissue sections were subsequently dehydrated with a series of ethanol washes, cleared by xylene and embedded in paraffin wax. The tissue wax was then cut into 5 µm slices by microtome, stained with Periodic Acid-Schiff (PAS) as per standard histological procedures, and photographed under a light imaging microscope (BX40F4, Olympus, Tokyo, Japan). The average of epidermis (Ep) thickness was measured from the three regions of each section and the number of epidermis goblet cells were counted in 1-mm length of the epidermis, following the methods of Heidarieh et al. (2013) and Sheikhzadeh et al. (2019).

5.2.7 Serum biochemical analysis

At the end of the feeding and challenge trial, six randomly chosen fish from each treatment (two/replicate) were anaesthetized (AQUI-S®, 8 mg l^{-1}) and blood was withdrawn from the caudal vein using 1-ml non-heparinized syringes fitted with 22G needles and then kept at room temperature for 4 h until coagulation. Serum was obtained from coagulated blood by centrifugation at 3000 rpm × 15 min at 4 °C and immediately stored at - 80°C prior to analysis of serum biochemical parameters and immune parameters.

The serum biochemical assays for two biological replicates and three technical replicates were performed as described in our previous study (Chaklader et al., 2020c). The samples were processed on an AU480 Clinical Chemistry Analyser (Beckman Coulter Australia Pty Ltd, Lane Cove West, NSW) to analyse alanine transaminase, ALT; total bilirubin, TB; urea; creatinine; cholesterol and total protein while Randox kits (Randox Australia Pty Ltd, Parramatta, NSW) were used for glutamate dehydrogenase, GLDH. Each sample was analysed in triplicate and results were expressed as an average.

5.2.8 Serum immune response

Serum lysozyme activity for both pre and post-challenge was determined for six fish/ treatment (two fish/replicate) following the turbidimetric method as described by Le Fotedar (2014b) and Bowden et al. (2004). Briefly, fifty microliters of each serum repeated in triplicate were pipetted in a 96-well plate (Iwaki, Tokyo, Japan) and then added fifty microliters of *Micrococcus lysodeiktikus* sus-pended in PBS (0.25 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) was added into each well. The absorbance was measured using an MS212 reader (Titertek Plus, Tecan, Austria) at 450 nm every 2 min for a total of 20 min at 25° C. One unit of lysozyme activity was defined as the amount of enzyme resulting in a decrease in absorbance of 0.001/min. The results were presented as U/ml.

Serum bactericidal activity for both before and after-challenge was determined for six fish/ treatment (two fish/replicate) following the protocol of Ueda et al. (1999) and Le Fotedar (2014b). Fifty microliters of *Vibrio anguillarum* obtained from the Department of Agriculture and Food, Perth, WA, Australia suspended in phosphate-buffered saline (PBS; 0.1 M, pH 7.2) was added to 50 µl serum and the same volume of bacterial suspension was added to 50 µl of PBS as control. The mixture was then incubated for 30 minutes at 25 °C. Following the reaction, 50µl from the mixture was plated onto triplicated tryptone soya agar and incubated for 24 h at 25 °C. Bactericidal activity was calculated as a decrease in the number of viable *V. anguillarum* cells, which is the log10 CFU/ml in the control minus the log10 CFU/ml in serum.

5.2.9 RNA extraction and gene expression analysis

Six fish per treatment (two/replicate) were euthanized using 175 mg l^{-1} AQUI-S® and the liver, spleen and head kidney were excised following 42 days post-feeding and also after 24 h post-challenge, samples were immediately preserved in RNA Later (Sigma-Aldrich, Germany) and then stored at - 80°C till extraction of RNA. Total RNA was extracted using 1 ml Trizol TM reagent (Invitrogen) based on manufacturers protocols from approximately 50-100 mg of the various tissue samples. The degradation and contamination of RNA were checked by gel electrophoresis and the

purity of RNA was monitored on a NanoDrop spectrophotometer 2000c (Thermo Fisher Scientific, USA). cDNA synthesis was carried out from 1 µg of total RNA by Omnicript RT kit (Qiagen, Hilden, Germany) as per the protocols of the manufacturer company. The specific primers of selected genes and reference genes used were as from the earlier published studies. RT-qPCR using PowerUp^{TM} Cyber Green Master Mix (Thermo Scientific, USA) with 7500 Real-Time PCR System (Applied Biosystems, USA) was conducted as described in our earlier study. The relative expression of target genes was normalised to the *18S rRNA* and *Ef1-a*, housekeeping genes (Table 5. 3), and calculated using $2^{-\Delta\Delta ct}$ method.

Table 5. 3 Primer sequence of heat shock related gene and immune related cytokines

Primer	Forward primer (5′-3′)	Reverse primer (5′-3′)	Reference
HSP90	ACCTCCCTCACAGAATACC	CTCTTGCCATCAAACTCC	Mohd-Shaharuddin et al. (2013)
IL-1β	ACAACGTCATGGAGCTCTGG	TCTTTGTCCTTCACCGCCTC	Siddik et al. (2019c)
IL-10	CGACCAGCTCAAGAGTGATG	AGAGGCTGCATGGTTTCTGT	Siddik et al. (2019c)
18S rRNA	TGGTTAATTCCGATAACGAACGA	CGCCACTTGTCCCTCTAAGAA	Mohd-Shaharuddin et al. (2013)
Ef1-a	AAATTGGCGGTATTGGAAC	GGGAGCAAAGGTGACGAC	Mohd-Shaharuddin et al. (2013)

5.2.10 Calculation and statistical analysis

Growth performance in terms of weight gain (WG), specific growth rate (SGR), feed intake (FI) and feed conversion (FCR), and biometry indices in terms of condition factor (CF), hepatosomatic index (HSI) and intraperitoneal fat index (IFI) were calculated as follows:

Weight gain (WG, g) = [(Mean final weight-Mean initial weight)/(Mean initial weight)]

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

Feed intake (FI, g/fish d^{-1}) = [(Dry diet given - Dry remaining diet recovered) /days of experiment)/ no. of fish]

Feed conversion ratio (FCR)= [(Dry feed fed)/(Wet weight gain)]

Condition factor (CF, %) = [Final body weight (g) / Body length cm^3)] ×100

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

Intraperitoneal fat index (IFI, %)= [Intraperitoneal fat weight (g)/Whole body weight (g)] ×100

The fillet lipid quality of barramundi fed control and HI larvae supplemented PBM were determined using two important lipid indexes, atherogenicity (AI) and thrombogenicity (TI), as follows:

AI= (aC12:0+bC14:0+C16:0)/(dp+eM+FM´)

Where P is the sum of n3 and n6 PUFA; M is the oleic acid and M´ is the sum of other monounsaturated fatty acids (MUFA); a, b, c, d, e, f are empirical constant; b = 4 and other constants = 1.

TI= (C14:0+C16:0+C18:0)/[(nM+nM´+p(n6)+q (n3)+(n3/6)]

Where M and M´ are as before; n, o, p, q are empirical constants; n, o, p = 0.5 and q = 3

Unless specified otherwise, all results are presented as mean ± SE. All data were subjected to Shapiro-Wilk's and Levene's tests to test the normal distribution and homogeneity of variances. A one-way ANOVA, followed by Dunnett's multiple comparisons test was performed on growth performance, biometry indices, heat shock-related gene expression, and adipocyte cell size to test the significant differences between the experimental groups. Survival of 42 days post-feeding groups and 14 days post-challenge groups were compared by the Kaplan-Meier method based on the pairwise multiple comparison Log-Rank (Mantel-Cox) test. Data on serum biochemistry and skin histomorphology after the challenge test were subjected to a two-way ANOVA using the General Linear Models (GLM) procedure where “diet” and “challenge” were used as the main factors. Data from the pre and post-challenge test was compared by paired t-test.

5.3 Results

5.3.1 Fish performance and feed utilization

Weight of barramundi at the termination of 6 weeks growth increased more than fourfold when compared to the initial weight, with no significant variations in growth performance as measured by final body weight (Figure 5. 1A), weight gain (Figure 5. 1B) and specific growth rate (Figure 5. 1C), feed intake (FI) (Figure 5. 1D) and feed conversion ratio (FCR) (Figure 5. 1E) between the three test diets. At the end of the trial, 60PBM+HI and 75PBM+HI fed groups showed a significantly higher survival rate than the control (Figure 5. 1F). None of the test diets had significant effects on the biometry indices including condition factor (Figure 5. 1G) and hepatosomatic index (Figure 5. 1H). However, the intraperitoneal fat index (IFI) decreased significantly in 60PBM+HI, and 75PBM+HI fed groups when compared with the control (Figure 5. 1I).

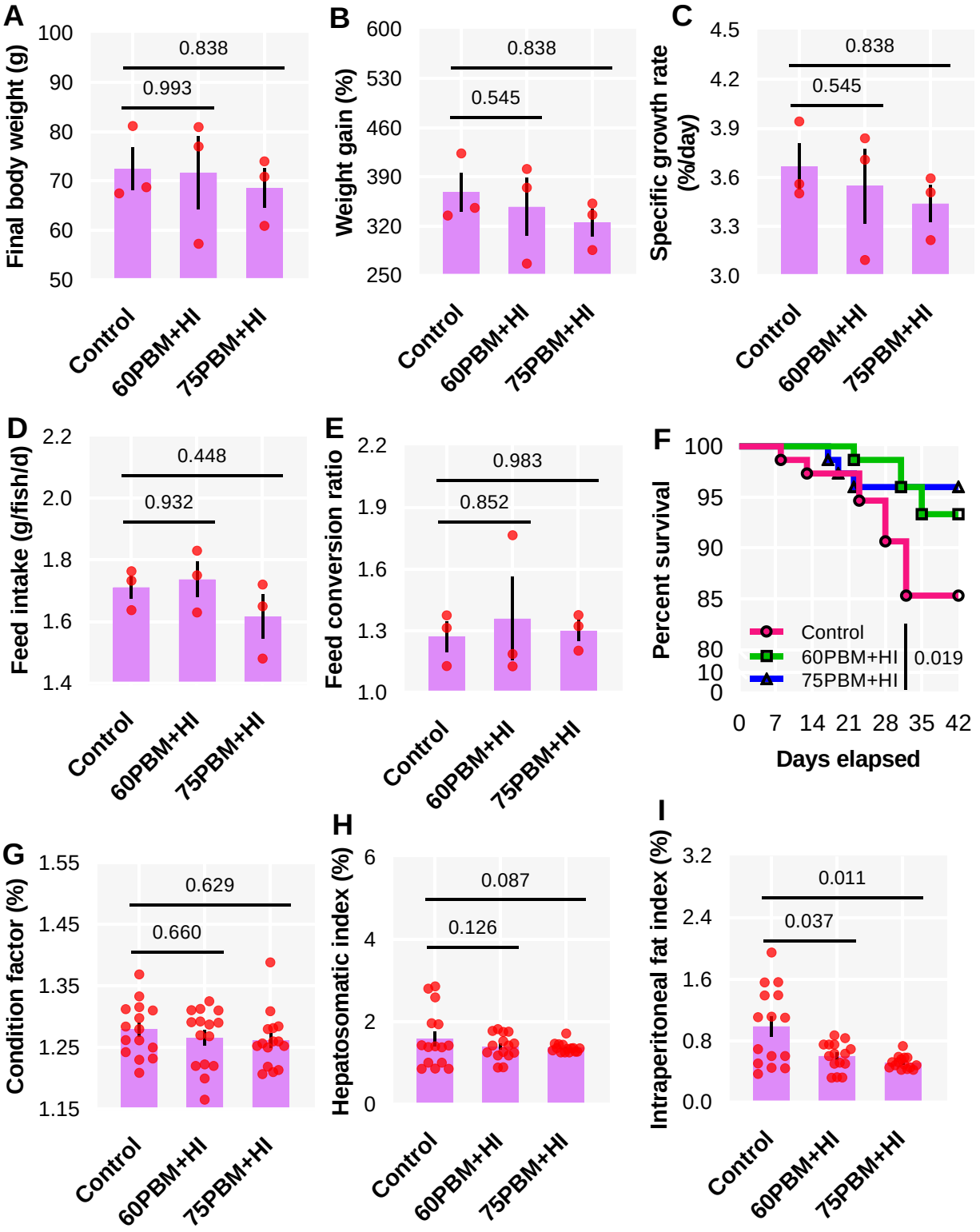

Figure 5. 1 Growth performance (A-C), feed utilization (D-E), survival (F) and biometry indices (G-I) of barramundi 6 weeks post-feeding with control, 60% FM replacement diet (60PBM+HI) and 75% FM replacement diet (75PBM+HI), supplemented with 10% full-fat HI larvae. Results represent mean ± SE of three values. *P*-values on the top of the bar with scatter dot plot denote significant differences between control- vs 60PBM+HI- and 75PBM+HI- fed fish (an ordinary one-way ANOVA, followed by Dunnett's multiple comparison test, $P < 0.05$). Asterisk denotes

significant differences between control- vs 60PBM+HI- and 75PBM+HI- fed fish (Kaplan Meyer survival method, followed by Log-rank test, $P < 0.05$).

5.3.2 Fillet fatty acids composition

The fatty acid profile of the barramundi fillets at 6 weeks post-feeding with the experimental diets are presented in Table 5. 4. Saturated fatty acid (SFA) content varied among dietary groups, increasing significantly from Control to 60PBM+HI and 75PBM+HI. The majority of the individual SFAs with the exception of C15:0 and C17:0 increased significantly in HI supplemented PBM diets when compared to the control. The majority of individual monounsaturated fatty acids (MUFA) were affected by diets, with most showing a significant increase in HI supplemented PBM diets compared to control. HI supplemented PBM diets showed an increased content of polyunsaturated fatty acids (PUFA) particularly α-Calendic acid (C18:3n6), Alpha-linolenic acid (C18:3n3), Stearidonic acid (C18:4n3), Dihomo-gamma-linolenic acid (C20:3n6), Arachidonic acid (C20:4n6), Eicosatrienoic acid (C20:3n3), whilst Adrenic acid (C22:4n6) and Docosahexaenoic acid (C22:6n3) declined significantly in HI supplemented diets when compared with the control. Regarding lipid quality, both atherogenicity (AI) and thrombogenicity (TI) increased in fish fed 60PBM+HI and 75PBM+HI.

Table 5. 4 Fillet fatty acid (mg/100g on dry matter basis) composition of barramundi fed control and HI larvae supplemented diets at the termination of the 6 weeks trial. Different superscript letters within the same row denote significant differences between control- vs 60PBM+HI- and 75PBM+HI- fed fish (an ordinary one-way ANOVA, followed by Dunnett's multiple comparison test, $P < 0.05$).

	Control	60PBM+HI	75PBM+HI	ANOVA P
C10:0	0.78±0.03^{b}	4.81±0.72^{a}	4.31±0.06^{a}	0.00
C12:0	1.02±0.09^{b}	295.03±33.71^{a}	294.63±2.47^{a}	0.00
C13:0	0.60±0.00^{b}	1.02±0.12^{ab}	0.92±0.00^{a}	0.01
C14:0	67.46±0.77^{b}	217.78±21.68^{a}	217.70±1.96^{a}	0.00
C14:1n5	0.97±0.03^{b}	6.43±0.73^{a}	6.73±0.07^{a}	0.00
C15:0	17.76±0.63	19.04±1.78	17.81±0.21	0.67
C15:1	0.00±0.00	0.00±0.00	0.18±0.20	0.42
C16:0	713.65±10.19^{b}	1253.87±105.68^{a}	1319.91±13.68^{a}	0.00
C16:1n7	130.78±1.90^{b}	281.81±27.55^{a}	287.55±2.38^{a}	0.00
C17:0	44.02±0.52	46.25±3.97	41.62±0.82	0.43
C17:1	22.70±0.31	27.71±2.80	23.65±1.08	0.18
C18:0	278.21±4.07^{b}	427.36±27.76^{a}	452.18±5.83^{a}	0.00
C18:1cis+trans	859.76±5.73^{b}	2698.63±240.32^{a}	2974.63±63.88^{a}	0.00
C18:2 trans	17.50±15.25	7.06±1.52	7.05±0.67	0.65
C18:2 cis	320.82±3.44^{b}	1169.13±101.94^{a}	1229.36±17.46^{a}	0.00
C18:3n6	17.51±1.42^{c}	48.60±0.80^{b}	58.10±1.30^{a}	0.00
C18:3n3	55.12±0.38^{b}	215.13±19.68^{a}	216.91±1.96^{a}	0.00
C18:4n3#	17.01±1.06^{b}	44.59±2.66^{a}	44.67±1.21^{a}	0.00
C20:0	8.95±0.22^{b}	17.81±1.00^{a}	18.59±0.21^{a}	0.00
C20:1	36.62±0.38^{b}	119.40±10.53^{a}	115.02±0.97^{a}	0.00
C20:2	8.84±0.03^{b}	16.73±1.26^{a}	17.02±0.26^{a}	0.00
C21:0	4.05±0.18^{b}	6.64±0.45^{a}	6.78±0.15^{a}	0.00
C20:3n6	25.68±0.57^{b}	46.67±3.26^{a}	52.49±0.53^{a}	0.00
C20:4n6	92.29±1.90^{b}	121.96±6.52^{a}	120.73±3.17^{a}	0.00
C20:3n3	4.61±0.09^{b}	6.83±0.52^{a}	6.77±0.33^{a}	0.01
C22:0	3.58±0.03^{b}	7.50±0.46^{a}	7.66±0.27^{a}	0.00
C20:5n3	109.31±1.78	126.12±9.98	113.69±1.31	0.19
C22:1n9	4.03±0.03^{b}	11.61±1.04^{a}	11.85±0.09^{a}	0.00
C22:2	0.00±0.00^{b}	0.93±0.09^{a}	1.00±0.00^{a}	0.00
C23:0	13.39±0.68^{b}	25.64±1.36^{a}	27.84±0.15^{a}	0.00
C22:4n6#	62.67±1.07^{a}	29.39±1.68^{b}	23.40±0.59^{c}	0.00
C22:5n3#	71.50±0.95	82.68±5.44	79.49±1.05	0.12
C24:1	11.81±0.12^{b}	14.76±0.91^{a}	14.72±0.17^{a}	0.01

C22:6n3	683.13±12.43[a]	447.58±23.47[b]	377.51±8.64[c]	0.00
∑SFA	1153.45±15.14[a]	2322.75±197.52[a]	2409.95±21.74[a]	0.00
∑MUFA	1066.66±8.25[b]	3160.35±283.49[a]	3434.32±67.55[a]	0.00
∑PUFA	1485.98±24.56[b]	2363.40±174.89[a]	2348.18±34.04[a]	0.00
∑n-3	940.67±15.92	922.93±61.54	839.03±11.05	0.20
∑n-6	198.15±3.87[b]	246.63±12.20[a]	254.72±4.82[a]	0.01
∑n-3/∑n-6	4.75±0.03[a]	3.74±0.15[b]	3.29±0.00[c]	0.00
AI	0.32±0.00[b]	0.34±0.00[a]	0.33±0.00[ab]	0.01
TI	0.27±0.00[c]	0.33±0.00[b]	0.34±0.00[a]	0.00

Hermetia illucens, HI; poultry by-product meal, PBM; saturated fatty acids, SFA; monounsaturated fatty acids, MUFA and polyunsaturated fatty acids, PUFA.

5.3.3 Histological analysis and expression of HSP90 in the liver

The internal architecture of the liver, intraperitoneal fatty tissue, heart, and muscle tissue was evaluated by histology, and results varied among test groups (Figure 5. 2A-K). The liver of fish fed control and test diets showed no histopathological changes with a higher amount of glycogen that was characterized by higher pigmented hepatic cytoplasm (Figure 5. 2A-C). Relative expression of heat shock protein-90 (HSP90) was not induced by the control and other test diets (Figure 5. 2D). All adipocytes sizes in intraperitoneal fatty tissue (Figure 5. 2E-G), in particular, HI larvae supplemented groups, showed a smaller size of adipocytes cells (Figure 5. 2H). The heart (Figure 5. 2I-k) and muscle (Figure 5. 2L-N) tissue results showed no significant difference between the test groups.

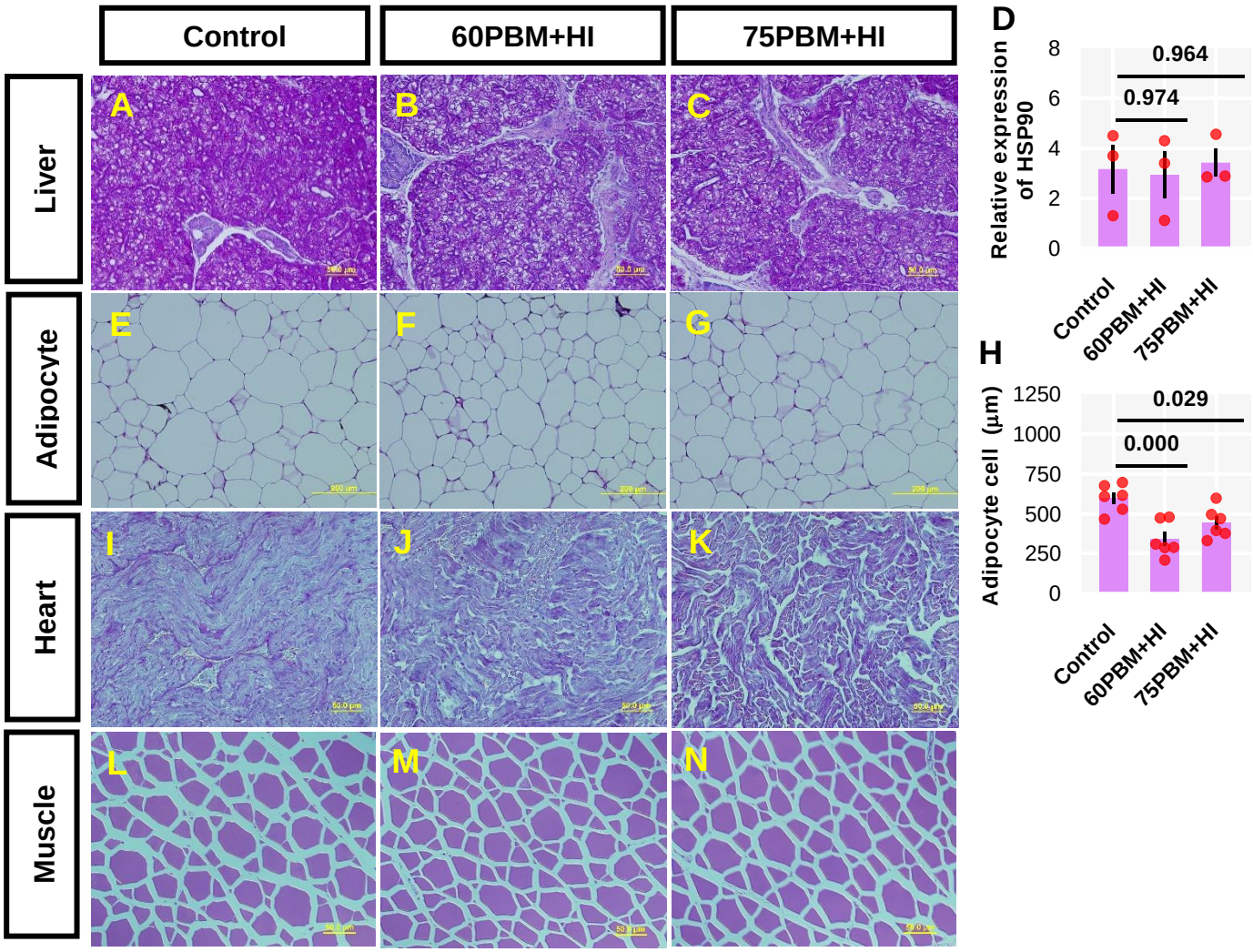

Figure 5. 2 Light microscopy of the liver (A-C) (PAS stain, 40 × magnification), adipose cell size (E-G) (PAS stain, 20 × magnification), heart (I-K) (PAS stain, 40 × magnification) and muscle (L-N) (PAS stain, 40 × magnification) of juvenile barramundi 6 weeks post-feeding with control, 60% FM replacement diet (60PBM+HI) and 75% FM replacement diet (75PBM+HI), supplemented with 10% full-fat HI larvae. (D and H) variation in the expression of hepatic HSP90 (n = 3) and adipocyte cell size (n = 6) in response to different levels of PBM supplemented with HI. Results represent mean ± SE. *P*-values on the top of the bar with scatter dot plot denote significant differences between control- vs 60PBM+HI- and 75PBM+HI- fed fish (an ordinary one-way ANOVA, followed by Dunnett's multiple comparison test, $P < 0.05$).

5.3.4 Resistance to *V. harveyi*

Supplementation of HI larvae with two different levels of PBM significantly modulated the infection rate, showing 64.52% and 60.00% asymptomatic fish in 60PBM+HI and 75PBM+HI, respectively when compared to 33.33% asymptomatic fish for the control fed groups (Figure 5. 3).

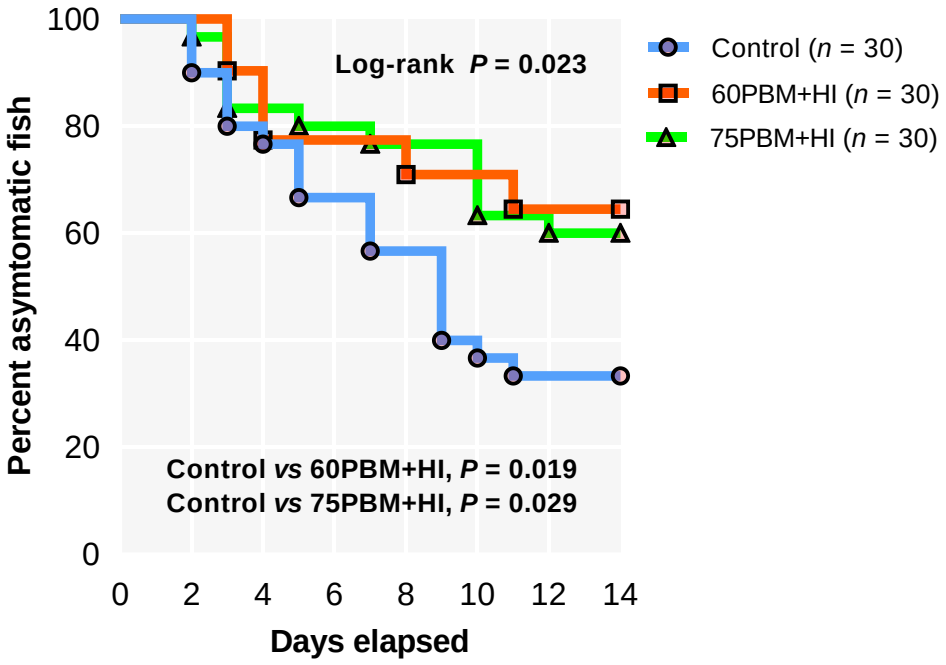

Figure 5. 3 Kaplan Meyer curve of barramundi fed with control, 60% FM replacement diet (60PBM+HI), and 75% FM replacement diet (75PBM+HI), supplemented with 10% full-fat HI larvae in response *V. harveyi* challenge over a period of two weeks. Control groups demonstrated infection at 2 days post-challenge whereas 60PBM+HI and 74PBM+HI groups demonstrated infection 3 days post-challenge. *P*-values denote significant differences between control- vs 60PBM+HI- and 75PBM+HI- fed fish (Kaplan Meyer survival method, followed by Log-rank test, $P < 0.05$).

5.3.5 Serum biochemistry

Considering serum biochemical parameters (Figure 5. 4A-G), the factors "diet and challenge" had no significant effects on all parameters with the exception of total bilirubin (TB) and cholesterol. Two-way ANOVA analysis demonstrated that the diet had a significant effect on TB, manifested by a significantly lower level of TB in HI larvae supplemented PBM diets compare to control ($P < 0.05$). Meanwhile, as reported in Figure 5. 4F, a significant effect with "diet and challenge" was only reported for cholesterol which decreased in post-challenge 60PBM+HI and 75PBM+HI groups ($P < 0.001$) compared to pre-challenge groups, as revealed by paired t-test. In both before and after the challenge test, cholesterol was significantly higher in HI supplemented PBM diets. Additionally, no significant interaction between "diet" and "challenge" was observed for all parameters ($P > 0.05$).

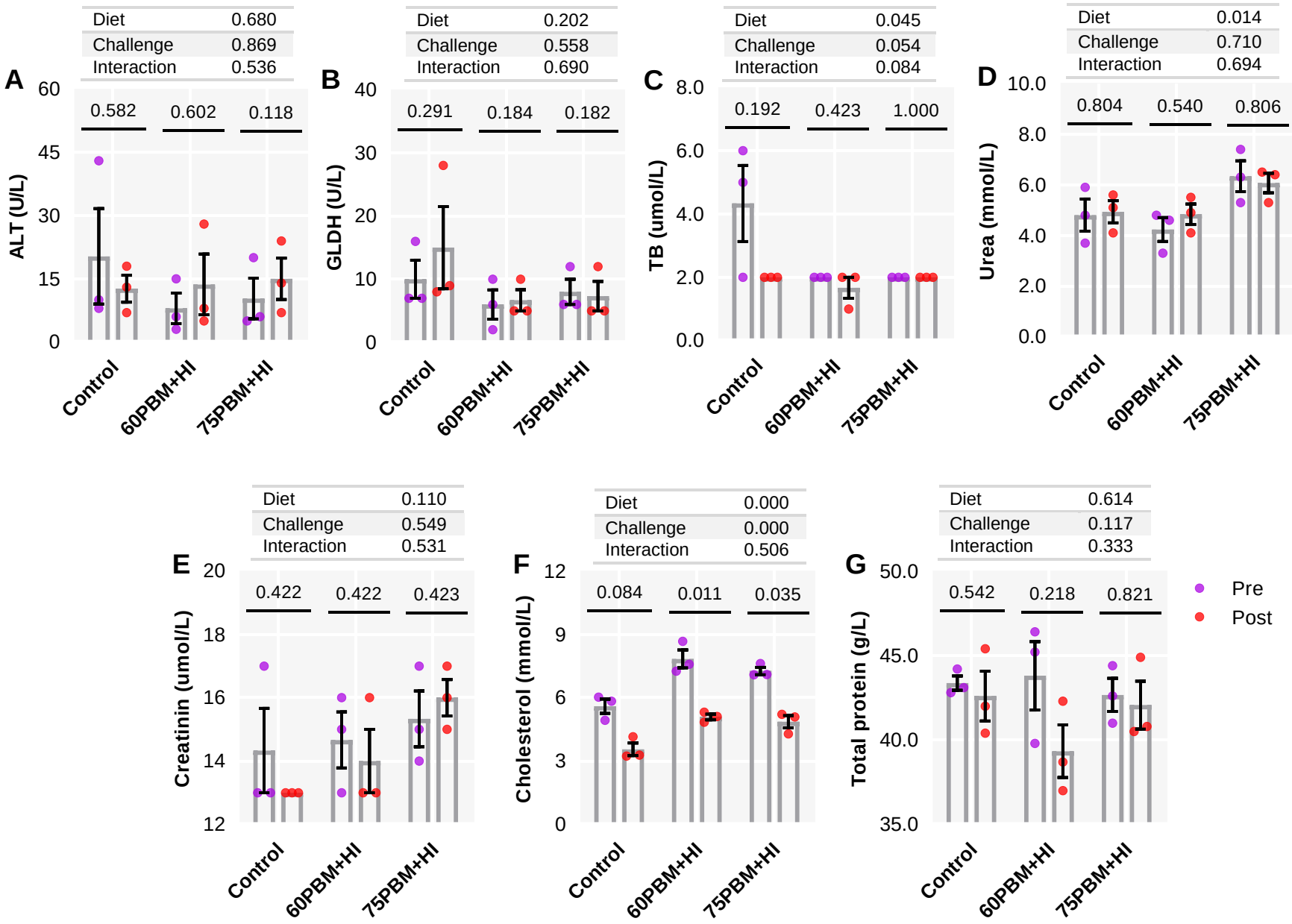
A
Diet 0.680
Challenge 0.869
Interaction 0.536
0.582 0.602 0.118
ALT (U/L)
B
Diet 0.202
Challenge 0.558
Interaction 0.690
0.291 0.184 0.182
GLDH (U/L)
C
Diet 0.045
Challenge 0.054
Interaction 0.084
0.192 0.423 1.000
TB (umol/L)
D
Diet 0.014
Challenge 0.710
Interaction 0.694
0.804 0.540 0.806
Urea (mmol/L)
E
Diet 0.110
Challenge 0.549
Interaction 0.531
0.422 0.422 0.423
Creatinin (umol/L)
F
Diet 0.000
Challenge 0.000
Interaction 0.506
0.084 0.011 0.035
Cholesterol (mmol/L)
G
Diet 0.614
Challenge 0.117
Interaction 0.333
0.542 0.218 0.821
Total protein (g/L)
Control
60PBM+HI
75PBM+HI
Pre
Post

Figure 5. 4 Serum biochemical changes (A-G) in pre-challenge and 24 h post-challenge juvenile barramundi fed control, 60% FM replacement diet (60PBM+HI), and 75% FM replacement diet (75PBM+HI), supplemented with 10% full-fat HI larvae. Bar represents the mean of three technical replicates. Violet and red markers (each marker represents the mean of two biological replicates) denote pre-challenge and post-challenge groups, respectively. *P*-values on the top of the bar with scatter dot plot denote significant differences between pre and post-challenge groups fed control and HI supplemented PBM diets (a paired t-test, $P < 0.05$). The effect of diet and challenge and their interaction was analysed by two way-ANOVA with Dunnett's multiple comparisons test.

5.3.6 Skin mucosal response

Skin epidermis thickness (Ep) and neutral mucins (NM) in pre-challenge and at 24 h post-challenge groups are presented in Figure 5. 5 (A-F). Two-way ANOVA demonstrated that both "diet" and "challenge" had significant effects on Ep thickness and NM and a significant interaction was also observed between the factors. As reported in Figure 5. 5 (G, H) both Ep and NM in response to 24 h post-challenge with *V. harveyi* significantly increased in all test diets groups when compared with all pre-challenge groups (paired t-test, $P < 0.01$ and 0.001). Considering the 24 h post-challenge groups, both Ep thickness and NM numbers increased significantly in fish fed HI supplemented PBM than the fish fed a control diet.

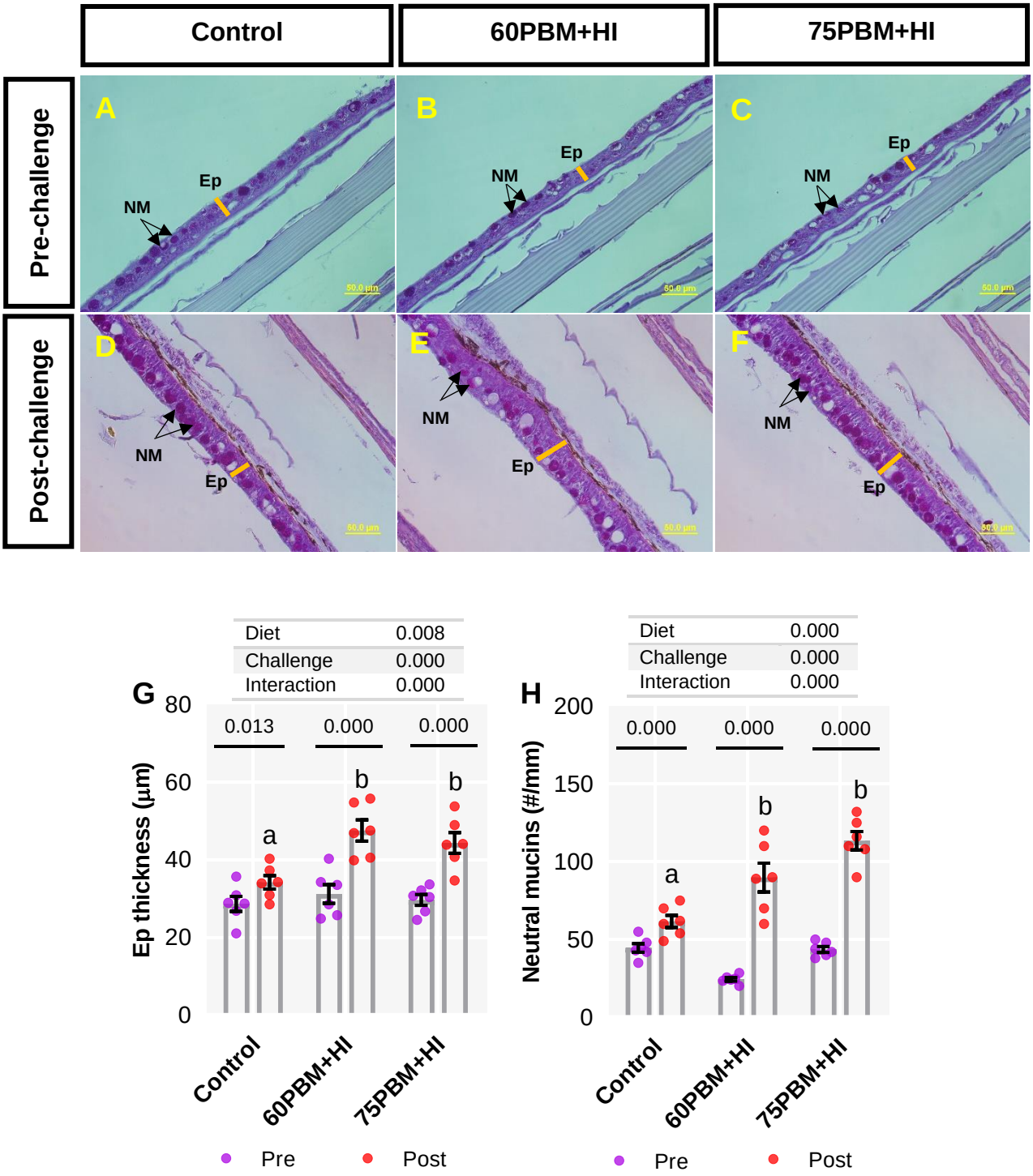

Figure 5. 5 Light microscopy of skin histometry and histochemistry (PAS, 40 × magnification) in pre-challenge (panel A-C) and at 24 h post-challenge (D-F) barramundi fed control, 60% FM replacement diet (60PBM+HI) and 75% FM replacement diet (75PBM+HI), supplemented with 10% full-fat HI larvae. Variation in the Ep thickness (G) and the number of goblet cells producing neutral mucins (H) in the skin of pre-challenge and post-challenge barramundi fed different test diets. *P*-values on the top of the bar with scatter dot plot denote significant differences between pre and post-challenge groups fed control and HI supplemented PBM diets (a paired t-test, $P < 0.05$). Different letters on the top of the bar with scatter dot plot denote significant differences

between control- vs 60PBM+HI- and 75PBM+HI- fed fish (a one-way ANOVA, followed by Dunnett's multiple comparison test, $P < 0.05$). The effect of "diet" and "challenge" and their interaction was analysed by two way-ANOVA with Dunnett's multiple comparisons test. Bar indicates the mean of six values. Violet and red markers denote pre-challenge and post-challenge groups, respectively. EP, epidermis, and NM, neutral mucins.

5.3.7 Serum immunity and cytokines expression

Serum immune response including lysozyme and bactericidal activity and cytokines expression including IL-1β and IL-10 in head kidney and spleen in response to diets and challenge are presented in Figure 5. 6 and Table 5. 5. After 24 h bacterial infection, serum lysozyme increased significantly in post-challenge groups fed HI supplemented diets (Figure 5. 6A) when compared with pre-challenge groups whereas dietary treatments and the bacterial challenge had no effects on bactericidal activity (Figure 5. 6B). Meanwhile, irrespective of the experimental diets, IL-1β expression level in the head kidney of 24 h post-challenge groups fed HI supplemented diets were down-regulated with respect to pre-challenge groups (Figure 5. 6C). These groups showed upregulation of *IL-10* (Figure 5. 6D). However, there were no significant effects on the expression level of IL-1β and *IL-10* in response to 24 h post-challenge in the spleen of barramundi fed control and HI supplemented diets (Figure 5. 6E-F).

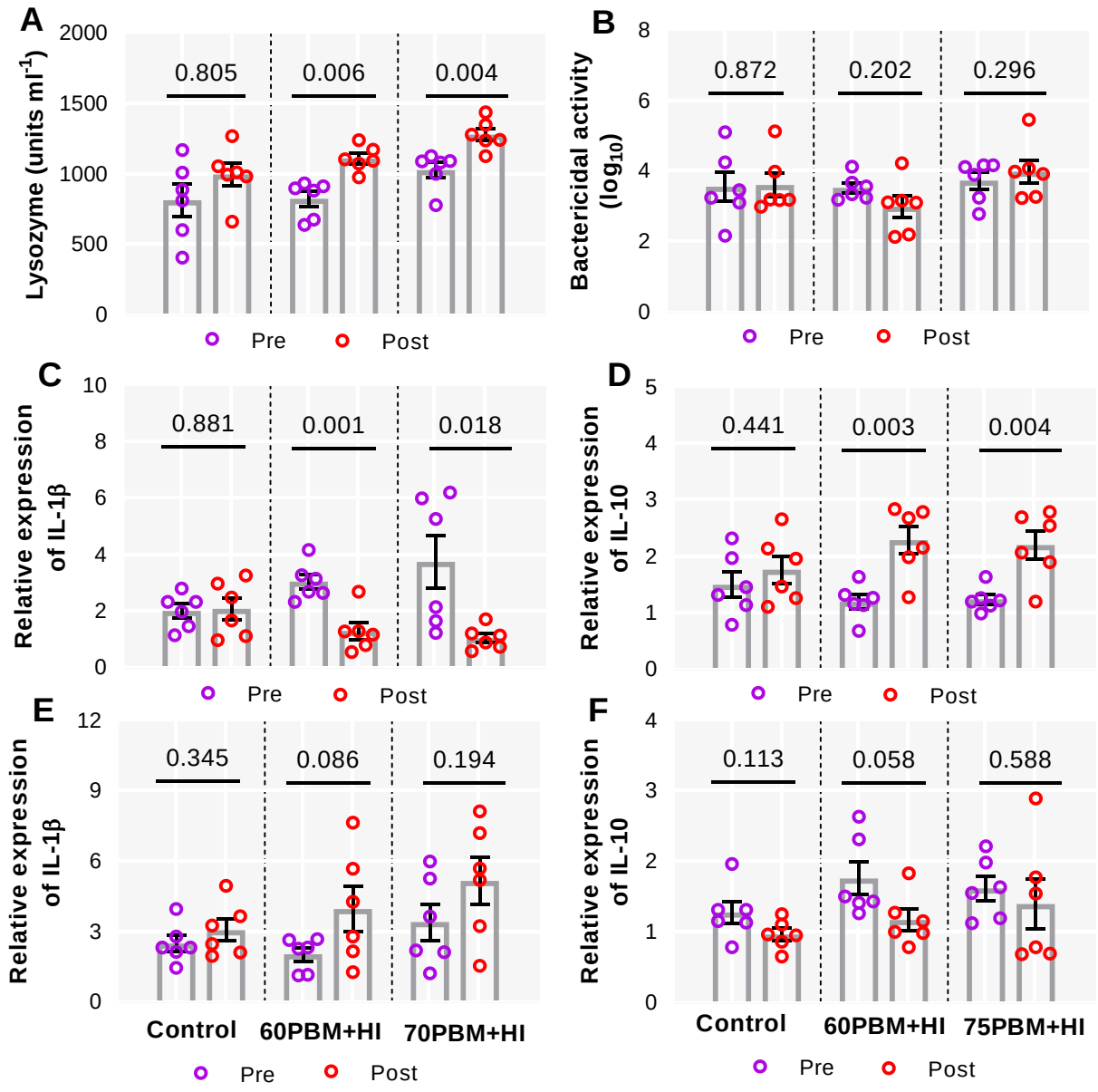

Figure 5. 6 Immune response in serum and cytokines expression participated in the inflammatory response in barramundi head kidney and spleen. Serum lysozyme (A) and bactericidal activity (B) as well as qPCR analysis of *IL-1β* and *IL-10* both in the head kidney (C-D) and spleen (E-F) isolated from barramundi pre-challenge control, 60PBM+HI and 75PBM+HI and post 24 h challenge with *V. harveyi*. *P*-values on the top of the scatter dot plot denote significant differences between pre and post-challenge groups fed control and HI supplemented PBM diets (a paired t-test, $P < 0.05$). Lower standard error in the bar indicates the adequacy of using minimum biological replicates for serum immunological assessment.

Table 5. 5 Factorial analysis on the effect of test diets and challenge time and their interaction on serum immune response (lysozyme and bactericidal activity) and cytokines expression (*IL-1β* and *IL-10*) both in kidney and spleen.

	Factors		Interaction
Parameters	Diet	Challenge	Diet × Challenge
Serum lysozyme	0.061	0.003	0.218
Serum bactericidal activity	0.164	0.774	0.418
IL-1β (kidney)	0.749	0.001	0.118
IL-10 (kidney)	0.850	0.001	0.119
IL-1β (spleen)	0.086	0.019	0.578
IL-10 (Spleen)	0.149	0.037	0.665

5.4 Discussion

The present study incorporated 60PBM+HI and 75PBM+HI and neither diets had significant effects on growth performance when compared to the control. Similarly, many previous studies have reported that dietary inclusion of HI larvae meal did not influence the growth performance of fish including Jian carp, *Cyprinus carpio* var. Jian (Li et al., 2017a), European seabass, *Dicentrarchus labrax* (Magalhães et al., 2017), Atlantic salmon, *Salmo salar* (Belghit et al., 2018), clownfish (Vargas-AbúNdez et al., 2019) and rainbow trout, *Oncorhynchus mykiss* Walbaum (Renna et al., 2017). However, it was also previously found that 45PBM+HI significantly influenced the growth performance, intestinal mucosal immunity, and resistance to *V. harveyi* compare to FM based diet, whilst 90PBM+HI negatively impacted the health of barramundi (Chaklader et al., 2019). Similar to Belforti et al. (2015), the HI inclusion increased the survival, probably meeting nutritional demands of the barramundi better than the control diet. The amelioration of survival rate might be due to the presence of immunomodulating components including chitin and antimicrobial peptide in HI larvae. However, 15% mortalities could be contributed to cannibalism as some partly eaten dead fish were found in the control treatment, similar to our previous studies (Chaklader et al., 2020c; Siddik et al., 2019a).

Fatty acid composition in a diet has been reported to influence the fish muscle fatty acid composition, which is an important trait for consumers since some fatty acids, in particular, MUFA and PUFA, promote health (Renna et al., 2017). Dietary inclusion of PBM to replace FM previously was reported to modify the muscle fatty acids composition of fish (Siddik et al., 2019a). In this study, high levels of lauric acid (C12:0), myristic acid (C14:0) and palmitic acid (C16:0) resulted in a higher total SFA content in the muscle of HI supplemented PBM fed fish, possibly owing to the consequence of the high abundance of C12:0, C14:0, and C16:0 in these respective diets. Similarly, feeding HI larvae meal-based diet increased total SFA due to a higher proportion of lauric acid in rainbow trout, *Oncorhynchus mykiss* (Renna et al., 2017), Jian carp, *Cyprinus carpio* var. Jian (Li et al., 2017a) and Atlantic salmon, *Salmo salar* (Belghit et al., 2019a; Belghit

et al., 2019b). In addition, a PBM based diet was reported to elevate the total SFA in juvenile black sea bass, as a consequence of a high abundance of palmitic acid (Dawson et al., 2018). The relatively lower levels of lauric acid in the muscle of HI-larvae-supplemented-PBM-fed fish in comparison to those respective diets may suggest that the fatty acid was readily utilized to produce energy by oxidation. However, adding 33, 67 and 100% PBM in the diet of juvenile totoaba, *Totoaba macdonaldi* decreased the levels of essential fatty acids in the muscle (Zapata et al., 2016) and similar results were reported in barramundi muscle when fed 75 and 100% PBM either unprocessed or bioprocessed (Siddik et al., 2019a). Interestingly, a significantly higher level of MUFA and PUFA in the muscle of barramundi fed HI larvae meal supplemented PBM diets than the control-fed fish was observed in this study. This was mainly due to the presence of a higher proportion of MUFA and PUFA in the respective diets. However, the substitution of FM with partially defatted HI larvae meal decreased the levels of PUFA in the muscle of rainbow trout (Renna et al., 2017). This heterogeneity might be due to the utilization of different growing substrate for HI larvae culture since nutritional profile largely depends on growing substrate. For instance, HI larvae fed with vegetable by-products showed no evidence of EPA and DHA (Renna et al., 2017) that was present in HI larvae fed carp mince utilised in this study. However, high levels of MUFA and PUFA in fish are associated with lessening the risk of neurological disease particularly myocardial infarction and cardiovascular disease. AI and TI are two important lipid indices used to determine the contribution of SFA, MUFA, and PUFA, and respected levels are associated with consumer health. Both the AI and TI values of muscle from barramundi in this study were less than 1.0 and were significantly increased by two different levels of PBM supplemented with 10% HI larvae, indicating the fish produced were healthier for human consumption (Renna et al., 2017).

As documented in many studies (Couto et al., 2016; Gu et al., 2014; Kokou et al., 2012; Martínez-Llorens et al., 2012; Yin et al., 2018), the dietary substitution of FM with alternative protein ingredients can impact the internal architecture of liver, in particular, increasing hepatic lipid deposition and lipid vacuoles, known as steatosis, in many fish species. The six weeks feeding trial reported here did not impose negative effects on the liver structure, which was further evidenced by the insignificant effect of HI supplemented PBM diets on the mRNA expression level of the stress-related gene, HSP90 in liver. In our earlier study, fish fed 45PBM+HI showed no obvious histopathological alteration and upregulation of HSP70 and HSP90 in the liver, whilst multifocal necrosis and upregulation of HSP70 and HSP90 were found in the liver of fish fed 90PBM+HI (Chaklader et al., 2019). However, hepatic vacuoles and lipid droplets, a sign of hepatic steatosis, increased in the liver of hybrid grouper, *Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂

when fed gradually increasing levels of an animal protein blend (20% to 80%) including PBM, shrimp meal and spray-dried blood meal (Ye et al., 2019a). The fact that there was no histopathological alteration, in the present study, in the liver of fish fed higher levels (up to 75%) of PBM might be due to the presence of chitin in HI larvae, as chitin and its derivatives have been reported to have a significant role in reducing the synthesis of fatty acids as well as boosting the hydrolysis of lipoproteins and triglyceride in the liver of fish and other animals (Li et al., 2016a; Li et al., 2017a; Zhang et al., 2008).

Feeding female tenches, *Tinca tinca* over a period of 86 days with graded levels of PBM (25.7 to 100%) increased the intramuscular fatty tissue deposition (Panicz et al., 2017). In the present study, dietary administration of 60 and 75% PBM, supplemented with 10% HI larvae in the diet of barramundi resulted in a significant decline in the intraperitoneal fat content which could be due to diminished adipose cells as revealed by the histology of the intraperitoneal fatty tissue. Regardless of PBM inclusion, dietary inclusion of HI larvae could influence the intraperitoneal fat content. For instance, a decrease in lipid deposition in the intraperitoneal fat tissue with concomitant upregulation of PPARα, a lipid metabolic relevant gene was found in juvenile Jian carp, *Cyprinus carpio* var. Jian fed 75 and 100% of HI larvae oil (Li et al., 2016b). There are several medium-chain fatty acids (MCFA), particularly C_6-C_{12} physiologically active fatty acids components in HI larvae, which have been reported to decrease the lipid deposition by enhancing the energy availability and reducing the deposition of adipose tissue (Hashim and Tantibhedyangkul, 1987; Li et al., 2016b). Similar results in terms of MCFA were observed in the HI-supplemented PBM diets and HI larvae meal in this study. In addition, the presence of chitin in HI larvae could modulate the intraperitoneal fat, as suggested by Hossain Blair (2007) who reported reduced body fat content in broiler chickens in response to dietary inclusion of chitin. It has also been reported that cardiac steatosis in the heart and necrosis and myodigeneration in the muscle may also occur due to nutritional deficiency (Courrèges et al., 2003; Rodger et al., 1991). In the present study, there were no histopathological changes in heart and muscle tissue, indicating no negative effects of higher inclusion of PBM aligned with HI supplementation.

As reported in earlier studies, feeding 2.5% maggot meal to black carp, *Mylopharyngodon piceus* modulated the survival rate against *Aeromonas hydrophila* (Ming et al., 2013) and addition of 5% housefly pupae meal protected 100% red sea bream, *Pagrus major* from *Edwardsiella tarda* infection (Ido et al., 2015). In this study, the infection rate of barramundi fed HI supplemented diets was influenced after 14 days challenge to *V. harveyi*. HI larvae was previously reported to improve the gut health of rainbow trout, *Oncorhynchus mykiss* by increasing the abundance of *Carnobacterium* genus, which are well-documented probiotics in salmonids with several

beneficial effects including in vitro growth inhibition of pathogens and in vivo improvement of disease resistance (Bruni et al., 2018). Such modulation of disease resistance following HI supplementation could be explained by the presence of bioactive compounds such as chitin, and antimicrobial peptides as well as MCFA, in particular, lauric acid (Skrivanova et al., 2005). Chitin is reported to hinder the growth of pathogens in fish by boosting the growth of beneficial intestinal bacteria (Karlsen et al., 2017) and lauric acid is also reported to be active against bacteria (Skrivanova et al., 2005). In addition, Elhag et al. (2017) and Park et al. (2015) extracted low molecular weight antimicrobial peptides, exhibiting antibacterial and antifungal activity from HI larvae.

ALT, GLDH, and TB are used as indicators of liver and kidney health in fish and increased levels may indicate cellular damage, characterized by degeneration, necrosis, and destruction of liver and kidney. Although there was no significant impact of diets and challenge on the serum ALT and GLDH levels, a decreasing tendency was observed in HI supplemented diets compared to control. Meanwhile, HI supplemented diets significantly decreased the TB levels of barramundi. These results may suggest that HI supplementation could hamper the negative effects caused by excessive levels of PBM and protect the liver and kidney from cell damage. Similarly, in this study no histopathological changes were observed in the liver, as determined by the histological evaluation. Conversely, it was previously reported that feeding barramundi with PBM (406 and 300 g/kg) replacing FM hampered the liver function by increasing plasma ALT and GLDH (Glencross et al., 2011). The dietary inclusion of APB likewise impaired the liver health of hybrid grouper by increasing the level of ALT and AST (Ye et al., 2019a). The heterogeneity between those studies and the current findings could be due to the presence of chitin or other functional components such as antimicrobial peptide and/or bioactive polysaccharides in HI larvae meal (Chaklader et al., 2019). Chitosan existed in the chitin of HI larvae meal contains cholesterol-lowering properties (known as hypocholesterolemia) in fish (Magalhães et al., 2017; Shiau and Yu, 1999). These polymers have been shown to have hypocholesterolemic effects by binding with lipid (cholesterol) micelles, hindering their absorption, elevating bile acids secretion, and interfering normal lipid digestion and absorption in the intestinal tract as well as biosynthesis of fatty acids in hepatocytes (Gasco et al., 2018; Khoushab and Yamabhai, 2010; Koide, 1998; Xia et al., 2011). Hypocholesterolemic effects were found in European sea bass, *Dicentrarchus labrax* fed 6.5-19.5% HI pre-pupae meal (Magalhães et al., 2017), juvenile mandarin fish, *Siniperca scherzeri* fed 30% yellow mealworm (Sankian et al., 2018) and Jian carp, *Cyprinus carpio* var. Jian fed 68-90% silkworm pupae (Ji et al., 2015), however, such effects were not observed in the present study. Both in pre-challenged and post-challenged conditions, cholesterol level increased

in HI-supplemented-PBM-fed barramundi, suggesting that barramundi could have chitinase activity, which remains to be studied. Moreover, cholesterol levels in diets may influence the level of cholesterol in fish (Li et al., 2017a). The full-fat HI larvae meal used in this study may contain more cholesterol than FM, therefore, resulting in higher cholesterol levels in the HI-supplemented-PBM-fed barramundi. However, the cholesterol levels of fish fed any of the test diets were within the normal range for barramundi. Interestingly, cholesterol levels in the serum of post-challenge HI supplemented groups had significantly lower levels than the pre-challenge HI supplemented groups, demonstrating hypocholesterolemic effects of challenge on barramundi. A similar effect was observed previously in 24 and 72 h post-challenge barramundi in response to *V. harveyi* infection (Chaklader et al., 2020c; Siddik et al., 2019b). Food deprivation has been reported to affect the fish's serum cholesterol which is usually compensated from the body reserves during fasting (Takahashi et al., 2011; Zhu et al., 2014). Hence, lower cholesterol in post-challenged fish could be hypothesised due to the lower feed supply during the challenged condition (Siddik et al., 2019b).

Skin in fish constitutes a large relative surface area when compared to other vertebrates, perhaps due to the fact that there is continuous contact with a variety of unfavourable biotic and abiotic hazards (Sheikhzadeh et al., 2019). Generally, infectious agents start the infection process in the mucous surface (McNeilly et al., 2008), leading to the production of goblet/mucous cells. The goblet cell densities in the skin are influenced by diet and stressors such as bacteria, viruses, and parasites, and therefore the enumeration of the skin goblet cells can be used to monitor the stress level in fish (N Vatsos et al., 2010). Moreover, goblet cells in the skin epidermis secrete mucus, which serves as a repository for a variety of biologically active substances and numerous defensive molecules which have been reported to exert an important role in both the innate and acquired immune systems in fish (Palaksha et al., 2008; Subramanian et al., 2007; Subramanian et al., 2008). In this study, both the thickness of the epidermis and the number of neutral mucins produced by goblet cells in the skin of post-challenge barramundi fed HI-supplemented PBM diets increased significantly. In comparison between pre- and post-challenge, the thickness of the epidermis and the number of goblet cells increased in all post-challenged groups, which suggests that HI larvae supplementation with PBM could boost skin barrier functions in barramundi. No other studies have been conducted on the skin associated neutral mucins of fish when fed HI larvae meal to allow comparison with our present findings. However, HI larvae supplementation with 45PBM significantly influenced the barramundi gut-associated acidic mucins in our previous study (Chaklader et al., 2019) and also resulted in a general increase in the number of mucous cells in

the intestine of Zebrafish, *Danio rerio* when fed with a diet containing 100% HI larvae meal (Vargas et al., 2018).

In teleost fish, serum lysozyme is an indicator of the immune response, protecting fish from infectious disease due to elevated levels decomposing the cell wall of Gram-positive and Gram-negative bacteria (Chaklader et al., 2019; Wu et al., 2019b). Dietary inclusion of 31.9% HI larvae, and 10% of the yellow mealworm, *Tenebrio molitor*, elevated lysozyme activity in the serum of yellow catfish, *Pelteobagrus fulvidraco* (Xiao et al., 2018) and European sea bass, *Dicentrarchus labrax* (Henry et al., 2018), respectively. In our earlier study, barramundi fed 45% PBM supplemented with 10% HI larvae showed elevated serum lysozyme activity at 24 h post-challenge with *V. harveyi* (Chaklader et al., 2019). In line with the previous study, lysozyme activity in response to 24 h post-challenge with *V. harveyi* reported here increased significantly in barramundi fed 60PBM+HI and 75PBM+HI compared to pre-challenge groups. The present findings contradict the results of Ye et al. (2019a), in which increasing levels of animal protein blend impacted the immune function of hybrid grouper, *Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂ by inducing the inflammatory response through upregulating the expression level of the inflammatory cytokines particularly *IL-8* and *IL-10*. Although supplementation of 10% HI larvae with 45%PBM modulated the bactericidal activity in 24h post-challenge groups in our earlier study (Chaklader et al., 2019), the bacterial challenge had no significant effect on bactericidal activity in this study. Elevated lysozyme activity in HI larvae supplemented groups might be due to the immunomodulating capacity of antibacterial peptides, chitin and other components found in HI larvae (Henry et al., 2018; Ido et al., 2015; Lee et al., 2008; Nogales-Mérida et al., 2018).

In teleost fish, the head kidney is an important immune organ participating in a wide array of functions including antigen processing (Dannevig et al., 1994) and the phagocytosis process (Brattgjerd and Evensen, 1996). The spleen is another large blood-filtering organ involved in trapping and processing antigens (Espenes et al., 1995). Hence, maintaining immune homeostasis in the head kidney and spleen is important in fish nutrition. Interleukin 1β (*IL-1β*), an immune-relevant proinflammatory cytokine is expressed early following microbial invasion and can stimulate the immune responses by enhancing different cellular responses such as phagocytosis, chemotaxis and lysozyme synthesis (Goetz et al., 2004; Tang et al., 2014). The expression of *IL-1β* is regulated by the expression of anti-inflammatory cytokines including *IL-10* (Moore et al., 2001). Apart from the beneficial effects of HI-larvae-supplemented diet on serum immunity, relative expression of *IL-1β* in post-challenge groups fed 60PBM+HI and 75PBM+HI decreased significantly compared to pre-challenge groups. In comparison to our findings, pro-inflammatory

cytokines (*IL-1β*, *IL-8*, and *TNF-α*) and anti-inflammatory cytokines (*IL-10*) responded strongly to bacteria mimic in the head kidney leukocytes isolated from Atlantic salmon, *Salmo salar* fed diets containing 66 and 100% of HI larvae meal (Stenberg et al., 2019). In considering anti-inflammatory cytokines in this study, up-regulation in the expression of *IL-10* was observed in the head kidney of HI supplemented groups after 24h post-challenge with *V. harveyi*, which may be associated with the decreased expression of *IL-1β*. The regulatory effect on the expression of *IL-1β by IL-10* has been reported in Indian major carp, *Catla catla* (Swain et al., 2012). *IL-10* plays a central role in suppressing inflammation through the inhibition of the production of pro-inflammatory cytokines (Rahim et al., 2005) and expression is reported to increase in several fish species following LPS stimulation and bacterial and parasitic infections (Chettri et al., 2014; Inoue et al., 2005; Zhang et al., 2005). Therefore, down-regulation of the expression level of *IL-1β* with concurrent up-regulation of *IL-10* at 24h challenge with bacteria indicates an active phase of anti-inflammatory effects and also suggests that HI supplemented diets indirectly intervene in the response of the host to the pathogen by upregulating the expression level of *IL-10*.

5.5 Conclusion

In summary, our research demonstrates that feeding juvenile barramundi with PBM and supplemented HI larvae diets for six weeks resulted in no significant impact on growth performances, biometry indices (except IFI), and also on the integrity of liver, heart, and muscle. The IFI index declined in fish fed HI-supplemented diets as reflected by a decrease in the size of adipocyte cells. In response to two-weeks *V. harveyi* infection, survival rate augmentation in HI-supplemented PBM diets was further supported by a significant increase in serum immunity, skin associated mucins production and controlling inflammatory cytokines in response to 24-h post-challenge with bacteria. Whilst the results are promising, further investigation is needed to decipher the role of chitin, antimicrobial peptides, and/or bioactive polysaccharides in HI larvae in influencing fish health.

CHAPTER 6: Transformation of fish waste protein to *Hermetia illucens* protein improves the efficacy of poultry by-products in culture of juvenile barramundi, *Lates calcarifer*

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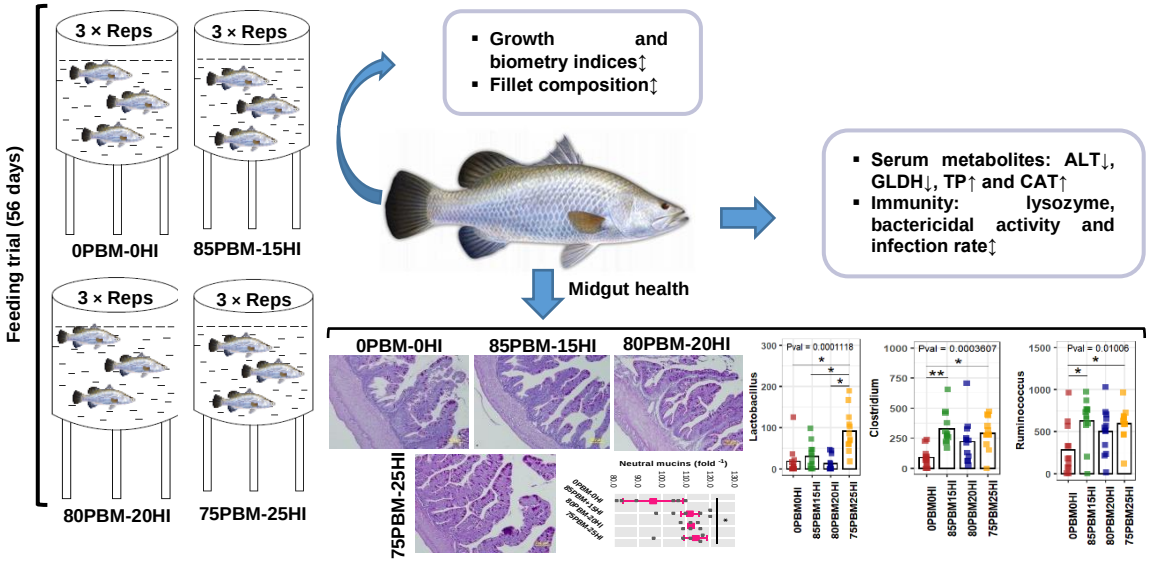
Graphical Abstract



Abstract

Promoting a circular economy via the transformation of food waste into alternative and high value protein sources for aquaculture diets is a novel approach to developing alternative raw materials to fishmeal (FM). This approach can reduce the ecological impact on the aquatic environment and simultaneously can provide an option for sustainable food waste management. In this context, we report a 56-day trial of feeding barramundi, *Lates calcarifer* on four iso-nitrogenous and iso-energetic diets where the control (0PBM-0HI) was a FM-based diet and the other test diets replaced FM protein with mixtures of a poultry by-product meal (PBM) and a full-fat *Hermetia illucens* (HI) larvae meal reared on fish waste: the test diets were 85% PBM + 15% HI (85PBM-15HI), 80% PBM + 20% HI (80PBM-20HI) and 75% PBM + 25% HI (75PBM-25HI). Fish fed PBM-HI-based diets showed an equal growth rate and amino acid profile when compared to the control group. Among all serum metabolites, alanine aminotransferase and glutamate dehydrogenase decreased in fish fed PBM-HI-based diets, whilst total protein levels improved in the same diets. Serum lysozyme and bactericidal activity were unchanged which supported the observation of similar infection rates against *V. harveyi*. Except for kidney and intestine, catalase activity in the serum and liver increased in fish fed PBM-HI-based diets. In assessing the gastrointestinal mucosal morphology, the goblet cells producing neutral mucins were higher in PBM-HI-fed fish than the control. PBM-HI diets also enhanced bacterial richness and diversity, and increased abundance for *Lactobacillus*, *Clostridium*, and *Ruminococcus*. In summary, combining full-fat HI with PBM allowed complete replacement of FM with no negative effects on growth whilst improving gut health. Such diets would be beneficial for the aquaculture industry, both ecologically and economically, as well as providing value adding to animal waste as alternative protein sources for aquafeed production.

Key words: Poultry by-products, black soldier fly larvae, circular economy, microbiota, mucin cells, *Lates calcarifer*

6.1 Introduction

Barramundi, or Asian seabass (*Lates calcarifer*) is one of the most important aquaculture finfish in Australia and Asia with production in combined freshwater and seawater of 76,843 tonnes, valued at USD320 million globally, in 2018 (Simon et al., 2019). Similar to other carnivorous fish, barramundi require a high proportion of dietary protein (450 to 550 g kg^{-1}) (Simon et al., 2019), which has traditionally been satisfied by fishmeal (FM). FM is a nutritionally balanced raw material mainly sourced from the wild capture marine fishery (Chaklader et al., 2020c). The ecological impact of the overexploitation of the marine aquatic environment, combined with an escalated price due to a reduction in FM availability, has driven academia and industry to investigate sources of alternative animal protein. Though research efforts coupled with technological advances have been able to replace FM partially with plant protein sources (Gai et al., 2016; Merrifield et al., 2011), the utilization of land-based plant protein source in this way is under review, due to the competition with production of human food (Gatlin et al., 2007). As well, factors such as depression of growth, immune response and digestibility, and disintegration of different organs have arisen when evaluating the complete replacement of FM with plant protein for barramundi diets (Ilham et al., 2016a; Ilham et al., 2016b; Ilham and Fotedar, 2017; Ilham et al., 2018; Van Vo et al., 2020b; Vo et al., 2020) and for other marine diets (Ye et al., 2019b; Yin et al., 2018), due to a nutritional imbalance in the terrestrial plant protein sources.

Food waste generation and disposal is a critical issue globally, associated with severe environmental degradation. Approximately 1.3 billion tons of edible food is lost or wasted each year (O'Connor et al., 2020) with Australia producing 7.3 million tonnes annually. Seafood industries in Australia contribute100,000 tonnes of waste annually (Peter and Clive, 2006) which require an estimated AUD 15 million per annum for disposal (He et al., 2013). Conventional food waste treatment options such as landfilling, composting, and incineration have been criticized with many detrimental environmental, social, and economic impacts (Xiong et al., 2019). Value-adding to food waste is a sustainable approach which supports aspirations for a circular economy. Although the transformation of food waste into biofertilisers, bioplastics, biofuels, chemicals, and nutraceuticals has been well-described (O'Connor et al., 2020), valorisation of fish waste into insect protein is a novel approach to assist aquaculture industries in achieving their sustainable goal by reducing the inclusion of FM in aquadiets. This approach can also reduce the cost burden for waste disposal by enhancing the value of material flows (Mak et al., 2020) while reducing the stresses on wild capture fisheries to produce FM.

In this regard, *Hermetia illucens* (HI), commonly as known black soldier fly larvae, are an excellent organic waste recycler as they can efficiently and quickly convert low-value organic

waste and by-products to animal biomass. This outcome has a lower net emission of greenhouse gas and a smaller water footprint than other recycling processes (Henry et al., 2015). The final insect biomass is characterized by a high fat and protein content with a favourable amino acid profile, suitable for farmed animal feeds including pig, chicken, and fish (Wang et al., 2019b). On the granting of approval to incorporate insects into diets as per Regulation (EC) No 2017/ of the European Commission, a wide spectrum of studies have investigated the potential of HI larvae meal as a protein source in aquaculture (Henry et al., 2015). However, utilization of full-fat black soldier fly larvae meal in fish nutrition is a less popular approach than using defatted HI larvae meal (Rawski et al., 2020). Whilst defatting may increase protein levels, allowing an increase in the inclusion level of HI larvae meal in aquadiets, defatting may lower the beneficial aspects of using HI larvae meal by imposing additional labour and processing costs, and the possible degradation of amino acids and antimicrobial proteins (Rawski et al., 2020). Furthermore, defatting may decrease the levels of lauric acid, an important fatty acid with antimicrobial activity in HI larvae (Skřivanová et al., 2006; Spranghers et al., 2018), as well as causing an increase in chitin levels. The presence of chitin in high amounts is considered as an antinutritional factor in fish diets. Full-fat HI larvae have already been proven as a functional feed ingredient that complemented and allowed higher inclusion of alternative protein ingredients such as PBM and soybean meal in barramundi (Chaklader et al., 2019; Chaklader et al., 2020b; Chaklader et al., 2021c), Atlantic salmon, *Salmo Salar* (Weththasinghe et al., 2021a) and rainbow trout (Kumar et al., 2020). Further, the partial substitution of FM with full-fat HI larvae improved the antioxidant activity, non-specific immune response, and resistance to the bacterial pathogen in European seabass, *Dicentrarchus labrax* (Abdel-Latif et al., 2021). Hence, HI larvae meal may supplement the lower quality animal-based protein sources such as poultry by-product (PBM) in aquadiets.

Poultry by-product (PBM) is produced from waste of slaughtered poultry, generally clean flesh, skin, head, and feet but excluding feathers and intestine (Dawson et al., 2018). This by-product is a sustainable animal protein source containing a high level of protein, and a favourable amino acid and mineral profile, with lysine, methionine, and phosphorous at higher levels than in plant meals (Simon et al., 2019). These characteristics make the ingredient a valuable alternative protein source for many species, in particular, for marine carnivorous fish. The potential of PBM as a substitute for FM has long been evaluated with various outcomes (Fowler, 1991): complete replacement of FM with PBM without affecting the welfare of juvenile gilthead seabream, *Sparus aurata* (Sabbagh et al., 2019), female tenches, *Tinca tinca* (Panicz et al., 2017), grouper, *Epinephelus fuscoguttatus* (Gunben et al., 2014) and humpback grouper, *Cromileptes altivelis* (Shapawi et al., 2007) has been reported. However, deleterious effects were observed in barramundi (Chaklader et

al., 2020a; Chaklader et al., 2019) and other fish species (González-Rodríguez et al., 2016; Rossi and Davis, 2012; Yigit et al., 2006; Zhou et al., 2011) when fed beyond a certain level of PBM. These contradictory results could be due to palatability issues, different fish species, experimental conditions, and variable nutritional composition from batch to batch and among supplier companies (Chaklader et al., 2019; Galkanda-Arachchige et al., 2020; Lewis et al., 2019). However, recently a number of studies have been conducted to increase the inclusion level of PBM by microbial fermentation or supplementing fish protein hydrolysate, insect larvae or minerals, and subsequently it has been reported that FM can be replaced with PBM completely in barramundi with an aligned improvement in growth, immune response and fillet quality (Chaklader et al., 2019; Chaklader et al., 2020b; Chaklader et al., 2020c; Siddik et al., 2019a; Siddik et al., 2019b; Simon et al., 2019). However, complete replacement of FM with a mixture of PBM and insect meal protein, particularly full-fat black soldier fly larvae, has not been reported.

The morphological study of the gastrointestinal tract (GIT) of fish through a histological approach is a key to understanding fish nutrition in aquaculture. The functionality and integrity of the GIT is largely influenced by dietary protein forms in fish such as barramundi (Chaklader et al., 2020a; Chaklader et al., 2019; Chaklader et al., 2020c; Chaklader et al., 2021c). Extensive studies have been conducted on the morphology of the GIT of livestock species such as poultry, ruminants, and pigs. However, in fish, a detailed morphological and functional characterization of the GIT starting from the stomach to the rectum has yet to be reported. Added to this, the feed ingredients may affect the structure and composition of fish intestinal microbiota. This microbiota can be strongly associated with the digestive functions of fish by producing an extensive range of enzymes, including amylase, cellulase, lipase, proteases, chitinase and phytase, and thereby influence the immune functions of the host (Wang et al., 2018). Recently, HI larvae meal has been shown to enhance the intestinal bacterial community of *Lactobacillales* and *Clostridiales* in rainbow trout, *Oncorhynchus mykiss* (Bruni et al., 2018; Huyben et al., 2019; Terova et al., 2019). The prebiotic effect of HI larvae has also been reported in laying hens (Borrelli et al., 2017).

In this study dietary FM was completely replaced with a mixture of full-fat HI larvae meal and PBM to investigate if this approach could improve the intestinal mucosal barriers and microbiota of fish. It was hypothesised that full-fat HI larvae would prevent the negative effects of complete replacement of FM by PBM on these parameters. The study reports on the growth, fillet amino acid composition, serum metabolites, gut health and immunity of barramundi.

6.2 Materials and Methods

6.2.1 Ethical statement

All experimental protocols for the handling of experimental fish were reviewed and approved by the Animal Ethics Committee of Curtin University (ARE2018-37) in strict accordance with the guidelines and regulations in Australia for the care and use of animals. Water quality parameters and visual observation of animal behaviour were checked daily to ensure optimal rearing condition and fish health and welfare. To minimize pain and discomfort during sampling and culling, a recommended dose of anaesthesia and euthanasia was used as per standard operating procedure (SOP) of Curtin Aquatic Research Laboratory (CARL).

6.2.2 Diets and experimental setup

Full-fat HI larvae meal produced on a mixture of carp mince and grain waste (70:30) were sourced from Future Green Solution, Western Australia whilst PBM was provided by Derby Industries Pty Ltd T/A, Talloman Lot Lakes Rd, Hazelmere WA 6055. The other ingredients were purchased from Specialty Feeds, Glen Forrest Stockfeeders, 3150 Great Eastern Highway, Glen Forrest, Western Australia 6071. Four isonitrogenous and isolipidic diets were formulated. A control diet (0PBM-0HI) containing FM was formulated and the other three diets were formulated by the complete replacement of FM protein with the mixture of full-fat HI larvae meal and PBM: 85%PBM and 15% HI (85PBM-15HI), 80%PBM and 20%HI (80PBM-20HI) and 75%PBM and 25%HI (75PBM-25HI) (Table 6. 1). The mixture of all ingredients, pellet production, drying, and storage were performed as reported by our previous study (Chaklader et al., 2019).

Juvenile barramundi with an average weight of 0.56 g were obtained from the Australian Centre for Applied Aquaculture Research (ACAAR), Fremantle, Australia and transported in plastic bags holding oxygenated seawater. Before the feeding trial began, all barramundi were acclimated for twenty-four days to adapt them to the CARL experimental conditions. Barramundi were then randomly stocked in four dietary experimental groups (75 fish/group,) consisting of triplicate tanks (25 fish/ tank). The tanks (73 × 84 cm) were equipped with a biological filter, heater and aerator. Water quality parameters were checked daily maintaining the values of the salinity, dissolved oxygen, ammonia, nitrite, and water temperature suitable for barramundi culture. The animals were kept under 14:10 h light: dark photoperiod, maintained by an automatic indoor timer (Clipsal, Australia).

Table 6. 1 Feed formulation, nutritional composition and amino acid composition of test diets and ingredients such as PBM and full-fat HI larvae meal.

Ingredients (g/100g)	0PBM-0HI	85PBM-15HI	80PBM-20HI	75PBM-25HI	PBM*	HI*
†FM[a]	72.00	0.00	0.00	0.00	-	-
‡PBM[b]	0.00	60.50	56.00	53.00	-	-
†Canola oil	1.00	3.00	2.00	1.10	-	-
†Full-fat HI[c]	0.00	16.70	23.00	28.70	-	-
†Corn/wheat starch	7.00	8.00	5.90	6.00	-	-
†Lecithin - Soy (70%)	1.00	2.00	2.00	2.00	-	-
†Vitamin C	0.05	0.05	0.05	0.05	-	-
†Dicalcium Phosphate	0.05	0.05	0.05	0.05	-	-
†Wheat (10 CP)	16.90	6.20	7.50	6.40	-	-
†Vitamin and mineral premix	0.50	0.50	0.50	0.50	-	-
†Salt (NaCl)	1.00	1.00	1.00	1.00	-	-
†Cod liver oil	0.50	2.00	2.00	1.20	-	-
Nutritional composition (%)[d]						
Crude protein	47.88	47.76	47.36	47.41		
Crude Lipid	12.59	13.29	13.78	13.41		
Moisture	4.56	4.68	4.53	4.36		
Ash	10.97	11.10	11.25	11.06		
Essential amino acid (% of total amino acid)						
Arginine	6.40	6.94	6.87	6.68	7.32	5.45

Histidine	3.24	2.40	2.46	2.50	2.96	3.27
Threonine	4.71	4.11	4.15	4.15	4.19	4.35
Lysine	7.50	6.53	6.69	6.63	6.58	7.10
Methionine	2.88	2.10	2.12	2.08	2.23	2.02
Valine	5.64	5.38	5.42	5.60	4.92	6.40
Isoleucine	4.91	4.39	4.43	4.50	4.07	4.86
Leucine	8.16	7.45	7.47	7.55	7.37	7.59
Phenylalanine	4.55	4.22	4.25	4.33	4.09	4.67
Non-Essential amino acid (% of total amino acid)						
Serine	4.39	4.25	4.24	4.29	4.34	4.48
Glycine	7.96	10.47	10.23	9.82	10.13	5.86
Aspartic acid	9.57	9.07	9.22	9.23	8.50	10.42
Glutamic acid	14.42	15.09	14.82	14.94	13.83	13.42
Alanine	7.10	7.38	7.40	7.30	6.61	6.59
Proline	5.65	7.09	6.96	6.95	6.63	6.24
Tyrosine	2.92	3.11	3.27	3.44	2.97	5.97

[†]Purchased from Specialty Feeds, Glen Forrest Stockfeeders, 3150 Great Eastern Highway, Glen Forrest, Western Australia 6071.

[‡]Kindly provided by Derby Industries Pty Ltd T/A, Talloman Lot Lakes Rd, Hazelmere WA 6055.

[†]Future green solution, Western Australia

[a]FM: Crude protein (64.00%), crude lipid (10.76%), moisture (11.23) and ash (19.12%)

[b]PBM: Crude protein (65.34%), crude lipid (15.04%), moisture (9.23) and ash (14.04)

[c]Full-fat HI larvae: Crude protein (44.95%), crude lipid (30.21%), moisture (9.23) and ash (10.72)

[d]Nutritional composition of diets and ingredients were analysed as per the standard method of Association of Official Analytical Chemists(AOAC) (AOAC, 1995)

*The amino acid data of PBM and full-fat HI larvae meal were published in our earlier study (Chaklader et al., 2021c) .

6.2.3 Sampling procedure

After 56 days of experimental feeding, fish in each tank were not fed for 24 h then anesthetized before bulk weighing. The number of fish was also counted to calculate the survival rate. For serum collection, four fish/tank were anaesthetized with 8 mg/l of AQUI-S®, and blood was collected by puncturing the caudal vein with a 1ml syringe fitted with a 22G needle, allowed to clot in ice for 4 h, centrifuged to separate serum and stored at - 80° C for further analysis. After collecting blood, fish were culled using euthanasia (175 mg/L) and then total length and weight, as well as wet weight of viscera, liver and intraperitoneal fat, were recorded to calculate the organo-somatic indexes. The same fish were filleted and fillets were freeze dried and stored at -80° C for further amino acid composition analysis. Total gut samples from two fish/tank were immediately preserved in 10% buffered samples for histological analysis. Four distal intestines/tank were stored at -80° C for intestinal microbiota analysis. A section of liver, kidney and intestine were immediately stored at -80° C for antioxidant activity analysis.

6.2.4 Growth and biometry indices calculation

The following equations were used to calculate the growth performance and biometry indices of barramundi at end of 56 days feeding trial-

Weight gain (WG, g) = [(Mean final weight-Mean initial weight)/(Mean initial weight)]

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

Feed intake (FI, g/fish

d^{-1}) = [(Dry diet given-Dry remaining diet recovered)/days of experiment)/ no. of fish]

Feed conversion ratio (FCR) = [(Dry feed fed)/(Wet weight gain)]

Survival (SR, %)=[(Final number of fish)/(Initial number of fish)] ×100

Condition factor (CF, %) = [Final body weight (g)/Body length cm^3)] ×100

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

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

Intraperitoneal fat index (IFI, %)= [Intraperitoneal fat weight (g) /Whole body weight (g)] ×100

6.2.5 Muscle amino acid composition analysis

Amino acid analysis was performed using liquid hydrolysis in 6M HCl to release the amino acids from the protein followed by quantification using pre-column derivatisation reversed-phase (RP) HPLC procedure. The samples were weighed out in duplicate or single into hydrolysis vials and 5 mL of 20% HCl was added. The vials containing samples and HCL were then incubated at 110 ºC for 24hrs. After hydrolysis, the samples were derivatised using AccQTag reagent (Waters Corporation, Milford, MA, USA) (Cohen and Michaud, 1993; Cohen and De Antonis, 1994) and then analysed using a high-resolution RP column on a UPLC system with 10 min run times. The instrument consisted of an ACQUITY UPLC system with UV detector (Waters Corporation, Milford, MA, USA) (Wheat et al., 2008). For all analyses, a Waters AccQTag Ultra column (BEH C18, 2.1 x 100mm; 1.7 µm) was used (Bosch et al., 2006). The column temperature employed was 57 ºC**,** detection was at 260 nm, and the flow rate 0.7 mL/min. This method does not analyse for tryptophan or cysteine which are acid sensitive and require special conditions for analysis.

6.2.6 Serum metabolites analysis

The serum biochemical panel was processed on a AU480 Clinical Chemistry Analyser (Beckman Coulter Australia Pty Ltd, Lane Cove West, NSW). Beckman Coulter clinical chemistry kits were used for the following panel components; albumin (catalogue code OSR6102), alanine aminotransferase (ALT; OSR6007), total bilirubin (OSR6112), calcium (OSR60117), Mg (OSR6189), inorganic phos (OSR6122), total protein (OSR6132), urea (OSR6134), Alb (OSR6102), cholesterol (OSR6116), creatine kinase (CK; OSR6179), creatinine (OSR6178),

gamma-glutamyltransferase (GGT; OSR6020) and iron (OSR6186), while Randox kits (Randox Australia Pty Ltd, Parramatta, NSW) were used for glutamate dehydrogenase (GLDH; GL 441). Separate serum haptoglobin was undertaken using a phase haptoglo-bin assay kit following the manufacturer's instructions (Tridelta Development Ltd, Co. Wicklow, Ireland) (Eckersall et al., 1999).

Lysozyme and bactericidal activity in barramundi serum were conducted following the standard protocol in our laboratory, as previously described in our study (Chaklader et al., 2020b; Le and Fotedar, 2014b).

6.2.7 Catalase activity analysis

Approximately 0.20 mg of liver, kidney, and intestine tissues from each replicate/treatment was weighed, homogenized with 2 mL of chilled PBS, immediately centrifuged at 10,000 ×g for 15 min at 4° C, and supernatant was collected and stored at - 80° C till analysis.

Catalase activity (CAT) in liver, kidney, and intestine homogenate were performed according to the manufacturer's company instruction (Bockit, BIOQUOCHEM SL, 33428 Llanera-Asturias, Spain).

6.2.8 GIT histology

Following fixation, different sections of the gut including stomach, pyloric caeca, anterior intestine, distal intestine, posterior intestine, and rectum were washed and dehydrated using series of alcohol, equilibrated in xylene, embedded in paraffin, sectioned at 5 microns, and stained with periodic acid-Schiff (PAS). Images from different sections were captured by light microscopy (BX40F4, Olympus, Tokyo, Japan). Mucosal morphology in terms of fold height and muscular wall was measured by ImageJ software, and goblet cells producing neutral mucin cell were counted as described by earlier studies (Chaklader et al., 2019; Elia et al., 2018).

6.2.9 DNA extraction and 16S rRNA sequencing

Following the trial, 48 fish, four fish per tank were euthanized, followed by the collection of whole gut and separation of hindgut with a biological safety cabinet. The gut samples in 1.5 ml Eppendorf were preserved immediately at -80 ºC until further processing. The gut samples including contents and mucosa were homogenized in tissue lyserII (Qiagen, Hilden, Germany) with 50 µl DEPC treated water (Thermo Fisher Scientific, USA) and sterile beads, and 250 mg samples were transferred into 1.5 ml Eppendorf for DNA extraction. Bacterial DNA from homogenized tissue samples was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The concentration and purity of extracted DNA were checked in

NanoDrop spectrophotometer 2000c (Thermo Fisher Scientific, USA), followed by dilution into 50 ng/µl even concentration for PCR. The V3V4 bacterial hypervariable regions were amplified with 2µl of template DNA, 1 µl of 0.2 µM forward and reverse primers, 21 µl of nuclease-free water, and 25 µl 2X Hot Start Taq 2X Master Mix (New England BioLabs Inc., Ipswich, MA, USA). 35 cycles of amplification was executed in an EP Gradient Thermal Cycler (Eppendorf, Hamburg, Germany) according to conditions stated in Hot Start Taq 2X Master Mix with 50 °C annealing temperature. Beads clean-up, amplicon barcoding, and pair-end (2 × 300 bp, 600 cycles, v3 chemistry) sequencing of the barcoded amplicons were performed according to Illumina standard protocol for 16S metagenomic sequencing library preparation (Part # 15044223 Rev. B).

6.2.10 Processing and analysing of pair-end sequences

FastQC pipeline (Andrews, 2010) was used to check the initial quality of raw sequences. BBduk (Bushnell, 2014) used for quality trimming and removing of adapter sequences and short reads with the following parameters: qtrim=r, trimq=20, ktrim=r, k=23, mink=11, hdist=1, minlen=200, tpe, tbo. The merging, filtering, deduplicating (fastq-uniques) and picking of amplicon sequence variants (ASVs) were performed using UPARSE and UNOISE3 implemented USEARCH pipeline (Edgar, 2010; 2013; 2016a). Chimeras and low-quality sequences were checked in the final set of ASVs using UCHIME2 (Edgar, 2016b). UNOISE3 workflow was used to map all the merge reads to the non-chimeric ASVs table. The representative ASVs were classified into different taxa levels against SILVA 132 release (Quast et al., 2012). Multiple sequence alignment (MSA) was performed using clustal omega (ClustalO) (Sievers and Higgins, 2014), followed by a rooted phylogenetic tree construction using micca (v1.7.0) root pipeline (Albanese et al., 2015). Each sample was set to an even depth of 12,254 bp, following analysis of alpha-beta diversity and microbial composition in QIIME (v1.9.1) (Kuczynski et al., 2012), microbiomeSeq https://github.com/umerijaz/microbiomeSeq), and phyloseq (McMurdie and Holmes, 2013) R packages. Bray-Curtis dissimilarity of weighted UniFrac metric was calculated as permutational analysis of variance (PERMANOVA) in vegan (Dixon, 2003) and ggplot2 R packages. Statistically significant bacteria at the genus level in different groups were identified using the Kruskall-Wallis test followed by Wilcoxon rank test and Bonferroni correction to avoid false discovery rate (FDR) in rarefied data. A *P*-value of ≤ 0.05 was considered statistically significant at all stages of data analysis. The raw sequence data in fastq format have been deposited to National Centre for Biotechnology Information (NCBI) and currently available under the BioProject accession number PRJNA726745.

6.2.11 Challenge test with *Vibrio harveyi*

10 fish with similar body weight from each tank were taken back to the respective tank and subjected to a challenge test in a similar manner as described in our earlier studies (Chaklader et al., 2020b; Chaklader et al., 2020c) to evaluate the efficacy of the mixture of full-fat HI larvae meal and PBM on the immunity of barramundi. Fish were injected intraperitoneally with 0.1 mL (1.1 x 10^8 cfu per 1 mL) of a pathogenic strain of *V. harveyi*, cultured and provided by Diagnostic and Laboratory Services, Department of Primary Industries and Regional Development (DPIRD), 3 Baron-Hay Court, South Perth WA 6151. A set of clinical signs of Vibriosis including a thick layer of mucous on the body surface, congestion of the fins, and haemorrhages and ulceration of the skin and muscle tissue were monitored twice daily and the trial lasted over 7 days. Fish with Vibriosis were immediately sacrificed with euthanasia following the protocol of CARL SOP.

6.2.12 Statistical analysis

Groups of fish/tank were used as an experimental unit to determine the growth performance whilst individual fish was considered as the experimental unit for serum metabolites, immune response, antioxidant activity, fillet amino acid composition, and intestinal histology. All data were compared by One-way variance analysis (ANOVA) with Dunnett's multiple comparisons test at a significance level of $0.05 < P < 0.001$ after checking the normality and equal variances by Shapiro-Wilk's and Levene's tests. Infection rates in response to the bacterial challenge were compared by the pairwise multiple comparison Log-Rank (Mantel-Cox) test using the Kaplan-Meier method. The results were expressed as mean ± SD (standard deviation).

6.3 Results

6.3.1 Growth, feed utilization and organo-somatic index

Barramundi fed FM-free diets had a similar final body weight (FBW), weight gain (WG) and specific growth rate (SGR) when compared with control fed barramundi (Table 6. 2). Similarly no variation was observed in feed intake (FI) and feed conversion ratio (FCR) among the test diets. Survival rate and biometry indices including CF, VSI, HIS and IFI were similar to the control diet.

Table 6. 2 Growth (n = 3) and somatic-indices (n = 15) from a 56-day feeding trial on barramundi fed iso-nitrogenous and iso-energetic diets that completely replace FM with the mixture of PBM and full-fat HI larvae meal.

	0PBM-0HI	85PBM-15HI	80PBM-20HI	75PBM-25HI
IBW (g)	7.44±0.17	7.23±0.15	7.34±0.23	7.19±0.04
FBW (g)	109.96±6.36	93.26±1.66	93.42±1.78	94.98±3.45
WG (g)	102.52±6.28	86.03±1.80	86.08±1.56	87.79±3.49
SGR (%/d)	4.80±0.09	4.57±0.03	4.54±0.03	4.61±0.05
FI (g/fish d^{-1})	1.96±0.11	1.67±0.03	1.67±0.03	1.70±0.06
FCR	0.94±0.04	0.80±0.05	0.81±0.02	0.81±0.04
SR (%)	90.67±5.81	100.00±0.00	100.00±0.00	97.33±2.67
CF (%)	1.34±0.06	1.38±0.04	1.41±0.08	1.34±0.02
VSI (%)	11.19±0.47	12.02±0.38	12.72±0.79	12.24±0.22
HSI (%)	5.35±0.20	6.14±0.18	5.86±0.54	6.24±0.44
IFI (%)	1.41±0.04	1.57±0.10	1.69±0.10	1.68±0.09

IBW, initial body weight; FBW, final body weight, FBW; weight gain, WG; specific growth rate, SGR; feed intake, FI; feed conversion ratio, FCR; survival rate, SR; condition factor, CF; viecerosomatic index, VSI; hepatosomatic index, HSI and intraperitoneal fat index, IFI.

6.3.2 Fillet proximate and amino acid composition

The effect of PBM-HI based diets on the retention of essential and non-essential amino acid composition of skinless fillet is presented in Table 6. 3. None of the test diets influenced the essential amino acids except for histidine which decreased in 80PBM-20HI and 75PBM-25HI when compared to 0PBM-0HI. Similarly, serine among all non-essential amino acid decreased in the same diet.

Table 6. 3 Amino acid composition (% of total amino acid) of fillet (skinless) from barramundi after 56 days on the mixture of PBM and full-fat HI larvae meal. Values are the average (± standard deviation) of six biological and three technical replicates. Different superscripts letter in the same row indicate significant difference between control (0PBM-0HI) and test diets.

	0PBM-0HI	85PBM-15HI	80PBM-20HI	75PBM-25HI
Essential amino acid (% of total amino acid)				
Arginine	6.30±0.03	6.32±0.05	6.33±0.05	6.28±0.04
Histidine	2.40±0.04^{a}	2.30±0.02^{ab}	2.27±0.03^{b}	2.27±0.03^{b}
Threonine	4.65±0.01	4.63±0.03	4.60±0.01	4.61±0.06
Lysine	9.71±0.02	9.67±0.08	9.67±0.09	9.58±0.14
Methionine	3.22±0.03	3.19±0.03	3.18±0.02	3.18±0.03
Valine	5.37±0.03	5.35±0.02	5.31±0.04	5.33±0.05
Isoleucine	5.12±0.03	5.12±0.02	5.05±0.04	5.08±0.07
Leucine	8.49±0.05	8.55±0.02	8.45±0.03	8.48±0.09
Phenylalanine	4.62±0.04	4.68±0.02	4.65±0.02	4.66±0.05
Total	49.87±0.23	49.81±0.15	49.50±0.23	49.47±0.36
Non-Essential amino acid (% of total amino acid)				
Serine	4.28±0.07^{a}	4.18±0.02^{ab}	4.15±0.03^{b}	4.14±0.04^{b}
Glycine	6.12±0.15	6.37±0.09	6.48±0.22	6.26±0.33
Aspartic acid	10.44±0.08	10.37±0.16	10.41±0.05	10.57±0.16
Glutamic acid	15.97±0.05	16.04±0.12	16.02±0.08	16.13±0.18
Alanine	6.40±0.05	6.36±0.05	6.47±0.07	6.51±0.13
Proline	3.58±0.05	3.55±0.08	3.67±0.09	3.60±0.09
Tyrosine	3.35±0.01	3.34±0.03	3.29±0.07	3.31±0.03
Total	50.14±0.14	50.19±0.15	50.49±0.23	50.53±0.36

6.3.3 Serum metabolites

Liver enzymes including ALT (Figure 6. 1B) and GLDH (Figure 6. 1D) decreased significantly in fish fed PBM-HI based diets, whereas total protein (Figure 6. 1L) level increased significantly in fish fed PBM-HI based diets respect to those fish fed 0PBM-0HI diet. The remaining serum metabolites including CK (Figure 6. 1A), GGT (Figure 6. 1C), TB (Figure 6. 1E), urea (Figure 6. 1F), creatinine (Figure 6. 1G), calcium (Figure 6. 1H), magnesium (Figure 6. 1I), phosphate

(Figure 6. 1J), cholesterol (Figure 6. 1K), albumin (Figure 6. 1M), haptoglobin (Figure 6. 1N), iron (Figure 6. 1O) and AG (Figure 6. 1P) were not influenced by none of the test diets.

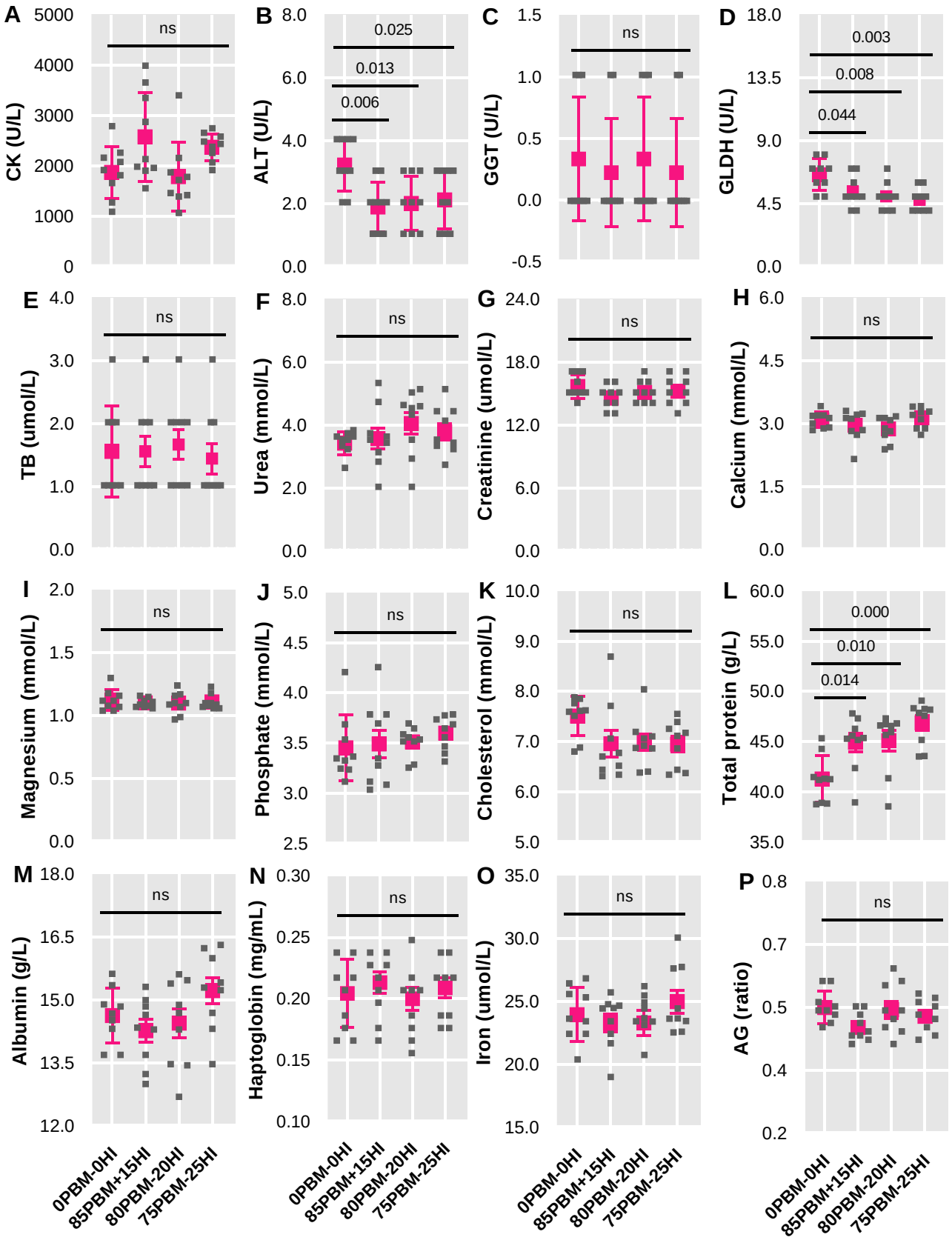


Figure 6. 1 Serum metabolites of barramundi after 56 days fed on the mixture of PBM and full-fat HI larvae meal. The results were demonstrated as mean ± SD (standard deviation) and compared test diets respect to control by one-way ANOVA analysis by followed by Dunnett's multiple comparisons test at $0.05 < P < 0.001$.

6.3.4 Immune response and antioxidant activity

Serum immune response and catalase activity in serum, liver, kidney and intestine are presented in Figure 3. Serum lysozyme (Figure 6. 2A) and bactericidal activity (Figure 6. 2B) did not differ among the test diets. Meanwhile, fish on PBM-HI showed a high catalase activity in serum (Figure 6. 2C) and liver (Figure 6. 2D) whereas same fish showed no differences in kidney (Figure 6. 2E) and intestinal catalase activity (Figure 6. 2F).

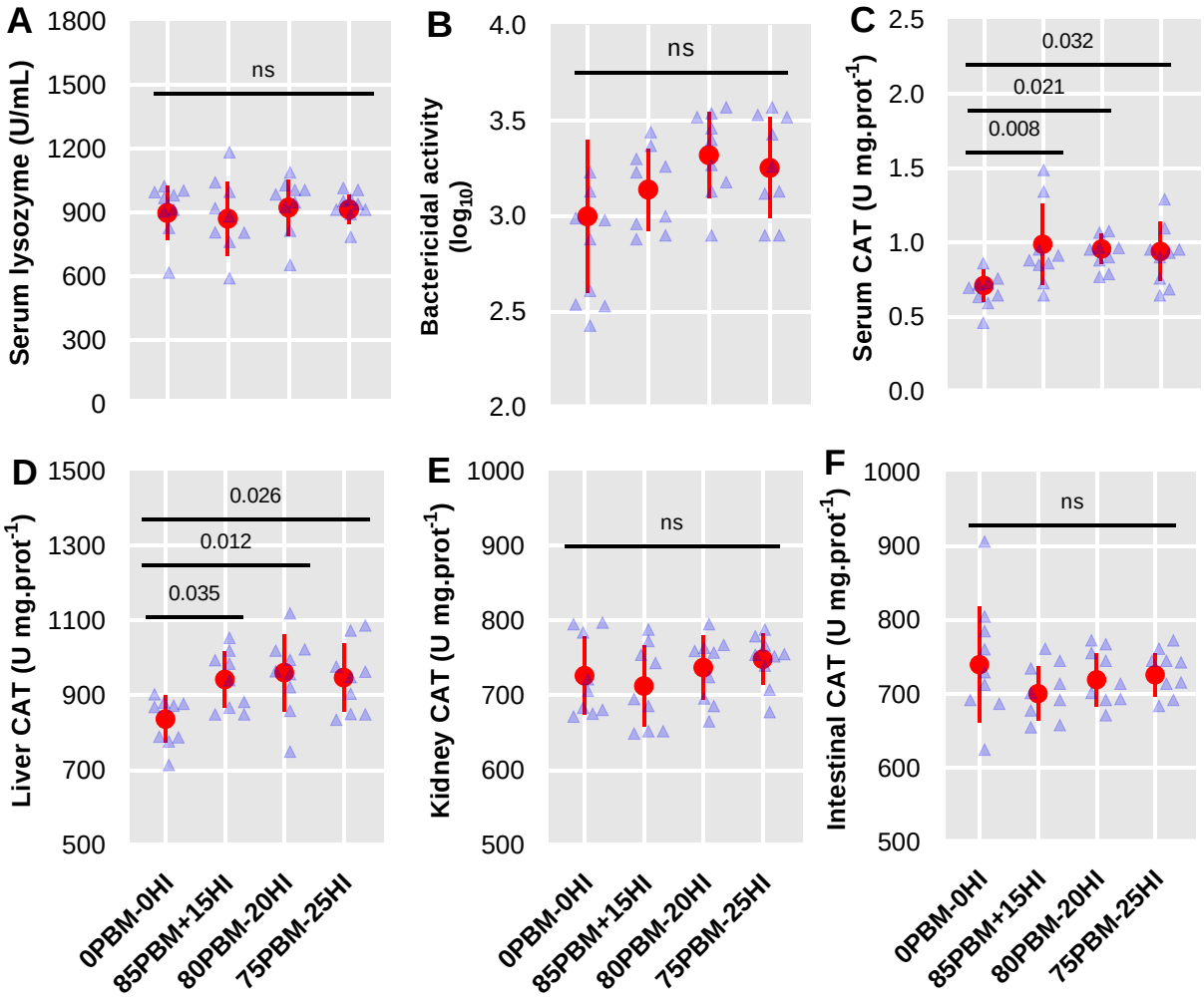


Figure 6. 2 Immune response and antioxidant activity of barramundi after 56 days on the mixture of PBM and full-fat HI larvae meal. The results were demonstrated as mean ± SD (standard deviation) and compared test diets respect to control by one-way ANOVA analysis followed by Dunnett's multiple comparisons test at $0.05 < P < 0.001$.

6.3.5 Gut mucosal barriers

Mucosal morphological changes of different parts of the gut including stomach, pyloric caeca, foregut, midgut, hindgut and rectum of barramundi fed mixture of PBM and full-fat HI larvae meal are presented in Figure 6. 3. The histometry of these tissues presented in Table 6. 4 showed that mucosal morphology were unchanged in all parts of the gut, except for midgut which showed a higher number neutral mucins in barramundi fed PBM-HI based diets.

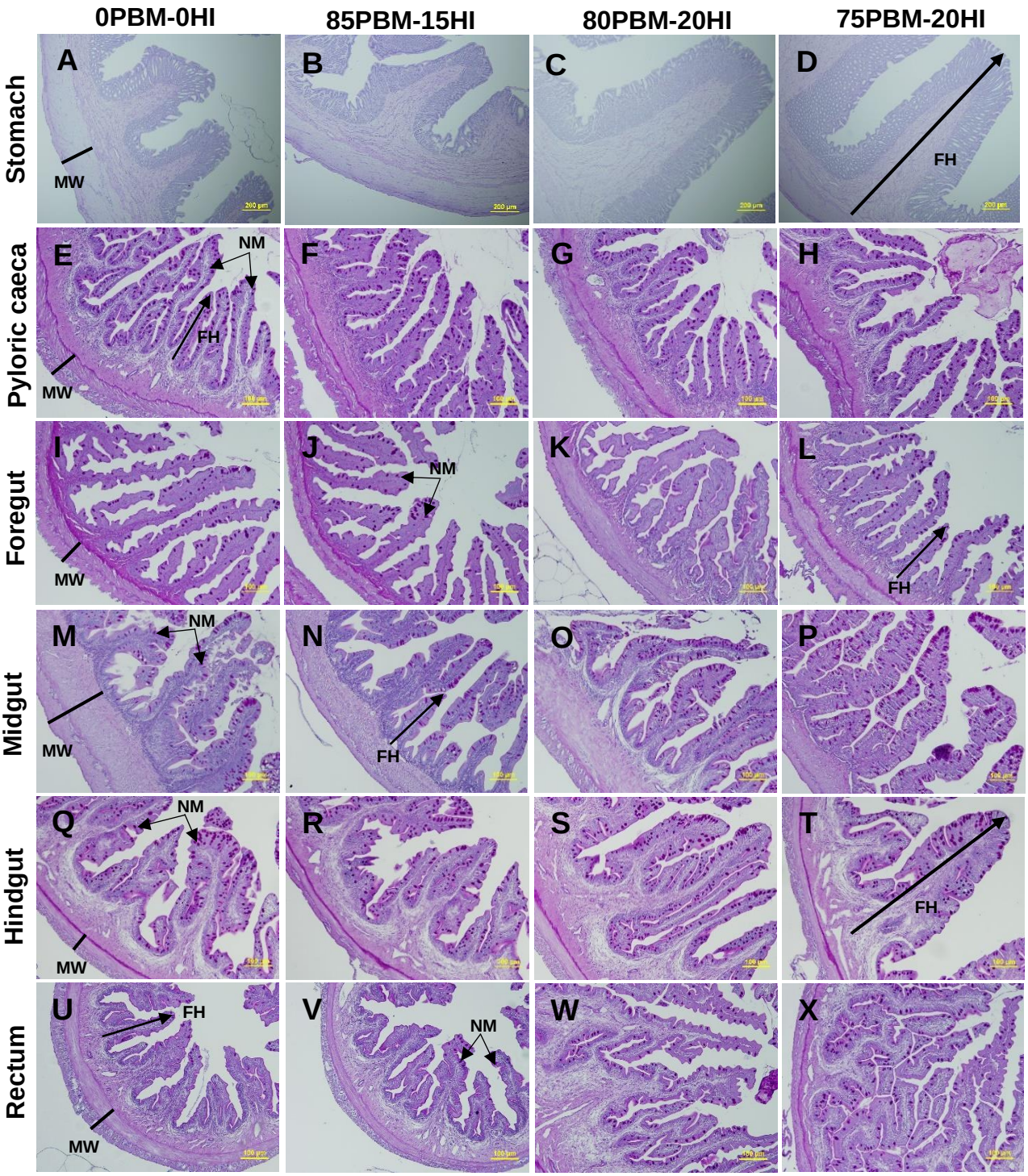


Figure 6. 3 Representative micrograph of GI tract (n = 6) including stomach (A-D), pyloric caeca (E-H), foregut (I-L), midgut (M-P), hindgut (Q-T) and rectum (U-X) of barramundi after 56 days on the mixture of PBM and full-fat HI larvae meal.

Table 6. 4 Histometry of GI tracts including stomach, pyloric caeca, foregut, midgut, hindgut and rectum of barramundi after 56 days on the mixture of PBM and full-fat HI larvae meal. Values are the average (± standard deviation) of six biological and three technical replicates. Different superscripts letter in the same row indicate significant difference between control (0PBM-0HI) and test diets.

	0PBM-0HI	85PBM-15HI	80PBM-20HI	75PBM-25HI
Stomach				
MW (µm)	251.24±5.83	262.02±10.93	248.48±11.25	241.32±6.69
FH (µm)	267.70±9.29	273.97±4.63	259.37±5.55	266.58±6.62
Pyloric caeca				
MW (µm)	88.12±1.44	85.04±1.80	84.23±2.31	87.35±2.51
FH (µm)	265.78±7.64	267.04±10.13	262.24±14.09	261.93±5.46
NM (fold^{-1})	61.33±2.03^{a}	53.33±0.88^{b}	57.67±2.40^{ab}	56.00±1.15^{ab}
Forgut				
MW (µm)	90.04±2.49	86.42±1.27	85.86±3.62	83.30±1.76
FH (µm)	654.70±16.57	621.23±15.00	609.20±4.87	611.93±6.64
NM (fold^{-1})	45.67±2.33	48.67±2.85	47.00±0.58	54.00±0.58
Midgut				
MW (µm)	258.34±3.73	259.86±2.30	261.61±4.73	242.32±5.90
FH (µm)	593.36±9.77	625.19±31.51	603.77±8.23	593.00±3.39
NM (fold^{-1})	93.00±6.25^{b}	131.67±15.90^{ab}	132.00±4.04^{ab}	138.00±2.65^{a}
Hindgut				
MW (µm)	69.84±3.10	62.89±2.21	61.38±2.26	61.13±0.84
FH (µm)	662.33±25.24	662.33±20.37	662.33±23.36	662.33±18.16
NM (fold^{-1})	134.33±6.17	126.67±4.41	135.00±3.21	139.00±5.03
Rectum				
MW (µm)	88.82±4.48	84.21±2.59	81.38±1.45	81.52±1.44
FG (µm)	357.64±10.38	350.51±9.72	330.53±8.26	326.78±3.21
NM (fold^{-1})	25.33±2.03	20.67±1.20	25.67±0.88	24.33±2.03

Muscular wall, MW; fold height, FH and neutral mucins, NM.

6.3.6 Sequence statistics

After filtering, trimming and adapter removal, a total of 2,100,364 high quality reads was obtained from 48 samples, ranging from 22,624 to 81,138. Subsequently, a total of 2,017,658 paired-end sequences were merged (96.1%), generated 6723 ASVs, ranging from 1,871 to 3,288 with UNOISE3 pipeline. The ASVs were assigned into 30 phyla, 236 families and 486 genera. A stationary rarefaction curve (Figure 6S. 1) and an average good's coverage index value of 0.997 indicated that each sample was sequenced at highest depth to capture maximum bacteria at different taxa level.

6.3.7 Alpha-beta diversity

Overall, all three treatment diets significantly improved species diversity, compared to control. However, Shannon and Simpson diversity was enriched in 75PBM25HI indicating higher abundance, richness and evenness of bacterial diversity (Figure 6. 4A). The diet 80PBM20HI generated most ASVs, yet the majority (97.5%) of them are shared by the other three diet groups, having the lowest unshared ASVs among four groups (Figure 6. 4B). Highest unshared ASVs were identified in the 85PBM15HI group, followed by 75PBM25HI (Figure 6. 4B). Beta-ordination NMDS plot showed separate clustering of ASVs for four different treatment groups, indicating that diet had significant impacts on gut microbiota of juvenile barramundi (R^2 = 0.113 and P = 0.003)) (Figure 6. 4C).

6.3.8 Microbial compositions

At phyla level, Proteobacteria (40.5%) was the most dominant bacteria in all groups, followed by Firmicutes (28.5%), Actinobacteria (7.6%) and Verrucomicrobia (4.1%). At genus level, all groups had high abundance of *Escherichia-Shigella* (30.5%). *Ruminococcus* (10.7%), Christensenellaceae R7 group (10.4%), and *Mycobacterium* (8.4%) were also profusely detected in all samples (Figure 6. 4D). A total of 30 genera were found to have at least 1% of read abundance in at least one of the diet groups in the present study (Figure 6S. 2). Differential data with Kruskal-Wallis test followed by Bonferroni correction identified four genera, including *Lactobacillus*, *Ruminococcus*, *Clostridium* and *Escherichia-Shigella* with significantly higher rarefied abundance in the gut of barramundi fed PBM-HI based diets (Figure 6. 5).

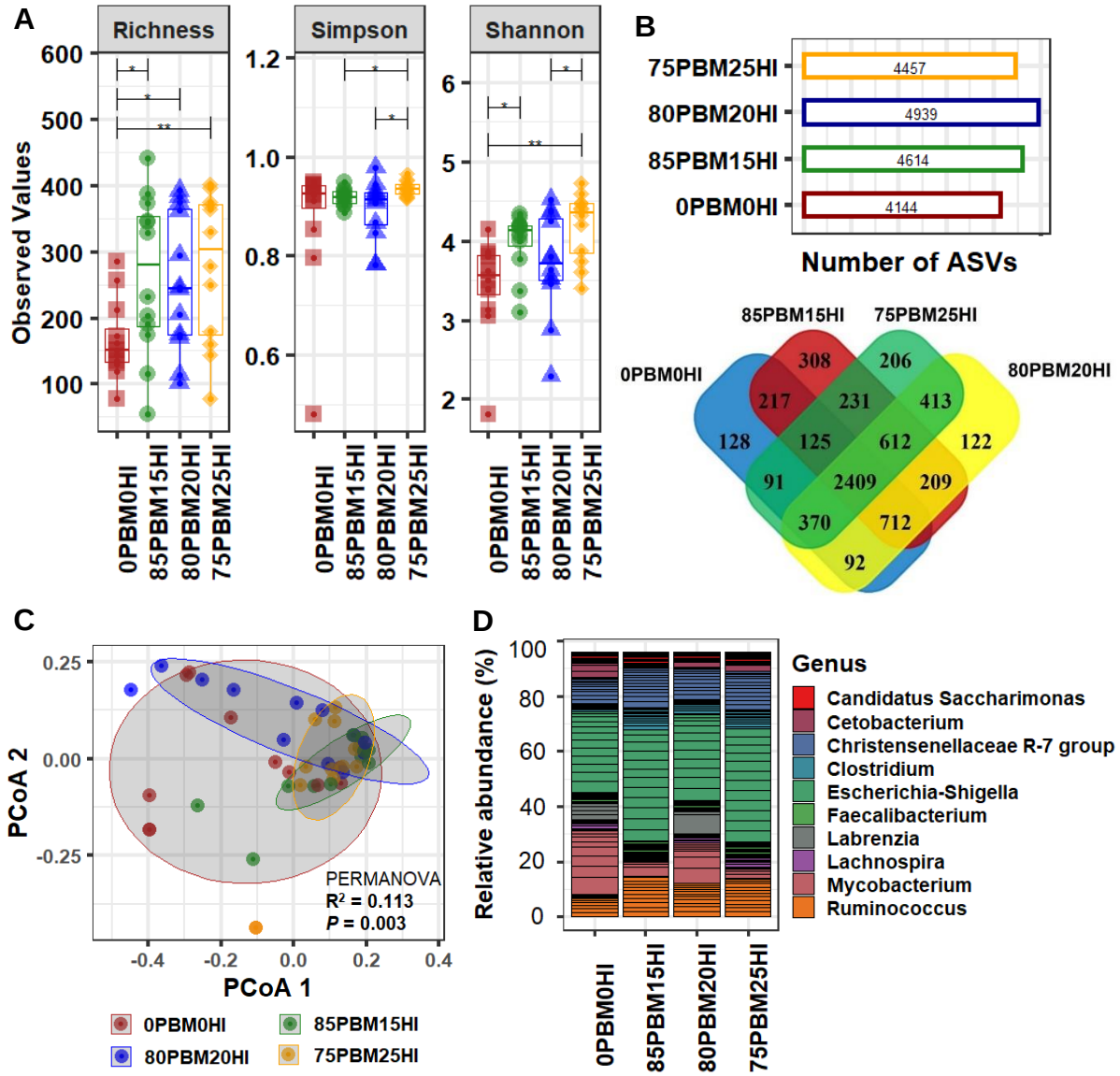


Figure 6. 4 Alpha-beta diversity of gut microbiota of barramundi after 56 days on the mixture of PBM and full-fat HI larvae meal. (A) Alpha diversity measurements in terms of richness, Shannon and Simpson. (B) Number and distribution of ASVs in four different treatments groups including shared and unique ASVs. (C) Beta-ordination in terms of PCoA based on Bray-Curtis dissimilarity of weighted Unifrac matric. (D) Relative abundance of bacteria at genus level (top 10).

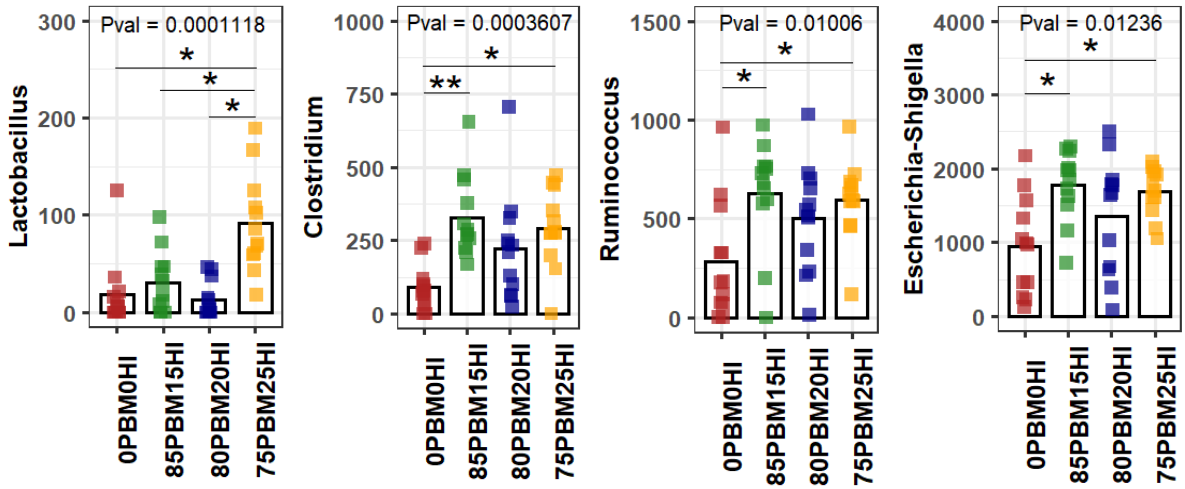


Figure 6. 5 Differential abundance of bacteria at genus level of barramundi after 56 days on four different treatments with Kruskal-Wallis test followed by Bonferroni correction.

6.3.9 Challenge with *V. harveyi*

The infection rate in 85PBM-15HI (45.2%, χ^2 = 3.095 and P = 0.079), 80PBM-20HI (35.3%, χ^2 = 2.110 and P = 0.146) and 75PBM-25HI (30.0%, χ^2 = 0.636 and P = 0.425) did not differ significantly when compared with the control (40.0%) (Figure 6. 6). However, fifty percent of fish were infected in 85PBM-15HI at day 5, 80PBM-20HI and 75PBM-25HI at day 6 and 0PBM-0HI fed fish at day 4.

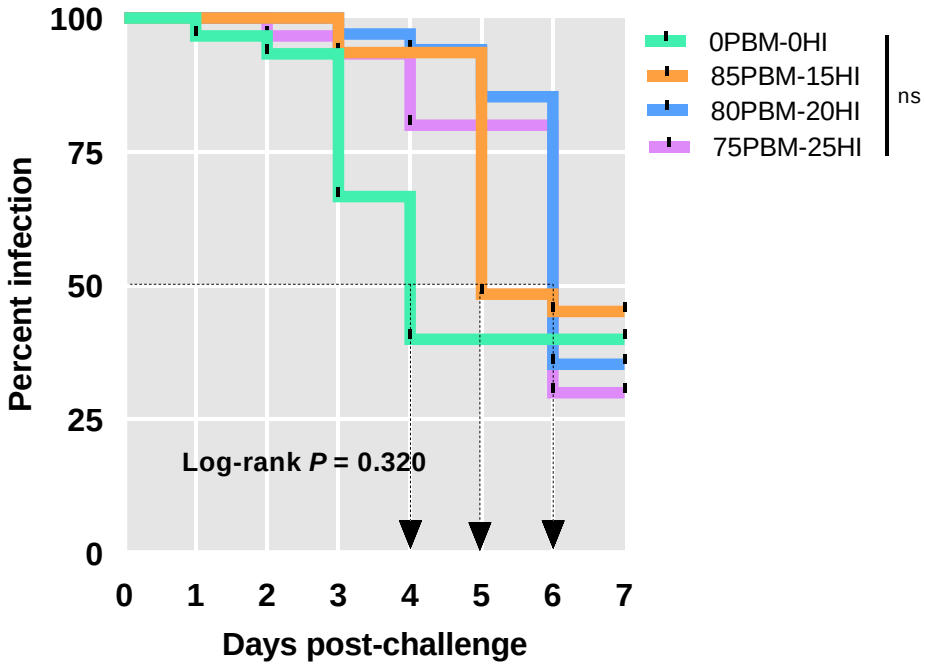


Figure 6. 6 Proportion of asymptomatic fish to *V. harveyi* when fed the mixture of full-fat and partially defatted HI larvae meal and PBM. Dotted line indicates Median survival time when 50%

fish was infected. Multiple comparisons among the test diets were performed by Mantel-Cox log rank test, performed by Kaplan Meier survival method at $p < 0.05$.

6.4 Discussion

6.4.1 Growth of fish and feed utilization

A considerable research effort into barramundi nutrition in the search for alternative protein ingredients has been undertaken in Australia, Indonesia, Malaysia, Philippines, Taiwan, and Thailand, since 1980. Some success has been reported in the total replacement of FM with PBM complemented with fish protein hydrolysates and insect larvae, with an aligned positive effect on growth performance and feed utilization (Chaklader et al., 2019; Chaklader et al., 2020b; Chaklader et al., 2020c; Siddik et al., 2019b). However, PBM alone at 50% or more inclusion in diets may have a deleterious effect on barramundi growth and feed utilization (Chaklader et al., 2020a; Siddik et al., 2019a). The present study recorded comparable growth and feed utilization of barramundi fed a mixture of full-fat HI larvae meal and PBM compared to control diets (Table 6. 2). A complementary effect of 10% full-fat HI larvae meal was reported in our earlier studies where up to 75% FM was replaced by PBM, with no deleterious effect on growth or feed utilization of barramundi (Chaklader et al., 2019; Chaklader et al., 2020b). Similarly, supplementation of 8 and 16% full-fat HI larvae meal prevented the negative effects of elevated levels of plant-based protein in rainbow trout (Kumar et al., 2020). The ameliorative effect of full-fat HI larvae on PBM in the present study (aligned with our earlier studies and that of Kumar) could be due to the presence of functional molecules such as anti-microbial peptides, lauric acid, chitin and novel polysaccharides such as choline, silkose and dipterose. In addition, the presence of low molecular weight peptides in HI larvae ranging mainly between 20 to 245 kDa may suggest a higher proportion of free amino acids, which are considered a fish attractant (Kanost, 2009; Kasumyan, 2016; Mintah et al., 2020; Rawski et al., 2020). These functional molecules may have been degraded by high temperature (Rawski et al., 2020) or other methods applied during the defatting of HI larvae. This requires further study. In support of the current study, no negative impact on growth, feed utilization and FCR has been reported for Siberian sturgeon (*Acipenser bareii*) fed a graded level of full-fat HI larvae (5-30%) at the expense of FM and fish oil (Rawski et al., 2020). However, Siberian sturgeon growth, feed utilization, and digestibility coefficients were affected by the inclusion of defatted HI larvae (Caimi et al., 2020a). The growth and feed utilization outcomes in our study indicate that barramundi may tolerate the total replacement of FM with the mixture of full-fat HI larvae meal and PBM.

6.4.2 Fillet amino acid composition

The total substitution of FM with PBM reduced the retention of most of the essential and non-essential amino acids in barramundi (Siddik et al., 2019a), although not in the fillets of juvenile gilthead seabream, *Sparus aurata* when fed 50 and 100% of PBM. In the latter study only threonine retention decreased (Sabbagh et al., 2019). In our study similar retention of the total essential amino acids were recorded (EAA) (Table 6. 3), implying that barramundi could utilize the amino acid from PBM along with the mixture of full-fat HI larvae meal. The improvement in the retention of fillet EAA could be attributed to the presence of free amino acids in insect haemolymph as well low molecular weight peptides which are considered as a fish attractant.

6.4.3 Serum metabolites

Serum biochemical parameters are an effective tool and an integral part of determining the effect of alternative ingredients on the health status of fish. CK (Figure 6. 1A) activity showed no variation among the fish fed test diets, indicating no damage of smooth and striated muscle (Chen et al., 2003; Glencross et al., 2011) of barramundi fed PBM-HI based diets. A significant decrease in ALT (Figure 6. 1B) and GLDH (Figure 6. 1D) activity together with no changes in GGT (Figure 6. 1C) and TB (Figure 6. 1E) suggested that the mixture of full-fat HI larvae and PBM could replace FM completely without affecting liver, kidney and spleen health. In contrast, complete replacement of FM with PBM alone elevated the level of two liver enzymes (AST and GLDH) and also impaired liver integrity (Chaklader et al., 2020a). The improvement in GLDH activity could be due to the inclusion of HI larvae meal as this has been reported in many studies of the liver enzymes (ALT, GLDH, AST, and ALP) of HI larvae fed fish (Chaklader et al., 2019; Chaklader et al., 2020b; Fawole et al., 2020). Also, fish fed PBM-HI based diets showed a higher level of total protein, implying an enhancement effect of HI larvae on the formation of ribosomes and stimulation of DNA and protein synthesis in liver tissues. This in turn elevated the serum albumin content, which is associated with stronger immunity in fish (Akrami et al., 2015; Hassaan et al., 2019). Likewise, an improvement in the serum total protein of African catfish, *Clarias gariepinus* was observed when fed 25% HI larvae meal, but beyond this level (50 and 75%) the total protein decreased (Fawole et al., 2020). Up to 25% HI larvae meal may provide an adequate level of chitin which may be related to the enhancement of total protein in the present study. The role of insect chitin as an immunopotentiator has been reported in fish and shrimp (Gasco et al., 2018; Motte et al., 2019; Xiao et al., 2018). We observed that the PBM-HI based diets did not influence urea, creatinine, calcium, magnesium, phosphate, total protein, and albumin levels (Figure 6. 1) which is similar to that reported by Glencross et al. (2016) in which complete replacement of FM and fish oil had no impact on those plasma metabolites in barramundi. Also,

complete replacement of FM with PBM resulted in no alteration in urea, creatinine, and total protein level in the serum of barramundi (Chaklader et al., 2020b; Chaklader et al., 2020c).

6.4.4 Immune responses

An evaluation of the effect of HI larvae meal on the immune response of fish is relatively uncommon. The inclusion of full-fat HI larvae enhanced the phagocytic activity, phagocytic index, nitroblue tetrazolium, and lysozyme activity in European seabass juveniles (Abdel-Latif et al., 2021). Similarly, Tippayadara et al. (2021) reported an elevated level of skin mucus lysozyme in Nile tilapia, *Oreochromis niloticus* when fed different levels of full-fat HI larvae (10-100%). However, defatted HI larvae meal did not induce the non-specific immune indices of juvenile Japanese seabass, *Lateolabrax japonicus* (Wang et al., 2019b). These results suggested that full-fat HI larvae meal may work as a functional material in improving the fish immune response. The present study recorded no changes in the serum lysozyme (Figure 6. 2A) and bactericidal activity (Figure 6. 2B) in the mixture of full-fat HI larvae meal and PBM-fed fish. This outcome was not aligned with our earlier studies (Chaklader et al., 2020a; Chaklader et al., 2019) in which the negative effect of complete inclusion of PBM on lysozyme and bactericidal activity of barramundi was reported. The outcomes of the present study imply that full-fat HI larvae had a complementary effect on PBM. Kumar et al. (2020) found an enhanced innate immunity in rainbow trout when fed full-fat HI larvae meal supplemented with soybean meal. Supplementation of 10% full-fat HI larvae meal showed a complementary effect on PBM, manifested by increased level of lysozyme and bactericidal activity (Chaklader et al., 2019; Chaklader et al., 2020b).

6.4.5 Antioxidant capacity

During normal metabolic processes, animal cells generate oxygen reactive species (ROS) such as superoxide anion radical, hydroxyl radical, and hydrogen peroxide (Guo et al., 2020) which are regulated by the activation of antioxidant enzymes such as SOD, CAT, GST, and MDA to maintain cell homeostasis. Dysregulation of ROS production and activation of antioxidant enzymes may cause oxidative stress, leading to an irreversible impairment of DNA causing serious damage to cells and tissues (Modesto and Martinez, 2010; Yonar et al., 2014). CAT has been reported as the first line of ther cellular antioxidant defence system to deter oxidative stress, by participating in the breakdown of hydrogen peroxide to water and oxygen and thus maintaining cellular homeostasis (Sharifinasab et al., 2016; Su et al., 2019). The antioxidant capacity of HI larvae meal has been reported via the elevation of CAT and SOD in African catfish, *Clarias gariepinus* (Fawole et al., 2020), MDA, CAT, and total antioxidant in Jian carp juveniles, *Cyprinus carpio* var. Jian (Li et al., 2017a; Zhou et al., 2018) and SOD in yellow catfish, *Pelteobagrus fulvidraco*

(Xiao et al., 2018). Similarly, an improved CAT activity in the serum (Figure 6. 2C) and liver (Figure 6. 2D) of barramundi fed the mixture of HI larvae meal and PBM was observed in the present study. Since an earlier study reported the impairment of antioxidant activity in barramundi in response to 100PBM (Chaklader et al., 2020a), we infer that HI larvae meal improved the antioxidant capacity in the present study, irrespective of PBM inclusion. In contrast, HI larvae meal has neither positive nor negative effects on SOD, CAT, and MDA in the liver and kidney of rainbow trout (Elia et al., 2018) and CAT, GSH-Px, and SOD activities in serum of Japanese seabass juveniles (Wang et al., 2019b). The mixed results from this group of studies regarding the utilization of HI larvae to maintain the equilibrium between antioxidant enzymes and ROS production could be attributed to different fish species, the inclusion of different stages and doses of HI larvae meal, different processed HI larvae meal, duration of trial and feeding regimes. However, the polysaccharides chitin and low molecular weight peptide (20 to 245 kDa) of HI larvae deserve attention since chitin and derivatives have antioxidative properties together with beneficial scavenging activity against ROS (Khoushab and Yamabhai, 2010; Ngo and Kim, 2014; Ngo et al., 2008; Ngo et al., 2009). The role of low molecular weight bioactive peptides on the antioxidant capacity has already been proven in fish. Nonetheless, the extraction of low molecular weight bioactive peptides from HI larvae and their role in the stimulation of antioxidant capacity is yet to be exploited.

6.4.6 Gut mucosal barrier functions

Total substitution of FM with PBM impaired intestinal integrity of barramundi, proven by the short length and diameter of microvilli in the distal intestine (Chaklader et al., 2020a). However, the observed no change in the integrity of barramundi stomach, pyloric caeca, foregut, and hindgut and rectum (Table 6. 4), whereas the improvement in the number of neutral mucin-producing goblet cells in the distal intestine (Table 6. 4), a major site for the digestion and absorption of macromolecule in most of the fish species (Verdile et al., 2020), such as barramundi (Chaklader et al., 2019) indicated a complementary effect of full-fat HI larvae on previously reported intestinal disruption, caused by exclusive inclusion of PBM (Chaklader et al., 2020a). Similarly, the goblet cells in rainbow trout fed on partially defatted HI larvae (25 and 50%) produced more neutral mucins than acidic mucins (Elia et al., 2018). Also, the beneficial effect of HI larvae meal on gut physiology has already been reported in different farmed fish species, as illustrated in the review of Gasco et al. (2020a). Recently, Weththasinghe et al. (2021a) found a reduction of enterocyte steatosis in pyloric caeca in salmon fed up to12.5% HI larvae meal or 6.7% paste, whilst reporting improvement distal intestine histology when fed up to 25%. The results were aligned with the findings of a recent study where full-fat HI larvae meal and oil prevented soybean meal-induced

inflammation caused by leucocytes in the villi and lamina propria and anti-inflammatory properties of HI larvae were further proven by the lower expression of intestinal proinflammatory cytokines such as *IRF-1/ProstD* (Kumar et al., 2020). The preventive effect of HI larvae on PBM-induced gut disruption in the present study could be ascribed to a rich source of choline in HI larvae, that has been reported to prevent excessive lipid accumulation in the proximal intestine of post-smolt salmon (Hansen et al., 2020; Krogdahl et al., 2020). Besides choline, another possible explanation for the improved intestinal health could be other functional molecules such as antimicrobial peptides, chitin, and lauric acid. In particular, lauric acid has been associated with anti-inflammatory properties, as proven in the intestine of gilthead seabream, *Sparus aurata* (Randazzo et al., 2021a). Further studies are needed to extract functional molecules from HI larvae meal and to determine their possible anti-inflammatory and pro-inflammatory effect on gut health.

6.4.7 Intestinal microbiota

The current study investigated the effects of the mixture of PBM and full-fat HI larvae meal on the gut microbiota of barramundi for the first time using a high-throughput sequencing technique. This method demonstrated the enrichment of intestinal bacterial diversity which may indicate a healthier gut microbiota in barramundi fed PBM-HI based diet (Figure 6. 4A). This is contradicting the findings of Moutinho et al. (2017) who found detrimental effects on the GIT microbial richness in gilthead sea bream, *Sparus aurata* fed high inclusion of animal protein particularly meat and bone meal (75%). The improved bacterial diversity in barramundi fed PBM-HI could be due to the inclusion of HI larvae meal since bacterial diversity improvement has previously been reported in barramundi (Chaklader et al., 2021c) and other fish species such as rainbow trout (Bruni et al., 2018; Huyben et al., 2019; Terova et al., 2019) fed HI based diets. The underlying reason for the gradual increase in the bacterial diversity may be a gradual increase in chitin content, which is not a typical component in commercial diets, and thereby might have stimulated the colonization and growth of less common bacteria to digest chitin as a source of nutrients (Huyben et al., 2019). In particular, HI larvae-based diets were reported to stimulate the abundance of lactobacillus (Terova et al., 2019) which explains an enrichment of lactobacillus (Figure 6. 5) in barramundi-fed PBM-HI-based diets in the present study. The enrichment of lactobacillus is of considerable interest since the reduction of lactobacillus was associated with poor growth or stress in salmonids (Hovda et al., 2012; Huyben et al., 2018; Huyben et al., 2017b). Also, *Lactobacillus* has been reported to produce lactic acid hydrogen peroxide and bacteriocins or biosurfactants that inhibit adherence of pathogens to the intestinal walls (Gudiña et al., 2015; Ringø and Gatesoupe, 1998). Chitin in HI larvae meal has been illustrated as a preferential substrate for lactic acid bacteria (Bruni et al., 2018) that has been proven in the gut of barramundi, and rainbow trout (Bruni et al., 2018; Terova

et al., 2019), and other animals such as egg lying hens (Borrelli et al., 2017). Another important role of adding HI larvae meal is the enhancement of butyrate-producing genus, *Clostridium* in barramundi (Chaklader et al., 2021c), and rainbow trout (Terova et al., 2019) which supported our findings as well (Figure 6. 5). Butyrate, an important short-chain fatty acid is well-documented due to its numerous positive roles such as bio-regulation and promotion of mucosal growth via direct or indirect mechanisms in the gut (Ptak et al., 2015), regulating the immune system together with anti-inflammatory properties (Hamer et al., 2008; Wong et al., 2006), as well as contributing to mineral uptake and providing extra energy to vertebrates (Kulshreshtha et al., 2014). Similarly, HI larvae meal enriched the total SCFAs propionate and butyrate by enriching butyrate-producing bacteria in egg laying hens (Borrelli et al., 2017). The increase of *Ruminococcus* levels (Figure 6. 5) in barramundi-fed PBM-HI-based diets is similar to the findings of our earlier study (Chaklader et al., 2021c) in which PBM-based diet supplemented with 5% tuna hydrolysate and 10% HI larvae increase the abundance of *Ruminococcus*. HI larvae chitin, a complex polysaccharide could be a reason since *Ruminococcus* is abundant in insect-eating carnivores (La Reau et al., 2016), converting polysaccharides into a variety of nutrients for their hosts (La Reau and Suen, 2018).

6.4.8 Resistance to *V. harveyi*

A recent study identified a novel polysaccharide, termed as silkrose from an insect, *Bombyx mori* which improved the survival rate of penaeid prawns such as *Litopenaeus vannamei* and *Marsupenaeus japonicas* by activating the innate immunity (Ali et al., 2018). Similarly, full-fat HI larvae improved the resistance against *Vibrio alginolyticus* in European seabass (Abdel-Latif et al., 2021), *V. harveyi* in barramundi (Chaklader et al., 2019; Chaklader et al., 2020b) and strongly induced the innate immune transcripts in the head kidney of salmon treated with bacterial mimic (lipopolysaccharide) (Stenberg et al., 2019). The positive effect on the immune response of the aforementioned studies could be attributed to the presence of a novel antimicrobial peptide in HI larvae meal that exhibited defense mechanism against Gram-positive and Gram-negative bacteria as well as fungus (Elhag et al., 2017; Park et al., 2015; Park et al., 2014). These studies cannot also overlook the potentiality of compounds in HI larvae meal, such as chitin and lauric acid, playing a critical in enhancing the immune response together with the antimicrobial peptides. Such effects were not found in the present study, proven by a similar infection rate (Figure 6. 6) with no changes in serum lysozyme and bactericidal activity. However, supplementation of full-fat HI larvae meal with soybean meal improved the serum lysozyme and peroxidase activity (Kumar et al., 2020). Hence, further studies are needed to make sure how and which components in HI larvae meal have potential roles in improving the immune response of farmed animals.

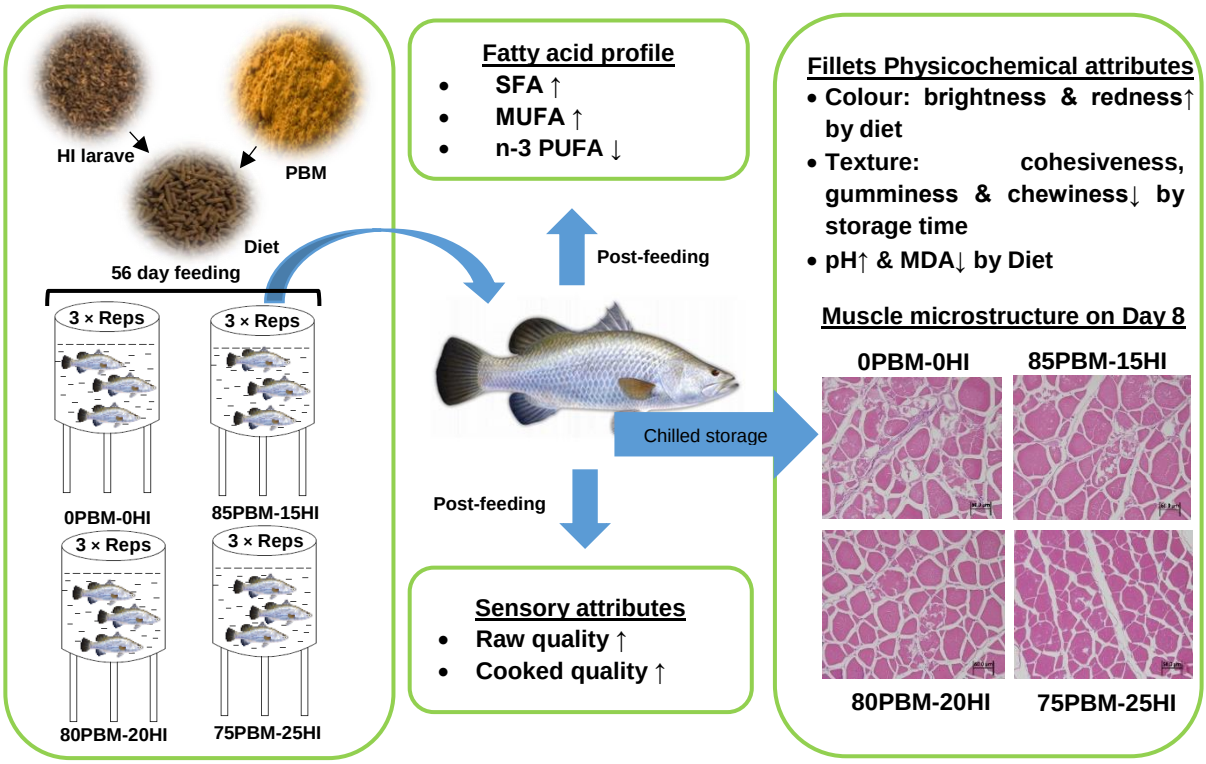
6.5 Conclusion

Barramundi could better utilize nutrients in the diets containing different levels of PBM when mixed with full-fat larvae meal. Growth, feed utilization, and somatic indices were unchanged with no negative effect on muscle amino acid composition. ALT and GLDH as well as total protein and albumin were improved by PBM-HI diets. The mixture of PBM and HI enhanced the antioxidant capacity of barramundi. The mucosal morphology of the GI tracts such as stomach, pyloric caeca, foregut, hindgut and rectum was not affected by PBM-HI-based diets, however, the number of goblet cells producing mucin cells was improved in the midgut. Similarly, the improved midgut health was further supported by the richness of microbiota, in particular stimulating the abundance of lactic acid bacteria and butyrate-producing bacteria. No variation in the infection rate to *V. harveyi* was detected, nor changes in serum lysozyme and bactericidal activity. Thus incorporation of HI larvae meal allowed the complete replacement of FM with PBM in the diet of barramundi, potentiating an easing of the disposal pressure of marine fishing waste, which is attractive from a circular economy perspective.

CHAPTER 7: A combination of *Hermetia illucens* reared on fish waste and poultry by-product meal improves acceptibility and physicochemical quality of aquacultured barramundi fillets

(Under review in Fronteirs in Nutrition)

Graphical Abstract



Abstract

The proximate composition, sensory attributes and shelf life of fillets from barramundi, *Lates calcarifer* were fed a fishmeal (FM) based diet (0PBM-0HI) and three test diets replacing FM protein entirely with 85% poultry by-products meal (PBM) and 15% *Hermetia illucens* (HI) larvae meal protein (85PBM-15HI), 80% PBM and 20% HI larvae meal (80PBM-20HI) and 75% PBM and 25% HI larvae meal (75PBM-25HI) were investigated. After 56-days feeding trial, crude protein, moisture and ash percentage were unchanged while crude lipid increased in barramundi fillet when fed PBM-HI based diets. Saturated fatty acid due to C12:0 (lauric acid) and C14:0 (myristic acid) and monounsaturated fatty acid due to C16:1n7 and C18:1cis+trans increased in the fillet of barramundi fed PBM-HI based diet. Whilst the decrease in total polyunsaturated (PUFA) content in PBM-HI based fed barramundi fillet was mainly due to essential fatty acid including C20:5n3 (eicosapentaenoic acid, EPA) and C22:6n3 (docosahexaenoic acid, DHA) when compared with the 0PBM-0HI fed barramundi fillet. Sensory quality were improved by PBM-HI based diets, manifested by the highest scores given by the panellists. Texture profile were not affected by diet but cohesiveness, gumminess and chewiness decreased with increasing storage time. On day 1 and 8, skin brightness decreased in the skin barramundi fed 85PBM-15HI and 80PBM-20HI compared to skin of 0PBM-0HI fed barramundi. Skin redness improved in fish fed PBM-HI based diets. Flesh brightness and yellowness increased significantly in barramundi when fed PBM-HI based diets. On day 1 and 4, flesh brightness of barramundi fed PBM-HI based diets demonstrated an increases compared to 0PBM-0HI. PBM-HI diets suppress the lipid oxidation while lipid oxidation increased over the storage time. In summary, complete replacement of FM with PBM-HI based diets improved sensory quality, colour and suppressed the lipid oxidation but reduced the synthesis of n-3 PUFA.

Keywords: *Lates calcarifer*, black soldier fly larvae, poultry by-products, Sensory evaluation, fillet brightness, lipid oxidation, shelf life

7.1 Introduction

The rapid expansion of the global aquaculture industry has exceeded the annual growth rate of other animal protein producing industries such as poultry, pork, dairy and beef industry (Troell et al., 2014). However, due to high cost and sustainability issues associated with fishmeal (FM), this resource is progressively being replaced by various plant and animal-based sources to fill the macronutrient void in aquafeed production, an approach also supporting provision of aquaculture sustainability. The high inclusion of plant-based sources featuring low protein content, imbalanced amino acid profile and biologically active antinutritional factors have been reported to stimulate a negative effect on carnivorous fish, such as barramundi, *Lates calcarifer* as demonstrated by a number of studies in our laboratory (Ilham et al., 2016a; Ilham et al., 2016b; Ilham and Fotedar, 2017; Ilham et al., 2018; Van Vo et al., 2015; Van Vo et al., 2020a; Van Vo et al., 2020b; Vo et al., 2020). Barramundi is an important tropical food fish farmed in South East Asian countries and Australia and its 40% annual production come from captive aquaculture production among the total global harvest estimated at around 164,000 t per annum where in Australia, 60% barramundi is produced by aquaculture out of the total national production (FAO, 2012; Jones and Carton, 2015; Skirtun et al., 2014). However, barramundi farming in Australia is heavily dependent on imported conventional protein source, consequently reducing the profitability of barramundi (Williams et al., 2003a). Recent innovations have successfully validated FM free diets for barramundi (Glencross et al., 2016; Simon et al., 2019) however, farmed barramundi of all sizes develop blue-greyish discolouration on the dorsal area of fillet flesh associated with the acceptance of consumers (Howieson et al., 2013). The mixture of poultry by-product and black soldier fly, *Hermetia illucens* (HI) larvae meal on flesh colour and other fillet quality traits beyond the growth and health performance at the exclusion of FM has not been thoroughly investigated.

PBM meal has been considered as one of the animal protein ingredients to replace FM in the diets of carnivorous and omnivorous aquaculture species (Klinger and Naylor, 2012). This is due to high production volume together with cheaper price, high protein, complete amino acid profile, good amount of essential fatty acids, vitamins, minerals and acceptable palatability (Cruz-Suárez et al., 2007; Galkanda-Arachchige et al., 2020; Gunben et al., 2014). Nevertheless, nutritional composition of PBM can vary and often lacks essential fatty acid and amino acid. PBM has been evaluated on numerous finfish and shellfish species with various success (Galkanda-Arachchige et al., 2020). In barramundi, PBM can completely replace FM without compromising growth, serum metabolites, immune response and integrity of various tissues when complemented with fish protein hydrolysate and/or insects larvae (Chaklader et al., 2019; Chaklader et al., 2020b; Chaklader et al., 2020c; Chaklader et al., 2021c). However, the potentiality of an ingredient cannot

be solely evaluated by effects on growth and other physiological and immunological parameters as dietary alteration may stimulate alteration in organoleptic characteristics and other biochemical composition function of fish fillet. Exclusive or complete inclusion of animal protein ingredients such as PBM or meat meal had no effect on the barramundi fillet texture and colour (Chaklader et al., 2021c; Williams et al., 2003b) though female tenches, *Tinca tinca* fillet quality were impacted by PBM (Panicz et al., 2017). Except for fillet organoleptic characteristics, PBM alone reduced the retention of nutrient composition such as essential amino acid and fatty acid for barramundi (Chaklader et al., 2020a; Siddik et al., 2019a), however, such effects were not observed for other species. Nonetheless, the effects that the mixture of HI larvae meal and PBM might have on barramundi fillet organoleptic traits have not yet been evaluated.

Recently, insects have received much attention in the light of circular economy principles being applied to aquaculture (Randazzo et al., 2021b). Besides valorising low-quality by-products or organic waste leaving less environmental footprint (Maiolo et al., 2020; Wang and Shelomi, 2017; Zarantoniello et al., 2020a), HI, one of the promising insects can accumulate good amount of proteins (31-59%) featuring minor deficiency in essential amino acids suitable for omnivorous and carnivorous fish. It is also a good source fat (11-49%), vitamins, minerals and even biologically active compounds such as chitin, antimicrobial peptide and short- and medium-chain fatty acids (SCFAs and MCFAs) (Nogales-Mérida et al., 2018), making this insect as novel alternative protein ingredients. These biologically active compounds have been associated with improved fish health by enhancing innate immune response and modulating the gut microbiota (Bruni et al., 2018; Osimani et al., 2019; Rimoldi et al., 2019; Terova et al., 2019). However, due to lower protein levels, utilization of full-fat HI larvae meal is a less popular approach than the use of defatted HI larvae meal (Rawski et al., 2020). It is worthy to mention that a number of recent studies have proven the ameliorative effect of full-fat HI larvae in PBM and plant protein on health of barramundi (Chaklader et al., 2021a) and rainbow trout (Kumar et al., 2020). Also, replacement of FM with full-fat HI larvae meal alone improved the feed utilization, gut health and immune responses of different fish species. What is more, utilization of full-fat HI larvae meal will reduce the additional cost incurred by defatting process which may degrade the nutritional quality and functional compounds (Rawski et al., 2020). However, evaluating fillet quality traits beyond the health aspects of fish is a research area of high interest while introducing new alternative protein ingredients in aquadiets. It has been reported that fillet qualitative traits of fish were influenced by the HI larvae (Hu et al., 2020), nevertheless, the effect of full-fat HI larvae meal in PBM replacing FM completely on the fillet quality traits has not been investigated till now.

The current study integrated the findings on growth performance and organo-somatic indices, serum metabolites, gastrointestinal mucosal morphology and gut microbiota and immunity of barramundi fed the mixture of full-fat HI larvae and PBM (Chaklader et al., 2021b), by exploring the influence of this mixture of two animal protein sources on the fillet quality traits in terms of fatty acids, texture, colour and muscle structure during shelf life for 8 days.

7.2 Materials and Methods

7.2.1 Ethical statement

Feeding trial and all the protocols conducted at Curtin Aquatic Research Laboratory (CARL) were performed in strict compliance with the guidelines and regulation of Australia and the acts were reviewed and approved by the Curtin University animal Ethics Committee (ARE2018-37).

7.2.2 Diets, fish husbandry, and sampling

The details of diets formulation and fish rearing are reported in our earlier study (Chaklader et al., 2021b). Briefly, four isonitrogenous and isolipidic were formulated: a FM based diet control (0PBM-0HI) diet and three diets with the complete replacement of FM protein with 85PBM and 15% HI larvae meal (85PBM-15HI), 80PBM and 20 HI larvae (80PBM-20HI) and 75PBM and 25 HI larvae (75PBM-25HI). The ingredients and chemical composition of diets are presented in Table 7. 1. After acclimatization with experimental condition, 300 barramundi with 7 g of mean initial weight were stocked into 12 tanks (300 L capacity) with 25 fish per tank. Each diet was assigned in triplicate and lasted for 56 days. Water quality parameters including dissolve oxygen, temperature, salinity, ammonia, nitrite and nitrate and photoperiod were monitored daily and maintained as illustrated in our earlier study (Chaklader et al., 2020c). After 56 days feeding trial, fish were not fed for 24 h and 8 fish/replicate were filleted immediately after stabbing the brain. For the remaining six fish, one side of the fillets were transported immediately post-mortem on sterilized ice to simulated display refrigerator for shelf-life study, while the other fillets were subjected to individual quick freezing (IQF) with liquid nitrogen and stored at -80 ◦C to preserve sensory quality until sensory evaluation.

Table 7. 1 Feed formulation and nutritional composition of control and test diets containing different proportion of PBM and HI larvae meal.

	Experimental diets				Ingredients	
Ingredients (g/100g)	0PBM-0HI	85PBM-15HI	80PBM-20HI	75PBM-25HI	PBM	FHI
FM	72.00	0.00	0.00	0.00	-	-
PBM	0.00	60.50	56.00	53.00	-	-
Canola oil	1.00	3.00	2.00	1.10	-	-
Full-fat HI	0.00	16.70	23.00	28.70	-	-
Corn/wheat starch	7.00	8.00	5.90	6.00	-	-
Lecithin - Soy (70%)	1.00	2.00	2.00	2.00	-	-
Vitamin C	0.05	0.05	0.05	0.05	-	-
Dicalcium Phosphate	0.05	0.05	0.05	0.05	-	-
Wheat (10 CP)	16.90	6.20	7.50	6.40	-	-
Vitamin and mineral premix	0.50	0.50	0.50	0.50	-	-
Salt (NaCl)	1.00	1.00	1.00	1.00	-	-
Cod liver oil	0.50	2.00	2.00	1.20	-	-
Nutritional composition (%)						
Crude protein	47.88	47.76	47.36	47.41	-	-
Crude Lipid	12.59	13.29	13.78	13.41	-	-
Moisture	4.56	4.68	4.53	4.36	-	-
Ash	10.97	11.10	11.25	11.06	-	-
Fatty acid (% of total FA)						

C10:0	0.04	0.32	0.44	0.57	2.31	0.04
C12:0	1.01	7.51	10.42	13.70	43.05	0.09
C14:0	2.32	2.11	2.61	3.04	6.80	0.68
C16:0	20.12	16.55	16.72	16.94	11.11	21.65
C16:1n7	2.80	3.45	3.61	3.70	1.55	5.01
C17:0	1.16	0.31	0.34	0.37	0.53	0.45
C18:0	6.75	5.11	5.05	5.08	3.51	7.07
C18:1cis+trans	20.50	37.23	34.69	31.93	16.05	40.88
C18:2 cis	10.17	16.42	15.33	14.92	12.23	16.09
C18:3n3	2.22	3.91	3.61	3.34	1.65	2.41
C20:1	1.41	1.57	1.56	1.21	0.09	0.56
C20:4n6	1.95	0.77	0.77	0.77	0.05	1.67
C20:5n3	3.66	1.07	1.20	1.12	0.05	0.16
C22:4n6#	1.80	0.08	0.07	0.07	0.00	0.04
C22:5n3#	1.13	0.34	0.35	0.28	0.01	0.34
C22:6n3	19.39	1.35	1.25	0.88	0.01	0.25
SFA	33.12	32.55	36.20	40.34	67.86	31.00
MUFA	25.47	42.65	40.26	37.17	17.92	46.88
PUFA	41.58	24.97	23.69	22.60	14.23	22.18
n-3 PUFA	26.95	7.04	6.86	6.16	1.75	3.33
n-6 PUFA	4.05	1.09	1.08	1.15	0.15	2.45

[†]Includes also C8:0, C10:0, C11:0, C13:0, C15:0, C17:0, C20:0, C21:0, C22:0, C23:0 and C24:0

‡Includes also C14:1n5, C15:1, C17:1, C22:1n9 and C24:1

ǂIncludes also C18:2 trans, C18:3n6, C18:4n3, C20:2, C20:3n6, C20:3n3, C22:2

*Includes also C18:4n3 and C20:3n3

†Includes also C18:3n6, C20:3n6

7.2.3 Proximate composition analysis

Crude protein, crude fat, ash and moisture were analysed following AOAC (1995) standard methods. For protein analysis, 1 g of sample, a boiling chip and a Kjeldhal catalyst tablet were placed in a digestion tube. Then, 8 mL of sulfuric acid-phosphoric acid mixture along with 4 mL of 35 % hydrogen peroxide were added to the tube and digestion completed using Kjeltec digester block (Foss Tecator 2020, Högänas, Sweden) at 420 ˚C. After distilling the digested samples using Kjeltec distilling unit (Foss Tecator 1002, Högänas, Sweden), distillate was collected into a flask with 25 mL of boric acid indicator containing bromocresol green and methyl red which was subjected to titration with hydrochloric acid and conversion factor of 6.25 was used to calculate crude protein content.

Crude fat content was determined using petroleum ether extraction method where fat from samples were extracted using the Soxhlet unit (Extraction unit E-816, BÜCHI Labortechnik AG, Flawil, Switzerland). Extracted fat was then dried at 105 ˚C till constant weight was obtained and crude fat percentage was calculated by dividing weight of extracted fat over sample weight.

For ash content determination, sample was weighed before and after heating overnight at 550 ˚C using a muffle furnace (Thermolyne muffle furnace, model 48000, Thermo Fisher Scientific Inc, Iowa, USA). Ash content was calculated by dividing post-heating sample weight over initial sample weight.

For moisture estimation, sample was weighed before and after oven drying at 105 ˚C until constant weight was obtained. Moisture content was then calculated by dividing post-drying weight over pre-drying weight.

7.2.4 Fatty acid analysis

Fatty acid of experimental diets and whole freeze-dried fillet (skinless) were analysed following the protocol of O'Fallon et al. (2007) as reported in our earlier study (Chaklader et al., 2020a).

7.2.5 Sensory quality

All the procedure related sensory trial was carried out in strict compliance with the Australian Code for the Responsible Conduct of Research and National Statement on Ethical Conduct in Human Research, and reviewed and approved by the Curtin University Human Research Ethic Committee (Approval number: HRE2020-0689).

Sensory quality of barramundi fillets at the end of the 56 days feeding trial was designated and evaluated following the method described by Gedarawatte et al. (2020) and Lawless Heymann (2010). 11 persons with no allergies, smoking habit, chronic health issues, visual impairment, respiratory issues, taste disorders, pregnant condition, not breast-feeding or on long-term medication, and who consume fish at least once every fortnight, were recruited, consented, and trained according to AS 2542.1.3:2014 (Australia, 2014) and CAC-GL 31-1999 (Alimentarius, 1991). Before starting sensory evaluation, screening on sensory sensitivity was conducted using multiple fish fillets with various quality. Three of the potential panellists were excluded following due to a low precision in determining fish quality after repeat exposures. After that, 9 participants in the age of 18 – 50, consist of 5 females and 4 males were screened and considered to be eligible and included in the study as semi-trained panellists. A labelled magnitude scale (Kalva et al., 2014) was used to evaluate the visual appearance, odour, and overall acceptability of the raw barramundi samples in order to understand the quality of barramundi in retail display.

Then, same samples were sous-vided at 74 ˚C for 6 min in vacuum-sealed boil-in pouches and served to panellists within 15 min to evaluate the appearance, odour, texture, taste and overall acceptability of cooked samples in similar manner to raw samples.

7.2.6 Simulated Retail display for shelf-life studies

Freshly filleted fish samples were immediately placed on ice inside an uncovered polystyrene box. The box was then placed in a 4 ˚C refrigerator to simulate condition in retail display. Melted ice was drained daily with new ice replaced. Six fillets from different fishes were analysed per treatment during each day on Day 1, 4, and 8 post-filleting.

7.2.6.1 Physical parameters

7.2.6.1.1 Texture profile analysis (TPA)

Prior to texture analysis, fish fillets were tempered at 24.5 ˚C for 30 mins. For each quality, A 5 × 5 cm fish cube sampled between the pelvic and anal fin portion along lateral line including dorsal and ventral portion, was then compressed using texture analyzer TVT 6700 (PerkinElmer, Inc., Waltham, Middx, USA) equipped with a 20 kg load cell and a 25 mm flat-ended cylindrical probe. Two consecutive cycles of 50 % compression with 5 sec in between were conducted under constant speed of 50 mm/min. Six texture parameters: hardness, cohesiveness, adhesiveness, springiness, gumminess, and chewiness were obtained from each analysis using Bourne (1978) calculation methods and TexCal 5.0 instrumental software.

7.2.6.1.2 Microscopic observation of fillet tissues

One portion of muscle at day 1, day 4 and day 8 stored at 0˚ C were cut from three fillets/treatment and immediately fixed in 10% buffered formalin before dehydrating with a series of alcohol. Then the samples were embedded in paraffin, sectioned to 5 µm thickness and stained with hematoxylin and Eosin (H & E) for observation under light microscope according to the standard histological procedure.

7.2.6.1.3 Drip loss

Drip loss was calculated from day 0 to day 8 by dividing weight loss over initial weight of fresh fish samples and expressed as a percentage.

7.2.6.1.4 Colour

Prior to colour measurement, HunterLab ColorFlex colourimeter (Hunter Association Laboratory Inc., Reston, VA, US) was calibrated using manufacturer standards. Surface colour coordinates (L*, a*, b*) were then obtained from portions along the lateral fillet line.

7.2.6.1.5 Quality Index (QI)

QI of barramundi fillets were determined following the method described by Fuentes-Amaya et al. (2016). In brief, skin, appearance and flesh of the fish were scored out of 10 based on quality parameters such as brightness, transparency, texture, blood colour, odour, and gaping.

7.2.6.2 Chemical parameters

7.2.6.2.1 pH

Approximately 1g of muscle tissue was homogenized with 10 mL of distilled water, mixed using a rotary suspension mixer (Ratek, Boronia, Vic, AU) for 30 min and pH values of the aliquot were determined using a three-scales calibrated Aqua-pH meter (TPS Pty, Ltd, Brendale, QLD, AU) (AOAC, 1995).

7.2.6.2.2 Lipid oxidation

Lipid oxidation was determined using 2-thiobarbituric acid reactive substances (TBARS) following the method of Raharjo et al. (1992) with some modifications. 10 g of muscle tissue were added to 40 mL of 5 % trichloroacetic acid (TCA) along with 1 mL of 0.15 % 2,6-di-teri-butil-4-methylphenol (BHT) in ethanol. The mixture was homogenized, filtered and adjusted with 5 % TCA to 50 mL. After which, 2 mL of 0.08 M 2-thiobarbutric acid (TBA) was added to 2 mL of sample in a screw cap test tube, heated at 100 ˚C for 10 min. Absorbance was then measured at 532 nm and concentration was calculated using standards prepared with 1,1,3,3-tetrathyoxypropane (TEP) in 20 % TAC at 1-10 µM.

7.2.7 Statistical analysis

All results are presented as mean ± standard error (SE). In order to determine the effect of diet and storage, two-way ANOVA was performed with tukey multiple comparison test if the data met normality and homogeneity of variance, checked by Kolmogorov-Smirnov and Levene's tests, respectively. If any of the factors was significant, one-way ANOVA was performed individually to compare the data among the diets and storage time. Data on proximate composition and fatty acid was compared using one-way ANOVA with tukey multiple comparison test. Variations were considered as significant at $0.05 < P < 0.001$.

7.3 Results

7.3.1 Fillet proximate composition and fatty acid profile

Proximate composition of barramundi fillet fed PBM-HI were similar to the 0PBM-0HI fed barramundi fillet, except for crude lipid which increased in the fillet of barramundi fed PBM-HI based diets (Table 7. 2). Total saturated fatty acid (SFA) increased significantly due to gradual increase in C12:0 (lauric acid) and C14:0 (myristic acid) in the fillet of barramundi fed PBM-HI based diets. A gradual increase of HI larvae meal in the diets also elevated the level C12:0 and C14:0 in the fillets from the respective diets. An elevation of C16:1n7 and C18:1cis+trans concentration resulted an increase the total concentration of monounsaturated fatty acid (MUFA) in the fillet of barramundi fed PBM-HI based diets. Lower concentration of total n-3 polyunsaturated fatty acid (PUFA) and n-6 PUFA, particularly, C20:5n3, C22:5n3, C22:6n3, C20:4n6 and C22:4n6 decreased the total PUFA in PBM-HI fed barramundi fillets. A similar tendency was observed in the respective diets (Table 7. 2).

Table 7. 2 Fillet proximate composition and fatty acid profile (% of total FA) of barramundi fed the mixture of PBM and full-fat black soldier fly larvae

	Experimental diets			
	0PBM-0HI	85PBM-15HI	80PBM-20HI	75PBM-25HI
Proximate composition (%, Wet basis)				
Moisture	76.10±0.25	76.23±0.35	75.57±0.05	76.40±0.35
Crude Protein	20.58±0.56	20.04±0.24	20.23±0.27	20.38±0.39
Crude lipid	1.77±0.15^{b}	2.04±0.35^{ab}	2.25±0.05^{a}	2.86±0.61^{ab}
Ash	1.10±0.07	1.12±0.05	1.17±0.03	1.07±0.02
Fatty acid (% of total fatty acid)				
C12:0	1.39±0.84^{c}	4.43±0.16^{b}	6.67±0.14^{a}	8.65±0.22^{a}
C14:0	2.30±0.14^{c}	2.28±0.03^{c}	2.87±0.04^{b}	3.47±0.06^{a}
C16:0	19.26±0.24^{a}	17.51±0.08^{b}	17.60±0.29^{b}	17.69±0.02^{b}
C16:1n7	3.35±0.15^{c}	3.64±0.05^{bc}	3.94±0.04^{ab}	4.17±0.04^{a}
C18:0	6.39±0.13^{a}	5.51±0.12^{b}	5.21±0.10^{b}	5.25±0.03^{b}
C18:1cis+trans	25.53±1.57^{b}	36.64±0.11^{a}	35.05±0.17^{a}	33.32±0.10^{a}
C18:2 cis	9.59±0.81^{b}	15.44±0.09^{a}	14.72±0.03^{a}	14.33±0.14^{a}
C18:3n3	2.00±0.12^{c}	3.33±0.06^{a}	3.21±0.44^{ab}	3.00±0.02^{b}
C20:1	1.20±0.03^{b}	1.38±0.01^{a}	1.40±0.00^{a}	1.13±0.02^{b}
C20:4n6	1.93±0.06^{a}	1.48±0.09^{b}	1.31±0.05^{b}	1.31±0.08^{b}

C20:5n3	2.51±0.20[a]	1.22±0.03[b]	1.35±0.04[b]	1.30±0.06[b]
C22:4n6	1.58±0.24[a]	0.16±0.01[b]	0.14±0.01[b]	0.14±0.01[b]
C22:5n3	1.71±0.09[a]	0.98±0.05[b]	0.95±0.04[b]	0.95±0.03[b]
C22:6n3	17.38±2.66[a]	2.99±0.18[b]	2.52±0.14[b]	1.86±0.10[b]
SFA[†]	31.20±0.56[b]	30.73±0.18[b]	33.40±0.31[a]	36.20±0.32[a]
MUFA[‡]	30.57±1.64[b]	42.03±0.13[a]	40.77±0.20[a]	39.00±0.12[a]
PUFA[ɻ]	38.37±2.20[a]	27.37±0.23[b]	25.97±0.35[b]	24.93±0.35[b]
n-3 PUFA[*]	24.10±2.81[a]	9.03±0.18[b]	8.63±0.23[b]	7.77±0.17[b]
n-6 PUFA[ŕ]	4.27±0.19[a]	2.60±0.06[b]	2.30±0.12[b]	2.53±0.09[b]

[†]Includes also C8:0, C10:0, C11:0, C13:0, C15:0, C17:0, C20:0, C21:0, C22:0, C23:0 and C24:0

[‡]Includes also C14:1n5, C15:1, C17:1, C22:1n9 and C24:1

[ɻ]Includes also C18:2 trans, C18:3n6, C18:4n3, C20:2, C20:3n6, C20:3n3, C22:2

[*]Includes also C18:4n3 and C20:3n3

[ŕ]Includes also C18:3n6, C20:3n6

7.3.2 Sensory attributes

Sensory evaluation of raw and cooked flesh of barramundi fed fishmeal-free diets containing the different proportion of PBM and HI larvae meal for 56 days is presented in Table 7. 3. PBM-HI based diets improved the raw fillet of barramundi, manifested by higher scores given by panellists for visual appearance, odour and overall quality. Compared to 0PBM-0HI, cooked odour was better in the fillet of barramundi fed PBM-HI based diets. Cooked texture and taste were unchanged by test diets, whereas cooked overall quality was better in the fillet of barramundi fed PBM-HI based diets when compared to the fillet of 0PBM-0HI fed barramundi.

Table 7. 3 Sensory quality of raw and cooked fillet of barramundi fed PBM and HI larvae meal replacing FM entirely for 8 weeks. Means are average of six biological replicates ± standard error (SE). Different superscript letters in the same row indicate significant differences while mean holding no superscript letters indicate no variation between 0PBM-0HI and test diets. Means were compared by one-way ANOVA with Dunnett's multiple comparisons test at $0.05 < P < 0.001$.

	Experimental diets			
	0PBM-0HI	85PBM-15HI	80PBM-20HI	75PBM-25HI
Raw visual appearance	3.64±0.41[b]	6.01±0.65[a]	5.07±0.48[ab]	6.55±0.55[a]
Raw odour	3.56±0.29[b]	6.60±0.46[a]	5.63±0.60[a]	6.37±0.71[a]
Raw overall quality	3.67±0.37[b]	6.04±0.63[a]	5.46±0.34[ab]	5.99±0.62[a]
Cooked visual appearance	3.93±0.58[b]	5.19±0.48[ab]	6.24±0.46[a]	5.99±0.71[ab]

Cooked odour	3.55±0.68[b]	6.14±0.49[a]	5.77±0.54[a]	6.23±0.49[a]
Cooked texture	4.90±0.61	5.24±0.31	5.94±0.61	6.02±0.69
Cooked taste	3.99±0.76	4.81±0.73	5.39±0.58	5.94±0.64
Cooked overall quality	3.48±0.72[b]	5.49±0.82[ab]	5.57±0.49[ab]	6.23±0.61[a]

7.3.3 Drip loss

Diet had no influence drip loss whilst storage time had significant effect on drip loss (Figure 7. 1). The percent of drip loss increased significantly over the storage time irrespective of diet. There were no interaction between diet and storage time.

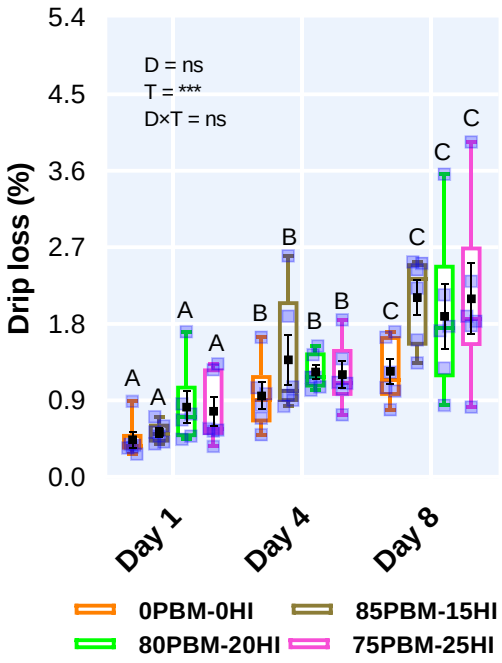


Figure 7. 1 Drip loss of barramundi fillet after 56 days of feeding PBM and HI based diets during chilled storage. Violin plot indicates the range and round shape marker indicates the number biological replicates used for analysis. Square shape back marker denotes the mean with standard error. The effect of factors and their interaction was conducted by two-way ANOVA.

7.3.4 Texture and colour of barramundi fillet

None of the texture profiles were influenced by the diet whereas storage time significantly influenced the cohesiveness, gumminess, and chewiness, as demonstrated by two-way ANOVA analysis (Table 7. 4). Cohesiveness, gumminess, and chewiness decreased in all test diets over the storage time. There were no interaction found between diet and storage time the texture profiles. Skin brightness and redness were significantly affected by diet. Brightness in the skin of barramundi fed 85PBM-15HI and 80PBM-20HI decreased significantly on Day 1 and 8. On Day 1, skin redness changed significantly in barramundi when fed PBM-HI based diets. Regardless of diet, skin redness over the 8 days storage time decreased. A similar change in the yellowness and chroma of skin was found over the storage time, though diet did not change the skin yellowness and chroma. Fillet brightness in response to test diets and storage time increased while flesh redness was not affected by diet and storage time. Flesh yellowness was increased by diet and storage time. Skin chroma was unaffected by diet and storage time. The micro-structure of muscle tissues in response to diets and over the storage time is presented in Figure 7. 2. On Day 1 (Figure 7. 2A-D) and 4 (Figure 7. 2E-H), muscle micro-structure were unchanged, supported by tight attachment and regular shape of myofibrils together with a uniform distribution and distinct connective tissues. However, on day 8, the muscle tissue of barramundi fed on 0PBM-0HI (Figure 7. 2I) showed muscle degeneration and atrophy whilst barramundi fed on PBM-HI-based diets (Figure 7. 2J-L) demonstrated mild structural changes in muscles with 75PBM-25HI showing least changes. It is also worth noting that increase in intermyofibrillar spaces, reduced fibre-fibre adhesion, ruptured fibre and loss of cell borders were observed on day 8.

Table 7. 4 Texture and colour changes of barramundi fed control and test diets containing different proportion of PBM and HI larvae meal during chilled storage. The effect of factors and their interaction was conducted by two-way ANOVA.

		Experimental diets				Two-way ANOVA		
		0PBM-0HI	85PBM-15HI	80PBM-20HI	75PBM-25HI	Diet	Time	Diet × Time
Texture								
Springiness (mm)	Day 1	0.99±0.01	0.99±0.00	0.99±0.01	0.99±0.00			
	Day 4	0.99±0.00	0.99±0.00	0.99±0.00	0.99±0.00	0.62	0.66	0.89
	Day 8	0.99±0.00	0.99±0.00	0.99±0.00	0.99±0.00			
Cohesiveness (ratio)	Day 1	0.33±0.02	0.34±0.01	0.35±0.01	0.33±0.01			
	Day 4	0.30±0.02	0.31±0.02	0.32±0.01	0.31±0.01	0.46	0.00	0.98
	Day 8	0.30±0.00	0.30±0.00	0.31±0.01	0.32±0.01			
Gumminess (g)	Day 1	1673.56±92.96	1493.84±88.88	1713.28±17.91	1711.11±136.02			
	Day 4	1268.03±175.40	1375.89±148.82	1299.16±93.19	1485.75±103.87	0.28	0.01	0.77
	Day 8	1297.75±51.85	1418.23±114.66	1585.28±107.74	1568.49±164.75			
Chewiness (g/mm)	Day 1	1668.96±93.86	1492.86±87.19	1709.05±175.85	1698.33±132.14			
	Day 4	1262.16±174.43	1371.71±148.42	1293.11±93.60	1477.99±102.87	0.29	0.01	0.77
	Day 8	1292.66±50.91	1407.80±113.14	1576.33±106.37	1559.56±164.11			
Adhesiveness (g/s)	Day 1	-39.60±10.22	-37.39±6.93	-42.00±11.16	-31.18±7.08			
	Day 4	-46.63±15.48	-38.80±5.15	-40.96±9.54	-32.75±7.65	0.67	0.93	0.88
	Day 8	-47.34±11.17	-49.58±20.25	-25.81±6.27	-39.63±15.15			
Hardness (g)	Day 1	5067.50±216.77	4353.00±284.39	4830.67±350.69	5146.67±354.13			
	Day 4	4170.00±341.12	4449.67±316.38	4138.67±283.77	4721.33±231.06	0.20	0.05	0.35

	Day 8	4324.17±116.64	4663.50±220.70	5014.00±229.68	4926.33±295.69		
Skin colour							
L*	Day 1	55.97±1.45^{a}	52.27±0.99^{ab}	50.30±0.93^{b}	53.06±1.82^{ab}		
	Day 4	54.75±1.30	52.35±0.79	52.52±0.51	54.06±0.90	0.00	0.73	0.42
	Day 8	54.41±1.14^{ab}	50.07±0.97^{b}	51.78±0.83^{ab}	54.97±1.69^{a}		
a*	Day 1	-0.73±0.05^{b}	-0.41±0.09^{ab}	-0.27±0.12^{a}	-0.16±0.15^{a}		
	Day 4	-0.63±0.09	-0.44±0.11	-0.54±0.04	-0.58±0.08	0.01	0.00	0.07
	Day 8	-0.77±0.07	-0.50±0.11	-0.69±0.10	-0.73±0.14			
b*	Day 1	-4.04±0.72	-4.95±0.46	-5.45±0.36	-4.21±0.34			
	Day 4	-5.39±0.63	-5.31±0.28	-5.32±0.20	-6.03±0.48	0.18	0.02	0.24
	Day 8	-4.75±0.37	-5.61±0.29	-5.49±0.21	-4.87±0.21			
C*	Day 1	4.40±0.62	4.98±0.45	5.47±0.36	4.25±0.34B			
	Day 4	5.44±0.63	5.34±0.28	5.61±0.27	6.06±0.48A	0.21	0.01	0.37
	Day 8	4.83±0.36	5.65±0.29	5.54±0.21	4.95±0.20AB			
Flesh colour							
L*	Day 1	44.00±0.98^{c}	49.37±0.46^{a}	48.15±0.63^{ab}	45.79±0.84^{bc}		
	Day 4	45.74±1.13^{b}	49.23±0.29^{a}	49.50±1.25^{a}	49.02±0.37^{ab}	0.00	0.00	0.02
	Day 8	46.74±0.45^{b}	48.66±0.62^{ab}	48.55±0.38^{ab}	50.30±0.63^{a}			
a*	Day 1	4.47±1.09	1.42±0.3	1.76±0.31	3.41±0.85			
	Day 4	2.01±0.44	2.06±0.24	2.84±1.00	2.26±0.53	0.23	0.18	0.03
	Day 8	1.80±0.33	1.96±0.58	2.10±0.42	2.09±0.26			
b*	Day 1	6.29±0.63	5.68±0.21	5.32±0.31	5.86±0.40			

	Day 4	5.15±0.47^{b}	6.45±0.27^{ab}	7.31±0.55^{a}	6.33±0.51^{ab}	0.00	0.06	0.00
	Day 8	4.79±0.31^{b}	6.77±0.44^{a}	7.29±0.30^{a}	7.02±0.22^{a}			
C*	Day 1	7.97±1.11	5.93±0.23	5.65±0.39	7.05±0.74			
	Day 4	5.63±0.56	6.80±0.32	8.10±0.96	6.81±0.36	0.26	0.88	0.00
	Day 8	5.18±0.37^{b}	7.18±0.61^{a}	7.66±0.40^{a}	7.36±0.26^{a}			

Two way ANOVA

	Days			Diets			
	Day 1	Day 4	Day 8	0PBM-0HI	85PBM-15HI	80PBM-20HI	75PBM-25HI
Texture							
Springiness (mm)							
Cohesiveness (ratio)	a	b	b				
Gumminess (g)	a	b	ab				
Chewiness (g/mm)	a	b	ab				
Adhesiveness (g/s)							
Hardness (g)	a	b	ab				
Skin colour							
L*				a	bc	c	ab
a*	a	ab	b	b	a	ab	a
b*	a	b	ab				
C*	b	a	ab				
Flesh color							
L*	b	a	a	b	a	a	a

a*				
b*	b	a	a	a
C*				

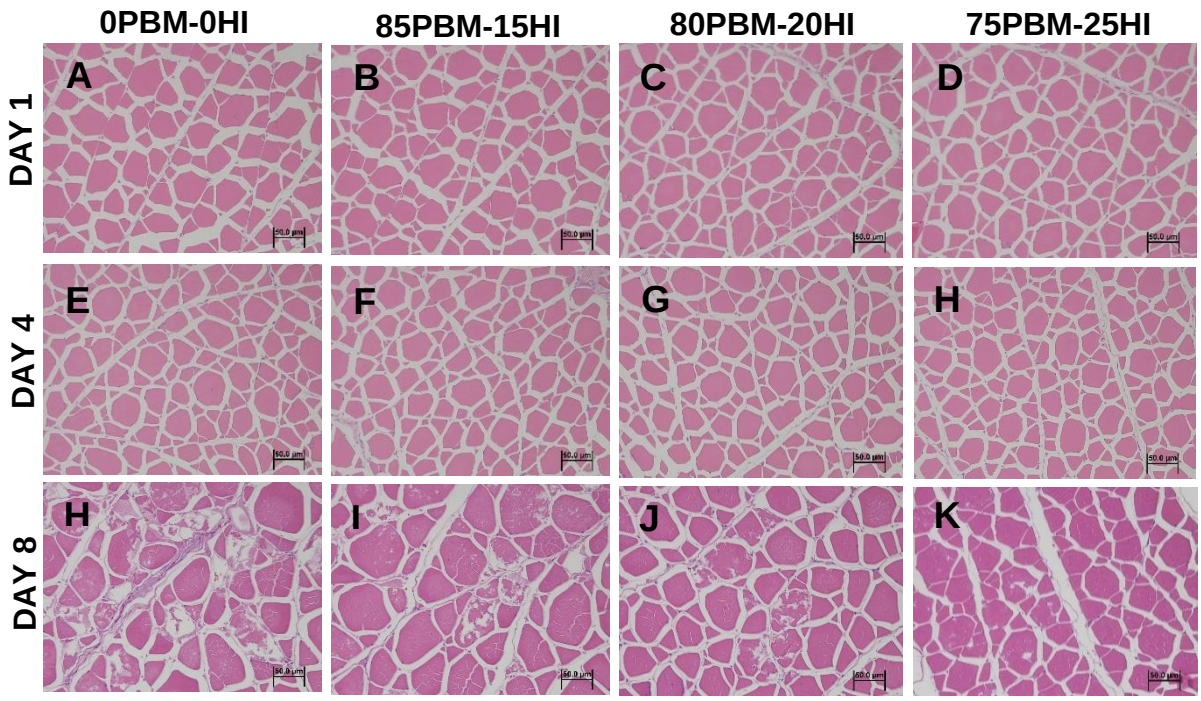


Figure 7. 2 Microstructure changes in barramundi muscle (H&E staining and 40 × magnification) when fed PBM-HI based diets during the chilled storage.

7.3.5 Linear relationship between QI score and post-days storage

Diet did not influence any of attributes including skin brightness, appearance transparency, flesh texture, flesh blood, flesh odour and flesh gaping used for QI schemes whilst storage time affected these attributes, as demonstrated by two-way ANOVA (Table 7. 5). The score of all attributes increased over the storage time regardless of diets. No significant interaction was found between diet and storage time. There were a strong linear relationship between storage time and QI score in 0PBM-0HI (R^2 = 0.934) (Figure 7. 3A), 85PBM-15HI (R^2 = 0.91) (Figure 7. 3B), 80PBM-20HI (R^2 = 0.91) (Figure 7. 3C) and 75PBM-15HI (R^2 = 0.91) (Figure 7. 3D).

Table 7. 5 Results of sensory parameters evaluated by QIM: skin brightness, appearance transparency, flesh texture, flesh blood, flesh odour and flesh gaping in barramundi fillet fed PBM-HI based diets during chilled storage.

		Experimental diets				Two-way ANOVA		
		0PBM-0HI	85PBM-15HI	80PBM-20HI	75PBM-25HI	Diet	Time	Diet × Time
Skin brightness	Day 1	-	-	-	-			
	Day 4	-	-	-	-	0.53	0.00	0.62
	Day 8	1.33±0.21	1.00±0.00	1.17±0.17	1.17±0.17			
Appearance transparency	Day 1	-	-	-	-			
	Day 4	0.50±0.22	0.67±0.21	0.83±0.17	0.33±0.21	0.44	0.00	0.32
	Day 8	1.00±0.00	0.83±0.17	1.00±0.00	1.00±0.00			
Flesh texture	Day 1	0.17±0.17	0.17±0.17	-	-			
	Day 4	0.83±0.17	0.67±0.21	0.50±0.22	0.67±0.21	0.48	0.00	0.92
	Day 8	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00			
Flesh blood	Day 1	0.17±0.17	-	-	-			
	Day 4	1.00±0.00	1.17±0.17	1.17±0.17	1.00±0.26	0.84	0.00	0.94
	Day 8	1.83±0.17	1.67±0.21	1.83±0.17	1.67±0.33			
Flesh odour	Day 1	-	-	-	-			
	Day 4	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	0.40	0.00	0.43
	Day 8	1.83±0.17	2.00±0.00	2.00±0.00	2.00±0.00			
Flesh gaping	Day 1	1.50±0.22	1.17±0.31	1.33±0.21	1.00±0.26			
	Day 4	2.00±0.00	1.83±0.17	1.83±0.17	1.50±0.22	0.14	0.00	0.78
	Day 8	2.00±0.00	2.00±0.00	2.00±0.00	2.00±0.00			

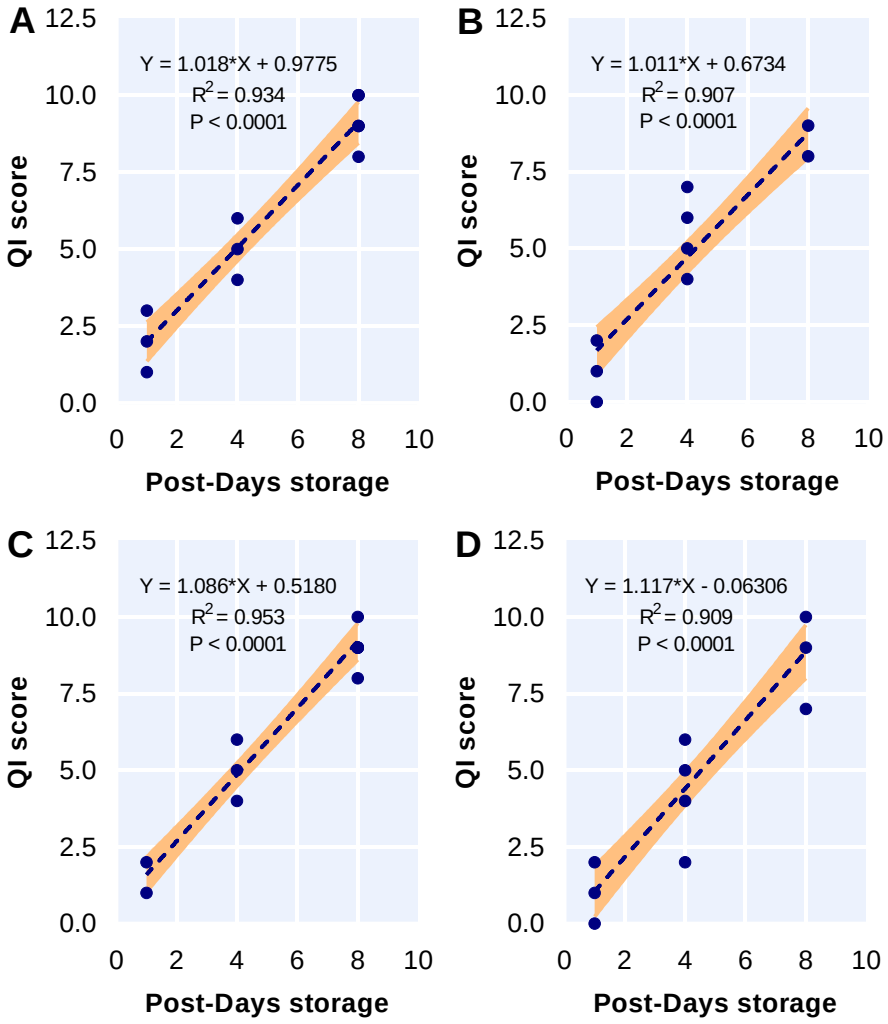


Figure 7. 3 Linear correlation between QI and storage time for test diets including 0PBM-0HI, 85PBM-15HI, 80PBM-20HI and 75PBM-25HI.

7.3.6 pH and lipid oxidation

The results of two-way ANOVA of pH and TBARs activity of barramundi fillet in response to diets and storage time are presented in Figure 7. 4. The pH of fillet was influenced by diet and showed an interaction between diet and storage time (Figure 7. 4A). pH of barramundi fillet upsurged at Day 1 and Day 4 when fed the mixture of PBM and HI larvae meal. However, storage time had no significant effect on pH. Malondialdehyde (MDA) content, measured by TBARS was influenced by both factors with a significant interaction (Figure 7. 4B). At day 8, PBM-HI based diets retarded the production of MDA in the flesh of barramundi but there was a gradual increase in lipid oxidation production over the storage time irrespective of diets.

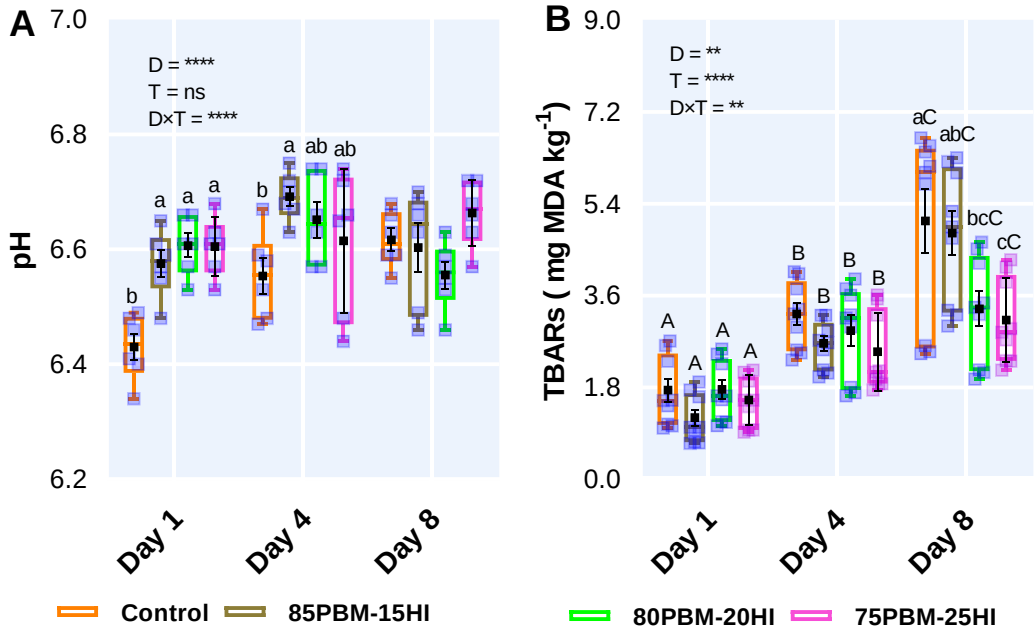


Figure 7. 4 The production of pH (n = 6) and rancidity (TBARS) (n = 9) of barramundi fillet after 56 days of feeding PBM and HI based diets during chilled storage. Violin plot indicates the range and round shape marker indicates the number biological replicates used for analysis. Square shape back marker denotes the mean with standard error. The effect of factors and their interaction was conducted by two-way ANOVA.

7.4 Discussion

The production data and other physiological responses to the different diet in terms of growth and body indices, muscle amino acid composition, gut mucosal morphology and microbiota, serum metabolites, antioxidant activity and resistance to *Vibrio harveyi* has been illustrated in our earlier article (Chaklader et al., 2021b). Briefly, growth performance in the test diets was comparable to the fish fed FM-based control diet, with no changes in body indices and muscle amino acid composition. However, the distal intestine mucosal barrier functions were improved by PBM-HI based diets, as further supported by the positive changes in the abundance of beneficial microbiota. The purpose of this study, however, was to the aligned fillet quality traits using multidisciplinary approaches to test parameters including proximate and fatty acid profile, sensory attributes, texture correlating with the muscle structure, and colour and lipid oxidation of barramundi fillet after 56 days feeding on diets representing a complete replacement of FM with animal based protein.

The crude protein levels was unaffected by the mixture of PBM and full-fat HI larvae meal, suggesting that barramundi can assimilate protein from PBM-HI similar as effectively as FM protein. Likewise, barramundi fed poultry protein concentrate alone (6.7-20%) or supplemented

with phosphorous (Simon et al., 2019), hybrid grouper fed blends of PBM, shrimp meal and spray-dried blood meal (20-80%) (Ye et al., 2019a) and juvenile black sea bass fed PBM (40-100) (Dawson et al., 2018) produced a similar whole body protein content to fish fed FM protein. However, a higher lipid concentration reported here indicated flesh quality improvement of barramundi flesh since lipid concentration has been reported to be associated with taste and appearance of cooked flesh (Grigorakis, 2007; Sabbagh et al., 2019). This result was mainly due to inclusion of FHI larvae which contain higher concentration of lipid.

Regarding muscle FA profile, an increase in total SFA in the muscle of barramundi fed 80PBM-20HI and 75PBM-25HI was due to the substantial increase in lauric acid (C12:0) and myristic acid (C14:0). HI larvae meal is a rich source of C12:0 and C14:0 which have been reported to enhance the total SFA concentration in barramundi (Chaklader et al., 2020b; Chaklader et al., 2021c) and other fish species such as Siberian sturgeon, *Acipenser baerii* (Caimi et al., 2020b), rainbow trout (Renna et al., 2017) and Eurasian perch, *Perca fluviatilis* (Stejskal et al., 2020). Oleic acid plus elaidic acid (C18: 1cis + trans), causing a higher concentration of MUFA in PBM-HI based duets clearly improved the MUFA concentration of barramundi in the present study. This is similar what has previously been reported in MUFA concentration of barramundi (Chaklader et al., 2020a; Chaklader et al., 2020b; Chaklader et al., 2020c; Chaklader et al., 2021c), juvenile coho salmon, *Oncorhynchus kisutch* (Twibell et al., 2012), Atlantic Salmon, *S. salar* (Higgs et al., 2006) and juvenile gilthead seabream, *Sparus aurata* (Sabbagh et al., 2019) fed PBM. PBM is a rich source of oleic acid, resulting in higher MUFA concentration in the aforementioned studies, however, it contains lower concentration of n - 3 LC-PUFA, EPA (20:5n-3) and DHA (22:6n-3). Similarly, lower concentration of n - 3 LC-PUFA, EPA and DHA decreased the total PUFA in PBM-HI based diets, perhaps underpinning the lower concentration PUFA in barramundi fillet fed PBM-HI based diets. Similarly, high inclusion of PBM resulted in lower concentration of PUFA in barramundi (Siddik et al., 2019a), totoaba juveniles, *Totoaba macdonaldi* (Zapata et al., 2016) and juvenile coho salmon, *Oncorhynchus kisutch* (Twibell et al., 2012).

Sensory quality is important to investigate the potentiality of new alternative protein ingredients and is associated with consumer acceptance. PBM-HI based diets improved the raw and cooked fillet quality of barramundi, perhaps suggesting that the aligned increase in fillet lipid might be aligned with the improved the sensory quality of barramundi fillet. Besides lipid content, chitin present in HI larvae might have played a role in improving sensory fillet quality as chitosan coating has been reported to improve the sensory scores in seafood (Baptista et al., 2020; Fan et al., 2009; Farajzadeh et al., 2016; Mohan et al., 2012), however, the mode of actions of chitin in HI larvae meal after feeding in improving the fillet quality is not well-understood. The present study provide

the first insight on the improvement of barramundi fillet quality when fed FM–free diets containing a mixture of PBM and HI larvae meal. However, total substitution of FM with PBM deteriorated the sensory quality of female tenches, *Tinca tinca* (Panicz et al., 2017) and meat meal did not affect the sensory quality of barramundi fillet (Williams et al., 2003b). Hence, further study is needed to identify the components in HI larvae meal that may improve the fillet quality of fish.

Evaluation of drip loss is of importance in determining the post-harvest fish quality due to the fact that it is directly related to lower water holding capacity caused by fibre shrinkage, cell damage, lower protein solubility and protein denaturation (Einen et al., 2002), leading to sensory loss including texture and flavour (He et al., 2014), loss of water soluble nutrients such as protein and amino acids (Kristoffersen et al., 2007) and financial implications (He et al., 2014). The unchanged drip loss in the fillet of barramundi fed PBM-HI based diets similar to the unchanged fillet water holding capacity of rainbow trout fed HI larvae prepupae meal (Bruni et al., 2020b) and blackspot sea bream, *Pagellus bogaraveo* fed mealworm larvae meal (Iaconisi et al., 2017). This finding is consistent with the results of texture and lipid oxidation. Regardless of diet, gradual increase in drip loss over the 8 days storage is consistent with the findings of Siah Ariff (2003) who reported increase in drip loss in barramundi flesh samples overtime during storage at 2 ± 2 °C but was not consistent with the findings of Wilkinson et al. (2008) who reported no differences in drip loss in barramundi flesh samples over 4 days storage at 2– 4 °C. Result is within expectation as degradation in structure and gradual increase in lipid oxidation was found, indicating that the integrity of cell membranes reduces with storage time, enhancing passage of sarcoplasmic fluid which lead to increase in drip loss (Halliwell and Gutteridge, 1999; Morrissey and Kerry, 2004). However, the changes in drip loss from 0.16 to 2.86% cannot be considered as high and therefore not a major problem in chilled barramundi, as reported by earlier studies (Duun and Rustad, 2008; Einen et al., 2002; Kaale and Eikevik, 2015).

A good number of studies have been evaluated the efficacy of FM replacement with PBM on 33 different fish species, though none of them have not been evaluated the flesh quality (Galkanda-Arachchige et al., 2020). However, our recent study found no changes in the final flesh quality of barramundi in terms of pH, texture and colour when fed PBM based diets, concurrently supplemented with tuna hydrolysate and HI larvae meal (Chaklader et al., 2021c). Similarly, in this study texture including springiness, cohesiveness, guminess, chewiness and hardness of barramundi fillets were not affected by the mixture of PBM and full-fat HI larvae meal. Regardless of PBM inclusion, HI larvae meal (33-100%) had no influence on the texture (hardness, cohesiveness, resilience and adhesiveness) of salmon (Bruni et al., 2020a). This is similar to what has been reported by Iaconisi et al. (2017) who found no variation in fillet hardness, cohesiveness,

gumminess and adhesiveness of sea bream, *Pagellus bogaraveo* fed mealworm, *Tenebrio molitor*. However, storage time had significant effect on the cohesiveness, guminess, chewiness and hardness, which decreased significantly at day 8. Similar result was found in shelf-life studies conducted in sea bream, *Sparus aurata*, where identical parameters reduced overtime (Ayala et al., 2010; Caballero et al., 2009). This phenomenon has shown to be highly related to histological structure, especially the increase in intermyofibrillar spaces, reduced fibre-fibre adhesion, ruptured fibre and loss of cell borders. Thus, it can be suggested that textural changes were a consequence of structural degradation over time. These results are further supported by elevated lipid oxidation over the storage time in the present study, suggesting that protein deterioration took place by the action of endogenous cathepsins and exogenous protease due to microorganisms (Yu et al., 2017; Zarandona et al., 2021). Hence, whole microbiome profile evaluation using modern tool rather than plate counting in barramundi fillet in response to PBM-HI based diet during shelf life study is needed to be thoroughly investigated.

Colour of fish is an important factor influencing the consumer preference and it is largely governed by dietary and post-harvest factors in fish since fish do not have *de novo* power to synthesis colour. There is a lack of studies concerning the effect of processed animal protein on the colour of fish. However, complete replacement of FM with PBM, concurrently supplemented with HI larvae meal and tuna hydrolysates did not change skin and fillet colour in barramundi (Chaklader et al., 2021c). In this study, the skin brightness (L*) was affected by diet and this variation in the present study could be due to light or lack of shading. Farmed fish are more exposed to light than wild fish, leading to darker skin chromophores. These suggest the farmed fish may be darker than wild fish (Adachi et al., 2005). This was proven by Howieson et al. (2013) who reported that barramundi reared in shaded tanks were less dark than the barramundi reared in unshaded tanks. PBM-HI based diets improved the redness (a*) in barramundi skin which could be due to presence of β-carotene in insects (Finke, 2002). A similar effect was observed in blackspot seabream, *Pagellus bogaraveo* skin when fed mealworm, *Tenebrio molitor* (Iaconisi et al., 2018).

Blue-grey discoloration due to melanosis is one of the common problems in farmed barramundi fillet, reducing the appeal of the raw farmed barramundi fillet on display to consumers and consequently impacting on sales in the retail sectors (Howieson et al., 2013). The same authors fed barramundi for six weeks by changing the levels of substrate (the amino acid tyrosine) or substrate competitors (the amino acid tryptophan), or limiting enzyme (tyrosinase) co-factors like copper in the experimental diets to manipulate the melanin synthesis pathway, and however none of these diets improved the greying issue. Irrespective of storage time, the improvement in barramundi fillet brightness when fed PBM-HI in the present study suggested that HI larvae meal

might have properties to reduce melanosis. This is not similar to the report of Moutinho et al. (2020) and Bruni et al. (2020b) who found no differences in the fillet brightness of European seabass and rainbow trout fed HI prepupae meal. However, brightness over the 8 days storage time increased irrespective of diet which is similar to the brightness of barramundi flesh over the 14 days of storage time (Jones and Carton, 2015). Also, brightening of fish flesh over the prolonged storage time has been frequently reported by many studies (Choubert and Baccaunaud, 2006; Guillerm-Regost et al., 2006; Robb et al., 2000; Ruff et al., 2002). It could be due to the elevated lipid oxidation during the storage time which has been reported to affect the flesh colour of fish. Flesh brightening may also be attributed to some other factors including alterations in light scattering properties in muscle during rigor (Erikson and Misimi, 2008; Guillerm-Regost et al., 2006) and light-absorbing and reflecting properties due to decreases in the translucency of fish muscle (Ozbay et al., 2006; Stien et al., 2005). Riboflavin (vitamin B2) a yellow-coloured pigment, variably found in most of the edible insects (Iaconisi et al., 2017) may also influence the skin and flesh color of fish. The improvement in yellowness (b*) in the flesh of barramundi fed PBM-HI based diets could be due the presence of 2.2 mg/100 g riboflavin in HI larvae meal (Nyakeri et al., 2017). In contrast, a six week feeding trial on barramundi fed PBM-based diets supplemented with 5 and/or 10% of HI larvae and tuna hydrolysate did not influence the skin and flesh yellowness (Chaklader et al., 2021c). A similar influence to previous study on the yellowness of European seabass, *Dicentrarchus labrax* juveniles fillets was found when fed pre-pupae larvae (Moutinho et al., 2020). These discrepancies in colour variation might also be attributed to different inclusion levels of HI larvae meal, trial duration and different fish species.

QI is a scoring system to estimate the freshness and quality of fishery products based on some attributes including appearance, odour and texture changing during the storage time. The score for all attributes increased gradually during the storage time but the score were below 3 (low freshness) (Fuentes-Amaya et al., 2016) for all attributes, indicating that barramundi fillet were acceptable until day 8. However, a significant linear relationship between QI score and storage time was expected since a similar trend has been reported in many fish species (Alexi et al., 2021; Boziaris, 2013; Calanche et al., 2019; Luten and Martinsdottir, 1997; Martinsdóttir et al., 2001; Mausse et al., 2000). After 8 days storage, the highest demerit score for QI for all diets were similar to results of textural attributes, muscle microstructure and lipid oxidation in the present study.

The final product quality deterioration of fish including colour degradation, muscle gaping, blood spotting, flesh texture alterations and drip loss is associated with reduction of pH (Chow et al., 2009; Jones and Carton, 2015; Wilkinson et al., 2008). An elevated level of pH in the fillet of barramundi fed PBM-HI based diet in the present study is similar to what has been reported by

Moutinho et al. (2020) in which pH increased in the fillet of European seabass fed pre-pupae meal after slaughtering and three days post-storage. However, pH over the storage time in the present study changed from 6.34 to 6.74 were within the normal pH range of barramundi muscle (Wilkinson et al., 2008). Although pH elevation may be attributed to formation of nitrogenous compounds such as ammonia, dimethylamine, trimethylamine, histamine, etc., produced by endogenous enzymes and microbial enzymatic actions (Li et al., 2017b), the increase in pH at certain levels has been reported to prevent production of volatile bases, leading bacteria to take energy from the oxidative product rather than glycogen and other normal substrate (Jones and Carton, 2015). Hence, an inverse association between pH and lipid has been reported in barramundi fillet after 5 months feeding of alpha-tocopherol acetate and following a two-week post-storage time (Jones and Carton, 2015). A similar association was found between pH and TBARS analysis in the present study. TBARS is widely used to quantify the degree of second stage lipid peroxidation of fish which produce aldehydes and ketones from the degradation of polyunsaturated fatty acids, associated with unpleasant odour (Wang et al., 2021b). PBM-HI based diets inhibited the rancidity production (MDA) compared to 0PBM-HI in the current study, the reduction in MDA level could be related to elevated SCFA level as it reduced the amount of double bond which is prone to be attacked by oxidant (Gibson and Newsham, 2018). A similar trend was also supported by Table 2 fatty acid profile, where decrease in TBARS were only found when SCFA was significantly higher. Previous study has suggested that HI larvae may possess antioxidant properties to halt the lipid oxidation. For example, Moutinho et al. (2020) reported the efficacy of HI larvae meal to reduce the production of TBARs in the fillet of European seabass. The less production of TBARS in the present study might be attributed to lower proportion of PUFA, in particular, EPA and DHA which are sensitive to peroxidation in contact with oxygen (Couturier et al., 2020).

Or, the presence of chitin in HI larvae might have retarded oxidation since the deacitylated form of chitin is known known as chitosan which has been reported to delay lipid oxidation by binding the free amino groups and hydroxyl radicals of the polymer with the metal ions (Fe^{2+}) and free radicals on food, thus making a stable macro-molecular structures (Baptista et al., 2020; Feng et al., 2008; Jeon et al., 2000). Another potential reason behind these improvements in rancidity is associated with the interaction between positively charged (NH_3^+) amino groups of chitosan with the negative carboxyl groups (COO^-) situated on the outer part of the membrane or the cell wall of bacteria and fungi (Baptista et al., 2020), which change the permeability of the membrane of by blocking the passage of nutrients and oxygen important for cellular metabolism, leading to cell death (Kong et al., 2010; Tsai et al., 2002). Although chitinolytic enzymes, chitinoclastic bacteria and the assimilation of chitosan have not been reported previously in barramundi, endogenous

production of chitinase has been previousy reported in marine carnivorous teleost fish (Fines and Holt, 2010; Kurokawa et al., 2004). Regardless of the diets, the flesh TBARS increase over the storage time in the current study is consistent with the reported TBARS production of barramundi flesh during two-weeks chilled storage (Jones and Carton, 2015) and in fillets of European seabass when fed different level of HI pre-pupae larvae meal (Moutinho et al., 2020). The range (0.70-6.69 mg MDA/kg) of TBARs production in the present study up to Day 8 were below the reported critical limits (7-8 mg MDA/kg) for fish (Öz, 2018; Varlik, 1993).

Complete replacement of FM with a mixture of PMB and full-fat HI larvae meal increased the lipid content while reducing the synthesis of essential fatty acid content. Interestingly, sensory quality in both raw and cooked fillet was improved by PBM-HI based diets. Drip loss in response to diet and storage time were within the acceptable range. PBM-HI based diets enhanced the brightness, redness and yellowness in skin and flesh, though diet did not influence the texture profile. A significant correlation between storage time and QI was observed for all diets. Test diets influenced the pH and reduced the production of rancidity over the 8 days storage time. Altogether, the mixture of PBM and HI could increase consumer acceptability and resolve blue-greyish coloration problem in farmed barramundi. However, further research is needed to better understand the complimentary effect of HI larvae functional molecules in improving the fillet quality. Also, enriching HI larvae with n-3 PUFA by changing feeding substrate could be recommended to increase the synthesis of n-3 PUFA in the flesh of barramundi.

CHAPTER 8: Growth, hepatic health, mucosal barrier status and immunity of juvenile barramundi, *Lates calcarifer* fed poultry by-product meal supplemented with full-fat or defatted *Hermetia illucens* larval meal

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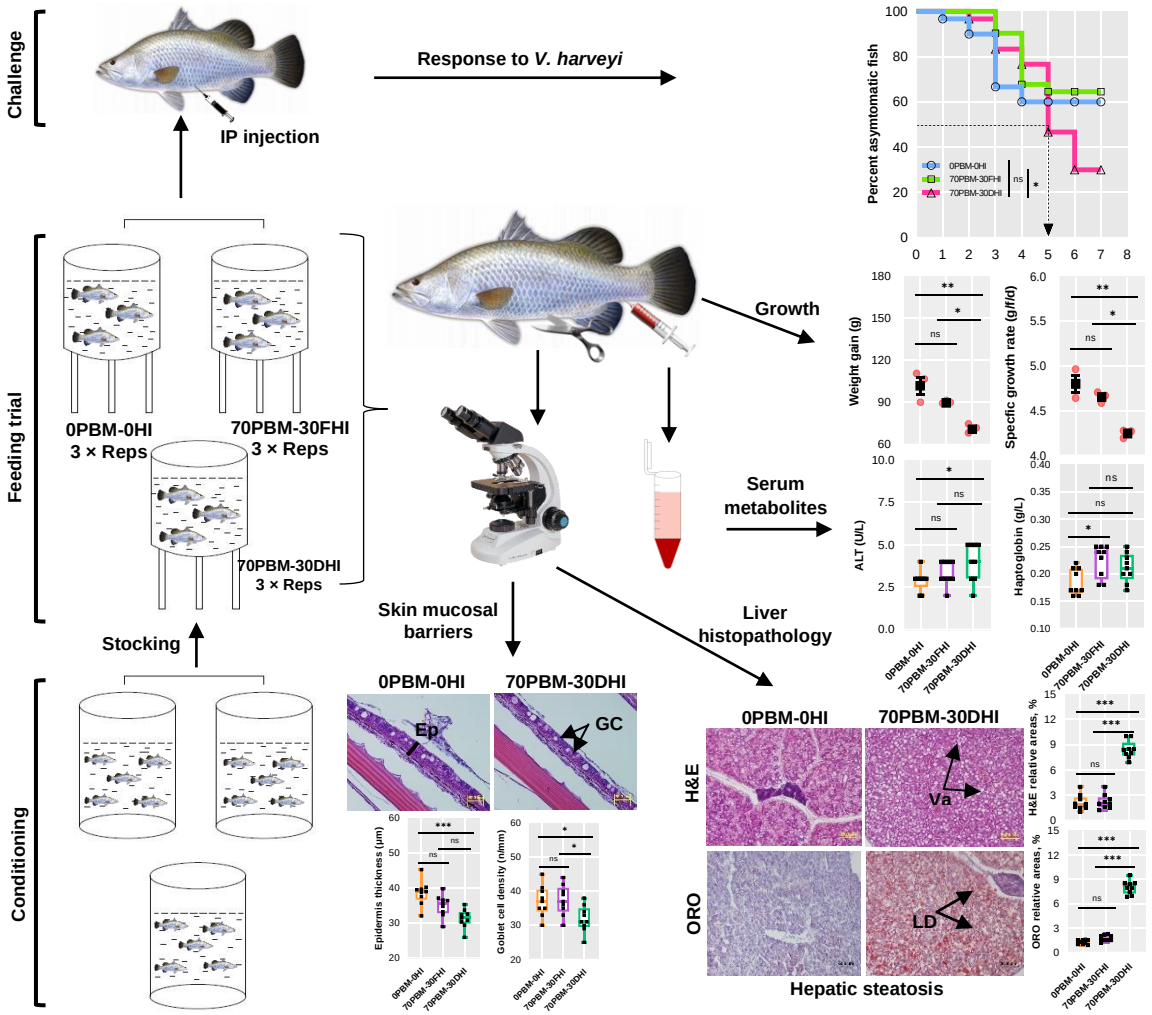
Graphical Abstract



Abstract

Poultry by-product meal (PBM) as a replacement for fish meal (FM), an expensive and unsustainable protein aquafeed ingredient, has been tested on different aquaculture fish species. However, the complete replacement of FM with a mixture of PBM and *Hermetia illucens* (HI) larvae in barramundi culture has not been previously investigated. In this study, results are presented on growth performance, fillet fatty acid composition, serum metabolites, skin mucosal barriers, hepatic steatosis, antioxidant activity, and immunity of juvenile barramundi, *Lates calcarifer* fed either a FM-based diet (0PBM-0HI) or two test diets in which total FM protein was replaced by a mixture of 70% PBM and 30% full-fat (FHI) and defatted HI larvae (DHI) meal (designated as 70PBM-30FHI and 70PBM-30DHI). After 56 days of feeding, the results showed that the growth was affected when fish were fed 70PBM-30DHI with a higher feed conversion ratio (FCR) with respect to 0PBM-0HI and 70PBM-30FHI diets. There was no variation in growth performance, feed utilization, and FCR between 0PBM-0HI and 70PBM-30FHI. The retention of total saturated and monounsaturated fatty acids increased in the fillet of juveniles fed 70PBM-30FHI and 70PBM-30DHI while total retention of polyunsaturated (PUFA), n-3 PUFA, and n-6 PUFA decreased than the control. Serum alanine transaminase (ALT) increased in fish fed 70PBM-30DHI whereas haptoglobin upsurged in 70PBM-30FHI compared to 0PBM-0HI. There were no significant effects in the serum immune response between dietary treatments whilst serum and liver CAT activity were negatively impacted by 70PBM-30DHI. The 70PBM-30DHI induced hepatic steatosis whilst 0PBM-0HI and 70PBM-30FHI showed no obvious change in the liver. Skin mucosal barriers were impacted by 70PBM-30DHI whilst 70PBM-30FHI fed fish showed a similar response to 0PBM-0HI. The fish fed on 70PBM-30DHI showed a higher proportion of infection rate with *Vibrio harveyi* than 70PBM-30FHI but showed no variation with the control-fed fish. In summary, FHI larvae meal could be a good complementary protein source, particularly when replacing FM completely with insect-based proteins.

Keywords: Black soldier fly larave, Poultry by-product, Hepatic steatosis, *Vibrio harveyi* and *Lates calcarifer*

8.1 Introduction

Industry and academia have dedicated substantial efforts in the last few decades to identify cheap and eco-sustainable alternative protein ingredients to replace fishmeal (FM), a resource that is unsustainable and predicted to be inadequate in the view of the expansion of aquaculture production (Chaklader et al., 2020c; Shafique et al., 2021). In recent years, the aquaculture industry has changed dietary formulations using plant-derived ingredients thereby decreasing the level of FM (Naylor et al., 2009; Tacon, 2020; Turchini et al., 2019) and improving the sustainability of the industry. However, plant-based ingredients have been reported to have secondary adverse health effects on carnivorous fish including growth depression with high mortality, enteritis in the distal intestine, histopathological changes in the immune organs, immune suppression, and hypertrophic mucus production (Gatlin et al., 2007; Glencross et al., 2007). For instance, higher replacement of FM protein with plant protein such as lupin meal, soybean meal, peanut meal, and algae has been reported to affect the growth performance of barramundi, *Lates calcarifer* by impacting the integrity of the liver, spleen, muscle, and other physiological responses (Ilham et al., 2016a; Ilham et al., 2016b; Ilham and Fotedar, 2017; Ilham et al., 2018; Van Vo et al., 2015; Van Vo et al., 2020a; Van Vo et al., 2020b; Vo et al., 2020). These adverse effects have mainly been attributed to anti-nutritional factors and imbalanced nutrient profiles of these plant ingredients. Processed terrestrial-based animal proteins including blood meal, poultry by-product meal (PBM), feather meal, meat, and bone meal have also been identified as FM substitutes due to their comparable nutrient profile and digestibility (Sogari et al., 2019).

In particular, the potential inclusions levels of PBM in aquafeeds have been evaluated in a wide range of finfish species for decades (Galkanda-Arachchige et al., 2020) as it invariably contains a higher concentration of protein than plant-based ingredients and amino acid profile comparable to FM (NRC, 2011). There has been significant research, particularly in Australia, with the commercial application of PBM and poultry by-product oil (Salini et al., 2015). However, a low utilization in comparison with plant protein has been largely attributed to a previous ban in the European Union, concerning the utilization of processed animal protein in aquadiets, and consumer acceptance due to social concerns related to the potential transmission of disease (Klinger and Naylor, 2012). Research on the utilization of PBM in the diet of carnivorous fish such as barramundi showed that the inclusion of PBM beyond certain levels was not possible due to palatability issues and other physiological problems such as growth depression, necrosis, and steatosis in the liver, elevated levels of liver enzymes, alterations in antioxidant activity and lower retention of essential fatty acids (Chaklader et al., 2020a; Chaklader et al., 2019). Recently, a number of studies have been able to replace FM exclusively and entirely with PBM either by the

supplementation of fish protein hydrolysate (Chaklader et al., 2020c; Siddik et al., 2019c) or a mixture of insect meal, in particular, black soldier fly, *Hermetia illucens* (HI) larvae meal (Chaklader et al., 2020b). However, the entire replacement of FM with a mixture of FHI and DHI larvae meal and PBM protein has not previously been investigated.

Recently, HI larvae have received attention as a replacement to conventional animal and plant-based aquafeed protein sources due to several advantages, most particularly, a capacity to assimilate and transfer nutrients from low-value by-products and wastes and turn them into favourable nutritional profiles, aligned with a low requirement for water, growing area and a decreased greenhouse gas emission (Chaklader et al., 2020b; Henry et al., 2015). As well, HI larval meal accumulates a favourable amount of protein and contains essential amino acids approximately similar to FM (Henry et al., 2015). The lipid content is highly variable (15-49%), depending on the substrate used for culture (Meneguz et al., 2018; Nogales-Mérida et al., 2018) and thus lipid profile can be improved by enriching the growth substrate. For example, feeding 10% of *Schizochytrium* sp in combination with coffee by-product and fish-offal has been reported to enrich polyunsaturated fatty acids in HI larvae meal (Sealey et al., 2011; Zarantoniello et al., 2020b). However, defatting of the meal may reduce the lipid content by up to 9% or less, which in turn may increase the protein content from 35% to 60% DM (Bußler et al., 2016; Schiavone et al., 2017) thereby allowing an increase in inclusion levels in aquafeeds. In addition, HI larvae have been characterized by useful levels of vitamins and minerals such as iron, zinc, potassium, phosphorus, manganese (Fisher et al., 2020; Henry et al., 2015; Van Huis, 2013), and also contains some novel antimicrobial peptides (AMPs), exhibiting defence mechanism against Gram-positive and Gram-positive bacteria and fungi (Chaklader et al., 2019; Elhag et al., 2017; Park et al., 2015; Park et al., 2014). The beneficial effects of FHI larvae meal have already been reported on various fish in terms of immune response and modulating gut mucosal morphology and microbiome (Abdel-Latif et al., 2021; Kumar et al., 2020; Rawski et al., 2021; Terova et al., 2019) and even FHI larvae meal has an ability to complement plant protein in rainbow trout diet (Kumar et al., 2020). Such effects were not found in other fish species (Tippayadara et al., 2021; Wang et al., 2019b).

Research conducted to date on the inclusion levels and efficiencies of DHI larvae in fish nutrition have been contradictory: promising results in the utilization of HI larvae meal have been reported in several finfish (Fawole et al., 2020), but deleterious outcomes were reported in others (Abdel-Tawwab et al., 2020; Guerreiro et al., 2020). The defatting process imposes high energy consumption, labour, and additional costs, which may lower the environmental sustainability and profitability of using insect meals (Rawski et al., 2020). What is more, defatting in high pressure

and temperature may negatively influence the nutritive and functional value of HI larvae due to the possibility of degradation of amino acids and AMPs and an increase in chitin share in the meal which, in high amounts, may be considered as an anti-nutritional factor, leading to impacts on the digestibility (Caimi et al., 2020b). In addition, fat separation reduces the potential beneficial aspects of fatty acids composition, in particular, the amount of lauric acid content (Wang et al., 2019b) which has been reported to have antibacterial activity. Hence, using full-fat HI larvae may be much more convenient from a functional and economic point of view.

The present study was aimed to investigate if FHI or DHI larvae meal mixed with PBM protein influenced the growth and muscle fatty acid profile of juvenile barramundi. The results were assessed in relation to other physiological parameters such as serum biochemistry, hepatic health, antioxidant status and immune responses.

8.2 Materials Methods

8.2.1 Ethical statement

A growth and challenge trial was conducted at the Curtin Aquatic Research Laboratory (CARL) at the School of Molecular and Life Sciences, Curtin University (Australia). All experimental protocols complied with the guidelines and regulations in Australia for the care and use of animals and were approved by the Animal Ethics Committee in Curtin University (ARE2018-37). Optimal rearing conditions were maintained during the trial, and all efforts were made to minimize suffering by using an anaesthetic (AQUI-S) according to the standard operating procedure (SOP) of CARL.

8.2.2 Experimental diets

Three experimental diets were formulated to have approximately 48%, 13%, and 20% of protein, lipid, and gross energy, respectively. All the ingredients were purchased from Specialty Feeds, Glen Forrest Stockfeeders, 3150 Great Eastern Highway, Glen Forrest, Western Australia 6071 except for PBM and HI larvae which were obtained from Derby Industries Pty Ltd T/A, Talloman Lot Lakes Rd, Hazelmere WA 6055, and Future Green Solutions, Perth, Western Australia, respectively. A diet containing FM was used as the control (0PBM-0HI), and the other two diets included total replacement of FM protein with 70% PBM and 30% FHI larval meal and 70% PBM and 30% partially DHI larvae meal which was designated as 70PBM-30HI and 70PBM-30DHI, respectively. HI larvae meal were partially defatted by a mechanical process using high pressure at low temperature. Different proportions of wheat, wheat starch, and canola oil were used to ensure the diets were isonitrogenous, isolipidic and isoenergetic. Diets were prepared as described

in our earlier study (Chaklader et al., 2020c), and respective nutritional compositions, and fatty acids composition are presented in Table 8. 1 and Table 8. 2 respectively.

Table 8. 1 Ingredient composition and proximate analysis of experimental diets

Ingredients (g/100g)	0PBM-0HI	70PBM-30HI	70PBM-30DHI
†FM[a]	72.00	0.00	0.00
‡PBM[b]	0.00	50.50	50.50
†Canola oil	1.00	0.50	0.50
ŧFull-fat HI[c]	0.00	35.00	0.00
ŧDefatted HI[d]	0.00	0.00	27.83
†Corn/wheat starch	7.00	5.90	11.00
†Lecithin - Soy (70%)	1.00	2.00	1.00
†Vitamin C	0.05	0.05	0.05
†Dicalcium Phosphate	0.05	0.05	0.05
†Wheat (10 CP)	16.90	4.00	7.07
†Vitamin and mineral premix	0.50	0.50	0.50
†Salt (NaCl)	1.00	1.00	1.00
†Cod liver oil	0.50	0.50	0.50
Nutritional Composition (%)[e]			
Dry matter	89.98	90.12	90.35
Crude protein	47.88	47.94	48.06
Crude lipid	12.59	13.61	13.96
Ash	11.65	11.78	11.85
Gross energy (MJ kg^{-1})	19.68	20.10	19.96

†Purchased from Specialty Feeds, Glen Forrest Stockfeeders, 3150 Great Eastern Highway, Glen Forrest, Western Australia 6071.

‡Kindly provided by Derby Industries Pty Ltd T/A, Talloman Lot Lakes Rd, Hazelmere WA 6055.

ŧFuture green solution, Western Australia

[a]FM: Crude protein (64.00%), crude lipid (10.76%), moisture (11.23) and ash (19.12%)

[b]PBM: Crude protein (65.34%), crude lipid (15.04%), moisture (9.23) and ash (14.04)

[c]Full-fat HI larvae: Crude protein (44.95%), crude lipid (30.21%), moisture (9.23) and ash (10.72)

[d]Defatted HI larvae: Crude protein (51.74%), crude lipid (19.03%), moisture (13.54) and ash (15.01%)

[e]Nutritional composition of diets and ingredients were analysed as per the standard method of Association of Official Analytical Chemists(AOAC) (AOAC, 1995)

Table 8. 2 Fatty acid composition of experimental diets and ingredients such as PBM, FHI and DHI

	0PBM-0HI	70PBM-30HI	70PBM-30DHI	PBM*	FHI*	DHI
C12:0	1.01	10.61	16.44	0.09	43.05	31.13
C14:0	2.32	2.55	3.33	0.68	6.80	5.74
C15:0	0.71	0.22	0.25	0.14	0.15	0.38
C16:0	20.12	17.95	17.20	21.65	11.11	15.08
C16:1n7	2.80	3.88	3.70	5.01	1.55	3.81
C17:0	1.16	0.34	0.37	0.45	0.53	0.57
C18:0	6.75	5.52	4.99	7.07	3.51	3.99
C18:1cis+trans	20.50	34.70	29.40	40.88	16.05	20.12
C18:2 cis	10.17	15.38	15.50	16.09	12.23	9.33
C18:3n6	0.13	0.10	0.09	0.21	0.08	0.08
C18:3n3	2.22	3.19	3.20	2.41	1.65	3.19
C18:4n3#	0.40	0.33	0.43	0.12	0.02	0.82
C20:0	0.36	0.16	0.14	0.24	0.09	0.13
C20:1	1.41	0.97	0.85	0.56	0.09	0.36
C20:3n6	0.17	0.17	0.14	0.53	0.02	0.08
C20:4n6	1.95	0.88	0.75	1.67	0.05	0.73
C20:5n3	3.66	0.93	1.03	0.16	0.05	1.95
C22:1n9	0.18	0.08	0.07	0.05	0.01	0.00
C22:2	0.00	0.13	0.12	0.01	0.00	0.10
C22:4n6#	1.80	0.06	0.07	0.04	0.00	0.11
C22:5n3#	1.13	0.27	0.22	0.34	0.01	0.16
C24:1	0.56	0.06	0.04	0.02	0.00	0.00
C22:6n3	19.39	0.61	0.52	0.25	0.01	0.40
∑SFA	33.12	37.94	43.59	31.00	67.86	58.50
∑MUFA	25.47	39.80	34.15	46.88	17.92	24.37
∑PUFA	41.40	22.26	22.24	22.18	14.23	17.08
∑n-3	26.95	5.38	5.45	3.33	1.75	6.57
∑n-6	4.05	1.21	1.05	2.45	0.15	0.99
∑n-3/∑n-6	6.66	4.44	5.17	1.35	11.67	6.61

*Fatty acid value for PBM and FHI from our earlier study (Chaklader et al., 2020b).

8.2.3 Feeding protocol and sampling

Four hundred barramundi weighing 0.56g each were obtained from the Australian Centre for Applied Aquaculture Research (ACAAR), Fremantle, Australia, transported using plastic bags holding oxygenated seawater and acclimated for twenty-four days at CARL. Fish were fed a commercial diet twice a day up to satiety during the acclimation period. Fish were starved for twenty-four hours before the commencement of the trial and visually healthy 225 fish were distributed randomly into nine tanks (25 fish/tank). During the acclimation and feeding trial, water quality parameters (salinity, dissolved oxygen, ammonia, nitrite, water temperature, and photoperiod) were maintained within the recommended ranges (Chaklader et al., 2020c). Each dietary treatment was fed in triplicate, and fish were hand-fed twice a day (9.00 and 18.00) to visual satiation for 56 days. Feed intake and mortalities, if any, for each experimental tank were recorded throughout the feeding trial to calculate feed efficiency and survival rate.

At the end of the trial (day 56), fish were fasted for 24 h, and fish from each tank were bulk weighed and counted to calculate the growth performance before being returned to the respective tank. To get sufficient blood for serum extraction, blood was collected from four randomly chosen anesthetized fish per tank by puncturing caudal vessels, allowed to clot at room temperature for 4 h, centrifuged at 3000 rpm for 15 min at 4 °C, and separated serum samples were immediately stored at -80 °C till biochemical analysis. The same two fish/tank were euthanized and then filleted, freeze-dried, pooled, and stored at -80 °C for fatty acids analysis. From the filleted fish and two more euthanized fish (4 fish/tank), viscera, liver, and intraperitoneal fat were collected and weighed to estimate the organo-somatic index. Also, liver, kidney, and intestine from four same fish were immediately stored at -80 °C for antioxidant activity analysis. A portion of liver and skin from three fish per tank was immediately preserved in 10% buffered formalin for histological analysis.

8.2.4 Growth and organo-somatic parameters

The following parameters were calculated to assess the growth performance and organo-somatic parameters-

Weight gain (WG, g)=[(Mean final weight-Mean initial weight)/(Mean initial weight)]

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

Feed intake (FI, g/fish
d^{-1}) = [(Dry diet given-Dry remaining diet recovered)/days of experiment)/ no. of fish]

Feed conversion ratio (FCR) = [(Dry feed fed)/(Wet weight gain)]

Condition factor (CF, %) = [Final body weight (g)/Body length cm^3)] ×100

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

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

8.2.5 Fatty acid analysis

The fatty acid of experimental diets and whole fillet freeze-dried barramundi muscle were analysed using methyl ester method as described by O'Fallon et al. (2007) and Chaklader et al. (2020a).

8.2.6 Serum biochemical analysis

Three biological replicates were processed on a AU480 Clinical Chemistry Analyser (Beckman Coulter Australia Pty Ltd, Lane Cove West, NSW) for serum biochemical panel analysis. Beckman Coulter clinical chemistry kits were used for the following panel components; albumin (catalogue code OSR6102), alanine aminotransferase (ALT; OSR6007), total bilirubin (OSR6112), calcium (OSR60117), Mg (OSR6189), inorganic phosphate (OSR6122), total protein (OSR6132), urea (OSR6134), Alb (OSR6102), cholesterol (OSR6116), creatine kinase (CK; OSR6179), creatinine (OSR6178), gamma-glutamyltransferase (GGT; OSR6020) and iron (OSR6186), while Randox kits (Randox Australia Pty Ltd, Parramatta, NSW) were used for glutamate dehydrogenase (GLDH; GL 441). Serum haptoglobin analysis was performed using the method of Eckersall et al. (1999).

8.2.7 Catalase activity analysis

For each replicate (tank)/per treatment, approximately 0.20 mg of liver, kidney, and intestine tissues were weighed and homogenized with 2 mL of chilled phosphate buffer solution (PBS). Immediately homogenized tissue was centrifuged at 10,000 ×g for 15 min at 4° C and supernatant was collected and stored at 80° C till analysis.

Catalase enzyme activities (CAT) in the liver, kidney and intestine homogenate of four fish/tank was analysed according to the manufacturer's company instruction (Bockit, BIOQUOCHEM SL, 33428 Llanera-Asturias, Spain).

8.2.8 Histological analysis of liver and skin

Liver and skin histological analysis was performed as described by our previous study (Chaklader et al., 2019) and (Ye et al., 2019a). Briefly, liver and skin tissues were dehydrated, cleared in xylene, and embedded in wax following fixation. Then the tissue wax was cut, stained with hematoxylin and Eosin (H&E) stain, and prepared for light microscopy. A set of liver tissues was

also stained with Oil Red O (ORO) to visualize lipid droplets and nuclei following the method of earlier study (Bancroft and Gamble, 2008). Following fixation, samples were placed into PBS for rinsing, and then place the sample was placed into 30% sucrose solution made up in PBS after rinsing for adequate cryoprotection. Samples were placed again in a 50:50 solution of 30% sucrose (in PBS) with optimal cutting temperature (OCT) overnight and then placed into pure OCT to remove any sucrose. 8µm thick sections were mounted on SuperFrost/Plus slides and the slides were directly filtered into 0.5% ORO in Dextrin and stained for 20 minutes. Hepatic vacuoles in H&E and lipid droplets in Oil Red O were quantified using Image J software.

8.2.9 *Vibrio harveyi* challenge test

Fish were subjected to *V. harveyi* infection following the protocol of earlier studies (Chaklader et al., 2020b). Briefly, 10 fish at the end of the trial were returned to the same tank after reducing water by 50% and fed with the same test diets for two days before injecting *V. harveyi*. *V. harveyi* culture was provided by Diagnostic and Laboratory Services, Department of Primary Industries and Regional Development (DPIRD), 3 Baron-Hay Court, South Perth WA 6151. 0.1 mL of PBS containing 1.1 x $10^{\wedge 8}$ CFU (colony forming unit)/mL of a pathogenic strain of *V. harveyi* were injected intraperitoneally and a set of symptoms of vibriosis such as a thick layer of mucous on the body surface, congestion of the fins, and haemorrhages and ulceration of the skin and muscle tissue were monitored daily for 7 days. Fish with vibriosis were euthanized as per the procedure of CARL SOP.

8.2.10 Statistical analysis

Before commencing statistical analysis, all dependent variables were subjected to Shapiro-Wilk's and Levene's tests to check the normality and equal variances. Individual fish was considered as an experimental unit to analyse organo-somatic indices, fillet fatty acid composition, serum biochemical and immune responses, and CAT, while the group of fish/tank were considered as an experimental unit to analyse growth performance. Dunnett's multiple comparisons test was applied to test the differences between each means of all dependent variables, and the significance was declared at $0.05 < P < 0.001$. The results were presented as the mean and standard error. The comparison in infection rate and median survival times among the test diets was performed by log-rank test and chi-square statistic, followed by the Kaplan–Meier survival method.

8.3 Results

8.3.1 Growth, feed utilization and biometry indices

The present study recorded a similar growth performance with respect to FM-based control diet in barramundi when FM was completely replaced by the mixture of FHI larvae meal and PBM (70PBM+30FHI) (Figure 8. 1A-C). In contrast, the mixture of partially DHI larvae meal and PBM (70PBM+30DHI) reduced the growth performance (Figure 8. 1A-C), and feed intake (Figure 8. 1D) with a higher FCR (Figure 8. 1E) compared to those fish fed 0PBM-0HI and 70PBM+30FHI diets. The survival rate (Figure 8. 1F) at the end of the trial was unaffected by any dietary treatment. Fish on 70PBM+30DHI showed a significantly lower condition factor (Figure 8. 1G) with a higher hepatosomatic index (Figure 8. 1H) than the 0PBM-0HI and fish fed 70PBM+30FHI. Viscerosomatic index (Figure 8. 1I) among the fish fed any test diets showed no variation.

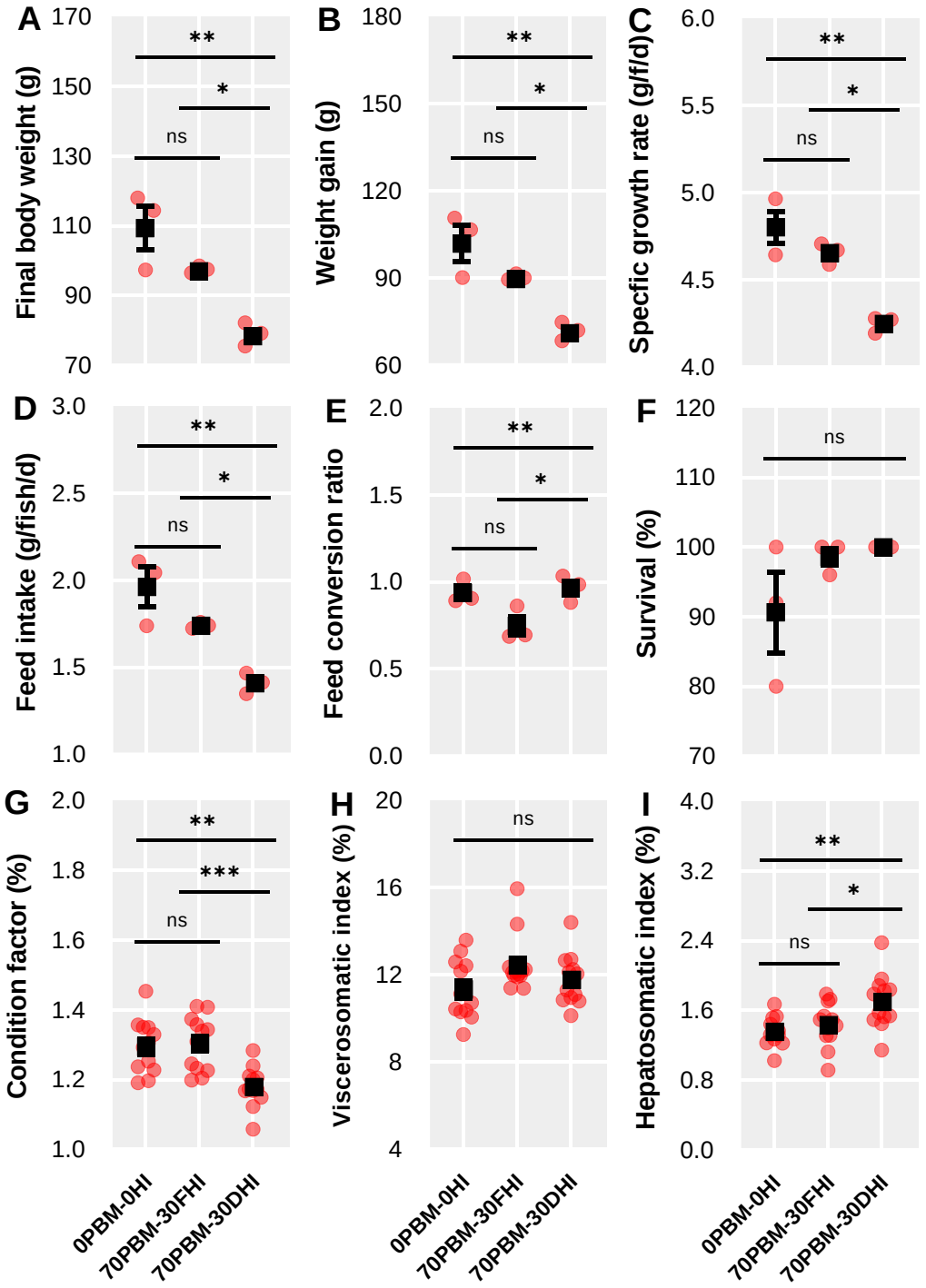


Figure 8. 1 Growth performance (n = 3), feed utilization (n = 3) and organo-somatic indices (n = 15) of barramundi fed mixture of FHI and partially DHI larvae meal and PBM for 56 days. Light orange round shape markers indicate the number of samples whereas black square shape marker indicates mean with standard error. Data were compared between experimental diets by Dunnett's multiple comparison test at $0.05 < P < 0.001$.

8.3.2 Fillet chemical composition and fatty acid profile

Individual fatty acid levels and summation of total fatty acid levels are presented in Table 8. 3 and Figure 8. 2. An increased level of C12:0 and C14:0 resulted in an elevation of the total saturated fatty acid (SFA) (Figure 8. 2A) in fish fed 70PBM-30FHI and 70PBM-30DHI than the 0PBM-0HI fed fish. The total monounsaturated fatty acid (MUFA) (Figure 8. 2B) content in the muscle of 70PBM-30FHI and 70PBM-30DHI was elevated due to the upsurge of C16:0, C16:1n7, and C18:1cis+trans. SFA and MUFA levels showed a similar trend in the diets (Table 8. 3). However, polyunsaturated fatty acid (PUFA) (Figure 8. 2C), n-3 PUFA (Figure 8. 2D), n-6 PUFA (Figure 8. 2E), and the ratio of n-3 and n-6 PUFA (Figure 8. 2F) decreased significantly in the muscle of fish fed 70PBM-30FHI and 70PBM-30DHI. This was mainly due to the lower contents of C20:5n3, C22:6n3, C22:5n3, and C22:4n6.

Table 8. 3 Fatty acid composition of barramundi fillet (skinless) fed the mixture of FHI and partially DHI larvae meal and PBM for 56 days. Results are represented as mean ± standard error (n = 3) and different superscript letters within the row indicates significant differences between 0PBM-0HI and test diets.

	0PBM-0HI	70PBM-30FHI	70PBM-30DHI	P-value
C12:0	1.39±0.85^{c}	10.29±0.15^{a}	6.52±0.35^{b}	0.000
C14:0	2.30±0.15^{c}	3.76±0.03^{a}	3.00±0.15^{b}	0.001
C15:0	0.54±0.03^{a}	0.28±0.00^{c}	0.24±0.00^{b}	0.000
C16:0	19.26±0.24^{a}	18.09±0.07^{b}	18.33±0.18^{b}	0.007
C16:1n7	3.35±0.15^{b}	4.17±0.03^{a}	4.13±0.17^{a}	0.006
C17:0	0.79±0.06^{a}	0.41±0.03^{b}	0.33±0.00^{b}	0.000
C18:0	6.39±0.12^{a}	5.37±0.09^{b}	5.94±0.30^{ab}	0.024
C18:1cis+trans	25.53±1.56^{b}	31.01±0.06^{a}	34.57±0.63^{a}	0.002
C18:2 cis	9.59±0.80^{b}	14.75±0.07^{a}	14.40±0.25^{a}	0.000
C18:3n6	0.42±0.09^{b}	0.76±0.03^{a}	0.99±0.03^{a}	0.002
C18:3n3	2.00±0.10^{b}	2.90±0.00^{a}	2.69±0.06^{a}	0.000
C18:4n3#	0.41±0.00^{b}	0.58±0.00^{a}	0.49±0.03^{b}	0.001
C20:0	0.25±0.03^{a}	0.18±0.00^{a}	0.19±0.00^{a}	0.422
C20:1	1.20±0.03^{a}	0.84±0.03^{b}	0.94±0.03^{b}	0.001
C20:3n6	0.36±0.03^{b}	0.42±0.00^{b}	0.66±0.09^{a}	0.011
C20:4n6	1.93±0.07^{a}	1.47±0.03^{a}	1.57±0.23^{a}	0.001
C20:5n3	2.51±0.20^{a}	1.37±0.00^{b}	1.31±0.10^{b}	0.001
C22:4n6#	1.58±0.25^{a}	0.16±0.00^{b}	0.14±0.03^{b}	0.001

C22:5n3#	1.71±0.10[a]	0.94±0.03[b]	1.07±0.12[b]	0.002
C24:1	0.32±0.03[a]	0.07±0.00[b]	0.03±0.03[b]	0.001
C22:6n3	17.38±2.65[a]	1.39±0.00[b]	1.74±0.22[b]	0.000

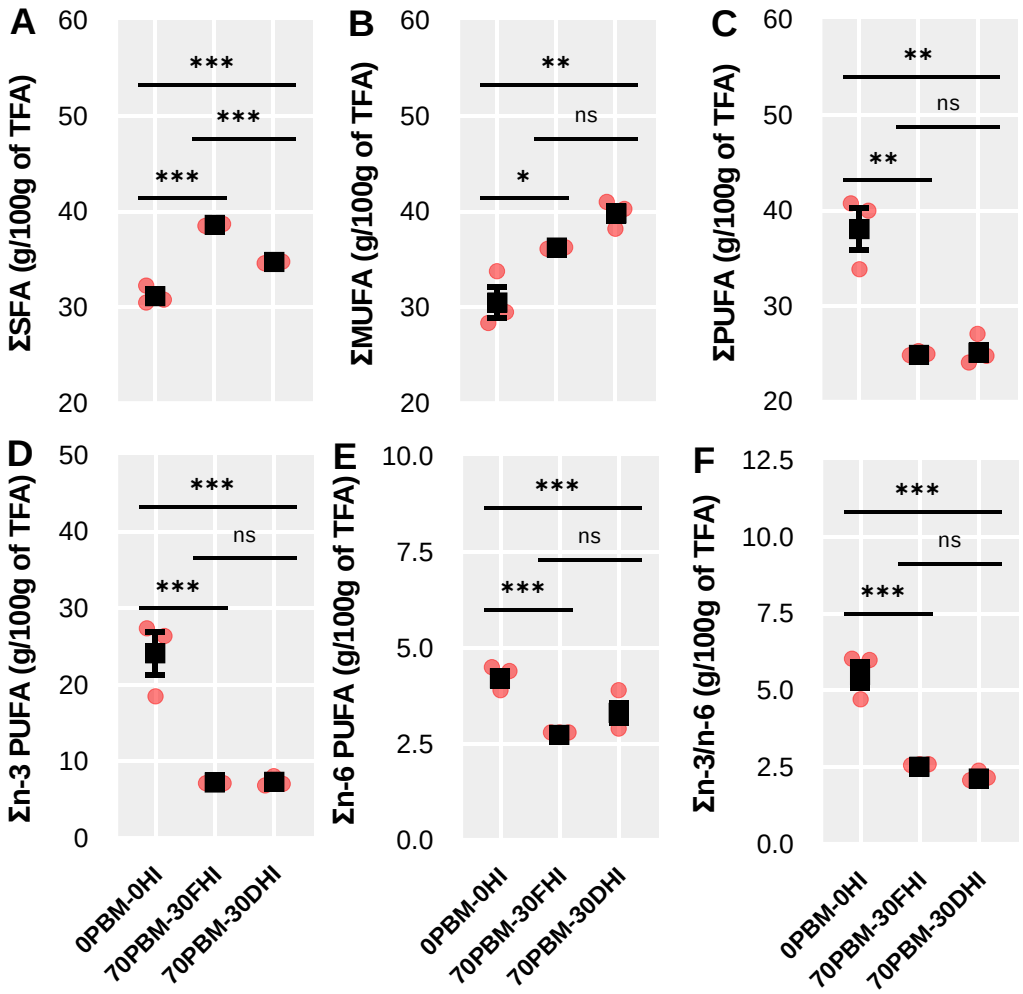


Figure 8. 2 Total SFA, MUFA, PUFA, n-3 PUFA, n-6 PUFA and n-3/n-6 ratio of barramundi fillet (skinless) when fed the mixture of FHI and partially DHI larvae meal and PBM for 56 days. Light orange round shape markers indicate the number of samples whereas black square shape marker indicates mean with standard error. Data were compared between experimental diets by Dunnett's multiple comparison test at $0.05 < P < 0.001$.

8.3.3 Serum metabolites

A panel of serum metabolite analysis in barramundi in response to the 0PBM-0HI diet and a mixture of FHI and partially DHI larvae meal and PBM is presented in Figure 8. 3(A-P). There were no significant differences between diets on the levels of CK (Figure 8. 3A), GGT (Figure 8. 3C), GLDH (Figure 8. 3D), TB (Figure 8. 3E), urea (Figure 8. 3F), creatinine (Figure 8. 3G), calcium (Figure 8. 3H), magnesium (Figure 8. 3I), inorganic phosphate (Figure 8. 3J), cholesterol (Figure 8. 3K), total protein (Figure 8. 3L), albumin (Figure 8. 3M), iron (Figure 8. 3O) and AG ratio (Figure 8. 3P) at the end of the feeding trial. However, barramundi fed 70PBM-30DHI showed a higher level of ALT (Figure 8. 3B) than 0PBM-0HI. The haptoglobin level (Figure 8. 3N) increased significantly in barramundi fed 70PBM-30FHI than in 0PBM-0HI.

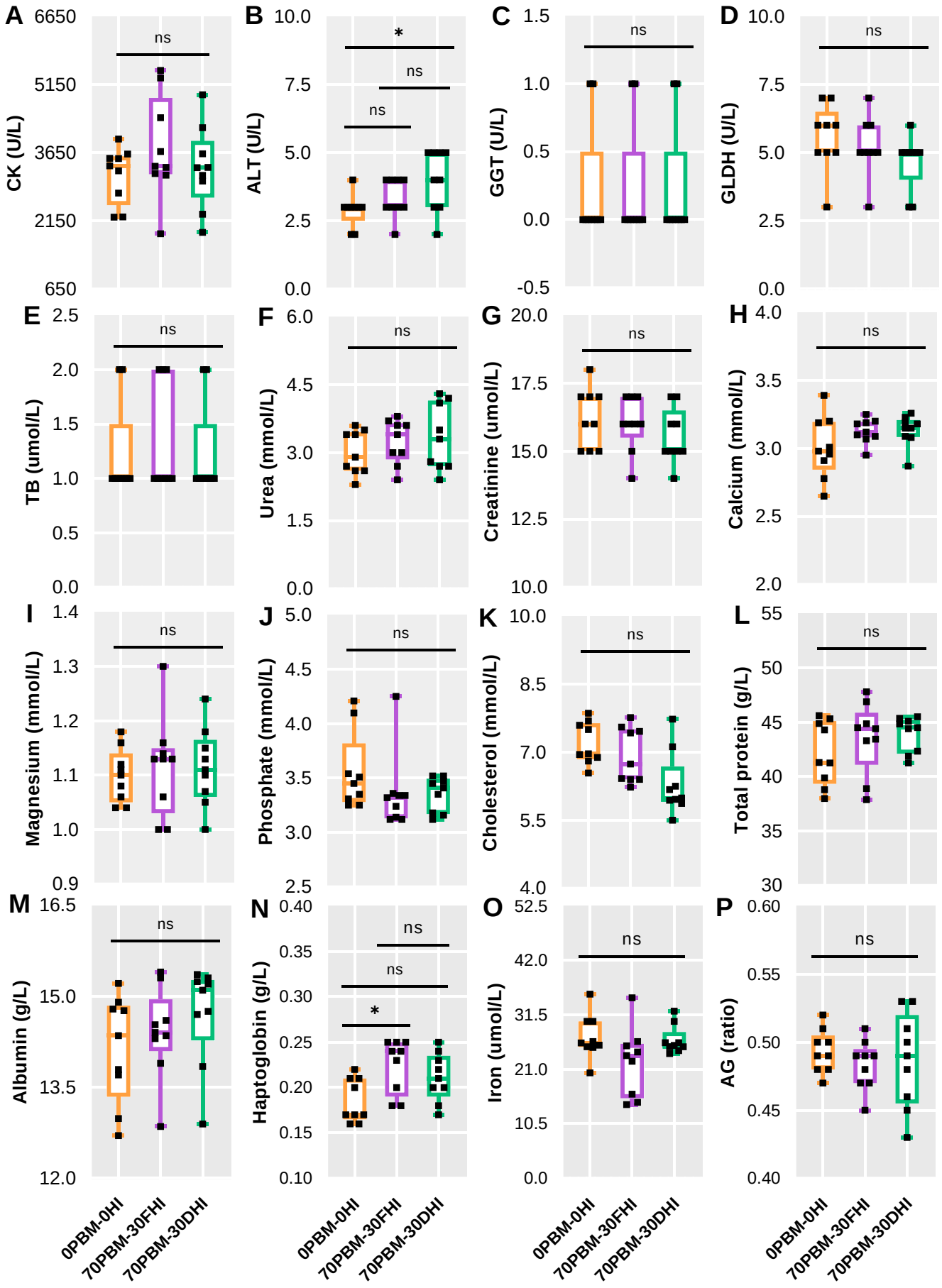

A
CK (U/L)
6650
5150
3650
2150
650
ns
B
ALT (U/L)
10.0
7.5
5.0
2.5
0.0
*
ns
ns
C
GGT (U/L)
1.5
1.0
0.5
0.0
-0.5
ns
D
GLDH (U/L)
10.0
7.5
5.0
2.5
0.0
ns
E
TB (umol/L)
2.5
2.0
1.5
1.0
0.5
0.0
ns
F
Urea (mmol/L)
6.0
4.5
3.0
1.5
0.0
ns
G
Creatinine (umol/L)
20.0
17.5
15.0
12.5
10.0
ns
H
Calcium (mmol/L)
4.0
3.5
3.0
2.5
2.0
ns
I
Magnesium (mmol/L)
1.4
1.3
1.2
1.1
1.0
0.9
ns
J
Phosphate (mmol/L)
5.0
4.5
4.0
3.5
3.0
2.5
ns
K
Cholesterol (mmol/L)
10.0
8.5
7.0
5.5
4.0
ns
L
Total protein (g/L)
55
50
45
40
35
30
ns
M
Albumin (g/L)
16.5
15.0
13.5
12.0
ns
N
Haptoglobin (g/L)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
ns
ns
*
O
Iron (umol/L)
52.5
42.0
31.5
21.0
10.5
0.0
ns
P
AG (ratio)
0.60
0.55
0.50
0.45
0.40
ns
0PBM-0HI
70PBM-30FHI
70PBM-30DHI

Figure 8. 3 Serum creatine kinase (CK), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), glutamate dehydrogenase (GLDH), total bilirubin (TB), urea, creatinine, calcium, magnesium, inorganic phosphate, cholesterol, total protein, albumin, haptoglobin, iron and albumin and globulin (AG) ratio of barramundi (n = 9) fed mixture of FHI and partially DHI larvae meal and PBM for 56 days. Multiple comparisons between experimental diets were performed by Dunnett's multiple comparison test.

8.3.4 Immune response and antioxidant activity

Serum lysozyme (Figure 8. 4A) and bactericidal activity (Figure 8. 4B) were unaffected by test diets whilst serum CAT (Figure 8. 4C) and liver CAT (Figure 8. 4D) activity decreased significantly in barramundi fed 70PBM-30DHI when compared with the 0PBM-0HI and 70PBM-30FHI. Meanwhile, the kidney (Figure 8. 4E) and intestinal CAT (Figure 8. 4F) were unaffected by fish fed either 0PBM-0HI or test diets.

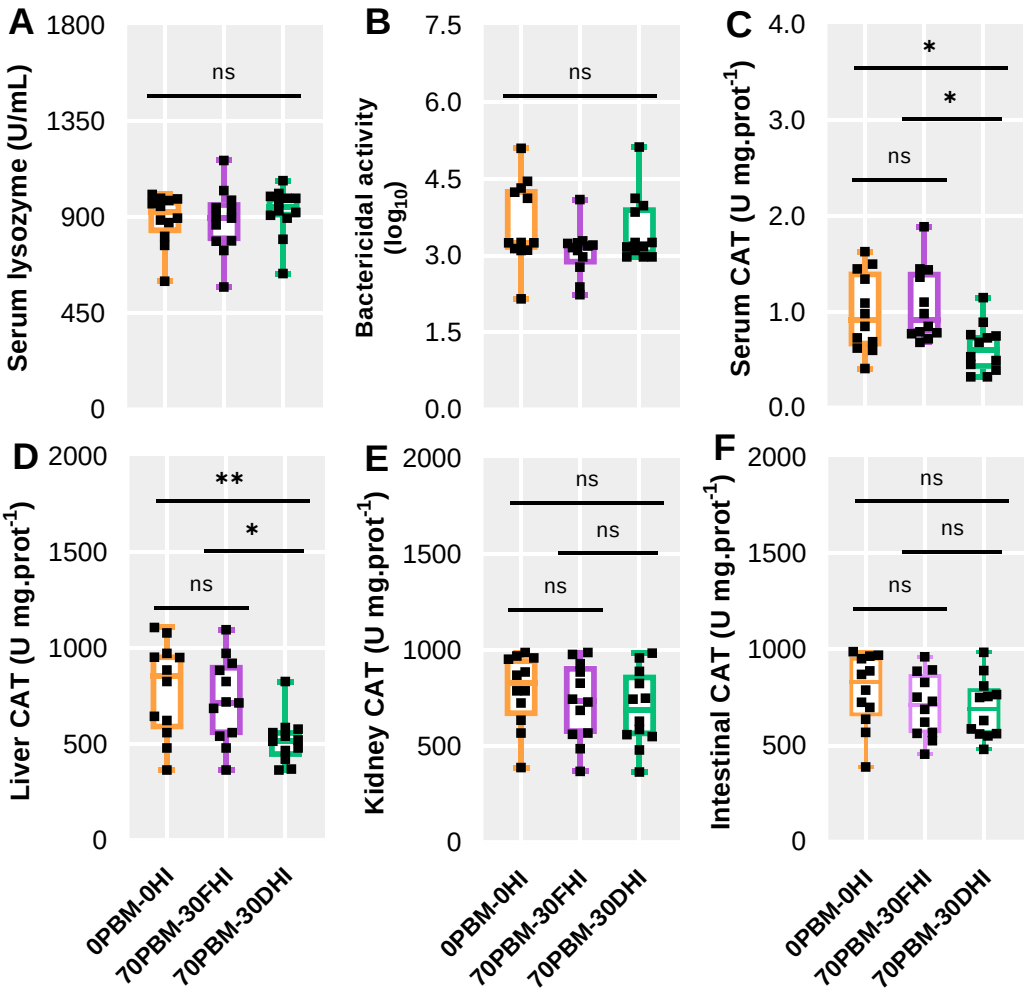


Figure 8. 4 Lysozyme, and bactericidal activities and serum, liver, kidney and intestinal CAT activities of barramundi (n = 12) fed the mixture of FHI and partially DHI larvae meal and PBM

for 56 days. Multiple comparisons between experimental diets were performed by Dunnett's multiple comparison test.

8.3.5 Skin mucosal barriers

The skin micrograph of barramundi fed different diets is presented in Figure 8. 5(A-C). Barramundi fed on 70PBM-30DHI recorded significantly thinner skin epidermis than 0PBM-0HI whilst no difference between 0PBM-0HI and 70PBM-30FHI dietary treatments was observed (Figure 8. 5D). The number of goblet cells in the skin of barramundi fed 70PBM-30DHI decreased significantly with respect to 0PBM-0HI and 70PBM-30FHI (Figure 8. 5E). Meanwhile, barramundi fed on 70PBM-30FHI showed a similar number of goblet cells when compared with the 0PBM-0HI (Figure 8. 5E).

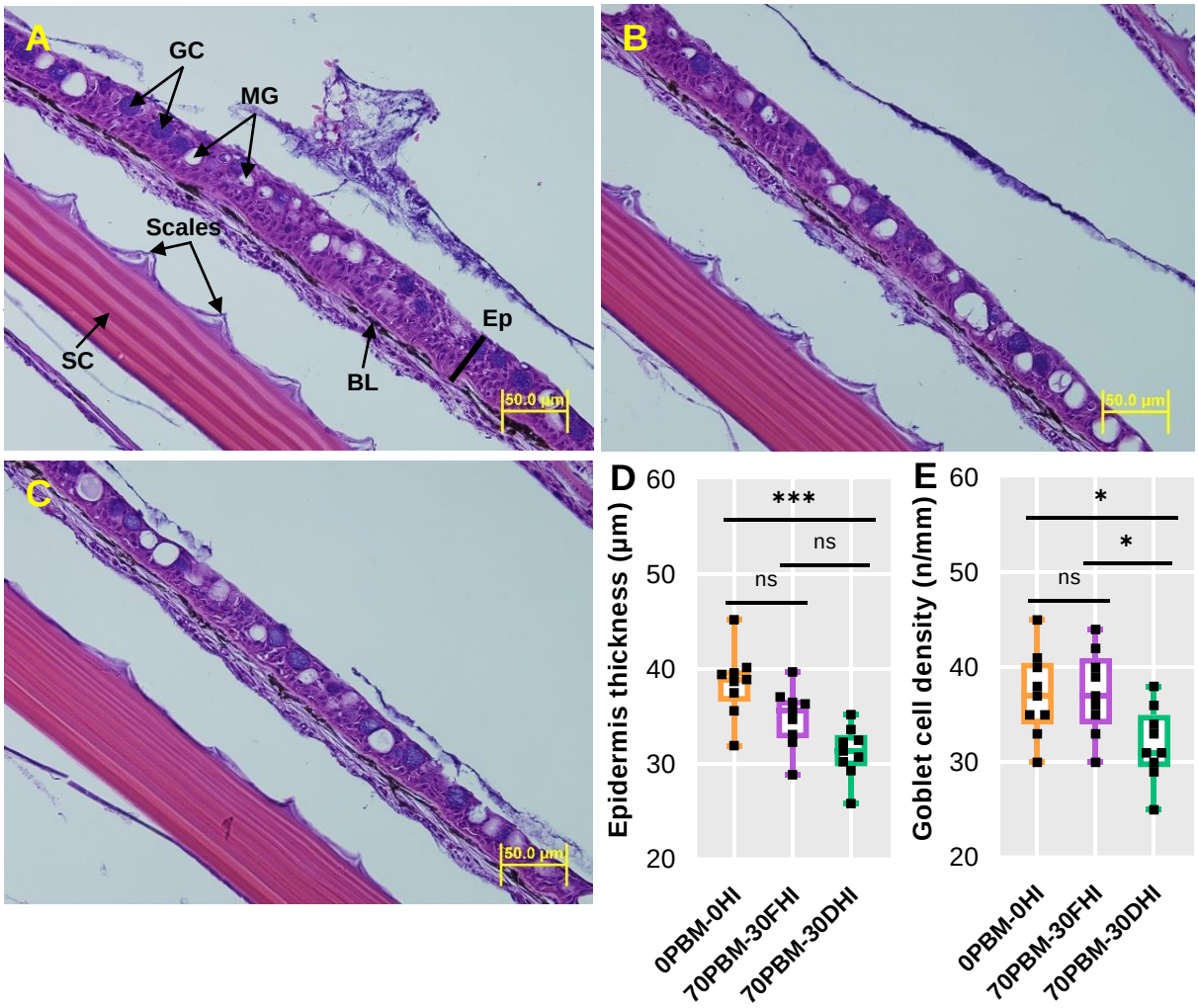


Figure 8. 5 Representative light microscopy of skin stained with H&E (A-C) of juvenile barramundi the mixture of FHI and partially DHI larvae meal and PBM for 56 days. Each box and whisker is the mean of three technical and six biological replicates. Multiple comparisons between

experimental diets were performed by Dunnett's multiple comparison test. Ep, epidermis; GC, goblet cells; MG, mucous gland; BL, basal layer and SC, stratum compactum.

8.3.6 Histopathology of liver

Liver histopathological changes stained with H&E and ORO in response to test diets were presented in Figure 8. 6(A-F). H&E stained micrographs showed the negative effect of the mixture of PBM and DHI larvae, manifested by the high occurrence of cytoplasmic vacuolation in the liver (Figure 8. 6C). Similarly, ORO staining showed an increased amount of lipid droplets in the liver of barramundi fed 70PBM-30DHI (Figure 8. 6F). These results were statistically proved by measuring the relative areas for hepatic vacuoles in H&E sections (Figure 8. 6G) and lipid droplets in ORO sections (Figure 8. 6H).

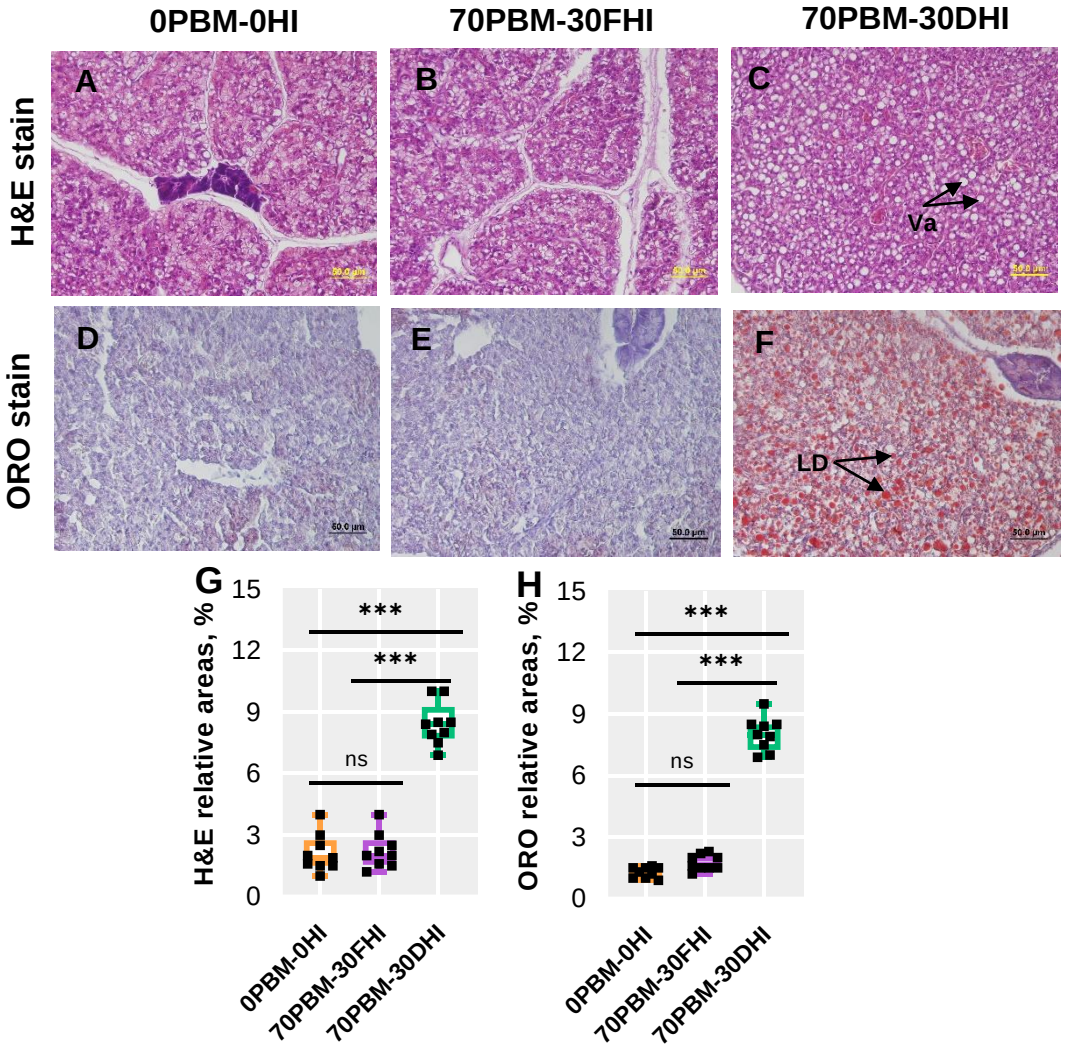


Figure 8. 6 Representative light microscopy of liver stained with H&E (A-C) and ORO (D-F) of juvenile barramundi the mixture of FHI and partially DHI larvae meal and PBM for 56 days. Relative area for hepatic vacuoles (Va) in H&E staining and lipid droplets (LD) in ORO staining

of the liver from juvenile barramundi fed test diets for 56 days. Each box and whisker is the mean of three technical and six biological replicates. Multiple comparisons between experimental diets were performed by Dunnett's multiple comparison test.

8.3.7 Infection rate against *V. harveyi*

Compared to 70PBM-30FHI fed fish, Kaplan-Meier survival analysis showed a lower proportion of asymptomatic fish fed 70PBM-30DHI where 50% fish were infected within 5 days (Figure 8. 7). However, there were no significant differences between 0PBM-0HI and test diets.

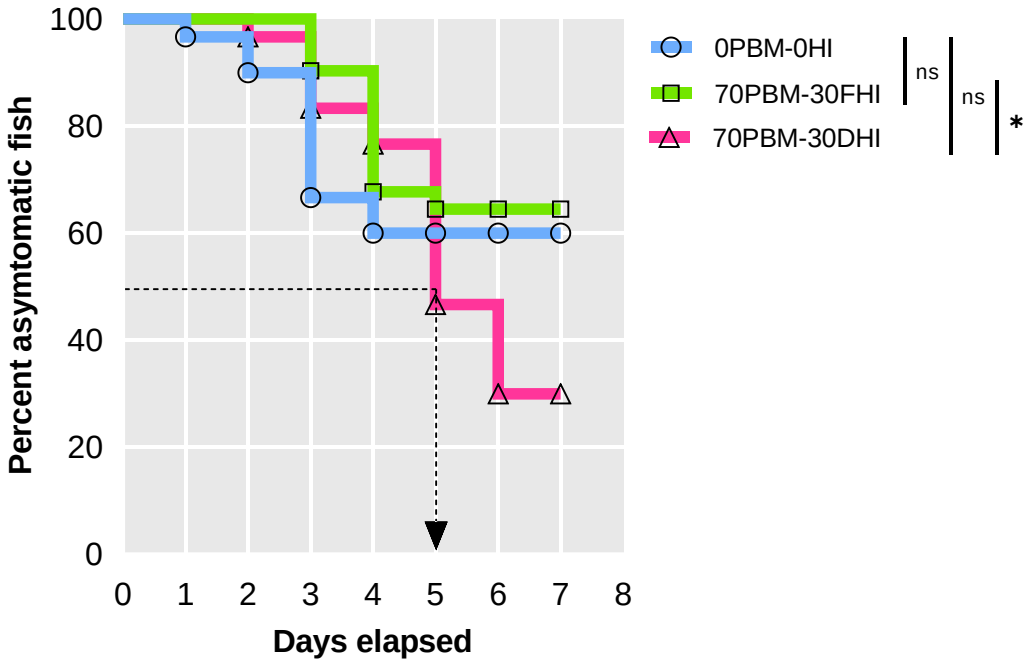


Figure 8. 7 The proportion of asymptomatic fish to *V. harveyi* when fed the mixture of FHI and partially DHI larvae meal and PBM. The dotted line indicates the median survival time when 50% of fish was infected. Multiple comparisons among the test diets were performed by Mantel-Cox log rank test, performed by Kaplan Meier survival method at $p < 0.05$.

8.4 Discussion

A reliable non-FM protein supply to ensure the continued development of sustainable aquaculture is required to hedge the volatility of global FM supply. Substantial research efforts have been able to replace FM partially (> 50%) at a commercial scale for most omnivorous and carnivorous species (Klinger and Naylor, 2012). However, the total replacement of FM with an alternative protein source, including PBM, has been reported to impose a negative effect on the growth performance of barramundi (Chaklader et al., 2020a; Chaklader et al., 2019) and other marine carnivorous fish (Dawson et al., 2018; Shapawi et al., 2007). The current research is a first attempt to compare the efficacy of FHI and DHI larvae meal when mixed with PBM protein to entirely replace the FM protein in barramundi diets. The growth improvement in fish fed 70PBM+30FHI over 70PBM+30DHI may be explained by the presence of AMPs and a high share of lauric acid in non-defatted HI larvae. HI larvae contain a high share of low molecular weight peptides, mainly between 20 to 245 kDa which are considered to act as a fish attractant (Mintah et al., 2020; Rawski et al., 2020), while lauric acid also plays a key role in stimulating immune response and stabilizing the microbiome of fish (Bruni et al., 2018; Lee et al., 2020; Terova et al., 2019). Complementary effects of FHI larvae in soybean and PBM-based diets have recently been reported in rainbow trout, *Oncorhynchus mykiss* (Kumar et al., 2020; Randazzo et al., 2021b), and barramundi (Chaklader et al., 2019; Chaklader et al., 2020b). Similarly, the replacement of FM with FHI (5-30%) larvae meal improved the growth, feed efficiency and gut health in Siberian sturgeon, *Acipenser baerii* (Rawski et al., 2020; 2021) whilst the inclusion of 50% FHI and DHI larvae retarded the growth, feed utilization, digestibility coefficients and gut health in the same species (Caimi et al., 2020a; Zarantoniello et al., 2021b). These results suggested that 30% FHI larvae meal may be worked as a functional material in sturgeon diet and also in barramundi. Defatting may reduce lauric acid content, degrade antimicrobial protein, and increase chitin share which could affect the nutritional and functional value of HI larvae meal. Such possible alteration together with the high inclusion of PBM might affect the growth performance in the current study. It, therefore, seems possible that the physical and chemical process involved in the defatting process can also degrade the protein biological value and functional benefits of AMPs, chitin, and fatty acid composition and this deserves further study.

In the present study, diet significantly influenced the total SFA, MUFA, PUFA, n-3, and n-6 PUFA of the fish flesh. The inclusion of HI larvae was mainly associated with significantly higher SFA content in barramundi muscle, reflecting that HI larvae are rich in SFAs (C:12, lauric acid; C:14, myristic acid, and C:16, palmitic acid). A similar result was observed in our earlier study in barramundi (Chaklader et al., 2020b) and has also been reported in other fish species such as Jian

carp, *Cyprinus carpio* var. Jian (Li et al., 2016b) and rainbow trout (Renna et al., 2017) fed HI larvae meal. The elevated total MUFA in barramundi fed the mixture of PBM, and FHI and DHI larvae were related to a higher dietary MUFA content. Several studies have already described the high amount of MUFA in PBM and poultry oil (Higgs et al., 2006; Panicz et al., 2017; Parés-Sierra et al., 2014; Zapata et al., 2016). Similarly, PBM-based diets increased the retention of MUFA in barramundi flesh (Chaklader et al., 2020b; Chaklader et al., 2020c). However, the lower retention of PUFA is likely to be due to the low content of individual omega-3 and omega-6 fatty acids, which were aligned with the total PUFA content in barramundi fed exclusively PBM (Chaklader et al., 2020a; Siddik et al., 2019a). Similarly, the decrease in essential fatty acids resulted in reduced retention of total PUFA in juvenile gilthead seabream, *Sparus aurata* (Sabbagh et al., 2019), juvenile black seabass, *Centropristis striata* (Sullivan, 2008) and coho salmon, *Oncorhynchus kisutch* (Twibell et al., 2012) when fed PBM based diets.

The micro-anatomical structure of skin mucosa, including epidermis containing goblet cells, serves as a physical barrier by forming a mucus layer, and has been considered the first line of defense against external aggressions in fish (Cabillon and Lazado, 2019; Chaklader et al., 2020b; Xu et al., 2013). Hence, manipulation of skin mucosal function through dietary modification is an important area of research to improve the overall health of fish. Dietary changes have been reported to influence the skin mucosal barrier functions in Atlantic salmon, *Salmo salar* (Leclercq et al., 2020), and barramundi (Chaklader et al., 2020c). The negative influence of 70PBM-30DHI on skin mucosal barriers of barramundi in the present study contradicts with our earlier study in which supplementation of 10% full-fat HI larvae meal with PBM improved the skin barrier functions, in particular, epidermis thickness and neutral mucins producing goblet cells (Chaklader et al., 2020b). The findings from our earlier study, together with no changes in skin barriers in barramundi fed 70PBM-30FHI than control, may suggest that full-fat HI larvae are functionally more active than defatted HI larval meal.

PBM at the total exclusion of FM induced the reduction of glycogen, multifocal necrosis, and vacuoles in the liver of barramundi (Chaklader et al., 2020a; Chaklader et al., 2019; Gupta et al., 2020; Siddik et al., 2019a) and other fish species such as hybrid grouper, *Epinephelus fuscoguttatus* ♀×*Epinephelus lanceolatus* ♂ juveniles (Zhou et al., 2020). Similarly, the mixture of DHI larvae and PBM influenced the hepatic steatosis, which attenuated significantly in barramundi fed the mixture of FHI larvae and PBM. The ameliorative effect of FHI larvae on PBM may be interpreted as being due to the higher amount of medium-chain fatty acids, in particular, lauric acid (C12:0), a major saturated fatty acid which is quickly oxidized by animals rather than stored in the liver (Kumar et al., 2020) and has led to the reduction of enterocyte steatosis in

Atlantic salmon (Weththasinghe et al., 2021a). A complementary effect of FHI larvae on soybean meal has also been observed in rainbow trout, characterized by enlarged bile ducts, which indicates more release of bile for lipid digestion (Kumar et al., 2020). Feeding low to moderate levels of FHI larvae meal and raw full-fat HI larvae paste for 7 weeks reduced the enterocyte steatosis of pyloric caeca of Atlantic salmon (Weththasinghe et al., 2021a). Besides lauric acid, we cannot exclude the potential contribution of choline, an important bioactive component rich in HI larvae like other insects, which assist in the transport of lipid across the intestinal mucosa of salmon (Krogdahl et al., 2020). Also, HI larvae meal and oil reduced the intraperitoneal fat index along with the reduction of adipocyte cell size, indicating more lipid metabolism in barramundi (Chaklader et al., 2019) and juvenile Jian carp (Li et al., 2016b). In contrast, it has been reported that FHI larvae induced hepatic steatosis in rainbow (Bruni et al., 2020b) and zebrafish (Zarantoniello et al., 2018) but it is noteworthy that pathological response was dose-dependent. Hepatic steatosis in the liver of barramundi fed 70PBM+30DHI can also be ascribed to the high inclusion of PBM protein and chitin content in HI larvae that may be increased during the defatting process. Feeding 25% of DHI larvae meal for 78 days caused severe and diffuse fatty changes in the liver of rainbow trout (Elia et al., 2018) while feeding 20% of DHI larvae meal alone or 30% of DHI larvae meal in combination with PBM did not influence lipid accumulation in the liver of gilthead seabream, *Sparus aurata* (Randazzo et al., 2021a). Hence, further work should consider the effect of the graded levels of lauric acid and various other bioactive components of HI larvae on the liver health and other physiological responses of fish.

A set of serum biochemical panels has been widely used and acknowledged as a valuable tool to evaluate the effect of aquafeed ingredients on the nutritional status and health status of different fish species. Increased ALT levels in 70PBM+3DHI fed barramundi supported the potential for liver damage, signifying that the mixture of defatted HI larvae meal and PBM protein could hamper liver tissue integrity. ALT is an important liver enzyme that often leaks abnormally into the blood if there is cell damage of the liver and bile obstruction (Fawole et al., 2020). Such negative results were inconsistent with the results of Wang et al. (2019b), Abdel-Tawwab et al. (2020), and Fawole et al. (2020) who found a lower level of this enzyme or no change in different fish species when fed different level of DHI larvae meal. The high inclusion of PBM may suppress the ameliorative effectiveness of DHI larvae meal as higher inclusion levels of PBM elevates the level of serum aminotransferase in barramundi (Chaklader et al., 2020a; Chaklader et al., 2019) and other fish species (Ye et al., 2019a). What is more, defatting may increase the chitin share which has been reported to influence the lipid accumulation in the liver (Zarantoniello et al., 2018). Haptoglobin, a rarely investigated serum biochemical metabolites in fish nutritional studies, produced by the liver similar to ALT, binds oxygenated cell-free hemoglobin to prevent the

accumulation of free radicals, which result in hemoglobin mediated tissue damage (Carter and Worwood, 2007). Also, haptoglobin level has been found to elevate to prevent inflammatory response (Matute-Blanch et al., 2018; Quaye, 2008; Sadrzadeh and Bozorgmehr, 2004) and mediate leukocyte activation, recruitment, migration, and migration along with modulating cytokines patterns and tissue repair (Jayasinghe et al., 2015; Wang et al., 2001). The elevated level of haptoglobin in 30PBM-30FHI indicates that FHI larvae meal may stimulate the anti-inflammatory response and prevent the oxidative damage induced by higher inclusion of PBM. In fact, Kumar et al. (2020) described the anti-inflammatory potency of FHI larvae in rainbow trout when supplemented with soybean meal. However, the potentiality of HI larvae in stimulating inflammatory-relevant cytokine expression induced by the higher inclusion of alternative animal protein sources requires further study to corroborate with observed serum biochemical responses.

A complementary effect of FHI larvae and oil-supplemented soybean meal on immunity has been reported in rainbow trout, manifested by an elevated level of lysozyme and peroxidase activity (Kumar et al., 2020). Similarly, DHI larval meal improved the immune response of yellow catfish, *Pelteobagrus fulvidraco* due to the contribution of chitin (Xiao et al., 2018). In the present study, neither FHI nor DHI larvae meal in PBM had an effect on the lysozyme and bactericidal activity of barramundi. However, our earlier studies (Chaklader et al., 2019; Chaklader et al., 2020b) reported an elevated level of lysozyme activity in barramundi when supplemented with 10% full-fat HI larvae. This study combined with our earlier studies (Chaklader et al., 2019; Chaklader et al., 2020b) and Kumar et al. (2020) suggests that the inclusion of higher-level HI larvae may exceed the optimum dose of chitin required for the stimulation of immune responses of barramundi.

The liver is a major lipid accumulation site in many marine fish species, and oxidative damage and cell death have been associated with lipid accumulation (Christian et al., 2013; Kawano and Cohen, 2013; Zhao et al., 2019). CAT produced in most of the tissues converts hydrogen peroxide, a molecule associated with oxidative damage, into oxygen and water, and elevated levels have been correlated with mitigating oxidative stress. A number of studies have reported the antioxidant potential of HI larvae meal in African catfish, *Clarias gariepinus* (Fawole et al., 2020), Jian carp (Li et al., 2017a), and juvenile Japanese seabass, *Lateolabrax japonicus* (Wang et al., 2019b). Such effects were not observed in the current study as measured by CAT activity both in the liver and serum, and indeed CAT levels were lower in 70PBM-30DHI, indicating that the mixture of DHI larvae and PBM protein mixture imposed oxidative stress on the barramundi. Similarly, our earlier study found an elevated level of MDA and a low level of GPx activity which are associated with oxidative stress in barramundi fed 100PBM (Chaklader et al., 2020a). The observed lack of

negative on the antioxidant activity of barramundi fed 70PBM-30FHI could be due to the inclusion of FHI larvae, attributed by different functional molecules such as lauric acid, polysaccharides (silkose and dipterose), and AMPs.

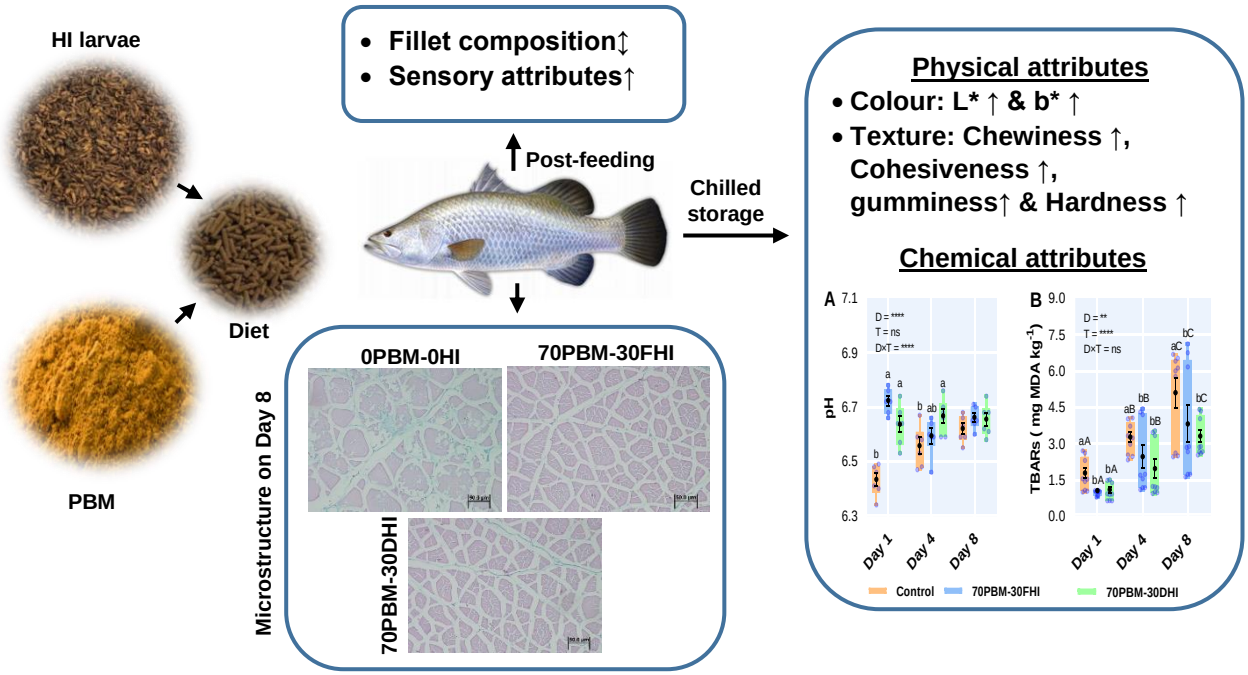
A recent study identified and purified a novel bioactive polysaccharides termed as silkrose from an insect, *Bombyx mori* that activated the innate immunity of penaeid prawns against *Vibrio penaecida* (Ali et al., 2018). Similarly, Elhag et al. (2017) obtained novel AMP genes from HI larvae with a high potential inhibitory activity on various Gram-positive bacteria, Gram-negative bacteria, and fungi. The inhibitory response of HI inclusion against bacteria has already been described in barramundi (Chaklader et al., 2019; Chaklader et al., 2020b) and other fish species including European seabass, *Dicentrarchus labrax* (Abdel-Latif et al., 2021), and rainbow trout (Kumar et al., 2020) when fed HI larvae meal alone or mixed PBM and plant protein-based diets. The predicted underlying reasons were the presence of chitin, AMPs, and polysaccharides. In the present study, the high infection rate in barramundi fed 70PBM-30DHI compare to 70PBM-30FHI could be due to the application of defatting which may increase the chitin share and degrade the functional properties of HI larvae (Rawski et al., 2020). No differences between 70PBM-30FHI and the 0PBM-0HI could be due to the inclusion of FHI larvae meal. The ability of FHI larvae to complement PBM and plant protein has already been proven by different studies (Chaklader et al., 2019; Chaklader et al., 2020b; Chaklader et al., 2021c; Kumar et al., 2020). However, further study is needed to evaluate how different functional molecules in HI larvae activate the immune system of fish or if defatting could degrade the functional properties of HI larvae.

In summary, barramundi can tolerate full FM replacement with a mixture of FHI larvae and PBM as proven by growth performance, biometry indices, and feed utilization, although there is a reduction in the polyunsaturated fatty acid content in the fish flesh. Also, serum haptoglobin level increased in barramundi fed the mixture of FHI larvae and PBM with no response on serum immunity and antioxidant activity. However, the mixture of DHI larvae and PBM affected the growth, feed utilization, liver health, and antioxidant capacity of barramundi. Despite having some positive effects of the mixture of FHI larvae and PBM on barramundi, further study is needed to enrich HI larvae fatty acid by changing feeding substrate that would improve the retention of essential fatty acids in barramundi flesh.

CHAPTER 9: Valorised full-fat and defatted *Hermetia illucens* protein combined with poultry by-product protein improves fillets quality traits of farmed barramundi, *Lates calcarifer*

(Under review in Food Control)

Graphical Abstract



Abstract

The physicochemical quality and shelf-life of fillets from barramundi which were fed for 56 days on a mixture of poultry by-product meal (PBM), full-fat (FHI), and defatted (DHI) *Hermetia illucens* (HI) larvae meal was investigated. Fillets compositions, and sensory evaluation were conducted to obtain baseline information. Physicochemical changes determined via quality index (QI), texture, microstructural histology, colour, drip loss, lipid oxidation, and pH in eight-day shelf life were then investigated under simulated retail display refrigerated conditions. Results indicated that PBM-HI diets enhance texture regardless of storage time and retarded microstructure degradation. Besides, colour traits were also found to be influenced by diet and storage time. *In vitro* data consensus with a sensory evaluation conducted in this study where fish fed PBM-HI diets scored higher in all parameters. Lipid oxidation also found to be lower in PBM-HI fillets, showing positive results in enhancing fish quality with a more sustainable non-fishmeal diet.

Keyword: *Lates calcarifer,* black soldier fly larvae, sensory evaluation, texture and colour, lipid oxidation, chilled storage

9.1 Introduction

In human nutrition, fish is an important source of lipid, in particular, omega-3 type polyunsaturated fatty acids (PUFA) and is also a good source of vitamins and minerals, therefore consumption is highly associated with promoting neural development in infants and preventing chronic diseases such as hypertension and cardiovascular diseases (Codabaccus et al., 2013; Haliloğlu et al., 2004; Han et al., 2018; Quiñones et al.; Xu et al., 2021). Such nutritional benefit along with the stagnation in volume of captured landing fisheries have resulted in an expansion of aquaculture to meet up the protein demand for increasing population and predicted growing population by 2050.

Barramundi, *Lates calcarifer*, also commonly known as Asian seabass is one of the important aquaculture carnivore fish species due to its rapid growth, high nutritional value, and suitability to cultivate both in freshwater and seawater environments, reaching 76,843 tonnes with a global value of USD 320 million (Chaklader et al., 2020a; Simon et al., 2019). Barramundi requires a high amount of protein which is largely obtained from fishmeal (FM). The sustainability of using FM in carnivore aquafeed production is questionable because of a stable production worldwide estimating roughly 6.3 million metric tons annually since 1980 (Dawson et al., 2018; FAO, 2012). For instance, barramundi farming in Australia has highly relied on imported FM which results in a 40% cost related to diet therby reducing the profitability (Williams et al., 2003b). Nutritional study of barramundi has been conducted since 1980 (Glencross, 2006) but alternative protein ingredients which can replace FM entirely with no deleterious effect on barramundi health, while

improving fillet quality are still limited. Poultry by-product meal (PBM) due to similar protein and amino acid profile as FM, availability, and relatively low cost could be a suitable protein ingredient for carnivorous aquafeed production particularly for barramundi (Badillo et al., 2014; Chaklader et al., 2020c; Chaklader et al., 2021c; Cheng and Hardy, 2002). PBM has been investigated on a wide variety of finfish and shellfish fish species with various success (Galkanda-Arachchige et al., 2020). A number of recent studies have allowed exclusive or even 100% FM substitution with PBM in the barramundi diet along with the supplementation of fish protein hydrolysate and insect larvae (Chaklader et al., 2019; Chaklader et al., 2020b; Chaklader et al., 2020c; Chaklader et al., 2021c). Those studies have extensively investigated the potential effects of PBM on growth performance, physiological and immunological response but have largely overlooked the food aspect of fillet which is commonly investigated via sensory, physical, and chemical evaluation (Cao et al., 2019).

Based on the concept of circular economy and onward the approval of utilizing insects in aquadiets European Union (Commission Regulation (EU) 2017/893) (Moutinho et al., 2020), research on including insects meal in the diet of different farmed fish at the expense of FM has increased over the recent years. This is mainly because of several considerable environmental and potential economic benefits including fast growth and reproduction rates together with minimal requirement of water and land, and a capability to biodegrade low-quality waste into high-value protein ingredients, and the produced frass can be used as soil ameliorants (Barroso et al., 2014; Henry et al., 2015; Van Huis et al., 2013). *Hermetia illucens* (black soldier fly) larvae meal have been regarded as one of the promising feedstuffs for aquafeeds as it contains comparable protein (~ 60%) and amino acid profile to that of FM, is a good source of energy (10-30% lipids), vitamins and minerals (Nogales-Mérida et al., 2018) and contains functional molecules including antimicrobial peptide and novel polysaccharides such as silkose and dipterose (Chaklader et al., 2019; Chaklader et al., 2020b). The inclusion of HI larvae meal in the diet of several fish species has been evaluated without negative effects on the growth and other physiological response. However, assessing the organoleptic characteristics beyond the growth and other physiological performance is important while introducing a new protein source into fish feed (Sealey et al., 2011) since the fillet sensory characteristics as well as physical and chemical composition during shelf life could be influenced by dietary manipulations (Borgogno et al., 2017; Iaconisi et al., 2017; Secci et al., 2019). Fillet quality of farmed fish is overwhelmingly influenced by pre-harvest factors such as feed, post-harvest processing, and storage method (Carton and Jones, 2014; Erikson et al., 2012; Jones and Carton, 2015; Wilkinson et al., 2008). Post-harvest storage time is associated with oxidative, enzymatic, and/or bacterial process which can degrade flavour, taste, and aroma qualities and also promote changes to texture, color, pH and nutritional status,

potentially making final fish products unfit for marketing and consumption (Bonilla et al., 2007; Gram, 1992; Gram and Huss, 1996; Jones and Carton, 2015; Olafsdottir et al., 1997). A recent study reported that full-fat HI larvae meal (6.5 and 19.5%) did not compromise fillet quality traits and shelf life at +2/4 ◦C juveniles European seabass, *Dicentrarchus labrax* (Moutinho et al., 2020). There is however limited information relating to the changes in barramundi fillet quality traits in response to the mixture of insect meal and PBM and during a shelf life study. Hence, the present study investigated the effect of the complete replacement of FM with the mixture of FHI + DHI larvae and PBM on the quality traits of barramundi fillet in terms of nutritional composition, texture, microstructure of muscle tissues, and lipid oxidation.

9.2 Materials and methods

9.2.1 The ethical statement, diets, animal husbandry, and fish sampling

The experimental procedure involving fish handling and care were carried out in strict compliance with the guidelines and regulations of Australia. The experimental protocols were reviewed and approved by the Curtin University Animal Ethics Committee (ARE2018-37).

The experimental design, diet formulation, and animal husbandry are illustrated in our previous trial (Chaklader et al., 2021a). Briefly, three test diets containing identical proximate composition were formulated to test FM based diet as control (0PBM-0HI) and two test diets containing 70% PBM and 30% FHI larvae meal (70PBM-30FHI) as well as 70% PBM and 30% partially DHI larvae meal (70PBM-30DHI) were formulated to replace FM completely. Ingredient composition and amino acid composition of test diets and ingredients such as PBM, FHI, and DHI are presented in Table 9. 1.To perform the trial, 225 fish with similar weight were stocked into 9 tanks with 25 fish per tank and fish were fed diet in triplicate. After 56 days feeding trial, fish were starved for 24 h and eight fish per tank were sacrificed by euthanasia (AQUI-S, 175 mg/l)) where six fish per tank (18 fish/treatment) were used for fillet quality traits analysis and two fish per tank (6 fish per tank) were used for amino acid analysis.

Among samples allocated for fillet quality analyses, all fishes were filleted by a trained researcher to ensure consistency between samples. Per each fish, one of the fillets was subjected to individual quick freezing (IQF) by immersing in liquid nitrogen with constant stirring and stored immediately at – 80 ˚C until the commencement of sensory evaluation. Another fillet was packed in a labelled resealable polyethylene bag on ice and transported within one hour to the simulated retail display storage.

Table 9. 1 Ingredients composition and nutrient content of test diets and different ingredients such as PBM, FHI and DHI. Ingredients and proximate composition were previously published in our article (Chaklader et al., 2021a)

	Test diets			Ingredients		
Ingredients (g/100g)	0PBM-0HI	70PBM-30HI	70PBM-30DHI	PBM*	FHI*	DHI
FM	72.00	0.00	0.00	-	-	-
PBM	0.00	50.50	50.50	-	-	-
Canola oil	1.00	0.50	0.50	-	-	-
Full-fat HI	0.00	35.00	0.00	-	-	-
Defatted HI	0.00	0.00	27.83	-	-	-
Corn/wheat starch	7.00	5.90	11.00	-	-	-
Lecithin - Soy (70%)	1.00	2.00	1.00	-	-	-
Vitamin C	0.05	0.05	0.05	-	-	-
Dicalcium Phosphate	0.05	0.05	0.05	-	-	-
Wheat (10 CP)	16.90	4.00	7.07	-	-	-
Vitamin and mineral premix	0.50	0.50	0.50	-	-	-
Salt (NaCl)	1.00	1.00	1.00	-	-	-
Cod liver oil	0.50	0.50	0.50	-	-	-
Nutritional Composition (%)						
Dry matter	89.95	90.21	90.16	-	-	-
Crude protein	47.88	47.94	48.06	-	-	-
Crude lipid	12.59	13.61	13.96	-	-	-

Ash	11.23	11.69	11.51	-	-	-
Essential amino acid (% of total amino acid)						
Arginine	6.40	6.66	6.64	7.32	5.45	5.31
Histidine	3.24	2.52	2.52	2.96	3.27	3.17
Threonine	4.71	4.16	4.18	4.19	4.35	4.39
Lysine	7.50	6.64	6.57	6.58	7.10	6.78
Methionine	2.88	2.10	2.07	2.23	2.02	1.97
Valine	5.64	5.63	5.62	4.92	6.40	6.70
Isoleucine	4.91	4.51	4.47	4.07	4.86	5.03
Leucine	8.16	7.52	7.44	7.37	7.59	7.63
Phenylalanine	4.55	4.36	4.33	4.09	4.67	4.88
Non-Essential amino acid (% of total amino acid)						
Serine	4.39	4.27	4.30	4.34	4.48	4.43
Glycine	7.96	9.62	9.79	10.13	5.86	6.01
Aspartic acid	9.57	9.41	9.21	8.50	10.42	10.93
Glutamic acid	14.42	14.73	14.94	13.83	13.42	13.46
Alanine	7.10	7.28	7.36	6.61	6.59	7.06
Proline	5.65	6.87	7.01	6.63	6.24	6.22
Tyrosine	2.92	3.71	3.55	2.97	5.97	6.05

9.2.2 Proximate composition

9.2.2.1 Crude protein

Crude protein analyses were conducted following AOAC (1995) method. In brief, 1 g of sample was weighed, and placed along with a Kjeldhal catalyst tablet and glass bead in a digestion tube. 8 mL of sulfuric acid and phosphoric acid mixture was then added to the tube, followed by 4 mL of 35 % hydrogen peroxide. After that, digestion occured Kjeltec digester block (Foss Tecator 2020, Hoganas, Sweden) at 420 ˚C. To determine the nitrogen content, the digested sample was distilled using a Kjeltec distilling unit (Foss Tecator 1002, Högänas, Sweden). The distillate was collected into a flask with 25 mL of boric acid indicator containing bromocresol green and methyl red. Following, titration with hydrochloric acid was conducted on final distillate and a conversion factor of 6.25 was used for calculation in crude protein content.

9.2.2.2 Crude fat

The petroleum ether extraction method described in AOAC (1995) was used to determine crude fat content. A Soxhlet unit (Extraction unit E-816, BÜCHI Labortechnik AG, Flawil, Switzerland) was used to extract fat from samples. Extracted fat was then dried at 105 ˚C until constant weight was achieved and crude fat percentage was calculated by dividing the weight of extracted fat over the raw material fat.

9.2.2.3 Ash

AOAC (1995) ash determination method was used in this study. The sample was weighed prior to and after heating at 550 ˚C using a muffle furnace (Thermolyne muffle furnace, model 48000, Thermo Fisher Scientific Inc, Iowa, USA) overnight. Ash content was calculated by dividing post-heating sample weight over initial sample weight.

9.2.2.4 Moisture

AOAC (1995) standard method was used to determine the moisture content of samples. The sample was weighed before and after oven drying until constant weight at 105 ˚C. Moisture content was then calculated by diving post-drying weight over pre-drying weight.

9.2.2.5 Amino acid analysis

Amino acid profile of test diets, DHI larvae, and barramundi muscle were analysed following the standard protocol of Australian Proteome Analysis Facility (APAF), Macquarie University, Sydney NSW 2109. Amino acid analysis was performed using liquid hydrolysis in 6M HCl to release the amino acids from the protein followed by quantification using pre-column derivatisation reversed-phase (RP) HPLC procedure. The samples were weighed out in duplicate

or single into hydrolysis vials and 5 mL of 20% HCl was added. These were then incubated at 110 ºC for 24hrs. After hydrolysis, the samples were derivatised using AccQTag reagent (Waters Corporation, Milford, MA, USA) (Cohen and Michaud, 1993; Cohen and De Antonis, 1994) and then analysed using a high-resolution RP column on a UPLC system with 10 min run times. Equipment - The instrument consisted of an ACQUITY UPLC system with a UV detector (Waters Corporation, Milford, MA, USA) (Wheat et al., 2008). For all analyses, a Waters AccQTag Ultra column (BEH C18, 2.1 x 100mm; 1.7 µm) was used (Bosch et al., 2006). The column temperature employed was 57 ºC**,** the detection was at 260 nm, and the flow rate 0.7 mL/min. This method does not analyse for tryptophan or cysteine which are acid sensitive and require special conditions for analysis.

9.2.3 Sensory quality

Sensory evaluation was designed based on Gedarawatte et al. (2020) and Lawless Heymann (2010). Eleven participants who consume fish at least once every fortnight were recruited, consented, and trained according to AS 2542.1.3:2014 (Australia, 2014) and CAC-GL 31-1999 (Alimentarius, 1991). People with allergies, smoking habits, chronic health issues, visual impairment, respiratory issues, taste disorders, pregnant, are breast-feeding or on long-term medication were excluded from sensory evaluation to reduce confounding factors and risks. Screening on sensory sensitivity was then conducted prior to the sensory evaluation. After both screenings, nine participants in the age of 18 – 50, consist of 5 females and 4 males were considered to be eligible and included in the study as semi-trained panellists.

In order to understand the quality of barramundi in retail display, visual appearance, odour, and overall acceptability were first evaluated using the Labelled Magnitude Scale (Kalva et al., 2014) in raw barramundi samples. After that, to investigate the cooked quality of fish fillets, the same samples given to the panellists were sous-vided at 74 ˚C for 6 min in vacuum-sealed boil-in pouches. Samples were served to panellists within 15 min. Appearance, odour, texture, taste and overall acceptability were then accessed in similar manner to raw samples.

The sensory trial was conducted in strict compliance with the Australian Code for the Responsible Conduct of Research and National Statement on Ethical Conduct in Human Research. The trial was reviewed and approved by the Curtin University Human Research Ethics Committee prior to all training and sensory evaluations conducted in this study (Approval number: HRE2020-0689).

9.2.4 Simulated Retail display protocols

Freshly filleted fish samples were unpacked and immediately placed on ice inside an uncovered polystyrene box. The box was then placed in a 4 ˚C refrigerator to simulate conditions in retail

display. Melted ice was drained daily with new ice replaced. Six fillets (2 fillet per tank) from different fishes were analysed per treatment during each day on day 0, 4, and 8 post-slaughter.

9.2.4.1 Physical parameters

9.2.4.1.1 Texture profile analysis (TPA)

For texture, all measurements were conducted under room temperature at 24.5 ˚C and fish fillets were tempered for 30 mins prior to analyses. The portion between pelvic and anal fin of barramundi was sampled. Fish flesh along the lateral line including dorsal and ventral portion was then compressed using texture analyzer TVT 6700 (PerkinElmer, Inc., Waltham, Middx, USA) equipped with a 20 kg load cell and a 25 mm flat-ended cylindrical probe. Two consecutive cycles of 50 % compression with 5 sec in between were conducted under a constant speed of 50 mm/min. Six texture parameters: hardness, cohesiveness, adhesiveness, springiness, gumminess, and chewiness were obtained from each analysis using Bourne (1978) calculation methods and TexCal 5.0 instrumental software.

9.2.4.1.2 Microscopic observation of fillet tissues

One portion of muscle at day 1, day 4, and day 8 stored at 4˚ C were cut from three fillets/treatment and immediately fixed in 10% buffered formalin before dehydrating with a series of alcohol. Then the samples were embedded in paraffin, sectioned to 5 µm thickness, and stained with Alcian blue for observation under a light microscope according to the standard histological procedure (Chaklader et al., 2019).

9.2.4.1.3 Drip loss

Fish samples were weighted daily from day 0 to day 8. Drip loss was then calculated by diving weight loss over the initial weight of the fresh fish sample and expressed in percentage.

9.2.4.1.4 Colour

Surface colour coordinates (L*, a*, b*) were obtained using HunterLab ColorFlex (Hunter Association Laboratory Inc., Reston, VA, US) with glass port insert. Colourimeter was calibrated prior to usage in the session. Colour was measured in the ventral and dorsal portion of barramundi fillet along the lateral line.

9.2.4.1.5 Quality Index (QI)

QI constructed by Fuentes-Amaya et al. (2016) was used to determine the quality of barramundi fillets. In brief, the skin, appearance, and flesh of the fish were scored based on quality parameters

such as brightness, transparency, texture, blood colour, odour, and gaping. The sample was then given a score out of 10.

9.2.4.2 Chemical parameters

9.2.4.2.1 pH

AOAC (1995) method was used to determine the pH value of the barramundi sample. 1 g of fish was homogenized with 10 mL of distilled water and mixed using a rotary suspension mixer (Ratek, Boronia, Vic, AU) for 30 min. Three-scales calibrated Aqua-pH meter (TPS Pty, Ltd, Brendale, QLD, AU) was then used to determine the pH values of the aliquot.

9.2.4.2.2 Lipid oxidation

The modified method by Raharjo et al. (1992) was used to determine 2-thiobarbituric acid reactive substances (TBARS) presented in the fish fillet. 40 mL of 5 % trichloroacetic acid (TCA) was added to 10 g of samples and homogenized along with 1 mL of 0.15 % 2,6-di-teri-butil-4-methylphenol (BHT) in ethanol. Homogenized sample was then filtered and adjusted to 5 % TCA to 50 mL. After that, 2 mL of sample was transferred to a screw cap test tube and 2 mL of 0.08 M 2-thiobarbutric acid (TBA) was added. The mixture was heated at 100 ˚C for 10 min to allow the reaction to occur. Absorbance was measured at 532 nm afterward. Concentration was calculated using standards prepared with 1,1,3,3-tetrathyoxypropane (TEP) in 20 % TCA at 1-10 µM concentration.

9.2.5 Statistical analysis

Tanks were considered as experimental units whereas fish represented the sample units. Results were represented as mean ± standard error (SE). Normality and homogeneity of variance for all data from individual observation were checked by Kolmogorov-Smirnov and Levene's tests, respectively. Results for proximate composition and amino acid profile were compared with the test diets with respect to control by one-way ANOVA. To determine the effect of diet and storage time and their interaction on the fillet texture and colour, two-way ANOVA was applied with diet and storage time as fixed factors. If the effect was significant, one-way ANOVA with Tukey post-hoc test was was subsequently performed for diet and storage to determine if the mean differed significantly among the test diets and storage time. Significant level in all cases was set $0.05 < P < 0.001$.

9.3 Results

9.3.1 Muscle composition

The moisture, protein, lipid, and ash content of barramundi fillet were unchanged at the end of the trial (Table 9. 2). Total essential amino acids were unaffected by the complete replacement of FM with the mixture of PBM and HI larvae meal (Table 9. 2). However, histidine was significantly lower in barramundi-fed PBM-HI-based diets than the 0PBM-0HI. Whereas in barramundi fed 70PBM-30DHI, lysine decreased significantly compared to 0PBM-0HI. The summation of non-essential amino acid in the fillet of barramundi on PBM-HI-based diets was similar to the fillet of barramundi fed 0PBM-0HI. Among all individual non-essential amino acids, serine was negative affected by PBM-HI-based diets compared to 0PBM-0HI.

Table 9. 2 Proximate composition and amino acid profile of the skinless whole fillet of barramundi fed complete replacement of FM with the mixture of PBM and FHI and DHI larvae meal. Values are the mean of six biological replicates ± standard error (SE). Mean with different superscript letters within the same row indicate significant differences while mean holding no superscript letters indicate no variation among the treatments. Means were compared by one-way ANOVA with Dunnett's multiple comparisons test at $0.05 < P < 0.001$.

	Test diets		
	0PBM-0HI	70PBM-30HI	70PBM-30DHI
Proximate composition (%, Wet basis)			
Moisture	76.10±0.25	76.71±0.42	76.70±0.41
Crude protein	20.58±0.56	19.82±0.03	20.33±0.30
Crude lipid	1.63±0.15	1.90±0.30	1.69±0.36
Ash	1.10±0.07	1.10±0.02	1.19±0.03
Essential amino acid (% of total amino acid)			
Arginine	6.30±0.02	6.32±0.03	6.33±0.03
Histidine	2.40±0.04[a]	2.28±0.02[b]	2.28±0.02[b]
Threonine	4.65±0.01	4.61±0.03	4.61±0.02
Lysine	9.71±0.02[a]	9.71±0.06[a]	9.49±0.13[b]
Methionine	3.22±0.03	3.19±0.03	3.17±0.02
Valine	5.37±0.02	5.31±0.02	5.34±0.02
Isoleucine	5.12±0.03	5.06±0.04	5.06±0.06
Leucine	8.49±0.03	8.46±0.04	8.43±0.06
Phenylalanine	4.62±0.03	4.64±0.02	4.64±0.03

Non-Essential amino acid (% of total amino acid)			
Serine	4.28±0.07[a]	4.15±0.03[b]	4.13±0.02[b]
Glycine	6.12±0.15	6.22±0.30	6.46±0.21
Aspartic acid	10.44±0.08	10.55±0.05	10.52±0.17
Glutamic acid	15.97±	16.12±	15.98±
Alanine	6.40±0.05	6.45±0.07	6.52±0.06
Proline	3.58±0.05	3.60±0.10	3.72±0.08
Tyrosine	3.35±0.01	3.33±0.06	3.33±0.02

9.3.2 Sensory evaluation

Fillet sensory attributes of barramundi fed the mixture of PBM and HI larvae meal are presented in Table 9. 3. The raw quality of barramundi fillets was improved in PBM-HI based diet, manifested by high merit points given by panellists for appearance, odour, and overall quality. A similar tendency was observed after cooking in all attributes but only cooked odour improved significantly in the fillets of barramundi fed PBM-HI based diets than the fillets of barramundi fed a control diet.

Table 9. 3 Sensory attributes of the fillet of barramundi fed complete replacement of FM with the mixture of PBM and FHI and DHI larvae meal. Values are the mean of nine biological replicates ± standard error (SE). Mean with different superscript letters within the same row indicate significant differences while mean holding no superscript letters indicate no variation among the treatments. Means were compared by one-way ANOVA with Dunnett's multiple comparisons test at $0.05 < P < 0.001$.

	Test diets		
	0PBM-0HI	70PBM-30FHI	70PBM-30DHI
Raw visual appearance	3.64±0.41[b]	5.88±0.46[a]	6.20±0.58[a]
Raw odour	3.56±0.29[b]	5.90±0.63[a]	5.89±0.55[a]
Raw overall quality	3.67±0.37[b]	5.57±0.55[ab]	6.10±0.67[a]
Cooked visual appearance	3.93±0.58	4.80±0.76	4.66±0.70
Cooked odour	3.55±0.36[b]	6.21±0.68[a]	5.71±0.39[a]
Cooked texture	4.90±0.61	6.05±0.60	6.33±0.63
Cooked taste	3.99±0.76	5.42±0.80	6.33±0.88
Cooked overall quality	3.48±0.72	5.43±0.79	6.02±0.82

9.3.3 Microstructure and texture profile of fillets

The changes in the microstructure of muscle tissues and texture profile including adhesiveness, chewiness, cohesiveness, gumminess, hardness, and springiness are presented in Figure 9. 1. Muscle structure at day 1 (Figure 9. 1A-C) and 4 (Figure 9. 1D-F) were unchanged in the fillet of barramundi fed any of test diets, manifested by tight attachment and regular shape of myofibrils together with a uniform distribution and distinct connective tissues. However, at day 8, severe muscle degeneration and atrophy in cells of 0PBM-0HI fed fish (Figure 9. 1G) while PBM-HI-based diets inhibited severe structural changes (Figure 9. 1H, I). Adhesiveness (Figure 9. 1J) and springiness (Figure 9. 1O) were not changed by diet and storage time while diets significantly changed the chewiness (Figure 9. 1K), cohesiveness (Figure 9. 1L), gumminess (Figure 9. 1M) and hardness (Figure 9. 1N) which were higher in the fillet of barramundi fed the mixture of PBM and FHI and DHI larvae meal. However, storage time did not influence on those parameters. There was no interaction between “diet” and “storage time” in all texture profile.

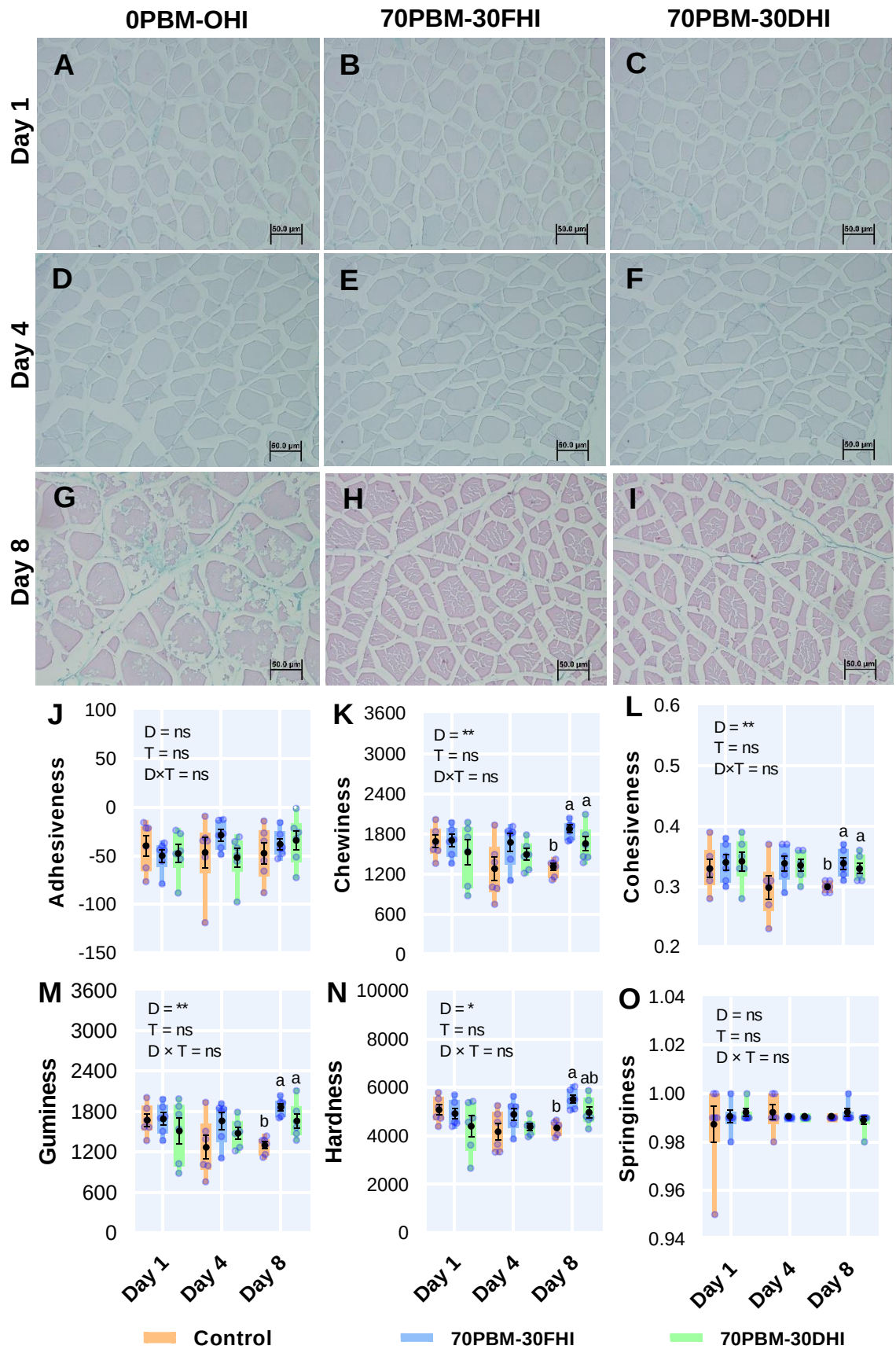


Figure 9. 1 Representative muscle histology at day 1 (A-C), day 4 (D-F) and day 8 (G-I) stored at 4° C and changes in the texture including adhesiveness (J), chewiness (K), cohesiveness (L),

gumminess (M), hardness (n) and springiness (O) of barramundi fed complete replacement of FM with the mixture of PBM and FHI and DHI larvae meal. Semi-transparent light blue round marker indicates individual data (n = 6), box-whisker of individual data and black marker denotes mean and standard error. Asterisks represents the significant difference at 0.05 < P < 0.0001 and ns denotes non-significant.

9.3.4 Drip loss

Drip loss was affected by diet, with a significantly higher drip loss in the fillet of barramundi fed PBM-HI based diet during the storage time (Figure 9. 2). Similarly, drip loss increased over the storage time in the fillet of barramundi-fed test diets. No interaction was observed between diet and storage time.

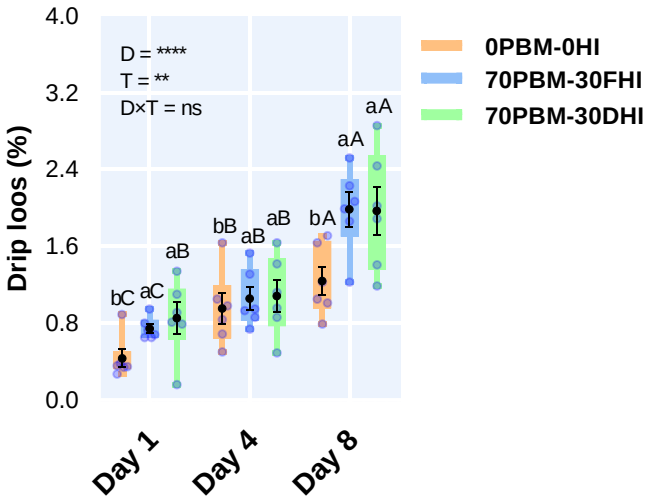


Figure 9. 2 Changes in drip loss after 8 weeks feeding trial and at day 1, day 4, and day 8 stored at 4˚ C of barramundi fed complete replacement of FM with the mixture of PBM and FHI and DHI larvae meal. Semi-transparent light blue round marker indicates individual data (n = 6), box-whisker of individual data and black marker denotes a mean and standard error. Lower cases indicate significant differences between the treatments while upper cases in the figure indicate significant differences between the storage days. Asterisks represents the significant difference at 0.05 < P < 0.0001 and ns denotes non-significant.

9.3.5 Skin colour

Two-way analysis showed that skin colors in terms of L* (brightness), a* (redness), yellowness (b*), and chroma were significantly influenced by the diet. L* at day 1 decreased significantly in the skin of barramundi fed 70PBM-30FHI compared to the skin of barramundi fed 0PBM-0HI and 70PBM-30DHI. Meanwhile, skin L* (Figure 9. 3A) was unchanged at days 4 and 8 when fed either

0PBM-0HI or test diets. Skin a* (Figure 9. 3B) was higher in barramundi fed 70PBM-30FHI and 70PBM-30DHI at days 1 and 8 while no variation at day 4 between the diets. On day 8, skin b* (Figure 9. 3C) decreased in barramundi fed 70PBM-30FHI whilst skin chroma (Figure 9. 3D) improved fed 70PBM-30FHI compared to control. However, there was no variation in skin b* and chroma between the test diets at 1 and 4. Meanwhile, storage time had no influence on the skin colours with no interactive effects.

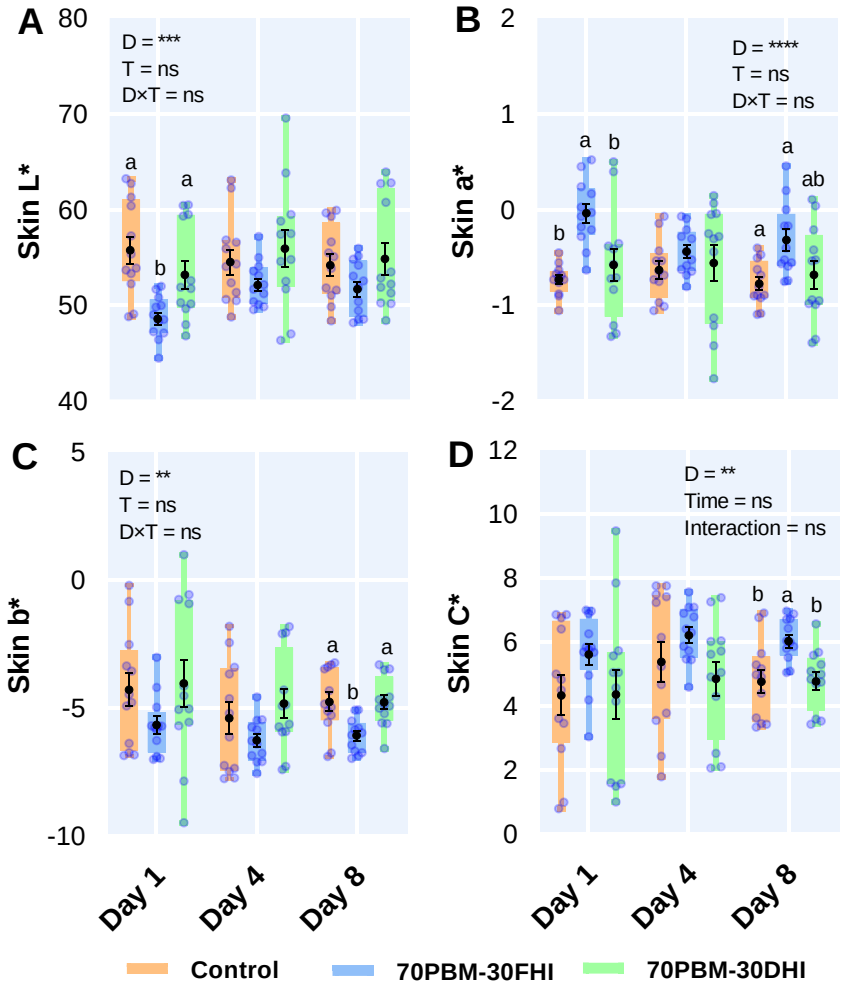


Figure 9. 3 Changes in the skin colour including lightness (L*), redness (a*), yellowness (b*) and chroma of after 8 weeks feeding trial and at day 1, day 4 and day 8 stored at 4° C of barramundi fed complete replacement of FM with the mixture of PBM and FHI and DHI larvae meal. Semi-transparent light blue round marker indicates individual data (n = 12), box-whisker of individual data and black marker denotes a mean and standard error. Asterisks represents the significant difference at $0.05 < P < 0.0001$ and ns denotes non-significant.

9.3.6 Flesh colour

Flesh colour in response to test diets and storage time are presented in Figure 9. 4. Two-way ANOVA showed that diet had a significant effect on flesh L* and b* (Figure 9. 4A, B), however, storage time influenced all colour attributes. On day 4 and 8, flesh brightness improved significantly in PBM-HI fed barramundi fillet (Figure 9. 4A) but flesh yellowness (Figure 9. 4C) increased only in barramundi fed PBM-HI based diets at day 8. Irrespective of diet, flesh lightness in all test diets increased with time while redness (Figure 9. 4A), yellowness (Figure 9. 4B), and chroma (Figure 9. 4D) decreased over the storage time.

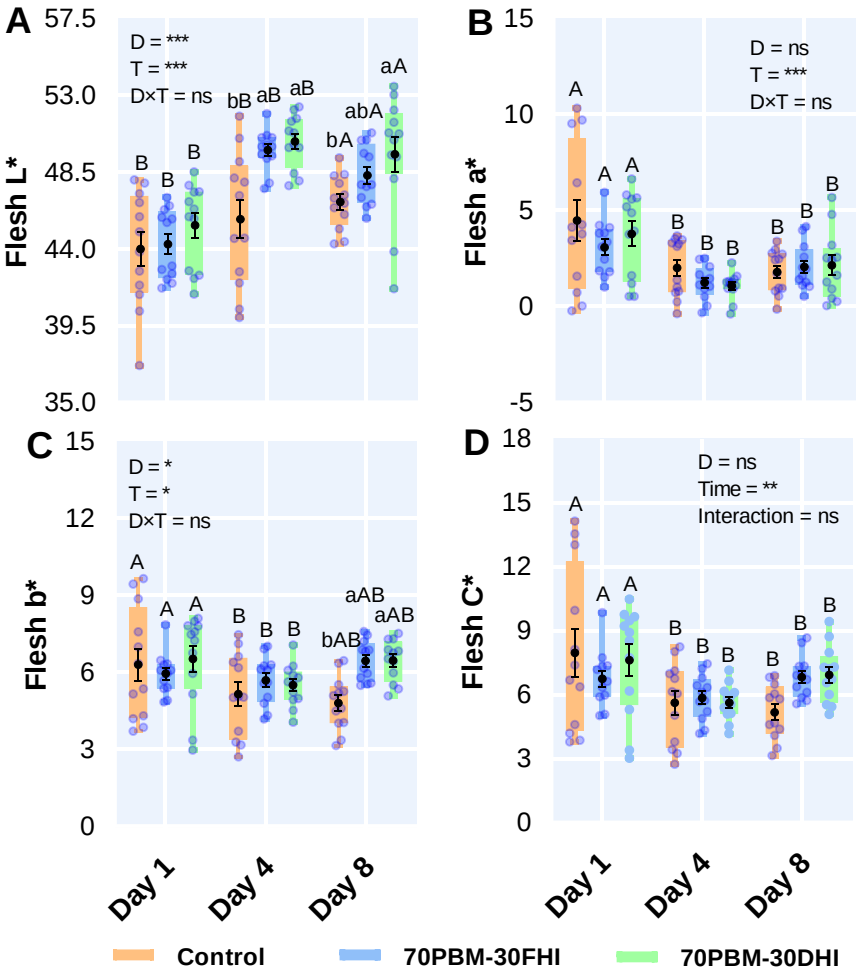


Figure 9. 4 Changes in the flesh colour including lightness (L*), redness (a*), yellowness (b*), and chroma (C*) of after 8 weeks feeding trial and at day 1 (A-C), day 4 (D-F), and day 8 (G-I) stored at 4° C of barramundi fed complete replacement of FM with the mixture of PBM and FHI and DHI larvae meal. Semi-transparent light blue round marker indicates individual data (n = 12), box-whisker of individual data and black marker denotes a mean and standard error. Lower cases indicate significant differences between the treatments while upper cases in the figure indicate

significant differences between the storage days. Asterisks represents the significant difference at $0.05 < P < 0.0001$ and ns denotes non-significant.

9.3.7 Quality index (QI) method

The changes in skin, appearance, and flesh of the barramundi based on quality parameters such as brightness, transparency, texture, blood colour, odour, and gaping in response to diets and storage time are presented in Table 9. 4. Diet had no significant effect on the QI while storage time influenced the QI attributes. There was no interaction between diet and storage time. The QI increased linearly with increasing storage time (Figure 9. 5), with a maximum demerit point 10 for 0PBM-0HI (Figure 9. 5A), 9 for 70PBM-30FHI (Figure 9. 5B), and 70PBM-30DHI (Figure 9. 5C), respectively.

Table 9. 4 Results of sensory parameters evaluated by QI method: skin brightness, appearance transparency, flesh texture, flesh blood, flesh odour and flesh gaping in barramundi fillet (n =6) fed PBM-HI based diets during chilled storage. Diet, D and Storage time, T.

		Test diets			Two-way ANOVA		
		0PBM-0HI	70PBM-30FHI	70PBM-30DHI	Diet	Time	Diet × Time
Skin brightness	Day 1	0.00 ± 0.00^{B}	0.00 ± 0.00^{B}	0.00 ± 0.00^{B}			
	Day 4	0.00 ± 0.00^{B}	0.00 ± 0.00^{B}	0.00 ± 0.00^{B}	0.33	0.00	0.34
	Day 8	1.33 ± 0.21^{A}	1.00 ± 0.00^{A}	1.17 ± 0.17^{A}			
Appearance transparency	Day 1	0.00 ± 0.00^{C}	0.00 ± 0.00^{C}	0.00 ± 0.00^{C}			
	Day 4	0.50 ± 0.22^{B}	0.33 ± 0.21^{B}	0.50 ± 0.22^{B}	0.83	0.00	0.94
	Day 8	1.00 ± 0.00^{A}	1.00 ± 0.00^{A}	1.00 ± 0.00^{A}			
Flesh texture	Day 1	0.17 ± 0.17^{B}	0.00 ± 0.00^{B}	0.17 ± 0.17^{B}			
	Day 4	0.83 ± 0.17^{A}	0.83 ± 0.17^{A}	0.67 ± 0.21^{A}	0.83	0.00	0.77
	Day 8	1.00 ± 0.00^{A}	1.00 ± 0.00^{A}	1.00 ± 0.00^{A}			
Flesh blood	Day 1	0.17 ± 0.16^{C}	0.00 ± 0.00^{C}	0.00 ± 0.00^{C}			
	Day 4	1.00 ± 0.00^{B}	1.50 ± 0.10^{B}	1.17 ± 0.13^{B}	0.22	0.00	0.20
	Day 8	1.83 ± 0.15^{A}	2.00 ± 0.21^{A}	1.83 ± 0.14^{A}			
Flesh odour	Day 1	0.00 ± 0.00^{C}	0.00 ± 0.00^{C}	0.00 ± 0.00^{C}			
	Day 4	1.00 ± 0.00^{B}	1.00 ± 0.22^{B}	1.00 ± 0.17^{B}	0.38	0.00	0.42
	Day 8	1.83 ± 0.17^{A}	2.00 ± 0.00^{A}	2.00 ± 0.00^{A}			
Flesh gaping	Day 1	1.50 ± 0.22^{B}	1.67 ± 0.21^{B}	1.17 ± 0.40^{B}			
	Day 4	2.00 ± 0.00^{A}	1.67 ± 0.21^{A}	1.83 ± 0.41^{A}	0.56	0.00	0.43
	Day 8	2.00 ± 0.00^{A}	2.00 ± 0.00^{A}	2.00 ± 0.00^{A}			

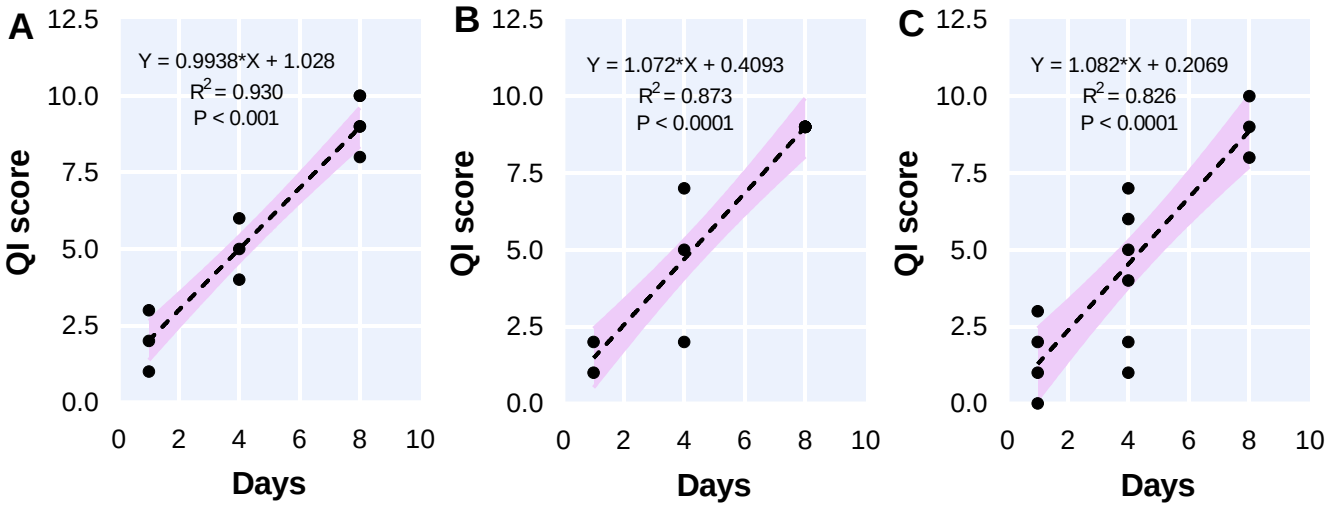


Figure 9. 5 Correlation between QI and storage time for test diets including 0PBM-0HI (A), 70PBM-30FHI (B) and 70PBM-30DHI (C).

9.3.8 pH and lipid oxidation

pH and lipid oxidation, measured by malondialdehyde (MDA) in response to test diets and storage time are presented in Figure 9. 6. Two-way ANOVA demonstrated that pH was influenced by test diets with a significant interaction between diet and storage time (Figure 9. 6A). pH increased significantly in the flesh of barramundi fed PBM-HI based diets at days 1 and 4 than the flesh of control fed barramundi. However, storage time did not influence the pH. Rancidity test evaluated by TBARs activity at all storage time was lower in the flesh of PBM-HI fed barramundi than the control (Figure 9. 6B). However, TBARs activity increased in all test diets with the storage time.

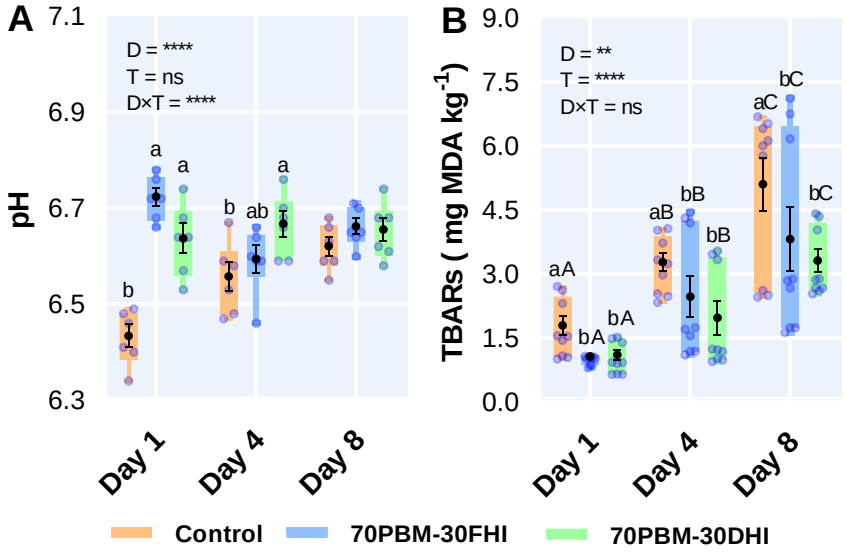


Figure 9. 6 Changes in the flesh pH and TBARS after 8 weeks feeding trial and at day 1, day 4, and day 8 stored at 4° C of barramundi fed complete replacement of FM with the mixture of PBM and FHI and DHI larvae meal. Semi-transparent light blue round marker indicates individual data (n = 9), box-whisker of individual data and black marker denotes a mean and standard error. Asterisks represents the significant difference at $0.05 < P < 0.0001$ and ns denotes non-significant.

9.4 Discussion

The retention of protein and essential amino acids of fish is related to dietary composition and has been used as one of the most sensitive indicators to evaluate the adequacy or inadequacy of the supply of amino acids (González-Rodríguez et al., 2016; Rodehutscord et al., 1995). The unchanged muscle amino acid profile of fish fed HI larvae based PBM diets in the present study were similar to the fillet amino acid profile of juvenile gilthead seabream, *Sparus aurata* fed 50 and 100% PBM (Sabbagh et al., 2019) and juvenile spotted rose snapper, *Lutjanus guttatus* fed PBM (up to 75%) (Hernández et al., 2014), however, inconsistent with the earlier study in our laboratory in which the retention of both essential and non-essential amino acids was impacted by the exclusive or complete inclusion of raw and bioprocessed PBM in the muscle of barramundi (Siddik et al., 2019a). No negative effect on the retention of amino acid could be ascribed to the inclusion of HI larvae meal which contains low molecular weight peptides mainly between 20 to 245 kDa, indicating more free amino acids (Rawski et al., 2020). It has been reported that free amino acids reach the intestine and are absorbed by enterocytes faster than the intact proteins (Chaklader et al., 2021c; Ganapathy, 1994; Ha et al., 2019).

The inclusion of PBM alone exclusively impacted the sensory quality of female tenches, *Tinca tinca* (Panicz et al., 2017) while meat meal did not affect the sensory quality of barramundi (Williams et al., 2003b). The highest score given by panellists to the raw and cooked fillets of barramundi-fed PBM-HI-based diets suggests that HI larvae containing chitin and other functional molecules might have improved fillet quality. Chitosan, the deacetylated form of chitin coatings has already been reported to improve the sensory quality of seafood (Fan et al., 2009; Farajzadeh et al., 2016; Mohan et al., 2012). Fillet sensory quality was consistent with instrumental texture and lipid oxidation results. However, in another study, the fillets of salmon fed complete replacement of FM with HI larvae meal were evenly appreciated, except for small weakness to colour intensity and textural attributes (Bruni et al., 2020a). The highly positive perception of panellists to PBM-HI-based fillet is very important from the consumer point of view in accepting the use of mixture PBM and HI larvae meal to replace FM completely from barramundi diets.

Consumers generally prefer more or less firm and elastic fish (Johnsen et al., 2011; Rasmussen, 2001), associated with the texture in terms of adhesiveness, chewiness, cohesiveness, gumminess, hardness, and springiness, all of which are broadly used to evaluate the meat quality of fish (Cai et al., 2018; Cao et al., 2019; Moreno et al., 2012; Wu et al., 2018). Among all external and internal factors, diet is one of the factors directly influencing the fish fillet texture (Andersen et al., 1997; Carbonell et al., 2003; Mørkøre et al., 2002; Trullàs et al., 2017) and tissue structure (Chaklader et al., 2020a). Previous shelf-life studies have indicated that bacterial protease acts on muscle protein and connective tissues post mortem which led to muscle disintegration in fish (Ando et al., 1995; Taylor et al., 2002; Taylor et al., 1995). A number of previous studies found antimicrobial peptides in HI larvae showing defensive mechanisms against microorganisms (Elhag et al., 2017; Park et al., 2014), though their ability to hinder the autolytic activity in fish muscle is not known. It can be hypothesized that the slower degradation during day 8 when comparing the structure of muscle tissue of fish fed PBM-HI base diets to control fed fish suggested that HI larvae bioactive properties might have influenced the fillet antimicrobial ability against autolytic microorganisms.

The alteration of muscle tissue structure during storage has shown to be associated with changes in texture profiles, especially in the declination of hardness, cohesiveness, gumminess and chewiness (Ayala et al., 2010; Caballero et al., 2009). Hence, the prevent in declination flesh texture, in particular, chewiness, cohesiveness, gumminess, hardness of barramundi fed PBM-HI based diets at day 8 indicated the ability of HI larvae to slow down degradation process and preserve texture better in comparison to control diet, aligns with results obtained from microstructure observation. There is a scantiness of information regarding the influence of HI larvae meal alone and/or in a mix with PBM on texture profile that allow comparison with the present study. However, the chitin in HI larvae meal might influence the flesh texture and also muscle tissue since chitosan, a derivative of chitin has been reported to have antioxidant (Pati et al., 2020) and antimicrobial properties and is widely used as a preservative agent in food application (Duan et al., 2019; Hafsa et al., 2016; Kong et al., 2010; Samar et al., 2013). Chitosan has successfully been transformed from the chitin of HI larvae meal (Liu et al., 2019) but their function in improving muscle texture is not well understood. However, the underlying hypothesis behind the antimicrobial mechanisms is the interaction between the positively charged NH_3^+ group of chitosan and the negatively charged microbial cell membranes which induce a change in the bacterial cell permeability (Kong et al., 2010; No et al., 2007; Riaz Rajoka et al., 2020; Sahariah and Masson, 2017). Also, lauric acid, a predominant medium-chain fatty acid and bioactive peptides in HI larvae might have influenced the texture quality, their function in improving flesh quality is not well understood. In contrast, feeding mealworm, *Tenebrio molitor* for 113 days did not influence on the hardness, cohesiveness, gumminess, and adhesiveness sea bream, *Pagellus*

bogaraveo fillet muscle (Iaconisi et al., 2017). The chemical composition of fish muscle particularly protein and lipid had a major influence on the flesh texture quality of sea bass but no differences in the lipid and protein were found among the experimental groups. PBM-HI-based diets had a higher content of glutamic acid and arginine which have been reported to improve the texture by increasing the muscle cell density and connective tissue (Kotzamanis et al., 2020; Østbye et al., 2018). Hence, further research should be conducted to decipher if chitin, lauric acid, choline, polysaccharides, and antimicrobial peptides in HI larvae meal could influence the flesh texture of barramundi.

Drip loss is an important quality parameter associated with juiciness, appearance, and colour of fish product (Kragten and Bee, 2010) as well as directly affecting the weight of fillet, thereby determining the economic revenue. On all days, the fillet of barramundi fed PBM-HI based diets showed a higher drip loss than the fillet of barramundi fed the FM control. Drip loss in meat has shown to be highly related to lipid oxidation as peroxidation can cause severe damage to membrane protein (Halliwell and Gutteridge, 1999; Morrissey and Kerry, 2004). However, TBARS result in this study does not align with this hypothesis as fish fed with the control diet were shown to have a much higher lipid oxidation. Thus, it could be suggested that such an observation could be related to the fatty acid profile of the fillet. This is supported by our earlier study (Chaklader et al., 2021a) in which muscle fatty acids compositions where barramundi fed with PBM-HI based diets shown to have lower content of polyunsaturated fatty acid (PUFA) in comparison to those fed with FM diet. As cell membrane is mostly composite with fatty acid, a reduction in PUFA proportion could reduce in the membrane fluidity and increase permeability of membrane bilayer (Morrissey and Kerry, 2004; Richter, 1987). Higher passage of sarcoplasmic fluid in barramundi fed with PBM-HI based diet result in higher drip loss. Regardless of diet, drip loss increased continuously with storage time in all test diets, results consistent with the lipid oxidation changes observed over the storage time. However, the drip loss ranging from 0.16 to 2.86% in this study cannot be considered as high and therefore not a major problem in chilled barramundi, supported by many earlier studies (Duun and Rustad, 2008; Einen et al., 2002; Kaale and Eikevik, 2015).

Colour is considered as one of the important attributes to assess the fillet quality of fish, being in direct association with the acceptance or rejection of product by the consumer. Farmed barramundi often look greyer than wild barramundi, directly impacting the consumer acceptance and making farmed barramundi position themselves with the lower market price. An effort has been paid in order to manipulating the melanin synthesis pathway by adding some precursors such as tyrosine, tryptophan and tyrosinase in diets which were fed barramundi for six weeks to prevent the greyish-blue colouration but did not resolve the greyish problem (Howieson et al., 2013). Both skin and

muscle lightness were significantly influenced by diet and storage time in the present study. The underlying reasons behind the improvement in the brightness (L*) barramundi fillet fed PBM-HI based diets could be related to reduction in melanosis as previous researches by Howieson et al. (2013), Cooper Midling (2007) and (Cooper et al., 2011) suggested the association of melanosis in greying of farmed fish. Currently, such topic has not been well studied and the exact biochemical pathway is unknown. However, it is possible that oxidation reduction-system, elemental and fatty acid composition difference could also be related to the lighter flesh colour described in the PBM-HI fed fish and the results are encouraging in resolving the greying issue in farmed barramundi. Valente et al. (2011) reported that different rearing conditions affect the brightness and skin colour of gilthead sea bream. The rearing condition of barramundi in the present study was uniform using constant photoperiod with a similar light intensity which might not affect fillet colour.

The gradual increase in flesh lightening over the storage time is similar to that reported by Jones Carton (2015). Since fish do not have *de novo* power to synthesize carotenoid, the presence of carotenoid in natural diets and supplemented with diets influence the fish colour. Generally, insects are a good source of β-carotene (Finke, 2002) which influenced the redness index (a*) of the skin colour of blackspot seabream, *Pagellus bogaraveo* fed 50% mealworm, *Tenebrio molitor* (Iaconisi et al., 2018). The same effects were observed in the skin of barramundi-fed HI larvae-based diets in the present study which may suggest that barramundi could utilize β-carotene from HI larvae. The unchanged flesh redness in the barramundi-fed PBM-HI-based diets was similar to muscle redness of European seabass, *Dicentrarchus labrax* (Moutinho et al., 2020) and rainbow trout (Bruni et al., 2020b) fed HI larvae and prepupae meal-based diets. Regardless of the diets, the decrease in the fillet redness over the storage time in all test diets fed fish was in line with the findings of Jones Carton (2015) who reported progressive colour changes in the fillets of barramundi subjected to two weeks storage at 2 °C irrespective of feeding commercial diets supplemented with α-tocopherol acetate for five months.

Also, most edible insects contain riboflavin (vitamin B2), a yellow-colored pigment and the quantity is extremely variable (Iaconisi et al., 2017) which may influence the skin and flesh color of fish fed insect based diets. For instance, feeding 25 and 50% of mealworm for 131 days increased the flesh yellowness blackspot sea bream, *Pagellus bogaraveo* (Iaconisi et al., 2017). HI larvae meal have been reported to contain 2.2 mg/100 g riboflavin (Nyakeri et al., 2017) which could have increased yellowness in the skin of fish fed 70PBM-30DHI and the flesh of fish fed insect-based PBM diets in the present study. However, in our previous study, feeding PBM-based diets supplemented with 5 and 10% of HI larvae and tuna hydrolysate for six weeks did not influence the skin and flesh color of barramundi (Chaklader et al., 2021c). A similar influence on

the yellowness of European seabass, *Dicentrarchus labrax* juveniles fillets was found when fed pre-pupae larvae (Moutinho et al., 2020).

A strong correlation was observed between QI and storage time 0PBM-0HI (R^2 = 0.93), 70PBM-30FHI (R^2 = 0.87), and 70PBM-30DHI (R^2 = 0.83) indicating that freshness gradually deteriorated with time with the control degrading the fastest, aligned with the texture microstructural results. The increase in QI overtime was consistent with previous report of QI of vacuum-packaged barramundi fillet stored at 4 ◦C (Fuentes-Amaya et al., 2016). The correlation between QI and storage time is expected since a similar correlation trend has been reported by many findings in several fish species (Alexi et al., 2021; Boziaris, 2013; Calanche et al., 2019; Luten and Martinsdottir, 1997; Martinsdóttir et al., 2001; Mausse et al., 2000). The maximum demerit score for 0PBM-0HI (10), followed by 70PBM-30FHI (9) and 70PBM-30DHI (9) at Day 8 was consistent with lipid oxidation and microstructure of muscle tissues. The demerit score at Day 4 for all test diets was below the QI score (7.5) of vacuum-packaged barramundi fillet stored at 4 ◦C (Fuentes-Amaya et al., 2016).

It has been reported that the reduction of fish muscle pH is associated with colour degradation, muscle gaping, blood spotting, flesh texture alterations and drip loss (Chow et al., 2009; Jones and Carton, 2015; Wilkinson et al., 2008). The increase in pH in the flesh of barramundi fed PBM-HI was similar to the changes in fillet pH after slaughtering and three days of storage of European seabass fed HI pre-pupae larvae meal (Moutinho et al., 2020). pH elevation in fish fillets has been reported to be associated with the production of alkaline compounds such ammonia and biogenic amines as a result of either bacterial activity or endogenous enzymes activity (Wang et al., 2017). Alternatively pH drop has been linked to the production of lactic acid caused by anaerobic fermentation of glycogen and liberation of inorganic phosphates from ATP degradation at the rigor stage (do Vale et al., 2020; Morachis-Valdez et al., 2017). It is noteworthy that the pH range in the present study irrespective of diet and storage time effect indicated no production of non-desirable acidic or alkaline compounds, suggesting that PBM-HI based diets can delay the spoilage process in barramundi muscle. Also, the pH range were within the range of fresh barramundi flesh (Wilkinson et al., 2008). The increases in pH at certain levels has been reported to prevent the production of volatile bases since bacteria can take energy from oxidative products resulted from lipid oxidation rather than glycogen and other normal substrates (Jones and Carton, 2015). Hence, there was an inverse correlation found between pH and degree of lipid oxidation in the flesh of barramundi over the storage of 14 days (Jones and Carton, 2015). Similarly, the pH of flesh in the present study appeared to be inversely proportional to the production of TBARS. TBARS, used to measure the carbonyl-like MDA concentration, can be formed primary and secondary oxidative

products from the degradation of polyunsaturated fatty acids, consequently generating unpleasant taste and rancid flavour in fat and protein-rich foods (Fernández-Segovia et al., 2012; Janero, 1990). Fatty acid results in our previous study have demonstrated that fish fed with PBM-HI based diet has less PUFA (Chaklader et al., 2021a) which is prone to oxidation due to unstable double bonds (Gibson and Newsham, 2018). Lower production in MDA could therefore be suggested to be related to lower PUFA content. Besides, previous study on European seabass conducted by (Moutinho et al., 2020) also suggested that HI antioxidant properties could positively improve the free radical scavenging ability of the fish, leading to inhibition of lipid oxidation. Similar to the assumption of Moutinho et al. (2020), the lower production of MDA might be attributed to the presence of chitin in HI larvae meal. In addition, several studies have already reported the antioxidant activity of chitosan, the deacetylated form of chitin, reduced the lipid oxidation in fish and fish products (Alak, 2012; Baptista et al., 2020; Fan et al., 2009; Farajzadeh et al., 2016; Shahidi et al., 2002). The increase in TBARS production in the fillet of barramundi fed test diets over the storage time was similar to a study by Moutinho et al. (2020) who found a similar increase in MDA production with increasing storage time in the fillet of European seabass when fed different level of HI pre-pupae larvae meal. However, MDA production levels in the present study was below the reported critical limits (7-8 mg MDA/kg) (Öz, 2018; Varlik, 1993). The results of pH and TBARS in barramundi fillet might be associated with microbial activity and hence, further studies are needed to evaluate the whole microbial profile in barramundi fillet using a modern tool to investigate if HI larvae can delay the production of spoilage bacteria.

Dietary inclusion of HI larvae meal either raw or defatted in combination with PBM can replace FM entirely without changing fillet proximate and amino acid composition but improved the sensory quality of barramundi fillet at the end of a 56 days feeding trial. There was a strong correlation observed between the storage time and QI in the fillet of barramundi-fed control and test diets. Skin redness and chroma as well as fillet brightness and yellowness were improved by PBM-HI-based diets during storage time. HI larvae inclusion in PBM diets was shown to effective in improving texture and delaying the alteration of muscle tissue structure. Also, lipid oxidation was significantly delayed by PBM-HI-based diets at the beginning of the storage and during chilled storage. Hence, inclusion of HI larvae either full-fat or defatted in PBM based diets could help in resolving the “grey” problem and improving the texture in farmed barramundi which would enhance the consumer acceptability to farmed fish. Further studies considering microbiological assessment would be beneficial to fully understand the beneficial effects of HI larvae inclusion on the final product quality of barramundi.

CHAPTER 10: The ameliorative effects of various fish protein hydrolysates in poultry by-product meal based diets on muscle quality, serum biochemistry and immunity in juvenile barramundi, *Lates calcarifer*

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Abstract

In an effort to reduce the use of fishmeal (FM), the effect of using protein from poultry by product meal (PBM) along with the supplementation of three different fish protein hydrolysate (FPH) including yellowtail kingfish, carp and tuna hydrolysate (designated as KH, CH and TH, respectively) were evaluated in juvenile barramundi for growth performance, fillet quality, mucosal immunity, serum biochemistry, immune response and infection against *Vibrio harveyi*. Fish were fed a FM based control diet + three isonitrogenous and isolipidic diets containing 90% of PBM protein supplemented with different types of hydrolysates: 90% PBM + 10% KH (90PBM+KH), 90% PBM + 10% CH (90PBM+CH) and 90% PBM + 10% TH (90PBM+TH). Growth performance and indices were unaffected by the hydrolysate supplemented diets when compared to the control. FPH supplemented PBM diets resulted in improved muscle quality by improving polyunsaturated fatty acids (PUFA), ∑n-3, ∑n-6 and ∑n-9, and health related lipid indexes were not affected. The internal architecture of spleen and kidney were not altered by test diets whilst FPH supplemented PBM modulated acidic mucins in intestine and skin of fish. Improved infection rate in response to two weeks post infection with *V. harveyi* in the FPH supplemented diets was further associated with an increased serum immune response and a concomitant regulation of proinflammatory and inflammatory cytokines in the head kidney. Serum biochemistry including alanine transaminase (ALT), glutamate dehydrogenase (GLDH) and total bilirubin (TB) showed a decreasing trend both in pre-challenge and post-challenge barramundi fed FPH supplemented diets whereas cholesterol level decreased significantly in post-challenge groups fed 90PBM+KH and 90PBM+TH than pre-challenge barramundi. This study signifies that supplementation of 10% with different three FPH, hydrolysed by an alcalase® enzyme in PBM-based diets for barramundi could be good strategies to overcome the negative consequences triggered by animal by-product ingredients.

Keywords: Hydrolysates, acidic mucins, immune response, cytokines, *Vibrio harveyi* and juvenile barramundi

10.1 Introduction

Despite FM being recognized as a nutritionally well-balanced ingredient and preferred protein source, in particular for carnivorous fish production (Glencross et al., 2007), limited supply, concomitantly increasing price and depletion of marine capture fisheries has led the aquaculture sector to seek widely available and inexpensive alternative ingredients. Poultry by-product meal (PBM) is one of the rendered animal protein ingredients which has been considered as a potential alternative in aquafeed production due to a high protein content and balanced amino acids profile (Chaklader et al., 2019). The quality of nutritional composition of PBM, however, vary from batch to batch, and among supplier companies, and may lack certain functional components (Chaklader et al., 2019). Though there are some promising results, research into the utilization of PBM in aquafeed for carnivorous species has identified some limitations, such as imbalanced amino acid and fatty acid profile, as well as poor digestibility which can affect the welfare of fish (Ye et al., 2019a). In addition, adding exclusive levels of PBM impacted the growth performance and immune status of barramundi (Chaklader et al., 2019; Siddik et al., 2019a) and also affected the growth of some other marine fish species including totoaba juveniles, *Totoaba macdonaldi* (Zapata et al., 2016), gilthead seabream, *Sparus aurata* (Karapanagiotidis et al., 2019) and hybrid grouper, *Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂ (Ye et al., 2019a). Due to the functional properties of FPH, supplementation of FPH with PBM could be a strategy to increase the inclusion level of PBM as well as to boost the immune response and induce improved serum biochemistry of fish.

The fishery and aquaculture industries generate a considerable amount of fish by-product, imposing a cost burden for waste disposal in the absence of productive utilization and/or value-added solutions. For instance, seafood industries in Australia are estimated to discard 100,000 tonnes of by-product annually (Peter and Clive, 2006) and spend an estimated AUD $15 million per annum on disposal (He et al., 2013). Recovery and value-addition by the application of enzyme technology could be an effective way of converting this by-product into acceptable protein ingredients for subsequent use in aquadiets. Enzymatic hydrolysis can produce fish protein hydrolysate (FPH) rich in free amino acids and biologically active immunomodulating low molecular weight peptides (Önal and Langdon, 2009; Ospina-Salazar et al., 2016) with potential for attractant and palatability enhancement (Kasumyan and Døving, 2003). The nutritional efficiency of dietary FPH in farmed fish diet has been evaluated and, in response to appropriate levels of FPH supplementation, many studies have reported modulatory effects on growth, feed intake, nutrient utilization, immune response, oxidative status and disease resistance in juvenile barramundi, *Lates calcarifer* (Chotikachinda et al., 2013; Siddik et al., 2019b; Siddik et al., 2018b),

Atlantic salmon, *Salmo salar* (Kousoulaki et al., 2012), red sea bream, *Pagrus major* (Khosravi et al., 2015b), European sea bass, *Dicentrarchus labrax* (Delcroix et al., 2015), Japanese flounder, *Paralichthys olivaceus* (Zheng et al., 2014), olive flounder, *Paralichthys olivaceus* (Khosravi et al., 2015a), large yellow croaker, *Larimichthys crocea* (Cai et al., 2015), Persian sturgeon, *Acipenser persicus* L. (Ovissipour et al., 2014), Japanese eel, *Anguilla japonica* (Masuda et al., 2013) and rainbow trout, *Oncorhynchus mykiss* (Aksnes et al., 2006b).

Incorporation of alternative protein ingredients, in many cases, may also negatively influence the internal architecture of various fish tissues including spleen, kidney, intestine and skin. Therefore, monitoring of the interaction between diets and internal tissues structure is important in developing new aquaculture diets. In particular, one area of investigation is gut and skin associated mucins which have been reported to be involved in digestion and absorption of nutrients as well as in the immune system of fish. Feeding and supplementation of FPH at appropriate levels influenced intestinal villi in silver catfish juveniles, *Rhamdia quelen* (Wosniak et al., 2016), and goblet cell, fold height, microvillous height and external circumference of serosa, as well as microvilli length and microvilli density in barramundi, *L. calcarifer* all of which are highly associated with disease resistance against pathogenic bacteria (Siddik et al., 2019b; Siddik et al., 2018b). Disease outbreaks are one of the factors limiting production in the aquaculture sector (Wallace et al., 2017). Due to rapid growth performance and flesh quality, barramundi, *Lates calcarifer* is one of the most widely cultured species, both in freshwater and seawater environment, especially in South East Asian countries and Australia. However, this species commonly suffers from Vibriosis due to *V. harveyi* resulting in large mortality and losses to fish farmers (Ransangan et al., 2012; Talpur and Ikhwanuddin, 2013). Application of antibiotics is a common traditional practice in aquaculture in controlling and preventing disease outbreaks, however a number of issues have risen in recent decades, such as development of antibiotic resistant bacteria and concern about the potential risk to the environment, public health and food safety (Suphoronski et al., 2019). Supplementation of an appropriate level of FPH could be an effective alternate way to modulate the immunity and defensive mechanism against bacteria in farmed fish by modulating gut and skin associated mucins.

As discussed previously, FPH supplementation could be an effective aquafeed strategy to maximize the utilization of animal by-product meal and enhance the welfare of fish. To further develop this hypothesis, various types of FPH supplemented with 90%PBM were fed to juvenile barramundi for six weeks, with the aim to determine the growth performance, muscle fatty acids composition, histopathology in liver and kidney, mucosal immunity, serum biochemistry, immune response, immune relevant gene expression and disease resistance to bacteria.

10.2 Material and methods

10.2.1 Ethical statement

The feeding trial procedures and protocols concerning animals were carried out in strict compliance with pertinent guidelines and regulation in Australia for the care and use of animals for experimentation. All procedures regarding handling or treatment of fish described in the current study, were reviewed and approved by the Animal Ethics Committee at Curtin University, Australia under Permit No. ARE2018-37. Pain management and killing methods were performed in accordance with the protocol of the Curtin Aquatic Research Laboratory (CARL) standard operating procedure (SOP) of anaesthetizing (AQUI-S®, 8 mg/l) and euthanizing (AQUI-S®, 175 mg/l) of fish.

10.2.2 Experimental diets

Four groups of iso-protein (around 48.00%) and iso-lipid (around 10.80%) diet were formulated using FM and PBM as the main protein source and canola oil and cod liver oil as the main lipid source, as presented in Table 10. 1. All the feed ingredients with the exception of yellowtail kingfish, *Seriola lalandi* hydrolysate (KH), carp hydrolysate, *Cyprinus carpio* (CH) and southern bluefin tuna, *Thunnus maccoyii* hydrolysate (TH) were purchased from Specialty Feeds, Glen Forrest Stockfeeders, 3150 Great Eastern Highway, Glen Forrest, Western Australia 6071. Liquid TH and CH was provided by SAMPI, Port Lincoln, Australia. KH was produced at CARL following the method of FRDC 2013/711.40. Briefly, kingfish frame were chopped into small pieces and placed in Sous vide appliance (Sunbeam), 2% alcalase® enzyme was added, the solution was covered and incubated at 55˚C for at least 2 hours with infrequent stirring until the completion of hydrolysis. The mixture was heated at 95˚C for 1 hour with stirring at 20 min intervals to deactivate the enzyme, sieved to remove bones/scales from hydrolysed solution, centrifuged at 3800 *g* for 10 minutes at room temperature to separate the liquid FPH solution and frozen for later use. All the dried ingredients were weighed and mixed homogenously in a food mixer (Hobart Food equipment, Australia). Thereafter, oil was added and mixed thoroughly and distilled water was gradually added to make a stiff dough before producing 3 mm long feed pellets by passing through a mincer. Pellets were dried in an oven for 36 h at 60 ˚C, broken up by hand after cooling at room temperature and sealed in a plastic bag before storing in the refrigerator until use in the feeding trial.

Table 10. 1 Formulation and proximate composition of PBM based diets, supplemented with different fish protein hydrolysates in a dry basis for juvenile barramundi.

	Diets			
Ingredients (g/100g)	Control	PBM+KH	PBM+CH	PBM+TH
†PBM[a]	0.00	61.40	61.40	61.40
‡KH	0.00	9.50	0.00	0.00
‡CH[b]	0.00	0.00	9.00	0.00
‡TH[b]	0.00	0.00	0.00	11.80
†Tuna FM[c]	72.00	0.00	0.00	0.00
Canola oil[c]	1.00	3.00	3.00	3.00
Cod liver oil[c]	0.50	6.00	6.00	6.00
Corn/wheat starch[c]	7.00	3.00	5.00	5.00
Lecithin - Soy (70%)[c]	1.00	1.00	1.00	1.00
Vitamin C[c]	0.05	0.05	0.05	0.05
Dicalcium Phosphate[c]	0.05	0.05	0.05	0.05
Wheat (10 CP)[c]	16.90	13.50	12.00	9.20
Vitamin premix[c]	0.50	0.50	0.50	0.50
Cholesterol[c]	0.00	2.00	2.00	2.00
Salt (NaCl)[c]	1.00	0.00	0.00	0.00
Proximate composition (dry matter)[d]				
Crude Protein (%)	47.88	48.00	47.71	47.87
Crude Lipid (%)	10.59	10.73	10.71	10.66

[a]Kindly provided by Derby Industries Pty Ltd T/A, Talloman Lot Lakes Rd, Hazelmere WA 6055

[b]SAMPI, Port Lincoln, Australia

[c]Specialty Feeds, Glen Forrest Stockfeeders, 3150 Great Eastern Highway, Glen Forrest, Western Australia 6071

† Poultry by-product meal (PBM): 67.13% crude protein, 13.52% crude lipid and 13.34% ash

† Fishmeal: 64.0% crude protein, 10.76% crude lipid and 19.12% ash.

‡ Kingfish hydrolysate (KH): 57.07% crude protein, 31.98% crude lipid, 2.55% moisture and 0.29% ash

‡ Carp hydrolysate (CH): 59.01% crude protein, 12.89% crude lipid, 0.73% moisture and 46.21% ash

‡ Tuna hydrolysate (TH): 58.4% crude protein, 1.05% crude lipid and 11.3% ash

[d]Determined according to Association of Official Analytical Chemists (AOAC), (AOAC, 1995)

10.2.3 Amino acids analysis

Amino acid profile (including hydroxyproline and taurine) analyses of all test diets and ingredients were performed as per the Australian Proteome Analysis Facility (APAF) SOP AAA-001. Briefly, samples underwent 24hr liquid hydrolysis in 6M Hydrochloric acid at 110 °C. Under these conditions, Asparagine is hydrolysed to Aspartic acid and Glutamine to Glutamic acid; therefore the reported amount of these acids is the sum of those respective components. After hydrolysis, all amino acids were labelled using the Waters AccQTag Ultra chemistry (following supplier's recommendations) and analysed on a Waters Acquity UPLC. Samples were analysed in duplicate and results are expressed as an average. Amino acids profile of PBM meal and test diets are shown in Table 10. 2.

Table 10. 2 Amino acids (g $100g^{-1}$ on dry matter basis) profile of four formulated experimental diets and PBM

	Diets				
Amino acids	Control	PBM+KH	PBM+CH	PBM+TH	PBM
Hydroxyproline	1.7	3.0	3.3	3.1	3.2
Histidine	2.4	2.0	1.8	1.8	1.8
Taurine	0.5	0.5	0.5	0.6	0.4
Serine	5.3	5.1	5.0	5.0	5.0
Arginine	4.5	4.9	4.8	5.0	5.1
Glycine	13.2	16.0	16.5	16.1	16.5
Aspartic acid	8.8	7.9	7.7	7.9	7.8
Glutamic acid	11.7	12.2	12.3	12.0	11.5
Threonine	4.9	4.3	4.2	4.3	4.3
Alanine	9.4	9.0	9.0	9.1	9.1
Proline	6.1	7.1	7.4	7.1	7.0
Lysine	6.2	5.5	5.2	5.4	5.5
Tyrosine	2.0	1.8	1.8	1.9	2.0
Methionine	2.4	1.9	1.8	1.8	1.8
Valine	5.6	5.1	5.3	5.2	5.1
Isoleucine	4.3	3.8	3.7	3.8	3.8
Leucine	7.6	6.9	6.8	6.8	6.9
Phenylalanine	3.3	3.0	3.0	3.0	3.0

10.2.4 Peptide size-exclusion chromatography analysis

Analysis was performed in accordance with the APAF SOP LC-004 (Size Exclusion Chromatography (SEC) of Proteins). Briefly, 5mL of the liquid hydrolysate samples was centrifuged at 9,000 x g for 10 mins. The supernatant was aspirated into an Eppendorf tube, avoiding the fat/oil layer. Hydrolysate samples were filtered through 0.22µm PES syringe filters, and analysed by size exclusion chromatography. A running buffer blank was also prepared. Size exclusion chromatography was performed using a Superdex Peptide HR 10/30 (GE Healthcare Life Sciences) column with dimensions 4.6 x 300mm, 3 µm. The chromatography conditions were: flow rate: 0.325 mL/min; stop time: 110 min; column temperature: room temperature. Peptide standards were run prior to sample analysis and showed the chromatographic conditions employed separated peptides over the molecular weight range of approx. 13,700 - 238 Da. Molecular weight of KH, CH and TH is shown in Table 10. 3.

Table 10. 3 Molecular weight (%) of kingfish hydrolysate, carp hydrolysate and tuna hydrolysate

	Hydrolysates		
Molecular weight (Da)	KH	CH	TH
>10,000	5.5	3.2	1.8
10,000 –5,000	1.9	11.1	6.8
5,000 – 1,000	22.3	45.0	36.8
1,000 – 500	31.5	13.1	22.7
500 - 238	18.1	15.9	16.8
<238.2	20.7	11.6	15.2

10.2.5 Experimental animals

Barramundi (N = 400) having an average weight of 7.25 g were transported from the Australian Centre for Applied Aquaculture Research (ACAAR), Fremantle, Australia to CARL in a large esky with oxygenated water. Upon arrival, the fish were kept in two 300L seawater tanks (recirculatory aquaculture system) and acclimated for 14 days feeding with a commercial diet twice a day to acclimatize them with the CARL facilities and condition. Fish were not fed for 24 h after two weeks acclimatization and 300 fish (15.13±0.08 g) following grading were randomly distributed into 12 tanks (250 litres, 3 tanks/diet; 25 fishes/tank) that were equipped with an external bio-filter (Astro® 2212, China), electric heater and aerator to maintain uniform water quality during the study period. Certain water indicators including oxygen (6.36±0.27, 5.92–7.42 mgL^{-1}), ammonia (0.28±005, 0.20-0.50 mgL^{-1}), nitrite (0.24±0.04, 0.20-0.40 mgL^{-1}), temperature (28.44±0.22, 27.90–29.20°C) and salinity (30.20±0.20, 30–31 ppt) were monitored on regular

basis. Fish were held in 14:10 h light: dark photoperiod, maintained by an automatic indoor timer (Clipsal, Australia).Throughout the study period, each diet was hand fed till apparent satiation twice daily at 8.00 am and 6.00 pm and any uneaten feed was collected by siphoning, and recorded on daily basis to calculate the feed intake (FI). Fish mortalities, if any, were monitored and recorded daily. At the termination of the trial, fish were bulk weighed and counted following fasting for 24 h and anesthetizing with 8 mg/l AQUI-S®. A further ten fish/tank with the exception of ten fish used for challenge trial were euthanized randomly to collect length and weight, viscera, intraperitoneal fat, liver, spleen and intestinal length for biometry indices.

10.2.6 Muscle fatty acids composition

Fish muscle (one fish/tank, three fish/dietary treatment) was used for fatty acids analysis. Fish were filleted and the muscle was wrapped with aluminium foil and freeze dried for 3 days at -48.4°C and 1.9×10^{-1} mB. The fatty acids profile of the experimental diets and fish flesh was carried out following the protocol of O'Fallon et al. (2007) and Siddik et al. (2019a).

The fillet lipid quality of the control and FPH supplemented PBM diets was determined using two health indexes, atherogenicity (AI) and thrombogenicity (TI), as follows:

$$AI = (aC12:0 + bC14:0 + C16:0) / (dp + eM + FM')$$

Where P is the sum of n3 and n6 PUFA; M is the oleic acid and M´ is the sum of other monounsaturated fatty acids (MUFA); a, b, c, d, e, f are empirical constant; b = 4 and other constants = 1.

$$TI = (C14:0 + C16:0 + C18:0) / [(nM + nM' + p(n6) + q\ (n3) + (n3/6)]$$

Where M and M´ are as before; n, o, p, q are empirical constants; n, o, p = 0.5 and q = 3

10.2.7 Histological analyses

At 42 days post-feeding, two fish/replicate were euthanized with an overdose of AQUI-S® (175 mg l^{-1}) and the spleen, kidney, intestine and skin collected aseptically. Collected tissues were immediately preserved in 10% neutral buffered formalin and processed using standard histological procedures. Spleen and kidney were stained with Periodic Acid-Schiff (PAS), while intestine and skin were stained with Alcian Blue (AB) pH 2.5 to visualize acidic mucins. Acidic mucins in intestine were counted following the method of our earlier study (Chaklader et al., 2019) and skin epidermis thickness and acidic mucins in one millimetre length of epidermis were estimated (Sheikhzadeh et al., 2019).

10.2.8 Bacterial infection

Bacterial strain culture and injection protocols were carried out as described in our previous study (Chaklader et al., 2019). In short, *V. harveyi* strain used for infection trial were obtained from Diagnostic and Laboratory Services, Department of Primary Industries and Regional Development (DPIRD), 3 Baron-Hay Court, South Perth WA 6151, after being cultured in trypticase soy broth medium (Oxoid, Basingstoke, UK) at 24ºC for 24 h and harvested by centrifugation (5000 × g, 15 min) at 4ºC. Discarded bacterial cells were suspended in PBS (phosphate-buffered saline, pH 7.2) and adjusted to 1.1 x 10^8 cfu/ml for infection trial. At the end of the trial, thirty fish from dietary treatments (10 fish/tank) were injected intraperitoneally with 0.1 mL containing 1.1 x 10^8 *V. harveyi* live cells and infection were observed three times daily for two weeks and infected fish were culled using 175 mg/L AQUI-S® for 20 minutes following the standard protocol of CARL.

10.2.9 Serum biochemistry and immunity

Fish were captured gently at 42 days post-feeding and 24 and 48 h post-challenge (six fish/dietary treatments) by *V. harveyi*, immediately dipped in a bucket containing 8 mg l^{-1} of AQUI-S® and blood was taken by puncturing caudal vessel using 1 mL non-heparinized syringes (22G). Blood was allowed to clot for 24h at room temperature, centrifuged for 15 min at 3000 rpm and 4 °C, the serum collected and stored immediately at - 80°C for the analysis of serum biochemical parameters and immune parameters (Chaklader et al., 2019). The serum clinical chemistry panel was processed on a AU480 Clinical Chemistry Analyser (Beckman Coulter Australia Pty Ltd, Lane Cove West, NSW). Beckman Coulter clinical chemistry kits were used for the following panel components: Alanine transaminase (ALT) OSR6107, Gamma-glutamyltransferase (GGT) OSR6219, Total Bilirubin (TB) OSR6112, Urea OSR6134, Creatinine OSR6178, Cholesterol OSR6116, Total Protein OSR6132 while Randox kits (Randox Australia Pty Ltd, Parramatta, NSW) were used for Glutamate dehydrogenase (GLDH) GL441. Serum immune related parameters were analysed according to the protocol of our earlier study (Chaklader et al., 2019).

10.2.10 RNA extraction and gene expression analysis

Six fish per treatment (2/replicate) were euthanized using 175 mg l^{-1} AQUI-S® and excised spleen and head kidney after 42 days post-feeding, 24 h and 48 h post challenge, were immediately preserved in RNA Later (Sigma-Aldrich, Germany) and then stored at - 80°C till extraction of RNA. Total RNA was extracted using 1 ml Trizol TM reagent (Invitrogen) based on manufacturers protocols from approximately 50-100 mg of the various tissue samples. The degradation and contamination of RNA was checked by gel electrophoresis and the purity of RNA was monitored on a NanoDrop spectrophotometer 2000c (Thermo Fisher Scientific, USA). cDNA synthesis was

carried out from 1 µg of total RNA by Omnicript RT kit (Qiagen, Hilden, Germany) as per protocols of manufacturer company. The specific primers of selected genes was used from our earlier published study (Siddik et al., 2019c). RT-qPCR using PowerUp^{TM} Cyber Green Master Mix (Thermo Scientific, USA) with 7500 Real-Time PCR System (Applied Biosystems, USA) was conducted as described in our earlier study. The relative expression of target genes were normalised to the *18S rRNA* and *Ef1-a*, housekeeping genes and analysed using REST© (http://www.gene-quantification.de) (Mohd-Shaharuddin et al., 2013).

10.2.11 Calculation and statistical method

Growth performance and biometry indices were calculated following the formula as described in our earlier study.

Weight gain (WG, g)= [(Mean final weight - Mean initial weight) / (Mean initial weight)]

Daily weight gain $\left(\text{DWG, gf}^{-1}\text{d}^{-1}\right)$ = [final body weight of fish (g) - initial weight of fish (g)] / days

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

Feed intake (FI,g/fish d^{-1}) = [(Dry diet given - Dry remaining diet recovered) / days of experiment) / no. of fish]

Feed conversion ratio (FCR) = [(Dry feed fed) / (Wet weight gain)]

Survival (SR, %) = [(Final number of fish) / (Initial number of fish)] ×100

Condition factor (CF, %) = [Final body weight (g) / Body length cm^3)] × 100

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

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

Intraperitoneal fat index (IFI, %) = [Intraperitoneal fat weight (g) / Whole body weight (g)] ×100

Spleen index (SI, %) = [(Weight of spleen (g)) / (Whole body weight (g))] × 100

Relative gut length (RGL, %) = [(Length of intestine (cm)) / (Length of fish $(CM)^3$] × 100

Results were expressed as mean values ± standard error and subjected to Shapiro-Wilk's and Levene's tests confirming the normality and homogeneity of variance, respectively, prior to performing one-way analysis of variance. One way-ANOVA with Dunnet's multiple comparison test was applied on the growth and biometry indices and fillet fatty acids composition to compare the test diets with respect to control. General Linear Models were performed on serum biochemistry as well as cytokines expression to test the significant difference among the dietary groups based on diet and challenge time, and their interaction. Infection data from the challenge trial was processed by the Kaplan-Meier method and difference between control and test diets was

compared by the Mantel-Cox log-rank test. Data from before and after the challenge test was compared by paired t-test.

10.3 Results

As presented in Table 10. 4, fish grew more than fourfold but growth performance (final body weight, FBW; weight gain, WG, specific growth rate, SGR and daily weight gain, DWG) and feed intake (FI) were not influenced in any of the test diets. Considering SR at 42 days, the survival rate ranged from 81.67% to 96.67%, and was increased significantly in FPH supplemented dietary groups than control. Feeding fish with control and FPH supplemented PBM diets did not have any effects on the biometry indices.

Table 10. 4 Effect of sole source of protein from PBM, supplemented with different kinds of FPH on the growth performance, feed utilization, survival and biometry indices of juvenile barramundi after six weeks trial. Values (mean ± SE) in the same row with different superscripts denote significant difference ($P < 0.05$).

	Diets				ANOVA	
	Control	PBM+KH	PBM+CH	PBM+TH	F-value	P-value
IBW (g)	15.48±0.19	15.87±0.15	15.81±0.13	15.73±0.17	1.401	0.312
FBW (g)	72.48±4.37	69.34±7.92	66.62±9.17	66.97±6.52	0.140	0.933
WG (g)	57.10±4.34	53.47±7.88	50.81±9.29	51.24±6.50	0.153	0.925
DWG (g/fish d^{-1})	1.36±0.10	1.27±0.19	1.21±1.21	1.22±1.22	0.153	0.925
SGR (%/d)	3.67±0.14	3.48±0.28	3.38±0.35	3.43±0.22	0.236	0.869
FI (g/fish d^{-1})	2.67±0.17	2.28±0.17	2.18±0.16	2.19±0.16	1.831	0.220
FCR	0.77±0.08	0.85±0.12	0.92±0.16	0.85±0.08	0.295	0.828
SR (%)	81.67±1.67^{b}	95.00±2.89^{a}	96.67±3.33^{a}	95.00±2.89^{a}	6.394	0.016
Biometry indices						
CF (%)	1.26±0.02	1.30±0.04	1.29±0.03	1.27±0.04	0.285	0.836

HSI (%)	1.49±0.28	1.68±0.10	1.35±0.11	1.42±0.06	0.915	0.449
VSI (%)	7.76±0.38	8.30±0.52	8.56±0.71	7.03±0.58	1.449	0.254
IFI (%)	0.75±0.17	0.98±0.22	0.74±0.09	0.75±0.10	0.573	0.638
SI (%)	0.07±0.02	0.10±0.01	0.13±0.04	0.10±0.01	1.059	0.386
RGL (%)	46.86±4.29	45.38±2.64	43.09±2.69	41.48±2.18	0.639	0.597

Initial body weight, IBW; final body weight, FBW; weight gain, WG; specific growth rate, SGR; feed intake, FI; feed conversion ratio; survival rate, SR; condition factor, CF; hepatosomatic index, HSI; viscerosomatic index, VSI; intraperitoneal fat index, IFI; spleen index, SI and relative gut length, RGL.

10.3.1 Muscle fatty acids composition

The total fatty acids content and lipid indexes, including AI and TI, from muscle from barramundi fed a control and three different FPH supplemented PBM diets are presented in Table 10. 5 and Figure 10. 1. Considering saturated fatty acids (SFA), 90% PBM supplemented with FPH increased the SFAs respect to control and a parallel increase in mono unsaturated fatty acids (MUFAs) and poly unsaturated fatty acids (PUFAs) content was observe for FPH supplemented PBM fed groups (Figure 10. 1A). A similar trend were observed for ∑n-3 and ∑n-9 whilst ∑n-6 only increased significantly in the CH supplemented groups (Figure 10. 1B). The majority of individual PUFA contents were modulated by FPH supplemented diets with the exception of C22:4n6 and C22:6n3 which decreased significantly in the muscle of fish fed FPH supplemented diets (Table 10. 5). AI (Figure 10. 1C) significant variations with respect to the control were found only in fish fed CH and TH supplemented PBM but there was no variation with KH supplemented PBM fed groups. Meanwhile, TI (Figure 10. 1D) were negatively induced by FPH supplemented diets when compared to the control.

Table 10. 5 Effect of sole source of protein from PBM, supplemented with different kinds of FPH on the muscle fatty acids (mg $100g^{-1}$ on dry matter basis) composition of juvenile barramundi after six weeks trial. Values (mean ± SE) in the same row with different superscripts denote significant difference ($P < 0.05$).

	Diets				ANOVA	
	Control	PBM+KH	PBM+CH	PBM+TH	F-value	P-value
C10:0	0.78±0.03a	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	529.00	0.00
C12:0	1.02±0.09^{c}	2.00±0.24^{ab}	2.40±0.03^{a}	1.8±0.03^{b}	20.46	0.00
C13:0	0.60±0.00^{c}	0.90±0.03^{b}	1.20±0.00^{a}	1.10±0.03^{b}	115.17	0.00
C14:0	67.46±0.77^{b}	172.1±6.53^{a}	186.4±0.78^{a}	176.9±1.93^{a}	262.01	0.00
C14:1n5	0.97±0.03^{d}	4.70±0.17^{c}	6.60±0.09^{a}	5.80±0.03^{b}	672.97	0.00
C15:0	17.76±0.63^{b}	19.30±0.74^{b}	23.50±0.38^{a}	23.00±0.23^{a}	26.71	0.00
C15:1	0.00±0.00^{c}	3.20±1.32^{b}	1.00±0.30^{bc}	6.60±0.09^{a}	18.45	0.00
C16:0	713.65±10.19^{c}	1271.8±51.16^{b}	1440.40±4.16^{a}	1399.10±16.01^{a}	150.95	0.00
C16:1n7	130.78±1.90^{c}	290.30±11.25^{b}	350.40±0.97^{a}	297.70±3.16^{b}	255.36	0.00
C17:0	44.02±0.52^{c}	42.10±2.95^{c}	53.20±1.64^{b}	62.3±1.00^{a}	27.33	0.00
C17:1	22.70±0.31^{b}	32.70±3.71^{b}	50.90±5.71^{a}	60.40±0.86^{a}	24.71	0.00
C18:0	278.21±4.07^{c}	381.2±20.45^{b}	423.0±3.93^{ab}	433.40±5.41^{a}	41.91	0.00
C18:1c+t	859.76±5.73^{c}	2128.3±84.09^{b}	2596.9±10.99^{a}	2425.10±27.82^{a}	308.99	0.00
C18:2 t	17.50±15.25	4.70±0.70	6.00±0.23	5.90±0.07	0.62	0.62
C18:2 c	320.82±3.44^{d}	860.0±34.31^{c}	1059.2±4.61^{a}	962.0±10.47^{b}	329.95	0.00
C18:3n6	17.51±1.42^{d}	25.8±1.52^{c}	39.0±0.67^{a}	31.2±0.25^{b}	67.78	0.00
C18:3n3	55.12±0.38^{d}	171.6±6.36^{c}	225.50±1.17^{a}	199.20±2.04^{b}	488.89	0.00

C18:4n3	17.01±1.06^{c}	71.20±2.92^{b}	80.10±0.75^{a}	80.80±0.82^{a}	341.09	0.00
C20:0	8.95±0.22^{c}	19.20±0.60^{b}	21.30±0.21^{a}	21.50±0.27^{a}	265.38	0.00
C20:1	36.62±0.38^{c}	187.6±7.05^{b}	221.00±1.36^{a}	210.50±2.41^{a}	513.96	0.00
C20:2	8.84±0.03^{b}	16.00±0.93^{a}	18.10±0.12^{a}	16.30±0.15^{a}	74.08	0.00
C21:0	4.05±0.18^{b}	8.50±1.25^{a}	8.60±0.06^{a}	8.10±0.09^{a}	12.04	0.00
C20:3n6	25.68±0.57^{c}	38.00±3.18^{ab}	43.70±0.12^{a}	36.00±0.35^{b}	21.51	0.00
C20:4n6	92.29±1.90^{b}	123.60±6.73^{a}	137.60±0.64^{a}	127.20±1.26^{a}	29.88	0.00
C20:3n3	4.61±0.09^{a}	6.40±0.67^{b}	7.70±0.12^{b}	6.30±.10^{b}	13.49	0.00
C22:0	3.58±0.03^{b}	6.50±0.50^{a}	7.00±0.00^{a}	7.00±0.09^{a}	41.88	0.00
C20:5n3	109.31±1.78^{c}	213.00±10.28^{a}	186.00±0.98^{b}	205.80±1.85^{ab}	79.85	0.00
C22:1n9	4.03±0.03^{b}	21.50±1.01^{a}	22.40±0.09^{a}	21.80±0.27^{a}	286.48	0.00
C22:2	0.00±0.00^{b}	0.90±0.09^{a}	1.10±0.03^{a}	0.90±0.03^{a}	97.19	0.00
C23:0	13.39±0.68^{ab}	8.00±6.67^{b}	24.60±0.19^{a}	23.90±0.41^{a}	5.89	0.02
C22:4n6	62.67±1.07^{a}	23.40±1.02^{b}	20.70±0.19^{b}	22.90±0.31^{b}	707.86	0.00
C22:5n3	71.50±0.95^{c}	118.60±4.63^{a}	111.70±0.52^{ab}	104.30±1.09^{b}	73.21	0.00
C24:1	11.81±0.12^{c}	21.10±0.67^{b}	22.20±0.10^{ab}	23.70±0.30^{a}	207.07	0.00
C22:6n3	683.13±12.43^{a}	575.90±27.08^{b}	464.30±1.70^{c}	605.20±5.84^{b}	35.54	0.00

Cis, c and trans, t.

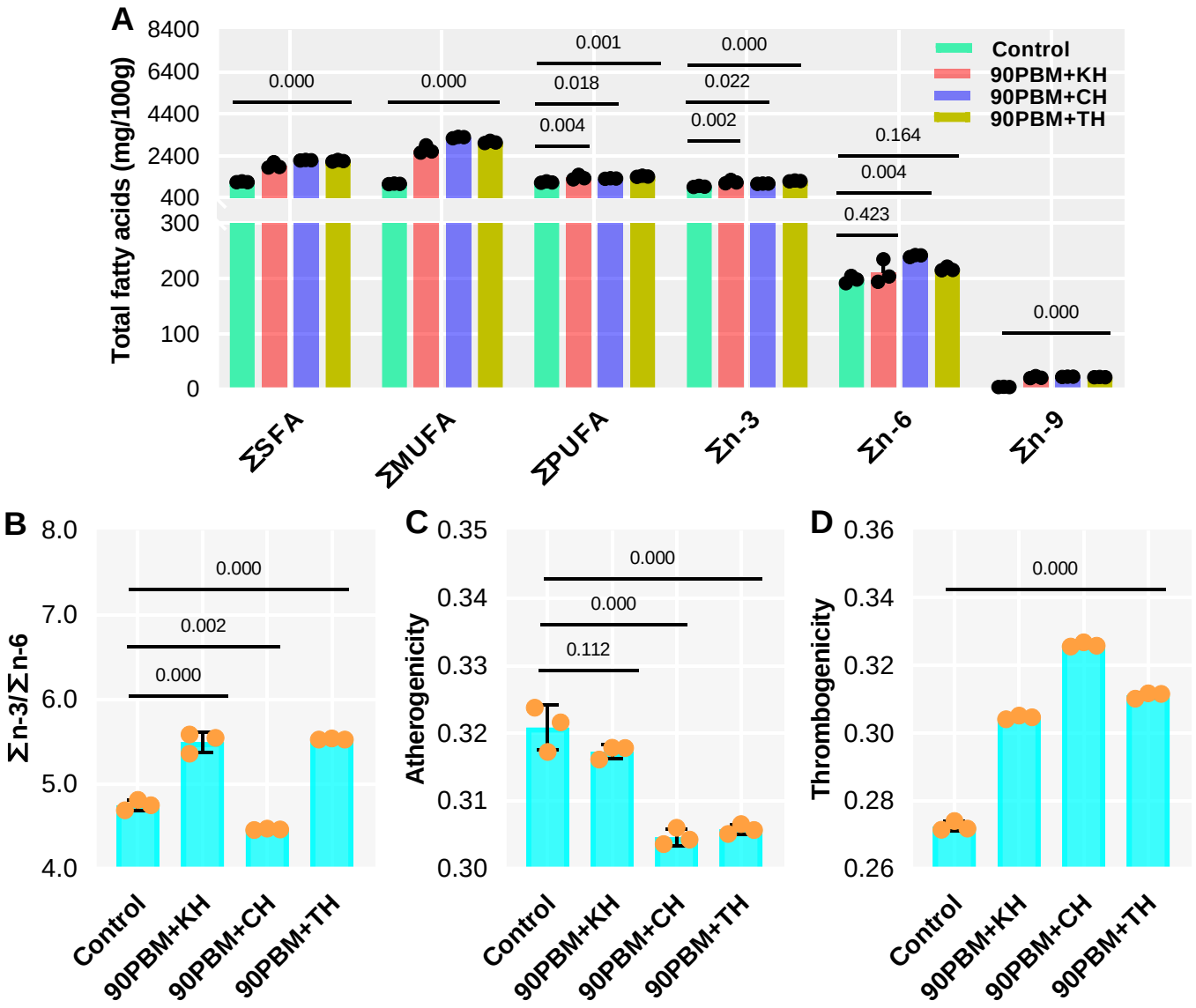

Figure 10. 1 Total fatty acids content (A), ∑n-3/∑n-6 ratio (B) and atherogenicity (C) and thrombogenicity (D) of barramundi fed control and FPH supplemented diets after six weeks trial. Kingfish hydrolysate, KH; carp hydrolysate, CH; tuna hydrolysate, TH; poultry by-product meal, PBM; saturated fatty acids, SFA; monounsaturated fatty acids, MUFA and polyunsaturated fatty acids, PUFA. *P* values reveal significant difference between the groups at $P < 0.05$, $P < 0.01$ and $P < 0.001$, as determined by an ordinary one-way ANOVA followed Dunnett's multiple comparisons test. Results displayed are mean ± SE (n = 3).

10.3.2 Spleen and kidney histology and acidic mucins in intestine and skin

The results of histology revealed no alteration between fish fed various diets with normal renal glomeruli and renal tubules in the kidney (Figure 10. 2A-D) and normal white and red pulp in the spleen (Figure 10. 2E-H). Histochemistry of intestine and skin in fish fed control and FPH supplemented diets is shown in Figure 10. 2(I-L) and Figure 10. 2(M-P), respectively. As shown

in Figure 10. 3(A-C), acidic mucins (AM) in the intestine and skin of fish fed FPH supplemented diets increased significantly while epidermis (Ep) thickness were affected by neither control nor test diets.

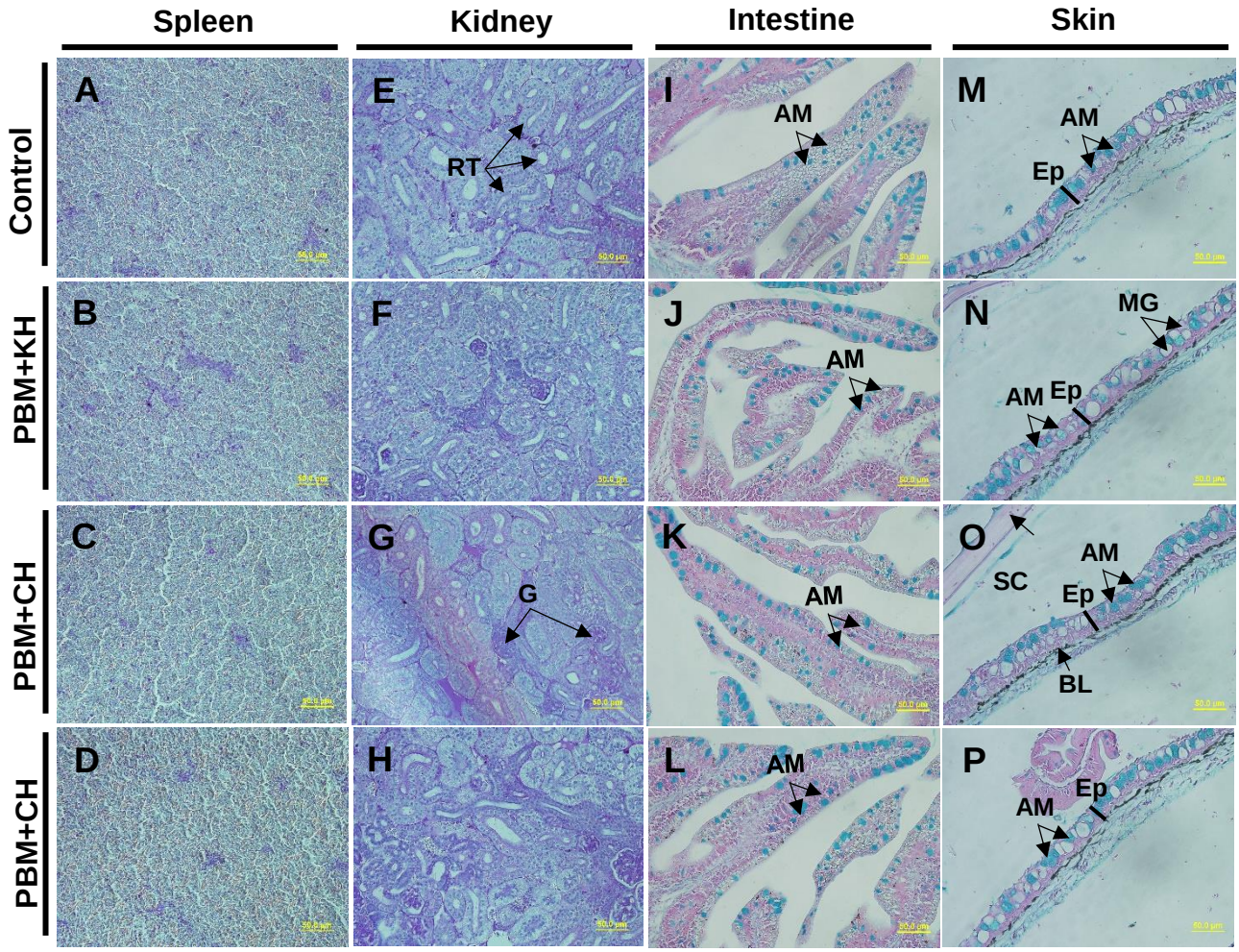

Figure 10. 2 Representative light microscopy of micrograph of spleen (A-D) (Periodic Acid-Schiff stain, 40 × magnification) and kidney (E-H) (Periodic Acid-Schiff stain, 40 × magnification) as well as histometric and histochemical changes in distal intestine (I-L) (Alcian Blue pH 2.5 stain, 40 × magnification), and skin (M-P) (Alcian Blue pH 2.5 stain, 40 × magnification) of juvenile barramundi fed PBM based diets, supplemented with different kinds of FPH after six weeks trial. RT, renal tubule; G, glomerulus; AM, acidic mucins; Ep, epidermis; MG, mucous gland; SC, stratum compactum and BL, basal layer

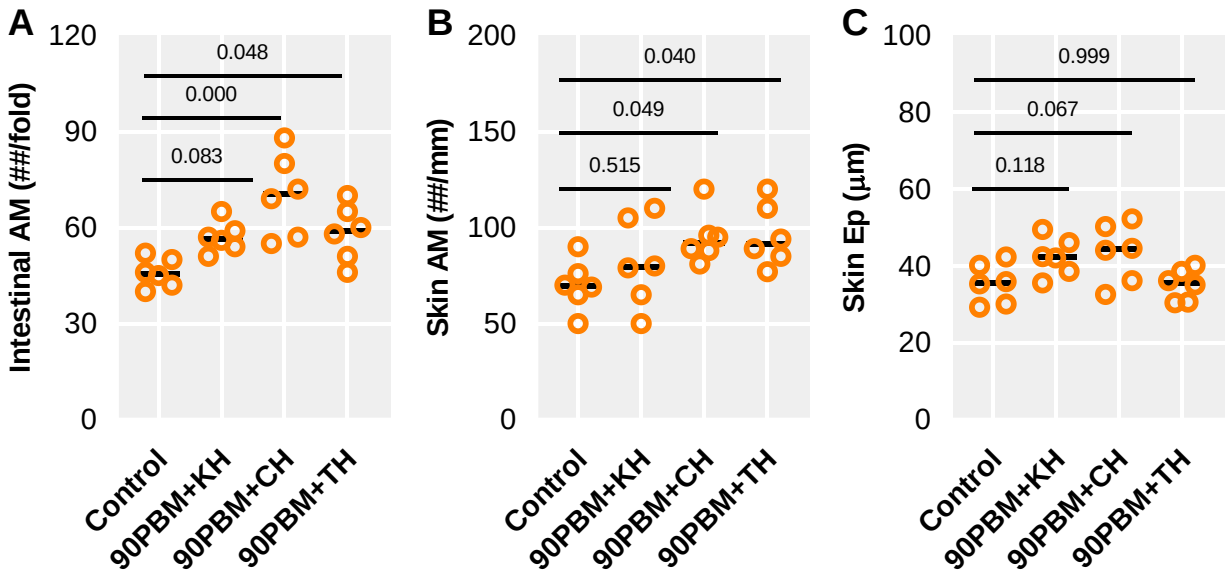

Figure 10. 3 Intestinal (A) and skin (B-C) morphology of juvenile barramundi fed PBM based diets, supplemented with different kinds of hydrolysate after six weeks trial. Orange marker denotes number of individuals and black line indicates mean. AM, acidic mucin and Ep, epidermis. *P* values asterisks reveal significant difference between the groups at $P < 0.05$ and $P < 0.001$, as determined by an ordinary one-way ANOVA followed by Dunnett's multiple comparisons test. Results displayed are mean ± SE (n = 6).

10.3.3 Infection against *V. harveyi*

Infection in the control started at 2 days post infection and infection in PBM+KH started at 2 days post infection, whilst both PBM+CH and PBM+TH first demonstrated signs of infection at 4 days post-infection (Figure 10. 4). Infection in the control group continued till 13 days post infection. Meanwhile, infection in PBM+KH, PBM+CH and PBM+TH stopped at 8, 9 and 10 days post infection, respectively. In comparison with the control (70.00%), the infection rate decreased significantly in PBM+KH (16.12%), PBM+CH (29.41%) and PBM+TH (23.33%) ($\chi^2 = 27.10$, df = 3, $p = 0.000$).

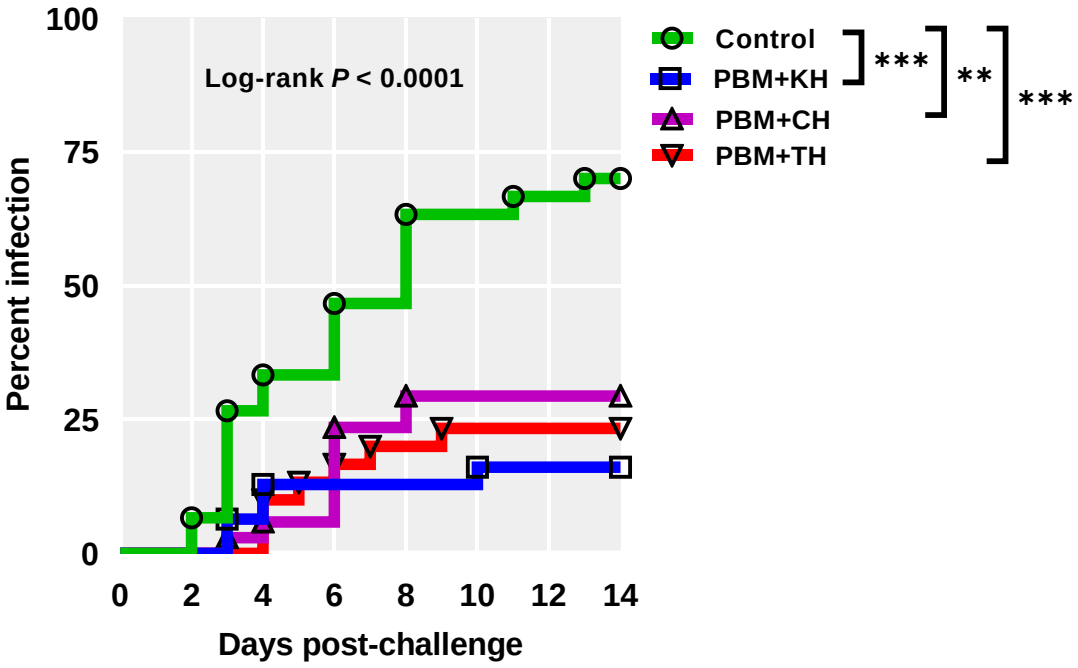

Figure 10. 4 Infection rate in fish fed PBM based diets, supplemented with different kinds of hydrolysate after 14 days post-challenge by injection with *V. harveyi*. Infection started in control at 2 days post challenge (dpc), 3 dpc in both PBM+KH and PBM+CH whereas 4 dpc in PBM+TH treated groups. Asterisks denote significant differences at *P* <0.01 and 0.001, respectively between control- vs 90PBM+KH-, 90PBM+CH- and 90PBM+TH- fed fish (Kaplan Meyer survival method, followed by Log-rank test, P <0.05).

10.3.4 Serum biochemistry

As demonstrated in Figure 10. 5, serum biochemical parameters including ALT (Figure 10. 5A), GLDH (Figure 10. 5B), urea (Figure 10. 5D), creatinine (Figure 10. 5E) and total protein (Figure 10. 5G) were not influenced by any diet or time following challenge time with the exception of TB (Figure 10. 5C) and cholesterol (Figure 10. 5F). Considering TB in pre-challenge condition, feeding fish FPH supplemented diets significantly declined its level respect to the control. As concern the cholesterol level, fish at 24 h post-challenge showed lower levels respect to the pre-challenge groups fed 90PBM+KH and 90PBM+TH.

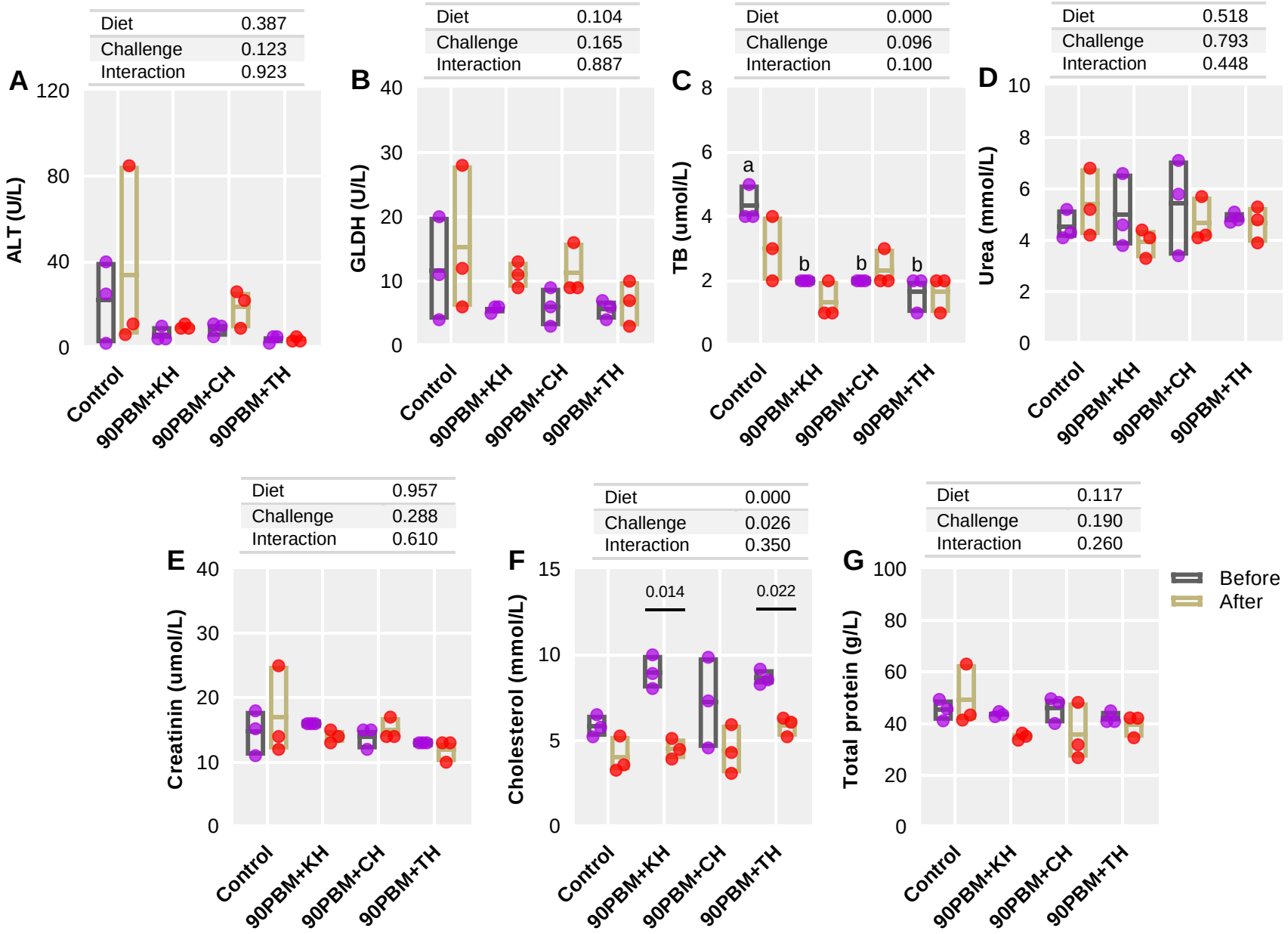
A
Diet 0.387
Challenge 0.123
Interaction 0.923
ALT (U/L)
B
Diet 0.104
Challenge 0.165
Interaction 0.887
GLDH (U/L)
C
Diet 0.000
Challenge 0.096
Interaction 0.100
TB (umol/L)
a
b
b
b
D
Diet 0.518
Challenge 0.793
Interaction 0.448
Urea (mmol/L)
E
Diet 0.957
Challenge 0.288
Interaction 0.610
Creatinin (umol/L)
F
Diet 0.000
Challenge 0.026
Interaction 0.350
Cholesterol (mmol/L)
0.014
0.022
G
Diet 0.117
Challenge 0.190
Interaction 0.260
Total protein (g/L)
Before
After
Control
90PBM+KH
90PBM+CH
90PBM+TH

Figure 10. 5 Biochemical assays changes in terms of aspartate aminotransferase, AST (A), glutamate dehydrogenase, GLDH (B), total bilirubin, TB (C), urea (D), creatinine (E), cholesterol (F) and total protein (G) in the serum of before and after challenge groups fed PBM based diets, supplemented with different kinds of hydrolysate. Two factors including "diet" and "challenge" as well as their interaction were analysed by Two-way ANOVA followed by tukey multiple comparison test. The results of before and after challenge test was compared by paired t-test. Results displayed are mean ± SE (n = 3).

10.3.5 Serum immunity and cytokines expression

The effect of dietary FPH supplementation with PBM following bacterial challenge at different time intervals on serum immunity, and pro-inflammatory and anti-inflammatory cytokines are presented in Figure 10. 6. Serum lysozyme and bactericidal activity as well as *TNF-α* and *IL-10* expression in the head kidney were induced by diets and challenge period. Meanwhile, expression level of those genes in the spleen were influenced by neither diet nor challenge period. No interaction between diet and challenge period were found in those serum immune parameters and gene expression both in head kidney and spleen. Lysozyme (Figure 10. 6A) increased significantly in 24 and 72 h post-challenge barramundi fed FPH supplemented PBM diets whilst in pre-challenge groups, bactericidal activity increased in fish fed TH supplemented PBM diet than control. TNF-α expression in the head kidney in response to 24 h and 72 h challenge (Figure 10. 6C) upregulated significantly in fish fed FPH supplemented diets respect to control while downregulation of *IL-10* (Figure 10. 6D) was observed in the same groups. None of the test diets and challenge had significant effect on TNF-α and IL-10 expression level in the spleen (Figure 10. 6E-F).

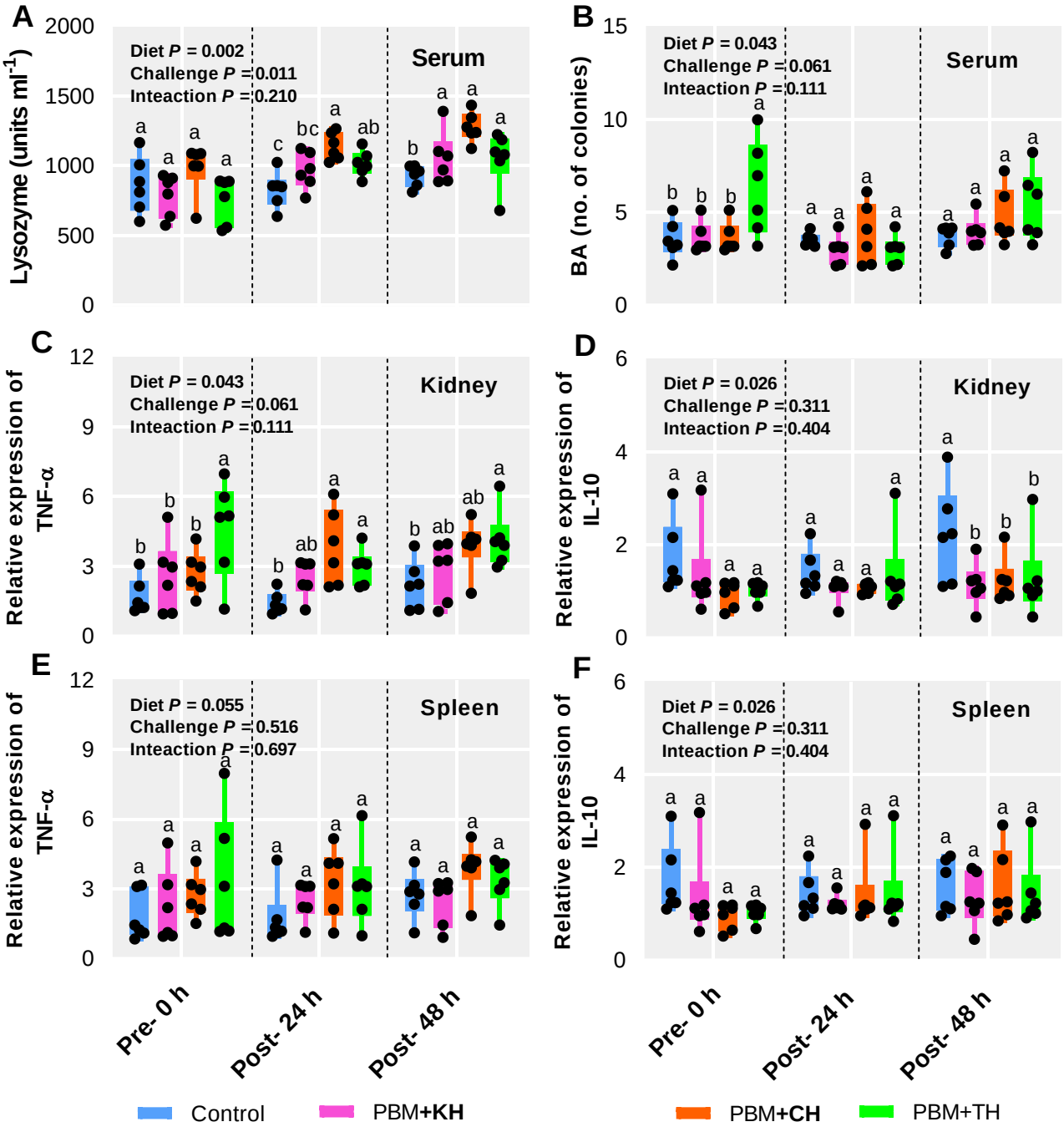

Figure 10. 6 Serum lysozyme and bactericidal activity as well as relative expression level of immune-related pro-inflammatory and inflammatory cytokines in the head kidney and spleen of juvenile barramundi at 24 and 72 h post-challenge with *V. harveyi* after feeding with control and FPH supplemented PBM diets. Two factors including "diet" and "challenge" as well as their interaction were analysed by Two-way ANOVA followed by Dunnett's multiple comparison test. Results displayed are mean ± SE (n = 6).

10.4 Discussion

Feeding PBM as the sole source of protein reduced the palatability, leading to decreased growth performance of barramundi, *L. calcarifer* (Chaklader et al., 2019) and other carnivorous fishes (Fowler, 1991; Webster et al., 2000; Yigit et al., 2006). However, supplementation of 10% FPH with 75% PBM and 90% bioprocessed PBM improved the growth performance of juvenile barramundi, *L. calcarifer* (Siddik et al., 2019b). Similarly, supplementation of an appropriate level of FPH had a positive effect on the growth performance of Atlantic cod, *Gadus morhua* (Aksnes et al., 2006a), rainbow trout *Oncorhynchus mykiss* (Aksnes et al., 2006b), Japanese sea bass, *Lateolabrax japonicas* (Liang et al., 2006), European sea bass, *Dicentrarchus labrax* (Kotzamanis et al., 2007), Japanese flounder, *Paralichthys olivaceus* (Zheng et al., 2012) and turbot, *Scophthalmus maximus* L. (Zheng et al., 2013). In the present study, all FPH supplemented PBM diets had a similar effect on the growth performance of barramundi which was in agreement with the report of Swanepoel Goosen (2018). It is well documented that survival of aquatic animal is strictly associated with the quality of formulated feed (Cahu and Zambonino Infante, 2001; Nguyen et al., 2012). Survival rate in our earlier study aggravated significantly in barramundi, *L. calcarifer* when fed 90% of PBM protein supplemented with 10% full fat black soldier fly larvae (Chaklader et al., 2019). In the present study, survival in response to dietary inclusion of PBM supplemented with different types of FPH increased significantly compared to control, indicating that FPH supplementation modulate the survival rate, which is consistent with the reported survival rate of Atlantic halibut larvae, *Hippoglossus hippoglossus* L. (Kvåle et al., 2002) and sea bass larvae, *Dicentrarchus labrax* (Cahu et al., 1999).

Feed management is important in the nutrition of aquaculture species, influencing the flesh quality characteristics of fish produced for food (Glencross, 2006). In the present study, an elevated level of total SFA content in the muscle of fish fed FPH supplemented PBM diets was mainly due to the presence of myristic acid (C14:0) and palmitic acid (C16:0). This was similar to the results of Dawson et al. (2018) who reported high abundance of palmitic acid resulting in an increase total SFA content in juvenile black sea bass fed 100PBM. FPH supplemented PBM diets in the present study increased the ∑MUFA content of barrmundi muscle. This could be the consequence of the higher concentration of MUFA content of PBM (Panicz et al., 2017; Zapata et al., 2016). Feeding exclusive levels of PBM negatively induced the essential fatty acids such as n-3 LC-PUFA, EPA and DHA in barramundi (Siddik et al., 2019a) and totoaba, *Totoaba macdonaldi* (Zapata et al., 2016). However, in the present study total PUFA, ∑n-3, ∑n-6, ∑n-9 and ∑n-3/∑n-6 increased significantly in barramundi muscle when fed PBM+FPH supplemented diets. The reason behind this retention of high level of PUFA may be due to the supplementation of various FPH with PBM. FPH supplementation has been reported to have modulatory effects on lipid accumulation and lipid

metabolism in turbot, *Scophthalmus maximus* (Xu et al., 2016) and fatty acid composition in some other animals including mice and rats (Bjørndal et al., 2013; Hosomi et al., 2011). This increase in flesh FA, in particular MUFA and PUFA, is desirable to human health and correlates with the lessening of the risk of neurological disease particularly myocardial infarction and stroke (Blondeau et al., 2015) as well as cardiovascular disease (Mensink, 2016; Michielsen et al., 2019). AI and TI are two important lipid quality indicators, which have been used to assess the contribution of SFA, MUFA and PUFA to consumer health (Renna et al., 2017) as the levels of AI and TI are highly associated with cardiovascular disorders in human (Siddik et al., 2019a). Although AI decreased in FPH supplemented groups, TI was increased by FPH supplementation. It is noteworthy that the calculated values from all dietary treatments were less than 1.0, indicating the muscle was still healthy for human consumption (Renna et al., 2017), however, very little data is available to further elucidate this modulation of muscle fatty acids by different types of FPH.

In our previous barramundi research, entire replacement of FM with PBM, supplemented with 10% full fat black soldier fly (BSF) larvae resulted in multifocal necrosis in the liver and negatively influenced the villi and enterocyte width and microvilli height (Chaklader et al., 2019). Similarly, hepatic vacuoles and lipid droplets in the liver of hybrid grouper was observed when fed with increasing levels of animal protein blend (APB) (Ye et al., 2019a). In the present study, feeding barramundi with PBM replacing FM entirely, supplementation with various FPH showed no histopathological alteration in spleen and kidney, indicating that FPH supplementation could ameliorate the nutritional quality of animal protein ingredients. Supplementation of FPH with FM and PBM-based diets has previously been reported to modulate the mucosal immunity of barramundi (Siddik et al., 2019b; Siddik et al., 2018b). Similarly, as reported here, all of the FPH supplemented PBM diets modulated the acidic mucins in the intestine and skin of barramundi. Fish mucosal surface produce neutral and acidic mucins which participate in preventing translocation of bacteria and also act as a physical barrier against other infectious pathogens (Ángeles Esteban, 2012; Estensoro et al., 2012; Sarasquete et al., 2001). Hence, the elevated acidic mucins in the intestine and skin of FPH added PBM groups may be related to the presence of low molecular weight antibacterial peptide in FPH, which may provide an increased defence mechanism against infectious agents.

FPH produced by enzymes have different functional properties including antioxidative and antimicrobial activity. A good number of studies have reported that certain level of FPH in the diet of fish can stimulate immune response, thereby, boosting the defensive power against pathogens (Khosravi et al., 2015b; Siddik et al., 2019b; Siddik et al., 2018b). Infection rate in response to *in vivo* injection of *V. harveyi* in the present study significantly decreased in 10%FPH supplemented

PBM diets when compared to the control. Similarly, 10% tuna hydrolysate (TH) supplemented with 75% PBM and 90% bioprocessed PBM modulated the disease resistance to *V. harveyi* in juvenile barramundi, *L. calcarifer*, whilst 90% PBM supplemented with 10%TH did not induce disease resistance in comparison to control (Siddik et al., 2019b). However, as reported in our earlier study (Chaklader et al., 2019), immune response and disease resistance was aggravated after administration of 90% PBM supplemented with 10% full fat BSF larvae. The antibacterial activity of different hydrolysates supplementation might be explained by the presence of a greater proportion of low molecular weight peptides. More than 95% of peptides in the present study from the various hydrolysates were smaller than 10 kDa, which are reported to be more biologically active peptides with health promoting capacity in fish (Aksnes et al., 2006a; Bui et al., 2014; Khosravi et al., 2015a). Also, less than 7000 Da with GAPDH-related antimicrobial peptides identified in the hydrolysates of yellowfin tuna, *Thunnus albacares* (Seo et al., 2012) and Atlantic mackerel, *Scomber scombrus* (Offret et al., 2019) were reported as having bacteriostatic activity against both Gram-postive and negative bacteria. In addition, FPH acts as a prebiotic by modulating lactic acid bacteria particularly *Lactobacillus sp.* in the digestive tract of American catfish, *Rhamdia quelen* (Ha et al., 2019). Augmented disease resistance due to FPH supplementation with PBM could also be further explained by either an improvement in the number of acidic mucins or by the influence of pro-inflammatory and anti-inflammatory cytokines.

Serum biochemical indices can be utilized as a part of fish health determination in response to alternative dietary ingredients and supplements. With a gradual increase of animal protein blend, liver enzymes, particularly ALT were reported to increase significantly in the plasma of hybrid grouper, *Epinephelus fuscoguttatus*♀× *Epinephelus lanceolatus*♂ (Ye et al., 2019a). Likewise, feeding 90%PBM disrupted the ALT and GLDH levels in the blood serum of barramundi (Chaklader et al., 2019). FPH supplementation has been reported to have hypolipidemic and hypocholesterolemic impacts on fish (Khosravi et al., 2015b). Similar effect was observed in the present study as indicated by a decreasing trend of ALT and GLDH in both before-challenge and after-challenge groups fed FPH supplemented PBM. Similar to the results of ALT and GLDH, TB demonstrated the same effect. An elevation of AST, GLDH and TB was reported in blood serum when cellular damage take place in liver and kidney due to stress and toxicity caused by dietary treatments (Bhardwaj et al., 2010; Chaklader et al., 2019; Kim and Kang, 2014). However, Siddik et al. (2018b) reported that dietary inclusion of a graded level of FPH (5-20%) did not influence the liver enzymes (AST and GLDH) in the serum of barramundi. Levels of cholesterol an important metabolite, can be influenced by the alteration of protein sources and is used to assess the nutritional condition of fish. Cholesterol in post-challenge groups fed 90PBM+KH and 90PBM+TH decreased significantly in comparison to pre-challenge groups fed the same diet.

Similarly, 8 weeks post-feeding with a FM based diet along with the supplementation of FPH based diet had hypocholesterolemic effect on barramundi and also reduced the serum total cholesterol, HDL-C and LDL-C in turbot, *Scophthalmus maximus* following 12 weeks experimentation (Xu et al., 2016). The mechanism behind hypolipidemic and hypocholesterolemic effects have been rarely studied but many researchers reported that FPH are rich in taurine, an active substance interfering with lipolysis and lipid oxidation by modulating the metabolism of bile acids and polyamine (Chatzifotis et al., 2008; Espe et al., 2012; Xu et al., 2016). In the present study, taurine concentration in FPH hydrolysate supplemented diets ameliorated compared to the PBM. Besides, FPH are also rich in betaine, choline, carnitine and creatine, which have also been reported to affect the lipid metabolism (Brosnan et al., 2004; Pegg, 2009), but these components were not analysed in the present study. Further study is recommended to isolate, identify and evaluate the functional role of these components on lipid metabolism. Therefore, lower levels of ALT, GLDH, TB and cholesterol suggested that FPH supplementation could reduce the negative effects of higher inclusion PBM-based diets in barramundi which were further evidenced by the histology of the kidney.

FPH produce smaller bioactive peptides with different biologically functional properties including immune-stimulating and antibacterial properties in aquaculture. FPH thus have been reported to have immune modulating capacity in fish. Administration of FPH stimulated the lysozyme and complement production in Atlantic halibut, *Hippoglossus hippoglossus* L (Hermannsdottir et al., 2009) and Japanese sea bass, *Lateolabrax japonicusas* (Liang et al., 2006) well as lysozyme activity, serum complement and immunoglobulin M in large yellow croaker, *Pseudosciaena crocea* R (Tang et al., 2008). In the present study, both diet and challenge had significant effects on serum immunity, which was characterized by increasing levels of lysozyme and decreasing number of bacterial colonies in the serum of barramundi fed FHP supplemented and post-challenge groups. Likewise, supplementation of 10% TH with FM and bioprocessed PBM significantly modulated the serum lysozyme and bactericidal activity and also increased at 72 h post-challenge compared to 24 h post-challenge and pre-challenge condition in barramundi (Siddik et al., 2019b). This stimulation of immune response might be due to the presence of a high percentage of small peptide size FPH. Molecular weight peptides ranging from 500 to 3000 Da have been reported to trigger fish macrophage activity (Bøgwald et al., 1996; Bui et al., 2014) which are concomitant with the present findings.

In teleost fish, the head kidney is one of the important lymphoid organs involved in antigen processing (Dannevig et al., 1994) and the phagocytosis process (Brattgjerd and Evensen, 1996). However, tumor necrosis factor-alpha (TNF-α), an important immune-relevant cytokine, mediates

powerful defensive mechanisms against microbes through lysing of either Gram-positive or negative bacteria, killing infected cells and hindering intracellular pathogen proliferation (Reyes-Cerpa et al., 2012; Roca et al., 2008). Fish at 24 and 72 h post-challenge with *V. harveyi* triggered upregulation in the expression of TNF-α in the head kidney of 90PBM+TH, 90PBM+KH and 90PBM+CH groups compared to pre-challenge condition, indicating that FPH supplemented diets may have stimulated defence mechanisms upon bacterial infection. Similarly, immune related genes, such as complement C4 and MHCIIa were expressed in the kidney of barramundi, *L. calcarifer* when infected with *Cryptocaryon irritans* (Mohd-Shaharuddin et al., 2013). Considering anti-inflammatory cytokines, such as *IL-10*, in this study a down-regulation in the expression level was observed in the head kidney of barramundi from FPH supplemented at 24 and 72 h post-challenge with *V. harveyi*, suggesting that FPH influenced the host response to pathogen through the reduction of anti-inflammatory cytokines after post-infection. Higher expression of IL-10 can lead to enhanced pathogen proliferation by suppressing the secretion of inflammatory molecules (Takatoshi et al., 2011). Stimulation of immune-relevant genes in response to bacterial infection in the FPH supplemented groups might be attributed to the presence of smaller size biologically active peptides. Siddik et al. (2019c) found an enhanced mRNA expression level of immune-related pro-inflammatory cytokines, including TNF-α and IL-1β, in the intestine of barramundi, *L. calcarifer* fed TH supplemented diets.

The present study demonstrated that feeding fish 90% PBM supplemented with three different types of FPH results in no alteration in growth performance and biometry indices associated with the normal structure of spleen and kidney. Mucosal immunity as determined by estimating the number of acidic mucins increased significantly in response to FPH supplemented PBM diets. In addition, FPH supplemented diets modulated the survival rate, reflected by a declining infection rate at 14 days post-challenge with *V. harveyi* in comparison with the control. ALT, GLDH, TB and cholesterol levels indicated no tissue damage of liver and kidney at day 14 post-infection with bacteria. Regulation of pro-inflammatory and inflammatory cytokines, both in spleen and head kidney, at 0, 24 and 72 h post-infection in FPH supplemented diets indicates that all kinds of hydrolysate supplemented with PBM in the present study modulated disease resistance and immunity of juvenile barramundi. Supplementation of KH, CH and TH to juvenile barramundi diets has the ability to ameliorate the negative effects on fish welfare caused by dietary inclusion of animal by-product compounds.

CHAPTER 11: Supplementation of tuna hydrolysate and insect larvae improves fishmeal replacement efficacy of poultry by-product in *Lates calcarifer* (Bloch, 1790) juveniles

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Abstract

The effects of feeding different levels of poultry by-product meal (PBM) replacing fishmeal (FM) protein, supplemented with tuna hydrolysate (TH) and *Hermetia illucens* (HI) larvae, on the growth, fillet quality, histological traits, immune status, oxidative biomarker levels and gut microbiota of juvenile barramundi, *Lates calcarifer* were investigated for six weeks. Barramundi were fed four isonitrogenous and isolipidic diets in which a FM based diet was used as the control diet (Diet1) and compared with other non-FM diets containing 80%, 85% and 90% PBM along with the concurrent supplementation of 5% and/or 10 % TH and HI larvae meal. These treatment diets were designated as $80PBM_{10TH+10HI}$ (Diet2), $85PBM_{5TH+10HI}$ (Diet3) and $90PBM_{5TH+5HI}$ (Diet4). The growth and condition factor of fish fed $80PBM_{10TH+10HI}$ and $85PBM_{5TH+10HI}$ were significantly higher than the control. Total saturated, monounsaturated and polyunsaturated fatty acid retention in the fish muscle increased in fish fed PBM-based diets, supplemented with TH and HI larvae meal, with no adverse effect on post-harvest characteristics such as texture and colour of fish fillets. Improvement in serum total bilirubin and total protein content was found in all fish fed TH and HI larvae supplemented PBM. Similarly, immune response showed a significant increase in fish fed non-FM test diets than the control. In the distal intestine, supplementation of any quantities of TH and HI larvae to PBM led to an increase in the microvilli density and neutral mucins while the number of goblet cells in the skin were unchanged. Liver, kidney, and spleen histology demonstrated a normal structure with no obvious changes in response to all test diets. Bacterial diversity increased in fish fed Diets 2 and 3 with a high abundance of *Proteobacteria* in Diets 1 and 4 and *Firmicutes* in Diets 2 and 3. The fish on test diets showed a lower abundance of genus *Vibrio*. Fish fed TH and HI larvae supplemented PBM diets showed lower infection rate to *V. harveyi* than the control. Collectively, concurrent supplementation of TH and HI larvae could improve the quality of PBM diets with positive effects on growth, fillet quality, intestinal health, immunity, and disease resistance.

11.1 Introduction

Due to a favourable nutritional profile, compatible with the nutritional requirement of most aquaculture species (Fawole et al., 2020), the aquaculture industry has traditionally relied on fishmeal (FM) as the main protein source (Henry et al., 2015). Since this dietary protein source is considered environmentally and ecologically unsustainable, there is societal and economical pressure on the aquaculture industry to search for alternative protein ingredients. Efforts have been dedicated over the past few decades to utilize plant-feedstuffs to replace FM (Fawole et al., 2020) however the resulting growth performance for carnivorous fish is inferior when compared to FM-based diets, likely due to the lower protein and imbalanced amino acids, anti-nutritional factors, and no taurine and hydroxyproline contents relative to FM in plant ingredients (Aksnes et al., 2008; Gatlin et al., 2007; Hassaan, 2017; Wang et al., 2019b).

Rendered animal by-products, particularly poultry by-product (PBM) could be a promising alternative protein source for carnivorous fish due to a higher protein content and lack of anti-nutritional factors (Allan et al., 2000; Ye et al., 2019a). The incorporation of 100% PBM replacing FM protein did not affect the welfare of a number of carnivorous fish such as humpback grouper (Shapawi et al., 2007), Nile tilapia, *Oreochromis niloticus* (El-Sayed, 1998; Hernández et al., 2010) and hybrid striped bass, *Morone chrysops* × *Morone saxatilis* (Rawles et al., 2011). However, feeding PBM at replacement levels more than 50% impacted the growth performance in barramundi, *L. calcarifer* (Chaklader et al., 2019), Florida pompano, *Trachinotus carolinus* L tench (Rossi and Davis, 2012), juvenile tench, *Tinca tinca* (González-Rodríguez et al., 2016), black sea turbot, *Psetta maeoticus* (Yigit et al., 2006) and cobia, *Rachycentron canadum* (Zhou et al., 2011). In addition, impairment of immunity and serum biochemistry were reported in largemouth bass, *Micropterus salmoides* (Subhadra et al., 2006a; Subhadra et al., 2006b) and barramundi fed PBM (Chaklader et al., 2020a; Chaklader et al., 2019) and hybrid grouper, *Epinephelus fuscoguttatus*♀× *Epinephelus lanceolatus*♂ fed an animal protein blend (Ye et al., 2019a). In our previous studies (Chaklader et al., 2020a; Chaklader et al., 2019; Siddik et al., 2019a), we have also shown that the exclusive inclusion of PBM protein at the expense of FM protein caused histopathological changes in the liver and impaired the integrity of intestinal micromorphology of barramundi. Though a number of studies have been conducted on re-formulating aquadiets supplementing fish protein hydrolysates (FPH) (Chaklader et al., 2020c; Siddik et al., 2019b; Siddik et al., 2018b; Siddik et al., 2019c) and other supplements (Talpur, 2014; Talpur and Ikhwanuddin, 2012; 2013) that complimented animal proteins and helped to satisfy the needs of barramundi, developing functional sustainable feeds that remain cost-efficient, while promoting fish welfare and maximising growth potential, is a foremost challenge for the aquaculture industry.

FPH due to presence of low molecular weight peptides and free amino acids, have been supplemented in aquafeed as immunostimulants, palatability enhancers and attractants (Kasumyan and Døving, 2003; Önal and Langdon, 2009; Ospina-Salazar et al., 2016). Promising results in terms of growth performance, immune response and disease resistance have been reported for many carnivorous fish species such as barramundi (Chotikachinda et al., 2013; Siddik et al., 2018b; Siddik et al., 2019c), Atlantic salmon, *Salmo salar* (Kousoulaki et al., 2012), European sea bass, *Dicentrarchus labrax* (Delcroix et al., 2015) and Persian sturgeon, *Acipenser persicus* L. (Ovissipour et al., 2014) when fed FPH at an appropriate level. Moreover, supplementation of FM and PBM with FPH modulated the intestinal microbiome of barramundi by increasing the number of *Bacillus*, *Lactococcus*, and *Cetobacterium* under phylum of *Firmicutes* and *Fusobacteria* and also regulated the inflammatory response (Siddik et al., 2019c).

Recently, insect have received much attention as an aquafeed ingredient due to rapid growth, and ease of reproduction (Guerreiro et al., 2020). Insects may be grown on low-value products and the final waste (frass) can be used as organic fertilizer, and insect production also has a lower risk of transmitting zoonotic infections, less greenhouse gas and ammonia emissions and less requirement for land and water than plant-feedstuffs production (Mertenat et al., 2019; van Huis et al., 2015; Wang et al., 2019b; Zarantoniello et al., 2019). Among all insects investigated so far, the larvae of *Hermetia illucens* (HI), commonly known as black soldier fly has been identified as a potential aquafeed ingredient due to the ability to assimilate nutrients from low-value wastes and by-products and transform such ingredients into high-quality animal biomass, representing a sustainable way to produce edible proteins for livestock production (Caimi et al., 2020b; Henry et al., 2015; Makkar et al., 2014). It is also noteworthy that the lipid content of HI larvae is highly variable, depending on the substrate used for their culture (Zarantoniello et al., 2020a) and easy to manipulate. Also, HI larvae as an ecological decomposer, dwelling in a harsh environment, infested with a high concentration of harmful microorganisms, can produce novel antimicrobial peptides (AMP) to gain protection from microbial infection (Elhag et al., 2017; Park et al., 2014). Indeed, a new defensin-like peptide4 AMP of 40 amino acids purified from immunized haemolymph of HI larvae has exhibited bactericidal activity to Gram-positive bacterial strains (Park et al., 2015). Hence, the dietary supplementation of HI larvae in the aquadiets could also play an important role in modulating immune response and defensive mechanisms against microbial pathogens.

Among all fish microbiota, bacteria are dominant in the fish intestine (Egerton et al., 2018). However, the bacterial composition is diverse in different parts of the intestine (Merrifield and Rodiles, 2015) and is influenced by diet, life stage, sex, habitat and season (Bano et al., 2007;

Cordero et al., 2015; Dhanasiri et al., 2011; Hansen and Olafsen, 1999; Hovda et al., 2012). Dietary manipulation of protein and lipid also has a selective pressure on the intestinal bacterial diversity in the digestive tract of fish (Egerton et al., 2018; Kim and Kim, 2013). Over the past few decades, many conventional methods have been applied to analyse the gut microbiota of fish (Wang et al., 2018) but recently with the development of molecular techniques such as next-generation sequencing (NGS), a rapid and low-cost technique, research on the gut microbiota of fish has evolved rapidly (Egerton et al., 2018). Like humans and other mammals, there is little doubt concerning the importance of the intestinal microbiota for fish health (Huyben et al., 2017a; Semova et al., 2012; Siddik et al., 2019c), however, dysbiosis of the intestinal microbiota could also be related to growth impairment, dysregulation of immune functions and disease development in fish (Estruch et al., 2015; Pérez et al., 2010). A recent study (Siddik et al., 2019c), has reported the modulatory effects of alternative protein sources on the intestinal microbiota in barramundi but possible links between diets, growth, and gut functions is fragmentary and incomplete. Hence, the profiling of gut microbiota is expected to be an important endpoint measurement to assess the effects of aquadiet in relation to growth and other health aspects.

As alternative protein ingredients could adversely affect the marketability of fish by altering fillet characteristics, a research evaluating the potential effects of extensive replacement of FM with other animal by-products on the post-harvest quality of fish flesh is highly desirable. Animal by-products particularly meat meal did not alter the organoleptic quality of barramundi fillet (Williams et al., 2003b), however, excessive inclusion of PBM negatively influenced the fillet quality of female tenches, *Tinca tinca* (Panicz et al., 2017). Nonetheless, the effects of animal based-diets at the exclusion of FM on the post-harvest quality of barramundi fillet have rarely been investigated with mixed results.

Barramundi is a commercially important euryhaline species, popularly cultured in South East Asian countries and Australia due to economic value and favourable flesh texture. The disease particularly Vibriosis, caused by *V. harveyi* is one of the common problems, causing mortalities in net-cages culture. A number of earlier studies have been conducted so far supplementing plant herbs (Talpur and Ikhwanuddin, 2013), FPH (Siddik et al., 2019b; Siddik et al., 2019c) and HI larvae (Chaklader et al., 2019) to improve the health status of barramundi. However, concurrent effects of TH and HI larvae meal have not been reported to date. Hence, the present study was designated to investigate the effects of total substitution of dietary FM with three different protein ingredients PBM and, TH and HI larvae meal on gut microbiota, health indices and fillet quality of barramundi. Protein from PBM was the main source of protein whereas TH and HI were supplemented in three different proportions to evaluate the replacement efficiency of PBM protein.

11.2 Materials and methods

11.2.1 Ethical statement

All experimental protocols involving handling and treatment of animals described in the present study were approved by the Animal Ethics Committee at Curtin University under Permit no. ARE2018-37 in full compliance with relevant guidelines and regulations in Australia for the care and use of animals. To minimize pain and discomfort during handling and culling of fish, anaesthesia (AQUI-S, 8 mg/l) and euthanasia (AQUI-S, 175 mg/l) were applied following standard operating procedure (SOP) of the Curtin Aquatic Research Laboratory (CARL).

11.2.2 Experimental diets

All the ingredients (Table 11. 1) excluding PBM, TH, and HI larvae were bought from Specialty Feeds, Glen Forrest Stockfeeders, 3150 Great Eastern Highway, Glen Forrest, Western Australia 6071. PBM was provided by Derby Industries Pty Ltd T/A, Talloman Lot Lakes Rd, Hazelmere WA 6055 and liquid TH was provided by SAMPI, Port Lincoln, Australia. Four animal protein based-test diets with similar proximate compositions were formulated (Table 11. 1). FM protein is substituted mainly by PBM followed by supplementing TH and/or HI. The control diet was formulated using FM as a protein source and the other three diets were formulated to replace 80%, 85% and 90% of FM protein with PBM along with the supplementation of 10% TH + 10% HI, 5% TH + 10% HI, and 5% TH + 5% HI, respectively. The four diets were designated as control (Diet1), $80PBM_{10TH+10HI}$ (Diet2), $85PBM_{5TH+10HI}$ (Diet3) and $90PBM_{5TH+5HI}$ (Diet4). The diets were formulated, packed and stored according to the common standard protocol in our laboratory (Chaklader et al., 2019). Fatty acid and amino acid composition of experimental diets and different ingredients are presented in Table 11. 2 and Table 11. 3, respectively.

Table 11. 1 Ingredients and nutritional composition of four experimental diets (% dry matter basis).

Ingredients (g/100g)	Diet1 (Control)	Diet2 ($80PBM_{10TH+10HI}$)	Diet3 ($85PBM_{5TH+10\ HI}$)	Diet4 ($90PBM_{5TH+\ 5HI}$)
†FM[a]	72.6	0.00	0.00	0.00
†PBM[b]	0.00	57.00	60.50	63.50
Canola oil[a]	2.20	4.00	4.00	4.00
Cod Liver Oil[a]	1.40	2.50	2.50	3.60
Corn/wheat starch[a]	6.7	8.00	10.00	10.80
wheat (10 CP)[a]	13.5	7.90	5.90	6.00
Lecithin – Soy (70%)[a]	2.00	2.00	2.00	2.00
Vitamin C[a]	0.05	0.05	0.05	0.05
Dicalcium Phosphate[a]	0.05	0.05	0.05	0.05
Vitamin and mineral premix [a]	0.50	0.50	0.50	0.50
Salt (NaCl)[a]	1.00	1.00	1.00	1.00
ǂHI full-fat larvae[c]	0.00	10.00	10.00	5.00
‡TH[d]	0.00	7.00	3.50	3.50
Total	100	100	100	100
Nutritional composition (%)				
Dry matter	92.90	90.35	91.35	92.14
Crude protein	46.20	45.92	46.20	46.72
Crude lipid	12.93	13.01	12.88	13.00
Ash	12.78	12.64	11.89	12.44

Gross energy (MJ kg^{-1})	20.02	19.98	19.88	19.46

[a]Purchased from Specialty Feeds, Glen Forrest Stockfeeders, 3150 Great Eastern Highway, Glen Forrest, Western Australia 6071.

[†]Fishmeal (FM): 64.0% crude protein, 10.76% crude lipid and 19.12% ash.

[†]Poultry by-product meal (PBM): 67.13% crude protein, 13.52% crude lipid and 13.34% ash

[ǂ]HI (*Hermetia illucens*) larvae: 40.04% crude protein, 28.30 % crude lipid, 7.81% moisture and 8.91% ash

[‡]Tuna hydrolysate (TH): 37.91% crude protein, 5.50% crude lipid, and 11.05% ash

Table 11. 2 Amino acid composition (g/100g DM) of PBM-based diets, supplemented with TH and HI larvae meal and different ingredients including PBM, TH and HI larvae meal

	Diet1 (Control)	Diet2 ($80PBM_{10TH+10HI}$)	Diet3 ($85PBM_{5TH+10\ HI}$)	Diet4 ($90PBM_{5TH+\ 5HI}$)	PBM	TH	HI
Hydroxyproline	1.79	3.14	3.16	3.37	3.48	1.81	0.24
Histidine	3.01	2.33	2.36	2.27	2.30	2.96	3.27
Taurine	0.48	0.59	0.56	0.53	0.44	2.84	1.08
Serine	4.50	4.22	4.26	4.19	4.34	4.55	4.48
Arginine	6.31	6.89	6.95	6.98	7.32	5.55	5.45
Glycine	7.99	9.62	9.74	9.98	10.13	8.79	5.86
Aspartic acid	9.45	8.84	8.71	8.81	8.50	9.07	10.42
Glutamic acid	13.90	14.55	14.31	14.49	13.83	11.69	13.42
Threonine	4.74	4.13	4.17	4.10	4.19	4.74	4.35
Alanine	6.72	6.65	6.65	6.66	6.61	7.01	6.59
Proline	5.67	6.70	6.70	6.71	6.63	5.64	6.24

Lysine	7.32	6.63	6.56	6.55	6.58	6.98	7.10
Tyrosine	2.87	2.90	2.86	2.79	2.97	2.90	5.97
Methionine	2.92	2.15	2.13	2.15	2.23	2.71	2.02
Valine	5.33	5.02	5.08	4.95	4.92	5.80	6.40
Isoleucine	4.54	4.20	4.24	4.14	4.07	4.61	4.86
Leucine	8.04	7.36	7.45	7.30	7.37	7.88	7.59
Phenylalanine	4.42	4.08	4.12	4.03	4.09	4.49	4.67

Table 11. 3 Fatty acids composition (g/100g DM) of PBM-based diets, combinedly supplemented with TH and HI larvae meal and different ingredients including PBM, TH and HI

	Diet1 (Control)	Diet2 ($80PBM_{10TH+10HI}$)	Diet3 ($85PBM_{5TH+10\ HI}$)	Diet4 ($90PBM_{5TH+\ 5HI}$)	PBM	TH	HI
C12:0	0.03	5.02	5.23	2.44	0.09	0.04	53.81
C14:0	1.32	2.10	1.96	1.66	0.74	4.75	8.87
C16:0	11.61	18.13	17.73	17.01	23.36	28.56	20.47
C18:0	4.49	5.73	5.55	5.34	7.62	10.92	4.65
∑SFA	19.81	33.03	32.41	28.13	33.45	48.41	93.05
C14:1n5	0.02	0.10	0.10	0.09	0.16	0.04	0.15
C16:1n7	1.65	3.93	3.90	3.71	5.40	5.70	6.21
C18:1cis+trans	11.59	43.54	43.93	42.49	44.11	33.34	23.37
C20:1	0.80	2.03	1.96	2.34	0.61	2.56	0.29
C22:1n9	0.09	0.20	0.19	0.25	0.06	0.31	0.00

∑MUFA	14.82	50.41	50.64	49.47	50.42	43.01	30.67
C18:3n3	1.20	4.28	4.37	3.99	2.60	1.53	5.17
C18:4n3#	0.30	0.55	0.47	0.42	0.13	2.25	1.84
C20:3n3	0.08	0.08	0.07	0.07	0.04	0.25	0.11
C20:5n3	1.78	2.15	1.68	1.77	0.17	12.12	3.67
C22:5n3#	0.63	0.66	0.53	0.60	0.37	3.41	0.28
C22:6n3	9.09	4.25	2.95	3.49	0.27	32.32	0.78
∑n-3 PUFA	13.09	11.97	10.06	10.34	3.59	51.88	11.85
∑n-3 LC PUFA	11.59	7.14	5.22	5.93	0.01	48.10	4.83
C20:2	0.15	0.18	0.17	0.17	0.20	0.35	0.12
C18:3n6	0.09	0.09	0.09	0.13	0.23	0.21	0.15
C20:3n6	0.15	0.30	0.30	0.30	0.57	0.47	0.23
C20:4n6	1.13	1.01	0.98	0.93	1.80	1.99	1.31
C22:4n6#	0.91	0.17	0.12	0.13	0.05	1.18	0.20
∑n-6 PUFA	2.28	1.58	1.50	1.49	2.65	3.86	1.88
∑PUFA	21.84	31.24	29.86	29.00	23.87	58.32	27.38

11.2.3 Experimental fish and feeding

The feeding trial was conducted in a recirculating aquaculture system at CARL, Curtin University, Australia. A total of 450 juvenile barramundi (mean weight, 3.33g) were provided by the Australian Centre for Applied Aquaculture Research, Fremantle, Australia and shipped to CARL in double-layered plastic bags filled with oxygenated 30 ppt saline water. Fish were housed in three 300L fiberglass tanks containing seawater for two weeks to adapt to the CARL conditions. Throughout the acclimation period, one-third of the water was changed daily and fish were hand-fed with a commercial diet. Fish were fasted for 24 h before commencing the trial and then 300 fish with almost similar weights were distributed randomly into 12 tanks (4 dietary treatments × 3 replicated tanks), with 25 individuals in each tank. Water quality parameters including temperature, dissolved oxygen, nitrite, nitrate, and temperature controlled by an electric heater, aerator and external bio-filter (ASTRO 2212, China), were maintained as described in our earlier study (Chaklader et al., 2019). 14:10 h light: dark photoperiod was maintained throughout the study period using an automatic indoor timer (Clipsal, Australia). Fish were hand-fed the experimental diets until apparent satiation, twice daily at 8.00 am and 6.00 pm for 6 weeks. After each feeding session, provided feed and uneaten feed weights were recorded to calculate FI on a daily basis. Fish health and mortality, if any, were monitored regularly to calculate the survival rate.

11.2.4 Sampling procedure

After the six weeks of feeding trial, fish were starved for 24 h and anesthetized with 8 mg/l AQUI-S prior to taking total biomass, and fish were also counted individually to estimate survival rate. Viscera and liver weight were recorded from randomly chosen ten fish from each treatment to determine viscerosomatic (VSI) and hepatosomatic (HSI) indices. For serum collection, four fish from each tank (n = 12) were anesthetized with 8 mg/l AQUI-S and blood was collected from the caudal vein using 1 mL syringes fitted with 22G needles. Serum was collected and preserved according to the protocols of our earlier study (Chaklader et al., 2019). Fish were then culled with a sharp blow to the head and dissected on trays to collect muscle, liver, and intestine samples. Pooled "fillet" muscle was collected and dried in a freeze dryer and stored at -80° C for further fatty acid analysis. Also, four fish/replicate were filleted to analyse texture and colour. The liver and intestine were also preserved immediately in -80° C until later analysis of antioxidant activity and gut microbiota. Four fish per tank were also euthanized to collect intestine, muscle with skin, liver, kidney, and spleen and immediately preserved in 10% buffered formalin for fixation for later histological examination.

11.2.5 Fillet fatty acids compositions and quality index

Fatty acids profile of four pooled dried muscle/replicate and experimental diets were performed according to the procedures of O'Fallon et al. (2007) and Siddik et al. (2019a).

Texture profile in terms of hardness, cohesiveness, adhesiveness, springiness, gumminess, chewiness of fish fillet (three fish/treatment) were analysed by texture analyser TVT 6700 (PerkinElmer, Inc., Waltham, Middx, USA) and TexCal texture analyser software (version 5.0) (Ayala et al., 2010). Each sample was run in triplicate and expressed as force per g. Fillet colour coordinates (L*, a*, b*) were determined by calibrated BYK spectro-guide sphere gloss (BYK-Gardener USA, Columbia, MD, USA) (Ozbay et al., 2018). 4 g of fillet were homogenized with 40 mL of distilled water and pH was determined in triplicate by Aqua-pH meter (TPS Pty Ltd, Brendale, QLD, AU) after calibrating at three-scale pH (pH 4, 7, 10) (AOAC, 1995).

11.2.6 Histological and scanning electron microscopy analysis

After fixation with 10% buffered formalin, fragments of intestine, liver, kidney, spleen, and skin tissue samples were dehydrated with a series of ethanol concentrations. Dehydrated samples were subsequently cleared in xylene, embedded in paraffin blocks, cut into around 5µm slices, and stained with haematoxylin and eosin (H&E) for histopathological assessment. Intestine and liver samples were stained with Periodic Acid-Schiff (PAS) to visualize neutral mucins and glycogen, respectively. Skin tissue was stained with AB-PAS stain to visualize goblet cells. Microphotographs of all histological slides were taken with a light imaging microscope (BX40F4, Olympus, Tokyo, Japan). The number of neutral mucins in intestine and skin was counted as described in our earlier study (Chaklader et al., 2019).

Scanning electron microscopy of distal intestine from four biological replicates were analysed according to the earlier study in our laboratory (Siddik et al., 2019b). Intestinal samples (5mm) were washed for 30s with 1 % S-carboxymethyl-L-cysteine to remove mucus and then fixed in 2.5 % glutaraldehyde in sodium cacodylate buffer (0.1 M pH 7.2). Samples were processed as described elsewhere, screened with JSM 6610 LV (Jeol, Tokyo, Japan) SEM and analysed with Image J 1.46r (National Institute of Health, USA).

11.2.7 Serum immunity and biochemical assays

Serum immunity including lysozyme and bactericidal activity were analysed as described in our earlier study (Chaklader et al., 2019).

The plasma clinical chemistry panel was processed on a AU480 Clinical Chemistry Analyser (Beckman Coulter Australia Pty Ltd, Lane Cove West, NSW). Beckman Coulter clinical chemistry kits were used for the following panel components: alanine transaminase (ALT

OSR6107) gamma-glutamyl transferase (GGT OSR6219) total bilirubin (TB OSR6112), urea (OSR6134), creatinine (OSR6178), cholesterol (OSR6116), total protein (TP OSR6132) while Randox kits (Randox Australia Pty Ltd, Parramatta, NSW) were used for glutamate dehydrogenase (GLDH GL441).

11.2.8 Serum and liver antioxidant activity

For each replicate/treatment, approximately 0.20 mg of liver tissues was weighed and homogenized with 2 mL of chilled PBS. Immediately homogenized tissue was centrifuged at 10,000 ×g for 15 min at 4° C and supernatant was collected and stored at 80° C till analysis.

Catalase activity (CAT) in serum and liver homogenate were performed according to the manufacturer company instruction (Bockit, BIOQUOCHEM SL, 33428 Llanera-Asturias, Spain).

11.2.9 Microbiome analysis

11.2.9.1 Amplicon sequencing

After the trial, two randomly selected fish per tank (n=6) were used for gut microbiota analysis. Processing of samples comprised of a collection of the whole gut, separation of the hindgut, homogenization of gut contents in tissue lyserII (Qiagen, Hilden, Germany), and pooling of homogenized samples from the respective tanks (n=3). Bacterial DNA from pooled the hindgut samples was extracted using Blood and Tissue Kit (Qiagen, Hilden, Germany). DNA concentration was measured in NanoDrop (Thermo Fisher Scientific, USA). PCR master mixture was prepared as 50 µl final concentration containing 25 µl Hot Start 2X Master mix (New England BioLabs Inc., USA), 2 µl of sample DNA, 1 µl of each V3V4 primers (Klindworth et al., 2013) and 21 µl of nuclease-free water. A total of 40 cycles of amplification reactions was performed in a thermal cycler (Bio-Rad Laboratories, Inc., USA). The library for 16S rRNA amplicons was prepared according to Illumina standard protocol (Part # 15044223 Rev. B). Samples were then sequenced on an Illumina MiSeq platforms (Illumina Inc., San Diego, California, USA using a v3 kit (600 cycles).

11.2.9.2 Sequence data processing and analysis

Low quality reads (phred score ≤ 20, > 6 homopolymers), adaptors, short bases (< 200 bp) were trimmed and cleaned using TrimGalore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The quality of sequences was checked before and after trimming in FastQC and MultiQC pipelines (Andrew, 2010; Ewels et al., 2016). MeFiT program was used for the merging of overlapping pair-end reads (Parikh et al., 2016). De novo assembly, picking of OTUs at 97% similarity threshold level and removing of singleton OTUs were performed in micca (v1.6.1)-qiime (v2.0) pipelines (Albanese et al., 2015; Caporaso et al., 2010). Phylogenetic assignment of OTUs at different taxa levels was performed

against SILVA 1.32 release (Quast et al., 2012). PASTA-aligned sequences were used for phylogenetic tree constructions under FastTree (version 2.1.8) GTR+CAT (Mirarab et al., 2015; Price et al., 2010). The rarefaction depth was set to 38,296 bp and subsequent measurements of alpha-beta diversity were performed using qiime (v1.9.1) and R packages. Alpha diversity was calculated in terms of observed OTUs and Shannon indices. Beta diversity was measured based on Bray-Curtis dissimilarity of Weighted UniFrac matric. Relative abundance (≥ 1% of read abundance) of phyla and genera was calculated phyloseq R package (McMurdie and Holmes, 2013) . Differential abundance was calculated using the linear discriminant analysis effect size (LEfSe) at LDA ≥2.0 and 0.05 level of significance (Segata et al., 2011).

11.2.10 Challenge test

After the feeding trial, 10 fish/tank (30 fish/treatment) with the same rearing condition were challenged with *V. harveyi*, provided by Diagnostic and Laboratory Services, Department of Primary Industries and Regional Development (DPIRD), 3 Baron-Hay Court, South Perth WA 6151. Fish were intraperitoneally injected with 0.1 mL of phosphate-buffered saline (PBS) containing 1.1 x 10^8 cfu/ml of a pathogenic strain of *V. harveyi* as elucidated in our earlier study (Chaklader et al., 2020c). Symptoms of vibriosis such as thick layer of mucous on the body surface, congestion of the fins, and haemorrhages and ulceration of the skin and muscle tissue were monitored every 8 h and lasted for 96 h. Fish with vibriosis were culled according to the protocol of the CARL SOP for fish euthanization.

11.2.11 Calculation and statistical analysis

Weight gain (WG), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), condition factor (CF), viscerosomatic index (VSI) and hepatosomatic index (HSI) were estimated using the following formulae:

Weight gain (WG, g) = [(Mean final weight - Mean initial weight) / (Mean initial weight)]

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

Feed intake (FI,g/fish d^{-1}) = [(Dry diet given - Dry remaining diet recovered) / days of experiment) / no. of fish]

Feed conversion ratio (FCR) = [(Dry feed fed) / (Wet weight gain)]

Condition factor (CF, %) = [Final body weight (g) / Body length cm^3)] × 100

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

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

All experimental results are presented as mean ± standard error (SE). Groups of fish per tank were used as experimental units for growth data. Individual fish were used as experimental units for organo-somatic assessment, biochemical assays, immune response, fatty acids, antioxidant activity, and histological analysis. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test was performed to the significant differences between treatments when data met the normality, checked by Shapiro-Wilk's and Levene's tests. Infection data from the challenge trial were analysed by the Kaplan-Meier method based on the pairwise multiple comparison Log-Rank (Mantel-Cox) test.

11.3 Results

11.3.1 Growth performance and organo-somatic indices

The effects of different levels of PBM concurrently supplemented with TH and HI on the growth performance and organo-somatic indices are shown in Table 11. 4. Fish fed Diet2 ($80PBM_{10TH+10HI}$) and Diet3 ($85PBM_{5TH+10HI}$) recorded significantly higher final body weight (FBW), weight gain (WG) and specific growth rate (SGR) after six weeks of feeding whereas fish fed Diet 4 ($80PBM_{5TH+5HI}$) was similar to control. No significant variation was observed in feed intake (FI) and feed conversion ratio (FCR) between control and test diets. The survival rate, ranging from 89.33 to 93.33%, was not influenced by the test diets. Condition factor (CF) increased significantly in fish fed supplemented TH and HI larvae diets, although hepatosomatic (HSI) and viscerosomatic (VSI) indices were not influenced by any test diets.

Table 11. 4 Growth performance, feed utilization, survival and organo-somatic indices of barramundi fed control (Diet1), $80PBM_{10TH+10HI}$ (Diet2), $85PBM_{5TH+10HI}$ (Diet3), and $90PBM_{5TH+5HI}$ (Diet4) for six weeks. Multiple comparisons were performed by one-way ANOVA analysis followed by Dunnett's multiple comparisons test.

	Diet1 (Control)	Diet2 ($80PBM_{10TH+10HI}$)	Diet3 ($85PBM_{5TH+10\ HI}$)	Diet4 ($90PBM_{5TH+\ 5HI}$)
FBW (g)	50.05 ± 2.05^{c}	67.60 ± 1.93^{a}	60.49 ± 2.37^{ab}	54.24 ± 2.57^{bc}
WG (g)	42.30 ± 2.05^{c}	59.84 ± 1.93^{a}	52.73 ± 2.37^{ab}	46.48 ± 2.57^{bc}
SGR (%/d)	4.28 ± 0.11^{c}	5.07 ± 0.08^{a}	4.73 ± 0.11^{ab}	4.42 ± 0.13^{bc}
FI (g/fish/d)	1.48±0.10	1.60±0.11	1.40±0.19	1.40±0.17
FCR	1.49±0.16	1.14±0.07	1.11±0.05	1.28±0.08
SR (%)	90.67±3.53	89.33±1.33	93.33±1.33	92.00±2.31
CF (%)	1.27 ± 0.01^{b}	1.38 ± 0.01^{a}	1.35 ± 0.01^{a}	1.37 ± 0.01^{a}
VSI (%)	9.86±0.46	10.69±0.40	11.06±0.30	11.08±0.30

HSI (%)	1.70±0.07	1.75±0.08	1.67±0.07	1.77±0.08

Results are expressed as mean ± SE (standard error) (n = 3). Means with different superscripts letters in the same row indicates significant difference at $P < 0.05$.

11.3.2 Muscle chemical and fatty acid composition

Moisture and ash were unchanged among the test diets at the end of the trial. Lipid in muscle of fish fed PBM based-diets supplemented with TH and HI larvae increased significantly while protein levels decreased significantly in PBM based diets (Table 11. 5). The concentration of C12:0, C14:0, C16:0, and C18:0 led to an increase in the total SFA in the muscle of fish fed PBM-based diets, concurrently supplemented with TH and HI larvae. Total MUFA content increased in the same diets and it was mainly due to the higher concentration of C14:1n5, C16:1n7, C20:1, C18:1cis+trans, and C22:1n9. TH and HI larvae supplemented PBM substantially improved the total PUFA, manifested by an elevated level of C18:3n3, C18:4n3, C20:3n3, C22:5n3, C18:3n6, C20:3n6 and C20:4n6. However, C22:6n3 and C22:4n6 decreased significantly in TH and HI larvae supplemented PBM fed fish than the control diet.

Table 11. 5 Chemical composition and fatty acids composition (mg g^{-1} DM) of barramundi muscle (skin less) after six weeks feeding with a gradient PBM diets, concurrently supplemented with TH and HI larvae meal.

	Diet1 (Control)	Diet2 ($80PBM_{10TH+10HI}$)	Diet3 ($85PBM_{5TH+10\ HI}$)	Diet4 ($90PBM_{5TH+\ 5HI}$)
Chemical composition (%)				
Moisture (WW)	75.22±0.37	74.88±0.44	75.67±0.15	75.12±0.33
CP (DM)	75.27±0.18^{a}	70.07±0.62^{b}	70.72±0.60^{b}	71.16±0.42^{b}
CL (DM)	5.70±0.13^{b}	10.70±0.38^{a}	10.58±0.53^{a}	10.74±0.25^{a}
Ash (DM)	5.69±0.02	5.61±0.13	5.67±0.08	5.65±0.10
Fatty acid composition, mg g^{-1} DM				
C12:0	0.03±0.01^{c}	1.55±0.09^{a}	1.63±0.10^{a}	0.74±0.04^{b}
C14:0	0.52±0.06^{b}	1.18±0.06^{a}	1.17±0.06^{a}	0.96±0.06^{a}
C16:0	5.81±0.31^{b}	10.99±0.53^{a}	11.13±0.41^{a}	10.92±0.40^{a}
C18:0	2.00±0.11^{b}	3.70±0.15^{a}	3.69±0.09^{a}	3.63±0.13^{a}
∑SFA	9.21±0.51^{b}	18.46±0.88^{a}	18.60±0.68^{a}	17.21±0.66^{a}
C14:1n5	0.01±0.00^{b}	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}
C16:1n7	0.86±0.05^{b}	2.27±0.14^{a}	2.32±0.10^{a}	2.20±0.11^{a}
C20:1	0.48±0.04^{b}	1.06±0.07^{a}	1.06±0.05^{a}	1.25±0.05^{a}

C18:1cis+trans	7.43±0.45^{b}	22.85±1.35^{a}	23.61±1.09^{a}	23.59±0.85^{a}
C22:1n9	0.05±0.01^{a}	0.10±0.01^{b}	0.10±0.00^{b}	0.12±0.01^{a}
∑MUFA	9.11±0.56^{b}	26.65±1.58^{a}	27.44±1.26^{a}	27.53±1.00^{a}
C18:3n3	0.72±0.05^{b}	2.03±0.12^{a}	2.11±0.10^{a}	1.91±0.08^{a}
C18:4n3#	0.11±0.01^{b}	0.31±0.02^{a}	0.28±0.02^{a}	0.26±0.02^{a}
C20:3n3	0.03±0.00^{b}	0.06±0.00^{a}	0.07±0.00^{a}	0.06±0.00^{a}
C20:5n3	0.80±0.03^{b}	1.24±0.02^{a}	1.06±0.04^{a}	1.10±0.07^{a}
C22:5n3#	0.47±0.04^{b}	0.80±0.04^{a}	0.74±0.02^{a}	0.69±0.03^{a}
C22:6n3	6.26±0.34^{a}	4.36±0.09^{b}	3.32±0.06^{c}	3.73±0.12^{bc}
∑n-3 PUFA	8.39±0.48	8.78±0.29	7.57±0.23	7.74±0.32
∑n-3 LC PUFA	7.56±0.42^{a}	6.45±0.16^{ab}	5.18±0.12^{c}	5.58±0.23^{bc}
C18:3n6	0.08±0.01^{b}	0.21±0.02^{a}	0.26±0.01^{a}	0.21±0.01^{a}
C20:3n6	0.13±0.01^{b}	0.28±0.02^{a}	0.33±0.01^{a}	0.28±0.01^{a}
C20:4n6	0.64±0.03^{b}	0.94±0.01^{a}	0.93±0.01^{a}	0.91±0.02^{a}
C22:4n6#	0.52±0.03^{a}	0.17±0.00^{b}	0.13±0.00^{b}	0.13±0.00^{b}
∑n-6 PUFA	1.37±0.07^{b}	1.60±0.04^{a}	1.65±0.04^{a}	1.53±0.04^{ab}
∑PUFA	6.73±0.41^{b}	15.19±0.65^{a}	15.61±0.63^{a}	14.68±0.68^{a}

Results are expressed as mean ± SE (standard error) (n = 12). Means with different superscripts letters in the same row indicates significant difference at $P < 0.05$.

11.3.3 pH, Texture and colour of fish fillet

At the end of the trial, pH, texture profile (springiness, cohesiveness, gumminess, chewiness, adhesiveness and hardness) and colour (L*, a*, b* and chroma) in skin and fillet flesh were similar in fish fed any diet (Table 11. 6).

Table 11. 6 Texture profile and colour of barramundi fillet after six weeks feeding with a gradient PBM diets, concurrently supplemented with TH and HI larvae meal.

	Diet1 (Control)	Diet2 (80PBM$_{10TH+10HI}$)	Diet3 (85PBM$_{5TH+10\ HI}$)	Diet4 (90PBM$_{5TH+\ 5HI}$)
P^{H}	6.90±0.05	6.91±0.04	6.90±0.02	6.91±0.02
Texture parameter				
Springiness	0.99±0.01	1.00±0.00	0.98±0.01	0.98±0.01
Cohesiveness	0.36±0.03	0.33±0.00	0.35±0.03	0.32±0.01
Gumminess (g)	957.76±82.30	1433.56±165.93	1147.32±149.53	1431.16±115.31
Chewiness (g)	957.53±	1430.60±	1144.52±	1426.03±

Adhesiveness (g.mm)	-13.51±2.17	-15.82±4.24	-16.07±2.42	-21.30±6.93
Hardness	2780.83±462.31	4427.33±477.80	3369.83±525.92	4453.17±494.24
Skin Colour				
L*	60.93±1.81	59.30±3.58	59.99±1.75	59.99±1.57
a*	-1.37±0.10	-1.30±0.24	-1.20±0.12	-1.45±0.07
b*	-4.06±0.26	-4.03±0.43	-4.76±0.39	-3.60±0.25
Chroma	4.30±0.23	4.26±0.44	4.93±0.36	3.90±0.22
Fillet muscle colour				
L*	56.55±1.12	57.11±1.03	60.25±1.03	58.61±1.52
a*	0.37±1.07	-1.04±0.32	-1.36±0.29	-1.21±0.56
b*	3.90±1.13	3.72±0.41	4.80±0.59	4.04±0.32
Chroma	4.36±1.32	4.00±0.28	5.08±0.52	4.40±0.34

L*, light/dark; a*, red/green and b*, yellow/blue.

11.3.4 Histological analysis of liver, kidney, and spleen

Hitopathological analysis did not demonstrate any substantial changes in the liver, kidney and spleen between diets at the end of the trial (Figure 11. 1A-L). Higher pigmented hepatic cytoplasm indicating higher amount of glycogen was examined in the liver of fish fed all test diets (Figure 11. 1A-D). In all dietary group, normal renal glomeruli and renal tubules in the kidney (Figure 11. 1E-H) and normal white and red pulp in the spleen (Figure 11. 1I-L) were observed.

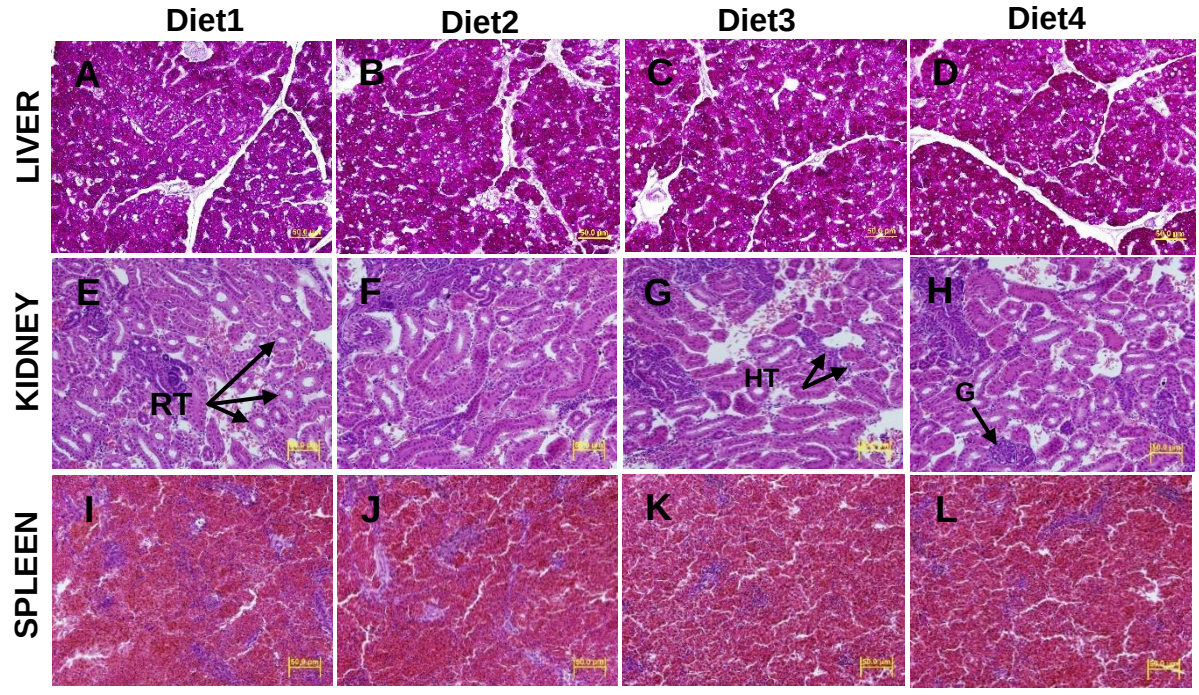

Figure 11. 1 Representative micrographs of staining liver (A-D), kidney (E-H) and spleen (I-L) sections of barramundi fed control (Diet1), 80PBM$_{10TH+10HI}$ (Diet2), 85PBM$_{5TH+10HI}$ (Diet3), and 90PBM$_{5TH+5HI}$ (Diet4) for six weeks using PAS and H&E (40 × magnification).

11.3.5 Intestinal mucosal morphology and histochemistry

The intestinal mucosal micromorphology of barramundi fed control and test diets were examined by SEM (Figure 11. 2A-D) and light microscopy (Figure 11. 2E-L). Microvilli count (density) (Figure 11. 2M) and neutral mucins (Figure 11. 2N) were significantly higher in barramundi fed TH and HI larvae supplemented at different levels of PBM when compared with those fed the control diet. However, neutral mucins producing goblet cells in rectum were unchanged (Figure 11. 2O).

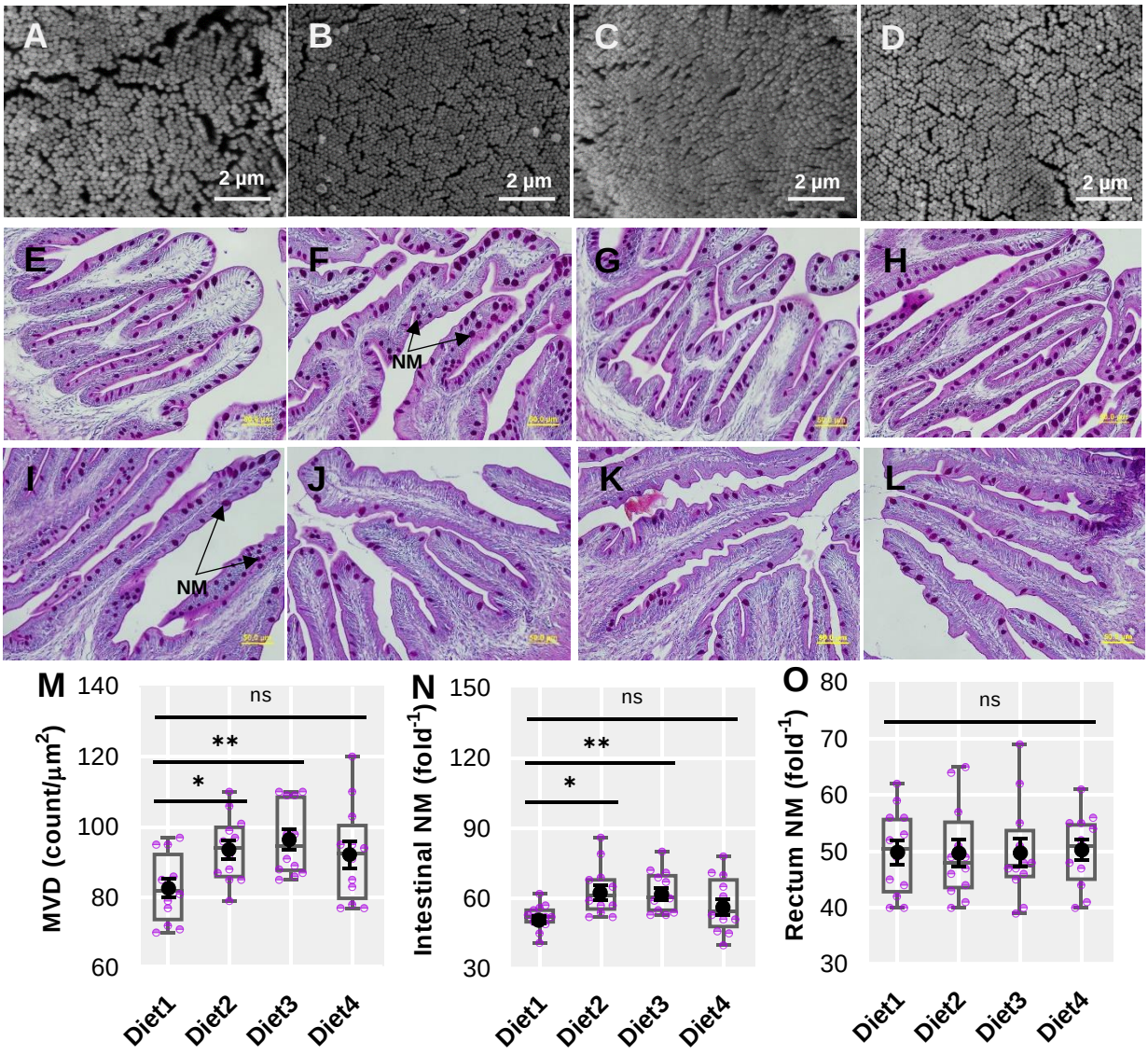

Figure 11. 2 Scanning electron micrographs (A-D) to visualize the microvilli density and goblet cells containing neutral mucins (E-H, PAS stain, 40 × magnification) in the distal intestine (E-H) and rectum (I-L) of barramundi fed control (Diet1), 80PBM$_{10TH+10HI}$ (Diet2), 85PBM$_{5TH+10HI}$ (Diet3), and 90PBM$_{5TH+5HI}$ (Diet4) for six weeks. Variation in the microvilli density (M) and neutral mucins in the distal intestine (N) and rectum (O) of barramundi fed test diets. Multiple comparisons were performed by one-way ANOVA analysis followed by Dunnett's multiple comparisons test. NM, neutral mucins and ns, not significant.

11.3.6 Skin histochemistry

The results of skin morphology in response to different levels of PBM, supplemented with TH and HI larvae are shown in Figure 11. 3(A-E). Mixed types of goblet cells were unchanged in fish fed control and test diets (Figure 11. 3E).

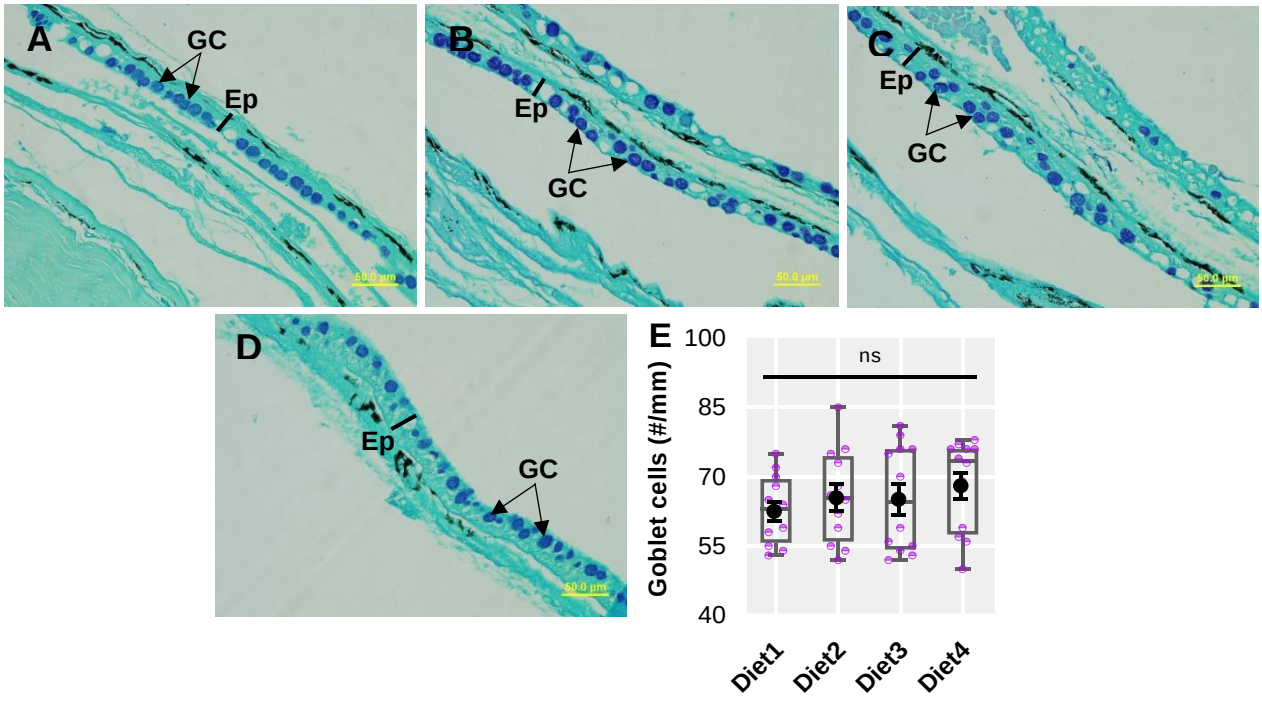

Figure 11. 3 Representative skin micrographs (A-D, AB-PAS stain, 40 × magnification) and number of mixed goblet cells of barramundi fed control (Diet1), 80PBM$_{10TH+10HI}$ (Diet2), 85PBM$_{5TH+10HI}$ (Diet3), and 90PBM$_{5TH+5HI}$ (Diet4) for six weeks. Variation in the mixed types of goblet cells in the skin (E) of barramundi fed test diets. Multiple comparisons were performed by one-way ANOVA analysis followed by Dunnett's multiple comparisons test. ns denotes not significant.

11.3.7 Biochemical assays, immune response and antioxidant activity

A significant decrease in the total bilirubin activity of fish fed PBM diets supplemented with TH and HI larvae meal was observed, whilst total protein content in fish fed 85PBM$_{5TH+10\ HI}$ increased significantly when compared to the control (Table 11. 7). The remaining biochemical assays including AST, GGT, GLDH, urea, creatinine, and cholesterol were not affected by any of the test diets. Concurrent supplementation of TH and HI larvae meal to PBM diets also modulated the serum lysozyme and bactericidal activity than control. CAT activity both in serum and liver was not affected by different levels of PBM supplemented with TH and HI larvae meal with respect to the control. Liver protein content did not differ significantly among the test diets.

Table 11. 7 Biochemical assays, immune response and antioxidant activity of barramundi fed with a gradient PBM diets, concurrently supplemented with TH and HI larvae meal for six weeks.

	Diet1 (Control)	Diet2 (80PBM$_{10TH+10HI}$)	Diet3 (85PBM$_{5TH+10\ HI}$)	Diet4 (90PBM$_{5TH+\ 5HI}$)
Serum biochemical assays				
ALT (U/L)	2.00±1.00	3.67±0.88	3.33±1.33	8.33±1.67
GGT (U/L)	0.00±0.00	0.67±0.33	0.67±0.33	0.67±0.33
GLDH (U/L)	8.00±0.57	6.00±0.00	6.67±0.33	6.33±0.88
Total Bilirubin (umol/L)	3.33±0.33^{a}	2.00±0.00^{b}	2.00±0.00^{b}	2.00±0.00^{b}
Urea (mmol/L)	4.07±0.47	5.47±0.59	5.50±0.12	5.90±0.28
Creatinine (umol/L)	17.33±0.88	21.00±1.00	19.00±0.00	18.67±0.33
Cholesterol (mmol/L)	8.08±0.16	13.08±1.24	10.77±0.37	11.38±1.43
Total Protein (g/L)	43.47±0.52^{b}	49.07±1.47^{ab}	47.60±0.90^{a}	45.87±0.22^{b}
Serum immune response and antioxidant activity				
Lysozyme (U/mL)	765.56±85.69^{bc}	1025.56±76.60^{a}	940.22±20.76^{ab}	668.89±66.45^{c}

BA (log_{10})	4.60±0.26[a]	2.50±0.30[b]	2.45±0.64[b]	3.76±0.68[ab]
CAT (U mg.prot^{-1})	0.82±0.32	0.74±0.12	0.72±0.22	0.56±0.06
Liver protein and antioxidant activity				
Total protein (mg/mL)	3.87±0.59	2.69±0.07	2.34±0.32	3.04±0.17
CAT (U mg.prot^{-1})	786.33±72.76	692.67±146.68	917.00±178.18	757.33±196.17

Results are expressed as mean ± SE (standard error) (n = 3). Means with different superscripts letters in the same row indicates significant difference at $P < 0.05$.

Alanine transaminase, ALT; gamma-glutamyl transferase, GGT; glutamate dehydrogenase, GLDH, bactericidal activity, BA and catalase activity, CAT.

11.3.8 Sequence stats and alpha-beta diversity

Quality trimming yielded total of 537,070 reads, ranging from 38,180 to 57,975 (44755.8 ± 28444.2), extracted from amplicon sequence data that assigned into 415 OTUs (286.8 ± 48.2), 19 phyla and 176 genera. The highest average OTUs (328.6 ± 32.4) and genera (132.8 ± 28.2) were obtained from the Diet3 group, in relation to other diets. The rarefaction plot indicated that each sample was sequenced at enough depth and up to saturation to capture most of the bacterial diversity (Figure 11. 4A). All samples also had high good's coverage index, ranging from 0.998 to 0.999, implying satisfactory bacterial coverage. Diets 2 and 3 had the highest ($P<0.05$) alpha diversity indices in term of observed OTUs and Shannon (Figure 11. 4B), indicating that this group had a different spectrum of bacterial diversity when compared to other diets. Beta ordination showed distinct clustering of bacterial OTUs and PERMANOVA R-value of 0.46 and P-value of 0.032 revealed that feeding had significant impacts on gut microbial diversity (Figure 11. 4C).

11.3.9 Microbial communities

Relative abundance at the phyla level showed that Proteobacteria was most dominant in Diet1 and Diet4 while *Firmicutes* richness was observed for Diet2 and Diet3 (Figure 11. 4D). At the genus level, Diet3 found to influence the colonization of more bacteria than other diets and therefore, a diverse range of genera was identified, including *Ruminococcus* (58%), *Psychrobacter* (12%), *Lachnospira* (11%), *Clostridium* sensu stricto (8%) and *Ruminoclostridium* (8%). Diet1, Diet2, and Diet4 were dominated by *Vibrio* (71%), *Staphylococcus* (44%), and *Rubritelea* (57%), respectively (Figure 11. 4E). Differential abundance with LDA 2.0 and above and at 0.05 level of significance identified *Ruminococcus* and *Lactobacillus* as significantly abundant genera in Diet3, and *Vibrio*, *Staphylococcus*, and *Rubritelea* in Diet1, Diet2 and Diet4 groups, respectively (Table 11. 8).

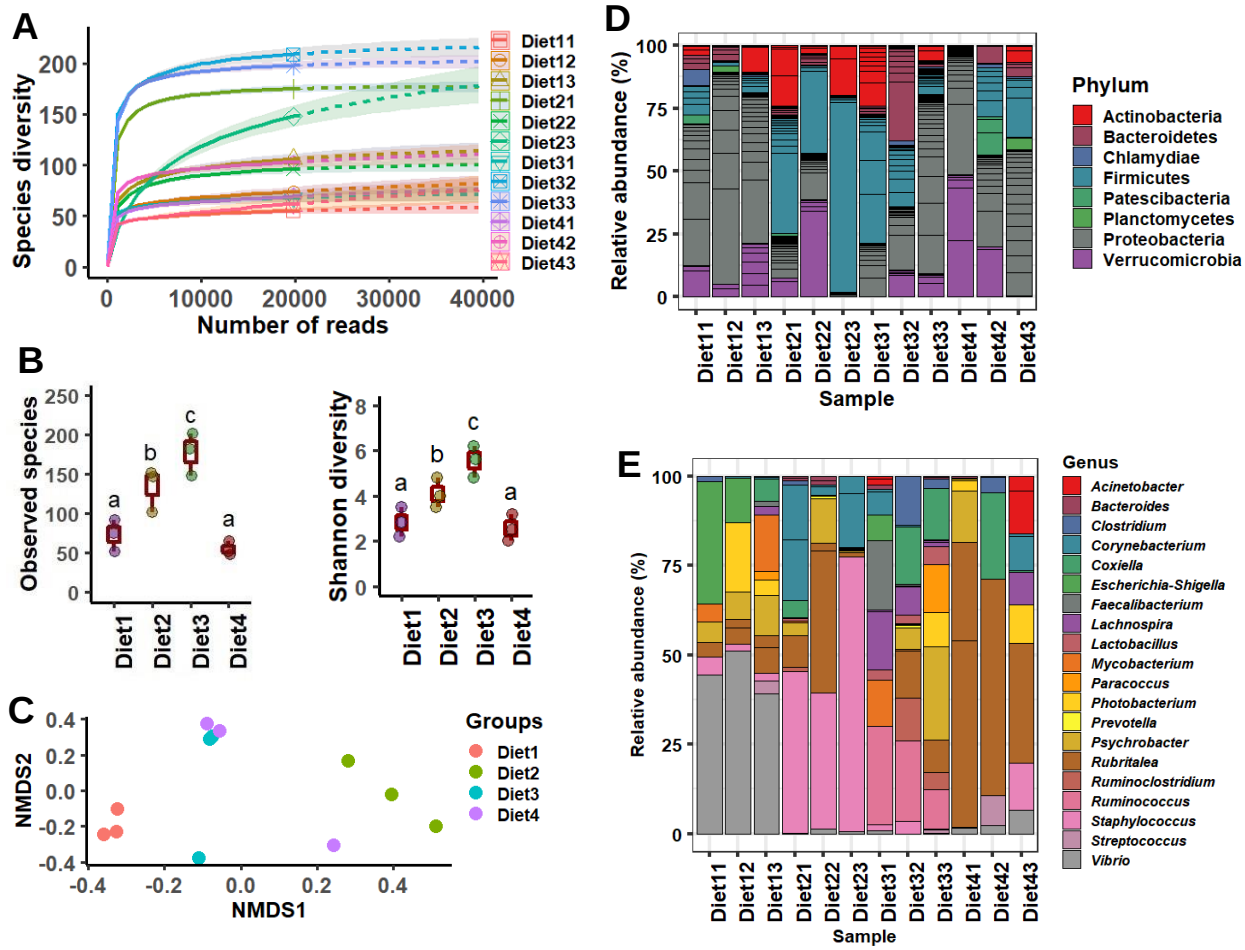

Figure 11. 4 Rarefaction curve (A) showing the depth of sequencing in terms of species diversity, observed species and Shannon diversity (B), Non-metric multidimensional scaling (nMDS) plot demonstrating clustering (C) and relative abundance of bacteria at phylum (D) and genus (E) level of different test diets fed to barramundi for six weeks.

Table 11. 8 Distinguishing bacterial genera in different diets after feeding trial

Genus	Diet1 (Control)	Diet2 ($80PBM_{10TH+10HI}$)	Diet3 ($85PBM_{5TH+10HI}$)	Diet4 ($90PBM_{5TH+5HI}$)	P value
Vibrio	43.08 ± 3.09	1.62 ± 0.63	1.02 ± 0.68	5.44 ± 2.2	<0.001
Ruminococcus	1.89 ± 0.92	0.68 ± 0.61	16.52 ± 4.22	0.81 ± 0.07	<0.001
Staphylococcus	2.34 ± 0.38	48.86 ± 9.98	2.33 ± 0.67	2.58 ± 1.78	<0.001
Rubritalea	10.05 ± 1.61	11.55 ± 4.18	3.68 ± 1.08	47.02 ± 13.01	<0.01

Each value displayed under the columns diet represents mean relative abundance with standard deviation.

11.3.10 Bacterial challenge

Infection rate in fish fed 80PBM and 85PBM supplemented with TH and HI larvae meal was significantly lower than the control (Figure 11. 5). Meanwhile, infection rate in fish fed TH and HI larvae meal supplemented 90PBM showed no significant variation than control.

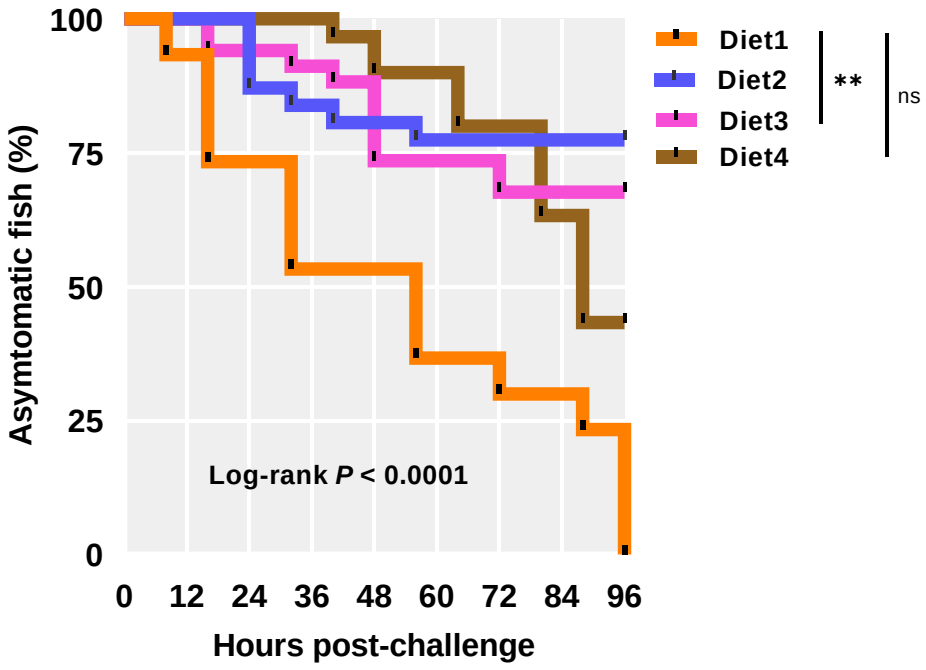

Figure 11. 5 Variation in the infection rate in fish fed different levels of PBM concurrently supplemented with TH and HI larvae after 96 h post-challenge by injection with *V. harveyi*. Infection started in control at 8 hours post challenge (hpc) and while infection started at 24 hpc in

80PBM$_{10TH+10HI}$, 16 hpc in 85PBM$_{5TH+10HI}$ and 40 hpc in 90PBM$_{5TH+5HI}$, respectively. Asterisks demonstrated significant variation between control- vs 80PBM$_{10TH+10HI}$, and 85PBM$_{5TH+10H}$ - fed fish at P <0.01 (Kaplan Meyer survival method, followed by Log-rank test).

11.4 Discussion

The ability of FPH supplementation to enhance growth performance has been reported in many species (Ha et al., 2019; Hevrøy et al., 2005; Siddik et al., 2018b; Tang et al., 2008), however, the concurrent supplementation of TH and HI larvae meal to non-FM based in the diet of barramundi has been reported for the first time in the present study. The total substitution of FM with PBM concomitantly supplemented with TH and HI larvae improved the growth and condition factor in barramundi. Our earlier (Chaklader et al., 2020a) and other study by Siddik et al. (2019a) evaluating the total FM substitution with PBM resulted in poor growth performance in barramundi. Also, many other studies reported negative outcomes in the growth of a number of fish when dietary inclusion of PBM exceeded >50% (González-Rodríguez et al., 2016; Yigit et al., 2006; Zhou et al., 2011). The ameliorative effects on growth and condition factor in the present study could be explained by the presence of greater amounts of low molecular weight peptides in TH and bioactive peptides in HI larvae. The molecular weight of peptides in TH in our earlier study showed that more than 90% of the peptides were less than 10 kda (Chaklader et al., 2020c) which have been reported as biologically active peptides acting as growth promoters (Aksnes et al., 2006a; Bui et al., 2014; Khosravi et al., 2015b; Robert et al., 2015). The hydrolysis process has been reported to elevate chemical and functional properties of feed as well as produce free amino acids, di- and try-peptides which reach the intestine faster than intact proteins and are easily absorbed by enterocytes (Ganapathy, 1994; Ha et al., 2019). Also, inclusion of insect meals at lower levels has been recommended by many researchers to promote the growth and health. For instance, Chaklader et al. (2019) reported a significantly higher growth performance in barramundi fed a 10% full-fat HI larvae supplemented PBM diet. Caimi et al. (2020b) recommended to include 18.5% HI larvae meal in the diet of Siberian sturgeon, *Acipenser baerii* without affecting antioxidant response, liver and gut health.

The taste and appearance of cooked flesh for the consumer is influenced by the lipid composition in the edible part of fish fillets (Grigorakis, 2007; Sabbagh et al., 2019). The higher lipid levels in PBM fed fish in the current study was similar to the reported lipid content in rainbow trout (Baboli et al., 2013) and tench (González-Rodríguez et al., 2016), however, contradict the results of (Siddik et al., 2019b) who found lower levels of lipid in barramundi fed PBM based diets supplemented with TH. The higher fillet lipid content in this study could be due to higher levels of lipid content in the added full-fat HI larvae meal. The unchanged moisture, protein and ash content is similar to

previous studies examining barramundi fed PBM based diets (Siddik et al., 2019a; Siddik et al., 2019b).

In this study, total SFA content increased in fish fed all inclusion levels of PBM concurrently supplemented with TH and HI larvae due to a rise in lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), and stearic acid (C18:0), and confirming that fatty acid composition of fish is generally a reflection of the fatty acids composition in the diets (Renna et al., 2017). HI larvae meal contain higher levels of lauric acid, causing an increase in lauric acid content in rainbow trout, *Oncorhynchus mykiss* Walbaum (Renna et al., 2017) and juvenile jian carp, *Cyprinus carpio* var. Jian (Li et al., 2016b) when fed HI larvae based-diets. Also, a rise in palmitic acid and stearic acid levels led to an elevated total SFA in the muscle of barramundi fed PBM based diets supplemented with various FPH (Chaklader et al., 2020c). However, lauric acid, palmitic acid, and stearic acid were relatively low in the muscle of fish fed TH and HI supplemented PBM than the dietary concentrations, perhaps indicating the utilization of these SFA for energy production. Similar to total SFA content, total MUFA content improved in the muscle of fish fed PBM diets, supplemented with TH and HI, similar to the muscle fatty acids of barramundi fed FPH supplemented PBM based diets (Chaklader et al., 2020c). This increase is mainly due to the higher proportion of MUFA content in the PBM based diets. Meanwhile, concurrent TH and HI supplemented PBM based-diets contained higher PUFAs content than the control, causing a corresponding augmentation in total PUFA content in the muscle of fish. However, feeding barramundi with exclusive levels of PBM reduced the total PUFA particularly n-3 PUFA in the muscle of barramundi (Siddik et al., 2019a). The amelioration of PUFA content in the present study could be due to the supplementation of TH and HI as they contain a higher amount of PUFA than PBM. Similarly, the modulatory effect of FPH on lipid accumulation, lipid metabolism, and fatty acid composition has been reported in barramundi (Chaklader et al., 2020c), turbot, *Scophthalmus maximus* (Xu et al., 2016) and some other animals (mice/rats) (Bjørndal et al., 2013; Hosomi et al., 2011). The FA results suggest that total replacement of FM with PBM supplemented with TH and HI improved the fillet FA composition, manifested by an increased level of MUFA and PUFA content. Such changes suggest that barramundi produced from feeds examined in this study would be beneficial to human health since MUFA and PUFA content are highly associated with reducing the risk of cardiovascular and neurological disease (Blondeau et al., 2015).

Besides the fatty acid profile, post-harvest fillet characteristics such as texture and colour are affected by dietary modification which are subsequently reflected in consumer acceptability and thus market demand. However, previous no information was available on the effects of total substitution of FM with PBM on the post-harvest quality of barramundi fillets. High inclusion of

meat meal (40% or more) at the expense of FM did not adversely affect the organoleptic quality of 66 days post-feeding pond-reared barramundi muscle (Williams et al., 2003b) which was similar to the current study. Whereas, 100% substitution of FM with PBM negatively influenced the sensory characteristics of female tenches, *Tinca tinca* (Panicz et al., 2017).

Histological damages of immune-sensitive organs including liver, kidney, and spleen could be easily assessed when host species are fed non-FM diets. For example, total substitution of FM with PBM resulted in lipid droplets and multifocal necrosis in the liver of barramundi (Chaklader et al., 2020a; Chaklader et al., 2019). Similarly, higher replacement of FM with PBM (50-70%) and animal protein blend containing PBM, shrimp meal and spray-dried blood meal (20-80%) induced hepatocytes steatosis in juvenile hybrid grouper, *Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂ (Ye et al., 2019a; Zhou et al., 2020). However, supplementation of TH and HI larvae meal appear to prevent such organ damage in the current study. FPH supplementation ameliorates the alternative protein quality and reduces the lipid accumulation coupled with increasing lipid metabolism in fish (Xu et al., 2016) and other animals. Also, the presence of chitin and its derivatives in HI larvae have been reported to boost the hydrolysis of lipoproteins and triglycerides, coupled with reducing the synthesis of fatty acids in the liver of fish and other animals (Li et al., 2017a; Li et al., 2016b; Zhang et al., 2008). Besides chitin, lauric acid (C12:0), one of the main fatty acids in HI larvae could also prevent liver damage induced by PBM in the current study since animals can quickly oxidize lauric acid after consumption rather than being stored in liver (Belghit et al., 2019b). A similar effect was observed by Kumar et al. (2020) who reported biliary duct hyperplasia in the liver of rainbow trout fed HI larvae oil, indicating more release of bile to facilitate lipid digestion.

Evaluating intestinal morphological structure is also important to understand the potential effects of alternative protein on any fish health, as such evaluation is reported to be highly correlated with nutrient assimilation and immunological function (Nicholson et al., 2012). An improvement in microvilli density intestine indicates elevated enterocyte absorptive surface area (Adeoye et al., 2016) and neutral mucins number in the intestine have been linked to the protection of the intestinal epithelium by lubricating, trapping and eradicating opportunistic pathogens (Padra et al., 2014; Sklan et al., 2004), prevent proteolytic damages and promoting enzymatic digestion and functionality of the gastric glands (Díaz et al., 2003; Gisbert et al., 2004; Ofelio et al., 2019). In this study, barramundi fed different levels of PBM with TH and HI larvae meal demonstrated a higher microvilli density and higher levels of neutral mucins than the control, indicating more uptake and transportation of amino acids and free fatty acids (Grosell et al., 2010). Such effects might modulate the growth performance and disease resistance via improving the serum immune

response. This could be due to the concurrent effect of TH and HI to confer a relatively higher benefit than when TH was used alone with 75 and 90% PBM in barramundi (Siddik et al., 2019b). Similarly, HI larvae meal supplementation alone with 90% PBM did not influence villus height and neutral mucins in barramundi (Chaklader et al., 2019). Regardless of TH supplementation, antimicrobial peptides, chitin, and lauric acid reported in HI larvae might have played a further key role in improving intestinal health since a number of recent studies have reported probiotic effects of HI larvae meal in rainbow trout (Bruni et al., 2018; Huyben et al., 2019; Terova et al., 2019).

Goblet cells synthesize biologically active substances and other numerous defensive molecules, generally known as mucus, which have been reported to exert an important role in both the innate and acquired immune systems in fish. Goblet cells enumeration in the skin revealed by AB-PAS showed that total substitution of FM with PBM supplemented with TH and HI larvae did not affect the number of goblet cells. However, supplementation of various hydrolysates with PBM improved the number of goblet cells in terms of acidic mucins in barramundi (Chaklader et al., 2020c).

Valuable information on internal organs, nutritional status, and metabolic state can be achieved through the investigation via a panel of serum biochemical assays. Dietary incorporation of exclusive levels of alternative animal protein, in particular PBM, has been reported to have negative impacts on the activity of fish liver enzymes such AST, ALT and GLDH (Chaklader et al., 2019; Ye et al., 2019a), which leak into the blood at abnormal levels when there is liver cell damage. In the present study, such a negative impact was not observed in the measured activity of ALT, GGT, and GLDH, indicating that supplementation of TH and HI larvae meal could prevent liver damage caused by higher inclusion levels of PBM. Total bilirubin, which at high levels can be associated with kidney damage, was significantly lower in fish fed TH and HI larvae supplemented PBM diets, suggesting no adverse effects of total substitution of FM with PBM on kidney function. Similar effects were observed in barramundi fed a TH supplemented PBM diet (Chaklader et al., 2020c). Serum TP is usually more stable in fish in well-nourished conditions (Peres et al., 2014) and elevation in levels is indicative of stronger innate immunity in fish (Rebl and Goldammer, 2018). A significant increase in the TP levels in fish fed concomitant TH and HI larvae supplemented PBM diets indicates an improvement in the immune system. However, TH and HI larvae meal supplementation separately with PBM did not influence the serum TP in barramundi (Chaklader et al., 2020c). Other unchanged biochemical assays including urea, creatinine, and cholesterol, were within the reported healthy ranges for barramundi (Chaklader et al., 2020c).

It is well established that FPH supplementation has been reported to improve diet quality (Egerton et al., 2020; Siddik et al., 2019b), coupled with immunostimulating properties due to the presence of biologically active di- and tripeptides and other oligopeptides (Hou et al., 2017; Martínez-Alvarez et al., 2015). Separate supplementation of FPH or insect larvae with PBM improved the immune response such as lysozyme and bactericidal activity in barramundi (Chaklader et al., 2019; Siddik et al., 2019b) but combined supplemental effects on barramundi immunity is reported here for the first time. In the present study, different levels of PBM supplemented with TH and HI larvae modulated the lysozyme and bactericidal activity of barramundi. Similarly, supplementation of FPH and crustacean hydrolysate have been reported to improve the nutritional quality of alternative protein source including PBM and plant protein with concomitant modulatory effects on immune responses such as lysozyme and complement activity in barramundi (Siddik et al., 2019b) and European sea bass, *Dicentrarchus labrax* (Gisbert et al., 2018). The modulatory effects of TH supplemented PBM diets could be due to a higher proportion of low molecular weight peptides in TH as earlier studies confirmed immune-stimulating properties of medium size-bioactive peptides (3000 Da > Mw > 500 Da) (Bøgwald et al., 1996; Gildberg et al., 1996). Besides the beneficial effects of TH, HI larvae supplementation could further boost the immunity of barramundi since earlier reports showed that HI larvae contain antibacterial peptides and some polysaccharides such as silkrose or dipterose which, along with chitin, play an important role in modulating the immune response. Elevated immune response was reported in gilthead seabream, *Sparus aurata* L. (Esteban et al., 2001) and common carp, *Cyprinus carpio* (Gopalakannan and Arul, 2006) when fed low levels of crustacean derived chitin which was not analysed in HI larvae meal in the present study. Similar with the present findings, an upsurge in lysozyme and bactericidal activity was reported in barramundi fed 10% HI larvae with a PBM based diet (Chaklader et al., 2019) and also enhanced lysozyme, complement 3 and complement 3 was found in black carp, *Mylopharyngodon piceus* fed 25 g kg^{-1} maggot meal in a FM-based diet (Ming et al., 2013).

Reactive oxygen species (ROS) including superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) are essential by-products of phagocytic processes, causing impairment of DNA and injury to lipids, proteins and nucleic acids membranes, or even death if there is no equilibrium between ROS production and antioxidant defence mechanisms. Like other terrestrial animals, fish have a similar manner of enzymatic and non-enzymatic antioxidant defence mechanisms to protect themselves from oxidative damage (Martínez-Álvarez et al., 2005). Radical scavenging enzymes particularly CAT protect cells from oxidative damage by converting hydrogen peroxide into water (Imlay and Linn, 1988). In the present study, CAT activity both in serum and the liver was not affected by PBM inclusion supplemented with TH and HI larvae,

suggesting that total replacement of FM with PBM supplemented with TH and HI larvae did not cause potential damage in the liver of barramundi. The present results agree with the findings of Siddik et al. (2019c) who reported no alteration of CAT activity in the serum of barramundi when fed TH supplemented PBM diets. However, feeding animal protein blends including PBM, shrimp meal, and spray-dried blood meal impaired the liver health of hybrid grouper, *Epinephelus fuscoguttatus*♀× *Epinephelus lanceolatus*♂ (Ye et al., 2019a). No impairment in the liver of barramundi fed PBM based diets reported here can be explained by the supplementation of TH and HI larvae since many studies have been reported the presence of antioxidative capacity of FPH and insect larvae.

Due to the advent of NGS technologies, knowledge of intestinal microbial ecosystem alteration in response to dietary modification has improved. High bacterial diversity in the intestine of fish fed $80PBM_{10TH+10HI}$ and $85PBM_{5TH+10HI}$ than the control may indicate a healthier gut since several studies have reported a number of beneficial effects of the rich bacterial community such as out-competition pathogens for nutrients and colonization and consequently resisting pathogen invasion and intestinal infection (Cerezuela et al., 2013; Huyben et al., 2019; Levine and D'Antonio, 1999). The possible reason for the highest bacterial richness may be the supplementation of HI larvae rich in chitin and lauric acid content which are similar to the bacterial diversity of rainbow trout, *Oncorhynchus mykiss* (Bruni et al., 2018; Huyben et al., 2019; Terova et al., 2019), and other animals such as laying hens (Borrelli et al., 2017) fed HI larvae meal-based diets. The unchanged bacterial diversity in $90PBM_{5TH+5HI}$ with respect to control could be due to the highest replacement of FM protein with PBM. The same effect is supported by an earlier study in our laboratory with no changes in intestinal bacteria diversity of barramundi fed 90PBM supplemented with FPH (Siddik et al., 2019c). In agreement with the earlier studies on barramundi, metagenomics results revealed that *Proteobacteria*, *Firmicutes,* and *Bacteroidetes* were the dominant phyla in all groups (Apper et al., 2016; Siddik et al., 2019c; Xia et al., 2014). Indeed these phyla constitute the "core gut microbiota" constituting up to 90% in the intestine of different marine water fish species (Givens et al., 2015; Li et al., 2014; Ringø et al., 2016). There is little information concerning the effects of dietary replacement of FM with alternative protein sources on the intestinal gut microbiota of barramundi. However, the dietary substitution of FM with a mix of terrestrial animal and plant proteins did not influence the intestinal microbiota of barramundi (Apper et al., 2016) whereas a recent study (Siddik et al., 2019c) found a modulatory effect of fermented PBM along with supplementation of TH on the intestinal microbial community. In this study, *Proteobacteria* abundance was improved in the intestine of fish fed control and $90PBM_{5TH+5HI}$ whilst *Firmicutes* were enriched in $80PBM_{10TH+10HI}$ and $85PBM_{5TH+10HI}$ fed groups. At the genus level, *Ruminococcus* and *Lactobacillus,* under *Firmicutes* phylum, were significantly more abundant in

fish fed 85PBM$_{5TH+10HI}$. The *Ruminococcus* genus is highly associated with the degradation of indigestible carbohydrate ingredients particularly resistant starch and dietary fibres, thereby contributing to a more efficient energy utilization of feed and this genus also plays an important role in the fermentation of dietary fibre, the subsequent butyrate end product is reported to influence the intestinal health of the host (Bonder et al., 2016; Rimoldi et al., 2020; Walker et al., 2011; Ze et al., 2012). Also the genera of lactic acid bacteria (LAB), in particular *Lactobacillus*, are beneficial microorganisms commonly used as probiotics for fish and other vertebrates (Ringø and Gatesoupe, 1998) due to an ability to create biofilms by producing bacterial compounds (lactic acid hydrogen peroxide, and bacteri-ocins or biosurfactants) that can prevent the adherence of pathogens to the intestinal surface (Gudiña et al., 2015). Similar to the present study, dietary inclusion of partially defatted HI larvae was reported to enrich the LAB in the intestine of rainbow trout, *Oncorhynchus mykiss* (Bruni et al., 2018; Terova et al., 2019). The presence of chitin and MCFA, particularly lauric acid, in HI larvae meal were reported to have a modulatory effect on LAB, as reported by the same authors. In addition, TH might have influenced the LAB abundance since FPH is a good source of nitrogen serving as a good medium for bacterial growth (Ha et al., 2019). However, the lower abundance of *Vibrio* in test diets in the present study could be due to the inclusion of HI larvae irrespective of TH supplementation. Similarly, the abundance of *Vibrio* decreased in the intestine of zebrafish when fed various levels of HI larvae meal (Zarantoniello et al., 2020b). The presence of chitin, lauric acid, and recently extracted novel antimicrobial peptides of HI larvae meal are active against Gram-negative and Gram-positive bacteria (Chaklader et al., 2019; Elhag et al., 2017; Terova et al., 2019) which could have reasonably negatively influenced the abundance of *Vibrio* in the current study. The *Staphylococcus*, and *Rubritelea* were abundant in control, 80PBM$_{10TH+10HI}$ and 90PBM$_{5TH+5HI}$ groups. The presence of *Staphylococcus* is common in the gastrointestinal tract of fish, have been documented in several studies over the last decade (Ringø et al., 2006a; Ringø et al., 2006b).

Supplementation of 3% anchovy and giant squid hydrolysates in the diet of European seabass, *Dicentrarchus labrax* showed bactericidal and bacteriostatic activities against a number of fish pathogenic bacteria including *V. parahaemolyticus*, *V. harveyi* and *P. damselae* subsp. *piscicida*, and *V. anguillarum* (Costa et al., 2020). A number of previous studies supplementing FPH have also proven the modulation of disease resistance against pathogenic bacteria in barramundi (Siddik et al., 2019b; Siddik et al., 2018b), red sea bream, *Pagrus major* (Bui et al., 2014; Khosravi et al., 2015a; Khosravi et al., 2015b) and European sea bass, *Dicentrarchus labrax* (Kotzamanis et al., 2007). In the present study, the improved infection rate against *V. harveyi* in the test diets could be due to the presence of bioactive peptides in FPH. Also improved infection rate may be due to the presence of low molecular weight peptides (<6500 Da) described in our earlier study in TH

(Chaklader et al., 2020c) with possible antibacterial properties (Offret et al., 2019), as supported by the improved lysozyme and bactericidal activity of barramundi fed TH supplemented PBM. Some peptides, in particular lactoferrin-derived peptides, have the ability to damage the bacterial membrane of different species and strains of *Vibrio* (Acosta-Smith et al., 2018), and this remains to be further studied. Besides the role of TH, the presence of antibacterial peptides in HI larvae meal has been demonstrated to have an inhibitory response against Gram-positive bacteria, Gram-negative bacteria and fungus (Elhag et al., 2017) and this may have also influenced the infection rate. In our earlier study, supplementation of 10% HI larvae meal with 45% PBM modulated the disease resistance in response to a two-week challenge with *V. harveyi* (Chaklader et al., 2019). However, the specific role of antibacterial peptides in TH and HI larvae in boosting the immune response of fish need to be further explored.

In summary, concurrent supplementation of 10 and/or 5% TH and HI larvae can facilitate the total replacement of FM with PBM in barramundi, including an improved growth performance and biometry indices, and improved microvilli density and neutral mucin levels in intestine. Fatty acid profiles such as MUFA and PUFA improved in TH and HI supplemented PBM fed barramundi with no significant effects on all tested post-harvest fillet quality characteristics. No histopathological changes in liver, kidney and spleen further proved the ability of TH and HI supplementation to hamper the negative effects previously reported to be caused by dietary animal protein ingredients. Whilst serum biochemical assays, particularly total bilirubin and total protein, improved in TH and HI larvae supplemented PBM groups, antioxidant activities in the serum and liver were unaffected. Concurrent supplementation of TH and HI larvae demonstrated bioactivity via modulating lysozyme and bactericidal activity which may elucidate higher survival rates in response to *V. harveyi* infection. Also, bacterial diversity on $80PBM_{10TH+10HI}$ and $85PBM_{5TH+10HI}$ increased but the presence of *Vibrio* decreased in barramundi fed PBM based diets concurrently supplemented with TH and/or HI larvae meal.

CHAPTER 12: General discussion, conclusion, recommendation and limitations

12.1 General discussion

The sustainability of aquaculture, in particular, carnivorous aquaculture, is questionable socially and environmentally because of high protein and lipid feed ingredient requirements which traditionally derive from FM and FO sourced from limited stocks of wild forage fish. Consequently, the production of these valuable ingredients has been declining over the past 20 years, causing a supply disruption and consequently inflating the price. The aforementioned issues have driven the aquaculture industry and researchers to investigate sustainable alternate protein sources and some success has already been achieved regarding the utilization of a variety of plant proteins in aquafeeds. However, plant protein sources beyond certain dietary inclusion levels have been reported to negatively influence the health of carnivorous fish due to an imbalance in the nutritional profile and anti-nutritional factors embedded in these plants. In addition, utilization of plant protein sources competes with the human need for food and even the predicted additional demand for aquafeeds by 2025 cannot be met by expanding plant protein production without imposing additional pressure on land, water, and phosphorous resources. Hence, multitude of aquafeed ingredients produced via recycling food waste and subsequent combination were investigated in the present study, for the first time with an aim to develop a nutritionally balanced novel aquafeed to ensure future environmental sustainability for the carnivorous finfish aquaculture industry.

12.1.1 Complementary effect of 10% FHI larvae meal supplementation in PBM

PBM as an alternative to FM has been tested on a variety of fish species since 1980 (Galkanda-Arachchige et al., 2020) and some success has been achieved regarding the inclusion of PBM in commercial diets (Glencross et al., 2011; Salini et al., 2015; Simon et al., 2019). In barramundi, validation of a zero FM diet has recently been described (Glencross et al., 2016; Siddik et al., 2019b), however, PBM alone has been associated with growth depression, poor digestibility, and other health-related issues in barramundi including histopathological changes in liver and alteration in mucosal barrier function (Glencross et al., 2016; Siddik et al., 2019a). Here first feeding trial (Chapter 3) in barramundi was conducted to better understand physiological and molecular effects of an entirely PBM-based diet that demonstrated a negative impact on growth, survival, and feed utilization (see Table 12. 1), similar to previously growth depression in barramundi (Siddik et al., 2019a) and juvenile Black Sea Bass (Dawson et al., 2018) fed 100% PBM and many other finfish species when fed >50% of PBM (González-Rodríguez et al., 2016; Rossi and Davis, 2012; Yigit et al., 2006). The poor growth performance was further supported by vacuolization in the liver (Figure 12. 1C), muscle necrosis (Figure 3. 2D), hyperplasia in gills

(Figure 3. 2F), short microvilli in the distal intestine (Figure 3. 2H), poor immune response (Figure 3. 4C, D) and antioxidant capacity (Figure 3. 5A, B) and higher expression of stress-related genes (Figure 12. 1D, E) in barramundi. The inferior physiological responses in barramundi reported here and from other previous studies in response to PBM-based diets might be due to poor digestibility caused by less palatability of PBM (Hu et al., 2008), presence of lower concentrations of essential fatty acids, and higher abundance of MUFA and n-6 PUFA (García-Pérez et al., 2018; Nengas et al., 1999; Yigit et al., 2006; Zapata et al., 2016), and lack of phosphorus and selenium (Glencross, 2006; Ilham et al., 2016a; Le et al., 2014; Simon et al., 2019).

Hence, the second (Chapter 4) and third trials (Chapter 5) were conducted to investigate if supplementation of 10% full-fat HI (FHI) larvae meal could prevent the negative effects caused by exclusive or complete replacement of FM with PBM using multidisciplinary approaches integrating biometric, microscopic, biochemical and molecular analysis. The results showed that with FHI supplementation up to 75% of FM protein can be replaced with PBM with an improved or equal growth performance to FM without impacting feed utilization (see Table 12. 1), fillet fatty acid composition (Table 5. 4), liver health (Figure 4. 6B and Figure 5. 2B, C) and heart (Figure 5. 2J, K) and muscle (Figure 5. 2M, N) tissue structure, suggesting that FHI larvae meal supplementation could complement PBM by preventing negative effects in barramundi diets. The improved or equal growth performance in barramundi fed up to 75% of PBM supplemented with PBM can be further supported by the enhancement of intestinal and skin barrier functions such as an increase in the measurement of villi (Figure 4. 3F) and enterocyte width (Figure 4. 3G), microvilli height (Figure 4. 3J) and the number of acid mucin-producing goblet cells (Figure 4. 3L and Figure 5. 5D-F) that are associated with absorption and digestion of nutrients (Siddik et al., 2018b) and lubricating, trapping and eliminating pathogens (Padra et al., 2014; Sklan et al., 2004). In support, a previous study by Kumar et al. (2020) has reported the ameliorative effect of FHI larvae meal or oil in releasing more bile to facilitate lipid digestion, manifested by bile duct hyperplasia in the liver and also in preventing pathological changes in the liver, caused by the inclusion of SBM in rainbow trout diet. Such impacts could be due to the presence of a higher concentration of lauric acid in FHI larvae meal, which is quickly oxidized rather than being stored in the liver and thus enhancing feed efficiency in Siberian sturgeon (Rawski et al., 2020). However, 90% of PBM along with the supplementation of 10% FHI showed a similar negative response in terms of growth and other physiological responses as with the barramundi fed 100% of PBM (see Table 12. 1 and Figure 12. 1B), suggesting that 10% supplementation may not be sufficient to suppress the negative effect of 90% of PBM.

Table 12. 1 Growth, body indices, mucosal barrier function and serum metabolites of barramundi fed PBM-based diets supplemented with 10% FHI larvae meal. ▲ increased significantly when compared FM-based diet (Control), ▼ decreased significantly when compared FM-based diet (Control), and ▬ unchanged when compared FM-based diet (Control).

	Experimental diets for Chapter 3, 4 and 5				
	45PBM+HI	60PBM+HI	75PBM+HI	90PBM+HI	100PBM
Growth performance and body indices					
FBW (g)	▲	▬	▬	▼	▼
WG (g)	▲	▬	▬	▼	▼
SGR (%/d)	▲	▬	▬	▼	▼
FI (g/fish d^{-1})	▬	▬	▬	▼	▼
FCR	▬	▬	▬	▬	
SR (%)	▬	▲	▲	▼	▼
CF (%)	▲	▬	▬	▼	**NA**
VSI (%)	▬	▬	▬	▬	NA
HSI (%)	▬	▬	▬	▬	**NA**
IFI (%)	▼	▼	▼	▼	**NA**
Mucosal barrier function	▲	▲	▲	▼	▼
Serum metabolites					
GLDH	▬	▬	▬	▲	▲
Cholesterol	▬	▲	▲	▬	▬

One of the encouraging results in the present study was the ability of FHI larvae meal in PBM to reduce the deposition of intramuscular fatty tissue, supported by a decrease in IFI (Table 12. 1) and adipocyte cell size (Figure 12. 2F) since the inclusion of different levels of PBM (25.7-100%) has been previously reported to enhance the intramuscular fatty tissue deposition in female tenches, *Tinca tinca* (Panicz et al., 2017). A similar positive response in terms of IFI and adipocyte cell size has been documented in juvenile Jian carp, *Cyprinus carpio* var Jian fed FHI larvae oil, and the results were further supported by the upregulation of a lipid metabolism-relevant gene (PPARα) in adipocytes isolated from intraperitoneal fat tissue (Li et al., 2016b). MCFAs, particularly, C_6-C_{12} were higher in FHI larvae meal supplemented PBM (see Table 5. 2), these are physiologically active compounds associated with decreasing lipid deposition by reducing the adipose tissue deposition (Hashim and Tantibhedyangkul, 1987; Hossain and Blair, 2007). Besides

the contribution of MCFAs, chitin in HI could influence fat deposition since chitin has been reported to reduce broiler chicken's body fat content (Hossain and Blair, 2007).

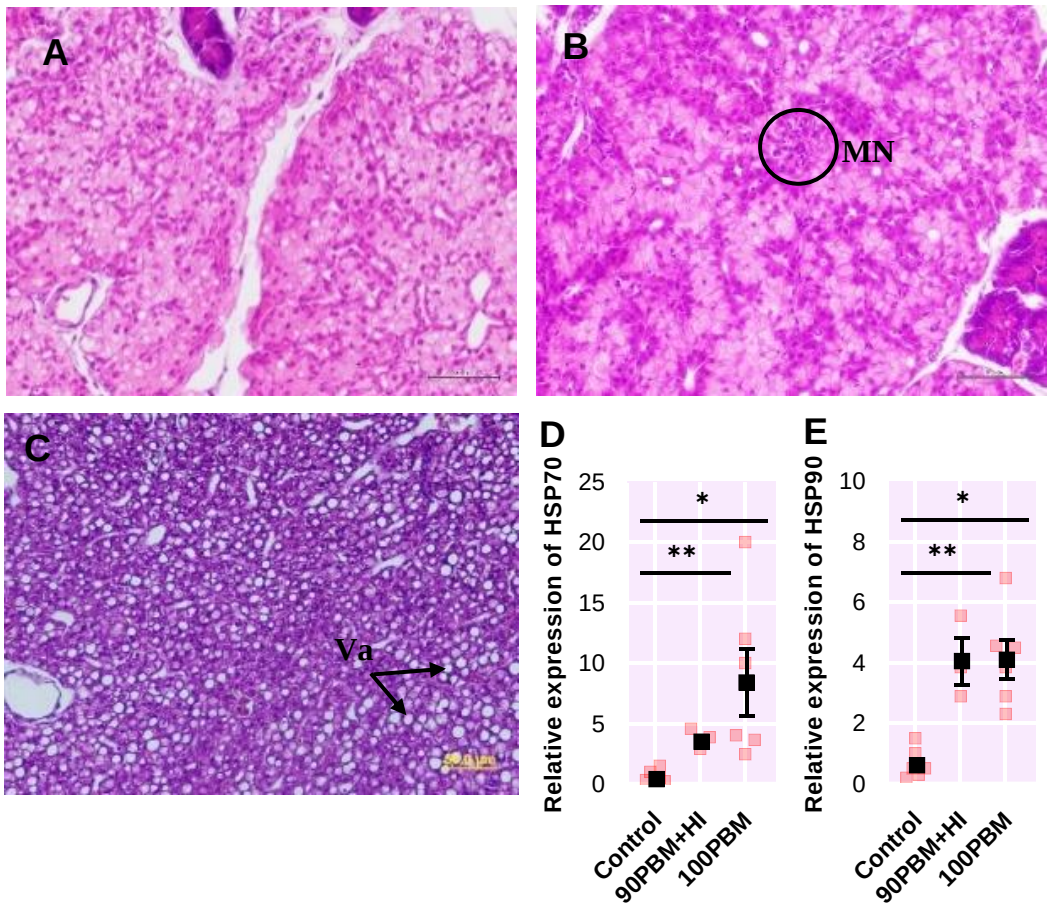

Figure 12. 1 Histopathological changes in the liver of barramundi fed 90% (B) and 100% (C) of PBM in comparison with control (A). Variation in the relative expression of heat shock proteins (HSP) including HSP70 (D) and HSP90 (E) in the liver of barramundi fed PBM-HI based diets 42 days. "*" and "**" indicate significant difference between the treatments at $P < 0.05$ and $P < 0.01$.

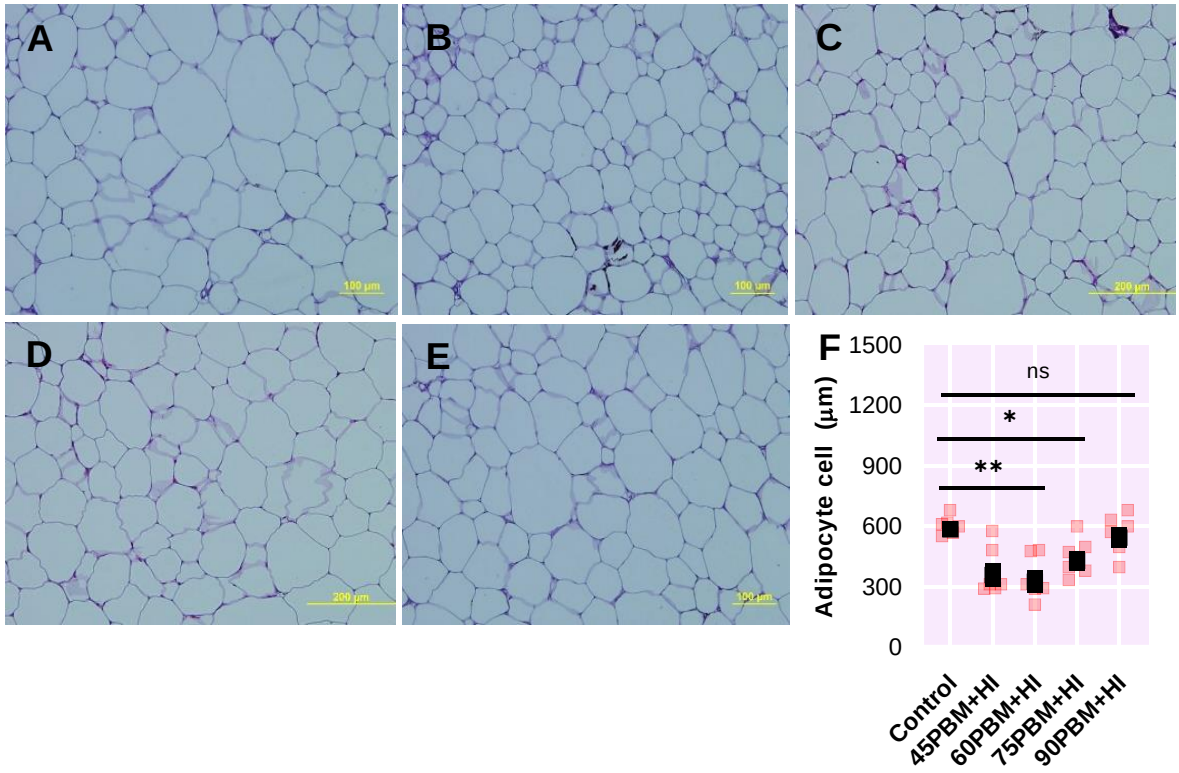

Figure 12. 2 Representative micrograph of adipocyte tissue of barramundi fed control (A) and graded levels of PBM, supplemented with 10% of FHI including 45PBM+HI (B), 60PBM+HI (C), 75PBM+HI (D), and 90 PBM+HI (E) over a period of 42 days. Variation in the cell size of barramundi fed PBM-HI-based diets (F). "*" and "**" indicate significant difference between the treatments at $P < 0.05$ and $P < 0.01$.

There is a lack of information on the effect of FHI larvae meal on the resistance of fish to bacteria. A two-week bacterial challenge trial at the end of the second (Chapter 4) and third (Chapter 5) feeding trials showed that 10% FHI larvae meal supplementation with PBM (up to 75%) stimulated the disease resistance against *V. harveyi* (Figure 12. 3A), implying that smaller quantities of FHI larvae meal could be potentially used as a functional ingredient in barramundi diet. The enhancement of disease resistance was aligned with improved serum immune responses including increased lysozyme (Figure 12. 3B) and bactericidal activity (Figure 12. 3C) in post-challenged barramundi fed FHI larvae meal based PBM. Similarly, supplementation of 16% FHI larvae meal in SBM and FHI larvae oil improved the serum lysozyme and peroxidase activity in rainbow trout (Kumar et al., 2020). The inclusion of different levels of FHI larvae meal (25-50%) also enhanced the survival rate against *Vibrio alginolyticus* after a two-week challenge trial (Abdel-Latif et al., 2021). The underlying reason for the enhancement of immune response could be attributed to the presence of lauric acid, chitin, bioactive peptides, and other polysaccharides including choline, silkrose, and dipterose. For instance, Ali et al. (2018) identified and purified silkrose from *Bombyx mori* which enhanced disease resistance by activating the innate immunity in penaeid prawns such

as *Litopenaeus vannamei* and *Marsupenaeus japonicas.* Also, a number of novel antimicrobial peptides screened and purified from HI larvae have been demonstrated to enhance defense mechanisms against Gram-positive and Gram-negative bacteria as well as fungi (Elhag et al., 2017; Park et al., 2015; Park et al., 2014). Along with the contribution of silkrose and antimicrobial peptides, chitin may act as an immunostimulant although it has a tendency to work as an anti-nutritional factor in others species. However, the smaller quantity of chitin has been reported to enhance the immune response of fish (Esteban et al., 2001; Gopalakannan and Arul, 2006). Also, it has been reported that lauric acid is associated with inflammatory and immune-boosting properties (Giorgini et al., 2018; Skřivanová et al., 2006; Zhou et al., 2013) in fish. However, such immunostimulating effects of HI larvae meal has not been reported in any other fish species (Hu et al., 2020; Wang et al., 2019b).

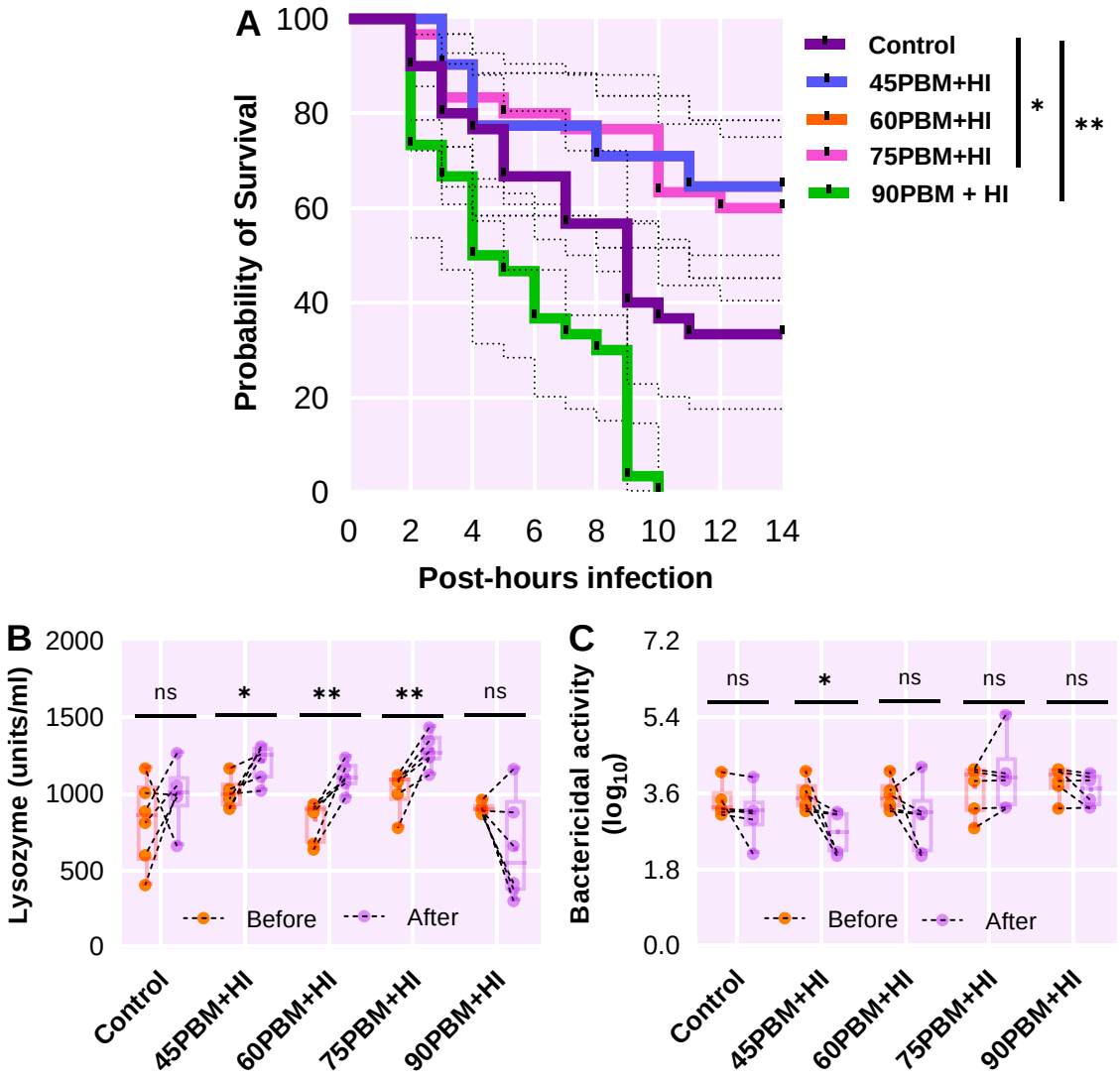

Figure 12. 3 Survival (A) and immune responses (B and C) of barramundi in response to *V. harveyi* challenge when fed graded levels of PBM supplemented with 10% FHI. "*" and "**" indicate significant difference between the treatments at $P < 0.05$ and $P < 0.01$.

12.1.2 Progressive inclusion of Full-fat and defatted HI larvae meal in PBM

Since 10% FHI larvae did not act effectively in combination with 90% of PBM, the fourth and fifth trials were conducted (covered in Chapter 6, 7, 8, and 9) using progressive levels of HI larvae meal including 15, 20, 25, and 30% in order to narrow the gap between 75 and 90% PBM inclusion in Chapter 4 and 5, and therefore to maximize the inclusion levels of PBM. The results showed that adding different levels of FHI (15-30%) larval meal with graded levels of PBM successfully replaced FM completely from the barramundi diet without imposing deleterious effect on barramundi health (see Table 12. 2). Also, the mixture of FHI larvae meal and PBM lowered the serum ALT (Figure 12. 4A) and GLDH (Figure 12. 4B) activity, which are two important liver enzymes, elevating abnormally in blood serum when liver or kidney cell damage occurs (Fawole et al., 2020). Similarly, the histological results showed no pathological changes in the liver and simultaneously improved antioxidant activity, as indicated by elevating haptoglobin and CAT (Figure 12. 4C, D) activity in serum and liver, implying the potentiality of FHI to prevent oxidative damage/stress caused by PBM-based diet and reported in Chapter 3. Zhu et al. (2020) hydrolysed HI larvae by alcalase and found low fractions of molecular weight peptides (<3 kDa) with high potential radical scavenging ability which may translate to higher antioxidant activity in the present study and also as reported in African catfish (Fawole et al., 2020), Jian carp (Li et al., 2017a) and Japanese seabass (Wang et al., 2019b). Also, PBM-FHI diets significantly improved the gut health in the present study by improving bacterial diversity, and the abundance of beneficial bacteria as well as improving the mucosal barrier functions (Figure 6. 3M-P and Table 6. 4). These changes have been reported to be linked with different functional digestion by producing amylase, cellulase, lipase, proteases, chitinase, and phytase (Clements et al., 2014; Ray et al., 2012), enhancing absorption and immune modulation thereby maintaining host gut homeostasis (Laparra and Sanz, 2010; Rombout et al., 2011; Sutili et al., 2018).

However, 30% of DHI larvae meal in 70% of PBM impacted the growth performance, feed utilization, and feed conversion ratio with respect to FM control and PBM-FHI based diets (Table 12. 2). The growth depression in barramundi fed 70PBM-30DHI were further reflected in the pathological condition of the liver, particularly liver steatosis (Figure 8. 6C, F) which has been associated with abnormal production of liver enzymes (ALT and GLDH) and oxidative damage and cell death (Christian et al., 2013; Kawano and Cohen, 2013; Zhao et al., 2019). Hence, 70PBM-30DHI fed barramundi enhanced the abnormal production of ALT (Figure 12. 4A) and showed oxidative damage by lowering serum CAT (Figure 12. 4D). Similarly, 30% FHI larvae meal worked as a functional ingredient in Siberian sturgeon, proven by improved growth, feed utilization, and gut health (Rawski et al., 2020; 2021), whereas the same inclusion levels of DHI

larvae impacted the health of the same species (Caimi et al., 2020a). The inferior performance of barramundi fed PBM-DHI to barramundi fed PBM-FHI can be attributed to the utilization of DHI larvae meal in combination with an exclusive level of PBM since it has been reported that defatting may decrease the nutritional and functional value of HI larvae meal by reducing lauric acid content, degrading antimicrobial protein, and increasing chitin share (Rawski et al., 2020). Such possible changes together with the exclusive inclusion of PBM can have negatively influenced the health of barramundi in the present study.

Table 12. 2 Growth, fillet proximate composition, and sensory attributes of barramundi fed PBM-based diets supplemented with FHI and DHI larvae meal. ▲ increased significantly when compared FM-based diet (Control), ▼ decreased significantly when compared FM-based diet (Control), and ▬ unchanged when compared FM-based diet (Control).

	Experimental diets				
	Chapter 6 and 7			Chapter 8 and 9	
	85PBM-15FHI	80PBM-20FHI	75PBM-25FHI	70PBM-30FHI	70PBM-30DHI
SGR (%/d)	▬	▬	▬	▬	▼
FI (g/fish d^{-1})	▬	▬	▬	▬	▼
FCR	▬	▬	▬	▬	▲
SR (%)	▬	▬	▬	▬	▼
HSI (%)	▬	▬	▬	▬	▲
Fillet proximate composition					
Crude protein	▬	▬	▬	▬	▬
Crude lipid	▲	▲	▲	▲	▬
Fillet amino acid composition (% of total AA)					
EAA	▬	▬	▬	▬	▬
NEAA	▬	▬	▬	▬	▬
Total fatty acid profile (% of total fatty acids)					
∑SFA	▲	▲	▲	▲	▲
∑MUFA	▲	▲	▲	▲	▲
∑PUFA	▼	▼	▼	▼	▼
∑n3-PUFA	▼	▼	▼	▼	▼
Fillet sensory quality					
Raw overall quality	▲	▲	▲	▲	▲
Cooked overall quality	▲	▲	▲	▲	▲

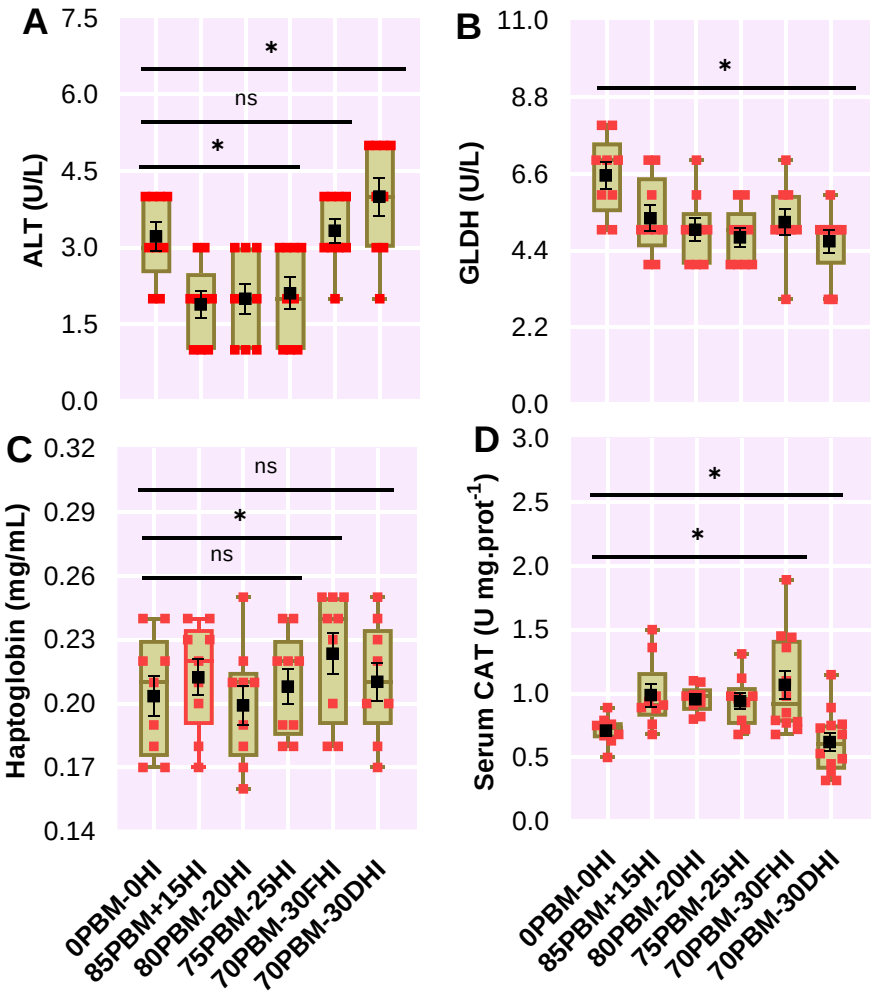

Figure 12. 4 ALT, GLDH, haptoglobin, and CAT activity in the serum of barramundi fed the mixture of the progressive levels of FHI and DHI larvae meal and PBM over the period of 56 days. "*" indicate significant difference between the treatments at $P < 0.05$.

Over the past two decades, a significant effort in aquafeed development has been dedicated to improving the nutritional aspects of barramundi diets but such research has largely overlooked the food aspect quality of the resulting barramundi product, including sensory quality and shelf life. Evaluation of sensory quality is important from the consumer's perspective while incorporating new dietary protein ingredients during its farming. Feeding female tenches, *Tinca tinca* with PBM negatively influenced the sensory quality (Panicz et al., 2017). The results in Chapter 7 and 9 demonstrated that inclusion of different levels of HI larvae meal, either full-fat or defatted, with different levels of PBM, improved the sensory quality of raw and cooked barramundi fillet at the end of the 8 weeks of feeding (see Table 12. 2). Such amelioration suggested that HI larvae could be used in the barramundi diet as one of the potential ingredients to improve fillet quality. A similar

positive effect was not found in the sensory attributes of salmon fillets but results were comparable to the fillets of salmon fed FM-based diet (Bruni et al., 2020a). The possible reasons for the highest scores given by panellists to the fillets of barramundi fed PBM-Hi based diet is difficult to elucidate, but the efficacy of chitosan, a derivative of chitin coating improving the sensory quality of different seafood, has been demonstrated by many studies (Fan et al., 2009; Farajzadeh et al., 2016; Mohan et al., 2012). These results suggested chitin in HI larvae meal or other functional molecules might have contributed to improving the fillet quality of barramundi.

The development of a blue-greyish colour in raw flesh is a common problem in farmed barramundi which is directly associated with consumer acceptance and thus impacting on sales in the retail sector (Howieson et al., 2013). Some futile attempts were made to resolve this problem by manipulating diets or adding different precursors such as tyrosine or tryptophan, or tyrosinase in the diets of barramundi so as to change the pathway of melanin synthesis (Howieson et al., 2013). Hence, a significant improvement in barramundi fillet brightness in response to the mixture of HI larvae meal and PBM (Figure 12. 5A) suggested that HI larvae meal could potentially have melamine synthesis changing properties to improve the colour. This finding is not aligned with the study of Moutinho et al. (2020) and Bruni et al. (2020b) in which fillet brightness of European seabass and rainbow trout were not influenced by HI prepupae meal, such discrepancies in fillet brightness might also be due to the utilization of different lifecycle stages for the HI meal. Currently, the lack of any research in this area elucidates the exact biochemical pathway. However, variation in lighter flesh colour in PBM-HI fed fish could also be associated with the oxidation reduction system, elemental, and variations in the fatty acid composition. The results in the present study are encouraging in resolving the greying issue in farmed barramundi. Fish colour is largely attributed to what they naturally consume or the following supplementation with formulated diets since fish do not have *de novo* power to synthesize colour. The increase in flesh yellowness in barramundi fed PBM-HI based (Figure 12. 5B) diet may be due to the presence of 2.2 mg/100 g riboflavin, a yellow-colour pigment in HI larvae meal (Nyakeri et al., 2017). This is similar to what has been found by other who reported yellowish flesh in sea bream, *Pagellus bogaraveo* when fed mealworm (Iaconisi et al., 2017) but was not similar to studies of Moutinho et al. (2020) and Chaklader et al. (2021c) who found no variation in the flesh yellowness of European seabass and barramundi fed HI pre-pupae larvae and larvae meal in a PBM-based, respectively.

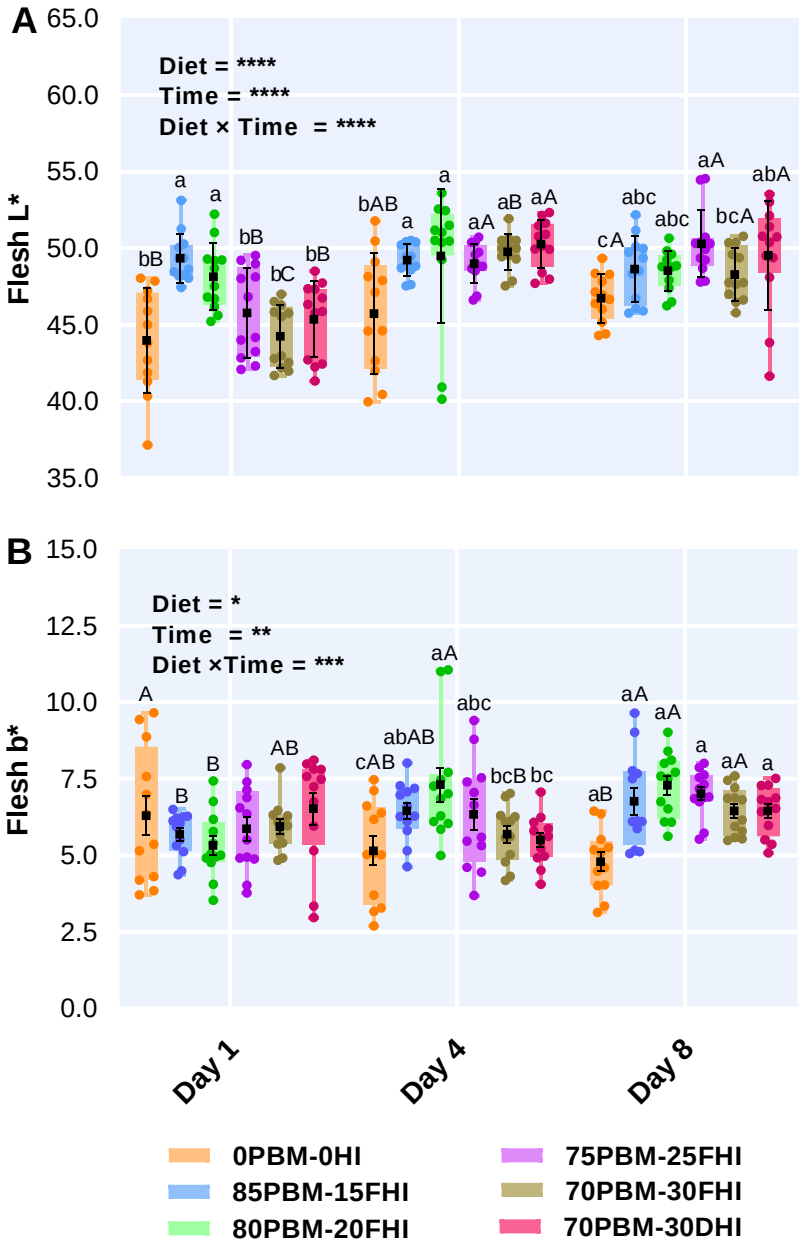

Figure 12. 5 Colour changes including brightness (A) and yellowness (B) in the flesh of barramundi fed graded level of HI larvae meal wither full-fat and defatted for 56 days under a simulated retail display. Round shape markers with box-whisker indicate the number of biological replicates (n = 12) while the black square shape marker indicates the mean of biological and technical replicates (n = 3) with standard error. The effect of two fixed factors "Diet" and "Storage time" were determined by Two-way ANOVA with Dunnett's multiple comparison test $0.05 < P < 0.0001$. Small letters on the top of the box-whiskers indicate significant difference between the treatments for each day whilst capital letters indicate significant between the storage times for each treatment.

Texture, evaluated by several parameters such as hardness, cohesiveness, springiness, chewiness, resilience, and adhesiveness, are crucial fillet product quality attributes directly associated with the freshness of fish and consumer acceptance (Cheng et al., 2014). In the texture profile analysis, hardness is closely associated with fish freshness (Cheng et al., 2014), most fillet's hardness increased significantly in the fillets of barramundi fed PBM-HI based diets on Days 4 and 8 (Chapter 7 and 9) (Figure 12. 6G). The texture changes were interlinked with observed microstructure changes of muscle tissue on Day 8, manifested by tight attachment and regular shape of myofibrils together with a uniform distribution and distinct connective tissues of muscles tissue of barramundi fed PBM-HI based diets (Figure 12. 6B-F) when compared with control. Similarly, 10 weeks post-feeding HI larvae meal improved muscle springiness, chewiness, hardness, and adhesiveness in eel, *Monopterus albus* (Hu et al., 2020) although such an effect was not observed in salmon fed HI larvae meal (Lock et al., 2016) and sea bream, *Pagellus bogaraveo* fed mealworm, *Tenebrio molitor* (Iaconisi et al., 2017) for 113 days. The possible reasons for the positive influence of the mixture of HI larvae meal and PBM on the texture of barramundi have not been well-studied. However, antimicrobial peptides present in HI larvae may play an important role in improving the texture profile since the antimicrobial activity of HI peptides has already been proven by several studies (Elhag et al., 2017; Park et al., 2015; Park et al., 2014).

In addition to antimicrobial peptides, chitin may also influence the autolysis process in barramundi flesh since it has been reported that chitosan, a derivative of chitin has been reported to have antioxidant and antimicrobial properties (Duan et al., 2019; Hafsa et al., 2016; Kong et al., 2010; Samar et al., 2013). Such properties may retard the rancidity production and enhance the texture profile of fillets through two different possible mechanisms: facilitating a stable macro-molecular structure through binding the free amino groups and hydroxyl radicals of the polymer with the metal ions (Fe_2^+) and free radicals on food; and killing of bacteria and fungi via the bonding of charged (NH_3^+) amino groups of chitosan with the negative carboxyl groups (COO^-) situated on the outer part of the membrane or the cell wall (Kong et al., 2010; Riaz Rajoka et al., 2020; Sahariah and Masson, 2017). Liu et al. (2019) have converted the chitin of HI larvae into chitosan but the application of this product on food preservation has not been studied. Also, it has been reported that marine carnivorous teleost fish can produce endogenous chitinase to digest chitin (Fines and Holt, 2010; Kurokawa et al., 2004) which may elucidate the improvement in texture and but the assimilation of chitosan in fish from the digestion of chitin has not been reported yet.

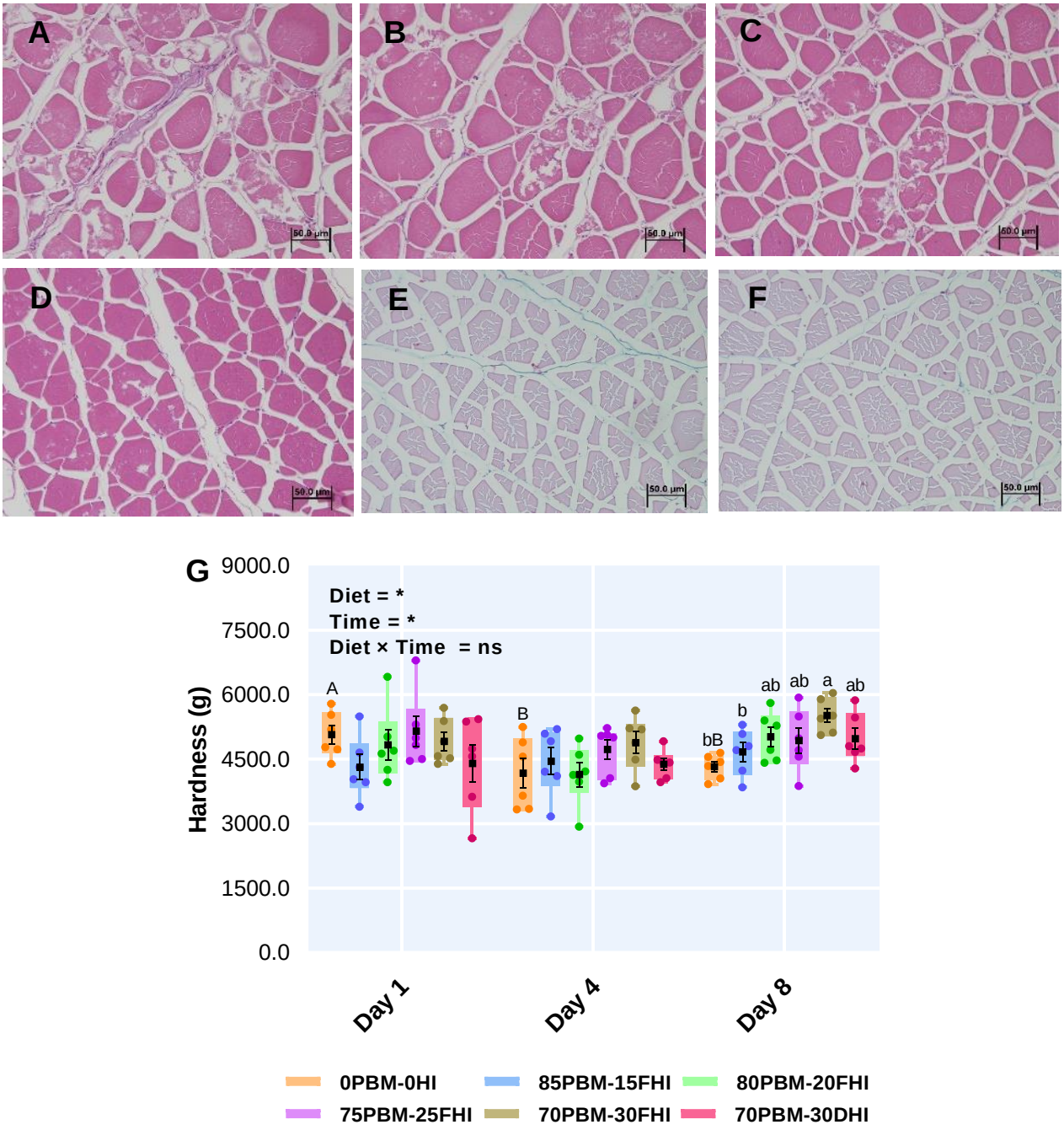

Figure 12. 6 Changes in the microstructure of muscle tissue on Day 8 and hardness in the flesh of barramundi fed graded level of HI larvae meal with full-fat and defatted for 56 days under a simulated retail display. Round shape markers with box-whisker indicate the number of biological replicates (n = 6) while black square shape marker indicates the mean of biological and technical replicates (n = 3) with standard error. The effect of two fixed factors "Diet" and "Storage time" were determined by Two-way ANOVA with Dunnett's multiple comparison test $0.05 < P < 0.0001$. Small letters on the top of the box-whiskers indicate significant difference between the

treatments for each day whilst capital letters indicate significant between the storage times for each treatment.

The pH values of the fillets in this study were impacted by the diets; pH showed a significant increase in the fillets of barramundi-fed PBM-based diets, supplemented with either FHI or DHI larvae meal (Figure 12. 7A). An elevation in fillet pH has been linked with bacterial activity or endogenous enzymes, resulting in the generation of alkaline compounds such ammonia and biogenic amines (Wang et al., 2017) whilst anaerobic fermentation of glycogen and liberation of inorganic phosphates from ATP degradation at the rigor stage was reported to result in the production of lactic acid, causing a drop in fillet pH (do Vale et al., 2020; Morachis-Valdez et al., 2017). It is imperative to note that regardless of diet and storage time, the pH of barramundi fillets described in Chapters 7 and 9 was within the normal range of fresh barramundi fillets, implying no generation of non-desirable acidic or volatile components during the eight days of storage. The increase in pH in the fillets of barramundi fed PBM-HI-based diets was aligned with the pH result from European seabass fillets during three days of storage following feeding with HI pre-pupae meal (Moutinho et al., 2020). There could be an association between pH and lipid oxidation process, reflected in a study (Jones and Carton, 2015) which reported a negative correlation between pH and the lipid oxidation process in barramundi fillets during two weeks storage periods. A similar association was found between pH and TBARS production, as measured by MDA activity, a secondary compound generated from the oxidation of PUFA (Fernández-Segovia et al., 2012; Janero, 1990). The lower MDA production in the fillets of barramundi-fed PBM-HI-based diets (Figure 12. 7B) may be due to a lower concentration of PUFA (Table 12. 1). Also, the presence of antioxidant properties of HI larvae chitin and lauric acid as well as antimicrobial activity of HI larvae bioactive peptide (Elhag et al., 2017; Park et al., 2015) have already been well-documented, could have caused the lower production of MDA in barramundi fillets in the present study. The ability of the derivative of chitin, known as chitosan to inhibit the lipid oxidation process has already been proven by many studies in fish and fish products (Alak, 2012; Baptista et al., 2020; Fan et al., 2009; Farajzadeh et al., 2016; Shahidi et al., 2002). Similarly, feeding HI larvae meal prevented the production of TBARS in the fillet of European seabass (Moutinho et al., 2020). Irrespective of diet, there was a significant increase in the production of MDA in barramundi fillets over the storage time, which were aligned with the microstructure changes in muscle tissues in the present study and MDA production in a two-week study of chilled stored barramundi fillets (Jones and Carton, 2015). It is noteworthy the production level of MDA did not exceed the food safety/quality critical limit (Öz, 2018; Varlik, 1993) in the present study.

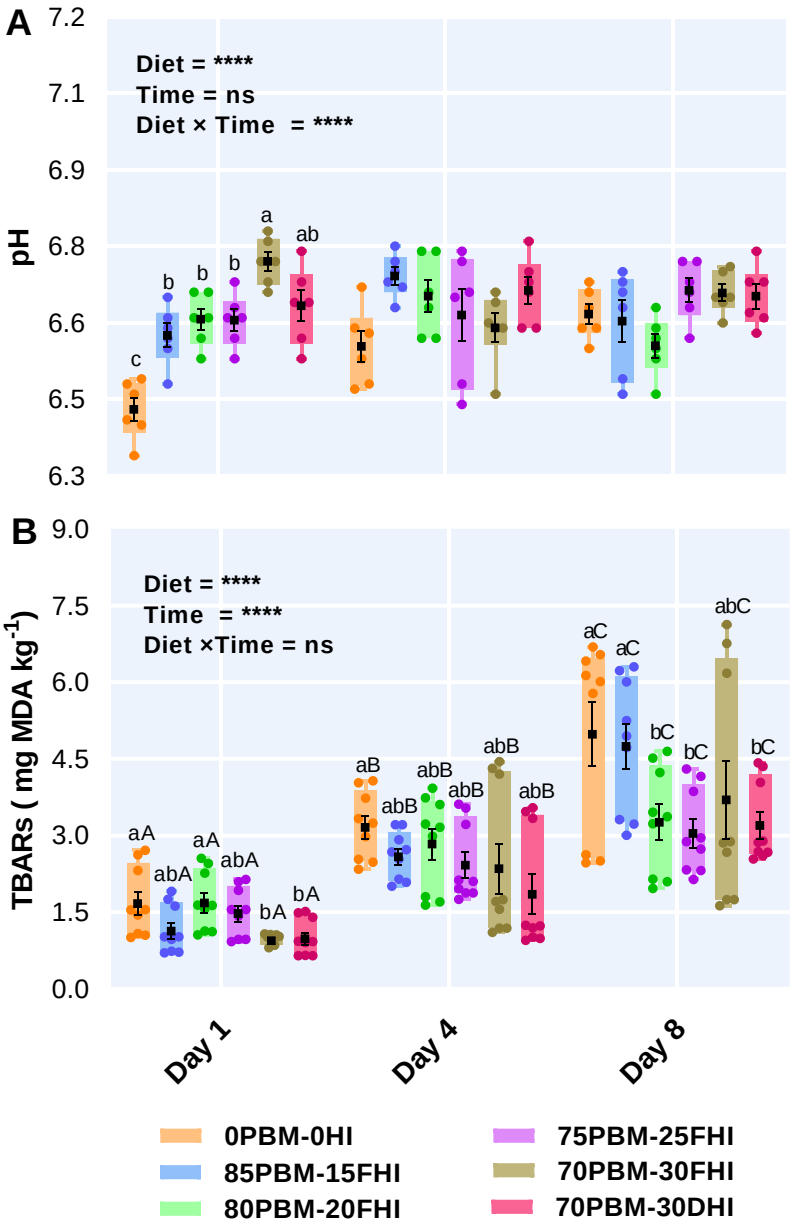

Figure 12. 7 The chemical composition including pH (A) and MDA (B) in the flesh of barramundi fed graded level of HI larvae meal wither full-fat and defatted for 56 days under a simulated retail display. Round shape markers with box-whisker indicate the number of biological replicates (n = 9) while black square shape marker indicates the mean of biological and technical replicates (n = 3) with standard error. The effect of two fixed factors "Diet" and "Storage time" were determined by Two-way ANOVA with Dunnett's multiple comparison test $0.05 < P < 0.0001$. Small letters on the top of the box-whiskers indicate significant difference between the treatments for each day whilst capital letters indicate significant between the storage times for each treatment.

12.1.3 Supplementation of FPHs alone or concurrently with full-fat HI larvae in PBM

The sixth trial (Chapter 10) was carried out to increase the inclusion of PBM at maximum levels (90%) along with the supplementation of 10% various FPHs, recovered from carp, tuna, and kingfish wastes. The results showed that various FPH supplementation complemented the PBM without impacting growth, biometry indices (Table 12. 3) and spleen (Figure 10. 2B-D), and kidney health (Figure 10. 2F-H). Based on the findings of all trials, the final trial (Chapter 11) was conducted to investigate if the concurrent supplementation of 10 and/or 5% FHI larvae meal and TH (selected among various FPHs based on a higher proportion of low molecular weight peptides) with graded levels of PBM could make the diets more functional than separate supplementation. The concurrent supplementation of TH and FHI facilitated the adding of PBM at a maximum level, with better results in terms of growth (Table 12. 3) and without affecting the liver (Figure 11. 1B-D), kidney (Figure 11. 1F-H), and spleen (Figure 11. 1J-L) health, suggesting that concurrent supplementation of TH and FHI larvae in PBM provided relatively higher benefits than when various FPH or HI larvae are supplemented separately with PBM in the previous trials.

An equal or improvement in growth performance of barramundi in response to various FPHs alone or concurrently with HI larvae was due to the presence of 90% less than 10kda molecular weight peptides (reported in Chapter 10) (Chaklader et al., 2020c) which have been reported to reach the intestine faster than intact protein and thus potentially working as growth promotors (Aksnes et al., 2006b; Bui et al., 2014; Khosravi et al., 2015b; Robert et al., 2015). This potentiality was reflected in mucosal barrier functions, manifested by the increasing number of acidic mucins (Figure 11. 2M, N) and microvilli density (Figure 11. 4B) in the distal intestine of barramundi as reported in Chapter 10 and 11. Various FPHs supplementation and concurrent supplementation of TH and FHI larvae also improved the synthesis of PUFA concentration (Table 10. 5 and Table 11. 5) in barramundi muscle when compared to other trials without changing flesh quality (Table 11. 6), which is important in consumer assessment and acceptance to zero FM-based diets. Also, it has been reported that PUFA in fish is related to lessening the risk of neurological and cardiovascular diseases (Blondeau et al., 2015; Mensink, 2016; Michielsen et al., 2019). The higher PUFA levels are likely to be with fillets due to the presence of a higher concentration of individual PUFA in various FPH.

Alongside growth improvement, immune response in terms of serum total protein, lysozyme and bactericidal activity (Figure 10. 6 A, B and) improved in barramundi fed PBM, supplemented with various FPHs or TH and FHI concurrently. The immune results were also aligned with disease resistance against *V. harveyi* (Figure 10. 4 and Figure 11. 5). Similarly, supplementation of FPH with FM or various alternative protein sources such PBM and plant protein improved the disease

resistance against pathogenic bacteria in barramundi (Siddik et al., 2019b; Siddik et al., 2018b), red sea bream, *Pagrus major* (Bui et al., 2014; Khosravi et al., 2015a; Khosravi et al., 2015b) and European seabass, *Dicentrarchus labrax* (Costa et al., 2020). Enzymatic hydrolysis produces a lower fraction of different bioactive peptides including di- and tripeptides and other oligopeptides which have immunostimulating properties (Bøgwald et al., 1996; Gildberg et al., 1996). The immunomodulatory effect in Chapter 10 and Chapter 11 aligned with the other previous studies might be attributed to the presence of medium size-bioactive peptides and low molecular weight peptides in FPH (Table 10. 3), as illustrated in Chapter 10 which have been reported to demonstrate antibacterial properties (Offret et al., 2019) by damaging the bacterial membrane of different species and strains of *Vibrio* (Acosta-Smith et al., 2018). The immunostimulatory properties of FHI has already been described in Chapter 4 and Chapter 5. Hence, the smaller quantity of FPHs alone and, concurrently with FHI could be used as potential immunostimulants to enhance the functionality of aquafeeds.

Table 12. 3 Growth, fillets of total fatty acid composition and quality traits of barramundi fed PBM-based diets supplemented with various FPHs or concurrently supplemented with TH and FHI larvae. ▲ increased significantly when compared FM-based diet (Control), ▼ decreased significantly when compared FM-based diet (Control), and ▬ unchanged when compared FM-based diet (Control).

	Experimental diets					
	Chapter 10			Chapter 11		
	90PBM-KH	90PBM-CH	90PBM-TH	80PBM$_{10TH+10HI}$	85PBM$_{5TH+10HI}$	90PBM$_{5TH+5HI}$
FBW (g)	▬	▬	▬	▲	▲	▲
WG (g)	▬	▬	▬	▲	▲	▲
SGR (%/d)	▬	▬	▬	▲	▲	▲
FI (g/fish d^{-1})	▬	▬	▬	▬	▬	▬
FCR	▬	▬	▬	▬	▬	▬
SR (%)	▲	▲	▲	▬	▬	▬
CF (%)	▬	▬	▬	▬	▬	▬
HSI (%)	▬	▬	▬	▬	▬	▬
IFI (%)	▬	▬	▬	▬	▬	▬
Total fatty acid						
∑SFA	▲	▲	▲	▲	▲	▲
∑MUFA	▲	▲	▲	▲	▲	▲
∑PUFA	▲	▲	▲	▲	▲	▲
∑n3-PUFA	▲	▲	▲	▬	▬	▬
Fillet quality traits						
pH	NA	NA	NA	▬	▬	▬
Texture	NA	NA	NA	▬	▬	▬
Skin colour	NA	NA	NA	▬	▬	▬
Flesh colour	NA	NA	NA	▬	▬	▬

12.1.4 The potentiality of FHI larvae meal and TH as prebiotics to modulate gut microbiota

Evaluation of gut microbiota is frontier research necessary to facilitate the selection of probiotics, prebiotics, and functional material for aquafeed formulation with the potential to improve the gut health of fish. Recently, HI larvae meal have received much attention due to a prebiotic effect on fish (Bruni et al., 2018; Terova et al., 2019), crustacea (Foysal et al., 2019), and other livestock animals (Borrelli et al., 2017). Here the inclusion of 15-30% of FHI larvae meal in graded levels of PBM improved the barramundi distal intestinal microbial diversity, with a higher proportion of beneficial bacteria including *Lactobacillus*, *Clostridium*, and *Ruminococcus* (Figure 6. 5) (Chapter 6). Even, the smaller quantity of FHI larvae meal and/or TH in PBM enhanced the abundance of bacteria, with a significant reduction of *Vibrio* spp (Table 11. 8) (Chapter 11). The richness of the bacterial community has been associated with out-competing the pathogens for nutrients and colonization and resisting pathogen invasion and intestinal infection thereby improving the gut health (Cerezuela et al., 2013; Huyben et al., 2019; Levine and D'Antonio, 1999). The enrichment of bacterial community identified in Chapters 6 and 11 could be attributed to chitin, an uncommon material in commercial diets, but may work as a substrate to colonize and grow less common bacteria that can digest chitin as a source of nutrients (Huyben et al., 2019).

Also, the probiotic effect of *Lactobacillus* has been demonstrated in fish and other vertebrates (Ringø and Gatesoupe, 1998) due to their ability to prevent the adherence of pathogens to the intestinal surface by creating biofilms hosting bacterial inhibiting components including lactic acid hydrogen peroxide, and bacteriocins or biosurfactants (Gudiña et al., 2015). Chitin in HI larvae has been reported as a preferential substrate for the proliferation of *Lactobacillus* whilst reducing the abundance of pathogenic microbes which may explain the increase and decrease in the abundance of *Lactobacillus* and *Vibrio* reported in Chapters 6 and 11, respectively. Similarly, HI larvae meal increased the abundance of *Lactobacillus* in rainbow trout (Terova et al., 2019) and egg laying hens (Borrelli et al., 2017). Whilst *Clostridium* produces butyrate, a short-chain fatty acid plays different important roles in the host body: bio-regulation and promotion of mucosal growth via direct or indirect mechanisms in the gut (Ptak et al., 2015), regulating the immune system together with anti-inflammatory properties (Hamer et al., 2008; Wong et al., 2006) as well as contributing to mineral uptake and providing extra energy (Kulshreshtha et al., 2014). *Ruminococcus* has been reported to convert polysaccharides into a variety of nutrients for their hosts (La Reau and Suen, 2018) and is common in insect-eating carnivores (La Reau et al., 2016) thereby enhancing *Ruminococcus* in the present study. Beside, the contribution of chitin, lauric acid, antimicrobial peptides, and other polysaccharides are also active microorganisms or in improving gut mucosal barrier functions may influence the gut microbiota in the present study.

Such improvement in the barramundi gut suggested that both HI larvae meal and TH could be used as potential prebiotics in the barramundi diet.

12.2 Conclusion

The following conclusions can be made based on the findings of this study:

I. Juvenile barramundi cannot tolerate total substitution of FM with PBM, reflected by experimental results of poor growth performance, feed intake, immune response, antioxidant activity, and synthesis of essential fatty acids. Also, the 100PBM diet caused histopathological changes in liver, muscle, and gill, impacted intestinal integrity, and resulted in a higher expression of stress-related genes in the liver of barramundi.

II. Supplementation of 10% FHI larvae prevented the negative effects caused by exclusive inclusion of PBM, allowing to inclusion of up to 75% of PBM, that either improved or did not influenced the growth performance with an increase in mucosal barrier functions in the intestine (villi width, enterocyte width, microvilli height, and acidic mucins) and skin (epidermis thickness and neutral mucins) and no histopathological changes in the liver, muscle, and heart. Also, supplementing HI larvae reduced the deposition of fat cells and stimulated the resistance to *Vibrio harveyi*. However, complete replacement of FM with 90% PBM and 10% full-fat HI larvae compromised the health of barramundi.

III. Progressive inclusion of FHI larvae meal of 15-30% allowed an inclusion of more than 75% of PBM (75-85%) without impacting growth and amino acid synthesis in juvenile barramundi fillets. Furthermore, the mixture of full-fat HI larvae and PBM enhanced gut health by improving the beneficial bacteria and mucosal barrier functions in the distal intestine and improved immunity in juvenile barramundi.

IV. Dietary inclusion of 30% of DHI larvae was unable to prevent negative effects caused by the higher inclusion level (70%) of PBM-based diet on the growth, liver health, antioxidant capacity, and immunity results in juvenile barramundi.

V. Both FHI and DHI larvae meal in PBM improved the raw and cooked fillet sensory quality, texture, and colour and suppressed the lipid oxidation in the juvenile barramundi fillet. The improvement in fillet brightness of barramundi is promising in the present study, which would help in resolving blue-greyish colour issues in farmed barramundi fillets.

VI. Supplementation of 10% of various FPHs also complemented the PBM-based diets and resulted in a comparable growth performance to FM-based diet. In addition, PBM supplemented with various FPHs increased the synthesis of omega-3 fatty acids, improved intestinal and skin mucosal barrier functions, and improved the anti-inflammatory response and immunity of juvenile barramundi.

VII. Finally, concurrent supplementation of TH and FHI larvae meal in PBM allowed to total replacement of FM from juvenile barramundi diet with an increase in growth performance and the synthesis of essential fatty acids whilst showing no impact on the fillet quality in terms of pH, texture, and colour of skin and flesh. Also, concurrent supplementation of TH and full-fat HI larvae meal increased the bacterial diversity in the intestine and immunity of juvenile barramundi.

VIII. Inclusion of FHI alone or in combination with TH enhanced the bacterial diversity coupled with an increase in the abundance of beneficial bacteria such as *Lactobacillus*, *Clostridium*, and *Ruminococcus*, suggesting that HI larvae meal or TH could be used as potential prebiotics to improve the gut health of barramundi.

12.3 Recommendations

I. Physical properties (pellet diameter, expansion ratio, pellet density, sinking velocity, water stability and volume increase) of PBM-based diets, supplemented with FHI larvae meal and/or FPHs need to be evaluated since these properties are associated with the manufacturing of practical aquafeed and management of water quality.

II. Since lipid profile in HI larvae meal can be manipulated, the possible enrichment procedures by changing feeding substrates to enrich limited PUFAs in HI larvae need to be further investigated.

III. FHI larvae-based PBM diet may be prone to lipid oxidation due to the presence of higher proportion of oil in HI larvae meal which needs to be further investigated.

IV. Further digestibility trials are needed to fully understand the reasons for the ameliorative of FHI larvae meal when compared to DHI larvae meal.

V. Further study should consider to evaluate chitinolytic enzymes and chitinoclastic bacteria to confirm if juvenile barramundi can digest and assimilate chitin in HI larvae meal.

VI. It is important to further study how different functional molecules such as bioactive peptides, lauric acid, chitin, and other polysaccharides including choline, silkrose, and dipetrose in HI larvae meal exert a beneficial effects in fish and also essential to examine if the defatting process may negatively affect the functional properties of HI larvae meal.

VII. The present study covered the juvenile stage of barramundi to evaluate fillet quality traits. Since the results are encouraging, further study is needed to evaluate the fillet quality of commercial size barramundi, following longer-term feeding trials to extend with the present findings. Also, it is crucial to identify the properties in HI larvae meal that improved sensory quality and retarded the lipid oxidation process.

VIII. The promising findings in the present study at the laboratory scale are needed to be further trialed at a commercial scale to investigate the feasibility of scaling up PBM-HI-FHI based diet in a barramundi or other carnivorous fish species.

12.4 Limitations

I. The present study only used three technical replicates for all analyses and more technical replicates might have provided more statistical robustness.

II. Due to animal ethical restrictions, the minimum number of fish for each replicate was used for the feeding trial. A larger number of fish may provide more accurate results.

III. The initial size of fish, and feeding trial duration, was not homogenous for all feeding trials.

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APPENDIX

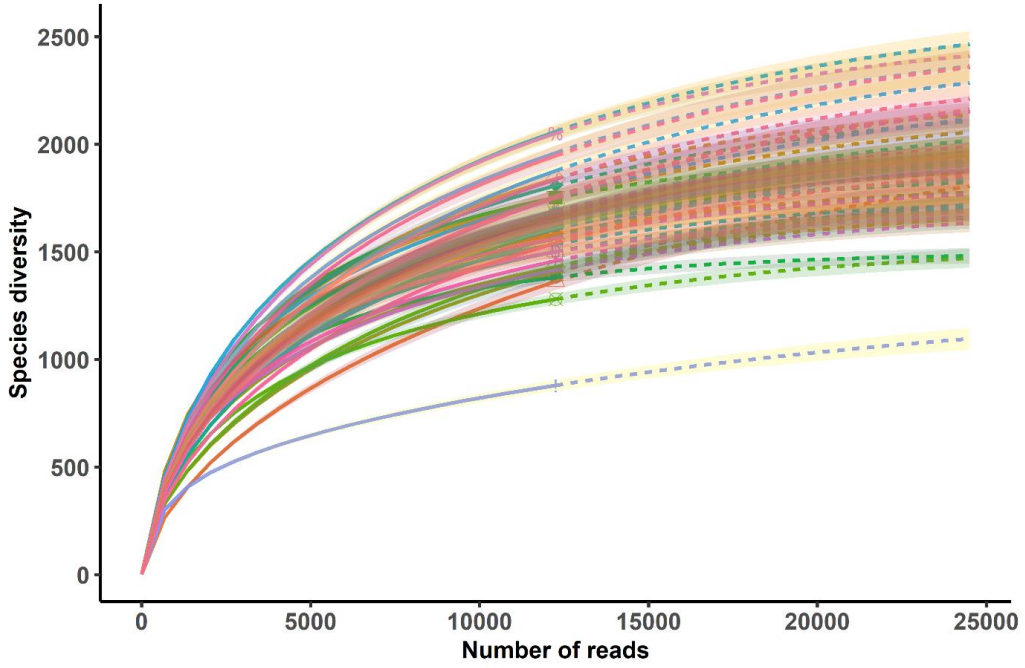

Figure 6S. 1 Rarefaction curve showing the depth of sequencing and species diversity in study samples.

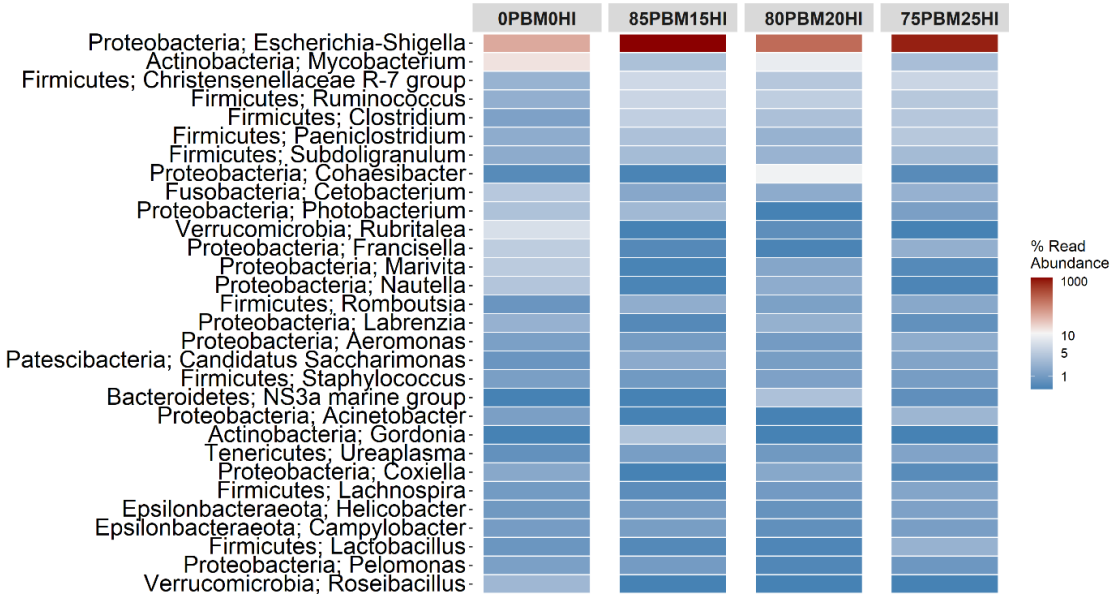

Figure 6S. 2 Relative abundance of bacteria representing at least 1% of the read abundance for a treatment.

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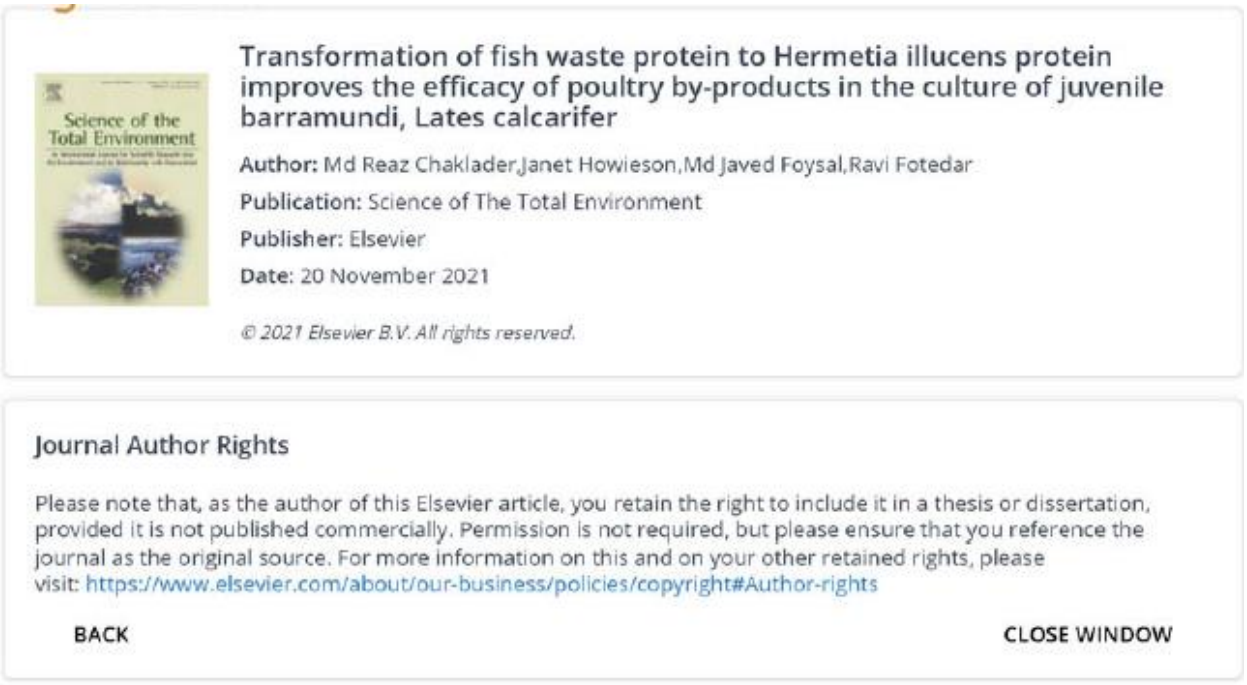
Science of the Total Environment (CHAPTER 6)



Transformation of fish waste protein to Hermetia illucens protein improves the efficacy of poultry by-products in the culture of juvenile barramundi, Lates calcarifer

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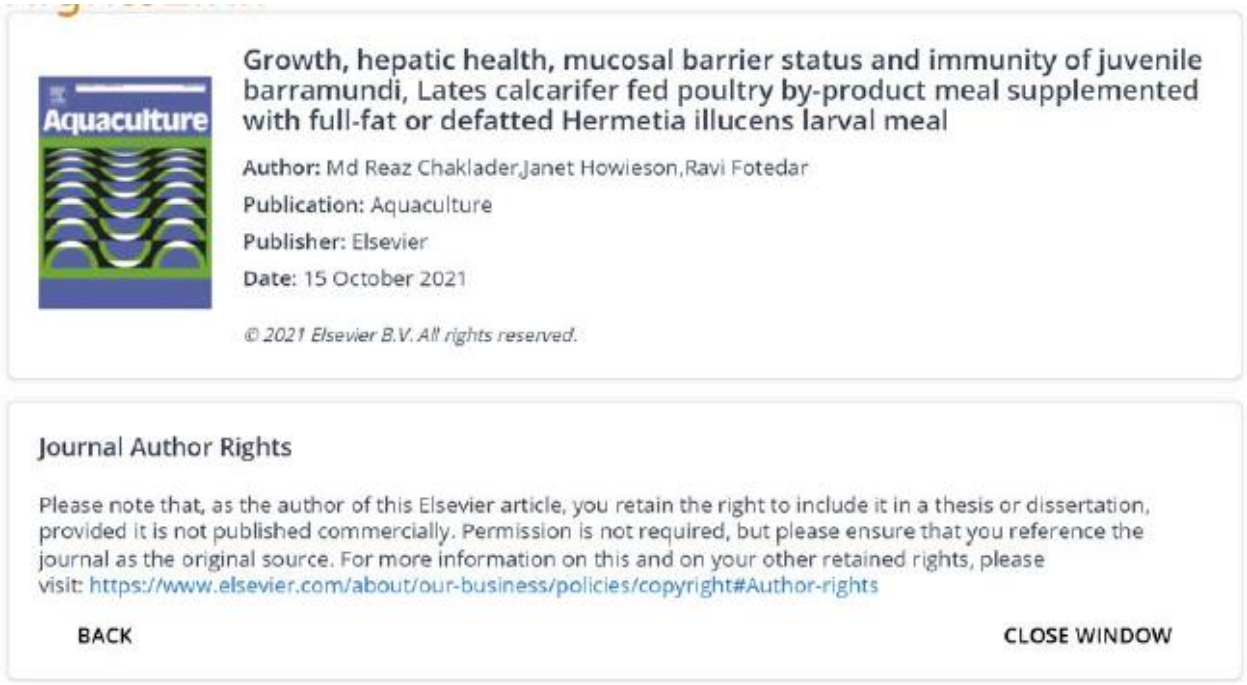
Aquaculture (CHAPTER 8)



Growth, hepatic health, mucosal barrier status and immunity of juvenile barramundi, Lates calcarifer fed poultry by-product meal supplemented with full-fat or defatted Hermetia illucens larval meal

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Fish and Shellfish immunology (CHAPTER 10)

The ameliorative effects of various fish protein hydrolysates in poultry by-product meal based diets on muscle quality, serum biochemistry and immunity in juvenile barramundi, Lates calcarifer

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Table S. 1 Author attribution statement

	Concepts	Funding	Methods	Data curation	Investigation	Software	Formal analysis	Validation	Writing: Draft	Writing: Reviewing	Resources	Supervision	Total % contribution
Chapter 2 (Paper 1) - Title: Opportunities and challenges of black soldier fly meal as a renewable protein source in aquaculture: a comprehensive review. Submitted to Review in Aquaculture.													
First Author Md. Reaz Chaklader	√		√	√		√		√	√				90
Co-Author 1 Ravi Fotedar	√											√	5
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Co-Author 2 Janet Howieson	√	√										√	5
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Total (%)													100
Chapter 3 (Paper 2) - Title: Total replacement of fishmeal with poultry by-product meal affected the growth, muscle quality, histological structure, antioxidant capacity and immune response of juvenile barramundi, *Lates calcarifer*. PLoS ONE, 15(11): e0242079. https://doi.org/10.1371/journal.pone.0242079													
First Author Md. Reaz Chaklader	√		√	√	√	√	√		√				85
Co-Author 1 Muhammad A. B. Siddik							√			√			2.5
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Co-Author 2 Ravi Fotedar	√									√		√	12.5
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Total (%)													100

Chapter 4 (Paper 3) - Title: Insect larvae, *Hermetia illucens* in poultry by-product meal for barramundi, *Lates calcarifer* modulates histomorphology, immunity and resistance to *Vibrio harveyi*. Scientific Reports, 9: 16703 (2019). https://doi.org/10.1038/s41598-019-53018-3													
First Author Md. Reaz Chaklader	√		√	√	√	√	√	√	√				87.5
Co-Author 1 Muhammad A. B. Siddik										√			2.5
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Co-Author 2 Ravi Fotedar	√									√		√	5
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Co-Author 3 Janet Howieson	√	√								√		√	5
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Total (%)													100
Chapter 5 (Paper 4) - Title: Supplementation of *Hermetia illucens* larvae in poultry by-product meal based barramundi, *Lates calcarifer* diets improves adipocyte cell size, skin barrier functions, and immune responses. Frontiers in nutrition, 7:613158 (2021). 10.3389/fnut.2020.613158													
First Author Md. Reaz Chaklader	√		√	√	√	√	√	√	√				85
Co-Author 1 Janet Howieson	√	√								√		√	7.5
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Co-Author 2 Ravi Fotedar	√									√		√	5
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Co-Author 3 Muhammad A. B. Siddik										√			2.5

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Total (%)													100
Chapter 6 (Paper 5) - Title: Transformation of fish waste protein to *Hermetia illucens* protein improves the efficacy of poultry by-products in barramundi, *Lates calcarifer* culture. Science of the Total Environment, 796, 149045 (2021). https://doi.org/10.1016/j.scitotenv.2021.149045													
First Author Md. Reaz Chaklader	√		√	√	√	√	√	√	√				85
Co-Author 1 Janet Howieson	√	√								√		√	7.5
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Co-Author 2 Md Javed Foysal							√			√			2.5
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Co-Author 3 Ravi Fotedar	√									√		√	5
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Total (%)													100
Chapter 7 (Paper 6) - Title: A combination of *Hermetia illucens* reared on fish waste and poultry by-product meal improves acceptibility and physicochemical quality of farmed barramundi fillets. Under review in Fronteirs in Nutrition.													
First Author Md. Reaz Chaklader	√		√	√	√	√	√	√	√				80
Co-Author 1 Wing H. Chung			√				√			√			10
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Co-Author 2 Janet Howieson	√	√	√							√		√	5
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Co-Author 3 Ravi Fotedar										√		√	5
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Total (%)													100
Chapter 8 (Paper 7) – Title: Growth, hepatic steatosis, mucosal barrier status and immunity of juvenile barramundi, *Lates calcarifer* fed poultry by-product meal supplemented with full-fat and defatted *Hermetia illucens* larval meal. Aquaculture, 543, 737026. https://doi.org/10.1016/j.aquaculture.2021.737026													
First Author Md. Reaz Chaklader	√		√	√	√	√	√	√	√				85
Co-Author 1 Janet Howieson	√	√								√		√	7.5
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 2 Ravi Fotedar	√									√		√	7.5
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Total (%)													100
Chapter 9 (Paper 8) – Title: Valorised full-fat and defatted *Hermetia illucens* protein combined with poultry by-product protein improves fillets quality traits of farmed barramundi, *Lates calcarifer*. Submitted to Food Control.													
First Author Md. Reaz Chaklader	√		√	√	√	√	√	√	√				80
Co-Author 1 Wing H. Chung			√				√			√			10
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 2 Janet Howieson	√	√	√							√		√	5
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Co-Author 3 Ravi Fotedar										√		√	5
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Total (%)													100
Chapter 10 (Paper 9) – Title: The ameliorative effects of various fish protein hydrolysates in poultry by-product meal diets on muscle quality, serum biochemistry and immunity in juvenile barramundi, *Lates calcarifer*. Fish & Shellfish Immunology, 104:567-578 (2020). https://doi.org/10.1016/j.fsi.2020.06.014													
First Author Md. Reaz Chaklader	√		√	√	√	√	√	√	√				80
Co-Author 1 Ravi Fotedar	√									√		√	5
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 2 Janet Howieson	√	√								√		√	10
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 3 Muhammad A. B. Siddik										√			2.5
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 4 Md Javed Foysal							√			√			2.5
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Chapter 11 (Paper 10) – Title: Supplementation of tuna hydrolysate and insect larvae improves fishmeal replacement efficacy of poultry by-product in *Lates calcarifer* (Bloch, 1790) juveniles. Scientific Reports, 11: 4997 (2021). https://doi.org/10.1038/s41598-021-84660-5													
First Author Md. Reaz Chaklader	√		√	√	√	√	√	√	√				85

Co-Author 1 Janet Howieson	√	√								√		√	5
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Co-Author 2 Muhammad A. B. Siddik										√			2.5
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Co-Author 3 Md Javed Foysal							√			√			2.5
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Co-Author 4 Ravi Fotedar	√									√		√	5
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