School of Molecular and Life Sciences

Characterization and role of the selected microbiome in water remediation and gut health of aquacultured marron (*Cherax cainii*, Austin 2002)

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DECLARATION

To the best of my knowledge, this thesis does not contain any previously published data including table(s) and figure(s) in the experimental chapters (chapter 4-8). Proper acknowledgement(s) or citation(s) have been made where data and information curated from published articles, books, conferences, thesis and other online sources.

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Signed | Md Javed Foysal

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List of common abbreviations and acronyms

WA	Western Australia
LAB	Lactic acid bacteria
HTS	High throughput sequencing
DO	Dissolved oxygen
FM	Fishmeal
PBP/PBM	Poultry-by-product meal
BSF	Black solder fly
SOY	Soybean
LPN	Lupin
THS	Tuna hydrolysate
BB	Bio-Ball
TWC	The Water Cleanser
WG	Weight gain
SGR	Specific growth rate
FCR	Feed conversion ratio
HO	Haemolymph osmolality
THC	Total haemocyte counts
bp	Base Pairs
OTU	Operational taxonomic unit
MICCA	MICrobial Community Analysis
QIIME	Quantitative Insights Into Microbial Ecology
NMDS	Non-metric Multidimensional Scaling
rRNA	Ribosomal RNA
RT-PCR	Real-time Polymerase Chain Reaction
LDA	Linear Discriminant Analysis
LEfSe	Linear Discriminant Analysis Effect Size
PCoA	Principal Coordinate Analysis
AMP	Antimicrobial peptides
KEGG	Kyoto Encyclopaedia for Genes and Genomes
NCBI	National Centre for Biotechnology Information
CARL	Curtin Aquatic Research Laboratories
AOAC	Association of Official Analytics Chemists
mg/L	Milligram per litre
g/L	Gram per litre
CFU/g	Colony forming unit per gram
mJ/kg	Mega joule per kilogram

List of common aquatic species names mentioned in the research

Common name	Scientific name
Marron	*Cherax cainii*
Red claw	*Cherax quadricarinatus*
Red swamp	*Procambarus clarkii*
Narrow clawed crayfish	*Astacus leptodactylus*
Giant Tasmanian crayfish	*Astacopsis gouldi*
Murray River crayfish	*Euastacus armatus*
Chinese mitten crab	*Eriocheir sinensis*
Mud crab	*Scylla paramamosain*
Tiger shrimp	*Penaeus monodon*
White shrimp	*Litopenaeus vannamei*
Freshwater prawn	*Macrobrachium rosenbergii*
River prawn	*Macrobrachium nipponense*
European lobster	*Homarus gammarus*
Spiny lobster	*Panulirus ornatus*
Signal crayfish	*Pacifastacus leniusculus*
Kuruma shrimp	*Marsupenaeus japonica*
Yabby	*Cherax destructor*
Atlantic salmon	*Salmo salar*
Barramundi/Sea bass	*Lates calcarifer*
Nile tilapia	*Oreochromis niloticus*
Rainbow trout	*Oncorhynchus mykiss*
Common carp	*Cyprinus carpio*
Turbot	*Scophthalmus maximus*
European flounder	*Platichthys flesus*
Grass carp	*Ctenopharyngodon idellus*

PREAMBLE

The aim of this research was to characterize the microbial communities in the gut and rearing water and their role on the health and immune response of freshwater crayfish, marron (*Cherax cainii*, Austin 2002).

The thesis consists of 9 chapters. **Chapter 1**, briefly outlines the current status of aquaculture and marron production; recent advancement in molecular techniques for evaluating health and immunity status of aquatic species; current research on probiotics, dietary protein ingredients and bacteria linked with water remediation in decapod crustacean aquaculture; and opportunities of omics technologies to improve marron health and immunity. This chapter also summarizes the aim, objectives and significance of the present study. **Chapter 2**, incorporates the review of relevant literature that commences with a brief review on the background research and the researched aquatic species, marron. The rest of the chapter reviews two main themes, namely (i) the research in the area of probiotics, and (ii) alternative dietary protein sources and various substrates in decapod crustacean nutrition. The review is with the background on the gut microbiota in decapods while emphasizing the most abundant and core bacteria in the gut of marron under different dietary and culture conditions. **Chapter 3** focusses on the general methodology describing the common methods used in the research chapters that follow this chapter. Chapter 4 to 8 are based on research findings. **Chapter 4** (objective 1) analyses the effects of nutrient deprivation on health status, gut microbiota and innate immune response of marron. Parameters assayed in this chapter, include the growth performance, tail muscle biochemistry, haemolymph parameters, gut microbial communities and genes associated with immune response of marron. **Chapter 5** (objective 2) evaluates the effects of different probiotics on marron growth, tail muscle, gut health and immunity. The probiotics include *Lactobacillus acidophilus* and *L. plantarum* mixture, *Clostridium butyricum* and *Bacillus*

mycoides. **Chapter 6** (objective 3) investigates the impacts of different protein diets from plant, animal and insect sources on gut health of marron. The protein sources tested were fishmeal, poultry-by-product meal, black soldier fly meal, soybean meal, lupin meal and tuna hydrolysate. **Chapter 7** (objective 4) evaluates the effects of substrate and non-substrate based biological filters on water quality and marron gut health. The substrates include the gravel, Bio-Ball, The Water Cleanser™ and zeolite. **Chapter 8** (objective 5) describes a trail based on the experimental findings from different marron diets used in chapter 6 and 7. *Lactobacillus plantarum* was mixed with black soldier fly meal-based marron basal diet, and its impacts on the marron growth, tail muscle, gut health and immunity was investigated. **Chapter 9** (objective 6 and 7) discusses the outcomes of present research comprehensively by comparing and contrasting data from all research based chapters included in this thesis and then compares them to the published literature. It also summarizes the conclusions, limitations and future recommendations of the current research. Every research based chapter of the thesis is formatted individually and differently as published research is structured according to the author guidelines of the journal.

A list of publications arising from the present research are outlined

Article 1. **Foysal MJ,** Fotedar R, Tay ACY, Gupta SK, 2020. Effects of long-term starvation on health indices, gut microbiota and innate immune response of fresh water crayfish, marron (*Cherax cainii*, Austin 2002). Aquaculture 514, 734444. https://doi.org/10.1016/j.aquaculture.2019.734444.
(**Chapter 4**: Effects of nutrient deprivation on marron health and immunity)

Article 2: **Foysal MJ,** Nguyen TTT, Chaklader MR, Siddik MAB, Tay CY, Fotedar R, Gupta SK, 2019. Marked variations in gut microbiota and some innate immune responses of fresh water crayfish, marron (*Cherax cainii*, Austin 2002) fed dietary supplementation of *Clostridium butyricum*. PeerJ 7, e7553. https://doi.org/10.7717/peerj.7553.

Article 3: **Foysal MJ,** Chua EG, Gupta SK, Lamichhane B, Tay CY, Fotedar R, 2020. *Bacillus mycoides* supplemented diet modulates the health status, gut microbiota and innate immune response of freshwater crayfish marron (*Cherax cainii*). Anim. Feed Sci. Technol. 114408. https://doi.org/10.1016/j.anifeedsci.2020.114408.

Article 4: **Foysal MJ,** Fotedar R, Siddik MAB, Tay A, 2020. *Lactobacillus acidophilus* and *L. plantarum* improve health status , modulate gut microbiota and innate immune response of marron (*Cherax cainii*). Sci. Rep. 10, 5916. https://doi.org/10.1038/s41598-020-62655-y.
(**Chapter 5**: Probiotics for marron aquaculture)

Article 5: **Foysal MJ,** Fotedar R, Tay CY, Gupta SK, 2019. Dietary supplementation of black soldier fly (*Hermetica illucens*) meal modulates gut microbiota, innate immune response and health status of marron (*Cherax cainii*, Austin 2002) fed poultry-by-product and fishmeal based diets. PeerJ 7, e6891. https://doi.org/10.7717/peerj.6891.
(**Chapter 6**: Protein diets for marron aquaculture)

Article 6: **Foysal MJ,** Fotedar R, Tay CY, Gupta SK, 2020. Biological filters regulate water quality, modulate health status, immune indices and gut microbiota of freshwater crayfish, marron (*Cherax cainii*, Austin, 2002). Chemosphere 125821. https://doi.org/10.1016/j.chemosphere.2020.125821.
(**Chapter 7**: Substrates for marron aquaculture)

Article 7: **Foysal MJ,** Fotedar R, Siddik MAB, Chaklader R, Tay A, 2021. *Lactobacillus plantarum* in black soldier fly (*Hermetica illucens*) meal modulates gut health and immunity of freshwater crayfish (*Cherax cainii*). Fish Shellfish Immunol. 108, 42–52. https://doi.org/10.1016/j.fsi.2020.11.020.
Chapter 8: Probiotics in alternative protein diets for marron aquaculture

ABSTRACT

Aquaculture is the fastest growing food sector that provides nutrient-dense food and nutritional security to the growing population of the world. Marron (*Cherax cainii*, Austin 2002) is one of the highly valued and iconic freshwater crayfish species of Western Australia. In spite of slight improvement in marron productivity by improved formulated diets and various substrates in the rearing environment, the farm production of marron has not significantly increased. The lack of research on the role of the microbial communities in the marron gut and water as a culture medium, on modulating health, physiology and immunity are a few bottlenecks to achieve the desirable productivity of marron in aquaculture operations. This current study was designed to characterize the gut microbial communities and their role in water quality and health of cultured marron when fed different supplemented probiotics and dietary protein ingredients, and reared on various commercially available water-remediation substrates. The first phase of the study investigated the effects of nutrient deprivation on the microbial communities in the marron gut and its health while second phase analysed the progressive impacts of different dietary proteins and probiotics using various substrates in the rearing environment on the dynamics of gut microbiome under laboratory conditions. The role of alternative to fishmeal protein ingredients, supplemented probiotics and microbial mat (biofilm) formed on various substrates used in the rearing environment, on the health and immunity of laboratory-cultured marron were also evaluated. In addition, the correlation between microbial communities and health indices of marron was drawn through systematic meta-analysis and bioinformatics to identify the beneficial bacteria for marron aquaculture. The final data showed that the marron gut microbiota are dominated by the phylum Tenericutes and genus *Candidatus* Bacilloplasma, while nutritionally deprived gut favoured *Vibrio* that negatively affected the immunity of marron. All the tested probiotics and potential alternative

to fishmeal protein ingredients including poultry-by-product meal and black soldier fly meal significantly reduced *Vibrio* abundance in the marron gut while *Lactobacillus acidophilus* and *L. plantarum* combinations positively influenced the higher number of immunological parameters, than any other test diets. The substrates Bio-Ball and Water Cleanser^{TM} exhibited positive impact on water quality by reducing nitrogen waste and improving gut microbial diversity of marron. Regardless of diets and substrates, phyla Firmicutes and Tenericutes, genera *Lactobacillus*, *Lactovum* and *Candidatus* lineages were found positively correlated to health and immunity of marron. *Lactobacillus* sp., and substrates such as Bio-Ball and Water Cleanser, therefore can be used to improve the health and immunity of marron.

CHAPTER 1: Introduction

1.1. Background

Crayfish are freshwater crustaceans with more than 600 species. The industry for the freshwater crayfish is rapidly expanding with value of more than $10 billion in 2018, and production increased by three fold within a decade (Vogt, 2020). Australia is known for crayfish culture and has one of the richest collection of freshwater crayfish fauna (DPI_Reports, 2000). The Giant Tasmanian crayfish (*Astacopis gouldii*) and Murray River crayfish (*Euastacus armatus*) found in the rivers, and three farming *Cherax* species, red claw (*C. quadricarinatus*), yabby (*C. destructor*) and marron (*C. cainii*) are some major crayfish found in Australia (Munasinghe et al., 2004, Sinclair et al., 2011, Zukowski et al., 2011). However, the first two have little aquaculture potential due to their slower growth rate and low meat ratio. Despite species richness and opportunities for crayfish aquaculture, Australia's crayfish production (166 tonnes) is far behind China (1,638,622 tonnes) and USA (72,682 tonnes) (Cai et al., 2019). Marron (*Cherax cainii*) is the third largest, omnivore, polytrophic crayfish from parastacidae family, native and iconic to Western Australia (WA) (Lawrence and Morrissy, 2000). Marron is the most cultured freshwater crayfish species in Australia, comprising of 40% (66 tonnes) of the total production in 2018, ahead of yabbies (31%) and red claw (29%). (Cai et al., 2019). Due to its larger size (2 kg), colour diversities, distinctive flavour and taste, marron commands a high global market demand (Saputra et al., 2019, Morrissy, 1979). Additionally, marron aquaculture possess several advantages and aquaculture attributes like high price over other crayfish, ease to culture, disease-resistance and prospects of long distance live shipment (Alonso, 2010, Alonso, 2009b). However, slow growth rates of marron under commercial farming (Lawrence, 2007) has resulted in stagnant production in the last two decades (Alonso, 2009a, MGAWA, 2020). Thus, there is an opportunity to increase the marron production through appropriate diets and by maintaining water quality under commercial farming.

Owing to the problem, research in improving growth and immune performance of marron with formulated, sustainable and non-fishmeal based diet, is warranted. In many crayfish aquaculture practices, probiotics have been used and lactic acid bacteria (LAB) are considered as most promising probiotic group for narrow clawed crayfish (*Astacus leptodactylus*) (Didinen et al., 2016, Valipour et al., 2019), red claw (*Cherax quadricarinatus*) (Lim et al., 2020) and red swamp (*Procambarus clarkii*) (Zhang et al., 2020) with beneficial effects on growth and immunity. In addition to health impacts, the selection of aqua-diet also depends on price and availability of the raw ingredients, and their environmental and ecological impacts. Switching from conventional fishmeal based aqua-diets to novel ingredients from cheap alternative sources would reduce the price of aqua-diets and pressure on wild fish-stocks (Cottrell et al., 2020). To make crayfish aquaculture more sustainable, researchers are evaluating soybean (*Glycine max*) and lupin (*Lupinus angustifolius*) from plants, poultry-by-product and tuna hydrolysate from animals and black soldier fly (*Hermetica illucens*) from insect sources for their suitability to be used as alternatives of fishmeal for signal crayfish (*Pacifastacus leniusculus*) (Fuertes et al., 2012), red claw (D'Agaro and Mastrocola, 2001, Garza de Yta et al., 2012), red swamp (Tan et al., 2018), yabby (*Cherax destructor*) (Jones et al., 1996) and marron (Saputra et al., 2019).

Alongside diets, management of water quality is another crucial factor for crayfish growth and development. Though some species can tolerate low dissolved oxygen (DO) level, however higher growth and immune performance can only be achieved with ideal rearing environment (Holdich, 2002). Among the crayfish cultured in Australia, marron required higher DO level (≥6 mg/L) compared to red claw and yabbies (4-5 mg/L) (Masser and Rouse, 1997, Morrissy et al., 1984). In addition, higher water temperature and pH are also limiting factors for marron growth, compared to yabbies and red-claw (Masser and Rouse, 1997, Morrissy et al., 1984). Recirculating aquaculture system (RAS), biological filters and various substrates have been

used frequently to treat organic wastes from various aquaculture practices. While no reports are currently available on crayfish, studies with other decapod crustacean including biological filters for shrimp (Tilley et al., 2002) and lobsters (Drengstig and Bergheim, 2013), and artificial substrate for prawns (Tidwell et al., 1998) have reported promising results. Considering the effects of water quality on health status of marron, addition of various substrates and biological filters could be a worthwhile approach to remediate the water quality. In recent times, development in sequencing technologies and computational biology has shown a strong connection between gut microbiota and biological processes including growth, digestion and immunity of decapod crustacean (Chen et al., 2020, Yang et al., 2019, Zhang et al., 2020). Compared to shrimp, prawn and lobster, only four published reports on crayfish gut microbial communities are currently available and have targeted only red swamp crawfish (Liu et al., 2020a, Shui et al., 2020, Zhang et al., 2020) and red claw (Liu et al., 2020b). These studies have found a significant links among gut microbial changes, dietary proximate composition, rearing environment and developmental stages. Based on high throughput sequencing (HTS) and comprehensive bioinformatics, authors have identified beneficial role of phyla Bacteroidetes, Firmicutes, and Lactic acid bacteria on the gut microbial communities, and subsequently augmenting these bacterial communities to have positive effects on digestion, stress response and immune performance of red swamp crawfish (Zhang et al., 2020) and red claw (Liu et al., 2020b). However, till now no information is available on the gut microbial structure of marron and their response to different dietary supplements including probiotics and protein sources, and various substrates that can act as biological filters.

Owing to the high market demand and low productivity, the use of advanced molecular technologies to analyse the impacts of diets and water quality on health and immunity are preferred ways to improve the production of marron. For instance, monitoring of gut microbiota can be a good indicator for crayfish health due to their role in digestion and immunity (Shui et

al., 2020). Using HTS and omics technologies, studies have identified some beneficial bacteria in the gut linked to the growth and development of red swamp crawfish (Liu et al., 2020a, Zhang et al., 2020). However, despite technological advancement, no information is yet available on the microbial communities in the gut of marron and their dynamics under different feeding regimes and culture conditions. On this backdrop, this study was aimed to identify and characterize the gut microbiota and their role in marron health under different feeding regimes and rearing conditions having different substrates.

1.2. Specific objectives

1. Identification of resident microbes in the gut of nutritionally deprived marron.

2. Investigating the response of gut microbiota and health status of marron fed different probiotics, and protein supplementations from plant, animal and insect sources.

3. Monitoring the quality and microbial changes in water with different substrates and subsequent effects on health and immune status of marron.

4. Evaluating the combinatory effects of potent probiotic and alternative proteins on gut microbiota and health status of marron.

5. Comparative analysis of gut microbiota in malnourished, probiotic and different protein sources fed marron, and marron with different substrates in the rearing environment to analyse the differences in microbial diversity and to identify the differentially abundant genera for the selective diet(s) and rearing condition(s) as well as the core bacteria.

6. Identification of microbes or microbial communities positively linked to improved health status of marron.

1.3. Significance of the study

- Present research will provide a baseline information about the gut microbiota and immunity of marron and its correlation(s) with water quality, growth and development.
- The study will identify beneficial gut bacteria for marron and their impact on health and immunity.
- The research results will enhance sustainable aquaculture practice for marron farming in Western Australia.
- The research outcomes will support farmers by lowering the feed dependency on fishmeal.

CHAPTER 2: Review of literature

2.1. Aquaculture industry and prospects of marron farming

Aquaculture plays a crucial role in feeding the proliferating world population and meeting the ever-rising protein demand. Aquaculture accounted for 46% of total production and 52% of fish for human consumption that valued of $250 billion where 20.5 million people directly involved in aquaculture for their primary sources of income (FAO, 2020). The fisheries and aquaculture value chain that includes culturing, harvesting, processing and marketing provides income and employment of about 250 million people globally (FAO, 2020). The total aquaculture production stranded at 82.1 million tonnes in 2018, and with 9.4 million tonnes of production, crustacean group comprised of 11.6% of the total production (Figure 2.1). Among the crustacean species cultured, crayfish market has expanded so fast globally due to high consumer demand and preferences. The taxonomy of crayfish are so diverse as 640 species have been reported so far (Crandall, 2016), and out of which only 10 species namely *Astacus astacus*, *Cherax cainii, C. destructor, C. quadricarinatus*, *Faxonius limosus, Pacifastacus leniusculu, Paranephrops planifrons, Procambarus clarkii*, *P. virginalis* and *P. zonangulus* are commercially cultured globally (Madzivanzira et al., 2020).

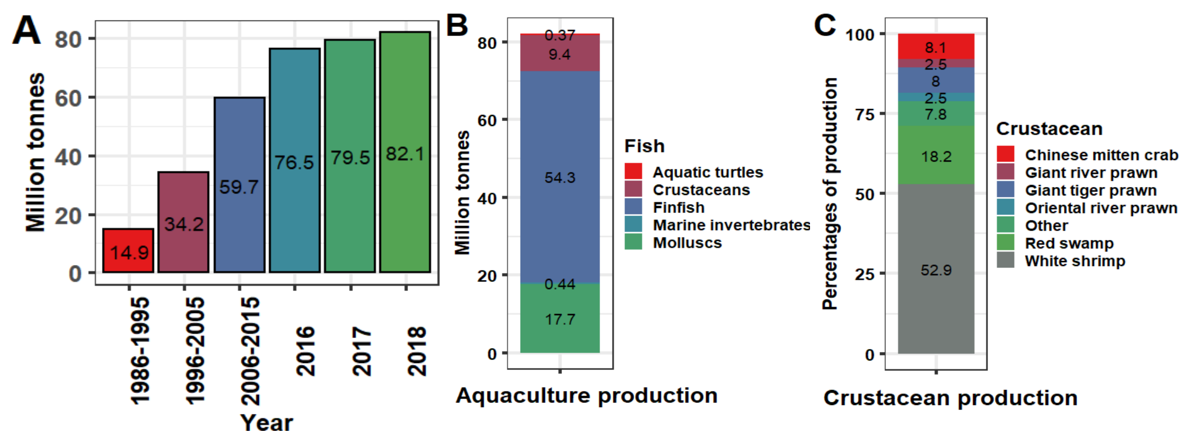

Figure 2. 1 Aquaculture production. (A) A summary of aquaculture production (in million tonnes) by year. (B) Aquaculture production (in million tonnes) by species in 2018. (C) Crustacean production (in percentage) in 2018. (Source: FAO 2020)

In Australia, the aquaculture production has doubled between 2000 and 2018 (ABARES, 2018), predicted to be doubled again by 2050 (Kearney et al., 2003). Alongside the production, the gross value of production has increased by 60% between 2003-04 and 2017-18 (Mobsby et al., 2020). However, the market value projected to fall by 12% due to COVID-19 outbreak and subsequent reduction of rock lobster export in China. In post-COVID era, the production value expected to reach rise by 21% between 2020-21 ($2.81 billion) and 2024-25 ($3.4 billion) (Mobsby et al., 2020). While aquaculture production accounted for 36% of the total volume in Australia, WA aquaculture contributes only 0.6% of the total volume (127 tonnes out of 22,846) (Mobsby et al., 2020). Marron aquaculture representing half of the total aquaculture production (66 tonnes out of 127) in WA (Cai et al., 2019). Considering the suitable land for ponds, market prices and sizes compared to yabbies (*C. destructor*), marron farming has immense opportunities in WA (DoF, 2010a, DoF, 2010b). Due to consumer preferences, high demand for commercial aquaculture, distinctive flavour and taste, marron has been translocated globally to China, Japan, USA, South Africa and Zimbabwe for aquaculture, beyond their natural habitat in WA (Morrissy et al., 1990, Rouse and Kartamulia, 1992, Sang and Fotedar, 2009). In WA, the number of active farms (170-180) and production of marron (60-70 tonnes) have not been increased as envisaged earlier (Tulsankar et al., 2020). Research groups from Curtin University lead by Professor Ravi Fotedar, Murdoch University lead by Dr Stephen Beatty and Associate Professor David Morgan, University of Western Australia lead by Dr Clodagh Guildea and Dr W. Jason Kennington, and Dr. Shannon Loughnan from Flinders University are working extensively on different and divergent areas of research including use of microbiological and biotechnological innovations to improve the growth and immunity of marron.

2.2. Marron: Taxonomy, biology, physiology and anatomy

Two species of marron are currently found in WA, smooth (*C. cainii*) and hairy (*C. tenuimanus*) marron (DoF, 2011). Smooth marron are widely distributed in rivers, dams and ponds of southwest of WA while hairy marron are categorized as critically endangered and their natural habitat is restricted to only Margaret river (Austin and Ryan, 2002, Bryant and Papas, 2007). Differences in allozyme electrophoretic mobilities differences differentiate smooth marron from hairy one (Austin and Ryan, 2002). The taxonomic classification of commercially farmed and widely distributed smooth marron as follows.

- Kingdom: Animalia
  - Phylum: Arthropoda
    - Sub-phylum: Crustacea
      - Class: Malacostraca
        - Order: Decapoda
          - Sub-order: Pleocyemata
            - Infra-order: Astacidea
              - Family: Parastacidea
                - Genus: *Cherax*
                  - Species: *Cherax Cainii* (Austin and Ryan, 2002)

In aquaculture, knowledge on biology of aquatic animals is crucial in providing the optimal growth conditions for the aquatic species. The survival and growth of marron depends largely on water quality and temperature as well as diets that directly affects physiology and metabolism (Ambas et al., 2017, Lawrence and Jones, 2002, Le Moullac and Haffner, 2000). The temperature ranges between 22-24 ºC and dissolved oxygen concentration of ≥6.0 mg/L are optimum for the marron growth (Bryant and Papas, 2007, Merrick and Lambert, 1991, Nugroho, 2014). Though marron are omnivorous in nature, scavenge and ingest everything

within their range, still composition of diets has significant impacts on growth parameters of marron (Fotedar, 1998, Jussila and Evans, 2002). Under intensive and semi-intensive culture systems, marron are subjected to a variety of stresses resulting into adverse environmental and health condition including susceptibility to various pathogens (Jussila, 1997). Hence, the change in immunity of aquacultured species is another health indicator. Unlike vertebrate, crustacean species like marron depends entirely on innate immune response elicited from cell surface or haemolymph proteins, antimicrobial peptides in response to the presence of pathogens (Vazquez et al., 2019, Wu, 2011). Though, currently marron is considered as disease-free species in Western Australia, but expansion of aquaculture, environmental pollution and outbreaks with *Aeromonas* and *Vibrio* in other *Cherax* species can pose threat to marron aquaculture (Eaves and Ketterer, 1994, Edgerton et al., 2002, Jiravanichpaisal et al., 2009, Raissy et al., 2014) in future. Adequate and balanced nutrition therefore are essential to achieve better performance of any aquatic species.

2.3. Aquaculture nutrition

In aquaculture, improved growth, stress and disease resistance are the most desirable outcomes for all cultured aquatic species (De Silva, 2000, Oliva-Teles, 2012). The ultimate goal of aquaculture nutrition is to provide balanced diets with ideal protein, fat, and micronutrients (amino acid, fatty acid, vitamins and minerals) ratios that could enhance the digestibility and growth of animals without affecting the surrounding environments (Craig et al., 2017, Hasan, 2001). In intensive and semi-intensive culture system, aquatic species are commonly cultured with high stocking densities that may reduce growth and increases physiological stresses leading to disease susceptibility, and cause major economic losses (Huang and Chiu, 1997). As aquaculture expanding globally, environmental sustainability is becoming another key challenges. The ultimate goal of sustainable aquaculture is to utilize our limited land and water resources effectively, enhance technology based and eco-friendly farming practices, improve

efficient feed management practices though intensification of current knowledge on aquaculture nutrition, specifically formulating effective feed ingredients and additives (Boyd et al., 2020). To enrich the knowledge of aquaculture nutrition taking sustainability, dietary supplementation of probiotics as a growth promoter and immune enhancer (Dawood et al., 2019), replacement of fishmeal from the aqua-diets (Ayadi et al., 2012), and biological filter based treatment of organic waste (Troell et al., 2005) are some of the potential research areas where enrichment of current knowledge on aquaculture nutrition will help improve the growth performance and disease resistance of aquatic animals as well as hasten sustainable aquaculture practice globally.

2.3.1. Probiotics

During 1980s, antibiotics were mostly used for disease control in aquaculture that led to antibiotic resistant bacterial isolates and transfer of antibiotic resistant genes to other bacteria (Smith, 2008). Subsequently European Union banned antibiotic uses in aquaculture industry in early twentieth century (2003) (Casewell et al., 2003). Since from then, probiotics has been used as a promising alternative to antibiotics to promote growth, immune performance and disease resistance of aquatic animals (Chauhan and Singh, 2019, Hoseinifar et al., 2018, Zorriehzahra et al., 2016). Probiotics are live microorganisms administered orally as a supplements that confer numerous health benefits to host. In shellfish aquaculture, most of probiotic research have been performed on white shrimp (*Litopenaeus vannamei*) (Chomwong et al., 2018, Karthik et al., 2015, Roomiani et al., 2018, Sha et al., 2016b, Sha et al., 2016c, Vieira et al., 2007, Zheng et al., 2017a, Zheng et al., 2018) and freshwater prawn (*Macrobrachium rosenbergii*) (Dash et al., 2014, Dash et al., 2016, Karthik and Bhavan, 2018, Khan and Mahmud, 2020, Mujeeb Rahiman et al., 2010, Seenivasan et al., 2014), while in crayfish only few studies are currently available (Ambas et al., 2015, Amrullah and Wahidah, 2019, Didinen et al., 2016, Valipour et al., 2019). In the studies with crayfish, *Micrococcus*

spp. with 200 and 300 g/L alginate supplementation showed increased growth and immune performance of red claw (Amrullah and Wahidah, 2019). Study on narrow clawed crayfish reported no positive influence of *Hafnia alvei* on growth and survival, however displayed potential inhibitory activity against *Aeromonas hydrophilla* (Didinen et al., 2016). A different study on same species showed significant influence of *Lactobacillus plantarum* at 10^{-8} CFU/g diet on hemolymph parameters, digestive enzyme activity and autochthonous lactic acid bacteria (Valipour et al., 2019). In marron, *Bacillus mycoides* reported to inhibit pathogenic *Vibrio mimicus* through production of protease enzyme (Ambas et al., 2015).

2.3.2. Alternative protein sources

In aquaculture, price and availability of raw ingredients are the two most important considerations for preparing aqua-diets (Rana et al., 2009). Both fin and shellfish aquaculture are highly depend on capture and supply of wild marine fish for fishmeal (FM), the most important and irreplaceable protein source for aqua-diets (Bandara, 2018). Fishmeal, as a primary protein sources of aqua-diets possesses a constant threat to wild fish stock and challenges the availability of low cost diets for aquatic animals (Allsopp et al., 2008, Olsen and Hasan, 2012). Expansion of aquaculture and high inclusion level in aqua-diets taking the price of FM beyond the affordable level for most of the farmers (Olsen and Hasan, 2012). To meet the burgeoning demand of global aqua-diets, researchers are trying to find cheap, readily available alternative proteins with balanced nutrients values from plant, animal and insect sources. In addition, environmental and ecological aspects of mass wild marine capture threaten the sustainability of global aquaculture (Allsopp et al., 2008). To reduce dependency on FM, several promising alternative proteins from plant, animal and insect sources are currently being investigated for shellfish aquaculture (Hua et al., 2019). Poultry-by-product meal (PBM) is the most promising alternative of FM due to its availability, low price and excellent nutritional profile (high protein and balanced amino acid profile) suitable for

crustacean aquaculture (Galkanda-Arachchige et al., 2020, Saputra et al., 2019). In decapod crustacean aquaculture, the effects of PBM supplementation has been tested on white shrimp (Cruz-Suárez et al., 2007, Markey et al., 2010), freshwater prawn (Nik Sin et al., 2020), black tiger shrimp (*Penaeus monodon*) (Phuong and Yu, 2003) and river prawn (*Macrobrachium nipponense*) (Yang et al., 2004). In crayfish, PBM effects on growth and immunity has been evaluated for red claw (Saoud et al., 2008) and marron (Saputra et al., 2019). Among these studies, 75% replacement of FM by PBM for freshwater prawn showed higher growth and survival (Nik Sin et al., 2020).

The insect black soldier fly, BSF (*Hermatics illucens*) is increasingly gaining attention among the researchers as a potential, promising and environmentally sustainable alternative to FM due to high protein (42-63%), lipid (35%), essential amino acids, vitamins, minerals and trace elements, and the ability of BSF larvae to utilize bio-waste effectively (Bondari and Sheppard, 1981, Müller et al., 2017, Wang and Shelomi, 2017). In addition, the gut of BSF larvae is rich in lactic acid bacteria (LAB) (Bruno et al., 2019, Klammsteiner et al., 2020), specifically *Lactobacillus* species that have positive role in health and immunity of aquatic animals. In fish, total replacement of FM with BSF have been reported without compromising gut health and fish fillet quality of Atlantic salmon (*Salmo salar*) (Bruni et al., 2020, Li et al., 2020a), however in white shrimp faster growth rate and feed conversion ratio (FCR) have been reported with ≤25% replacement of FM by BSF meal (Cummins Jr et al., 2017). In crayfish, no published data yet available on substitution of FM with BSF.

Among the plant protein, soybean (SOY) and lupin (LPN) meal are most widely used in aquaculture due to their availability, nutritional consistency and stability, and relatively low market price (Robaina et al., 1995, Yang et al., 2015). Compared to PBM and BSF, a substantial numbers of studies have been conducted on decapod crustacean with SOY and LPN meal. For LPN meal, while one study reported impaired growth and phenoloxidase activity with >10%

LPN supplements (Weiss et al., 2020) other described up to 50% FM replacement without compromising growth in white shrimp (Molina-Poveda et al., 2013). Longer duration of trial (57 days), and alkaloid and fat free lupin used in Molina-Poveda et al. (2013) trial, compared to 42 days trail in recirculating aquaculture system (RAS) could be associated with the differences in growth performances depicted in Weiss et al. (2020) study.

In black tiger shrimp two studies stated up to 40% (Smith et al., 2007) and 75% (Sudaryono et al., 1999) replacement of FM by LPN without affecting growth and tail muscle biochemistry. For SOY meal, 0% and 33% (Yun et al., 2017) replacement of FM and 50% (Sookying and Davis, 2011) replacement of FM, PBM and pea meals had similar effects on growth performance and body biochemical composition of white shrimp. Two further studies on SOY meal reported up to 25% replacement of FM with significant differences in growth parameters for kuruma shrimp (*Marsupenaeus japonica*) (Bulbul et al., 2015, Bulbul et al., 2013). However, lower essential amino (AA) and fatty acid (FA) profile in SOY and LPN required inclusion AA and FA from exogenous sources, and hence negative attributes on health and immunity at higher inclusion level or as sole protein sources has been reported for the plant proteins in decapod crustacean's aquaculture (Fox et al., 2004, Gatlin III et al., 2007, Lim and Dominy, 1990, Weiss et al., 2020).

In red claw, a study with seven different alternative protein sources (30% inclusion) namely FM, PBM, meat and bone meal (MBM), SOY, canola meal (CM) and brewer's yeast (BY) from plant and animal sources showed significant higher digestibility and tail muscle biochemical compositions with SM, compared to other diets while MBM exhibited lowest values for all parameters (Pavasovic et al., 2007). Another study on red claw showed no differences in survival, weight gain (WG) and feed conversion ratio (FCR) among four different dietary groups where SOY meal mixed with FM, PBM, ground pea meal (GPM) and distillers dried grains with solubles (DDGS) (Garza de Yta et al., 2012). In marron, on the other

hand, animal protein PBM showed positive influence on gut microvilli and survivability in a study fed five different proteins namely FM (30%), PBM (30%), feather meal (FEM) (22%), LPN (36%) and MBM (24%) from plant and animal sources (Saputra et al., 2019). A separate study with yabby, feeding with different percentages of SOY meal revealed that 40%-60% FM replacement had significant negative effects on lipid and protein of carcass whereas maximum weight gain achieved with 20% replacement (Jones et al., 1996).

2.3.3. Substrates and biological filters in aquaculture

Development of environmental friendlier technologies enhanced sustainable and profitable aquaculture practice by recirculating organic wastes (mainly nitrogen and ammonia) and reducing nutrient pollution discharge. To cope with the limited water sources and supplies, suitable low cost land for farming, water discharges facilities and environmental impacts, use of substrates or bio-filters are some common practices in nutrient and water recycling in past few years (Gutierrez-Wing and Malone, 2006). Biological filters in recirculating aquaculture system (RAS) have been used to treat dissolved organic waste and ammonia by favouring growth of nitrifying bacteria, and reducing water use in commercial aquaculture (Bartelme et al., 2017). Diverse microbial communities associated with decomposition of chemical pollutants by utilizing organic and inorganic compounds as a primary electron source for the reduction of nitrate and oxidation of ammonia (Kamira et al., 2018, Lu et al., 2014). The water remediation approaches in aquaculture include use of bio-filters (Guerdat et al., 2010), bio-filters with microbial enrichment (Kamira et al., 2018), artificial substrates with microbial succession (Li et al., 2017b), novel field-scale aquaculture waste water treatment system (FAWTS) (Li et al., 2020b), rice husk biochar (Mopoung et al., 2020) and peat based media (Kamauddin et al., 2019).

2.4. Gut microbiota of decapod crustacean and its link to health and immunity

Microbiome refers to the set of genome from various microorganisms present in a specific environment like gut/intestine, gill, skin and hemolymph. Gut microbiota of animal plays a key role in various biological functions including digestion and immunity. Gut microbial communities of aquatic animals are dense and sensitive to various factors including developmental stages, rearing environment, various stresses and diets (Banerjee and Ray, 2017). The gut microbial communities largely depend on feeding nature of aquatic animals; protease-producers are dominant in carnivores that break down complex protein into simple amino acids while herbivores have higher abundance for amalyse and cellulase producing bacteria (Ray et al., 2012). Omnivores eat a variety of food, both from plant and animal origin. Due to omnivorous nature of decapod crustacean, their gut microbiota are more diverse than carnivores and herbivores. Therefore, higher microbial diversity and up-regulated metabolic functions are key requirements to digest lipids and proteins from different sources, and to exhibit tolerance against physical, physiological and environmental stressors (Senghor et al., 2018). In addition to that, rearing environment, including habitat, types of water (freshwater and saltwater) and water quality parameters (temperature, pH, dissolved oxygen, nitrogenous compound) were reported to modulate gut microbiota of decapod crustacean (Chen et al., 2017, Giatsis et al., 2015, Holt et al., 2020b, Tzeng et al., 2015). Compared to fish gut which is dominated by Proteobacteria, Bacteroidetes and Firmicutes (Ghanbari et al., 2015), decapod crustacean's gut microbial communities have higher abundance for Tenericutes, Firmicutes and Proteobacteria (Cheng et al., 2019b, Dong et al., 2018). Proteobacteria reported as the most dominant bacteria in shrimp, prawn and lobster (Fan et al., 2019, Holt et al., 2020b, Tzeng et al., 2015). The phylum a physiologically, morphologically and genetically diverse group consisting of three major sub-types– Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria. The largest sub-type Gammaproteobacteria is the most common

bacteria in shrimp gut comprised of *Vibrio*, *Photobacterium* and *Aeromonas* that accounted for 70% of the total bacteria (Rungrassamee et al., 2014, Rungrassamee et al., 2016, Zheng et al., 2017b). The dominancy of *Vibrio* species can be correlated with the ability to utilize higher salt in marine water (Munro et al., 1994), and to produce chitinolytic enzymes in chitin-rich environment of the crustaceans' gut that act as a substrate for their growth in host tissue (Sugita and Ito, 2006). The enzymatic potentials of *Vibrio* species including *V. harveyi*, *V. parahaemolyticus, V. alginolyticus, V. anguillarum*, *V. vulnificus*, and *V. splendidus* bring negative outcomes for host carapace and linked to several diseases of decapod crustacean's including vibriosis, red diseases, tail necrosis and loose shell syndrome (Jayasree et al., 2006). However, chitinolytic enzyme producer *V. scophthalmi* and *V. ichthyoenteri* are the native harmless species in the gut, and live harmoniously with other bacteria in the gut of decapod crustacean (Holt et al., 2020a, Sugita and Ito, 2006).

In freshwater crayfish, the gut microbiota are only investigated for red-claw and red-swamp under different dietary conditions, developmental stages, cultural environment seasons and salinities regimes (Liu et al., 2020a, Liu et al., 2020b, Shui et al., 2020, Zhang et al., 2020). Unlike in shrimp, prawn and lobster, crayfish gut microbiota are dominated by Tenericutes and *Candidatus* lineages at phylum and genus level that are very similar to Chinese mitten crab (*Eriocheir sinensis*) and mud crab (*Scylla paramamosain*) (Dong et al., 2018, Su et al., 2020, Tang et al., 2020, Wei et al., 2019). Beside phyla Tenericutes and *Candidatus* lineages, Gammaproteobacteria, specifically *Aeromonas*, *Citrobacter*, *Hafnia*, *Shewanella* and *Vibrio*, are most abundant bacteria in crayfish gut (Liu et al., 2020b, Shui et al., 2020, Zhang et al., 2020), similar to shrimp, prawn and lobster (Table 2.1). Prior to this research, no gut microbiome information is available for marron and hence no past literature has compared it with the gut microbial communities of other decapods.

Table 2. 1 Diversity of gut microbiota in crayfish under different culture system and diets

Species	Study design	Dominant phyla	Dominant species	Key findings	References
Red swamp	Effects of developmental stages, geographical location and fermented diets on gut microbiota.	Proteobacteria Bacteroidets Firmicutes Teniricutes RsaHF231	*Candidatus* Bacilloplasma *Tyzzerella* *Citrobacter* *Bacteroides*	Development stage and diets are two key factors in driving gut microbial shift in red swamp crayfish. Diversity reduced significantly with the ages of crayfish. *Hydrogenophaga* was only detected in early stages whereas *Candidatus Bacilloplasma* and *Tyzzerella* in developmental stages. Fermented feed increase the diversity of Bacteroidetes and polysaccharide degradation function.	Zhang et al. (2020)
Red swamp	Gut microbiota was investigated under crayfish-rice cultivation (CR) model.	Actinobacteria, Proteobacteria, Tenericutes, Firmicutes Bacteroidetes	*C.* Bacilloplasma *Vibrio* *Tyzzerella* *Shewanella* *Citrobacter*	Growth conditions have significant impact on gut microbiota. *C. Bacilloplasma* and *Ornithinibacter* was predominant in all samples under CR model with major gene functions for amino acid and carbohydrate metabolism and membrane transport.	Shui et al. (2020)
Red swamp	Intestinal microbiota of crayfish in rice field and pond cultivation system were compared in summer and autumn.	Bacteroidetes, Firmicutes, Proteobacteria, Tenericutes	*Clostridium* *Citrobacter* *Shewanella* *RsaHF231*	In summer, microbial abundance was higher in pond than rice field, opposite to autumn. Higher species diversity in the pond model than the rice model in both seasons. Microbial changes correlated to season not to cultivation model.	Liu et al. (2020a)
Red claws	Changes in gut microbiota and immune response of crayfish under different salinities (0, 5, 10 and 15 psu).	Tenericutes Proteobacteria Fusobacteria	*C.* Bacilloplasma *Aeromonas* *Hypnocyclicus* *Citrobacter* *Vibrio*	Gut microbiota modulated significantly with salinities. Higher salinities decreased alpha diversity (15 psu), increased diversity for Tenericutes and *C. Bacilloplasma* while decreased Firmicutes and *Aeromonas*.	Liu et al. (2020b)

A systemic meta-analysis was performed using decapod crustacean's gut microbiome data from 27 different studies (Table 2.2) in order to understand the nature and composition of gut microbiota, and to find out the core bacteria in different species. The relative abundance (rarefied) at genus level in different decapod crustacean species is shown in Figure 2.2. *Candidatus* lineages that consist of *C.* Bacilloplasma and *C.* Hepatoplasma is the main core bacteria of 8 out of 10 decapod crustacean species, Chinese mitten crab, mud crab, red swamp, red claw, white shrimp, freshwater prawn, European lobster and spiny lobster, except tiger shrimp and river prawn that have *Vibrio* and *Cupriavidus*, as their main core.

Table 2. 2 Studies and databases reporting gut microbiota in decapod crustaceans using HTS

DC	Condition	Year	Accession	Reference
Tiger shrimp	Health	2016	SRP059721	Oetama et al. (2016)
Tiger shrimp	Development	2020	PRJNA540737	Angthong et al. (2020)
Tiger shrimp	Growth	2020	PRJNA553862	Uengwetwanit et al. (2020)
FW prawn	Development	2016	SRR1502207	Mente et al. (2016)
River prawn	Environment	2015	PRJNA280489	Tzeng et al. (2015)
River prawn	Environment	2017	PRJNA354668	Chen et al. (2017)
River prawn	Environment	2018	PRJNA381860	Zhao et al. (2018)
White shrimp	Diet	2020	Provided	González et al. (2020)
White shrimp	Diet	2020	PRJNA600113	Cao et al. (2020)
White shrimp	Environment	2018	PRJNA422950	Zoqratt et al. (2018)
White shrimp	Diet	2019	SRP128484	Shao et al. (2019)
White shrimp	Development	2019	SRP136220	Fan et al. (2019)
White shrimp	Growth/Health	2020	PRJNA578594	Yu et al. (2020)
White shrimp	Environment	2019	PRJNA505962	Deng et al. (2019)
White shrimp	Development	2017	SRX2946975	Zeng et al. (2017)
White shrimp	Environment	2019	PRJNA522274	Landsman et al. (2019)
White shrimp	Environment	2018	PRJNA352369	Gainza et al. (2018)
EU lobster	Environment	2019	PRJNA577421	Holt et al. (2020b)
CMC	Health	2018	SRP110849	Dong et al. (2018)
CMC	Environment	2020	PRJNA646327	Su et al. (2020)
CMC	Environment	2020	PRJNA530094	Sun et al. (2020)
Mud crab	Environment	2020	SRP215842	Tang et al. (2020)
Red-Swamp	Development	2020	PRJNA609648	Zhang et al. (2020)
Red-Swamp	Environment	2020	PRJNA557576	Shui et al. (2020)
Red-Claws	Environment	2019	PRJNA573062	Unpublished
Red-Claws	Environment	2020	PRJNA634222	Liu et al. (2020b)
Spiny lobster	Development	2017	PRJNA396648	Ooi et al. (2017)

Abbreviation: DC, Decapod crustacean; CMC, Chinese mitten crab; FW, Freshwater; EU, European.

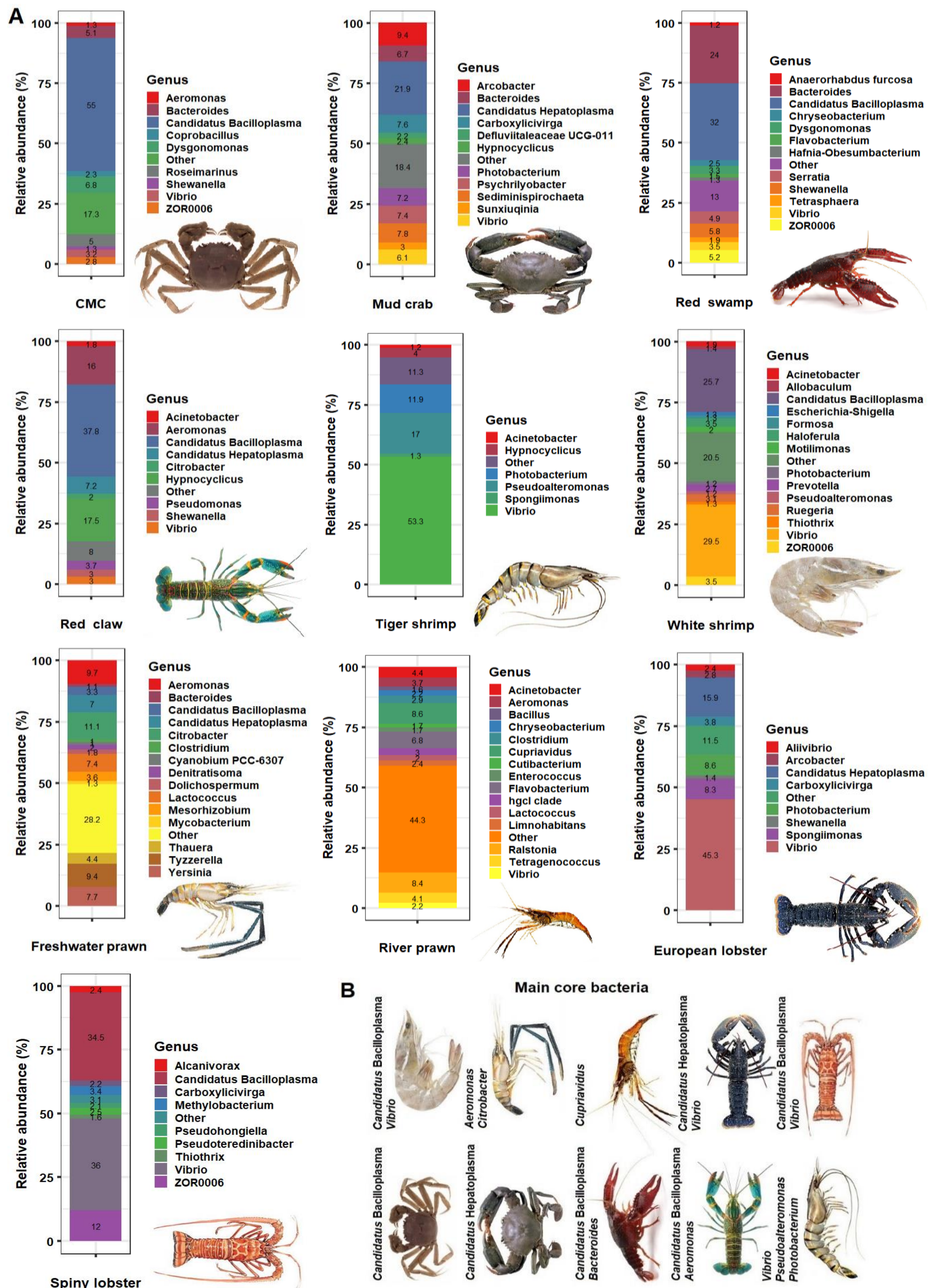

Figure 2. 2 A systemic meta-analysis of major decapod crustacean's gut microbiota using HTS data of 26 published articles. (A) Rarefied relative abundance of genera. (B) Core bacteria in

the gut of decapod crustacean identified in all samples, regardless of diets, growth, health, developmental stages and environmental conditions. Abbreviation: CMC, Chinese mitten crab.

Shift or modulation of gut microbiota resulting from physical and environmental stresses, pathogen invasion, stages of life cycle, changes in diets, differences in rearing conditions and habitats, can be either beneficial or harmful for the aquatic animals. Most of the bacteria, especially symbionts exert beneficial role to host for better physiological and metabolic functions and thereby influencing immunity (Banerjee and Ray, 2017). In decapod crustacean, Bacteroidetes, Firmicutes and Tenericutes are reported to play a positive role in digestion and immunity (Chen et al., 2015). The bacteria belong to Tenericutes are less likely to grow in the laboratory using traditional microbiological techniques, and therefore, their biological function mostly remained unknown. Most of the probiotic bacteria including *Bacillus*, *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Bifidobacterium* and *Clostridium* belong to Firimicutes, and therefore any shift of gut microbiota that increases relative abundance of Firmicutes is considered as beneficial for all aquatic species including decapod crustacean. These bacteria associated with the production of wide range of enzymes including protease, amylase, cellulose, lipase, phytases for the degradation of lipid, protein, chitin, cellulose and phytate, and play role in digestion and nutrition of animals (Banerjee and Ray, 2017). Some reports are also available on transmission of bacteria from rearing environment including water and sediment into aquatic animal resulting in positive modulation of gut microbial communities (Giatsis et al., 2015, Zoqratt et al., 2018). Compared to higher vertebrates like human and fish, a very few reports are currently available on HTS-based analysis of decapod crustacean's gut microbiota modulation through dietary incorporation or changing rearing conditions, and not surprisingly, due to widely cultivated species, most of the studies have been conducted on white shrimp (Table 2.3).

Table 2. 3 Modulation of decapod crustacean's gut microbiota in response to diet and environmental conditions

Inclusion	Modulation	References
Lactobacillus sp. in feed *Nitrosomonas* and *Nitrobacter* in water	Dietary supplementation of *Lactobacillus* sp., increased abundance for heterotrophic bacteria and lactic acid bacteria (LAB) while reduced *E. coli*, *Salmonella* and *Shigella* in the gut of white shrimp. In addition to *Lactobacillus* in the feed, inclusion of *Nitrosomonas* and *Nitrobacter* in water after 30 days significantly improved growth performance and reduced organic waste in the tank.	Karthik et al. (2015)
Bacillus coagulans	Dietary supplementation of *B. coagulans* reduced the abundance of opportunistic pathogen *Vibrio*, *Photobacterium* and *Tenacibaculum*, in white shrimp. An increased abundance of *Pseudoalteromonas* found linked to higher growth, immunity and disease resistance against *V. parahaemolyticus*	Amoah et al. (2019)
Bacilli and Gamma-Proteobacteria	Probiotic mixture increased abundance for Proteobacteria and Firmicutes. Eight bacterial species found novel in probiotic mix diet that transmitted to white shrimp gut where *B. subtilis* was the only bacteria known as probiotic in shrimp aquaculture.	Vargas-Albores et al. (2017)
Lactobacillus pentosus Enterococcus faecium	White shrimp gut was investigated after feeding (i) basal + strain of *L. pentosus* (H), (ii) basal + strain of *E. faecium* (N), (iii) basal diet + bacteria free supernant of *L. pentosus* (S), and (iv) control (C) diets. Diet H increased diversity with 202 unshared genera and increased abundance for *Paracoccus*, *Polymorphum gilvum*, *Rhodobacter* and *Methylobacterium*, compared to others (H>N>C>S).	Sha et al. (2016a)
Bacillus subtilis	White shrimp larvae after saline treatment were subjected to addition of *Bdellovibrio* and like organisms (BALOs) (BD) and *B. subtilis* (SD) in the rearing water, and gut microbiota was investigated compared to control (CD). In day 1, Gammaproteobacteria was found dominant in all three groups, BD, SD and CD with 99.4%, 85.6% and 83.3%, respectively. In day 7, abundance for Gammaproteobacteria found consistent for SD and CD, while dropped to 8.4% in BD. The alpha diversity increased significantly for BD (0.93 to 7.4) while dropped sharply for control (2.94 to 0.94).	Cao et al. (2020)
Bacillus sp., *Lactobacillus graminis*, *Streptomyces* sp.,	Gut microbiota of white shrimp was investigated after feeding *Streptomyces* sp., (RL8), a mixture of *Streptomyces* sp., and *Lactobacillus graminis* (Lac-Strep), and a mixture of *Bacillus* sp., and *Streptomyces* sp., (Bac-Strep). After challenge with *V. parahaemolyticus*, RL8 and Bac-Strep produced highest bacterial diversity with significant augmentation of *Bacteriovorax* that linked to antimicrobial peptides (AMPs) in the gut.	Mazón-Suástegui et al. (2020)

2.5. Microbiome in the rearing environment and their role in health of decapod crustacean

In aquaculture, the quality of water is important and interacts largely by the water microbial communities. Several factors, including culture conditions and production system types, water quality and physico-chemical properties, water exchange, time or duration of culture and geography, play a role in changing water microbial communities and subsequently re-shaping the gut microbiota of aquatic animals including decapod crustaceans (Dodd et al., 2020, Giatsis et al., 2015, Heyse et al., 2020). In tilapia culture, the water microbial communities were found sensitive to culture systems (recirculating aquaculture and activated sludge) and physico-chemical properties, such as pH, dissolved oxygen, nitrate, nitrite, phosphate of water, and observed changes were reflected by the duration of culture (Giatsis et al., 2015). During larval development, the shift of gut microbial communities were found significantly correlated to differences in water microbial communities between two systems whereby the bacterial correlation between feed and gut was found to be minor compared to water and gut.

In decapod crustacean, a most recent study on white shrimp reported that 37% of the bacterial communities in the rearing tank water were introduced through feed or water exchange (Heyse et al., 2020). Live feed with algal supplementation produced highest diversity of microbiome in water, followed by live feed supplemented with *Artemia*, water exchange and dry feed, respectively, revealed the positive influence of algal population on water bacteria. Another recent study reported microbial communities of white shrimp intestine, rearing water and sediment under "Aquamimicry" culture system, an environmental friendly method for better shrimp production (Zeng et al., 2020). Results revealed that gut microbial communities of white shrimp are distinctly different from rearing environment (water and sediment) and the changes are subjected to various culture stages, from stage 1 (day15) to stage 5 (day 75). A few of the OTUs were found shared between shrimp gut and rearing environment from various

culture stages (stage 1 - stage 5) suggesting that microbial shift in the gut was not consistent as water and sediment, and independent of microbiome in the rearing environment. The findings suggested that dissimilar gut and surrounding microbiome could be a potential indicator for better health status and improved production of shrimp under "Aquamimicry" culture system. Another study on white shrimp revealed that microbial diversity in ponds were significantly higher than gut while fewer ubiquitous genera, mostly *Vibrio* and *Photobacterium*, dominating the intestinal microbial communities (Zoqratt et al., 2018). The study (Zoqratt et al., 2018) also found no differences of microbial communities in the rearing water between Malaysia and Vietnam, and geographical differences also did not have any impact on gut microbiota of adult shrimp, which were much similar and dominated by *Vibrio*.

CHAPTER 3: General methodology

3.1. Marron collection and animal husbandry

All marron were procured from Blue Ridge Marron Farm, Manjimup, Western Australia (34.2019 S, 116.0170 E). Marron were transported in live condition to Curtin Aquatic Research Laboratories (CARL), Turner Avenue, Bentley (32.0010 S, 115.9240 E). Marron were nurtured in 200 L capacity tanks filled with 150 L underground freshwater. Fixed temperature (20 ± 2.0 ºC) and constant aeration were maintained with submersible thermostat (Aqua One, Perth, Australia) and air stones (Aqua One, Perth, Australia). Marron were reared in specially prepared plastic mesh cage (0.8-8.0 mm thickness) to avoid cannibalism. Twelve hours of dark and light photoperiod was maintained. The temperature and pH of water were checked using portable digital C/mV/pH meter (CyberScan pH 300; Eutech Instruments, Singapore), and dissolved oxygen (DO) was monitored with digital DO meter (YSI55; Perth Scientific, Australia). Nitrate (NO_3^-) and nitrite (NO_2^-) concentration in water were monitored using Hach DR/890 Colorimeter (Hach, Loveland, CO, USA) following cadmium reduction and diazotization method, respectively as described previously (Hoang et al., 2016). Phosphate (PO_4^-) concentration in water was tested by ascorbic acid standard 4500-PE method, as described previously (Mai et al., 2010). The concentration of ammonia (NH_3^+) was measured using ammonia test kit (Hach, Loveland, CO, USA).

3.2. Analysis of haemolymph parameters

The haemolymph osmolality of marron was measured at the end of the experiment according to method described earlier (Sang and Fotedar, 2004). With 0.5 mL syringe, 0.05 mL of haemolymph was extracted carefully from the pericardial cavity of each marron in 0.1 mL of precooled anticoagulant (0.1% glutaraldehyde in 0.2M sodium cacodylate, pH 7.0±0.2). The osmolality of the mixed solution was measured by Cryoscopic Osmometer-Osmomet 030 (Gonotec, Berlin, Germany). The lysozyme activity of marron haemolymph from each tank

was calculated using turbidimetric assay, as described previously (Mai and Fotedar, 2018). Fifty microliters of anticoagulant added hemolymph sample was pipetted in 96-well micro-plate (Iwaki, Tokyo, Japan). After 15 min incubation, 50 µL of PBS (0.25 mg/mL) suspended *Micrococcus lysodeiktikus* (Sigma-Aldrich, St. Louis, MO, USA) solution was added to individual well. The absorbance of the solution for each well in the plate was then monitored at 450 nm wavelength for each 2 minutes intervals for 20 minutes in a MS212 reader (Titertek Plus, Tecan, Grodig, Austria). For total haemocyte counts (THC), 0.2 mL of haemolymph was suspended in 0.2 mL of anticoagulants. THC was calculated for each of the marron under a haemocytometer (Nauabuer, Germany) with 100X magnification (Ambas et al., 2017).

3.3. Analysis of tail muscle biochemistry

The amount of protein, energy and fat in the tail muscle of marron was calculated using methods described by the Association of Official Analytical Chemists, AOAC international (AOAC, 2006). The protein concentration in tail muscle was measured using sulfuric acid (H_2SO_4) and copper catalyst tablets in Kjeltec Auto 1030 analyzer (Foss Tecator, Höganäs, Sweden) following the Kjeldahl method (N× 6.25). The percentage of crude fat was calculated using using Soxtec System HT6 (Tecator, Höganäs, Sweden). The tail muscle gross energy was determined by using bomb calorimeter (Heitersheim, Germany).

3.4. DNA extraction, PCR amplification and 16S rRNA sequencing

At the end of the experiment, marron gut samples were collected inside class-II biological safety cabinet. Gut content including mucosa were homogenized using tissue lyser (Qiagen, Hilden Germany). DNA extraction from homogenized samples was performed using Qiagen Blood and Tissue Kit (Qiagen, Hilden, Germany) following manufacturer’s instructions. DNA quality including concentration and 260/280 ratio were assessed in NanoDrop Spectrophotometer 2000c (Thermo Fisher Scientific, USA). Subsequent dilution was performed to make 50 ng/µl final concentration. The V3-V4 (5′F-

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; 5′R-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) regions of the bacterial hypervariable regions was amplified according to Illumina 16S metagenomic sequencing protocol (Part # 15044223 Rev. B). PCR amplification was performed in a total of 50 µl master mix volume containing 25 µl Hot Start 2X Master Mix (New England BioLab Inc., USA), 2 µl of template DNA, 1 µl of each V3 (forward) and V4 (reverse) primers and 21 µl of DEPC treated water (Thermo Fisher Scientific, USA). Amplification reactions was executed in a S1000 thermal cycler (Bio-Rad Laboratories, Inc., USA). Before clean-up, all PCR products were visualized in 1% agarose gel. The amplicon libraries were generated using a secondary PCR with indexing primers. AMPure XP beads was used to clean-up primary PCR products and amplicon libraries. DNA concentration of final pool was measured using Qubit Fluorometer (Life Technologies Inc., USA). Samples were pooled to equimolar concentrations and sequenced on an Illumina MiSeq platforms (Illumina Inc., San Diego, California, USA) with 2 × 300–base pair paired-end reads (v3 chemistry).

3.5. Bioinformatics

The initial quality of extracted 16S rRNA sequences was checked in fastQC pipelines (Andrews, 2010). The sickle windowed adaptive trimming tool was used for quality trimming of reads and subsequent trimming, reads of less than 200 bp were discarded (Joshi and Fass, 2011). The overlapping pair-end reads were then merged using Merging and Filtering Tool (MeFiT) with default parameters (Parikh et al., 2016). Filtering of chimeric sequences, *de novo* greedy clustering of 16S rRNA sequences into Operational Taxonomic Units (OTUs) at 97% similarity threshold and removal of singleton OTUs were performed using micca otu (version 1.7.0) (Albanese et al., 2015). Taxonomic assignment of the representative OTUs was performed using MICCA classify against SILVA 1.32 database collected at 97% identity

(Quast et al., 2013). PASTA algorithm was used for multiple sequence alignment of the representative OTUs (Mirarab et al., 2015).

3.6. Gene expression analysis

Genes associated with the immune response of crayfish (Appendix 4) were selected for gene expression analysis (Dai et al., 2017, Liu et al., 2020b, Liu et al., 2013). For RNA extraction from tissue samples, whole intestine samples were stored in RNA *Later* solution (Sigma-Aldrich, Germany) immediately after collection following manufacturer's instructions until further use. Then samples were homogenized into fine powder using tissue lyser (Qiagen, Hilden, Germany). For prophenoloxidase (proPO) and cytosolic manganese superoxide dismutase (cytMnSOD), haemocyte pellet from centrifuged haemolymph samples (in pre-cooled anticoagulant) was used for RNA extraction (Liu et al., 2013). Approximately, 5 mg of tissue and pellet samples were used for RNA extraction using RNeasy Mini Kit (Qiagen, Hilden, Germany). Extracted RNA was then treated with RNase free DNase-I (Qiagen, Hilden, Germany) for eliminating DNA associated impurities. The quality and quantity of the extracted RNA was checked in gel electrophoresis using 1% agarose in TAE buffer and NanoDrop spectrophotometer 2000c (Thermo Fisher Scientific, USA), respectively. The cDNA library was prepared from 1 µg of RNA using Omnicript RT kit (Qiagen, Hilden, Germany). Real-time PCR amplification was performed using PowerUp™ Cyber Green Master Mix (Thermo Scientific, USA) with 7500 Real-Time PCR System (Applied Biosystems, USA). The data of qRT-PCR was analysed using $2^{-\Delta\Delta CT}$ method, after normalisation against β-actin reference gene (Livak and Schmittgen, 2001).

3.7. Calculations

At the end of the experimental trial, the marron growth performance was calculated by using the following formulae:

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

$$\text{Specific growth rate (SGR, \%/day)}$$

$$= \left[\frac{\ln\text{ (final body weight)} - \ln\text{ (initial body weight)}}{\text{days}}\right] \times 100$$

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

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

CHAPTER 4: Effects of nutrient deprivation on marron health and immunity

Effects of long-term starvation on health indices, gut microbiota and innate immune response of fresh water crayfish, marron (*Cherax cainii*, Austin 2002)

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Abstract

Present study aimed to investigate the physiological and molecular response of 28-days starved marron (*Cherax cainii*), an economically important freshwater crayfish species. Thirty two marron were randomly distributed into two distinct groups in quadruplicated tanks with a density of 4 marron per tank. The feeding- group of marron was fed the marron basal diet on every day while the starved- group was deprived of feed for four weeks. The results showed a significant (P<0.05) reduction in protein, energy and fat contents in the tail muscle of marron due to starvation. The microbiome data showed significant modulation of bacterial abundance at both genus and species level in post-starved marron where core microbiota was replaced by *Vibrio*. The predicted KEGG metabolic pathway based on 16S rRNA data showed a notable shifting of biosynthesis capability towards nutrients stress response activity in starved marron. Significant impact of starvation on the relative expression level of immune genes was observed in starved marron where pro and anti-inflammatory cytokine genes were significantly down-regulated in fourth weeks. This study provides an insights into the immune response and health of marron exposed to starvation under farming conditions.

Keywords: Marron; starvation; health and immune indices; microbial structure; gene expression

4.1. Introduction

There is no unique response for any animals to transformed feeding conditions, including starvation. Animals can quickly adapt to short-term (<7 days) starvation by adjusting physiological and metabolic responses as well as modifying their intestinal microbes (Furet et al., 2010, Xia et al., 2014). However, prolonged (>7 days) absence of supplemented nutrients through feeding, may lead to rapid shift of microbial diversity and relative abundance of particular bacterial taxa (Kohl et al., 2014). Prolonged starvation not only affects the health status but also restructures the gut microbiota of all living species (Eslamloo et al., 2017, Sacristán et al., 2016) and has negatively affected the growth parameters, biochemical compositions and immune response in finfish (Bar, 2014, Namrata et al., 2011). In addition, significant changes in the core microbiota in response to different stressors, energy crisis, nutritional status, age, antibiotic exposure, poor water quality and infection have also been validated (Dehler et al., 2017c, Karl et al., 2018, Kers et al., 2018, Mir et al., 2019, Zha et al., 2018). A transformed gut microbiota can lead to an altered immune function of the host, and also increase the risk of disease predisposition by the pathogenic bacteria (Brown et al., 2012).

Finding a universal response against starvation is difficult as tolerance level differs even within a group of animals including aquatic species (Bar, 2014). Despite similar body mass, some aquatic species can survive under limited nutrient conditions by utilizing alternative energy sources containing ketone bodies, fatty acids and nitrogenous compounds produced by the gut microorganisms during starvation (Barreto-curiel et al., 2017, Egerton et al., 2018, Kohl et al., 2014, Kohl and Carey, 2016). DNA sequence based studies of starvation effects reported significant enrichment of *Bacteroidetes* but depletion of *Proteobacteria* in Asian seabass (*Lates calcarifer*) whereas in Nile tilapia (*Oreochromis niloticus*), a differential microbial shift of *Coprobacillus* and *Ruminococcus* have documented (Kohl et al., 2014, Xia et al., 2014). This type of study provided baseline information about the resident microbes of host and their

activity during the food shortage. Most of the studies on effects of starvation have been performed on human and other land animals, compare to the aquatic animals. Some microbes like *Akkermansia* genus ingest host-directed mucus and rapidly increase their relative abundance level to extend the survivality of hamster and python in nutrient-deprived condition (Costello et al., 2010, Derrien et al., 2004, Sonoyama et al., 2009). Although some studies have been conducted on the response of fish microbiota due to starvation (Kohl et al., 2014, Mekuchi et al., 2018, Xia et al., 2014), however, no study has yet been recorded for any crayfish species.

Marron (*Cherax cainii*) is one of the largest and economically important parastacidae crayfish cultured in south-western of Western Australia (Cole et al., 2019). Previous research study mainly focused on the growth and immune responses of post feeding marron and plate-based identification of bacteria from the intestinal tract (Ambas et al., 2015, Ambas et al., 2017). Therefore, the information on the physiological, immunological and molecular responses of marron during starvation is yet to be decoded to understand the effects of nutrient deprivation or under-feeding during the farming conditions.

Advancement in 16S rRNA based high throughput sequencing technology has facilitated precise detection of bacterial community and their metabolic functions (Ooi et al., 2017, Rimoldi et al., 2018). However, this technology can only provide phylogenetic compositions of bacteria in the intestine, while other important health parameters like protein, stored fat, and energy need to be analysed subsequently in relation to gut microbiota in order to gain holistic understanding on impacts of starvation in marron. In this study, we have applied various molecular and immunological techniques, and sequence based bioinformatics to analyse the gut microbiota, health and immune responses of marron after starvation.

4.2. Materials and Methods

4.2.1. Experimental set-up

The experiment with 32 marron was designed as described in general methodology (chapter 3.1). Approximately 30% of water was exchanged from each tank on daily basis through siphoning. During acclimation and experimental trial, the marron were fed every afternoon with commercial formulated fishmeal based diet (Table S4.1, Appendix 3) named *marron pellet* (Glenn Forrest, Perth, Australia) composed of 29.9% of crude protein, 7.5% ash, 7.5% lipid, calcium 1.5%, phosphorus 1.0%, and 18.2 kJ gross energy. The number of moults in each tank was monitored carefully and recorded every day before feeding.

4.2.2. Monitoring of water quality

The temperature, pH, dissolved oxygen (DO), nitrogenous compound including nitrate (NO_3^-) and nitrite (NO_2^-), phosphate (PO_4^-) and ammonia (NH_3^+) of each tank were monitored as described in general methodology (chapter 3.1).

4.2.3. Marron sampling

For haemolymph parameters and health indices, eight marron, one marron randomly selected from each tank was used. However, for DNA extraction from gut content, two randomly selected marron from each tank per treatment, were selected at the end of the experiment. Marron were then taken into biosafety cabinet followed by careful excision of gut. Then the hindgut were separated and its contents were homogenized and pooled together and transferred in to 1.5 mL of Eppendorf tube. The hind gut was selected because of its potential role in digestion and immunity of crayfish (Wang et al., 2018). For gene expression analysis, intestine tissue from one marron per tank at first week (7 days) and fourth week (28 days) was collected, hence a total of 16 samples were used in two different weeks.

4.2.4. Analysis of haemolymph parameters

The haemolymph parameters of marron including osmolality, lysozyme activity and THC were calculated following methods described in general methodology (chapter 3.2).

4.2.5. Analysis of tail muscle biochemistry

The amount of protein, energy and fat in the tail muscle of marron was calculated using methods described in general methodology (chapter 3.3).

4.2.6. High-throughput sequencing

DNA extraction, PCR amplification and meta-barcoding for high throughput sequencing were performed following methods described in general methodology (chapter 3.4).

4.2.7. Gene expression analysis

Eight genes associated with immunity of crayfish species based on previous studies (Dai et al., 2017; Jiang et al., 2015; Liu et al., 2013; Shekhar et al., 2013) were selected. The selected primers are listed in Table S4.2. RNA extraction, cDNA library, Real-time PCR (RT-PCR) and data analysis were performed as described in general methodology (chapter 3.6).

4.2.8. Bioinformatics

Unless any modifications mentioned, the “pipelines” for downstream sequence analysis and bioinformatics were used according to general methodology in chapter 3.5 with default parameters. The rarefaction depth value was set at 12550 and subsequent computation of alpha and beta diversities was performed using QIIME (v1.9.1) (Kuczynski et al., 2012). Briefly, alpha diversity was evaluated based on the following metrics: observed species and Shannon diversity. Principle coordinates analysis (PCoA) using Bray Curtis and jaccard abundance metrics was performed in ampvis2 R package (Andersen et al., 2018). Non-parametric statistical analysis of the distance metric was performed using ANOSIM with 1000 permutations. Venn diagram for bacterial abundance regarding bacterial diversity at genus level was generated using FunRich (v3.1.3) (Benito-Martin and Peinado, 2015). The Shannon,

Simpson, and Fisher alpha diversity index were calculated using "vegan" package in R (Oksanen et al., 2018). Chao1 diversity was calculated using formula $S_{chao1} = S_{obs} + (n1)^{\surd}/2n2$, where S_{obs} = number of observed genera, n1 = number of singletons, n2 = number of doubletons (Militon et al., 2010). Linear Discriminant Analysis Effect Size, LEfSe was applied to find the indicator bacterial groups in two different groups with a minimum logarithmic LDA cut-off value of 3.0 (Segata et al., 2011). For predicting differentially abundant metabolic pathway from KEGG database in two different groups, Piphillin algorithm (http://secondgenome.com/Piphillin) was used with supports of BioCyc (v21) and LEfSe (LDA 3.0) (Iwai et al., 2016, Segata et al., 2011). Independent sample T-test was used to calculate any significant differences ($P<0.05$) among variables in two different groups.

4.3. Results

4.3.1. Marron health and immune indices

At the end of the trial, although the growth rate was not reduced significantly ($P>0.05$), protein, energy and fat contents in the tail muscle of starved marron decreased significantly. Lysozyme and THC values in the haemolymph also dropped significantly in the starved marron. Fasting had no effects ($P>0.05$) on hepatopancreas moisture and hemolymph osmolality and number of moults in marron (Table 4.1).

Table 4. 1 Mean ± SE of some health and immune parameters of marron after trial

Parameters	Feeding	Starved	P-value
WG (g %)	21.65 ± 1.78	19.24 ± 0.81	0.07
SGR (g/day %)	0.45 ± 0.034	0.41 ± 0.031	0.07
Protein (%)	84.4 ± 0.57	79.7 ± 0.83	0.01
Energy (kJ/kg)	21038.8 ± 275.6	20241.1 ± 136.5	0.04
Fat (%)	8.8 ± 0.09	6.5 ± 0.06	0.006
HM (%)	32.5 ± 1.52	27.5 ± 1.56	0.07
Lysozyme (U/mL)	0.48 ± 0.02	0.41 ± 0.01	0.008
THC (cells/mL)	8.2 ± 0.07	7.8 ± 0.21	0.01
HO (U/mL)	0.41 ± 0.004	0.40 ± 0.011	0.16
Moults	3	6	0.09

WG, weight gain; SGR, specific growth rate; HM, hepatopancreas moisture; THC, total haemocyte count; HO, haemolymph osmolality. (n=4)

4.3.2. Microbial diversity in the hindgut

The 16S rRNA sequence data generates 551,674 raw reads after removing singleton that were classified into 102 OTUs, 10 phylum and 74 genera. Starvation found to have significant effects ($P<0.05$) on modulation of gut microbiota. Principal coordinate analysis based on relative abundance (weighted) and Bay-Curtis dissimilarity showed a distinct pattern of bacterial clustering in two different groups (Figure 4.1A). Besides 40 shared genera in two different groups, additional 34 unshared genera were enriched in the fed groups (Figure 4.1B). At phylum level, *Firmicutes* (51.7%) was the most abundant bacteria in the fed group whereas starved group was dominated by uncharacterized (52.8%) bacteria (Table 4.2). At genus level, *Streptococcus* comprised of 28.9% OTUs in the fed group while sharp decrease of 25.2% was in the starved group. On the contrary, starvation accelerated the growth of *Vibrio* by 11.7%, from 2.5% in the fed group to 14.2% in the starved group. (Figure 4.2). The Shannon, Simpson, Fisher-Alpha and Chao1 diversity index declined significantly ($P<0.001$) in starved marron while more pronounced effects were observed for Shannon and Fisher-Alpha (Table 4.3). Wisconsin non-parametric t-test at 0.05 level of significance and stringent LDA cut-off value of 3.0 identified *Streptococcus*, *Clostridium* and *Corynebacterium* as the indicator bacterial groups in the gut of fed marron whereas other (uncharacterized) and *Vibrio* were the signature bacteria in the starved marron (Figure 4.3).

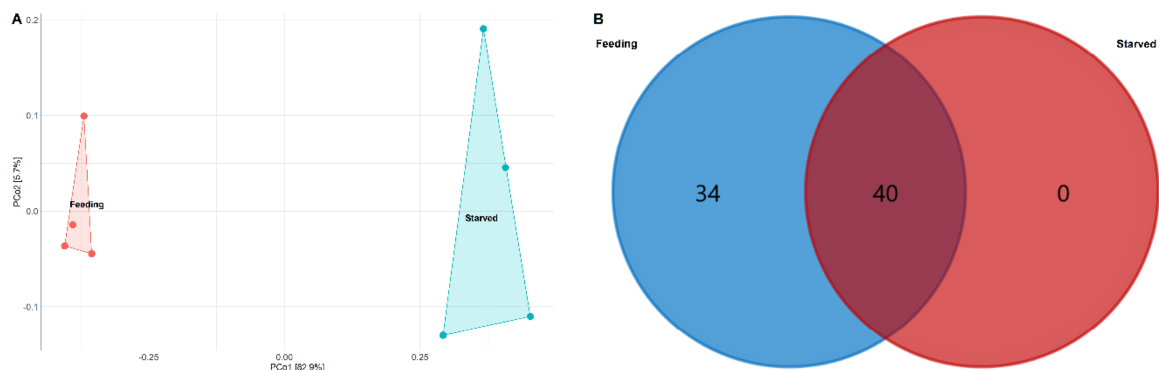

Figure 4. 1 (A) PCoA plot presenting the clustering of samples in two different conditions based on relative abundance of bacterial OTUs. (B) Venn diagram contrasting the number of

shared and unshared genus in two different groups (feeding and starved) after four weeks of trial. (n=8).

Table 4. 2 Relative abundance (>1%) of bacteria at genus level in two different groups (n=8)

Phylum	Feeding (%)	Starved (%)	P-value
Firmicutes	51.7	10.2	0.04
Proteobacteria	26.4	36.9	0.18
Actinobacteria	9.7	0.1	0.005
Bacteroidetes	6.0	0.1	0.01
Other	4.7	52.8	0.03

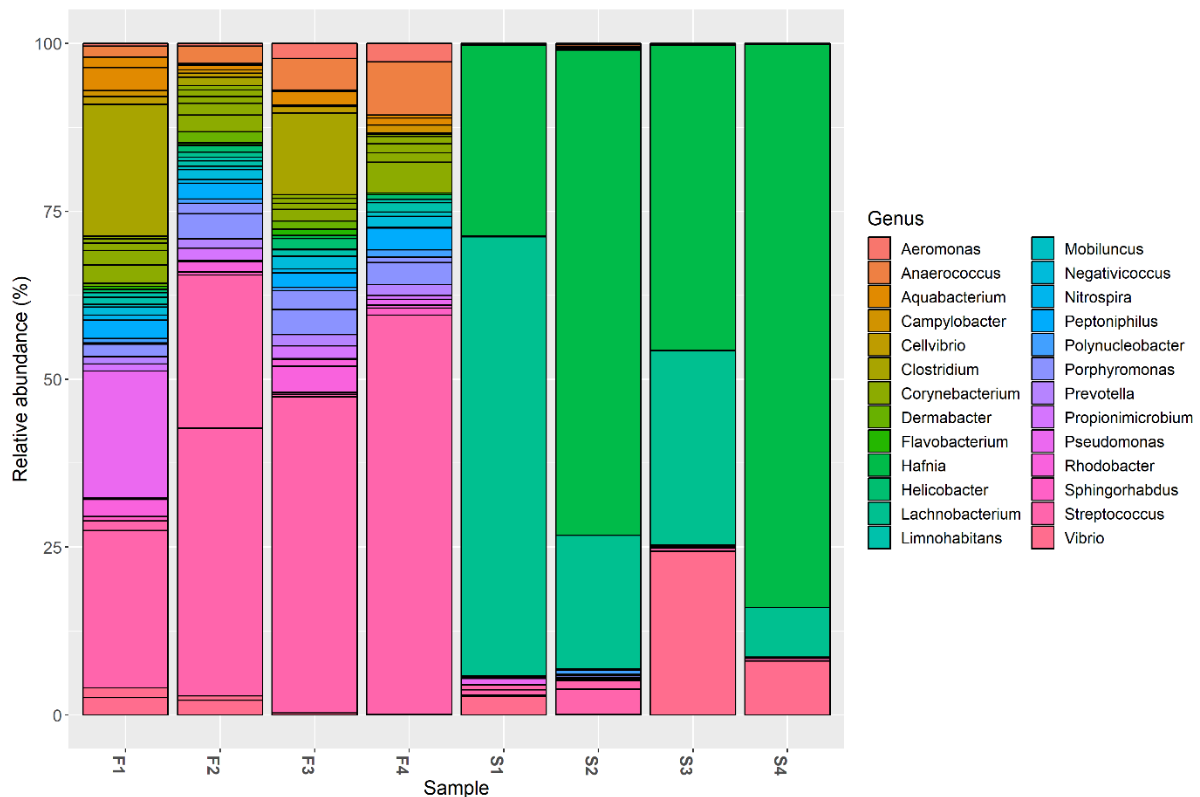

Figure 4. 2 Relative abundance of bacterial OTUs at genus level in two different groups (feeding and starved) after four weeks of experimental trial. Abbreviation: F = Feeding; S = Starved. (n=8).

Table 4. 3 Mean ± SE, major diversity indices at genus level in the marron gut (n=8)

Diversity	Feeding (SE)	Starved (SE)	P-value
Shannon	2.73 (0.10)	0.96 (0.10)	0.0008
Simpson	0.85 (0.03)	0.51 (0.05)	0.01
Fisher alpha	10.25 (0.78)	3.56 (0.49)	0.0009
Chao1	49.77 (5.8)	24.90 (8.6)	0.006
Good's index	96.05%	93.88%	–

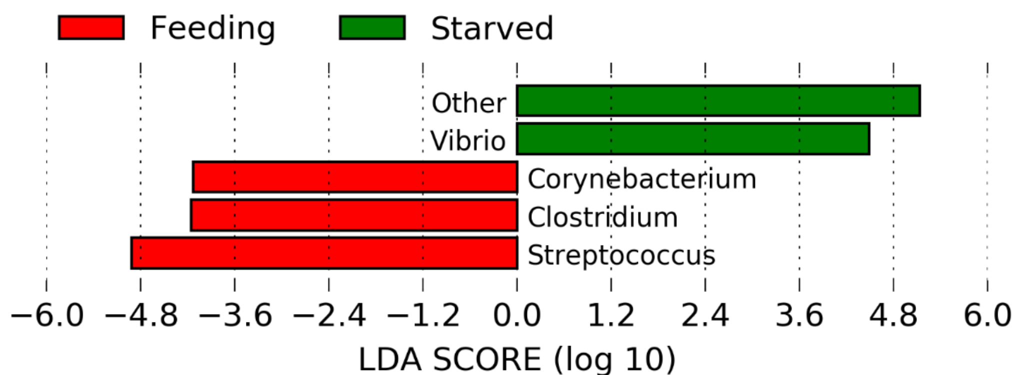

Figure 4. 3 Indicator microbial groups at genus level in two different treatment groups (feeding and starved) after four weeks of trial. The groups were compared using Linear Discriminant Analysis (LDA) at strict LDA cut-off value of 3.0 and above. (n=8).

4.3.3. Impacts on metabolic pathways

Piphillin and KEGG based observation of metabolic pathway based on 16S rRNA data revealed a significant shift of pathways in starved marron. With strict LDA cut-off value of >3.0, we predicted 10 up-regulated pathways in the fed group than 13 in the starved marron. The fed group comprised of pathways, including biosynthesis of amino acids, amino-acyl tRNA, ribosome etc. mostly related to biosynthesis of amino acids, proteins, secondary metabolites including antibiotics and cell wall components. On the other hand, the identified metabolic pathways in the starved group (i.e. flagellar assembly, two-component system, biofilm formation, microbial metabolism, ABC transporter etc.) were commonly associated with

energy consumption, virulence factors mediated cell wall invasion and stress related response (Figure 4.4).

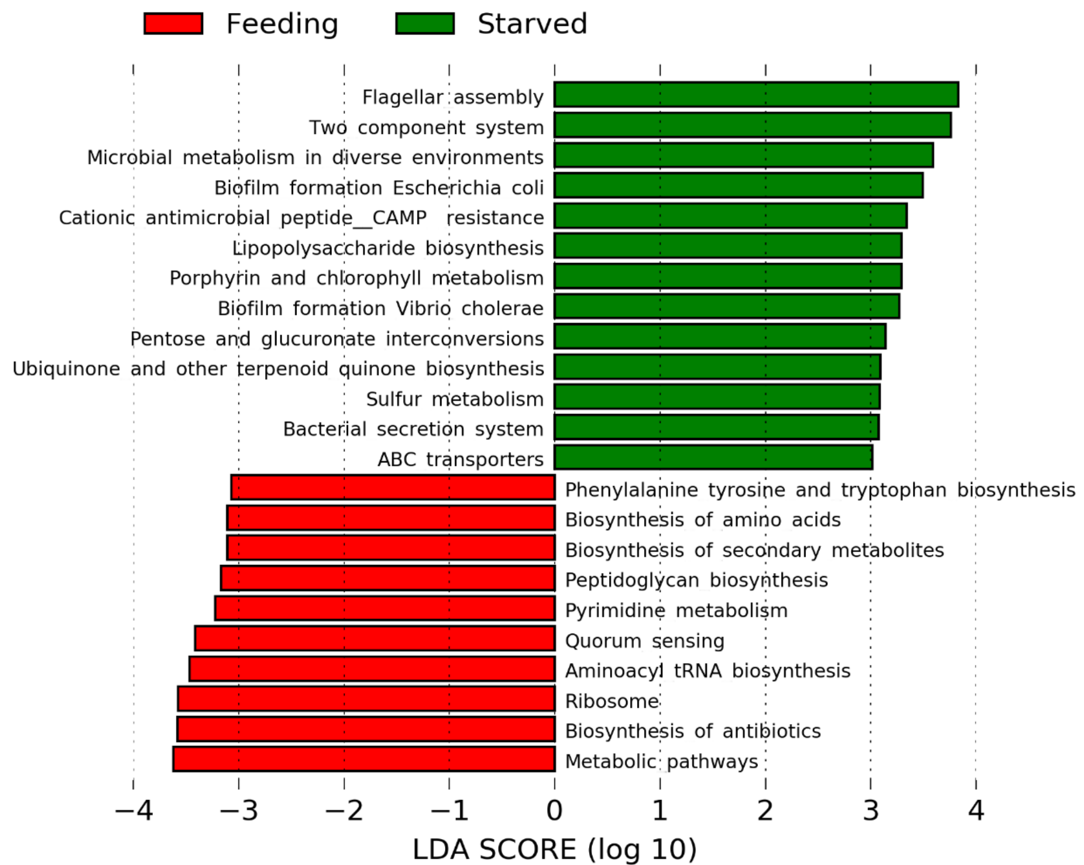

Figure 4. 4 Differentially abundant metabolic pathways based on 16S rRNA data in two different treatment groups (feeding and starved) after four weeks of trial. (n=8)

4.3.4. Change in relative expression level of immune genes

The results of qRT-PCR gene expression associated with marron innate immune response demonstrated a significant (P<0.05) down-regulation of pro-inflammatory cytokine (interleukin 17, IL-17) and anti-inflammatory cytokine (interleukin 10, IL-10). After four weeks of starvation, the relative expression level of IL-17 and IL-10 were decreased by 3.78 and 3.33 folds, respectively (Figure 4.5). The genes linked to innate immune response of marron such as prophenoloxidase (proPO) and cytosolic manganese superoxide dismutase

(cytMnSOD) were also down-regulated (*P*<0.05) at the end of starvation period. The expression level of interleukin 1β and 8, and cathepsin L (PcCTSL) genes were relatively static (*P*>0.05) during the post starvation period.

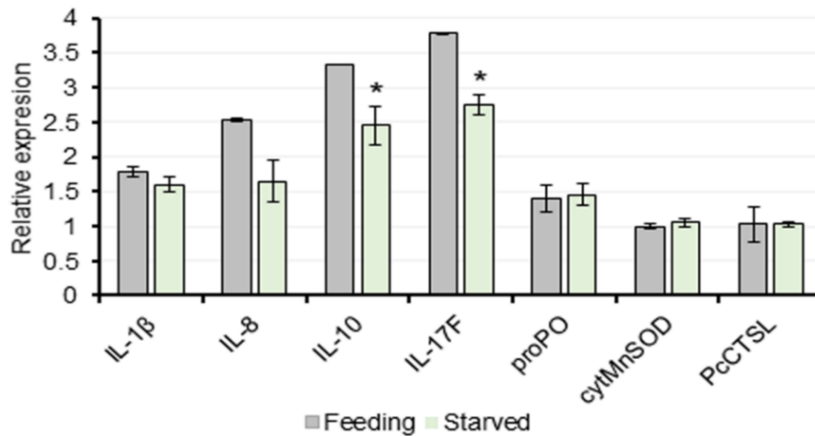

Figure 4. 5 Relative expression level (Mean ± SE) of genes associated with inmate immune response of marron after four weeks of trial. Means with asterisk superscripts on top of bar are statistically significant between two groups at α-level of 0.05. (n=4).

4.3.5. Data availability

The FASTQ files are currently available in National Centre for Biotechnology Information (NCBI) BioProject under the accession number PRJNA549030.

4.4. Discussion

Long term starvation can provides valuable base line information about immunological and molecular aspects of alteration and adjustment in animals. In this context, some published research are available on physiological and biochemical responses of Pacific white leg shrimp, (*Litopenaeus vannamei*) against short-term starvation, and gut microbial changes of Nile tilapia and Asian seabass against long-term starvation (Kohl et al., 2014, Sánchez-paz et al., 2007, Xia et al., 2014). However, reports on the impacts of long-term starvation on physiological and molecular aspects of crayfish has yet to be studied. In present study, a significant drop (P<0.05) in health and immune indices of marron was observed following four weeks of starvation.

Consistent to our findings Sanchez-Paz et al., (2007) reported a non-significant ($P>0.05$) effects on growth parameters of pacific white leg shrimp in response to short term starvation. In contrast to our results, one study reported significant effects of starvation ($P<0.05$) on the growth of early and late juvenile of red claw crayfish, *Cherax quadricarinatus* where early juvenile recovered growth after feeding from 60 days (Calvo et al., 2012). The growth rate for late juvenile was dropped significantly after 27 days of starvation and could not compensated after feeding. In a separate study, Sacristán et al. (2016) reported significant ($P<0.05$) negative impacts of long-term starvation on growth rate, lipid content, number of moults, and energy level of juvenile red claw. The insignificant effects ($P>0.05$) of prolonged starvation in this study was probably due to significant weight difference in experimental crayfish, Calvo and Sacristan (Calvo et al., 2012, Sacristán et al., 2016) studies used 1g and 6.27g of juvenile red claw wherein present study used 69.7g of adult marron. The survival ability of marron differs in various life stages where adult marron can tolerate more stresses including nutrient deficiencies, salinity, high temperature and other parameters (deGraaf et al., 2010). Besides growth, starvation also led to significant drop of hemolymph parameters, especially lysozyme and THC of marron. A similar effects have been reported for serum lysozyme activity in tinfoil barb (*Barbonymus schwanenfeldii*) (Eslamloo et al., 2017) and THC in white shrimp (Lin et al., 2012) from the second week of starvation.

The gut microbiota a plays vital role in digestion and immunity, and interact with environment to supports host cell to quickly adapt and response to any kinds of stressor (Wang et al., 2018). As intestinal microbial communities of crayfish consist of diverse and vast range of bacteria from different phylum and genus (Cornejo-granados et al., 2017, Skelton et al., 2017), hence, it is imperative to understand as how the bacterial population respond against long term fasting. Previous studies on impacts of long-term starvation on fish and shellfish demonstrated a significant shift of intestine microbial communities and reported both positive and negative

impacts on bacterial diversity (Kohl et al., 2014, Mekuchi et al., 2018, Xia et al., 2014). Significant decrease ($P<0.05$) of microbial density and diversity in the starved marron of present study is supported with decrease of microbial the diversity in zebrafish and grass carp (Semova et al., 2013, Xia et al., 2014). However, in contrast to our findings, starvation led to increase microbial diversity in tilapia and locusts (Dillon et al., 2010, Kohl et al., 2014). Therefore, fasting does not appear to have a universal effect on gut microbial communities of host species. Present study found significant ($P<0.05$) drop of *Firmicutes* and *Actinobacteria*, and an increase of *Vibrio* in starved marron. The phylum *Firmicutes* reported to be associated with improved digestibility and health status of fish to counteract against pathogenic bacteria (Egerton et al., 2018). Decrease in relative abundance of Firmucutes cause dysfunction of mucosal immune system that influence other microbial community in the gut (Shi et al., 2017). On the other hand, *Actinobacteria* are widely used as probiotic strain for fish and crayfish demonstrating beneficial role against disease causing bacteria (Das et al., 2008, Tan et al., 2016). Contrarily, *Vibrio* reported to be associated with numerous diseases in crayfish and also regarded as emerging pathogen for marron (Ambas et al., 2013, Foysal et al., 2019a, Momtaz et al., 2019). The shift in the relative abundance of bacteria in the gut might have strong association with the poor immune status of starved marron, and thus needs further research.

To identify indicator microbial groups in two different conditions, we applied Linear Discriminant Analysis Effect Size (LEfSe), a platform that can efficiently predict high-dimensional biomarkers in diverse conditions from 16S rRNA data set (Huang and Jiang, 2016). Results of LEfSe analysis suggested a significant drop of *Corynebacterium*, *Streptococcus* and *Clostridium* in the starved marron in comparison to increment of *Vibrio* and uncharacterized bacteria in fed marron. Dietary supplementation of *Clostridium* revealed to have positive impact on health and immune status of marron (Foysal et al., 2019d) and white shrimp (Duan et al., 2017). Additionally, lactic acid bacteria (LAB), *Corynebacterium* and

Streptococcus reported to improve the growth rate of turbot (*Scophthalmus maximus*) (Martínez Cruz et al., 2012). Excluding *Corynebacterium diphtheria*, most species of *Corynebacterium* genus are beneficial to animal gut and possess ability to prevent the colonization of pathogenic bacteria *C. pseudodiphthericum* in the host (Uehara et al., 2000). Considering previous studies, it can be concluded that the significant drop of LAB might associated with poor health and immune condition of starved marron.

Analysis of metabolic pathways is an effective approach to understand the health condition of animals (Arai, 2014, Pietro et al., 2018). Ingested food materials are utilized as main sources of energy by animals through various physio-biochemical processes including digestion, absorption, metabolism, and different types of transport systems (Arai, 2014). The identified up-regulated stress response metabolic pathways in starved marron suggest physiological and immunological imbalance leading to poor immune and health status. For instances, up-regulation of biofilm formation is an energetically costly processes which are regulated tightly by cellular organelles in response to various environmental stress (Cairns et al., 2014). In other pathways, bacterial secretion system triggers the invasion of host cell by the virulence factors of pathogenic bacteria whereas two-component regulatory system allows microbes to respond quickly to altered environmental condition or stress (Coburn et al., 2007, Ribet and Cossart, 2015). The up-regulation of metabolic pathways found to be linked with microbial abundance in this study. *Vibrio*, the significant abundant genus in starved marron forms microbial biofilm for long term persistent in the intestinal cells and stated as emerging pathogen for marron in earlier studies (Ambas et al., 2013, Foysal et al., 2019a, Sengupta et al., 2016). *Virbio* can also adapt rapidly to ever-changing environmental stresses including temperature, salinity and nutrient concentrations (DeAngelis et al., 2018). Therefore, shift in *Vibrio* abundance can be linked with up-regulation of biofilm formation, bacterial secretion system and two-component regulatory system in starved marron.

Innate immune response exerted from various tissues and cellular membranes have specific response against certain types of environmental stress. Among the various immune-responsive genes, interleukin family, cathespsin L (PcCTSL), prophenoloxidase (proPO) and cytosolic manganese superoxide dismutase (cytMnSOD) are the major genes associated with innate immune response of crayfish (Dai et al., 2017, Foysal et al., 2019b, Jiang et al., 2015a, Liu et al., 2013). The results of present study showed significant down-regulation of genes associated with innate immune response of crayfish. When the anti-inflammatory cytokine IL-10 up or down-regulated, the pro-inflammatory cytokine IL-17 follow the similar expression pattern to prevent animals from inflammation and stress related damage (Miao et al., 2018). In addition, proPO and cytMnSOD reported to have antimicrobial activity against crayfish pathogen i.e. *Vibrio* spp., shrimp white spot virus (WSSV) etc. and play a key role in boosting host immune defence capability (Foysal et al., 2019b, Liu et al., 2013). Inhibition of *V. harveyi* and *V. parahaemolyticus* shown elicitation of strong immune response by enhancing antibody production in white shrimp (Gao et al., 2016b). Increased relative abundance for *Vibrio* species and downregulation of proPO and cytMnSOD therefore can be correlated with poor immune response in post starved marron. Down-regulation of immune genes in short (5 days) and long starvation (> 21 days) have been recorded for white shrimp and rainbow trout (*Oncorhynchus mykiss)* in two separate studies (Lin et al., 2012, Salem et al., 2007). Hence starvation can be correlated with down-regulation of genes linked to immune response of crayfish in this study. In overall, although the effects of long-term starvation was significant on growth and immunity of marron, however, the response of gut microbial communities was found to be different in marron compared to other aquatic species. Further research is needed to find out the exogenous and endogenous factors linked to bacterial community shift and their role in health and immune performance of marron.

CHAPTER 5: Probiotics for marron aquaculture

EXPERIMENT 1: Marked variations in gut microbiota and some innate immune responses of fresh water crayfish, marron (*Cherax cainii*, Austin 2002) fed dietary supplementation of *Clostridium butyricum*

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Abstract

This study aimed to investigate the effects of *Clostridium butyricum* as a dietary probiotic supplement in fishmeal based diet on growth, gut microbiota and immune performance of marron (*Cherax cainii*). Marron were randomly distributed into two different treatment groups, control and probiotic fed group. After 42 days of feeding trial, the results revealed a significant ($P<0.05$) increase in growth due to increase in number of moults in marron fed probiotics. The probiotic diet also significantly enhanced the total haemocyte counts (THC), lysozyme activity in the haemolymph and protein content of the tail muscle in marron. Compared to control, the 16S rRNA sequences data demonstrated an enrichment of bacterial diversity in the probiotic fed marron where significant increase of *Clostridium* abundance was observed. The abundance for crayfish pathogen *Vibrio* and *Aeromonas* were found to be significantly reduced post feeding with probiotic diet. Predicted metabolic pathway revealed an increased activity for the metabolism and absorption of carbohydrate, degradation of amino acid, fatty acid and toxic compounds, and biosynthesis of secondary metabolites. *C. butyricum* supplementation also significantly modulated the expression level of immune-responsive genes of marron post challenged with *Vibrio mimicus*. The overall results suggest that *C. butyricum* could be used as dietary probiotic supplement in marron aquaculture.

Keywords: Marron, Probiotic bacteria, Health and immune indices, High throughput sequencing, Bioinformatics, Gene expression profiling

5.1(1). Introduction

Crayfish harbour complex bacterial communities in the intestine that stimulates various host functions like digestion, absorption, immunity and disease resistance (Skelton et al., 2017). Augmentation in the growth and immunity of fish and crayfish with dietary probiotic supplements has long been studied (Ambas et al., 2017, Ambas et al., 2013, Didinen et al., 2016, Panigrahi et al., 2007). Probiotics are live microorganisms that can improve the digestive function by influencing the growth of beneficial microorganisms in the gut when administered in adequate amounts (Dawood et al., 2016, Irianto and Austin, 2002). Probiotic bacteria can improve the growth, immune response, feed utilization and stress response of fish in the host species (Dawood et al., 2016, Iwashita et al., 2015, Kesarcodi-Watson et al., 2008). Among the bacterial supplements, majority of the studies focussed on the beneficial effects of Lactic acid bacteria (LAB), especially *Lactobacillus* species on growth, immunity and disease resistance of fish and crayfish (Didinen et al., 2016, Pirarat et al., 2006, Zheng et al., 2017a). *Clostridium butyricum*, a short-chain butyric acid producing bacteria that resides in healthy intestinal flora, has been used as diet supplements to enhance the growth and immune response of fish and broiler chicken (Gao et al., 2013, Ramírez et al., 2017, Song et al., 2006, Zhang et al., 2014). In addition, *C. butyricum* as probiotic supplement demonstrated to have inhibitory effects against the colonization of pathogenic bacteria including species from *Vibrio* and *Aeromonas* in the fish gut (Gao et al., 2013, Pan et al., 2008). However, the impacts of *C. butyricum* on crayfish, especially on marron (*Cherax cainii*) growth performance, gut microbiota and immune gene expression has yet to be explored.

Marron is the third largest freshwater crayfish and iconic to Western Australia (WA). The global demands of marron is very high due to its large harvest size (up to 2 kg), distinctive flavour, disease-free status, high consumer preference, and ability of live transport (Ambas et al., 2013). Slow growth of marron has long been a bottleneck in the expansion of marron

aquaculture in WA (Alonso, 2010, DoF, 2017, Lawrence, 2007). Although there has not been any current report of infection in marron, future expansion of marron industry could bring this threat especially infections by *Vibrio* species (Bean et al., 1998, Eaves and Ketterer, 1994, Sherry et al., 2016). Therefore, enhancing growth parameters and minimizing the possible incidences of *Vibrio* infections are the two utmost challenges in the expansion of marron aquaculture.

Integration of traditional growth performance analysis methods with recently developed modern technologies like 16S rRNA based high throughput sequencing and bioinformatics pipelines have enabled more comprehensive analysis of feeding effects at a cellular and molecular level of fish (Allali et al., 2017, Miao et al., 2018). In addition, quantitative real-time polymerase chain reaction (PCR) has been widely used to measure the relative expression level of immune responsive genes for fish (Mahanty et al., 2017). The present investigation was designed to evaluate the effects of dietary supplementation of *C. butyricum* on health, gut microbial community and immune related gene expression of marron.

5.2(1). Materials and methods

5.2(1).1. Experiment set-up

The experiment with 24 marron (69.65 ± 1.04 g) was designed as described in general methodology (chapter 3.1). Marron were then randomly distributed into six tanks (4 marron/tank) and acclimatized for two weeks before starting the feeding trial. During acclimatization, marron were fed with commercial basal diet named *marron pellet* (Glenn Forest, Perth, Australia) (Table S4.1, Appendix 3). After acclimatization, marron were disseminated into two distinct treatment groups' viz. control and probiotic fed group with a stocking density of four marron per tank. Approximately 30% of water was exchanged every day from each tank to remove uneaten feed and faecal debris by siphoning.

5.2(1).2. Probiotic feed formulation

The probiotic feed was formulated at CARL according to the procedure used by Ambas et al. (2013). The fishmeal based basal diet was used for probiotic feed formulation (Table S4.1, Appendix 3). The ingredients of the commercial basal feed were gently passed through 100 µm mesh sieve and homogenized to get uniform particle size. *C. butyricum* purchased from Advanced Orthomolecular Research (AOR, Calgary, Canada) was cultured on Clostridial Agar (Sigma-Aldrich, MO, USA) followed by sub-culture on Clostridial agar. Then a serial dilution and subsequent plate counts were performed to obtain the desire bacterial concentration. The bacteria (*C. butyricum*) were then added at 10^7 CFU/g of feed as described in previous studies (Ramírez et al., 2017) and mixed uniformly. Pellet were prepared with mince mixture followed by vacuum drying oven at 37 °C for overnight and storage at 4 °C until used. Final proximate compositions of protein 29.9%, lipid 7.1% and gross energy 18.2% Mj/kg, and bacterial concentration of 1.01 x 10^7 CFU/ml in the diet were determined as per the method of Association of Official Analytical Chemists, AOAC (AOAC, 2006) and plate counts on Clostridial agar. During experimental trial, each marron were fed once every day at 5 PM for 42 days at a rate of 1.5% of total marron biomass per tank (Ambas et al., 2017). Control group fed basal diet and probiotic group served *C. butyricum* supplemented diet.

5.2(1).3. Monitoring of water quality parameters

The water quality including temperature, pH, dissolved oxygen (DO), nitrate (NO_3^-), nitrite (NO_2^-), phosphate (PO_4^-) and ammonia (NH_3) were monitored as described in general methodology (chapter 3.1).

5.2(1).4. Marron sampling

For analysis of haemolymph parameters (lysozyme, haemolymph osmolality and total haemocyte count) health indices (protein and energy in tail muscle), one randomly selected marron from each tank (N = 6) was selected. However, for DNA extraction and microbiome

analysis, two randomly selected marron from each tank (N = 12) were scarified followed by careful separation of hindgut. The hindgut contents of two samples from each tank were homogenized and pooled together (N = 6), and then transferred into 1.5 mL Eppendorf. Finally, for immune gene expression, the whole intestine tissue sample from one randomly selected marron from each tank (N = 6) was used after challenge test. Sample from each marron was used for the haemolymph parameters (before sacrifice), biochemical assay (after sacrifice), and molecular analysis (microbiome and gene expression).

5.2(1).5. Analysis of growth and immune parameters

Before starting the experiment and at the end of the trial, the weight of each marron was recorded. The weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) were measured according to calculations mentioned in general methodology (chapter 3.6). The number of moults in each tank was monitored carefully. Immune parameters including haemolymph osmolality (HO), lysozyme and total haemocyte counts (THC) were analysed according to methods described earlier in general methodology (chapter 3.2).

5.2(1).6. Analysis of biochemical composition

The protein, lipid and energy content in the tail muscle were measured according to methods described in chapter 3.3, general methodology.

5.2(1).7. High throughput sequencing

At the end of the experiment, gut samples prepared as described in marron sampling were used for DNA extraction. Due to special role in digestion, absorption and immunity, hindgut was selected for 16S rRNA sequencing (Wang et al., 2018). Rest of steps were performed according to methods described in general methodology of chapter 3.4.

5.2(1).8. Gene expression analysis in challenged marron

Six genes linked to innate immune response of crayfish (Dai et al., 2017, Jiang et al., 2015a, Liu et al., 2013, Miao et al., 2018) were selected as listed in Table S5.1.1 (Appendix 4).

Crayfish pathogen *Vibrio mimicus* was collected from the Department of Food and Agriculture, Western Australia. At the end of the feeding trial, each selected marron was injected with previously prepared 2×10^8 CFU/mL stock solution of *V. mimicus* (Ambas et al., 2013). Fifty microliters of bacterial solution were injected through the base of the fifth thoracic leg of marron (Ambas, Suriawan, & Fotedar, 2013). Control marron were injected with 50 µL of phosphate buffer saline (PBS). Injected marron were subjected to RNA extraction from intestine tissue after 24 h of bacterial challenge. For RNA extraction, intestine tissue samples from challenged marron were initially stored at -80 °C with *RNA Later* solution (Sigma-Aldrich, Germany). Rest of the steps were performed according to methods described in general methodology (chapter 3.6).

5.2(1).9. Bioinformatics

Unless any modifications mentioned, the “pipelines” for downstream sequence analysis and bioinformatics were used according to general methodology in chapter 3.5 with default parameters. The rarefaction depth value was set at 4672 and subsequent calculation of alpha and beta diversities was performed using QIIME pipeline (version 1.9.1) (Kuczynski et al., 2012). Briefly, the alpha diversity was calculated based on observed species and diversity indices (Shannon, Simpson, Alpha Fisher and Chao1) using vegan package in Rstudio (Oksanen et al., 2018). Non-parametric *t*-test for two samples was performed to compare the alpha diversity metrics between the control and probiotic-fed samples. Principle coordinates analysis (PCoA) was performed to visualize separation of samples using Bray-Curtis and jaccard abundance metrics. Non-parametric statistical test of the distance metric was done using ANOSIM (1000 permutations). PCoA plots were generated using PhyloToAST software (Dabdoub et al., 2016). The assigned bacterial genus from two different feeding groups were plotted in Venn diagram, generated using FunRich tool (Benito-Martin and Peinado, 2015). Indicator bacterial genus in two different conditions was differentiated from rarefy 16S rRNA

sequence data using Linear Discriminant Analysis Effect Size, LEfSe (Segata et al., 2011). For predicting the metabolic pathways through 16S rRNA sequences of two different fed groups, Piphillin pipeline (http://secondgenome.com/Piphillin) was applied with supports of KEGG (May, 2017 release), BioCyc 21.0 databases and LEfSe algorithm (Iwai et al., 2016, Segata et al., 2011). One way analysis of variance (ANOVA) was used to calculate any significant differences ($P<0.05$) among the numerical data including growth (WG, SGR, FCR), biochemical compositions (protein, fat, energy), lysozyme, haemolymph osmolality and THC obtained from two different feeding conditions.

5.2(1).10. Calculations

Weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) were calculated according to methods described in general methodology (chapter 3.7).

5.3(1). Results

5.3(1).1. Water quality parameters

The water quality parameters were recorded within the normal range for optimum growth of marron according to Nugroho & Fotedar, (2013). Temperature was maintained at 22.3 ± 0.014 – 22.4 ± 0.01 °C, pH was recorded within the range of 7.5 ± 0.01 – 7.6 ± 0.01, dissolved oxygen levels between 6.51 ± 0.01 and 6.65 ± 0.01 (mg/L), nitrate and nitrite concentration of 0.046 ± 0.01 – 0.049 ± 0.01 (mg/L) and 0.015 ± 0.01 – 0.018 ± 0.01 (mg/L) were recorded, phosphate concentration was found to be varied within the range of 0.36 ± 0.01 – 0.52 ± 0.02 (mg/L), and ammonia concentration of 0.20 ± 0.01 – 0.22 ± 0.01 (mg/L) was observed in water during the experiment.

5.3(1).2. Probiotic supplement enhanced the growth and haemolymph parameters

After 42 days, the marron in the probiotic fed tanks gained significant ($P<0.05$) weight gain (WG) and specific growth rate (SGR) than the control (Table 5.1.1). However, a total of 11 marron were moulted in the probiotic fed tanks compared to three in the control tanks, shown

significant (P<0.05) increase of moult number in probiotic fed marron. Multiple regression analysis revealed a strong “Pearson” correlation (0.99) between the number of moults and weight gain (Figure 5.1.1 A). PCoA plot also revealed a complete separation of probiotic fed group based on moulting numbers and weight gain (Figure 5.1.1 B). Probiotic diet also exhibited significant (P<0.05) impacts on two out of three haemolymph parameters, total haemocyte counts (THC) and lysozyme. Among two tail muscle parameters, significant (P<0.05) increase in protein content was observed in probiotic fed marron while the variations were insignificant (P>0.05) for total gross energy, and fat in the hepatopancreas in the marron fed probiotic feed (Table 5.1.1).

Table 5.1. 1 Mean ± SE of some health parameters of marron after feeding trials

Parameters	**Control**	**Probiotic**
WG (g %)	21.66 ± 1.78	26.24 ± 0.81*
SGR (g/day %)	0.45 ± 0.034	0.53 ± 0.031*
Protein (%)†	84.4 ± 0.57	88.7 ± 0.83*
Energy (kJ/kg)†	20038.8 ± 56.06	20241.1 ± 88.25
Fat (%)⁋	8.8 ± 0.09	8.4 ± 0.06
Lysozyme (U/mL)	0.42 ± 0.005	0.48 ± 0.009*
THC (cells/mL)	8.2 ± 0.09	9.5 ± 0.21*
HO (U/mL)	0.41 ± 0.004	0.44 ± 0.011

Abbreviation: WG = Weight gain; SGR = Specific growth rate; HM = Hepatopancreas moisture; THC = Total haemocyte count; HO = Haemolymph osmolality. *Significantly different at α-level of 0.05. †From tail muscle. ⁋From hepatopancreas.

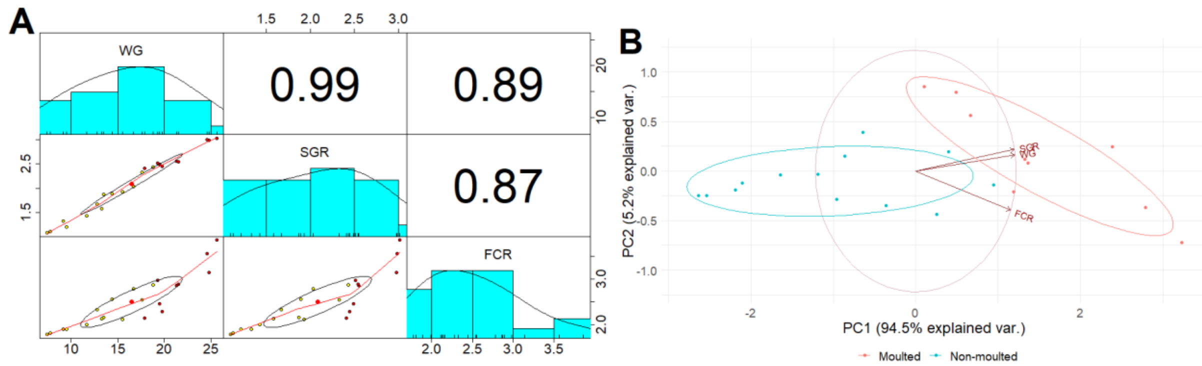

Figure 5.1. 1 (**A**) Multiple regression analysis showing the relationship between the number of moults and three health index parameters of marron after trial; (**B**) PCoA plot displaying the separation of marron regarding moult counts in two different feeding conditions.

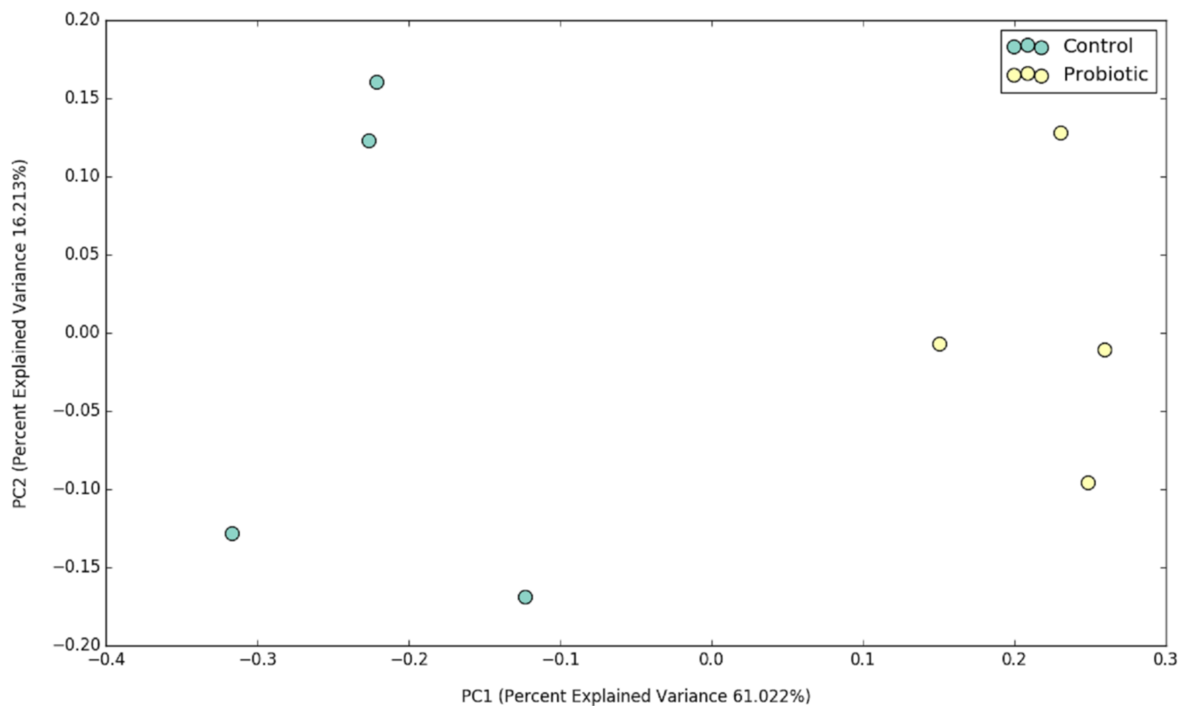

Figure 5.1. 2 PCoA plot representing the impacts of two different feeds on gut microbial communities of marron after 42 days of feeding trial.

5.3(1).3. Probiotic diet modulated the microbial communities in the distal gut

The probiotic supplemented diet exhibited a distinct effect on gut microbial community of marron after feeding trial (Figure 5.1.2). The 16S rRNA sequence from eight pooled hindgut samples generated 128,567 high quality reads after quality trimming and removing singletons, that were classified into 83 OTUs and 49 genus. Besides 18 shared genus in both groups

(control and probiotic), the probiotic feed group had an additional 31 unique genus compared to four in control diet group, indicating higher species diversity (Figure 5.1.3). At phylum level, control diet group dominated by the *Proteobacteria* (96.5%) while *Fusobacteria* (53.4%) and *Proteobacteria* (44.1%) shared 97.5% of OTUs in the probiotic fed group (Figure 5.1.4 A). At genus level, control group demonstrated more abundance for *Aeromonas* (47.1%) and *Vibrio* (31.8%) whereas in probiotic fed group, *Hypnocyclicus* (57.7%) was the most dominant bacteria, followed by *Vibrio* (13.1%) and *Aeromonas* (9.7%). A significant reduction of *Vibrio* (13.1%) and *Aeromonas* (9.7%) counts was noticed in the probiotic fed group at the end of the trial (Figure 5.1.4 B). Although the values for major diversity indices such as Shannon, Simpson, Fisher Alpha and Chao1 were significantly ($P<0.05$) higher but more pronounced effects was observed only for Shannon ($P<0.001$) and Simpson ($P<0.005$) indices in the probiotic fed group (Table 5.1.2).

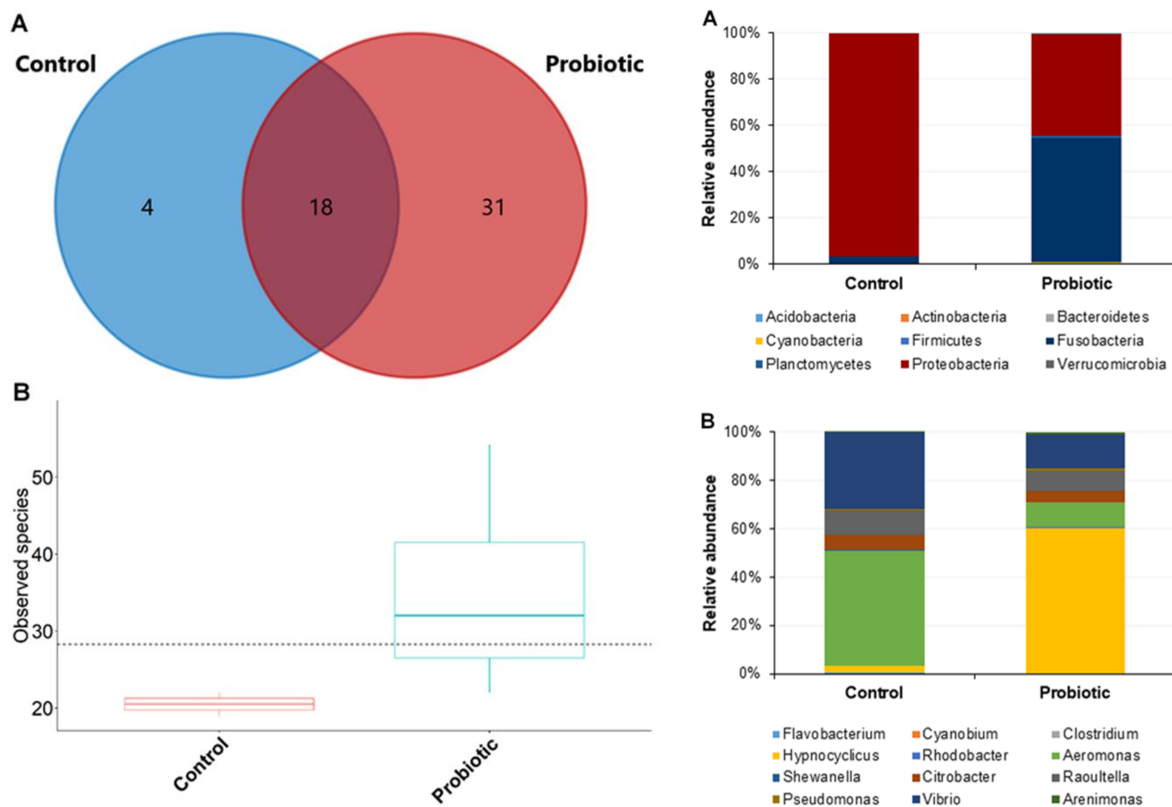

Figure 5.1. 3 **(Left)**. (**A**) Venn diagram showing shared and unshared genus in two different groups; (**B**) Box plots exhibiting the species richness in two different feeding conditions.

Figure 5.1. 4 **(Right)**. Microbial communities in two different fed groups after trial; **(A)** Relative abundance of bacterial OTUs at phylum level; **(B)** Relative abundance of bacterial OTUs at genus level.

5.3(1).4. Probiotic diet modified the microbial lineages and metabolic pathways

Linear discriminant analysis effect size (LEfSe) based on Wisconsin non-parametric t-test at 0.05 level of significance revealed three bacterial genus namely, *Illumatobacter*, *Clostridium* and *Cyanobium* to be enriched with *C. butyricum* supplementation in the marron (Figure 5.1.5 A). No significant (P>0.05) enrichment of bacteria was observed in the control group. Piphillin and LEfSe based metabolic pathways extracted from KEGG database predicted an increased pathway activities for carbohydrate metabolism and absorption, amino acid metabolism, catabolism of fatty acid, degradation of toxic and synthetic compounds and biosynthesis of secondary metabolites in the probiotic fed group. In contrast, the analysis predicted significantly higher galactose, hormone and vitamin metabolism activities in the control group (Figure 5.1.5 B).

5.3(1).5. Probiotic feed up-regulate the expression profile of immune genes

The results of qRT-PCR for the selective genes associated with crayfish immune response post *in-vivo* challenge demonstrated significant (P<0.05) up-regulation of pro-inflammatory cytokine (interleukin 17, IL17), anti-inflammatory cytokine (interleukin 10, IL10), cytosolic manganese superoxide dismutase (cytMnSOD) and prophenoloxidase (proPO). After 24 h of challenge, results revealed that the relative expression level of IL-17, IL-10, cytMnSOD and proPO were increased by 2.8, 2.5, 2.2 and 1.9 folds, respectively (Figure 5.1.6). The expression level of cathepsin L (PcCTSL) and interleukin 8 (IL-8) genes were relatively static during post challenge test.

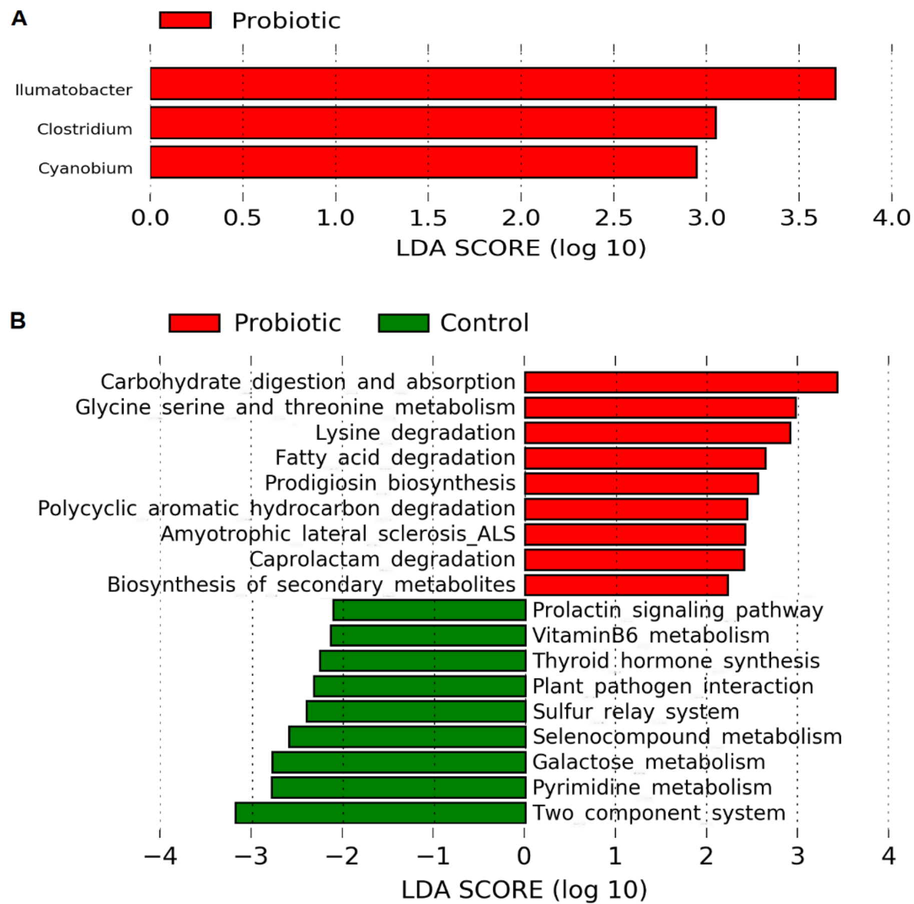

Figure 5.1. 5. (**A**) Indicator bacterial genus in two different conditions (control and probiotic) with LDA value 2.0 or more; (**B**) Predicted metabolic pathways in two different fed groups revealed from 16S rRNA sequence data using Piphillin and LEfSe packages.

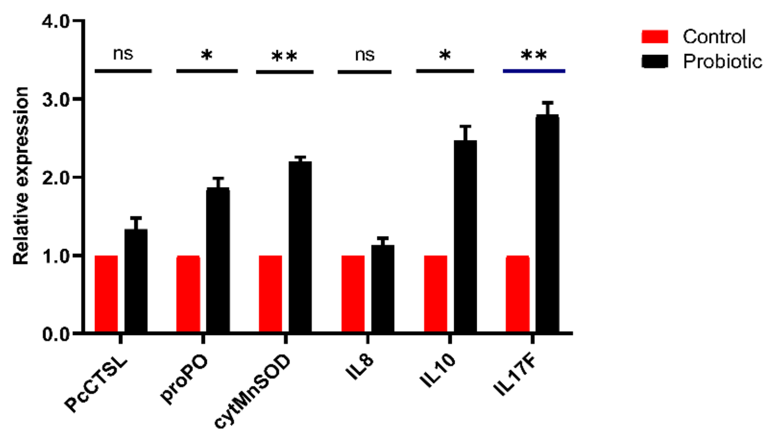

Figure 5.1. 6 Bar plot showing the relative expression level of immune genes in marron intestine tissue after feeding trial. *Significantly different at α-level of 0.05. **Significantly different at α-level of 0.005.

Table 5.1. 2 Diversity index (Mean ± SE) of bacterial genus in marron gut after trial

Condition	OTUs (SE)	Shannon (SE)	Simpson (SE)	Fisher alpha (SE)	Chao1 (SE)
Control	15.8 (1.9)	1.51 (0.1)	0.69 (0.1)	7.59 (0.2)	86.74 (5.8)
Probiotic	30.3 (11.5)	3.19 (0.1)***	0.82 (0.1)**	16.7 (2.1)*	128.48 (8.6)*

5.3(1).6. Survival rate

At 24 and 96 h post-challenge, all marron injected with *V. mimicus* and PBS remained alive and started to respond to the feed given on day 5. The PBS injected marron, however, remained actively responsive to the feed given earlier (day 3) than bacteria injected marron. No signs of diseases were observed in any of the injected marron.

5.3(1).7. Data availability

The raw FASTQ files are currently available in National Centre for Biotechnology Information (NCBI) BioProject under the accession number PRJNA515886.

5.4(1). Discussion

Probiotics as a dietary supplement have been in vogue for past 10 years for crayfish aquaculture to promote growth, disease resistance and stress tolerance (Ambas et al., 2017, Ambas et al., 2013, Li et al., 2009, Zheng et al., 2017a). Most of the earlier researches on probiotics studies in aquatic animals were performed with the dietary inclusion of *Bacillus* and *Lactobacillus* species (Ambas et al., 2017, Ambas et al., 2013, Kongnum and Hongpattarakere, 2012, Zheng et al., 2017a). However, a very few studies highlighted the importance of using *C. butyricum* as possible dietary probiotic candidate for crayfish (Ramírez et al., 2017). Results conducted with *C. butyricum* as dietary probiotic supplements demonstrated positive influence on growth and immune parameters of fish, chicken and piglet (Abdel-Latif et al., 2018, Chen et al., 2018, Gao et al., 2013, Pan et al., 2008, Song et al., 2006). Reports also have shown that *C. butyricum* supplementation promote growth performance, body protein content of white leg shrimp, *Lotopenaeus vannamei* fed (Duan et al., 2017). Based on the findings of Duan et al. (2017)

study, we aimed to analyse the effects of *C. butyricum* on growth, microbial composition in gut and immune response of marron for the first time. Consistent with previous study by Duan et al. (2017), we found significant ($P<0.05$) improvement of growth and protein content in tail muscle of marron after 42 days of feeding trial. Besides this, *C. butyricum* supplementation in the diet also led to significantly increase the lysozyme and total haemocyte counts (THC) of haemolymph, indicating the possibility of using *C. butyricum* as a probiotic supplements in marron aquaculture.

Intestine of crayfish is regarded as the centre for digestion and absorption of nutrients whereas microbial communities present in the distal intestine play vital role in digestion and immunity (Duan et al., 2017). Besides the available core microbiota, enrichment with some bacterial communities facilitate to enhance the growth and immune performance of crayfish (Hoseinifar et al., 2018, Li et al., 2018a). *C. butyricum* is a spore-forming butyric acid producing bacteria, common in animal gut that has many beneficial characteristics to be used as probiotic in poultry and aquaculture industry (Pan et al., 2008, Takahashi et al., 2018, Van Immerseel et al., 2010). Butyric acid has been widely used for animal nutrition owing to its positive effects on growth, intestinal digestion, absorption, metabolism and disease resistance (Bourassa et al., 2016, Levy et al., 2015, Robles et al., 2013). The dietary supplementation of *C. butyricum* in present study significantly ($LDA>2.0$, $P<0.05$) increased the abundance for *Clostridium* genus in the marron gut leading to enhanced growth and immune performance. In addition to *Clostridium*, the probiotic supplement also increased the richness of genus *Cyanobium* and *Illumatobacter*. Both of these bacteria identified from the gut and water demonstrated to have positive influence on remediation of water quality through the detoxification of pollutants and minimization of hypoxia (Homonnay et al., 2012, Matsumoto et al., 2013, Naoum, 2016, Wilhelm et al., 2014, Yamaguchi et al., 2016). The interactions among bacterial communities at phylum to species level are very complex, a single probiotic bacteria can influence the growth of whole microbial

communities in the intestine of fish (Stubbendieck et al., 2016). A probiotic bacteria can influence a wide range of biological processes in fish and crayfish including digestive enzymes, immune systems (phagocyte activity, clearance efficacy), pH of gastrointestinal tract and nutrient availability (Nayak, 2010b, Sullam et al., 2012). And hence, in a study, inclusion of single probiotic bacteria *Bacillus subtilis* in tilapia (*Oreochromis niloticus*) culture significantly ($P<0.05$) modulated the diversity of six genus (Giatsis et al., 2016), a very similar findings to present study. At genus level, *C. butyricum* supplement significantly ($P<0.05$) reduced the *Vibrio* and *Aeromonas* abundance in marron gut. *Vibrio* species has been associated with a number of diseases in crayfish and also considered as emerging pathogen for marron (Ambas et al., 2013, Bean et al., 1998, Momtaz et al., 2019). *Aeromonas hydrophila* is widely reported as the pathogen for many crayfish species including signal crayfish, *Pacifastacus leniusculus,* and freshwater prawn, *Macrobrachium rosenbergii* (Abdolnabi et al., 2015, Jiravanichpaisal et al., 2009). Therefore, reduction of abundance for these two bacteria from the fish gut represents positive impact of probiotic diet on marron gut.

Although the use of probiotic bacteria for the growth and development of aquatic animals are widespread, however, the mode of action of these bacteria in crayfish gut is yet not clear. To investigate the effects of dietary *C. butyricum* supplementation on marron metabolic pathway, we applied simple, commonly used and straight-forward online tool, Piphillin (Iwai et al., 2016). The analysis predicted significantly higher activities for protein and energy metabolism, detoxification and secondary metabolites synthesis in the probiotic fed group. Furthermore, the positive effects of *C. byturicum* on white shrimp growth, as reflected in terms of significant increase in enzymatic pathways activities for the metabolism of carbohydrate and protein were recorded (Duan et al., 2017). Consistent to this, our results predicted up-regulation of carbohydrate metabolism and absorption, amino acids (glycine, serine, threonine and lysine) metabolism and fatty acid metabolism. In addition, we also projected increased activities for

biosynthesis of secondary metabolites including prodigiosin, degradation of toxic compounds including organic (polycyclic aromatic hydrocarbon, PAHs) and synthetic (caprolactam) waste. High PAHs concentration is harmful for aquatic life as they can persist for long time without being degraded by the natural system while microbial mediated biological degradation has been reported in the aquatic systems (Takarina et al., 2013). The present study was designed to nurture marron without any filtration system, rather 30% water exchanged regularly. The untreated organic waste accumulated from the faecal and excess feed therefore mostly settled in the button of the tanks. The up-regulation of degradation pathway suggests bacterial decomposition of toxic organic waste might also be associated with higher growth in the probiotic fed marron.

The profiling of immune responsive genes post bacterial challenge test were performed to understand the role of probiotic feeding with the infection. Interleukins (IL) are the major class of cytokines associated with the immunity of crayfish (Jiang et al., 2015a). Dietary supplementation of probiotic bacteria modulated the expression level of cytokine genes in fish (Panigrahi et al., 2011, Selim and Reda, 2015, Zokaeifar et al., 2012). Present study also revealed significant upregulation of IL-17F and IL-10 expression in pathogen challenged marron. When pro-inflammatory cytokine (i.e., IL-17F) are up-regulated in challenged fish, the anti-inflammatory cytokine are also over-expressed to prevent the damage from inflammation (Miao et al., 2018). Up-regulation of cytokine genes after feeding probiotic bacteria have been widely authenticated as positive influence to enhance immune performance of fish (Miao et al., 2018, Panigrahi et al., 2007, Yang et al., 2014). Two other significantly overexpressed genes, prophenoloxidase (proPO) and cytosolic manganese superoxide dismutase (cytMnSOD) reported to inhibit the growth of some crayfish pathogen i.e. shrimp white-spot virus (WSSV), *Vibrio* spp., and *A. hydrophilla* (Liu et al., 2013). Boosting immune performance by inhibition of *Vibrio* and *Aeromonas* after dietary administration of *C.*

butyricum is in accordance with the finding of Liu et al. (2013). Cathepsin L (PcCTSL) showed discrepancy in the expression level with previous study (Dai et al., 2017) which possibly due to differences in the composition of feed and shorter length of the previous study (two weeks).

5.5(1). Conclusion

In summary, cellular and molecular based study revealed that supplementation of *C. butyricum* as a probiotic in feed improved growth performance, gut microbiota and immune response of marron. Therefore, *C. butyricum* could be utilised as potential probiotic supplement in the diet of marron. Further studies are required to reveal out the molecular mechanism of metabolic signalling pathways for improving the immune performance induced by *C. butyricum* in marron.

CHAPTER 5: Probiotics for marron aquaculture

EXPERIMENT 2: *Bacillus mycoides* supplemented diet modulates the health status, gut microbiota and innate immune response of freshwater crayfish marron (*Cherax cainii*)

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Abstract

This study evaluated the effects of dietary supplementation of probiotic bacteria, *Bacillus mycoides* on the gut microbiota and health status of marron (*Cherax cainii*) under laboratory conditions. A total of 40 marron were randomly distributed into eight different tanks and two different groups in quadruplicates. The control group were fed only basal marron pellet while the second group were fed 10^8 CFU/g of probiotic bacteria in the diet. After 60 days of feeding trial, the health and immune indices of marron including protein and energy in the tail muscle, and total haemocyte counts in the haemolymph were positively influenced (p<0.05) by the probiotic diet. The 16S rRNA sequence data revealed distinctly different microbial communities in the hindgut of marron with two different feeds after trial where *Vibrio* and *Holdemania* were the significantly abundant bacteria in the control and probiotic fed groups, respectively. In addition, the qRT-PCR analysis revealed upregulation of cytokine genes associated with the immunity and health status of crayfish indicated the beneficial effects of probiotic bacteria on the immune status of marron. Our results suggest that the diet supplemented with probiotic bacteria *B. mycoides* positively influenced the health status, immune indices and microbial composition of the marron gastrointestinal tract.

Keywords: Marron; probiotic; *16S rRNA* gene sequencing; diversity; expression level

5.1(2). Introduction

Aquaculture is one of the fastest and promising growing sectors around the globe. Augmenting the growth and boosting immune status of aquatic animals with safer dietary supplements are the major concerns for the farmers associated with aquaculture industries. Application of probiotics in aquaculture is now widespread as they have proven to be beneficial in improving fish health and immunity by modulation of gut microbiota (Hemarajata and Versalovic, 2013). An ideal probiotic candidate should be able to colonize rapidly the host gut, protect host gut against colonization by a broad range of pathogens, and exert positive regulatory effects on metabolic pathways and the expressions of cytokine genes (Asaduzzaman et al., 2018, Panigrahi et al., 2007). Notably, depending the species of probiotics being used and the aquatic animal species being tested, in addition to water quality, dosages and durations of feeding, the beneficial outcomes may differ (Lakshmi et al., 2013, Zorriehzahra et al., 2016).

Marron (*Cherax cainii*) is the third largest freshwater crayfish in the world and is endemic to Western Australia (WA) (Cole et al., 2019). Marron aquaculture is an iconic WA tradition and has several advantages over other crayfishes such as its disease-free status, capability of surviving long-distance shipping, high consumer demand and good market price (Ambas et al., 2013). The growth can be boost up by improving the quality of aquaculture water and finding of proper diet that can influence the growth and immune performance of marron (Ambas et al., 2015, Foysal et al., 2019a). To improve the production figure, use of probiotic as feed supplement to improve the growth and immune status of marron would be an worthwhile approach, however it is yet to determine which bacterial species would be a suitable probiotic candidate for WA marron and under what conditions should the probiotic be applied to maximise the beneficial outcomes.

Bacillus species have been reported for their potential probiotic attributes in human and aquatic organisms (Elshaghabee et al., 2017, Zokaeifar et al., 2012). Dietary supplements with *B.*

amyloliquefaciens and B. subtilis as probiotic candidates were shown to modulate the gut microbiota of turbot and tilapia, respectively (Giatsis et al., 2016, Jiang et al., 2018). In addition, the administration of *B. subtilis* to white shrimp had significantly up-regulated host immune and inflammatory genes (Zokaeifar et al., 2012). *Bacillus mycoides* is a gram-positive spore-forming environmental bacterium revealed to have beneficial plant growth promoting (PGP) and biocontrol activities (Yi et al., 2017b). While previous marron studies using *B. mycoides* as probiotic feed-additive were capable of boosting bacterial populations and several haemolymph parameters (Ambas et al., 2017, Ambas et al., 2013), no study has been performed to examine the alterations in the hindgut bacterial community and changes in immune-related gene expression.

Hence the study aimed to investigate the effects of *B. mycoides* supplemented diet on marron health and biochemical composition of tail muscle, identification of the microbial community composition of the hindgut, the changes in immune status and quantification of the expression levels of different genes associated with immune response.

5.2(2). Materials and methods

5.2(2).1. Experiment set-up

The experiment with 40 marron (68.8 ± 0.5g) was designed as described in general methodology (chapter 3.1). The marron were acclimated in eight tanks (five marron per tank) for 14 days in laboratory conditions before commencement of the experiment. Approximately 30% of water was exchanged from each tank on daily basis along with siphoning of faecal waste from the bottom of the tank.

5.2(2).2. Feed formulation and feeding frequency

Commercial fishmeal based basal feed named *marron pellet* (Table S4.1), was purchased from Glenn Forest (Perth, Australia). *B. mycoides*, provided by Department of Agriculture and Food, Western Australia, was cultured overnight on nutrient agar (Sigma-Aldrich, MO, USA) at 35

°C. For preparation of probiotic supplemented feed, the commercial basal feed was homogenized with *B. mycoides* bacterial culture at 10^8 CFU/g as described previously (Ambas et al., 2013). The resultant pellets from mince mixture were vacuum-dried at 37 °C for overnight and then stored at 4 °C until use. The proximate compositions of the feeding pellets (crude protein 29.9%, lipid 7.1% and gross energy 18.2 MJ kg^{-1}) were determined according to the standard protocols of Association of Official Analytical Chemists, AOAC (AOAC, 2006). Bacterial colony counting (1.05 x 10^8 CFU ml^{-1}) was performed on nutrient agar using spread plate method. During acclimation period , marron were fed only with basal diet while after acclimation, control group fed basal diet and probiotic group served *B. mycoides* supplemented diet at 1.5% of the total biomass per tank for 60 days.

5.2(2).3. Water quality analysis

In this study, suitable range of water quality for optimum growth of marron were maintained according to previous studies (Nugroho and Fotedar, 2013). The water quality including temperature, pH, dissolved oxygen (DO), nitrate (NO_3^-), nitrite (NO_2^-), phosphate (PO_4^-) and ammonia (NH_3) were monitored as described in general methodology (chapter 3.1).

5.2(2).4. Marron sampling

At the end of the experiment, one marron per tank (n=4) was selected for biochemical analysis including health and immune parameters. For microbiome analysis, two randomly selected marron from each tank were sacrificed, where the hindgut contents were extracted, homogenized and pooled together for genomic DNA extraction. Finally, one marron per tank was used to analyse the relative expression level of immune genes in the intestinal tissue. The whole intestinal tissue samples were stored in RNA Later (Sigma-Aldrich, Germany) at -80 °C until further processing.

5.2(2).5. Measurement of growth and haemolymph parameters

The weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) were measured according to calculations mentioned in general methodology (chapter 3.7). Immune parameters including haemolymph osmolality (HO) and total haemocyte counts (THC) were analysed according to methods described earlier, general methodology (chapter 3.2).

5.2(2).6. Measurement of biochemical composition in the tail muscle

The protein, lipid and energy content in the tail muscle were measured according to methods described in chapter 3.3 of general methodology.

5.2(2).7. Genomic DNA extraction and 16S rRNA gene sequencing

The extraction of bacterial genomic DNA from pooled samples, PCR amplification of v3v4 hypervariable regions, library preparation and pair-end sequencing were performed according to methods described in general methodology of chapter 3.4.

5.2(2).8. Gene expression analysis

Six genes linked to immune response of crustaceans were selected for the expression study (Foysal et al., 2019b, Jiang et al., 2015a). The primers for the selected genes are listed in [Table S5.1 (2)]. For RNA extraction, intestinal tissue samples stored initially in RNA Later (Sigma-Aldrich, Germany) were used for gene expression analysis as described in general methodology (chapter 3.6).

5.2(2).9. Bioinformatics

The initial quality of 16S sequencing reads was checked using FastQC (Andrews, 2010). Quality trimming was performed using Sickle with the following parameters: -q 20 –l 200 (Joshi and Fass, 2011). Following merging of overlapping paired-end reads using MeFiT with default parameters, Micca (version 1.7.0) was employed for filtering of chimeric sequences, *de novo* greedy clustering of 16S sequences into Operational Taxonomic Units (OTUs) at 97% similarity threshold and removal of singleton OTUs (Albanese et al., 2015, Parikh et al., 2016).

Taxonomic assignment of each OTU was performed using the Bayesian LCA-based taxonomic classification method with a 1e-100 cut-off e-value and 100 bootstrap replications, against NCBI 16S microbial database (Gao et al., 2017, Sayers et al., 2019). At each level, to accept a taxonomic assignment, we applied a minimum confidence score of 80. For phylogenetic tree construction, PASTA-aligned representative OTU sequences were subjected to FastTree (version 2.1.8) under the GTR+CAT model (Mirarab et al., 2015, Price et al., 2010). The rarefaction depth value was set at 3159 and subsequent computation of alpha and beta diversities was performed using QIIME (version 1.9.1) (Kuczynski et al., 2012). Briefly, alpha diversity was evaluated based on observed species and Shannon diversity indices, followed by the one-way analysis of variance (ANOVA) test for comparison between the control and probiotic-fed samples. Principle coordinates analysis (PCoA) based on weighted UniFrac distance metric was performed to visualize separation of samples (Dabdoub et al., 2016). Non-parametric statistical analysis of the distance metric was performed using ANOSIM with 1000 permutations.

5.2(2).10. Statistical and correlation analyses

LEfSe was employed to identify indicator bacterial genera in both groups. For statistical comparison of different marron health parameters between two feeding groups, student independent *t-test* was employed, with P-values less than or equal to 0.05 considered as statistically significant.

5.3(2). Results

5.3(2).1. *B. mycoides* supplemented feed improved marron's health

No significant differences in weight gain and specific growth rate was observed between the probiotic fed marron and the control group after 60 days. However, compared with the control group, in probiotic-fed marron significant improvements were observed in total haemocyte count (P-value<0.001), as well as both energy and protein content of the tail muscle (P-values of <0.01 and 0.045, respectively) (Table 5.2.1). Moderate increase in haemolymph osmolality

and decrease in tail muscle lipid content were (P-values of 0.07 and 0.059, respectively) were also measured in the probiotic-fed group.

Table 5.2. 1 Marron health and immune indices after trial

Parameters	Control	Probiotic-fed	P-value
HO (U/mL)	0.41 ± 0.01	0.44 ± 0.02	0.07
THC (cells/mL)	8.07 ± 0.18	9.62 ± 0.34	0.00018*
HM (%)	63.5 ± 2.24	51.75 ± 0.81	0.19
Lipid (%)	8.65 ± 0.39	7.88 ± 0.54	0.059
Energy (kJ/kg)	21244.1 ± 295.67	22297.82 ± 269.42	0.0019*
Protein (%)	85.76 ± 2.53	89.27 ± 1.16	0.045*
WG (g %)	37.78 ± 7	35.5 ± 2.4	0.56
SGR (g/day %)	0.69 ± 0.03	0.73 ± 0.01	0.5

Abbreviation: HO, haemolymph osmolarity; THC, total haemocyte count; HM, hepatopancreas moisture; WG, weight gain; SGR, specific growth rate. The (*) symbol denotes statistically significant difference at P-value less than 0.05.

5.3(2).2. *B. mycoides* supplemented diet enhanced microbial richness and diversity

A total of 57891 reads were generated for 8 samples. Following quality trimming and merging of overlapping paired-end sequences, 50111 sequences with an average length of 407.8 ± 2.2 bp were retained. The number of sequences per sample ranged from 3312 to 9445. Taxonomic classification of 84 *de novo* OTUs revealed 7 phyla and 22 genera, respectively, for further analysis. While alpha diversity analysis revealed no significant differences in bacterial species richness and diversity between both groups, PCoA based on weighted UniFrac distance metric demonstrated that the hindgut microbial communities of the control and probiotic-fed marron are distinctly different, when compared against each other (P-value=0.027, *R*=0.844) (Figure 5.2.1). Both control and probiotic-fed groups shared Firmicutes, Fusobacteria and

Proteobacteria as the major bacteria phyla in the hindgut microbiota. In control group, the relative abundances were detected at 3.1%, 3.1% and 64.2%, respectively (Figure 5.2.2 A). In probiotic-fed marron, there were significant increases of both Firmicutes and Fusobacteria, at 46% and 19.6%, respectively, and decrease of Proteobacteria (9.9%), in comparison to the controls (Figure 5.2.2 A). Additionally, three significant bacterial phyla including Chloroflexi, Cyanobacteria and Planctomycetes were also detected in probiotic-fed marron. At genus level, four distinguishing bacterial genera were identified. As compared with control group, in probiotic-fed marron there were significant enrichments in *Hypnocyclicus*, *Holdemania* and *Cyanobium*, and reduction in *Vibrio* (Figure 5.2.2 B).

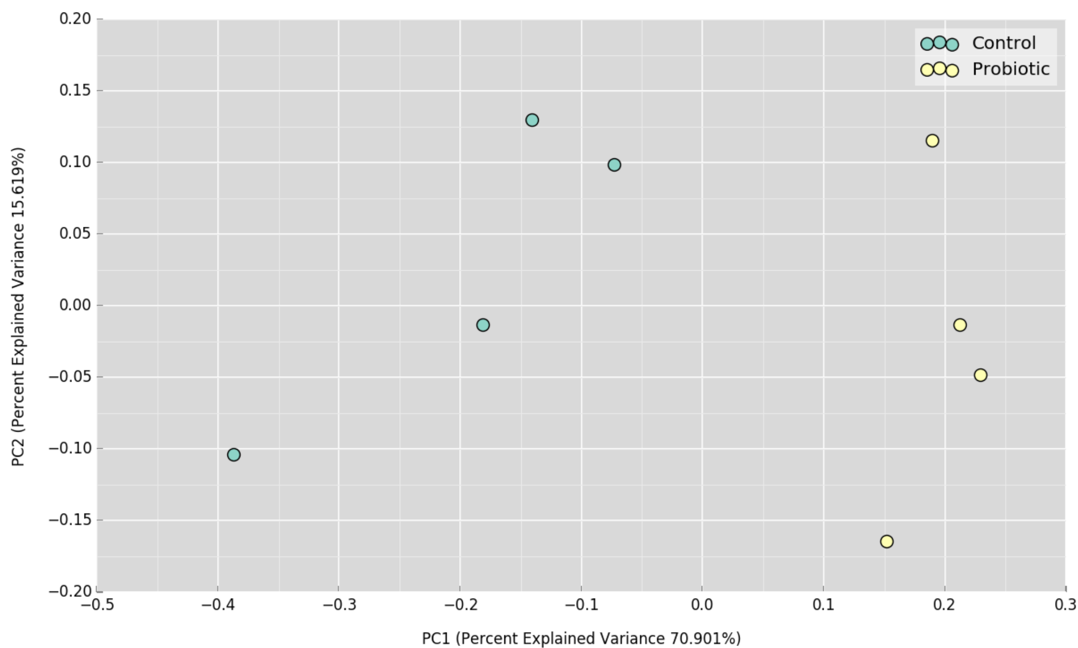

Figure 5.2. 1 PCoA plot based on weighted UniFrac distance metric in two different feeding conditions.

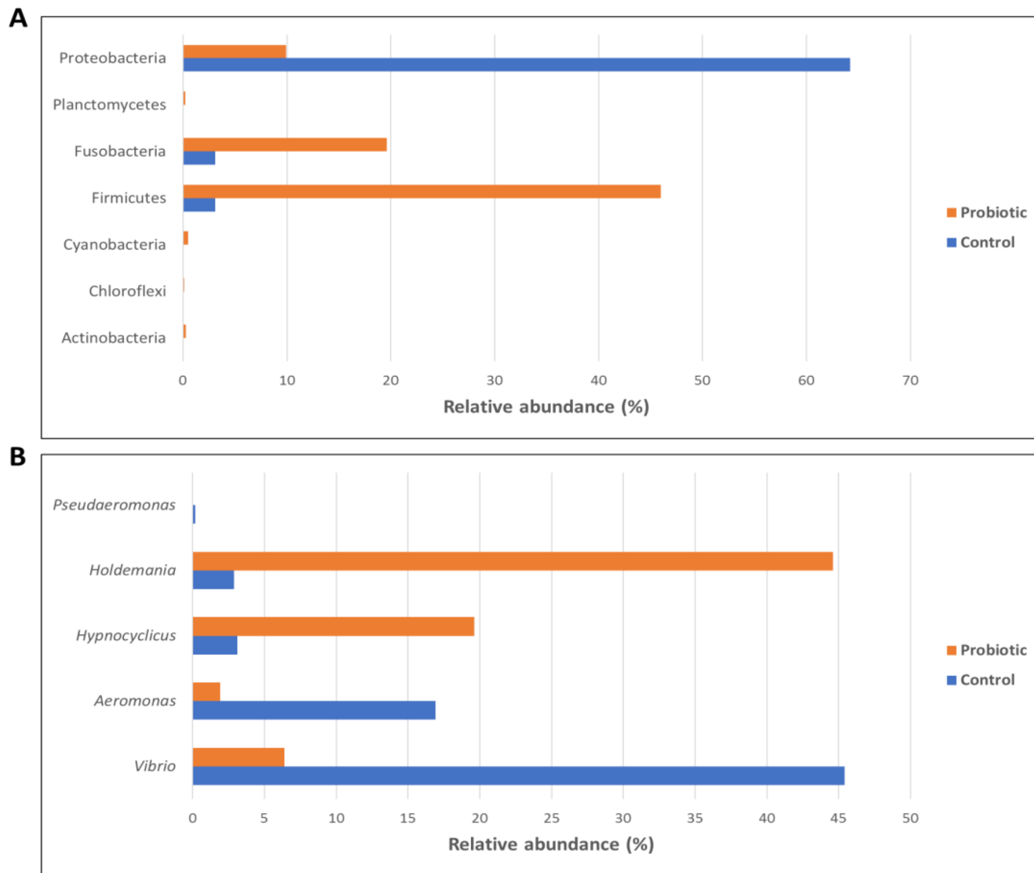

Figure 5.2. 2 Bacterial phyla (A) and genera (B) compositions in both control and probiotic-fed groups after trial.

5.3(2).3. *B. mycoides* supplemented diet influenced the expression level of genes

The relative expression level of seven different genes in control and probiotic-fed groups are shown in Figure 5.2.3. After two months of feeding trial, the probiotic-supplemented marron gut tissue displayed significant (P-values of <0.05) up-regulated expression of IL-10 (2.8 fold changes) and IL-17F (3.2 fold changes) while *Vg* gene expression was significantly down-regulated. No significant (P>0.05) expression changes was observed for IL-1β, IL-8 and TNF-α genes after feeding trial.

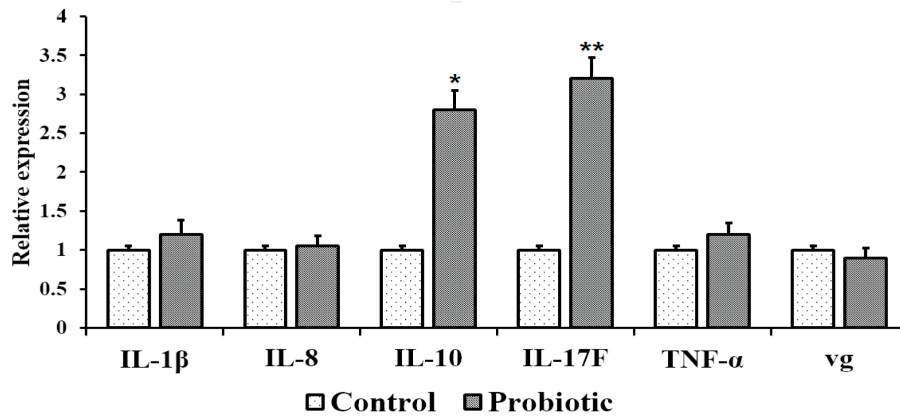

Figure 5.2. 3 Relative expression level of genes in marron gut tissue after feeding trial.

5.3(2).4. Water quality

The water quality parameters recorded during the study are as follows: temperature, 22 °C; pH , 8.0 ± 0.5; dissolved oxygen, 8.5 ± 0.5 mg/L; nitrate, 2.0 ± 0.5 mg/L; nitrite, 0.3 ± 0.15 mg/L; ammonia, 0.15 ± 0.05 mg/L; and phosphate, 0.4 ± 0.25 mg/L.

5.3(2).5. Data availability

The sequence data in FASTQ files are currently available at National Centre for Biotechnology Information (NCBI) BioProject under the accession number PRJNA558040.

5.4(2). Discussion

In this study we examined the efficacy of dietary *B. mycoides* as a prospective probiotic candidate for marron aquaculture. The dietary supplementation using *B. mycoides* significantly improved the health indexes of marron and modulated the microbial compositions in the hindgut. Haemolymph osmolality, THC, HM, protein, lipid and energy, and fat content are considered as important parameters to determine the immunity and health conditions of crayfish (Ambas et al., 2017, Sang et al., 2009). In this study, the specific growth rate and weight gain of marron were not significantly improved at the end of the experiment, in agreement with the findings of previous studies (Ambas et al., 2017, Foysal et al., 2019b, Saputra et al., 2019). Compared to other crayfish species, marron has a longer life cycle and that could be further extended under farming condition, hence a two-month feeding trial is

likely to be insufficient to properly examine the growth effect of dietary supplements on marron and a longer feeding trial should be considered in future study. On the other hand, both protein and energy contents were significantly higher in the probiotic-fed marron, that can be stated as beneficial because of their crucial role in growth, building muscle and repairing broken tissues in human (Pal et al., 2018, Tidwell and Allan, 2001). It is thought that the inclusion of probiotic *B. mycoides* in marron's diet could have boosted the tail muscle properties through competitive exclusion in the marron's gut (Rengpipat et al., 1998, Rengpipat et al., 2000). Furthermore, it is tempting to speculate that the presence of probiotic bacteria may facilitate the production of growth-promoting factors and better digestion and nutrient absorption in the gut (Gullian et al., 2004).

Dietary supplementation with *B. mycoides* also significantly altered the hindgut microbial communities in marron. No significant increase of *Bacillus* species was observed with dietary probiotic supplementation in present study. Because marron gut is not a natural host, hence probiotic bacteria could not able to colonize and might get expelled through faeces. Following probiotic treatment, a significantly higher Firmicutes abundance was observed. In a recent study where marron were fed with black soldier fly meal, a significant increase in Firmicutes abundance was also recorded and such increase was shown to improve marron health and immune conditions (Foysal et al., 2019b). At genus level, we found significant decreases in the abundance levels of *Vibrio* and *Aeromonas* in the probiotic fed marron. *Vibrio* species, especially *V. cholerae*, *V. harveyi*, *V. mimicus* and *V. parahaemolyticus*, have been described as emerging pathogen for crayfish including marron in near future (Ambas et al., 2013, Foysal et al., 2019a). Among these, *V. cholerae*, the causative agent of cholera (watery diarrhoea), can be transmitted from water and aquatic sources to human (Hossain et al., 2018). The genus *Aeromonas*, on the other hand, contains pathogenic members such as *A. hydrophilla*, *A. sobria*, *A. veronii* and *A. salmonicida* that are associated with a number of crayfish diseases (Foysal et

al., 2019c, Hayakijkosol et al., 2017). Notably, *Holdemania* and *Hynocyclicus* were also significantly enriched in the probiotic-fed marron. While the members of both genera have been detected in fish gut and ocean water, and were reported as non-pathogen, their functional role in fish or crayfish is yet to be explored (Borsodi et al., 2017, Roalkvam et al., 2015).

Probiotic supplementation in feed has been reported to have the ability to improve the innate immune response in fish by exerting positive regulatory effect on cytokine gene expressions (Miao et al., 2018, Selim and Reda, 2015). Consistent with previous findings, we found significant up-regulations of both IL-10 and IL-17F in the probiotic fed group. While up-regulation of pro-inflammatory IL-17F could play an important role in bacterial defence (Wang et al., 2014), the increased expression of anti-inflammatory IL-10 is likely to prevent damages from long term inflammation as a defensive mechanism (Miao et al., 2018). In this study, interestingly, we also found down-regulation of *Vg* mRNA level in probiotic fed marron. *Vg* is an important protein precursor and energy reserve for eggs development in oviparous animals (Matozzo et al., 2008). Non-homogenized male: female ratio in the tanks might affect the expression level of *Vg* gene in the probiotic group. Otherwise, the reduced expression of *Vg* gene may have a detrimental effect on marron fertility and this merits further investigation.

In conclusion, dietary supplementation with *B. mycoides* significantly improved the immunity and health status of marron, possibly by modulation of intestinal microbiota. However, the interaction mechanism of *B. mycoides* with marron gut and associated factors, and the effect of reduced Vg expression on marron reproductive health require further investigations.

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EXPERIMENT 3: *Lactobacillus acidophilus* and *L. plantarum* improve health status, modulate gut microbiota and innate immune response of marron (*Cherax cainii*)

(This chapter is published from Scientific Reports. https://doi.org/10.1038/s41598-020-62655-y)

Abstract

This study aimed to investigate the combined effects of two most potent probiotic bacteria *Lactobacillus acidophilus* and *Lactobacillus plantarum* on overall health and immune status of freshwater crayfish, marron under laboratory conditions. A total of 36 marron were distributed into six different tanks and two different feeding groups, control and probiotic-fed group. After acclimation, control group was fed with basal diet while probiotic group was fed 10^9 CFU/g of bacterial supplemented feed for 60 days. The results showed no significant differences in weight gain, however, probiotic feed significantly enhanced some hemolymph parameters and biochemical composition of tail muscle. Histology data revealed better hepatopancreas health and higher microvilli counts in the marron gut fed probiotic diet. The probiotic bacteria triggered significant shift of microbial communities at different taxa level, mostly those reported as beneficial for crayfish. The probiotic diet also enriched the metabolic functions and genes associated with innate immune response of crayfish. Further correlation analysis revealed significant association of some taxa with increased activity for hemolymph and immune genes. Therefore, dietary *Lactobacillus* supplementation can modulate the overall health and immunity as well as gut microbial composition and interaction network between gut microbiota and immune system in crayfish.

Keywords: Crayfish; marron; probiotic bacteria; health and immune response; gut microbiota; bioinformatics; correlations

5.1(3). Introduction

Aquaculture has become an important food sector for meeting the overall protein demand for growing population. The global consumption of crustaceans has increased greatly and especially those with live transport abilities are increasingly becoming popular (FAO, 2018, Saputra et al., 2019, Yi et al., 2018). Marron (*Cherax cainni*) is one of the largest freshwater crayfish farmed in Western Australia (WA) that has high nutritive value and widespread consumer preferences (Cole et al., 2019, Foysal et al., 2019a). In addition, long distance live transportation ability of marron further increases its international demand, and thus has become an ideal crayfish species for commercial farming (Ambas et al., 2013, Foysal et al., 2019b). However, the production of marron in WA has remained stagnant for a long time (Foysal et al., 2019b). Selection of proper diets and maintaining the optimum water quality are the two most crucial factors required for marron farming (DoF, 2010b, Foysal et al., 2019a, Foysal et al., 2019b). Although no disease outbreaks have yet been reported, the interest and intend of intensification in marron farming can expose marron to possible crayfish pathogens including *Vibrio*, *Aeromonas* and *Rhodobacter* (Ambas et al., 2013, Foysal et al., 2019a). In the past, several laboratory based trials have been conducted (Ambas et al., 2017, Ambas et al., 2013, Foysal et al., 2019b, Foysal et al., 2019d, Saputra et al., 2019), however, finding a suitable diet and identifying the beneficial bacteria that potentially can influence the growth and immune performance of marron has still remained unknown.

Towards sustainable development of aquaculture, the use of feed additives including probiotics, prebiotics, synbiotics, parabiotics and phytogenics in crustacean's diet to boost biological indices has gained extensive attention from the researchers and farmers. Probiotics are microorganisms associated with health and immune benefits for the host when administered in adequate amounts or numbers (Nayak, 2010a). Among the probiotic bacteria, lactic acid bacteria (LAB) are considered as the most promising candidates for boosting the growth, gut

health, immune defence mechanism against pathogenic bacteria (Gatesoupe, 2007, Martínez Cruz et al., 2012, Ringø and Francois-Joel, 1998). *L. acidophilus* and *L. plantarum* are the two major bacterial species of LAB used as probiotics in aquaculture (Gatesoupe, 2007). Improving growth and immune performance with dietary incorporation of *Lactobacillus acidophilus* has been reported in Nile tilapia (*Oreochromis niloticus*) (Lara-Flores et al., 2003, Villamil et al., 2014), rainbow trout (*Oncorhynchus mykiss*) (Enferadi et al., 2018), common carp (*Cyprinus carpio*) (Adeshina, 2018), stripped catfish (*Pangasianodon hypophthalmus*) (Akter et al., 2019) and black swordtail (*Xiphophorus helleri*) (Hoseinifar et al., 2015) as well as in crustaceans; white shrimp (*Litopenaeus vannamei*) (Wang and Gu, 2010) and tiger shrimp (*Penaeus monodon*) (Hoseinifar et al., 2018). *L. plantarum* is another important probiotic species that is known to produce various active compound like plantaricin with outstanding ability to counteract toxicity caused by various aquatic pathogenic bacterial species (Cebeci and Gürakan, 2003). *L. plantarum* has shown potential to be used as probiotic in Nile tilapia (Zhai et al., 2017), common carp (Kazuń et al., 2018, Soltani et al., 2017), rainbow trout (Soltani et al., 2019), silver pomfret (*Pampus argenteus*) (Gao et al., 2016a), African hybrid catfish (*Clarias gariepinus* Male × *Clarias macrocephalus* Female) (Butprom et al., 2013), and also in crustaceans including narrow clawed crayfish (*Astacus leptodactylus*) (Valipour et al., 2019), white shrimp (Kongnum and Hongpattarakere, 2012, Vieira et al., 2010) and giant fresh water prawn (*Macrobrachium rosenbergii*) (Dash et al., 2014). Studies also reported that combination of two or more probiotic bacteria including species from *Lactobacillus* can induce higher growth and immune performance of the host aquatic animals (Alishahi et al., 2018, Wang and Gu, 2010). However, despite the beneficial role of LAB in finfish and crustaceans, their effects on overall health and immune status of marron is still unknown.

Recent development in "omics" technologies has enabled in-depth analysis of feeding effects on health and immunity of fish and crayfish (Foysal et al., 2019d). In addition, advancement in

information technology, data analysis packages and repository system allows to correlate various data from several trials and make interpretation easier and comprehensive (Li et al., 2017a). This form of integrated data analysis packages can be used to explore the contributions of feed additives on growth performance, gut microbiota, innate immune response and disease resistance of crustaceans (Cheng et al., 2019a, Shui et al., 2020). The aim of the present study was, therefore to investigate the impacts of dietary *L. acidophilus* and *L. plantarum* on health status, hemolymph parameters, intestine morphology and microbiota and innate immune responses of marron.

5.2(3). Materials and methods

5.2(3).1. Ethics statement

Formal ethics approval is not necessary for the laboratory trial with invertebrates at Curtin University. However, the study was performed according to the guidelines of Animal Welfare Act, Western Australia and the Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2004).

5.2(3).2. Experimental set-up

The experiment with 36 marron (70.2 ± 0.6g) was designed as described in general methodology (chapter 3.1). The marron were acclimated into six tanks (6 marron/tank) for 7 days in laboratory conditions, then distributed into two different dietary groups, control and probiotic. During acclimation, both groups were served with standard basal diet (Table S4.1).

5.2(3).3. Bacterial culture, diet formulation and feeding

L. acidophilus and *L. plantarum* were purchased from Nature Way Probiotic (Warriewood, New South Wales, Australia) in powdered form. The bacteria were then cultured in MRS broth (Sigma-Aldrich, Germany) overnight at 37 °C. Bacterial cells were then harvested by centrifugation at 4000 rpm for 10 min, washed twice with phosphate buffer saline (PBS) followed by re-suspension in the same buffer. The colony forming units (CFU) of respective

bacteria was calculated following standard serial dilution method. One hundred microliter of bacterial broth from each dilution was cultured in MRS agar (Sigma-Aldrich, Germany), incubated for 48 h at 37 °C under anaerobic conditions. Subsequently the dilution containing 10^9 CFU/mL *L. acidophilus* and *L. plantarum* counts in culture plate was selected based on results of previous study on crayfish (Siddik et al., 2020b). The ingredients of basal diet (Table S4.1) was purchased from the commercial feed supplier (Glenn Forest, Perth, Australia). The probiotic diets were formulated and prepared at CARL following previously described method (Ramírez et al., 2017). Briefly, the ingredients were passed gently though 100 µm mesh sieve and rigorously homogenized to get uniform particle size. The suspension of *L. acidophilus* and *L. plantarum* in water were then added at 10^9 CFU/g of feed using sprayer until the bacterial suspension for 1 kg diet was finished. Feeding pellet was prepared using a mince mixture followed by vacuum drying oven at 37 °C for overnight and then stored at 4 °C in air tight jars before the use. The proximate composition of final diet (Crude protein 29.9%, lipid 7.4%, gross energy 18.9 Mj/kg) was determined according to the method of Association of Official Analytical Chemists, AOAC (AOAC, 2006). Bacterial CFUs, 1.01×10^9 (CFU/g) for *L. acidophilus* and 1.05×10^9 (CFU/g) for *L. plantarum* were calculated on MRS agar plate. Throughout the trial, marron were fed based on their satiation level, once every day at 6 PM for 60 days at a rate of 1% of total biomass per tank (Ambas et al., 2017). Control group fed basal diet (Table S4.1) while probiotic group served with *Lactobacillus* supplemented diet.

5.2(3).4. Sampling

For analysis of haemolymph parameters, health and immune indices, 12 marron, two randomly selected from each tank were used. For DNA extraction and microbiome analysis, total 24 marron, four randomly selected marron from each tank were selected. The hindgut content of two randomly selected marron from each tank were homogenized and pooled together, eventually prepared two pools of sample from each tank and six for each treatment. Finally,

for gene expression analysis, the whole intestine of two randomly selected marron from each tank (n = 12) was used for RNA extraction.

5.2(3).5. Growth parameters

The weight gain (WG), specific growth rate (SGR), feed intake (TFI) and feed conversion ratio (FCR) were measured according to calculations mentioned in chapter 3.7 of general methodology.

5.2(3).6. Haemolymph parameters

Haemolymph parameters including osmolality (HO), lysozyme and total haemocyte counts (THC) were analysed according to methods described in general methodology (chapter 3.2).

5.2(3).7. Biochemical composition of tail muscle

The biochemical composition of tail muscle including protein, lipid and energy content were measured according to methods described in chapter 3.3 of general methodology.

5.2(3).8. Hepatopancreas and intestinal mucosal morphology

After 60 days of feeding trial, six randomly selected marron were selected from each treatment for histology of hepatopancreas and scanning electron microscopy of intestinal microvilli. Hepatopancreas samples were dehydrated in ethanol, equilibrated in xylene and embedded in paraffin wax following standard histological techniques. By using a rotary microtome, a section of approximately 5 µm in size was cut from each paraffin block and stained with Hematoxylin-Eosin (H&E) solution followed by histological examination under a light microscope (BX40F4, Olympus, Tokyo, Japan).

For SEM analysis, intestinal samples were prepared according to previously described standard method for marron with slight modifications (Saputra et al., 2019). Briefly, the dissected transverse segments (~1 mm long) of intestinal specimens were bathed immediately in 3% glutaraldehyde containing 0.1 M cacodylate buffer followed by overnight (24 h) incubation at

4°C. Samples were then rinsed briefly with cacodylate buffer and PBS prior to secondary fixation using 1% OsO_4, followed by three consecutive washes in deionized distilled water for 5 min, followed by dehydration in ethanol (50, 70, 95 and 100% at 250W, 5 min each). The samples were dried by washing in a series of 50%, 75% and 100% (twice) hexamethyldisilizane (HMDS) in ethanol solutions for 5 min. The processed samples were then dried at room temperature and mounted on a stub using carbon tape, coated with gold and viewed under a pressure scanning electron microscope (SEM, model Phillips XL 30, FEI, Hillsboro, OR, USA). The inner part of the digestive tract was assessed under 5000 X magnification for distribution and densities of microvilli. The images acquired from SEM were used to calculate the number of hindgut microvilli by counting and averaging microvilli on each slide (n = 3) using digital imaging software (Adobe Photoshop CC 2015, Adobe System Incorporated, USA).

5.2(3).9. Illumina sequencing

The extraction of bacterial genomic DNA from pooled samples, PCR amplification of v3v4 hypervariable regions, library preparation and pair-end sequencing were performed according to methods described in general methodology of chapter 3.4.

5.2(3).10. Gene expression analysis

In this study, ten (10) genes (Table S5.1.3, Appendix 4) associated with innate immune response of crayfish were selected for expression analysis after trial (Dai et al., 2017, Foysal et al., 2019b, Jiang et al., 2015a, Liu et al., 2013). For RNA extraction, intestinal tissue samples stored initially in RNA Later (Sigma-Aldrich, Germany) were used for gene expression analysis as described in general methodology (chapter 3.6).

5.2(3).11. Bioinformatics

Unless any modifications mentioned, the “pipelines” for downstream sequence analysis and bioinformatics were used according to general methodology in chapter 3.4 with default

parameters. Then the rarefaction depth point was set at 13950 bp and subsequent measurement of alpha beta diversities were performed in QIIME pipeline (version 1.9.1) (Kuczynski et al., 2012) and different R packages. Briefly alpha diversity was calculated in terms of species richness and Shannon index using student independent *t-test*. Non-parametric statistical analysis of the distance metric was performed with 1000 permutations using ANOSIM. The beta diversity analysis was performed as nonmetric multidimensional scaling plot (NMDS) using permutational analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarity matrix. Differential abundance at genus level, global similarity, pairwise microbiome-metadata correlations, multivariate regression were performed using LEfSe (Linear Discriminant Analysis Effect Size) and MMCA microbiome pipeline (Ni et al., 2020, Segata et al., 2011). Differentially expressed metabolic pathways in two different groups based on 16S rRNA data were predicted using Piphillin (http://secondgenome.com/Piphillin) in support of KEGG database (May, 2017 release), BioCyc 21.0 and LEfSe (Iwai et al., 2016, Segata et al., 2011). In all cases, p value of <0.05 was considered as statistically significant.

5.2(3).12. Data availability

The raw sequence data in FASTQ files are currently available at National Centre for Biotechnology Information (NCBI) BioProject under the accession number PRJNA579035.

5.3(3). Results

5.3(3).1. Growth and health parameters

At the end of 60 days of feeding trial, growth was not significantly different between probiotic fed marron and the control. The probiotic fed marron did efficiently (P<0.05) utilise the feed. The THC was positively influenced (P<0.05) by the probiotic diet while no impacts on osmolality and lysozyme were recoded. The probiotic diet significantly (P<0.05) improve the tail muscle crude protein and gross energy while the crude lipid content remained unchanged (Table 5.3.1).

Table 5.3. 1 Health parameters of marron after 60 days of feeding trial

Parameters	**Control**	**Probiotic**	**P-value**
Weight gain (g %)	6.55 ± 0.41	7.35 ± 0.55	0.059
Specific growth rate (g/day %)	0.69 ± 0.04	0.86 ± 0.04	0.001
Feed conversion ratio	4.36 ± 0.17	3.83 ± 0.07	0.045
Muscle crude protein (%)	85.40 ± 0.75	88.2 ± 0.46	0.007
Muscle crude fat (%)	8.50 ± 0.19	8.40 ± 0.11	0.278
Muscle gross energy (MJ/kg)	20.44 ± 0.21	20.84 ± 0.09	0.029
Hemolymph lysozyme (U/ml)	0.48 ± 0.02	0.51 ± 0.02	0.184
Haemolymph osmolality (mOsm/kg)	406.3 ± 2.65	408.8 ± 3.61	0.228
Total haemocyte count (cells/ml)	8.40 ± 0.23	10.20 ± 0.64	0.002

5.3(3).2. Gut microvilli and hepatopancreas structure

Histologically, healthy and balanced structure of hepatopancreas were found in the probiotic fed marron characterized by normal hexagonal hepatocytes and rare cytoplasmic vacuolization. Also, the lumen of hepatopancreatic tubule and hepatocyte vacuole were found comparatively smaller in probiotic fed group when compared to control (Figure 5.3.1 A). SEM analysis showed that the probiotic diet enhanced the number and density of microvilli in the distal gut of marron (Figure 5.3.1 B).

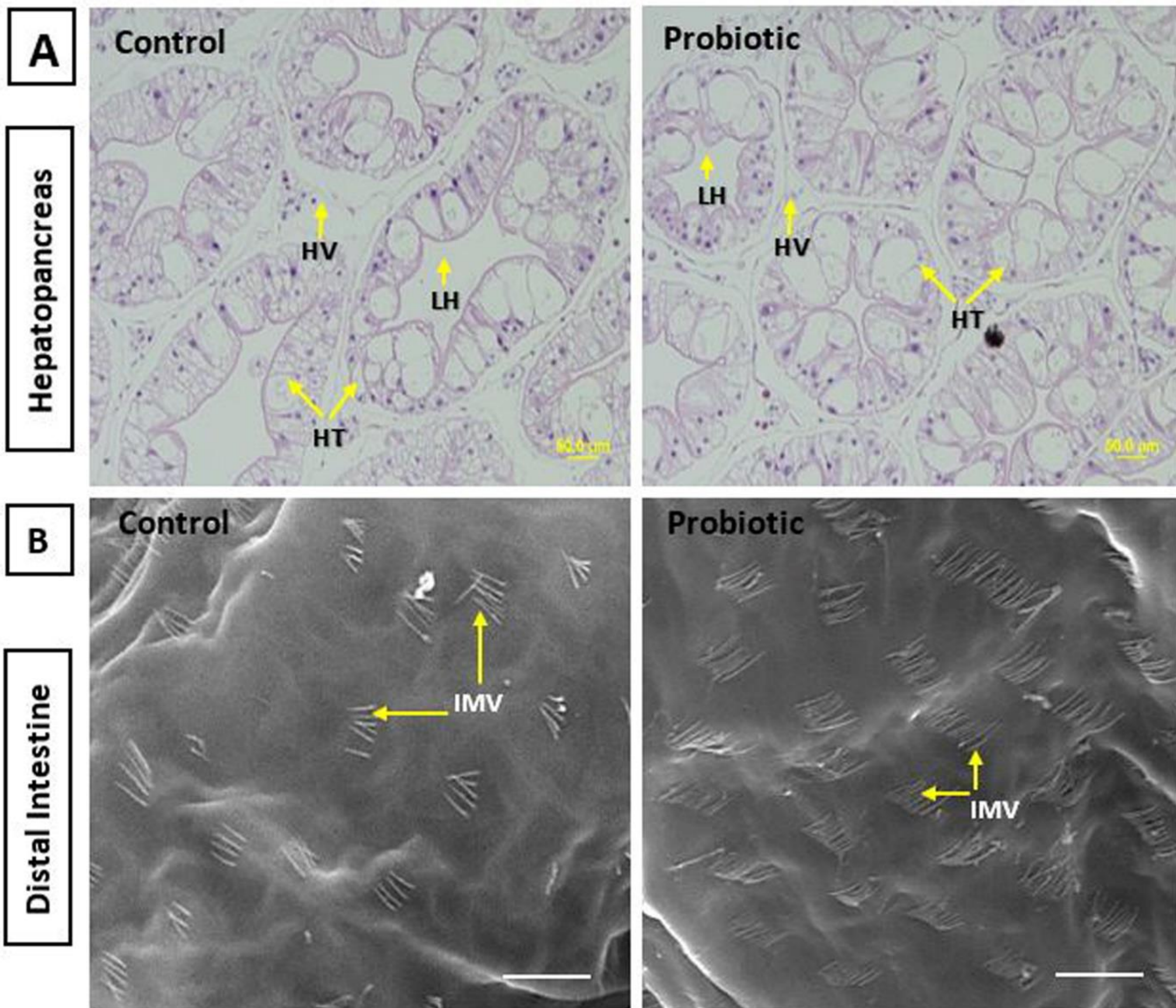

Figure 5.3. 1 (**A**) Hepatopancreatine and intestinal morphology of freshwater crayfish, *Cherax cainii* fed control and probiotic diets for 60 days. Images are arbitrarily chosen from the micrograph observed in control and probiotic fed marron. Transverse section of hepatopancreas tubules showing reduced hepatopancreatic lumen and vacuole in probiotic fed group (H&E stain at 40×, scale bar =50µm). (**B**) High magnification (x 50,000) electron micrograph showing increased number of microvilli in the distal gut of marron fed probiotic diet (scale bar=20 µm). (HT: hepatopancreatic tubule, HV: hepatocyte vacuole, LH: lumen of hepatopancreatic tubule, IMV: intestinal microvilli).

5.3(3).3. Alpha-beta diversity of gut microbiota

After filtering, 12 samples generated 804,713 quality reads that were classified into 984 OTUs, 16 phyla and 182 genera. The rarefaction curve indicated that each sample was sequenced at a higher depth and nearing about saturation to capture enough diversity (Figure 5.3.2 A). The rarefaction curve also showed that samples from probiotic fed marron had higher bacterial population than the control group. Further analysis revealed a significant increase (P<0.001) of alpha diversity in terms of observed species, Shannon, Simpson and Chao1 diversity indices in the probiotic fed marron gut (Figure 5.3.2 B-E). A NMDS plot based on the relative abundance of bacterial OTUs and Bray-Curtis dissimilarities is shown in Figure 5.3.2 F. An R^2 value of 0.82238 and P value of 0.002 revealed significant beta dispersion of bacterial communities and distances between the control and probiotic fed marron gut.

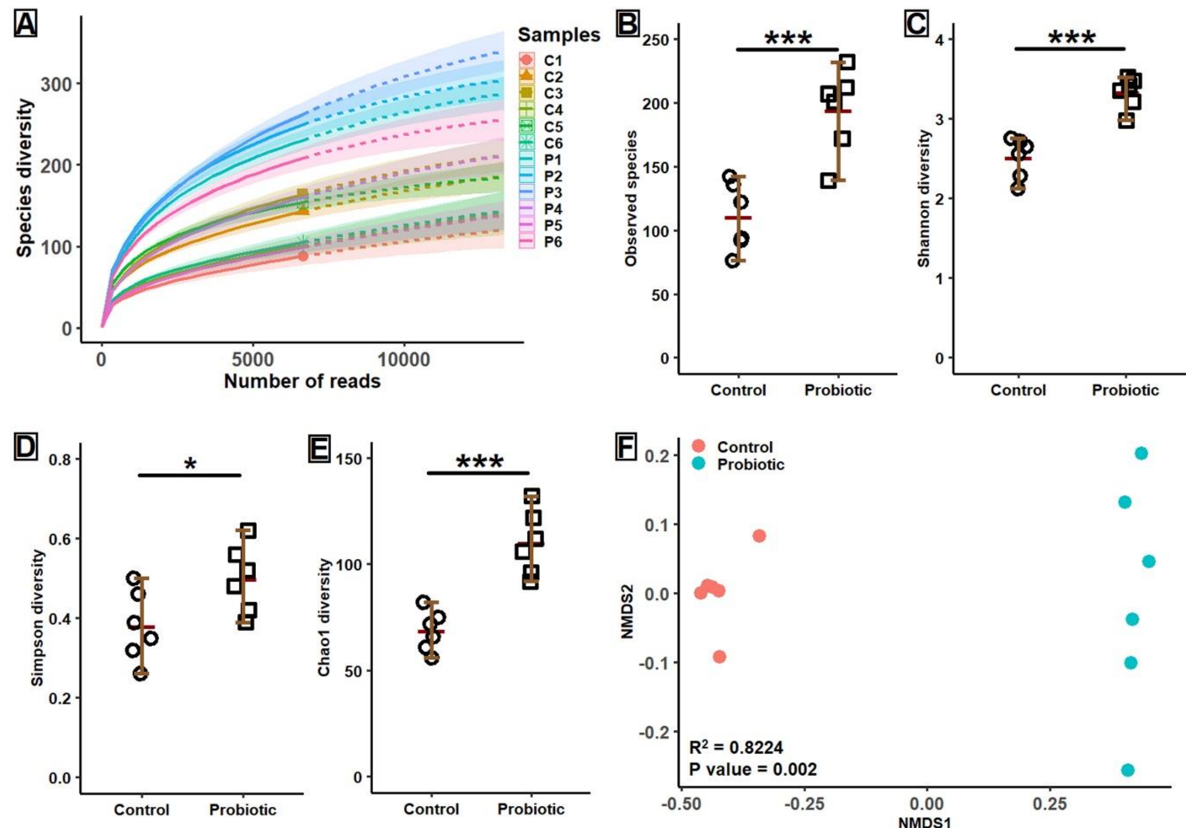

Figure 5.3. 2 Alpha-beta diversity measurements of gut microbiota. (**A**) Rarefaction curve presenting the saturation level of sequencing in terms of observed species (**B-E**) Alpha diversity measurements in terms of observed species, Shannon, Simpson and Chao1 diversity.

(**F**) Non-metric multidimensional scaling (NMDS) plot showing the clustering of samples. *Significant at α-level of 0.05. ***Significant at α-level of 0.001.

5.3(3).4. Differential abundance of microbial communities

Proteobacteria (87.7%) was the most dominant (P<0.05) phylum in the control group, followed by Tenericutes (11.12%) and Firmicutes (1.1%) phyla. While in the probiotic fed group, the relative abundance for Tenericutes (14.1%) and Bacteroidetes (1.2%) was significantly (P<0.05) higher along with Actinobacteria, Planctomycetes and Verrucomicrobia (Figure 5.3.3). Use of non-parametric *t-test* at 0.05 level of significance identified 9 genera including *Lactobacillus* that significantly were enriched in the probiotic fed marron. The other genera were AlphaI cluster, *Luteolibacter*, *Paracocccus*, Pir4 lineage, *Pirellula*, *Reyranella*, *Planctomyces* sp. SH-PL14 and *Terrimicrobium* (Figure 5.3.4). Further analysis using LEfSe identified 17 taxa that were differentially expressed (P<0.05) in two different groups. Among these, 10 including phyla Teniricutes, Firmicutes, class Bacilli, and genus *Candidatus* Hepatoplasma and *Lactobacillus* were significantly enriched in probiotic group while Proteobacteria (phylum) and *Vibrio* (genus) were the dominant bacteria in the control group (Figure 5.3.5).

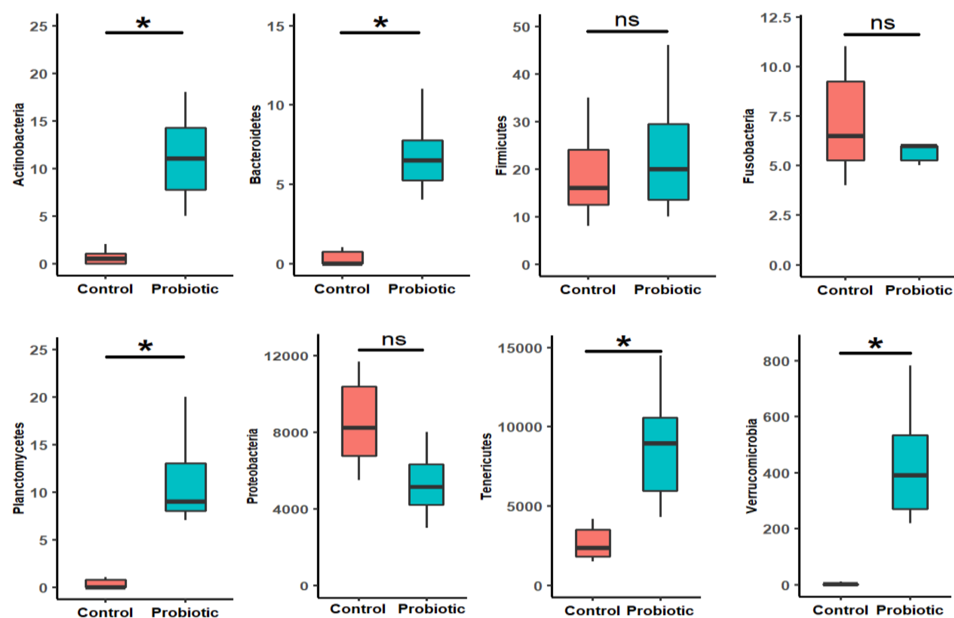

Figure 5.3. 3 Differential abundance (P<0.05) of bacterial communities in control and probiotic fed groups at phylum level.

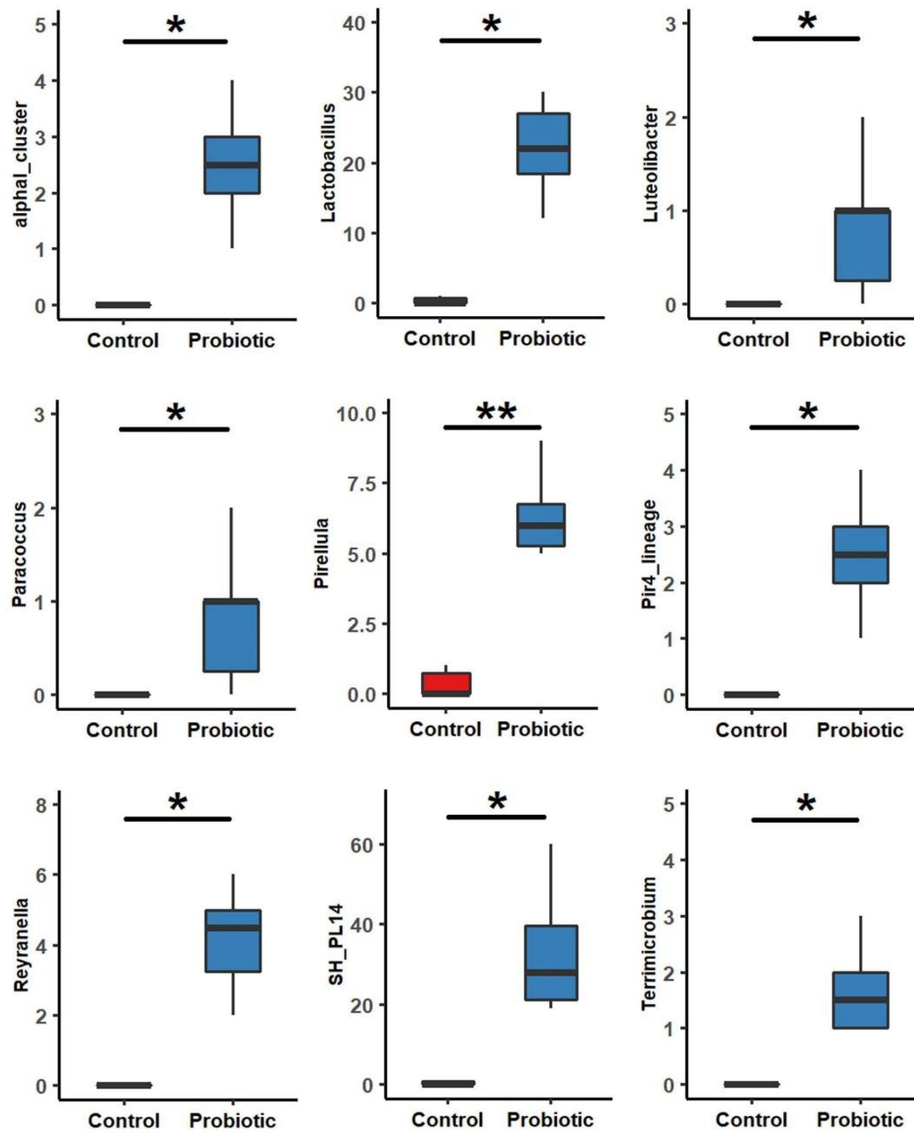

Figure 5.3. 4 Differential abundance (P<0.05) of bacterial communities in two different feeding groups at genus level.

5.3(3).5. Metabolic pathways and genes associated with immunity

The predicted metabolic pathways from 16S rRNA data using Piphillin and KEGG database showed that probiotics significantly influenced the enrichment of pathways related to amino sugar and nucleotide sugar metabolism, interleukin 17 signalling pathway and quorum sensing, whereas, glyoxylate and dicarboxylate metabolism was influenced in the absence of probiotics

(Figure 5.3.6 A). The results of qRT-PCR from intestine tissue showed significant up-regulation of cytokine gene families (IL1β, IL8, IL10 and IL17F), proPO, and cytMnSOD in the probiotic fed marron. However, significant effects ($P<0.001$) were observed for IL1β (3.8 fold), IL10 (9.2 fold) and IL17F (14.8 fold), followed by ($P<0.005$) IL8 (6.5 fold) and proPO (4.6 fold), respectively. Significant ($P<0.05$) upregulation was also observed for cytMnSOD (4.0 fold) while the expression level was static ($P>0.05$) for TNF-α (1.7 fold), vg (1.8 fold), pcna (1.1 fold) and PcCTSL (1.4 fold) in the probiotic fed marron (Figure 5.3.6 B).

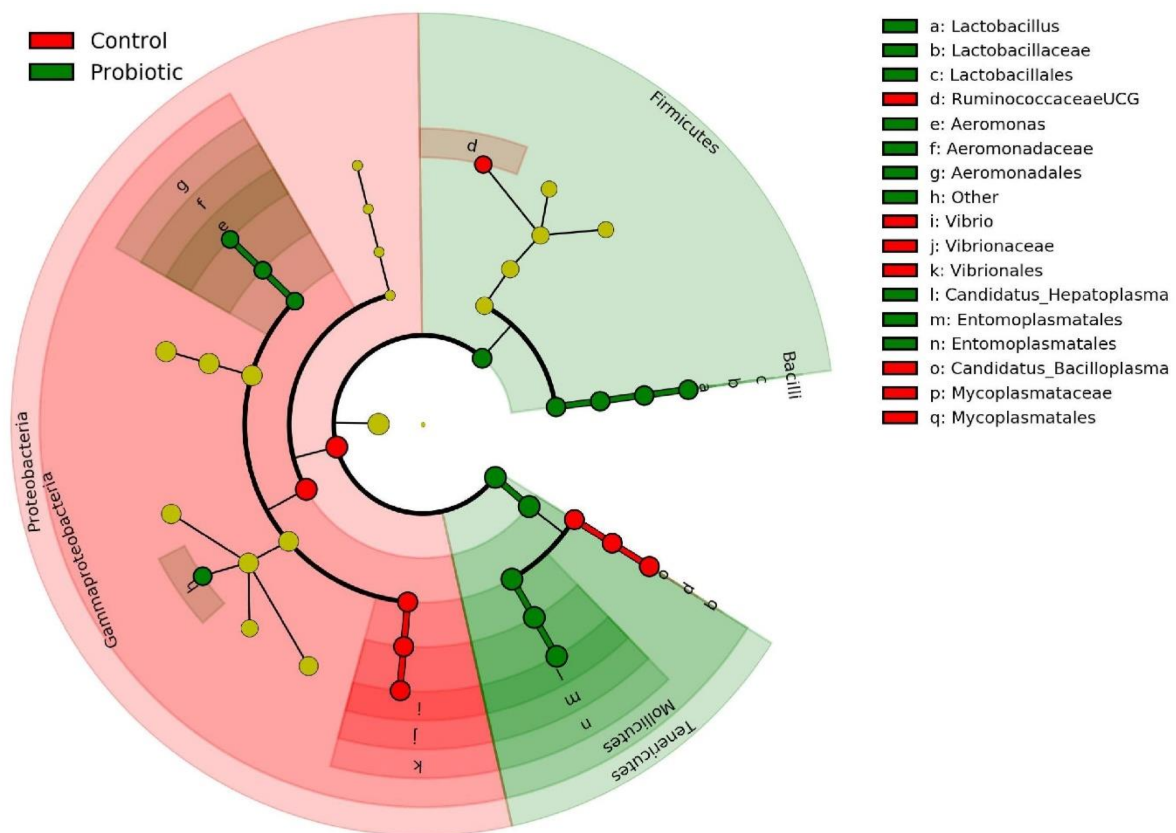

Figure 5.3. 5 Cladogram representing the indicator bacteria at different taxa level in control and probiotic groups with LDA score ≥ 2.0 and at 0.05 level of significance.

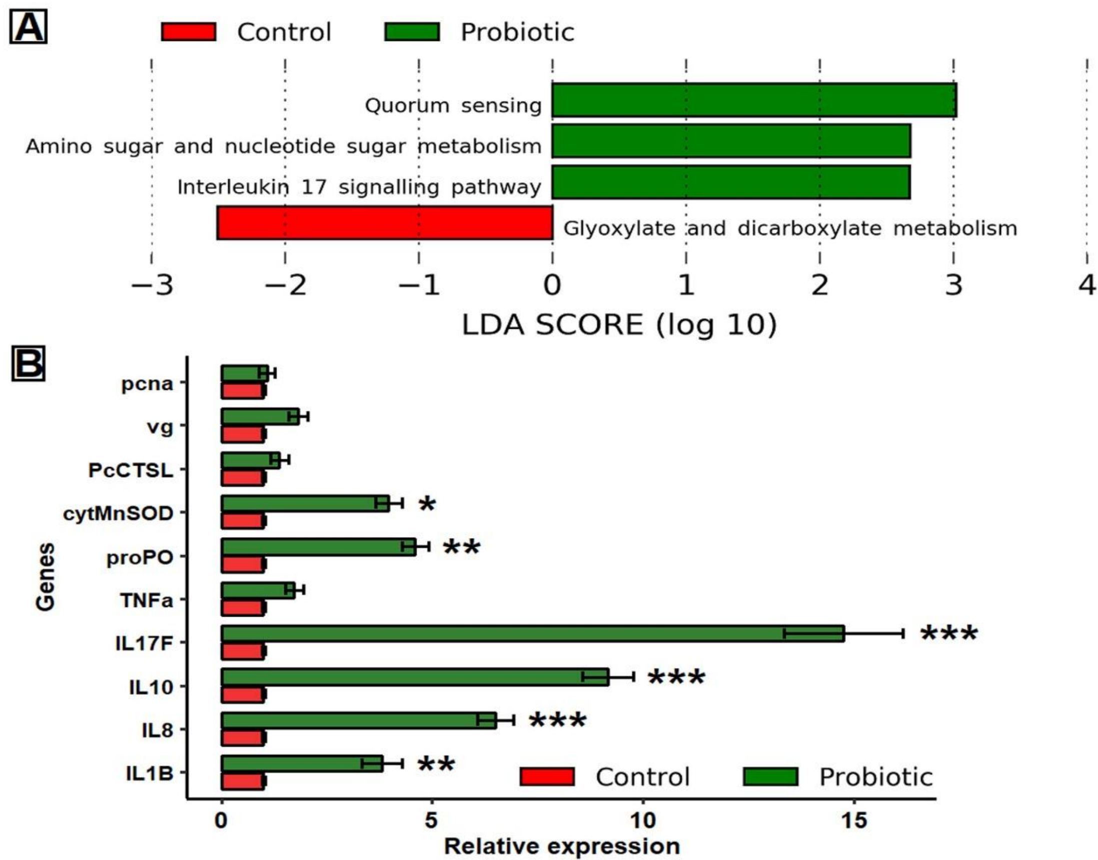

Figure 5.3. 6 (**A**) Differentially abundant metabolic pathways based on 16S rRNA data in control and probiotic fed marron. (**B**) Relative expression level (mean ± SE) of crustacean genes associated with innate immune response of marron. *Significant at α-level of 0.05; **Significant at α-level of 0.005; ***Significant at α-level of 0.001.

5.3(3).6. The role of microbial communities in health and immunity

Pairwise "Spearman" correlation analysis among the microbial abundance and metadata, including health and immune indices showed a significant positive correlation between enriched bacteria at different taxa levels and immune indices of marron. Most of the enriched bacteria in the probiotic fed marron including *Lactobacillus*, *Candidatus* Hepatoplasma, *Terrimicrobium, Pirellula*, *Reyranella* and *Luteolibacter* were found strongly correlated to up-regulation of immune genes (Figure 5.3.7 A). *Vibrio*, *Shewanella* and *Candidatus* Bacilloplasma were found negatively correlated to immune response while positively correlated to gross energy in the tail muscle. The correlation network also revealed strong

positive relationships between Firmicutes and Teniricutes with immune gene expression while hemolymph parameters correlated to Plactomycetes (Figure 5.3.7 B). In particular, *Candidatus* Hepatoplasma was strongly associated with IL17F and cytMnSOD upregulation, *Lactobacillus* and *Pirellula* with IL10 expression, *Reyranella* amd *Luteolibacter* with prePO up-regulation in the probiotic fed marron.

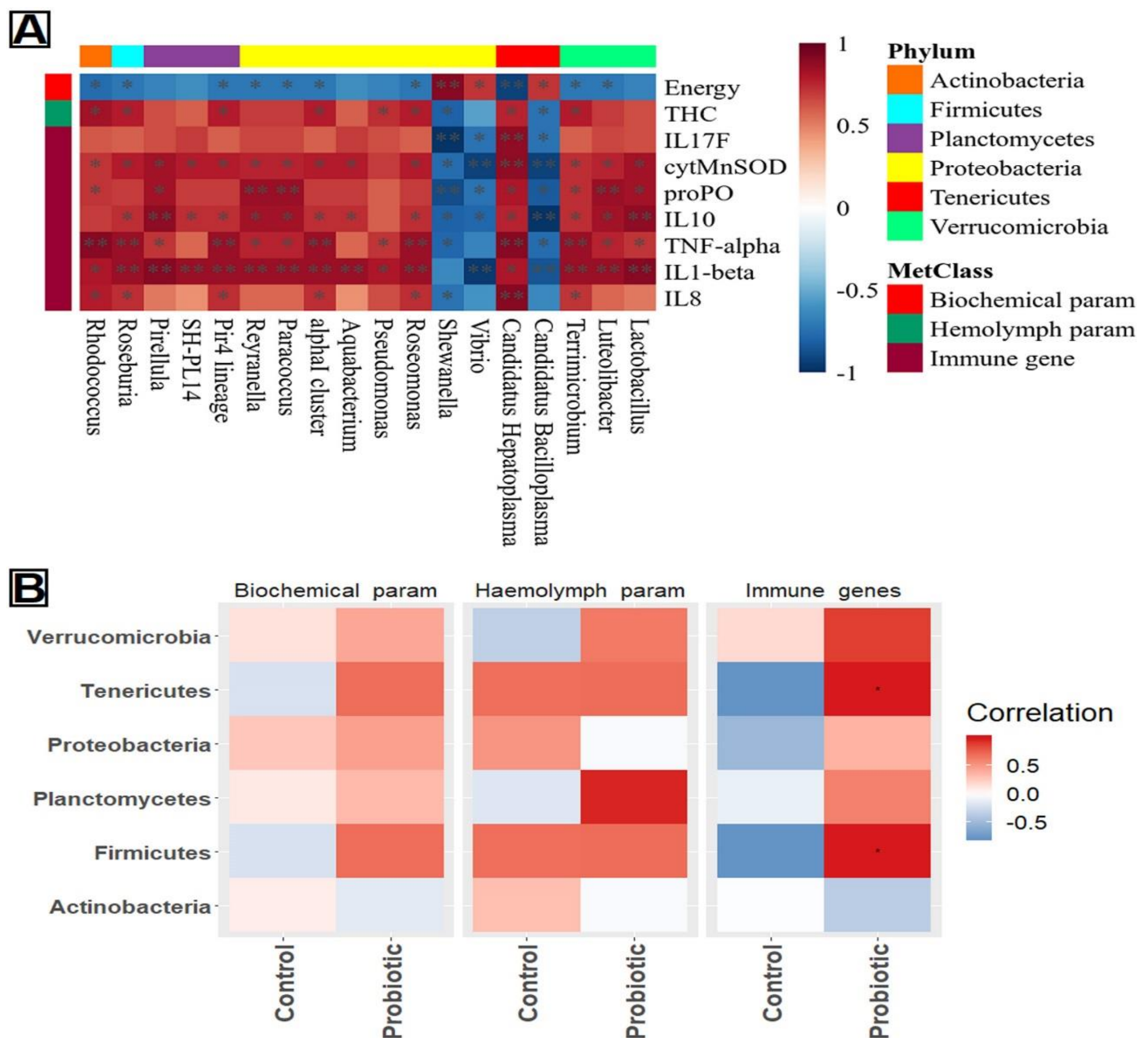

Figure 5.3. 7 (**A**) A "heat map" showing Spearman correlation between microbial abundance and health and immune indices of marron. (**B**) Correlation "heat map" displaying the interactions between bacterial groups at phylum level and biological indices of marron after trial. Red representing positive interactions while green demonstrating negative interactions. *Significant at α-level of 0.05; **Significant at α-level of 0.005.

5.4(3). Discussion

Dietary supplementation of feed additives have significant impacts on growth, immunity and disease resistance of crustaceans (Foysal et al., 2019b, Foysal et al., 2019d, Safari and Paolucci, 2017b, Safari and Paolucci, 2017a, Safari and Paolucci, 2018, Saputra et al., 2019, Valipour et al., 2019). Probiotic in aqua diets, has positive influence on the growth and immune performance of commercially farmed shrimp and crayfish species. Among the probiotic bacteria, *Lactobacillus* species are widely used for their beneficial role in health and immunity of narrow clawed crayfish, *Astacus leptodactylus* (Valipour et al., 2019) and marron (Siddik et al., 2020b). Present study evaluated the combined effects of two most potent LAB on growth, hemolymph parameters, biochemical compositions of tail muscle, intestine and hepatopancreas structure, gut microbiota and immune genes of freshwater crayfish marron. Additionally, with the aid of bioinformatics, we investigated the correlations between microbial abundance and other metadata associated with growth and immunity. Therefore, this is a comprehensive study that combined biochemical, immunological, molecular and bioinformatic approaches to analyse the probiotic effects on overall health performance of marron. Although the growth data showed non-significant effects of probiotic diet, however, a P-value of 0.059 suggest that higher growth performance could be achieved by this probiotic combinations, under a trial involving a longer feeding duration. Nevertheless, the growth data obtained with probiotic diet are really promising in considering the slow growing nature of marron under commercial farming conditions and when none of the previous studies found significant growth improvement with any of the diet formulations (Ambas et al., 2017, Ambas et al., 2013, Foysal et al., 2019b, Foysal et al., 2019d). The augmented THC counts in hemolymph, and enhanced crude protein and gross energy in the tail muscle further revealed the beneficial role of probiotic diet. These parameters are crucial for determining the health and immune status of crayfish including marron (Ambas et al., 2017, Ambas et al., 2013, Sepici-Dinçel et al., 2013).

Increasing evidence has found a close association between the intakes of diet-supplemented probiotic strains and gut health of fish and crustacean (Amoah et al., 2019, Du et al., 2019a, Du et al., 2019b, Foysal et al., 2019d). A proper orientation of gut microvilli, villi length, and healthy structure of hepatopancreas cell are associated with proper nutrient absorption and utilization, and thus higher growth and immune function (Dimitroglou et al., 2009). The SEM and histology image revealed better morphology and orientation of gut and hepatopancreas in the probiotic fed marron than the control. The results is consistent and compatible with other previous studies that fed dietary supplementation of *Lactobacillus* bacteria including *L. plantarum* (Siddik et al., 2019, Wang et al., 2016). In crayfish, no published data are currently available, however, a previous study found that dietary supplementation of *Lactobacillus pentosus* in the commercial diet of shrimp protect the hepatopancreas from pathogen invasions (Du et al., 2019b). Enferadi et al. (2018) reported a significant increase of intestine enzyme activity, and higher digestibility and absorption of feed in rainbow trout fed *L. plantarum* supplemented diet. Therefore, better gut and hepatopancreas structure in the probiotic fed marron may be attributed to the beneficial effects of *Lactobacillus* bacteria on digestion, absorption and nutrient utilization.

Modulation of gut microbiota and their interactive networks results in production of several types of metabolites, vitamins and antimicrobial agents that affect largely on the host physiology and immune response (Levy et al., 2016, Li et al., 2018b, Ringø et al., 2016). The gut microbes of freshwater fish are commonly dominated by phyla Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteria (Huang et al., 2014, Michl et al., 2017). Firmicutes, Bacteroidetes, and Teniricutes are defined as the beneficial bacteria of the gut and their positive role in growth performance, immunity, digestion and disease resistance of aquatic animals (Costantini et al., 2017, Egerton et al., 2018, Mekuchi et al., 2018, Miao et al., 2018). Additionally, they also play an influential role in augmentation of other beneficial bacteria in

the gut (Borrelli et al., 2016, Vargas-Albores et al., 2017, Wang et al., 2018). The enriched bacteria at genus level in the probiotic fed marron can be correlated with better growth and immune performance and water quality from previous studies. For instances, *Pirellula* abundance was found correlated to essential amino acids such as isoleucine, leucine and valine production in grass carp (*Ctenopharyngodon idellus*) and blunt snout bream (*Megalobrama amblycephala*) (Li et al., 2017a). Another genus *Reyranella* that is phylogenetically very close to Rhodospirillaceae, also reported to play a crucial role in nitrate reduction from soil and freshwater (Cui et al., 2017, Kim et al., 2013). The bacteria recently been characterized from Tropical gar fish (*Atractosteus tropicus*) where its positive role on fish adaptation and survival has been described (Mendez-Perez et al., 2019). *Paracoccus* species identified from turbot (*Scophthalmus maximus*), European flounder (*Platichthys flesus*) and wild common dab (*Limanda limanda*) gut were found strongly correlated to high content of polyunsaturated fatty acids (PUFA), especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) that have potential health benefit effects on aquatic animals as well as on humans, especially the role of PUFA in liver and brain function, inflammation, cardiovascular diseases, obesity and diabetes has been widely documented (Sokoła-Wysoczańska et al., 2018, Wanka et al., 2018, Zárate et al., 2017). Another genus *Luteolibacter* is described to play a positive role in *Lactobacillus* colonization in the fish gut, however, the mechanism is not clearly stated yet (Baldo et al., 2015, Webster et al., 2018). The positive role of *Lactobacillus* in the gut, health and immune status, and disease resistance of aquatic animals has also been investigated (Du et al., 2019b, Enferadi et al., 2018, Giri et al., 2018, Roomiani et al., 2018, Vieira et al., 2010). In a most recent study, dietary supplementation of 10^9 CFU/mL of *L. plantarum*, a same concentration to the present study significantly increased the haemolymph parameters, enzymatic activity and LAB counts in the gut of narrow clawed crayfish (Valipour et al., 2019). Therefore, addition of *L. acidophilus* and *L. plantarum* in diet generated an augmented

community of beneficial bacteria for aquatic animals that might be associated with improved health and immune status of marron in this study.

Unlike fish and other vertebrates, crustaceans lacks adaptive immune system and therefore solely rely on innate immune response generated primarily from the immunocompetent cells and mucus of intestinal mucosal membrane (Foysal et al., 2019d, Fredrick and Ravichandran, 2012, Lieschke and Trede, 2009). In the present study, we selected 10 genes that are reported to play a crucial role in innate immune response of crayfish earlier. We found upregulated expression of pro-inflammatory cytokines, and crustacean's hemolymph genes in the probiotic fed marron. To prevent inflammation related damages due to upregulated expression of pro-inflammatory cytokines, the anti-inflammatory cytokines (IL10) was also stimulated in the probiotic fed marron to neutralize inflammation. This upregulation was significant due to the pro and anti-inflammatory mechanisms, aquatic animals balanced the immune response for better defence against stress and infection (Miao et al., 2018). Though the presence of cytokine family genes in crustacean tissue including crayfish are evident (Beschin et al., 2001, Gerber et al., 2007, Huang et al., 2015, Liang et al., 2017, Siddik et al., 2020b, Yi et al., 2017a), however, their role have not been studied yet. Hence, further transcriptomic analysis of mRNA is recommended to identify the expressed genes under cytokine family and its level of expression in the intestinal tissue of marron.

The predictive role of microbial communities and correlating them with the health and immune indices of marron is the most significant findings of this study. Firstly, the predicted enriched IL17 signalling pathway from 16S rRNA data has been validated by upregulation of IL17F gene in qPCR assay. We also investigated the correlations between microbial abundance and biological indices and found the significant role of Firmicutes, Bacteroidetes and Teniricutes in enhancing the innate immune response while Proteobacteria was mostly linked to health performance. The positive role of Fimicutes and *Lactobacillus* on innate immune response

including cytokine expression, and Bacteroidetes, Teniricutes in expression of anti-microbial peptides (AMPs), gut health and immunity has been reported in northern snakehead (*Channa argus*) (Miao et al., 2018) and in Chinese mitten crab (*Eriocheir sinensis*) (Dong et al., 2018), respectively. However, although reported abundant in gut and considered as beneficial bacteria for the health of crustaceans (Chen et al., 2015, Foysal et al., 2019b, Ooi et al., 2017, Wang et al., 2019), the role of *Candidatus* species including *Candidatus* Bacilloplasma and *C*. Hepatoplasma, have not been investigated yet. In this regard, its role in health and immunity of crayfish could be useful in future diet preparations, disease resistance and metabolomics studies, and thus need further investigations.

The above findings demonstrated that the supplementation of potential probiotic *L. acidophilus* and *L. plantarum* to diet, significantly promote the gut and hepatopancreas health, immune response as well as microbial composition and interaction network in marron. This work will help to understand the probiotic mechanism and possibility of using *L. acidophilus* and *L. plantarum* as potent probiotic bacterial combinations in marron aquaculture.

CHAPTER 6: Protein diets for marron aquaculture

EXPERIMENT 1: Dietary supplementation of black soldier fly (*Hermetica illucens*) meal modulates gut microbiota, innate immune response and health status of marron (*Cherax cainii*, Austin 2002) fed poultry-by-product and fishmeal based diets

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Abstract

The present study aimed to evaluate the dietary supplementary effects of black soldier fly (*Hermetia illucens*) (BSF) meal on the bacterial communities in the distal gut, immune response and growth of fresh water crayfish, marron (*Cherax cainii*) fed poultry-by-product meal (PBM) as an alternative protein source to fish meal (FM). Sixty four marron were randomly distributed into 16 different tanks with a density of four marron per tank. After acclimation, a 60-days feeding trial was conducted on marron fed isonitrogenouts and isocalorific diets containing protein source from FM, PBM, and a combination of FM+BSF and PBM+BSF. At the end of the trial, weight gain and growth of marron were found independent of any dietary treatment, however, the two diets supplemented with BSF significantly (P<0.05) enhanced haemolymph osmolality (HO), lysozyme activity, total haemocyte counts (THC), and protein and energy contents in the tail muscle. In addition, the analysis of microbiota and its predicted metabolic pathways via 16s rRNA revealed a significantly (P<0.05) higher bacterial activity and gene function correlated to biosynthesis of protein, energy and secondary metabolites in PBM+BSF than other dietary groups. Diets FM+BSF and PBM+BSF were seen to be associated with an up-regulation of cytokine genes in the intestinal tissue of marron. Overall, PBM+BSF diet proved to be a superior diet in terms of improved health status, gut microbiota and up-regulated expression of cytokine genes for marron culture.

Keywords: Aquaculture, Marron, Protein supplements, Health indices, Gut microbiota, Gene expression profiling, Bioinformatics

6.1(1). Introduction

Fish meal (FM) is one of the major sources of dietary protein for cultured aquatic animals, posing an increasing challenge on the reduction of feed cost and wild fish stocks (Tacon and Metian, 2008). Recent reports revealed the global decline of fishmeal production due to the increasing demand of wild stocks in contrast to amount of fish harvested for trashing (Cashion et al., 2017, Pauly and Zeller, 2017). It is an utmost priority to find a suitable, cheap and widely available alternative to fishmeal to meet-up the burgeoning demand of feed for the sustainable aquaculture industry. On the other hand, poultry-by-products meal (PBM) is a solidified by-product from poultry processing industry that has high potential to be incorporated into aqua-diets as a substitute for fishmeal (Saoud et al., 2008). PBM is readily available worldwide and is more economical when compare with FM (Emre et al., 2004, Wu et al., 2018). PBM alone or in combination with bone, feather, and blood meal, can be one of the major protein source in aqua-diets owing to high protein, suitable fatty acids, vitamins, and minerals, (Dozier et al., 2003, Fuertes et al., 2013). In addition, PBM is reported to have positive effect on the growth rate, digestibility, and immune status of fish and crayfish (Bransden et al., 2001, Saoud et al., 2008, Yang et al., 2006). The black soldier fly (BSF) larvae, another alternative to fishmeal protein source, is gaining increasing popularity for aquaculture industry (Stamer et al., 2014). BSF contains high protein and fat, rich in trace elements, and more importantly, has a lesser impact on the environment and hence named as “savior” in the food-insecure world (Wang and Shelomi, 2017). The efficacy of BSF as an alternative protein source for warm-water fish has been validated in earlier studies (Stamer et al., 2014, Stankus, 2013, Wang and Shelomi, 2017), however limited data are currently available on the suitability of using BSF as alternative

protein source and its supplementary effect on other sources of protein, including FM and PBM in aqua-diets (Kroeckel et al., 2012, St-Hilaire et al., 2007).

Marron (*Cherax cainii*) is one of the largest freshwater crayfish endemic to Southern part of Western Australia (WA) (DoF, 2010b, Foysal et al., 2019a). Marron is popular for its distinctive flavour and taste, and therefore considered as an iconic freshwater crayfish in WA. Marron farming possesses a number of advantages over other crayfish species (*Cherax destructor*, *C. preissii* and *C. crassimanus*) including disease resistance, high market demand and price, and possible live shipment to long distances (Lawrence, 2007). Yet, the production has remained stagnant over the decade (Mai and Fotedar, 2018). To improve the production potential, several investigations have been conducted to find the suitable combination of feed for marron (Ambas et al., 2015, Ambas and Fotedar, 2015, Ambas et al., 2013). As an omnivorous animal, the protein requirement for marron is moderate (approximately 30%) (Ambas et al., 2017). Therefore, a combination of PBM and BSF can be ideal alternative protein source in marron diet.

Crayfish harbour complex bacterial communities in the gut that influences various host functions like digestion, nutrition, immunity, and disease resistance (Zoqratt et al., 2018, Skelton et al., 2017). Due to their vital roles, enrichment of some bacterial communities in the gut with dietary feed supplementation has been validated to accelerate the growth and immune status of the crayfish (Anuta et al., 2011, Safari and Paolucci, 2018). High relative abundance of lactic acid bacteria, especially *Bacillus*, *Lactobacillus* in the gut are described to have beneficial influences on the health and immunity of white shrimp (*Liptopenaeus vannamei*) (Zokaeifar et al., 2012), zebrafish (*Danio rerio*) (Falcinelli et al., 2015), Atlantic salmon (*Salmo salar*) (Gajardo et al., 2017) and northern snakehead (*Channa argus*) (Miao et al., 2018).

Recent advancement in 16S rRNA based high throughput sequencing techniques has enabled precise detection of gut microbial communities of fish. This technology has been successfully employed to analyze the feeding effects on the gut bacterial populations of fish in earlier reports (Gajardo et al., 2017, Miao et al., 2018). In addition, quantitative real-time PCR is routinely used for the measurement of relative expression level of immune genes to infer the effect of feed on the immunity of crayfish (Jiang et al., 2015b, Shekhar et al., 2013). This study aimed to characterize the bacterial communities in the distal gut of marron fed FM, PBM, a combination of FM+BSF and PBM+BSF and analyze the expression patterns of some cytokine genes.

6.2(1). Materials and methods

6.2(1).1. Experiment set-up

The experiment with 64 marron (65.01 ± 5.09g) was designed as described in general methodology (chapter 3.1). Marron were then distributed into 16 different tanks and acclimated for 14 days before commencement of the trial. Water quality parameters including pH, dissolved oxygen, nitrate, nitrate, and ammonia were monitored according to methods mentioned in general methodology (chapter 3.1). During acclimation, marron were fed every day at afternoon with standard basal marron diet (Table S4.1), prepared by Glenn Forest, Western Australia.

6.2(1).2. Test diets and feeding

Four isoproteic and isocalorific diets containing fishmeal (FM), poultry-by-product meal (PBM), fishmeal supplemented with black soldier fly meal (FM+BSF), and poultry-by-product meal supplemented with black soldier fly meal (PBM+BSF) were prepared (Table S6.1.1, Appendix 3). The ingredients were supplied by Glenn Forrest, Western Australia and after feed formulation the test diets were also prepared by the same company. Proximate compositions of each diet was determined as per the method of Association of Official Analytical Chemists,

AOAC (AOAC, 2006). Four randomly assigned tanks, with four individually held marron in cages, were given each test diet, hence using a design of four dietary treatments X 4 replicates per dietary treatment. Each marron in a cage was fed the respective diets, once every day at 12 PM.

6.2(1).3. Marron sampling

For haemolymph, health indices and gene expression analysis, 16 marron, one marron randomly selected from each tank was used. However, for DNA extraction, nine randomly selected marron from three randomly selected tanks from each treatment, were selected. No samples were collected from one of the tanks from each treatment.

6.2(1).4. Analysis of growth, hemolymph parameters and health indices

Marron growth performance including weight gain (WG), specific growth rate (SGR), feed intake (TFI), feed conversion ratio (FCR), and haemolymph parameters including osmolality (HO), lysozyme and total haemocyte counts (THC) were analysed according to methods described earlier, general methodology chapter 3.7 and 3.2, respectively. The tail muscle protein, lipid and energy content were measured according to methods described in chapter 3.3 of general methodology. The hepatopancreas index of marron after feeding trial was calculated using the formula: Hepatopancreas index (HI) = Hepatopancreas dry weight/ (body dry weight excluding large cheliped x 100).

6.2(1).5. DNA extraction, PCR, and high throughput sequencing

At the end of the experiment, randomly selected marron were taken into biosafety cabinet followed by careful excision of the guts. Then the hindgut were separated and the gut contents of three marron from each tank were homogenized, pooled, and transferred in to 1.5 mL Eppendorf. The extraction of bacterial genomic DNA from pooled samples, PCR amplification of v3v4 hypervariable regions, library preparation and pair-end sequencing were performed according to methods described in chapter 3.4 of general methodology.

6.2(1).6. Gene expression analysis

In this study, five genes from interleukin family, IL-1β, IL-8, IL-10 IL-17F and TNF-α along with crayfish tissue gene vitellogenin (*Vg*) and proliferating cell nuclear antigen (*Pcna*) were selected (Jiang et al., 2015a, Miao et al., 2018) for qRT-PCR assay (Table S6.1.2, Appendix 4). For RNA extraction, intestinal tissue samples stored in RNA Later (Sigma-Aldrich, Germany) were used for gene expression analysis as described in general methodology (chapter 3.6).

6.2(1).7. Bioinformatics

Unless any modifications mentioned, the "pipelines" for downstream sequence analysis and bioinformatics were used according to general methodology in chapter 3.5 with default parameters. Non-metric multidimensional scaling (nMDS) of bacterial OTUs from four different groups was done in PAST statistical software package (Hammer et al., 2009). The Shannon, Simpson, and Fisher alpha diversity indices were calculated using "vegan" package in R (Oksanen et al., 2018). Chao1 diversity in the samples was calculated using formula $S_{chao1} = S_{obs} + (n1)^{\surd}/2n2$, where S_{obs} = number of observed genera, n1 = number of singletons (genus captured once), n2 = number of doubletons (genera captured twice) (Militon et al., 2010). Venn diagram for bacterial abundance regarding diversity at genus level was generated using FunRich (v3.1.3) (Benito-Martin and Peinado, 2015). Linear Discriminant Analysis Effect Size, LEfSe was applied to find the indicator bacterial groups in four different feeding groups with a minimum logarithmic LDA cut-off value of 8.0 (Segata et al., 2011). For predicting differentially abundant metabolic pathway in four different groups, Piphillin algorithm (http://secondgenome.com/Piphillin) was used with supports of KEGG database, BioCyc (v21), and LEfSe (LDA 3.0) (Iwai et al., 2016). Numerical growth and health indices data for marron were analyzed using SPSS IBM (v23, 2017). One way analysis of variance (ANOVA)

was used to calculate any significant differences (P<0.05) among variables in four different dietary treatments.

6.3(1). Results

6.3(1).1. Effects of four different feed on marron health indices

No significant (P>0.05) weight gain and growth rate were observed in PBM and two BSF supplemented diet (FM+BSF and PBM+BSF) compared to FM (Table 6.1.1). The effects of dietary PBM was found to be almost similar to FM on marron health and immunity. Both BSF (FM+BSF and PBM+BSF) supplemented diets showed significant enhancement in protein, energy, lysozyme, and THC. However, the diet PBM+BSF showed a significant (P<0.05) influence on the energy content in tail muscle, and significantly pronounced (P<0.005) effects of PBM+BSF was recorded for tail muscle protein, lysozyme, THC and HO (Table 6.1.1). Dietary incorporation of BSF with PBM also showed a significant (P<0.05) decline in HI and fat content of hepatopancreas whereas both BSF (FM+BSF and PBM+BSF) supplemented diets showed significant enhancement in protein, energy, lysozyme, and THC.

Table 6.1. 1 Mean ± SE of some health parameters of marron at the end of feeding trial

Parameters	**FM**	**PBM**	**FM+BSF**	**PBM+BSF**
WG (g %)	24.33 ± 1.38	23.91 ± 0.77	25.35 ± 0.85	26.49 ± 0.84
SGR (g/day %)	0.51 ± 0.26	0.49 ± 0.12	0.51 ± 0.13	0.53 ± 0.14
Protein (%)†	82.4 ± 0.67	83.2 ± 0.56	85.4 ± 1.01*	89.4 ± 0.95**
Energy (kJ/kg)†	20010.3 ± 75.1	20242.3 ± 89.2	21672.8 ± 311.5*	21778.5 ± 356.9*
Fat (%)⁋	8.9 ± 0.11	8.4 ± 0.11	6.4 ± 0.50*	6.1 ± 0.40*
HI	38.5 ± 0.52	36.9 ± 0.27	34.5 ± 0.31*	32.2 ± 0.72*
Lysozyme (U/mL)	0.42 ± 0.006	0.4 ± 0.003	0.47 ± 0.011*	0.59 ± 0.036**
THC (cells/mL)	8.0 ± 0.09	7.9 ± 0.09	8.2 ± 0.13*	9.5 ± 0.15**
HO (U/mL)	0.4 ± 0.003	0.41 ± 0.005	0.42 ± 0.011	0.46 ± 0.010**

Abbreviation: FM = Fish meal; PBM = Poultry-by-product meal; FM+BSF = Fish meal + Black soldier fly meal; PBM+BSF = Poultry-by-product meal + Black soldier fly meal; WG = Weight gain; SGR = Specific growth rate; HI = Hepatopancreas index; THC = Total haemocyte

count; HO = Haemolymph osmolality. *Significantly different at α-level of 0.05. **Significantly different at α-level of 0.005. †From tail muscle. ⁋From hepatopancreas.

6.3(1).2. Bacterial diversity in the hindgut after feeding trial

At the end of the trial, all the four experimental diets displayed distinct effect on different bacterial populations of marron hindgut (Figure 6.1.1 A). Quality filtering obtained 377,848 high-quality reads which were assigned into 119 genera. The number of the shared and unique genera were found to be significantly (P<0.05) higher in PBM+BSF fed marron than the other marron groups (Figure 6.1.1 B).

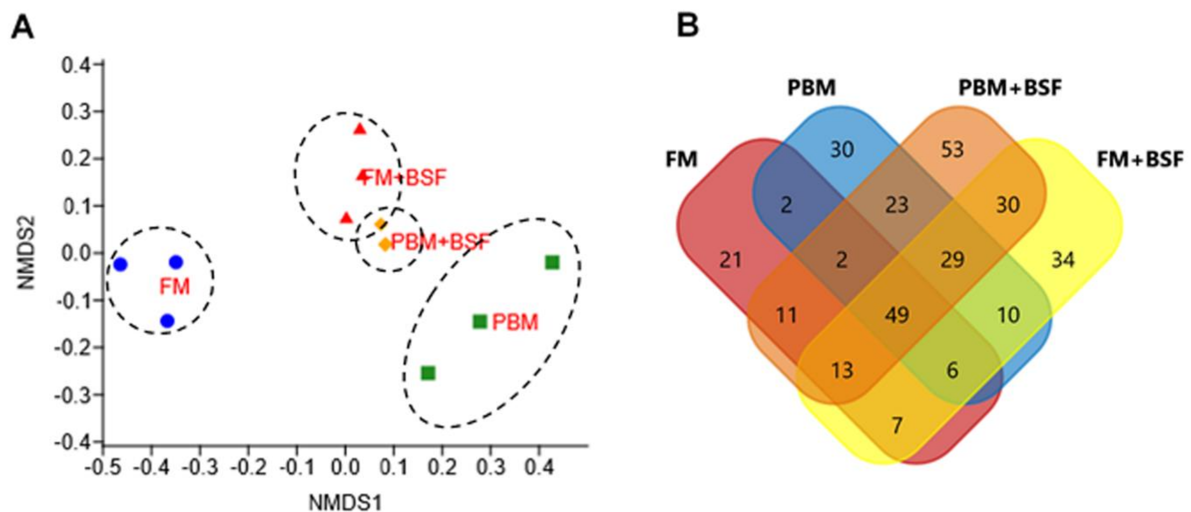

Figure 6.1. 1 Effects of four different dietary protein on marron distal gut. (A) Non-metric multidimensional scaling (nMDS) plot showing feeding effects on microbiota of distal gut in marron. (B) Number of shared and unique genus in four different protein fed groups of marron. Abbreviations: FM = Fishmeal, PBM = Poultry-by-products meal, BSF = Black soldier fly.

At genus level, *Proteobacteria* (46.62–92.88%) was found to be dominant in FM, PBM, and FM+BSF dietary groups whereas significantly (P<0.05) higher abundance for *Firmicutes* (63.52–92.88%) was recorded in PBM+BSF fed group (Figure 6.1.2 A). Next to *Proteobacteria*, *Fusobacteria* (36.74–39.15%) and *Tenericutes* (20.28–39.77%) were the second abundant phyla in FM and FM+BSF fed marron, respectively. In the PBM+BSF fed marron, *Fimicutes* profusion (25.18–42.37%) was recorded, followed by *Proteobacteria* (28.4–

33.6%), and *Bacteroidetes* (10.54–11.8%). Compared to FM, the other diets showed a significant (P<0.05) increase in the diversity of bacteria at genus level whereas, the BSF supplemented diet, the marron displayed higher abundance. At genus level, *Hypnocyclicus* (46.31–47.81%), *Aeromonas* (39.93–58.5%), *Candidatus Bacilloplasma* (27.88–40.65%), and *Streptococcus* (17.77–24.38%), were the most dominant bacteria in FM, PBM, FM+BSF, and PBM+BSF fed marron, respectively (Figure 6.1.2 B). The relative abundance level for *Vibrio* was found to decrease significantly in PBM+BSF (1.13–2.42%) groups than FM (16.6–38.9%) fed group. The abundance of *Serratia* and *Enterobacter* dropped to zero in PBM+BSF fed marron whereas, FM fed marron had *Serratia* and *Enterobacter* in abundance of 2.99–5.0% and 1.58–2.57%, respectively. Among the top 20 abundant genera, *Polynucleobacter*, *Limnohabitans*, *Flavobacterium*, *Shewanella*, *Corynebacterium*, *Ezakiella*, *Porphyromonas*, *hgcl clade*, *Anaerococcus*, *Rhodobacter*, and *Lactovum* were present in PBM+BSF group than FM fed group. The Fisher Alpha and Chao1 diversity indices of PBM+BSF diet group were significantly (P<0.05) higher than the FM group. The Simpson and Shannon indices were also found to be augmented in PBM+BSF fed marron, however, at higher significance level, α-level of 0.005 and 0.001, respectively (Table 6.1.2). In contrast, only Chao1 diversity index was significantly improved in PBM and Fisher alpha in FM+BSF fed marron, respectively.

Table 6.1. 2 Major diversity indices (Mean ± SE) of bacteria genera in the marron gut

Treatment	Shannon (SE)	Simpson (SE)	Fisher alpha (SE)	Chao1 (SE)
FM	1.51 (0.13)	0.69 (0.03)	7.59 (1.36)	86.74 (10.33)
PBM	1.77 (0.36)	0.69(0.07)	11.34 (1.30)	116.68 (4.6)*
FM+BSF	1.89 (0.27)	0.76 (0.04)	12.3 (1.28)*	103.99 (3.08)
PBM+BSF	3.19 (0.06)***	0.82 (0.006)**	16.7 (1.91)*	128.48 (6.2)*

Abbreviation: FM = Fish meal; PBM = Poultry-by-product meal; FM+BSF = Fish meal + Black soldier fly meal; PBM+BSF = Poultry-by-product meal + Black soldier fly meal.

*Significantly different at α-level of 0.05. **Significantly different at α-level of 0.005. ***Significantly different at α-level of 0.001.

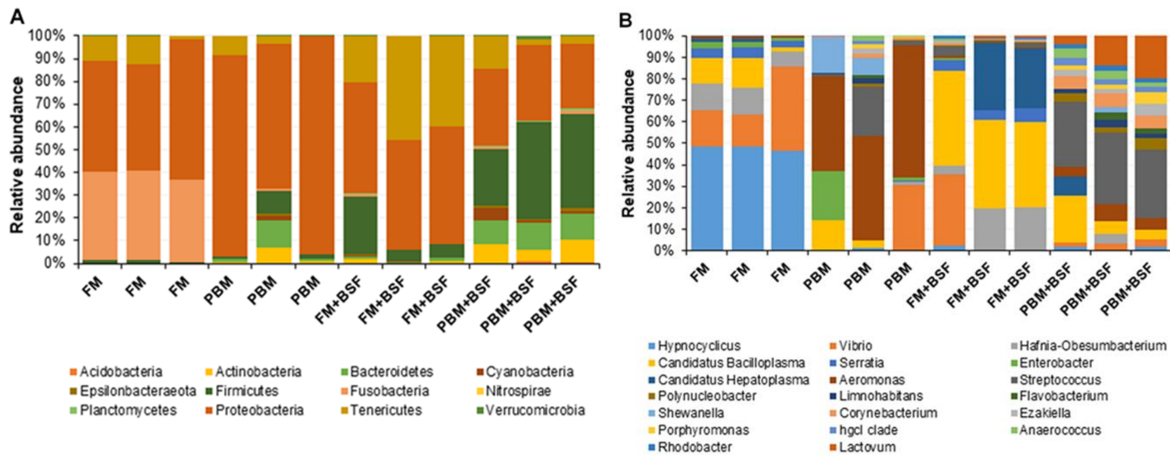

Figure 6.1. 2 (A) Relative abundance of bacteria at phylum level. (B) Relative abundance of bacteria at a genus level. Abbreviation: FM = Fish meal; PBM = Poultry-by-product meal; FM+BSF = Fish meal + Black soldier fly meal; PBM+BSF = Poultry-by-product meal + Black soldier fly meal.

6.3(1).3. LEfSe based microbial lineages and metabolic pathways

The results of Linear Discriminant Analysis Effect Size (LEfSe) revealed 21 genus which were significantly modulated in three different dietary groups (FM, PBM, and PBM+BSF) at LDA cut-off value of 8.0 based on Wilcoxon non-parametric *t-test* corrected for multiple hypothesis testing (P<0.05). Out of 21, 16 genera showed to be enriched in PBM+BSF fed marron including species from *Lactobacillus*, *Streptococcus*, *Bacteroidetes*, *Aquabacterium*, *Actinobacteria* (Figure 6.1.3). Significantly (P<0.05) increased bacteria at genus level in PBM fed group were *Aeromonas* and *Limnohabitans*. *Hafnia Obesumbacterium*, *Citrobacter* and *Serratia* were found to be enriched in FM fed marron while no genus was exhibited to be significantly (P>0.05) higher in FM+BSF fed marron at stringent cut-off value. LEfSe cladogram revealed significantly (P<0.05) enriched 46 taxa (phylum to genus) in three different

dietary groups. No significant enrichment was observed in FM+BSF group at a strict cut-off value (LDA 4.0), where 39 were significantly enriched after fed PBM+BSF diet (Figure 6.1.4). The microbial lineages in PBM+BSF fed marron were exclusively enriched from the phylum *Firmucutes*, *Bacteroides*, and class *Bacilli*, *Negativicutes* whereas in the FM and FM+BSF fed group, the enriched lineages were mostly from the *Proteobacteria* phylum. Differences in predicted functional pathways based on the bacterial abundance exhibited to be associated with carbohydrate metabolism and transport among the FM fed marron, fatty acid biosynthesis and metabolism among PBM fed marron , galactose metabolism and aminoacyl tRNA biosynthesis among FM+BSF feed group, and amino acid biosynthesis and energy metabolism in PBM+BSF fed marron (Figure 6.1.5).

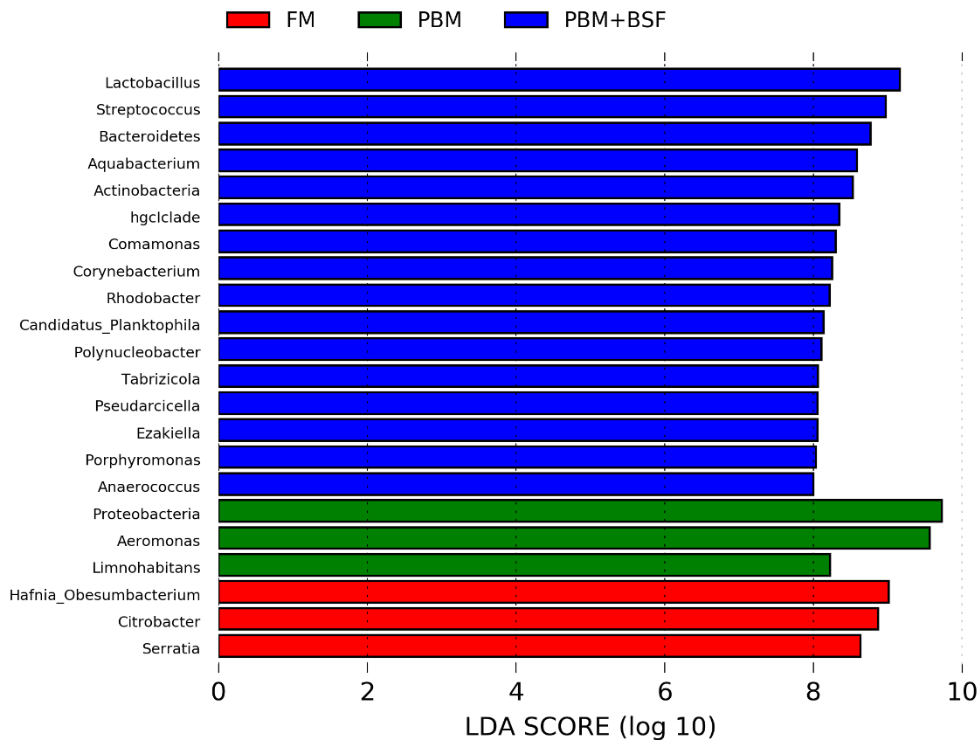

Figure 6.1. 3 Indicator bacteria at genus level in three different proteins fed marron groups. FM+BSF had no significant indicator at LDA cut-off value of 8.0. Abbreviation: FM = Fish meal; PBM = Poultry-by-product meal; FM+BSF = Fish meal + Black soldier fly meal; PBM+BSF = Poultry-by-product meal + Black soldier fly meal.

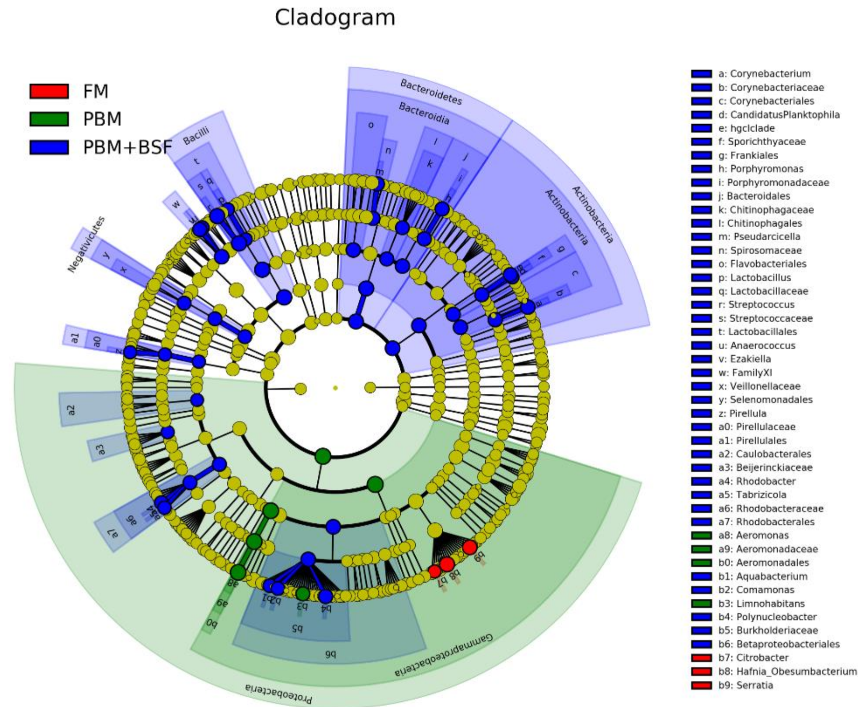

Figure 6.1. 4 Circular LEfSe cladogram representing the phylogenetic distribution of bacterial lineage in three different proteins fed marron groups. The lineage with LDA scores of 3.0 or above are displayed here. The order, family, and genus that are significantly different between two groups are given in the upper right corner with respective colour codes.

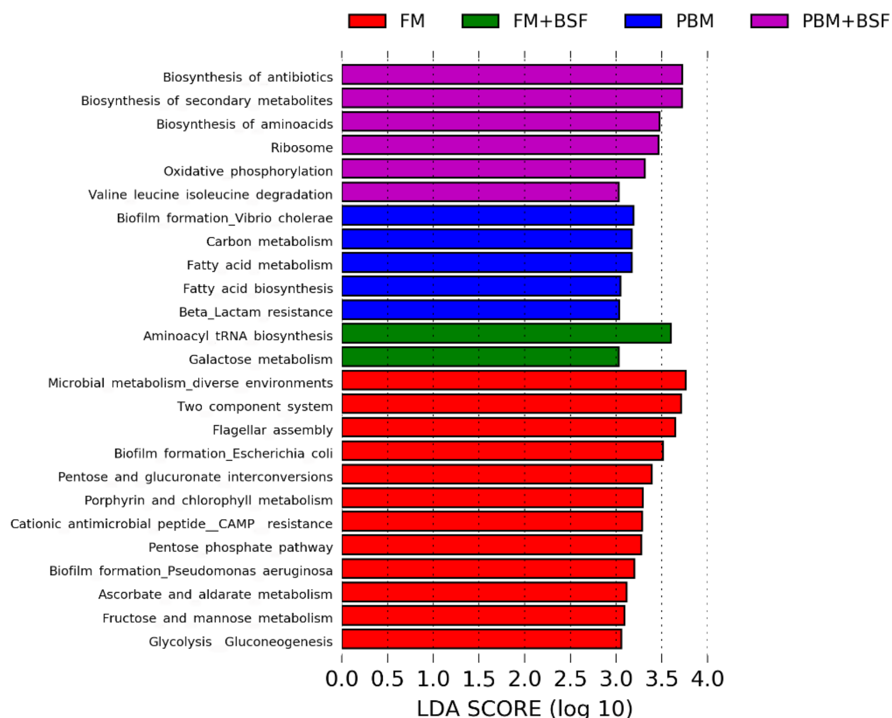

Figure 6.1. 5 Predicted differentially expressed KEGG metabolic pathway in four different protein fed marron groups identified by Piphillin and linear discriminant analysis (LEfSe) (LDA>3.0, P<0.05).

6.3(1).4. The relative expression level of genes

The relative expression level of seven different genes in four different dietary groups are shown in Figure 6.1.6. Compared to FM dietary group, the BSF supplemented (FM+BSF and PBM+BSF) marron gut displayed significant (P<0.05) up-regulation of IL-1β, IL-10, IL-17F, TNF-α, and down-regulation of *Vg* genes in terms of fold change in contrast to β-actin reference gene after 60 days of feeding trial. The expression of cytokine genes, however, was found to be significantly up-regulated in PBM+BSF fed group. The normalised data showed the highest mean expression for IL-17F, followed by IL-10, IL-1β, and TNF-α, respectively. The relative expression level of IL-8 and *Pcna* were relatively stable in three different protein fed groups while *Vg* expression was found to be decreased significantly with PBM and two BSF supplemented diets at the end of the trial.

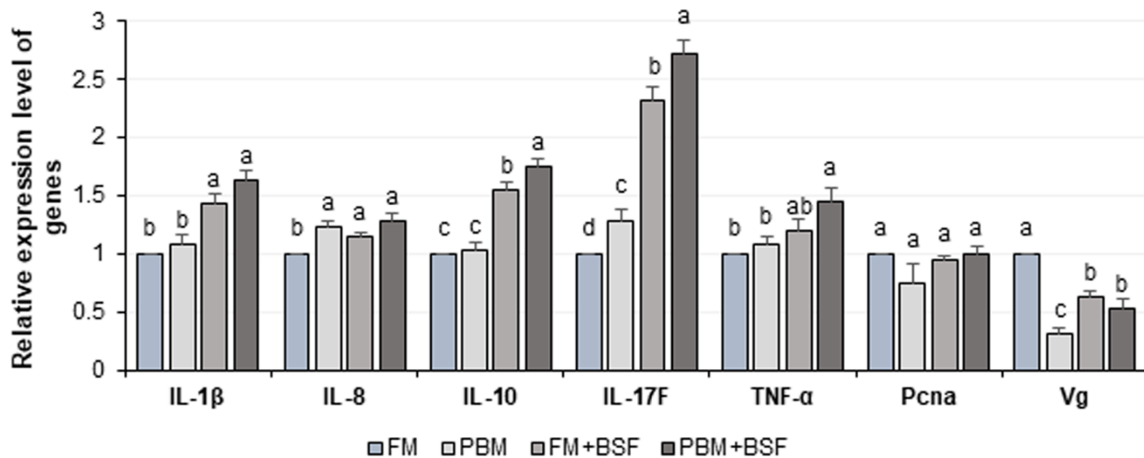

Figure 6.1. 6 Relative expression level (Mean ± SE, n = 3) of major cytokines and crustacean reference genes in marron intestine at the end of 60 days feeding trial. Means with different superscripts are statistically significant at α-level of 0.05 with Duncan's multiple range test.

6.3(1).5. Data availability

The raw sequence data in FASTQ files are currently available at National Centre for Biotechnology Information (NCBI) BioProject under the accession number PRJNA504966.

6.4(1). Discussion

In aquaculture practice, replacement of dietary fishmeal with alternative cheap protein sources is one of the sought-after research issue that has gained significant momentum in the last couple of decades. Both PBM and BSF have all the ideal properties to be used as a substitute for FM (Rimoldi et al., 2018, Zhou et al., 2018). To date, several trials have been conducted to analyze the effect of dietary PBM and BSF on the growth performance of aquatic species but yet no research reported the impact on gut microbial structure and cytokine gene expression patterns of commercially important species including crayfish (Badillo et al., 2014, Gajardo et al., 2017, Zhou et al., 2018). Therefore, the information obtained from this study on the effects of PBM and BSF supplemented diets on gut microbiota and intestine tissue genes will add novel findings that will have a vital contribution to our existing knowledge.

The present study found no significant impacts ($P>0.05$) of other three diets (PBM, FM+BSF and PBM+BSF) on growth parameters of marron, compared to FM. A feeding trial with different concentration of BSF supplemented diet on Jian carp (*Cyprinus carpio* var. Jian) showed a similar results where the growth rate was independent of BSF supplementation (Zhou et al., 2018). As the life cycle of marron under farming conditions is longer than other decapods (Lawrence, 2007), and the present experiment lasted for only two months, hence, no significant growth rate was noticed. There was no significant change in the health indices between FM and PBM fed marron in this study, but the BSF supplemented diets resulted in significant ($P<0.05$) positive effects on HO, serum lysozyme, protein and THC, and the impact was more pronounced ($P<0.005$) with PBM+BSF diet. BSF larvae contains high percentages of protein (≥40%), and inclusion of BSF in the diet reported to increase the protein concentration of fish

and poultry (Wang and Shelomi, 2017). We found positive impacts of BSF on the percentage of protein in the tail muscle, may be BSF has improved protein assimilation efficiency in marron, however this cannot be compared with any existing publication. In fish and crayfish, very limited data are currently available for the effects of BSF on the immune response of the host species. A study on boiler chicken had a significant increase in serum lysozyme and other immune parameters including T-lymphocyte, cell proliferation, and disease resistance after 56 days of feeding on BSF supplemented diet (Lee et al., 2018).

The gut microbiota of freshwater fish is dominated by *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria* (Huang et al., 2014, Michl et al., 2017). The *Proteobacteria* in addition with *Firmicutes* and *Bacteroidetes* comprises 90% of bacteria in the fish gut (Ghanbari et al., 2015). Like human, the majority of beneficial bacterial species are from *Firmicutes* and *Bacteroidetes* in fish (Egerton et al., 2018, Rajilić-Stojanović et al., 2007). *Firmicutes* and *Bacteroidetes* possess the ability to improve the digestibility and immune status of fish to counteract the effects of pathogenic bacteria (Costantini et al., 2017). Dietary supplementation of protein sources, poultry and soybean meal reported to have significant effects on the modulation of gut microbiota in fish (Miao et al., 2018, Zarkasi et al., 2016). In the present study, the BSF supplemented diets showed to have significant effects on the bacterial diversity of marron distal gut. Besides *Proteobacteria*, the results showed the abundance of *Fusobacteria* and *Terericutes* in FM and FM+BSF fed marron while *Firmicutes* was dominant in PBM+BSF marron. The 16S rRNA sequence data of BSF larvae gut showed to have higher percentage of *Bacteroidetes* (≥50%), *Proteobacteria* (≥25%), and *Firmicutes* (≥15%) (Bruno et al., 2019). Dietary protein sources can have positive effects on gut microbiota of fish where *Firmicutes*, *Bacteroidetes*, and *Teniricutes* are positively correlated with growth and immune parameters of fish (Egerton et al., 2018, Mekuchi et al., 2018, Miao et al., 2018). In addition, these bacterial groups also have an influential role to colonize the beneficial

bacteria in the fish gut (Borrelli et al., 2016, Vargas-Albores et al., 2017, Wang et al., 2018). The results of the current study found *Firmicutes*, *Teniricutes*, and *Bacteroidetes* richness in BSF supplemented diets that possibly come from the *H. illucens* larvae and associated with higher growth and immunity of marron. At genus level, all four diets triggered the relative abundance of four different genera, *Hynocyclicus* (FM), *Aeromonas* (PBM), *Candidatus* Bacilloplasma (FM+BSF), and *Streptococcus* (PBM+BSF). *Hypnocyclicus* commonly found in the ocean but its role in fish and crayfish gut has yet to be revealed (Roalkvam et al., 2015). *Aeromonas* abundance has been reported for freshwater fish and some of *Aeromonas hydrophila*, *A. veronii*, *A, sobria*, and *A, salmonicida* have a potential association with fish diseases (Foysal et al., 2019c, Wang et al., 2018). *Candidatus* Bacilloplasma identified in large scale from all crustacean species including shrimp, lobster, Crab, a hepatopancreatic symbiont helps isopods to survive in nutrient stress, promotes digestion process and up-regulated the expression of immune genes (Chen et al., 2015, Dong et al., 2018, Kostanjšek et al., 2007, Meziti et al., 2010). The present study found enrichment of lactic acid bacteria (LAB) in the BSF supplemented marron, especially in PBM+BSF more than 50% of bacteria were recorded from genus *Streptococcus*, *Lactovum*, and *Lactobacillus*. LAB are promising probiotic candidates in aquaculture whose health benefits have been widely validated (Balcázar et al., 2008, Ringø and Francois-Joel, 1998, Ringø et al., 2018).

To analyze the indicator microbial lineages, we applied Linear Discriminant Analysis Effect Size (LEfSe), a tool that can effectively predict high-dimensional biomarkers in different conditions from 16S rRNA sequence data. This tool can precisely provide biological class explanation to define statistical effects, biological consistency, and effect size estimation of the classified biomarkers (Huang and Jiang, 2016). With the aid of 16S rRNA data, we identified diverse bacterial lineages in three different feeding regimes where no biomarker was identified for FM+BSF fed marron. Sixteen out of 21 indicator bacterial lineages from the PBM+BSF

fed group indicate the enrichment of bacterial population from the selected genus. Besides LAB, *Bacteroides*, *Aquabacterium* and *Actinobacteria* were also found to be improved with PBM+BSF supplemented diet. *Bacteroides* abundance in fish and human gut have been described widely while a lower level of *Bacteroides* reported to have a strong correlation in the progression of several diseases in human (Egerton et al., 2018, Wang et al., 2018, Zhou and Zhi, 2016). The genus *Aquabacterium* has been identified from water and insect (walking sticks, *Phasmatodea*) gut, however, the exact role of this bacteria is yet to be decoded (Kalmbach et al., 1999, Lin et al., 2009, Shelomi et al., 2013). A study demonstrated the inhibitory role of *Actinobacteria* in the fish gut against nine common fish pathogenic bacteria (Jami et al., 2015). The genus *Hafnia* reported from healthy crayfish, *Astacus astacus* (L.) showed to have an association with haemorrhagic disease of brown trout (*Salmo trutta*) (Orozova et al., 2014). In addition to significantly enriched energy metabolism pathway in all four dietary groups, the PBM+BSF diet also enhanced the antibiotic and amino acid biosynthesis pathways. Limited resources are currently available on the metabolic capabilities of different feeds in the fish gut. However, a study by Wang et al. (2017), found increased protein concentration in the diet had a positive influence on carbohydrate, protein and energy metabolism. Contrarily, antibiotics provide a frontline defense mechanism against the pathogen, investigated to have beneficial function raised from dietary pectin and inulin supplementation (Johnson et al., 2015). The PBM+BSF diet, thus, had a positive impact on pathways associated with amino acid biosynthesis, carbohydrate and energy metabolism, driven by bacteria from *Firmicutes* and *Streptococcus*, as previously reported (Atasoglu et al., 1998, Besten et al., 2013, Bhute et al., 2017, Dai et al., 2014).

The immune response of crayfish is primarily generated from the immunocompetent cells and mucus of intestinal mucosal membrane (Ángeles Esteban, 2012, Lieschke and Trede, 2009). Among the factors, interleukin (IL), interferon (IF), tumour necrosis factor (TNF) are the major

cytokine candidates associated with immunity and inflammation of crayfish (Araki et al., 2004, Calderón-Rosete et al., 2018, Goins, 2003, Jiang et al., 2015a). The present study revealed significant ($P<0.05$) up-regulation of pro-inflammatory cytokine genes (IL-1β, IL-17F, and TNF-α) in BSF supplemented diet groups while similar expression patterns were observed for FM and PBM fed groups. To counteract the adverse effect of overexpressed pro-inflammatory cytokines on intestinal tissues, anti-inflammatory cytokines (IL-10) also need to be up-regulated to neutralize inflammation (Miao et al., 2018). Enrichment of *Fimicutes* with dietary protein supplementation reported to have a positive role on cytokine gene expression resulting into the improvement of the immune status of fish (Miao et al., 2018, Panigrahi et al., 2007, Selim and Reda, 2015). The bacteria from *Firmicutes* phylum was found to be abundant in PBM+BSF followed by FM+BSF diet groups, and thus we discovered a link between the expression level of cytokine genes and richness of *Firmicutes*. The improved expression level of cytokine genes was recorded with dietary soybean meal where phylum *Firmicutes*, especially *Bacillus* and *Lactobacillus* reported to be increased (Miao et al., 2018). Lactic acid bacteria (LAB) associated with the production of pro and anti-inflammatory cytokines, IL-1β, IL-6, IL-8, TNF-α, and IL-10 in the intestine tissue have been reported (Ringø et al., 2018). The results of the present study showed a significant up-regulation of intestinal cytokine genes of marron after 60 days of feeding trial in the BSF supplemented groups. The higher abundance (>50%) of *Firmicutes* and LAB in PBM+BSF fed marron might be associated with overexpression of cytokine genes.

6.5(1). Conclusion

The overall findings of present study showed that the dietary supplementation of BSF significantly improved the intestinal microbiota, health and immune status of marron. However, compared to other three diets, significant effects of PBM+BSF were recorded for gut

microbiota and cytokine genes. However, the interaction mechanism of BSF supplemented diets with bacterial abundance in the gut and associated factors needs to be further investigated.

CHAPTER 6: Protein diets for marron aquaculture

EXPERIMENT 2: The effect of various dietary non-fishmeal protein sources on the water and gut microbial communities in marron (*Cherax cainii*, Austin 2002) culture

(The findings of this chapter has been submitted to Science of the Total Environment)

Abstract

Alternative to fishmeal protein sources from plant, animals and insects sources have been evaluated constantly on growth performance and gut microbiota of aquatic species. In a 60-days feeding trial, the present study investigated the effects of different protein sources on the gut microbiota and rearing aquatic environment of marron (*Cherax cainii*) as well as the correlations among protein sources, microbial communities and growth performance of this species. Six different protein diets were formulated from fishmeal, black-soldier-fly meal, lupin meal, poultry-by-product meal, soybean meal and tuna hydrolysate. Analysis of 16S rRNA data showed significant differences of microbial communities between gut and water microbiota during marron aquaculture. Bacterial diversity in the gut were found sensitive and selective in plant based diets, where LPN and SOY influenced the growth of same bacterial groups. *Aeromonas*, *Candidatus* Bacilloplasma, *Hafnia* Obesumbacterium, *Shewanella* and *Vibrio* were identified in all gut samples regardless of dietary protein source, suggesting that they could be the core microbiota in marron gut. The growth of *Aeromonas*, *Hafnia* Obesumbacterium and *Vibrio* in the marron gut was significantly increased by soybean, tuna hydrolysate and fishmeal diets, respectively. In water, black-soldier-fly meal favoured the growth of Firmicutes and lactic acid bacteria (LAB) including *Clostridium*, *Lactobacillus* and *Streptococcus*. The higher relative abundance of *Aeromonas* in both gut and reared water signifying the ubiquitous nature of the genus in the environmental samples. Only a few of the genera including *Acinetobacter-Pseudomonas*, *Acinetobacter-Aeromonas* and *C. Bacilloplasma-Enterobacter* were found to have positive correlation between gut and water,

indicating that the bacteria from these two environments are less likely to interact. The animal dietary protein sources, higher abundance of *C.* Bacolloplasma in poultry-by-product meal is linked to better marron gut health while insect diet black-soldier-fly augmented beneficial LAB in the rearing water. The overall results suggest that these two protein diets can be used as an alternative of fishmeal in marron aquaculture.

Keywords: Marron; microbial communities; high throughput sequencing; bioinformatics

6.1(2). Introduction

The fishmeal (FM) from wild marine stocks has been used for the culture of aquatic animals for many years. However, considering the limited stocks of FM (Olsen and Hasan, 2012), environmental sustainability and ecological stability (Malcorps et al., 2019), the focus of attention has been shifted to find a cheap, suitable and sustainable alternative protein sources for the global aquaculture in recent times. To meet the burgeoning demand of FM, researchers are evaluating proteins from plant, animal and insect sources for their suitability to replace FM from the aqua-diets. A significant breakthrough already achieved for some of the finfish (Snout bream, *Megalobrama amblycephala*) (Ahmed et al., 2019) and crustaceans (Pacific white shrimp, *Litopenaeus vannamei*) (McLean et al., 2020) by replacing 100% FM from the aqua-diets without affecting growth parameters. Approaches to replace 50% of FM by the soybean (Tan et al., 2018) and poultry-by-product (Saoud et al., 2008) meal was also successful for the red claw (*Cherax quadricarinatus*) crayfish without impacting growth parameters.

As an alternative to FM, protein from different sources have been tested on aquatic animals, especially on fish. Among them, soybean meal (SOY) (Catalán et al., 2018, Miao et al., 2018) from plant feedstuffs, poultry-by-products (PBM) (Rimoldi et al., 2018) and tuna hydrolysate (TSH) (Siddik et al., 2018) from animal, and black soldier-fly (BSF) (Huyben et al., 2019) from insect sources are most commonly tested alternative protein sources in aqua-diets. These proteins have modulatory effects on gut microbiota of fish (Gajardo et al., 2017), bacterial

community that play a key role in growth, digestion and immunity of aquatic animals (Butt and Volkoff, 2019, Wang et al., 2018). In crayfish on the other hand, dietary effects of BSF and PBM has been investigated only for the marron (*Cherax cainii*) where significant shift of gut microbial communities and gene functions were observed with PBM+BSF diet (Foysal et al., 2019b). The knowledge regarding the interaction and correlations between protein sources and gut microbiota is incomplete and fragmentary. Furthermore, considering the steep increase of global production (400000 tonnes in 2008 vs 9500 t in 2000) (Gherardi, 2011), and advantages of crayfish aquaculture including high consumer preferences, distinctive favour and taste, easy culturing methods, less disease susceptibility and long distance live shipment (Goyert, 1978), the study of gut microbiota is immensely important to understand the impacts of protein diets on intestinal health status, nutrients digestibility and functionality.

The composition of gut microbiota strongly affected by bacterial communities colonized in rearing water (De Schryver et al., 2008, Gutierrez-Wing and Malone, 2006). Bacterial interaction between gut and external environment also depend on experimental settings (bio-floc vs recirculating aquaculture systems), feeding regime and cultured species (Giatsis et al., 2015). Static active suspension culture with no water recirculating facilities can transfer more bacteria from surrounding water into the gut of aquaculture species (Giatsis et al., 2015). Still now, no information is available for the symbiosis between the gut bacteria of crayfish and rearing water. On this backdrop, we employed Illumina based 16S rRNA amplicon sequencing followed by massive downstream bioinformatics to generate information about the interaction between gut and water microbial communities and their correlations to crayfish growth.

6.2(2). Materials and methods

6.2(2).1. Experimental set-up and animal husbandry

The experiment set-up was designed by our colleague as a part of another trial. A total of 170 marron (71.2 ± 0.4 g) were distributed into 18 different tanks at a density of 9 marron per tank (X 3 replicates per dietary treatment), and acclimated for 14 days before commencement of the

trial. The rest of the experimental set-up along with water quality analysis including temperature, pH and dissolved oxygen (DO) were performed as described in general methodology (chapter 3.1). Considering the aims of the study, no water exchange was performed during trial. Uneaten feedstuffs and faecal wastes were removed once in a week using high density filter net.

6.2(2).2. Feed formulation and feeding trial

Six isoproteic, isolipidic and isocalorific diets containing fishmeal (CTL), black soldier fly meal (BSF), lupin meal (LPN), poultry-by-product meal (PBP), soybean meal (SOY) and tuna hydrolysate meal (TSH) were prepared (Table S6.2.1, Appendix 3). The ingredients were supplied by Glenn Forrest, Western Australia and after feed formulation the test diets were also prepared by the same company. Proximate compositions (Table S6.2.1, Appendix 3) of each diet was determined as per the method of Association of Official Analytical Chemists, AOAC (AOAC, 2006). Each marron in a cage was fed the respective diets, once every day at 12 PM at 1.5% of the body weight.

6.2(2).3. Sampling

Extraction of DNA from water samples was conducted following method described earlier (Hinlo et al., 2017, Jeunen et al., 2019) for the higher recovery of environmental DNA (eDNA). Water samples (1L/tank) were collected in sterile plastic bottle at day 58, 59 and 60 from each tank. Before sample collection, water in the tank was mixed properly using tank stirrer (AstralPool, WA, Australia). Water samples were concentrate first by centrifuging at 8000 rpm for 10 min, followed by membrane filtration in 0.2-µm polycarbonate filters. The filters were then cut into small pieces (~1 mm) and transferred into 2-ml Eppendorf tube. For gut microbiota analysis, 54 marron were collected from 18 tanks, 9 from each treatment (3 marron/tank) at the end of trial. Extraction of whole gut was performed under biological safety

cabinet. Marron gut with mucosa and pellet from water were homogenized in a tissue lyser with sterile beads (Qiagen, Hilden, Germany) in 2-ml Eppendorf tube.

6.2(2).4. DNA extraction, PCR amplification and 16S rRNA sequencing

DNA extraction from 108 samples was performed using Qiagen Blood and Tissue Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The PCR amplification of v3v4 hypervariable regions, library preparation and pair-end sequencing were performed according to methods described in general methodology (chapter 3.4).

6.2(2).5. Sequence data processing

TrimGalore (v0.6.6) (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) was used for quality trimming of reads with following parameters. FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and MultiQC (Ewels et al., 2016) were used for checking the quality of reads before and after trimming. Overlapping pair-end reads were merged using NGmerge (Gaspar, 2018). Micca pipeline (v1.7.2) was used for the filtering of merged sequences and *de novo* greedy clustering into Operational Taxonomic Units (OTUs) at 97% similarity threshold level. Phylogenetic assignment of the representative OTUs at different taxa level was performed against SILVA 1.32 release (Quast et al., 2012). The rarefaction depth value was set to 17,796 bp and subsequent calculations of alpha beta diversity were performed in QIIME (v1.9.1) (Caporaso et al., 2010) and calypso pipeline (Zakrzewski et al., 2017). Alpha diversity of samples was calculated in terms of Richness and Shannon index. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity of relative abundance was used for the beta diversity analysis. Relative abundance of bacterial OTUs at phyla and genus level was calculated in ampvis2 (Andersen et al., 2018) and phyloseq (McMurdie and Holmes, 2013) R packages.

6.2(2).6. Statistical analysis

Statistical analysis was performed in R statistical environment (v3.6.1) (https://www.r-project.org/) and calypso pipeline (Zakrzewski et al., 2017). One-way ANOVA with Tukey's post-hoc test was used to compare the growth parameters among six different treatment groups. Clustering differences between and among groups was measured in terms of Permutational multivariate analysis of variance (PERMANOVA) using anosim/adonis with 999 permutations in vegan. To identify the differentially abundant bacteria at phylum and genus level, we performed non-parametric Wilcoxon rank test (for two groups) and Kruskal-Wallis test (for more than two groups) with Bonferroni correction. Correlation analysis of top 15 taxa and growth parameters of crayfish was measured as "Pearson" correlation coefficient following Benjamini and Hochberg *P*-value adjustment for multiple comparisons. At all stages, *P*-value of 0.05 was considered as statistically significant and annotated as P<0.001 (***), P<0.005 (**) and P<0.05 (*) to express the level of significance.

6.2(2).7. Calculations

At the end of the experimental trial, the marron growth performance was calculated as described in chapter 3.7, general methodology.

6.3(2). Results

6.3(2).1. Marron growth performance

End data showed no significant improvement in weight gain for any of the alternative protein source (APS) diets, compared to SOY and LPN. However, specific growth rate (SGR) and feed conversion ratio (FCR) were significantly ($P<0.05$) higher with PBM and BSF diets compared to fishmeal (CTL) (Table 6.2.1).

Table 6.2. 1 Growth performance of marron with six different protein diets

Growth	CTL (FM)	PBM	BSF	SOY	LPN	TSH
WG (g %)	18.5 ± 1.1^{a}	19.2 ± 1.3^{a}	18.8 ± 0.9^{a}	17.6 ± 1.4^{a}	17.7 ± 1.3^{a}	18.2 ± 1.1^{a}
SGR (g/day)	0.7 ± 0.1^{ab}	0.8 ± 0.01^{b}	0.8 ± 0.01^{b}	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}	0.7 ± 0.1^{ab}
FCR	3.0 ± 0.1^{ab}	2.8 ± 0.1^{b}	2.8 ± 0.1^{b}	3.1 ± 0.1^{a}	3.1 ± 0.1^{a}	3.0 ± 0.1^{ab}

Letters with different superscripts indicating significantly different mean standard values

Abbreviations: CTL, control (fishmeal); BSF, black-soldier-fly meal; PBP, poultry-by-product meal; SOY, soybean meal; LPN, lupin meal; TSH, tuna hydrolysate meal; WG, weight gain; SGR, specific growth rate; FCR, feed conversion ratio.

6.3(2).2. Sequence statistics: Water microbial communities are more diverse than gut with lower read abundance

After quality filtering, a total of 4.5 M reads (41458.4 ± 1480.6) were obtained from 108 samples. The rarefaction curve revealed that each sample was sequence at high depth to capture maximum diversity (Figure 6.2.1 A). Collectively, gut and water samples generated 6034 OTUs (2865 shared), 26 phyla (21 shared) and 420 genera (176 shared). For the gut, 1.5 M reads (28365.5 ± 1315.5) and 745 OTUs were obtained from 54 samples that were phylogenetically assigned into 21 phyla and 229 genera. On the other hand, water samples yielded 3.0 M reads (36868.8 ± 1586.4) and 5732 OTUs that were classified into 26 phyla and 367 genera. The average rarefied reads for the top most abundant genus in the gut (5070.4 ± 867.3) found significantly higher (P-value< 0.001) than the most enriched genus in water (1260.8 ± 253.1) (Figure S6.2.1 A, Appendix 2).

6.3(2).3. Diversity indices: Gut and water microbial communities are distinctly different

The richness and Shannon measurements of alpha diversity were significantly higher in water, in relation to gut (Figure 6.2.1 B-C). In addition, the number of unique OTUs (3083 unshared) [Figure 6.2.1 D] and genera (191 unshared) (Figure S6.2.1 B, Appendix 2) was found higher in water samples, compared to gut. Beta-ordination showed distinct clustering of bacterial

OTUs wherein PERMANOVA R^2 value of 0.8232 and *P*-value of 0.0012 revealed a very different microbial diversity in the gut and water (Figure 6.2.1 E).

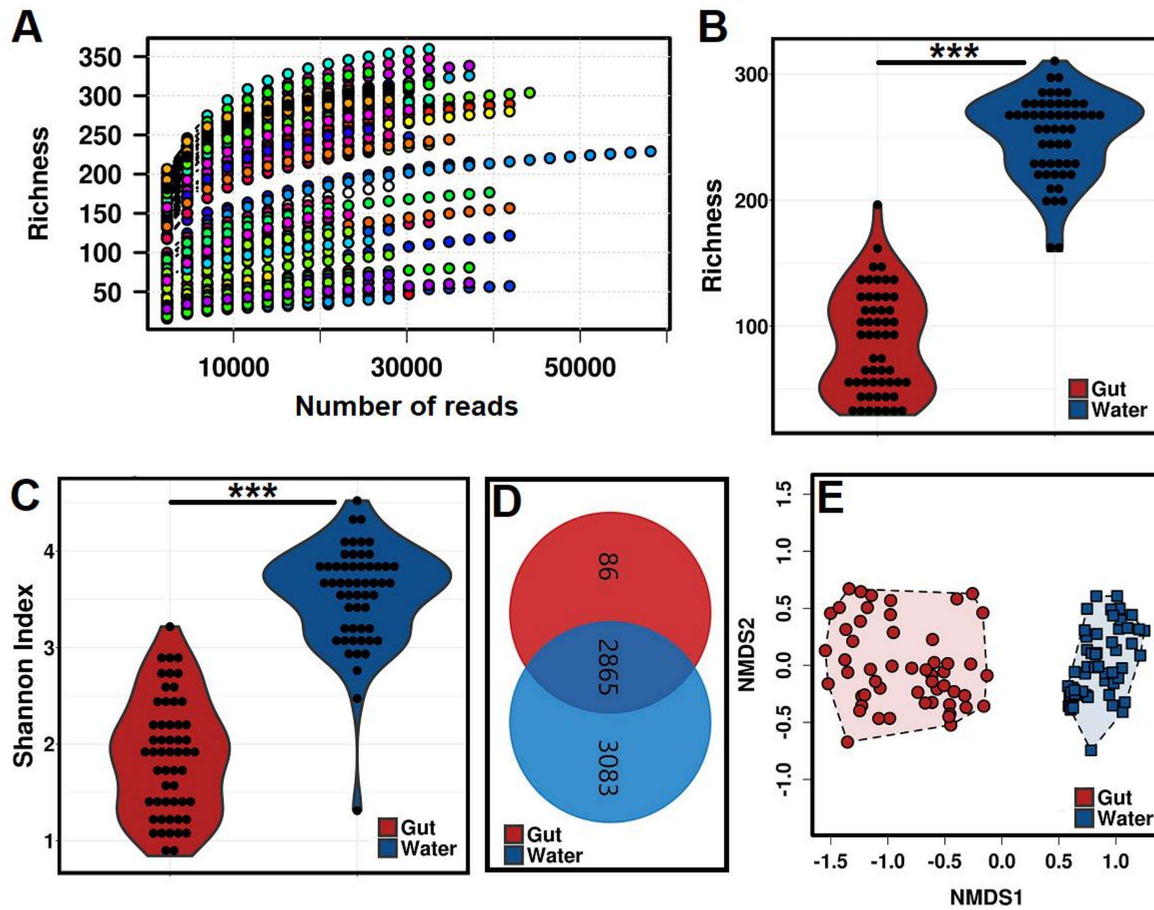

Figure 6.2. 1 Sequence statistics and alpha-beta diversity indices. (A) Rarefaction curve showing depth and saturation level of sequences. (B) Alpha diversity, richness. (C) Alpha diversity, Shannon index. (D) Number of shared and unique OTUs in two different environments. (E) NMDS plot showing clustering of OTUs from two different environments.

6.3(2).4. Microbial communities in the gut and water

Four bacteria genera, *Acinetobacter*, *Aeromonas*, *Flavobacterium* and *Pseudomonas* had more than 1% read abundance in both gut and water samples for at least one of the treatment group. A total of 191 unshared genera in water, compared to 53 in the gut (Figure S6.2.1 B, Appendix 2) suggesting that feeding aquatic animals with protein diets influences a complex bacterial interactions in water. Similarity in OTUs abundance between gut and water samples was observed only for the *Aeromonas* in SOY (73% in the gut vs 66.6% in the water) and BSF

(37.4% in the gut vs 17.7% in the water) groups. Surprisingly, only *Candidatus* Hepatoplasma and *Vibrio* had significantly higher abundance in the gut samples, compared to water (Figure 6.2.2 A-B). Differentially abundant water bacteria were *Bacteroides*, *Dechloromonas*, *Acidovorax*, *Lactococcus*, *Haliangium*, *Prevotella,* *Devosia,* *Pajaroellobacter* and *Polunucleobacter* (Figure 6.2.2 C).

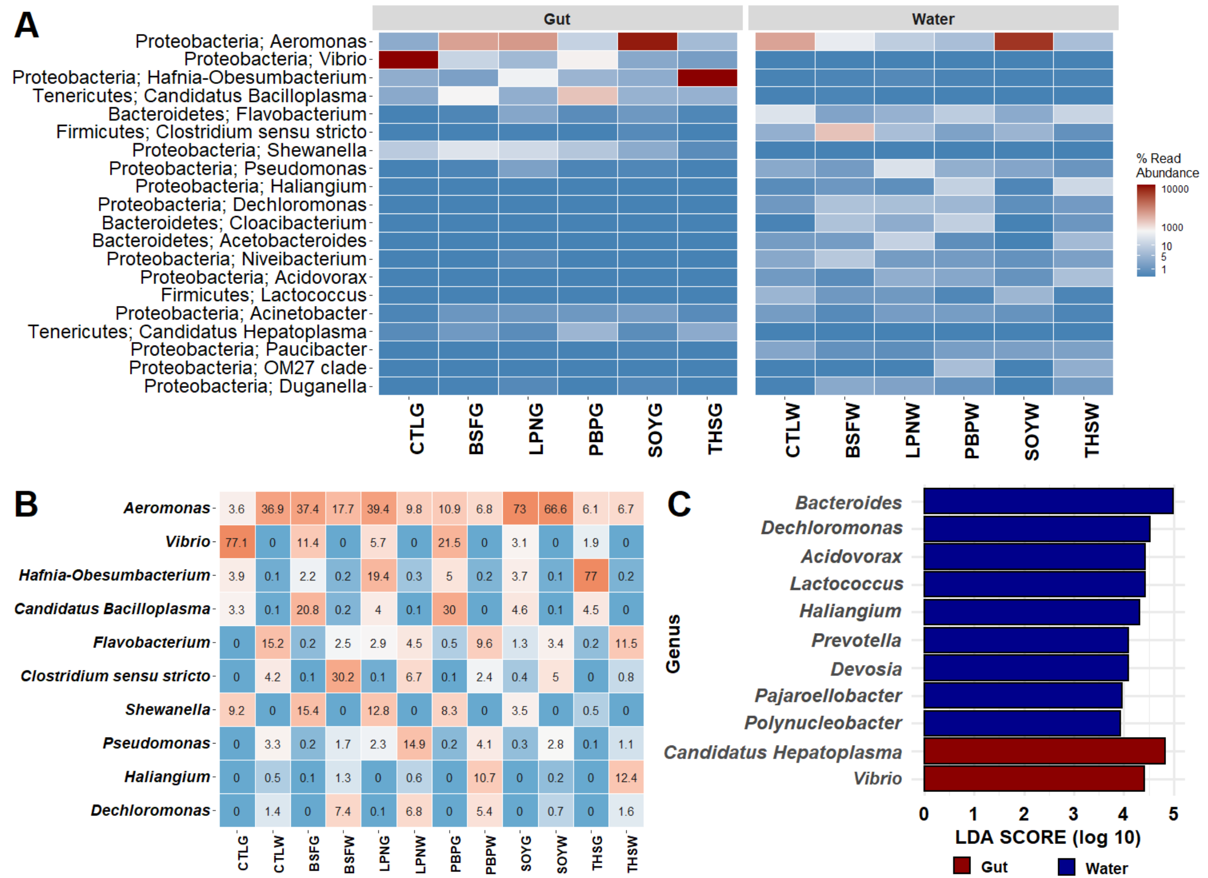

Figure 6.2. 2 Microbial communities in the gut (G) and water (W) samples with different APS diets. (A) Relative abundance of bacteria at genus level (top 20 genera based on read abundance). (B) Percentages of bacterial abundance at genus level (top 1% OTUs) in the gut and water samples. (C) Differentially abundant bacteria at genus level in the gut and water with APS diets. Abbreviations: CTL, control (fishmeal); BSF, black-soldier-fly meal; PBP, poultry-by-product meal; SOY, soybean meal; LPN, lupin meal; TSH, tuna hydrolysate meal.

6.3(2).4.1. Microbial communities: protein diets modulate gut microbial communities

The alpha diversity measurements showed improvement of Shannon index only by the LPN diet while richness was not impacted by any of the diets (Figure 6.2.3 A-B). Out of 745, only 21 OTUs (2.8%) were shared by the all diet groups. Majority of OTUs generated for the CTLG, BSFG, LPNG and TSHG groups were found to be shared within and among the groups. Lowest OTUs counts recorded for CTLG (81) group while PBPG (303) diet group generated maximum OTUs and majority of them were unique across the sample groups (Figure 6.2.3 C-D). However, most of the unshared OTUs from PBPG group were classified into same taxonomic clades, mostly belong to *Vibrio* and *C*. Bacilloplasma. The clustering of gut bacterial OTUs with different APS diets in terms of Non-metric multidimensional scaling plot is shown in Figure 6.2.3 E. Centroid analysis within the beta-ordination demonstrates exhibited clustering was statistically significant with observed dissimilarity (R^2) score of 0.983 and PERMANOVA P-value<0.001. The dispersion of samples for three different protein sources (plant, animal and insect) also found significant (R^2 = 0.722, PERMANOVA P-value 0.0223) (Figure 6.2.3 F). The relative abundance profile of gut bacteria showed Proteobacterial dominance in all samples. The phylum Tenericutes augmented in PBP and BSF feed groups, representing 42.8% and 36.4% of the read abundance (Figure S6.2.2 A, Appendix 2). Nevertheless, Proteobacteria and Tenericutes comprised of more than 98% of read abundance in all sample groups. Several genera were limited to only specific treatment groups whereas *Aeromonas*, *Candidatus* Bacilloplasma, *Hafnia* Obesumbacterium, *Shewanella* and *Vibrio* were identified from all samples of marron gut, regardless of treatments (Figure S6.2.2 B, Appendix 2). We compared the read abundance only for the lower taxonomic level and found 12 genera were significantly differed in six treatment groups. *Aeromonas*, *Clostridium* and *Rheinheimera* were enriched with SOY diet whereas LPN diet favoured the growth of *Aquitalea*, *Flavobacterium* and *Pseudomonas*. In contrast, CTL and THS groups augmented the abundance for *Vibrio* and *Hafnia* Obesumbacterium, respectively (Figure S6.2.3, Appendix 2).

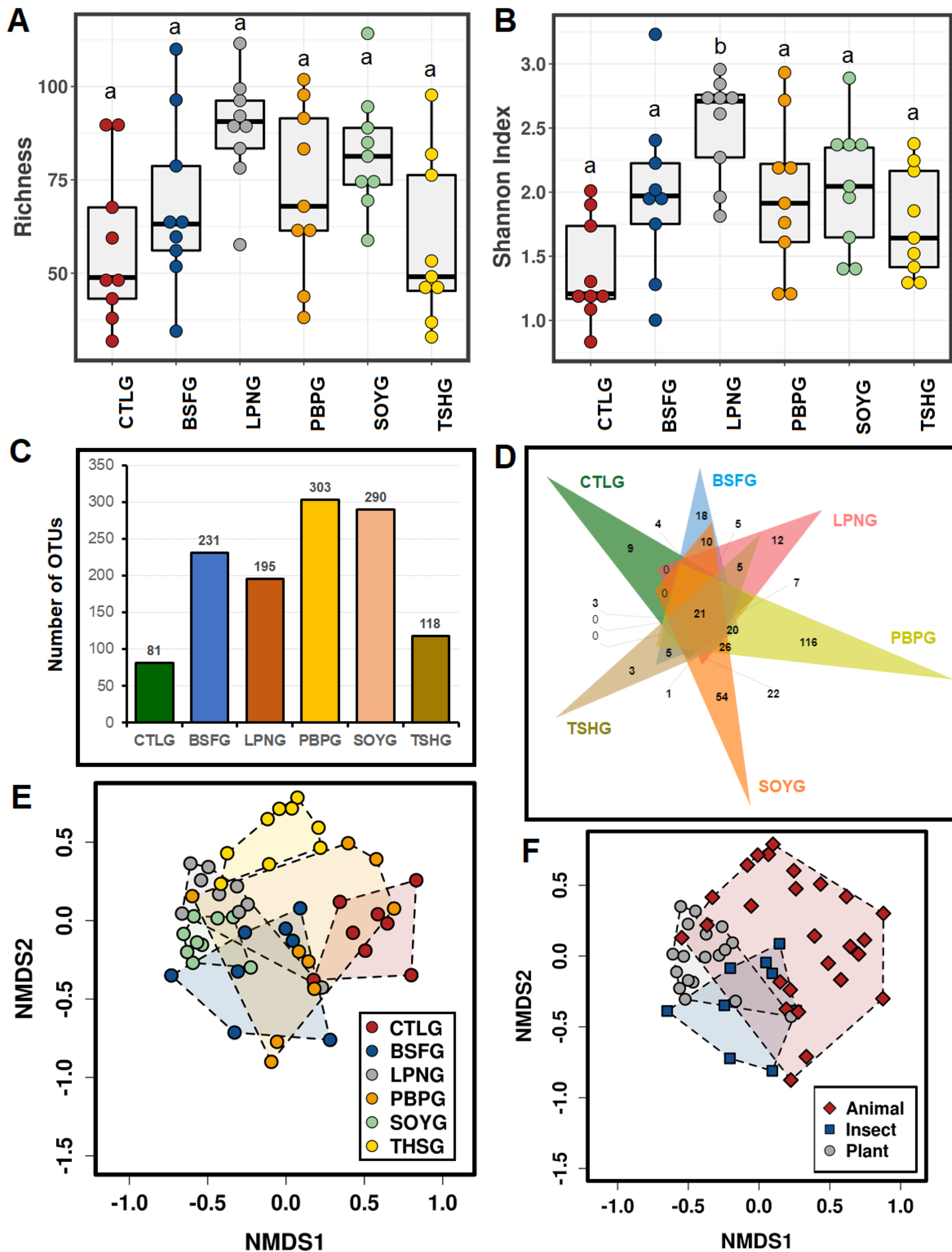

Figure 6.2. 3 Alpha-beta diversity indices of gut (G) microbiota fed APS diets. (A) Richness. (B) Shannon. (C) Number of OTUs. (D) Number of shared and unique OTUs. (E) NMDS plot showing clustering of bacterial OTUs in six different protein fed groups. (F) NMDS plot for three different protein sources, plant, animal and insect. Abbreviations: CTL, control

(fishmeal); BSF, black-soldier-fly meal; PBP, poultry-by-product meal; SOY, soybean meal; LPN, lupin meal; and TSH, tuna hydrolysate meal.

6.3(2).4.2. Microbial communities: Protein diets alter microbial communities in water

The alpha diversity measurements found no differences (P>0.05) in richness and Shannon index among the groups of six different water samples (Figure 6.2.4 A-B). The number of generated OTUs (total 5732) also did not differ much for six treatment groups, ranging from 984 for the CTLW to 1190 for the BSFW. Overall, only 2.6% (150) of the OTUs were shared by the water samples from all six groups whereas 5.4% (310), 4.8% (276) and 3.6% (205) of the OTUs were found unique for the TSHW, BSFW and PBPW groups, respectively, revealing the sensitivity of water microbial communities to protein diets (Figure 6.2.4 C-D). PERMANOVA (R^2 = 0.722, P-value 0.032) and Bray-Curtis revealed that protein diets contributed significantly to the variation in microbial communities in the water (Figure 6.2.4 E). The ordination also found significant (R^2 = 0.542, P-value 0.045) based on sources (animal, insect and plant) of protein diets (Figure 6.2.4 F), where the ordination for BSFW (insect source) found different from other groups. Alike gut, the majority of water bacteria at phylum level are belong to Proteobacteria (59.8%), Bacteroidetes (23.2%) and Firmicutes (16.4%). Interestingly, compared to Proteobacteria abundance (41.6%), high Firmicutes abundance (36.6%) was observed for the BSFW group (Figure S6.2.4 A, Appendix 2). Unlike in the gut, *Aeromonas* dominated in the water for all groups at genus level (Figure S6.2.4 B, Appendix 2). *Auitalea* and *Clostridium* along with lactic acid bacteria (LAB) *Lactobacillus* and *Streptococcus* had significantly (P<0.05) different abundance in the BSFW group (Figure S6.2.5, Appendix 2). In contrast, *Lactococcus* in CTLW, *Aeromonas* and *Enterobacter* in SOYW, and *Desulfovibrio* and *Propinovibrio* in LPNW had significantly higher abundance. *Acidovorax* with some uncommon genera including *Bryobacter*, *Fimbriiglobus*, *Gemmatimonas* and *Stella* enriched in the TSHW group.

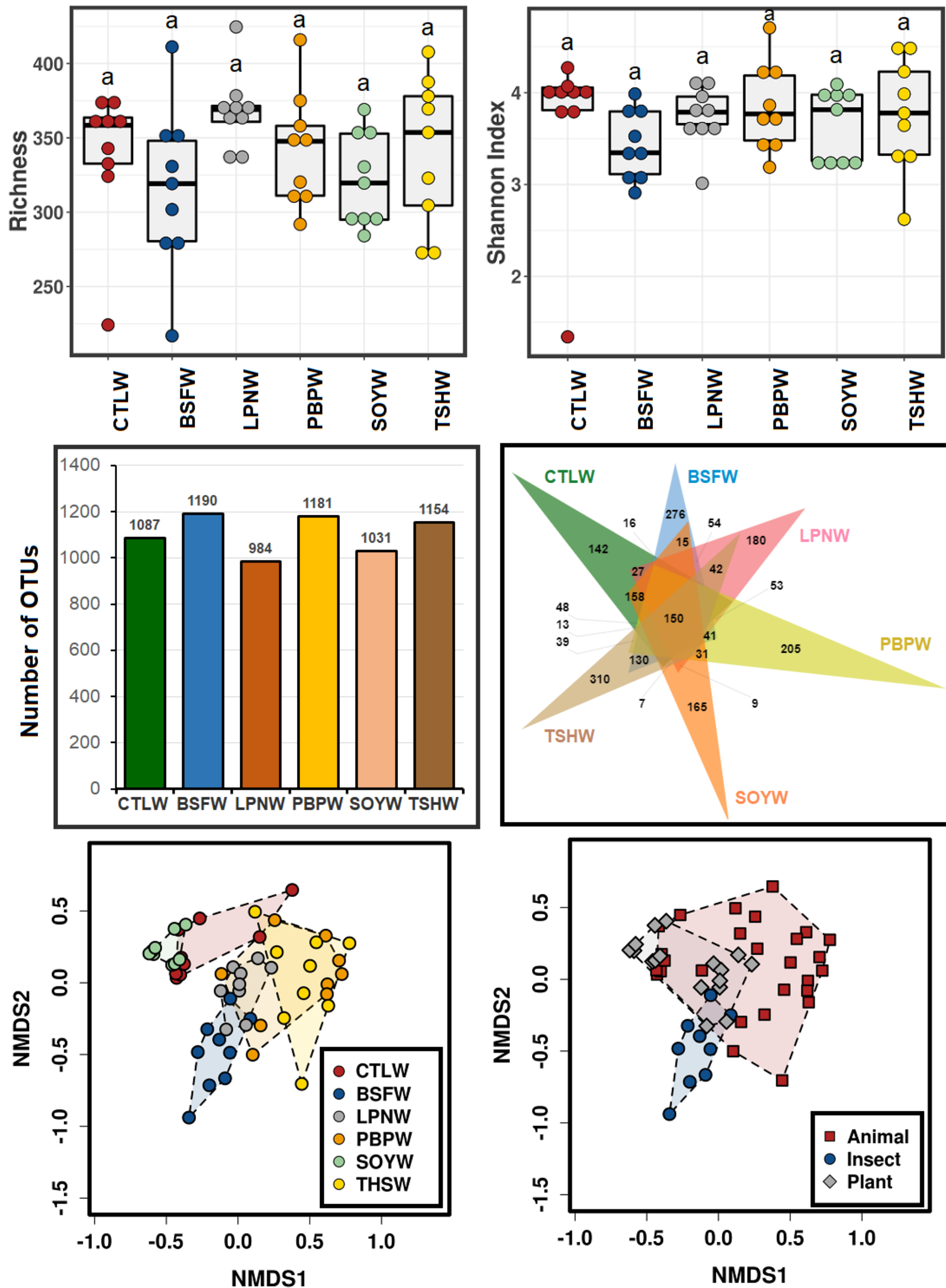

Figure 6.2. 4 Alpha-beta diversity indices of water microbiota with protein diets. (A) Richness. (B) Shannon. (C-D) Number and distribution of OTUs in six different groups. (E) NMDS plot showing clustering of bacterial OTUs in the water samples. (F) NMDS plot showing clustering

of bacterial OTUs in the water samples for plant, animal and insect protein sources. Abbreviations: CTL, control (fishmeal); BSF, black-soldier-fly meal; PBP, poultry-by-product meal; SOY, soybean meal; LPN, lupin meal; and TSH, tuna hydrolysate meal.

6.3(2).5. Correlation: Gut and water microbiota are unlikely correlated

To investigate the correlation between gut and water bacteria, we have selected only those 11 genera that have at least 1% of the read abundance either in gut and water samples for any of the diet group. Strong positive correlation ($P<0.005$) was observed only between OTUs of *Hafnia* Obesumbacterium and *Candidatus* Hepatoplasma (gut), and *Shewanella* and *Enterobacter* (gut). Positive correlations ($P<0.05$) also observed between *Acinetobacter* and *Pseudomonas* (gut-water), *C*. Bacilloplasma and *Enterobacter* (gut), and *C*. Bacilloplasma and *Shewanella* (gut). Negative correlations observed between *Acinetobacter* and *Vibrio* (gut), and *Flavobacterium* and *Shewanella* (gut) (Figure S6.2.6, Appendix 2).

6.3(2).6. *C. Bacilloplasma* and *Cloacibacterium*: Key bacteria for marron

The relative read abundance for *C*. Bacilloplasma and *Cloacibacterium* was positively correlated ($P<0.005$) to the SGR of crayfish. *Dechloromonas* in water had also positive ($P<0.05$) impact while *Aeromonas* and *Lactococcus* found inversely correlated ($P<0.001$) to the growth parameters (Figure 6.2.5 A). *C*. Bacilloplasma enriched in animal based diets positively correlated to the health parameters of crayfish. An inverse relationship was observed between *Aeromonas* from plant based diets and *Lactococcus* from animal based diets (Figure 6.2.5 B). Further diet based analysis showed positive correlation between *C*. Bacilloplasma in PBPG and weight gain of marron (Figure 6.2.5 C). Top 15 water bacteria in six different groups had no correlations ($P>0.05$) to growth parameters of marron (Figure 6.2.5 D).

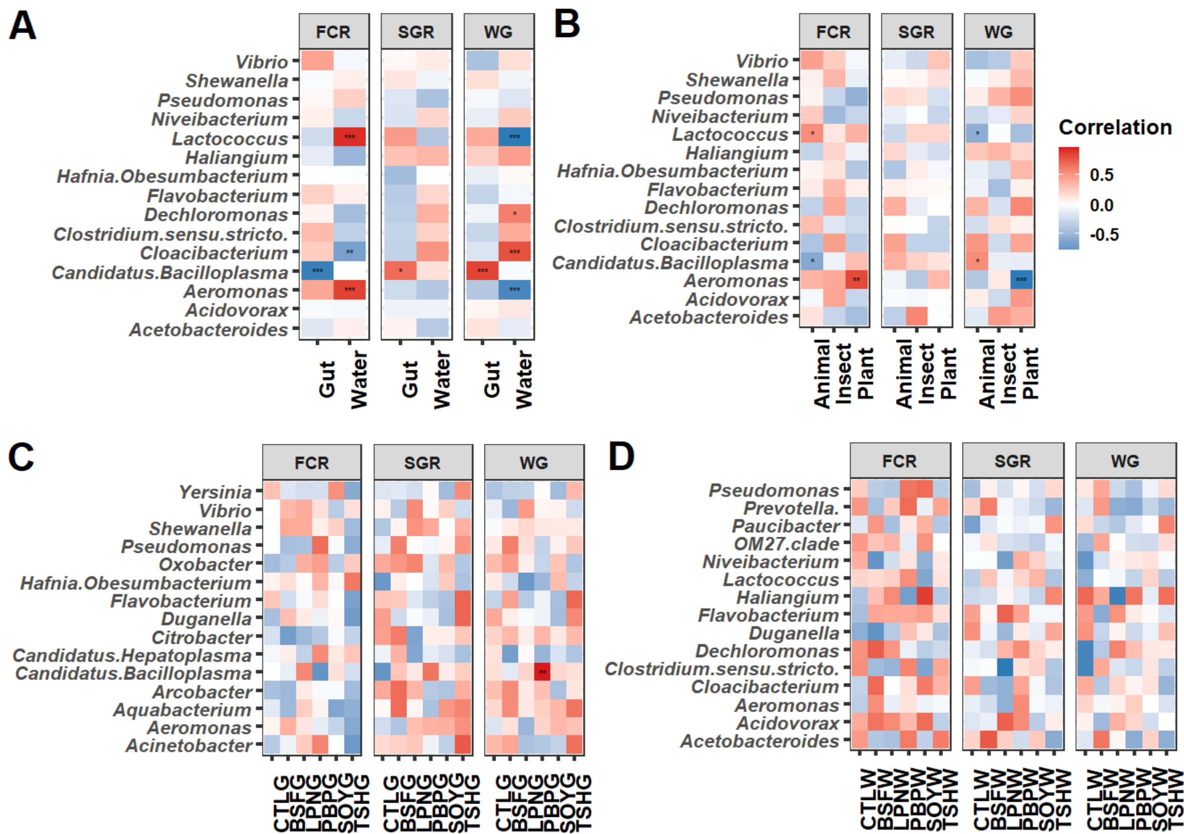

Figure 6.2. 5 Pearson plot showing correlation between bacterial abundance (gut and water) and growth performance of marron. The correlation values are in the upper right corner where red indicates positive correlation and blue specifies inverse correlation. ***Significantly different at α-level of 0.001. **Significantly different at α-level of 0.005. *Significantly different at α-level of 0.05. Abbreviations: CTL, control (fishmeal); BSF, black-soldier-fly meal; PBP, poultry-by-product meal; SOY, soybean meal; LPN, lupin meal; and TSH, tuna hydrolysate meal.

6.3(2).7. Data availability

The sequence files have been deposited in the National Centre for Biotechnology Information (NBCI) resources and will be available on specified date.

6.4(2). Discussion

Microbiome analysis has become a most popular tool to evaluate the dietary impacts of aquatic animals. In present study, marron were reared under fixed environmental conditions including photoperiod, water temperature, dissolved oxygen, pH and nitrogenous compounds (nitrate, nitrite and ammonia). Hence, the microbial differences in the gut and water are primarily generated due to the differences in the dietary composition. Effects of alternative protein sources have been investigated on fish gut microbial communities, however this is the first in-depth study where impacts of five different alternative to fishmeal protein diets from plant, animal and insect sources on the gut and water microbial communities and correlations among them were investigated for marron aquaculture. A very different microbial communities in the gut and water while *Aeromonas* was found by observing the only genus comprised of more than 5% of the read abundance in all samples. This results further reveal the ubiquitous nature of *Aermonas* in environmental samples reported earlier (Janda and Abbott, 2010). A previous study on impacts of feed and water on the gut microbiota of tilapia reported no similarity between gut and water samples as only two of the OTUs found shared between them (Giatsis et al., 2015). In contrast, 2865 out of 6034 common OTUs suggesting resemblances between gut and water microbial communities in marron, compared to tilapia. Nevertheless, most of the predominant bacteria in water were absent in the gut and the results are very much consistent with the Giatsis et al., (Giatsis et al., 2015). In every way, the microbial communities in both gut and water are distinctly dissimilar in present study with marron, compared to previous study with tilapia. Therefore, differences in physiology of aquatic animals, gut morphology and biochemistry, nutritional preferences, composition or ingredients of feed, sources of raw materials and type of aquaculture species influence the gut and water microbial communities.

Present study identified no differences in richness of gut microbial communities with APS diets while higher Shannon diversity in in LPNG suggesting that the species distributions are even

and uniform in this group. Only 21 shared OTUs among diets groups and significant beta-dispersion suggested that the gut microbiota of marron are very sensitive to protein sources. The highest number of unshared OTUs in PBPG advocated that this group produced more diversified microbial communities in the marron gut, and can be correlated to better marron health from previous studies (Saputra et al., 2019). Nevertheless, the number of unshared OTUs in marron is far behind fish, as a study stated 60 unique OTUs in Atlantic salmon (*Salmo salar*) with five different protein diets (Gajardo et al., 2017). The dissimilarities in processing of gut samples could be the reason of OTUs differences in marron and Atlantic salmon as the gut contents (digesta) and mucosa processed separately in Gajardo et al., (2017) study wherein we collectively homogenized gut contents and mucosa. Furthermore, the differences in physiology and culture environment (Dehler et al., 2017a, Wong and Rawls, 2012) have significant impact on gut microbiota that also linked to microbial community differences between marron and Atlantic salmon. Regarding gut microbial communities, the differential abundance showed a clearest differences between plant proteins (LPN and SOY) and other sources (animal and insect). This variation caused mainly by pathogenic and toxin producing bacteria *Clostridium* (*perfringens*) (Cai et al., 2008), *Pseudomonas* (Ardura et al., 2013), *Flavobacterium* (Rahman et al., 2010), *Rheinheimera* (Chiellini et al., 2019), and peptidoglycan-chitin degrading *Vogesella (*Jørgensen et al., 2010*)* and toxin degrader *Paucibacter* (Rapala et al., 2005). Among these *C. butyricum* reported to have positive impacts on crayfish gut (Foysal et al., 2019d). The inability to generate species level information due to shorter read lengths (≤300 bp) is one of the major constrains of Illumina sequence and therefore we could not differentiate between beneficial and pathogenic bacteria of *Clostridium* in this study. The anti-nutrition factors (ANFs) in plant based proteins cause intestinal dysfunction and dysbiosis in aquatic animals, and plant proteins are recommended to be used at lower concentration (Krogdahl et al., 2010, Veron et al., 2016). The selective, and differential effects of plant proteins on gut microbial

communities of crayfish therefore raise further concern on the use of plant protein sources at higher concentration in aqua-diets. Other genera having differential expression including *Aeromonas*, *Citrobacter*, *Shewanella* and *Vibrio* found in all groups however at various concentrations suggesting the genus specific response of certain diets on common bacterial communities in the intestine of crayfish. Nevertheless, the overwhelming abundance of some selective genera in SOYG and LPNG groups need further investigations and amplification of long reads is recommended for the some of the genera having both positive and negative impacts on fish health and immunology.

In the present study, we found that *Aeromonas*, *Candidatus* Bacilloplasma, *Hafnia* Obesumbacterium, *Shewanella* and *Vibrio* are the inhabitants of marron gut and capable of growing independently despite changes in diets and environmental conditions. The observations of these five core microbiota are consistent with three previous studies where these five genera were present in all type of dietary treatments (Foysal et al., 2019b, Siddik et al., 2020a) and altered environmental conditions (Foysal et al., 2020c). Two very recent studies on red-swamp crayfish (*Procambarus clarkii*) under different feeding regimes (Shui et al., 2020) and developmental stages (Zhang et al., 2020) reported *C*. Bacilloplasma rich gut communities along with *Aeromonas*, *Shewanella* and *Vibrio* while *Hafnia* was absent in both cases. Therefore, *Hafnia*-Obesumbacterium can be used to differentiate the core microbiota between marron and red-swamp crayfish. However, considering the various abundance level of these five core genera in different diet groups, it is not clear to what extent and under which environmental conditions host plays active role in promoting the growth of a selective core microbiota.

Beside gut microbial alteration by feeding APS, insect diets also significantly altered the microbial communities in water. The difference regarding lactic acid bacteria (*Lactobacillus* and *Streptococcus*), *Aquitalea*, *Clostridium*, *Cloacibacterium* and *Prevotella* in water with

insect diet was prominent for the BSF meal. Interestingly, plant diet LPNW had similar but low abundance for these five genera except *Aquitalea*. We have identified *Clostridium*, *Lactobacillus*, *Streptococcus* and *Prevotella* from marron gut fed PBM+BSF diet (Foysal et al., 2019b). That specifies positive correlation between insect diet and LAB for the crayfish aquaculture. Our study can be supported further by comparing the microbiome of BSF gut from most recent study (Klammsteiner et al., 2020), where fly fed on grass-cuttings had 75% of abundance for three Bacilli genera, *Lactococcus*, *Lactobacillus* and *Weissella*. With no water exchange, the bacteria from uneaten feedstuff might colonize in water and increase their relative abundance. TSH and Acidobacter-Gemmata positive correlations in water is another interesting findings of this study. Acidobacteria (*Acidovorax*, *Bryobacter* and *Stella*) and Gemmataceaefam (*Fimbriiglobus* and *Gemmatimonas*) are most widely distributed bacteria with very little information about phylogeny, nature, functions (Kulichevskaya et al., 2010, Vasilyeva, 1985, Zhang et al., 2003).

A very week or no correlations was observed between genera in the gut and water. Higher SGR is strongly correlated with the abundance of *C. Bacilloplasma* in the gut and *Cloacibacterium* in the water. *Candidatus* lineages are the key bacterial groups for the crustaceans, highly dominant in red-swamp crayfish (*Procambarus clarkii*) (Shui et al., 2020), lobster (*Nephrops norvegicus*) (Meziti et al., 2010), Gammarus (*Gammarus pulex*), Chinese mitten crab (*Eriocheir sinensis*) (Chen et al., 2015) and marron (*C. cainii*) (Foysal et al., 2020b). Although lineages are highly diversified and play significant role in crustacean gut health and immunity (Foysal et al., 2020b, Shui et al., 2020), no isolate yet characterized in the laboratory. The water bacteria *Cloacibacterium* on the other hand is Gram-negative and facultatively anaerobic in nature, predominantly colonize in waste-water and reported to associated with heavy metal decomposition (Nouha et al., 2016) and biological phosphate removal (Gay et al., 2016). However, alike *Candidatus* lineages, *Cloacibacterium* bacteria also non-culturable, and no

isolated laboratory strain is available yet to validate the data generated by culture independent method. Considering their role, a culture-based approach therefore is necessary to grow the bacteria belong to *Candidatus* lineages and *Cloacibacterium* in the laboratory.

By investigating the overall gut and water microbiota it is evident that sources of dietary protein have significant impacts on gut and water microbial communities, and animal sources are better alternative to FM for the marron diet. Although PBM reported as potential alternative of FM for marron aquaculture (Saputra et al., 2019), the results of DNA sequencing-based microbiome analysis further supports the beneficial effects of PBM from earlier studies and hence scale up the reliability, replicability and reproducibility of data. Insect diet also showed promising results, especially in augmenting beneficial microbes in water. Since the impacts of feed and water have significant impacts on shaping the gut microbiota of aquatic species (Giatsis et al., 2015), a mixture of animal and insect diets (PBM+BSF) showed better performance for marron (Foysal et al., 2019b), and therefore could be a diet of interest for other crayfish aquaculture. However, the variation of feeding in different life cycle of crayfish (Zhang et al., 2020), shift of microbiome from juvenile to adult phage, and limitations of Illumina sequences and databases to generate species level information (Alberdi et al., 2019) are some of the major concerns that deliver insights into the importance of scale up sequencing-based diet studies to create a solid framework from where major ecological conclusions can be drawn. Nevertheless, the study design, number of replicates used, volume of data generated and comparative analytical procedures used in this study endorse the consistency, reliability and replicability of the eDNA results.

CHAPTER 7: Substrates for marron aquaculture

EXPERIMENT 1: Biological filters regulate water quality, modulate health status, immune indices and gut microbiota of freshwater crayfish, marron (*Cherax cainii*, Austin 2002)

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Abstract

Water quality has significant impacts on the health and immune responses of aquaculture species. This study aimed to analyse and compare the effects of two biological filters namely, gravel and, Bio-Ball with a recently developed filter called The Water-cleanser ™ on regulation of water quality parameters, health and immune response of marron reared in plastic tanks for 60 days. Results showed that addition of Bio-Ball significantly ($P<0.05$) reduced the concentration of ammonia, nitrate and phosphate while Water-cleanser showed the ability to reduce ammonia and nitrate from water in aquaculture tanks. Although the biological filters had no significant effect on marron growth but inclusion of Bio-Ball and Water-cleanser positively influenced the biochemical composition of tail muscle and some hemolymph parameters of marron. The next generation sequence data demonstrated higher bacterial diversity in the hindgut of marron with Water-cleanser, followed by Bio-Ball and gravel, respectively. In addition, the predicted metabolic pathways revealed a significantly higher bacterial activity and gene function correlated to metabolism and biosynthesis of protein, energy and secondary metabolites in Bio-Ball and Water-cleanser. Bio-Ball and Water-cleanser were also associated with up-regulation of innate immune responsive genes of marron gut. Overall, Bio-Ball and Water-cleanser proved to have higher water remediation and immune response modulation capabilities, and therefore could be used as preferred filters for growth of beneficial bacteria in crayfish culture.

Keywords: Aquaculture; marron; biological filters; water quality; health and immune indices; gut microbiota

7(1). Introduction

Globally, unplanned and unregulated expansion of aquaculture industry has negatively impacted on the aquatic environment (Roriz et al., 2017). At the same time, increase in aquaculture production by means of improving the water quality has become an utmost priority among the fish farmers. Regulation of temperature, pH, dissolved oxygen (DO), nitrogenous compounds including nitrate, nitrite and ammonia, phosphorus in form of phosphate, suspended solids (SS) are critical for the growth of aquatic organisms (Camargo and Alonso, 2006, Yildiz et al., 2017). Poor water quality in any stagnant or flow-through production systems can lead to the accumulation of organic waste, deterioration of the surrounding ecosystem, enhanced growth of pathogenic microbes, and increase in the cost of production (Gutierrez-Wing and Malone, 2006). Recirculating aquaculture systems (RAS), on the other hand, can be a good solution for maintaining optimum water quality and enhanced environmental sustainability. However, high capital and operational cost of RAS are a few constraints for its economic viability (Ngoc et al., 2016). In addition, accumulation of nitrogenous wastes and organic load in any RAS can compromise the performance of the cultured species (Ngoc et al., 2016).

Some bacterial communities shown to have positive impact on the growth and immune performance of cultured species meditated through improving the water quality (Chun et al., 2018, Foysal et al., 2019a). However, there is a limited knowledge available on the performance of the key microbial species in water remediation. Different kinds of substrates and filters are used in aquaculture systems are known to establish a biofilm that has an ability to play a significant role in improving water quality (Cole et al., 2019, Khatoon et al., 2007, Lananan et al., 2014, Viau et al., 2012). Conventional biological filters using gravel and sand

as substrates reported to have positive effects on water quality during the culture of various aquatic species (Huang et al., 2018, Rogers and Klemetson, 1985, Summerfelt, 2006). However, slow filtration, trapping of organic debris, manual hand cleaning and limited activity against nitrates and heavy metals are some of the major limitation of this type of filters (Odell, 2014). Conversely, some non-substrate based biological filters come with more surface area for the growth of probiotic and denitrifying bacteria that demonstrated to have positive effects on water quality (Cole et al., 2019, Foysal et al., 2019a). However, none of the studies have yet analysed the impacts of different biological filters on growth, gut microbiota, and immune indices of any crayfish under laboratory condition.

Marron (*Cherax cainii*) is the largest commercially farmed parasticid crayfish in Western Australia (Cole et al., 2019). In spite of the best intentions from the farmers, the total production of marron has remained stagnant (Cole et al., 2019; Foysal et al., 2019a). Excessive dissolved unionised ammonia, nitrite and nitrates have harmful physiological effects on crayfish including marron (Cole et al., 2019, Roessink et al., 2017, Svobodova et al., 2012). Although marron farming is devoid of any major diseases till today, the poor water quality can hasten accumulation and growth of pathogenic microbes including *Vibrio* species, a possible future pathogen (Ambas et al., 2013, Foysal et al., 2019a). Certain advances in molecular techniques have recently validated the positive claims made by a few biological filters on the water quality and the health of the farmed species (Andre et al., 2007, Castine et al., 2013, Cole et al., 2019, Foysal et al., 2019a, Permatasari et al., 2018). Hence, this study was conducted to evaluate the performance of three selected biological filters on the water quality, health and immune performance of the targeted host crayfish species, marron.

7.2(1). Materials and methods

7.2(1).1. Ethics statement

Ethics approval is not mandatory for the study performed with invertebrates at Curtin University, Australia. However, the laboratory trial, crayfish handling and sample collection were performed according to the guidelines of Animal Welfare Act, Western Australia and the Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2004).

7.2(1).2. Experimental set-up and sampling

A total of 84 marron (71.8 ± 0.7 g) were distributed into 16 different tanks at a density of 5 marron per tank (X 4 replicates per dietary treatment), and acclimated for 14 days before commencement of the trial. Unless mentioned, the rest of the experimental set-up along with water quality analysis including temperature, pH, dissolved oxygen (DO), nitrate, nitrite, ammonia and phosphate were performed as described in general methodology (chapter 3.1). After acclimation, 16 tanks were randomly divided into four different treatment groups, namely, control, gravel, Bio-Ball and Water-cleanser. Gravel was collected from Bibra Lake Soils, WA, Australia (32.1023° S, 115.8240° E), while Bio-Ball (38 mm in diameter) and Water-cleanser (200g block size) were purchased from Serene Aquarium, NSW, Australia and Marine Easy Clean, WA, Australia, respectively. Gravel, Bio-Ball and The Water-cleanser ™ were added in 12 tanks according to manufacturer's instructions: 10 kg/150 L gravel, 1 gallon of Bio-Ball (approximate 60 pieces, 40 mm size) per 50 gallon of water, and one 200 g block Water-cleanser per tank (for <1000 L water). Control tanks had no filtration system. Throughout the trial including acclimation period, marron were fed fishmeal based basal diet (Glenn Forest, Perth, Australia) (Table S4.1) containing 29.9% protein and 7.5% lipid, every day at 6 PM and 1.5% of the total biomass per tank. The water quality in each tank was measured every day. The faecal waste of marron in each cage and tank was removed carefully using a hand-held net once a week.

7.2(1).3. Sampling

To determine marron health indices and haemolymph parameters, two marron from each tank (n = 8, N = 32) were randomly sampled. For DNA extraction and microbiome analysis, two randomly selected marron from each tank (n = 8, N = 32) were used followed by careful aseptic excision of whole gut and separation of anterior, mid and hindgut inside a biological safety cabinet. The contents from two marron hindgut samples from each tank were then pooled together (N =16), homogenized, and transferred to 1.5 mL Eppendorf tube for DNA extraction. Finally, two randomly selected marron from each tank (n = 8, N = 32) was used for the gene expression analysis with specific primers. The whole intestine samples were chopped into small pieces, suspended into RNA later solution (Sigma-Aldrich, Germany) and stored at -80 °C until further use. Same marron was used for health and hemolymph parameters as well as for gene expression analysis.

7.2(1).4. Analysis of growth, immune and biochemical parameters

A mid-term sampling was performed on day 30th and at the end of the trial on day 60th in order to calculate weight gain (WG), specific growth rate (SGR). Haemolymph osmolality (HO), lysozymal activity and total haemocyte counts (THC) of marron were analysed as described earlier in general methodology (3.2). The amount of protein, energy and fat in the tail muscle was measured according to methods described in chapter 3.3, general methodology.

7.2(1).5. Microbiome study of hindgut

The hindgut was selected for bacterial diversity analysis due to its role in digestion, and immunity of crayfish (Wang et al., 2018). The extraction of bacterial genomic DNA from pooled samples, PCR amplification of v3v4 regions, library preparation and pair-end sequencing were performed according to methods described in chapter 3.4.

7.2(1).6. Gene expression analysis

Primers used for gene expression analysis in this study are listed in (Table S7.1.1, Appendix 4). Only those genes, associated with health and immunity of crayfish were selected (Dai et al., 2017, Foysal et al., 2019b, Foysal et al., 2019d, Jiang et al., 2015a, Liu et al., 2013). For analysis of proPO and MnSOD, 0.5 mL of haemolymph was diluted in 0.5 mL of anticoagulant and centrifuged immediately at 8000 rpm for 20 min to collect hemocytes (Liu et al., 2013). The collected haemocytes and 5 mg of homogenized tissue samples were used for RNA extraction. The gene expression analysis was performed as described in chapter 3.6 of general methodology.

7.2(1).7. Bioinformatics and statistics

Unless any modifications mentioned, the "pipelines" for downstream sequence analysis and bioinformatics were used according to general methodology in chapter 3.4 with default parameters. The rarefaction depth value was set at 17,796 and consequent calculation of alpha and beta diversities were accomplished using QIIME (version 1.9.1) (Kuczynski et al., 2012). Concisely, alpha diversity was calculated based on the following metrics: species richness, Shannon, Chao1 indices and goods coverage. Non-parametric statistical analysis of the distance metric was performed using ANOSIM with 1000 permutations. One way ANOVA with Tukey's HSD was used to compare the water quality, health indices and alpha diversity measurements among the groups in Rstudio. Beta dispersion in terms of non-metric multidimensional scaling (NMDS) and bacterial diversity at phylum and genus level were calculated and visualized using microbiomeSeq and phyloseq packages in Rstudio. Venn diagram regarding bacterial diversity among groups at genus level was generated using FunRich tool (v3.1.3) (Benito-Martin and Peinado, 2015). To find out the indicator bacterial genus in different groups, Linear Discriminant Analysis Effect Size, LEfSe was applied with stringent LDA cut-off value of ≥3.5 (Segata et al., 2011). Differentially abundant metabolic

pathways in four different groups were predicted from the 16S rRNA sequence data using Piphillin algorithm (http://secondgenome.com/Piphillin) with supports of KEGG database, BioCyc (v21), and LEfSe (LDA 3.5) (Iwai et al., 2016; Segata et al., 2011). The numerical data for growth, health and immune indexes of marron were analysed using SPSS IBM (v23, 2017). At all stages alpha level of 0.05 was considered as statistically significant.

7.2(1).8. Calculations

Growth performance and was measured according to calculations mentioned in general methodology (chapter 3.7).

7.3(1). Results

7.3(1).1. Water quality and marron health

Among some major water quality parameters such as pH, dissolved oxygen, nitrate, nitrite, ammonia and phosphate, significant ($P<0.05$) reduction of nitrate and ammonia concentration were observed for both Bio-Ball and Water-cleanser treated tanks, and phosphate reduction noticed in Bio-Ball added tanks (Figure 7.1.1). No significant ($P>0.05$) impacts of different filters was recorded for weight gain (WG), specific growth rate (SGR), tail muscle protein and fat contents. However, one-way ANOVA revealed significant positive influence ($P<0.05$) of Bio-Ball and Water-cleanser on the lysozymal activity and THC, and gross energy of tail muscle, compared to control and gravel (Figure 7.1.2). Hemolymph osmolality (HO) was significantly ($P<0.05$) influenced by Water-cleanser in relation to control, gravel and Bio-Ball. Gravel as a filter had no effects on marron health and immune response compared to any of the filters used in this study.

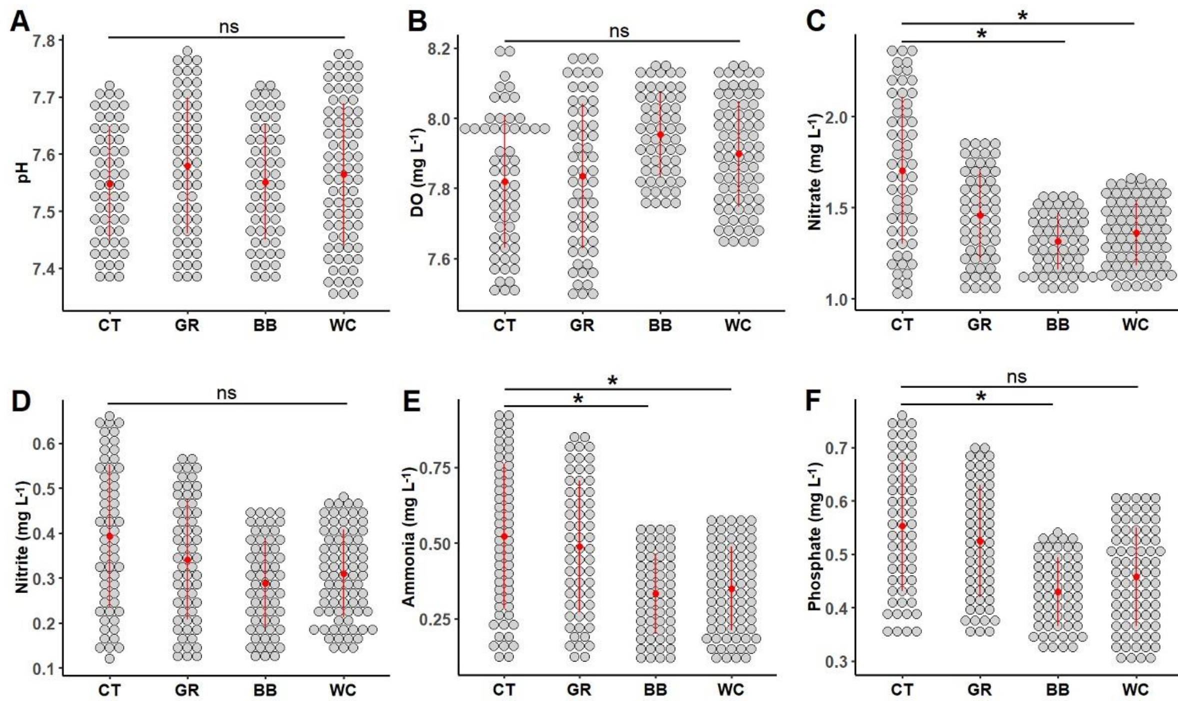


Figure 7.1. 1 Water quality parameters of tanks for four different treatment groups. (**A**) pH; (**B**) Dissolved oxygen; (**C**) Nitrate; (**D**) Nitrite; (**E**) Ammonia; (**F**) Phosphate. Data gathered from four tanks of each group are expressed as mean ± SE for 60 days. Abbreviation: BB = Bio-Ball; CT = Control; GR = Gravel; WC = Water-cleanser. *Significant at α-level of 0.05.

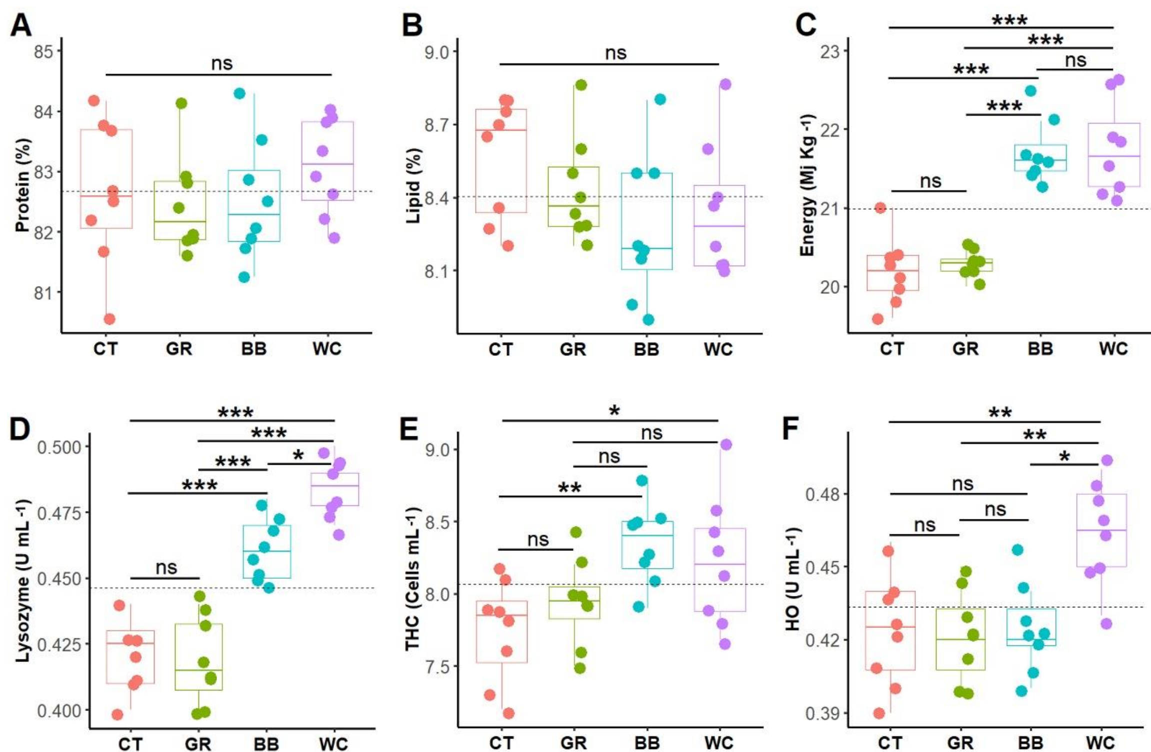


Figure 7.1. 2 Effects of biological filters on growth, health and immune indices of marron. (**A**) Protein; (**B**) Lipid; (**C**) Gross energy; (**D**) Lysozyme activity; (**E**) Total haemocyte count; (**F**) Hemolymph osmolality. Data expressed as mean ± SE. *Significant at α-level of 0.05; **Significant at α-level of 0.005; ***Significant at α-level of 0.001. Abbreviation: BB = Bio-Ball; CT = Control; GR = Gravel; WC = Water-cleanser.

7.3(1).2. Microbial diversity in marron gut

The study generated 577,808 raw reads from 16 samples from four different treatment groups. After removing single-tone, the reads were classified into 348 OTUs, 12 phyla and 149 genera. The rarefaction curve indicated that the 16S rRNA sequence captured enough depth and diversity for 16 samples of four different treatment groups (Figure 7.1.3 A). The curve revealed significant influences of Bio-Ball and Water-cleanser on bacterial communities in the marron hindgut. ANOVA measurements of alpha diversity showed significantly higher species richness, Shannon and Chao1 indices in Bio-Ball and Water-cleanser groups compared to control and gravel (Figure 7.1.3 B-D). However, the number of genera and unshared genera were higher with Water-cleanser compared to other groups. The goods coverage index of 0.95-0.99 suggested that the coverage degree of sequences were high and anticipant. The non-metric multidirectional scaling (NMDS) plot based on PERMANOVA analysis of rarefied data exhibited a distinct clustering of bacterial OTUs, and R value of 0.8221 and P value of 0.002 revealed significant effects of biological filters on marron gut microbiota (Figure 7.1.4 A). Proteobacteria was found to be more abundant in control (77.7%) and gravel (92.1%) while Firmicutes profusion was observed for Bio-Ball (55.7%) and Water-cleanser (46.7%) at phylum level (Figure 7.1.4 B). Whereas at genus level, *Citrobacter* (86.9%), *Vibrio* (61%), *Clostridium* (49.2%) and *Streptococcus* (49.6%) were recorded to be the most abundant bacteria in the hindgut of marron in control, gravel, Bio-Ball and Water-cleanser groups, respectively (Figure 7.1.4 C).

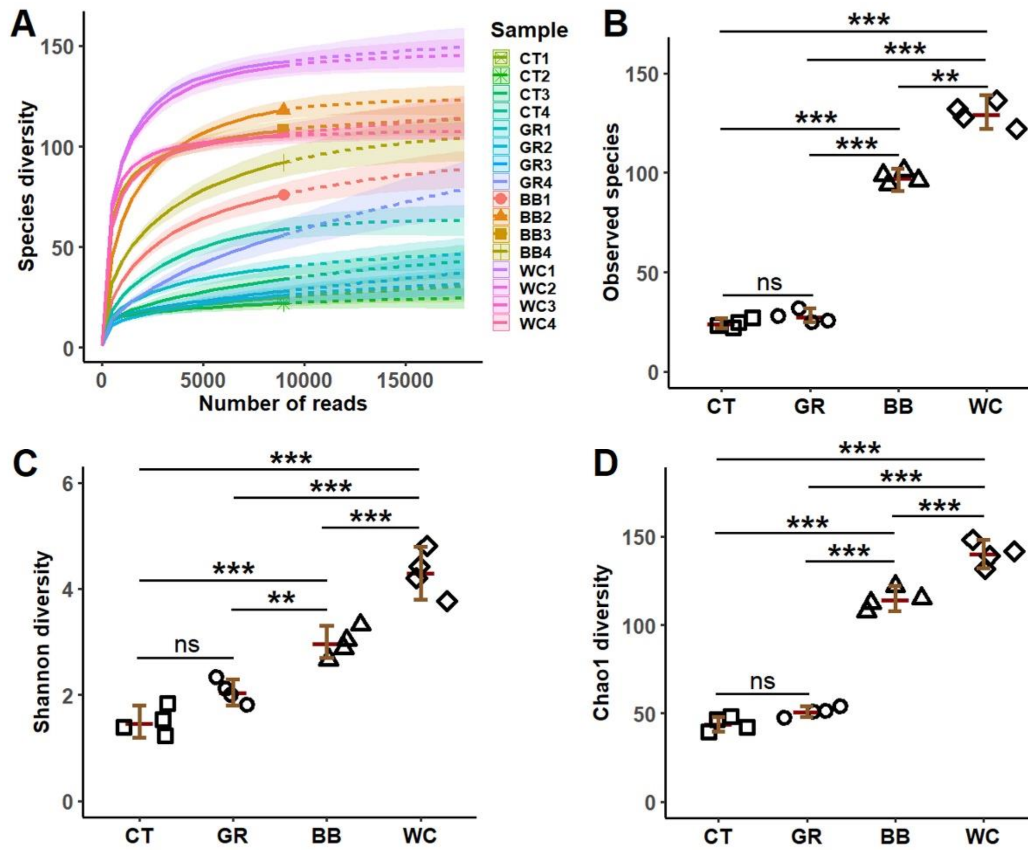


Figure 7.1. 3 Alpha diversity measurements of samples in four different treatment groups. (**A**) Rarefaction curve showing the depth of sequencing in terms of species diversity; (**B**) Observed species; (**C**) Shannon index; (**D**) Chao1 index. Data expressed as mean ± SE. *Significant at α-level of 0.05; **Significant at α-level of 0.005; ***Significant at α-level of 0.001. Abbreviation: BB = Bio-Ball; CT = Control; GR = Gravel; WC = Water-cleanser.

7.3(1).3. Indicator bacterial groups and metabolic pathways

Linear discriminant analysis revealed 20 significantly abundant signature bacterial genera in four different treatments at strict LDA cut-off value of ≥3.0. Out of 20, 14 enriched in Water-cleanser group followed by three, two and one from control, Bio-Ball and gravel groups, respectively. In control, *Citrobacter* was found to be the most enriched signature bacteria (LDA ≥ 5.0) while *Vibrio, Clostridium* and *Streptococcus* were the indicator genera for gravel, Bio-Ball and Water-cleanser, respectively (Figure 7.1.5 A). Out of significantly abundant 52 taxa in all four treatment groups, 39 were found to be enriched with Water-cleanser (Figure 7.1.5

B). The predicted KEGG metabolic pathways indicated that Water-cleanser stimulated the biosynthetic pathways for amino acids, amino-acyl tRNA, antibiotics, secondary metabolites etc. Relative to Water-cleanser, the substrate in gravel filter up-regulated the expression of two-component system, biofilm formation (*Vibrio cholerae*) and bacterial chemotaxis. In control group, the significantly overexpressed predicted pathways were flagellar assembly, microbial metabolism in diverse environment and ABC transporter. Compared to these three, no pathway was found to be up-regulate with Bio-Ball in this study (Figure 7.1.6 A).

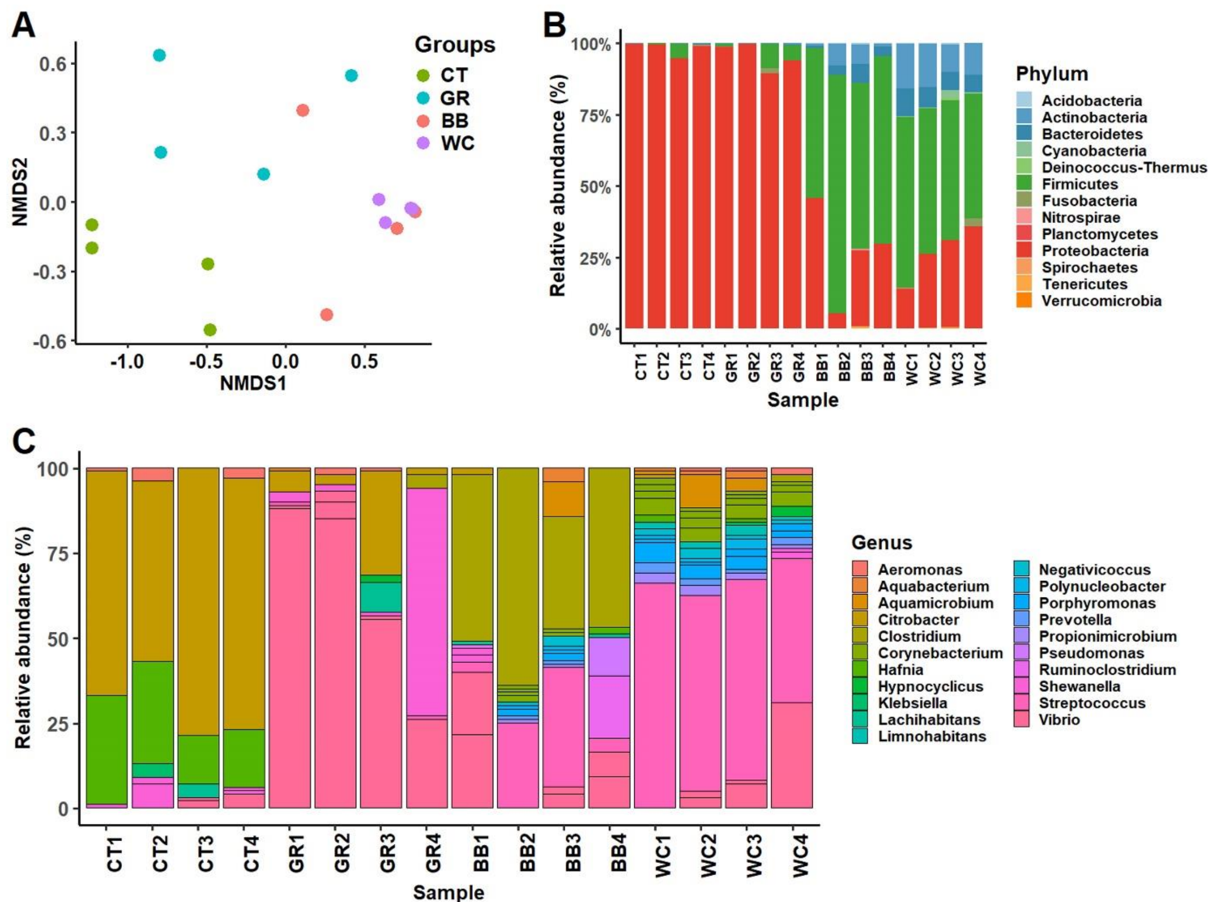


Figure 7.1. 4 Beta diversity and bacterial communities in four different treatment groups. (**A**) Non-metric multidimensional scaling (nMDS) plot showing clustering of marron gut samples; (**B**) Relative abundance of bacteria at phylum level in the hindgut of marron; (**C**) Relative abundance of bacteria at genus level in the hindgut of marron. Abbreviation: BB = Bio-Ball; CT = Control; GR = Gravel; WC = Water-cleanser.

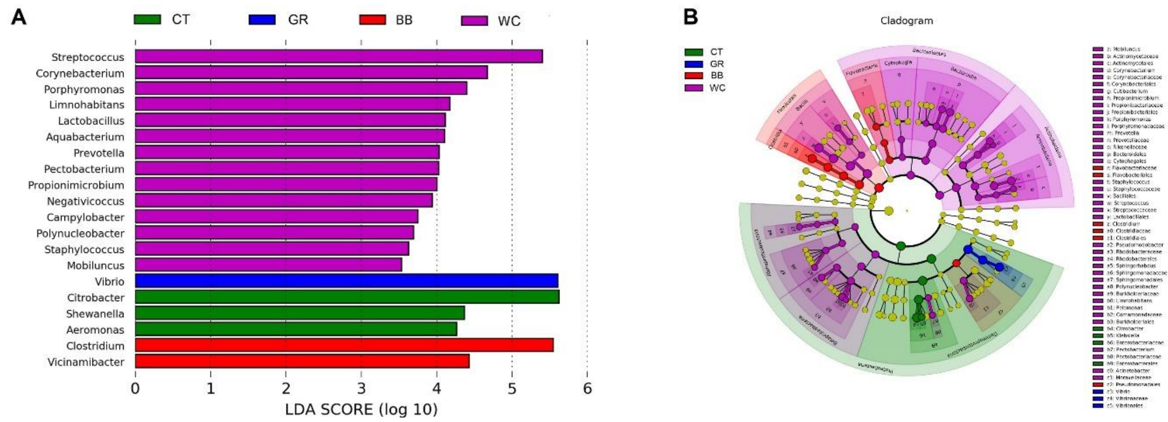


Figure 7.1. 5 Indicator microbial groups in the marron gut. (**A**) Differentially abundant bacteria at genus level with LDA cut-off value of ≥3.5 and at 0.05 level of significance. **(B)** Circular LEfSe cladogram representing the phylogenetic distribution of bacterial lineage. The lineage with LDA scores of ≥3.5 are displayed here. The order, family, and genus that are significantly different between two groups are given in the upper right corner with respective colour codes. Abbreviation: BB = Bio-Ball; CT = Control; GR = Gravel; WC = Water-cleanser.

7.3(1).4. Impacts on immune gene expression

Relative to control, the results of qRT-PCR showed the up-regulation of innate immune responsive genes in the Bio-Ball and Water-cleanser groups. The expression level of immune genes in the marron with gravel had almost similar patterns to the control treatment. There was 3.0 and 2.8 fold increase in the expression of pro-inflammatory cytokine, interleukin-17F (IL-17F); 1.6 and 1.8 fold increase in the expression of anti-inflammatory cytokine, interleukin 10 (IL-10); 2.0 and 1.8 fold up-regulation of prophenoloxidase (proPO); both 1.6 fold increase expression for cathepsin L (PcCTSL); 2.3 and 2.5 fold increase expression for cytosolic manganese superoxide dismutase (cytMnSOD) with Bio-Ball and Water-cleanser, respectively (Figure 7.1.6 B). Compared to control and gravel, the relative expression level of interleukin 1β (IL-1β), interleukin 8 (IL-8) and tumour necrosis factor (TNF-α) genes were relatively static with Bio-Ball and Water-cleanser group.

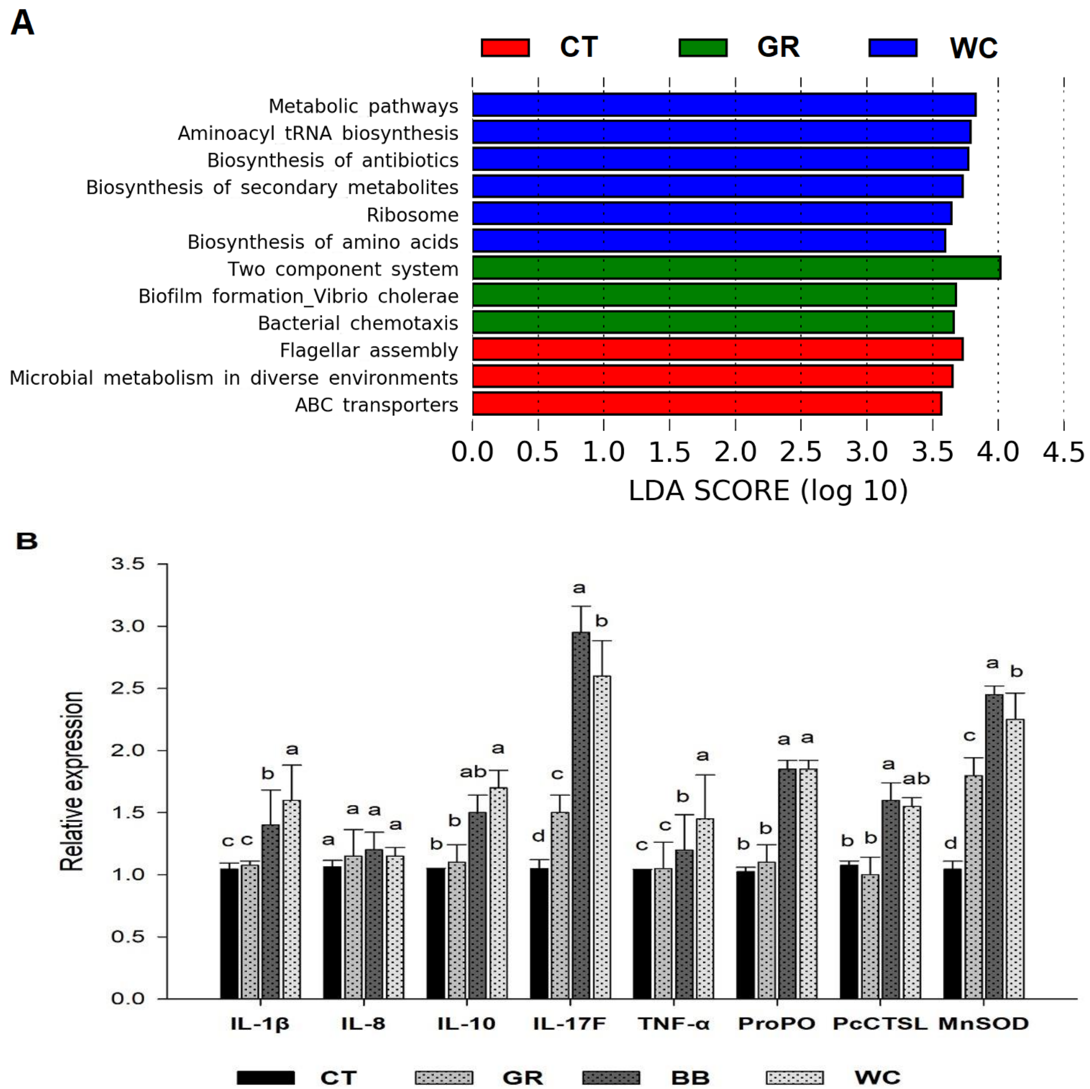


Figure 7.1. 6 Metabolic functions and immune response in hindgut of marron after trial. (**A**) Predicted differentially expressed KEGG metabolic pathway in four different treatment groups (LDA>3.5, P<0.05). (**B**) Relative expression of genes in the gut and haemolymph of marron. Means with different superscripts are significant at α-level of 0.05. Data expressed as mean ± SE. Abbreviation: BB = Bio-Ball; CT = Control; GR = Gravel; WC = Water-cleanser.

7.3(1).5. Data availability

The raw sequence data in FASTQ files are currently available at National Centre for Biotechnology Information (NCBI) BioProject under the accession number PRJNA549032.

7.4(1). Discussion

Many intensive aquaculture practices result in accumulation of organic wastes that are linked to water quality deterioration and environmental pollution. Poor water quality favours the growth of pathogenic microbes in the aquatic environment that can be transmitted to aquatic species (Bhateria and Jain, 2016; Foysal et al., 2019a; Yildiz et al., 2017). Therefore, a cost effective approach for maintaining optimum water quality is a major challenge for the farmers and researchers. Biological filter system has been successful in treating organic waste from both fresh and marine farming operations (Foysal et al., 2019a; Gutierrez-wing and Malone, 2006; Permatasari et al., 2018). The three different filters used in present study (gravel, Bio-Ball and Water-cleanser) were reported to be effective in improving the quality of aquaculture water (Castine et al., 2013; Cole et al., 2019; Foysal et al., 2019a). Studies also reported the augmentation of bacterial communities in water with two commercial biological filters, Bio-Ball and Water-cleanser (Cole et al., 2019; Foysal et al., 2019a). However, none of the above study analyse the impacts of commercial substrate and non-substrate based biological filters on the gut microbiota health and immune indices of any aquatic species.

Both Bio-Ball and Water-cleanser improved the water quality parameters, whereas marron growth was not influenced by any filter. Both of the biological filters are claimed to provide substrates for the growth of denitrifying bacteria that can remove nitrogenous compound and reactive phosphorus from water (Cole et al., 2019; Foysal et al., 2019a; Permatasari et al., 2018). Earlier studies with Bio-Ball, reported significant improvement of bacterial diversity of Firmicutes, a phylum that is known to be involved in denitrification process in the aquatic environment (Foysal et al., 2019a; Sharmin et al., 2013). The bacterial abundance at phylum level in this study showed significant enrichment of Firmicutes in Bio-Ball and Water-cleanser treatments, similar to previous reports where Firmicutes mediated waste water treatment established (Sharmin et al., 2013). The information relating to the impacts of biological filters

on growth performance of crayfish is very limited, however, a most recent study found no significant differences in weight gain of marron with Water-cleanser (Cole et al., 2019). Previous studies on marron with different diets reported insignificant growth changes in laboratory trial for 56-60 days (Ambas et al., 2017, 2013; Foysal et al., 2019). Alike previous studies, present study also lasted for two months– a very short time to achieve significant growth rate for a species having fairly long life cycle under farming conditions. However, some water quality parameters in this study were positively impacted after application of Bio-Ball and Water-cleanser, identical to recent studies where nitrate and ammonia reduction from aquaculture water were reported (Foysal et al., 2019a; Permatasari et al., 2018).

The gut microbiota of aquatic species are commonly dominated by Proteobacteria, Firmicutes and Bacteroidetes (Ghanbari et al., 2015; Huang et al., 2014; Michl et al., 2017). Firmicutes and Bacteroidetes are mostly beneficial bacteria for both human and animals including crayfish (Egerton et al., 2018, Rajilić-Stojanović et al., 2007). Firmicutes can improve the digestibility, health and immune performance of fish and marron, and counteract the damaging effects of pathogenic bacteria in the gut (Costantini et al., 2017, Foysal et al., 2019b). Present study revealed significant enrichment of phylum Firmicutes and genus *Clostridium* in the marron gut with the Bio-Ball. *Clostridium butyricum,* a probiotic can modulate the gut microbiota and immune gene expression of marron and white pacific leg shrimp (*Litopenaeus Vannamei*) where significant increase in the relative abundance of Firmicutes and Bacteroidetes (Duan et al., 2017, Foysal et al., 2019d). Water-cleanser on the hand favoured the growth of microbes from diverse genera in the marron gut, mostly from lactic acid bacteria (LAB). *Streptococcus* is one of the core bacterial group in the marron gut while *Lactobacillus* enrichment in the fish and crustacean gut plays a key role in health and immunity of white shrimp and common carp (*Cyprinus carpio*) (Foysal et al., 2019b, Giri et al., 2018, Vieira et al., 2008). Increase in relative abundance of *Corynebacterium*, *Porphyromonas* and *Limnohabitans* were associated with

better health and immune response of marron fed black soldier fly (*Hermetica illucens*) supplemented with poultry-by-product meal (Foysal et al., 2019b).

Besides, the bacterial communities, information on metabolic pathway could be an effective way to understand the health condition of animals. The Water-cleanser and Bio-Ball as bio-filters enhanced the metabolic capability of marron and up-regulated the pathways for the synthesis of amino acids, antibiotics, secondary metabolites. Previous reports demonstrated *Firmicutes* driven up-regulation of amino acid biosynthesis and metabolic pathways in fish, mostly by lactic acid bacteria (LAB) *Streptococcus* and *Lactobacillus* (Besten et al., 2013, Bhute et al., 2017, Dai et al., 2014). The significant enrichment of LAB in marron gut with Water-cleanser, therefore associated with the modulation of these metabolic pathways. The predicted up-regulated pathways in the gravel are mostly stress response elements that protect bacteria against host immune defence, antimicrobials, and adverse physiological and environmental conditions (Tiwari et al., 2017, Wang et al., 2013). The conditions created by two-component systems and biofilm pathways enforced the host cell to respond quickly to a changed environmental condition resulting release of more energy, and help bacteria in chemotaxis, cell wall invasion– thereby flagging the immune response of host (Falke et al., 2010, Rutherford and Bassler, 2012). Finally, flagellar assembly, ABC transporter and microbial metabolism in control marron commonly play an important role in regular metabolic process of aquatic species including adhesion, various metabolic processes including transport, metabolism, drug resistance under different environmental conditions (Tripathy et al., 2014, Wang et al., 2015, Zhou et al., 2009). The use of Bio-Ball and Water-cleanser is therefore beneficial for digestion and immunity of marron in compared to gravel.

The intestinal mucosal membrane plays a significant role in immunity of fish and crayfish (Ángeles Esteban, 2012, Lieschke and Trede, 2009). Among the various factors, cytokines (interferon, interleukin, and tumour necrosis factors), lysosomal peptidase (pcCTSL), and

innate immune response genes, prophenoloxidase (proPO) and cytosolic manganese superoxide dismutase (cytMnSOD) play a pivotal role in regulation of the immune response of crayfish (Dai et al., 2017, Foysal et al., 2019b, Jiang et al., 2015a, Liu et al., 2013). Over-expression of IL-1B in fish modulates the expression of IL-17F, a gene essential for antibacterial defence (Wang et al., 2014). Administration of IL-1B in rainbow trout (*Oncorhynchus mykiss*) also known to be associated with antibody production thereby improving immunity (Taechavasonyoo et al., 2013, Yin and Kwang, 2000). Upregulated expression of pro-inflammatory cytokine (IL-1B, IL-17F) regulates the expression of anti-inflammatory cytokines in order to respond quickly to prevent the damages associated with inflammation (Foysal et al., 2019b, Miao et al., 2018). Hence in the present study, over-expression of IL-10 could be linked to the expression profile of major cytokine genes. The gene PcCTSL have been shown to boost the growth, lysosomal activity and facilitate in antigen processing (Dai et al., 2017). The enhanced lysosomal activity in the Bio-Ball and Water-cleanser group is in accordance with the results of previous study. Prophenoloxidase system helps to improve the innate immune response of crayfish by controlling the growth of pathogens whereas cytMnSOD is an antioxidant that transport oxygen in the haemolymph (Gómez-anduro et al., 2012, Liu et al., 2013).

7.5(1). Conclusion

Addition of Bio-Ball and Water-cleanser into the culture system resulted in significant reduction of crayfish pathogen *Vibrio*. Due to the huge species diversity of microbial communities, the pathogenic properties and virulence of the respective species are dictated by the species type, species abundance and available stressors. Hence, the pathogenicity of any selected microbial species in the gut is difficult to predict without any *in vivo* challenge test under the culture conditions. Still, the overall results suggested that, Bio-Ball and Water-cleanser could be used as a potential water treatment biological filters in aquaculture tanks for

better health and immune status of marron. However, the physiological interactive mechanism between microbes in biological filters and crayfish gut is a study of future. The surface area of biological filters could play a crucial role in augmentation of microbial community in water by producing bacterial biofilm that can hasten the process of organic waste decomposition. Therefore, extensive research studies are needed to characterize the microbial communities at species level, and *in vivo* challenge tests. The enrichment of beneficial bacteria on the biofilm is our next phase of research.

CHAPTER 7: Substrates for marron aquaculture

EXPERIMENT 2: Zeolite increases microbial diversity, gene functions and innate immune response of freshwater crayfish, marron (*Cherax cainii*, Austin 2002)

Abstract

Zeolite uptake toxic heavy metal and filters ammonia from aquaculture water and has been routinely used in South Asia for shrimp aquaculture. The Western Australian native crayfish marron (*Cherax cainii*) grows fairly slow under commercial culture and its growth is positively correlated to water quality and gut microbiota. The present study aimed to investigate the impacts of zeolite in three different forms of administration to the rearing systems, dietary zeolite (DZ), suspended zeolite (SZ) and in combination of both (DZSZ) on gut microbiota and immune genes of marron. End trial 16S rRNA data showed significant improvement of bacterial diversity including restoration of core OTUs with SZ, compared to other two ways of administration (DZ, DZSZ) and control in the absence of zeolite (CT). Suspended zeolite also increased the number of unshared OTUs and genera, and improved gene functions for biosynthesis and metabolism of proteins, amino acids, fatty acids and hormones. Out of 21 up-regulated metabolic pathways, only three were positively influenced by CT (1) and DZSZ (2) where SZ stimulated highest (10) number of pathways. Further analysis showed that SZ resulted in upregulation of genes associated with innate immune response of crayfish. Overall results suggest that bagged zeolite can be used for better gut health and immunity of marron.

Keywords: zeolite; aquaculture, marron, gut microbiota and immunity, bioinformatics

7.1(2). Introduction

Use of immunostimulants has gained attention in sustainable aquaculture practice over the decade. Probiotic, prebiotic, and herb included diet as an immunostimulants can improve growth performance and humoral and cell mediated immunity of aquatic animals in specific and non-specific ways (Amenyogbe et al., 2020, Dawood et al., 2018, Mona et al., 2015). In addition, discharge of agricultural, industrial and household wastes and excretion of nitrogenous faecal wastes by the aquacultured animals raise major concern about environmental pollution (Dauda et al., 2019). Tackling the environmental pollution without compromising the growth performance of aquatic species, therefore is a major challenge in aquaculture. Some of the feed additives can improve the digestibility and water quality and thereby improving growth, immune response, disease resistance, and impacts of aquaculture on the environment (Guerreiro et al., 2018, Martínez Cruz et al., 2012).

Natural zeolites are crystalline, consisting diverse mineral groups, hydrated aluminosilicates of alkali (Na^+, K^+) and alkaline (Ca^{2+}, Mg^{2+}) earth cations, comprising of 3D-frameworks of SiO^- ^{4} and AlO^{-6} connected through the shared O_2-atoms (Papaioannou et al., 2005). Zeolites are linked to ion-exchange, mainly sodium to ammonium, and shift the ammonia equilibrium away from toxic un-ionized ammonia to ionized form (Soetardji et al., 2015). In addition, zeolites increase dissolved oxygen (DO) concentration in water and improve feed digestibility ratio (Mumpton, 1999). Dietary zeolite supplementation have positive impact on water quality, growth, digestibility, nutrient utilization and feed conversion ratio of rainbow trout (*Oncorhynchus mykiss*) (Obradović et al., 2006); growth and muscle biochemical composition of common carp (*Cyprinus carpio*) (Khodanazary et al., 2013); immunity and disease resistance of snakehead murrel (*Channa striatus*) (Jawahar et al., 2016). In crayfish however, no study has yet investigated the effects of zeolite on growth and immune performance.

Marron (*Cherax cainii*) is the largest freshwater crayfish in Western Australia (DoF, 2011, Lawrence and Morrissy, 2000). Marron culture possess several unique advantages like easy culture methods, disease-free status, long-distance live shipment (Ambas et al., 2013, DoF, 2010b, DoF, 2011, Lawrence and Morrissy, 2000, Saputra et al., 2019). Due to high aquaculture interest and consumer preferences, the species is rapidly translocated within and outside Australia (Mikkola, 1996, Morgan et al., 2004).

Gut microbiota are good indicator of health and immune status of aquatic animals as they play crucial role in digestion and immunity (Butt and Volkoff, 2019, Egerton et al., 2018). In recent times, advances in high throughput sequencing and computational biology enable precise identification of microbial communities and their metabolic functions in the gut (Foysal et al., 2020b). This combined approaches allow comprehensive, in-depth analysis of dietary impacts on overall health and immunity of aquatic animals (Yu et al., 2019). Despite the role in nutrient absorption, the impacts of zeolite on gut microbiota of aquatic animals has yet to be studied. Considering the effects on zeolite on rearing water and host, we have designed the experiment with zeolite supplementation in feed, water, and both in feed and water, and aimed to investigate the gut microbial response of marron.. The study also aimed to investigate the correlation between altered microbial communities and genes associated with immune response of marron.

7.2(2). Materials and methods

7.2(2).1. Experimental set-up and animal husbandry

The laboratory trial was performed by two masters by research student of aquaculture, Curtin University. The methodology has been re-used with their permission. This study was a carry-on study after original trial was completed, and focussed only on micobiome analysis. The experiment with 128 marron (70-100 g) was designed as described in chapter 3.1 of general methodology. The marron were acclimated into 16 tanks (8 marron/tank), acclimatized for 7 days in laboratory conditions, then distributed into four different treatment groups, absence of

zeolite as control (CT), dietary zeolite (DZ), suspended zeolite (SZ), and combination of dietary and suspended zeolite (DZSZ). During acclimation, all four groups were served with standard basal diet.

7.2(2).2. Feed formulation and feeding

In commercial fishmeal based marron pellet (Table S4.1) prepared by Glenn Forrest, WA, zeolite was mixed at a concentration of 2% in the feed and suspended in water. Marron were fed each day afternoon at 1.5% of their body weight.

7.2(2).3. DNA extraction, barcoding and high throughput sequencing

After trial, two randomly selected marron from each tank (n = 8) were used for microbiome analysis. Dissection of marron, collection of gut were performed under biological safety hood. The whole gut was lysed with sterile beads in 2 ml Eppendorf using TissueLyserII (Qiagen, Hilden, Germany). DNA extraction, PCR amplification, library preparation and sequencing were performed according to methods described in chapter 3.4 of general methodology.

7.2(2).4. Sequence data processing

The pipelines used for bioinformatics are the same as mentioned in chapter 3.5 of general methodology except trimming and merging of reads. Briefly, TrimGalore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and NGmerge (Gaspar, 2018) were used for quality trimming and merging of pair-end reads (Gaspar, 2018, Krueger, 2012). Even rare-depth value of 17,587 was used for statistical analysis of samples from four different groups. For the calculation of alpha-beta diversity, we have processed sequence and metadata in Calypso microbiome (Zakrzewski et al., 2017), microbiomeseq (https://github.com/umerijaz/microbiomeSeq) and FunRich tool (Pathan et al., 2015). Briefly, alpha-diversity were calculated regarding richness, Shannon and Simpson while beta-diversity was calculated based on Bray-Curtis dissimilarity of weighted UniFrac matric. Number of OTUs, goods coverage and Chao1 diversity were calculated in QIIME (v1.9.1) (Caporaso et

al., 2010). Functional predictions of metagenome was performed using PICRUSt2 (Douglas et al., 2020) against KEEG databases.

7.2(2).5. Statistical analysis

Kruskal-Wallis with Bonferroni correction were used for correcting false discovery rate (FDR) in multiple comparison testing of genus and functional predictions data. At all stages, P-value of < 0.05 was considered as statistically significant.

7.2(2).6. Immune gene expression analysis

The primers used for gene expression analysis are listed in Table S7.1.2, Appendix 4. Marron intestine (one marron per tank) stored at -80 ºC in RNA-*later* (Thermo Scientific, USA) and hemolymph samples were processed accordingly as described in chapter 3.6 of general methodology. RNA extraction, cDNA preparation and qRT-PCR were performed following methods described in chapter 3.6 of general methodology. One-way ANOVA with Duncan multiple range test was used to calculate significantly different

7.3(2). Results

7.3(2).1. Sequences statistics

Illumina sequencing of 32 samples yielded 1,910,495 high quality reads, with mean average of 59,703 ± 3011 reads per sample, ranging from 21,443 to 84,973. The reads were assigned into 1103 OTUs (865 ± 76.8) at 97% similarity threshold level. After assignment of phylogeny, more than two third of the OTUs (76.8%) were classified into genus level, 88.8% into family and 99.8% into phylum level. A total of 12 phyla, 86 family and 188 genera was obtained from four different treatments and 32 samples. Rarefaction results showed that the curve reached close to saturation (Figure 7.2.1 A), and thus the depth of sequence was adequate and good's coverage of microbial community was obtained for each sample (Table 7.2.1).

Table 7.2. 1 Sequence statistics and alpha diversity measurements

Parameters	CT	DZ	SZ	DZSZ
Observed OTUs	133.5 ± 18.2^{b}	129.0 ± 7.8^{b}	278.6 ± 8.1^{a}	137.4 ± 11.4^{b}
Good's coverage	0.998 ± 0.0^{a}	0.997 ± 0.0^{a}	0.996 ± 0.0^{a}	0.997 ± 0.0^{a}
Chao1	166.8 ± 20.1^{b}	181.5 ± 8.3^{b}	351.3 ± 11.4^{a}	192.1 ± 17.2^{b}

7.3(2).2. Bacterial diversity and composition

When comparing the alpha diversity, suspended zeolite (SZ) had the highest microbial richness, compared to other three diets. The Simpson and Shannon diversity however found higher only in DZSZ, compared to DZ (Figure 7.2.1 B). In addition, the number of unique OTUs (Figure 7.2.1 C) that reflected by the presence of unique species (chao1 index) in the marron gut reared with suspended zeolite (Table 7.2.1). Beta-ordination analysis based on weighted UniFrac metric of 32 samples displayed divergent clustering of samples as shown in NMDS plot (Figure 7.2.1 D). PERMANOVA R value of 0.495 and P-value of <0.001 revealed significant impacts of zeolite on gut microbial communities of marron.

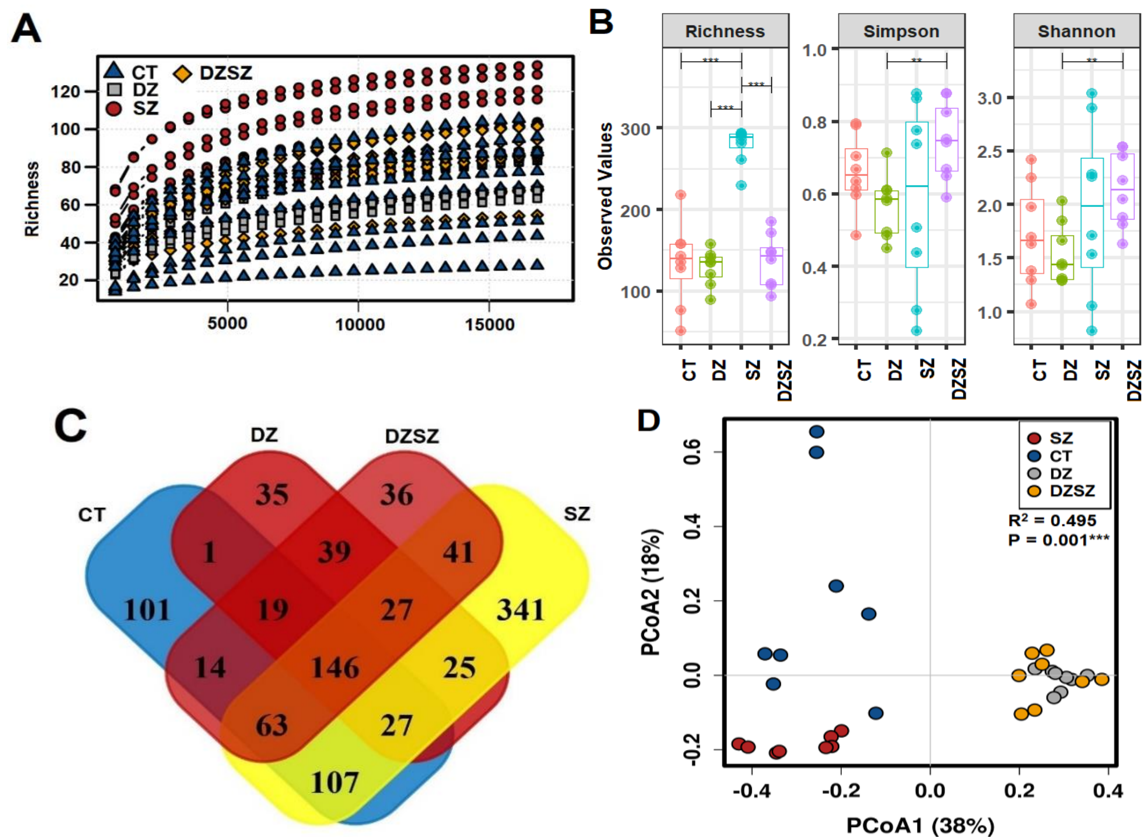


Figure 7.2. 1 Alpha-beta diversity of gut microbial communities in four different groups. (A) Rarefaction curve showing the saturation level of the sequence; (B) Alpha-diversity measurements of gut microbial communities; (C) Number of shared and unique OTUs; (D) NMDS plot displaying the clustering of samples. Abbreviations: CT, control; DZ, dietary zeolite; SZ, suspended zeolite; DZSZ, dietary zeolite + suspended zeolite. **Different at alpha level of 0.005. ***Different at alpha level of 0.001.

In all groups, Proteobacteria was most dominant bacteria at the phylum level, comprising of 70.3% of total OTUs in the distal gut. However, the relative abundance for Firmicutes and Tenericutes were significantly ($P<0.05$) higher in CT and DZSZ groups, respectively. At genus level, regardless of the treatment, *Aeromonas*, *Candidatus* Bacilloplasma, *Hafnia* Obesumbacterium and *Vibrio* were identified in all samples and therefore can be categorized as core microbiota of marron gut. However, high abundance of *Aeromonas* (70.7%), *Hafnia* Obesumbacterium (84.9%), *Candidatus* Bacilloplasma (57.3%) and *Vibrio* (30.3%) were detected in DZ, SZ, DZSZ and CT, respectively (Figure 7.2.2). These four bacteria comprised of 55% of the total classified reads at genus level in 32 samples. Kruskall-Wallis test with Bonferroni correction identified 10 genera that differentially present in four different groups. Out of the these 10, five genera namely *Aeromonas*, *Bosea*, *Dechloromonas*, *Phreatobacter* and *Fimbriiglobus* were enriched in SZ group, *Hafnia* Obesumbacterium and Plesiomonas in DZ, Bacteroides and ZOR0006 in CT and *Candidatus* Bacilloplasma in DZSZ (Table 7.2.2).

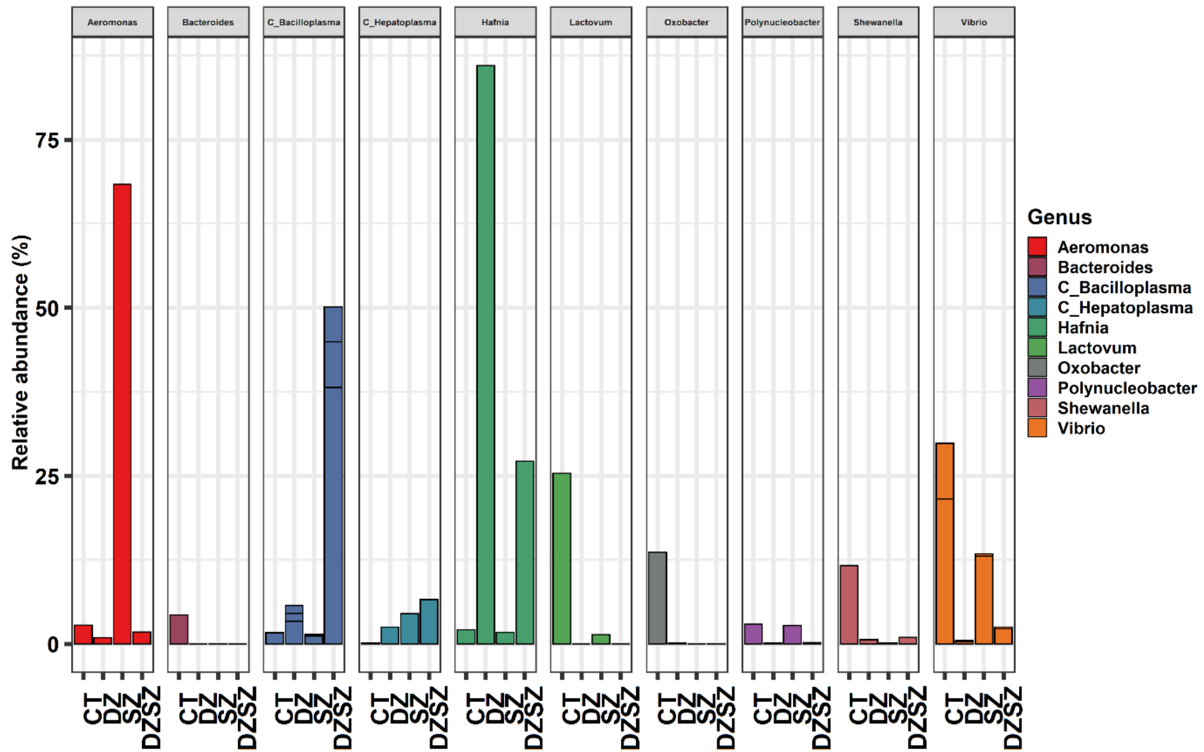


Figure 7.2. 2 Top 10 bacteria at genus level based on their relative abundance in four different groups. Abbreviations: CT, control; DZ, dietary zeolite; SZ, suspended zeolite; DZSZ, dietary zeolite + suspended zeolite.

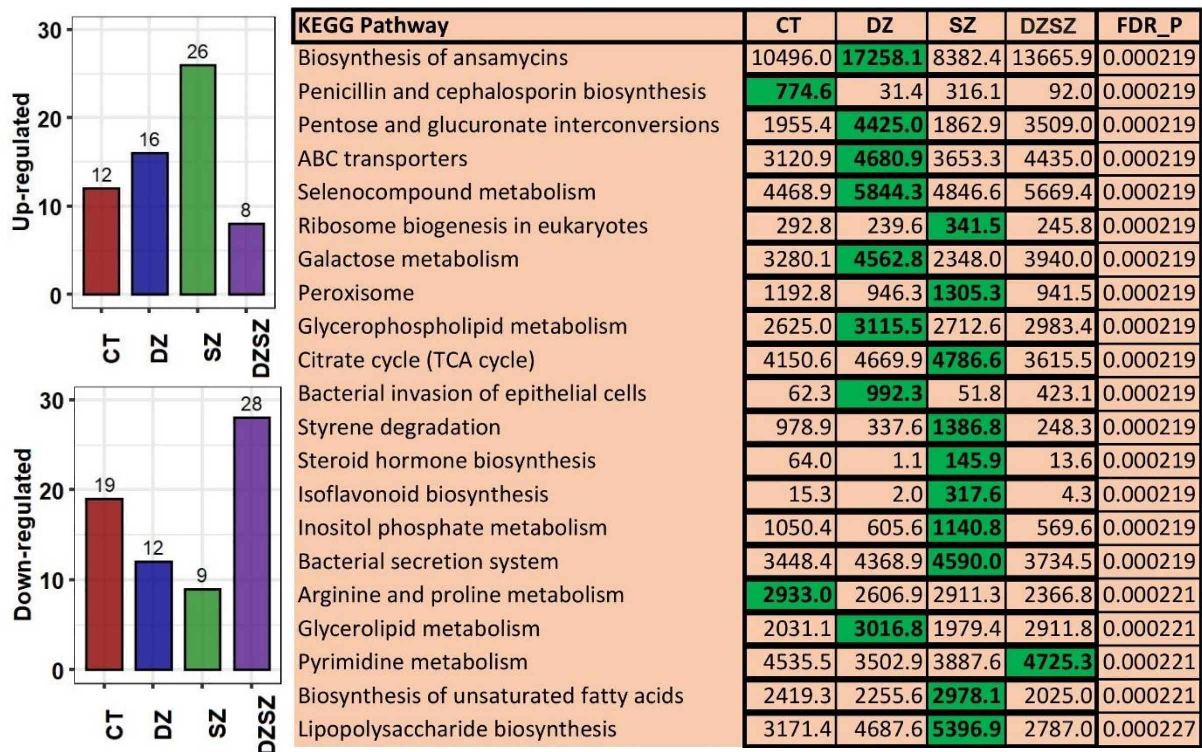
Table 7.2. 2 Differential abundance of bacteria at genus level in four different groups

Genus	CT	DZ	SZ	DZSZ	Adjusted *P*
Hafnia Obesumbacterium	1.01±0.2	**7.99±1.7**	1.15±0.3	4.23±1.1	0.00084
Bosea	0.01	0	0.18	0.039	0.0024
Candidatus Bacilloplasma	1.07	2.32	1	**6.6**	0.0053
Aeromonas	1.78	1.05	**7.51**	1.18	0.0054
Phreatobacter	0.029	0.044	0.19	0.034	0.023
Bacteroides	**1.35**	0	0.12	0.024	0.026
Dechloromonas	0.06	0.014	0.22	0.01	0.029
ZOR0006	0.4	0	0.062	0.01	0.031
Plesiomonas	0.05	0.44	0.4	0.3	0.043
Lactobacillus	0.04	0.08	0.18	0.06	0.047

Each value displayed under the "CT", "DZ", "SZ" and "DZSZ" columns represents mean relative abundance with standard deviation.

7.3(2).3. Prediction of functional metagenome

Prediction metabolic functions are large in groups having highest and lowest bacterial diversity. A total of 21 KEGG pathways were found that could have differential regulation in four different groups. Out of 21, 10 pathways, mostly associated with biosynthesis, metabolism and degradation predicted to have significant higher activity in suspended zeolite marron group. In DZ, alongside pathways linked to biosynthesis and metabolism, ABC transporters and bacterial invasion of epithelial cell were predicted to be changing in the host. Only one pathway (pyrimidine metabolism) showed up-regulation with mixture of dietary and suspended zeolite (DZSZ) while in control higher activity for arginine and proline metabolism and antibiotic biosynthesis pathway were predicated using PICRUSt2 (Figure 7.2.3).



KEGG Pathway	CT	DZ	SZ	DZSZ	FDR_P
Biosynthesis of ansamycins	10496.0	**17258.1**	8382.4	13665.9	0.000219
Penicillin and cephalosporin biosynthesis	**774.6**	31.4	316.1	92.0	0.000219
Pentose and glucuronate interconversions	1955.4	**4425.0**	1862.9	3509.0	0.000219
ABC transporters	3120.9	**4680.9**	3653.3	4435.0	0.000219
Selenocompound metabolism	4468.9	**5844.3**	4846.6	5669.4	0.000219
Ribosome biogenesis in eukaryotes	292.8	239.6	**341.5**	245.8	0.000219
Galactose metabolism	3280.1	**4562.8**	2348.0	3940.0	0.000219
Peroxisome	1192.8	946.3	**1305.3**	941.5	0.000219
Glycerophospholipid metabolism	2625.0	**3115.5**	2712.6	2983.4	0.000219
Citrate cycle (TCA cycle)	4150.6	4669.9	**4786.6**	3615.5	0.000219
Bacterial invasion of epithelial cells	62.3	**992.3**	51.8	423.1	0.000219
Styrene degradation	978.9	337.6	**1386.8**	248.3	0.000219
Steroid hormone biosynthesis	64.0	1.1	**145.9**	13.6	0.000219
Isoflavonoid biosynthesis	15.3	2.0	**317.6**	4.3	0.000219
Inositol phosphate metabolism	1050.4	605.6	**1140.8**	569.6	0.000219
Bacterial secretion system	3448.4	4368.9	**4590.0**	3734.5	0.000219
Arginine and proline metabolism	**2933.0**	2606.9	2911.3	2366.8	0.000221
Glycerolipid metabolism	2031.1	**3016.8**	1979.4	2911.8	0.000221
Pyrimidine metabolism	4535.5	3502.9	3887.6	**4725.3**	0.000221
Biosynthesis of unsaturated fatty acids	2419.3	2255.6	**2978.1**	2025.0	0.000221
Lipopolysaccharide biosynthesis	3171.4	4687.6	**5396.9**	2787.0	0.000227

Figure 7.2. 3 Predictions of metagenomic functions from 16S rRNA data for four different dietary groups. Numbers (only significant at 0.05 level of significance) of up and down-regulated pathways on the left and group significant pathway descriptions with Kruskal-Wallis and Bonferroni adjustment (only P-value of < 0.005 are presented here).

7.3(2).4. Immune gene expression are higher in suspended zeolite marron group

The fold-changes expression of genes associated with crayfish immune response showed up-regulation only in the SZ group. Prophenoloxidase (proPO), Cathepsin L (PcCTSL) and astacidin (Ast) showed 4.1, 2.4 and 1.6 times higher fold changes expression in the hemolymph and intestine of SZ group marron than the control (CT). Downregulation of c-type lectin (CTL) in the DZSZ group, compared to control and SZ, and further down-regulation of CTL in the DZ marron, in relation to all three groups were recorded in qPCR (Figure 7.2.4). In addition, the expression of lysozyme in the hemolymph also down-regulated in the DZSZ group, compared to control. No changes (P>0.05) in the expression of cytosolic manganese superoxide dismutase (cytMnSOD) was observed for all groups after zeolite trial.

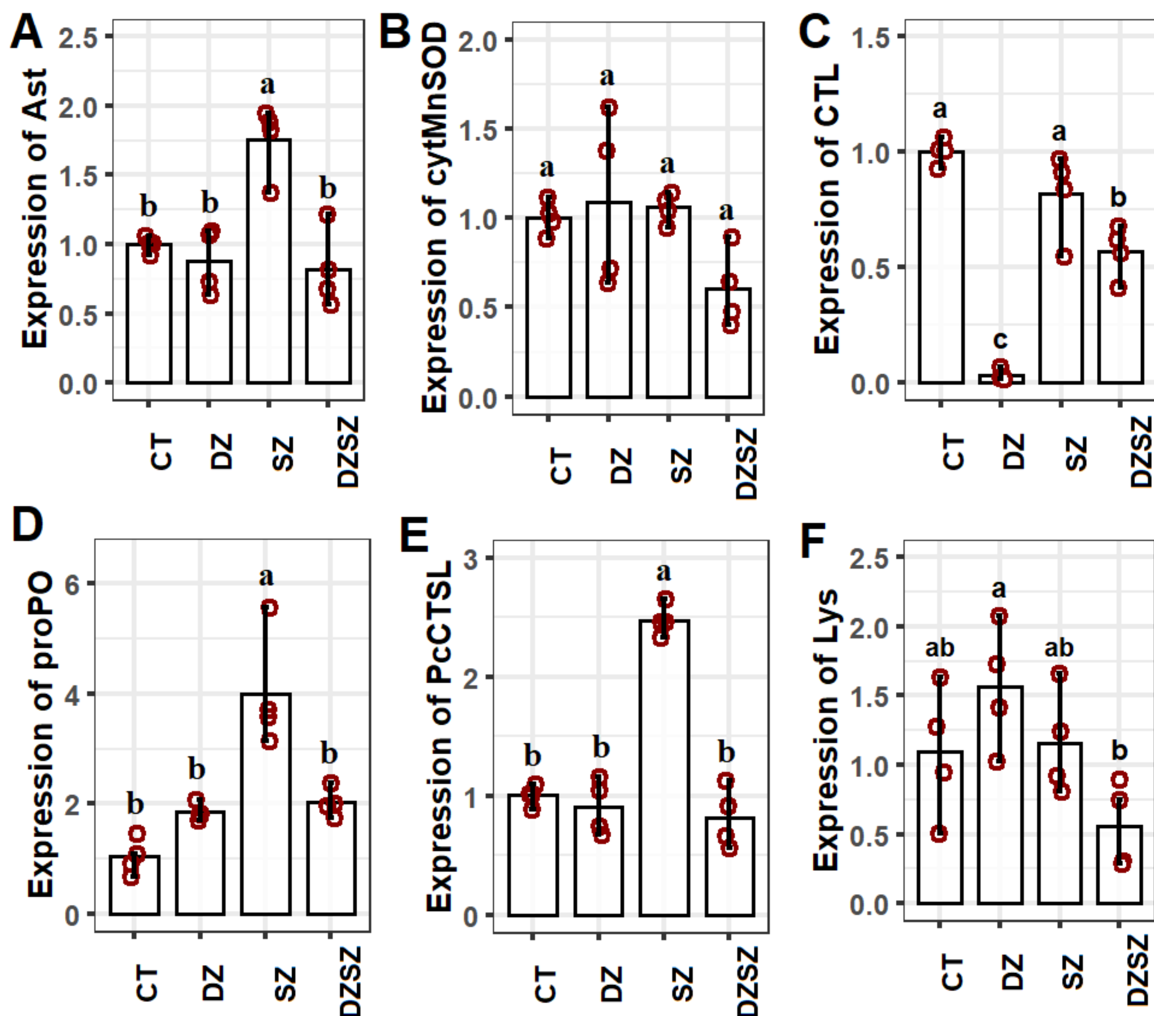


Figure 7.2. 4 Expression of genes associated with immune response of crayfish in four different dietary groups after trial. Abbreviations: CT, control; DZ, dietary zeolite; SZ, suspended zeolite; DZSZ, dietary zeolite + suspended zeolite. Letters with different superscripts indicating significantly different expression in terms of fold-changes at 0.05 level of significance.

7.4(2). Discussion

Considering the role of gut microbiota in digestion and immunity, DNA meta-barcoding has become an indispensable tool to investigate the feeding effects on overall health and immune performance of aquatic animals. In aquaculture, water quality is one of the most important factors linked to digestion, immunity and growth of aquatic species. Some studies already reported significant influences of the external environment including rearing water on growth performance and gut microbial communities of aquatic animals (Giatsis et al., 2015, Shui et al., 2020). In recent times, use of biological and non-biological substrates for aquaculture water remediation are gaining much attention (Foysal et al., 2019a, Gutierrez-Wing and Malone, 2006, Permatasari et al., 2018). Zeolite consists of large one, two and three dimensional cavity that is 30-50% of the total volume (Jawahar et al., 2016). These spores are capable of trapping small molecules and contaminants, and favoured the growth of nitrifying bacteria under different aquatic conditions to remediate water (Grismer and Collison, 2017, Ivankovic et al., 2019, Widiastuti et al., 2008). We found that suspended zeolite significantly increased bacterial communities, gene functions and up-regulate some immune genes associated with defence mechanism of crayfish, compared to other forms (DZ and DZSZ) and control. Considering the porous nature and mode of action of zeolite and based on previous studies on water remediation using dissolved and water-soaked substrates under fish and crustacean aquaculture (Azim et al., 2002, Foysal et al., 2020c, Keshavanath et al., 2012, Pechenik et al., 2015), suspended zeolite assumed to work better than the dietary form. Additionally, omnivores and nocturnal

crayfish usually ingest everything by their claws within their range. Therefore, alongside the daily supplied feed, marron were also attracted by the suspended zeolite that significantly influence their gut microbial communities. On the other hand, higher concentration of zeolite in DZSZ group favoured the growth of *Candidatus* species in the marron gut, a novel lineages of Mollicutes (Kostanjsek et al., 2007). Regardless of diets and culture conditions, *Candidatus* Bacilloplasma was identified from all the gut samples in previous studies (Foysal et al., 2019; Foysal et al., 2020a; Foysal et al., 2020b; Foysal et al., 2020c) and therefore can be defined as the core microbiota of marron. As high zeolite concentration can cause *in-vivo* toxicity to animal organs (Petushkov et al., 2010, Thomas and Ballantyne, 1992), the DZSZ culture condition favoured OTUs abundances for the resident microbiome *C.* Hepatoplasma.

On SZ group, beside *Aeromonas*, three other significantly abundant bacteria in the SZ group are *Bosea*, *Phreatobacter* and *Lactobacillus*. *Bosea* and *Phreatobacter* are phylogenetically close genera identified from ultrapure water suggesting their role in water remediation (Tóth et al., 2014). Due to the role of natural zeolite in waste water treatment, the correlation between these two bacteria and better water quality can be envisaged. On the other hand, the positive role of *Lactobacillus* in health and immune performance of aquatic animals (Foysal et al., 2020a, Hoseinifar et al., 2015) including crayfish (Foysal et al., 2020b) has been deeply investigated earlier. Hence based on the activity of zeolite and feeding nature of marron it is predicted that the gut microbial diversity was positively influenced by the rearing environment rather than diet and combined effects of diet and water.

Predicting metagenomic functions from 16S rRNA are key in investigating the feeding impacts on host tissue and defence mechanisms (Gao et al., 2019, Koo et al., 2017b). However, database and pipelines limitations for the environmental samples, functional prediction of metagenomes are currently limited to only human samples. Thus PICRUSt (v1.0), Phipillin and Tax4Fun generated significantly lower correlation coefficient for the environmental samples, compared

to human (Iwai et al., 2016, Koo et al., 2017a). However, in most recent PICRUSt2 release reported to have high and competent correlation coefficient for the eDNA samples, in relation to previous three prediction methods (Douglas et al., 2020). PICRUSt2 prediction identified that the most significantly up-regulated pathway in SZ group are associated with digestion and reproduction of aquatic animals. TCA cycle associated with breakdown of glucose, sugar, amino acids, fatty acids and production of energy for calls. Unlike in animals, bacterial TCA cycle happened in cytosol however performed similar functions like animals (Jurtshuk, 1996). Peroxisome similarly linked to metabolism toxic hydrogen peroxide and other reactive oxygen species in animals. Steroid and moulting hormones are essential for the growth and development of decapod crustaceans (Chang et al., 2001) while styrene has potential negative effects on reproduction and development (Brown et al., 2000). Biosynthesis of steroid and degradation of styrene are therefore two crucial pathways for the growth and reproduction of animals.

In animals, low concentration of reactive oxygen species (ROS) and peroxisome are synthesized regularly, and required for homeostatic while higher concentration has potentially damaging effects on DNA and proteins. Some dietary compounds have intrinsic capabilities to metabolise and eliminate excessive ROS and peroxisomes generated from external stimuli (Su et al., 2019). Zeolite increase superoxide dismutase activity (Aseervatham et al., 2013) and scavenges ROS and peroxisomes, and maintained homeostasis in animals.

Crayfish immune-defence mechanism depends solely on innate immune response that arise mainly from antimicrobial peptides (AMPs). AMPs are primary immune elicitors and of crayfish. Two major AMPs, prophenoloxidase (proPO), a primary AMP in crustaceans that promote immune defence system through production of melanin and phagocytosis of foreign particles (Ashida et al., 1984; Cerenious et al., 2008). Augmented bacterial diversity, enriched bacterial pathways for the metabolism, growth, reproduction and toxic compound degradation

could be linked to the upregulated expression of cytMnSOD and proPO in the gut of SZ group. Among the six tested genes, only CTL is downregulated in DZ and DZSZ group, compared to control. CTLs are protein linked carbohydrate, plays role in cell-adhesion and destroy foreign particles directly prior to infection (Ewart et al., 2001, Vazquez et al., 2019).

Overall, based on molecular analysis of gut health and immunity, present study revealed that zeolite can be used in bagged form to increase gut microbial diversity, digestive functions and some innate immune response of marron. However, considering its effects on water remediation, the changes in water microbial communities after inclusion of zeolite in various form is required to identify any correlations between aquatic and gut microbial communities of marron. Furthermore, a subsequent field trial is necessary to investigate the efficacy of zeolite in different form and optimize the concentration for large scale commercial application.

Chapter 8: Probiotics in alternative protein diets for marron aquaculture

EXPERIMENT: *Lactobacillus plantarum* in black soldier fly (*Hermetica illucens*) meal modulates gut health and immunity of freshwater crayfish (*Cherax cainii*)

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Abstract

Probiotic supplements are being used to improve the growth and immune performance of aquaculture species over the last couple of decades. In recent times, black soldier fly (BSF) is considered as one of the promising sources of alternative protein to fishmeal protein in aqua-diets. Since the freshwater crayfish, marron (*Cherax cainii*), a Western Australian's native and iconic freshwater crayfish species, grows fairly slow under commercial farming environment, this study was aimed to investigate the supplemental effect of BSF and BSF with probiotic bacteria *Lactobacillus plantarum* (BSFLP) on overall health and immune performance of marron after 56 days of feeding under laboratory conditions. The post-trial data revealed insignificant influences of any diets on growth performance, however, both BSF and BSFLP based diets significantly improved some hemolymph parameters and gut health of marron. High throughput sequence data revealed that both BSF and BSFLP diets significantly improved the diversity of microbial communities including some beneficial bacteria for crustaceans in the hindgut of marron. Further analysis showed that both BSF and BSFLP diets upregulated the expression of some genes in the gut tissue and haemocytes associated with the innate immune response of marron at 48 h post injection. The up-regulation of some immune genes in BSFLP diet group was found significantly linked to OTU abundance for *Lactobacillus*. The findings of this study could be helpful for improving overall health status of marron.

Keywords: Aquaculture; marron; black soldier fly; probiotic bacteria; gut microbiota

8.1. Introduction

Fishmeal (FM) has always been used as the major protein source for the aquaculture species. Therefore, with the intensification of aquaculture, the demand for FM in aquaculture industry has increased over the years, resulting in greater misuse of wild stocks (Jennings et al., 2016, Tidwell and Allan, 2001). To cope with the burgeoning demand of seafood and inclination towards the sustainability practices, aquaculture nutritionists are trying to find alternative protein sources for aqua-diets. Recently, the interest in finding alternative protein sources has shifted from plant and higher animal products to insect meal that has an ability to confer nutritional suitability and environmental sustainability (Chaklader et al., 2019, Gasco et al., 2020). Among the insects, black soldier fly (BSF) has emerged as one of the most promising candidate species due to its balanced nutritional compositions, consistent protein, lipid and amino acids contents, and carriers of essential vitamins, minerals and trace elements (Belghit et al., 2019, Katya et al., 2017). In addition to attractive nutritional composition and feedstuff for aquaculture species, the BSF larvae has the outstanding ability to convert the diverse ranges of organic wastes into high quality protein and fat biomass that can be easily digested and assimilated by the animal body (Müller et al., 2017, Wang and Shelomi, 2017). Additionally, BSF has complex polypeptide system and probiotic bacteria have the ability to break down the peptide bonds into smaller molecules that can be easily digested and absorbed by the aquatic animals (Chaklader et al., 2019, Müller et al., 2017). The efficacies of BSF supplemented diet on growth and immune performance of aquatic animals including freshwater crayfish has been validated in some earlier studies (Biancarosa et al., 2019, Chaklader et al., 2019, Foysal et al., 2019b), however, limited knowledge is available on the suitability of using probiotic bacteria in BSF based diet for fish and crayfish.

Marron is one of the largest crayfish, native and iconic to Western Australia, and popular for its distinctive flavour and taste (Ambas et al., 2013, DoF, 2010b, Lawrence and Morrissy,

2000). Due to its disease-free status, live shipment opportunity over long distances and high market demand, marron are considered as an ideal crayfish for commercial farming (Ambas et al., 2013, Saputra et al., 2019). However, due to its slow growing nature, marron's overall production has remained stagnant over the decades (Cai et al., 2019, Foysal et al., 2019b, Mosig and Fallu, 2004). Past studies on crayfish have shown that the probiotic supplementation significantly modulated the gut microbiota and immune parameters whereas BSF inclusion reduced emerging pathogen like *Vibrio* and *Aeromonas* in the gut (Ambas et al., 2017, Ambas et al., 2013, Foysal et al., 2019b). However, none of the previous studies has supplemented probiotic bacteria in BSF diet and investigated the impacts on health and immunity of aquatic species. In the field of probiotic research in aquaculture, lactic acid bacteria (LAB) are the most promising candidates that can promote growth, immunity and disease resistance of crayfish (Enferadi et al., 2018, Ringø et al., 2018). Within LAB, *Lactobacillus plantarum* supplementation in aqua-diets has improved the growth and immune performance of some crustaceans including narrow clawed crayfish (*Astacus leptodactylus*) and white shrimp (*Litopenaeus vannamei*) (Valipour et al., 2019, Vieira et al., 2008). Therefore, the inclusion of *L. plantarum* in the BSF diet could be a diet of interest for marron and other aquatic organisms that has not been investigated yet.

Gut microbiota of animals plays a significant role in health and immunity of animals, therefore, a vast majority of research, nowadays is focused on microbial diversity analysis in the gut of aquatic species in response to dietary supplementation in aqua-feed. In addition to microscopic investigation of gut tissue, recent development in high throughput sequencing (HTS) and bioinformatics warrant an in-depth analysis of feeding effects on any animal (Michl et al., 2019, Rimoldi et al., 2018). Furthermore, HTS analysis leads to identification of bacteria linked to growth and immune performance as well as microbes with antagonistic activity against aquatic pathogens (Xiong et al., 2019). This study was therefore aimed to investigate the effects of

BSF diet alone and in combination with *L. plantarum* on gut microbial communities and innate immune response of marron under laboratory condition.

8.2. Materials and Methods

8.2.1. Experimental animals

A total of 75 marron 1+ size (70–100 g) were distributed into 15 different tanks at a density of 5 marron per tank (X 5 replicates per dietary treatment), and acclimated for 14 days, and assigned into three different feeding groups viz. CTL (control), BSF (black soldier fly) and BSFLP (black soldier fly + *L. plantarum*). The rest of the experimental set-up along with water quality analysis including temperature, pH and dissolved oxygen (DO) were performed as described in general methodology (chapter 3.1).

8.2.2. Diets and feeding

The probiotic bacteria *L. plantarum* was procured from Quest *L. plantarum*, Nutra Pharma, West Yorkshire, UK in powdered form. The bacterial suspension was first resuspended in de Man, Rogosa and Sharpe (MRS) broth (Sigma Aldrich, Darmstadt, Germany) and incubated overnight at 37 °C in a MTH-100 shaker incubator (Thermo Scientific, Waltham, MA, USA). From broth culture, the bacteria was then re-cultured overnight in MRS agar using sterile L-shape glass rod following serial dilutions (10^2-10^9), and dilution containing bacterial cell number of 1.0×10^9 CFU mL^{-1} on plate was collected. The probiotic feed was then formulated according to method described by Ramírez et al. (2017). The ingredients of basal feed (Table S4.1) was purchased from commercial feed supplier (Glenn Forest, Perth, Australia) and feed prepared at CARL. Firstly, the ingredients were passed gently through 100 µm mesh sieve and thoroughly homogenized to achieve uniform particle size. BSF based diet was prepared by inclusion of 12% protein from BSF into commercial basal diet for marron. During pellet preparation in mince mixture, the bacterial suspension was added to the feed ingredients at 1:1 ratio (1 mL suspension into 1 kg feed) using hand sprayer. The pellet was prepared using mince

mixture, followed by drying at 37 °C for overnight in an oven to reduce the moisture content and finally stored at 4 °C in air tight jars before use in feeding trial. The proximate compositions of final diet including 29.9%, 30.1%, 30.6% crude protein, and 7.1, 7.6, 7.6% lipid in , CTL, BSF and BSFLP, respectively, was measured according to AOAC method (AOAC, 2006), and bacterial concentration of 10^9 CFU/ml was determined via plate count in MRS agar. During acclimation, all marron were fed only fishmeal based basal diet while after acclimation, CTL, BSF and BSFLP groups were fed fishmeal based basal, BSF and BSFLP diets, respectively. Marron were fed everyday afternoon at 1.5% of their body weight (Foysal et al., 2020b).

8.2.3. Sampling

At the end of the trial, one randomly selected marron per tank (5/treatment) was used for the analysis of haemolymph parameters, gut microscopy, microbial diversity, and immune gene expression. For gut microbiota, marron were taken inside biological safety cabinet followed by excision of whole gut and separation of hindgut. The gut content with mucosa was homogenized using TissueLyseII (Qiagen, Hilden, Germany) and transferred to 1.5 mL Eppendorf for DNA extraction. Finally, for the gene expression analysis, the whole intestine tissue of marron were chopped into fine pieces after removing the contents and then stored in RNA Later solution (Sigma Aldrich, Darmstadt, Germany) at -80 °C according to manufacturer's instructions until further use. Same marron was used for hemolymph, gut histology and microbiome analysis.

8.2.4. Haemolymph parameters

Haemolymph osmolality (HO), lysozymal activity and total haemocyte counts (THC) of marron were analysed as described earlier in general methodology (chapter 3.2). DHC was calculated using methanol, May-Grunwald and Giemsa staining followed by cell counts on cover slip based on size and shape. Haemolymph osmolality of marron hemolymph solution

(anticoagulant added) was measured using Cryoscopic Osmometer-Osmomet 030 (Gonotec, Berlin, Germany).

8.2.5. Gut histomorphology

Mid gut sample of marron were used for light microscopy analysis as described earlier (Saputra et al., 2019). The dissected gut samples were dehydrated in ethanol, equilibrated in xylene and embedded in paraffin wax following the standard histological techniques. Each sample were cut into approximately 5 µm thickness and stained with haematoxylin and eosin (H&E). The histological images were captured with 40x magnification and analysed to assess intestinal fold height (IFH) by measuring them on each slide using digital imaging software (Adobe Photoshop CC 2015, Adobe System Incorporated, USA).

8.2.6. High throughput sequencing

The extraction of bacterial genomic DNA from pooled samples, PCR amplification of v3v4 regions, library preparation and pair-end sequencing were performed according to methods described in chapter 3.4.

8.2.7. Downstream bioinformatics

Unless any modifications mentioned, the "pipelines" for downstream sequence analysis and bioinformatics were used according to methods described in chapter 3.5. PASTA-aligned representative OTU sequences were subjected to FastTree (version 2.1.8) under the GTR+CAT model for phylogenetic tree construction (Mirarab et al., 2015, Price et al., 2010). Rarefaction depth value was set at 17,996 bp and subsequent calculation of alpha-beta diversities were performed using QIIME and microbiomeSeq (https://github.com/umerijaz/microbiomeSeq), microbiome (https://microbiome.github.io/tutorials/), phyloseq (McMurdie and Holmes, 2013), iNEXT (Hsieh et al., 2016), vegan (Dixon, 2003) in R-packages. In brief, alpha diversity was calculated in terms of observed species, Shannon, Simpson and Chao1 index followed by non-parametric Bonferroni correction at 0.05 level of significance. Non-metric

multidimensional scaling (NMDS) test was used for beta ordination and clustering of samples based on Bray-Curtis dissimilarity and permuational multivariate analysis of variance (PERMANOVA) of weighted UniFrac. Differential abundance of bacterial abundance at phylum level was identified using Linear Discriminant Analysis Effect Sizes (LEfSe) test at 0.05 level of significance in Galaxy server (http://huttenhower.sph.harvard.edu/galaxy/). Significant different bacteria in three different diet groups was identified using five-samples Kruskall-Wallis test in QIIME with Bonferroni corrections for multiple comparisons.

8.2.8. Challenge test

In-vivo challenge test was performed on 45 marron, 15 per group against *Vibrio mimicus* isolate. After the termination of the trial, each marron was injected with 50 µL of previously prepared 2×10^8 CFU/mL stock solution of *V. mimicus,* reported as median lethal dose (LD50) (Ambas et al., 2013), into the fifth thoracic leg. Injected marron were fed with respective diets and subjected to collection and storage of whole gut (5/treatment) after 48 h of challenge. Marron survival (10/treatment) analysis with BSF and BSFLP diets, compared to control was performed using Kaplan-Meier survival estimator and log-rank test at 0.05 level of significance in survival (https://github.com/therneau/survival), survminer (http://www.sthda.com/english/rpkgs/survminer/) and ggplot2 in Rstudio.

8.2.9. Gene expression analysis

A total of 12 genes (Table S8.1, Appendix 4) that reported to be associated with innate immune response of crayfish were selected for this study (Dai et al., 2017, Liu et al., 2020b, Liu et al., 2013). For analysis of proPO and MnSOD, 0.5 mL of haemolymph was diluted in 0.5 mL of anticoagulant and centrifuged immediately at 8000 rpm for 20 min to collect hemocytes (Liu et al., 2013). The collected haemocytes and 5 mg of homogenized tissue samples were used for RNA extraction. The gene expression analysis was performed as described in chapter 3.6. Student t-test was used for the paired comparison of qRT-PCR data. The “Pearson” correlation

of gene expression data with top 40 genus abundance was performed using "microbiomeSeq", and "phyloseq" (McMurdie and Holmes, 2013) R packages at 0.05 level of significance.

8.2.10. Calculations

The growth performance was measured according to calculations mentioned in chapter 3.7 of general methodology.

8.3. Results

8.3.1. Growth and hemolymph parameters

None of the diets had significant (*P*>0.05) impacts on growth performances, however, THC was positively influenced by both BSF and BSFLP while the activity of hemolymph lysozyme was increased with BSFLP (Table 8.1).

Table 8. 1 Growth and immune performance of marron after 56 days of the feeding trial

Parameters	CTL	BSF	BSFLP
WG (g)	6.21 ± 0.38	6.88 ± 0.42	6.91 ± 0.45
SGR (%/day)	0.49 ± 0.04	0.51 ± 0.04	0.52 ± 0.05
FCR	3.02 ± 0.16	2.91 ± 0.15	2.92 ± 0.18
Lysozyme (U/mL)	0.43 ± 0.01^{a}	0.45 ± 0.02^{a}	0.48 ± 0.01^{b}
THC (cells/mL)	7.95 ± 0.11^{a}	8.81 ± 0.15^{b}	8.87 ± 0.15^{b}
DHC (cells/mL)	21.67 ± 0.23	22.09 ± 0.19	21.95 ± 0.25
HO (U/mL)	0.41 ± 0.01	0.43 ± 0.02	0.43 ± 0.01

Values are presented as mean ± SE. Different superscript alphabetical letters (a, b) indicate significantly different means for different treatments at 0.05 level of significance in Tukey's post-hoc test. WG, weight gain; SGR, specific growth rate; FCR, feed conversion ratio; THC, total haemocyte count; DHC, differential haemocyte count; HO, haemolymph osmolality.

8.3.2. Gut micromorphology

The histological images of mid gut surface of marron fed control, BSF and BSFLP diets, and their statistics are shown in Figure 8.1. The intestinal fold height (IFH) of marron fed both BSF and BSFLP diets was significantly (P<0.05) higher than the control with the size ranges from 38.26 µm in control to 79.95 µm in BSFLP fed marron.

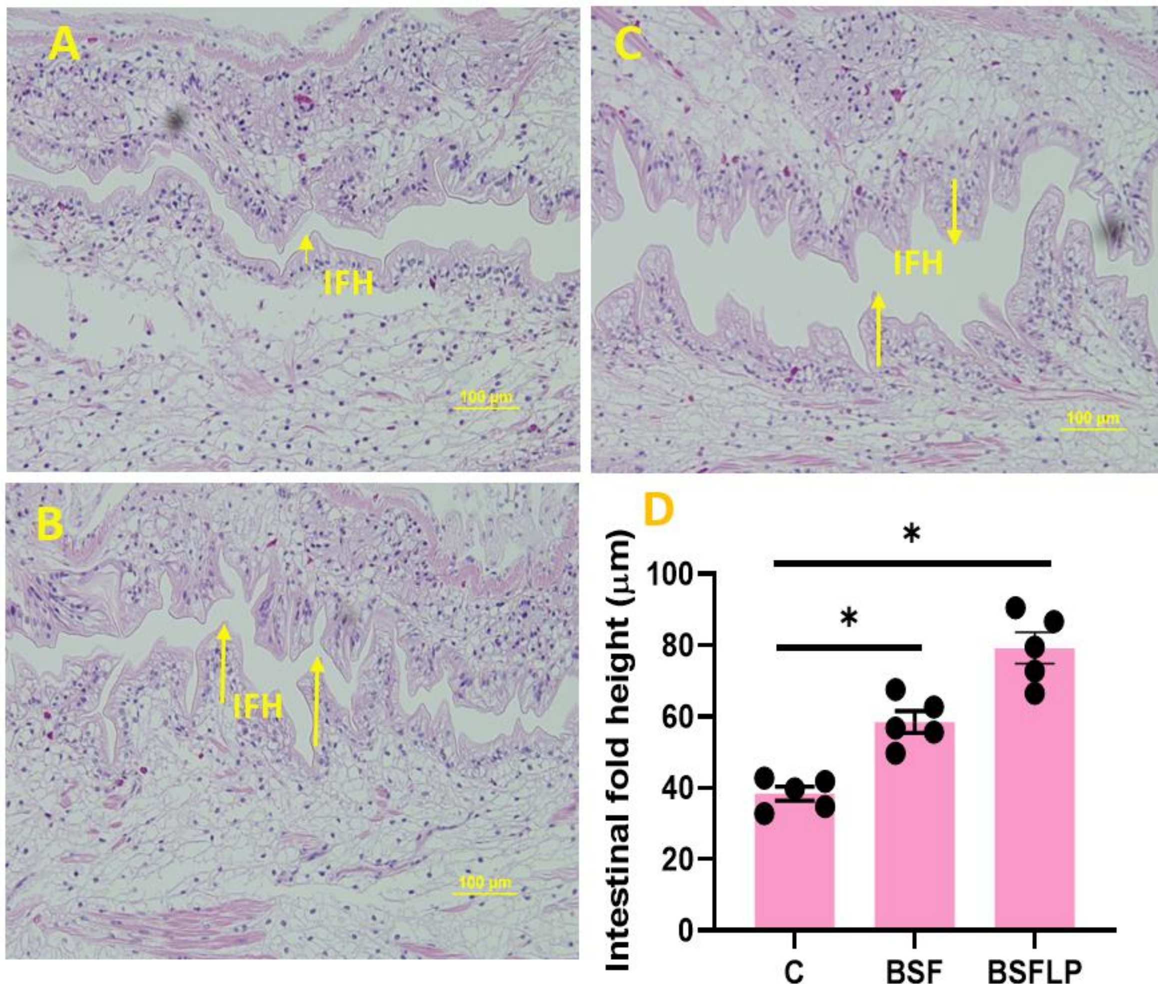

Figure 8. 1 Mid gut histo-morphological structure of marron fed control, BSF and BSFLP diets (Panel A-C, respectively) (Haematoxylin and Eosin, 40 x magnification, scale bar = 100µm). Arrow indicates intestinal fold height (IFH) from base to top of the individual fold. Variation in IFH of marron feeding test diets for 56 days (D). *Significantly different at α-level of 0.05 (one-way ANOVA with Dunnett's multiple comparisons test).

8.3.3. Alpha-beta diversity of gut microbial communities

A total of 885,669 high quality sequences were obtained from 15 samples. After removing of chimeras and singleton, filtered high quality sequences were assigned to 212 OTUs, 17 phyla and 152 genera. The rarefaction curve showed that each sample was sequenced at saturated level to capture all the major microbial diversity including rare species (Figure 8.2 A). The alpha diversity measurements revealed significant (P<0.05) enrichment of observed species, Shannon and Chao1 indices in BSFLP fed marron compared to BSF and control wherein Simpson index remained unchanged (Figure 8.2 B-E). A NDMS plot based on Bray-Curtis dissimilarities of relative abundance (weighted) and PERMANOVA is shown in Figure 8.2F. An R value of 0.9831 and P value of 0.0001 revealed significant effects of feeding on beta dispersion of gut microbial communities in the gut.

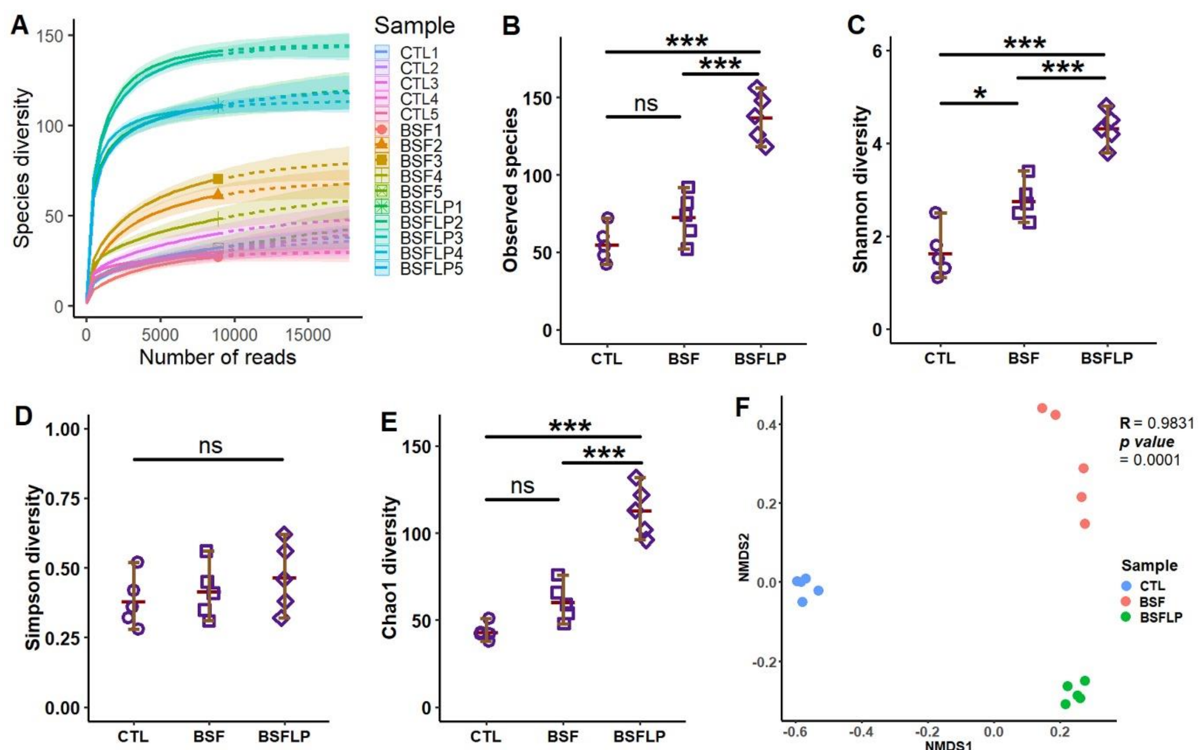

Figure 8. 2 Alpha-beta diversity measurements of marron hindgut microbial communities after feeding trial. (A) Rarefaction curve showing the depth and saturation level of sequencing. (B) Observed species; (C) Shannon diversity; (D) Simpson diversity; (E) Chao1 diversity; (F) NMDS plot based on Bray-Curtis dissimilarity showing clustering of samples after feeding

trail. *Significantly different at α-level of 0.05. ***Significantly different at α-level of 0.001 (one-way ANOVA with Tukey's honest significant differences, HSD).

8.3.4. Relative and differential abundance of microbial diversity

At phylum level, five taxa were considered as dominant phyla as their relative abundance were higher than 1% in at least one sample. Among them, Proteobacteria (84.5± 8.7%) was predominant in control group while BSF and BSFLP groups were dominated by Tenericutes (69.7 ± 6.6%) and Firmicutes (48.9 ± 2.9%), respectively (Figure 8.3 A). In addition to Firmicutes, Actinobacteria (10.8 ± 1.5%) and Bacteroidetes (7.1 ± 0.7%) were identified as the second and third most abundant phyla in BSFLP fed marron. At genus level, *Aeromonas*, *Candidatus* Bacilloplasma, *Candidatus* Hepatoplasma, *Citrobacter*, *Hafnia*, *Shewanella* and *Vibrio* comprised of 95.7%, 91.5 and 71.8% of total classified sequences in control, BSF and BSFLP fed marron, respectively (Figure 8.3 B). The number of shared and unshared genus were significantly higher in BSFLP fed marron, compared to BSF and control groups (Figure 8.3 C). At phylum level, LEfSe identified Proteobacteria and Tenericutes as the significantly abundant phyla in the control and BSF fed group, respectively, wherein BSFLP group enriched ($P<0.05$) the colonization of Actinobacteria, Acidobacteria, Bacteroidetes, Epsilonbacteraeota and Firmicutes bacteria in the marron gut (Figure 8.3 D). At genus level, *Aeromonas*, *Candidatus* Bacilloplasma, *Shewanella* and *Vibrio* were the significantly ($P<0.05$) abundant group in control revealed by more strict five-samples Kruskal-Wallis test, and these four species comprised 88.6% of total read abundance. In BSF fed marron, differentially abundant ($P<0.05$) genera were *Candidatus* Hepatoplasma, *Citrobacter*, *Flavobacterium* and *Pseudomonas* that composed of 68.7% of total sequence reads. Finally, BSFLP diet significantly enriched *Corynebacterium*, *Streptococcus*, *Rhodobacter*, *Limnohabitans* *Staphylococcus* and *Lactobacillus* genera that represented 48.2% of classified sequences (Table 8.2).

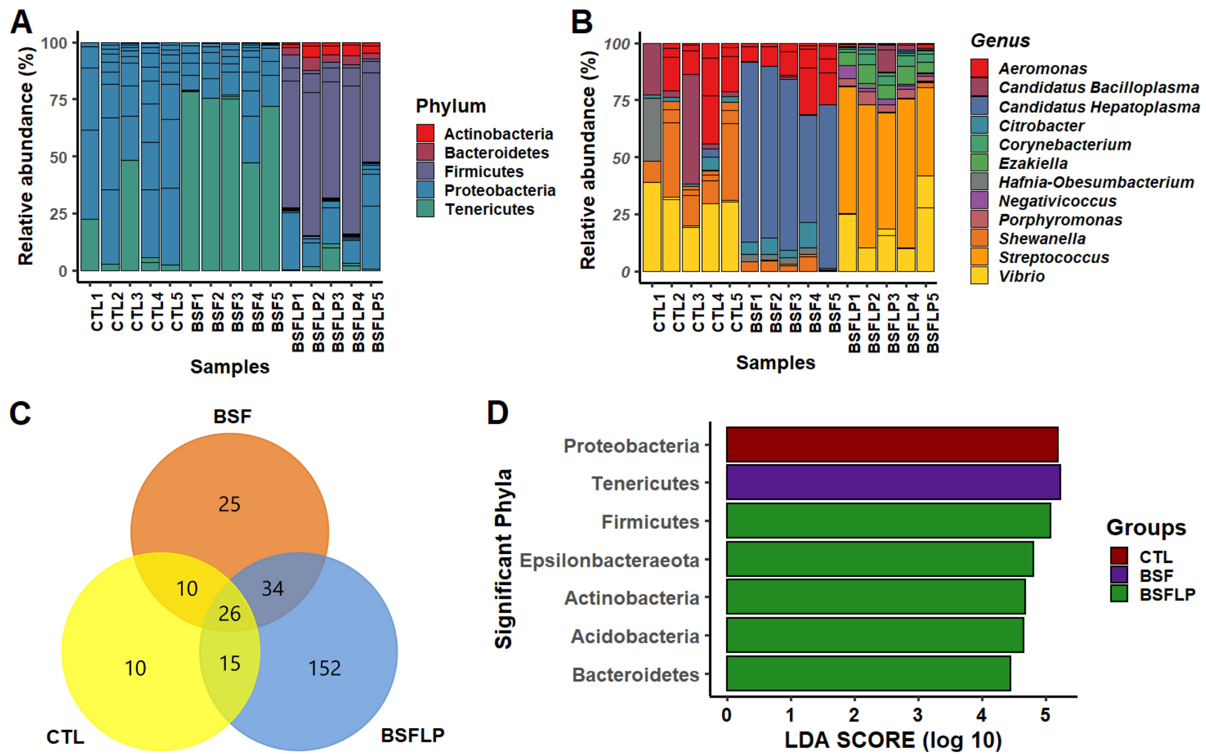

Figure 8. 3 Microbial communities in marron gut after trial. Relative abundance at (A) phylum and (B) genus level; (C) Number of shared and unshared genera in different groups; (D) Differentially abundant bacteria at phylum level (one-way ANOVA with Tukey's HSD).

Table 8. 2 Differential abundance of bacterial communities at genus level in different groups

CTL	BSF	BSFLP	P-value	Genus
0.4	0.6	623.6	0.0013	*Corynebacterium*
5.1	12.8	3695.4	0.0013	*Streptococcus*
0.1	1.1	58.6	0.0013	*Rhodobacter*
0.4	1.8	183.4	0.0013	*Limnohabitans*
1.8	75.2	10.6	0.002428	*Pseudomonas*
12383	36	780	0.002428	*Vibrio*
10006.6	330.6	59.8	0.003058	*Shewanella*
0.2	0.2	30.6	0.003981	*Staphylococcus*
0.1	3.6	0.4	0.007146	*Rhodococcus*
0.1	0.1	25	0.007146	*Lactobacillus*
313.6	23630	12.8	0.008167	*Candidatus* Hepatoplasma
0.6	63	41.8	0.008631	*Flavobacterium*
8649.4	4733.6	83.4	0.012155	*Aeromonas*
7331.8	160.6	245.6	0.012778	*Candidatus* Bacilloplasma
1062	1301	35.4	0.013168	*Citrobacter*

Data expressed as mean of reads.

8.3.5. Expression of immune genes after trial and post-challenge

Among the 12 genes, seven including Toll groups (Toll 2, 3, 4, 5), proPO, C-type lectin (CTL) and anti-lipopolysaccharide (ALF1) showed differential expression (DE, $P<0.05$) at 48 h post injection (hpi) in BSF and BSFLP fed marron in the gut tissues compared to pre-challenged marron. In addition, upregulation of PcCTSL was observed in BSFLP and Ast in BSF fed post-challenged marron. (Figure 8.4). However, the relative expression level of cypA and cytMnSOD remained static ($P>0.05$) in BSF and BSFLP fed marron, and lysozyme expression downregulated in control marron group at 48 hpi. In BSFLP fed marron, the expression level of ALF1, Toll 3, Toll 2, Toll 4, CTL, Toll 5 and proPO reached to 6.4, 5.8, 5.4, 4.6, 4.4, 4.1 and 3.8 fold changes at 48 hpi with *V. mimicus*. On the contrary, BSF fed marron showed 6.5, 5.6, 4.8, 4.2, 3.5, 3.2 and 2.1 fold changes for ALF1, Toll 3, Toll 2, CTL, Toll 4, Toll 5 and proPO, respectively. The expression level of PcCTSL and Ast increased by 2.2 and 1.6 fold in BSFLP and BSF fed post-challenged marron. The expression level of PcCTSL and proPO in BSFLP group revealed to have significant ($P<0.05$) positive correlations with the number of OTUs for *Lactobacillus* genus. Additionally *Vibrio*, *Lactovum* and *Candidatus* Bacilloplasma were found positively correlated to CTL, cypA, proPO, PcCTSL and Lys in the BSF feed group, however their interactions were found non-significant (Figure 8.5).

8.3.6. Post-challenged marron survival

The first mortality was observed at day 3 in the control group while in BSF and BSFLP, marron survived up to day 5 and day 6, respectively. Marron started to uptake feed from day 4 of post-challenge and no mortality was observed for BSFLP group after day 8 and day 9 for the BSF fed group while last mortality was recorded at day 12 for the control group. Compared to control, significantly higher survival was observed in the BSFLP ($P<0.005$), followed by BSF ($P<0.05$) fed marron (Figure 8.6).

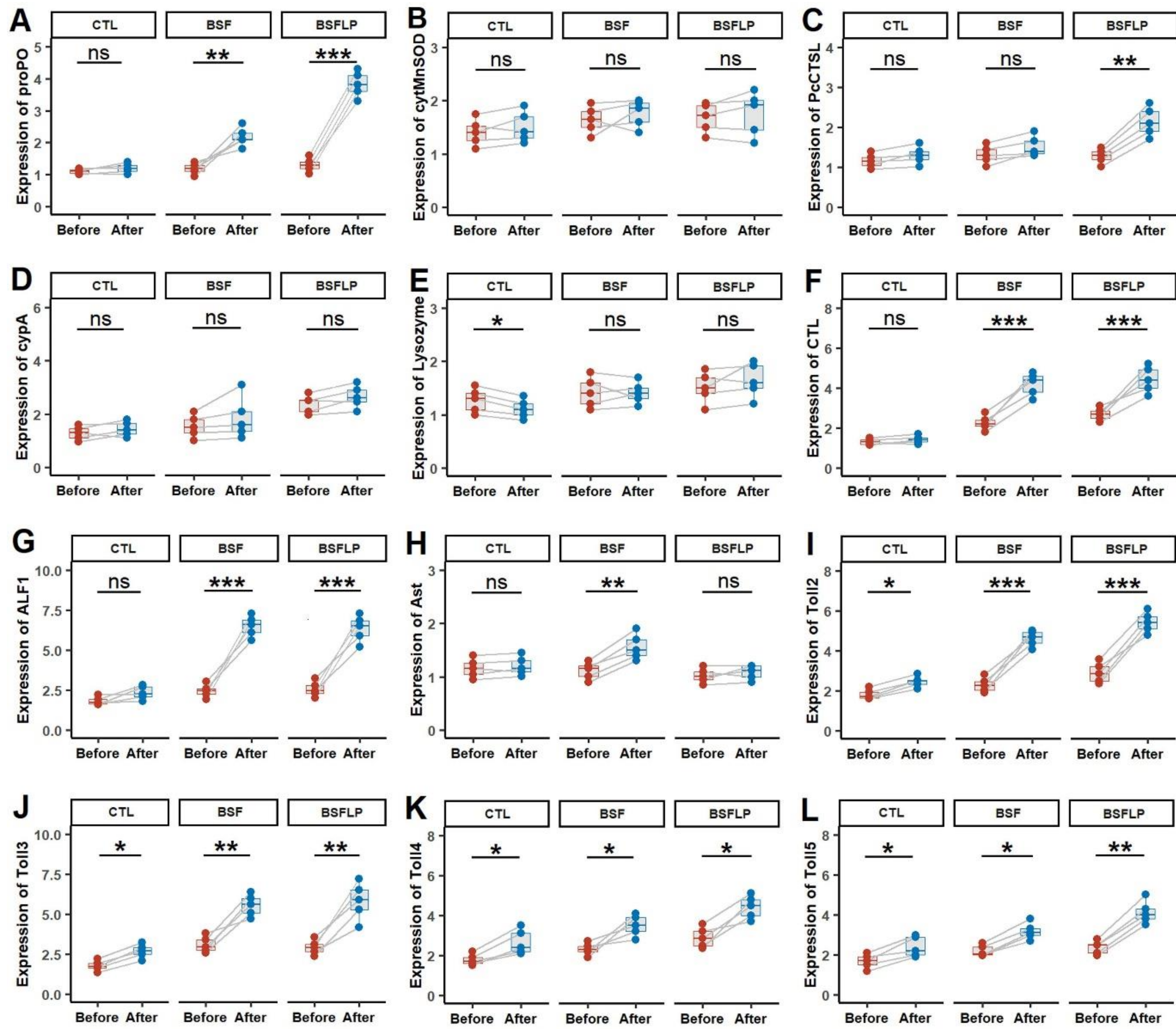

Figure 8. 4 Relative expression level of genes in terms of fold changes in the intestine tissue and haemocytes associated with innate immune response of crayfish. (A) Prophenoloxidase, *proPO* (haemocytes); (B) Cytosolic manganese superoxide dismutase, *cytMnSOD* (haemocytes); (C) Cathepsin L, *PcCTSL* (intestine); (D) Cyclophilin A, cypA (intestine); (E) C-type lysozyme, lysozyme (haemocytes); (F) C-type lectin, CTL (intestine); (G) Anti-lipopolysaccharide factor 1, *ALF1* (intestine); (H) Astacidin, *Ast* (intestine); (I) Toll like receptor 2, Toll2 (intestine); (J) Toll like receptor 3, Toll3 (intestine); (K) Toll like receptor 4, Toll4 (intestine); (L) Toll like receptor 5, Toll5 (intestine). *Significantly different at α-level of 0.05. **Significantly different at α-level of 0.005. ***Significantly different at α-level of 0.001 (paired student t-test).

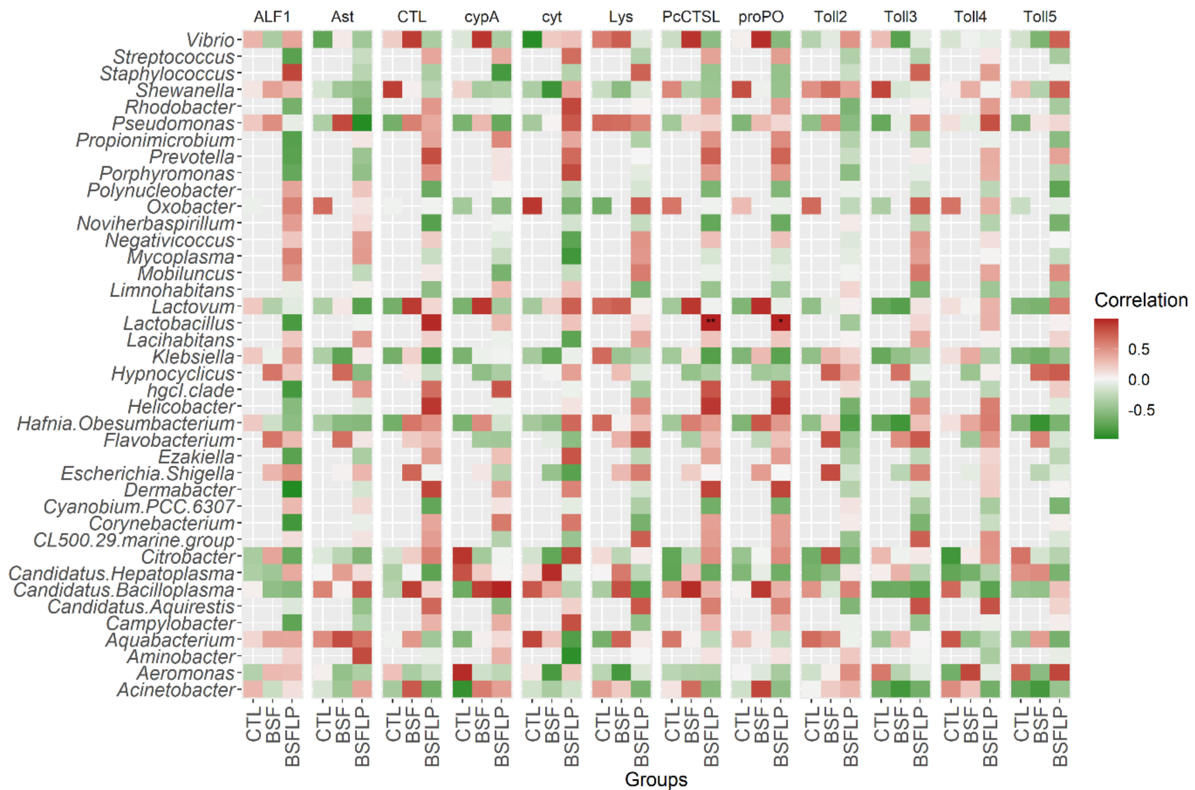

Figure 8. 5 Pearson correlation plot showing the relationship between top 40 taxa (at genus level) and gene expression data. Abbreviations: as mentioned in Figure 8.4. *Significantly different at α-level of 0.05. **Significantly different at α-level of 0.005.

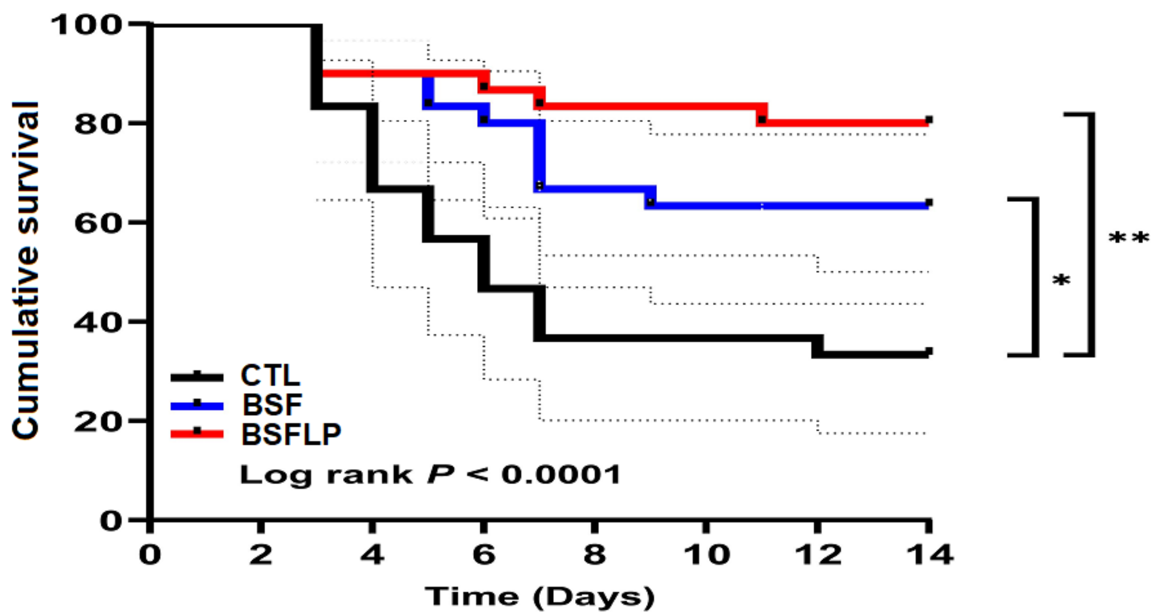

Figure 8. 6 Cumulative survival of marron following 14-days challenge test with *V. mimicus*.

8.3.7. Data availability

The relevant fastq sequence files used for microbial community analysis are currently available at National Centre for Biotechnology Information (NCBI) BioProject under the accession number PRJNA609769.

8.4. Discussion

In aquaculture, replacing fishmeal with cheap alternative protein sources without affecting the growth and immune performance of aquatic species has always paid the most attention. The choice of raw ingredients mostly depends on sources with high protein contents, availability and cost. In this regards, insect BSF protein has shown significant potential to replace other plant and animal based proteins from aqua diets (Bruni et al., 2020, Chaklader et al., 2019, Foysal et al., 2019b). Furthermore, considering the impacts of LAB on growth and immune performance of aquatic animals (Enferadi et al., 2018, Foysal et al., 2020b, Giri et al., 2018, Valipour et al., 2019), the combined effects of BSF and LAB could be an interesting and significant study in the perspective of sustainable aquaculture. To investigate the impacts of diets, beside growth parameters, we have assessed haemolymph constituents, histomorphology, gut microbial communities, and genes associated with immune response of crayfish. The incorporation of *L. plantarum* into BSF based diet significantly increased some biological indices for marron. Though growth performance was not significantly improved, however, *P*-value of 0.075 suggests that significant growth improvement could be achieved with optimum concentration of *L. plantarum* in BSF diet. Additionally, due to long life cycle of marron in commercial farming, longer experimental trial with juveniles could be an interesting study to compare the growth with BSFLP diet under laboratory conditions.

In addition to growth, digestion and nutrient absorption that are principally linked to gut health should also be considered in selecting any aqua-diets (Le et al., 2019). The histological structure of intestine has been considered as a good indicator to evaluate the nutritional

condition of fish and crustacean (Raskovic et al., 2011, Siddik et al., 2018). Dimitroglou et al. (2009) stated that longer intestinal fold and microvillus size are associated with the higher nutrient absorptive efficiency leading to improved health and immune functions whereas shorter intestinal fold as an indication of poor nutrient utilization and absorption result in reduced immune functions (Raskovic et al., 2011). In the present study, significantly enhanced IFH was found in marron fed BSF singly and conjointly with probiotic diets than control. So far, no research has been reported the influence of insect-based diet on histological changes in crustaceans, however in fish, it is found that the supplementation of insect larvae in PBM diet can increase intestinal microvillus height and enterocyte width in relation to fishmeal-based diet that was linked to BSF protein, amino acids, minerals and vitamins (Chaklader et al., 2019). In addition, Nogales-Mérida et al. (2019) reported that insect's larvae contain biologically active peptides, which not only promote growth performance and immunity but also act against pathogenic bacteria in gastro-intestinal tract of animals. In present study, the highest IFH was observed in marron fed BSFLP diets might be due to the production of extracellular digestive enzymes including amylases, proteases and lipases by *L. plantarum* which aid in nutrition, and that could be a reason behind enhanced IFH of marron (Mukherjee et al., 2019).

In recent times, the role of gut microbiota in fish and crustacean health has been validated with the aid of next generation sequencing. Gut bacterial communities of aquatic animals are mostly composed of Proteobacteria, Fusobacteria, Bacteroidetes and Firmicutes while crustaceans have high percentage of Tenericutes in their gut (Dong et al., 2018, Egerton et al., 2018, Mukherjee et al., 2019, Wang et al., 2018). Alike in fish, most of the Bacteroidetes and Firmicutes in crustaceans are categorized as beneficial microbes for the gut health and immunity (Foysal et al., 2019b, Wang et al., 2019). Bacteroidetes is predominant in human gut and some other higher vertebrates while relatively less abundant in aquatic species (Foysal et

al., 2019b, Xia et al., 2014). Most of the probiotic bacteria including LAB are classified into Firmicutes, a phylum playing a crucial role in growth, digestion and immunity of aquatic animals (Foysal et al., 2020b, Giri et al., 2018). In addition, Tenericutes is shown to have positive influence on digestion, metabolism and immunity of crustaceans (Chen et al., 2015, Dong et al., 2018, Foysal et al., 2019b). In present study, we found augmentation of bacterial OTUs for Tenericutes with BSF diet, and Firmicutes and four others with BSFLP diet. The improved haemolymph parameters and gut health of marron fed BSF diet therefore can be correlated to Tenericutes, and this results are consistent with some previous studies (Chen et al., 2015, Foysal et al., 2019b, Liu et al., 2020b). However, BSFLP diet enriched five different phyla where apart from Bacteroidetes and Firmicutes, the role of Acidobacteria, Actinobacteria and Epsilonbacteraeota has yet to be understood for crustaceans. The positive correlation of Bacteroidetes and Firmicutes on haemolymph and biochemical properties of marron has been reported with *L. acidophilus* and *L. plantarum* in a recent study (Foysal et al., 2020b). At genus level, the representative OTUs from *Aeromonas*, *Candidatus* Bacilloplasma, *Rhodobacter*, *Shewanella* and *Vibrio* in all samples suggesting that they are the core microbiota of marron gut. This findings is further supported by some recent studies on gut microbiota of some crustacean species under different experimental conditions (Cheng et al., 2019a, Foysal et al., 2019b, Shui et al., 2020). Beside core microbiota, the intensification of LAB, including *Lactobacillus* and *Streptococcus* in the BSFLP fed marron can be correlated with the inclusion of probiotic bacteria in the diets and colonization of *L. plantarun* in the gut. However, *Candidatus* bacterial lineage although reported as abundant genera in some crustacean gut, their role yet fully understood (Chen et al., 2015, Dong et al., 2018, Foysal et al., 2019b). By investigating the nature of gut microbiota in three different groups, it is evident that *Lactobacillus* in insect meal generated a positive and complex bacterial interactions that provided the environment for the growth diverse bacteria in the BSFLP fed marron.

Crayfish are considered as model organism for the study of innate immunity of crustaceans. Challenges with pathogenic bacteria causes pathological and immunological changes in the tissues or organs, and host cell counteract pathogens to protect them by various immune defence mechanism (Ambas et al., 2013). Unlike higher vertebrates, crayfish depends solely on innate immune system for protection against foreign particle (Vazquez et al., 2019). Among the various aspect of innate immune system, antimicrobial peptides (AMPs) are the principal immune elicitors of the host defence system in crayfish (Jiravanichpaisal et al., 2007). Toll like receptors (Toll) in crustaceans activate downstream signalling cascades that play crucial role in generating innate immune response, especially bacterial clearance (Han-Ching Wang et al., 2010). The upregulation of Toll receptors in BSF and BSFLP groups in present study suggests that they are the primary and key anti-microbial defence system in crayfish following infections by the foreign particles. The present study also revealed that the mRNA of three AMP genes (proPO, CTL, ALF1) were differentially expressed in the examined tissue and haemolymph of BSF and BSFLP fed marron at 48 hpi. Prophenoloxidase (proPO) in haemocytes, a melanin producer that serves as the innate defence mechanism of crustaceans and associated with phagocytosis of foreign particles (Ashida and Söderhäll, 1984, Cerenius et al., 2008). ALF, commonly found in hepatopancreas, intestine and gill- an AMP with anti-microbial activity against a broad range of pathogen including virus, bacteria and fungi. Over expression of ALF has been reported to protect shrimp (*Penaeus vannamei*) from *Vibrio penaeicida* and *Fusarium oxysporum* infections (de la Vega et al., 2008). CTLs, a protein shared with carbohydrate that associated with cell-cell adhesion, innate immune response against pathogens and infections (Cambi and Figdor, 2009, Drickamer, 1999). Some of the CTLs have been testified as capable to kill bacteria directly prior to infection or damages (Cambi and Figdor, 2009). Hence it can be concluded that upregulation of proPO, ALF1 and CTLs were mainly linked to bacterial challenge and the protective response primarily generated from BSF and *L. plantarum* in the

BSF diets. Other differentially expressed genes after challenge were Ast in BSF and PcCTSL in BSFLP fed groups. Ast is a hypertensive AMP that upregulates during bacterial infection and plays crucial role in bacterial clearance while PcCTSL, a protease predominantly found in haemolymph of crayfish, plays crucial role in innate immunity (Dai et al., 2017, Shi et al., 2014). However, the inhibitory effects Ast on BSFLP fed marron and links between upregulation of PcCTSL and LAB needs further investigation. Finally, we found downregulated expression of intestinal lysozyme in control that was consistent to a recent study on red swamp crayfish (*Procambarus clarkii*) where lower lysosomal activity was recorded in the gut but higher activity was documented in the gills and hepatopancreas (Liu et al., 2020c). The lower expression patterns of lysozyme in the intestine therefore linked to lesser cell wall cleaving ability of post-challenged control marron.

The correlation study between top 40 genus and post-challenge gene expression data revealed the association of lactic acid bacteria, *Lactobacillus* and *Lactovum* to host-defence mechanisms of marron in the formulated diets. In addition, the overwhelming abundance of *Vibrio* and *Candidatus* lineage and their effects on marron health in the BSF and BSFLP groups (compared to control) demonstrated that they are the core microbiota of marron with an influential role in digestion and immunity. Therefore, *Vibrio* plays a differential role in marron gut compared to other decapod crustaceans where the bacteria reported to linked with major diseases (Jayasree et al., 2006, Wong et al., 1995).

The overall findings of the present study indicated that the dietary supplementation of BSF and BSF with *L. plantarum* significantly improved the intestinal microbiota, health and immune status of marron. Although growth performance was not significantly improved, the *P*-value of WG, SGR and AMP genes in BSF and BSFLP diet groups suggest that these two could be the diets of interest for commercial crayfish farming, especially the impacts of BSFLP was promising. However, the augmentation of differentially expressed ($P<0.05$) bacterial species

and their correlations to diet ingredients, mechanism of microbial interactions in the gut, and correlation of AMP gene expression in relation to microbial community modulation need further investigations.

CHAPTER 9: General discussion, conclusions, limitations and future recommendations

9.1. General discussion

Technological advancement in sequencing and bioinformatics revolutionize some of the critical aspects of aquaculture. In some cases, technological innovations and applications improve aquatic-animal health managements, expand sources of raw materials and additives in aqua-diets, and enhance sustainability practices. On this way, tremendous achievements have been made in finfish aquaculture in terms of disease prevention through precise monitoring of health status, screening of alternative proteins and oils from various sources, selection of probiotic specific to growth and immunity, identification of bacteria associated with better health, and aquaculture water remediation. Unfortunately, the applications of sequencing technologies are very limited for crayfish aquaculture reflected by fewer and fragmented published documents. While a good number of published articles describe gut microbiota of white shrimp (Holt et al., 2020a), and their changes with different probiotics, alternative protein diets and under various rearing conditions, still we have very limited data for marron despite their high market demand and consumer preference. On this backdrop, present research investigated the gut microbiota of marron under different feeding regime and rearing conditions to identify bacteria linked to gut health and immunity that would help to find cost-effective diet(s) for marron aquaculture.

In present study, irrespective of diets and culture conditions, marron gut microbial communities found dominated by Tenericutes (46.5%) and *C*. Bacilloplasma (35.3%) at phylum and genus level. In addition to *C*. Bacilloplasma, *C*. Hepatoplasma also found in copious numbers for some of the probiotic and substrate added marron gut samples. The representative OTUs for *Aeromonas* and *Hafnia* were found inconsistent where *Hafnia* (8.8%) abundance increased and *Aeromonas* (4.6%) growth inhibited by most of the probiotic bacteria, especially with *Lactobacillus* mixture in present research. Similar variation is reported in a study where

screening of 110 isolates from crayfish *Astacus leptodactylus* gut from juvenile and adult growth stages revealed significant inhibition of *Aeromonas hydrophila* by *Hafnia* and hence authors stated the possibility use this bacteria as potential probiotic for crayfish aquaculture alongside *Bacillus* and *Lactobacillus* (Didinen et al., 2016). In present study, excluding starvation, most of the trials were performed with probiotic, APS and biological substrates, therefore the higher abundance for the core microbiota *Hafnia* (Table 9.1) in the marron gut can be positively correlated while *Aeromonas* and *Vibrio* found negatively correlated to health status (Figure 9.1). Contrarily, substrates and APS studies favoured the growth of *Aeromonas* (9.5%) compared to *Hafnia* (3.2%). The negative interaction between *Aeromonas* and lactic acid bacteria (LAB) in aquatic animals is responsible for the *Aeromonas* inhibition by the LAB (Balcázar et al., 2008, Fečkaninová et al., 2017). Irrespective of diets and conditions, only *Lactobacillus* and *C.* Hepatoplasma were found positively correlated to tail biochemical composition, haemolymph parameters and genes associated to immune response (Figure 9.1).

Table 9. 1 Core gut microbiome of marron (based on SILVA 1.32) in various trials

Nutrient deprivation	**Dietary Probiotics**	**Dietary protein sources**	**Substrates of rearing environment**
C. Bacilloplasma	*Aeromonas*	*Aeromonas*	*C.* Bacilloplasma
Hafnia	*C.* Bacilloplasma	*C.* Bacilloplasma	*Aeromonas*
Limnohabitans	*Hafnia*	*Hafnia*	*Hafnia*
Vibrio	*Vibrio*	*Vibrio*	

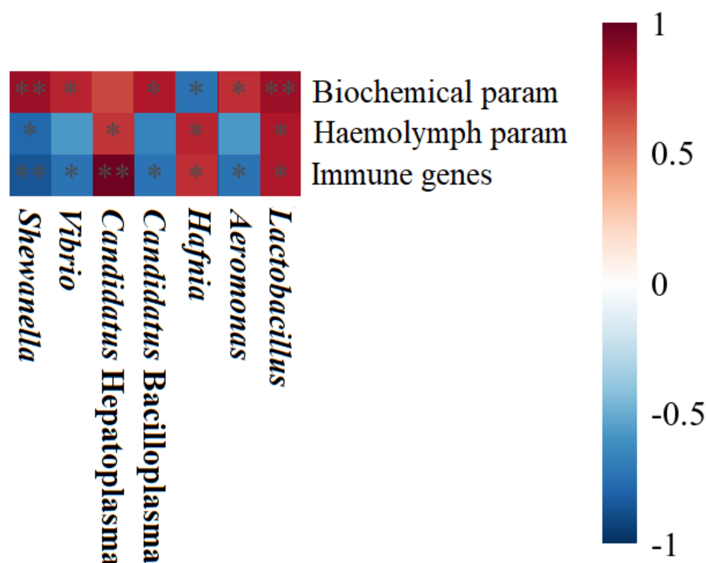

Figure 9. 1 Predicted correlation between the gut microbiota and health-immune indices of marron exposed to various parameters as detailed in various chapters.

Long term nutrient deprivation reduces microbial diversity and immune response of marron, but did not affect (P>0.05) the survival rate. The result is consistent with some previous studies where starvation reduced microbial diversity and Bacteroidetes phyla in fish (Michl et al., 2019, Xia et al., 2014). Omnivores can adjust food habitat and survive longer in nutrient deprivation, compared to carnivores and herbivores (Zhang et al., 2018). Marron are "benthic omnivores" therefore can survive longer time without food supplements and possibly utilize recycled nutrients from organic waste and uneaten feedstuffs decomposed by bacteria.

In present study, none of the diet improved marron growth significantly. The higher growth results with *Clostridium butyricum* are due to significantly higher moults in probiotic feed group. However, *Lactobacillus* combination was very close (P=0.059) to significant in terms of marron weight gain. As marron grows slowly (Lawrence, 2007), a longer culture time is required to achieve market size marron. Moreover, the size (1+) and weight (70-100 g) of the experimental animal are the two crucial growth limiting factors that restrained marron to gain weight rapidly. An experiment with juvenile marron could have different results as juvenile experimental animal are preferred for growth performance analysis (Valente et al., 2013).

One of most interesting finding in present study is similar effects of probiotics on haemolymph parameters and tail muscle biochemistry while gut microbial communities were distinctly different (Table 9.2). However, in depth analysis of gut microbiota identified a common drift for all probiotic trails. *L. acidophilus* + *L. Plantarum*, *B. mycoides* and *C. butyricum* increased abundance for LAB and core microbiota of marron including *Candidatus* lineages and *Hafnia* while reduced *Vibrio* significantly (Table 9.3). Based on that findings, we have identified a negative correlation of *Vibrio* with marron health indices (Figure 9.1). Again, compared to other probiotics, significantly higher gut microbial diversity, better health and immune indices of marron was achieved in trials with *Lactobacillus* strains (Table 9.2 and 9.4). This results can be positively correlated to a recent study on *L. plantarum* effects on narrow clawed crayfish

(*Astacus leptodactylus*) where probiotic supplementation improved haemolymph parameters including total haemocyte counts, phenoloxidase, superoxide dismutase, lysozyme, digestive enzyme activity (protease, amylase, phosphatase) as well as LAB in the gut of crayfish (Valipour et al., 2019). *Lactobacillus* elevates health and immune parameters by releasing immunostimulants from peptidoglycan in the cell wall and thereby improving the resistance of host against pathogen (Dash et al., 2015). It is therefore evident that the mechanism and role of *Lactobacillus* on crayfish gut health, immunity and disease resistance are very similar.

Table 9. 2 Health and immune indices and gut health of marron in present research

	Starvation	***L. acidophilus* + *L. plantarum***	***Bacillus mycoides***	***Clostridium butyricum***	**PBM+BSF**	**BB**	**TWC**	**BSFLP**
Health + immunity (+)		SGR, FCR, protein, energy, THC, 6 immune genes	THC, protein, energy, 2 immune genes	Protein, Lys, THC, 4 immune genes	Protein, energy, Lys, THC, HO, 3 immune genes (+)	Energy, Lys, THC, 5 immune genes	Energy, Lys, THC, HO, 5 immune genes	Lys, THC 6 immune genes
Health + immunity (-)	Protein, energy, lipid, Lys, THC, 4 immune genes	-			Lipid, one immune genes			
Gut health (+)	*Vibrio* spp., Stress response pathway activities	Microvilli length and counts Alpha div, Bactroidetes Firmicutes *Lactobacillus*	Firmicutes, *Holdemania/ Hafnia Hypnocyclicus*	Alpha div, Unshared OTUs Fusobacteria *Hypnocyclicus*	Alpha div Unshared OTUs Firmicutes *Lactobacillus*	Aplha div Firmicutes *Clostridium*	Aplha div Species Firmicutes *Streptococcus*	Aplha div Unshared OTUs Species Firmicutes *Streptococcus*
Gut health (-)	Alpha div, unshared OTUs, Firmicutes	*Vibrio*	*Aeromonas Vibrio*	*Aeromonas Vibrio*	*Vibrio Serratia Enterobacter*	Proteobacteria	Proteobacteria	*Aeromonas*

Table 9. 3. Comparative analysis of gut microbiome data of marron exposed to various parameters as detailed in various chapters

Studied Parameters	**Sub-parameters**	**Nutrient deprivation**	**Dietary Probiotics**	**Dietary protein sources**	**Substrates of rearing environment**
Differentially abundant genera	*Vibrio*	3.2 ± 0.2^{a}	0.4 ± 0.1^{c}	1.8 ± 0.8^{b}	1.3 ± 0.6^{b}
	Aeromonas	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	1.2 ± 0.6^{a}	0.4 ± 0.1^{b}
	Hafnia	1.8 ± 0.2^{a}	1.6 ± 0.5^{a}	1.3 ± 0.8^{ab}	0.8 ± 0.6^{b}
	C. Bacilloplasma	0.8 ± 0.1^{b}	0.7 ± 0.3^{b}	1.6 ± 0.4^{a}	1.2 ± 0.5^{a}

The rarefied read abundance was converted into fold changes (log1p). Different superscript letters indicate significantly different mean fold changes of bacteria in the marron gut exposed to various parameters.

Table 9. 4 Comparative analysis of gut microbiome data of marron exposed to various parameters as detailed in various chapters

Diversity	**Nutrient deprivation**	***L. acidophilus + L. plantarum***	***Bacillus mycoides***	***Clostridium butyricum***	***Fishmeal***	***PBM***	***BSF***	***PBM+BSF***	***BB***	***TWC***	***BSFLP***
Observed species	39 ± 4.2^{e}	162 ± 16.6^{a}	62 ± 8.2^{cd}	52 ± 6.8^{cd}	66 ± 5.9^{cd}	82 ± 8.2^{c}	104 ± 10.6^{b}	138 ± 12.4^{ab}	102 ± 4.6^{b}	102 ± 9.6^{b}	146 ± 18.8^{ab}
Shannon	0.96^{d}	3.2 ± 0.2^{a}	1.6 ± 0.2^{c}	1.5 ± 0.2^{c}	1.6 ± 0.2^{c}	1.8 ± 0.2^{c}	$2.2 \pm 0.2b^{c}$	3.1 ± 0.2^{a}	2.8 ± 0.2^{ab}	3.5 ± 0.3^{a}	3.8 ± 0.3^{a}

In addition to cost of diets, regular recycling of water in the ponds can also increases the production cost. Furthermore, chemical treatments of water and subsequent discharge of effluents have serious human health and environmental impacts. In recent time, substrate based biological filters have been reported successful in treating organic waste by improving nitrifying bacterial communities in the water (Permatasari et al., 2018). In present study, Bio-ball (BB) and water cleanser (TWC) augmented the growth of Actinobacteria, Bacteroidetes

and Firmicutes in the water and marron gut, the bacterial group that reported to play role in water remediation (Kruglova et al., 2020, Sharmin et al., 2013). A correlation between water and gut bacteria therefore can be seen at phylum level revealing transmission of water bacteria into the gut through feeding. At genus level, higher abundance of *Aquabacterium, Dechloromonas, Polynucleobacter, Prevotella*, *Rhodobacter* and *Streptococcus*, bacterial communities linked to water remediation in previous studies (Numberger et al., 2019, Shahid et al., 2020, Yang et al., 2020). Therefore, BB and TWC generated an ideal microbial communities in the substrate and water for the decomposition of organic waste as reported in different studies. In present research, the effect of bagged zeolite and BB on gut microbial communities of marron are quite similar reflected by similar effects on innate immune response (Figure 9.2, Table 9.2 and 9.4). The reason behind that the mechanism of water remediation by BB and zeolite is similar, ammonia and nitrite reduced to nitrate by nitrifying bacteria (Al-Amshawee et al., 2020, Grismer and Collison, 2017). Currently, there is no available microbiome data for BB, TWC and zeolite to compare with our study findings and hence, more research is recommended for substrate based water remediation.

Replacement of fishmeal from aqua-diets is one of the major challenges to enhance sustainable aquaculture globally. Considering the ample of approaches on fish to replace fishmeal using proteins from various alternative sources (Hua et al., 2019), not much of studies can be found for crayfish (discussed in literature review). Our research group tested five different protein sources on marron (Saputra et al., 2019) while another study evaluated different form of soybean meal for red-claw (Saoud et al., 2012). However, none of the study yet reports response of gut microbiota with different diets on marron. Considering the importance and role of gut microbiota in health and immunity of crayfish, dietary effects have been investigated deeply for marron in this study including the correlation between the gut bacteria and immune parameters, functional features of predicted metagenome. Consistent with previous study of

Saputra et al., 2019, poultry-by-product meal (PBM) and black soldier fly meal (BSF) generated better results in terms of health and immunity and gut microbial diversity. Trial with PBM alone or in combination to BSF favoured the growth of Bacteroidetes, Firmicutes and Tenericutes that positively correlated to growth and immunity of marron. Inadequate data for crayfish gut microbiota with different protein sources constrained us to correlate our findings, yet the community in marron gut was found much consistent (except Tenericutes) compared to fish when taking growth and immunity as the two major criteria for selecting protein sources in aqua-diets (Dam et al., 2020, Gajardo et al., 2017, Michl et al., 2019, Pérez-Pascual et al., 2020, Rimoldi et al., 2018). Tenericutes on the contrary, only abundant in crustacean gut, more specifically hindgut where they reported to associated with elevated expression of antimicrobial peptides and genes associated with innate immune response (Dong et al., 2018). Trials with probiotics, alternative protein diets (APS), substrates and probiotic in APS lead to a common observation that better gut health and immunity are correlated to increased abundance for Firmicutes and Tenericutes. In this regards, *Lactobacillus* strains, BSF supplementation in PBM, and substrate based biological filters Bio-Ball and Water Cleanser influenced more beneficial bacteria from Firmicutes and Tenericutes in the marron gut.

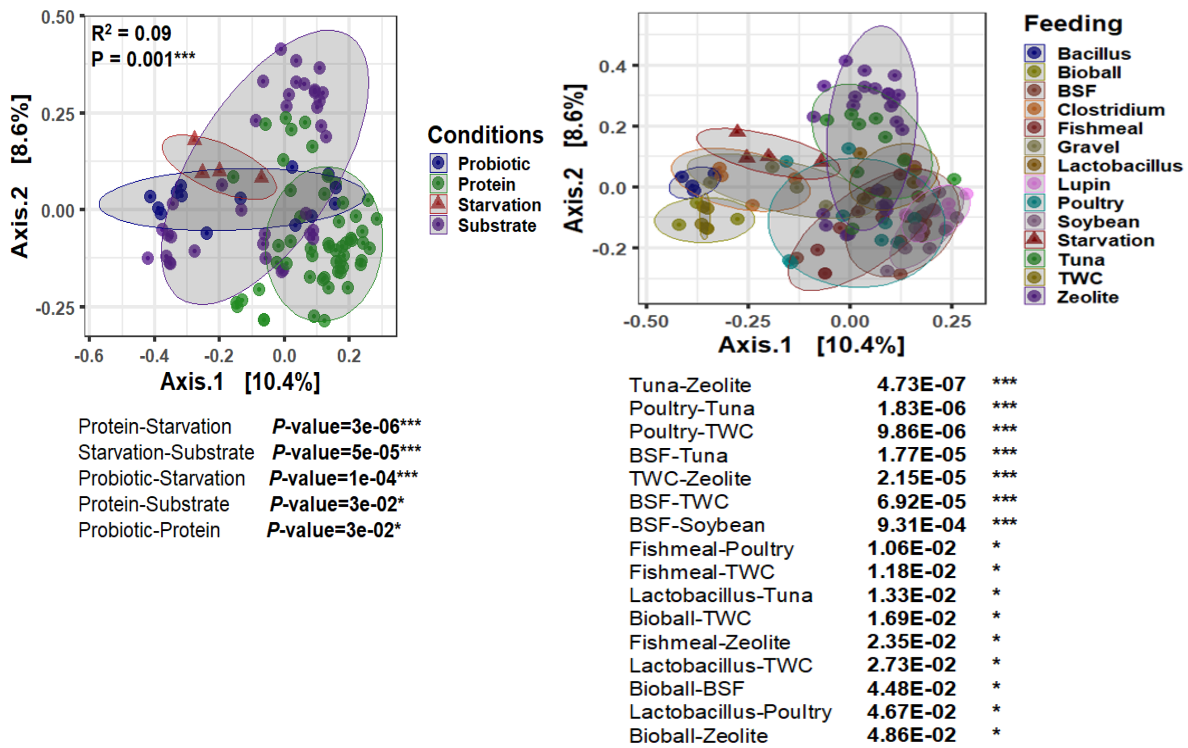

Figure 9. 2 Beta-ordination of bacterial OTUs in the marron gut exposed to various parameters as detailed in various chapters.

As a final point, the analysis of marron gut microbiota with currently available crayfish and decapod crustacean sequence database showed that marron gut microbiota are distinctly different from red-swamp ($P<0.001$) red-claw ($P<0.005$). Present research identified *Hafnia*, *Hypnocyclicus* and *Bacteroides* as an indicator genus in marron, red-claw and red-swamp, respectively (Table 9.5) meaning that those bacteria can be used as identification marker for specific crayfish species. The analysis also revealed that unlike Proteobacteria, Firmicutes, Fusobacteria and Bacteroidetes in fish (Liu et al., 2016, Talwar et al., 2018, Wang et al., 2018), crayfish gut microbiota composed of high percentages of Tenericutes, regardless of culture conditions and diets. *C.* Bacilloplasma and *Hafnia* Obesumbacterium are the core microbiota of marron gut whereas *Bacteroides* and *Hypnocyclicus* for the red-swamp and red-claw. However, regardless of diets and culture conditions, *C.* Bacilloplasma comprised of one third (32%-37.8%, average 35.03%) of total reads revealing the consistency and dominancy of

Tenericutes in crayfish (Figure 9.3). The differences in physiology, geography and culture conditions are probably associated with the differences in gut microbiota and core genera in these three commercially cultured crayfish.

Table 9. 5 Differentially abundant genera in three commercially cultured crayfish

Marron	Red-Claw	Red-Swamp	Adjusted P	Genus
4.4	11.6	**7636.7**	2.18E-12	*Bacteroides*
47.6	**6603.6**	2.9	1.06E-10	*Hypnocyclicus*
1197.4	9.6	405.4	1.28E-05	*Hafnia*

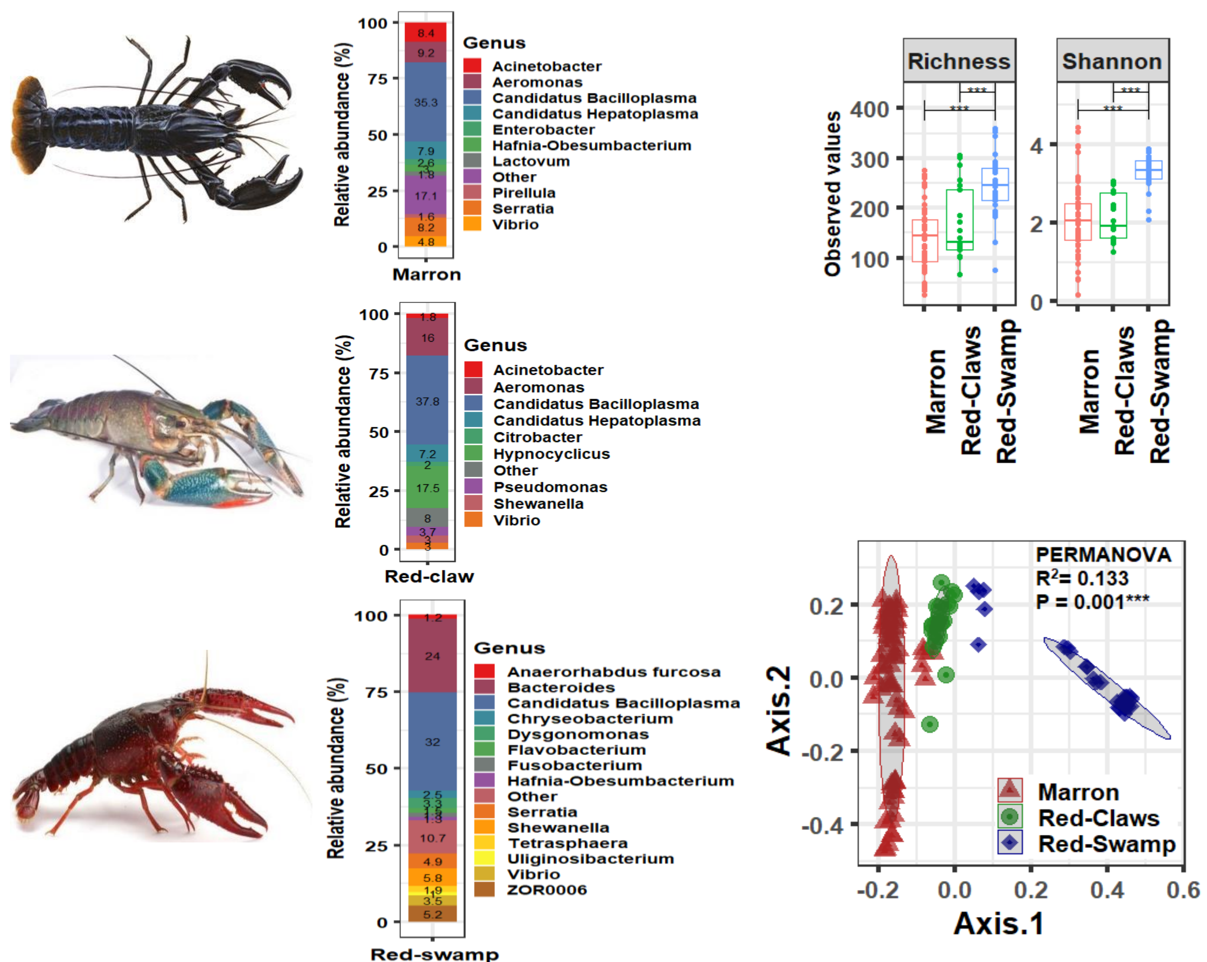

Figure 9. 3 Differences in gut microbial diversity and composition in three commercially cultured crayfish– marron, red-claw and red-swamp.

In decapod crustacean, marron and crayfish gut microbiota are more similar to crab, especially Chinese mitten crab and prawn, however highly distant from shrimp, particularly tiger shrimp (Table 9.6, Figure 9.4). To understand these differences, present study curated meta-data from associated studies from previous chapter (Table 2.3) and found gut microbiota of decapod crustacean reared in freshwater including marron, red claw, red swamp, freshwater prawn, Chinese mitten crab are significantly different than saltwater shrimp, lobster, and the findings can be supported with some previous reports on differential response of gut microbiota for Atlantic salmon (Dehler et al., 2017b) and river prawn (Chen et al., 2017) in freshwater and saltwater culture system.

Table 9. 6 Similarity and dissimilarity between gut microbiota in terms of beta-ordination in decapod crustacean emphasizing crayfish and marron.

Decapod crustacean	**PERMANOVA**	**Decapod crustacean**	**PERMANOVA**
Crayfish-shrimp	*P*-value=6e-10***	Marron-tiger shrimp	P-value=2e-19***
Crayfish-prawn	*P*-value=4e-07***	Marron-river prawn	P-value=5e-09***
Crayfish-lobster	*P*-value=6e-06***	Marron-freshwater prawn	P-value=4e-07***
Crayfish-crab	*P*-value=3e-03**	Marron-CMC	P-value=6e-02**

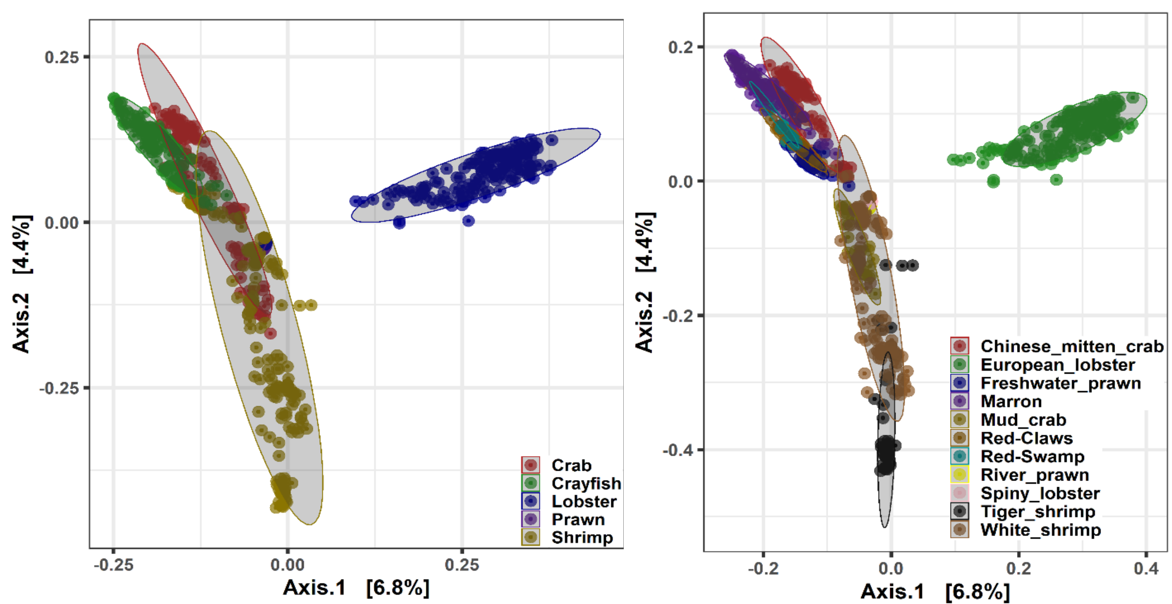

Figure 9. 4 Beta-ordination displaying similarity and dissimilarity of gut microbial communities in decapod crustacean.

9.2. Limitations

There were some technical limitations of the present research, expressed below:

- Resolution of HTS: The size of v3v4 hypervariable regions of the 16S is around 500 bp, and eventually cannot generate species level resolutions precisely. Therefore, identification of bacteria was entirely limited up to genus level in cases of HTS data analysis.
- Databases for eDNA: The information about environmental DNA (eDNA), especially information about taxonomy and metagenome functions are partial and fragmented. However, with the increasing number of research on eDNA in recent time, the databases like SILVA, RDP classifier, NCBI are updating their resources routinely.
- Gene expression: Only a set of genes associated with innate immune response of crayfish have been tested for their relative expression in present study. The number is only <1% of the total genes present in a crayfish species like red claw and marbled crayfish.

9.3. Conclusions

Besides, conclusions depicted in every research chapters (Chapters 4-8), following is the summary of additional conclusions based on the current research:

- Marron gut microbial communities are sensitive to the dietary protein sources and the use of substrates in the rearing environments. Nevertheless, *Aeromonas*, *Candidatus* Bacilloplasma and *Hafnia* are the core gut microbiota of marron, irrespective of dietary protein source and culture conditions.
- The significant abundance of *Candidatus* lineages in marron gut exhibits its similarity to red claw, freshwater prawns and crab species, all cultured in freshwater environments, but are different from shrimp and lobster reared in saltwater.
- Long-term nutrient deprivation significantly decreases marron gut microbial diversity and abundance of core genera of microbial communities in marron gut with a significant increase of *Vibrio* that are linked to poor immune response.

- Although the effects and mode of actions of probiotic bacteria are different, three of the probiotics *Bacillus mycoides*, *Clostridium butyricum* and *Lactobacillus* mixture from different family have similar type of impacts on the tail muscle biochemistry, haemolymph parameters and genes associated with immune response of marron. More importantly, *Vibrio* species in the marron gut can be replaced or reduced by the dietary supplementation of *Bacillus mycoides*, *Clostridium butyricum* and *Lactobacillus* mixture. *Lactobacillus* spp. can produce higher diversity of microbial communities in the marron gut and can influenced greater number of health and immune parameters than any other dietary probiotics, and thus can be used as potential probiotics for marron aquaculture.
- Poultry-by-product (PBM) from animal and insect black soldier fly (BSF) are better alternative sources of fishmeal for marron aquaculture. PBM alone or in combination with BSF, and BSF with *L. plantarum* can significantly increase the gut microbial diversity, and improve the health and immune status of marron, compared to fishmeal. In addition, BSF supplemented diet increases Firmicutes and lactic acid bacteria (LAB) in the rearing water.
- The effective substrates in the rearing environment of the marron culture have an ability to decompose nitrogenous wastes in the water and increase gut microbial diversity by augmenting Firmicutes resulting marron with improved gut health and immunity.
- Most of the bacteria from phylum Firmicutes including lactic acid bacteria (LAB) are beneficial for marron gut health and immunity. Some species from Tenericutes, particularly the *Candidatus* lineages also positively correlated to health and immunity of marron.

9.3. Recommendations

Based on findings of the present study, two further research themes are recommended:

1. To investigate the patterns of global gene expression and selectively identify the gene(s) associated with growth and immunity of marron *via.* transcriptome analysis of gut tissues fed various dietary probiotics and exposed to different substrates

2. To evaluate the effects of enrichment of Firmicutes, lactic acid bacteria and *Candidatus* lineages in the substrates for marron aquaculture by providing the optimum growth conditions with selective nutrients.

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	Concepts	Funding	Methods	Data curation	Investigation	Software	Formal analysis	Validation	Writing: Draft	Writing: Reviewing	Resources	Supervision	Total % contribution
Paper 1- Title: Effects of long-term starvation on health indices, gut microbiota and innate immune response of fresh water crayfish, marron (*Cherax cainii*, Austin 2002). Aquaculture 514, 734444. https://doi.org/10.1016/j.aquaculture.2019.734444.													
First Author Md Javed Foysal	√	√	√	√	√	√	√		√				87.5%
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Paper 2- Title: Marked variations in gut microbiota and some innate immune responses of fresh water crayfish, marron (*Cherax cainii*, Austin 2002) fed dietary supplementation of *Clostridium butyricum*. PeerJ 7, e7553. https://doi.org/10.7717/peerj.7553.													
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Paper 3- Title: *Bacillus mycoides* supplemented diet modulates the health status, gut microbiota and innate immune response of freshwater crayfish marron (*Cherax cainii*). Anim. Feed Sci. Technol. 114408. https://doi.org/10.1016/j.anifeedsci.2020.114408.													
First Author Md Javed Foysal	√	√	√	√	√		√		√				70%
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Paper 4- Title: *Lactobacillus acidophilus* and *L. plantarum* improve health status , modulate gut microbiota and innate immune response of marron (*Cherax cainii*). Sci. Rep. 10, 5916. https://doi.org/10.1038/s41598-020-62655-y.													
First Author Md Javed Foysal	√	√	√	√	√	√	√		√				85%
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Paper 5- Title: Dietary supplementation of black soldier fly (*Hermetica illucens*) meal modulates gut microbiota, innate immune response and health status of marron (*Cherax cainii*, Austin 2002) fed poultry-by-product and fishmeal based diets. PeerJ 7, e6891. https://doi.org/10.7717/peerj.6891.													
First Author Md Javed Foysal	√	√	√	√	√	√	√		√				87.5%
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Appendix 1

(Author contributions in published articles)

	Concepts	Funding	Methods	Data curation	Investigation	Software	Formal analysis	Validation	Writing: Draft	Writing: Reviewing	Resources	Supervision	Total % contribution
Paper 6- Title: Biological filters regulate water quality, modulate health status, immune indices and gut microbiota of freshwater crayfish, marron (*Cherax cainii*, Austin, 2002). Chemosphere 125821. https://doi.org/10.1016/j.chemosphere.2020.125821.													
First Author Md Javed Foysal	√	√	√	√	√	√	√		√				87.5%
Co-Author 1 Ravi Fotedar		√						√		√	√	√	5 %
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 2 Alfred Tay								√		√	√	√	5%
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 3 Sanjay Kumar Gupta										√			2.5 %
I acknowledge that these represents my contribution to the above research output. Signed:													
Total %													100

Appendix 1

(Author contributions in published articles)

	Concepts	Funding	Methods	Data curation	Investigation	Software	Formal analysis	Validation	Writing: Draft	Writing: Reviewing	Resources	Supervision	Total % contribution
Paper 7- Title: *Lactobacillus plantarum* in black soldier fly (*Hermetica illucens*) meal modulates gut health and immunity of freshwater crayfish (*Cherax cainii*). Fish Shellfish Immunol. 108, 42–52. https://doi.org/10.1016/j.fsi.2020.11.020.													
First Author Md Javed Foysal	√	√	√	√	√	√	√		√				85%
Co-Author 1 Ravi Fotedar		√						√		√	√	√	5 %
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Co-Author 2 Muhammad M.A.B. Siddik							√						2.5 %
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 3 Md Reaz Chaklader							√						2.5 %
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 2 Alfred Tay								√		√	√	√	5%
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Total %													100

Appendix 1

(Author contributions in submitted articles)

	Concepts	Funding	Methods	Data curation	Investigation	Software	Formal analysis	Validation	Writing: Draft	Writing: Reviewing	Resources	Supervision	Total % contribution
Paper 8 (Submitted) - Title: Valorised poultry-by-product and insect protein improve the microbial diversity and composition of gut and water microbiota in aquacultured marron (*Cherax cainii*). Submitted to Science of the Total Environment													
First Author Md Javed Foysal	√	√	√	√	√	√	√		√				70%
Co-Author 1 Thi Thanh Thuy Dao			√		√								10 %
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Co-Author 2 Ravi Fotedar		√						√		√	√	√	5 %
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Co-Author 3 Alfred Tay								√		√	√	√	5%
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Co-Author 4 Sanjay Kumar Gupta										√			2.5 %
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 5 Md Reaz Chaklader							√						2.5 %
I acknowledge that these represents my contribution to the above research output. Signed:													
Total %													100

Appendix 2
(Supplementary data)

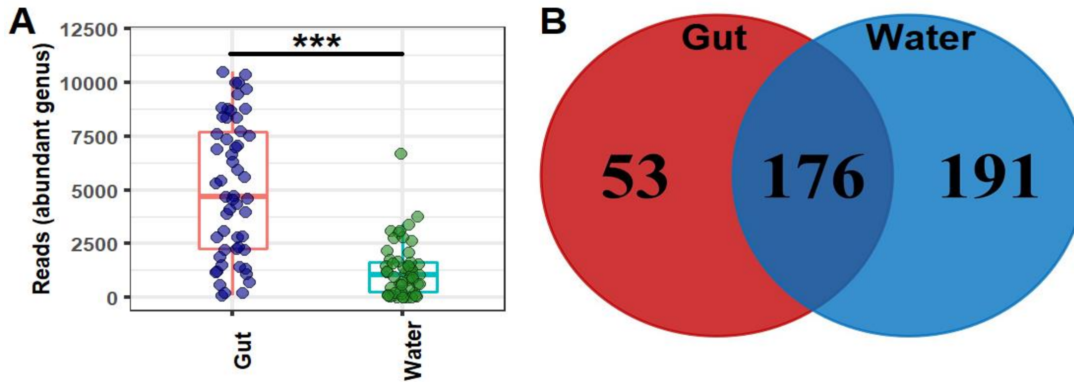

Figure S6.2. 1 (A) The read abundance for the most abundant genus in the gut and water. (B) Number of shared and unique genera in the gut and water samples.

A
Relative abundance (%)
Phylum
Bacteroidetes
Firmicutes
Proteobacteria
Tenericutes
Sample

B
Relative abundance (%)
Genus
Acinetobacter
Aeromonas
Candidatus Bacilloplasma
Candidatus Hepatoplasma
Citrobacter
Clostridium sensu stricto
Flavobacterium
Hafnia-Obesumbacterium
Klebsiella
Pseudomonas
Shewanella
Vibrio
Sample

Figure S6.2. 2 Relative abundance of bacteria in the gut at Phylum (A) and genus (B) level.

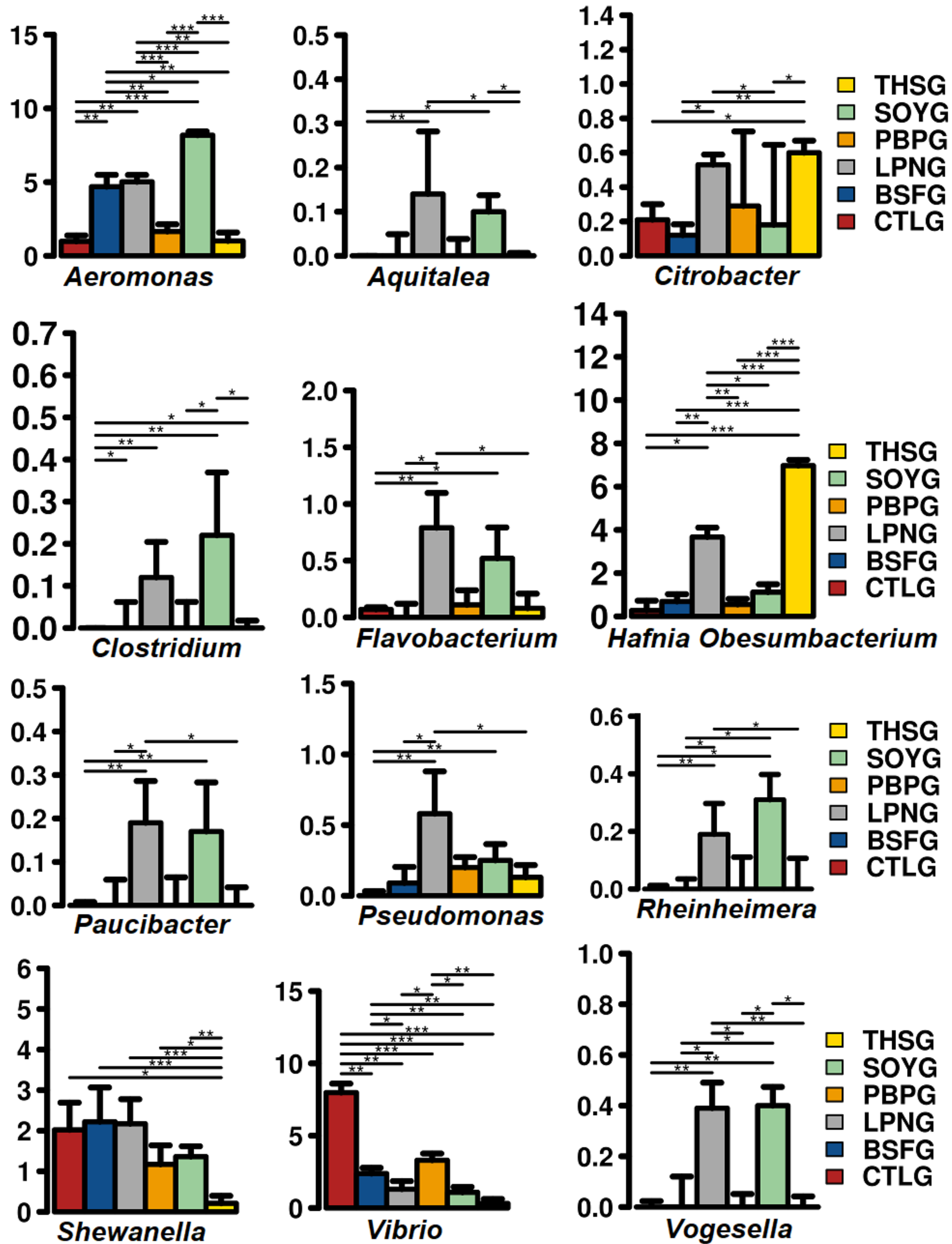

Figure S6.2. 3 Differential abundance of bacterial OTUs at genus level in the gut of marron fed different APS diets with non-parametric Kruskal-Wallis test followed by Bonferroni corrections. ***Significantly different at α-level of 0.001. **Significantly different at α-level of 0.005. *Significantly different at α-level of 0.05.

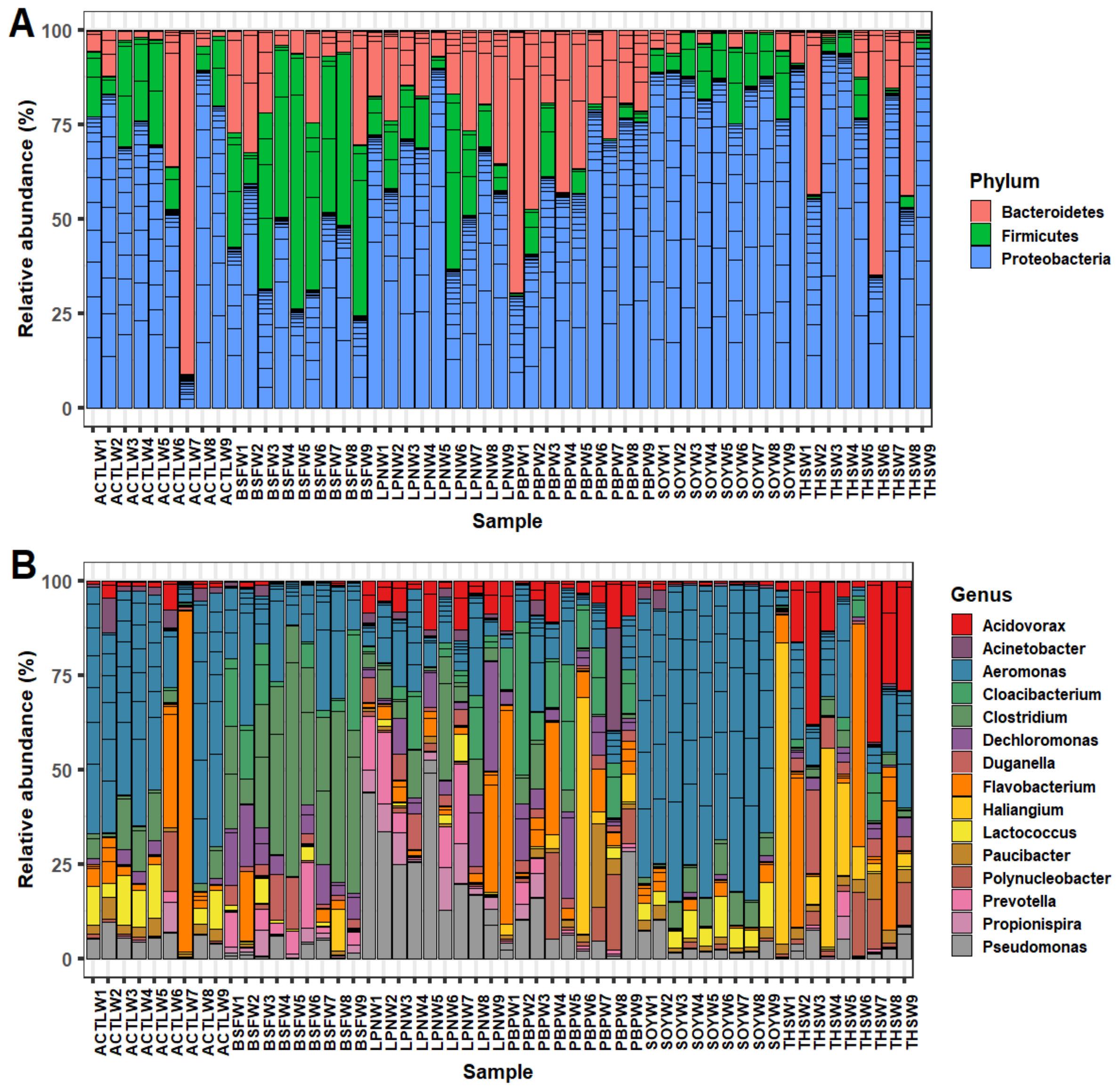

Figure S6.2. 4 Relative abundance of bacteria in the water at Phylum (A) and genus (B) level.

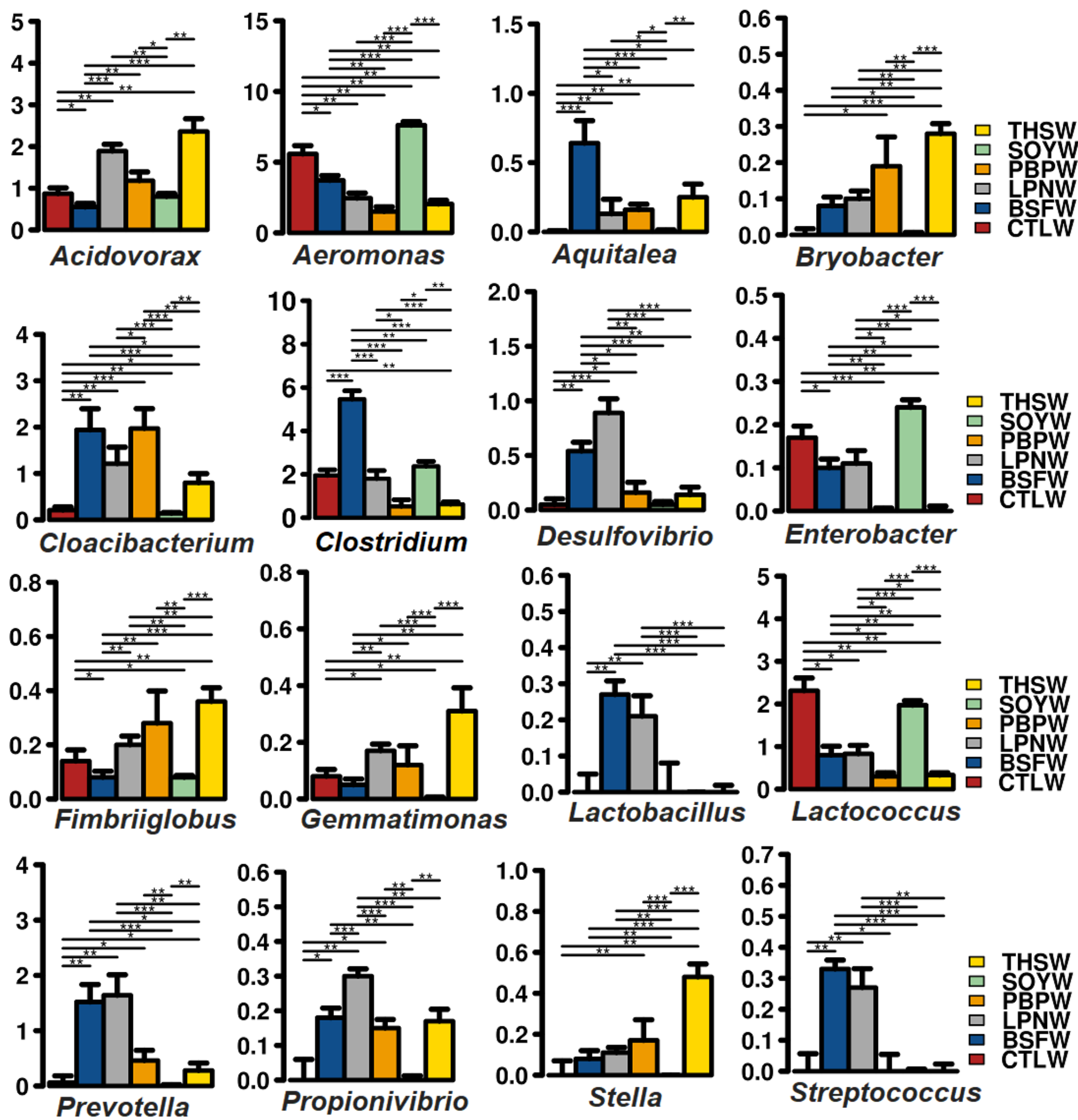

Figure S6.2. 5 Differential abundance of bacterial OTUs at genus level in the water with different APS diets with non-parametric Kruskal-Wallis test followed by Bonferroni corrections. ***Significantly different at α-level of 0.001. **Significantly different at α-level of 0.005. *Significantly different at α-level of 0.05.

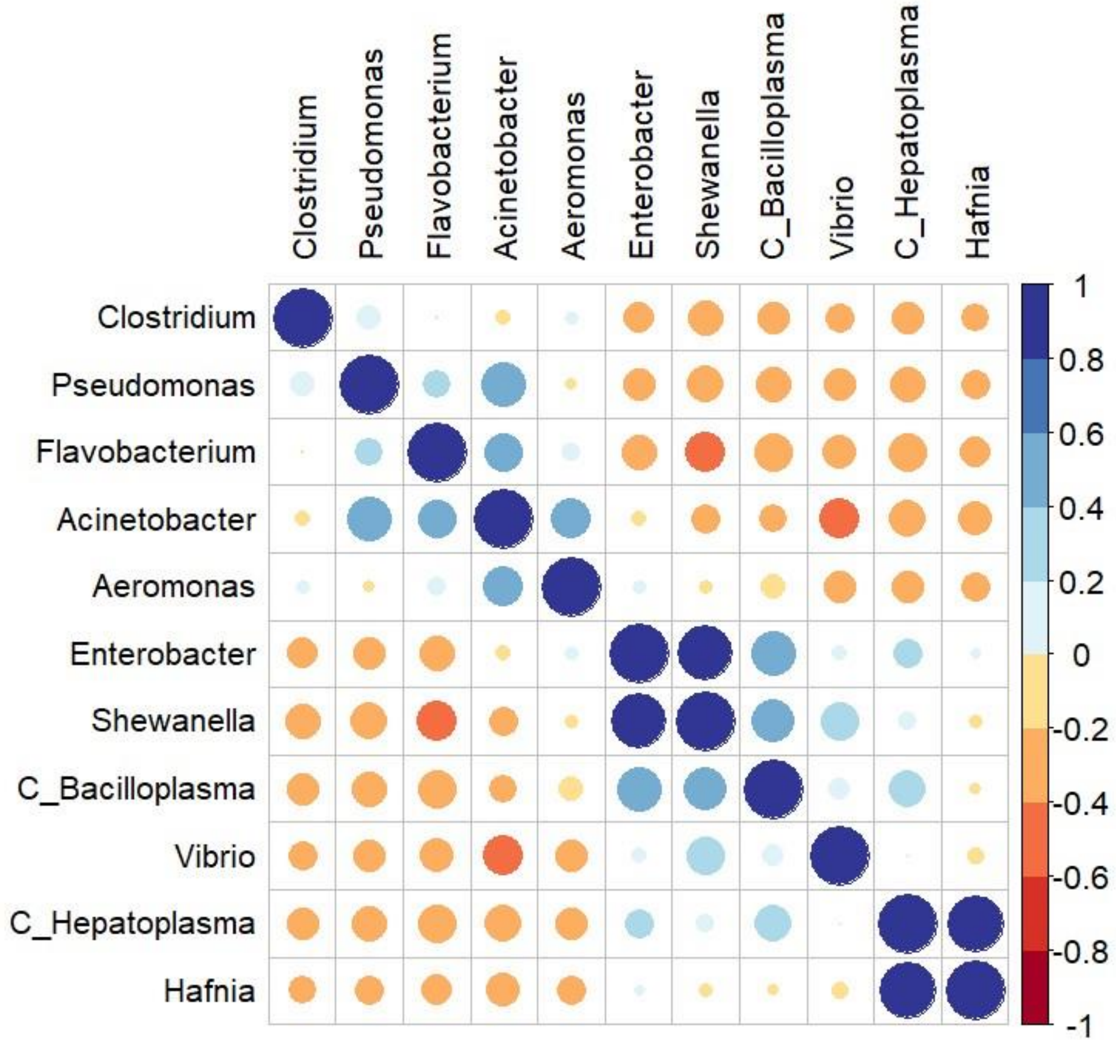

Figure S6.2. 6 Pearson correlation plot showing the relationship between microbial communities (at genus level) in the gut and water. Genera with more than 1% read abundance were considered (excluding unclassified).

Appendix 3

(Ingredients and proximate composition of the final diets used in this study)

Table S4. 1 Ingredients and proximate composition of the experimental diet used in chapter 5, methods 5.2(3).3.

Ingredients (g/100g)⁋	**Basal diet**
Fishmeal (Anchovy)	41.00
Soya bean meal	10.00
Wheat flour	37.00
Corn starch	4.80
Cod liver oil	4.20
$CaCO_3$	0.02
Vitamin premix	0.23
Vitamin C	0.05
Cholesterol	0.50
Lecithin-Soy	1.00
Betacaine	1.20
Nutrient composition (% dry weight)	
Crude protein	29.85
Crude lipid	7.45
GE MJ kg^{-1}	18.2

Note: ⁋All ingredients were procured and feeds were formulated by Glen Forest Specialty Feeds, Western Australia.

Table S6.1. 1 Feed ingredients and proximate composition (% dry weight) of the marron diets used in chapter 6, methods 6.2(1).2.

Ingredients*	**FM**	**PBM**	**FM + BSF**	**PBM+ BSF**
Fishmeal	41	0	32	0
Poultry by product meal	0	39	0	31.5
Black soldier fly meal	0	0	12	11
Soya bean meal	10	10	10	10
Wheat	37	38	36	36
Corn starch	4.80	4.80	4	4.80
Cod liver oil	4.20	5.20	3	3.70
$CaCO_3$	0.02	0.02	0.02	0.02
Vitamin premix	0.23	0.23	0.23	0.23
Vitamin C	0.05	0.05	0.05	0.05
Cholesterol	0.50	0.50	0.50	0.50
Lecithin-Soy	1	1	1	1
Betacaine	1.20	1.20	1.20	1.20
Total	100	100	100	100
Proximate composition of (% dry weight basis)				
CP%	29.93	29.61	30.07	30.20
Lipid %	7.12	7.32	7.56	7.48
GE MJ kg^{-1}	18.21	18.75	18.45	18.53

*All ingredients were procured and diets were prepared by Glen Forest Specialty Feeds, Western Australia. Abbreviation: Abbreviation: FM = Fish meal; PBM = Poultry-by-product meal; FM+BSF = Fish meal + Black soldier fly meal; PBM+BSF = Poultry-by-product meal + Black soldier fly meal; CP =Crude protein; GE = Gross energy; MJ =Mega joule

Table S6.2. 1 Ingredients (in percentages) and proximate composition of final diets mentioned in chapter 6, methods 6.2(2).2.

Ingredients	**FM**	**PBP**	**BSF**	**SOY**	**LPN**	**TSH**
Fishmeal	46	40	0	0	0	0
Poultry by product	0	42	0	0	0	0
Soybean	0	0	0	62	0	0
Black soldier fly larvae	0	0	33.6	0	0	0
Soybean	0	0	0	0	0	0
Lupin	0	0	0	0	70	0
Tuna hydrolysate	0	0	0	0	0	27
wheat (10 CP)	30	34.5	33.4	12	7	35
Corn/wheat starch	11	11	11	10	11	11
Cholesterol	0.5	0.5	0.5	0.5	0.5	0.5
Canola oil	2	1.5	0	4	2	0
Cod liver oil	3	2	0	5	2.5	0
Vitamin premix	0.3	0.3	0.3	0.3	0.3	0.3
Vitamin C	0.1	0.1	0.1	0.1	0.1	0.1
Dicalcium Phosphate	0.1	0.1	0.1	0.1	0.1	3
Lecithin-Soy (70%)	3	3	3	3	3	4
Barley	4	5	5	3	3.5	19
Casein			13			
Total	100	100	100	100	100	100
Proximate composition (% dry basis) of the final diet						
Crude protein	30.5	30.5	30.0	30.4	30.2	30.7
Crude lipid	12.5	12.8	12.5	12.8	12.6	12.6

Appendix 4

(Set of primers used for gene expression analysis in various trials)

Table S4. 2 Primers used for gene expression analysis in chapter 4, methods 4.2.7

Primers	**Forward sequence (5′-3′)**	**Reverse sequence (5′-3′)**
proPO	GCCAAGGATCTTTGTGATGTCTT	CGGCCGGCCAGTTCTAT
cytMnSOD	AGGTCGAGCAAGCAGGTGTAG	GTGGGAATAAACTGCAGCAATCT
PcCTSL	CGGATCACTGGAGGGTCAAACACTT	GCAATTTTCATCCTCGGCATCAT
IL-8	CTATTGTGGTGTTCCTGA	TCTTCACCCAGGGAGCTTC
IL-10	CAGTGCAGAAGAGTCGACTGCAAG	CGCTTGAGATCCTGAAATATA
IL-17F	GTCTCTGTCACCGTGGAC	TGGGCCTCACACAGGTACA
β-actin	TTGAGCAGGAGATGGGAACCG	AGAGCCTCAGGGCAACGGAAA

Table S5.1. 1 Primers used for gene expression analysis in chapter 5, methods 5.2(1).8.

Primer	**Forward sequence (5′ to 3′)**	**Reverse sequence (5′ to 3′)**
proPO	GCCAAGGATCTTTGTGATGTCTT	CGGCCGGCCAGTTCTAT
cytMnSOD	AGGTCGAGCAAGCAGGTGTAG	GTGGGAATAAACTGCAGCAATCT
PcCTSL	CGGATCACTGGAGGGTCAAACACTT	GCAATTTTCATCCTCGGCATCAT
IL-8	CTATTGTGGTGTTCCTGA	TCTTCACCCAGGGAGCTTC
IL-10	CAGTGCAGAAGAGTCGACTGCAAG	CGCTTGAGATCCTGAAATATA
IL-17F	GTCTCTGTCACCGTGGAC	TGGGCCTCACACAGGTACA
β-actin	TTGAGCAGGAGATGGGAACCG	AGAGCCTCAGGGCAACGGAAA

Abbreviation: IL = Interleukin, PcCTSL = Cathepsin L, proPO = Prophenoloxidase, cytMnSOD = Cytosolic manganese superoxide dismutase

Table S5.1. 2 Primers used in gene expression analysis in chapter 5, methods 5.2(2).8.

Primer	Forward sequence (5′ to 3′)	Reverse Sequence (5′ to 3′)
IL-1β	GTTACCTGAACATGTCGGC	AGGGTGCTGATGTTCAGCCC
IL-8	CTATTGTGGTGTTCCTGA	TCTTCACCCAGGGAGCTTC
IL-10	CAGTGCAGAAGAGTCGACTGCAAG	CGCTTGAGATCCTGAAATATA
IL-17F	GTCTCTGTCACCGTGGAC	TGGGCCTCACACAGGTACA
Vg	CCAGAAGACGCCACAAGAA	CAGAAGGCATCAGCCAATC
β-actin	TTGAGCAGGAGATGGGAACCG	AGAGCCTCAGGCAACGGAAA

Table S5.1. 3 Primers used for gene expression analysis in chapter 5, methods 5.2(3).10.

Primer	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
proPO	GCCAAGGATCTTTGTGATGTCTT	CGGCCGGCCAGTTCTAT
cytMnSOD	AGGTCGAGCAAGCAGGTGTAG	GTGGGAATAAACTGCAGCAATCT
PcCTSL	CGGATCACTGGAGGGTCAAACACTT	GCAATTTTCATCCTCGGCATCAT
IL1β	GTTTACCTGAACATGTCGGC	AGGGTGCTGATGTTCAGCCC
IL-8	CTATTGTGGTGTTCCTGA	TCTTCACCCAGGGAGCTTC
IL-10	CAGTGCAGAAGAGTCGACTGCAAG	CGCTTGAGATCCTGAAATATA
IL-17F	GTCTCTGTCACCGTGGAC	TGGGCCTCACACAGGTACA
TNF-α	TGGAGGGGTATGCGATGACACCTG	TGAGGCCTTTCTCTCAGCGACAGC
vg	CCAGAAGACGCCACAAGAA	CAGAAGGCATCAGCCAATC
pcna	AGAGGCGGACTGAAGAGG	TTGATGGCATCCAGCACT
β-actin	TTGAGCAGGAGATGGGAACCG	AGAGCCTCAGGCAACGGAAA

Table S6.1. 2 List of primers used in gene expression analysis in chapter 6, methods 6.2(1).6.

Primer	Forward sequence (5′ to 3′)	Reverse Sequence (5′ to 3′)
IL-1β	GTTACCTGAACATGTCGGC	AGGGTGCTGATGTTCAGCCC
IL-8	CTATTGTGGTGTTCCTGA	TCTTCACCCAGGGAGCTTC
IL-10	CAGTGCAGAAGAGTCGACTGCAAG	CGCTTGAGATCCTGAAATATA
IL-17F	GTCTCTGTCACCGTGGAC	TGGGCCTCACACAGGTACA
TNF-α	TGGAGGGGTATGCGATGACACCTG	TGAGGCCTTTCTCTCAGCGACAGC
Vg	CCAGAAGACGCCACAAGAA	CAGAAGGCATCAGCCAATC
Pcna	AGAGGCGGACTGAAGAGG	TTGATGGCATCCAGCACT
β-actin	TTGAGCAGGAGATGGGAACCG	AGAGCCTCAGGCAACGGAAA

Table S7.1. 1 Primers used for gene expression analysis in chapter 6, methods 6.2(1).6.

Primer	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
proPO	GCCAAGGATCTTTGTGATGTCTT	CGGCCGGCCAGTTCTAT
cytMnSOD	AGGTCGAGCAAGCAGGTGTAG	GTGGGAATAAACTGCAGCAATCT
PcCTSL	CGGATCACTGGAGGGTCAAACACTT	GCAATTTTCATCCTCGGCATCAT
IL-1β	GTTACCTGAACATGTCGGC	AGGGTGCTGATGTTCAGCCC
IL-8	CTATTGTGGTGTTCCTGA	TCTTCACCCAGGGAGCTTC
IL-10	CAGTGCAGAAGAGTCGACTGCAAG	CGCTTGAGATCCTGAAATATA
IL-17F	GTCTCTGTCACCGTGGAC	TGGGCCTCACACAGGTACA
TNF-α	TTGAGCAGGAGATGGGAACCG	AGAGCCTCAGGGCAACGGAAA

Table S8. 1 Primers used for gene expression analysis in chapter 8, methods 8.2.9.

Primer	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
proPO	GCCAAGGATCTTTGTGATGTCTT	CGGCCGGCCAGTTCTAT
cytMnSOD	AGGTCGAGCAAGCAGGTGTAG	GTGGGAATAAACTGCAGCAATCT
PcCTSL	CGGATCACTGGAGGGTCAAACACTT	GCAATTTTCATCCTCGGCATCAT
cypA	AGGGCAACAGTGGTATCAAC	GAAAGTGGACCCTTTGTAGC
Lysozyme	GGACGTCCTCAGGAAAGGTG	TTGTTAGTAGCGGCCGTGTT
CTL	GATCGGGCAGGAGCAAAGT	ATAAAGGAACACGACCAGGCA
ALF1	ACTGGGCGGGTTATGGAAAG	TGCAGCTTCTTCAGCGGTAA
Ast	AGAAACCAAGGTGGTCAGGC	TGATGGGCAGGTGAACCTCT
Toll 2	GCAATGCCGTCTTTACGCTC	TTCTGCTGTTCGGAGGCTAC
Toll 3	AGAAGCTCATGTGAACAGGCA	CACATCTTTGCATCCAGCTCC
Toll 4	GCCTCACATGGATATTGTGCC	AAGAGCTGCATCTCCCTGGTA
Toll 5	GTATCCTCTGACCCCGGACT	CCTGCCCTTGACTGGTTAGG
18S	TCTTCTTAGAGGGATTAGCGG	AAGGGGATTGAACGGGTTA

Abbreviations: *proPO*, Prophenoloxidase; *cytMnSOD*, Cytosolic manganese superoxide dismutase; *PcCTSL,* Cathepsin L; *cyp*A, Cyclophilin; *CTL*, C-type lectin; *ALF1,* Anti-lipopolysaccharide factor 1; *Ast,* Astacidin; *Toll2,* Toll like receptor 2; *Toll3*, Toll like receptor 3; *Toll4*, Toll like receptor 4; *Toll5*, Toll like receptor 5.

Appendix 5

(List of publications from this thesis)

SL	Details of publication
1	**Foysal, M.J.,** Fotedar, R., Tay, A.C.Y., Gupta, S.K., 2020. Effects of long-term starvation on health indices, gut microbiota and innate immune response of fresh water crayfish, marron (*Cherax cainii*, Austin 2002). Aquaculture 514, 734444. https://doi.org/10.1016/j.aquaculture.2019.734444. [SJR **Q1**, Impact factor **3.022**]
2	**Foysal, M.J.,** Nguyen, T.T.T., Chaklader, M.R., Siddik, M.A.B., Tay, C.Y., Fotedar, R., Gupta, S.K., 2019. Marked variations in gut microbiota and some innate immune responses of fresh water crayfish, marron (*Cherax cainii*, Austin 2002) fed dietary supplementation of *Clostridium butyricum*. PeerJ 7, e7553. https://doi.org/10.7717/peerj.7553. [SJR **Q1**, Impact factor **2.359**]
3	**Foysal, M.J.,** Chua, E.G., Gupta, S.K., Lamichhane, B., Tay, C.Y., Fotedar, R., 2020. *Bacillus mycoides* supplemented diet modulates the health status, gut microbiota and innate immune response of freshwater crayfish marron (*Cherax cainii*). Anim. Feed Sci. Technol. 114408. https://doi.org/10.1016/j.anifeedsci.2020.114408. [SJR **Q1**, Impact factor **2.69**]
4	**Foysal, M.J.,** Fotedar, R., Siddik, M.A.B., Tay, A., 2020. *Lactobacillus acidophilus* and *L. plantarum* improve health status , modulate gut microbiota and innate immune response of marron (*Cherax cainii*). Sci. Rep. 10, 5916. https://doi.org/10.1038/s41598-020-62655-y. [SJR **Q1**, Impact factor **4.011**]
5	**Foysal, M.J.,** Fotedar, R., Tay, C.Y., Gupta, S.K., 2019. Dietary supplementation of black soldier fly (*Hermetica illucens*) meal modulates gut microbiota, innate immune response and health status of marron (*Cherax cainii*, Austin 2002) fed poultry-by-product and fishmeal based diets. PeerJ 7, e6891. https://doi.org/10.7717/peerj.6891. [SJR **Q1**, Impact factor **2.19**]
6	**Foysal, M.J.,** Fotedar, R., Tay, C.Y., Gupta, S.K., 2020. Biological filters regulate water quality, modulate health status, immune indices and gut microbiota of freshwater crayfish, marron (*Cherax cainii*, Austin, 2002). Chemosphere 125821. https://doi.org/10.1016/j.chemosphere.2020.125821. [SJR **Q1**, Impact factor **5.108**]
7	**Foysal, M.J.,** Fotedar, R., Siddik, M.A.B., Chaklader, R., Tay, A., 2021. *Lactobacillus plantarum* in black soldier fly (*Hermetica illucens*) meal modulates gut health and immunity of freshwater crayfish (*Cherax cainii*). Fish Shellfish Immunol. 108, 42–52. https://doi.org/10.1016/j.fsi.2020.11.020. [SJR **Q1**, Impact factor **3.289**]

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Article 1: Foysal, M.J., Fotedar, R., Tay, A.C.Y., Gupta, S.K., 2020. Effects of long-term starvation on health indices, gut microbiota and innate immune response of fresh water crayfish, marron (*Cherax cainii*, Austin 2002). Aquaculture 514, 734444. https://doi.org/10.1016/j.aquaculture.2019.734444.

Effects of long-term starvation on health indices, gut microbiota and innate immune response of fresh water crayfish, marron (Cherax cainii, Austin 2002)

Author: Md Javed Foysal,Ravi Fotedar,Alfred Chin Yen Tay,Sanjay K. Gupta

Publication: Aquaculture

Publisher: Elsevier

Date: 1 January 2020

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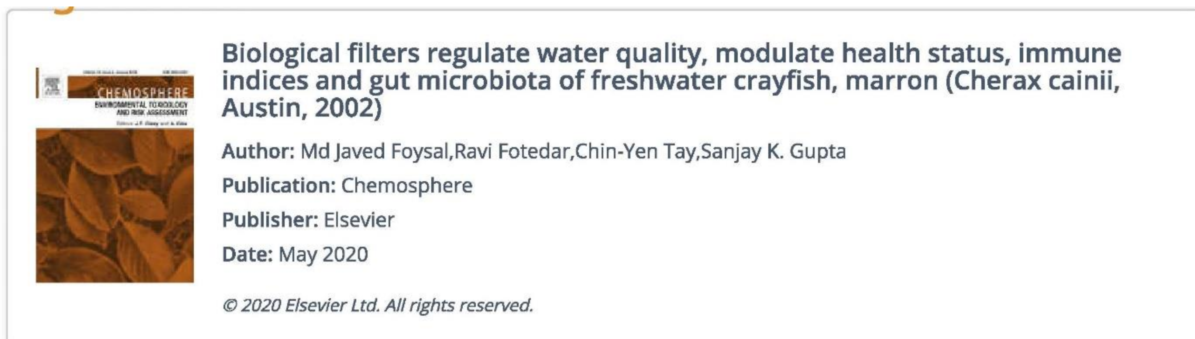

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Lactobacillus plantarum in black soldier fly (Hermetica illucens) meal modulates gut health and immunity of freshwater crayfish (Cherax cainii)

Author: Md Javed Foysal,Ravi Fotedar,Muhammad A.B. Siddik,Md Reaz Chaklader,Alfred Tay

Publication: Fish & Shellfish Immunology

Publisher: Elsevier

Date: January 2021

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